

Review

Detecting Biothreat Agents: From Current Diagnostics to Developing Sensor Technologies

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Detecting Biothreat Agents:
From Current Diagnostics to Developing Sensor Technologies

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Keywords: Biothreat, bacteria, virus, toxin, biosensor, FDA, diagnostic, assay, pandemic, Ebola.

Abstract

Although a fundamental understanding of the pathogenicity of most biothreat agents has been elucidated and available treatments have increased substantially over the last decades, they still represent a significant public health threat in this age of (bio) terrorism, indiscriminate warfare, pollution, climate change, unchecked population growth, and globalization. The key step to almost all prevention, protection, prophylaxis, post-exposure treatment, and mitigation of any bioagent is early detection. Here, we review available methods for detecting bioagents including pathogenic bacteria and viruses along with their toxins. An introduction placing this subject in the historical context of previous naturally-occurring outbreaks and efforts to weaponize selected agents is first provided along with definitions and relevant considerations. An overview of the detection technologies that find use in this endeavor along with how they provide data or transduce signal within a sensing configuration follows. Current “gold” standards for biothreat detection/diagnostics along with a listing of relevant FDA approved *in vitro* diagnostic devices is then discussed to provide an overview of the current state of the art. Given the 2014 outbreak of Ebola virus in Western Africa and the recent 2016 spread of Zika virus in the Americas, discussion of what constitutes a public health emergency and how new *in vitro* diagnostic devices are authorized for emergency use in the U.S. are also included. The majority of the review is then subdivided around the sensing of bacterial, viral, and toxin biothreats with each including an overview of the major agents in that class, a detailed cross-section of different sensing methods in development based on assay format or analytical technique, and some discussion of related microfluidic lab-on-a-chip/POC devices. Finally, an outlook is given on how this field will develop from the perspective of the biosensing technology itself and the new emerging threats they may face.

Vocabulary

Biothreat agent. Microorganism-based pathogens and/or their toxic products that pose a substantial threat to human health, livestock, or agriculture.

Pandemic. An infectious disease epidemic that has spread across a large region or population.

Anthrax. An infectious disease caused by *Bacillus anthracis* that can target the skin, lungs, or intestines. One of a number of agents that underwent military weaponization to be spread by spore dissemination.

Ebola. One of several viral hemorrhagic fevers affecting humans that is spread by direct contact and characterized by high degree of transmissibility, infectivity, and lethality.

Ricin. A highly toxic protein found in castor oil plant seeds. Microgram dosages can be fatal. Famous for being utilized to assassinate Soviet era dissidents.

Biosensor. An analytical device that combines a biological component such as an enzyme or antibody with a detection element to perform assays on chemicals and related analytes.

USFDA. The United States Food and Drug Administration, an agency of the Department of Health and Human Services, is tasked with protecting and promoting public health through the control and supervision of medication, medical devices, and animal foods and feeds among other similar oversight responsibilities under the Federal Food, Drug, and Cosmetic Act.

Table of Contents

Abstract

Vocabulary

Table of Contents

Introduction

 Important Considerations

 Definitions

 Agents, Their Classes, and Categories

 Knowing When to Deploy Sensors

 Environmental *versus* Clinical Matrices

Overview of Detection Methodologies

 Immunoassays and ELISAs

 PCR

 Whole Genome Sequencing

Medical Diagnosis of Biothreat Agents

 Current Gold Standards

 Clinical Laboratory Considerations and the Role of the Laboratory Response Network

 FDA, Medical Countermeasures, EUAs, and IVDs

 Infectious Disease IVD Testing Beyond the Traditional Clinical Laboratory

1	
2	
3	WHO and Emerging Infectious Diseases
4	Bacteria
5	Overview of Some Common Bacterial Biothreats
6	Anthrax
7	Plague
8	Glanders/Melioidosis
9	Tularemia
10	Brucellosis
11	Q Fever
12	Bacteria as Food and Water Safety Threats
13	Physical Sensors
14	Direct Mass Spectrometry
15	Chromatography with MS Detection
16	Waveguide Based Sensors
17	Surface Plasmon Resonance
18	Fiber Optic Biosensors
19	Quartz Crystal Microbalances
20	Surface Acoustic Wave Sensors
21	Microcantilevers
22	Magnetic Sensors
23	Biochemical Sensors
24	Antibodies
25	Enzyme-Linked Immunosorbent Assays
26	Lateral Flow Assays
27	Phage Display
28	PCR
29	Isothermal Amplification Techniques
30	Functional Nucleic Acids
31	DNA Sequencing
32	Electrochemical Sensors
33	Amperometric Sensors
34	Impedimetric Sensors
35	Potentiometric Sensors
36	Spectroscopic Sensors
37	Microscopy-based Detection Methods
38	LED-Based Microscopy
39	Light-Scattering-Based Culture Analysis
40	Colorimetric Nanoparticle Sensors
41	Photoluminescent, Fluorescent, and FRET-Based Sensors
42	Quantum Dots, Polymer Dots, Carbon Dots
43	Alternative Nanoparticulate Materials
44	Dipicolinic Acid Detection in Bacterial Spores
45	Chemiluminescent Sensors
46	Vibrational Spectroscopy and Surface-Enhanced Raman Spectroscopy
47	Infrared Spectroscopy
48	Flow Cytometric Sensing
49	
50	
51	
52	
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2	
3	Microfluidic Sensor Formats
4	E-nose
5	Alternative Approaches
6	Viruses
7	Overview of Common Viral Biothreats
8	Smallpox
9	Viral Hemorrhagic Fevers
10	Influenza
11	Coronaviruses and Acute Respiratory Syndrome
12	Physical Sensors
13	Direct or Label Free Detection
14	Quartz Crystal Microbalance
15	Mass Spectrometry
16	Biochemical Sensors
17	Immunoassay-Based Detection of Viral Agents
18	Lateral Flow Assays for Viral Detection/Identification
19	Recognition Elements
20	Reporter Elements
21	Enzyme-Based Reporters
22	Quantum Dot Reporters
23	Improved Gold Nanoparticle Reporters
24	Improved Design Elements
25	Next Generation Analysis
26	Advanced PCR Techniques for Viral Detection
27	PCR and Smallpox
28	PCR and Influenza
29	PCR and Livestock Monitoring
30	PCR Combined with Other Techniques
31	PCR with Electrospray Ionization Mass Spectrometry
32	PCR and Magnetic Beads
33	Viral Detection using Next Generation or Whole Genome Sequencing
34	Electrochemical Detection of Viruses
35	Amperometric Sensors
36	Conductometric Detection
37	Potentiometric Sensors
38	Impedimetric Sensors
39	Spectroscopic Sensors
40	Colorimetric
41	Fluorescence-based Detection Strategies
42	Other Optical Sensors
43	Integrated Microdevices
44	Toxins
45	Overview of Some Common Toxin Agents
46	Botulinum Neurotoxin
47	Ricin
48	Abrin
49	
50	
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3	Staphylococcal Enterotoxin B
4	Mycotoxins
5	Epsilon Toxin
6	Tetrodotoxin
7	Other Toxins
8	
9	Physical Techniques
10	Direct Mass Spectrometry
11	Chromatographic Separation with Mass Spectrometry Analysis
12	Other Chromatographic Analysis
13	Surface Plasmon Resonance
14	Quartz Crystal Microbalances/Cantilever/Piezoelectric
15	
16	Biochemical Sensors
17	Antibody-Based Toxin Detection
18	Electrochemical Toxin Detection
19	Polymerase Chain Reaction
20	
21	Spectroscopic Sensors
22	Colorimetric Based Detection
23	Fluorescent Detection
24	Raman Spectroscopy
25	Flow Cytometry
26	Chemiluminescence
27	
28	Integrated Microdevices
29	Outlook and Perspective
30	Acknowledgements
31	Abbreviations
32	Figure Legends
33	References
34	
35	
36	
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Biological threat agents or, more colloquially, biotreats or bioagents are pathogens and/or their toxic products that pose a substantial threat to human health.¹⁻⁴ They are a diverse group that includes viruses, bacteria, and toxins from biological sources, and indeed that diversity is reflected in the extraordinary range of transmissibility, infectivity, and lethality that they exhibit.^{1,2} Bioagents encompass both naturally occurring and engineered pathogens and the threat they pose originates from natural outbreaks as well as from their intentional release. Examples run the gamut from a naturally-occurring, aerosol-borne emerging virus that can establish in a host exposed to just a few virions (*e.g.*, Ebola virus) to a bacterium with an infectious dose of 10,000 spores that rarely poses a threat to human health unless it has been processed for intentional release such as *Bacillus anthracis* (anthrax), for example.^{1,2,5-7} **Figure 1** presents some sample images of patients who have been infected with smallpox virus or succumbed to Ebola virus along with those diagnosed with plague (*Yersinia pestis*) and anthrax. The starkness of these and other images (see the Public Health Image Library provided by the Centers for Disease Control and Prevention - phil.cdc.gov/phil/bt.asp) effectively reminds us of how serious these threats continue to be.

While apocalyptic viruses may seem the stuff of science fiction, there is an ample and accumulated experience that has accrued over time that forms the basis for our modern concern.^{1,8-11} Historically, smallpox was so ubiquitous and lethal that it was responsible for ~10% of deaths annually.¹⁰ Even 150 years after introduction of the smallpox vaccine, in the 1950s there were still *ca.* 50 million cases annually resulting in tens of millions of fatalities, with 15 million cases remaining in the late 1960's until the virus's successful eradication a decade later in 1979 following a concerted effort by the World Health Organization (WHO).^{10,12} Less pervasive than smallpox, outbreaks of the plague or Black Death, so called due to the visible blackened coloration from gangrenous tissue, arose less frequently but with no less deadly effect. Indeed, one of the worst known pandemics, that of plague in Europe between 1346 and 1352, is estimated to have killed 25-40% of the continent's population.^{12,13} That pandemic is thought to have been seeded by an act of biological warfare in which a Mongol army laying siege to the Crimean city of Kaffa (now known as Feodosia) flung plague-infected corpses over the city walls.^{1,2} This event highlights the truly horrific nature of bioagents, invisible killers that target friend and foe alike and against which weapons of traditional warfare are useless. A more recent pandemic is that of the Spanish flu (influenza virus) which is estimated to have killed 20-50

million people in the early 20th century.^{14,15} Interestingly, this virulent influenza variant did not originate in Spain but is so called because Spain was, paradoxically, one of the only countries to openly report the real results of the pandemic with veracity. Thus, in comparison to other affected countries, Spain's infection rate and death toll seemed abnormally high suggesting it to be the initial outbreak site. The actual source of the virus is believed to have been a duck farm in Kansas where an avian flu mutated and mixed with a human flu at a nearby army camp. Due to growing U.S. involvement in World War I, the camp was engaged in continuous movements of large numbers of soldiers and recruits and this contributed to initially seeding the first of several consecutive outbreaks that soon began to spread.¹⁶ Reoccurrence of pandemic Spanish flu or the emergence of a new pandemic strain (*e.g.*, H7N9) remains amongst one of the greatest modern public health threats. Other historical examples of biological warfare include Napoleon's intentional flooding of the plains of Mantua in Italy in 1797 to enhance the localized spread of malaria and the selling of yellow fever and smallpox patient clothing to Union troops by Confederate agents during the American Civil War.¹⁷ Riedel and others provides an excellent overview of the use of biological warfare in recent history for the interested reader.^{9,17-19} Epidemics can also just as easily occur naturally as highlighted by the 2014 Ebola outbreak in Africa and the recent spread of Zika virus in the America's in 2016.

The advent of modern microbiological techniques meant that instead of relying on the natural propagation of disease, bioweaponers were now able to isolate, culture, characterize, and look to eventually re-engineer the bioagents themselves for more targeted, concerted, and lethal use especially as a weapon of terror, war, or mass destruction. See **Figure 2A,B** for representative images of *Clostridium* sp. Gram-positive bacteria and Arenavirus, respectively, both of which can be pathogenic to humans.^{1,5,9,11} After limited research during World War I, more robust bioweapons programs emerged during World War II. The Japanese, for example, operated several research facilities, including the infamous Unit 731, where the effects of agents such as anthrax were studied on prisoners. Field tests of bioagents included the dropping of plague-infected fleas from Japanese airplanes in regions of China and Manchuria, where plague epidemics subsequently broke out.^{1,2} The U.S. responded to the threat of bioweapons from hostile forces by beginning its own offensive bioagent research program at Camp Detrick (later Fort Detrick) in 1942. From then until President Nixon's 1969 executive order to halt bioweapons development, a variety of antipersonnel agents were researched and seven ultimately

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3 weaponized, including *Bacillus anthracis* (anthrax), *Clostridium botulinum* (botulism),
4 *Francisella tularensis* (tularemia), *Coxiella burnetii* (Q fever), Venezuelan equine encephalitis
5 (VEE), *Brucella suis* (brucellosis), and staphylococcal enterotoxin B (SEB).^{1,2}
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8 The tapering off of large, state-run bioweapons research programs continued with the
9 United Nations 1972 Biological Weapons Convention (BWC), signed by the US, United
10 Kingdom, USSR, and more than 150 other nations. This treaty prohibited development,
11 production, and stockpiling of bioweapons (except for prophylactic or protective research) along
12 with bioweapons delivery systems.²⁰ Although stockpiles of conventional chemical, and nuclear
13 weapons constituted a formidable arsenal next to the relatively untested bioagents that were
14 outlawed under the BWC, a substantial Soviet bioweapons program that predated the convention
15 remained in effect, and the consequences of an accidental release of anthrax in Sverdlovsk in
16 1979 underscored the unpredictability and high human cost associated with their
17 development.^{1,2,20-22} While the Soviet program was carried out in relative secrecy, perhaps the
18 most public example of bioagent development post-BWC took place in Iraq in the 1980's and
19 1990's, where Saddam Hussein's regime produced thousands of liters of botulinum neurotoxin
20 (BoNT), *B. anthracis* spores, and aflatoxin.^{1,2,23}
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31 In spite of the BWC and rapid developments in modern medicine, the threat that bioagents
32 pose is perhaps higher now than at any point in history as a result of a number of compounding
33 factors.^{1-3,7,21,23-27} Lowered barriers to international travel have resulted in the global spread of
34 naturally occurring diseases that would once have been geographically confined (*e.g.*, the severe
35 acute respiratory syndrome or SARS outbreak in 2002 and the Ebola outbreak in 2014).^{6,24,26}
36 **Figure 3** highlights some currently emerging and reemerging viruses that present serious public
37 health threats along with some of the generalized scientific approaches being pursued to help
38 contain them. The lowered cost of equipment for microbiology and genetic engineering coupled
39 with greater accessibility, widespread technical know-how, and ease of use has increased the
40 potential that a malfeasant group could culture an existing strain of bioagent or develop new
41 strains; critically, the resources of a state-run program are no longer required.²³ The latter
42 possibility is compounded by our increased understanding of pathogens, including the
43 publication of the genome sequences of smallpox and Spanish flu (and subsequent synthesis of
44 the Spanish flu strain from that sequence). Indeed, much controversy surrounded the publication
45 of work with an engineered strain of H5N1 avian influenza that had been optimized for
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3 pathogenicity and which could serve as a “recipe” for bioterrorists.²⁸⁻³³ These developments have
4 arisen in concert with a shift in conflict away from overt warfare between state actors focused on
5 military targets and towards localized conflicts by non-state actors such as rebels or terrorist
6 organizations which target civilians with the aim of inciting fear rather than attaining tactical
7 military victories. Thankfully, such groups have had relatively little success in deploying
8 bioagents to date. Yet when bioterrorism succeeds, the social disruption and cost from even
9 small-scale incidents can be enormous.^{1,9} For example, after nine separate unsuccessful releases
10 of *B. anthracis* spores and BoNT by the Japanese doomsday cult Aum Shinrikyo, the group
11 succeeded in carrying out two sarin nerve agent attacks on the Tokyo subway system. While the
12 total death toll from the two attacks numbered only twenty, more than 250 were injured, and
13 thousands sought medical attention.¹ In the U.S. in 2001, several letters containing *B. anthracis*
14 spores were mailed to media agencies and the Hart Senate Office building in Washington D.C.,
15 resulting in 22 cases of anthrax and 5 deaths. In the wake of this attack, 10,000 people were
16 offered prophylactic antibiotics and over 1 million environmental samples were tested. In
17 addition, national, state, and local agencies were forced to pursue numerous reports of
18 unidentified ‘white powders’ ultimately confirmed not to be anthrax, drawing resources away
19 from the primary investigation and decontamination processes.^{1,34}

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21 It is also important to remember that the targets of biothreat agents, whether naturally
22 occurring or engineered, do not have to be exclusively humans and this can still have a
23 substantial impact on the public welfare regardless of context. For example, evidence indicates
24 that German agents attempted to infect horses and livestock with Glanders (*Burkholderia mallei*)
25 and anthrax prior to them being shipped from the U.S. to Allies and similarly infected Romanian
26 sheep prior to their export to Russia.¹⁷ Similar attempts were carried out in France, Spain,
27 Argentina and Norway but all were without any military consequences. Additionally, many in
28 the West do not realize that rabies is still a serious public health concern in Asia with more than
29 30,000 human deaths occurring each year.^{35,36} The main source and continued reservoir of this
30 virus appear to be unvaccinated feral and domestic dogs. In another stark example, cattle stocks
31 in the United Kingdom and the European Union were decimated by bovine spongiform
32 encephalopathy (BSE) in the late 1990’s.³⁷ The causative agent here is believed to be a prion or
33 miss-folded protein that induces other proteins in the brain to do the same. From a more
34 agricultural perspective, the taro plant is the source for the fourteenth most consumed food in the

world and production of this crop has been significantly affected by taro leaf blight since the early 1990's, see **Figure 4**.³⁸⁻⁴⁰ Serious outbreaks affecting local food production have occurred in Nigeria, Ghana, and Samoa in the Pacific. Two Nenet reindeer herder deaths, hundreds of hospitalizations, and the deaths of thousands of reindeer due to anthrax were very recently reported in the Northern Russian Yamal-Nenets autonomous region.⁴¹ The source of this outbreak was traced to a 75 year old reindeer carcass that thawed due to unusually high temperatures in the region.²⁷ Interestingly, Russian chemical warfare troops were part of the response team sent to help contain the outbreak, vaccinate local inhabitants, and dispose of the dead reindeer by high-temperature burning to kill any remaining spores. This example also effectively highlights how climate change has now become a factor in the overall biothreat equation.

All told, this increase in the threat posed by bioagents, either naturally occurring or intentionally deployed, underscores the need to develop sensors capable of detecting them with high sensitivity and specificity, and to do so rapidly, in different matrices, and across multiple dispersion modes. Here, we present an overview of a variety of what are considered to be emergent or threatening bioagents, provide a historical and clinical context for them, and examine the current methods for their detection and identification. We also focus on the current state of biosensor development and explore recent advances and future prospects for the field of biosensors in this endeavor. Our aim is to provide a representative overview of the state of the field and imagine where it may be headed as developing assay techniques mature and are integrated into the suite of established methodologies. We begin by offering a listing and some background on the properties of bioagents that are pertinent to this discussion along with important considerations for their detection. The subsequent section provides a high-level overview of detection methodologies and a discussion of considerations for public health emergencies and U.S. Food and Drug Administration (FDA) Emergency Use Authorization (EUA) for *in vitro* diagnostic (IVD) devices. Later sections examine detection paradigms for bacteria, viruses, and toxins across a broad range of methodologies. We recognize the many other excellent publications that precede this one and, in fact, draw heavily from them in many cases.^{4,23,27,34,42-46 47,48} Some demonstrative examples are also included where pertinent that utilize a non-threat agent (*e.g.*, HIV) since the technology or experimental format is still relevant to the current context. We also point out to the reader that this article is written primarily from a

U.S. centric perspective. Given the breadth of this field, we realize that there will be omissions in the description of particular methodologies or pertinent citations and our apologies are extended for any unintended oversight.

Important Considerations

For our purposes, bioagents are defined as being biological in nature or from a biological source as opposed to completely synthetic or artificial chemical agents such as nerve agents; sensors for the latter have been reviewed elsewhere.⁴⁹⁻⁵² Bioagents typically fall into three categories: bacteria, viruses, and toxins, the latter of which originates from the first two or can include other biologicals such as prion proteins. The defining attributes of each of these will be covered in detail in devoted sections that include an overview of the most important bioagents in each of those categories and the historical context that places them in that position, their pathogenesis, and the methods employed for their detection. Here we provide a brief primer on some of the important definitions and considerations required for the identification and detection of bioagents.

Definitions. The bioagents that have been studied over the course of the past century were selected for a variety of attributes that made them especially suited to weaponization. Primary among these are *infectivity*, *pathogenicity*, *virulence*, *transmissibility*, and *stability*, although additional characteristics such as *toxicity*, *incubation period*, and *lethality* may have also played a critical role. In combination, these attributes determine which of the many different niches a given biothreat agent will fill in an arsenal, from incapacitation to mass fatalities, from localized attack to an autonomous epidemic. *Infectivity*, or the ease with which a microorganism establishes itself in the host, is not necessary related to *pathogenicity*, which is the pathogen's ability to cause disease in the host, nor to *virulence*, which refers to the severity of that disease. *Lethality*, referring to the ease with which the pathogen causes death, is different still. Consider the incapacitating agent *Brucella*, causing brucellosis, which requires only 10-100 bacteria to establish itself in a host making it highly infective, but is lethal in fewer than 5% of cases, even if untreated.^{2,3} *Toxicity* reflects the severity of the illness precipitated by a toxin. For example, in cases of botulism, it is the toxicity of the BoNT released by the bacteria rather than the infection itself that is lethal. *Incubation period* is the time that elapses between exposure to a bioagent and

the appearance of symptoms. *Transmissibility* of the pathogen directly from one person to another either through casual contact, *e.g.*, Ebola, or intimate contact, *e.g.*, HIV, or indirectly through vectors such as mosquitos or fleas, *e.g.*, malaria or plague, is required to seed an epidemic, while *stability* refers to a pathogen’s ability to survive environmental factors and plays a critical role in determining the nature and effectiveness of the dispersal method. *Morbidity* refers to a person or the number of people in a population who have a given disease while *mortality* generally refers to death on a large scale. *Zoonotic* describes diseases that are present in animals that can, under certain circumstances, be transmitted to humans. *Etiology* refers to the cause(s) or the manner in which a disease, illness, or condition arises. A detailed description of each of these characteristics can be found in the *NATO Handbook on the Medical Aspects of NBC Defensive Operations*.⁵

Taken together, the characteristics of a pathogen determine not only how it will function as a bioagent but also how the disease it causes sustains itself within a population. An *endemic* disease is one that exists without requiring reinfection of the population. For example, malaria which is caused by infection from any one of five *Plasmodium* parasitic protozoans, requires an arthropod vector (typically an infected female *Anopheles* mosquito), and is endemic only in regions where the carrier mosquito is present and rates of infection in those places are steady. An *epidemic* occurs when cases of a disease are unusually large in community or region, or when disease rates climb above the baseline level that is known for an area.¹ The annual arrival of “flu season,” for example, marks the start of a seasonal epidemic.⁵³ The term *pandemic* is applied when the disease becomes more widespread, appearing over a wide area, affecting large numbers of people, and, in some cases, spreading worldwide.⁵³ Smallpox provides an illustrative example since before its eradication, it had occurred at all three levels. In large populations, smallpox was an endemic disease, accounting for ~10% of annual deaths. Much like chickenpox, most of the ill were children (hence the “small” in “smallpox”) because older individuals had acquired immunity from previous exposure to the disease. Populations too small to support endemic smallpox lacked immunity and would experience occasional epidemics when an infected individual introduced the disease into the community. An epidemic in Boston in 1752 resulted in the infection of all but 174 susceptible individuals (those who had not been inoculated or previously had smallpox).¹⁰ Introduction of smallpox in the Americas in the 16th Century to an extensive native and naïve population with no acquired immunity led to a pandemic across an

enormous geographical area that, by some estimates, eventually reduced indigenous populations by 50-90%.^{10,54}

Emerging diseases are those that have shown increased incidence in the past twenty years or exhibit signs of a likely increase in the near future.^{24-27,55} These include both newly developed or newly identified diseases (*e.g.*, SARS) as well as re-emerging diseases that are increasing in prevalence for reasons that may include environmental changes, increased population density, and pathogen mutation (*e.g.*, new strains of influenza, antibiotic-resistant tuberculosis). While emerging diseases appear naturally, in the context of bioagents the possibility of genetically engineered or modified strains must also be noted. Last, and certainly not least, a *patient* is usually defined as someone who has signs and symptoms of exposure or who has a high risk of epidemiological exposure.

Agents, Their Classes, and Categories. From a predominantly U.S. perspective, there are three primary listings of the pathogens, diseases, and toxins that could threaten the health and safety of the public, animals, and/or plants. These are maintained by select U.S. government entities, specifically the Department of Health and Human Services (HHS) Centers for Disease Control and Prevention (CDC), Animal and Plant Health Inspection Service (APHIS), and the National Institute of Allergy and Infectious Diseases (NIAID). While the contents of the lists are somewhat similar, they serve different purposes ranging the gamut from safe and secure handling through funding priorities, as outlined in **Table 1**. One list is maintained by the Federal Select Agent Program (FSAP), which is a partnership between the CDC and the U.S. Department of Agriculture's (USDA's) APHIS. FSAP regulates the possession, use, and transfer of biological select agents and toxins so that important work with potentially dangerous and deadly pathogens is conducted as safely and securely as possible. The FSAP list is split into 4 groups; (1) HHS Select Agents and Toxins; (2) Overlap Select Agents and Toxins; (3) USDA Select Agents and Toxins; and (4) USDA Plant Protection and Quarantine (PPQ) Select Agents and Toxins. Groups (1) and (2) pose the most severe threat to human health. The list is, of course periodically reviewed and updated. Changes to these regulated lists typically requires publishing a notice of proposed and final rulemaking in the *Federal Register* (<https://www.federalregister.gov/>), and providing an opportunity for the public to comment on the notice of proposed rulemaking. A 2016 Federal Register Notice proposed the removal of

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Coxiella burnetii, *Rickettsia prowazekii*, *Bacillus anthracis* Pasteur strain, *Brucella abortus*, *Brucella melitensis*, and *Brucella suis* from the HHS list of select agents. However, after review of the public comments received as part of the Advanced Notice of Proposed Rulemaking, HHS subsequently decided not to finalize the proposed changes at that time.^{56,57} Effective October 2016, *Bacillus cereus* biovar *anthracis* was added to the HHS Select Agents List as a Tier 1 agent (see **Table 2**).^{57,58}

In addition, CDC also maintains The National Notifiable Diseases Surveillance System (NNDSS) which includes a list of Nationally Notifiable Conditions [<https://wwwn.cdc.gov/nndss/>] and FDA maintains a list of qualifying pathogens in the Code of Federal Regulations or CFR (found under 21 CFR 317.2) that have the potential to pose a serious threat to public health [https://www.ecfr.gov/cgi-bin/text-idx?SID=4b9cce11e73ec49a1b75d5ed97f9ae8b&mc=true&node=se21.5.317_12&rgn=div8]. While these lists serve a different purpose from the biothreat lists outlined in this review, there are a number of overlapping pathogens and diseases.

Table 1. Overview of U.S. Government Pathogen/Disease Lists and Their Purpose

List	U.S. Government Agency	Purpose
Biological Select Agents and Toxins (Select Agents)	CDC* and APHIS**	FSAP oversees the possession, use, and transfer of biological select agents and toxins. Subject to federal regulations [§] developed to satisfy the requirements of the Uniting and Strengthening America by Providing Appropriate Tools Required to Intercept and Obstruct Terrorism (USA PATRIOT) Act (2001) and Public Health Security and Bioterrorism Preparedness and Response Act (2002). FSAP maintains a select agent database. Link: https://www.selectagents.gov/SelectAgentsandToxinsList.html
Bioterrorism Agents/Diseases	CDC*	Maintained as part of CDC's emergency preparedness and response list of pathogens and diseases thought to present a great threat to public health and national security. Not associated with a Congressional mandate and/or Federal regulations. Link: https://emergency.cdc.gov/agent/agentlist-category.asp
Category A, B, and C Priority Pathogens	NIAID*	Created for extramural and intramural program management within the NIAID Biodefense/Emerging Infectious Diseases (EID) mission, to prioritize research and funding on specific pathogens and diseases. Link: www.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/Pages/CatA.aspx

*Under the Department of Health and Human Services (HHS) **Under the Department of Agriculture (USDA)

[§]See Code of Federal Regulations (CFR): 7CFR Part 331, 9CFR Part 121, 42CFR Part 73 [The Code of Federal Regulations can be searched electronically at: <http://www.ecfr.gov/cgi-bin/ECFR?page=browse>]

The pathogens and diseases outlined in these lists are typically grouped based on their risk to public health, as described in **Table 2**. Highest risk pathogens and diseases are typically those that can cause high mortality rates, are easily spread or transmitted, and have the ability to cause mass casualty/public health events and/or public panic and social disruption.

Table 2. Risk Categorization of the Pathogens and Diseases

List	Select Agent List	CDC Bioterrorism and NIAID Priority Pathogen Lists		
Class/ Category	Tier 1* (T1)	Category A (A)	Category B (B)	Category C (C)
Summary	Pose severe threat to public health and safety	Pose the highest risk to public health and national security	Second highest priority pathogens	Third highest priority and includes certain emerging pathogens that could be engineered for mass dissemination in the future
Description	○ Significant potential for mass casualties or devastating effect to the economy, critical infrastructure, or public confidence ○ Communicability ○ Low infectious dose ○ History of or current interest in weaponization based on threat reporting	○ Easily spread or transmitted ○ High death rates ○ Major public health impact ○ Can cause public panic and social disruption ○ Require special action for public health preparedness	○ Moderately easy to disseminate ○ Moderate morbidity rates and low mortality rates ○ Require specific enhancements of CDC’s laboratory capacity and enhanced disease monitoring	○ Easily available ○ Easily produced and disseminated ○ Potential for high morbidity and mortality rates and major health impact

*Select agent regulations were revised in 2012 to designate Tier 1 agents with a documented risk of causing the highest consequence event relative to the other select agents.⁵⁹ Website link last accessed 7/6/2018 <https://www.gpo.gov/fdsys/pkg/FR-2012-10-05/pdf/2012-24389.pdf>

The tables below list human pathogens and diseases grouped by bacteria (**Table 3**), viruses (**Table 4**), toxins (**Table 5**), and others (**Table 6, 7**) as drawn from the three main lists outlined in **Table 1**.

Table 3. Select Bacterial Biothreat Agents

Pathogen (Disease)	HHS and Overlapping Select Agents	CDC Bioterrorism/ Agents List	NIAID Priority Pathogens List
<i>Bacillus anthracis</i> (Anthrax)	Yes (T1) ^a Pasteur strain not T1	Yes (A) ^a	Yes (A)
<i>Bacillus cereus</i> Biovar <i>anthracis</i> ^b	Yes (T1)	No	No
<i>Brucella</i> spp. (Brucellosis)	Yes <i>B. abortus</i> , <i>B. melitensis</i> , and <i>B. suis</i>	Yes (B) ^a	Yes (B)
<i>Burkholderia</i> spp. ○ <i>B. mallei</i> (Glanders) ○ <i>B. pseudomallei</i> (Meliodiosis)	Yes (T1)	Yes (B)	Yes (B)
<i>Chlamydia psittaci</i> (Psittacosis)	No	Yes (B)	Yes (B)
<i>Clostridium</i> spp. – Botulinum neurotoxin producing (Botulism)	Yes (T1)	Yes (A)	Yes (A)
<i>Coxiella burnetii</i> (Q-fever)	Yes	Yes (B)	Yes (B)
<i>Francisella tularensis</i> (Tularemia)	Yes (T1)	Yes (A)	Yes (A)
<i>Mycobacterium tuberculosis</i> (Tuberculosis-TB)	No	No	Yes (C) ^a including drug resistant TB
<i>Rickettsia</i> spp. ○ <i>R. prowazekii</i> (Typhus Fever)	Yes	Yes (B)	Yes (B) and other <i>Rickettsias</i> (C)
<i>Yersinia pestis</i> (Plague)	Yes (T1)	Yes (A)	Yes (A)

^aRisk categorization of the organism, for definitions of T1, A, B, and C, see Table 2. ^bAdded as part of an interim final rule effective October 14th, 2016.⁵⁸

Table 4. Select Viral Agents

Pathogen (Disease)	HHS and Overlapping Select Agents	CDC Bioterrorism/ Agents List	NIAID Priority Pathogens List
Chikungunya Virus	No	No	Yes (C) ^a
Coronavirus (CoV)	Yes: SARS-CoV	No	Yes (C): SARS-CoV, MERS-CoV and other pathogenic CoV
Hendra Virus	Yes	No	Yes (C)
Influenza Viruses	Yes: Reconstructed 1918	No	Yes: Reconstructed 1918 (C)
Mosquito-Borne Encephalitis Viruses (EV)	Yes: Easter equine (EEE) and Venezuelan (VEE)	Yes (B): alphaviruses, EEE, VEE and Western equine (WEE)	Yes (B) ^{a,b}
Nipah Virus	Yes	Yes (C)	Yes (C)
Orthopoxviruses	Yes: Monkeypox, Variola Major (Smallpox) (T1) ^a and Variola minor (Alastrim) (T1)	Yes: Variola Major (A)	Yes: Variola major (A)
Rabies Virus	No	No	Yes (C)
Tick-Borne Encephalitis Complex Flaviviruses	Yes Subtypes: Far Eastern, Siberian	Yes (B): under viral encephalitis	Yes (C) Subtypes: Tickborne EV, European, Far Eastern, Siberian, Powassan/Deer Tick virus
Viral Hemorrhagic Fever (VHF) ○ Arenaviruses VHF ○ Bunyaviruses VHF ○ Filoviruses VHF ○ Flavivirus VHF	Yes: South American Haemorrhagic Fever viruses Arenaviruses: Junin, Machupo, Guanarito, Chapare, Lassa, Lujo, Sabia Bunyaviruses: Rift Valley Fever, Crimean Congo Filoviruses (T1): Ebola, Marburg Flavivirus: Omsk HF, Kyasanur Forest	Yes: Arenaviruses (A): Machupo, Lassa Bunyaviruses: Hantaviruses ^c (C) Filoviruses (A): Ebola, Marburg	Yes: Arenaviruses (A): Junin, Machupo, Guanarito, Chapare, Lassa, Lujo Bunyaviruses: Hanta ^c (A), other Hanta (C), Rift Valley Fever (A), Crimean Congo (A), SFTSV ^d (C), Heartland (C) Filoviruses (A): Ebola, Marburg Flavivirus: Dengue (A), Omsk HF (C), Alkhurma (C), Kyasanur Forest (C)
Yellow Fever Virus	No	No	Yes (C)

^aRisk categorization of the organism, for definitions of T1, A, B and C, see Table 2. ^bSpecific Mosquito-borne EV: EEE, California, Japanese (JE), LaCrosse (LACV), St. Louis (SLEV), VEE, West Nile (WNV) and WEE. ^cSpecifically Hantaviruses causing Hanta Pulmonary Syndrome and ^dSevere Fever with Thrombocytopenia Syndrome virus.

Table 5. Selected Toxin Agents of Biological Origin

Toxin (pathogen)	HHS and Overlapping Select Agents	CDC Bioterrorism/ Agents List	NIAID Priority Pathogens List
Abrin	Yes	No ^a	No
Botulinum neurotoxins	Yes (T1) ^b	Yes (A)	Yes (A)
Conotoxins	Yes	No	No
Epsilon toxin – <i>Clostridium perfringens</i>	No	Yes (B) ^b	Yes (B)
Ricin	Yes	Yes (B)	Yes (B)
Saxitoxin	Yes	No*	No
Staphylococcal enterotoxins	Yes	Yes (B) Type B - SEB	Yes (B) Subtype B - SEB
Tetrodotoxin	Yes	No	No
2 Type A Trichothecenes			
○ Diacetoxyscripenol	Yes	No	No
○ T-2 toxin	Yes	No	No

^aIncluded in CDC's Chemical Agent list (<https://emergency.cdc.gov/chemical/index.asp>)

^bRisk categorization of the toxin of biological origin, for definitions of T1, A, B and C, see Table 2.

Note: Shiga toxin and Shiga-like ribosome inactivating proteins have been removed from these lists.

Table 6. CDC and NIAID Pathogens/Diseases Not on the Select Agents List That Are Still Characterized as a Group of Pathogens/Diseases

Group	CDC Bioterrorism/ Agents List	NIAID Priority Pathogens List
Food- and Water- borne Pathogens /Safety Threats	Yes (B), examples include: Bacteria: Diarrheagenic <i>E. coli</i> , pathogenic <i>Vibrios</i> , <i>Shigella</i> spp., <i>Salmonella</i> , <i>Listeria monocytogenes</i> , <i>Campylobacter jejuni</i> , <i>Yersinia enterocolitica</i> Viruses: Caliciviruses, Hepatitis A Protozoa: <i>Cryptosporidium parvum</i> , <i>Cyclospora cayetanensis</i> , <i>Giardia lamblia</i> , <i>Entamoeba histolytica</i> , <i>Toxoplasma gondii</i> , <i>Naegleria fowleri</i> , <i>Balamuthia mandrillaris</i> Fungi: Microsporidia	
Antimicrobial Resistance	No	Yes (C) – however, excludes sexually transmitted organisms unless the resistance is newly emerging
Emerging Infectious Diseases	Yes (C) e.g., Nipah virus and Hantavirus	Yes (C) – see previous pathogen specific tables*
Prions	No	Yes (C)
<i>Coccidioides</i> spp. (fungi)	No	Yes (C)

*NIAID lists specific many emerging pathogens under Category C but in addition keeps an additional list of Emerging Infectious Diseases/Pathogens that are not presently considered part of their NIAID Priority Pathogens List (Table 7).

Table 7. NIAID Additional Emerging Infectious Diseases/Pathogens

Organism	Pathogen (Disease)
Bacteria	<i>Anaplasma</i> spp. (Anaplasmosis), <i>Bartonella henselae</i> (bartonellosis), <i>Bordetella pertussis</i> (Whooping cough), <i>Borrelia miyamotoi</i> , <i>Clostridium difficile</i> , <i>Cryptococcus gattii</i> (cryptococcosis), <i>Ehrlichia</i> spp. (Ehrlichiosis), <i>Enterococcus faecium</i> and <i>faecalis</i> , <i>Leptospira</i> spp. (Leptospirosis), <i>Borrelia miyamotoi</i> , <i>Lyme borreliosis</i> (Lymes disease), <i>Streptococcus</i> Group A, <i>Staphylococcus aureus</i> .
Viruses	Australian bat lyssavirus, BK virus, Enterovirus 68 and 71, Hepatitis C and E, Human herpesvirus 6 and 8, JC virus, Measles virus (Rubeola – measles), Mumps virus, Poliovirus, Zika virus
Protozoa	<i>Acanthamoeba</i> (Acanthamebiasis), <i>Babesia</i> –atypical (babesiosis),
Fungi	<i>Aspergillus</i> spp., <i>Mucorales</i> spp. (Mucormycosis)

Research in the life sciences that include investigation of pathogens and diseases that could potentially threaten the health and safety of the public, animals and/or plants introduces the biosecurity issue of dual use research of concern. As part of the U.S. Government's oversight of federally conducted or supported dual use biological research, the National Science Advisory Board for Biosecurity (NSABB) was founded as a federal advisory committee and is composed of subject matter experts who are not full-time employees of the Federal Government.⁶⁰ The objective of the NSABB is to provide advice and guidance concerning the legitimate scientific benefits of research in this area while considering the risk of misuse if the insights gained during research pose a biologic threat to public health and/or national security.

Knowing When to Deploy the Sensors. Successful detection of a bioagent requires, above all else, deployment of a sensor. This may be in the form of continuous environmental monitoring, as sensors that detect bioagents prior to human exposure (or development of symptoms) provide obvious benefits, including the ability to initiate a preemptive prophylactic response in potentially exposed individuals and to reduce the number of individuals exposed. The range of pathogens and toxins to be detected, including many with extremely small infectious or toxic doses, however, poses a serious challenge that is further exacerbated by the complexity of the ambient environment, including background particulate matter and nonpathogenic biological background.^{23,46,61} Currently available automated monitoring systems are both expensive and limited in their detection capabilities (sensitivity and number of bioagents). For a more in-depth description of these systems and their components, the reader is encouraged to refer to *An Introduction to Biological Agent Detection Equipment for Emergency First Responders*.⁶¹

The human body, with all the symptoms of disease that it presents, is an excellent indicator of the presence of a bioagent, and the identification of a diseased patient may well be the impetus to collect the clinical samples necessary to identify a growing public health threat. An example of how this situation plays out is common in food-borne illness. For example, an unusual number of cases of *E. coli* infection were reported in the states of Washington and Oregon in October 2015. The high incidence of the illness led to an investigation that revealed Shiga toxin-producing *E. coli* O26 to be responsible and the source was determined to be the restaurant chain Chipotle Mexican Grill, and a number of the locations were closed while the investigation continued.⁶² Whereas the ideal scenario involves identifying and mitigating the

threat of bioagents before human exposure, the difficulty in doing so means that a reactive approach is often the more realistic option. However, even this late-stage recognition of a threat relies on the diligence of medical care professionals to notice the need for specialized tests.¹ The most sensitive and selective sensor conceivable will do little good if it fails to be deployed.

An additional consideration – and one crucial in the development of an appropriate response – lies in ascertaining whether an outbreak is the result of intentional dissemination or natural causes.^{2,55} The fact that many of the diseases caused by bioagents respond to treatment only before the patient becomes symptomatic underscores the importance of rapid identification of the nature of the threat in order to begin prophylactic care for those in need of it. Moreover, intentional attacks may result in larger numbers of simultaneous casualties, necessitating measures to prevent overwhelming the medical care infrastructure. In addition to casualties, “worried well” may inundate hospitals, as occurred subsequent to the Aum Shinrikyo sarin attack on the Tokyo subway system in 1995, when thousands of “worried well” sought emergency care hindering the ability of medical professionals to triage, diagnose, and treat individuals who had actually been exposed.⁶³ An additional concern is if the hospital(s) have mechanisms in place to deal with a patient that tests positive for one of these biothreat agents, especially if they are highly infective. Indications of an intentional attack such as the appearance of a rare disease may fail to raise alarm because of the possibility of natural occurrence. Subtle cues such as unusually large outbreaks of a naturally occurring disease (*e.g.*, a flu epidemic in July when these typically occur in November through March in North America) or higher than usual morbidity or mortality again rely on the ability of medical care providers to recognize and report unusual patterns. Identifying point source outbreaks, another possible cue of a biological attack, requires sufficient suspicion to initiate an investigation.¹ The services provided by state health tracking agencies such as the CDC to collect, consolidate, analyze, and track individual health department reporting have proven to be invaluable in the search for the first clues and cues of an outbreak along with its potential source.

Environmental versus Clinical Matrices. The difficulty in identifying bioagents prior to human infection is highlighted not only by the example of the 2015 *E. coli* outbreak in Chipotle restaurants but, more generally, by the number and regularity of food-borne illness-related recalls that occur every year. While specific sensor methodologies are discussed below, detection of

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3 bioagents in real-world scenarios is substantially more complex than in the clean conditions and
4 with the pure samples that we associate with a laboratory. Analytical samples are likely to arrive
5 in environmental or clinical matrices that may contain biological, organic, and/or inorganic
6 particulate matter present at much higher concentrations than the analyte of interest.⁴⁵
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8 Environmental samples, arriving in matrices such as soil or water, are the variety that might be
9 used to recognize intentional dissemination of an aerosolized bioweapon, inadvertent
10 contamination of the food supply, or a natural outbreak such as that of tularemia on the National
11 Mall in Washington D.C., for example.⁴ Preparing samples for use with a typical sensor requires
12 cleanup/processing steps such as centrifugation, filtration, dielectrophoresis, immunogenic
13 separation, nucleic acid extraction, and concentration. These tasks become especially arduous
14 when multiplied by the sheer number of bites of food and breaths of air we humans take that are
15 potentially interesting samples for testing.^{45,61} Thus any detection process that can eliminate a
16 sample preparation step will save time many times over. The use of magnetic nanoparticles
17 (MNPs), for example, as a means of scavenging a bioagent from an analytical sample and
18 concentrating it with a magnetic field can vastly simplify the subsequent detection process.⁶⁴
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29 Clinical samples may be collected more judiciously (*i.e.*, only from patients or
30 individuals with a high risk of exposure), but the matrices in which they arrive – blood, urine,
31 stool, gastric contents, and sputum – are no less complex. Moreover, clinical samples may suffer
32 from the medical treatments offered to the patient. A dose of antibiotics will prevent the
33 detection of many bacterial pathogens in blood, even while the infection continues to develop
34 elsewhere.¹ Indeed, appearance of the pathogen in the bloodstream is a late development in
35 many infections and therefore of minimal utility in screening for exposure.¹ Working with both
36 environmental and clinical samples requires the utmost caution, as testing for the presence of
37 bioagents inherently involves the risk of handling dangerous pathogens. Otherwise routine
38 laboratory procedures are therefore more arduous and must to be carried out in properly
39 equipped facilities.⁶⁵ Although some environmental sensors are discussed here where
40 appropriate, the primary focus is on sensors that target more clinically-oriented and similarly
41 related samples.
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Overview of Detection Methodologies

This section provides a brief working description of concepts that are directly related to how sensing events occur. Amongst the many principles associated with all forms of sensors in general, those utilized frequently here and/or directly relevant to the current discussion include direct/label free detection, biorecognition, biochemical detection, biosensor, and signal transduction. Beyond a cursory explanation of how a particular sensing approach, technique, process, detector or device works, and only when specifically warranted, we do not typically describe these as this is not the goal of this discussion. For example, we describe sensors based on applying surface plasmon resonance (SPR), polymerase chain reaction (PCR), reverse-transcriptase PCR (RT-PCR) or some form of DNA-based molecular beacon (MB), but we do not delve into their intrinsic mechanistic processes. The interested reader is referred to relevant textbooks on the subject.^{66,67}

Consider what exactly is required to detect a biothreat agent from the most basic level. The biothreat agent must first be present, then either it or something originating from it must interact with a sensing or recognition element or its presence must be detected using some other form of interrogation. This can include an antibody binding directly to it, light or other energy interacting with it spectroscopically, or even an electronic instrument determining its mass or mobility in a given phase. The signals or changes in signals from these interactions must then be transduced and typically amplified into an output that can be recorded or viewed. Ideally, the interaction with the sensing or recognition element would occur with as much specificity, selectivity, and sensitivity as possible and it is here that biorecognition process very often comes into play.

The simplest definition of biorecognition is the application of a biological molecule to recognize some other (bio)chemical entity spanning the gamut from a small ion to a cellular membrane. This usually means that the biological molecule will also bind to that chemical entity which will most likely be another biological molecule in this context. Biorecognition elements can include, but are certainly not limited to, antibodies and all their derivatives, proteins, peptides, nucleic acid aptamers and all their derivatives, carbohydrates, and so on.^{47,67-69} The primary source of biosensor targeting diversity originates from the library of molecular recognition elements which provide the physical recognition or binding interface between the bioagent(s) and the sensor device itself.⁷⁰ The vast majority of biosensors utilize either protein-

or nucleic acid-based molecules as their recognition elements and each of these come with their own set of benefits and liabilities for this role. Based on just the number of possible combinations (~20 natural amino acids *versus* 4 DNA/RNA bases), proteins intrinsically provide a much larger pool of structural and chemical diversity than that of nucleic acids. This diversity is far more complex and nuanced than just the simple assumption of a multiplicative property as many residues do not make major contributions to a recognition motif while others have some redundancy in terms of the chemical functionality that they contribute. Using proteins to recognize and bind surface proteins displayed either on bacterial membranes or viral capsids may reduce the number of required purification/capture steps, while nucleic acids often requires additional extraction to properly access the genetic material. However, the Watson-Crick base pairing of nucleic acids enables accurate prediction of nucleic acid hybridization, stability, and secondary structure. Nucleic acids can also be more stable, relying predominantly on primary structure rather than the tertiary structure of protein folding that can be disrupted and degraded without refrigerated storage. Antibodies and their many related functional analogs also do not guarantee high affinity binding towards every desired epitope every time and thus considerable efforts are still being invested in selecting and improving them.⁷¹

Single domain antibodies (sdAb or nanobodies, the smallest size immunorecognition element), derived from sharks as well as llamas and other camelids, offer an alternative approach to traditional antibodies.⁷²⁻⁷⁴ These antibodies consist only of a heavy chain and either two (shark) or three (camelid) small, antigen-binding domains. The removal of the light chain and the cysteine bridges greatly simplifies the antibody structure both physically and chemically. This, in turn, improves the thermostability of these proteins, having been shown to bind their respective antigens at over 90°C while also being capable of refolding after heat denaturation. They have also shown promise in bacterial detection,⁷⁵ although there has only been limited use against biothreat agents.⁷⁶ Nevertheless, one of the most notable examples is the use of llamabodies for the detection of *B. anthracis* spores.^{77,78} In addition to its innate sensitivity in this role, these proteins were also shown to be flexible in bioassay development, demonstrated *via* creation of a maltose-binding protein fusion and coupling to gold nanoparticles (AuNPs) for increased detection sensitivity by SPR.⁷⁹ A hybrid methodology falling between biorecognition and chemical detection would be, for example, the use of a molecularly imprinted polymer to bind to a threat agent.⁸⁰ Biorecognition is differentiated from chemical recognition which may occur

between a purely chemical “entity” binding to a biological one such as, for example, a polymer or polyelectrolyte binding to DNA.

The International Union of Pure and Applied Chemistry (IUPAC) defines a biosensor as “a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals” and a reagentless biosensor as one that accomplishes the same without any external input and thus functionally incorporates both the recognition element and the transduction element that signals a binding event.⁸¹⁻⁸⁴ **Figure 5** brings to together the description of what is required to detect a biothreat agent with biorecognition in the context of a biosensor and effectively highlights the flow of events and some possible components in this process. Here, we see that the recognition elements are interfaced with the other major element of the biosensor, the transducer whose primary role is that of reporting the event. Signal transduction, for all intents and purposes, can be defined as how a detector or biosensor tells us that it is working and there is a change in its state. As shown in **Figure 5**, the transducer senses a change in a physical, chemical or optical properties, and then signals that to the user. We, more commonly, recognize this portion of the process as monitoring changes in absorbance, fluorescence, color, conductance, and the like, and this is carried out by a detector. The latter is usually coupled to a computer and/or user interface in most analytical instrumentation.

Here, we define direct or label free detection to be synonymous with physical detection that is dependent upon sensing and transducing some physical property of the biothreat agent or the agent itself directly and not the presence of a label attached to a chemical or biorecognition element. For example, determining the presence of a toxin by confirming its molecular weight using mass spectral analysis.⁸⁵ Biorecognition elements can be a part of direct sensing and come into this equation when a biological molecule is used to bind to the agent or otherwise detect it. For example, attaching antibodies to an SPR probe to capture a particular agent that is then sensed by the change in plasmon resonance.⁴⁷ Utilizing a biochemical process as a key part of sensing a biothreat agent is exemplified by the application of PCR to amplify up select portions of a given pathogens’ genome for subsequent analysis and/or sequence confirmation.⁶⁷ Biorecognition elements can be coupled to biochemical processes in the form of an enzyme-linked immunoassay (ELISA), where an antibody captures some analyte and then an enzyme attached to the primary or secondary antibody catalyzes a reaction that creates a distinct,

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3 amplified signal from a substrate present in high concentration.⁶⁷ Spectroscopic detection is
4 epitomized by detecting the presence of a toxin by its unique Raman signature.⁸⁶
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7 Electrochemical sensors function through the measurement or monitoring of events that
8 occur at or near an electrode surface.⁸⁷ Most often electrochemical biosensors are monitoring the
9 interactions between a receptor and its ligand which leads to an accumulation at the electrode
10 surface and, in turn, to a change in some property of the device such as current or resistivity.⁸⁸
11 Electrodes now vary quite widely and include carbon nanotubes, graphene oxide, AuNPs,
12 AgNPs, magnetic beads, Au nanorods (AuNRs), and Zn nanowires amongst many other
13 materials.⁸⁹⁻⁹¹ Amperometric sensors monitor the production of current associated with a
14 reaction while conductometric sensors measure changes in the conductive properties of the
15 medium between two electrodes. Charge accumulation at an electrode surface is measured with
16 potentiometric devices. Potentiometric sensors and, in particular, ion selective electrodes (ISE)
17 are typically used for chemical analysis to determine the analytical concentration of a substance
18 in solution. These devices measure the potential of an electrode in the absence of voltage,
19 essentially monitoring the electromotive force. Typically the device is designed in such a way as
20 to monitor the electromotive force of a single sample or ion species. Devices of this type have
21 had significant success in the detection of ions such as ammonia in environmental samples and
22 biological ions such as sodium and calcium in clinical samples.⁹² As the technology has
23 advanced, potentiometric sensors have gradually been adapted for the detection of antigen-
24 antibody interactions making them viable platforms for biosensor development. Finally,
25 impedimetric systems measure changes in resistance. Similar to other electrochemical sensors,
26 impedimetric sensors allow for direct detection of biorecognition events such as receptor ligand
27 binding without additional enzymes or reporters to generate a signal. Impedimetric sensors are
28 based on electrochemical impedance spectroscopy (EIS) which is capable of monitoring enzyme-
29 substrate interaction, antibody-antigen binding, and other specific binding events that occur
30 between biomolecules.⁸⁸ Impedimetric sensors work through monitoring both the resistive and
31 capacitive properties of a material allowing for their use with a wide range of materials which are
32 selected based on specific experimental or application requirements. This versatility has allowed
33 impedimetric biosensors to be developed for numerous applications which have been the subject
34 of several excellent reviews.^{88,93} For each type of electrochemical assay, the electrode chosen,
35 the material from which it is derived, and the surface modifications required for assembling the
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sensing element all contribute to its success as a tool for detection. Though all the parameters incorporated into the assay design are critical they will not be the focus of the subsequent sections. Rather, successful demonstrations of electrochemical sensors will be highlighted with a focus on limit of detection (LOD), specificity, and other properties of the device that make them suitable candidates for deployable assays. Electrochemical sensors have great potential to satisfy many of the rapid, low-cost, and portability requirements necessary for a capable pathogen detection device, however, the conversion of a biological signal to an electrochemical one is still challenging for researchers. Additionally, the variability within biological samples such as ionic strength and pH contributes to the limitations of this assay platform. Advances in new materials that allow for measurements at the nanoscale along with integrated sample cleanup and processing in so called point-of-care (POC) devices have allowed researchers to overcome some of the limitations encountered with the building of a functional electrochemical sensor.

Other techniques which are commonly used in biothreat detection, such as culturing or growing the agent, for example do not fall neatly into the above classes although PCR and DNA sequencing may be used to confirm a particular agent following culture. While the gold standards of detection have performed admirably to date and will continue to see widespread use, the large diversity of biochemical, clinical, and engineering considerations in biothreat agent handling and detection ensures that no single sensor technology will optimally satisfy all possible constraints or potential scenarios. As the threat of bioagent weaponization, release, and transmission continue to evolve, either naturally or artificially, the continued development of an expansive and varied sensor design landscape will remain an imperative public health directive. As highlighted below, there have been an incredible number of proposed methods for bioagent detection, each with their own advantages and limitations, from the direct detection of the bioagent to the detection of pathogen-specific biomolecules, along with their associated proteins, and nucleic acids.

Immunoassays and ELISAs. In response to the presence of a foreign antigen, vertebrate immune systems produce a barrage of chemical signals and proteins to combat the invader and limit infection. Antibodies produced from activated B-cells are a primary mechanism of defense capable of binding target antigens and sequestering them until they can be removed from the host through phagocytosis, excretion, or other mechanisms. A process of maturation that occurs by

genetic recombination of hyper-variable domains coding for the antigen-binding portion of the antibody allows them to develop an incredibly high degree of specificity, sometimes even attaining a binding constant in the nanomolar range or better for their target antigens.⁹⁴ This property of antibodies has made them invaluable components of various immunological assays that are routinely employed for scientific research, clinical diagnostics, and as therapeutics. In addition to assays based solely on antibody binding, such as agglutination and ELISAs, they are also widely used in surface-based techniques such as SPR as well as NP-based approaches (*i.e.*, lateral flow assays).⁹⁵ As antibody applications spans nearly all biosensing formats, we have grouped sensors instead based on their detection modality to better highlight the diversity of technology that they contribute to.

The commercialization of antibodies, including their evolution for desired binding properties, functionalization through the addition of chemical compounds or reporter molecules, and manufacture have been optimized over decades of work by both industry and laboratory researchers. Today, antibodies can readily be acquired for a considerable number of targets and modified to serve in any number of immunological assays (immunoassays). In most immunoassays, antibodies serve as capture reagents, reporter molecules, or both. Immunoassays typically work through the formation of the so-called immunocomplex which is based on the interaction of the antibody and the antigen. Formation of the immunocomplex can then be measured in an assay specific manner. Antibodies are also easily labelled to serve as reporters and allow for quantitation of the immunocomplex. Antibodies can be dye-labeled, conjugated to fluorescent proteins, or are often conjugated to an enzyme to enhance the signal output.

One of the most common immunoassay formats is the ELISA which has been extensively used in clinical diagnostics in the fields of bacteriology, parasitology, and virology for its simplicity and reliability.^{96,97} Though other versions exist, there are four main ELISA formats available; direct, indirect, sandwich, and competitive (**Figure 6**). In the direct format, a solid support is coated with a target antigen which is subsequently detected with a labeled antibody specific for the antigen. Consequently, the signal produced is relative to the amount of target within the sample and can therefore be used for quantitative analysis. The indirect format ELISA usually requires the participation of a secondary antibody which is labelled and recognizes the antibody forming the immunocomplex with the target. As the secondary antibody is usually polyclonal and not specific for a single epitope on the primary antibody, multiple, labelled

secondary antibodies can often bind a single primary antibody leading to an overall amplification of the signal. This secondary antibody is typically linked to a readout enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase and if the antigen is present and the antibody remains attached to the assay surface, the enzyme’s substrate can be continuously converted to a colorimetric product. This not only provides a positive readout for the sensor but also serves to functionally amplify the signal as a small amount of enzyme can convert a great deal of substrate to product over a short time in a confined volume such as a microtiter well. The sandwich format utilizes both a capture and reporter antibody that is specific for the target antigen. Here a capture antibody is first immobilized to a well of the microtiter plate to which sample is directly added and binds to. Following iterative washes to remove non-bound material a second, labeled antibody containing a reporter of some sort is added to the well. Once non-bound secondary antibody is removed through washing, antigen levels can be quantitated based on the signal produced by the label in comparison to that of a calibration curve. Finally, a competitive assay is a modified version of a direct ELISA in which antigen is immobilized and labeled antibody then added and bound to the well *via* the antigen. With this format of immunoassay, a sample containing unknown antigen is then added which displaces the antibody if present. Addition of substrate or measuring the output of the reporter molecule indicates how much antibody was displaced allowing for indirect quantitation of the unknown antigen.⁹⁸

Immunological methods have been extensively improved over the years, resulting in a vast collection of methods that allow for the rapid identification of potential threats in clinical and environmental monitoring as well as public health surveillance. Combined with advances in molecular biology techniques that have allowed for the production of monoclonal antibodies at a fraction of the cost, researchers and clinicians have been able to utilize immunoassays as a rapid method of sample analysis capable of detecting a range of targets from small molecules to intact pathogens such as bacteria and viruses.⁹⁹ The diversity of ELISA formats have allowed for assays to be developed that accommodate a range of sample types and outputs. For the purpose of this review, we focus mostly on ELISA assays for pathogen detection which provide diagnostic capabilities by detecting bacterial or viral proteins along with toxins directly,¹⁰⁰ or the corresponding antibodies in the patient sera.¹⁰¹

PCR. Unlike the complex tertiary interactions between antibody variable regions and the antigens, nucleic acid based recognition is incredibly simple, yet very powerful and effective. The predominant form of nucleic acid structure and its detection is based on Watson-Crick base pairing, driven by hydrogen bonding and base pair (bp) stacking of the four bases – adenine, thymine (uracil in RNA), guanine, and cytosine. The simplicity of this system makes it possible to predict energy, stability and secondary structure of nucleic acid hybridization, although there are some exceptions (such as the G-T wobble base pairing and rare bases such as inosine).¹⁰² PCR is an incredibly sensitive method for sensing nucleic acid sequences, with a proven detection limit of a single target copy.^{103,104} In essence, positive or negative PCR results are very often interpreted in the context of a positive or negative test result. The nature and reliability of PCR has made it the gold standard technique used for most sample screening and amplification in nucleic acid detection within a host of different assay formats.¹⁰⁵⁻¹⁰⁸

PCR also has many functional variants, including real-time or quantitative PCR (qPCR) and RT-PCR, which converts RNA transcripts or viral RNA genes to a DNA template. Due to the quantitative nature of qPCR, it is often more desirable for use in biosensor assays over standard PCR. In qPCR, a fluorescent signal is generated and proportionally increases as copies are made usually through the presence of a DNA intercalating dye or a MB. Readout is reported as either the C_q (quantitation cycle), C_t (threshold cycle), or ΔR_n (normalized reporter fluorescence), which are all variations on the detection of the fluorescent signal over the baseline control. As with antibody-based ELISAs, PCR is another mature technology that has decades of scientific development behind it and will continue to remain a mainstay of the biosensing field.

Whole Genome Sequencing. Along with PCR, genomic sequencing is one of the major scientific developments of the last century. Although it generally remains too time-consuming and expensive to run for large sample sets or constant environmental monitoring, sequencing technology continues to develop at an incredible pace with decreasing cost, increasing read lengths, and sample throughput growing at a nearly exponential rate.¹⁰⁹⁻¹¹² It is little wonder then that sequencing is often considered the next major frontier in biosensor design and development, capable of providing the exact sequence and identity of any pathogen. This is often critical, as many biothreat agents, including both bacteria and viruses, undergo rapid genetic evolution and can have large variability, resulting in significant changes to the underlying

genome. This has also proven useful for epidemiological analysis of pathogen evolution, allowing the tracking of what genetic mutations are conferred to particularly deadly strains.¹¹³ Sanger sequencing was the original method for sequencing DNA. Although slow and costly it remains, however, a powerful method for *de novo* sequencing. Once an organism is sequenced, its genomic sequence can be used as a reference from which to determine variations in strain, type, and other genetic characteristics. Pyrosequencing has since replaced Sanger as the current method for DNA sequencing, as it represents both a 100-fold increase in throughput and a 10-fold decrease in cost when compared to the electrophoresis-based Sanger method. Pyrosequencing is done by the real-time monitoring of phosphate release following base incorporation into a growing DNA strand. Two main commercial ventures employing this technology are the Roche 454 system, which can achieve a 700 mega bp (Mb) run with an individual read length up to 1000 bp in 23 hrs with their GS FLX+ system, and the Illumina Solexa system, which can achieve a 100-120 giga bp (Gb) run with an individual read length of 2x150 bp in 29 hrs. Two new sequencing methods, SOLiD and the IonTorrent (10-15 Gb with 200 bp read length in 2.5 hrs) have also become recently available.¹¹⁰⁻¹¹²

In the not too distant past (~20 years ago), the idea of sequencing an entire genome required significant monetary investment, large research teams, large arrays of instrumentation in dedicated facilities, and countless hrs both in and away from the laboratory to generate and assemble the millions of nucleic acid fragments into a usable format. Today, instrumentation for genome sequencing can be found in larger academic and national laboratories where the genomes from single or even multiple organisms can be elucidated with a day's labor using commercially available kits and reagents. These advances in sequencing technologies have had enormous impacts on numerous fields of biological study including the development of new therapeutic strategies and diagnostics.^{112,114} Next generation sequencing (NGS) and whole genome sequencing (WGS), as their names imply, utilize a form of PCR to generate millions of short nucleic acid fragments that can then be assembled into a full genome sequence. While the technology varies slightly between manufacturers and instrumentation, it still remains quite similar in the protocols for sample preparation and for downstream processing and assembly of the target genome or genomes. In all instances, purified nucleic acids are fragmented and attached to an adapter sequence that facilitates subsequent PCR reactions. During template amplification, integration of complementary nucleotides to the template DNA strand produces a

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3 signal that can be measured by the instrument. Unlike Sanger sequencing that produced single
4 reads,¹¹⁵ NGS devices can run highly parallel reactions enabling hundreds of Gb of DNA
5 sequences to be generated in a single run. These advances have significantly driven down the
6 labor and cost associated with obtaining sequence information from both pure and complex
7 biological samples.^{112,114}

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11 The use of NGS technologies for viral detection is rapidly emerging as an efficient and
12 accurate method for identifying viral pathogens in a variety of matrices.¹¹⁶ To date, this
13 technology has been utilized mostly within the medical community for the development of
14 diagnostic tools, treatment strategies, and the design of vaccine candidates. However, NGS and
15 WGS has significant potential for monitoring the molecular epidemiology of known viral
16 pathogens and for the identification of unknown or poorly characterized viral species. For well
17 characterized viral agents, NGS/WGS can be used to monitor genetic drift and variability, to
18 track transmission through populations, and to identify genetic variations that contribute to
19 resistance to antibiotic or anti-viral therapeutics.^{112,114,116}

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22 Today, bench-top instruments such as the Illumina MiSeq, Oxford Nanopore
23 Technologies MinION, ThermoFisher IonTorrent, and others have made sequencing applications
24 achievable in moderate sized laboratories everywhere from academia to industry. Paired with the
25 consistent decline in the cost per base to sequence, which has decreased significantly over the
26 past decade (from multiple dollars to <1 cent/bp), NGS/WGS technologies are rapidly emerging
27 as a viable platform for detection of viral pathogens.¹¹⁷ Successful NGS/WGS reactions are
28 affected more by experimental design than instrumentation and the expense once associated with
29 gene sequencing. Similar to traditional PCR reactions, care must be taken with the methods
30 chosen for sample preparation, probe/primer design, reaction conditions, and the number of reads
31 necessary for accurate identification of targets.¹¹⁶ Finally, identifying a low abundance viral
32 genome or genome fragment in the Gb of collected data can prove difficult. In parallel to
33 optimization of NGS/WGS protocols, bioinformaticians have developed software tools capable
34 of identifying the proverbial needle in a haystack to help accomplish this.

51 **Medical Diagnosis of Biothreat Agents**

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53 Medical diagnosis of any disease typically starts with a patient's visit to a healthcare
54 provider, where based on the reported signs and symptoms, medical/travel history, and physical
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examination, various medical tests, including IVD tests, may be ordered to aid in the diagnosis.¹¹⁸ While some infectious diseases are clinically distinct, many of the biothreat agents discussed here present with non-specific symptoms such as fever, pain, myalgias, and/or rash. Therefore, the ability to identify the etiologic agent quickly and respond with appropriate treatments for the management of patients and to guide infection control is an essential first step in any emergency response chain. First responders, healthcare providers, public health, and clinical laboratories all play an essential role in identifying and responding to these types of threats. Clinical laboratories rely on a variety of techniques to aid in identifying the etiologic agent of an illness, including for example, traditional gold standard techniques and FDA approved/cleared IVDs as discussed in more detail below.

Current Gold Standards. To aid in the medical diagnosis of disease resulting from exposure to a biothreat agent, medical diagnostic tests are based either on direct detection of the pathogen itself, and/or the toxins/agents it produces, or indirect methods such as measuring the human humoral response resulting from exposure (*e.g.*, immune responses such as IgM and/or IgG). The choice of medical diagnostic testing is dependent on the timing of the patient presentation at the healthcare facility relative to the point of exposure. Specimens collected should reflect the disease process and should be of sufficient quantity and quality to facilitate complete medical diagnostic testing. Traditional microbiologic tests often look for typical phenotypic characteristics of the etiologic agent such as morphology, growth, antigens, phage susceptibility, and various biochemical tests (*e.g.*, Gram staining, nitrate reduction, carbohydrate fermentation, oxidase production, *etc.*) using laboratory techniques such as wet chemistry, assay kits/dedicated instrumentation, microscopy, ELISA for antigen detection, and culture. For more than a century, suspected tuberculosis (TB) diagnosis has been done with a simple microscopic observation of a sputum smear sample, a diagnostic test that can be completed within mins but typically only identifies 30-35% of positive cases. Culture traditionally plays a pivotal role in the clinical laboratory but it can be time-consuming and often require specialized conditions, which in some cases yields a diagnosis too late to be of help to the patient. Nevertheless, culture methods are still generally considered the gold standard for diagnosis of infection with many bacterial pathogens. Bacterial culture takes a long time relative to disease progression even for bioagents that are easily cultured such as *B. anthracis* (1-2 days),^{119,120} *Y. pestis* (>24 hrs for barely visible

colonies), *B. mallei* (2 days, under enhanced circumstances), and *B. pseudomallei* (3-5 days). However, some bacterial species grow poorly on culture medium; *F. tularemia* is typically identified with chocolate agar, but grows poorly even under optimized conditions,¹²¹ *M. tuberculosis* requires 9 days or more,^{122,123} and *Brucella* diagnosis takes up to 21 days *via* culture (with a highly variable 10-90% accuracy), whereas the agent responsible for Q Fever cannot be reliably cultured.^{1,124}

Detection of microbial antigens or the human immune response to the pathogen using immunoassays can be especially useful when the suspected microbial agent either cannot be isolated in culture or is difficult to culture. This is typically performed using immunologic-based agglutination, coagglutination, or ELISA assays. Antibodies are used to bind antigenic proteins on the surface of the microorganism and can be very specific, leading to identification of the etiologic agent. Detection of the human immune response with ELISAs or neutralization serology tests can also aid in the diagnosis of a disease. However, serological testing also has significant drawbacks. Most prominently, it often takes days (IgM response) to weeks (IgG response) for the immune system to develop detectable levels of antibodies, limiting the effectiveness of such testing for diagnosis in the initial timeframe of an outbreak.¹²⁵ Other complicating factors include potential cross-reactivity with antibodies to other related microbial agents, differential responses from patients who have received antibiotic treatment or immunosuppressed patients who are unable to produce significant antibody levels (due to ongoing treatments for cancer, arthritis, or rheumatologic disorders), and the presence of persistent antibodies from prior endemic exposure to the same or a related microbial agent can affect the antibody titers resulting from the current microbial infection.^{121,126}

Genotypic testing technologies such as PCR can be used to detect nucleic acids (DNA or RNA) from microbial agents and have transformed clinical virology and have transformed clinical virology given inherent difficulties in culturing most viruses. Many pathogens have well defined genetic markers, either specific mutations or sequence domains within the genome, or carry plasmids encoding virulence factors, that produce the unique proteins that confer pathogenicity; the latter of which can also be detected *via* PCR in most cases. This can be used not only to precisely identify the microorganism, but also to differentiate it from non-pathogenic strains. To access the genetic material, the microorganism must be lysed and the nucleic acids purified. PCR provides notable advantages over the more traditional serological-type tests. PCR

can be very sensitive and relies on short, easily synthesized nucleic acid primers which optimally have little to no secondary structure. This makes PCR relatively straightforward to design, cheap, easy to run, and can provide positive identification within hours. In addition, the stability of nucleic acids removes the need for refrigeration with some of the sample reagents, a major limitation of many antibody testing technologies. PCR can also be applied in a multiplexed format allowing the clinical laboratory to test a sample simultaneously for more than one potential microorganism. PCR is, of course, not without its own limitations, however. PCR as a process relies upon well conserved and well defined target areas to indicate pathogenicity.¹ Certain biothreat agents, such as *Francisella tularensis*, have poorly defined and highly variable regions of pathogenicity, yielding a high degree of uncertainty. Although not currently confirmed as an issue, PCR would be more susceptible to evasion by genetic engineering. It would likely be easier to genetically alter the identifying sequence to evade known PCR primers than it would be to alter the protein display on the viral/bacterial surface in a directed and predictable fashion.

Clinical microbiological laboratories continue to evolve as advances in testing and instrument technology come to fruition and thus what is considered the current gold standard testing is also likely to change. Each test/technique has its own unique benefits and disadvantages and it is the role of the clinical laboratory to balance these when trying to identify the clinically relevant microorganisms in the patient specimen. There are an increasing number of automated and semi-automated instruments that are making it easier for clinical laboratories to run not only some of the more routine traditional microbiologic tests, but also the more complex multi-step immunoassays and PCR-based procedures. Advances in sequence-based testing and mass spectrometry (MS) are also likely to have impact on clinical laboratories and potentially become part of their routine testing repertoire in the future.^{118,127} Obviously, this is just an extremely brief overview of a staggeringly complex area that requires sophisticated knowledge of the agent, the disease etiology, and the diagnostic process in conjunction with the actual resources that are available for a given scenario.^{128,129}

Clinical Laboratory Considerations and the Role of the Laboratory Response Network. When testing for the presence of biothreat agents there are a number of important inter-related issues that clinical laboratories have to consider including the rarity of the pathogen(s) and the ability of the laboratory staff to identify and correctly handle such agents.¹¹⁸ Biothreat agents are often

associated with significant biological hazards and therefore specimens should be handled and testing procedures followed appropriately, which can require sophisticated laboratory environments (*i.e.*, biosafety level 3 or 4 facilities) and significant training of personnel. It is not practical or cost-effective for smaller local and/or hospital clinical laboratories to store reagents and maintain the proficiency testing required to perform some of the specialized assays used for identifying biothreat agents. As became apparent during the 2014 Ebola virus outbreak, clinical laboratories should establish preparedness plans/procedures for collection and handling of clinical specimens as well as effectively interfacing with federal, state, local, tribal, and territorial government stakeholders in the event one of these rare biothreat agents is presumptively diagnosed.¹³⁰ The hospital laboratory is likely going to be the initial location for identification of biothreat agents. Familiarization with the American Society for Microbiology (ASM) Sentinel Level Clinical Laboratory Protocols for Suspected Biological Threat Agents and Emerging Infectious Diseases is essential for the safe handling of potential biothreat agents in a clinical laboratory setting.¹³¹ In addition to instituting the ASM Sentinel Protocols in clinical laboratories, it is important that these laboratories participate in proficiency testing for biothreat agents such as the College of American Pathologists (CAP) Laboratory Preparedness Exercise (LPX) surveys which consist of three challenge specimens sent twice per year.¹³²

In an effort to improve the public health laboratory infrastructure and ensure an effective response to biological, chemical, and other public health threats, the U.S. established the Laboratory Response Network (LRN) through a collaborative effort involving the CDC, Federal Bureau of Investigation (FBI), Department of Defense (DoD), and the Association of Public Health Laboratories (APHL).¹³³⁻¹³⁵ The LRN became operational in 1999 and is an integrated network of state and local public health, federal, military, and international laboratories that currently has two primary focus areas: (1) the Laboratory Response Network for Biological Threats Preparedness (LRN-B), and (2) the Laboratory Response Network for Chemical Threats Preparedness (LRN-C). The LRN-B uses a 3-tiered system comprising sentinel, reference, and national laboratories, represented in **Figure 7**, with the thousands of sentinel clinical laboratories at the base of the tier playing an integral role in the initial identification, rule-out, and/or referring of specimens up to the 2nd tier reference laboratories.

FDA, Medical Countermeasures, EUAs, and IVDs. IVD devices have now become a key and essential initial test in any emergency response chain. The availability of IVDs targeting biothreat agents enable health care staff to identify the causative agent quickly and respond with appropriate treatment for the management of patients and to guide infection control. The Center for Devices and Radiological Health (CDRH), part of the FDA, is responsible for the regulation of medical devices, including IVD devices, as defined in the Federal Food, Drug and Cosmetic Act (FD&C Act) and outlined in the regulations in Title 21-Code of Federal Regulations (21 CFR) Parts 1-58, 800-1299. The regulatory oversight of IVDs is dependent on the risk of the test to the population in which it is meant to be used, described in its Intended Use (IU) and/or Indications for Use (IFU). Risk in the context of most IVDs means the risk to a patient stemming from actions taken based on a false-positive or a false-negative result (*e.g.*, unnecessary surgery, treatment delay, *etc.*). The FD&C Act defines three classes of devices based on risk: Class I designation is for devices of least risk and Class III, the highest risk class, is reserved for devices that in general ‘*are of substantial importance in preventing impairment of human health*’ or ‘*for which insufficient information exists to determine that general and special controls are sufficient to provide reasonable assurance of the safety and effectiveness of such device*’. Overall, device classification is determined according to the claimed IU and risk to the patient, and governs the regulatory pathway and the types of controls to which the medical device will be subject.¹³⁶

Class I (lowest risk) devices are subject to general controls but are mostly exempt from premarket submission and review by FDA. Class II (moderate risk) devices are subject to both general and, where applicable, “special controls.” Most Class II devices are reviewed through the 510(k) premarket notification process, where the new device must demonstrate substantial equivalence to a predicate device (*i.e.*, an already legally marketed device) in terms of IU, technological characteristics, and performance testing Class III (highest risk) devices are subject to general controls and premarket approval where valid scientific evidence is used to establish a reasonable assurance of safety and effectiveness for the IU in a premarket approval application (PMA). Certain novel devices that do not have a legally marketed predicate device, and historically would therefore automatically be designated class III, may be eligible for the *de novo* process, outlined in the FDA guidance document “*De Novo Classification Process (Evaluation of Automatic Class III Designation)*”.¹³⁷ Devices that are classified, through a risk-based

analysis, into class I or class II through a *de novo* classification request may be marketed and used as predicates for future 510(k) premarket notification submissions.

For many of the biothreat agents discussed here, IVD device classification, depending on the technology, would likely be Class II or Class III, requiring a 510(k), *de novo* or PMA submission to the FDA. Developers of IVDs, in general, are encouraged to engage early with the FDA through the Pre-Submission program, outlined in the FDA guidance document “*Requests for Feedback on Medical Device Submissions: The Pre-Submission Program and Meetings with Food and Drug Administration Staff*” which is an informal nonbinding mechanism for potential developers to request FDA’s feedback on IU, study design, and other relevant information prior to a premarket device submission.¹³⁸ In addition to the Pre-Submission program, CDRH has a number of resources available to IVD developers on its website, some of which are summarized in **Table 8**.

Table 9 outlines current FDA cleared or approved IVDs for the Tier I and category A pathogens. In addition, in 2017 FDA granted BioFire Defense, LLC a *de novo* approval for their FilmArray NGDS Warrior Panel (DEN160048/K170883), a multiplexed device that tests for several biothreat agents simultaneously. Unfortunately, experience has shown that there is reluctance on the part of the commercial sector to develop IVD devices for many of the biothreat pathogens because exposure to these agents is perceived as a rare event resulting in a lack of sustainable market and commercial incentive from the developer perspective. This has proven problematic in the past, as demonstrated during the 2014 Ebola and the 2016 Zika virus outbreaks, where lack of commercial IVDs initially hampered the emergency response. Section 564 of the FD&C was first added to the FD&C Act by the Project BioShield Act of 2004 (Public Law 108-276) and, taking into account existing delegations of authority (found in the FDA Staff Manual Guide 1410.10), permits the FDA Commissioner to authorize the emergency use of an unapproved medical product or an unapproved use of an approved medical product in certain circumstances after the HHS Secretary has made a declaration of emergency, significant potential of emergency, or threat justifying authorization of emergency use. FDA’s Emergency EUA authority along with details about pre-EUA submissions and formal EUA requests are outlined in the recent FDA guidance document “*Emergency Use Authorization of Medical Products and Related Authorities*”.¹³⁹

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Since its enactment, the HHS Secretary has made a total of six declarations relating to IVD devices, summarized in **Table 10**, that have resulted in a total of 50 IVD EUAs granted by FDA to address a lack of commercial IVDs at the time of the declaration. Interestingly, to date, these declarations have all been for viral pathogens. Of the six emergency declarations, five remain active as of June 2018. IVD EUAs for the recent Ebola and Zika outbreaks are summarized in more detail in **Table 11**. Once an IVD has been granted an EUA from the FDA Commissioner, the FDA makes public on its website (<http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/default.htm>) the Authorization Letter, any Fact Sheets, and the assay Instructions for Use (under “Labeling”) that describes how the assay is carried out and outlines all the analytical and clinical studies performed to support the EUA request.

To facilitate emergency preparedness, FDA similarly encourages early engagement about any potential EUA products through a pre-EUA submission. The pre-EUA submission allows IVD developers, particularly those with a potential EUA product at an advanced stage of development, the opportunity to interact with FDA even prior to an emergency use declaration. FDA and the developer may discuss the likely IU, abbreviated analytical and clinical study designs, and other relevant information that would be required to support a formal EUA submission to the agency should the requisite HHS declaration be made. Ultimately, the EUA is not a short cut for the traditional FDA clearance or approval of these IVD devices, but facilitates the emergency response by allowing the EUA holder to market the IVD device in the U.S. temporarily until the HHS declaration is terminated or the EUA is revoked sooner by the FDA. Upon EUA termination or revocation, the EUA holder must remove the IVD device from the market. At any time, the IVD developer can submit information to FDA for review in consideration of traditional clearance or approval. The product’s performance during its EUA period can, potentially, be utilized in the traditional approval or clearance process.

Table 8. FDA Resources for Device Developers

Resource	Comment	Website Link
Device Advice: Comprehensive Regulatory Assistance	Device Advice is intended to provide industry with information that is accurate, timely, comprehensive, and useful about device regulation. Page includes a link for IVD Regulatory Assistance.	https://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/default.htm
Guidance Documents for Industry and FDA Staff	Guidance documents provide FDA staff and industry with non-binding recommendations that relate to many aspects of device regulation. Searchable database that includes final and draft guidance documents.	https://www.fda.gov/RegulatoryInformation/Guidances/default.htm
Medical Device Databases	Includes all searchable databases maintained by CDRH concerning medical devices.	https://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/Databases/default.htm
PMA Database	Searchable database of PMA devices, helps to know the device name or company (sponsor name). Clicking on the resulting <i>de novo</i> product number takes you to information about the product, click the “original PMA” link to find a summary of the safety and effectiveness data provided in support of the PMA submission.	https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMA/pma.cfm
De novo	Searchable database of <i>de novo</i> classification orders, helps to know the device name or company (sponsor name). Clicking on the resulting <i>de novo</i> product number takes you to information about the product, including the FDA Decision Summary that outlines the analytical and clinical performance studies provide in support of the regulatory submission.	https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/denovo.cfm
510(k)	Searchable database of 510(k) premarket notifications, helps to know the device name or company (sponsor name). Clicking on the resulting 510(k) product number takes you to information about the product, including the FDA Decision Summary that outlines the analytical and clinical performance studies provide in support of the regulatory submission.	https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm
Find All <i>In Vitro</i> Diagnostic Products and Decision Summaries Since November 2003	Provides link and recommendations for searching the FDA’s In Vitro Diagnostic Product Database	https://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/LabTest/ucm126189.htm
FDA Counterterrorism and Emerging Threats	Webpage that provides an overview of FDA’s emergency preparedness activities. Includes links to the FDA Medical Countermeasures Initiative (MCMi) that is coordinated by the Office of Counterterrorism and Emerging Threats.	https://www.fda.gov/EmergencyPreparedness/Counterterrorism/default.htm

(Websites accessed 07/06/2018)

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Table 9. Current FDA Cleared or Approved IVDs for Tier I and Category A Pathogens (as of May 2018)

Pathogen	Gold Standard	Technology	FDA Cleared/Approved Submission Numbers
BACTERIA			
Bacillus anthracis	Isolation/culture B. anthracis from clinical specimens.	Gamma phage lysis assay	K143592
		PCR	K051713, K071188, K140426
		ELISA	K040407
		Gas chromatography	K052485
		Immunochromatography lateral flow	K030370
Bacillus cereus Biovar anthracis	Isolation/blood culture from clinical specimens	N/A	N/A
Burkholderia spp. (B. mallei, B. pseudomallei)	Isolation/culture from clinical specimens	N/A	N/A
Clostridium sporogenes (neurotoxin producing)	Test clinical specimens for the presence of toxins, in addition detection of bacteria can be performed to support diagnosis.	N/A	N/A
Francisella tularensis	Blood tests, PCR or immunohistochemical staining – presumptive. Follow up with culture from clinical specimens, serology.	PCR	K072547
Yersinia pestis	Isolation/Culture from clinical specimens	PCR	K072631
VIRUSES			
Variola virus (major and minor)	PCR in clinical specimen or isolation of the virus on live cell cultures from clinical specimen with PCR confirmation	PCR	DEN160016
Viral Hemorrhagic Fevers (VHF – Ebola, Marburg, Machupo, Lassa, Junin, Guanarito, Chapare, Lujo, Hanta, Rift Valley Fever, Crimean Congo)	Testing clinical specimens with ELISA, PCR, serological testing and virus isolation, electron microscopy	N/A	N/A Ebola – Emergency Use Authorization Assays, PCR and Antigen, see Table 11
Dengue	PCR or NS1 antigen detection during the acute phase or serological IgM testing.	PCR	K113336
		IgM capture ELISA	K100534
TOXINS OF BIOLOGICAL ORIGIN			
Botulinum neurotoxins	Mouse lethality test	N/A	N/A

Table 10. Summary of FDA Granted EUAs and Related HHS Declarations (as of June 2018)

Pathogen	Influenza H1N1	Influenza H7N9	MERS-CoV	Ebola Virus	Enterovirus D68	Zika Virus
HHS EUA Declaration Date	April 26, 2009	April 19, 2013	May 29, 2013	August 4, 2014	February 6, 2015	February 26, 2016
EUA Termination Date	June 23, 2010	N/A	N/A	N/A	N/A	N/A
Details	Emergency use of certain <i>in vitro</i> diagnostic, antiviral, and personal respiratory protection products accompanied by emergency use information	Emergency use of <i>in vitro</i> diagnostics for detection of the avian influenza A (H7N9) virus	Emergency use of <i>in vitro</i> diagnostics for detection of Middle East Respiratory Syndrome Coronavirus	Emergency use of <i>in vitro</i> diagnostics for detection of Ebola virus	Emergency use of new <i>in vitro</i> diagnostics for detection of Enterovirus D68	Emergency use of <i>in vitro</i> diagnostic tests for detection of Zika virus and/or diagnosis of Zika virus infection
EUA IVDs						
Molecular EUA	17	2	2	9	1	15^
Antigen EUA	1	1	0	3*^	0	0
Serological EUA	0	0	0	0	0	5

*Includes one product that was authorized for two different intended uses

^Includes one product that was authorized but subsequently withdrawn by the manufacturer.

<http://www.fda.gov/EmergencyPreparedness/Counterterrorism/MedicalCountermeasures/MCMLegalRegulatoryandPolicyFramework/ucm182568.htm>

<http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm>

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Table 11. Ebola and Zika EUAs for IVDs (as of June 2018)

Pathogen	Technology	Assay Name	Authorized Testing Environment	Target(s) [§]	Authorized Specimen Types	Comments [^]
Ebola Virus	Real-time reverse transcription polymerase chain reaction (RT-PCR)	U.S. DoD EZ1 Real-time RT-PCR Assay	Laboratories designated by the U.S. DoD	Ebola Zaire virus	Whole blood, plasma, Trizol inactivated whole blood, or Trizol-inactivated plasma	Manual extraction & manual PCR setup (multiple instruments), 12-44 specimens, results in ~3 h
		CDC Ebola Virus NP Real-Time RT-PCR Assay	Laboratories designated by CDC	Ebola Zaire virus	Whole blood, serum, plasma, and urine	Multiple automated & manual extractions, manual PCR set-up, ~33 samples/ plate in ~3 h
		CDC Ebola Virus VP40 Real-Time RT-PCR Assay	Laboratories designated by CDC	Ebola Zaire virus	Whole blood, serum, plasma, and urine	Multiple automated & manual extractions, manual PCR set-up, ~33 samples/ plate in ~3 h
		BioFire Defense LLC FilmArray Biothreat-E test	CLIA* moderate and high Complexity Laboratories	Ebola Zaire virus	Whole blood and undiluted urine	Fully automated, 1 sample/ pouch, 1 pouch/ instrument, results in ~1 h.
		BioFire Defense, LLC FilmArray NGDS BT-E Assay	Laboratories designated by the U.S. DoD	Ebola Zaire virus	Whole-blood, plasma, and serum	Fully automated, 1 sample/ pouch, 1 pouch/ instrument, results in ~1 h.
		Altona Diagnostics GmbH RealStar Ebola virus RT-PCR Kit 1.0	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Ebola viruses (such as Zaire, Sudan, Tai Forest, Bundibugyo, Reston)	EDTA plasma	Multiple automated & manual extractions, manual PCR setup on multiple instruments ~96 samples/plate in ~4 h
		Roche Molecular Systems, Inc. LightMix Ebola Zaire rRT-PCR Test	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Ebola Zaire virus	EDTA whole blood or TriPure-inactivated EDTA whole blood	Automated extraction, manual PCR set-up, 96 samples in 2 h
		Cepheid Xpert Ebola Assay	CLIA moderate and high complexity U.S. laboratories or by similarly high complexity qualified non-U.S. laboratories	Ebola Zaire virus	EDTA venous whole blood	Fully automated 1 sample/ cartridge, different GeneXpert instruments can hold different numbers of cartridges (up to 80) results in 1 h.
		BioCartis Idylla Ebola Virus Triage Test	CLIA moderate and high complexity U.S. laboratories or by similarly high complexity qualified non-U.S. laboratories	Ebola Zaire virus	EDTA venous whole blood	Fully automated 1 sample/ cartridge, 1 cartridge/ instrument, results in ~2 h.

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	Rapid chromatographic immunoassay	OraSure Technologies, Inc. OraQuick Ebola Rapid Antigen Test	Laboratories or facilities adequately equipped, trained, and capable of such testing (including treatment centers and public health clinics)	Ebola viruses (such as Zaire, Sudan, Bundibugyo)	Venipuncture whole blood and fingerstick whole blood	Visual interpretation: positive results may be interpreted as soon as lines are visible at the Test and Control lines. Negative results must be read 30 mins.
		OraSure Technologies, Inc. OraQuick Ebola Rapid Antigen Test for cadaveric oral fluid	Laboratories, facilities, and in field surveillance and response teams acting under the direction of public health authorities	Ebola Zaire virus	Cadaveric oral fluid	Visual interpretation: positive results may be interpreted as soon as lines are visible at the Test and Control lines. Negative results must be read 30 mins.
Zika Virus	RT-PCR	CDC Triplex Real-time RT-PCR Assay (Triplex rRT-PCR)	Laboratories designated by the Centers for Disease Control and Prevention (CDC)	Zika virus (ZIKV), dengue virus (DENV), chikungunya virus (CHIKV)	ZIKV, DENV, CHIKV: human sera, whole blood (EDTA) [#] or cerebrospinal fluid [#] ZIKV: urine [#] and amniotic fluid [#]	Multiple automated & manual extractions, manual PCR set-up, ~24 samples/ plate in ~3 h
		Quest Diagnostics Infectious Disease, Inc. Zika Virus RNA Qualitative Real-Time RT-PCR test	Laboratories designated by Quest Diagnostics Infectious Disease, Inc. that are CLIA high complexity U.S. laboratories	Zika virus	Serum or urine [#]	Automated extraction, manual PCR set-up, 30 samples/plate in ~3 h
		Altona Diagnostics GmbH RealStar Zika Virus RT-PCR Kit U.S.	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum, EDTA plasma or urine [#]	Multiple automated & manual extractions, manual PCR setup on multiple instruments ~96 samples/plate in ~4 h
		Viracor Eurofins. Zika Virus Real-time RT-PCR	Viracor-Eurofins designated Laboratories that are CLIA high complexity U.S. laboratories	Zika virus	Serum, plasma, or urine [#]	Automated extraction, manual PCR set-up, ~91 samples/plate in ~3 h
		Siemens Healthcare Diagnostics Inc. VERSANT Zika RNA 1.0 Assay (kPCR) Kit	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum, EDTA plasma and urine [#]	Automated & manual extraction and automated PCR setup, ~190 samples in ~8 h
		Luminex Corporation xMAP MultiFLEX Zika RNA Assay	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum, plasma, or urine [#]	Automated extraction, manual PCR set-up, ~96 samples/plate in ~4 h
		Vela Diagnostics USA, Inc. Sentosa SA ZIKV RT-PCR Test	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum, EDTA plasma and urine [#]	Automated extraction and automated PCR set-up, 24 samples in ~3 h
		ARUP Laboratories Zika Virus	Laboratories designated by ARUP	Zika virus	Serum, EDTA plasma and	Automated extraction,

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		Detection by RT-PCR	Laboratories that are CLIA high complexity U.S. laboratories		urine [#]	automated PCR, ~96 on 3 h
		Abbott Molecular, Inc.	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum, EDTA plasma, EDTA whole blood [#] and urine [#]	Automated extraction, automated PCR, 48-96 samples in 5-7 hrs
		ELITechGroup Inc. Molecular Diagnostics Zika ELITe MGB Kit U.S.	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum and EDTA plasma	Fully automated 12 samples in 2.5 h
		Nanobiosym Diagnostics Inc. Gene-RADAR Zika Virus Test	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum	Manual extraction, automated PCR set-up, 1 sample/instrument, about 2 hrs
		Thermo Fisher Scientific TaqPath Zika Virus Kit	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum and urine [#]	Automated extraction and manual PCR set-up, 564 samples in ~8 hrs
		Columbia University CII-ArboViroPlex rRT-PCR Assay	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus (ZIKV), dengue virus (DENV), chikungunya virus (CHIKV), West Nile virus (WNV)	ZIKV, DENV, CHIKV, WNV: serum ZIKV: urine [#]	Automated extraction and manual PCR set-up, 88 samples in ~6 hrs
	Transcription-mediated amplification (TMA)	Hologic, Inc. Aptima® Zika Virus assay	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Zika virus	Serum, plasma, processed urine [#] , and processed whole blood K2EDTA [#] .	Fully automated 275 samples in 8 hrs
	IgM antibody detection assay	InBios International, Inc. ZIKV Detect 2.0 IgM Capture ELISA	CLIA high complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Human IgM antibodies against Zika Virus	Sera	~7 hrs for MAC-ELISA, 28 samples/plate
		CDC Zika MAC-ELISA	Laboratories designated by the Centers for Disease Control and Prevention (CDC)	Human IgM antibodies against Zika Virus	Sera or cerebrospinal fluid (CSF) [#]	~48 hrs including plate preparation for MAC-ELISA, 8 samples/plate
		DiaSorin Incorporated LIAISON XL Zika Capture IgM Assay	CLIA high and moderate complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Human IgM antibodies against Zika Virus	Sera	Automated system, 30 samples per 1 hrs
		Siemens Healthcare Diagnostics Inc. ADVIA Centaur Zika test	CLIA high and moderate complexity U.S. laboratories, or by similarly qualified non-U.S. laboratories	Human IgM antibodies against Zika Virus	Serum and plasma (EDTA or lithium heparin) [#]	Automated system, 120 samples per 1 hrs
		ChembioDiagnostic Systems,	CLIA high and moderate complexity	Human IgM	Serum (plain or separation	Single use lateral flow format,

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		Inc. DPP Zika IgM Assay System	U.S. laboratories, or by similarly qualified non-U.S. laboratories	antibodies against Zika Virus	gel), fingerstick whole blood [#] , EDTA venous whole blood [#] , or EDTA plasma [#]	15-20 mins/sample
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*CLIA = Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a
§IVD assays for the qualitative detection of the pathogen
[#]Specimen must be collected alongside a patient-matched serum (or plasma specimen - if the assay claims plasma as part of its intended use for molecular-based assays only)
^Estimated from protocol details provided in published instructions for use

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Infectious Disease IVD Testing Beyond the Traditional Clinical Laboratory. Developments in assay and instrument technology are pushing testing outside of the traditional clinical laboratory paradigm with the desire to test near the patient, often referred to as POC testing, driving these advancements. While there are various definitions of POC testing, it can be broadly defined as testing that is performed near a patient or where patient care is given, outside of laboratory testing facilities. At a minimum, POC diagnostics should be easy to use, have a small footprint and give rapid, easy to interpret results. The WHO developed the **ASSURED** criteria to benchmark new and developing diagnostics and sensors detailing the need for the technology to be **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free and **D**eliverable to the end-users.¹⁴⁰ While this WHO criteria was developed with diagnostic tests for sexually transmitted infections in mind, it is clearly applicable to POC testing in general. POC diagnostics are typically based on rapid lateral flow assays, PCR and/or ELISA's, and can play a key role when used in conjunction with clinical assessment, epidemiological considerations, and other laboratory findings as an aid in the initial or presumptive diagnosis. This presumptive diagnosis can then be followed-up by more traditional techniques performed in a clinical laboratory for the definitive confirmatory diagnosis. Early diagnosis in a POC setting can facilitate appropriate patient management in a far timelier fashion which may be a key feature in outbreak or intentional exposure event situations. In addition, while the previous sections reflect processes in place within the U.S. healthcare system, POC testing can be extremely beneficial in countries that do not have an extensive healthcare infrastructure and where access to clinical laboratories is extremely limited. WHO has a number of programs and mechanisms in place to address diagnostic testing in resource-limited settings and while not the focus of this review, some highlights are provided below, since many of the infectious diseases the WHO member states are routinely confronted with are considered biothreats.

WHO and Emerging Infectious Diseases. Under the auspices of the United Nations, the WHO is concerned with international public health and communicable/infectious diseases, and preparedness, surveillance, and response are considered some of the key focus areas for this agency. WHO has a number of programs covering diagnostics for infectious diseases, including their prequalification assessment program that facilitates WHO procurement of safe, reliable and appropriate IVDs for priority diseases

(http://www.who.int/diagnostics_laboratory/evaluations/en/). WHO convenes the R&D Blueprint (<http://www.who.int/blueprint/en/>), which as a global coalition aims to improve coordination, accelerate research and development, develop norms and standards, and streamline responses in the context of identified priority diseases that pose a public health risk because of their epidemic potential and absence of or insufficient countermeasures. The list of priority diseases is reviewed annually and includes a number of biothreat agents and Disease X, which represents a serious international epidemic caused by a pathogen currently unknown to cause human disease. With respect to situations that are considered public health emergencies, WHO works with countries to respond (<http://www.who.int/emergencies/diseases/en/>) through their Health Emergencies Programme, which coordinates a number of networks including the Emerging Diseases Clinical Assessment and Response Network (EDCARN: <http://www.who.int/csr/edcarn/en/>). In the case of diagnostics, WHO have the Emergency Use Assessment and Listing (EUAL) procedure for IVDs, akin to the prequalification program for pharmaceutical products that can be used for any disease that has been declared by the WHO to be a Public Health Emergency of International Concern (PHEIC).

The remaining portions in this document are divided by the type of threat, *i.e.*, viral, bacterial or toxin, starting with some overview of each for context. Sensing technologies under development for each of the major threat types are then divided into sub-categories based on some common analytical mechanism or process. The first category is based primarily on physical detection methods. These are typically instrumentation-based, such as MS, chromatography, and various wave-based methods (*i.e.*, SPR, fiber optics, quartz crystal microbalance, surface acoustic waves, microcantilevers, and magnetic sensors). The second category details biochemical methods. These methods encompass approaches such as detection *via* antibodies, DNA (including PCR, non-PCR methods, and sequencing techniques), phage, and electrochemical approaches, including amperometric, impedimetric, and potentiometric sensors. Third are spectroscopic methods, including microscopy, colorimetry, fluorescence, chemiluminescence, vibrational spectroscopy such as Raman or infrared (IR), and flow cytometry (FC)/microfluidics. Lastly, where applicable some integrated devices or other techniques are discussed. It is important to note that many biosensors often employ hybrid approaches and could easily be placed into multiple categories. While this makes straightforward classification more difficult, it again serves to highlight the vast diversity within the biosensor

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field, in general. Lastly, these are not meant to be strict definitions or classes, but rather to help organize different types of sensing based on some common foundational criteria in order to help provide structure to the presentation.

Bacteria

Bacteria are unicellular organisms that can cause disease either through host cell invasion, systemic invasion, infection, and/or through toxin release. In this section, we focus on the bacteria directly implicated in disease, while proteins and other toxins, including those from bacteria, are covered later in the Toxin sections. The diversity of bacteria is reflected in the wide range of diseases they cause, infectious doses, routes to infection, *etc.* While some bacteria almost invariably produce lethal outcomes if untreated, others are incapacitating agents, resulting in relatively low mortality/morbidity ratios. Both the availability of bacteria and ease of culturing potentially contribute to their appeal as bioagents, and those with sporulating forms add another degree of long-term environmental stability necessary for dispersion. The development of antibiotics is undoubtedly one of the most important advances in human health in the 20th Century, and antibiotic use is prescribed in the event of infection with any of the bacterial agents covered in this review.^{1,2,5} However, the fact that treatment exists does little to mitigate the threat posed by a biological attack using a bacterial agent. Often, mitigation by antibiotics is most effective only before the onset of symptoms, and in many cases, aggressive treatment is still insufficient. Moreover, the possibility of natural and artificial development/selection of antibiotic-resistant strains may potentially negate one of the strongest front line defenses against bacterial infection.

Overview of Some Common Bacterial Biothreats

Anthrax. Reports of naturally occurring anthrax outbreaks date back to antiquity as described by Homer in *The Iliad* (700 BC) and Virgil in the First Century BC.¹⁴¹ In modern times, it has remained an agriculturally-important disease in less developed countries, as was the case in Zimbabwe in 1978-1980, where an epidemic of human anthrax contracted from infected animals resulted in approximately 10,000 cases.^{1,2} Anthrax is also notable for being the first state-sponsored bioweapon. Used during World War I, German agents infected animals shipped from the U.S and other neutral countries to Allied Forces with anthrax and glanders.²¹ Subsequent development for militarized use focused on dispersion to people through the spores of the causative bacteria, *Bacillus anthracis*, with interest stemming from its high infectivity and high mortality rate (for inhalation anthrax, greater than 90% if untreated, 45% in aggressively treated cases in the U.S. after terrorist attacks in 2001), though risk of person-to-person transmission is

low.^{1,3} Although the infectious dose for inhalation of *B. anthracis* spores is higher than the effective doses of a number of other bacterial bioagents (10,000 spores, ~0.01 µg), their tolerance for extreme conditions make them, along with smallpox, the greatest potential for mass casualties and civil disruption.^{3,21}

B. anthracis became one of the core bioagents researched by the U.S., the UK, and the USSR, where in 1979 an accidental release of *B. anthracis* spores from a Soviet military microbiology facility in Sverdlovsk (now Yekaterinburg) led to the deaths of at least 66 residents.¹ Along with botulinum toxin and aflatoxin, *B. anthracis* was produced by Saddam Hussein's Iraqi regime and loaded into bombs and missile warheads that were deployed but not used during the Persian Gulf War.¹ The Japanese cult Aum Shinrikyo attempted to spread *B. anthracis* spores using sprayer-based dispersion systems on two occasions, resorting to the use of the chemical neuroagent sarin when both that and their attacks with BoNT failed. More recently, letters containing spores were mailed to members of the press and the U.S. Congress in 2001, resulting in contraction of cutaneous or inhalation anthrax by 22 people.¹ Treatment of anthrax with antibiotics can be effective, but only during the 1-7 day incubation period.⁵ After this, initial onset shows generic symptoms, including fever, malaise, and coughing, followed by the sudden development of severe respiratory problems and death from sepsis or shock within 24-36 hrs.^{3,5} A vaccine exists, but the extent of research and probable genetic manipulation that has been done in the weaponization of *B. anthracis* raises concerns over the efficacy of both the vaccine and antibiotics.⁵

Plague. Plague has played a pivotal role through human history; its natural occurrences were responsible for three great pandemics, including the Black Death of the Middle Ages. Although many groups pursued weaponization of plague (including the U.S. and USSR), the most notable offensive use was in WWII, in which the Japanese biowarfare Unit 731 began experimenting with the plague-causing bacteria *Yersinia pestis*.²¹ When dissemination by bomb proved ineffectual, fleas were recruited as a means of both protecting the bacteria from the bomb blast and enabling subsequent transmission.¹ Air-dropping plague-infected fleas over cities in China and Manchuria resulted in the appearance of bubonic plague in regions where the disease was not endemic. Plague manifests in humans in one of three forms. Bubonic plague, characterized by swollen lymph nodes when infection by flea bite occurs, can develop into septicemic and/or

pneumonic plague, with the latter being the form expected from an aerosolized delivery of the bacteria.² Bubonic plague has a fatality rate around 5% with immediate treatment (60% if left untreated) and is non-transmissible from person to person. Pneumonic plague is a more potent biothreat, with a 50% fatality rate (close to 100% fatality rate if not treated within 18-24 hrs of symptom onset), and is known to spread from person to person.² Primary pneumonic plague, developed from the inhalation of *Y. pestis* rather than as a secondary disease from flea inoculation, has an incubation period of 1-3 days. Flu-like symptoms rapidly progress to pneumonia and death from respiratory failure and circulatory collapse.⁵ No vaccine is currently available, and the formerly available killed vaccine was not effective against aerosolized *Y. pestis*. The infectious dose for inhaled *Y. pestis* in nonhuman primates falls between 100 and 20,000 organisms, but can be as low as 1-10 organisms for other exposure routes.^{1,2,21,142,143} Interestingly, occasional outbreaks of plague still occur *via* flea/rodent vectors in the Southwestern U.S. as it is endemic there.^{144,145}

Glanders/Melioidosis. Glanders is an ancient disease caused by the bacterium *Burkholderia mallei*, and is most commonly associated with horses, but can cause serious illness in humans as well. Up until the 20th century, glanders was a significant concern due to the reliance on horses for transportation both by the civilian population as well as the military. Naturally occurring infections are no longer an issue in first world countries (eradicated in Great Britain in 1928 and the U.S. in 1942), although it is still problematic in underdeveloped countries. Transmission is also rare, with the last naturally contracted human case in 1934, however, glanders is still considered a high risk biothreat agent due to its high infectivity and availability.^{146,147} It is also closely related to *Burkholderia pseudomallei*, the causative agent in melioidosis, and unlike glanders, *B. pseudomallei* has been found outside its natural animal reservoir in soil and water samples in subtropical regions. These diseases also pose a unique threat due to long term latency - in a singular instance, melioidosis was reactivated 62 years after initial exposure in a man taken prisoner by the Japanese during WWII.¹⁴⁸ The rarity of these diseases in the human population makes their study and prediction of outbreak behavior exceedingly difficult.

Tularemia. Tularemia, caused by the bacterium *Francisella tularensis*, is another disease that can infect both animals and humans and is considered a significant threat for weaponization.

Rodents along with rabbits appear to be especially susceptible and have died in large numbers during natural outbreaks. There have been reports of naturally occurring infections in all states within the continental U.S. Humans are usually exposed through contact with infected animals and from tick and certain fly bites.¹⁴⁹ Although there has been speculation about the use of tularemia as a biological weapons in both WWII as well as in Kosovo in the Balkans, it is known that the Japanese also conducted experiments with tularemia, however, there has yet to be a confirmed use of tularemia as a weapon.^{150,151} It's potential for weaponization stems from a serious disease prognosis, with both high morbidity and mortality, yet generic onset symptoms making early diagnosis a significant challenge. In addition, infection can be obtained with extremely low doses – as little as 10 bacteria for subcutaneous injection and 10-50 for aerosolized inhalation.^{149,152}

Brucellosis. Another one of the more recognized bacterial bioagents that has no record of use in a bio attack, the bacteria that cause brucellosis was nevertheless loaded into munitions and tested on animal targets in the U.S. in the mid-1940's.³ As with a number of the other bacteria that were researched for weaponization, infections from *Brucella* species typically manifest as zoonotic diseases, with human infection resulting from contact with or consumption of infected animals or animal products, especially unpasteurized dairy products. Six of the eight *Brucella* species are known to infect humans, with *B. suis*, *B. melitensis*, and *B. abortus* exhibiting the highest human pathogenicity.^{2,3,21,124} The natural incubation period for brucellosis is relatively long (7 days to several months, likely shorter in high doses of aerosol); however the low fatality rates (2-5% if untreated), the low infectious dose (10-100 organisms), and the viability of the bacteria even after several years in storage make brucellosis a viable incapacitating agent. Symptoms vary among the *Brucella* species and differ among patients, and their onset may be either gradual or sudden.^{2,124}

Q Fever. Originally discovered in Australia and the U.S. before WWII, Q Fever has now been recorded in over 50 countries.¹ Caused by *Coxiella burnetii* (originally *Rickettsia burnetii*), it is an effective incapacitant although rarely deadly. Animal reservoirs include domesticated ungulates such as cows and sheep with inhaled aerosolized particulates being the primary mode of transmission; interestingly, humans are the only species to show symptoms.^{153,154} It is

considered a moderate risk for bioweapon development, primarily for its high infectivity (with infection caused by <10 organisms) and hardy stability in the environment, being resistant to both bleach and Lysol.¹⁵⁵ Q Fever can also be both acute or chronic, further complicating identification of infection.

Bacteria as Food and Water Safety Threats. Although many of the Category A select agents are known for their high infectivity and transmissibility to and from other humans, the Category B agents representing the food and water safety threats are worth specific mention as they are almost exclusive to bacteria. Per the CDC, these include *Clostridium botulinum* (Botulism), Shiga-toxin producing *E. coli* O157 (STEC), *Salmonella typhi* (Typhoid fever), *Shigella dysenteriae*, and *Vibrio cholera* (cholera); other foodborne bacterial pathogens, such as *Listeria monocytogenes* and *Campylobacter jejuni*, pose similar threats. As *C. botulinum* detection typically focuses on the identification of their toxins (botulinum neurotoxin, BoNT) rather than the bacteria itself, discussion of biosensors will be reserved to the Toxin section. There are many strains of *E. coli*, both disease-causing and benign. Disease is caused by the secretion of the Shiga-toxin (or Shiga-like toxin); the most prominent example being the *E. coli* O157:H7 strain. However, there are many non-O157 strains that have since been discovered as well.¹⁵⁶ There are also many different strains of *Salmonella* bacteria with *S. enterica* being one of the strains that cause common food poisoning, while *S. typhi* (and the murine version *S. typhimurium*) cause Typhoid Fever. Both *E. coli* and *Salmonella* are often used as model organisms for the development of bacterial biosensors, and as such, nearly every biosensor variant and implementation has seen characterization of these threats, which also allows for a more accurate and direct comparison between biosensor performances. While many of the Category A biothreats more vividly capture the public and scientific imagination, in the age of globalization and nationalized food distribution system, the threats posed by highly virulent foodborne pathogens should not be underestimated.^{113,157-162}

Physical Sensors

Direct Mass Spectrometry. One of the best examples of the instrumentation-based approach, MS has long been used to determine the chemical species present in a sample. Unlike the other approaches in this category, MS detects (bio)molecules based on their intrinsic mass-to-charge

(m/z) ratio, which provides a unique biochemical signature for each bioanalyte.¹⁶³ Traditional MS consisted of three steps. First, the (bio)analytes are fed into the MS and broken down into ions for conversion into a gas *via* an ionization source. Then, a mass analyzer separates the resulting ions based on their m/z ratio, which are finally ‘read out’ by a detector. Due to the nature of the molecular degradation, initial MS instrumentation worked well for small molecules, whose degradation patterns were fairly simple. However, the harsh ionization resulted in irregular and unreliable degradation for larger biomolecules such as proteins. Thus, two soft ionization techniques were developed to accommodate these biomolecules, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). In MALDI, the bioanalyte is embedded in a matrix, which is then irradiated to produce ions, whereas in ESI, the analyte is dissolved in a solvent, which is then passed through a capillary and exposed to high voltage, whereupon it is converted into an ionized gas through a series of pressure changes. These systems are often coupled with time-of-flight (TOF) mass analyzers. As the rate at which (bio)analytes pass through the detector is proportional to the square root of the m/z ratio, it is possible then to separate the bioanalytes based on their time of flight through the detector. Of course, this is a very brief and simplified overview of MS technology with far more comprehensive and definitive sources available.¹⁶⁴

The development of alternative ionization techniques and macromolecule-specific sample preparation has made pathogen sensing with MS more viable.¹⁶⁵⁻¹⁶⁸ Indeed, MS has been shown to be highly versatile for bioagent detection, capable of detecting proteins, genomic nucleic acids, and PCR products.^{165,169} MALDI-TOF-MS is capable of detecting many different protein signatures from bacteria or spores,^{170,171} and this strategy has been shown effective for a number of bioagents, including *B. anthracis*,^{172,173} *B. mallei* and *pseudomallei*,¹⁷⁴ *E. coli* O157,¹⁷⁵ *C. burnetii*,¹⁷⁶ and *Y. pestis*.^{177,178} MALDI-TOF also allows for direct detection of bacteria.¹⁷⁹ Several alternative surfaces have been shown effective in the capture and readout sensitivity of bacterial detection, including AuNPs¹⁷⁵ and graphene.¹⁸⁰ In one impressive example, Lasch *et al.* used MALDI-TOF-MS to characterize all of the 146 known strains of the *Yersinia* species, along with 35 common background strains of enterobacteria.¹⁷⁸ Classification analysis using neural networks was able to identify *Y. pestis* with 100% accuracy. See, for example, **Figure 8** for some MS spectra collected from three different *Yersinia* species in this study. Regarding commercial systems, the BioTyper by Bruker Daltonics is one of the better known MALDI-TOF-MS

system, and it has been successfully demonstrated for biothreat agent detection.¹⁸¹ However, bacterial genomes often show a high degree of variability which, along with the presence of plasmid-based virulence factors, can complicate the unique biochemical signature used to denote a positive identification.

An alternative approach, ESI-TOF has been shown uniquely suited for detection of PCR amplicons. In this method, PCR-amplified targets from various sections of the bacterial genome are amplified, including both generic targets as well as species-specific targets.^{182,183} The PCR products are fed into the ESI-TOF, which is able to provide the exact nucleotide composition of the product, although it cannot determine the specific sequence. This method has been most prominently demonstrated in the IBIS T5000 from IBIS Biosciences.¹⁸⁴⁻¹⁸⁶ This system has roughly 1,400 pathogenic standards and clearly highlights the capabilities of MS for biothreat detection. In one initial study using this system, samples taken from patients with acute respiratory infections were spiked with biothreat agents such as *F. tularensis*, *B. anthracis*, *Y. pestis*, *Brucella*, *Burkholderia*, and *Rickettsia* bacteria and RNA amplified using reverse transcriptase PCR, with detection specificity of the mock bacterial samples ranging from 95-100%.¹⁸⁷ Hannis *et al.* also demonstrated the IBIS T5000 for the detection of *Campylobacter jejuni*, by detecting eight different housekeeping genes *via* PCR, providing highly specific strain typing, see **Figure 9**.¹⁸³ Indeed, many other groups have also used this system with a high degree of success.^{167,188}

Chromatography with MS Detection. Although direct MS analysis is highly useful in the detection of biothreat agents, samples often require additional preparation, purification, and especially target enrichment steps. This is particularly important in whole cell bacterial detection where the target pathogen may only be present in trace amounts within the complex background of benign bacterial species and other nucleic acid materials found in biological or environmental samples. Some type of chromatography is then typically used to augment the MS analysis by providing a sensitive and powerful method of sample separation and purification to quickly reduce background. Gas chromatography (GC), liquid chromatography (LC), and affinity chromatography are the most prominent methods by which to fractionate and concentration samples before detection by MS.^{166,189}

In one representative example, GC-MS was used for the detection and differentiation of two very similar pathogens, *B. mallei* and *pseudomallei*, with an LOD down to 4000 cells.¹⁹⁰

LCMS-MS has also been used in the detection of *C. burnetii*,¹⁹¹ and species-specific detection of *B. anthracis* spores along with *Y. pestis*.^{192,193} In the latter approach, an initial immunocapture step was performed to enrich the sample of intact *Y. pestis* cells, see **Figure 10**. Magnetic beads coated with an antibody directed towards Pla, a membrane protein expressed on the surface of *Y. pestis*, were added into the sample solution and extracted *via* magnetic separation. Target proteins were extracted using a previously characterized trifluoroacetic acid method of MS-compatible inactivation of pathogenic bacteria and spores directly from the magnetic bead surface followed by enzymatic digestion to produce short peptide products.¹⁹⁴ The peptides were then analyzed using LC-tandem MS, which first separates the peptides on a LC column, then analyzes the products by MS. In the latter examples, sensitivities of 7×10^3 (*B. anthracis*) and 2×10^4 CFU/mL (*Y. pestis*) were reported in a complex matrix such as milk with no additional purification steps required.^{192,193} These values are quite promising given that the minimal infection dose for *B. anthracis* is in the range of 10^3 up to 5×10^4 spores and the LD₅₀ is 8000 CFU.¹⁹²

Although the primary role of chromatography in this context is in sample preparation and enrichment, denaturing high performance liquid chromatography (D-HPLC) has been shown to be an effective means of biosensing in its own right.^{195,196} This is achieved through the size analysis of PCR amplicons generated from pathogen specific-genes, as in the case of *C. botulinum*,¹⁹⁷ or of the 16S ribosome for *B. anthracis*.¹⁹⁸ This approach has also been used to identify genes conferring antibiotic resistance in biothreat pathogens, such as rifampicin-resistance in *M. tuberculosis*¹⁹⁹ and ciprofloxacin-resistance in *Y. pestis*.²⁰⁰ Magnetophoretic chromatography, whereby particles are separated in the presence of a magnetic field, has also been used for the colorimetric detection of *E. coli* O157.²⁰¹ It is important for the reader to appreciate that this is just the briefest overview of a vast and technically complex area of work.

Waveguide Based Sensors. Another major group of physical biosensors are the so called “wave”-based sensors. These sensors typically rely on the capture of a target biomolecule to the sensor surface, usually through an attached biorecognition element such as antibodies or nucleic acid hybridization. This capture then alters the physical parameters of the system, which results in a change of the interrogating waveform. Six generalized classes of waveguide biosensors are discussed here: surface plasmon resonance (SPR), fiber optic biosensors (FOBS), quartz crystal

microbalances (QCM), surface acoustic waves (SAW), microcantilevers (MCL), and magnetoelastic/magnetorestrictive (ME/MR) resonators. This family of sensors typically measures binding as a function of mass at the biosensor surface. Because of their mass sensitivity, these sensors are also considered label-free, with the exception of fluorescence FOBS, which instead measures binding based on fluorescent tags instead of mass. These sensors also vary based on wave type: an evanescent wave excited from an external light source, as in the case of SPR and FOBS, or through measuring frequency changes in acoustic waves, induced either by piezoelectric transducers (QCM, SAW, MCL sensors) or by a magnetic field (ME/MR sensors).

Surface Plasmon Resonance. SPR is the most widely used and best characterized of these systems and is commercially available in systems such as the BiaCore from GE Healthcare and has matured to use in clinical settings.^{202,203} A typical SPR system consists of a thin gold surface on top of a highly refractive glass surface. Light is reflected off of the metal film, and this reflection excites the surface plasmons of the metal, forming an evanescent wave. This evanescent wave is highly sensitive to the angle of refraction of the original light wave. Antibodies to target a bioanalyte are then usually displayed on the gold surface in the assay format. Analytes are flowed over the surface and targets present in the sample are captured by the antibodies. This capture changes the mass present on the surface, altering the refractive index of the surface and thereby changing the plasmon resonance and evanescent wave propagation at the surface. As these changes are directly proportional to the mass change at the surface, one can calculate the total amount of sample bound in a highly specific, sensitive, and label-free manner.

SPR has been used to detect a variety of pathogenic bacteria and related proteins, including *B. anthracis*,²⁰⁴⁻²⁰⁶ *M. tuberculosis*,²⁰⁷ *S. typhimurium*,²⁰⁸ and *E. coli* O157.^{209,210} While SPR technology is both powerful and versatile, it still has some notable limitations. Changes in the refractive index are a function only of mass, thus collecting any additional information on the target would require a different sensor. The sensor surface chemistry is also generally limited to gold or silver. Furthermore, SPR is often sensitive to buffer conditions and passive adsorption of off-target molecules may result in a higher background in crude samples. Variations on SPR technology have sought to overcome these limitations and improve signal. In demonstrating detection of a protein indicative of TB, Chen *et al.* employed nickel oxide NPs in a sandwich

assay-based format targeting increases in the mass on the surface. This approach greatly enhanced the angle of refraction, and therefore the SPR signal, resulting in a more sensitive sensor.²¹¹ Analogous approaches have used semiconductor quantum dots (QDs) for similar signal amplification purposes.²¹² In a different approach, Huang *et al.* combined SPR with fluorescence spectroscopy, resulting in a hybrid approach termed ‘Long-Range Surface Plasmon-Enhanced Fluorescence Spectroscopy’ (LRSP-FS), see **Figure 11**. This approach extended the mass sensitivity of the system to several microns from the sensor surface, enough to encompass the entirety of the pathogenic *E. coli* O157:H7 cells, and demonstrated an LOD down to 10 CFU/mL within 40 mins.²¹³

Fiber Optic Biosensors. FOBS are another class of waveguide-based biosensors, using the optical propagation of an evanescent wave traveling through a fiber optic cable *via* total internal reflection to detect target pathogens.^{214,215} These biosensors operate on a similar principle to SPR, in which surface-based changes caused by the binding of an analyte result in a change in the evanescent wave. There are several types of FOBS including those that are intensity-, absorption-, and fluorescence-based. While the first two are label-free, fluorescence FOBS requires a fluorescent tag, as it primarily uses the standard sandwich immunoassay for bioanalyte detection. Here, an antibody is coated on the sensor surface and captures target biomolecules present in the sample. Next, a second, fluorescently-labeled antibody specific to the same biomolecule is then added, which binds to the captured biomolecule resulting in a sandwich format, and the resulting change in fluorescence is transmitted *via* fiber optics. The standard ELISA setup has been demonstrated with a number of biothreat pathogens in FOBS sensors, including *B. anthracis*,²¹⁶ *E. coli* O157,²¹⁷⁻²¹⁹ *M. tuberculosis*,²²⁰ *Y. pestis*,^{221,222} and *Salmonella*.²²³⁻²²⁵ While the use of fluorescent tags can allow for more sensitivity over SPR by ignoring any passive adsorption to the surface, distal interactions outside of the evanescent wave may be missed.²²⁶

The use of fiber optics also provides a straightforward mechanism to multiplexed bioanalyte detection, either by arrays, channels, or wells. This approach has been successfully implemented to detect many biothreat pathogens simultaneously.²²⁷⁻²²⁹ As with other technologies, microspheres can provide an alternative approach to multiplexing, for example, incorporating DNA binding probes that correspond to a large panel of biothreat pathogens, but

requires initial PCR amplification and sample processing to generate the necessary sequences.²³⁰ One of the most mature demonstrations of FOBS has come from the Naval Research Laboratory in Washington D.C., which has led the development of several fully integrated systems over the past 20 years, including the RAPTOR²³¹⁻²³⁵ and the NRL Array Biosensor.²³⁶⁻²⁴² In one demonstration of the RAPTOR's multiplexing capabilities, six biohazard agents were simultaneously detected, including the bacterial biothreats *B. anthracis* (7.1×10^4 CFU/mL), *B. abortus* (1.5×10^5 CFU/mL), and *F. tularensis* (7.3×10^6 CFU/mL), while demonstrating a false-negative rate of 2.4% and a false-positive rate of 5.2%.²³⁹ Other utility demonstrated included detection of *Campylobacter* and *Shigella* subspecies in food samples, see **Figure 12**.²³⁷ Another example from Wei *et al.* demonstrated the detection of *Y. pestis* in infected animal species using a different fiber optic system, the FOB-3.²²² Using polystyrene probes with different antibodies, this system was sensitive down to 60 CFU/mL of stock cells, while sampling of homogenized infected animal spleen tissue was able to generate a positive signal from only 150 CFU/mL. Overall, these systems have demonstrated some impressive capabilities for the detection of a variety of biothreat targets in multiplexed fashion without requiring significant sample pre-processing.

Quartz Crystal Microbalances. QCM are a class of piezoelectric-based devices, often in the form of targeted immunosensors, in which mass changes are detected by changes in frequency of the quartz resonator. QCM can be used for biological or chemical sensing techniques in air or liquid environments making it a potentially robust and versatile sensor. The QCM measures mass per unit area as a function in changes to the resonance frequency of the quartz resonator. Therefore, it can also be used to evaluate and characterize surface modifications, adsorption processes, and the interaction of biomolecules with the sensor. Most frequently, the quartz crystal is layered with a gold electrode that serves to both induce the crystal oscillation and function as the scaffold for assembly of the antigen recognition element which is typically an antibody but can also be nucleic acids or other biomolecules. These devices can be highly sensitive, capable of detecting mass change as low as $1 \mu\text{g}/\text{cm}^2$. In theory, given their small size, individual QCM sensors can be functionalized for different targets and incorporated into a microfluidic system for easy automation and multiplexed analysis. QCM sensors propagate the acoustic waves within the entire piezo substrate, and as such, may also be referred to as bulk

acoustic wave or thickness shear mode sensors. Although the waves propagate within the whole substrate (typically within a frequency range of 5-30 MHz), the most notable displacement occurs at the edges, providing a surface sensitive to changes in mass. For a deeper insight into the theory behind piezoelectric devices, we refer interested readers to an excellent discussion by Janshoff *et al.*²⁴³

QCM devices have generated considerable interest as biosensors, second in the class of waveguide-based devices only to SPR. QCM devices have been demonstrated for a wide range of biothreat agents including for Tularemia detection,²⁴⁴⁻²⁴⁷ *B. anthracis*,^{248,249} *Salmonella*,²⁵⁰ *E. coli* O157,²⁵¹ and other *E. coli* antigens.²⁵² QCM has also been augmented with NPs for detection, particularly for *E. coli*, which provides for enhanced mass changes at the MCL surface.^{253,254} One of the primary drawbacks of QCM devices comes from manufacturing constraints, which imposes limits on the substrate thickness and therefore the frequency range available to the device. Excellent demonstrations of QCM capabilities come from Hao *et al.* where QCM devices were used to detect anthrax, first using a QCM immunosensor to detect *B. anthracis* spores, and then using a DNA-probe based QCM to detect PCR amplicons generated from *B. anthracis* genomic material, with AuNPs incorporated for signal amplification.^{248,249} In the first example, the QCM immunosensor were able to detect 10³ CFU/mL of spores while the DNA biosensor was able to detect 3.5×10² CFU/mL, see **Figure 13** for a schematic and some representative data.

Surface Acoustic Wave Sensors. SAW sensors operate based on the same piezoelectric resonator as QCM devices, however, the sensor setup and instrumentation display some important differences, thereby changing the sensitivity and output of the device. SAW sensors have a few unique advantages within the waveguide sensor class. First, they are not only sensitive to mass changes like SPR, but also to density, viscosity, and acoustic coupling phenomena. Second, while QCM waves propagate through the entire substrate, which decreases the energy and sensitivity at the surface, SAW sensor waves (typically from 50 MHz to several GHz) propagate only in the waveguide layer at the top of the substrate, referred to as ‘shear-horizontal’ waves (SH-SAW) thus making these sensors independent of substrate thickness and highly sensitive to surface changes.²⁵⁵ There are three types of SAW sensors, Rayleigh-SAW, Lamb-mode (measuring the Lamb waves which are the acoustic modes that propagate along the

sensor plate), and Love-mode (shear horizontal wave), each with various advantages and sensor design characteristics. Love-mode sensors present the predominant form of these biosensors in the literature and several have been demonstrated for bacterial detection,^{256,257} although very few have directly been used for biothreat detection.²⁵⁸ Of these, *E. coli* O157 has again been a primary target,^{259,260} with one notable study by Branch *et al.* in the detection of a *B. anthracis* simulant (the non-pathogenic spore-forming *B. thuringiensis*).²⁶¹ In this study, they tested Love-wave sensors made out of both polyimide and polystyrene as coated with antibodies. These were able to detect a total of 1764 spores/mL, with the polyimide material determined to have an increased sensitivity over polystyrene. Overall, despite some useful properties, development of SAW sensors for pathogen detection still lags considerably behind interest in SPR and QCM-based devices.²⁵⁵

Microcantilevers. MCL-based sensors offer yet another physical, piezoelectric-based approach for the direct detection of pathogenic bacteria. Small, lightweight, and with a high surface-to-volume ratio, MCLs hold considerable promise for the direct detection of bacteria and spores. Although MCLs offer the same label-free approach as other wave-based sensors, the primary advantage of MCL technology is the ability to approach single organism sensitivity. The cantilever design has one end capable of bending while the other end is fixed to a surface. These devices usually operate in either a “static” or “dynamic” mode. In a static mode configuration, a single side binds the analyte, changing the mass and inducing a strain on the MCL, thus acting like a weighing scale. In dynamic mode, both sides of the MCL can bind the analyte. The MCL is oscillated at a given frequency, which is subsequently dampened in proportion to the additional mass on the MCL surface. Their use in pathogen detection has been well reviewed.²⁶²⁻²⁶⁴ While there have been efforts to detect *E. coli* with MCL devices,²⁶⁵ a significant focus has been direct detection of *B. anthracis*.²⁶⁶⁻²⁷⁰ Of these, the piezoelectric-excited millimeter-sized cantilever (PEMC) sensor described by Campbell *et al.* is rather intriguing due to its ability to capture airborne particulates of *B. anthracis* spores, with a detection limit of 38 spores/L while sampling at a rate of 267 L/min.²⁶⁷ In this system, antibodies targeted to the analyte are bound to the glass surface. Spore capture leads to a mass change on the surface, and the subsequent decrease in resonance frequency is measured. **Figure 14A** highlights some representative results of this sensing format.

Magnetic Sensors. Magnetic-based separation techniques, particularly using magnetic micro- and NPs, have been around for a long time and their benefits are well documented.²⁷¹ Here, we highlight the application of magnetic beads as direct sensors as such applications are generating considerable interest.^{272,273} For example, magnetic beads have been shown to be effective when combined with apoferritin nanoprobe, in both detection and separation of biothreat agents, as recently demonstrated by Seo *et al.*²⁷⁴ Apoferritin is a protein with a functional domain that can bind the Fc region of antibodies; the latter are the (fragment crystallizable) antibody tail region that can interact with some cell surface receptors and also certain complement system proteins. With genetic modification, this protein can also be engineered to contain a hexahistidine or 6×His-tag, for the binding of nitrilotriacetic acid (NTA)-labeled fluorescent dyes.²⁷⁵ In this system, apoferritin was coupled to both a dye and pathogen-specific antibody, targeted to inactivated forms of *F. tularensis*, *Y. pestis*, and *B. anthracis*. Antibodies were also conjugated to magnetic beads for the concentration and enrichment of pathogen detection, in a sandwich assay format. This system was used to detect each of the pathogens independently, as well as a dual-detection scheme for both *Y. pestis* and *B. anthracis*, with an LOD around 10^3 - 10^4 CFU/mL as determined from the absorbance of the coupled dye. A schematic overview of the process is provided in **Figure 14B**. Magnetic beads can also be used in direct sensing through AC susceptometry, (*i.e.*, dynamic magnetic measurement) which report on absorption-induced changes in Brownian relaxation as demonstrated in the detection of *B. abortus*.⁶⁴ An alternative approach, referred to as “frequency mixing” was described in the detection of *F. tularemia* and *Y. pestis*,^{276,277} in which the magnetic bead response was measured at a given frequency directly proportional to the two excitation frequencies.

There are also two classes of wave-based magnetic sensors, magnetoelastic (ME) and magnetoresistive (MR), each of which has been used in various biothreat detection applications.²⁷⁸ ME devices are ribbon-like ferromagnetic strips (typically constructed from commercially available Metglas 2826MB alloy) that act as acoustic wave resonators, similar to the piezoelectric MCL devices, but instead resonate in the presence of a magnetic field.²⁷⁹ These devices are magnetostrictive, meaning that a strain is exerted on the material in the presence of the magnetic field. A number of examples have successfully utilized ME devices for biothreat detection, mostly commonly targeting *B. anthracis*²⁸⁰⁻²⁸² and *S. typhimurium*,^{281,283,284} although

several studies have demonstrated sensing of other bioagents such as *E. coli* O157.^{285 286} ME sensors have also been highly amenable to use with a landscape phage-based library, which has advantages over antibodies in specificity, stability, cost, and storage.²⁸⁷⁻²⁹⁰ MR have been used for biodetection, although far less than ME devices.²⁹¹ These devices change resistance in the presence of a magnetic field and have been shown to be effective in bacterial detection as well.²⁹² The most prominent use of MR devices is with giant magnetoresistive (GMR) sensors, which are large arrays that have been applied to biothreat sensing.²⁹³⁻²⁹⁶ In one interesting study, Pal and Alocilja used MNPs coated in polyaniline, an electrically active conducting polymer (EAPM), to concentrate and detect *B. anthracis* spores from food samples in a direct-charge transfer biosensor setup.²⁹⁷ Here, detection was performed *via* a sandwich immunoassay; bacterial spores were captured from the food sample matrix, concentrated and washed, then the spore-EAPM NP complex was captured by surface-bound antibodies. Binding of the electrically active NP resulted in a decreased resistance across silver electrodes as shown by the schematic and representative data of **Figure 15**. This system demonstrated an LOD of 4.2×10^2 spores/ml in roughly 15 min.

Biochemical Sensors

Antibodies

Enzyme-Linked Immunosorbent Assays. Simple, sensitive, and highly specific, ELISA-based assays have been developed for many biothreat agents and are too numerous to fully list here.⁹⁹ Representative examples include *B. anthracis*,²⁹⁸⁻³⁰⁰ *Burkholderia* sp.,³⁰¹ *F. tularensis*,^{302,303} *S. typhi*,³⁰⁴ *Campylobacter jejuni*,³⁰⁵ *Coxiella brunetii*,³⁰⁶ *Brucella*,³⁰⁷ and *Y. pestis*.³⁰⁸ As with other mature technologies, development of ELISAs has moved well beyond assay design and now looks to alternative methods for implementation. This includes biothreat detection with NPs (see **Figure 16**),^{309,310} assay variations like the Amplified Luminescent Proximity Homogeneous Assay (AlphaLISA) which utilizes singlet oxygen as an energy donor to a fluorescent acceptor moiety,³⁰⁰ and, more recently, utilizing rapid technological advancements to move the ELISA format into low-cost, portable device setups.^{311,312} It is clear that the use of this mature and reliable technology as a core sensor component designs stands to benefit highly as the rapid advancement of technology continues to open up new application frontiers.³¹³⁻³¹⁷

Lateral Flow Assays. One of the most commonly used antibody-based bioassay formats is the lateral flow assay (LFA), a chromatographic approach that is often implemented in a competitive or sandwich format. This assay often relies on inducing a localized SPR with labeled AuNPs to quickly and cheaply detect the presence of a bioanalyte by eye.³¹⁸ These assays are supported on a variety of substrates such as gels and more recently paper.³¹⁹ Once a sample with a suspected analyte is added to the paper, the fluid is carried down the strip, enabling the analyte to interact with the capture molecules. This complex then interacts with the AuNPs, for example, resulting in a colorimetric readout due to aggregation. As with ELISAs, LFAs have long been used and commercialized, and are considered one of the prime candidates for field-based and third-world assays.³²⁰ Traditional LFA formats have been developed for many biothreat targets, such as *S. typhi*,³²¹ *B. anthracis*,³²² and *E. coli* O157,³²³ for example.

Alternative NP materials are also being incorporated into LFAs. Vyas *et al.* used fluorescein-doped silica nanospheres coated with lipopolysaccharides from *B. abortus* to test for the presence of *Brucella* antibodies using only a few microliters of sample in under 15 mins.³²⁴ Another one of the newer developments in LFAs is the use of upconverting phosphors which are typically lanthanide-doped ceramic particles generally several hundred nanometers in size. These particles are unique in that they “upconvert” an infrared excitation into emission in the visible spectrum in a non-linear manner.³²⁵ This technology can provide a substantial increase in sensitivity over colloidal AuNPs in a quantitative format.³²⁶ Upconverting phosphor LFAs have now been demonstrated for *Y. pestis*,³²⁷ *Brucella*,³²⁸ *E. coli* O157,³²⁹ and *B. anthracis*.³³⁰ In the latter study, the authors explored the upconverting phosphor-LFA design for the detection of all three biowarfare agents. These LFA’s operated with two lines of detection, based on a sandwich assay format. Addition of the analyte-containing solution is wicked along the paper and, if present, binds to upconverting phosphor-conjugate antibodies during this movement. This complex is then captured by antibodies in the “positive” strip of the LFA. Phosphore-labeled antibodies are also captured by a secondary line, the “control” strip, which binds regardless of whether pathogenic analytes are present or not. In this system, the 50 nm diameter phosphor particles were made of NaYF₄:Yb³⁺Er³⁺ and required 980 nm excitation wavelength to produce an upconverted ~540 nm emission wavelength. This system demonstrated an LOD of 10⁴ for *Y. pestis*, 10⁵ for *B. anthracis*, and 10⁶ CFU/mL for *Brucella*, respectively.³³⁰ See **Figure 17** for some representative data highlighting the specificity of the *B. anthracis* assay using this system.

Phage Display. As mentioned, due to the limitations of traditional antibodies, a number of alternative amino acid based recognition elements have been explored that remove the immunoglobulin scaffold. One such alternative with growing interest is phage display where recent efforts have been focused on transitioning to clinical detection.^{331,332} Phage display offers an alternative backbone by using the bacteriophage particle for selection while providing a variable region for binding similar to that of antibodies. Phage have an immense sequence pool, and are estimated to be the most abundant organism on the planet numbering roughly 10^{31} !³³³ Use of this format also provides the ability to potentially differentiate between live and dead cells with bacteriophage replication assays, which require a viable host bacteria for growth; this is a unique diagnostic tool not achievable using DNA hybridization assays.³³⁴ One of the most significant drawbacks is that typical phage typing assays still require the growth of isolated bacteria to achieve a sufficiently high bacterial load, thereby increasing the number of preparatory steps between infection and detection, and putting it more on par with longer cell culture timelines. A more expanded and insightful discussion of these limitations can be found in a detailed review by Schofield *et al.* regarding phage display for clinical detection.³³⁵

Phage display while not as prevalent as antibodies still has generated considerable interest for use in biothreat sensors.³³⁶ Recent advances in this area have seen phage used for the generation of peptide and antibody-based capture sequences to a number of biothreat pathogens including *B. anthracis* cells,³³⁷ toxins,^{298,338} spores,²⁹⁰ *Brucella* spp.,³³⁹ *Burkholderia* spp.,³⁴⁰ and *Salmonella*.³⁴¹ Use of lytic bacteriophages as direct reporters for detection, sensing, and potentially therapeutic treatment of bacterial biothreats has also been extensively reviewed.³⁴²⁻³⁴⁵ Phage display has also been used in the selection of alternative recognition domains, such as affibodies.³⁴⁶ The backbone of an affibody is derived from the staphylococcus protein A, giving it a small, highly thermostable scaffold with rapid folding capabilities and good solubility.³⁴⁷

Phage display is now most often used to select for short peptides sequences which are quite desirable in this context given that they represent the smallest and perhaps simplest possible peptide-based solution to antigen binding. Small peptides benefit from a simple, robust, and scalable synthesis, high stability, access to diverse attachment chemistry with alternative biological or material scaffolds, and can be easily modeled with molecular dynamic software.³⁴⁸⁻

³⁵¹ In addition to selection from phage display libraries, peptide sequences can also be derived

from known functional peptides such as antimicrobial peptides (AMPs), cell-penetrating peptides, or peptide substrates of protein enzymes while still being produced in bulk *via* solid state synthesis.³⁵²⁻³⁵⁴ With these advantages, peptide-based biosensors generated in this manner continue to garner considerable interest.^{355,356}

PCR. A large number of publications have detailed the use of PCR and qPCR for the detection of biothreat agents including many reviews,^{22,105-108,357,358} only a cursory overview is provided here. Representative examples of PCR-based detection of bacterial threats include those targeting *F. tularensis*,³⁵⁹ *Y. pestis*,^{360,361} *B. anthracis*,³⁶² *Burkholderia* spp.,^{363,364} *E. coli* O157,³⁶⁵ *S. typhi*,^{366,367} *C. jejuni*,³⁶⁸ *C. burnetii*,^{369,370} and *Brucella* spp.³⁷¹⁻³⁷³ These assay formats can be extremely sensitive and amplify quite minute amounts of target template as shown in **Figure 18A**. In addition, one of the primary advantages of PCR is the ability to multiplex sample analysis or perform multiplexing, due to the specificity of the PCR primers targeting unique genomic or transcript sequences from the pathogen. Many examples within the literature are able to target multiple biothreat agents simultaneously, providing the capability for detecting multiple high risk targets in a single assay.^{374,375} There are also commercial products available to accomplish the same, including the EntericBio Panel II (automated results from fecal samples in 3 hrs screening for 9 or more enteric bacteria across 1-32 samples), LightCycler, RAPID, and Smart Cycler platforms.^{358,376} However, PCR utility in this role does have some limitations, such as was evidenced by a study of bovine brucellosis.³⁷⁷ This study showed that effective detection *via* PCR is still dependent on many other factors including a reliable amplification target, availability of the pathogen in the clinical samples (in this case, blood samples that are tested for an intracellular pathogen), and sample preparation steps such as DNA extraction. As mentioned, there is no question that PCR will remain a primary technique in a myriad of assays targeting biothreat agents either in a stand-alone modality or as incorporated into some other process or device.

Isothermal Amplification Techniques. Several DNA-based alternatives exist that seek to address one of the primary limitations to PCR, the reliance on thermocycling to provide amplification. These technologies instead exploit isothermal amplification.³⁷⁸ The most prominent examples, loop-mediated isothermal amplification (LAMP)³⁷⁹ and nucleic acid

sequence-based amplification (NASBA),³⁸⁰ have generated considerable interest, although many other approaches have also been characterized, including rolling circle amplification (RCA), recombinant polymerase amplification (RPA), signal mediated amplification of RNA technology (SMART), helicase-dependent amplification (HDA), self-sustained sequence replication (3SR), isothermal multiple displacement amplification (IMDA), and catalytic hairpin assembly (CHA).^{381,382}

LAMP uses four DNA primers which, in combination with a DNA polymerase, undergo a series of hybridization, extension, and displacement steps to produce additional primer binding domains to continue amplification. LAMP has been demonstrated across a variety of assay designs and setups to be quite effective for the detection of biothreat agents.³⁸³ From the detection of *S. typhi*,³⁸⁴⁻³⁸⁶ and *Brucella*,^{387,388} to the detection of *B. anthracis* using a disposable pocket warmer,³⁸⁹ LAMP has provided a cost-effective and alternative way to achieve nucleic acid sequence amplification and is also one of the few alternative (non-PCR/ELISA) sensor technologies used in the detection of *C. burnetii* (Q Fever).³⁹⁰⁻³⁹² Using LAMP, Chen *et al.* demonstrated the detection of 25 gene copies (~1 organism) of *C. burnetii* extracted from human plasma samples.³⁹²

In contrast, NASBA is slightly more complex and consists of the avian myeloblastosis virus, reverse transcriptase protein, T7 RNA polymerase and RNase H, along with two DNA primers. In this system, the primer binds to an RNA strand, which is degraded by RNase H after extension by the reverse transcriptase enzyme. The second primer binds, forming a dsDNA complex for the T7 RNA polymerase, at which point cycling can begin. NASBA has been used in biothreat detection for *E. coli*,³⁹³ *M. avium* subvar *paratuberculosis*,³⁹⁴ and has been commercialized for other pathogenic organisms (*i.e.*, Leishmania OligoC-Test from Coris BioConcept), while RPA has been used for the detection of *Y. pestis* as shown by the schematic in **Figure 18B**.³⁹⁵

Functional Nucleic Acids. An alternative approach to depending upon some type of nucleic acid hybridization-based approach is the use of functional nucleic acids (FNA) which encompass both DNA and RNA materials. Here, nucleic acid sequences have been artificially selected to have additional functionality using, for example, the systematic evolution of ligands by exponential enrichment (SELEX) process.³⁹⁶ After the initial discovery of naturally occurring ribozymes,

equivocally proving that nucleic acids can catalyze reactions previously assumed to be the exclusive domain of protein enzymes, artificial selection has seen the discovery of a vast multitude of FNAs with activities that include RNA transesterification (known as DNazymes or deoxyribozymes), RNA ligation, adenylation, and more.³⁹⁷ Aptamers, on the other hand, occupy a distinct class of FNAs. Instead of displaying catalytic-type activity, these nucleic acid sequences are selected as binders to molecular targets, similar to the antigen-binding regions of antibodies. Although aptamers are FNAs, their use and development in pathogen detection far outstrip any other type of FNA available.³⁹⁸ Given their nucleic acid basis, they are cheap to synthesize, and the vast potential sequence space that can be addressed by their capability to assume complex 3-dimensional structures ensures a continuity for new aptamers to be discovered. Many groups have used aptamers in the role of the biorecognition element within biothreat detection assays; for clarity, these are included in the section based on their detection modality, which is predominantly either electrochemical or fluorescence based.^{399,400} Along with recognizing and binding to a wide variety of small molecules, many aptamers have been discovered that also bind to larger protein targets as well.^{399,401} Aptamers have been generated to target most of the major biowarfare agents including *F. tularensis*,⁴⁰² *B. anthracis*,⁴⁰³ *S. typhi/typhimurium*,^{404,405} *C. jejuni*,⁴⁰⁶ *B. pseudomallei*,⁴⁰⁷ and *E. coli* O157.⁴⁰⁸ Several aptamer candidates have advanced to clinical trials, showing their high potential for both sensing and therapeutic applications, possibly providing the bridge to true theranostic applications – devices or technologies capable of both diagnosis and treatment.⁴⁰⁹ A highly diverse set of integrated assays have been developed using FNA's for biothreat detection by also incorporating MBs⁴¹⁰ or DNazymes,⁴¹¹ into their activity. Molecular logic processing has even been proposed for the potential to add a “smart” decision making component to such complex pathogen sensing.⁴¹²⁻⁴¹⁷

Beyond DNA and RNA, several xeno nucleic acid (XNA) alternatives have been investigated for this particular role.⁴¹⁸ Because XNA molecules alter the normal nucleic acid backbone they are highly resistant to nucleases and other hydrolytic degradation that can affect standard nucleic acids and this holds considerable promise for therapeutics or applications in challenging environments. Spiegelmers are another type consisting of L-RNA molecules, the enantiomeric form of natural D-RNA, as nucleases are evolved to only recognize the D-RNA form.⁴¹⁹ Other chemistries place the nucleic acid bases on a modified backbone, as opposed to the standard phosphate backbone. These backbone modifications include peptide bonds, forming

peptide nucleic acids (PNA), glycol (GNA), methylenemorpholine (morpholinos), and therosene (TNA). Locked nucleic acids (LNA) connect the 2' oxygen and the 4' carbon, 'locking' it into the "North" conformation.^{420,421} As with Spiegelmers, these modified backbones are highly resistant to chemical degradation by cellular nucleases, making them excellent candidates for clinical trials, and indeed, some are currently underway.^{409,422} XNA versions can still encode information and engage in bp hybridization suggesting they could find use in siRNA knockdown assays where their resistance to degradation would ensure a longer intracellular lifetime while use in aptamers would provide an excellent method to reliably bind and sequester its ligand. However, XNA versions are often difficult to synthesize due to their artificial backbones and generally are far more expensive to obtain due to patented and licensed technologies along with often requiring far more complex and specialized chemistries. Gomez *et al.* provided a notable example of the strong potential such technologies have to offer, combining PNA, RCA, and DNazymes into a single sensor that enabled visual detection of the bacterial pathogens *E. coli*, *S. typhimurium*, and *C. difficile*.⁴²³ In this approach, PNA was used to specifically detect target sequences within the bacterial genomes. Once bound, a "padlock" DNA-based probe sequence was ligated to the PNA, forming a loop which would be amplified by RCA. The extended DNA is then nicked and the short DNA pieces are displaced upon primer extension by the DNA polymerase allowing them to form G-quadruplex DNazymes capable of providing a colorimetric output in a HRP-mimicking reaction, see **Figure 19**.

DNA Sequencing. Many bacterial biothreats have now been sequenced and a variety of sensors based on these sequences have, in turn been developed. Because sequencing is time consuming, expensive, and requires substantial instrumentation, it is rarely used as a direct or initial screening method. Rather, sequencing functions as the ultimate confirmation in identifying pathogens. Indeed, it is helpful in identifying pathogens such as *F. tularensis*,^{424,425} *E. coli* O157,⁴²⁶ and *B. anthracis*,^{427,428} which are often quite closely related to several other non-pathogenic strains, and thus can present significant challenges to alternative modes of detection, see **Figure 20**. Strain typing of bacterial pathogens is typically achieved through the sequencing of the 16S ribosomal RNA as demonstrated in the detection of *Brucella* and *Burkholderia*.^{429,430}

Sequencing in conjunction with MS-proteomic analysis can also help understand the molecular origins and genetic drift in the rare outbreaks of poorly understood agents, as in the

case of Q Fever (*C. brunettii*).⁴³¹ However, the biggest advantage in sequencing-based identification of pathogens is through the detection and determination of antibiotic or drug-resistant strains, a critical yet still widely underappreciated aspect of biothreat detection. While most sensors detailed within this review focus on detecting the biothreat agent, determining other properties such as the presence of resistance genes is equally as important for the containment and treatment of patients during an outbreak. It is this aspect towards which most sequencing efforts have been directed, particularly with rifampicin and isoniazid resistance in *M. tuberculosis*⁴³²⁻⁴³⁴ along with resistance to other therapeutics now found in both *B. anthracis* and *Y. pestis*.^{435,436} As the technology and cost of genomic sequencing continues to improve with next generation techniques, use of sequencing in direct biosensing and confirmation of initial screening tests can be expected to increase dramatically.

Electrochemical Sensors. Electrochemical sensors typically convert biochemical changes, such as bioanalyte capture, to electrical changes within the sensor often with the goal of accessing and directly integrating into the vast library of available electronic devices for reporting.^{437,438} Of all the sensors detailed herein, electrochemical sensors offer perhaps the best example of the potential breadth and depth available in biosensor design and construction along with simplicity in many cases.

Amperometric Sensors. Amperometric devices operate by detecting changes in current, typically achieved through an enzyme-based redox reaction, and they make up the predominant mode of detection by electrochemical means.⁴³⁹ However, the biochemical basis for modulating the electrochemical readout varies widely. Some electrochemical devices use DNA hybridization-based approaches for the detection of pathogenic bacteria.⁴⁴⁰ For example, del Río *et al.* used solid phase isothermal DNA amplification with a recombinant polymerase amplification in both a colorimetric and amperometric setup capable of detecting an *F. tularensis* genomic DNA primer at an LOD of 3.3×10^{-14} M.⁴⁴¹ Alternative approaches to tularemia sensing have utilized immuno-based setups rather than DNA hybridization.^{245,442,443} Similar electrochemical immunosensors have also been used for other pathogens, including *E. coli* O157,^{444,445} *S. typhi*,⁴⁴⁶ *S. typhimurium*,⁴⁴⁷ and *Y. pestis*.⁴⁴⁸ Cortina *et al.* recently demonstrated an electrochemical immunosensor that utilized magnetic beads, referred to as electrochemical

magnetic microbead-based biosensor (EMBIA) which was capable of detecting multiple pathogens including *Brucella* and *T. cruzi* (the agent of Chagas disease), see schematic and representative data in **Figure 21**.⁴⁴⁹

Impedimetric Sensors. Another category of electrochemical sensors, impedimetric sensors have also been explored for pathogen detection.⁴⁵⁰ Impedimetric sensors are typically label-free, cost-efficient, and have low background due to their insensitivity to non-target analytes within the sample, although in the past, they have been challenged by high limits of detection and non-specific binding.¹⁵⁹ However, impedance based sensors are starting to gain in popularity, as the majority of these biosensors have been developed only within the last ten years, although EIS has been around for nearly 100 years.⁴⁵¹ EIS applies small amplitude sine wave perturbations over a range of frequencies, in which electrical current is plotted as a function of frequency. This approach then employs a straightforward mathematical conversion to determine changes in impedance. Impedimetric sensors have similarly been applied to the detection of several bacterial biothreat agents, such as *S. typhimurium*,⁴⁵² *E. coli* O157,^{453,454} and *C. jejuni*.⁴⁵⁵ These sensors have also heavily benefitted from the integration of other nanotechnology advancements. Physical modifications have incorporated various nanomaterials and surfaces such as graphene,^{456,457} self-assembled monolayers,⁴⁵⁸ magnetic beads,⁴⁵⁹ gold-tungsten microwires,⁴⁶⁰ microelectrodes,⁴⁶¹ and polymeric films.^{462,463} Other biochemical augmentation such as incorporation of a phage element,⁴⁶⁴ carbohydrates,⁴⁶⁵ and LAMP-based reactions have also been explored.⁴⁶⁶ Impedance-based electrochemical sensing has also been demonstrated in other detection formats such as in conjunction with SAW sensors as demonstrated for the detection of *M. tuberculosis*.⁴⁶⁷

Potentiometric Sensors. Potentiometric sensors are less widely used for electrochemical sensing than are the amperometric or impedimetric devices. These sensors function based on the difference in electrical potential between a test and reference electrode. The use of differential pulse voltammetry and square wave voltammetry have been the primary methods for biosensing, being used to effectively detect several biothreat agents. Studies have reported on the detection of *E. coli* O157,⁴⁶⁸ as well as *B. anthracis*-like markers from *B. subtilis* for whole cell sensing,⁴⁶⁹ the characteristic surface protein protective antigen for peptide sensing,⁴⁷⁰ and short oligos

corresponding to genomic sequences for DNA-based sensing.⁴⁷¹ In one fascinating example, Rahi *et al.* demonstrated direct detection of *Brucella* genomic material at zeptomolar concentrations with no sample processing or amplification steps through the use of gold “nanoblooms” deposited on gold nanoribbons, see schematic and data in **Figure 22**.⁴⁷² The gold nanostructures were formed by hydrogen seeding of nanogold through sonoelectrodeposition.⁴⁷³ These, and some other similar examples, often rely on a hybrid approach combining enzymes with DNA recognition techniques.^{474,475}

Spectroscopic Sensors

This class of sensors detects the presence of target analytes through spectroscopic means, by measuring changes in the electromagnetic energy intensity or profile of the system. Spectroscopy can be used to measure various innate physical characteristics of the target analyte by looking at, for example, fluorescence, light scattering, and vibrational properties with Raman and infrared techniques.^{476,477} Fluorescence spectroscopy and Förster resonance energy transfer (FRET) use organic and other types of fluorophores to provide a simple and straightforward means of identifying targets and monitoring molecular interactions.^{477,478} NPs have also been extensively used in a variety of spectroscopic measurements, including colorimetric/visual, photoluminescent, fluorescent, and FC-based readouts.⁴⁷⁹⁻⁴⁸¹ These sensors generally employ the same setup, with a light source (either arc lamp or laser) for an excitation source and a collector (*i.e.*, CCD camera or PMT) to determine the emission wavelength. Some types of sensors have even been coupled to protein-based chemiluminescent readouts and are effectively able to generate their own light.⁴⁸²⁻⁴⁸⁶ While it is impossible to cover all topics in this area, certain advancements require particular mention, carrying the potential to significantly improve or augment current technologies in new and interesting ways. This is particularly relevant to microscopy techniques, which remain one of the key instruments in microbial analysis, and will always remain relevant as a confirmatory or supplemental technique. The ability to obtain visual confirmation, along with the use of older but established protocols in resource-poor areas, ensures that the development of improved microscopy techniques still has the potential to vitally enhance diagnostic efforts.

Microscopy-based Detection Methods. As long as bacterial culture remains an essential diagnostic technique, particularly in resource-poor areas, improvements (whether time, cost,

reagent stability, or sensitivity) to the detection assay repertoire will continue to be of substantial value to the biothreat community. Here, we highlight a few notable yet disparate examples, demonstrating the diversity available to microscopy-based methods. As expected, many groups have used immunofluorescence microscopy to detect bacterial pathogens.⁴⁸⁷ In one representative example, Wuthiekanun *et al.* reported a substantial improvement in an immunofluorescence microscopy assay for *Burkholderia pseudomallei*, in which specificities exceeded 99% (although only 66% sensitivity).⁴⁸⁸ By incorporating a blocking agent (5% skim milk in phosphate buffered saline) in the assay, complexity was significantly reduced and assay time was cut from 2 hr to ~10 min. It is worth noting from this that even apparently trivial modifications may have substantial consequences, and the importance of reducing assay time an order of magnitude cannot be overstated. For the interested reader, the use of microscopy in this application has been extensively reviewed elsewhere.⁴⁸⁹⁻⁴⁹²

LED-Based Microscopy. Along with culture-based techniques, sputum smear microscopy remains another of the predominant gold-standard diagnostic techniques, particularly for diagnosing TB. This method, unlike cell culture, relies on analysis directly from the patient samples, ensuring minimum time from sample to potential diagnosis. However, high variability associated with these samples occur due primarily to a lack of proper equipment and analysis time.⁴⁹³ Providing low cost equipment has the potential to overcome many of these issues in resource poor environments. The use of cheap light emitting diodes (LEDs) for TB microscopy is currently being explored and commercialized,⁴⁹⁴ and indeed, in 2009 the WHO specified that LEDs replace other light sources in conventional fluorescence microscopy (CFM).⁴⁹⁵ As the potential for use of cost-efficient microscopes extends far beyond TB, LED-FM will likely find uses in conjunction with other techniques described herein for a variety of diagnostic and identification applications.

Light-Scattering-Based Culture Sensing. Culturing remains one of the gold standards for confirmatory diagnosis of many bacterial biothreat agents, however, the similarities in colony morphology between closely-related bacterial species still remains one of the primary limitations to this approach. Sensing of optical light scattering from bacterial colonies has the potential to dramatically increase the sensitivity of this technique. The most well-characterized of these

methods is the BARDOT system (BACTERIAL Rapid Detection using Optical light-scattering Technology), originally developed at Purdue University, and now commercially available by Advanced Bioimaging Systems (West Lafayette, IN), see **Figure 23A**. This technology has been used to separate serovars based on differential light scattering from colony morphology.⁴⁹⁶ In this system, a 635 nm laser beam passes through the colony as grown on agar, which produces a unique scatter profile. This profile can then be compared to a database to find the appropriate match. This approach has been successfully employed for the detection of *Salmonella*,¹⁶² *Vibrio*,⁴⁹⁷ *E. coli*,⁴⁹⁸ *Campylobacter*,⁴⁹⁹ and *Listeria*,⁵⁰⁰ see **Figure 23B**. This technique still retains the same limitations of bacterial culture in general, primarily that of long growth times and difficult-to-culture pathogens. However, considering the ubiquity of cell culture and its role as a primary diagnostic, application of the BARDOT system ensures that the maximum possible information can be extracted from the use of culture, further justifying the latter's continued use.

Colorimetric Nanoparticle Sensors. Colorimetry is one of the simplest forms of sensor readout, in which the presence of a target analyte results in a stark change in color that can often be discerned by the naked eye. This approach is highly advantageous for many reasons. First, a visible readout removes the need for more sophisticated interrogation and detection equipment, removing upstream external light sources such as arc lamps or lasers for excitation along with downstream light collection and light amplifying instruments. These optics can typically account for the bulk of a sensor's cost. Of the colorimetric sensors, AuNPs and AuNRs are by far the most widely used and characterized materials, due to their innate biophysical properties that make them well suited for use in such biochemical assays.⁵⁰¹ AuNP surfaces display a high surface-to-volume ratio, are amenable to many bioconjugation techniques, and have visual properties that are tunable to the local biochemical environment.³⁵¹ The optical signatures of these particles is determined by their physical properties, particular the particle radius, however, due to ease of synthesis, AuNPs are typically 13-20 nm in diameter.⁵⁰² In this context, changes in the visual properties arise from the inducement of localized surface plasmon resonance (LSPR) due to AuNP aggregation, which is induced by the addition of a biochemical analyte. Unaggregated particles typically exhibit a reddish color, which turns to blue upon aggregation. AuNP assays can be done with or without requiring the particle to undergo functionalization with biorecognition elements. Functionalization using covalent attachment is typically done using

gold-thiol chemistry. Numerous studies have used AuNPs for the detection of biothreat pathogens, with the variations based primarily by conjugation strategy and the molecular target.⁴⁸ AuNPs have been used to detect pathogenic bacteria *via* genomic targeting, as demonstrated for the detection of *Listeria* and *Salmonella*,^{503,504} *Brucella*,⁵⁰⁵ *B. anthracis*,⁵⁰⁶ *Salmonella*,⁵⁰⁷ TB,⁵⁰⁸ and *E. coli* O157.⁵⁰⁹ These types of assays can be augmented sometimes with rather simple chemistry. For example, it is well known that unmodified AuNPs will aggregate in the presence of salt compounds.⁵⁰² However, the addition of DNA, often amplified by PCR, will coat the AuNPs, preventing their charged aggregation. This principle was utilized by Deng where asymmetric PCR was used to amplify long (508+ bp) target genomic sequences from *B. anthracis*. Upon addition of NaCl, the aggregated control sample turned blue, while the presence of the target amplicons, amplified from 10 pg of starting genomic material, remained red, see **Figure 24**. DNAzymes have also been shown in the detection of *Salmonella* genes, demonstrating the potential for hybrid approaches.⁴¹¹

Surface modified AuNPs in all their forms have similarly been used in a wide variety of ways to detect pathogenic bacteria. Antibody-labeled AuNPs have been used in the immunodetection of *Salmonella*,⁵¹⁰ *C. jejuni*,⁵¹¹ and antibiotic resistance genes commonly found in *E. coli* and *B. cereus*; the latter is a rather commonly used model for *B. anthracis*.⁵¹² AuNPs have also proven useful in whole cell capture and detection. Su *et al.* demonstrated the binding of *E. coli* O157 by AuNPs labeled with mercaptoethylamine (MEA) through electrostatic interactions where their aggregation on the bacterial surface promoted the colorimetric change.⁵¹³ This group also demonstrated an alternative approach, again detecting *E. coli* O157 by 4-mercaptophenylboronic acid-functionalized Au-shelled platinum (Au@Pt) NPs.⁵¹⁴ These particles exhibit a peroxidase activity, which catalyzes the conversion of substrate into a colored product in the presence of H₂O₂. A related study showed bacterial association and colorimetric detection could be achieved with positively charged polyethyleneimine-coated AuNPs.⁵¹⁵ AuNPs have even been incorporated into several paper-based devices which are distinct from the function of LFA's. Several groups have explored the use of AuNPs patterned on paper surfaced for bacterial detection,⁵¹⁶ including *Salmonella*,⁵¹⁷ TB,⁵¹⁸ and *E. coli* O157 along with *H. pylori*.⁵¹⁹ This method has also been demonstrated in conjunction with MNPs as well.⁵²⁰ Other noble metal NPs have been used in biothreat sensing applications as well, but these are far less

popular due to their poorer plasmonic characteristics and a tendency to rapidly form oxides in some cases such as that seen with silver NPs.⁵²¹

Photoluminescent, Fluorescent, and FRET-Based Sensors. Photoluminescence- (PL) and fluorescence-based methods likely represent the most widely employed detection metrologies of all sensor technologies. They are widely used in most technologies covered here, from microscopy assays to PCR reactions to microfluidics.⁵²²⁻⁵²⁹ As many of these sensors are covered in sections that are more assay or instrument specific, the approaches briefly described in this section represent only a fraction of the total number of such sensors. In addition, many of the more recent sensors are NP-based and these are bringing with them many interesting and unique properties suitable for a wide range of sensor applications beyond just biothreat sensing.^{530,531} Here, we briefly highlight QD-based sensors along with use of various other NPs (polystyrene, AuNPs, *etc.*) and some alternative fluorescent assay formats that exploit aptamers and targeted dipicolinic acid (DPA) sensing.

Quantum Dots, Polymer Dots, Carbon Dots. The use of QDs in biological assays has greatly expanded over the past decade. The significant advancements in attachment chemistry, materials diversity, and the bioconjugation chemistries that have facilitated this have been widely reviewed.^{348,349,351,480,481,532,533} QDs are semiconducting nanocrystals, roughly 1-10 nm in diameter, most commonly constructed with a CdSe core and a ZnS shell. They have a broad excitation spectrum yet a comparatively narrow PL emission spectrum, which can be tuned as a function of the particle size and quantum confinement effects. This provides QDs with a wide color palette highly beneficial in luminescent and fluorescent applications, where they also commonly act as donors in FRET-based assays.^{478,533} Additionally, there are a wide range of functionalization chemistries available to QDs, making them now suitable for many biochemical assays.^{349,351,534} Their small size, high quantum yield, photonic stability, and ability to display diverse biochemical elements continue to make them an exceedingly attractive candidate for use in biothreat assay development.

QDs have already been used in a wide variety of assays targeting biothreat agent detection.⁵²⁹ Several groups have demonstrated QD detection of *E. coli* O157 in a variety of formats including array-based,⁵³⁵ antibody-based,⁵³⁶⁻⁵⁴⁰ and even using a cell phone-capable

readout.⁵⁴¹ The latter, example in particular, provides an excellent demonstration of the future potential of low-cost, portable assay detection setups. Here the authors constructed an antibody sandwich assay to detect whole bacterial cells in a 10× parallel glass capillary array, using excitation by inexpensive LEDs, and with a smartphone camera lens for readout. Using this format, a detection limit of ~5-10 CFU/mL was achieved in a complex, milk-protein background, highlighting the potential of simple and efficient assays that rely on non-scientific instrumentation.

The bioassay recognition and enrichment modalities exploited in these QD assays range quite widely based on the biomolecule displayed on their surface and its signature. In addition to antibodies, QDs have also incorporated aptamers,^{542,543} other types of nucleic acids,⁵⁴⁴ and even carbohydrates⁵⁴⁵ for whole-cell bacterial detection. QDs are quite often utilized in conjunction with MNPs for separation and enrichment, a hybrid approach particular well-suited for the detection of low concentrations of foodborne pathogens in complex real-world sample backgrounds.⁵⁴⁶ This approach has been demonstrated for the detection of *E. coli* O157,⁵⁴⁷ *Listeria*,²⁵³ *C. jejuni*,⁵⁴² and *Salmonella*.^{548,549} The ability to perform multiplexed sample detection in such formats is often critical for increasing sample throughput.^{540,550} For example, Bruno *et al.* sought to detect and discern *C. jejuni* from other related foodborne pathogens such as *Listeria*, *E. coli* O157, and *Salmonella*.⁵⁴² By using specific and selective DNA aptamers attached to QDs and concentrating the sample using magnetic beads, they were able to detect 2.5 CFU/mL in buffer and 10-250 CFU/mL in various food matrix backgrounds; these are both well below the infectious dose levels of 400-500 CFU/mL as per CDC guidelines. In another example, Kim *et al.* used the combination of magnetic bead capture with apoferritin-QD-antibody nanoprobe to detect and differentiate amongst *F. tularensis*, *B. anthracis*, and *Y. pestis*, see **Figure 25**.⁵⁵¹ The limit of detection was determined to be 10⁴ CFU/mL for all three pathogens, although this limit for *F. tularensis* is lower than some other antibody-fluorophore detection methods, *albeit* in a single, targeted assay format.

Carbon dots (Cdots) and polymer dots (Pdts) are two other types of more recently developed QD-like NP materials and they differ substantially from their more well-known semiconducting brethren. Cdots are crystalline graphitic NPs with a quasi-spherical shape similar to that of standard QDs. They share many similar advantages to QDs, with multiple excitation/emission wavelengths available, high quantum yield, chemical stability, ready

surface functionalization, and low photobleaching.⁵⁵² They have a broader emission wavelength profile than standard QDs but demonstrate fluorescent upconversion properties.⁵⁵³ Cdots have already been shown effective in many detection assays,^{554,555} including coupled detection using aptamer sensors.⁵⁵⁶ Pdots are much newer and less well characterized, but consist of semiconducting polymers which provide many of the same advantageous fluorescent characteristics of QDs and Cdots.^{314,557} Pdots have already been applied for the detection of *Bacillus* spores as discussed in more detail below.⁵⁵⁸

Alternative Nanoparticulate Materials. Silica NPs are one of the fluorescent alternatives to QDs and this is primarily due to their ability to be doped with large quantities of fluorescent dyes. They have seen a variety of applications within direct bacteriological detection, including *E. coli*,⁵⁵⁹ *Salmonella*,⁵⁶⁰ DPA detection, LFAs, and FC.^{324,561,562} Other NP materials utilized in biothreat agent detection include polystyrene,⁵⁶³ fluorescent AuNPs,⁵⁶⁴ upconverting rare earth metal NPs,⁵⁶⁵ and micelles.⁵⁶⁶ As mentioned, MNPs are often used in the detection of bioagents,⁵⁶⁷ in conjugation with optical probes such as TiO₂ for the detection of *Salmonella*⁵⁶⁸ or fluorescently-labeled antibodies for *E. coli* O157.⁵⁶⁹

Dipicolinic Acid Detection in Bacterial Spores. DPA is one of the signature biochemical compounds found in all *Bacillus* spores, including *B. anthracis*.⁵⁷⁰ Found almost exclusively in the core of the spore, it comprises roughly 5-15% of the spore's dry weight. It is often complexed with a divalent metal cation, usually calcium (CaDPA). As one of the major *Bacillus* spore components, significant emphasis has been devoted to the detection of DPA in assays. One of the primary methods for sensing DPA is the use of lanthanide elements, such as erbium, europium, neodymium, and terbium. DPA is a tridentate molecule which can form a metal complex with selected metal ions, such as the lanthanides which, in turn, can serve as a luminescent reporter that benefits from their large Stokes shifts and long-lived luminescent emissions.^{480,571-574} However, standard assays using TbCl₃ still have several drawbacks as described by Barnes *et al.*⁵⁷⁵ They note that "luminescence-based quantitative analysis of spore counts or DPA concentration are unnecessarily complicated, as complexes of mixed DPA stoichiometry, emission brightness, and emission lifetimes are formed under biological conditions".

Developments and improvements to DNA assays are highly desirable and thus groups continue to explore alternative lanthanide elements and formulations⁵⁷⁵⁻⁵⁷⁷ as well as various other nanomaterial and NPs to improve DPA detection bioassays.^{558,561,578,579} Chen *et al.* demonstrated the use of Tb-based fluorescent Cdots for the detection of CaDPA, in which binding of the Tb receptor by DPA resulted in a ratiometric fluorescent change, see **Figure 26**.⁵⁷⁸ Many of these NPs employ Eu rather than Tb, which provides for an increased Stokes shift and redder emission. Ai *et al.* showed the detection of CaDPA *via* europium-labeled and fluorescent (fluorescein isothiocyanate or FITC-doped) silica NPs, using primarily fluorescence-based detection.⁵⁶¹ However, they also noted the potential colorimetric use of this approach, as higher CaDPA concentrations lead to visible changes in the solution color. Indeed, the colorimetric-only approach was corroborated in an alternative format by Gonçalves *et al.* where the betanin pigment from the red beet plant was complexed with solution-phase EuCl₃ to act as a competitive ligand to the Eu chelation complex.⁵⁸⁰ Addition of CaDPA, derived from *B. anthracis* spores, displaced the betanin pigment, resulting in a noticeable color change.

Chemiluminescent Sensors. Although organic fluorophores and fluorescent NPs dominate the field of fluorescence-based pathogen detection due to their small size, stability, and broad biochemical compatibility, chemiluminescent sensors have also been explored for their high sensitivity, low background, and circumvention of the need for an external light source.⁵⁸¹ This particular phenomenon is generally based on a redox process in which the reaction product has a different chemical structure from the initial reactant. As a consequence of a chemical reaction, a light emission is produced. Several hundred organic and inorganic compounds are at the origin of chemiluminescence reactions which can occur in liquid or solid phases.⁵⁸² Radical species formed during the process may interact and produce unstable intermediates that decompose with the formation of excited species that either deactivate to the ground state or transfer energy to other luminophore molecules of relatively high quantum yield. Given its great sensitivity, rapidity, safety and controllable emission rate, applications in food safety⁵⁸³ and clinical diagnosis are common.⁵⁸⁴ Several assays using some form of chemiluminescence output have been developed for the detection of *Salmonella*,⁵⁸⁵ *C. botulinum*,⁵⁸⁶ and *E. coli* O157.⁵⁸⁷ Chemiluminescence is often used as a readout in other sensor formats, such as microfluidic arrays.⁵⁸⁸ In one demonstrative report, Liebes *et al.* used a chemiluminescent optical fiber

immunosensor to detect *Brucella* spp. including *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis*.⁵⁸⁹ The use of an optical fiber format enabled the detection of a lower analyte concentration (1.95×10^4 CFU/ml for colorimetric ELISA, 2.44×10^3 CFU/ml for chemiluminescent ELISA, and 3.05×10^2 CFU/ml for optical fiber methodologies), while retaining the same sensitivity. See **Figure 27** for a schematic of the sensor format and some representative data.

Vibrational Spectroscopy and Surface-Enhanced Raman Spectroscopy. These methodologies in all their different manifestations represent another widely used method for the detection and characterization of bacterial pathogens. Typically, the sample is externally excited, which results in vibration of specific molecular bonds within the sample. These electronic oscillations are then picked up by the detectors, giving a unique peak signature based on the number of types of bonds that were excited. Raman spectroscopy (RS) and infrared (IR) spectroscopy are the two most prominent vibrational spectroscopy techniques for identifying bacteria.⁵⁹⁰⁻⁵⁹³ Most simplistically, IR measures the absorption of energy while RS measures an energy exchange referred to as the inelastic light scattering effect. However, the differences in these measurements also means these approaches can be complementary, capable of being run on the same sample simultaneously.⁵⁹⁴ RS was originally described in 1929 by Raman and Krishnan,⁵⁹⁵ however, due to the complexity of the measurements, IR became the dominant mode of chemical characterization, supplemented by large spectroscopic libraries for quick comparison. Many improvements since the 1990s have drastically expanded the capabilities of both these techniques, and due to their non-invasive and label-free capacity, they are still being widely explored for bacterial biothreat speciation determination.

RS is widely used for its ability to non-invasively determine the chemical structure of biological samples. Although the standard Raman signal is quite weak, it can be detected over a broad range of wavelengths. It can even be used in the IR, and unlike mid-IR spectroscopy, RS is much less sensitive to absorption by water, reducing the background signal generated in direct analysis of bacterial samples in the IR region.⁵⁹⁶ However, the Raman signal will decrease as a function of λ^{-4} (Rayleigh's law), resulting in a rapid drop-off in signal intensity. While this can be compensated for with higher energy, the latter often disrupts and degrades biological samples. Thus, it appears the visible spectrum represents the best portion of the spectrum to conduct

experiments for bacterial sensing to some extent.⁵⁹⁷ Furthermore, and in contrast to some of the other less utilized sensor approaches, RS has been used to detect many of the highest priority bioterror agents, whose spectral profiles are being added to a database for future reference.⁵⁹⁸ There have been several notable examples of RS detection of specific bioterror agents. A popular variation of RS is micro-RS, which combines conventional light microscopy with RS to enable single-cell identification. The Popp lab in Germany demonstrated this principle targeting the pathogens causing Brucellosis and Glanders. In the first instance, Meisel *et al.* used micro-RS to detect *Brucella* spp. with an accuracy of 92% in medium and 94% in milk, see **Figure 28**.⁵⁹⁹ Stockel *et al.* also used micro-RS to detect *Burkholderia* in feedstuff, with an accuracy between 93-98% in BSL1 conditions after inactivation.⁶⁰⁰ Another noteworthy report from Fort Detrick, MD, in conjunction with ChemImage Corp. and Applied Perception, Inc., demonstrated a ground-based robot capable of detecting chemical, biological, and explosive agents using RS, including bacterial agents such as *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, *Brucella abortus*, and *Francisella tularensis*.⁶⁰¹

The discovery of NP surface-enhanced RS (SERS) provided perhaps the largest advance in the field of bacterial detection, which brought the signal generated by a single molecule on par with that of fluorescent dyes.^{602,603} Now, many sensors incorporate a specific SERS component, which can detect bacterial bioterror agents based on their own unique spectral profile.^{604 598,605} There are now several approaches for detecting bacteria in this manner including exploiting solid substrate, colloidal suspension, direct NP-bacteria association, and discrete SERS tags.⁵⁹² SERS tags use metal NPs (often Au or Ag) to produce enhanced Raman signals specifically targeted to a biological analyte.⁶⁰⁶ Several such SERS-enhanced sensing studies have been conducted for the detection of foodborne pathogens such as *E. coli*⁶⁰⁷ and *Salmonella*.⁶⁰⁸ Papadopoulou *et al.* used SERS to strain-specifically detect *Y. pestis* using PCR amplicons to discriminate single nucleotide polymorphisms (SNPs) found within two of the *Yersinia* genes.⁶⁰⁹ Other prominent applications of SERS include the detection of DPA and direct detection of *B. anthracis* spores.⁶¹⁰ Impressive detection limits have been shown down to 10 spores/mL in as low as 10 min.^{611,612}

Infrared Spectroscopy. Bacterial detection using infrared (IR) spectroscopy has been around since the 1950s.⁶¹³ Although the IR spans a large part of the electromagnetic spectrum (10-12,500 cm⁻¹), most biological assays use the mid-IR (400-4000 cm⁻¹), due to the commonality of

absorbance frequencies and molecular vibrations at these wavelengths. While there has been some use of the 4000-12,500 cm^{-1} range, this often results in reduced absorptivity and higher background.⁶¹⁴ The advent of powerful computational analysis brought about by the use of Fourier transform IR (FT-IR), has since greatly expanded the usage of IR for bacterial detection.^{615,616} The ability to discern samples in complex backgrounds has resulted in FT-IR being widely explored for the detection of foodborne pathogens.^{614,617} Studies have explored FT-IR for the detection of *E. coli*,⁶¹⁷⁻⁶²⁰ *Salmonella*,^{621,622} and *Listeria*.^{623,624} In an interesting derivative technique, Wang and Irudayaraj coupled MNPs to AuNRs, combining both optical and magnetic properties. Using the MNPs for separation, detection was accomplished by monitoring changes in the UV-Vis-NIR spectrum, while NIR irradiation enabled pathogen ablation.⁶²⁵

Flow Cytometric Sensing. FC is a widely used analytical technique, most often used for studying and characterizing fluorescently labeled eukaryotic cells. In a standard flow cytometer, a pre-processing step fluorescently tags the target analyte with either a fluorescent dye (often on an aptamer or antibody) or capture particle. The solution is then run through a flow cell, and the analyte is interrogated with a laser, scattering light at a 0° angle (forward scatter) and a 90° angle (side scatter), along with generating fluorescence. In general, forward scatter *versus* side scatter is used to group similar particles together (gating), the fluorescence of which can be monitored within the gated population. The fluorescent tags are excited *via* lasers; the most common laser lines are blue (488 nm) and red (~640 nm), although multiple other laser lines are available depending on the system, even down into the UV (~375 nm). Once the fluorescent tag is excited, the emitted fluorescence is collected with a series of filters. Thus, on a single particle, a flow cytometer is able to generate a substantial amount of information from multiple scatter, excitation, and emission properties. This, coupled with the fact that flow cytometers can record hundreds of events per second, makes FC a promising technology for the detection of very low concentrations of biothreat agents originating from clinical samples and complex matrices.^{626,627}

Although FC has been used in the detection of bacterial biothreats, the detection itself still poses significant challenges. To start, bacteria are typically an order of magnitude smaller in size than most eukaryotic cells, which translates to a 1000× reduction in analytical volume comparatively. This smaller volume means a significant reduction in the number of potential

cellular fluorophore tags, resulting in a much lower fluorescent signal output. Bacterial cells are also less physiologically complex, reducing the amount of scatter generated from the lasers. Thus, bacterial detection often requires modified electronics and flow cells capable of extracting meaningful signals from the smaller bacterial cells. One method that has seen considerable use is the introduction of fluorescent NPs, particularly QDs, as a method of labeling target bacteria.^{628,629} The strong QD PL naturally lends itself as an excellent tag for the detection of the smaller bacterial cells.

As with FT-IR, the ability to detect rare events in complex backgrounds makes FC a strong candidate for detecting foodborne biothreats. In this vein, the detection of *E. coli* O157 has been demonstrated in numerous instances,⁵³⁷ along with a variety of supplemental techniques including microscopy,⁶³⁰ bioconjugated luminescent silica NPs,⁵⁶² MNP enrichment,^{631,632} dual-color FC,⁶³³ background reduction,⁶³⁴ and even in combination with MS.⁶³⁵ Other foodborne bacterial pathogens such as *Listeria*⁶³⁶ and *Salmonella*⁶³⁷ have also been similarly detected.

The most prominent use of FC for biothreat detection targeted *B. anthracis* and anthrax spores.⁶³⁸ Here, detection of the *Bacillus* species was done by labeling a generic *Bacillus* surface peptide (ATYP) with *R*-phycoerythrin (RPE), an extremely bright and stable light harvesting complex. Specific detection of infectious *B. anthracis* spores targeted the protective antigen protein, a surface marker considered to be a primary virulence factor. Although this study provided promising results on the usage of FC for *B. anthracis* speciation, there were still some notable limitations. First, there appeared to be a small but significant population which demonstrated a positive stain for PA, but not for the generic ATYP marker, indicating substantial surface heterogeneity within spores with a high virulence likelihood. Second, it is important to note that this method in its current iteration cannot distinguish between virulent PA⁺ *B. anthracis* strains and those that are nonvirulent (Ames versus Sterne strains, respectively). In a follow on study, RPE was found to be sufficient to label surface antigens, however, in this case QDs did not demonstrate the ability to bind to the spores, possible due to the complexity of the surface fibers, see **Figure 29**.⁶³⁹ These issues provide a useful reminder that there remains a large technological gap between a potential sensor in development and a final commercial product.

Microfluidic Sensor Formats. One of the primary drawbacks to traditional FC approaches for bacterial biothreat detection is the equipment setup itself. FC often requires both large sample

and sheath fluid volumes, with sheath fluid often requiring a high degree of purity. Additionally, the long travel distance of the sample, from intake to waste collection, makes containment of highly virulent pathogens problematic. Microfluidics and lab-on-chip (LOC) approaches have the potential to alleviate many of these concerns while retaining the advantages of traditional FC. These approaches can also incorporate many different types of sensing formats making them quite versatile.⁶⁴⁰⁻⁶⁴² Such microfluidic devices are part of the larger microelectromechanical systems (MEMS) family. Typical versions are fabricated using photolithography to etch the channels into polydimethylsiloxane (PDMS) and these are attached to a glass base, for example.^{643,644} Of course, there are many other materials iterations, fabrication techniques, and final functional formats. Such LOC systems, also known as micro-total analysis systems (μ TAS), can couple sample processing capabilities (including enrichment, lysis, amplification, and/or purification) to standard microfluidic detection regimes, with all necessary steps hopefully contained within a single device. These systems have several unique advantages that hold considerable promise for biosensor development. Their small size reduces much of the sample volume necessary for detection, while many of the sensor devices fully incorporate sample processing steps, reducing potential exposure. Moreover, while reusable devices for continuous sample monitoring are highly desirable, many microfluidic devices are often designed to be disposable, again decreasing exposure risk. Finally, microfluidics offer a straightforward method for multiplexed detection, with the use of multiple parallel channels, along with demonstrated single-molecule analytical capabilities.^{104,645-647}

Microfluidic/MEMS platforms have been used to detect many different biothreat agents using a wide variety of experimental setups.⁶⁴⁰ Due to full sample processing integration, they have been explored for on-site detection of foodborne pathogens,^{641,642} including, *E. coli* and *Salmonella*.⁶⁴⁸⁻⁶⁵⁰ Such devices have also been used for the capture of both sputum and airborne bacteria, as was demonstrated in the case of *M. tuberculosis*.^{651,652} Other efforts have used MEMS in the detection of *B. anthracis* Sterne spore germination⁶⁵³ or the detection of PCR amplicons targeting this same pathogen's genetic material.⁶⁵⁴

One of the biggest advantages of MEMS devices is the capacity for field portable detection of multiple pathogens. Dietzsch *et al.* demonstrated the detection of PCR amplicons targeting both *Yersinia pestis* and *Brucella melitensis*.⁶⁵⁵ Complete chip-based PCR processing reduced the time required from a conventional 3 hr run down to 1.25 hrs and demonstrated LODs

of 1 pg/ μ l and 10 pg/ μ l, translating roughly to 1.9×10^2 *Y. pestis* and 2.8×10^3 *B. melitensis* genomic copies, respectively. Although this remains far above standard infectious loads in clinical detection, it still serves as a powerful proof-of-concept. Other efforts represent more commercially viable approaches. An integrated microfluidic system with the capabilities of continuous air monitoring using both ELISA and PCR from ChipShop GmbH in Germany, has shown positive detection capabilities for *B. anthracis*, *Brucella abortus*, *B. melitensis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Coxiella burnetii*, *Francisella tularensis*, and *Yersinia pestis*.⁶⁵⁶⁻⁶⁵⁸ A second such device consists of a 4-plex optical microchip array named the SpectroSensTM (Stratophase Ltd), which demonstrated the simultaneous detection of *B. anthracis* spores (5×10^7 CFU/ml), *F. tularensis* (2×10^8 CFU/ml), *Vaccinia* virus (10^7 PFU/ml) and ricin toxin (250 ng/ml), showing the potential biochemical diversity available to multiplexed targeted analysis.⁶⁵⁹ The latter approach used an optical waveguide array, in which antibodies were able to capture the target analytes in a label-free system and which resulted in detectable changes to the refractive index, see **Figure 30**. Another well-characterized approach is the autonomous pathogen detection system (APDS), developed at Lawrence Livermore National Laboratory.⁶⁶⁰ This system employs an immunoassay-based format with individual Luminex-PCR confirmation and has been demonstrated for the detection of *B. anthracis* and *Y. pestis* cells, spores, lysates, and genomic material. More importantly, this system was tested in a real-world scenario, testing air samples in continuous operation over 74 days in a high-traffic mass-transit subway system.⁶⁶⁰

E-nose. Another promising avenue for the detection of multiplexed biothreat signature detection is the use of E-nose systems. These systems are colorimetric arrays which can detect volatile organic compounds (VOCs) released by bacterial growth in contained environmental setups such as culture. The presence and concentration of the VOC profile can provide a species-specific signature to identify the target bacteria.^{661,662} They have been used to identify *M. tuberculosis*⁶⁶³ and food-borne pathogens,⁶⁶⁴ along with discriminating between other biothreats. In one study, volatile sulfur compounds were used to differentiate between strains of *Burkholderia*,⁶⁶⁵ while in another report they were used to differentiate between strains of *B. anthracis*, *M. pseudotuberculosis*, *E. coli* and *Y. pestis*.⁶⁶⁶ In the latter, compound profiles were detected through the use of a panel of 80 different molecular dye indicators, which detected differences

based on various reactivity parameters including acid/base reactivity, pH, and redox potential, amongst others. In the case of two strains, one of *B. anthracis* and another of *Y. pestis*, this approach was able to detect single digit CFUs/plate, each achieving 100% accuracy and specificity, see **Figure 31**. Although a colorimetric approach, the variability of colors still requires substantial electronic detection capabilities as opposed to naked eye visualization along with appropriate data processing software. This is likely not a substantial limitation, as laboratories capable of culturing high BSL pathogens would almost assuredly have access to the needed imaging modalities.

Alternative Approaches. Finally, there are a number of less utilized or less developed spectroscopic approaches that still demonstrate potential for biosensing. These include, for example, Rayleigh scattering spectroscopy and spectroscopic ellipsometry. *E. coli* O157 was detected by Rayleigh scattering spectroscopy through the use of AuNRs.⁶⁶⁷ This example exploited the LSPR of the AuNRs where multiple AuNRs attach to the surface of the bacterium *via* antibody affinity. This results in an increase in the Rayleigh scattering intensity even at 50 CFU/mL, resulting in a sensitivity increase of nearly two orders of magnitude over similar colorimetric assays. Another approach used a photonic biosensor to detect *F. tularensis* using both interferometric and long period fiber grating sensing structures.⁶⁶⁸ In this approach, antibodies were coated on a nanostructured, fiber optic surface, and minimal capture of 10⁵ CFU of *F. tularensis* resulted in a refractive index change, recorded by spectroscopic ellipsometry. A fiber-based interferometric sensor was then used to detect nanogram quantities of DNA for strain typing.

It is worth noting that the goal of biosensing technology is to provide clearly defined discrimination between positive and negative samples. This is easily performed when the target analyte exists in concentrations that are well above the sensor's LOD, and a simple, binary readout is easily obtained. However, in dilute samples, discrimination between positive samples may not be readily observable, in which case mathematical analysis may provide an alternative approach to data analytics. This was achieved using the fractal analysis of the CANARY biosensor,⁶⁶⁹ by discriminating between *F. tularensis*, *Y. pestis*, *B. anthracis*, and other species.⁶⁷⁰ Morris and colleagues used this biosensor to obtain additional kinetic information regarding sensor detection, including binding and dissociation constants, important parameters

that are rarely explicitly accounted for in sensor design. In the dawning era of cheap yet powerful computing, it may be that data analysis becomes as critical to robust performance and information gathering as the underlying sensor design itself.

Viruses

Most simplistically, viruses are infectious agents consisting of DNA or RNA genetic material encased in a protein shell and require a host for replication.^{43,671} The latter also makes it debatable as to whether they strictly meet the criteria for being a true life form. The inability to grow outside of a host makes viruses more challenging to culture than bacteria, although this has not been an insurmountable impediment to their weaponization. Their highly infectious nature, dearth of treatment options, and potential for genetic manipulation have made viruses attractive candidates for offensive bioweapons programs. Moreover, with the exception of pneumonic plague, bacterial bioagents are poorly transmissible from person to person and their efficacy relies on an initial dissemination event, whereas viruses often have a much higher degree of transmissibility.

Outbreaks of endemic viruses often follow a seasonal trend. An infection is spread from person to person throughout the year, with disease becoming more common when a season peaks. A certain percentage of the population has immunity from previous infection and a certain percentage remains unexposed. The risk of a pandemic emerges with new pathogenic strains or results from increased virulence, increased transmissibility, or a jump from an animal reservoir; these scenarios often arise from genetic mutation or genetic reassortment.⁶⁷¹ When a virulent strain encounters a population without immunity from prior exposure, the infection rates can be astounding. In Boston in 1752, only 174 of the total population of susceptible people (*i.e.*, those who had not been previously infected or inoculated and remained in the city) escaped infection with smallpox.¹⁰ In cases of such extraordinary virulence, viruses may kill a host more quickly than they are transmitted to a new one and therefore fail to garner a foothold or leave behind so many survivors in the thinned population that herd immunity precludes establishment of an endemic disease.¹⁰ While pandemics of even the last hundred years have decimated populations, with the Spanish Flu of 1918-19 causing an estimated 20-50 million or likely more deaths, the threat of the next pandemic looms even larger in the age of international travel.⁶⁷² Diseases can now quickly hop around the globe, as occurred with the most recent outbreak of Ebola in West

Africa in 2014 in which travel of infected individuals resulted in infections in the U.S., Spain, and Italy. The potential for catastrophic viral outbreaks arising from either natural origins or a bioterrorism event make this a significant class of biothreat agent. Other viruses migrate far more slowly and can take decades to move from continent to continent, with modern travel again facilitating an outbreak after introduction into a naïve community, see **Figure 32**.

Overview of Common Viral Biothreats

Smallpox. Smallpox has been a shaping force in human history. The origin and first human encounter with variola virus cannot easily be determined. The historians of ancient Egyptian, Chinese, and Indian cultures report epidemics that included the formation of pustules as early the 3rd century of the Common Era. The first definitive identification of variola virus is far more recent, as reported by Duggan *et al.* with their detection of the pathogen in the remains of a 17th century mummified child.⁶⁷³ Regardless of its origin, variola virus spread around the globe, often moving with trade routes while devastating populations along the way. Smallpox is highly virulent and on average kills 3 of every 10 infected. In places with sufficiently large populations that supported endemic smallpox, as many as 10% of deaths in a non-epidemic year might be attributable to the disease.^{10,54} In non-endemic regions, the devastation was far worse. It was responsible for the decimation of the indigenous population in America after Spanish colonization in the 16th century, by some estimates leading to a 95% reduction in the native population, and was therefore a critical facilitating factor in the conquest of the Aztecs, Incans, and other pan-American tribes by European colonists.¹⁰ The threat posed by smallpox persisted well into modernity, killing 500 million people in the 20th century, in spite of near elimination of the disease in developed nations.¹ In 1966, the WHO began the Smallpox Eradication Programme, and in 1980, the program was declared a success, leaving only two declared stocks of the virus (at the CDC in Atlanta, Georgia, and Vector in Novosibirsk, Russia).

However, the virus’s stability, infectivity, and person-to-person transmissibility, coupled with the severity of the disease it produced made smallpox an ideal candidate for weaponization.^{1,5} Moreover, the end of endemic smallpox marked the end of wide-scale vaccination efforts, effectively increasing the virus’s viability as a biothreat. During the peak of its bioweapons program, the Soviet Union was believed to be producing up to 100 tons of weaponized smallpox annually. Accidental exposure of a civilian to smallpox during field tests in Aralsk in 1971

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3 resulted in ten cases, including three fatalities. With extraordinary effort, including
4 administration of 50,000 vaccines and a total quarantine of the city, an outbreak was averted.¹
5 Because the genetic sequence of smallpox is known, current concern over its use in bioterrorism
6 stems not only from possible leftovers from the Soviet weaponization program but also from the
7 potential for an individual or organization to recombinantly synthesize and reconstitute the virus.
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11 Smallpox is the disease caused by the variola virus, a member of the family of orthopox
12 viruses that is exclusive to humans. Other orthopox viruses with animal reservoirs are also
13 known to infect humans, including monkeypox and cowpox, though these diseases are less
14 severe in human hosts. Smallpox is most known for the appearance of lesions on the face, hands,
15 and forearms that gradually spread to cover the whole body (see **Figure 1**), although lesions
16 don't appear until 2-3 days after onset of flu-like symptoms (fever, vomiting, headache, *etc.*).^{1,2,21}
17 The disease is associated with what was described in the medical literature as 'toxemia,'
18 referring to the apparent severity of the illness that in many cases led to death. Because
19 vaccination programs made smallpox relatively uncommon in Western countries even well
20 before the complete eradication in 1980, modern medicine is largely unfamiliar with the disease,
21 and thus the origin of 'toxemia,' possibly a result of hypotension and shock, is not known. Basic
22 symptoms aside, the disease manifests in several clinical forms, detailed descriptions of which
23 can be found in the WHO's publication *Smallpox and its Eradication*.¹⁰
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26 While the horror smallpox engenders has permeated human history, even influencing
27 inheritance law in some cultures, such fears have largely been forgotten thanks to the
28 development of a smallpox vaccine by Edward Jenner. A century before viruses were
29 discovered, in 1796, Jenner recognized that exposure to cowpox prevented subsequent infection
30 by smallpox and proposed the idea of vaccination using cowpox. Due to poor record-keeping
31 over decades of propagation and viral mutation, it was recognized that the virus used in vaccines
32 could no longer be considered cowpox and was thus aptly renamed *Vaccinia*.^{10,671} Serious
33 vaccine-related health problems, including myopericarditis and often-fatal encephalitis, occurred
34 with sufficient frequency that targeted vaccination of those who had come into contact with
35 infected patients replaced full-scale population prophylactic vaccination in 1971. Nevertheless,
36 due to the risk of bioterrorism, military and healthcare workers in the U.S. are still sometimes
37 vaccinated (hiatus 1989-2003).^{1,2} The vaccine, which can offer protection even when
38 administered as many as four days after exposure, is part of the Strategic National Stockpile
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maintained by the CDC.^{1,2,5} Treatment for smallpox is mainly supportive, though there is some hope - and mixed evidence - that antiviral drugs may prove effective.^{1,2,5,21} Mortality rates from endemic smallpox with the ordinary (most common) presentation were historically 30% in unvaccinated individuals.²¹ The infectious and lethal doses have not been formally established.³

Viral Hemorrhagic Fevers. Viral hemorrhagic fevers (VHFs) are caused by a taxonomically diverse group of single-strand RNA viruses from four different viral families. The viral species are typically characterized by a geographic region where the disease is endemic and all are zoonotic in nature.^{1,2} Ebola and its cousin Marburg are the most well-known of all hemorrhagic viruses. They belong to the *Filoviridae* family, named for their filament-like shape, and are endemic to Africa, see **Figure 33**. Their natural reservoir is unknown, although bats are the most likely source.⁶⁷⁴⁻⁶⁷⁶ *Filoviridae* have some of the highest mortality rates of any virus, ranging from 20% to >90% and are thus considered especially significant threats for weaponization.⁶⁷⁷ Variability in mortality rates is known among the five documented strains of Ebola, which include Ebola Zaire (60-90% mortality), Ebola Sudan (40-60%), Bundibugyo Ebola (~25%), Côte d'Ivoire (Ivory Coast) Ebola (unknown), and Ebola Reston (non-pathogenic to humans). Ebola Zaire accounts for the majority of cases, including the 2014 West Africa outbreak in Guinea, Sierra Leone, and Liberia, which caused more than 11,000 deaths from nearly 27,000 probable and confirmed cases (as of May 2015, see **Figure 34**).⁶ The original outbreak of Marburg in Germany had a mortality of 22%, but subsequent outbreaks in Africa have been in the range of 70-85%.

Three other families of viruses also cause hemorrhagic fever (HF) in humans. *Arenaviridae* include a number of South American HFs, including Junin (Argentine HF) and Machupo (Bolivian HF), along with Lassa fever, which is endemic to West Africa. Rodents serve as the natural reservoir of the viruses on both continents. *Flaviviridae*, including Dengue, West Nile, and yellow fever, are transmitted by arthropods, *i.e.*, mosquitoes. The final family, *Bunyaviridae*, include three different genres, each with its own animal reservoir: Crimean-Congo HF (*Nairovirus*, tick-borne), Rift Valley fever (*Phelovirus*, mosquitoes and sandflies), and Sin Nombre (Hantavirus, rodents).^{1,678} Symptoms specific to HFs arise from changes in vascular permeability and microvascular damage, eventually leading to shock. Early symptoms of VHF infection, such as headache, fatigue, and muscle stiffness, are similar to other, more

common illnesses like malaria, making early clinical identification challenging. The molecular mechanisms of pathogenesis are still being studied, and uncertainty in this area has led to little success in specific antiviral treatments, though the recent Ebola outbreak has invigorated study on that disease. Instead, treatment consists largely of supportive care, which was shown in the 2014 West African outbreak to substantially lower mortality rates.⁶ The only VHF for which a vaccine is currently available in the U.S. is yellow fever. Experimental vaccines for Ebola, Argentine HF, and Rift Valley Fever are currently under development.^{1,2,679}

Due to the exceedingly high mortality, the dramatic nature of their clinical presentation, and their potential aerosol-based transmission, it is perhaps unsurprising that VHFs have been targeted for their potential as a bioweapon.^{1,2,21} It is believed that Ebola, Rift Valley fever, and yellow fever were developed for weaponized use by the US, USSR (later Russia), and possibly North Korea.¹

Influenza. The viruses causing influenza are from the taxonomic family *Orthomyxoviridae*. Three genera within the family consist of influenza A, B, and C types, which are in combination responsible for influenza in humans. Types A and C also infect other animals, including birds and other mammals.⁶⁸⁰ Influenza A viruses are broken down into subtypes based on variations in two key proteins, the surface hemagglutinin (H1-H18) and the enzyme neuraminidase (N1-N11). Influenza B viruses are indicated by lineages and strains.⁶⁸¹ One of the defining characteristics of the virus is its segmented genome, typically comprised of eight single-stranded RNA segments.⁶⁸² While RNA viruses are known for their high mutation rate, which leads to the rapid evolution of strains, the segmented genome of influenza enables reassortment, in which whole segments are rearranged to form a new virus. This ability is especially potent for strains that can infect multiple species to form hybrid influenza viruses. Reassortment is thought to be the mechanism responsible for recent appearances of avian (H5N1 and H7N9) and swine (H1N1 and N3N2) flus, which combined avian/human and swine/human strains to form the variant strains. The 2009 outbreak of H1N1 “Swine Flu” variant was recognized by the WHO as the first flu pandemic in over 40 years.⁶⁸¹ Initial reports suggested a higher-than-normal mortality, particularly in the under-30 age group in which mortality is rare, however, later reports emerged of lower-than-normal mortality compared to seasonal flu strains.^{683,684} This poor understanding underscores the challenge in assessing an emerging disease; even after the fact, some were

critical of an overly aggressive response from the WHO, while other reports indicate initial severity may have been underestimated.^{685,686}

Vaccination is the primary means of controlling the spread of influenza, but rapid changes in the protein that elicits an immune response means that the vaccine needs to be reformulated annually. Because production requires six months, experts must predict well ahead of time which strains will be most important in the upcoming 'flu season' based on the currently circulating strains.^{680,687} Three or four of the most important A and B strains are identified by the WHO and are then included in the annual vaccine batch. Complicating this process, influenza viruses can exhibit antigenic changes in the window between vaccine virus selection and the beginning of the influenza season or during the course of the influenza season, rendering the vaccine less effective. Moreover, the predominant strains at the time of vaccine formulation may well have been replaced by emergent strains by the time 'flu season' is underway, as was the case with the 2009 H1N1 pandemic.^{684,687} As a result, vaccine effectiveness can vary dramatically from year to year.

While influenza is not a traditional biothreat agent in that it has not been targeted for weaponization, it is nevertheless a substantial threat to human health. An estimated 250,000-500,000 people worldwide die annually from influenza, and the emergence of a pandemic strain like the 1918 "Spanish Flu," could have a far greater impact.⁶⁸⁸ The 1918 H1N1 influenza virus had fatality rates greater than 2.5%, vastly higher than the < 0.1% mortality rate associated with other flu pandemics. In 2009, a variant of H1N1 again emerged causing 185,000 laboratory confirmed deaths. This number is seen as a considerable underestimate of the true number of deaths associated with this pandemic as many cases were likely not reported.⁶⁸⁹ In addition to the H1N1 influenza virus, other subtypes such as avian influenza H5N1 and H5N7 are seen as looming threats that could potentially cause casualties beyond the 1918 pandemic should they develop the ability to be transferred from human to human.

In 2005, sequencing and subsequent reconstruction of the 1918 Spanish Flu strain allowed researchers to study the high pathogenicity of that strain.^{30,31} Unsurprisingly, the ability to resurrect such a deadly virus was met with public consternation, similar to when the sequence of the polio virus was released in 2002.^{690,691} Indeed, in instances like these, the availability of rapid and cheap full genome sequencing and synthesis can challenge the ethical balance between developing invaluable scientific knowledge and leaking a potential terrorist blueprint.^{672,692}

Coronaviruses and Acute Respiratory Syndrome. Two coronaviruses that cause acute respiratory syndrome emerged in the early 2000's. SARS was first described in 2003 after a widespread outbreak in Asia.⁶⁹³ After 8,000 cases and a 10% mortality rate reported in the first year, the outbreak fizzled out; and no cases have been reported since 2004.⁶⁹⁴ In 2012, another novel coronavirus with similar symptoms and epidemiology was discovered in an outbreak in Saudi Arabia; the disease was named Middle East Respiratory Syndrome (MERS).⁶⁹⁵ Since then, hundreds of cases have been documented annually, the majority of which have occurred in Saudi Arabia. In 2015, a large-scale outbreak involving at least 186 people in the Republic of Korea (South Korea) was traced to a single exported case from Saudi Arabia.

SARS-associated coronavirus (SARS-CoV) and MERS-associated coronavirus (MERS-CoV) belong to the *Coronaviridae* family and are enveloped viruses with a positive-sense, single-stranded RNA genome, which at 27-32 kb is the largest known RNA genome.^{696,697} The non-structural proteins are derived from two large polyproteins comprising the first two-thirds of the genome; the last third contains the structural proteins, including a spike glycoprotein that protrudes from the viral envelope, giving it the appearance of a corona. Both SARS-CoV and MERS-CoV are in the beta-coronavirus subfamily which inhabits predominately mammalian reservoirs.⁶⁹⁸ In the aftermath of the 2003 SARS outbreak, it was determined that the most likely source was a jump from either a bat or civet reservoir. Although several close relatives of MERS have also been found in bats, genetic differences have prevented precise determination of the original animal reservoir.⁶⁹⁹ The extraordinarily recent emergence of these viruses, within the last 5 years in the case of MERS, serves as an excellent reminder that new threats to human health regularly arise from unknown or unexpected sources. This may be due to natural evolution, antigenic shift within hosts, or a jump from a different species.

Physical Sensors

Direct or Label Free Detection

In an emergency or crisis situation, techniques that require no sample preparation, are robust, and which are incorporated into intuitive devices can greatly improve care and limit the spread of disease. To develop systems to satisfy these requirements researchers have begun to access so-called 'label free' techniques for the detection of viruses. These systems can demonstrate sensitivities equivalent to PCR, are highly portable, and typically require minimal

sample preparation making them appropriate for incorporation into POC diagnostic devices. Ideally, such systems would provide single virion detection in biological fluids, however, similar to most other biosensing approaches, these techniques have limitations to their sensitivity. In the case of direct physical detection methodologies, the binding of a single small viral particle can be a challenge as it is on a similar size scale as the wavelength of light that may be used to interrogate the system. Moreover, single virion binding events occur at what would be equivalent to a working concentration that is far below a binding or biorecognition element's effective binding constant.⁷⁰⁰ In the subsequent sections we highlight several platforms that are, despite their use of antibodies as capture elements, classified as label-free viral detection systems.

Quartz Crystal Microbalance. Kleo *et al.* utilized QCM-based analysis in conjunction with PCR amplification to detect DNA amplicons from Vaccinia virus, see **Figure 35**.⁷⁰¹ These studies demonstrated that the analysis time could be drastically reduced down to 15 min, a significant improvement when compared to other techniques such as PCR and ELISA alone. While they still employed initial PCR amplification of the target DNA, they only required ten PCR cycles to generate enough ssDNA material when using ~25 ng of starting DNA material. To further improve the frequency shift and the sensitivity of the assay, AuNPs were employed which were functionalized with ssDNA complementary to the viral amplicons. Using this method they were able to increase the frequency shift associated with viral detection twofold. The added mass of the NP is behind this increase in signal and sensitivity and this type of approach can be accomplished with many different types of NP materials.^{212,702,703} In another related example, Owen *et al.* developed a QCM-based strategy for detection of aerosolized influenza A virions.⁷⁰⁴ In these studies, the authors were able to detect as few as 4 virions/mL suggesting the device could be used for real-time monitoring scenarios.

QCM-based detectors have several advantages over other conventional viral detection platforms. Through the immobilization of proteins specific for different viral targets, QCM detectors are easily adapted to multiplexed formats.⁷⁰⁴⁻⁷⁰⁶ Additionally, the self-assembled monolayers deposited on the Au electrodes are typically stable under most conditions as are many antibody recognition elements. This allows these systems to be regenerated multiple times, decreasing the overall cost associated with each device when performing multiple assays.

Mass Spectrometry. Proteotyping is probably one of the most exploited features of MS for pathogen and especially for viral identification in clinical samples or within environmental applications.^{169,707} The signature peptides for a given virus can allow for rapid identification of the seasonal circulating subtypes in a global context along with identifying potential therapeutic targets.⁷⁰⁸ This was especially relevant in the H1N1 pandemic episode in 2009 as shown by Schwahn *et al.*⁷⁰⁹ Given the rapid evolution of influenza virus, the unique peptide fingerprint for the 2009 pandemic (H1N1) virus enabled an expeditious differentiation of it from the seasonal type A circulating virus. In addition, the highly pathogenic avian influenza strains responsible of high mortality rates can be identified according to mutations in the hemagglutinin protein. To accomplish this, Yea and coworkers included an *in vitro* translation of RT-PCR products from the virus and its transcripts followed by peptide mapping using MALDI-TOF.⁷¹⁰ As a result, they could detect mutant strains with pandemic potential at a much earlier stage. Jang and coworkers implemented an enhancement protocol for viral subtyping of the avian influenza and Newcastle Disease Virus by application of a prefractionation step utilizing detergent-based isolation which was followed by differential centrifugation; this served to simplify the MS spectral analysis and subsequent identification.⁷¹¹ Other sample pretreatment steps involved the use of functionalized MNPs coated with anti-hemagglutinin for H5N2 viral isolation. The captured hemagglutinins were later separated by acrylamide gel electrophoresis and subjected to peptide analysis by LC-MS/MS. This method provided results in less than 1 hr with good sensitivity and selectivity while also allowing for the subtyping of different influenza strains when different capture antibodies were used.⁷¹² Peptide mapping can also be quite relevant if a clinical sample is suspected of containing different respiratory viruses, where the use of MALDI or LC/MS makes it possible to identify certain viruses at the subtype level rapidly.⁷¹³ Furthermore, MS techniques are powerful enough to study not only a single viral proteome, but also provide related virus-host interaction maps.⁷¹⁴ In support of these applications, the bioinformatics algorithm FluClass was developed to help classify influenza virus from mass spectral data.⁷¹⁵ This software utilizes a novel random resampling function for the scoring of leaf nodes as part of the phylogenetic classification. Initial testing showed this algorithm correctly classifying both seasonal and regional influenza strains.

Biochemical Sensors

Immunoassay-Based Detection of Viral Agents. The use of immunoassays to detect viral agents still remains quite common and has been reviewed in detail elsewhere.⁷¹⁶⁻⁷¹⁹ The focus here is a brief overview of some recent advances with mostly ELISA-based methods and some remaining issues with this approach. Very specific detection through sandwich ELISA (sELISA) for different viruses is typically achieved through the use of monoclonal antibodies. For example, Luo *et al.* demonstrated discrimination of the H5 subtype influenza from a panel of fifteen avian influenza subtypes (H1-H15) in clinical veterinary samples using collected swabs and tissues obtained from animals suspected of infection.⁷²⁰ Additionally, they observed very low LODs down to approximately 1 ng of viral protein. Relying on this sandwich format ELISA, bird carriers of disease could be detected in as little as four hrs potentially limiting the consequences of an outbreak. This type of surveillance tool is especially convenient for field application in poultry that are meant for human consumption.⁷²¹ While avian influenza H5N1 is by far the better known strain, avian influenza virus H7N9 shows a higher rate of transmissibility to humans leading Yu *et al.* to develop an sELISA for the detection of this viral pathogen.⁷²² To generate the antibodies required, mice were immunized with a DNA-based vaccine encoding the neuraminidase open reading frame followed by boosts with recombinantly produced N9 protein. From these successive vaccinations, an antibody pair was isolated that allowed for discrimination of closely related influenza viruses. In general, due to their specificity and lower cross reactivity, use of monoclonal antibodies can often result in better LODs.^{722,723}

Not all sandwich immunoassays require the isolation of monoclonal antibody (mAb) pairs. Palaniyappan and coworkers recombinantly produced the nucleocapsid protein from SARS-CoV, and used it for immunization of mice and chickens to develop antibodies for use in an ELISA targeting the virus.⁷²⁴ The assay was designed to use a hetero-sandwich format with a mouse mAb as the capture antibody and the polyclonal IgY collected from the yolks of immunized chicken eggs as the detecting antibody. The authors were able to detect nucleocapsid protein over a dynamic concentration of range of 9.2 pg/mL - 2.5 µg/mL.

Competitive ELISA formats are often used to improve the specificity of diagnostic assays. Dea *et al.* used a DNA-based vaccine to generate an immune response to the porcine reproductive and respiratory syndrome virus (PRRSV) that is considered an economically relevant pig pathogen.⁷²⁵ Here, two monoclonal antibodies isolated from the ascites fluid of

pristane-primed mice were shown to be viable partners for a competitive ELISA. The nucleocapsid protein of PRRSV was produced recombinantly in bacteria and served as the antigen. Sera of infected and control pigs were tested with a competitive ELISA which showed a specificity and sensitivity (95%) comparable to immunofluorescence based indirect assays. Elevated specificity and sensitivity was also shown by Fukushi *et al.* for the detection of MERS-CoV.⁷²³ Here, monoclonal antibodies for the spike protein of MERS-CoV were paired and tested with sera obtained from Ethiopian dromedary camels. The authors observed a 98% sensitivity and 100% specificity in the tested samples.

Indirect and direct ELISA formats commonly provide for a rapid method of testing clinical samples for exposure to the presence of viral antigens. Typically, abundant proteins of the viral capsid or immunodominant proteins that are encountered during infection are produced recombinantly and immobilized to microtiter plate wells to serve as capture reagents. Given the voracity of viral infection and the need for a rapid assay for diagnosis, the direct ELISA format has been extensively explored for the development of quick and inexpensive immunoassays for various filovirus infections. Nakayama and coworkers produced His-tagged forms of the transmembrane glycoprotein from nine Ebola and one Marburg virus species to develop a diagnostic assay for antibodies specific for filovirus. While successful, the authors did observe some cross reactivity with glycoproteins from multiple filovirus species, a complication that is often encountered with closely related viral species.⁷²⁶ As an alternative to depending on the glycoprotein antigen, Huang *et al.* used His-tagged recombinant nucleoproteins of Ebola and Marburg virus to capture antigen-specific antibodies in human sera.⁷²⁷ In an attempt to eliminate cross-reactivity and achieve the greatest level of sensitivity and specificity, Krähling *et al.* used inactivated *Zaire ebolavirus* particles as their capture antigen. This format allowed the researchers to capture and detect IgG specific for all of the viral proteins simultaneously.⁷²⁸

Large-scale production of animals for human consumption comes with the potential for deadly, rapidly-evolving viruses that can decimate the flock and potentially jump to human workers. With this in mind, the differentiation of vaccinated specimens in poultry or livestock is key to understanding and controlling the spreading of diseases and, in particular, (avian) influenza. To achieve this, continuous serological studies must be carried out and, although different alternatives are available,⁷²⁹ ELISA remains one of the most widely extended approaches. As an example, surveillance of poultry for avian influenza in countries where it is

highly endemic such as Indonesia is mandatory. Wibowo and coworkers compared two ELISA formats to detect antibodies for the influenza M2e structural protein, allowing for differentiation of immunized and non-immunized birds.⁷³⁰ Although its immunogenicity is relatively low, M2 protein is expressed in infected cells, triggering an immune response and producing antibodies which can be detected. In this particular case, the authors used monomeric and tetrameric M2e antigen as capture reagents and tested sera from various flocks around the country. The tetrameric version of the antigen was found to be more conducive to this assay format and could be used to identify fowl that had been exposed to the H5N1 virus.

Recombinant production of viral proteins is often challenging and in some cases even impossible with standard laboratory bacterial and mammalian expression strains. In some instances, short peptide fragments for specific viral proteins can prove sufficient as capture reagents. Mavrouli and coworkers used an ELISA method based on synthetic peptides derived from the hemagglutinin and neuraminidase originating from the 2009 pandemic H1N1 strain to study the overall immunization of a large Greek population.⁷³¹ Careful design of their synthetic peptides was key to showing that an assay could be developed that discriminated between the pandemic H1N1 influenza strain and the less deadly seasonal flu that was circulating the country at the time study. A similar approach could be followed for the serologic surveillance of human and poultry against the H5N1 influenza virus.^{732,733} In these studies the focus was on demonstrating the cost effectiveness of a synthetic peptide ELISA approach which could be a limiting factor for the development of an immunoassay for use in developing countries and rural areas. In some instances, diagnostic and detection assays can be developed using peptides specifically designed from the antigen-antibody interaction. These synthetic peptides, often referred to as “mimotopes,” can be employed as surrogates for the natural epitopes of a viral antigen. Typically, these anti-idiotypic peptides are isolated from large random libraries using some form of high-throughput selection system such as phage display. This technique was exploited by Chen *et al.* to isolate a mimotope for the avian H5N1 neutralizing antibody 8H5.⁷³⁴ In addition to mapping the neutralizing epitope of the virus, this peptide could also be used to detect other antibodies that bound this epitope.

A continuing focus of many research efforts in this area is obtaining improved sensitivities within diagnostic immunoassays. To this aim, Jian-umpunkul *et al.* explored the use of an immunoassay that employed three monoclonal capture antibodies, each specific for a

different influenza protein.⁷³⁵ They generated monoclonal antibodies specific to the nucleoprotein, matrix protein, and non-structural protein of influenza A virus as each is considered highly abundant in infected cells. With this approach, sensitivities 4-fold higher than those achieved for individual antigen tests were achieved. Similarly, Chen *et al.* developed an immunoassay for detecting MERS that utilized two monoclonal antibodies isolated against a nucleocapsid protein to aid in diagnosis of infection.⁷³⁶ As an alternative to the more traditional method of diagnosis through PCR, this latter alternative is cheaper, faster and simpler making it a viable alternative for use in challenging Middle Eastern regions.

Although ELISA is a powerful tool for rapid diagnostic and serologic surveillance studies, it still presents some drawbacks, especially in terms of cross-reactivity when looking for certain antibodies. This phenomenon leads to an overall decline in sensitivity and specificity in some assays. As an example, Stelzer-Braid and coworkers demonstrated how a commercial ELISA test, designed to detect H5 hemagglutinin antigen antibodies can cross-react towards seasonal H3N2 and H1N1 antibodies in human sera from vaccinated patients.⁷³⁷ Such cross-reactivity is not actually uncommon, therefore in designing ELISA assays substantial effort needs to be initially expended to limit these possibilities. Inclusion of numerous controls with both target and cross-reactive antigens are also necessary to ensure proper interpretation of experimental results. While sensitivity is often a point of concern, methods are constantly evolving to improve immunoassays. As an example, sub-fM LODs can be achieved by the use of digital-ELISA. By isolating and detecting single immunocomplexes in arrays of femtoliter-volume wells, digital ELISA enables clinically important proteins in complex matrices to be detected at very low concentrations.⁷³⁸ This ultrasensitive platform has recently been used to detect influenza nucleoprotein in nasopharyngeal swabs at fM level, see **Figure 36** for a schematic.⁷³⁹ While ELISA does have limitations, its incredible versatility, ease of use, amenability to incorporating both monoclonal and polyclonal antibodies from many different sources, and relatively low-cost will ensure that immunoassay technologies remain viable for detecting viral pathogens for years to come.

Lateral Flow Assays for Viral Detection/Identification. LFAs are currently being developed for viral detection in a wide range of applications including monitoring livestock, crops, and human diseases, see **Table 12** for a representative overview. For a number of reasons, LFAs are

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attractive as POC diagnostics especially in resource limited locales because they are easy to manufacture and store along with deploy, cheap to make, usually require no special equipment or sample preparation, and yield intuitive results. For some viral pathogens for which there are no available treatments or vaccines, such as Ebola, the only recourse in a bioattack or emergent epidemic is to rapidly identify and quarantine infected persons thereby limiting further spread within the local population. To aid in identification, highly sensitive, rapid, and easily deployable diagnostics are needed with LFAs having the ability to potentially fulfill this role. Despite their many advantages, LFAs often suffer from low levels of sensitivity compared to PCR-based assays. Research efforts have thus focused intensely on all aspects of the LFA assay format to improve sensitivity without sacrificing its overall design simplicity to a large extent. This includes creating extremely sensitive yet robust recognition elements, brighter reporter elements, and even engineering the fluidics or the device itself to maximize signal.

Table 12. Lateral Flow Assays for Viral Detection

Type	LOD	Time	Reporter Probe	Detection Probe	Analyte Probe	Viral target	Multiplexing	Ref.
Wicking	47 TCID ₅₀ mL ⁻¹	15 min	AuNP (15,40 nm)	Antibody	Antibody	Influenza A	No	740
Electrophoresis	---	60 min	Dye (AF488)	Antibody	Antibody	Hepatitis C	Yes	741
Wicking	0.13 ng/mL	10 min	Enzyme	Antibody	Bacteriophage	--	No	742
Wicking	250 ng/mL	30 min	Cy5-silica NP	Antibody	mAb	Influenza A	No	743
Wicking	0.1 nM	15 min	AuNP aggregates	Nucleic acid	Streptavidin capture probe	HIV-1	No	744
Wicking	5x10 ⁷ PFU/mL	25 min	AuNP	Antibody		M13 bacteriophage	No	745
Wicking	0.5-2.5 nM		AuNP	Antibody	Antibody	Dengue	No	746
				Nucleic acid	Nucleic acid			
Wicking	0.016 (H5) 0.25 HAU (H9)	15 min	QD	Antibody	Antibody	Influenza A H5, H9	Yes	747
Wicking	10 ² IU/mL	60 min	AuNP	Nucleic acid	Nucleic acid	Hepatitis B	No	748
Wicking	6.7 ng/mL	15 min	Au nanostars	Au nanostars	Antibody	Influenza A	No	749
Wicking	0.24 pg/mL	---	AuNP	Nucleic acid		HIV-1	No	750
Wicking	10 ⁶ copies/mL	45 min		Nucleic acid		Influenza A	No	751
Wicking	2.5 ug/mL	15 min	AuNP	Nucleic acid	Nucleic acid	Nucleic acid	No	752

TCID₅₀ - median tissue culture infective dose, mAb - monoclonal antibody, HAU - hemagglutinating unit, IU - International Unit

Recognition Elements. For any detection technique, sensitivity will ultimately rely on the specificity of the binding/detecting element. Because a lot of POC diagnostics rely on antibodies as such an element, there are substantial research efforts underway to find antibodies that can identify both the type of virus along with its subtype if applicable. The isolation of antibodies with such specificity is often challenging due to the genetic diversity of viral species and their rate of mutation which can often lead to changes in viral epitopes previously recognized by the antibodies. To develop successful immunological tools – a balance between finding antibodies for highly conserved areas of the virus and which still recognize unique signatures of different viral subtypes must be attained. Ou *et al.* developed antibodies to detect and differentiate various strains of Ebola virus utilizing the surface envelope glycoprotein GP_{1,2} which is involved with cell entry as a target.^{753,754} They showed these antibodies could be used in a number of diagnostic assays including ELISA, FACS, or Western blots. Though the LFA format was not specifically examined, the antibody pairs identified in these studies were shown as effective detection elements and could readily be adapted to an LFA format.

Most LFAs usually incorporate only one target element or antibody for detection. To increase the sensitivity of an LFA, multiple viral specific detection elements can be incorporated. For example, Wiriyachaiporn *et al.* used influenza A as a model antigen in a LFA that incorporated two types of AuNPs and two types of detection elements.⁷⁴⁰ The detector probe was composed of AuNPs conjugated to monoclonal antibodies against nucleoprotein and matrix protein which are two of the most abundant influenza A proteins present. The use of two antibodies simultaneously with each specific for a highly abundant viral protein contributed to increased accumulation of the 15 nm AuNP at the test line of the LFA strip. Biotinylated BSA was also immobilized to the 15 nm AuNP to serve both as a blocking agent and as the capture antigen for the second AuNP layer. Here, 40 nm AuNP functionalized with anti-biotin monoclonal antibodies served to form the second layer amplifying the LFA output signal intensity. This approach demonstrated an LOD of 47 TCID₅₀/mL (50% tissue culture infectious dose) within 15 mins and performed better than a conventional assay that contained only one type of antibody target by a factor of 8.

Reporter Elements. In traditional LFA formats, antigens bind to antibodies or other capture elements immobilized on a solid support such as nitrocellulose. Visualization of capture antigen

is then achieved through the accumulation of AuNP functionalized with antibodies capable of binding epitopes on the antigen, effectively sandwiching the virus to the test line of the LFA. When enough bound antigen has accumulated at the test line a positive result can be discerned by the naked eye. While this LFA format is tried and true, researchers continuously seek to improve this format to boost the overall LOD of their assay.

Enzyme-Based Reporters. The catalytic activity of enzymes makes them useful tools for improving the signal and LOD in a large number of assay formats including LFAs. Indeed, enzymes conjugated to monoclonal or polyclonal antibodies have long been used as reporters in ELISA and Western blot assays.^{755,756} With these assays, as with LFA, the goal is to accumulate enzyme at a specific location where its continuous catalytic activity toward a precipitating or other type of substrate can generate enough signal to allow visualization. When employing antibodies as the recognition element that allow for accumulation of reporter enzymes, the latter are usually conjugated or somehow functionally coupled to the antibody, however, the total number of enzymes that can be localized to the test line is limited to the number of available epitopes on the target antigen. To overcome this limitation and improve the overall LOD of their assay, Adhikari *et al.* utilized an HRP enzyme that was assembled to the coat proteins (pVIII) of an M13 filamentous bacteriophage.⁷⁴² The goal was to achieve a high recognition area and signal output leading to greater LODs over conventional AuNP assays. The assay was developed for the detection of IgE, a model analyte that does have some clinical relevance in allergic responses. In practice, an M13 phage which had been modified with an aptamer recognition element and an HRP reporter element was used. The IgE aptamer bound to the bacteriophage could also bind to the anti-IgE antibody already bound to the test line of the LFA. The M13 protein coat provided numerous sites for enzyme attachment yielding higher sensitivities. The authors were able to achieve an LOD of 0.13 ng/mL IgE which was about ~100 times more sensitive than other IgE assays.

Though not an enzyme, Duan *et al.* used Fe₃O₄ MNPs as a nanozyme probe for the detection of the Ebola virus glycoprotein within a LFA format.⁷⁵⁷ They exploited the intrinsic peroxidase activity of MNPs to develop an immunochromatographic LFA test strip using a substrate buffer containing 3,3'-diaminobenzidine and H₂O₂. In the presence of Ebola virus, a colored reaction was generated at the test line. This assay format enhanced the signal at the test

line yielding an LOD of 1 ng/mL Ebola virus glycoprotein which was discernable by the naked eye. This achieved a 100-fold increase in sensitivity over the standard strip method and is as accurate as an ELISA test. This LFA could also be performed in under 30 mins without any special equipment required for analysis.

Quantum Dot Reporters. QDs are another emerging visualization reporter for use in LFAs. Additionally, because of their unique size dependent photophysical properties,⁵³³ QDs have the ability to be quite sensitive detection agents, sometimes approaching the single molecule level in standard assays,⁷⁵⁸ and can also be easily integrated into multiplexed assays.^{550,759-762} Wu *et al.* utilized QDs in an LFA for the simultaneous detection of both influenza A virus H5 and H9 subtypes.⁷⁴⁷ Using a traditional LFA format, influenza virions were captured at the test line *via* a mAb and then sandwiched with an antibody-labeled QD. When excited with UV light, a bright fluorescent band appeared at the test line. Using relatively low cost instrumentation, the amount of bound QD could be quantified based on the fluorescence intensity and correlated to the amount of virus present. In these studies, the LOD for the influenza virus ranged from 0.016 to 0.25 HAU depending on the virus subtype with results being obtained within 15 mins.

Improved Gold Nanoparticle Reporters. AuNPs are the most commonly employed reporter element in LFAs due to their low-cost and ease of use. In traditional LFA formats, AuNPs are functionalized with a biomolecule that recognizes and binds the target antigen leading to an accumulation at the test line which can be discerned by the naked eye. However, AuNPs are also able to be detected by SERS at extremely low concentrations making them unique reporter molecules for use in LFAs. In this vein, Maneepakon *et al.* developed a SERS-LFA for the detection of influenza A nucleoprotein.⁷⁴⁹ They utilized multi-branched Au nanostars coated with 4-aminothiophenol, a Raman active molecule, and monoclonal antibodies specific to influenza nucleoprotein. The bioconjugate served as both a SERS signal reporter and a detection probe. The multiple arms and the surface roughness of the Au nanostars increased the SERS performance because of the hot spot regions and the multiple sites available for bioconjugation. They achieved an LOD of 67 ng/mL for influenza A nucleoprotein by visual inspection and a 10-fold better LOD of 6.7 ng/mL utilizing SERS detection. Detection of the predominant seasonal circulating influenza A (H1N1) pdm09 variant at 5.6×10^3 TCID₅₀/mL in allantoic fluid was also

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3 shown. Fu *et al.* similarly utilized SERS and Raman reporter labeled-AuNPs in a LFA for the
4 detection of HIV-1 DNA, achieving an LOD of 0.24 pg/mL.⁷⁵⁰ This was ~1000 times more
5 sensitive than colorimetric or fluorescent detection methods routinely used in LFAs. Though
6 both methods demonstrate improved sensitivity, it is important to remember that the SERS
7 portion relies on additional equipment for signal readout and thus requires far more than the
8 prototypical “bare-bones” LFA.
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Increasing the sensitivity of an LFA sometimes means incorporating more steps to detect a signal which can mean more reagents and greater complexity in device operation. To amplify the signal while simultaneously detecting the target virus, Hu *et al.* utilized AuNP aggregates that formed as a result of complimentary oligonucleotide binding.⁷⁴⁴ To achieve the aggregates, the authors employed two types of AuNPs. The first type of AuNP was functionalized with a DNA amplification probe while the second type of AuNP was functionalized with a complimentary oligonucleotide sequence and a detector probe sequence which is designed to recognize a specific target sequence, see **Figure 37** for a process overview. Aggregates form when the two AuNP populations are combined as the amplification probe sequence hybridizes with the complimentary sequence forming an aggregate which can be visually observed. This method provided for a 2.5-fold improvement in LOD compared to conventional AuNP-based LFAs. Mao *et al.* also employed AuNPs in combination with HRP to probe for DNA in their LFA design.⁷⁵² Here, ssDNA complementary to target nucleic acid sequences was conjugated to AuNPs to facilitate capture. Test and control line nucleic acid sequences were also designed to be complementary to the target sequences facilitating accumulation of the AuNPs. A peroxide substrate was degraded by the HRP-labeled AuNPs to improve overall assay sensitivity. Using this method, detection of as little as 50 pM of target DNA was achieved. Although no specific virus was targeted in these studies, the authors were able to improve the overall LOD in an assay that provided results in as little as 15 mins which certainly suggests its relevance to this field. Finally, Mashayekhi *et al.* utilized a two-phase micellar system comprised of Triton X-114 to concentrate a model virus (bacteriophage M13) into the micelle-poor phase.⁷⁴⁵ Using this technique they were able to increase the LFA LOD by 10-fold to 5×10^7 PFU/mL with colloidal AuNP-antibody conjugates.

Improved Design Elements. Design features such as the length and width of the LFA, the location of the test and control lines, *etc.*, must all be considered when fabricating an LFA to increase diagnostic effectiveness. Choi *et al.* incorporated a simplistic fluid control strategy into the construction of their LFA device with the goal of increased sensitivity.⁷⁴⁸ This consisted of adding a paper-based shunt and a PDMS barrier allowing for fluidic delays to occur altering the reaction conditions and yielding a 10-fold enhancement in signal. They used this device to detect Hepatitis B virus in clinical blood samples as a prototype. This exercise confirmed the supposition that by understanding the underlying fluidic dynamics of a simplistic LFA, minor design changes could be fairly easily incorporated and these would act to significantly increase the sensitivity of the test. This result also suggests that many LFAs are not optimally configured nor functioning at peak efficiency (yet).

Environmental conditions such as temperature and relative humidity can also broadly affect the performance of LFAs. For LFAs utilizing nucleic acid hybridization or antigen/antibody interaction, Choi *et al.* created a device that could maintain a relative humidity of 60% with elevated temperatures of 55-60°C and 37-40°C for optimum nucleic acid hybridization or antigen antibody interactions, respectively.⁷⁴⁶ This provided for a 10-fold and 3-fold signal enhancement over ambient conditions, respectively. While this did improve signal and therefore sensitivity, success did require additional instrumentation which again complicated the overall assay and equipment simplicity desired from LFAs.

The majority of LFAs typically rely on wicking process(es) to move the analyte over the test and control sites. In contrast to this norm, Lin *et al.* used directed electrophoretic transport in a manner that they termed a lateral e-flow assay for multiplexed detection.⁷⁴¹ Utilizing simple lithographic techniques, antigens were immobilized along a microchannel filled with a polyacrylamide hydrogel in a barcode-like pattern. Low-power electrophoresis was then used to drive the analyte through to the capture antigen for the detection of Hepatitis C. Detection of this virus within human serum samples was accomplished in 60 mins. This technology appears to be well suited toward a setting that is near the patient while still having access to electricity and other equipment.

Next Generation Analysis. Beyond modifications to the assay itself, improvements in analysis that move beyond a simple “naked eye” evaluation can have a dramatic effect on the achievable

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3 LODs, sensitivity, and general utility of LFAs. In one pertinent example, Mudanyali *et al.*
4 developed a reader platform that connects to a smartphone for the analysis of a number of
5 common LFAs.⁷⁶³ One of the main goals here was to provide high-throughput detection in a
6 resource limited environment by incorporating an LED capable of imaging in both reflection and
7 transmission modes into the device. Smart application of the phone allowed digital processing of
8 test results within 0.2 seconds and also uploaded the results to a central server for presentation of
9 the geo-tagged results on a world map. In a similar study that employed commercial electronic
10 devices for LFA analysis, Feng *et al.* utilized Google Glass as their rapid diagnostic test reader
11 with the goal of achieving a deployable system that could be used for high-throughput analysis
12 (**Figure 38**).⁷⁶⁴ A smart application was written for Google Glass that could qualitatively and
13 quantitatively assess a number of different rapid diagnostic tests. As long as these tests contained
14 so called “Quick Response” codes (*i.e.*, a digital barcode), more than one test at a time could be
15 imaged with Google Glass’s camera. In practice, the collected images were digitally transmitted
16 to a server for processing before the results were returned to the user. The information was also
17 geo-tagged and stored on a spatiotemporal map to provide real-time statistics. This example
18 employed a qualitative (yes/no) HIV and a quantitative (0-200 ng/mL) prostate-specific antigen
19 test as prototypical tests. Such devices could enable healthcare professionals to monitor and track
20 outbreaks spurring rapid preventative measures while also providing testing capabilities in
21 resource limited environments. It is also important to note that the technologies in both of these
22 examples could be used with a wide range of currently available LFAs or other rapid POC
23 diagnostic tests.
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41 ***Advanced PCR Techniques for Viral Detection.*** PCR is considered one of the gold standard
42 methods for detecting and identifying viral agents. Researchers have demonstrated that through
43 the careful design of target-specific primers, optimization of reaction conditions, and use of
44 signal-amplifying reporters, viral agents can be detected to extremely low levels in a variety of
45 different sample matrices. Despite success in the development of laboratory and field diagnostic
46 devices and assays, PCR detection of viral agents is plagued by a number of limiting factors. As
47 a diagnostic tool, PCR requires well-controlled reactions that are often sensitive to polymerase
48 type, reaction buffer, cycling parameters, and the concentration and stability of key reaction
49 components. Additionally, variability of the viral genome that occurs through mutation or
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3 evolutionary divergence often requires assays that utilize nested primer pairs or probes to
4 accurately detect and quantitate viral load. Despite these limitations, PCR-based detection assays
5 are more amenable to large-scale screening of samples, multiplexing, consistent achievement of
6 very sensitive LODs, and can often be adapted to a field deployable format.⁷⁶⁵ For example,
7 Sikazwe *et al.* demonstrated the advantage of well-designed primer/probe combinations in their
8 efforts to detect and quantify rhinovirus-C *via* the amplification of a 296 bp region of the viral
9 genome.⁷⁶⁵ They were able to optimize the LOD and the linear dynamic range of the assay
10 detecting a range of rhinovirus-C genotypes in clinical samples. The key here was *in silico*
11 analysis of available rhinovirus-C sequences along with subsequent design of probes to provide
12 100% homology for targeting the corresponding viral genotypes
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21 Viral pathogens and the assays to detect them are an ever-changing area of scientific
22 research. This is not only due to new pathogens emerging from natural reservoirs to cause
23 regional epidemics but also due to the rapid evolution of viral genomes that, in turn, require the
24 redesign of once successful diagnostic tools. In the rest of this section we discuss a range of viral
25 detection assays starting with traditional PCR techniques and extending to new diagnostic tools
26 that combine PCR with other analytical tools for quantitation.
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32 Researchers have developed and implemented a significant number of PCR-based assays
33 for the detection and quantification of both viral DNA and RNA depending upon the virus type
34 in question. Real-time and/or qPCR, which utilizes a fluorescent reporter or beacon to monitor
35 and evaluate the formation of a target DNA sequence, is by far one of the most commonly
36 employed PCR-based assays for viral detection. For example, Liu *et al.* employed the DNA
37 intercalating dye SYBR-Green-I as a fluorescent probe in quantitative nested RT-qPCR for the
38 detection of two different hantaviruses in murine serum and blood samples. Detection of ~100
39 copies/ μ L without measureable cross-reactivity between the two hantavirus examined was
40 achieved.⁷⁶⁶ In addition to being highly sensitive, RT-PCR can be used to both detect viral
41 pathogens and survey for specific genetic markers simultaneously. Pachucki *et al.* designed
42 primer combinations capable of identifying the influenza A virus with greater sensitivity than the
43 Directigen Flu A test while also simultaneously screening viral genomes for the presence of the
44 amantadine-resistance genomic marker.⁷⁶⁷ This ability to both detect and characterize samples in
45 parallel is not usually easily achievable with immunoassay formats. The large number of
46 available dyes and reporters that can be conjugated to oligonucleotide probes has also allowed
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RT-PCR assays to be expanded to a multiplexed format for the detection of multiple viruses simultaneously. Schroeder *et al.* incorporated multiple probes in a multiplex PCR format using fluorescence to simultaneously detect and differentiate human-pathogenic poxviruses, validating their methodology with thirty-six human clinical samples. The LOD for this method was dependent on the type of the poxvirus targeted and the specificity of the probe but they were still able to achieve an LOD down to a remarkable 9.7 copies/assay for selected targets.⁷⁶⁸ The results from these types of multiplexed assays are typically visualized using either fluorescence or gel separation or even both.^{115,769} From an optical perspective, dense multiplexing with fewer fluorophores in an energy transfer configuration rather than relying on a lot more distinct dyes along with spectral decoding can limit the required photonic component while still enhancing this capability.⁷⁷⁰ High-speed massively parallel separations, such as those achieved with capillary electrophoresis can also dramatically increase sample analysis throughput.⁶⁴⁶

MBs have shown great promise for the development of viral detection assays that do not require more costly thermocyclers capable of real-time integrated fluorescence monitoring. MBs are hybridization probes designed to bind specific sequences of the target amplicon. These single stranded oligonucleotides are labeled with a fluorescent reporter at one end of the molecule and a quencher at the opposing end (schematic in **Figure 39**). The nucleotide sequence is designed in such a way as to ensure the formation of a stem-loop structure that brings the reporter and quencher within proximity. Hybridization occurs between the amplified target DNA sequence and the probe sequence that is located in the central loop structure. As this hybridization occurs, the reporter and quencher are separated restoring the fluorescence of the reporter.⁷⁷¹ At its root, MB incorporating PCR methodologies rely on a high degree of complementarity between the probe and the target sequence and this can allow for detection of gene sequences that vary by as little as a single nucleotide.⁷⁷² Under ideal conditions, MBs identify single target sequences with a high degree of accuracy and sensitivity. This method, however, occasionally suffers leaky signal due to destabilization of the probe structure as a result of reaction conditions or temperature constraints.⁷⁷³ To minimize this effect Lee *et al.* developed twinned MBs which activated upon base-pairing with PCR product that targeted the presence of the H5N2 influenza hemagglutinin and neuraminidase genes as template. A positive signal required MB binding to both amplicons, the product of a well-designed “AND” logic gate. This method of sample analysis was able to achieve an LOD of 120 pM in their laboratory trials. Additionally, they

demonstrated that this high specificity combined with the use of dual probes could also provide for a rapid method of subtyping influenza samples. The speed and sensitivity of MB-based diagnostic assays make them valuable tools for highly virulent viral pathogens where the time of diagnosis is critical to limiting viral spread through large populations of animals. McKillen *et al.* used MB technology to develop a fieldable diagnostic that could quickly identify and discriminate between swine viruses.⁷⁷⁴ In this instance, they showed that the sensitivity of the MB assay was as sensitive as current PCR methods and vastly superior to ELISA-based immunoassays. Integrating dynamic DNA technology into these types of assays could potentially allow for more complex “processing” or interpretation of the results using Boolean type logic.^{416,417,775,776} This would be especially helpful in multiplexing approaches or where both viral typing and the presence of other particular characterization markers are being sought.

LAMP is another PCR method that is well suited for field-based assays for viral detection. Unlike other PCR methods that require a thermocycler to achieve the melting, annealing, and elongation temperature commonly associated with DNA amplification, LAMP PCR is performed isothermally at a constant temperature between 60°C and 65°C. Here, a combination of reverse transcriptase and DNA polymerase facilitates the conversion of RNA sequences to DNA template and subsequent DNA amplification in a single reaction tube. This method can attain sensitivity levels equal to or even above traditional PCR and RT-PCR. For their detection of H5N1 influenza viremia, Tang *et al.* utilized immunoassay based-RT-LAMP to detect the viral particles in whole blood samples.⁷⁷⁷ They enriched for the viral particles by capturing virions onto PCR tubes using an immobilized mAb specific for the hemagglutinin protein on the capsid surface. Following thermal lysis of the capsid, RT-LAMP was then used to analyze the collected samples. Ge *et al.* also utilized RT-LAMP combined with a lateral flow device to detect H7N9 virus in clinical samples.⁷⁷⁸ In this study, primers for LAMP were conjugated to FITC and biotin which ensured integration of the reporter and capture moiety into the amplicons. Mobilization of amplicons across the lateral flow device allowed for accumulation at streptavidin coated sites which were then visualized *via* the FITC label.

With most PCR based methods of detection, accurate quantitation of target gene sequences requires that a standard curve be generated with each new assay to ensure comparability between individual runs. Digital droplet PCR (ddPCR), in contrast, exploits the dilution and partitioning of samples into numerous individual reactions or droplets which are

interrogated simultaneously. The reactions are typically assembled using reporters, primers, and conditions identical to real-time PCR. In individual reaction droplets there is a distribution of nucleic acid template ensuring a Poisson distribution across the field of samples, see **Figure 40** for an example of such data collected from a nanofluidic device. Statistical analysis of signal intensity across the hundreds of samples allows for a highly accurate determination of template concentration without the need of a standard curve.⁷⁷⁹⁻⁷⁸¹ An important advantage of ddPCR is its ability to identify mutant or low abundance nucleic acid sequences in a sample containing a high background of non-target sequences.⁷⁸² This is highly relevant to the detection of viral pathogens whose target genome may often comprise a very small proportion of a clinical sample. Larsson *et al.* employed ddPCR to screen clinical samples for the presence of 28 different genotype variants of the human papilloma virus (HPV).⁷⁸³ They were able to show LODs down to 1.6 copies of viral genome though this LOD did vary with viral genotype. Despite this impressive specificity, the authors did indicate that qPCR assays run in parallel showed a 1 to 31 times higher signal intensity. A similar observation was made by Hayden *et al.* in their efforts to quantitate cytomegalovirus.⁷⁸⁴ While real-time PCR methods were more sensitive, the ddPCR methods showed less variability across samples and again did not require standards for reliable quantitation of viral nucleic acids.

It should be noted that, in general, PCR techniques suffer from some similar drawbacks including requiring skilled technicians, access to thermocyclers and/or other analytical instruments which are likely to be expensive and centrally located in a laboratory setting, and maintenance of sterile and non-contaminating conditions. Though there are exceptions, most PCR-based assay require a significant time and labor investment in isolation of sample material along with the time to acquire the results. Therefore, using PCR even with a second technique might not be appropriate to screen large populations of people in a suspected bioterrorism event unless it is very carefully implemented. However, given PCR's high specificity and sensitivity it will always remain a tool for confirming suspected viral exposure. Paradoxically, one of the main advantages of PCR is also a drawback in some situations. Careful design of PCR primers allow very low levels of viral DNA to be detected, classified, and also subtyped in many instances. However, without exiting knowledge of the target pathogen or some unique sequences within its genome, the design of specific PCR primers can be very difficult to near impossible.

In these types of situations, use of degenerate primers that can potentially amplify up a variety of different target sequences may help to enrich a potential target sequence for further analysis.⁷⁸⁵

Numerous viral pathogens are classified as potential biothreats due largely to their lethality or lack of a suitable treatment strategy. Filoviruses such as Ebola and Marburg periodically emerge and become a source of global concern. While less lethal, Chikungunya, Dengue, and a number of encephalitic viruses such as West Nile and VEE virus are carefully monitored due to the rapidity at which they spread and the debilitating nature of their infection. Of greatest concern are the variola virus, the causative agent of smallpox, and the influenza virus both of which have long histories of devastation within human societies.

PCR and Smallpox. Differentiating variola virus from other closely related Orthopoxviruses often proves quite challenging for many assay formats. Through the design and utilization of highly specific primers in PCR-based assays, researchers are often able to distinguish smallpox from other species. Fedele *et al.* described a method employing real-time PCR based on TaqMan 3'-minor groove binder chemistry to detect variola and to differentiate it from other Orthopoxviruses such as cowpox or monkeypox.⁷⁸⁶ They incorporated an internal control to weed out the possibility of false negatives while their primers were designed to target the highly conserved genomic region of the cytokine response-modifying protein B (*crmB*) gene. Detection of 10 to 100 copies of variola virus per sample tube was achieved with their methodology with ~100 copies of variola virus being routinely found. One of the disadvantages of designing these probes is that false positives could occur if the probe can still bind but has a few mismatches, something that has been noted before in forensic PCR-based genotyping assays.⁷⁸⁷ Therefore stringent parameters are usually needed regarding probe design and melt temperature. Scaramozzino *et al.* also developed a methodology utilizing TaqMan chemistry to detect and distinguish variola from other Orthopoxviruses.⁷⁸⁸ They utilized nested PCR primers that first amplified a conserved genetic sequence encoding a 14-kD protein common among pathogenic Orthopoxviruses. Iterative rounds of PCR were used to first down-select for Orthopoxviruses positive samples which were then identified in the subsequent rounds of amplification. Using primers that differed by only a few nucleotides they were able to discriminate between the more pathogenic smallpox virus and the closely related cow and monkeypox viruses. A careful analysis of the signal intensity as compared to control reactions was critical to obtaining

consistently low LODs. This impressive study assessed 85 different Orthopoxviruses species and strains at concentrations of 100 ng/L to 1 μ g/L with a minimum of 0.05 fg of DNA (corresponding to 25 copies of DNA) being detected. While the work of Scaramozzino *et al.* highlights the amazing ability of PCR-based assays to detect very low levels of viral DNA with a high degree of specificity, these studies again allude to a common shortcoming of this technique. Subtle changes in genomic sequences of either the target pathogen or closely related viruses can dramatically affect the results of existing detection assays as shown by Kondas *et al.* in their analysis of assays directed against two genetic markers of variola virus.⁷⁸⁹ Here, two newly identified cowpox viruses were shown as the source of false positive results in PCR-based smallpox assays. Alignment of the new cowpox viral genomes with other members of the orthopoxvirus family revealed the confounding variations that gave rise to the false results. Primer design and target sequence information are critically important factors in the development of assays for viral pathogens that inherently undergo consistent genetic drift, recombination, and selection pressure. Put simply, reliance on a single or consistent method of detection will invariably lead to inaccurate detection assays that suffer from false positive/negative results or an inability to detect new strains that emerge from minor genetic mutations.

PCR and Influenza. While immunoassays are reliable for the identification of influenza virus and their discrimination into individual subtypes, this platform is not conducive to rapid analysis nor field deployable systems that can screen large populations of infected individuals or animals. For these purposes, PCR remains the gold standard. Additionally, researchers have shown that through careful experimental design, PCR-based methods can be as good as or even better than immunoassays for detecting and even subtyping influenza viruses. In the quest to go small, Angione *et al.* developed a methodology and prototypical device utilizing a droplet of clinical sample for real time, RT-PCR.⁷⁹⁰ RNA extracted from clinical samples was utilized in a droplet sandwich platform, a modified version of ddPCR, to detect and subtype influenza virus from the nasal swabs of 40 patients, see **Figure 41** for a schematic of the platform and process. Using this method, they were able to differentiate between H1N1p (p-pandemic), H1N1s (s-swine), and H3N2 with a 96% sensitivity. Their LOD was $\sim 10^4$ copies/mL which is two orders of magnitude lower than the viral load seen in typical patients. Of note, the sandwich platform they developed required very low voltage allowing thermal cycling to be controlled. Paired with USB-linked

software for control and data acquisition, the system was well suited for field distribution. Such distributable assays would be useful not only for the rapid analysis of human clinical samples but also the high-throughput testing of farm animals, a likely reservoir of future influenza pandemics.

PCR and Livestock Monitoring. Diagnostic targeting of viral pathogens has significant utility beyond the identification of biothreat agents. Today, facilities that house domesticated animals for human consumption typically harbor hundreds to thousands of animals in extremely close proximity to one another. In these facilities a viral infection can decimate a herd, costing a farmer undue financial hardship, and impacting the general population that is dependent on these food sources. Additionally, throughout history viral pathogens have repeatedly shown the ability to evolve into zoonotic diseases, able to jump from their animal reservoir to a human host.

Influenza viruses, particular avian influenza subtypes H5N1 and H5N7, have existed on the periphery of pandemic status for some time. The rapidity at which these strains spread through and decimate poultry flocks combined with the ever increasing number of cases of human fatalities in farm workers, has led to growing concern for the potential mutation that allows for human to human transmission. Researchers continue to develop assays for monitoring livestock with the dual-purpose use of rapid analysis of human clinical samples should a pandemic develop. As an example of these efforts, Deng *et al.* developed an assay based on immunological recognition of viral capsids and PCR amplification of a nucleic acid reporter sequence.⁷⁹¹ Their goal was to detect ultra-low concentrations of H5N1 such that infected chickens could be identified and removed from the rest of the population before spreading disease. By combining immunology with PCR, the authors were able to increase the overall detection sensitivity 100 to 1000 fold compared to ELISA or traditional PCR, respectively. In their approach, a mouse mAb specific for the H5 subtype of hemagglutinin served as the capture component to improve specificity for the H5N1 influenza strain. Additionally, restriction enzyme cleavage and purification of the DNA reporter molecule was also necessary to improve the subsequent PCR analysis of individual samples. Under optimized conditions, detection of a 10^{-4} EID₅₀ mL⁻¹ in a 100 μ L sample was achievable (EID₅₀ is a logarithmic measure of agent infectivity or potency). Samples taken from chickens and eggs demonstrated the utility of this methodology in mass screenings. While clearly very sensitive, the methodology, however, had a

number of disadvantages. The technique requires specialized equipment and trained personnel, therefore, direct commercialization for routine use at the point of concern is not currently feasible. The method also required numerous rounds of washing which contributes significantly to analysis time. Finally, the reaction required a target-specific mAb and optimized PCR reaction conditions, all of which would need to be developed for each new viral target.

While influenza virus is a target of grave concern for its pandemic potential other viral pathogens are no less devastating to the regions in which they are endemic. Foot-and-mouth disease caused by a picornavirus has a relatively low mortality rate but severely impacts food and trade.⁷⁹² Rift Valley fever is a zoonotic disease that has historically plagued Africa and the Middle East but is emerging as a pathogen of concern in European livestock as well (Mansfield 2015). The need for rapid, low-cost, high-throughput assays for the screening of livestock and poultry continues to increase as the concentration of animals increases with larger industrial farms. At present few assays that satisfy all the necessary requirements have been fully developed.⁷⁹³

PCR Combined with Other Techniques. In the quest to develop methodologies that have better LODs and improved accuracy with fewer false positives or negatives, biosensing assays have been developed that combine PCR with other analytical techniques. In most instances, the goal is to not only attain extremely low LODs but also to accomplish viral strain identification and perhaps even further subtyping if warranted. This capability could potentially allow for the identification of infected animals and individuals at preclinical levels of infection even before the onset of symptoms.

PCR with Electrospray Ionization Mass Spectrometry. Traditional PCR for the most part relies on highly specific primers that have been designed to detect a known viral pathogen. The successful use of PCR-based diagnostics in this scenario require that a healthcare professional or veterinarian has some *a priori* knowledge of what the causative agent is suspected to be. Alternatively, diagnosis may require large panels of assays capable of detecting the presence of many different causative agents. However, in the event of a bioterrorist attack, infected persons might present symptoms to health care officials difficult to attribute to a specific viral disease or pathogen. Given the sheer number of biothreat agents that exist and the need to quickly identify

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3 them, researchers have begun to develop shotgun approaches that combine PCR with ESI-
4 MS.^{186,794} The possibility of achieving ultralow LODs and the potential ability to detect a broad
5 range of pathogens without *a priori* sequence knowledge makes this approach useful for the
6 generalized detection of a biothreat or infectious agents. Here, we describe the approach in
7 relation to viruses but the technique has far reaching application beyond this to a plethora of
8 other infectious bacterial and fungal (bio)agents.
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13 At first description, the combinatorial methodology combining PCR and ESI-MS was
14 called Triangulation Identification for the Genetic Evaluation of Risks (TIGER) and was
15 suggested as a universal identification strategy.¹⁸⁶ The method, now referred to as PCR/ESI-MS,
16 utilizes broad range PCR primers that are designed to amplify genetic sequences that are
17 common in organisms of a taxonomic group.⁷⁹⁵ Amplified nucleic acid products are then
18 analyzed using ESI-MS to determine precise masses which are then used to calculate base
19 composition. Results are compared to databases containing sequence information for members of
20 the target taxonomic group allowing for identification of the source organism from which the
21 amplicon originated, see **Figure 42** for an overview. In contrast to many other sensing
22 modalities, detection here is based on the nucleotide composition and not the primary sequence.
23 For viral detection, primers would be designed for highly conserved regions that are found in
24 most, if not all members, of a given viral family. These “intelligent PCR primers” would target
25 the evolutionarily conserved regions that are flanked by variable genetic regions.
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36 In theory, one of the most important advantages of the PCR/ESI-MI technique is that a
37 healthcare professional need not have any idea what type of organism is present (viral, bacterial,
38 or fungal, *etc.*). This technique offers the ability to not only identify a pathogen down to strain
39 level in some instance but also the potential to identify novel organisms whether they be
40 naturally occurring or even recombinantly engineered. Given the ever evolving nature of viruses
41 this is definitely an advantage when compared to the many other biosensing approaches
42 discussed here. To validate PCR/ESI-MS for the detection of biothreat agents, Jeng *et al.* used
43 this technique to identify bacterial, fungal, and viral agents in bronchoalveolar lavage samples.¹⁸⁷
44 In addition to the pathogens present in the clinical samples, the authors spiked individual
45 samples with known viral threat agents to further assess specificity and LOD of the PCR/ESI-MS
46 method. In these studies, they showed a 99.0% concordance with biothreat spiking of samples
47 and a 96.6% concordance with viral pathogens. In addition to the high degree of accuracy in
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pathogen detection, the PCR/ESI-MS method significantly reduced analysis time by eliminating the need for cell culture. Despite this successful demonstration, interference from the background microbial populations present is always possible making pathogen identification difficult in some instances. This is especially true when a more populous organism is present within a sample leading to preferential amplification of certain gene sequences. Simultaneous pathogen detection, therefore, is only reliable when the original concentration of the organisms are of similar magnitude.¹⁸⁷

PCR/ESI-MS capabilities appear to be especially suited to detecting influenza virus. This capability is clinically relevant since, as mentioned, the influenza virus sees dramatic yearly changes that contribute to its seasonal variety along with that of other zoonotic strains that are closely monitored in large-scale livestock farms. Sampath *et al.* developed a high-throughput PCR-ESI-MS method that used eight parallel RT-PCR reactions to simultaneously survey for all influenza viruses.⁷⁹⁶ The assay not only provided clade-level resolution, but also allowed for the identification of viruses in mixed populations while also identifying genetic re-assortments. The utility of this assay was demonstrated through the direct analysis of more than 600 human clinical samples in which 92 mammalian and avian influenza isolates representing 30 different H and N subtypes were identified. Detection and identification was accomplished with a 97% sensitivity and specificity. Clearly, these capabilities provide a highly useful tool for viral surveillance.

PCR and Magnetic Beads. A limiting factor with many PCR-based assays is the need to isolate/purify source nucleic acids of sufficient concentration and purity for subsequent amplification. This is especially important for viral detection as the viral genome often is present as only a very small component of the total nucleic acid material recovered. In many instances, scientists have employed solid matrices such as resins or NPs that are functionalized with some capture element to allow for enrichment of virions or ideally, viral nucleic acids. Magnetic beads are often employed with these capture elements as a coupled method of separating biomolecules from complex samples.⁷⁹⁷

Their low cost and the simplicity of separation using magnets have long made magnetic bead useful tools for high-throughput applications such as SELEX and phage display. Huang *et al.* utilized a combination of magnetic beads and AuNPs to separate and identify RNA and DNA

genomic sequences from porcine viruses *via* PCR.⁷⁹⁸ Samples from pigs were first boiled in a lysis buffer to release the nucleic acid material from both virions and host cells. Viral nucleic acids were then captured by magnetic beads coated with a viral DNA probe sequences through complementary base pairing (**Figure 43**). AuNPs functionalized with virus specific oligonucleotides were then added and captured forming a complex that could readily be separated using a magnet. The probe nucleic acid could be released from the magnetic bead complex using a reducing agent such as dithiothreitol. A subsequent PCR reaction using the concentrated nucleic acid probe allowed for a 250-fold improvement in viral detection capability. Xing *et al.* also developed a methodology employing magnetic beads while utilizing rolling circle amplification as the basis for H1N1 detection.⁷⁹⁹ Here, magnetic beads were chemically functionalized with an oligonucleotide probe designed to capture H1N1 DNA. A second reporter primer which was complementary to regions of the influenza genome and serves as the template for rolling circle replication was then added. These components spontaneously assemble through base pair complementarity resulting in the formation of a complex that can then be isothermally amplified using phi29 polymerase (**Figure 44**). Rolling circle replication produces an extended ssDNA molecule that can be 1000 times more abundant than the starting viral genomic segment. Subsequent incubation of reaction products with unmodified AuNPs allowed for confirmation of the rolling circular amplicons which were correlated to presence or absence of influenza genetic sequences. The negatively charged DNA backbone served to also localize the AuNP which can subsequently be monitored spectroscopically and even visually at higher amplicon abundance by changes to their surface plasmon. In this protocol, as little 1 pM of viral nucleic acid could be detected spectroscopically while 100 pM or greater concentrations could be visually detected by the naked eye as the AuNPs aggregated producing a purple color. While not demonstrating the effectiveness of this assay with clinical samples *per se*, this method is notable for by its low cost, simplicity, and use of isothermal amplification which eliminates the need for more costly PCR equipment.

Viral Detection using Next Generation or Whole Genome Sequencing. As mentioned in other sections, identification of viral pathogens using PCR is most often accomplished through the amplification and detection of a limited number of genes specific for the viral targets. Though

these detection platforms are successful a majority of the time, careful design of primers/probes is crucial to success and can lead to false negative results if sufficient genetic variability or variation exists. As the cost of NGS/WGS technologies have rapidly declined, the potential use of these systems for viral detection and identification has become far more feasible. Though they come with limitations of their own, NGS/WGS methods eliminate the need for careful primer/probe predesign and also allow for the identification of any genetic variations or drug resistance markers that may have been acquired to be identified. Houldcroft *et al.* demonstrated the advantage of WGS for the identification of resistance markers in human cytomegalovirus samples from immunocompromised patients.⁸⁰⁰ Over the course of their analysis, they identified specific genetic mutations in the viral population that contributed to the emergence of drug-resistant variants which, in turn, contributed to a poorer prognosis for the patient. These results were consistent with others examining human cytomegalovirus infection in hospital infections.^{801,802}

Sequencing and identification of viral genomes can be complicated by both low viral abundance within collected samples and the presence of contaminating host nucleic acids.^{114,803,804} Often, researchers or clinicians that are seeking to identify specific viral pathogen utilize designer protocols to either capture and enrich the virions themselves or amplify conserved viral genes that can then be sequenced for more detailed identification. Such protocols, however, are not always applicable particularly when specific viral pathogens are not known *a priori* or if the viral genome is prone to genetic variability that make probe/primer design difficult. In the broadest sense, this type of sample preparation and experimental design can be categorized as either metagenomic sequencing, PCR amplification sequencing, or target enrichment sequencing as shown in **Figure 45**. Each of these methods have their advantages and disadvantages and these are discussed in detail in ref.¹¹⁴

Clinical identification of viral pathogens has traditionally been accomplished through a variety of biochemical and molecular biology techniques including cell culturing, microscopy, immuno-based assays and PCR.⁸⁰⁵⁻⁸⁰⁷

Through iterative methods of analysis, researchers have had significant successes using these techniques to identify known viruses. However, when novel or divergent viruses or variants are the source of infection, these methods can often fail to identify the viral pathogen.⁸⁰⁸ In contrast to these methods that often rely on identification of a specific virus or viral family, metagenomic

analysis of collected samples can identify any and all viruses contained within the sample. While language varies in the literature, here we define metagenomic sequencing as NGS/WGS of raw nucleic acid material without enrichment of viral particles or viral nucleic acids. In these instances, nucleic acid preparations contain total RNA and DNA from all host, bacteria, viruses, and fungi that are found within the sample. Within these samples, viral sequences comprise only a very small percentage of the total reads obtained; this is often well below 0.01% of the total nucleic acid material.^{809,810} Despite the low concentration of available viral material, such metagenomic analytical methods are now routinely used to identify both novel and established viral pathogens from a variety of sample types. In their seminal 2007 report, Cox-Foster *et al.* used an unbiased metagenomics approach to identify the bacterial and viral pathogens likely contributing to colony collapse disorder (CCD) experienced by honey bee hives.⁸¹¹ Metagenomic data was collected from large pools of bees from CCD hives and control hives identifying the microflora in each while attempting to correlate CCD occurrence with specific microflora profiles. Of the identified organisms, two viral species in particular, Kashmir bee virus and Israeli acute paralysis virus, were determined to be prevalent in CCD samples and a possible contributor or correlator to the phenomenon.

Metagenomic sequencing for identification and diagnosis of viral pathogens can also prove valuable when symptoms, such as acute febrile illness, can be associated with a broad range of pathogens including viruses, bacteria, and many other parasites. From a public health perspective, metagenomic NGS analysis of clinical samples would allow for the rapid identification of the causative agent(s) and speed the time of diagnosis and development/implementation of an appropriate treatment plan. To highlight this capability, Greninger *et al.* showed unbiased metagenomic detection of three viral pathogens in human blood samples using the MinION nanopore sequencer.⁸¹² This not only highlighted the ability of the MinION system to more rapidly identify viral targets, but also to do so with significant accuracy and at low levels of viral load. See **Figure 46** for a representative and comparative workflow between this and the directly competitive Illumina MiSeq system. In these studies, analysis of a Hepatitis C positive blood sample identified the viral genomes presence with only six aligned sequences of the 85,647 reads (0.0070 %). Metagenomic analysis is not without its limitations, however. Without a preconcentration or amplification of viral nucleic acids there is often a low sensitivity for target pathogen. Additionally, the coverage is often proportional to

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3 viral load which can lead to complications with detection and increased costs as more reads are
4 needed.
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7 Clearly, the relative abundance of viral nucleic acids in a given sample can often be quite
8 low compared to other “contaminating” nucleic acids from the host along with other organisms
9 that may be present. One alternative to metagenomic sequencing is the enrichment of targeted
10 viral sequences through an initial PCR reaction. Referred to here as PCR amplification
11 sequencing, this method utilizes primers complementary to specific sequences in the target
12 virus(es) designed to amplify either the entire genome, in the case of relatively small genomes
13 like norovirus,^{813,814} or amplicons that can be assembled into an intact genome, as necessary
14 with viruses that have variable or larger genomes.^{114,815} Unlike traditional laboratory PCR
15 reactions that have primer sequences tailored for the amplification of specific genes, PCR
16 amplification sequencing most often utilizes random or degenerate primers to amplify the viral
17 genome. This type of primer design is necessary to ensure the greatest probability of capturing
18 the subtle changes that can occur between variants or viruses prone to mutation during genome
19 replication. In 1991, Reyes and Kim described a method termed sequence-independent single-
20 primer amplification which they used to amplify and manipulate viral genomes.⁸¹⁶
21 Cumulatively, modifications to this technique for specific applications improved its utility in
22 viral identification and genome sequencing.^{817,818} Later, Djikeng *et al.* combined several of these
23 methods with NGS/WGS technologies to sequence positive stranded ssRNA viruses, negative
24 stranded ssRNA viruses, and dsDNA viruses.⁸¹⁹ PCR amplification based sequencing has also
25 been used to analyze and barcode multiple viral genomes simultaneously as a method of tracking
26 genome variability and evolution. Neill *et al.* designed amplification primers that consisted of 20
27 bp of known sequence and terminated with 8 random nucleotides that served as a barcode for
28 identification of each viral genome following sequencing.⁸²⁰ Full or nearly full sequence
29 coverage could be obtained for each of the viruses without other prior sequence knowledge.
30 PCR amplification has further been shown effective in the detection and sequencing of numerous
31 viral targets in clinical and environmental samples, but the methodology is not without its
32 limitations. The approach itself can be labor intensive and time consuming compared to other
33 sequencing methods. Additionally, while the quality of polymerases has increased substantially
34 over the years, the potential for introducing mutations with the numerous rounds of amplification
35 and skewing sequencing results is still a possibility. The random primer amplification approach
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has and will still continue to have success, but without prior knowledge of viral genomic sequences this methods will still have difficulties in identifying novel viral genomes.

Enrichment of viral nucleic acids can readily be accomplished using immobilized complementary nucleic acid sequences that serve to capture or “pull down” viral nucleic acids contained within a sample. Capture sequences can be comprised of overlapping sequences that cover the entire genome. Once isolated, the captured nucleic acids are then available for amplification which is typically accomplished using an adaptor ligation and amplification strategy to further increase the quantity of material available for sequencing. Enrichment of target nucleic acids and amplification for library generation can often be accomplished in a single tube which lends itself to high-throughput methods. Combined with improved specificity compared to other approaches such as metagenomic analysis, this method often comes with reduced overall cost and speed. Finally, target enrichment sequencing will also accommodate a broad range of sample sources. Depledge *et al.* utilized a target enrichment protocol to identify *Herpesviridae* in clinical blood, saliva, cerebrospinal fluid, and other samples.⁸⁰⁴ Members of the *Herpesviridae* family are often considered extremely difficult to work with due largely to their cell association and variability within the family. Nevertheless, the authors identified 13 distinct genomes in the samples tested. In this example, the targeting of a specific viral family simplified the design of capture nucleic acids, however, researchers often require a collection of probes with broader range capability of capturing both known targets and novel virus species. Wylie *et al.* used a computational approach to condense ~1 billion viral reference sequences into less than 200 million bp of unique nucleic acids sequences that could be used to capture a broad range of viral species.⁸²¹ This was based on the assumption that the nucleic acid capture reagent ViroCap could be used to capture 34 viral families that comprised 190 annotated viral genomes and 337 species, see **Figure 47** for an overview. By tiling capture probes across the genomes of many different viral species, the ViroCap collection was able to capture genetic sequences from highly divergent viral species. Several publications that compare currently available techniques and reagents used for capture and identification are available.^{822,823} Target enrichment sequencing, despite its successes, can again prove limiting in the detection of novel viral genomes as some of knowledge of target sequence is required. Additionally, the design of a functional capture system can be quite labor intense and some degree of technical expertise is necessary for sample preparation.

Regardless of the method chosen for obtaining sequence information, NGS/WGS endeavors typically generate huge data sets that must then be analyzed to identify individual viral genomic sequences. The development of computational tools for rapidly and efficiently identifying and compiling viral genomes is a continually evolving aspect of bioinformatics. These highly complex undertakings must account for variables such as chromosomal insertion, viral source genomes, and how these bias the search results, along with non-biological factors such as user interface and CPU requirements. One common method for analyzing NGS/WGS data collections for the detection of viral agents involves identification of non-human genomic sequences which greatly simplifies the later analysis. Software tools such as PathSeq⁸²⁴ and rapid identification of non-human sequences (RINS)⁸²⁵ apply the concept of computational subtraction through different approaches. PathSeq relies on an unbiased comparison to human genomic sequences which allows for a significant proportion of the sequences reads to be ignored when attempting pathogen detection. As shown by Kostic, this computational method could be used to successfully identify viral genomic sequences with a high degree of reliability.⁸²⁴ After subtraction of the human-derived reads, greater than 97% of all non-mutated, virus-derived reads were correctly identified. Additionally, only 0.78% of viral genomic sequences were discarded in the initial subtraction step. This success, however, does not come without cost. The PathSeq software package requires heavy computational capabilities and is therefore driven by a cloud-based server. As an alternative, Bhaduri *et al.* developed the RINS system that utilizes user provided reference sets that serve as a first discriminator of the dataset.⁸²⁵ The RINS software reduces the computational burden allowing the analysis to be run locally decreasing the overall analysis time. Both PathSeq and RINS have been used for successful identification of viral genomic sequences but both have short-comings with identifying viral integration into human chromosomes. This limits their capability to detect viral pathogens that utilize host genome integration as part of their life cycle. To overcome these limitations, software such as VirusFinder,⁸²⁶ VERSE,⁸²⁷ VirFinder,⁸²⁸ and VirSorter⁸²⁹ have emerged. These typically scan NGS data sets for viral specific sequences such as integration sites that further reduce the need for heavy computational methods and CPU power. These tools and others are continuously evolving to meet the challenges encountered by researchers and the ever growing NGS/WGS data collections that continue to accumulate.

Clearly, NGS/WGS technologies are well positioned to greatly improve clinical diagnosis of disease attributed to viral infection. This applies not only to known viral pathogens but also those that have yet to be identified or highly divergent variants of those previously identified. This can extend well beyond clinical applications to agricultural studies and crop monitoring as discussed extensively by Massart *et al.* who focused on plant virus diagnostics with NGS technologies.¹¹⁷ Additionally, NGS/WGS methods will inevitably contribute to epidemiological studies including outbreak analysis and disease monitoring. An early example of this was the identification of a novel version of the Ebola virus.⁸³⁰ Here, clinical samples examined during an Ebola outbreak in Uganda gave conflicting results with immunological assays showing positive results while traditional RT-PCR assays for common versions of this filovirus failed to accurately identify the causative strain. Metagenomic pyro-sequencing of total RNA extracted from patients yielded a reference genome that was then used to develop RT-PCR primers for the nucleoprotein gene of the novel virus aiding in subsequent patient diagnosis. Similar examples of this type of approach have been recently reviewed.^{827,831}

At present, NGS/WGS technologies have not been transitioned to full or classical biosensor applications. With the exception of the Oxford Nanopore MinION system, most of the instrumentation necessary for library assembly and sequencing is of laboratory scale and complexity and not conducive to field distribution. Additionally, the sheer volume of data generated and the computing power and time necessary to process the information does not yet translate to a rapid sensing platform.

Electrochemical Detection of Viruses

Amperometric Sensors. One of the earliest electrochemical sensors was based on the conversion of glucose to glucono-1,5-lactone by the enzyme glucose oxidase (GOx).⁸³² During this bioconversion, GOx reduces O₂ to H₂O₂ which is measured electrochemically using an amperometric sensor platform. This format of glucose meter has become one of the most widely distributed amperometric sensors along with it probably being the most distributed biosensor in the world due to its ability to reliably quantitate blood glucose levels. This successful demonstration led other researchers to pursue the development of new biosensors targeted towards viruses based on the same type of electrochemical detection principles.

The Mark's Group has exploited GOx in several different amperometric assay formats for the detection of West Nile virus.^{833,834} To accomplish this, Ionescu *et al.* immobilized a DNA aptamer displaying complementary to viral specific sequences to a polypyrrole-functionalized electrode surface which served as the recognition element for target sequences originating only from the viral genome.^{833,834} A second, biotinylated nucleic acid complementary to the 3' end of the viral RNA was then captured to the nucleic acid complex on the surface. The biotin conjugated to this second nucleic acid sequence facilitated the localization of the GOx reporter to the electrode surface *via* a biotin-streptavidin interaction. In this initial demonstration, the authors used synthetic nucleic acids rather than actual viral material as sample. The assay, however, was able to successfully and reliably detect target down to 1 fg/mL of nucleic acid. In later efforts, the group shifted to detection of antibodies specific for a West Nile capsid protein rather than the virus itself. Up until that point, immunodiagnostics for West Nile virus relied on the detection of host immunoglobulins as the mainstay technology for diagnosis of infection; such methods, while successful, suffered from limitations common to many immunoassays. The Mark's group approach here relied on the production of T7 bacteriophage expressing a 15 amino acid peptide similar to an immunodominant West Nile viral envelope protein.^{833,834} To facilitate sensor assembly, photoactivatable polymers were used to generate a poly(pyrrole-benzophenone) surface for the immobilization of the modified T7 phage capsids.⁸³⁴ This served as a simple method of reagentlessly grafting the protein to the electrodes. Subsequently, an amphiphilic pyrrole ammonium mixture was used to facilitate the deposition of the recombinant phage at the electrode surface.⁸³³ In proof-of-concept experiments, the viral peptide on the phage capsid served to capture West Nile-specific antibodies from solution. An HRP-conjugated, anti-human IgG was then used to generate electroactive quinolones in the presence of substrate and peroxide. This assay was able to detect West Nile specific antibodies down to 36 pg/mL which is an order of magnitude better LOD than other comparable immunodiagnostic assays.

Cui *et al.* developed a sensor for influenza virus based on detecting the presence of an active enzyme originating from the virus.⁸³⁵ The sensor design here relies on the premise that the influenza neuraminidase is capable of cleaving galactose bearing substrates to release galactose which can be monitored with a glucose monitoring strip and reader; the latter utilizes a dehydrogenase enzyme rather than GOx for the production of electroactive compounds. The assay was tested using a considerable number of viral neuraminidases (NA1 - 9) and in each

instance the viral neuraminidase was able to cleave the modified galactose substrates with varying levels of efficiency. Clinical samples were also tested in order to validate the assay as a reliable method of viral detection that meets many of the WHO’s ASSURED requirements and to determine the assays ability to detect influenza virus in complex samples comprised of diverse microbial flora. In samples spiked with influenza the researchers observed an LOD of 10^2 CFU per sample with analysis times of ~15 mins.

Conductometric Detection. Though successes have been shown with bacterial pathogen detection, conductometric detection systems have seen less application in viral detection than some of the other electrochemical detection methods.^{161,836,837} Conductometric sensors typically employ a conductive polymer such as polyaniline which serves as a transducer and which converts a biological event such as antigen-antibody interaction into an electrical signal that can be measured. Muhammad-Tahir *et al.* expanded upon previous successes with bacterial detection and transitioned their conductometric sensor to one capable of detecting bovine viral diarrhea virus (BVDV), a model for many agricultural viruses that are considered potential economic bioweapons.⁸³⁶ Similar to the previously developed bacterial assays, the BVDV detection device employed a polyaniline-coated electrode and antibody-based capture and reporter elements. A variety of assay parameters such as antibody and polyaniline concentration were tested and optimized ultimately resulting in an average LOD of 10^3 CFU/mL. Parallel comparisons of their conductometric sensor to other immunodiagnostic assays and PCR-based methods were, however, difficult to accurately make in a meaningful way due to the stark differences in process and detection. Nevertheless, the relatively low-tech requirements and portability of this approach do suggest that this system would be a valuable tool for monitoring large livestock herds.

Potentiometric Sensors. These sensors typically monitor the flow of ions across a membrane electrode. To indirectly detect biological interactions, researchers monitor the accumulation of ions that occur as a part of, for example, immunobinding at the membrane surface which, in turn, leads to a resistance in the mass transport of the marker ion, see **Figure 48**.⁸³⁸ This type of biosensor format was demonstrated by Ozdemir *et al.* who developed a POC device for the detection of respiratory syncytial virus (RSV).⁸³⁸ Here, the ISE surface was first functionalized with a biotinylated PEG that facilitated the immobilization of streptavidin. Distal biotin binding

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3 sites on the streptavidin were then backfilled with a biotinylated anti-RSV antibody to facilitate
4 capture of RSV virions on the electrode surface. Subsequent antigen-antibody interaction within
5 the assays resulted in a dose responsive change in potential. This system was able to achieve an
6 LOD of 10^3 PFU and the authors suggested that miniaturization of the device could perhaps even
7 further improve performance.
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11 As with the development of any electrochemical biosensor, researchers must pay
12 particular attention to the capture moiety, the electrode properties, and the ionic strength of the
13 solution. Additionally, since potentiometric sensors are measuring ion accumulation, preliminary
14 studies of pH sensitivity under the defined assay conditions are important towards optimizing the
15 final assay format. For example, to assess the effects of pH on their ability to detect a dengue
16 biomarker, Figueiredo *et al.* first conducted a series of experiments with solutions of varying pH
17 to ascertain the role this parameter would play in the analysis of their samples.⁸³⁹ They also
18 examined and optimized the surface chemistry used for electrode functionalization and antibody
19 immobilization as well as the ionic strength of the samples. The optimized sensor was able to
20 detect as little as 90 ng/mL of the nonstructural protein 1 that is secreted by dengue virus during
21 the first days of infection.
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32 ***Impedimetric Sensors.*** In an attempt to significantly improve the LOD towards influenza A
33 H1N1, Bonanni *et al.* paired a DNA-based impedimetric sensor with signal amplification
34 achieved through a second interaction between the viral DNA and AuNPs.⁸⁴⁰ A screen-printed
35 carbon nanotube-based electrode functionalized with carboxylic acid groups was reacted with
36 amine-labeled ssDNA molecules complementary to viral RNA sequences to create the biosensor
37 surface. For their proof-of-concept demonstration, the authors used biotinylated nucleic acid
38 sequences based on those found within the H1N1 genome. In direct binding assay formats, 405
39 fmols of the target nucleic acids could be detected. As expected, subsequent incubation with
40 streptavidin-conjugated AuNPs led to a 10-fold improvement in LOD, sensing the presence of
41 7.5 fmol of target nucleic acid material.
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50 Nucleic acid aptamers offer an alternative to the classical and direct DNA-DNA
51 complementarity-based detection and these binders can often be selected to have additional
52 functionality for these types of formats. For example, Labib *et al.* isolated DNA aptamers that
53 were able to discriminate between viable and non-viable Vaccinia virus.^{841,842} To accomplish
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3 this, the team modified their selection protocol so that the later part of the selection process used
4 heat inactivated virus to sequester non-target aptamers. Nonbound aptamers, which recognized
5 epitopes present only on non-heat-treated-viable Vaccinia could then be amplified for additional
6 rounds of selection towards higher affinity. The isolated aptamers for viable Vaccinia were then
7 thiolated, immobilized to a gold electrode, and tested for their ability to bind live virions (**Figure**
8 **49**).⁸⁴² This proof-of-concept sensor demonstrated an LOD of ~ 60 PFU/ μ L and may represent
9 the first direct aptamer-based sensing system capable of discriminating between live and dead
10 viral samples.
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17 Influenza viruses target specific cell-surface glycans to facilitate cellular uptake and
18 infection and in many instances the glycan targeted is specific for a particular influenza strain.
19 Therefore, sensors that utilize glycans as the capture moiety could theoretically discriminate
20 between pathogenic and non-pathogenic strains of the virus. This theory is the basis of the
21 glycan-based impedimetric sensor developed by Hushegyi *et al.*^{843,844} In experimental trials, their
22 glycan-based sensor was able to detect as few as 13 H3N3 viral particles per μ L with a
23 selectivity ratio of 30 for H3N2 *versus* H7N7 influenza strains.⁸⁴⁴ These results suggest this
24 sensor as the most sensitive achieved to date that relies on glycan binding and this also places it
25 amongst the most sensitive antibody or aptamer based biosensing devices, in general, as well.
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33 Electrochemical sensors in all of the aforementioned formats offer several advantages
34 over many of the more traditional immunodiagnostic and PCR-based methods for viral detection.
35 As a class, these devices can typically be mass produced, miniaturized, and developed to satisfy
36 many of the WHO's ASSURED requirements.^{140,845} They are also highly versatile and can be
37 adapted to a wide range of recognition elements including traditional antibodies, nucleic acids,
38 lectins, glycans, and many others. Additionally, with minimal instrumentation and technical skill
39 these devices have been able to achieve LODs in the fM range which can translate to 10's of
40 virions in a very low sample volume. One limitation of note is that, in general, electrochemical
41 sensors do require very specific environments (*e.g.*, ionic strength, pH, *etc.*) to function
42 efficiently and slight changes in the solution conditions can dramatically affect the results. Given
43 the complexity of many biological samples this can prove quite limiting. Efforts by researchers
44 to continuously address and overcome these limitations have had some success with
45 improvements to electrode materials, immobilization layers, and even the reference
46 electrodes.⁸⁴⁶⁻⁸⁴⁸ Moreover, integration of an electrochemical sensor component into a
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microfabricated or POC device can help overcome this issue as it can allow a sample to be preprocessed, cleaned up, undergo target enrichment, and adjust the final sample conditions (*e.g.*, pH) to allow for optimal assay function.

Spectroscopic Sensors

Colorimetric-based Detection Strategies. Colorimetric assays are a valuable tool which can usually provide qualitative assays results and sometimes even quantitative information about a sample. Thanks to their simplicity, colorimetric assays have gained relevance as a rapid screening strategy for viral presence within a range of applications including clinical surveillance and POC utility. Simple platforms, such as the paper-based immunoassay proposed by Lei *et al.* to detect influenza virus are of especial interest and can be quite powerful tools.⁸⁴⁹ Here, a wax printer was utilized to generate an 8 x 6 array of microzone plates on a paper substrate that was then used as a testing platform for a sandwich type immunoassay. The assay utilized polyclonal capture antibodies and monoclonal primary antibodies targeted to components of the influenza viral capsid. An HRP-conjugated secondary antibody allowed for visualization of antigen capture providing for the reliable detection of both the H1N1 and H3N2 influenza strains. Paper-based colorimetric approaches do offer some advantages over more common microtiter-based formats. Shorter analysis time and lower reagent usage combined with simplicity of use have made these a viable format for the future development of low-cost, distributable diagnostics and sensors.

Despite the catalytic advantages of enzyme reporters, sensitivity can often be a limiting factor with colorimetric assays. Lin *et al.* sought to overcome this limitation through the utilization of enzyme filled liposomes anchored to the secondary antibody in a sandwich type assay for influenza virus.⁸⁵⁰ It was shown that HRP could be encapsulated within a synthetic liposome and then released with the addition of peroxide and the 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Rapid formation of the blue product highlighted this method as a low-cost alternative to other strategies that rely on complex and expensive instrumentation. Additionally, thanks to the large amount of visualizing enzyme present, the H5N1 viral strain could be detected in the low ng/mL level by the naked eye. Ahmed *et al.* demonstrated that AuNPs could replace traditional HRP-conjugated antibodies in an immunoassay format.⁸⁵¹ This approach exploited the fact that AuNPs exhibit peroxidase-like activity toward the standard HRP substrate,

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3 TMB. Again using a sandwich type immunoassay, they were able to reliably detect H1N1 viral
4 proteins down to low pg/mL levels.
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7 As highlighted previously, AuNPs are widely used in a plethora of colorimetric assays
8 due in large part to the color switching plasmon phenomenon that is dependent on their
9 aggregation state.⁸⁵² The ability to observe results with the naked eye is obviously especially
10 useful when performing a screening assay and is certainly advantageous over other sensor types
11 which utilize chemical reporters that may require special instrumentation or the addition of a
12 substrate to an enzyme such as HRP to obtain a measurable output. Additionally, by means of
13 specific capture probe conjugation on the NP surface single step procedures can be developed,
14 for example, to detect influenza A virus (**Figure 50**) *via* a targeting antibody⁸⁵³ or a peptide.⁸⁵⁴
15 These examples also exploit the repeat copies of the capsid protein targets to accumulate AuNPs
16 on the capsid surface which can, in and of itself, enhance the AuNP plasmonic coupling. The
17 antibody and peptide conjugation elements target epitopes or receptors of the viral surface
18 leading to an aggregation event that can be measured spectrophotometrically or visualized with
19 the naked eye at high viral titers. Natural cell surface receptors such as glycans that are often the
20 targets of viral surface proteins, can also be immobilized onto AuNP surfaces to similarly
21 facilitate aggregation and viral detection. For example, Zheng *et al.* coated AuNPs with sialic
22 acids designed to mimic cell surface glycans targeted by the hemagglutinin of influenza viruses
23 to develop a colorimetric assay capable of discriminating between different influenza strains.⁸⁵⁵
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27 Despite their advantages over reporter molecules, the sometimes poor monodispersity of
28 AuNPs (which can produce broad and non-homogeneous plasmon peaks) may limit the
29 sensitivity of its applications.⁸⁵⁶ To overcome this limitation, Xu and coworkers employed AU
30 nanobipyramids (NBPs) which are highly uniform nanostructures.⁸⁵⁷ In this particular case, a
31 traditional sandwich immunoassay was used employing an alkaline phosphatase-conjugated
32 secondary antibody as a component of the detection system. Here, alkaline phosphatase converts
33 silver nitrate to metal silver through chemical formation of 4-aminophenol from 4-aminophenyl
34 phosphate. The free metallic silver deposits to the surface of the Au NBPs causing a visual color
35 transition in the nanostructures (**Figure 51**). In addition to antibodies and peptides, aptamers can
36 also be used as capture probes on NPs for virus targeting. When immobilized on MNPs, aptamer
37 bioconjugates could capture targets such as the H3N2 strain, for example.⁸⁵⁸ Following capture
38 with the decorated MNPs, the addition of concavalin A-GOx conjugated AuNPs formed a
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sandwich complex with the viral target. Addition of glucose as enzyme substrate allowed for colorimetric detection based on the reduction of gold ions with H_2O_2 .

Fluorescence-based Detection Strategies. The use of fluorescence for visualization in viral immunoassays is quite common and discussed elsewhere.^{806,859} Fluorescence is also widely used as a signal transduction strategy to provide information about biomolecular complex formation and this, too, is commonly exploited for viral sensing in the context of FRET. For example, Chou and coworkers designed a QD-DNA system with two oligonucleotides sequences that are specifically targeted to two different regions of the hemagglutinin H5 gene for avian influenza detection.⁸⁶⁰ One of the oligonucleotides sequences acted as a capture probe when conjugated to 655 nm QD donors and the second one, labelled with the fluorescent acceptor dye Alexa Fluor (AF) 660, acted as the reporter probe following the scheme shown in **Figure 52**. In the presence of target and subsequent complex formation, FRET from the QD to AF660 was measurable even at a very low LOD of 0.5 nM. Higher sensitivities could be achieved by using dsDNA probes assembled onto microgel particles.⁸⁶¹ To achieve this, viral DNA (following reverse transcription of virus RNA) was captured by complementary strands confined within the polymeric hydrophilic network of the microgel particles which occupied fL volumes. The detection mechanism was based on a dye-labeled capture probe on the microparticle's surface which was prequenched by a complementary strand. In the presence of viral DNA, the quencher was displaced after target hybridization resulting in a fluorescence increase from the probe. This microparticle format apparently led to faster interaction kinetics and provided for a higher capacity of biomolecule immobilization which, in turn, enhanced target-probe interactions and minimized non-specific interactions. Because of this, the LOD achieved for SARS, hepatitis C, and HIV was in the fM range and could be detected within a multiplexed format. Another quenching-based sensor format included glycan-conjugated QDs, with the former providing the natural target for the hemagglutinin protein, and an anti-HA antibody modified with AuNPs. In the presence of the viral target, both NP materials are forced closer and the QD emission was quenched by a non-Förster energy transfer type process.⁸⁶² This assay was able to detect and discriminate between human and avian influenza virus hemagglutinin in serum samples. It is worth noting that the latter AuNP quenching interaction is often mistakenly described as FRET in many reports due to some similar characteristics, but, in reality it is not with nanosurface

energy transfer (NSET) being the most probable mechanism.^{478,533,863-865} NSET displays a inverse 4th power dependency on donor acceptor separation.

QDs have also contributed to viral sensing in a number of other quite distinct formats. In one interesting approach, viral gene sequences could be detected without any separation steps and high sample throughput by means of exploiting optical tweezers which consist of a tightly focused Gaussian laser beam designed for noncontact/noninvasive micromanipulation.⁸⁶⁶ This innovative approach could detect neuraminidase and hemagglutinin gene sequences in the pM range directly in whole human serum samples. To achieve this, solid phase polystyrene and QDs both displaying complementary DNA to the target were employed. In the presence of both target DNAs, a hybridization event generated a macrostructure which was individually captured by means of the tweezer, keeping the complex in the laser focal point for later excitation and generating a measurable emission signal. The tweezers also maintained the complex in the focal point in such a confined 3D space that any background interference is avoided, resulting in high sensitivity detection. Detection of intact, viable virus could also be achieved by labelling virions with specific aptamer-QDs conjugates.⁸⁶⁷ Moreover, fluorescence imaging of these labeled virions allowed tracking of the infection pathway in living cells. Another unique approach relied on localized pH changes to develop a pH-sensitive QD-based detection platform.⁸⁶⁸ To implement this, a smart molecular machine was designed that utilized the proton flux generated by molecular machinery or ATP synthase in conjunction with QD-labelled chromatophores and an antibody-target biorecognition event for detection. Following binding to the target, ADP is added to initialize the reaction and protons are pumped out of the chromatophores, resulting in a fluorescent intensity change of the CdTe QDs. The rate of the fluorescent intensity change further reflected the activity of the ATP synthase. The target capture process takes place over the β -subunit of the ATP synthase and this enhances the activity resulting in a faster change in the fluorescence intensity.⁸⁶⁸ By measuring this rate, influenza and murine gammaherpesvirus presence could be detected⁸⁶⁹ along with the avian influenza H5N1 strain.⁸⁷⁰

The advantages of biosensing by immunochromatographic methods such as LFAs converge with the advantages of fluorescence immunoassays in the SOFIA method.^{871,872} This technique is a novel fluorescence-based lateral flow immunoassay marketed by Quidel (San Diego, CA) which not only demonstrates some of the highest sensitivities available compared to other in class diagnostics, but also extraordinary rapid testing times since results can be obtained

in ~15 min. One initial evaluation report analyzed 241 nasopharyngeal swab samples from influenza A/B patients and found that SOFIA outperformed other available diagnostic tests, even RT-PCR, and more importantly showed positive results where others gave false negatives.⁸⁷² Similar studies showed that SOFIA provided no false positive results and better sensitivities than other kits such as Quickvue or Directigen colorimetric assays.⁸⁷³ This assay could also be used with saliva specimens which are less invasive than swabs.⁸⁷⁴ Because of this, SOFIA has become a great tool for POC influenza diagnostics within clinical environments.⁸⁷² Using some similar materials with an analogous type of biosensing approach, it was shown that optimized conjugation of antibodies and fluorescent beads could lead to an improved fluorescent immunochromatography method with lower LODs than other immunochromatography counterparts when targeting influenza A/B in clinical samples.⁸⁷⁵ Fluorescent signal in this format was enhanced by conjugation of latex beads with antibodies and a unique Red Dye-53 to allow detection of influenza A in human nasopharyngeal samples in just 15 mins incubation time, see **Figure 53** for a schematic highlighting this sensing process.⁸⁷⁶ One of the primary advantages of this type of approach is the ability to bypass sample pretreatment and provide diagnostic results rapidly with minimal equipment constraints.^{875,877}

Other Optical Sensors. Many optical sensors require precise alignment of the light source and the sample for accurate analysis which often limits the field applicability of such devices. Optofluidic nanoplasmonic devices circumvent these limitations coupling perpendicularly incident light while minimizing the requirements for alignment of the light source in relation to the sample. Yanik *et al.* employed an optofluidic nanoplasmonic sensor capable of detecting intact virus from biological media at clinically relevant concentrations.⁸⁷⁸ Their device relied on a light transmission effect in plasmonic nanoholes and utilized group-specific antibodies targeted against different evolving viral strains. Within the device there is a reference sensor that is unfunctionalized for comparison and a detection sensor that has been functionalized with antibodies for specific groups of viruses. This enabled detection of both large DNA and small enveloped RNA viruses within a dynamic range of 10^6 to 10^9 PFU/mL and a projected LOD of $<10^5$ PFU/mL. Because the device does not destroy the captured virus, the sample could potentially be studied for further genomic or other useful marker information. Lu and colleagues also pursued a 'label-free' biosensing strategy by adapting a microcavity sensor that measured

perturbation in optical properties through whispering gallery mode (WGM) measurements.⁸⁷⁹ These microcavity-based sensors rely on signal production from particles or molecules such as viruses binding to the surface of a microcavity which perturbs its optical properties causing a resonant wavelength shift.⁸⁸⁰ The magnitude of the shift is dependent on the particle's polarizability which is also proportional to the volume of macroscopic particles used. This WGM approach could detect single 12.5 nm diameter particles and was able to detect influenza A at a concentration of 1 pM with a signal-to-noise ratio of 38:1. Zhu *et al.* also utilized WGM for label free viral detection in an optofluidic ring resonator.⁸⁸¹ The device here consisted of a thin-wall micro-sized capillary which also served as the microfluidics for transporting the viral sample. The surface of the capillary was coated with virus-specific antibodies providing the device with target specificity. The glass portion of the capillary itself formed the ring resonator, and by passing a tunable diode laser perpendicular through the capillary, antibody/virus interactions could be monitored in real time. The set-up yielded an LOD of 2.3×10^4 PFU/mL and had an impressive dynamic range spanning seven orders of magnitude.

Using a slightly different approach, Ymeti created a sensor based on a Young's interferometer for the detection of herpes simplex virus type 1 (HSV-1).⁷⁰⁶ While this virus is not a potential biothreat *per se*, the principles this approach demonstrates can certainly be applicable to sensing other viruses. This sensor design included a reference channel and a test channel coated with virus-specific antibodies against the target. The total test time for detection of HSV-1 was approximately one hr and they were able to detect 850 HSV-1 particles/mL. Interestingly, the authors were able to detect virus binding to the sensor surface after only a few mins suggesting the possibility for a more rapid analysis. Additionally, the signal produced by the Young's interferometer sensor could easily be transitioned to a readable output that, when combined with strategies to functionalize different channels with alternate target-specific antibodies, would allow for multiplexing. Cumulatively, these observations suggest a strong possibility for the future development of a portable or handheld screening device based on the same technology.

Integrated Microdevices

The development of integrated microdevices for viral detection is a rapidly developing field given the dire need from public health agencies especially in the developing world. The

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3 devices range from simple to quite complex with the latter requiring specialized fabrication and
4 utilizing multiple preparatory and analytic steps along with being interfaced into phones or
5 computers. The vastness and depth of this field does not justify a cursory overview here and so,
6 in this particular case, the reader is directed to the many available focused and specialized review
7 articles.^{294,316,378,882-889}
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Toxins

Toxins are most simplistically poisons that are produced biologically and originate from diverse sources including animals, plants, and microbes. They are also found in both proteinaceous and molecular or chemical form. They are generally defined first by their biological effects and subsequently differentiated from other harm-inducing chemicals (*e.g.*, chemical agents) in that they are neither man-made nor typically volatile. Given their lack of self-replication, the effectiveness of toxin-based biothreats is tightly tied to toxicity, lethality, dosage, and delivery method. Because of this, the use of toxins in a terroristic attack requires production capabilities and consideration of their stability, their aerosol toxicity, and their accessibility even more than other biological agents. In addition, while toxins can be extracted and concentrated from their biological source, they inherently lose any protection this source may provide. Thus, while BoNT is one of the most toxic substances known and is relatively easily obtained from a cultured bacterial source, it is quickly denatured by environmental factors such as heat and UV radiation. Ricin, is more stable yet nearly three orders of magnitude less toxic, requiring potentially far larger quantities to carry out an attack of similar intended magnitude.²

Overview of Some Common Toxin Agents

Botulinum Neurotoxin. This is one of the most researched bioagents, having been extensively studied in the U.S., the Soviet Union, and Japan during the First and Second World Wars. As early as the 1930’s, Japan’s Unit 731 was experimenting with BoNT on prisoners of war, and even subsequent to the signing of the BWC, the Soviet Union (and later Russia) maintained biowarfare programs that included weapons development and production of BoNT. More recently, BoNT was one of three bioagents that was extensively researched and subsequently produced by Saddam Hussein’s regime in Iraq. By 1990, 13 SCUD missiles and 100 R-400 bombs had been loaded with a concentrated solution of BoNT.¹ Although the weapons were deployed during the Gulf War, they were never actually used. For bioterrorism purposes, the Japanese cult Aum Shinrikyo succeeded in cultivating the bacteria and producing BoNT and experimented with dispersion systems. In three instances, the group used sprayer systems to deploy the toxin in public spaces, including the Tokyo subway, although no cases of botulism were reported to result from any of these attacks.¹

Botulism is the neuromuscular disease that results from exposure to the toxin produced by the spores of the *Clostridium botulinum* species of bacteria. Though the spores are ubiquitous, they only germinate under specific conditions, including in the anaerobic environment encountered in canned foods, which is the most common source of botulism in humans. Of the seven antigenic types of neurotoxin that *C. botulinum* and three related *Clostridium* species are capable of producing, cases of botulism in humans are typically caused by the A, B, E, and F subtypes.² Type A (BoNT/A) is most commonly considered for biological warfare, although inhalation botulism could potentially be caused by any of the above types.^{1,3} Botulism can develop from consumption, inhalation, or contact (in wounds) with BoNT. Among those, inhalation botulism is likeliest in the event of a terrorist attack, although a *Gedanken* experiment involving contamination of the milk supply suggests that foodborne botulism could also be an effective attack method.^{1,890}

The BoNT protein itself consists of disulfide linked 50- and 100 kDa light and heavy chains, respectively. Following endosomal uptake by cells especially into presynaptic nerve cells, the disulfide bond is cleaved, releasing the light chain into the cytosol where it acts as a Zn-dependent endopeptidase.^{1,2,21} Functionally, BoNT inhibits the release of acetylcholine, causing paralysis.^{1,2,21} Although early symptoms are nonspecific, developing between 12 hrs and several days after exposure, the onset of paralysis is a tell-tale sign of botulism, with death typically occurring as a result of respiratory failure due to paralysis of the respiratory muscles.^{2,5} The toxin is extremely lethal, with an LD₅₀ of approximately 0.1 µg/70 kg intravenously, 1 µg/70 kg by inhalation, and 70 µg/kg orally.¹ Although an antitoxin is available, it does not reverse any paralysis but only prevents progression. Many cases therefore require prolonged life support *via* mechanical ventilation even up to several months to allow neuronal recovery.^{2,21}

Ricin. Ricin is a cytotoxic protein found in the seeds of the castor bean plant *Ricinus communis*. The mature or active glycosylated protein toxin consists of A and B chains with molecular weights of 32 (267 amino acids) and 34 kDa (262 amino acids), respectively, joined together by a single disulfide bond. The ready availability of ricin originates from the widespread cultivation of castor beans, where it is present in the endosperm, for the production of castor oil and from the toxin's subsequent presence in the waste mash (3-5% by weight) that is a by-product of the

oil extraction process.¹⁻³ Though less potent than BoNTs or Staphylococcal enterotoxin B (SEB), which also fall in the protein toxin class, both the stability and world-wide availability of ricin make it a potential bioterrorism agent.¹ Delivery of the agent can be by oral, inhalation, or intramuscular routes, and both the toxicity and symptoms that develop differ based on the route of intoxication, with oral ingestion exhibiting lower toxicity than other routes. The cause of death varies by exposure route, but ricin's mechanism of toxicity originates from its inhibition of protein synthesis by altering ribosomal RNA.^{2,5,21} The LD₅₀ for humans is not known, though in mammals exposed to aerosolized ricin, the range is from 3-15 µg/kg.¹

Ricin is perhaps most infamously known for its role in murder and assassination than maximizing casualties. Although part of the U.S. biological warfare research program where it was developed and tested as part of an Allied effort to create a ricin bomb during WWII, perhaps the most well-known example of its use was in the assassination of Georgi Markov, a Bulgarian defector. Using a device disguised as an umbrella, a 2 mm pellet loaded with ricin was injected into his leg in London in 1978. Although the use of ricin was not directly confirmed, a survivor of an earlier, similar attack in Paris upon hearing of the incident with Markov was able to locate a pellet in his leg by X-ray. The survivor tested positive for anti-ricin antibodies and the subsequently removed pellet still contained a small amount of ricin enclosed by a waxy substance and some scar tissue.^{891,892} Ricin has only been used or its use intended in biocrimes which are delineated from bioterrorism in their more personal nature rather than being politically motivated. Its ease of production or procurement relative to other bioagents means that even individuals with non-technical backgrounds have used it.¹ Possession of ricin is restricted not only under the BWC but also under the Chemical Weapons Convention and Biological Weapons Anti-Terrorism Act.

Abrin. Similar to ricin, abrin toxin (AB) is a type-II ribosome-inactivating protein found in the seeds of the rosary pea plant *Abrus precatorius*.⁸⁹³ Its structure is composed of two different A and B subunits linked by a disulfide bond. AB is close to ricin in terms of structure and properties, but can be found in three groups (I, II and III) with different levels of toxicity, ranging from 0.1 to 1 µg/kg, with type-II being the most toxic. AB exhibits a similar toxic profile as ricin, which confers to it the same level of threat as a bioterrorism agent, but it is considered 75 times more toxic.⁸⁹⁴ After ingestion, the initial stages of poisoning concur with

gastrointestinal issues, neurological effects, and finally multi-organ failure. AB inhalation can also lead to death.⁸⁹⁵ To date, there is no evidence of weaponized AB, but there are examples of its use in criminal actions, one as a poison in beverages and the other from an illegal sale including instructions on how to use it to poison food and drinks.⁸⁹⁶

Staphylococcal Enterotoxin B. This is another protein toxin that was a significant part of the U.S. offensive bioweapons research program, pursued because it was an effective incapacitating agent at very low doses, while the dose required to produce lethal effects was substantial, giving it an excellent “safety ratio.”¹ A common source of food poisoning, Staphylococcal Enterotoxin B (SEB) was developed as an incapacitating agent to be delivered by aerosol, though an attack on a food supply was also considered possible.² SEB is one of several exotoxins (*i.e.*, excreted from the organism) produced by the bacterium *Staphylococcus aureus*. The term ‘enterotoxin’ refers to the fact that the toxin typically affects the intestines after ingestion of the bacteria. The severity of the effect it has on the immune system classifies SEB, among a number of other bacterial proteins, as a superantigen.¹ The illness they cause stems from the physiological overreaction of the immune response to their presence in the system.²¹ Symptoms differ based on the route of exposure, with flu-like symptoms developing as early as 4 hrs after inhalation or ingestion. Gastrointestinal symptoms and distress develop from ingested exposure, while inhalation exposure leads to pulmonary edema and can result in pulmonary failure.² Exposure to 1.5 μg constitutes an approximate lethal dose (by inhalation) while 0.03 μg is considered incapacitating.^{1,21} This rather small dosage reflects how potent this toxin can potentially be.

Mycotoxins. The term ‘mycotoxin’ collectively refers to a class of toxins produced by fungi. Possible use of a sub-class of these, trichothecenes (T2 toxins), may have occurred between 1975 and 1981 in regions of conflict arising from Cold War tensions causing as many as 10,000 suspected deaths, see **Figure 54A** for the chemical structure.² For example, in Laos reports of ‘yellow rain’ targeted at Hmong villagers and resulting in more than 6,000 deaths have been ascribed to possible use of T-2 toxins deployed primarily by rocket, although the possibility that a chemical rather than biological agent was responsible for those attacks has also been proposed.^{1,5} Other reported use of mycotoxins in this era include in Afghanistan and Cambodia.

Aflatoxin, another mycotoxin, was one of three bioagents later weaponized by Saddam Hussein's regime in Iraq.

Filamentous fungi (molds) are responsible for the production of mycotoxins, which, unlike the previous examples in this section, are low molecular weight compounds, not proteins.^{1,2} Fungi that produce mycotoxins are endemic worldwide, frequently contaminating grain and sometimes evoking mortality rates of up to 60%.¹ Culturing fungi in order to extract their toxins is accomplished using methods that are already well-developed for the production of antibiotics, and the toxin itself is highly stable under a wide range of environmental conditions, surviving even standard autoclaving.^{1,2} Desired predominantly for the effect they have on the skin as a blister agent, mycotoxins also differ from other bioagents in that droplets rather than the smaller aerosols optimized for inhalation are the more likely route of dissemination.²¹ Interestingly, tricothecenes are 400 times more potent than mustard agents in causing skin injury, and concentrations of nano- to micro-grams per cubic meter would be expected to disrupt military operations.¹

Initial symptoms of mycotoxin poisoning, which affect most eukaryotic cells, depend on the route of exposure, with irritation focused on the skin and eyes (inflammation, lesions, necrosis) in cases of skin contact or with the upper gastrointestinal tract after ingestion. Subsequent systemic symptoms arise from the toxin's interference with protein and DNA synthesis, resulting in radiomimetic suppression of immune function, central nervous system toxicity, hypotension, and shock.^{1,5} While percutaneous exposure is more toxic than inhalation, with respective LD₅₀ values of 2-12 mg/kg and 200-5800 mg min/m³, inhalation of large doses can be fatal in mins to hrs, a time-frame more typical of chemical agents than other bioagents.^{1,61} Unlike many bioagents, which are 'invisible threats,' the use of mycotoxins is associated with yellow rain-like events, meaning individuals can prevent contact and exposure through the use of protective clothing and decontamination of skin, both of which provide substantial protection. After exposure, use of superactivated charcoal can mitigate the effects of oral ingestion, but other treatments are limited to alleviation of symptoms.^{1,2,5} In the event of natural exposure from food sources, no treatment is available beyond general supportive/symptomatic care.

Epsilon Toxin. Epsilon toxin (ETX) is produced by *Clostridium perfringens* and is considered a potential biothreat agent, although no human poisonings have been reported. The toxin is

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3 produced by this anaerobic, spore-forming bacteria as a protoxin that is later cleaved to a 14
4 residue polypeptide with necrotizing activity. ETX causes toxicity mostly in animals, especially
5 in herbivores, after *C. perfringens* colonizes the gastrointestinal tract and produces lethal toxemia
6 by pulmonary edema, renal failure, and cardiovascular collapse.⁸⁹⁷ ETX can potentially be used
7 as an aerosol or water/foodborne agent to rapidly interact with the gastrointestinal and
8 respiratory mucosa and acts as an incapacitating agent.^{898,899} ETX is classified as a category B
9 agent because of its potential lethality. It has a determined LD of 100 ng per kilo in rodents with
10 an estimated value of 7 μg in humans by direct ingestion.⁸⁹⁷ There is no human vaccine available
11 and the treatments are merely supportive in order to increase the survival ratio after contact.⁹⁰⁰
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20 **Tetrodotoxin.** Tetrodotoxin (TTX) is a deadly toxin found in pufferfish which is popular in
21 traditional Japanese cuisine and commonly known as *fugu*, see **Figure 54B** for the chemical
22 structure. Contrary to popular belief, TTX is not endogenously produced in the fish but rather by
23 the presence of either symbiotic or infective bacterial. The lethal amount of this substance found
24 in those fish, requires very specialized licensed chefs to prepare it for consumption. The interest
25 in this risky meal is bound to the very small amount of the deadly toxin remaining in the
26 appropriately prepared food, which leads to a very special taste due to the blocking of certain
27 sensory nerves of the mouth. If the amount present is too high, the result can be death.⁹⁰¹ While
28 pufferfish is the main source of TTX, it can be also found in other fishes and animals such as
29 salamanders, frogs, octopus, starfish, and some mollusks.⁹⁰²
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38 TTX is a potent non-protein neurotoxin with a relatively low molecular mass (319.27
39 g/mol) and low water solubility (at pH 7). Its structure consists of a cage with four charged N
40 atoms and OH groups which confer great reactivity in water at pH's above 8.⁹⁰³ The lethality of
41 TTX is one of the highest in nature, just 2 mg are enough to kill a human being. It causes severe
42 nerve paresthesia by blocking Na^+ ion voltage dependent channels in neurons and this
43 subsequently causes death by pulmonary muscle paralysis.⁹⁰⁴ While it has not been weaponized
44 yet, Unit 731 of the Japanese army did make some initial attempts during WWII.⁹⁰⁵ Its use as a
45 bioweapon could be as an aerosol and due to its high toxicity it is still considered a threat.
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53 **Other Toxins.** Saxitoxin (**Figure 54C**) was first isolated from the *Saxidomus* butter clam but
54 actually constitutes more than 50 structurally related compounds typically found in shellfish that
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have been contaminated by toxic algal blooms.⁹⁰⁶ It functions as a relatively potent neurotoxin that acts as a selective Na⁺ channel blocker and which produces paralytic shellfish poisoning in humans following consumption. The lethal dose for humans is estimated to be < 0.75 mg or close to a small fraction of the size of a grain of sand.⁹⁰⁷ Brevetoxins (**Figure 54D**) are cyclic polyether compounds produced by certain species of dinoflagellates and similarly bind to voltage-gated Na⁺ channels leading to neurotoxic shellfish poisoning. They are found primarily in an A and B form with multiple functional substitutions leading to many subtypes. Interestingly, the Nicolaou Group reported that the initial synthesis of the B subtype brevetoxin-1 required 123 steps with an overall yield of 9×10^{-6} %.⁹⁰⁸ Cholera toxin consists of a multisubunit protein complex that is produced and secreted by the bacteria *Vibrio cholerae*.⁹⁰⁹ This toxin is directly responsible for the massive and watery diarrhea which is characteristic of a cholera infection. As such it is considered more of an incapacitating agent although if not properly treated, especially in the young or infirm, it can be deadly. Breakouts of cholera are common in environments where human (and other) waste intermingle with a local water source such as, for example, refugee camps lacking proper sanitary conditions.

Physical Techniques

Direct Mass Spectrometry. MS-based detection of toxins in the current context can be a powerful and versatile analytical technique capable of overcoming the limitation of other molecular-based approaches. One of the great advantages of MS applications in this context is the capacity to provide relevant structural information about the target analyte thus improving detection capability. This is not to say that other (bio)molecular techniques are still not valuable in this context as they can be used as a first screening approach in a toxin-related bioterrorism scenario since it is highly probable that the toxin may not be pure and will be contaminated and carry some of the parent organisms genetic material or other biomarkers of interest.¹⁶⁵ Although only an overview of MS-based techniques and their application to toxin detection are presented here without in-depth explanation of all factors, it is important to appreciate the wide variety of equipment available, the different types of MS detectors in use, along with the large number of preparatory techniques and sample purification strategies that are applied for these purposes.⁹¹⁰⁻

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MALDI-MS is one of the most extensively used techniques in environmental and clinical analysis. Its robustness, efficiency and rapidity can permit the identification of intact microorganisms or their toxic products. MALDI-MS techniques utilize a lot of different MS detectors ranging from TOF^{914,915} to high resolution Orbitrap detectors.⁹¹⁶ Most of these come with a tandem configuration, allowing not only for fingerprinting based on the identity of the toxin, but also partial sequencing of the target metabolite(s) in many cases. To support reliable and reproducible protein toxin identification, MALDI approaches many times incorporate some form of identification of tryptic peptides, which result from the digestion of the parent protein toxin. Given the complex matrices which form the basis of samples typically analyzed for toxins, a sample pre-treatment step is usually required in order to enrich sample presence and increase analytical selectivity before proteolysis. Interestingly, immunocapture is one of methods for purification and enrichment that is most exploited in this situation.^{917,918}

Protein toxin identification by MS can also allow for multiplexed detection meaning that in a single sample analysis, unambiguous identification of several different types of toxins may be achievable. In this regard, the Kamboj Group proposed a multidetection method for BoNTs, ETX, SEB, and Shiga toxin using a bioaerosol-based sample collection strategy meant to be applied in an air-dispersed bioterrorism attack.⁹¹⁹ To prototype their method, they generated an aerosol in a controlled environment and collected proteins from the gaseous phase. The crude extract was then digested generating tryptic peptides which were analyzed by MALDI-TOF-TOF and identified by comparison to a bioinformatics peptide database. In this approach, no further purification or separation method was required and every toxin could be detected in a single run. Moreover, no false positives were detected with a demonstrated 0.2 ppb LOD in aerosol. There are other examples of peptide profiling for toxin detection. For example, Liu *et al.* proposed a method to validate ricin intoxication in mice.⁹²⁰ They first incorporated a selective enrichment step consisting of magnetic microparticle-based solid phase extraction (SPE) in order to avoid serum matrix interferences. Laser desorption was then applied over the extracted material allowing them to obtain the peptide profile by TOF detector. The use of different matrix coatings such as hydrophobic interaction (C8 and C18), weak cation ion exchange, and immobilized metal-affinity chromatography containing Cu ions (IMAC-Cu) allowed for the validation of the model animal system even at very low dosages.

In contrast to the sample preparation described above, Tisilia *et al.* relied on a classic in-gel proteomics approach for the detection of enterotoxin.⁹²¹ They performed a precipitation and subsequent PAGE separation of the proteins excreted by different *B. cereus* strains. The separated protein bands were further analyzed by MALDI-TOF allowing identification of the enterotoxins cytotoxin K1 (CytK1) and non-haemolytic enterotoxin (NHE) which are responsible for diarrheal food poisoning.^{922,923} Although capable of detecting these and other toxins, for which there is no commercial kit available, this approach still lacked a quantitation capacity which is a fundamental need in risk evaluation during an outbreak. The Wils Lab used a different approach based on a MS (MALDI and ESI) detection method targeting botulism.^{924,925} Using commercial BoNT samples with different serotypes, they were capable of identifying intact toxin and even assignment of the different A-F serotypes. Later, the same group managed to identify different strains with MALDI-TOF based on their endogenous endopeptidase activity.⁹²⁶ Enhanced selectivity was achieved by using an immunoaffinity extraction before the enzymatic cleavage allowing identification of all known-human botulism serotypes.^{927,928} The Barr group also used a similar endopeptidase-MS approach for botulism detection in food⁹²⁹ and stool⁹³⁰ with high sensitivity and specificity, see **Figure 55**. Further improvements on this method allowed very sensitive amol/mL quantification of each serotype as well.⁹³¹ This was accomplished by creating a two-stage method that optimized the enzyme incubation times. An initial short incubation of 30 min was first performed to monitor for higher toxin concentrations and, if this proved negative, the cleavage reaction was then allowed to continue for another 3.5 hours allowing for LODs of 55 amol/mL to be achieved. Overall, by implementing this two-stage approach, 4-5 orders of magnitude dynamic range in sensing could be achieved.⁹³¹

Others have included an ultrafiltration (UF) step to detect BoNT/A by MS in highly diluted samples (such as environmental water samples), detecting the presence of toxin at 7 pg/mL in a large volume sample (100 L of municipal tap water).⁹³²

The presence of interferents is a constant drawback for many analytical methods including especially MS. In the case of an outbreak, fast identification of the causative agent(s) is desired and quickly eliminating any interferents that may be in samples is clearly necessary. Bourne *et al.* proposed a modified approach to detecting intact ricin from castor beans in the presence of other related seed proteins.⁹³³ To achieve this, they included a final concentration of 0.01% Tween 80 detergent in the sampling media which reduced the interference signal in the

MS and allowed a 31 fmol LOD (4 $\mu\text{g/mL}$ in wet *on site* sample). The method was robust and quick as no proteolytic digestion was required for peptide fingerprinting^{934,935} and appears to be easily integrated and automatable within environmental monitoring stations.⁹³⁶

In some cases, the presence of a given toxin can also be determined in an indirect way with MS. For example, ricin shows a natural deanylase activity that can be assessed using a MS-based approach. For this purpose, the Barr group developed a method which starts with a magnetic immunoseparation of intact ricin from different beverages (milk, water and fruit juice). After that, a synthetic DNA substrate is added to evaluate the deanylase activity.⁹³⁷ Differences are monitored between the MS spectra of the original oligonucleotide and its depurynated product following ricin activity; deadenylase activity increases in proportion to the amount of ricin extracted. The authors were able to combine the activity assay with peptide mapping quantification using isotopic dilution tandem MS for absolute quantification of ricin. This combined strategy provided for a very sensitive LOD of 10 fmol/mL (0.64 ng/mL). While most sophisticated analytical procedures require a multistep approach with time consuming steps, the Direct Analysis in Real Time with Mass Spectrometry (DART-MS) can drastically reduce sample treatment steps and the overall analysis time.⁹³⁸ This approach is an ambient-pressure ionization MS technique which takes advantage of the excited-state of He atoms to induce the ionization of the target analytes. DART-MS has been successfully applied to the determination of aflatoxin M1 and B1 (AFM1 and AFB1) in complex matrices such as milk and corn.^{939,940} Others have used a similar approach to perform a multiplexed mycotoxin analysis in cereals.⁹⁴¹ In general, DART-MS can provide good sensitivity with very low noise from the background matrix.⁹⁴¹

Chromatographic Separation with Mass Spectrometry Analysis. Despite the great potential of direct MS-based strategies, MS analysis is more often coupled with different chromatographic separation techniques to extend their toxin sensing capabilities, selectivity and overall sample throughput. The hyphenation of chromatography (gas or liquid) with MS (and tandem MS) combined with an appropriate sample treatment can allow for multiple qualitative and quantitative analyses in almost every sample matrix. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) with ESI is the configuration most often employed in the analytical field. The potential of this technique can be quite remarkable when the sample matrix is

complex or the number of target analytes is large. The latter is clearly the case for the multiplexed analysis of mycotoxins, a family of compounds with a vast number of potential analytes. Addressing this issue, the Humpf Group found contamination of nine mycotoxins in different food products ranging from vegetables to oils. They developed a fast 12 min method which included a very simple sample pretreatment followed by HPLC-MS/MS analysis.⁹⁴² The method was further used to evaluate human exposure to the target mycotoxins in Germany. The same group had previously evaluated the presence of 26 mycotoxins in 42 different maize samples in South Africa based on a microLC method which used less than 0.3 mL of solvent.⁹⁴³ In both reports, a parent ion and the transition to qualifying and quantifying ions for each mycotoxin was evaluated by MS analysis. Others have used a similar approach to evaluate the presence of different mycotoxins in broiler chicken feed in Thailand in a single HPLC run following a simple sample pretreatment⁹⁴⁴ and in traditional Chinese medicine products.^{945,946} Other human consumption products analyzed with analogous strategies targeted to mycotoxins are coffee,⁹⁴⁷ milk including an online clean-up step to reduce matrix effects,⁹⁴⁸ cereals,⁹⁴⁹ fish,^{950,951} and meat.⁹⁵² Within these types of analyses, immunoaffinity columns are the preferred method for clean-up and sample preparation in food matrices.^{953,954}

Nano chromatography has also been used for the identification of BoNT based on a full proteomic approach.⁹⁵⁵ Demonstrating improvements over the reports mentioned above, this process began with generating tryptic digests of commercial BoNT standards, then after extensive dialysis, separated the peptide population by capillary chromatography and assigned the ID of each peptide based on MS and MASCOT database comparisons. Thanks to the LC system employed, the sample amount was approximately 40% smaller than that previously required for the same type of characterization. The same principle was applied by Klaubert and coworkers to analyze culture supernatants instead of commercial samples.⁹⁵⁶ Given the expected complexity of the sample, a second chromatographic dimension (2D-nano-LC-ESI MS) was included here. They further suggested that this adapted protocol may be applicable for wide-ranging BoNT detection in many foods and beverages.

Another tryptic peptide-based digestion application was applied by Callahan *et al.* to the detection, confirmation, and quantification of SEB in apple juice.⁹⁵⁷ With a simple sample treatment consisting of UF to remove low molecular weight components before the trypsin digestion, this method allowed SEB determination at a concentration of 5 parts per billion in a 10

mL sample. Others have included immunocapture and/or in-gel digestion to detect SEB in cheese and coco-pearls⁹⁵⁸ or milk.⁹⁵⁹ An alternative acetylation strategy with an acetic anhydride isotope ($\text{Ac}_2\text{O}/^2\text{H}_6\text{-Ac}_2\text{O}$), which is cheaper than commercial isotopic tagging, allowed for sensitive determination of SEB in chicken meat meant for human consumption.⁹⁶⁰ A simple precipitation with⁹⁶¹ or without⁹⁶² filtration also appears to be enough to determine the presence of SEBs based on the tryptic peptide population or the presence of whole intact proteins in food matrices. For the specific case of TTX, very low LODs are required in order to ensure the safety of many foods. To address this, Rodriguez and coworkers designed a strategy of low energy collision dissociation tandem mass spectrometry (CID-MS/MS) combined with an LC-ESI technique.⁹⁶³ They were able to detect the presence of TTX (and some of its analogues) in five different tissues originating from puffer fish at a level of 0.08 $\mu\text{g/g}$. This method has higher sensitivity than some previously reported LC-MS methods designed for the same role.⁹⁶⁴

Many times identifying the source or type of a given toxin during an outbreak is not enough on its own as a large cross-section of the general population may have been exposed to the pathogen. For this reason, the development of reliable methods to detect toxins within different human fluids and especially patient samples is mandatory. In the case of TTX, the poisoning generally occurs after deliberate or accidental ingestion of contaminated food. Although poisoning symptoms may be evident, the final diagnosis may still require identification of the toxin in the patient. Combinations of gas and LC^{965,966} have been considered for the analysis of TTX in blood and urine. Both examples, however, usually require an intensive clean-up of the sample using SPE or C18 reverse phase purification cartridges. Jen *et al.* employed a SPE/LC-MS approach to a real case of TTX poisoning in Kaohsiung, Taiwan.⁹⁶⁷ Urine remains the preferred sample matrix to evaluate TTX exposure in humans since the toxin will accumulate there during systemic excretion and be present at higher concentrations than in blood, even if several days have elapsed since ingestion or exposure.⁹⁶⁸ Sample throughput was increased by coupling SPE sample pretreatment to a hydrophobic interaction liquid chromatography (HILIC-LC) MS system, reducing the overall analysis time while maintaining its analytical performance.⁹⁶⁹ In response to other real-life events, the aforementioned endopeptidase LC/MS-MS assay was used as an alternative to the standard *in vitro* assay for BoNT/A detection during two separate botulism outbreaks in France in 2008 and 2010. Here, Mazuet *et al.* employed immunocapture in patient sera with polyclonal antibodies to also protect the enzymatic activity

of the toxin for subsequent assay, see **Figure 56**.⁹⁷⁰ The method showed a similar analytical performance to the *in vitro* method, but with the advantage that the results (even from contaminated food) could be obtained in hrs instead of days.

Clearly, screening and fast evaluation of toxin poisoning events can be a great advantage of instrumental techniques. Focusing on this specific property, different approaches to evaluate human samples for the presence of mycotoxins have been developed. Taevernier and coworkers design a method to evaluate skin exposure to the mycotoxins beauvericin and some enniatins.⁹⁷¹ They evaluated the interaction of the mycotoxin with skin using a transdermal Franz diffusion cell in an *in vitro* experimental format. The migration of the mycotoxins and interaction with skin was evaluated using a HPLC-MS/MS system based on the ion transitions of the different compounds. They were able to achieve an LOD of 10-17 pg/mL with an analysis time of less than 5 mins. Others have also detected the same mycotoxins in urine and plasma with MS.⁹⁷² Given the nature of the latter samples, a clean-up step was still required, but the optimized protocol allowed for detection of the target analytes at 20-40 ng/L in plasma and 5-20 ng/L in urine with no matrix effects. Beyond reliance on detecting the toxins directly themselves, the presence of certain biomarkers reflecting a poisoning event can also be relevant. Huybrechts and co-workers evaluated the presence of several mycotoxins and their glucuronide metabolites in urine samples in Belgium.⁹⁷³ The overall analysis time was 30 mins with a sensitivity below 200 pg/mL achieved for all the 32 different mycotoxins included. Surprisingly, this study found that exposure to mycotoxins (but not actual poisoning) is a relatively common phenomena in the general population.

Other Chromatographic Analysis. Many times coupling chromatography to MS detection is not easily available since it can be an expensive and requires sophisticated equipment. However, chromatographic techniques can be easily coupled to many other detectors types at a reduced fraction of the cost. LC coupled to UV/Vis detection is one of the most commonly implemented analytical systems, and has been successfully applied to the determination of mycotoxins in foods,⁹⁵⁴ crops,⁹⁷⁴ and fruit products.⁹⁷⁵ Using a similar approach, TTX has been detected in urine and plasma after a SPE step⁹⁷⁶ along with the proteolytic products of BoNT/A.⁹⁷⁷ Fluorescence detectors can increase the overall selectivity of sensing procedures and have been applied to more complex matrices such as patient urine or serum in the case of TTX

poisoning,⁹⁷⁸ for screening animals,⁹⁷⁹ or within automated methods analyzing for the presence of mycotoxins in edible oils.⁹⁸⁰

Surface Plasmon Resonance. SPR can sometimes offer a quick and portable alternative for differential analyte detection while functioning as an effective screening tool. As will be highlighted, the Biocore platform is one of the most popular commercial instruments for such applications.⁹⁸¹ The success of SPR sensing in this context relies directly on the biorecognition element which selectively interacts with the target analyte; in the case of toxins these are primarily antibodies.⁹⁸² Given the commonality of their source and origins, mycotoxins have been extensively analyzed by SPR in agricultural products intended for human consumption. For example, mycotoxins in wheat were detected using a deoxynivalenol (DON)-casein conjugate immobilized on the SPR chip surface.⁹⁸³ The target analyte was extracted from contaminated wheat samples using acetonitrile. An excess of anti-DON was added to the sample and then introduced onto the chip for a competition assay between immobilized DON conjugate on the surface and free analyte molecules in the sample. The sensor allowed for the determination of target analyte in a range of 2.5 to 30 ng/mL and was comparable to the results obtained by LC/MS approaches. More importantly, the sensor could be regenerated and reused up to a remarkable 500 times! Similar principles were applied by Kadota *et al.* to detect DON and nivalenol in wheat⁹⁸⁴ and by Meneely for HT2 and T2 mycotoxins in maize-based baby food and breakfast cereals.⁹⁸⁵ A related assay format was used to develop a patulin biosensor with in-house sourced polyclonal antibodies allowing detection of this toxin with an LOD of 100 nM.⁹⁸⁶

Multiplexed mycotoxin detection is also feasible as demonstrated by Joshi and co-workers.⁹⁸⁷ A two-chip system was targeted towards the most important mycotoxins with significant contamination in barley including DON, zearalenone (ZEA), T2, ochratoxin A (OTA), fumosin B1 (FB1) and AFB1, see **Figure 57**. The mycotoxins were first immobilized on the chip surface *via* amine/ovoalbumin conjugates for competition assays. With this strategy, the demonstrated LODs for DON, T2, ZEA, and FB1 fulfilled EU regulatory criteria. However, when the assay was implemented on a single microfluidic device using an image-SPR format, the overall sensitivity was observed to decrease. Rather than multiplexing, Naimushin and coworkers focused on designing a portable SPR sensor capable of detecting *S. aureus* enterotoxin B in complex matrices such as seawater and milk at a cost of less than a

dollar/sensor.⁹⁸⁸ The sensor device itself is constructed with two channels (reference and sample), a temperature control module and operated by 12V batteries. It allowed for the determination of target analyte at femtomolar levels in a detection mode called amplification which used a secondary antibody after the toxin antibody binding step. The Biacore platform has also been used to detect enterotoxin in milk at low ppb levels in less than 15 mins per sample.⁹⁸⁹

Detection of paralytic toxins such as saxitoxin^{990,991} and TTX⁹⁹² have been a common focus with SPR technology. Further efforts in improving a saxitoxin sensor yielded a two channel system which provided triplicate results in less than 5 mins on a single platform with acceptable sensitivity.⁹⁹³ For TTX, an inhibition assay with a chemically immobilized target provided a 0.3 ng/mL LOD with an easy-to-regenerate SPR device. Both designs provided better results than previously reported ELISA approaches.⁹⁹⁴ TTX has been detected using different SPR approaches in varied food matrices such as milk and even directly in the puffer fish itself.⁹⁹⁵ A first type SPR sensor for direct antibody detection has also been developed.⁹⁹⁶ The method here focuses on the toxin itself instead of the unbound antibody as a result of the inhibition process. Thanks to this technique, TTX detection at a concentration level of 2ng/mL in puffer fish matrix (0.09 ng/mL in buffer, four times more sensitive than the inhibition format) could be accomplished in 3 mins. Different food matrices such as honey have been investigated for the presence of BoNTs using SPR. According to the FDA, honey can be a source of potential botulism poisoning especially in children.⁹⁹⁷ Ladd and coworkers developed a SPR method to detect the presence of BoNT serotypes A, B and F in honey matrix in one hr, see **Figure 58A**.⁹⁹⁸ The system used a three detection channel design plus one reference channel and a secondary antibody to enhance the sensitivity of the procedure for each serotype which ranged from 0.5 to 1 ng/mL.

In the event of a poisoning event (intentional or accidental), on site determination and identification of the toxic agent can be critical to coordinate an appropriate response. With this in mind, Feltis *et al.* designed the first field-deployable ricin sensor based on SPR technology.⁹⁹⁹ As shown in **Figure 58B**, the device was designed to be hand held, self-operated, and independent of an external power source or outside software. Using immobilized anti-ricin antibody and a secondary antibody for confirmation, the device is capable of detecting traces (200 ng/mL) of ricin in ten mins of total operational time. This initial design also seems quite promising for expanding the capabilities to target other toxins or contaminants. Ricin was also detected using

the Biacore X commercial platform with monoclonal antibodies.¹⁰⁰⁰ This system was used to detect the commercial and environmental (horticultural) presence of ricin with great sensitivity (0.5 ng/mL) and reproducibility.

The existence of naturally occurring isoforms or homologs of a toxin can sometimes make it quite difficult to achieve unambiguous identification and/or quantification. Such is the case of the protein agglutinin which is a closely related to ricin but less toxic and hard to differentiate between when using antibodies. In an attempt to address this, Stern *et al.* design a SPR alternative using monoclonal antibodies.¹⁰⁰¹ The success of the method relies on the different interaction of the antibodies with both proteins; while the immobilized primary mAb (R109) interacts with both toxins, a secondary mAb (R18) recognizes only the B-chain of ricin. Evaluation of the SPR response for both antibodies allowed identification of both target toxins with a ricin LOD of 3 ng/mL and 6 ng/mL for agglutinin in castor beans, food, and environmental samples, see **Figure 59**. This sensitivity is comparable to MS methods and the authors further suggest that this approach could be easily incorporated into the aforementioned Feltis hand-held platform.⁹⁹⁹

If a toxic episode is suspected, a reliable diagnosis in patients then becomes mandatory. If an original source sample is not available (*e.g.*, a contaminated meal), the patient(s) biofluids must be tested for the presence of toxin or its metabolites. Given its rapidity, sensitivity, and specificity, SPR methods are especially useful for this purpose and indeed Botulism has been successfully detected in patients with this approach. For example, Ferracci *et al.* developed a method to detect BoNT/B in food and human serum.¹⁰⁰² The basis of this sensing is indirect, detecting the cleavage of vesicle associated membrane protein 2 (VAMP2), one of the toxin's natural substrates. Briefly, the sensor surface is coated with anti-VAMP2 antibodies and, in the presence of BoNT, the substrate is cleaved from the synaptic vesicles of patient serum and no SPR signal is detected as shown schematically in **Figure 60A**. With the optimized protocol, toxin can be detected at the fM level in buffer medium while the analysis of patient serum requires a previous immunoprecipitation step to reach acceptable sensitivity. Others have also taken advantage of the endoprotease activity of BoNT/A/E on SNAP-25, a presynaptic protein that is another of the toxin's substrates.^{1003,1004} In this case, SNAP-25 is immobilized on the SPR chip surface and later incubated with patient sera suspected of BoNT presence to assay for proteolytic activity. Subsequent to this, a monoclonal antibody (mAb10F12) for one of the

SNAP-25 epitopes generated by proteolytic activity is injected to detect the toxin activity products; shown schematically in **Figure 60B**.¹⁰⁰³ The LOD for this approach is a rather remarkable 0.5 fM from just 100 μ L of diluted patient sera sample.

SPR-based biosensors have also been utilized to detect TTX in the sera of intoxicated patients. Using an antibody inhibition format assay, Taylor *et al.* were able to detect TTX in all confirmed patient samples and in contaminated foods. Moreover this data correlated well with that obtained by LC-MS/MS techniques, demonstrating that SPR could be as reliable and robust as other more complex analytical techniques.¹⁰⁰⁵ Beyond use of antibodies as the SPR biorecognition element, aptamers have also seen concerted application.¹⁰⁰⁶ For example, Zhu and coworkers evaluated an SPR platform to detect OTA in wine and peanut oil.¹⁰⁰⁷ For this, a Biacore chip was coated with an anti-OTA biotinylated aptamer *via* streptavidin/biotin interactions. The optimized protocol allowed the detection of spiked wine and peanut oil sample at a 0.005 ng/mL LOD with minimum sample pretreatment (liquid-liquid extraction to eliminate interferences from the sample matrix) in seconds.

Quartz Crystal Microbalance/Cantilever/Piezoelectric. Most biorecognition processes are candidates for evaluation as a mass transfer process over a sensing interface. As already mentioned, such gravimetric measurement processes have been applied extensively within the biosensing field and present several advantages over other techniques, as extensively highlighted by Montagut *et al.*¹⁰⁰⁸ For example, Bergantin *et al.* combined QCM technology (piezoelectric transduction) with toxin-receptor interactions to detect saxitoxins.¹⁰⁰⁹ They immobilized isolated sodium channels from electric eels (*Electrophorus electricus*) on the surface of a quartz crystal *via* a hydrophobic mixed bilayer/gold electrode surface. Later, the solution containing the toxin was injected into the flowcell to measure the interaction with the modified electrode. With an optimized procedure, the authors detected the toxin at an LOD of 0.275 mg/mL. This approach is an interesting proof of concept of how a toxin targeting a biological ligand can be selectively bound on a sensor surface and use the underlying interaction as a detection method. However, the more common sensing interface found with these types of sensors still primarily rely on antibodies.¹⁰⁰⁸

Salmain and coworkers proposed a direct, label-free immunosensor for the detection of Staphylococcal enterotoxin A (SEA) using QCMs.¹⁰¹⁰ The sensing principle relied on the high

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3 affinity association between the target analyte and the immobilized antibody. For this, they
4 immobilized an anti-SEA polyclonal antibody on a gold electrode surface by a chemisorption
5 process. The interaction generated a decrease of the quartz crystal chip's resonance frequency as
6 a result of mass uptake. This assay allowed determination of SEA at an LOD of 20 and 7 ng/mL
7 in direct or sandwich format, respectively, with an overall analysis time of less than 20 mins in
8 buffer. The same authors later demonstrated applicability to contaminated milk samples.¹⁰¹¹
9 Others have used a similar piezoelectric immunosensor based on monoclonal antibodies to detect
10 SEA in chicken meat and milk with improvements to sensitivity (0.4 ng/mL).¹⁰¹²
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15 Cantilever devices are also capable of multiplexed and sensitive detection with minimal
16 sample waste and usage time. Microcantilever sensors can similarly respond to surface stress
17 changes produced by a biorecognition event.¹⁰¹³ Ricciardi and coworkers developed a
18 microcantilever immunoassay to detect different mycotoxins at the ng/mL concentration level
19 and to determine OTA with no cross-reactivity in the presence of other mycotoxins.¹⁰¹⁴ LODs for
20 these devices could be as low as the picogram level as reported by Gosset *et al.*¹⁰¹⁵ A PEMC
21 was fabricated from a piezoelectric ceramic (lead zirconate titanate, PZT) and a borosilicate glass
22 layer a few millimeters in length; the latter of which is especially suitable to measure protein
23 adsorption.¹⁰¹⁶ The polyclonal-antibody functionalized layer allowed detection of SEB at under
24 50 pg/mL. The PEMC could also be regenerated and re-used at least twice without significant
25 loss in antibody activity, extending the potential usability. Aptamers have also been employed as
26 biorecognition elements in piezoelectric based detection. Zhao and coworkers developed a SEB
27 detection method using aptamers to overcome some common limitations of antibody-based
28 alternatives.¹⁰¹⁷ A sensing layer was created by immobilizing thiol-modified anti-SEB aptamers
29 on the gold-coated surface of a cantilever. The aptasensor response was linear with SEB
30 concentration in saline buffer and skim milk (6-300 ng/mL), and although sensitivity can be
31 higher when using antibodies,¹⁰¹⁸ this proposed method is fast, cheap, and sensitive enough for
32 on-site preliminary screening measurements.
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51 Biochemical Sensors

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53 ***Antibody-Based Toxin Detection.*** As noted repeatedly, antibodies have been extensively used in
54 the biosensing field to provide the critical biorecognition capabilities within many assay formats
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and even in concentration and clean up approaches.^{953,1019} Among the many applications of ELISA technology in all its formats, toxin detection continues to be an area of strong research and application interest. The vastness of this endeavour again only allows us to mention a few representative examples. For example, Zhou *et al.* developed an indirect competitive ELISA (icELISA) assay for the determination of TTX.¹⁰²⁰ Using a very simple approach, a microtiter well plate was coated with 0.1 µg of TTX and later incubated with mAb which competes with any toxin subsequently added in a test sample. Signal transduction from a sensing event was obtained from a second peroxidase-conjugated antibody allowing for detection at 0.05 ng/well in less than 2 hrs. A direct competitive ELISA format (dcELISA) was less sensitive but much faster.¹⁰²¹ The latter derivative methodology includes immobilization of the anti-toxin antibody and a second incubation with enzyme-labelled antigen, eliminating any possible cross-reactivity due to the use of secondary antibodies. Both methods are suitable for TTX detection at a fraction of the cost of instrumental techniques such as HPLC.⁹⁷⁸

Significant efforts continue to be made to replace the mouse bioassay for detecting BoNT activity due to ethical considerations, questions about sensitivity levels, timeliness, along with general access and implementation. With an aim of increasing sensitivity, Brooks *et al.* proposed a culture enrichment method to assist sELISA procedures for diagnosis in mammals.¹⁰²² In this assay protocol, samples are first heated and cultured anaerobically. After this treatment step, previous sELISA and/or mouse bioassay samples that tested negative due to the presence of toxin below the LOD became positives, thus improving the overall diagnostic capabilities. However, other samples suspected of toxin poisoning could require other treatments, such as UF of drinking water to increase the sensitivity of the analytical process. Raphael and co-workers demonstrated how UF of large municipal tap water samples could improve ELISA sensitivity.⁹³² Using tangential-flow UF with hollow fibre dialyzers, they were able to detect BoNT/A in 100 L of spiked water samples at a concentration level below 7 pg/mL, where both ELISA and Endopep-MS method without UF failed to detect the presence of the toxin. BoNT/B was detected by Scotcher *et al.* using an engineered mAb against this toxin in a sandwich immunoassay with a remarkable LOD of 100 fg/mL in buffer and 39 pg/mL in milk without any sample pretreatment.¹⁰²³

Methods based on ELISA technology can be directly employed in complex matrices such as milk to detect different Staphylococcal enterotoxins (SE). Rahimi employed ELISA to detect

the presence of different SEs in raw milk collected from different mammals with an LOD of 0.1 ng/L for at least one SE (within an array of A-E enterotoxins tested).^{1024 865} This allowed them to conclude that the overall quality of the milk in the test region (Iran) was rather poor and created an existing risk of poisoning in the general population. The presence of non-classical enterotoxins, but with similar toxin potential can be missed with typical ELISA approaches. Nagaraj *et al.* highlighted this importance for detecting the presence of Staphylococcal enterotoxin G (SEG) in a selective assay and this could also act as a potential indicator of the presence of other SEs.¹⁰²⁵ SEG in this assay was detected by sELISA using chicken anti-SEG (IgY) to capture the target toxin, rabbit anti-IgY as a revealing antibody, and peroxidase conjugated goat-anti rabbit antibody to generate a visible reaction. With the optimized procedure, the authors were able to detect the presence of SEG in milk at an LOD of 1 ng/mL. Furthermore, analysis of 89 real-world samples revealed a total of 23 positive results. The authors also suggested that analytical performance could be improved by implementation of lateral-flow assays using QDs or AuNPs to enhance the signal.

As mentioned before, early detection of a toxic agent in the environment can be as important as early diagnosis. This is especially true in the case of ricin, where most of the sensing methods are focused on environmental samples. Chen *et al.* proposed a diagnostic test based on an ELISA kit valid for both scenarios.¹⁰²⁶ In this assay, a biotinylated polyclonal antibody is used to first coat the ELISA plate, and following sample exposure, HRP-conjugated streptavidin was used to reveal the positive samples indirectly by loss of signal. This method was used as a diagnostic tool in environmental (aerosol), human (faeces and blood), and infected animal samples (faeces, blood and urine). This allowed identification in blood (0.277 ng/mL) and faeces (0.205 ng/mL) at a level comparable with previously reported procedures which had required longer analysis time or tedious and more expensive sample treatments.¹⁰²⁷ These results also suggested that fecal samples may be a more suitable sample for ricin detection due to better reproducibility and sensitivity when compare to urine and blood, along with providing access to longer useful detection time periods after a poisoning event most probably due to the bioconcentration of toxin during the natural elimination process.

In some cases, a single mAb can exhibit strong affinity for different but related targets such as mycotoxins. Ertekin *et al.* took advantage of this to develop an IgA-based sELISA assay and an immune affinity column (IAC) method for detection of five different mycotoxins.¹⁰²⁸ In

both cases, no orientation of the antibodies was required, which is a major advantage of IgA's since it is a multivalent antibody. Both systems were further applied to food matrices (corn and hazelnut) where the sELISA was able to detect ricin in the range of 2-50 $\mu\text{g/L}$ in 40 mins of analysis time whereas the IAC showed a binding capacity from 70-114 ng depending on the mycotoxin. Phage display techniques have been used to isolate high affinity antibodies from a large population of ligands in order to improve ricin detection. It has been used with sdAb for sensitive detection of ricin by ELISA or Luminex assays,¹⁰²⁹ and with single chain variable fragments (scFv, single chain recombinant binding elements) for the detection of SEA in an sELISA format.¹⁰³⁰ Use of sdAbs was later applied to ultrasensitive ricin detection, with a fg/mL LOD using a single-molecule array with paramagnetic beads.¹⁰³¹

An ideal toxin assay kit should be able to perform a multiplexed screening for different toxins, and with this in mind, Jenko and co-workers developed an ELISA microarray capable of detecting ten biothreat toxins simultaneously.¹⁰³² This method was able to detect BoNT/A-F, ricin, shiga toxins 1 and 2, and SEB in clinical and environmental samples. To accomplish this, biotinylated capture antibodies for each toxin were first printed on a glass slide as an array. The slide was then incubated with the samples and later with HRP-streptavidin-antibody conjugates and Cy3-streptavidin conjugates following a similar procedure previously described by Woodbury *et al.*¹⁰³³ The optimized protocol permitted toxin detection in biological (serum, plasma, nasal fluid, urine, saliva, and stool) and food (milk, apple juice, and beef baby food) samples with great sensitivity (pg/mL range) and precision which suggested it as a powerful tool to provide fast multiplexed screening and identification in a potential bioweapons event.

Electrochemical Toxin Detection. Potentiometry is the most commonly used technique for electrochemical detection of toxins. Fetter and coworkers applied an aptamer scaffold electrochemical sensor (named E-DNA) design developed by Lubin¹⁰³⁴ for the detection of different oligonucleotides in complex matrices for targeting of BoNT/A and ricin.¹⁰³⁵ The E-DNA configuration consisted of an aptamer/DNA-scaffold containing an electrochemically active reagent (methylene blue) bound to an electrode surface. The interaction between target analytes and aptamer/scaffold modified the position of the methylene group moiety, generating a change in signal for voltammetric analysis. The optimized system allowed for the detection of

BoNT and ricin at an LOD of 0.4 and 0.7 nM respectively, see **Figure 61** for a schematic and some associated data.

BoNT/E was also detected by Narayanan *et al.* using a more complex electrochemical immunosensor.¹⁰³⁶ The experimental set up in this report consisted of a graphene nanosheet covalently immobilized on a glass carbon electrode surface covered with a capture layer of anti-BoNT/E rabbit antibody. For implementing a sandwich immunoassay with this sensor, the assay sequence was carried out stepwise with: sample containing the target analyte is first put in contact with the electrode followed by a second incubation with mouse anti-BoNT/E; a revealing step is then implemented consisting of a third incubation with rabbit anti-mouse IgG-conjugated with alkaline phosphatase and functionalized with AuNPs; the enzyme substrate 3-indoxylphosphate (3-IP) is then added along with AgNO₃ solution to evaluate silver deposition on the electrode surface *via* linear sweep voltammetry. As 3-IP reduces the Ag ions, the deposition of AgNPs responds to the presence or absence of BoNT/E. Little-to-no cross reactivity with other BoNT isoforms was observed when tested directly. Overall, BoNT/E could be detected at an LOD of 5 pg/mL in buffer and 0.1 ng/mL in milk and orange juice in 65 mins. Rather than rely on full antibodies, ricin chain-A was detected using sdAbs in a deployable potentiometric sensor with enhanced durability and stability.¹⁰³⁷ The use of thiolated antibodies on gold electrodes in a disposable unit format has also allowed detection of 5 pg of SEB in a 5 μ L sample.¹⁰³⁸

Electrochemical immunosensors have been used to target mycotoxins such as OTA in a variety of different matrices. Alarcon *et al.* employed direct and indirect ELISA on disposable screen-printed electrodes decorated with anti-OTA monoclonal antibodies.¹⁰³⁹ A simple liquid extraction of wheat samples followed by a direct immunosensor assay allowed detection of the target analyte at an LOD of 0.4 μ g/kg. Further efforts were made to increase the rate of electron transfer to the electrode by using antibodies immobilized on chitosan-Fe₃O₄ NPs achieving an improved LOD of 5 pg/mL. The inclusion of Fe₃O₄ NPs increased the electroactive surface enhancing the antibody loading capacity.¹⁰⁴⁰ Other platforms have employed reduced graphene oxide and AuNPs to enhance electrical compatibility and antibody immobilization, resulting in a high sensitivity/selectivity sensor for mycotoxins with applicability to food products.¹⁰⁴¹

In contrast to potentiometry, amperometric detection may require a variety of different types of electrodes based on the specific target desired. For example, a rapid method for

detection of seafood toxins, including TTX, employed screen-printed carbon electrodes and alkaline phosphatase-tagged antibody¹⁰⁴² while ricin assays utilized multiwalled carbon nanotubes and graphite paste electrodes;¹⁰⁴³ sensitivities in the ng/mL levels were achieved in both cases. To implement an impedimetric sensor for SEB detection, Wu and colleagues utilized a smart design combining SEB conjugated with magnetosomes, which enhances the immunoreaction by providing a larger specific surface area for the antibodies to form a smooth and dense film on the gold electrode, and an ionic liquid to improve electron transfer to the electrode.¹⁰⁴⁴ This approach allowed SEB detection in milk at an enhanced LOD of 0.017 ng/mL with good repeatability and stability. Impedimetry has also been implemented to detect OTA in plant extracts by immobilization of anti-OTA antibody on BSA-modified gold electrodes.¹⁰⁴⁵ Some examples of capacitive sensors have been reported for the detection of SEB¹⁰⁴⁶ and SEA.¹⁰⁴⁷ In these formats, SEB is detected using HRP-labeled antibody with an LOD of 0.3 pg/mL in 10 min, whereas SEA sensing was in a label free format with an LOD of 1 pg/mL. The latter was applied to contaminated food matrices such as tea, milk, fried chicken, and orange juice.

PCR. As described previously, PCR and its many functional derivatives target genomic or plasmid DNA in pathogenic organisms along with their transcripts when coupled into a RT-PCR format. Since the toxins under consideration here are a biosynthetic product or enzyme from the pathogen, this approach is generally not applicable. There are, however, some applications of PCR within toxin detection that still merit mentioning. For example, Umeda *et al.* directly targeted the BoNT/A gene cluster of *C. botulinum* for PCR amplification allowing correct classification in human samples and food suspected of BoNT contamination;¹⁰⁴⁸ a similar approach was also later reported for detecting BoNT/C/D.¹⁰⁴⁹ Single tube nested PCR has been used to increase the specificity of PCR amplicons by inclusion of a second pair of anti-sense primers in order to detect avian botulism in the cecal contents of chicken.¹⁰⁵⁰ Real-time PCR has been performed on liver samples to detect BoNT/C/D presence in an avian botulism outbreak.¹⁰⁵¹ Real-time PCR has helped with the simultaneous determination of ricin and abrin in contaminated food.¹⁰⁵² Kwoon *et al.* used an ingenious strategy combining antibody recognition and PCR amplification to allow BoNT detection at the fM level in honey matrix.¹⁰⁵³ The target BoNT protein is first recovered from a sample through immunoprecipitation and a sialyllactose-

conjugated DNA is then added to the sample where the sialyllactose interacts with the BoNT's binding domain and acts as a binding probe. After several washing steps, Glyco-qPCR analysis of the toxin-bound DNA is used to detect aM to fM concentrations of BoNT in the honey samples, see **Figure 62** for a schematic of the assay. Synthesis of multivalent sialyllactose-DNA conjugates was used to improve the assay performance even further.

Spectroscopic Sensors

Colorimetric Based Detection. Colorimetric assays are highly desired for toxin detection as they can provide simplicity, rapid analysis time, low-cost, and are suitable for on-site detection. Given their prominence within the diagnostics field, direct AuNP-based sensors are continuously being incorporated into a large number of detection strategies for chemical and biological threat detection including toxins.^{48,501,1054} As indicated, depending upon their preparation, AuNPs can manifest peroxidase-like activity which can provide extraordinary advantages for the development of simple analytical methods.⁵⁰² In the context of toxin bioagents, this principle has been successfully applied to the detection of ricin.¹⁰⁵⁵ To achieve effective and sensitive detection in this format, the AuNP surface was first activated by conjugation with an anti-ricin aptamer which increased the NPs innate peroxidase activity. In the presence of the target analyte, the aptamer was released from the NPs surface reducing the catalytic activity (dark blue color/light blue color in absence/presence of target). The proposed method was shown to be suitable for the detection of ricin in raw milk and carbonated beverages with a sensitivity in the nM range. Interestingly, the authors also indicated that the presence of H₂O₂ for the catalytic reaction also controlled the AuNP dispersion properties leading to a color switch between red (highly dispersed) to blue (aggregated). Using a different approach, Zhou and coworkers exploited the growth of AuNPs as mediated by a hemin/G-quadruplex DNAzyme to design a colorimetric detection method for SEB.¹⁰⁵⁶ The color shift in this assay was easily observed by the naked eye, or measured spectrophotometrically yielding an LOD of 1 pg/mL in human serum.

Color transition of AuNPs generated by their aggregation state have been tested for the detection of BoNT. In a very intelligent design, Liu and coworkers used the proteolytic activity of the active neurotoxin on a biotinylated peptide substrate (designed to mimic its natural target, SNAP-25) to induce changes on the aggregation state of the particles.¹⁰⁵⁷ The optimized design,

with an LOD of 0.1 nM for BoNT/A (light chain), involved a doubly- biotinylated peptide which, in the absence of the neurotoxin, induced the aggregation of neutravidin-modified AuNPs (blue color). The presence of active neurotoxin releases one of the biotin from the peptides and induced the dispersion of the NPs (red color). Lower detection limits could be achieved (1.67 pM) by using peptide cleavage products and Cu²⁺ induced AuNP aggregation.¹⁰⁵⁸ The authors suggested a mechanism by which peptide product-induced aggregation is enhanced by the presence of Cu ions given the strong affinity of this metal for the amine groups on the cleaved peptides. Other AuNP aggregation-based assays using aptamers against target analytes were proposed by Liu *et al.* for use in food matrices to detect SEB¹⁰⁵⁹ and ricin in milk powder and soda with great sensitivity.¹⁰⁶⁰ In the latter example, the presence of ricin in samples could be visualized by eye and confirmed by UV-vis absorption spectroscopy even when using unmodified AuNPs, see **Figure 63**. Pharmaceutical samples were also tested for the presence of BoNT/A by following the toxin's proteolytic activity.¹⁰⁶¹ SNAP-25 coated AuNPs allowed the analysts to follow the color switch induced in the presence of active toxin. Furthermore, the procedure appears to be adaptable to more complex samples by application of a simple sample pretreatment step.¹⁰⁶²

On-site detection of target toxin is crucial for rapid decision making, but it's especially important in the case of products intended for human consumption. In this vein, Arduini and coworkers took advantage of a portable optical device to detect the presence of OTA and AFB1 mycotoxins in millet and wine.¹⁰⁶³ The novelty of this approach was derived from the device's ability to perform both spectrophotometric and fluorimetric measures. In addition, an immunoaffinity clean up step was also implemented making it possible to reach an LOD in the µg/kg range. The fluorescence mode was employed to determine and quantitate the presence of OTA whereas AFB1 was detected based on its inhibition of acetylcholinesterase by Ellman's colorimetric method. Sapsford and coworkers presented a similar scheme using a multimodal portable device with both fluorometric and spectrophotometric capabilities to detect the presence of SEB.¹⁰⁶⁴ In this case, the platform of choice was a miniaturized 96-well ELISA chip functionalized with anti-SEB antibodies. This portable device allowed detection of SEB within a sample volume as low as 5 µL in different food matrices with LODs in the ng/mL range.

In scenarios where a toxin poisoning has already occurred, accurate detection of directly related biomarkers is crucial. To realize such an objective, Cho *et al.* designed a colorimetric

strategy to detect the presence of N-methyl L-tryptophan in urine as an abrin poisoning detection method.¹⁰⁶⁵ The enzyme N-methyl tryptophan oxidase (MTOX) was first immobilized on the surface of magnetic microparticles. MTOX oxidizes L-abrin present in urine samples to produce tryptophan and H₂O₂ as a byproduct. As a result, H₂O₂ can be colorimetrically detected by following the color generated from TMB substrate oxidation with addition of HRP. The MTOX-magnetic microparticles could be reused more than six time while providing for an LOD of 4 μ M of L-abrin in contaminated urine samples.

Fluorescent Detection. From an analytical perspective, fluorescence-based detection provides a well-established, fast, and potentially sensitive approach to toxin sensing. The most common format, of course, is exploitation of a fluorescently-labelled antibody with recognition specificity towards a given toxin and their application in some type of immunoassay be it direct, indirect, sandwich, *etc.* In one representative example, Taitt *et al.* developed a sandwich immunoassay supported on a hydrogel matrix to detect cholera and SEB toxins with high sensitivity.¹⁰⁶⁶ An anti-toxin IgG capture antibody was used with a secondary tracer-dye modified antibody in a sandwich format aided by the 3-dimensional nature of the polyacrylate hydrogel. The detection event was measured by confocal microscopy over the hydrogel matrix yielding an LOD in the ng/mL range. Labeled-aptamers are also commonly found in use as fluorescent probes for toxins and these formats can get quite sophisticated. For example, OTA was targeted using a structure-switching signaling aptamer.¹⁰⁶⁷ This assay design was based on a duplex-to-complex structural transition using two strands where one was labeled with a quencher and the other recognition strand was labeled with a fluorophore. In the presence of target, the quencher is released and a simultaneous increase in fluorescence intensity takes place. Under optimized conditions, OTA could be detected in corn with an LOD of 0.8 ng/mL without any interference from structurally related mycotoxins in only one min of analysis time, see **Figure 64**. Due to its simple design, easy operation, fast response, low cost, and analytical performance, the proposed strategy strongly suggests itself for integration into portable systems for use in on-site mycotoxin screening applications. This would, of course, necessitate availability of equivalently functioning aptamers that extend beyond just one mycotoxin target.

Deeply multiplexed toxin analysis is a straightforward extension of the immunoassay approach as demonstrated by Ligler *et al.*¹⁰⁶⁸ This assay design relied on specific capture

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3 molecules, typically antibodies, which were immobilized in stripes on the surface of a planar
4 optical waveguide, forming a "bar code" with each stripe in the "bar code" directed towards a
5 different analyte. Flow chambers placed at right angles over the waveguide provided for sample
6 administration along with subsequent visualization by using labeled primary and/or secondary
7 detection antibodies specific towards the targets. This semiautomated approach allowed both
8 identification and quantification of SEB, ricin, BoNTs and mycotoxins, along with other analytes
9 such as cholera toxin and even TNT at low ng/mL levels. Actual fluorescence was captured by
10 means of a CCD camera with a laser providing evanescent illumination of the waveguide. Later
11 image analysis in comparison to a calibration standard allowed for quantitation when required.
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14 For many toxin detection applications, it is impossible to discuss fluorescence without
15 mentioning the unique contributions QDs can bring to this endeavor and especially in the context
16 of multiplexed analysis.^{212,481,533,550,759,770,1069-1072} Within the specific context of biothreats and
17 toxins, Goldman and coworkers developed a multiplexed immunoassay for cholera and SEB
18 toxin using capture antibodies immobilized in a microtiterplate with corresponding detection
19 antibodies coupled to the QD surface.¹⁰⁷³ The assay format was later expanded by the same
20 group to the multiplexed detection of four different toxins - cholera toxin, ricin, shiga-like toxin
21 1, and SEB.⁵⁴⁰ Multiplexing was facilitated in this case by using 4-different QD colors with
22 emission maximums spectrally separated by at least 20 nm. This allowed the emission profile to
23 be resolved through a simple deconvolution scheme while still providing acceptable sensitivity
24 for individual toxins within the mixture. Utilizing QDs engaged in FRET with spectrally distinct
25 acceptor fluorophores could also help provide an extended pallet of emission colors especially if
26 only a limited number of QD colors are available to begin with.¹⁰⁷⁴
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29 As mentioned, for BoNTs not only is detecting the presence of the protein important but
30 also if it is active. Sapsford *et al.* highlighted this by showing that an acceptor dye-labeled
31 SNAP-25 peptide substrate could be digested in the presence of BoNT/A and that this would
32 significantly alter the rate of FRET upon subsequent assembly of the peptide to a QD donor.¹⁰⁷⁵
33 The reported sensitivity (17.5 ng/mL) achieved was comparable with previously reported
34 methods based on immuno- or radioassay detection. This format was recently extended to
35 include both the BoNT/A and B serotypes.¹⁰⁷⁶ QDs provide not only improved fluorescent
36 properties while their non-trivial size and mass can also enhance SPR detection capabilities as
37 shown for ricin detection.²¹² New synthetic methods for producing improved water soluble QDs
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have also contributed to mycotoxin detection.¹⁰⁷⁷ Here, a reverse microemulsion process was used to produce silica-encapsulated QD (QD@SiO₂) which were later bioconjugated to antibodies and used in a microtiter plate based immunoassay and quantitative immuno-column analysis yielding LODs of 473 and 20 ng/mL, respectively for DON. Not only are improved QD materials important to their use in toxin detection but also improved methods for bioconjugating the biorecognition elements such as the antibodies or aptamers to their surfaces with a high degree of control and concerted efforts continue here as well.^{348,349,351,481,534}

Beyond classical QD materials, the use of lanthanide doped NPs as fluorescent probes has been suggested for developing a new generation of detection probes.¹⁰⁷⁸ Their preliminary application to toxin detection was demonstrated by Huang *et al.* in a multicolor platform targeting different enterotoxins in milk samples, with sensitivity achieved in the ng/mL level. Specificity in this assay was provided by the use of specific aptamers against the target toxins immobilized on Ln-doped KGdF₄ nanoparticles (NPs). AMPs have also been investigated as semi-specific biorecognition elements in a sandwich immunoassay array format for different targets, including BoNTs with sensitivities comparable to other reported methods.¹⁰⁷⁹ Switching to a custom synthesized peptide to detect toxin endopeptidase activity allowed detection of BoNT/A in rat serum with an LOD of 100 pM in 20 uL sample in a FRET assay format.¹⁰⁸⁰ Rather than relying on a commercial SNAP 25 FRET substrate peptide, the custom peptide in this case decreased the donor acceptor spacing from 13 residues to 3 resulting in more efficient energy transfer which, in turn, allowed for more sensitivity due to a larger fluorescent signal change upon cleave.

Raman Spectroscopy. More complex techniques, such as SERS have been undergoing continuous evaluation for toxin detection applications. There are many examples in the literature that implement different analytical approaches with SERS-based sensing.¹⁰⁸¹ The overall quality of the procedure is, however, almost always dependent on the fabrication of the SERS substrate. With this in mind, Wen-Chi and coworkers fabricated a SERS substrate using nanosphere lithography to create AgNP arrays that were used in TTX detection.¹⁰⁸² Analysis of the AgNP SERS spectrum when in contact with different TTX concentrations in solution allowed detection of the target analyte at an impressive LOD of 0.9 ng/mL; this LOD is in the range of chromatographic techniques with other detection modalities but required a much shorter analysis

time. If sample matrices are complex, interpretation of the SERS spectrum can be quite difficult and steps have been added to these assay formats in order to selectively isolate the target of interest from any potential interferents. He and coworkers demonstrated this with an anti-ricin B aptamer conjugated on silver dendrites for toxin detection in food.¹⁰⁸³ In their two-step procedure, the target toxin is first captured by the silver nanostructure and then measured from its SERS spectrum to quantitate the amount of toxin present in milk or orange juice in less than 40 min. In terms of sensitivity, the method was capable of detecting 50-100 ng/mL, depending on the sample. An additional immunomagnetic separation step reduced the analysis time to 20 mins in milk, with acceptable sensitivity (4 $\mu\text{g/mL}$).¹⁰⁸⁴ Silver dendrites have also been explored as a valuable SERS substrate for the detection of ricin even on letter paper. A non-destructive extraction procedure yielded an LOD of 0.04 μg ricin B chain surrogate in less than 10 mins.¹⁰⁸⁵ This bodes well for using this approach as a rapid screening tool of any substances found in mail or other similar sample types along with most surfaces in general. Other SERS formats such as aptamer-based sandwich assays have achieved ricin sensitivities in the fM range.¹⁰⁸⁶ The assay format for this is based on AgNPs surface-immobilized with 4,4'-bipyridyl and an anti-ricin B aptamer that is used with a hybrid silicon modified substrate that also displays the anti-ricin B aptamer, see **Figure 65** for a schematic and representative data. The high selectivity of this assay format allowed detection of the target toxin in complex samples such as orange juice, milk, blood or urine. Human blood has similarly been probed for the presence of ricin toxin using SERS based on immobilizing ricin specific aptamers on other silver substrates.¹⁰⁸⁷

Some toxin analytes exhibit certain natural activities that can also serve as an indirect target for detection. Tang and coworkers created a SERS chip that monitored the depurination of AuNP-conjugated oligonucleotide substrates for detecting ricin depurination activity.¹⁰⁸⁸ This substrate was deposited over a Si surface where, following ricin depurination, signal was enhanced and changes between samples observed by the subsequent growth of an Ag-nanoshell over the AuNPs. This chip approach was able to detect ricin in complex food and biological samples at an LOD of 9 ng/mL with excellent stability (up to three months). Others have exploited the natural binding target of ricin toxin (*N*-acetyl-galactosamine) as a capture layer on a SERS substrate for toxin detection in fruit juice matrices with comparable sensitivities.¹⁰⁸⁹ In conjunction with targeting different types of toxin activity, different NP materials have also been

explored as SERS substrates including a variety of upconversion, Ag-Au, Au@C@Pt, and other core-shell bimetallic NPs.¹⁰⁹⁰⁻¹⁰⁹³

SERS has been further employed as a sensitive detection strategy for targeting enterotoxins. The Tamer group developed two different sandwich assay formats using magnetic AuNR particles functionalized with peptide-aptamers conjugates¹⁰⁹⁴ or antibodies¹⁰⁹⁵ as SEB target capture probes. Their methodology incorporates a second gold SERS tag modified with either aptamer or antibody to perform the actual measurement. In both scenarios, the sensitivities were outstanding with LODs of 768 and 224 aM for antibodies and aptamers; these values corresponds to only *ca.* 9250 and 2697 copies of the target toxin in 20 μ L of sample. These results are far more sensitive than many other ELISA approaches and, more importantly, can be applied in complex samples such as milk, blood and urine. Other sandwich strategies for the detection of SEB in milk involved the design of a Au@Ag core@shell structure decorated with 4-nitrophenol as an electromagnetic stabilizer of the Raman reporter along with anti-SEB antibodies.¹⁰⁹⁶ The carefully tailored SERS reporter demonstrated sensitive detection of the target toxin at low pg/mL levels.

LFA devices are of great interest in the toxin biosensing field due to their facile handling requirements and fast analysis time, as mentioned, but their general lack of sensitivity or quantification capabilities are considered a major limitation. To address these deficiencies, a SERS based LFA biosensor was improved by Hwang *et al.* for the detection of SEB.¹⁰⁹⁷ They replaced the typical AuNPs used in lateral flow strips with hollow gold nanospheres (HGNs) to help overcome the sensitivity limitations. HGNs have been widely used in recent years in biosensing applications thanks to their unique SPR properties.¹⁰⁹⁸ In this alternative configuration, the color change in the test zone in the presence of toxin generates the qualitative analytical information, while SERS analysis reveals the quantitative information, see **Figure 66**. The detection is based on a sandwich antibody/antigen/antibody-conjugated HGN reaction in the strip conjugation zone and subsequent migration through the pad up to the test zone, which has the secondary capture antibodies. This strategy provided a remarkably sensitive LOD of 0.001 ng/mL which was three times more sensitive than an ELISA format tested alongside. These results are also encouraging enough to suggest the development of a portable Raman device for on-site detection which is something that has been quite challenging. Other types of HGNs, in this case a silica-encapsulated and magnetic bead SERS platform, were used for detection of

AFB1 in water.¹⁰⁹⁹ For this, two types of NPs were incorporated, MNPs with an anti-AFB1 capture antibody and the SERS substrate itself which consisted of the silica-encapsulated HGN decorated with anti-AFB1 antibody. The two metal NPs form a sandwich immunocomplex in the presence of the target toxin while non-specific complexes are washed away, see **Figure 67**. The SERS intensity was then evaluated and compared with two other mycotoxins (FMB and OTA) functioning as negative controls. AFB1 was sensitively and selectively detected with an LOD of 0.1 ng/mL while also outperforming HPLC-based methods for the same samples. SERS exploiting AgNPs has also been successfully applied to monitoring the presence of mycotoxins in active cultures within mins.¹¹⁰⁰

Flow Cytometry. FC analyses generally detects individual entities *via* scattering or fluorescence while other non-particulate biomolecules including many small molecule toxins are not normally detected directly.¹¹⁰¹ To overcome this limitation, conjugated particle-based approaches have been developed to detect small targets and toxins and bind to them.^{1102,1103} If multiplexing is desired, different coded particles conjugated with a specific recognition ligand or binding moiety for each target analyte must be employed. Using a microcytometer and fluorophore labelled microspheres conjugated with target specific antibodies allowed the detection of multiple targets in a sandwich immunoassay format with good sensitivity including cholera toxin (1.6 ng/mL), SEB (0.064 ng/mL), ricin (1.6 ng/mL), and even bacteria.¹¹⁰⁴ Later, the same methodology was applied to more bacterial targets and toxins in clinically relevant spiked samples (serum and nasal wash) with analytical performance similar to other antibody-based assays.¹¹⁰⁵ Working with more complex matrices like food can be quite confounding for cytometric analysis and sometimes require more ingenious solutions. Moreover, in most sensing schemes, antibodies are the main biorecognition element, but for many targets it is quite difficult to obtain high quality, sensitive and specific binders. As a result of both of these issues, unspecific interaction or cross-reactivity could reduce the analytical performance of the method. Tallent and coworkers bypassed these limitations by developing a capture entity for SEB based on a specific T-cell receptor superantigen called V β -TCR.¹¹⁰⁶ This superantigen is immobilized on uniquely labelled paramagnetic microspheres to create a capture probe, while antibodies are also immobilized to complete a sandwich format assay. Thanks to the small size of the superantigen, the capture density over the microspheres is high and, as a result, the assay efficiency is better than antibody-

only versions. This method was further tested in food matrices such as milk, mashed potatoes, vanilla pudding, and cooked chicken with LODs in the low ng/mL level achieved. A multiplexed assay for staphylococcal and streptococcal toxins based on V β domains conjugated to beads and using antibodies as reagents was also demonstrated by Sharma *et al.* with sensitivities in the pg/mL level for the testing of culture supernatants.¹¹⁰⁷

Chemiluminescence. Compounds used in chemiluminescent reactions can be employed in different formats, including as a substrate for ELISAs, giving origin to what is referred to as a chemiluminescence enzyme immunoassay (CLEIA) which has been applied for the detection of BoNT/A in milk.¹¹⁰⁸ Liu *et al.* also applied CLEIA for the sensitive detection of SEB in different matrices such as sewage, tap water, river water, roast beef, peanut butter, cured ham, 10% nonfat dry milk, milk, orange juice, and human urine/serum with great sensitivity (0.01 ng/mL) and no cross reactivity from other related toxins.⁵⁸³ The chemiluminescent reaction format implemented in this case was based on a sandwich immunoassay where the secondary antibody is HRP-labelled and in the presence of H₂O₂ generates luminescence from Luminol reagent. The same authors later applied a similar principle for the detection of BoNT/A in beef and milk with greater sensitivity than an ELISA counterpart.¹¹⁰⁹ Beyond the use of Luminol systems, oxalate-based chemiluminescent systems tend to be more sensitive and their chemiluminescence quantum yield is higher while still adaptable to the sandwich format as shown by Xue *et al.*¹¹¹⁰ In this case, the sensitivity was considerably higher than the ELISA/Luminol system with a low pg/L LOD for SEB detection.

In many scenarios, multiplexing is necessary in order to identify the toxic agent involved in a certain episode from amongst many potential candidates.¹¹¹¹ Szkola *et al.* designed a chemiluminescence microarray platform targeting different toxins¹¹¹² and this was later applied to ricin, SEB and saxitoxin detection in a biothreat context.¹¹¹³ In this rapid methodology, requiring as little as 18 mins of analysis time, the authors were able to detect target toxin in the low μ g/L level. To achieve this sensitivity, anti-toxin antibodies were immobilized on a glass slide and a cocktail of biotinylated detection antibodies were set in contact with the sample to create a sandwich complex with the immobilized group of antibodies. The biotin-conjugated antibodies then interact with streptavidin-HRP for subsequent chemiluminescence visualization in the presence of Luminol and H₂O₂. This principle was shown useful for protein toxins like

ricin and SEB, whereas the small size of saxitoxin required a competitive format using an anti-idiotypic antibody which was recognized by the detection antibody set.

When complex matrices are analyzed, sample preparation/enrichment procedures through immunoreaction and/or NPs have been successfully utilized for target extraction.^{1114,1115} In combination with NPs and immunoassays, these methods actually revealed some unique sensitivity and selectivity merits due to signal amplification and selective biorecognition processes. Kim and coworkers implemented an extraction step for OTA in rice by using amine-functionalized MNPs. The extracted target was later tagged with anti-OTA antibody while the unreacted amino groups were chemically capped with propanal after which an HRP-tagged secondary antibody is used to enhance detection *via* Luminol-H₂O₂ system.¹¹¹⁶ Although requiring several sequential steps, the LODs obtained were as low as 1.39 pg/mL.

Bioimmobilization over silica nanomaterials has gained relevance in toxin sensing over the past few years as it can be used as a functional part of a chemiluminescent label.¹¹¹⁷ Tao *et al.* developed a sandwich immunoassay for enterotoxin using dye-encapsulated SiNPs.¹¹¹⁸ Here, the mesoporous silica NPs generate a hydrophobic environment to retain the selected dyes. Microplate formats were used to capture the target SEC by immobilized antibodies and the sandwich was created by the introduction of a secondary SiNPs-Ab labelled with rhodamine 6G and fluorescein. A chemiluminescence reaction takes place by introduction of bis(2,4,6-trichlorophenyl)oxalate, H₂O₂, and imidazole in proportion to the amount of SiNPs present. The presence of the SiNPs thus allows signal amplification yielding an LOD of 19 pg/mL and can potentially be subjected to automation.

Integrated Microdevices. Sensing technologies typically follow the main trends occurring in analytical chemistry.¹¹¹⁹ This has shaped the desire for a reduced sample size, automated instrumentation, simplicity in cost and use, and integration for on-site/field deployment, all of which are also critical considerations for toxin detection.¹¹²⁰ LFA devices, in particular, fulfill almost all of these requirements and naturally lend themselves to these needs. The Garber Group prototyped a rapid detection alternative to screening for the presence of ricin in cosmetic products, using 2.5% non-fat milk powder as a buffer modifier which reduced non-specific interactions with the sample matrix that arose from the presence of lectins. This detection

platform was based on the commercially available BioThreat Alert® chip from Tetracore, Inc.¹¹²¹ Alternatives for toxin detection in this format target the more generalized effects that would be expected from the presence of the toxin itself. Fan and coworkers developed a microfluidic device for monitoring cell-free protein synthesis with the target of this strategy being to verify the effects of several target toxins on protein synthesis.¹¹²² The device used either Western blot or luminescence for measuring the yield of protein expression, depending on the property of the proteins expressed in the system. Protein synthetic routes could be selected to detect specific toxin(s) enabling multiplexing in an array format. As proof of concept, they demonstrated the detection of ricin by inhibition of luciferase synthesis.¹¹²³ Later improvements on the device by addition of passive pumping microfluidics reduced the cost, analysis time, and increased sensitivity, allowing detection of ricin in beverages with an LOD of 0.1 ng in 1 μ L of sample in less than 35 min.¹¹²⁴ Relying on a fluorescence microplate reader, Babrak and coworkers reported a simplified method to detect BoNT/A in sera.¹¹²⁵ This system consisted of a 96-well microfluidic plate and double sandwich immunoassay using an HRP system and QuantaRed fluorescent substrate. Overall, the assay was suitable for direct automation in other formats since it only required a plate device, compatibility with most detectors, extraordinary sensitivity (<30 pg/mL), fast analytical times (approx. 75 min), while requiring only small volumes as it typically used only 5 μ L of serum sample.

An emerging technique for the detection of both proteins and nucleic acids is fluidic force discrimination (FFD) assays. This flow-based technique requires the use of biorecognition elements as capture probes which are again mostly antibodies. In general, the test sample is mixed with secondary antibodies and conjugated microbead labels in solution. The conjugated sample is later incorporated into a microarray where it is captured by means of primary antibody and controlled fluidic forces are applied to preferentially remove nonspecifically bound beads. The remaining beads are counted to determine identity and quantify the target analyte. This innovative technique has been applied to the detection of SEB at fg/mL levels and ricin toxoid in complex matrix such as blood or serum, see **Figure 68**.¹¹²⁶ Yakes *et al.* developed a modified inhibition immunoassay combined with FFD technology for TTX detection.¹¹²⁷ Given the small size of the TTX molecule (**Figure 54B**), traditional sandwich immunoassay formats are typically not suitable in FFD platforms. To overcome this, TTX was immobilized onto the surface of the biosensor platform allowing an exposed part of the analyte molecule to be later recognized by

the anti-TTX antibody. The actual inhibition immunoassay was performed where mouse anti-TTX was first captured and subsequently labeled by anti-mouse microbeads. Increases in TTX concentration in samples led to less free antibodies binding to the substrate, and consequently fewer microbeads observed upon readout in the FFD assay. In application, TTX toxin was detected with an LOD of 15 ng/mL.¹¹²⁷

Microfluidic devices can also readily act as scalable platforms suitable for multiplexing. Simultaneous detection of SEB, BoNT/A, and ricin was demonstrated by Weingart and co-workers using a microfluidic platform and dedicated detection instrumentation.¹¹²⁸ Their platform was based on an Inca Bioanalytical System which is a microarray-based system for use in diagnostics and environmental control. The core part of the system is the so-called IncaSlide, a microchanneled slide printed out to display the capture anti-toxin antibodies. Sample containing target analytes was then pumped through the microchannel followed by biotin-labelled antibodies and then Cy5 dye-labeled streptavidin to enable complex detection. The slides were scanned and the fluorescent signals captured from the slide allowing quantitation of each individual array feature. The LOD in contaminated samples such as raw milk was between 1 and 5 ng/mL. Other platforms for multiplexed detection of bacterial toxins for medical diagnosis purposes have also been developed relying on electrophoretic microarrays.¹¹²⁹ Here, the sample is electrophoretically pumped into the device where capture antibodies catch the target toxins and then biotinylated antibodies are passed through the cell using shear flow. Finally, visualization of the microarray-bound biotin labels is achieved by flowing across the microarray surface with streptavidin-coated magnetic beads in a shear flow and a magnetic field applied to reveal the presence of toxins. The LOD for SEB/A toxins were in the pg/mL range with a total analysis time of less than 10 min. The device could also be used to detect cholera toxin and the *E. coli* heat-labile toxin in water and meat samples. It is worth considering that electrophoretic devices are quite amenable to miniaturization while still being massively parallel and accommodating integrated sample preparation technologies.^{104,115,645-647,1130}

Outlook and Perspective

The focus of the preceding discussion has been to provide a wide cross-section of current diagnostics and, more importantly, recent research describing how biothreat agents are sensed along with some pertinent information on how they are classified and viewed from the perspective of public health officials. The continuing importance of this field is directly reflected in the depth and breadth of ongoing investment and the different research approaches currently being explored. Clearly, in the near term we can expect that there will be a lot more progress in sensor development in the same vein as discussed above. That is both incremental and significant improvements to a particular sensing approach, assay, sensor, biorecognition element, device, and the like. We can expect the development of more multiplexed assays that are cheaper, faster, more sensitive, more robust, and tolerant of a lot more extraneous material (*i.e.*, requiring less sample cleanup and preparatory steps). Of course, this will be in conjunction with the development of simplified or field-portable devices capable of rapid on-site detection. Indeed, every new issue of *Analytical Chemistry*, *Biosensors and Bioelectronics*, *ACS Sensors*, *ACS Applied Materials and Interfaces*, and many other similar scientific journals serves to continuously verify this.¹¹³¹⁻¹¹³³ We also note that there are many, many resources available to the interested reader on almost any subject related to sensing biothreat agents and the best approach to finding these in many cases is to do a key word internet search using Google or other related search engines. Such searches typically provide information on specifically focused review articles and/or available textbooks.¹¹³⁴⁻¹¹³⁵ It is not always easy to predict how such a broad field will develop in the longer-term. In this case, however, we believe that is highly probable that three other areas of inter-related research currently making significant progress and receiving much scientific attention will have the most impact; namely microfabricated or POC devices, disposable paper devices/wearable sensors, and synthetic biology.

Progress in engineering and fielding integrated microfabricated POC devices represents the culmination of more than 30 years of research. Qiao recently provided an excellent and timely overview of the state of the art in microfluidics based analysis in the context of detecting microorganisms using such devices.¹¹³¹ The integration in these devices represents the interfacing of disparate sample input, sample preparation and processing, microfluidic, analytical, detection, electronic, control, power, and reporting components into a single device.¹¹³⁶⁻¹¹³⁹ Cost, robustness, simplicity, and sensitivity all have to be carefully factored into

this equation in pursuit of meeting necessary requirements. A common theme amongst the devices coming to fruition suggests that they are capable of multiplexed detection of several targeted pathogens and that they switch between different targets using the equivalent of different disposable coupons or modular test strips. For example, the FilmArray[®] is a commercial, portable PCR-based detection system that includes integrated sample preparation along with data analysis.¹¹⁴⁰ One of its modules or coupons is the Biothreat Panel which is a multiplexed PCR assay for 17 pathogens/toxins with an LOD down to 25 genomic equivalents for some of the targeted pathogens. **Figure 69** presents some recent results that evaluated this device's ability to detect *B. anthracis*, *F. tularensis* and *Y. pestis*. This system is based on a "Lab-in-a-Pouch" system which contains all the lyophilized reagents required and usually only needs addition of water to activate the kit. The FilmArray's[®] Respiratory Panel pouch, which targets a panel of 15 different respiratory pathogens, has already received FDA clearance.¹¹⁴⁰

These same types of POC bioassay/sensor devices are now having significant impact in rapid, on-site diagnostics by healthcare providers especially in resource challenged environments such as the Third World; in many cases this was the original intended use for these devices.⁸⁸⁴ Continuing issues that still require improvement within this class of device are removing the need for refrigeration and improving the shelf-life and storage times of the biological components. Achieving sensitivities comparable to most dedicated laboratory instrumentation regardless of the target pathogen remains a continuous goal to allow the device to be fully diagnostic rather than just serving as an initial screening tool. Access to 3-D printing will greatly aid development in this effort as it can allow devices to be rapidly prototyped and produced.¹¹⁴¹ The consumer electronic and communication revolution also has much to offer for device application as it allows hand-held phones to act as both imaging devices for assays while providing computational resources as well. Indeed, if properly implemented, coupling of hand-held assay devices with cell phone technology and cloud computing could potentially allow for real-time geospatial tracking of an outbreak event.^{316,1142,1143}

Paper-based devices represent perhaps the ultimate in disposable sensor technology and are an emerging paradigm continuing from the same philosophy that inspired integrated POC devices.¹¹⁴⁴⁻¹¹⁴⁶ This fascinating field has been recently reviewed in detail by the Henry Group.¹¹⁴⁷ Attesting to the exploding interest in this approach, they point out that the number of papers reporting on this technology continues to grow with more than 1,000 reported within the

2014-2016 time period alone. Magro and colleagues recently showed what this area has to offer for pathogen sensing when they applied paper-based detection to Ebola virus diagnostics.¹¹⁴⁸ They implemented isothermal reverse transcription and recombinant polymerase amplification (RT-RPA) of synthetic Ebola RNA virus with a paper-based microfluidics device. They then applied this methodology in Guinea to detect the presence of Ebola virus in human RNA sample extracts, with minimal facilities using a hand-held detection device and freeze-dried reagents on paper. In a test set of 43 patient samples, they were able to demonstrate a 90.0% sensitivity compared to gold-standard RT-PCR comparison. A fascinating analog to paper-based devices has also recently appeared in the form of the “foldscope” (<https://www.foldscope.com/>), a foldable microscope made mostly of paper with a predicted net cost of < \$1.¹¹⁴⁹ The ability to create these and similar viable diagnostic hardware from such cheap and disposable components will be essential for providing health services, biosurveillance, and diagnostics across the undeveloped portions of the world along with empowering locals to provide this.

In the same vein as disposable POC devices, disposable-wearable sensors, sometimes referred to as Lab-on-Skin or disposable tattoo sensors, offer up the ability to monitor a patient directly by integrating to the human body’s largest organ.¹¹⁵⁰⁻¹¹⁵³ **Figure 70** highlights some representative technologies under development to integrate such thin and soft electronic devices onto skin. As mentioned several times above, detecting the presence of a pathogen or toxin is usually the first critical step, but then this needs to be followed by determining if it is active, and then finally if a person has been infected or exposed and is starting to become symptomatic or needs prophylaxis. These types of disposable sensor devices can allow for the monitoring of potential patients or large groups of exposed people. The devices can also be interfaced wirelessly and monitored continuously for geospatial tracking over time as with the POC’s above. Perhaps most importantly, they can report on slight changes in a person’s physiology at the very early stage of infection or poisoning when they start to become symptomatic but before they are even self-aware. Although the commercial focus for these types of sensors are currently on chronic monitoring or diagnostics, appropriate development and implementation of this technology can certainly help augment the toolset available for biothreat sensing in general.

Last, but certainly not least, the burgeoning field of synthetic biology potentially has much to offer for sensing biothreat agents. The long-term possibilities here are epitomized by several recent reports coming out of the Collins Lab at MIT.¹¹⁵⁴⁻¹¹⁵⁶ They developed a cell-free

system based on so called reaction pellets constituting freeze-dried, cell-free transcription/translation components which are hydrated and then utilized for target biosynthesis through the addition of a DNA initiator; the latter encodes the desired synthetic product. Preliminary demonstrations of the versatility inherent to this platform included the manufacture of AMPs, vaccines, and combinatorially-derived antibody conjugates and small molecules.¹¹⁵⁵ A subsequent report described a paper-based sensor for Zika virus specifically targeting its RNA genome where the initial sensor design, development, and prototyping required only 5 days and produced a diagnostic that had greater than 1 year stability and could yield assay results in around 3 hrs, see **Figure 71** for a schematic highlighting this workflow and some representative Zika assay results.¹¹⁵⁴ The continuing development of this disruptive technology clearly bodes well for producing the biorecognition entity, sensor format, and even therapeutics as needed on-site in a modular, disposable fashion from reagent packs that can be distributed and stored for long periods of time. There are other types of more bare-bones, cell-free synthetic biology systems also under development that can enhance the activity of selected enzymes when attached to nanoscaffolds and these may also prove useful in similar but more targeted roles such as sensing or hydrolyzing a given threat agent.¹¹⁵⁷⁻¹¹⁶⁰ Nanoscaffold materials utilized in this role range from NPs to even DNA.^{1161,1162}

Overall, from a technological perspective, we are clearly more prepared than ever to detect and respond to a biothreat event even as compared to just 10-15 years ago.^{45,1163} However, many question if this is even sufficient given the nature of these threats in an economic reality of political issues and decreasing investment in research and preparedness.¹¹⁶⁴ Further investment in this arena will only increase this capability while also having a significant and beneficial impact in resource poor environments and the Third World where many biothreats originate from as witnessed by the recent Ebola and Zika outbreaks. Important issues extend even beyond research and preparedness. For example, budgetary cuts and rising costs have severely curtailed the number of autopsies that are performed in hospitals and by public health providers.¹¹⁶⁵ This is a critical and perhaps last level of vigilance that must remain in use for detecting an initial outbreak event. We are also keenly aware of some emerging threats looming on the horizon. For example, the rapid evolution of multidrug resistant or even completely drug resistant pathogenic bacterial strains coupled to decades of poor investment in discovering and developing new alternative classes of antibiotics are already beginning to produce fatalities. This

scenario remains a largely underrated but potentially devastating threat.¹¹⁶⁶⁻¹¹⁶⁸ Second to this is the ongoing susceptibility of industrial scale farming and agriculture to many different types of biothreats which could imperil local food sources. Lastly, the possibility always remains for an event arising from a new, previously undescribed pathogen that has either mutated or migrated or, more ominously, something that has been nefariously engineered. The latter is becoming more of a possibility given the recent development of custom gene editing tools such as CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) which are available to the public in commercial kit form.¹¹⁶⁹ Indeed, the many potential possibilities this can engender have already been recognized as a U.S. and international security threat.¹¹⁷⁰ How to prepare for the surveillance, detection, and response to such a scenario is still not understood and represents perhaps the greatest challenge our society may face in this arena.

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Abbreviations

Note: in mentioning certain infectious agents, the source pathogen and/or illness is provided and sometimes used interchangeably.

AB	abrin toxin
AF	Alexa Fluor
AFB1	aflatoxin B1
AFM1	aflatoxin M1
AgNP	silver nanoparticle
AlphaLISA	Amplified Luminescent Proximity Homogeneous Assay
AMP	antimicrobial peptides
anthrax	<i>Bacillus anthracis</i>
APDS	autonomous pathogen detection system
APHIS	Animal and Plant Health Inspection Service
APHL	Association of Public Health Laboratories
ASM	American Society for Microbiology
ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable
AuNP	gold nanoparticle
AuNR	gold nanorod
<i>B.</i>	<i>Bacillus</i> and sometimes <i>Brucella</i>
BARDOT	BActerial Rapid Detection using Optical light-scattering Technology
botulism	<i>Clostridium botulinum</i>
BoNT	botulism neurotoxin
bp	base pair
brucellosis	<i>Brucella suis</i>
BSE	bovine spongiform encephalopathy
BVDV	bovine viral diarrhea virus
BWC	Biological Weapons Convention
CaDPA	calcium dipicolinic acid
CAP	College of American Pathologists
CBRN	chemical, biological, radiological and nuclear
CCD	charge-coupled device or colony collapse disorder
CDC	Centers for Disease Control and Prevention
Cdot	carbon dot
CDRH	Center for Devices and Radiological Health
CFM	conventional fluorescence microscopy
CFR	Code of Federal Regulations
CFU	colony forming units
CHA	catalytic hairpin assembly
CID-MS/MS	collision dissociation tandem mass spectrometry
CLEIA	chemiluminescence enzyme immunoassay
CLIA	Clinical Laboratory Improvement Amendments
CoV	coronavirus
CPU	central processing unit
CSF	cerebrospinal fluid

CytK1	cytotoxin K1
DART-MS	direct analysis in real time with mass spectrometry
ddPCR	digital droplet PCR
D-HPLC	denaturing high performance liquid chromatography
DNA	deoxyribonucleic acid
DNAzymes	deoxyribozymes
DoD	Department of Defense
DON	deoxynivalenol
DPA	dipicolinic acid
ds	double stranded
EAPM	electrically active conducting polymer
EDTA	ethylenediaminetetraacetic acid
EMBIA	electrochemical magnetic microbead-based biosensor
EEV	Eastern equine virus
EIS	electrochemical impedance spectroscopy
ELISA	enzyme-linked immunoassay
ESI	electrospray ionization
ETX	Epsilon toxin
EUA	Emergency Use Authorization
FB1	fumosin B1
FBI	Federal Bureau of Investigation
FC	flow cytometry
FD&C Act	Federal Food, Drug and Cosmetic Act
FDA	U.S. Food and Drug Administration
FFD	fluidic force discrimination
FITC	fluorescein isothiocyanate
FNA	functional nucleic acids
FOBS	fiber optic biosensors
FRET	Förster resonance energy transfer
FSAP	Federal Select Agent Program
FT-IR	Fourier transform infrared (spectroscopy)
Gb	giga basepair
GC	gas chromatography
GHz	gigahertz
Glandars	<i>Burkholderia mallei</i>
GMR	giant magnetoresistive
GOx	glucose oxidase
HxNx	strain type of influenza by genotype variants of the H-hemagglutinin (x = 1-18) and N-neuraminidase (x = 1-11) genes
HAU	hemagglutinating unit
HDA	helicase-dependent amplification
HGN	hollow gold nanospheres
HHS	Department of Health and Human Services
HILIC	hydrophobic interaction liquid chromatography
HPLC	high performance liquid chromatography
HPV	human papilloma virus

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3	h/hr(s)	hour(s)
4	HRP	horseradish peroxidase
5	HSV-1	herpes simplex virus type 1
6	IAC	immune affinity column
7	IFU	indications for use
8	IgG / IgM	immunoglobulin G / immunoglobulin M
9	IMAC	immobilized metal-affinity chromatography
10	IMDA	isothermal multiple displacement amplification
11	3-IP	3-indoxylphosphate
12	IR	infrared
13	ISE	ion selective electrodes
14	IU	intended use or International Units
15	IUPAC	International Union of Pure and Applied Chemistry
16	IVD	<i>in vitro</i> diagnostic
17	LAMP	loop-mediated isothermal amplification
18	LC	liquid chromatography
19	LC-MS/MS	liquid chromatography-tandem mass spectrometry
20	LD ₅₀	lethal dose that kills 50% of the test sample
21	LED	light emitting diodes
22	LFA	lateral flow assay
23	LNA	locked nucleic acids
24	LOC	lab-on-chip
25	LOD	limit of detection
26	LPX	Laboratory Preparedness Exercise
27	LRN	Laboratory Response Network
28	LRSP-FS	long-range surface plasmon-enhanced fluorescence spectroscopy
29	LSPR	localized surface plasmon resonance
30	mAb	monoclonal antibody
31	MALDI	matrix-assisted laser desorption/ionization
32	MALDI-MS	matrix-assisted laser desorption ionization mass spectrometry
33	Mb	mega bp
34	MB	molecular beacon
35	MCL	microcantilevers
36	MCMi	Medical Countermeasures Initiative
37	meliodosis	<i>Bacillus pseudomallei</i>
38	ME/MR	magnetoelastic/magnetorestrictive
39	MEMS	microelectromechanical systems
40	MERS	Middle East Respiratory Syndrome (coronavirus)
41	MHz	megahertz
42	min(s)	minute(s)
43	MNP	magnetic nanoparticle
44	MS	mass spectrometry
45	MTOX	<i>N</i> -methyl tryptophan oxidase
46	<i>m/z</i>	mass-to-charge ratio
47	NASBA	nucleic acid sequence-based amplification
48	NBP	nanobipyramids
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3	NGS	next generation sequencing
4	NHE	non-haemolytic enterotoxin
5	NIAID	National Institute of Allergy and Infectious Diseases
6	NNDSS	National Notifiable Diseases Surveillance System
7	NP	nanoparticle
8	NSABB	National Science Advisory Board for Biosecurity
9	NSET	nanosurface energy transfer
10	NTA	nitrilotriacetic acid
11	OTA	ochratoxin A
12	PCR	polymerase chain reaction
13	PCR/ESI-MS	polymerase chain reaction/electrospray ionization mass spectrometry
14	PDMS	polydimethylsiloxane
15	Pdot	polymer dots
16	PEMC	piezoelectric-excited millimeter-sized cantilever
17	PFU	plaque forming units
18	PL	photoluminescence
19	plague	<i>Yersinia pestis</i>
20	PMA	premarket approval application
21	PMT	photomultiplier
22	PNA	peptide nucleic acid
23	POC	point-of-care
24	PPE	personal protective equipment
25	PPQ	Plant Protection and Quarantine
26	PRRSV	porcine reproductive and respiratory syndrome virus
27	Psittacosis	<i>Chlamydia psittaci</i>
28	QCM	quartz crystal microbalances
29	QD	quantum dot
30	Q fever	<i>Coxiella burnetii</i>
31	qPCR	quantitative PCR
32	RCA	rolling circle amplification
33	RINS	rapid identification of non-human sequences
34	RNA	ribonucleic acid
35	RPE	<i>R</i> -phycoerythrin
36	RPA	recombinant polymerase amplification
37	rRNA	ribosomal ribonucleic acid
38	RSV	respiratory syncytial virus or respiratory syndrome virus
39	RS	Raman spectroscopy
40	RT-PCR	reverse-transcriptase PCR
41	3SR	self-sustained sequence replication
42	SARS	severe acute respiratory syndrome
43	SAW	surface acoustic waves
44	scFv	single chain variable fragments
45	sdAb	single domain antibodies or nanobodies
46	SE	staph enterotoxins
47	SEA/SEB	staphylococcal enterotoxin A/B
48	SEG	staphylococcal enterotoxin G
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Select agents	Biological Select Agents or Toxins
SELEX	systematic evolution of ligands by exponential enrichment
sELISA	sandwich ELISA
SERS	surface-enhanced Raman spectroscopy
siRNA	small interfering RNA
SMART	signal mediated amplification of RNA technology
SNP	single nucleotide polymorphism
SPE	solid phase extraction
spp.	<i>species pluralis</i>
SPR	surface plasmon resonance
STEC	Shiga-toxin producing <i>E. coli</i> O157
T1	Tier 1
TB	tuberculosis / <i>Mycobacterium tuberculosis</i>
TCID ₅₀	median tissue culture infective dose
TIGER	triangulation identification for the genetic evaluation of risks
TNT	trinitrotoluene
TOF	time-of-flight
TTX	tetrodotoxin
Tularemia	<i>Francisella tularensis</i>
Typhus fever	<i>Rickettsia</i> spp.
U.S.	United States (of America)
USDA	United States Department of Agriculture
USSR	Union of Soviet Socialist Republics
μTAS	micro-total analysis system
UF	ultrafiltration
UV	ultraviolet
VAMP2	vesicle associated membrane protein 2
VEE	Venezuelan equine encephalitis
VHF	viral hemorrhagic fever
VOC	volatile organic compounds
WGS	whole genome sequencing
WGM	whispering gallery mode
WHO	World Health Organization
XNA	xeno nucleic acid
ZEA	zearalenone

Figure Legends.

Figure 1. Representative images of patients infected with selected viral or bacterial biothreat agents. (Top left) This young girl in Bangladesh was infected with smallpox in 1973. **(Top right)** King Tom Cemetery in Sierra Leone's capital city of Freetown, during the country's Ebola outbreak. The image illustrates how the burial process had improved in order to prevent the spread of the viral disease, showing us how the burial team was now appropriately using personal protective equipment (PPE), how a burial shroud for a deceased person of the Muslim faith was now included in the burial process, and how the use of designated grave markers were now being installed throughout the cemetery. **(Bottom Left)** Anthrax infection of the eye on day 25. **(Bottom right)** *Yersinia pestis* infection highlighting the blackened coloration from gangrenous tissue with gave rise to the name 'Black Death.' Image 3265, 20927, 14280 and 4505 from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL) and are in the public domain.

Figure 2. Representative images of bacterial and viral biothreat agents. (A) This photograph depicts a colony of *Clostridium* sp. Gram-positive bacteria, which had been grown on a 4% blood agar plate (BAP) over a 48 hr time period. A number of *Clostridium* spp. are highly pathogenic to humans. Members including *C. perfringens*, and *C. septicum*, are known to be a cause of gas gangrene in humans, due to their production of a potent exotoxin, and *C. botulinum* is the cause of botulism food poisoning. **(B)** This transmission electron micrograph depicted eight virions (viral particles) of a newly-discovered virus, which was determined to be a member of the genus, *Arenavirus*. A cause of fatal hemorrhagic fever, it was confirmed that this virus was responsible for causing illness in five South Africans, four of whom died having succumbed to its devastating effects. Image 12050 and 10839 from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL). These are in the public domain.

Figure 3. Map of recently emerging viral diseases. Shown are examples of sites where diseases caused by newly emerging viruses (those not known to have infected humans before, or not previously recognized as human pathogens; yellow) and reemerging viruses (those increasing in incidence, emerging in new locations or exhibiting enhanced pathogenicity; orange) have appeared. (Bottom) Listed are modern developments that facilitate virus emergence, such as commercial air travel and urbanization, and research advances that are helping to control emerging viruses, including genomic sequencing, rapid diagnostics, and new approaches to vaccine and therapeutic design. Reprinted with permission from ref 27. Copyright 2014 The American Association for the Advancement of Science.

Figure 4. Examples of taro leaf blight disease. Note the progressive wasting of the leaf material which increases with time. Image in the Public domain, *via* Wikimedia Commons.

Figure 5. Biosensor components. Configuration of a biosensor showing the common biorecognition, interface, and transduction elements. Reprinted with permission from ref 1171. Copyright 2008 Caister Academic Press.

Figure 6. ELISAs. Schematic highlighting different ELISA formats including those that are direct, indirect, sandwich, and competitive.

Figure 7. Structure of the Laboratory Response Network for Biological Threats Preparedness (LRN-B). Adapted from ref 133-135.

Figure 8. Mass spectral identification of bacterial threat cells. Typical MALDI-TOF mass spectra between m/z 2500 and m/z 12000 from three different *Yersinia* species. (A) *Y. enterocolitica*, (B) *Y. pseudotuberculosis*, and (C) and *Y. pestis*. Preparation/inactivation of the microbial samples was carried out as described.¹⁷⁸ Reprinted with permission from ref 178. Copyright 2010 American Chemical Society.

Figure 9. Representative mass spectra of PCR amplicons. Deconvoluted, ESI-TOF mass spectra of PCR amplicons derived from the *tkt* housekeeping genes from six different *C. jejuni* strains. Both the forward ($\diamond$) and the reverse ($\#$) strands of the PCR amplicons from each strain are clearly evident in the spectra (*e.g.*, for strain RM4197, the forward strand is A49, G22, C26, and T45 and the reverse strand is A45, G26, C22, and T49). As can be observed in the stacked spectra, differences due to variations in the sequence (and, thus, the base composition) are readily discernible. Reprinted with permission from ref 183. Copyright 2008 American Society for Microbiology.

Figure 10. LC tandem MS analysis for detection of *Y. pestis*. Overview of the steps involved in preparing samples for detection of the *pestis* Pla surface protein. TFA is trifluoroacetic acid and SRM refers to the use of the MS for selected reaction monitoring. Reprinted with permission from ref 193. Copyright 2014 American Chemical Society.

Figure 11. Long-range surface plasmon-enhanced fluorescence spectroscopy (LRSP-FS) biosensor. (A) Optical setup of a LRSP-FS biosensor, surface architecture, and detection assay. (B) The angular reflectivity (left axis) and fluorescence (right axis) spectra measured after the analysis of *E. coli* O157:H7 at the concentrations between 10^1 and 10^6 CFU mL^{-1} (curves clearly indicated in the graph). Reprinted with permission from ref 213. Copyright 2011 American Chemical Society.

Figure 12. The NRL Array Biosensor. (A) Physically-isolated patterning and sample analysis leads to formation of an array of fluorescent spots on the waveguide surface. Image adapted from ref²⁴² under open source publishing agreement. (B) The final CCD image after the slide was exposed to *S. dysenteriae*, 0.5×10^6 CFU mL^{-1} . (C) The image was converted into intensity values, and the resulting standard dose-response curve is shown for each species of Shigella as a plot of net intensity versus bacterial cell concentration; *S. dysenteriae* ($\bullet$), *S. flexneri* ($\circ$), *S. boydii* ($\blacktriangle$), and *S. sonnei* ($\triangle$). The net intensity values are an average of eight or more data squares $\pm$ SD; CVs for these points average at 20%. Reprinted with permission from ref 237. Copyright 2004 American Chemical Society.

Figure 13. QCM DNA sensor with gold nanoparticle amplification. (A) Schematic illustration of the QCM surface, followed by hybridization with a target DNA sequence, and subsequent signal amplification by DNA probe 2 functionalized with gold NPs. (B) Real-time response of the anthracis DNA probe functionalized QCM biosensor to target DNA (0.1 mM of final concentration) and sequential signal amplification with gold NP (GNP) immobilized DNA

probe. (C) Frequency shift of the anthracis DNA probe functionalized QCM biosensor to the target DNA sequence amplified by asymmetric PCR to 3.5×10^7 CFU/ml of *B. anthracis*, *E. coli*, *B. thuringiensis*, *B. subtilis*, and *B. cereus*. Reprinted with permission from ref 248. Copyright 2011 Elsevier.

Figure 14. Detection of pathogens by cantilever and magnetic sensors. (A) Detecting airborne *Bacillus anthracis* spores with a cantilever sensor. By recirculating the sample and spore attachment, one can follow the transient response of the sensor to the binding of *B. anthracis* spores. Reprinted with permission from ref 267. Copyright 2007 Elsevier. (B) Scheme for the sensitive detection of inactivated high-risk pathogens. The sandwich immunoassay for detection of pathogens with apoferritin nanoprobe and magnetic beads. Reprinted with permission from ref 274. Copyright 2016 The Royal Society of Chemistry.

Figure 15. Electrically active polyaniline coated magnetic nanoparticle-based biosensor (A) Schematic representation of the biosensor detection system and sensing process. (B) Comparison of the EAPM NP-based direct-charge transfer biosensor resistance response between pure spore suspensions of *B. anthracis* and pure cultures of generic *E. coli* and *S. enteritidis*. Reprinted with permission from ref 297. Copyright 2009 Elsevier.

Figure 16. Schematic of *Salmonella enterica* serovar Typhimurium (STM) detection. This system used a colorimetric enzyme-linked antibody-aptamer sandwich (ELAAS) format, chemiluminescent ELAAS, or AuNP-ELAAS. (A) Preparation of nanoprobe. (B) Preparation of magnetic microparticle (MMP)-aptamers. (C) Comparison of the three signal amplification strategy methods (not to scale). The AuNP-ELAAS proved most sensitive under optimized reaction conditions showing a quantitative detection range from 10^3 - 10^8 CFU mL⁻¹, a limit of detection of 10^3 CFU mL⁻¹, and a selectivity of >10-fold for STM in samples containing other bacteria at higher concentration with an assay time less than 3 hr. Reprinted with permission from ref 310. Copyright 2014 American Chemical Society.

Figure 17. Specificity of an LFA for *B. anthracis* using upconverting phosphor-conjugated antibodies. 50 nm diameter phosphor particles made of NaYF₄:Yb³⁺Er³⁺ were conjugated to antibodies and used in a LFA for *B. anthracis* spores. Reprinted under the Creative Commons license from ref 330.

Figure 18. Sensitivity of qPCR and solid phase recombinant polymerase amplification. (A) Real-time PCR amplification curve of *B. melitensis* strain 16M genomic DNA. A 10-fold dilution series from 10 ng to 10 fg was used as a template. Reprinted with permission from ref 371. Copyright 2008 American Society for Microbiology. (B) Schematic of solid-phase RPA on maleimide-activated microtiter plate with amplification using ssDNA. Reprinted with permission from ref 395. Copyright 2010 Springer.

Figure 19. Visual detection of bacterial pathogens via PNA-based padlock probe assembly and isothermal amplification of DNazymes. Scheme of the bacterial DNA detection method. (A) A pair of bis-PNA openers binds to one strand of bacterial pathogen DNA, leaving the other strand free for padlock probe hybridization. (B) PD-loop formation is limited to the preselected 20–30 bp target site. (C) Hyperbranched RCA allows for exponential signal amplification. (D)

The product is double-stranded, so a nicking enzyme is used to create a gap in one of the strands and the nicked pieces are subsequently displaced by primer extension. This process causes an accumulation of ss-DNA pieces that fold into G-quadruplex structures. **(E)** Results of method validation on pathogen DNA samples. The y axis represents intensity. Cutoff for positive identification was defined as the average of the negative control plus three negative control standard deviations (dashed line at 0.59 in Table 2). E = *E. coli* 0157:H7; S = *S. typhimurium*; and H = human samples. Reprinted with permission from ref 423. Copyright 2014 American Chemical Society.

Figure 20. Sequence-based single nucleotide variation analysis of an *F. tularensis* isolate. **(A)** Alignment of the concatenated sequences of the 66 single-nucleotide variation (SNV) alleles of each *F. tularensis* strain. The nucleotide positions of the alleles in *F. tularensis* SCHU S4) are shown above the alignment. **(B)** Phylogenetic analysis of the alignment sequence. The indicated distinct clades of *F. tularensis* ssp. were previously divided into categories such as types A.I, A.II, and B. Reprinted with permission from ref 424. Copyright 2012 American Society for Microbiology.

Figure 21. EMBIA platform for serodiagnosis. **(A)** Schematic representation of the electrochemical enzyme-linked magnetic beads immunoassay (EMBIA) and detection principle. Antigen-coated magnetic particles are incubated with the serum samples, washed, and then incubated with HRP-conjugated secondary antibodies. After washing, the particles are magnetically collected and placed onto the surface of the electrode. Peroxidase activity is amperometrically recorded using an 8-channel portable potentiostat after adding H₂O₂ and hydroquinone as the substrate and redox mediator, respectively. All incubation and washing steps are performed in 8-tube strips using a magnetic rack without the need of centrifugation. **(B)** EMBIA platform current transients of positive and negative sera. Representative time-current curves for positive and negative serum samples used as control sera for human brucellosis. **(C)** Dot plot analysis of the results obtained with the EMBIA platform for diagnosis of Chagas disease, human brucellosis. The mean and standard deviation for each group are indicated. ***, P<0.0001, Mann–Whitney test. Reprinted with permission from ref 449. Copyright 2016 Elsevier.

Figure 22. Electrochemical detection of *Brucella* genome using gold nanoribbons covered by gold nanoblossoms. **(A)** Fabrication protocol of the genosensor and detection of target or t-ssDNA. **(B)** Zepto-molar electrochemical detection of *Brucella* genome based on gold nanoribbons covered by gold nanoblossoms. Reprinted under the Creative Commons license from ref 472.

Figure 23. Light-Scattering based bacterial detection. **(A)** Major components of the BARDOT system. Reprinted under the Creative Commons license from ref 498. **(B)** Comparison of light scatter patterns of *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. Each of the scatter images was acquired from a different strain of these pathogens. Reprinted with permission from ref 499. Copyright 2015 Elsevier.

Figure 24. Gold nanoparticle based bacterial detection. **(A)** Schematic illustration of the colorimetric detection of DNA with naked AuNPs. The genomic DNA serves as a template for

asymmetric-PCR generating ssDNA–dsDNA mixtures. Dispersed NPs remain red in color in salt solution after amplicons adsorption. Non-target control fails to produce amplified products, aggregated NPs turn to blue-gray upon adding NaCl. **(B)** Initial genomic DNA (1 ng–1 pg) from *B. anthracis* was used to run asymmetric-PCR for colorimetric detection as mentioned before. Correlation of starting template DNA quantities and maximum absorbance shift with triple replicates. Reprinted with permission from ref 506. Copyright 2013 The Royal Society of Chemistry.

Figure 25. Magnetic nanoparticles combined with quantum dots. **(A)** Scheme for the sensitive detection of inactivated *F. tularensis*. Sandwich immunoassay for the detection of *F. tularensis* with apoferritin nanoprobe and magnetic beads. **(B)** Visualization of inactivated *F. tularensis* capture by apoferritin nanoprobe and magnetic beads. Red fluorescence corresponds to the QDs from apoferritin-pathogen-magnetic bead immuno-constructs, thereby indicating the presence of *F. tularensis*. Reprinted with permission from ref 551. Copyright 2015 Elsevier.

Figure 26. Dipicolinic acid detection with Cdots and terbium. **(A)** Single-step synthesis of Cdots with carboxyl and amine groups; operating principle of the ratiometric fluorescent Cdots-Tb nanoprobe for DPA recognition. **(B)** Evolution of the fluorescence (FL) spectra of Cdots-Tb upon increasing the DPA concentration. Inset: photographs of the CDs-Tb dispersion taken under a UV lamp before (left) and after (right) addition of 50 μM DPA. **(b)** Fluorescence responses of Cdots-Tb toward different potentially interfering aromatic carboxylic acids or amino acids, and common biorelevant ions. The blue bars represent addition of 10 μM of interfering acid or ion to a 30 $\mu\text{g mL}^{-1}$ solution of Cdots-Tb. The green bars represent subsequent addition of 10 μM DPA to the solution. Reprinted with permission from ref 578. Copyright 2015 The Royal Society of Chemistry.

Figure 27. Chemiluminescent optical fiber immunosensor. **(A)** Biosensor assembly for the detection of *Brucella abortus* particles resulting in chemiluminescent signal generation and fiber optic tip used as a waveguide for the light measurement. **(B)** Calibration curve for detecting inactivated *B. abortus* strain 1119 in spiked milk. **(C)** Analysis of the specificity of the test with different bacterial strains. Reprinted with permission from ref 589. Copyright 2009 Elsevier.

Figure 28. Raman detection of *Brucella* spp. The mean spectra of *Brucella* spp. (Ab, Bb) from media compared to the mean spectra of *Escherichia* (Aa) and *Ochrobactrum* (Ba) spp. isolated from medium by calculating the loading vector (Ac, Bc). Reprinted with permission from ref 599. Copyright 2012 American Society for Microbiology.

Figure 29. Flow cytometry detection of spores. Flow cytometric histograms and confocal images for the one-step (a) ATYP-RPE and (b) ATYP-Qdot585 conjugate spore assays. Flow cytometric statistical data is as follows: ATYP-RPE conjugate, 73% positive with MCF = 128; ATYP-Qdot585 conjugate, 0.52% positive with MCF = 96. Confocal xy λ scans showing that the fluorescence measured from confocal images was due to (a) RPE or (b) spore autofluorescence. Ten randomly selected spores were used for each xy λ analysis. For comparison, the solution fluorescence spectra for (a) RPE and (b) *B. anthracis* Sterne spores are presented in the insets. Reprinted with permission from ref 639. Copyright 2009 Elsevier.

Figure 30. Optical microchip array biosensor for multiplexed detection of biohazardous agents. (A) Schematic illustration of the operating principle of a SpectroSens™ sensor for biological detection. Light travels through a waveguide within the microchip sensor to the Bragg grating, which reflects a precisely defined wavelength of light. Interaction of target agents with antibodies immobilized on the sensing region results in localized changes in refractive index, which manifest as changes in sensor reflected wavelength. Each chip contains multiple sensing channels operating independently enabling assay multiplexing. (B) Real-time sensorgrams demonstrating specific detection of (left) *B. anthracis* spores (5×10^7 CFU/ml) and (right) *Francisella tularensis* cells (2×10^8 CFU/ml). Binding interactions across all 16 sensor channels were monitored for ~30 min for each antigen before returning to PBS-T running buffer. Note: only 8 channels (4 specific channels, 4 control channels) are displayed on each graph for clarity. Reprinted with permission from ref 659. Copyright 2011 Elsevier.

Figure 31. Colorimetric E-nose sensor arrays to discriminate between pathogenic bacteria. (A) The colorimetric sensor array consists of 80 different chemically responsive nanoporous pigments. (B) Examples of four classes of chemically-responsive dyes: (i) metal-ion-containing dyes that respond to Lewis basicity, (ii) pH indicators that respond to Brønsted acidity/basicity, (iii) dyes with large permanent dipoles (*e.g.*, solvatochromic dyes) that respond to local polarity, and (iv) redox indicators that respond to electrochemical reaction. (C) Color difference maps of *B. anthracis* Ames, *Y. pestis* CO92, *Y. pseudotuberculosis*, and *E. coli*. Each color represents the difference between the indicator color intensity measured before exposure and the intensity measured at the indicated detection time for each species. Reprinted under the Creative Commons license from ref 666.

Figure 32. Spread of Zika virus. Estimated pathway of Zika virus spread over the last *ca.* seventy years. Maps sourced from <http://ian.mackay.net/pat/map/world.html> and are in the Public Domain.

Figure 33. Ebola virus. TEM micrograph of the Ebola Virus Reston strain. Photo Credit: CDC/ Cynthia Goldsmith in the Public domain, *via* Wikimedia Commons.

Figure 34. Mortality of the 2014 Ebola virus outbreak. Estimated number of Ebola cases and confirmed deaths in Guinea, Sierra Leone, Liberia, and Nigeria as of April 2016 (<https://www.cdc.gov/vhf/ebola/outbreaks/2014-west-africa/case-counts.html>).

Figure 35. Detection of Vaccinia virus DNA by quartz crystal microbalance. (A) Flow cell device and principle of detection. The analysis is based on hybridization of complementary ssDNA, generated by PCR and denaturation, with capture DNA in a microfluidic system. The hybridization event is detected as mass increase on the quartz by frequency measurements. (B) Frequency change after hybridization of an amplified product by PCR with different cycle numbers (0–20) (gray column) to the capture probe and the total signal increase after a second hybridization with a gold NP-labeled enhancer sequence (black column). Reprinted with permission from ref 701. Copyright 2011 Elsevier.

Figure 36. Femtomolar detection of influenza virus. Schematic highlighting the detection of a digital ELISA platform capable of ultrasensitive detection of the nucleoprotein in

nasopharyngeal swabs. Reprinted with permission from ref 739. Copyright 2016 American Chemical Society.

Figure 37. Oligonucleotide-linked gold nanoparticle aggregate for enhanced sensitivity in lateral flow assays. (A) Preparation of oligonucleotide-linked AuNP aggregates. (B) Design of nucleic acid lateral flow test strips. (C) Principle of the AuNP aggregates based assay. AP: amplification probe; CP: complementary probe; DP: detector probe. AP and CP are complementary to each other. Reprinted with permission from ref 744. Copyright 2013 The Royal Society of Chemistry.

Figure 38. Labeled Google Glass and demonstration of imaging a rapid diagnostic test. (A) Front-profile view of the Google Glass with various hardware components labeled. (B) Example of using the Glass for taking an image of a rapid diagnostic test as part of the reader application. (C) Examples of rapid diagnostic tests used in this work. (i) JAJ International free PSA test; (ii) JAJ International total PSA test; (iii) OraQuick ADVANCE Rapid HIV-1/2 test; (iv) OraQuick In-Home HIV test. Reprinted with permission from ref 764. Copyright 2014 American Chemical Society.

Figure 39. Schematic of molecular beacon function in the context of PCR product formation. The MB displays a dye reporter and a dark quencher in this example. The unbound MB structure results in dye quenching as the stem-loop structure brings the dye and quencher into very close proximity. PCR amplification of target molecule allows it to hybridize with the MB and open up the structure moving the quencher away from the dye and providing for dye fluorescence upon excitation.

Figure 40. Digital PCR. (A) Human genomic DNA NA10860 (left 5 panels) and the RPP30 synthetic construct (right 5 panels) were quantitated using the RPP30 (FAM) assay on this digital array. (B) Results of actual experiments on the digital array with varying number of copies of the target gene. Reprinted under the Creative Commons license from ref 780.

Figure 41. Microdroplet sandwich real-time PCR. (A) Drawings of platform: 3D drawing of the droplet sandwich platform displaying the ITO coated glass (a) with a compound droplet (b) surrounded by a spacer (c) and covered with a coverslip (d), which is fully assembled to sandwich the compound droplet in a reaction chamber (e). The ITO surface heats radially, as displayed by the modeled heating profile for the ITO glass when 15 V is applied to the resistive surface as generated by COMSOL Multiphysics (f). The dimension of the slide is 40 mm×40 mm and the compound droplet is approximately 2.8 mm in diameter. (B) Workflow and representative data: Sample isolation is done from nasopharyngeal swabs and the RT-PCR mix is transferred to the droplet sandwich platform for thermal cycling. Temperature cycling occurs at the center of the radial profile as displayed by the plot where the black line represents the controlled surface temperature and the red line is the calibrated droplet temperature. Fluorescence is collected in real-time during the extension phase of PCR, with DNA amplification of positive samples displayed in green, negative samples with no change over time in blue and calculated threshold in black. Reprinted under the Creative Commons license from ref 790.

Figure 42. Schematic highlighting the step by step TIGER process. Reprinted with permission from ref 186. Copyright 2005 Elsevier.

Figure 43. Schematic for magnetic bead-based capture and amplification of viral nucleic acids. Reprinted under the Creative Commons license from ref 798.

Figure 44. Colorimetric method for H1N1 DNA detection using rolling circle amplification. (A) Scheme of the RCA-based assay for H1N1 DNA detection. (B) Photograph of the AuNP aggregates after incubation with different concentrations of the target DNA RCA products. (C) Plot of discrimination factor vs. target DNA concentration. Reprinted with permission from ref 799. Copyright 2013 The Royal Society of Chemistry.

Figure 45. Methods for sequencing viral genomes from clinical specimens. All specimens originally comprise a mix of host (in blue) and pathogen (in red) DNA sequences. For pathogens that have RNA genomes, RNA in the sample is converted into complementary DNA (cDNA) before PCR and library preparation. Direct metagenomic sequencing provides an accurate representation of the sequences in the sample, although at high sequencing and data analysis and storage costs. PCR amplicon sequencing uses many discrete PCR reactions to enrich the viral genome, which increases the workload for large genomes substantially but decreases the costs. Target enrichment sequencing uses virus-specific nucleotide probes that are bound to a solid phase, such as beads, to enrich the viral genome in a single reaction, which reduces workload but increases the cost of library preparation compared with PCR. Reprinted with permission from ref 114. Copyright 2017 Macmillan Publishers Ltd.

Figure 46. Metagenomic sequencing workflow for MinION nanopore sequencing compared to Illumina MiSeq sequencing. (A) Overall workflow. (B) Steps in the MetaPORE real-time analysis pipeline. The turnaround time for sample-to-detection nanopore sequencing, defined here as the cumulative time taken for nucleic acid extraction, reverse transcription, library preparation, sequencing, MetaPORE bioinformatics analysis, and pathogen detection, was under 6 hr, while Illumina sequencing took over 20 hr. The time differential is accounted for by increased times for library quantitation, sequencing, and bioinformatics analysis with the Illumina protocol. *Assumes a 12-hr 50-bp single-end MiSeq run of ~12-15 million reads, with 50 bp the minimum estimated read length needed for accurate pathogen identification. The stopwatch is depicted as a 12-hr clock. Reprinted under the Creative Commons license from ref 812.

Figure 47. Taxonomic distribution of target genomes included in ViroCap. Shown are the viral groups and families included in the ViroCap targeted sequence capture panel. A highlighted subset illustrates the underlying genera. Reprinted under the Creative Commons license from ref 821.

Figure 48. Schematic of a potentiometric sensor. (A) Left to right. A potentiometric device measures the flow of ions across a membrane or barrier. Equilibrium is reached and establishes an initial baseline. Antigen-antibody interaction at the surface (purple circles) leads to an increase in ion concentration at the surface. A measurable potential is detected which eventually equilibrates. (B) Data acquired from specific (left) and a group of 3 types of nonspecific (*i.e.*,

control, right) electrodes. The specific electrode was a PEG-B membrane modified with mAb-RSV. Reprinted with permission from ref 838. Copyright 2013 American Chemical Society.

Figure 49. Schematic of the electrochemical detection for virus viability. (A) A thiolated DNA primer is hybridized with a complementary end of a Vaccinia virus (VACV)-specific aptamer and the hybrid was self-assembled onto an AuNP-modified screen-printed carbon electrode (GNPs-SPCE). Binding of the virus to the immobilized aptamer causes an increase in the redox current, measured *via* square wave voltammetry. Nyquist plot ($-Z_{im}$ vs. Z_{re}) of impedance spectra obtained using (a) 150 PFU, (b) 300 PFU, (c) 450 PFU, (d) 600 PFU, (e) 750 PFU, (f) 900 PFU of VACV in serum. The impedance spectra were recorded from 100 kHz to 0.1 Hz and the amplitude was 0.1 V vs. pseudo Ag reference. The inset represents the circuit employed to fit to the EIS measured data. The circuit consists of the ohmic resistance, R_s , of the electrolyte solution, electrolyte/film interface resistance R_{ef} and capacity CPE_{ef} , film resistance R_f and capacity CPE_f , and the Warburg impedance, Z_w , resulting from diffusion of the redox probe. Reprinted with permission from ref 842. Copyright 2013 The Royal Society of Chemistry.

Figure 50. AuNP-virus composites. (A) Representative TEM images of AuNP composites. Scale bar = 50 nm. (B) Absorption spectra of AuNP probes in response to H3N2 in the presence of 2 nM specific probes (a), 2 nM specific probes without H3N2 (b), and 2 nM nonspecific probes (c). The inset shows the corresponding color changes. Reprinted with permission from ref 853. Copyright 2015 The Royal Society of Chemistry.

Figure 51. Gold nanobipyramids for ultrasensitive colorimetric detection of influenza virus. (A) Schematic illustration of the principle of the immunosensor. (B) (i) Original Au NBPs were standard bipyramid shape with a brownish red color. (ii) After the deposition of Ag, the bipyramid nanostructures had a cylinder like shape and the solution color was green. (iii) When almost all silver precursors were reduced, the nanostructures were “walnut-like”. The solution color was red. Simultaneously, the LSPR peak blue-shifted from 750 to 580 nm. Reprinted with permission from ref 857. Copyright 2013 American Chemical Society.

Figure 52. Schematic illustration of the sandwich hybridization assay with a QD-induced FRET reporter system for H5 target DNA detection. (A) Sandwich hybridization with label-free H5 sequence (target) by the capturing probes conjugated on QD655 (FRET donor) and the reporter probes labeled with Alexa Fluor 660 (FRET acceptor). (B) FRET emission shift before and after the sandwich hybridization. Reprinted under MDPI Open Access license from ref 860.

Figure 53. Schematic representation of fluorescent immunochromatography. First is application of the conjugate and sample to the rapid diagnostic strips. After 15 min, the presence of virus was measured with a portable fluorescence reader. Reprinted with permission from ref 876. Copyright 2017 Elsevier.

Figure 54. Chemical structures of selected toxins. (A) Trichothecene mycotoxin. (B) Tetrodotoxin. (C) Saxitoxin. (D) Brevetoxin B.

Figure 55. MS analysis of anthrax lethal factor activity. (A) Sequence of substrate and cleaved product and expected m/z of each. Peptide cleavage products indicating the presence of

the anthrax lethal factor are marked with asterisks. X = norleucine. (B) MALDI-TOF mass spectrum indicating the presence of anthrax lethal factor. Reprinted under MDPI Open Access license from ref 926.

Figure 56. Endopep-MS assay. Typical LC-MS/MS chromatograms obtained for N-terminal and C-terminal peptides resulting from botulinum neurotoxin A activity after immunocapture-Endopep-MS assay in serum samples. Negative-control serum sample (A); positive-control serum sample spiked with 4 minimal lethal dose (MLD₅₀)/ml of BoNT/A (B); serum sample from patient 912 (C); serum sample from patient 943 (D). Two selected reaction monitoring (SRM) transitions of N-ter (Nter-1 and Nter-2) and C-ter (Cter-1 and Cter-2) peptides are represented. The last SRM transition corresponds to the internal standard (I.S.). Reprinted with permission from ref 970. Copyright 2008 American Society for Microbiology.

Figure 57. Multiplexing mycotoxin detection. (A) Left, portable imaging SPR (iSPR) setup with nanostructured gold as sensor surface thus omitting the need for any prism and right, chip microfluidic array for 6-plex assay (single chip with OVA as reference and six toxin conjugates). (B) Calibration curves in (left) buffer and (right) barley extract for the six mycotoxins measured in a 6-plex format measured using a prototype nanostructured iSPR instrument. Reprinted with permission from ref 987. Copyright 2016 The Royal Society of Chemistry.

Figure 58. Illustration of the custom-built SPR for detection of botulinum neurotoxins and portable SPR. (A) A gold-coated glass slide is pressed onto a prism. A collimated light beam passes through the prism and excites a surface plasmon upon incidence with the metal-dielectric interface. Four independent flow channels create four sensing channels. Reprinted with permission from ref 998. Copyright 2008 Elsevier. (B) Portable SPR sensor operating in hand held mode. Reprinted with permission from ref 999. Copyright 2008 Elsevier.

Figure 59. Immunological SPR-sensor for simultaneous differentiation of ricin and agglutinin. (A) Immobilized monoclonal antibody mAb R109 binds both ricin and agglutinin while differentiation takes place by injection of mAb R18. (B) Schematic binding curves. (C) Ability to differentiate ricin from agglutinin in a concentration independent manner. Reprinted with permission from ref 1001. Copyright 2016 Elsevier.

Figure 60. SPR-sensors for botulinum neurotoxin. (A) Schematic representation of the SPR biosensor method to assay BoNT/B activity. Synaptic vesicles were incubated in the presence or absence of BoNT/B and then injected on sensor chips functionalized with anti-VAMP2 antibodies or reference antibodies (anti-synaptophysin). BoNT/B treatment induces a decrease of synaptic vesicle binding only to anti-VAMP2 antibodies. Reprinted with permission from ref 1002. Copyright 2011 Elsevier. (B) A sample containing BoNT/A was injected over the SNAP-25 sensor chip. MAb10F12 was then injected to detect epitopes generated by BoNT/A activity. The difference between red and black traces corresponds to specific mAb binding, and indicates the number of neo-epitopes produced. Reprinted with permission from ref 1003. Copyright 2013 Elsevier.

Figure 61. Aptamer scaffold electrochemical DNA (E-DNA) biosensors. (A) Schematic. A specific target-binding aptamer (red) is inserted into an oligonucleotide scaffold (black). A thiol

group (S) on the scaffold attaches it to the gold electrode surface (yellow). The position and dynamics of a methylene blue molecule (blue star, attached to the scaffold) relative to the electrode surface changes in response to aptamer-target binding, producing a measurable electrical current change. Biosensors directed against botulinum toxin (BoNTA) and ricin toxin (RTA) are shown. **(B)** Dose-responsive curves for the biosensors performed in a mixture containing 10% bovine blood serum (BSA) and 90% 1× PBS. Peak current vs. toxin concentration is shown for botulinum neurotoxin variant A (BoNTA) and ricin toxin chain A (RTA). Reprinted with permission from ref 1035. Copyright 2015 The Royal Society of Chemistry.

Figure 62. High-sensitivity detection of BoNT with Glyco-qPCR. Tiny amounts of BoNT are captured by sequential immunoprecipitation, binding of sialyllactose- (SL) -DNA conjugate, washing, and signal amplification by PCR. Reprinted with permission from ref 1053. Copyright 2014 American Chemical Society.

Figure 63. Ricin detection by eye with gold nanoparticle based colorimetric assays. **(A)** The mechanism of the colorimetric detection of ricin utilizing Ricin binding aptamer (RBA) and unmodified AuNPs. **(B)** UV-vis absorption spectra of AuNPs in the presence of 0.138 M NaCl under different experimental conditions, cRBA = 83 nM, cAuNPs = 2.78 nM. **(C)** Visual color changes corresponding to data in B. Reprinted with permission from ref 1060. Copyright 2014 The Royal Society of Chemistry.

Figure 64. Biosensor for ochratoxin A based on a structure-switching signaling FRET aptamer. **(A)** Schematic illustration of the fluorescent aptamer sensor for OTA, based on the displacement of a quencher-labeled DNA by the target. F = fluorophore and Q = quencher. **(B)** Fluorescence spectra of aptameric assay system in the absence (blank) and presence of target. Reprinted with permission from ref 1067. Copyright 2012 Elsevier.

Figure 65. SERS detection of ricin. **(A)** Schematic illustration of the fabrication process of SERS aptasensor for ricin protein toxin recognition. **(B)** AFM images of ricin B aptamer bound silicon surface with ricin B. **(C)** SERS spectra of silicon surface without SERS probe (a), and without and with Ag enhancement (b and c); the concentration of ricin B is 10 fM. Reprinted with permission from ref 1086. Copyright 2015 The Royal Society of Chemistry.

Figure 66. Lateral flow assay with SERS detection of SEB. **(A)** SERS mapping images acquired using a peak intensity at 1650 cm^{-1} for nine different SEB concentrations over a range of 0.1 pg mL^{-1} to 1000 ng mL^{-1} . The scale bar at the bottom displays the color decoding scheme for different SERS intensities. **(B)** Average SERS spectra for the 1600 pixel points of the SERS mapping zones. Reprinted with permission from ref 1097. Copyright 2016 The Royal Society of Chemistry.

Figure 67. SERS based immunoassays of aflatoxin. SEM images of sandwich immunocomplexes with **(A)** $1.0\text{ }\mu\text{g/mL}$ of AFB1 and **(B)** without AFB1. Corresponding TEM images shown underneath. Average SERS spectra for the 1600 pixel points of the SERS mapping zones. **(C)** SERS spectra for AFB1, FMB, OTA, and some of their mixtures in distilled

water. **(D)** Comparison of the relative Raman peak intensities at 1616 cm^{-1} . Reprinted with permission from ref 1099. Copyright 2015 Elsevier.

Figure 68. Fluidic force discrimination assays. **(A)** Reaction schemes for sequential and semi-homogeneous fluidic force discrimination immunoassays. In sequential assays, biotinylated capture antibodies are arrayed on a NeutrAvidin-functionalized substrate. The immobilized antibodies then capture target, secondary antibodies (2°Ab), and Ab-conjugated microbead labels in sequential exposures. In semi-homogeneous assays, the target, 2°Ab , and beads are first mixed in solution, and then applied to the microarray. Finally, for both assays, nonspecifically bound beads are preferentially removed by controlled, laminar fluidic forces and the remaining beads counted to determine target identities and concentrations. **(B)** Comparison of sequential and semi-homogeneous FFD assays for the detection of SEB in buffer. **(C)** Semi-homogeneous FFD detection signal for 35 pM ($\square$) and 35 fM ($\circ$) SEB in buffer as a function of the homogeneous mixing time (1–20 min). Reprinted with permission from ref 1126. Copyright 2009 Elsevier.

Figure 69. FilmArray® performance. LOD analysis for the *B. anthracis* (Ba), *F. tularensis* (Ft) and *Y. pestis* (Yp) panels. Summary of the instrument calls for each sample. Grey boxes indicate positive and white boxes are negative calls for the target organism; a hatched box indicates an instrument call of ‘Bacillus species.’ For the Ba test, all three targets must be present for a *B. anthracis* call; if less than three targets are identified, the sample is called as ‘Bacillus species.’ For *F. tularensis* and *Y. pestis*, only one of the targets is required for a positive call. Reprinted with permission from ref 1140. Copyright 2013 Society for Applied Microbiology.

Figure 70. Strategies to integrate thin and soft electronics on skin. **(A)** Three categories of skin integration strategies for lab-on-skin devices. **(B)** Tattoo with bare die chip mounted on an acrylic adhesive film. **(C)** Soft radio sensor with commercial chips encapsulated in a fluid/Ecoflex package. **(D)** Sweat sensor on silicone foam. Reprinted with permission from ref 1151. Copyright 2017 American Chemical Society.

Figure 71. Paper-based, low cost synthetic biology derived diagnostics. **(A)** Using sequence database information, primers for isothermal RNA amplification and toehold switch-based RNA sensors were designed by purpose-built algorithms. Once synthesized, the resulting sequence-specific toehold sensors can be assembled and validated in less than 7 hr. In under a day, validated sensors can be embedded into paper and freeze-dried along with a cell-free transcription and translation system to be deployed in the field as stable diagnostics. For the diagnostic test, extracted RNA is isothermally amplified *via* nucleic acid sequence based amplification (NASBA) and used to rehydrate the freeze-dried paper sensors. The detection of the appropriate trigger RNA is indicated by a color change in the paper disc from yellow to purple. **(B)** Zika RNA fragments diluted in water or 7% human serum were amplified using NASBA with input concentrations ranging from 30 pM down to 3 fM . A 1:7 dilution of the NASBA reaction in water was then used to rehydrate freeze-dried, paper-based reactions containing sensors 27B and 32B. Reprinted with permission from ref 1154. Copyright 2016 Elsevier.

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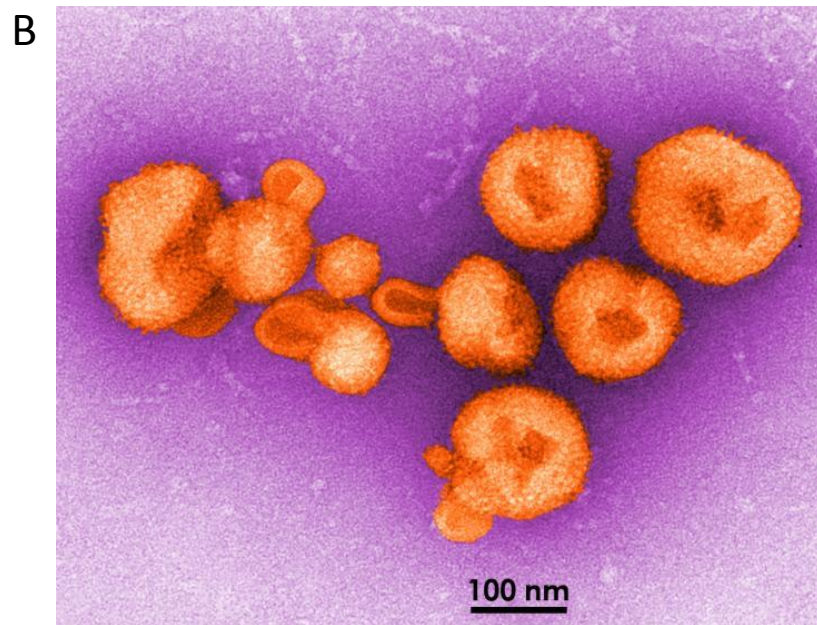
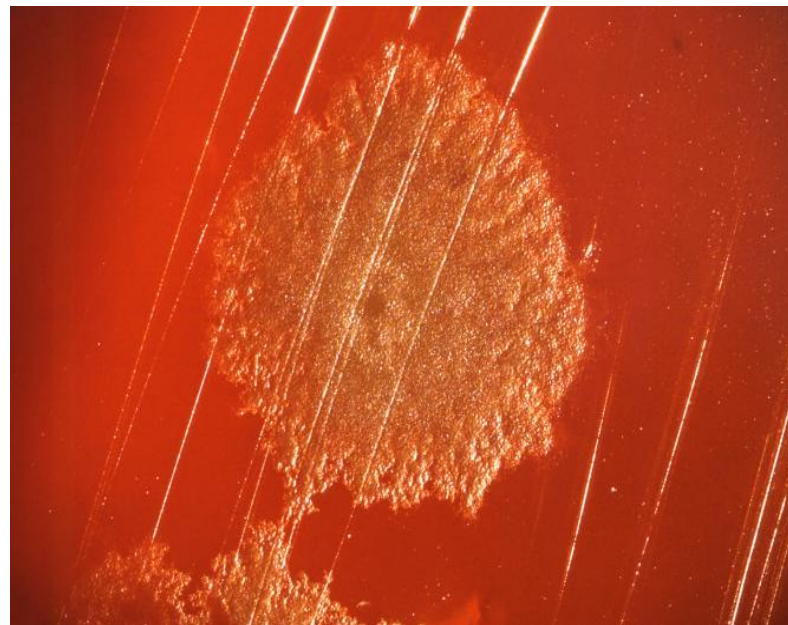
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Figure 1



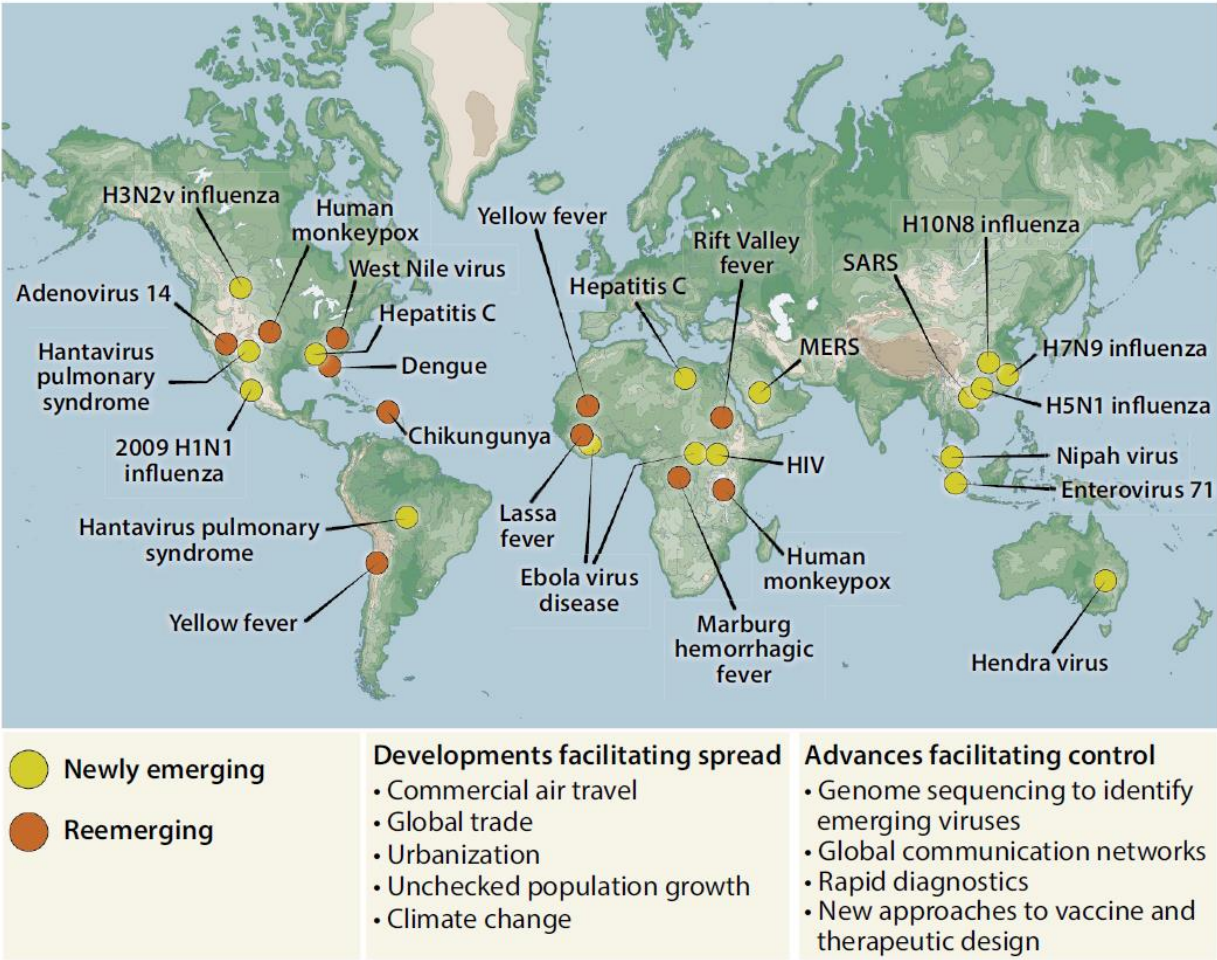


Figure 3



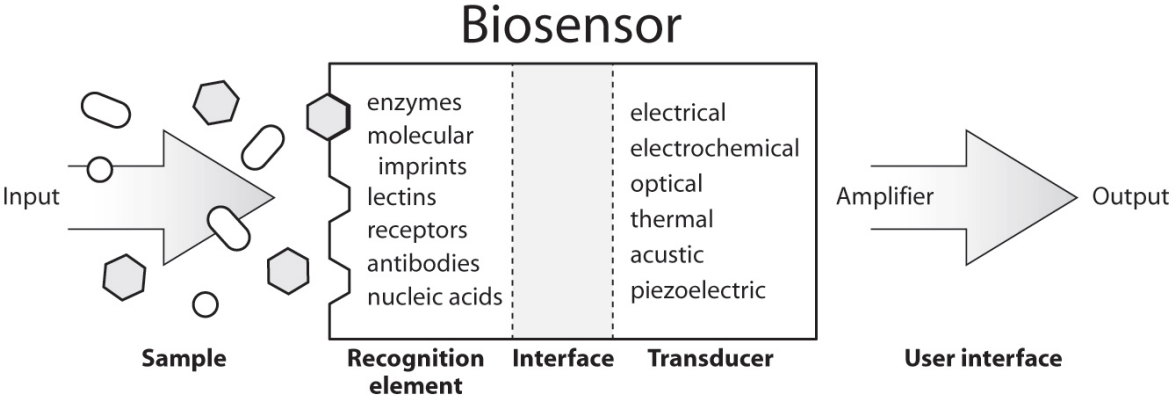
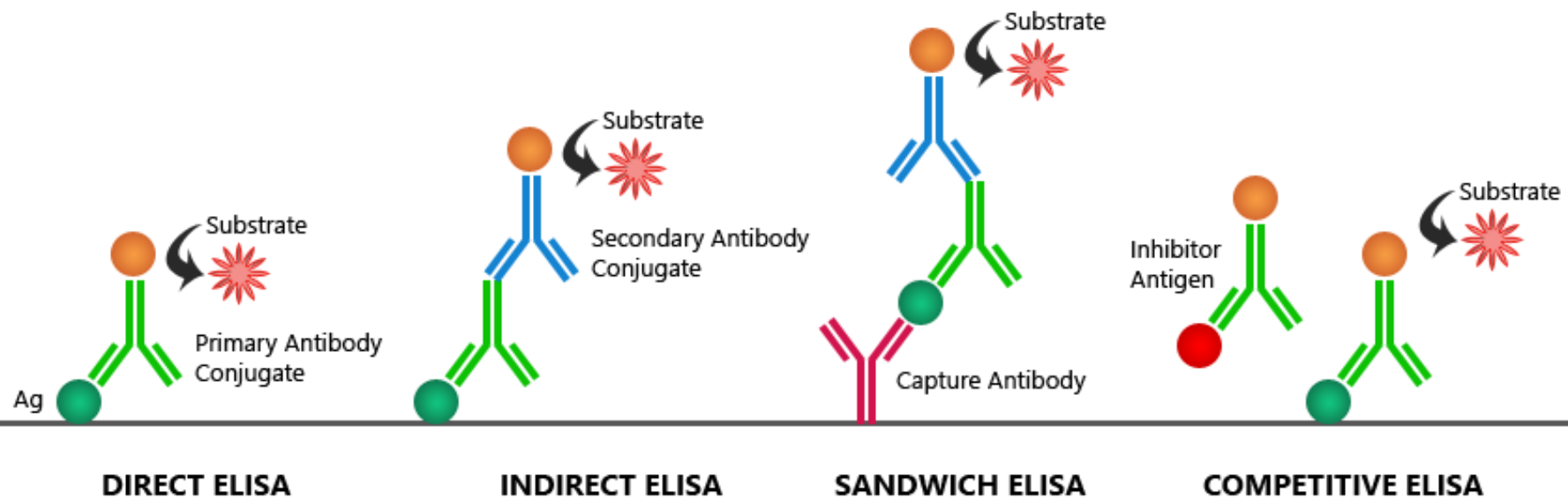


Figure 5



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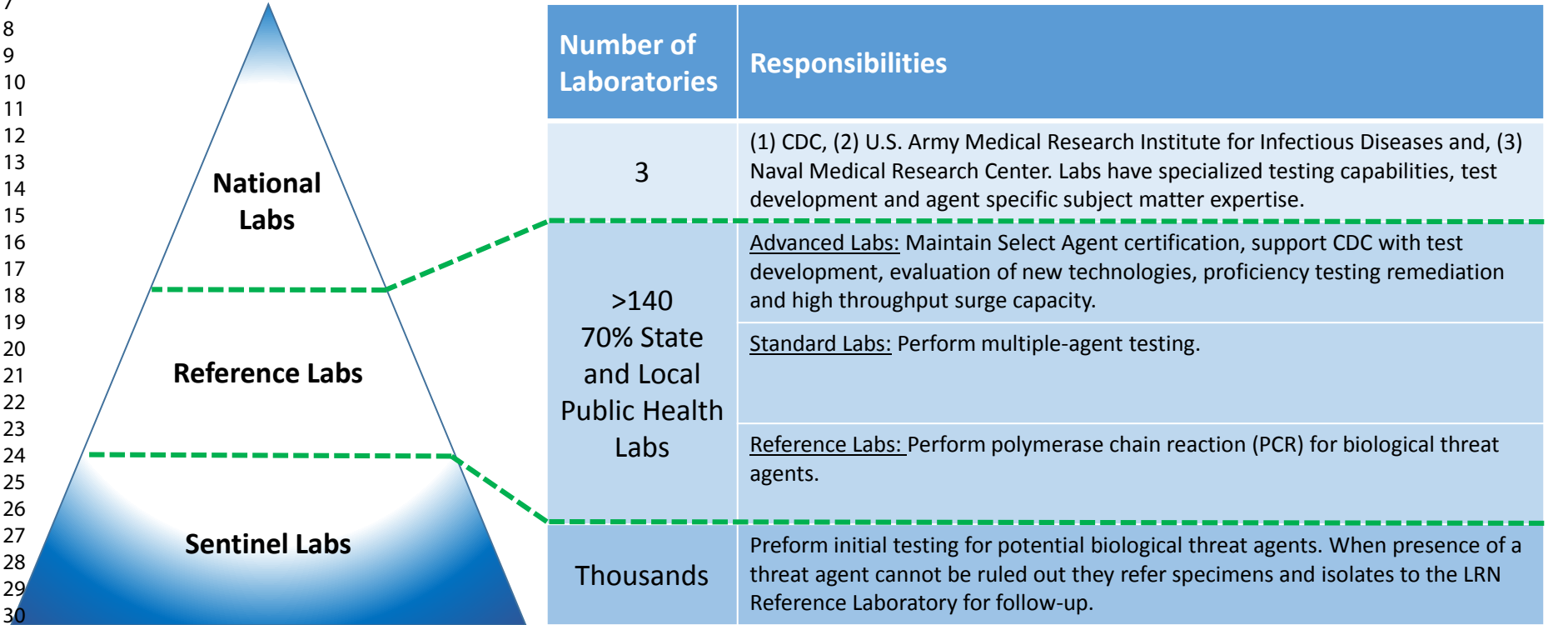


Figure 7

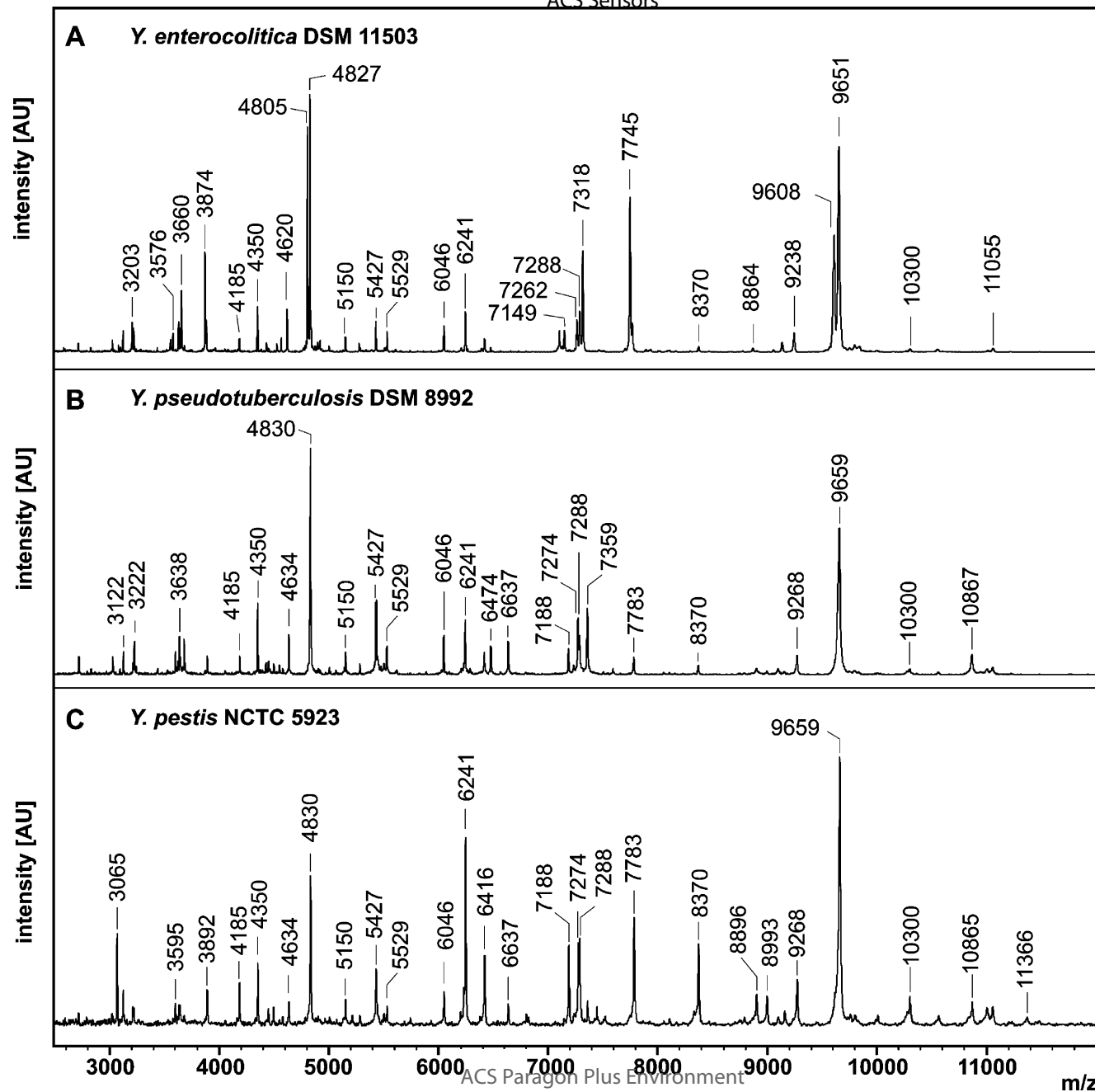
Target: *Y. pestis*/*C. jejuni*

Figure 8

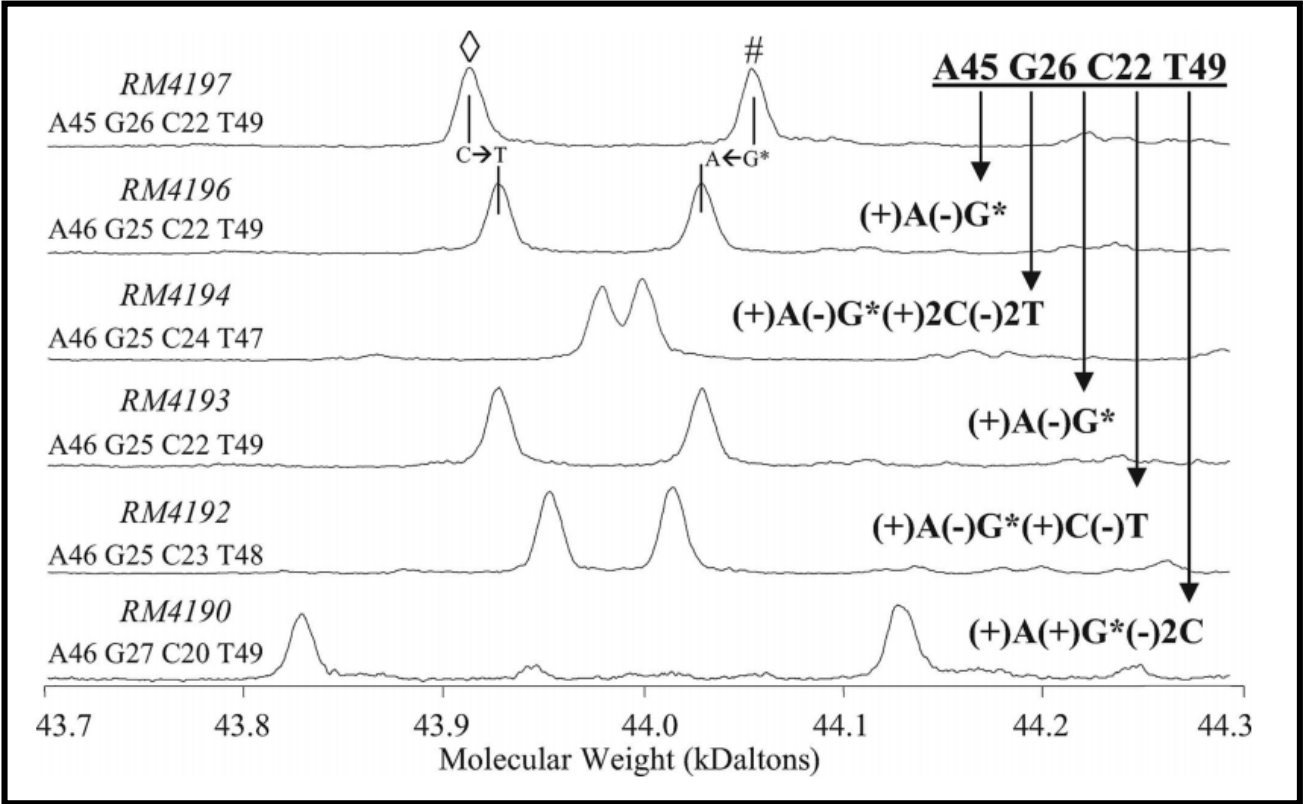
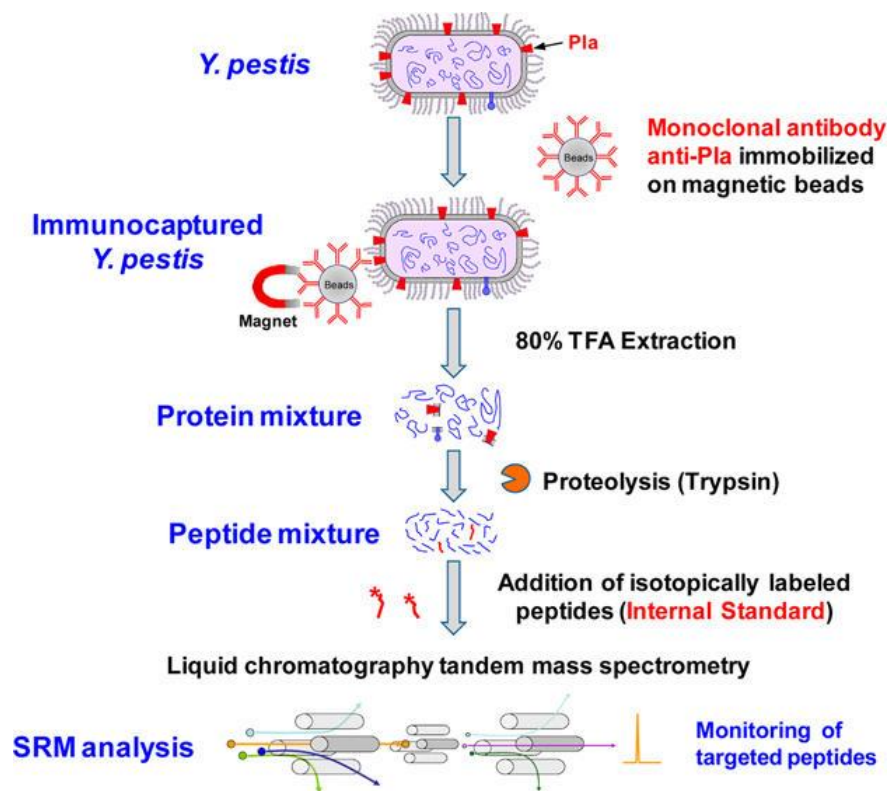


Figure 9



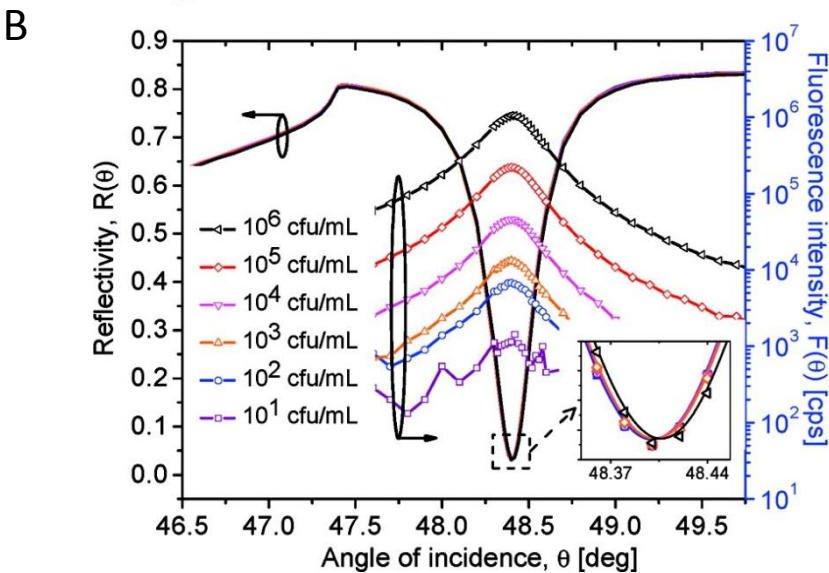
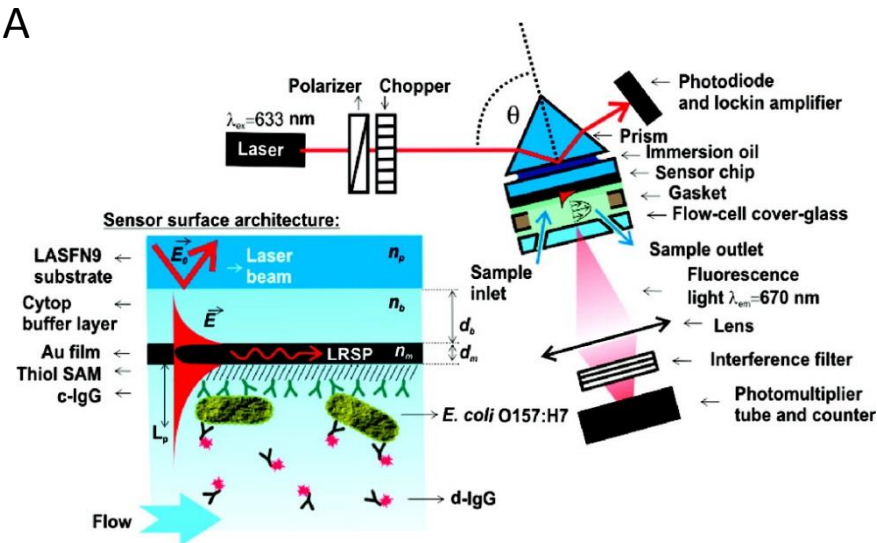
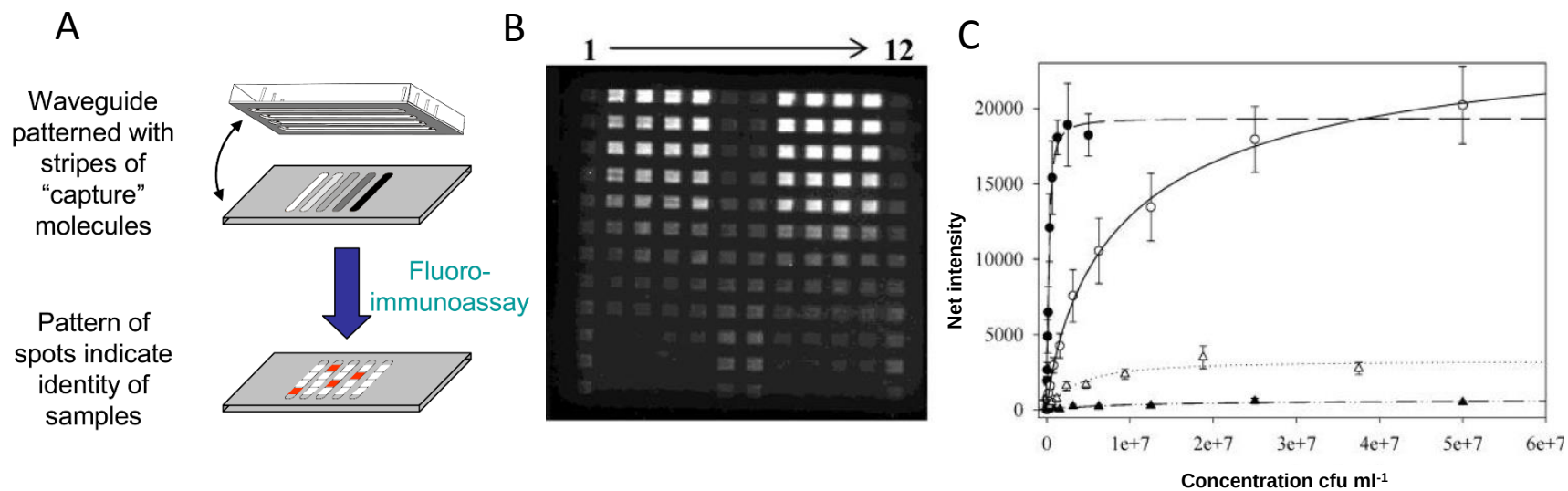


Figure 11



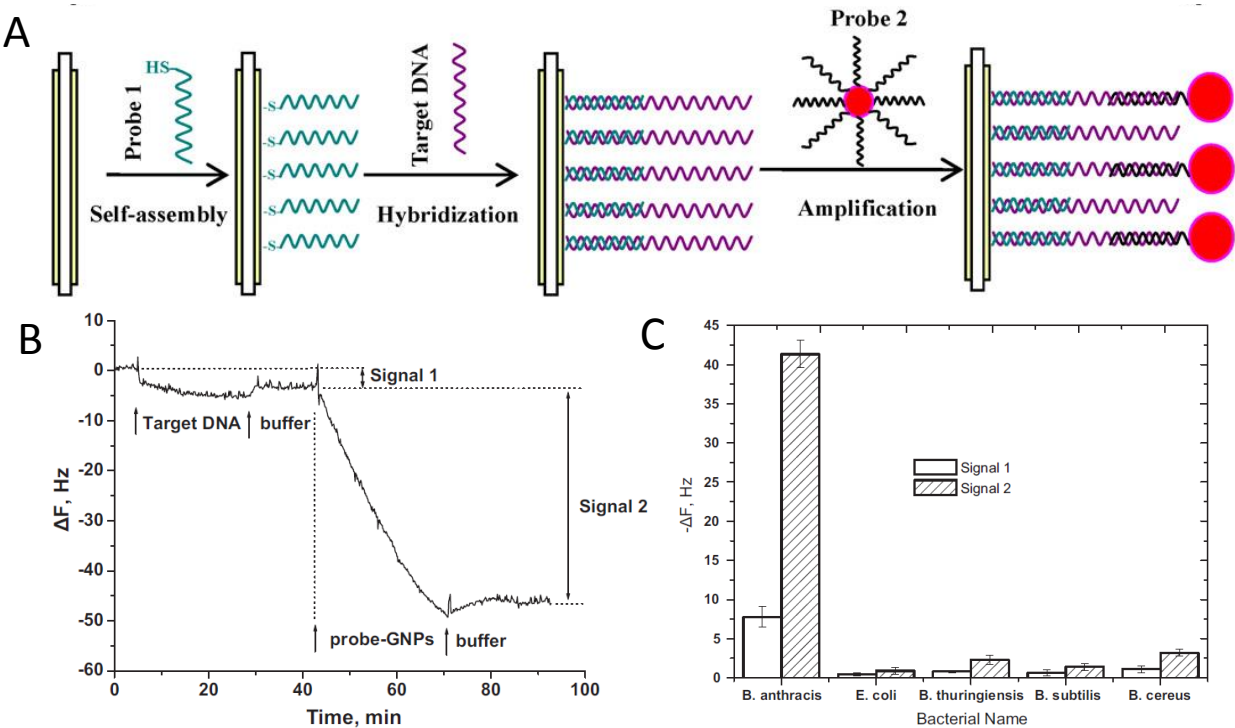
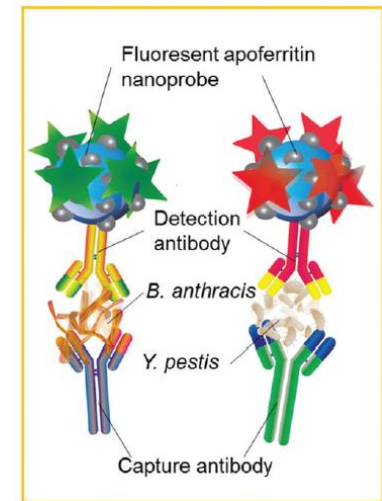
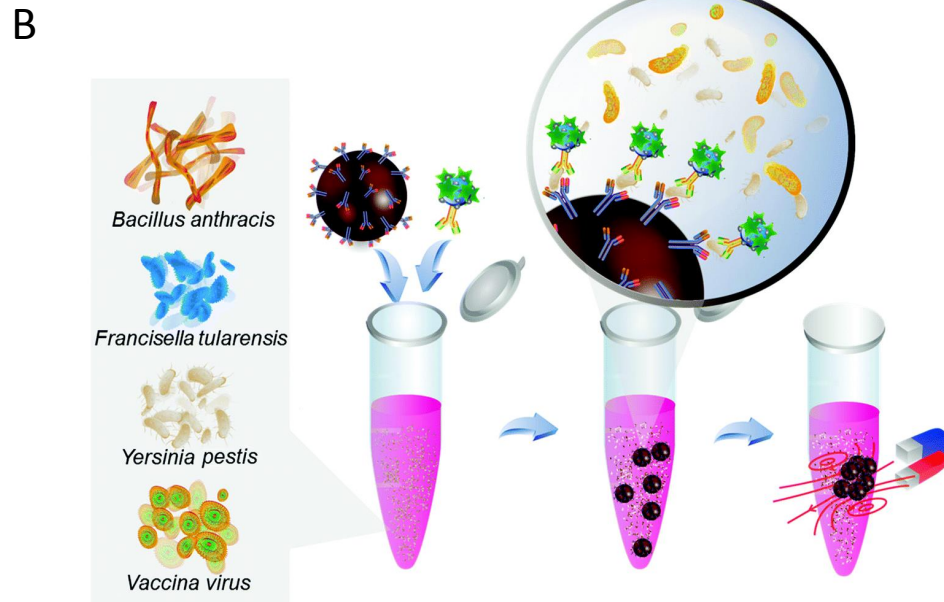
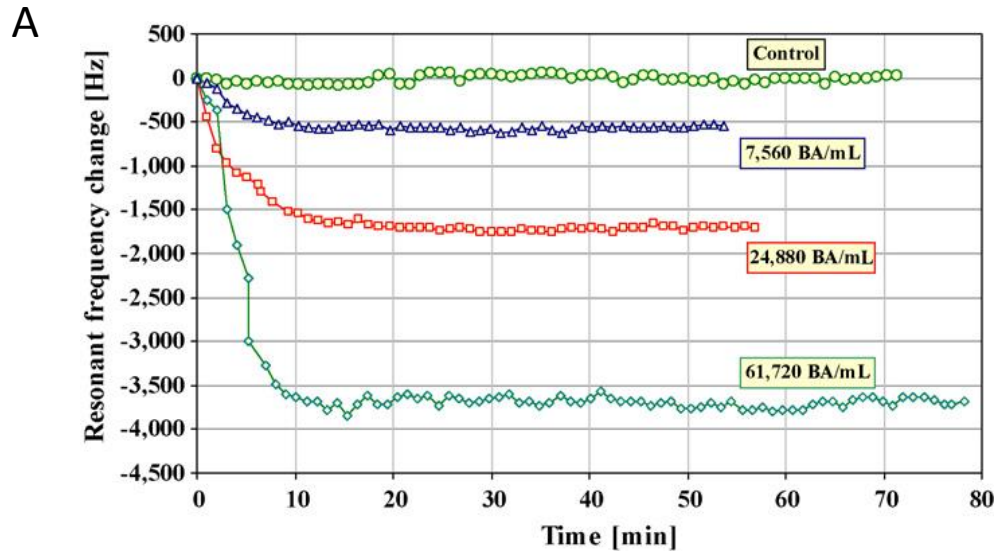


Figure 13



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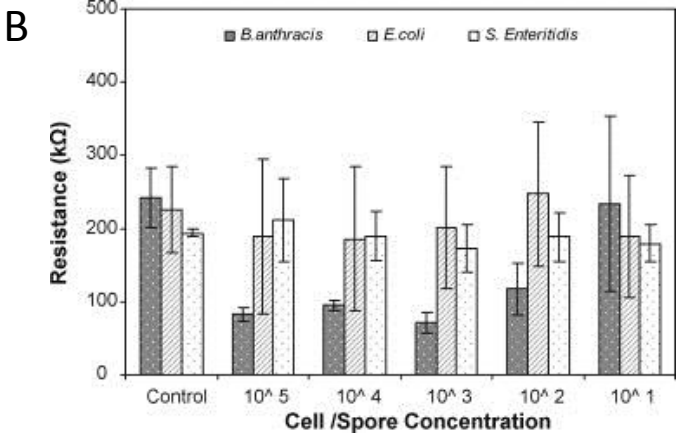
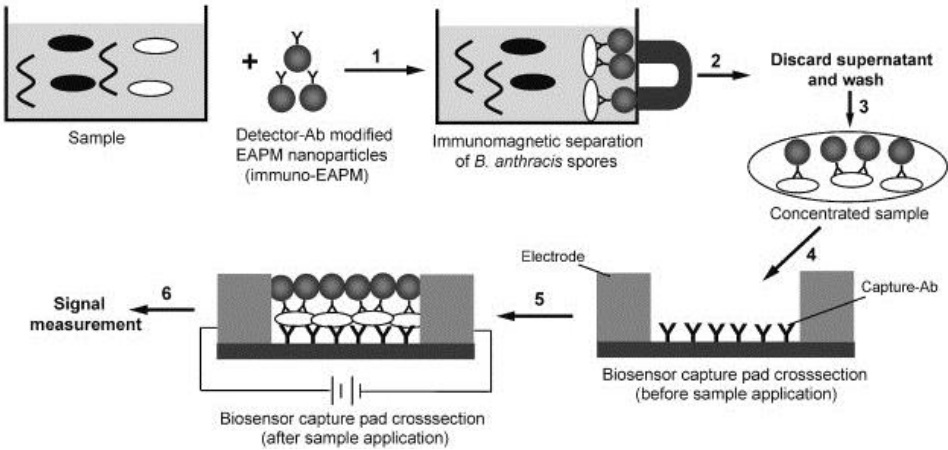
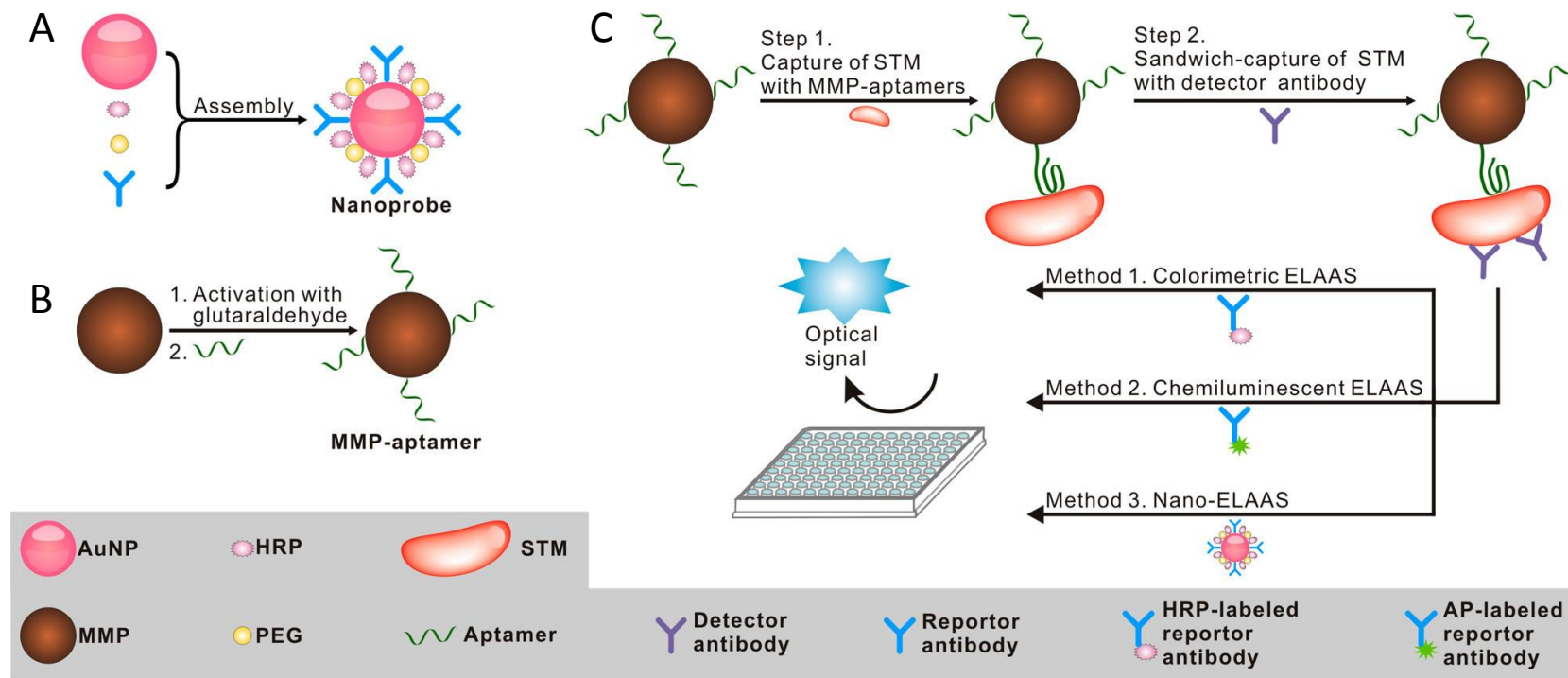


Figure 15



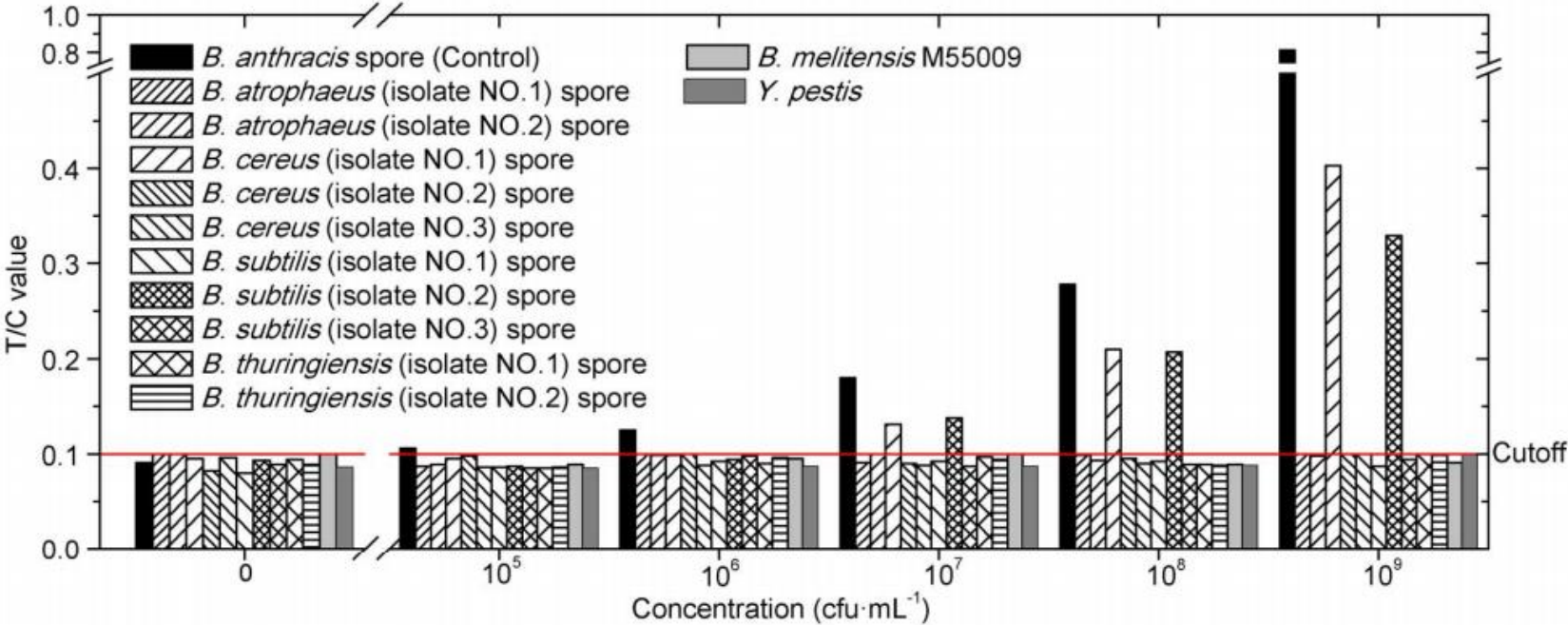
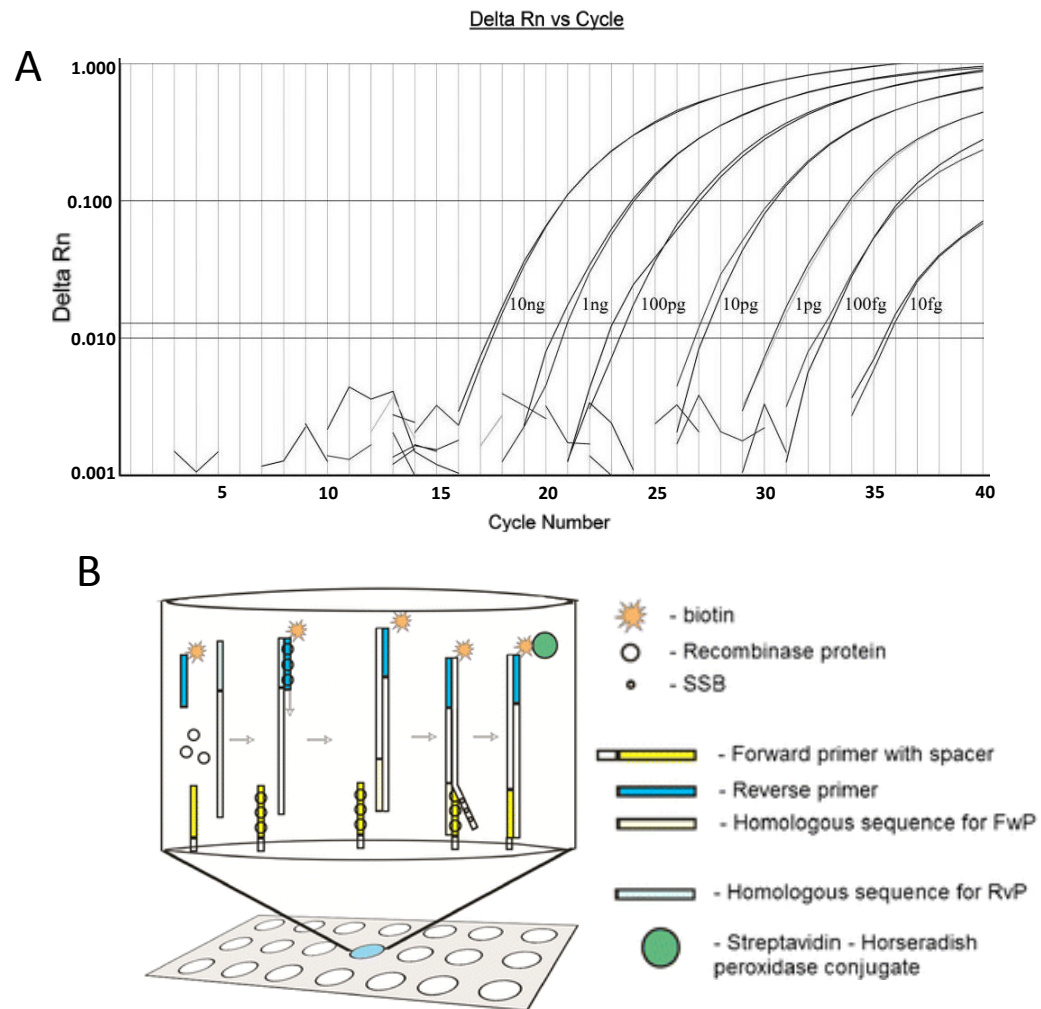


Figure 17



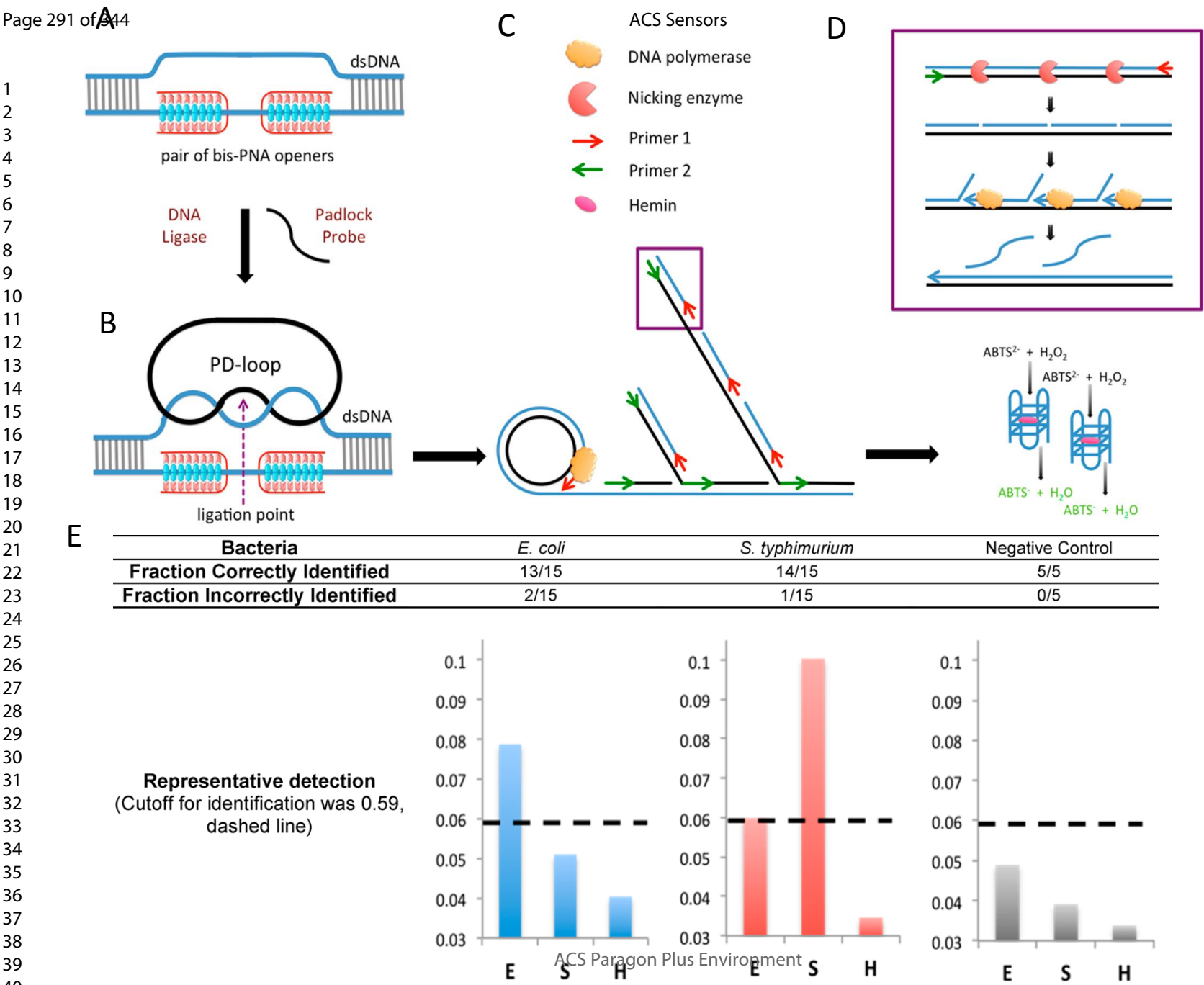
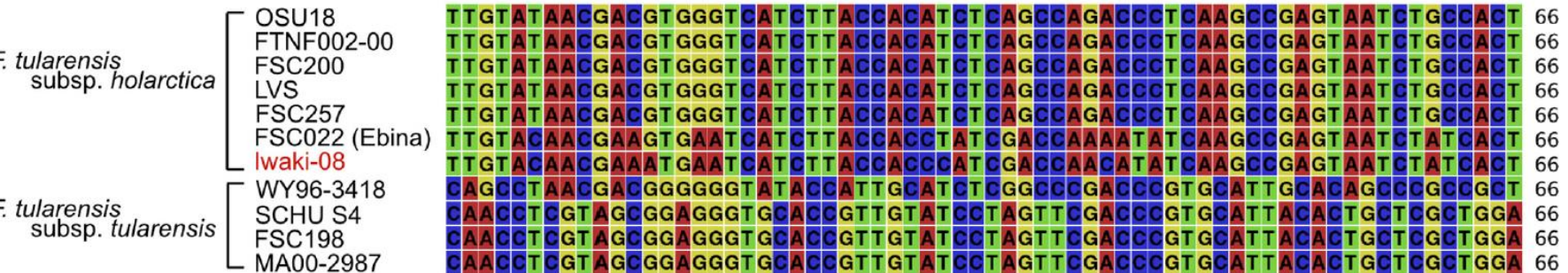
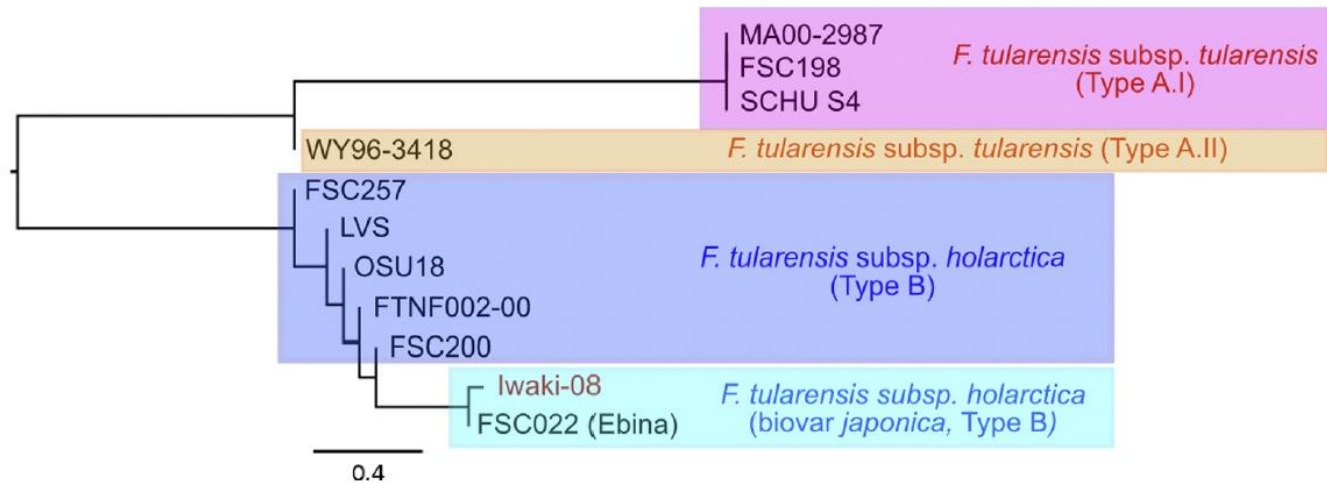


Figure 19

A

Nucleotide position of *F. tularensis* subsp. *tularensis* SCHU S4

B



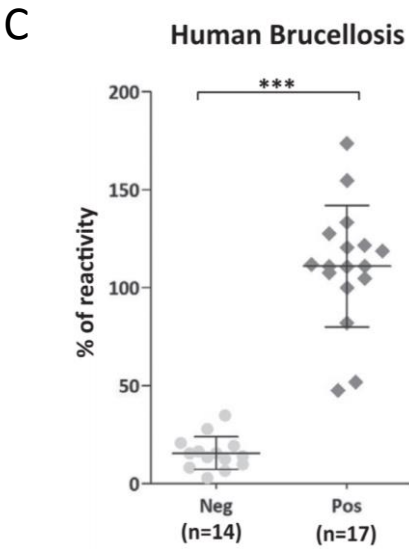
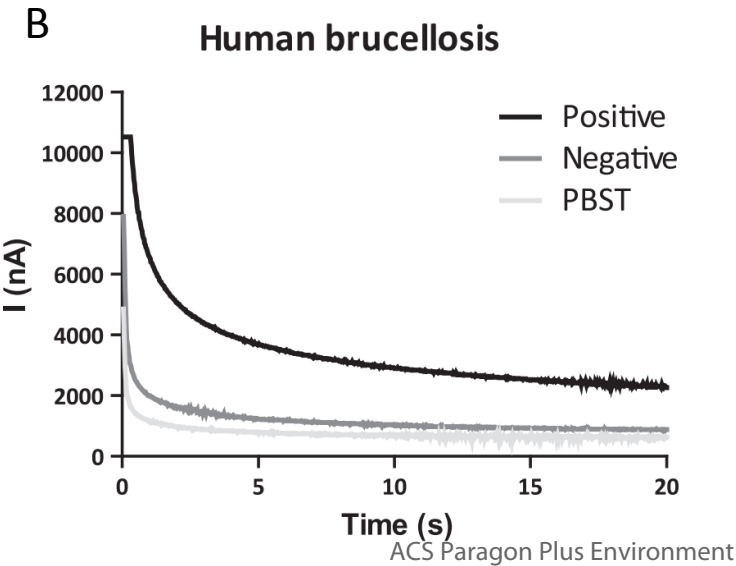
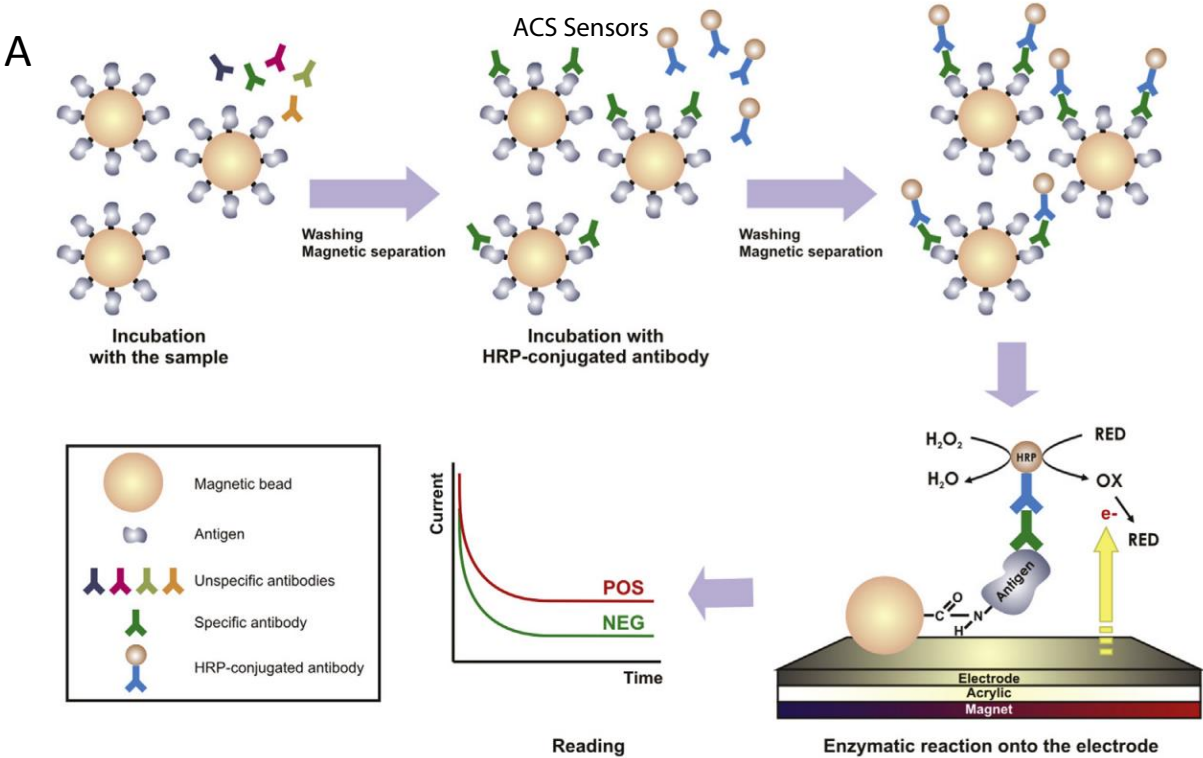
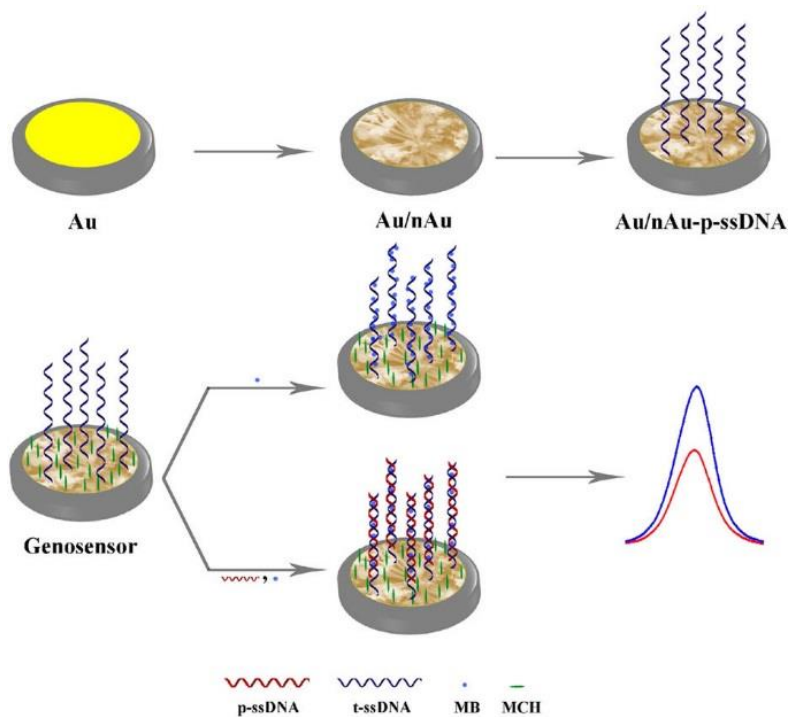
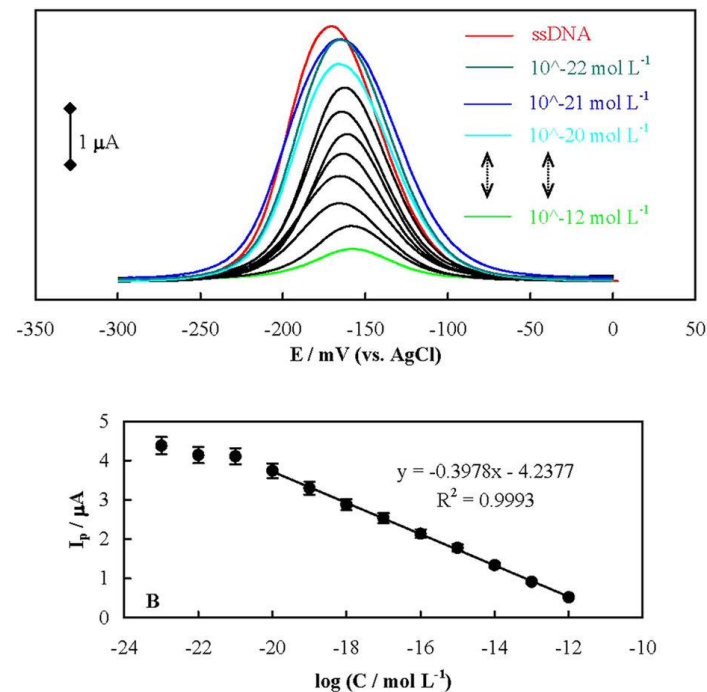


Figure 21

A



B



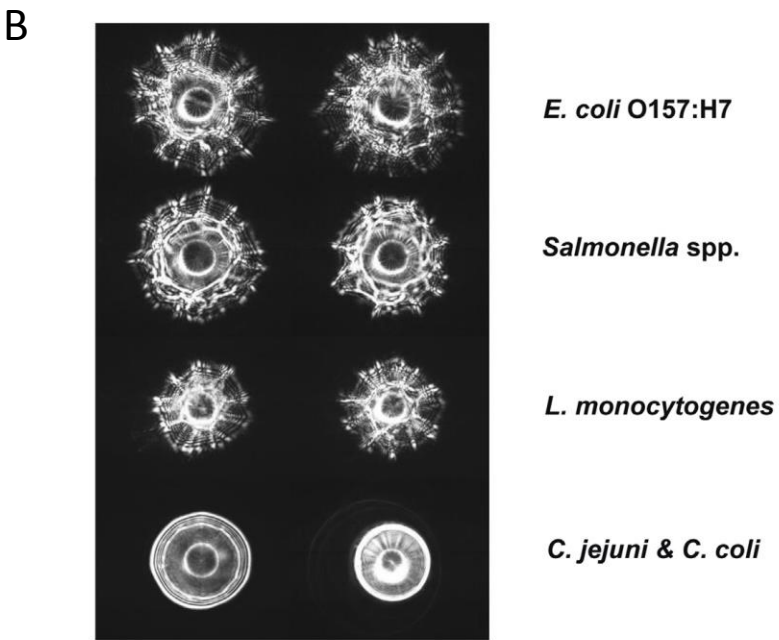
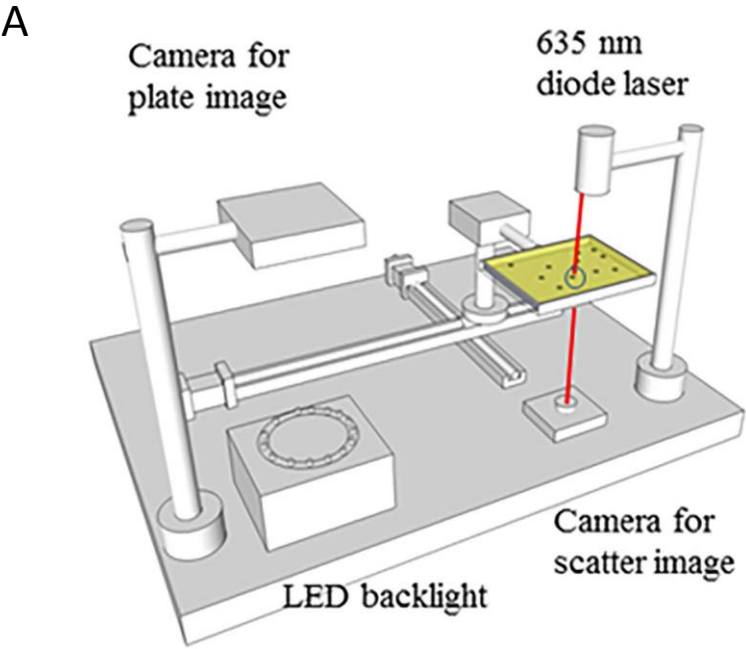
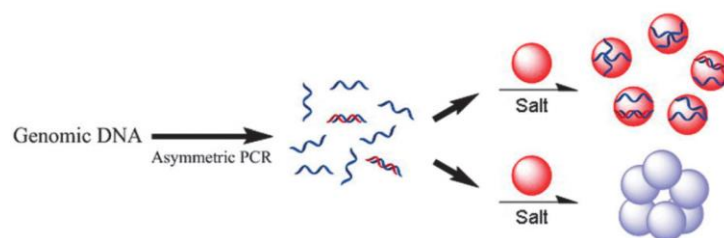
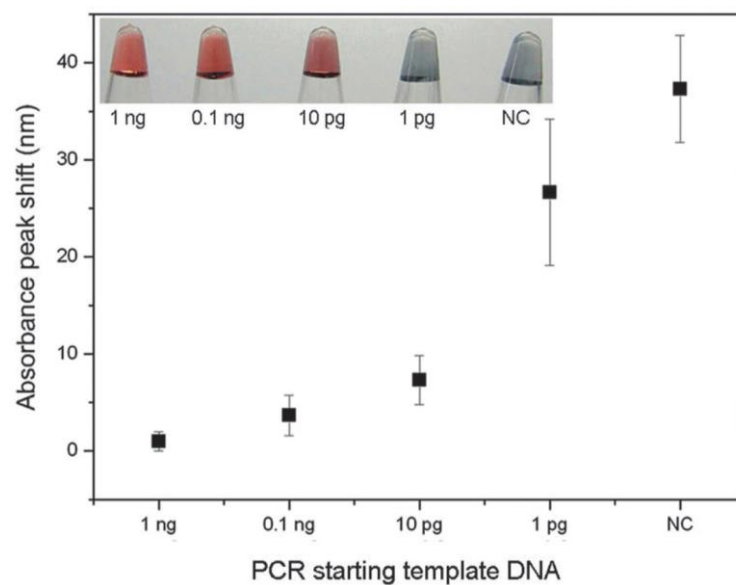


Figure 23

A



B



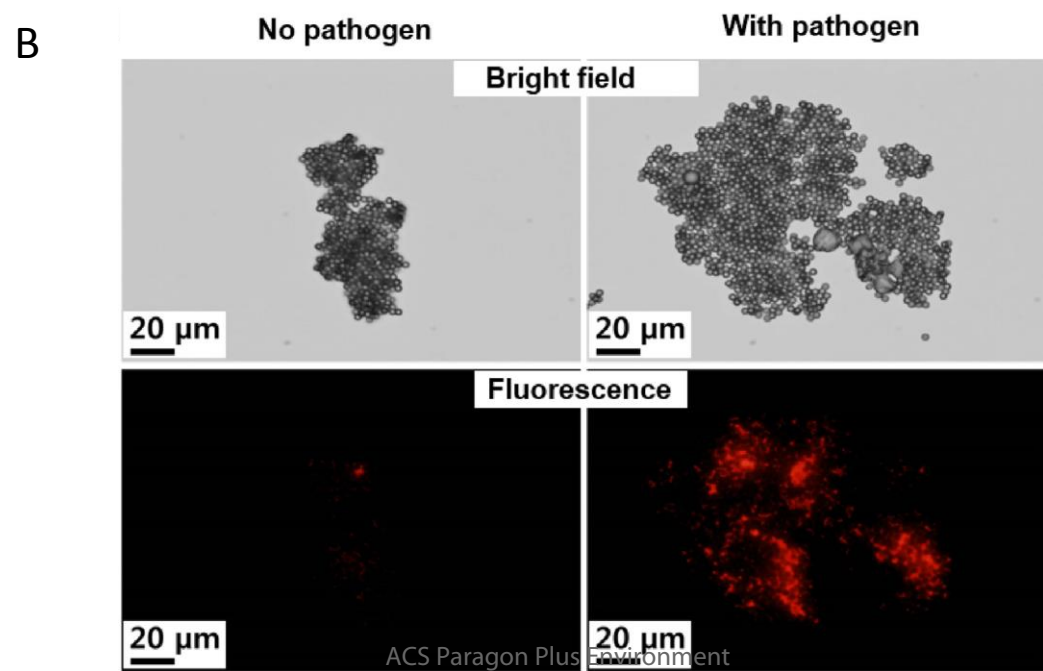
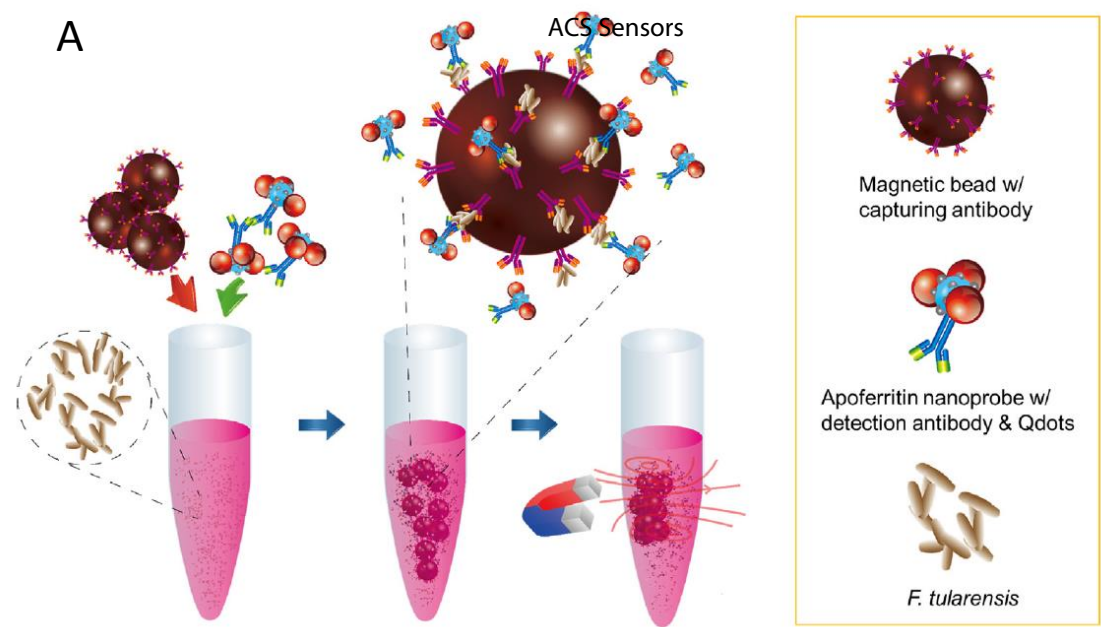
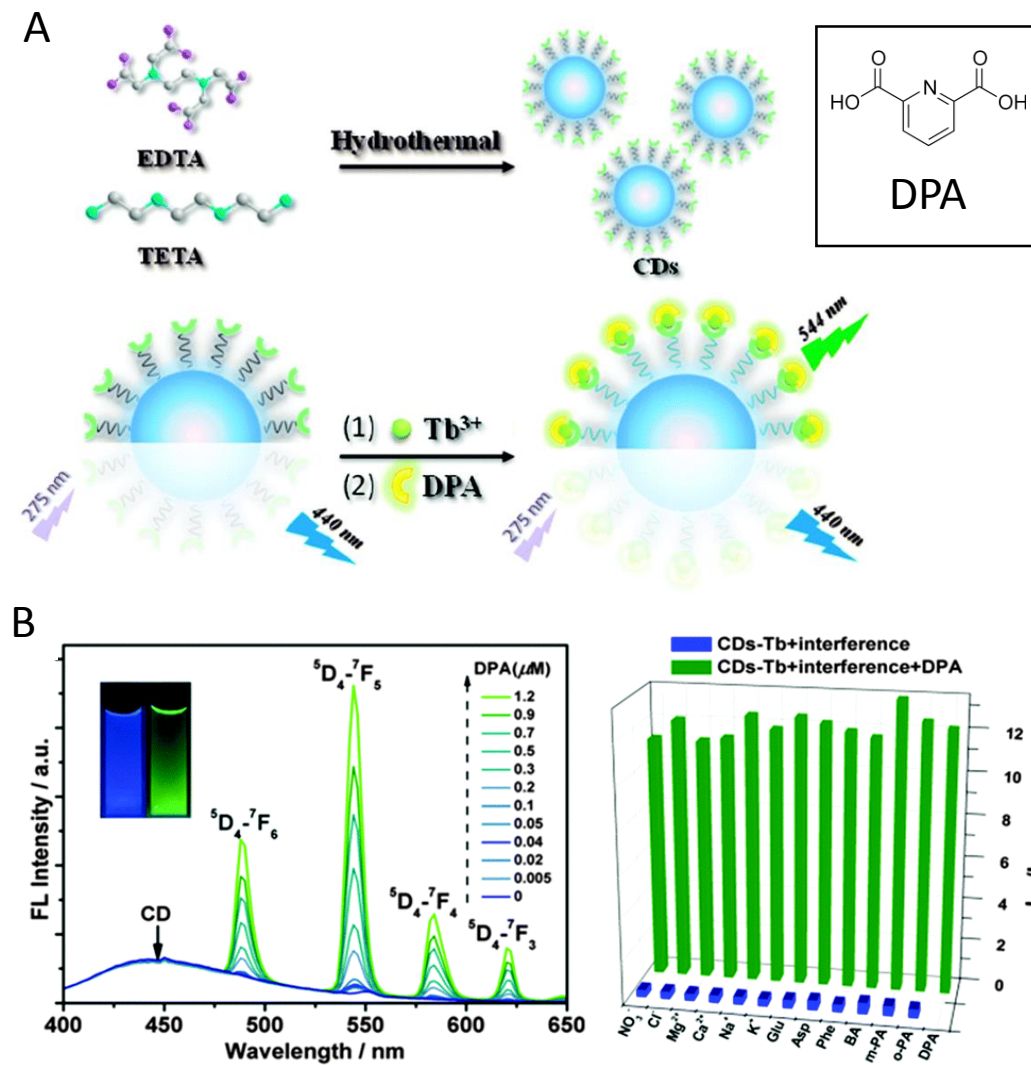
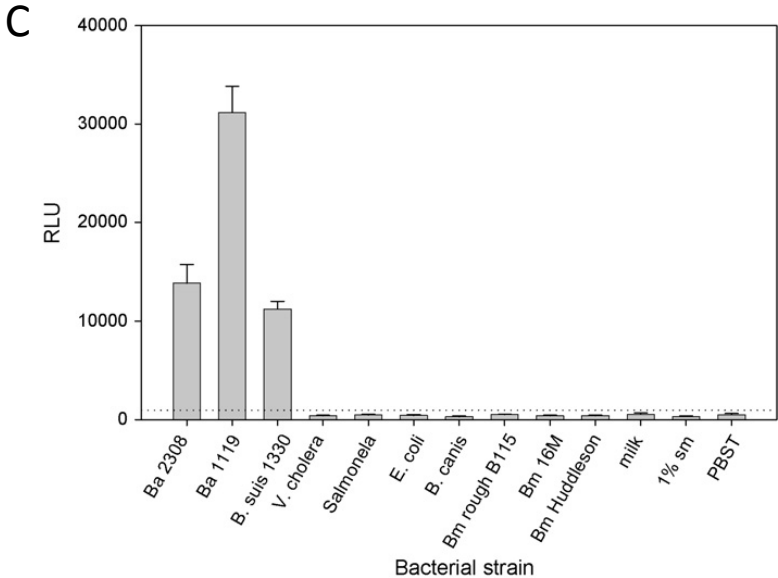
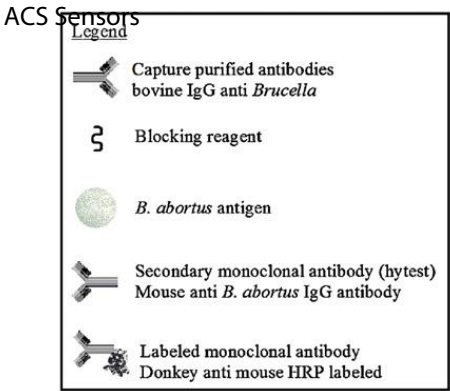
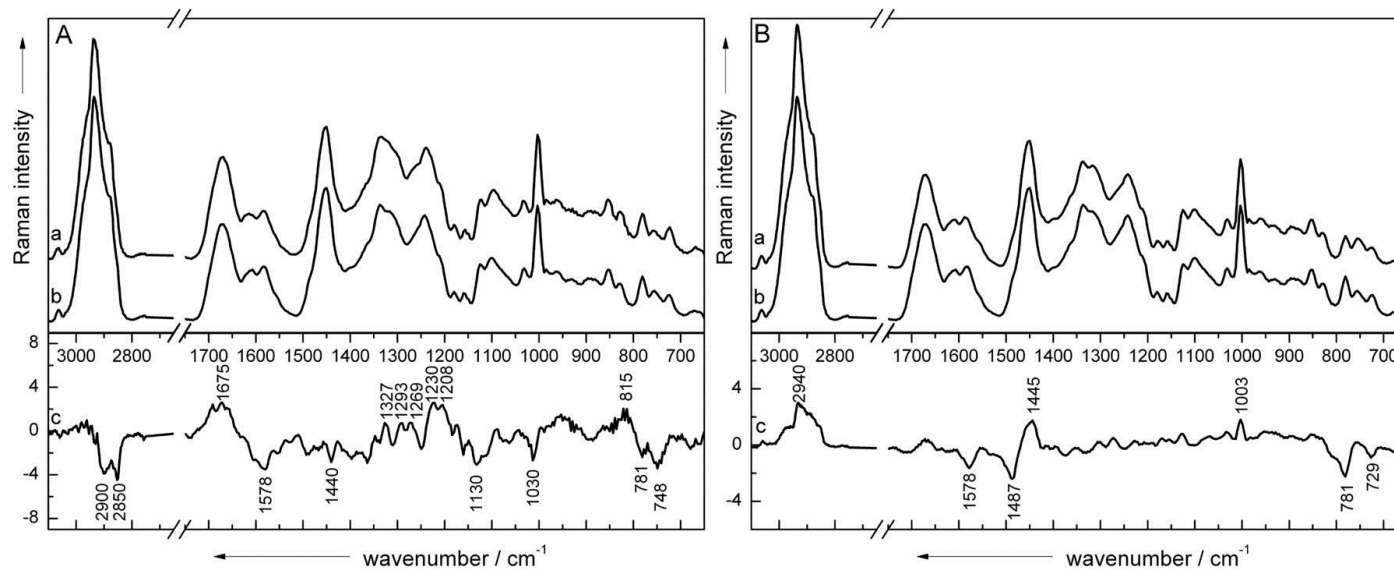


Figure 25







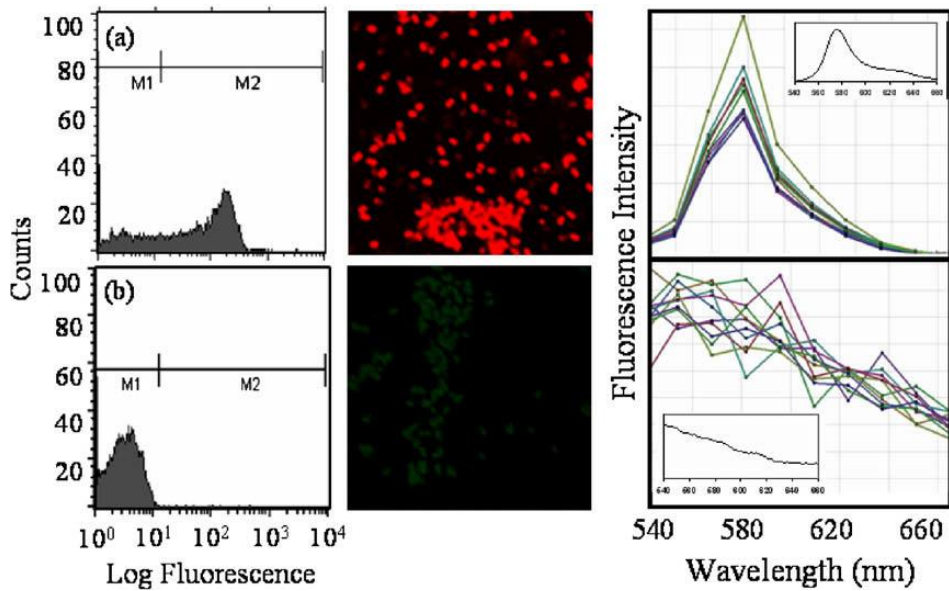
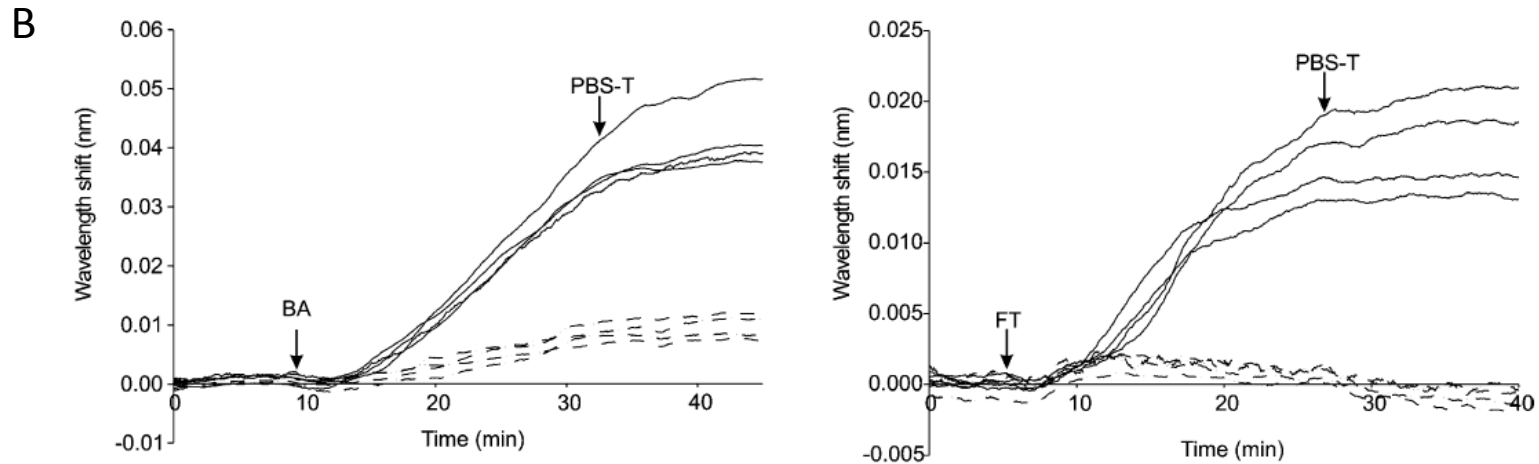
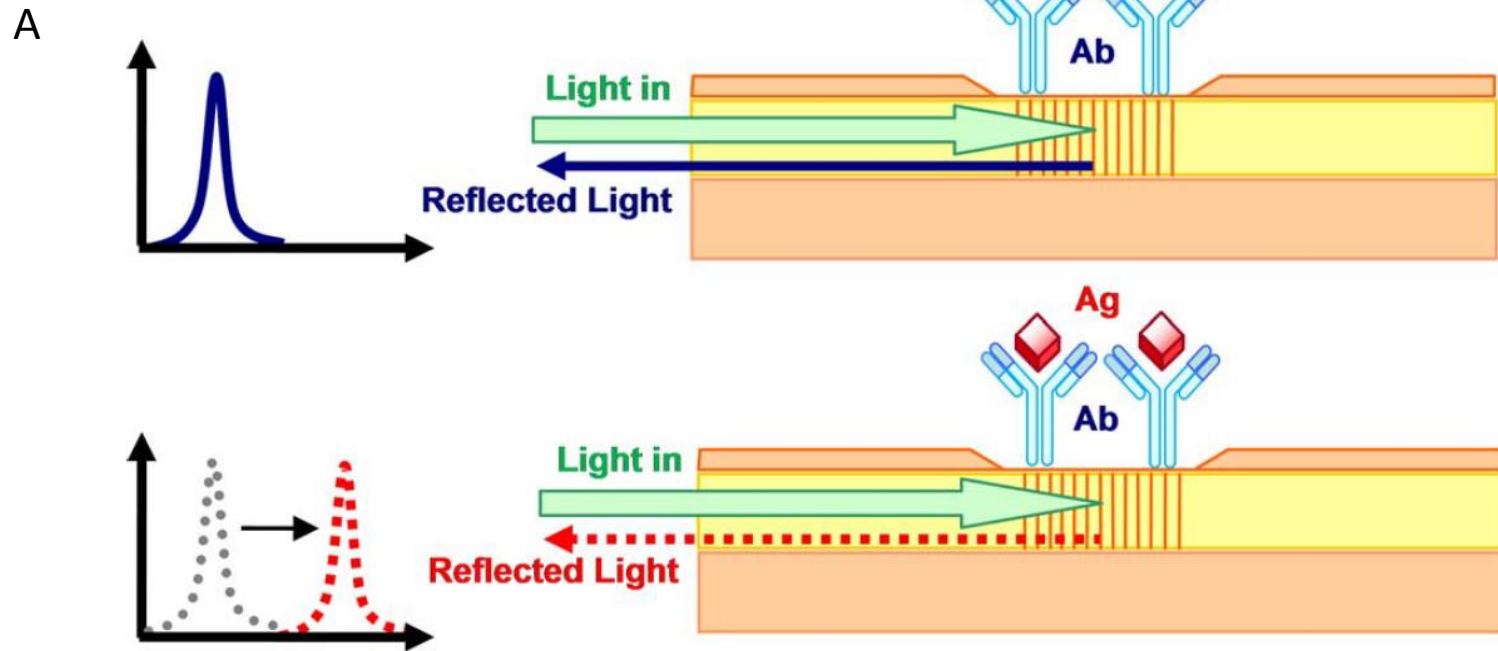


Figure 29



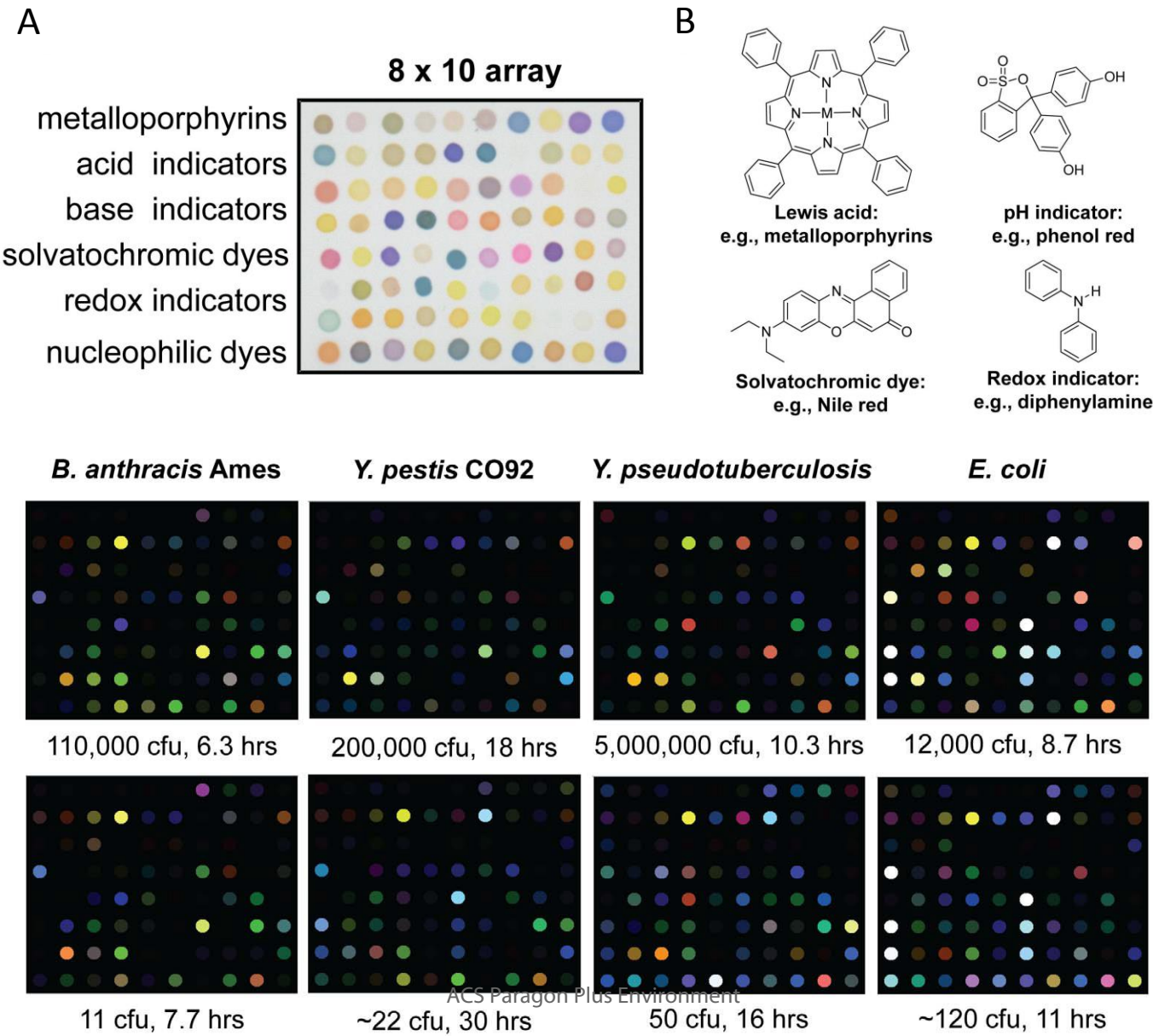


Figure 31

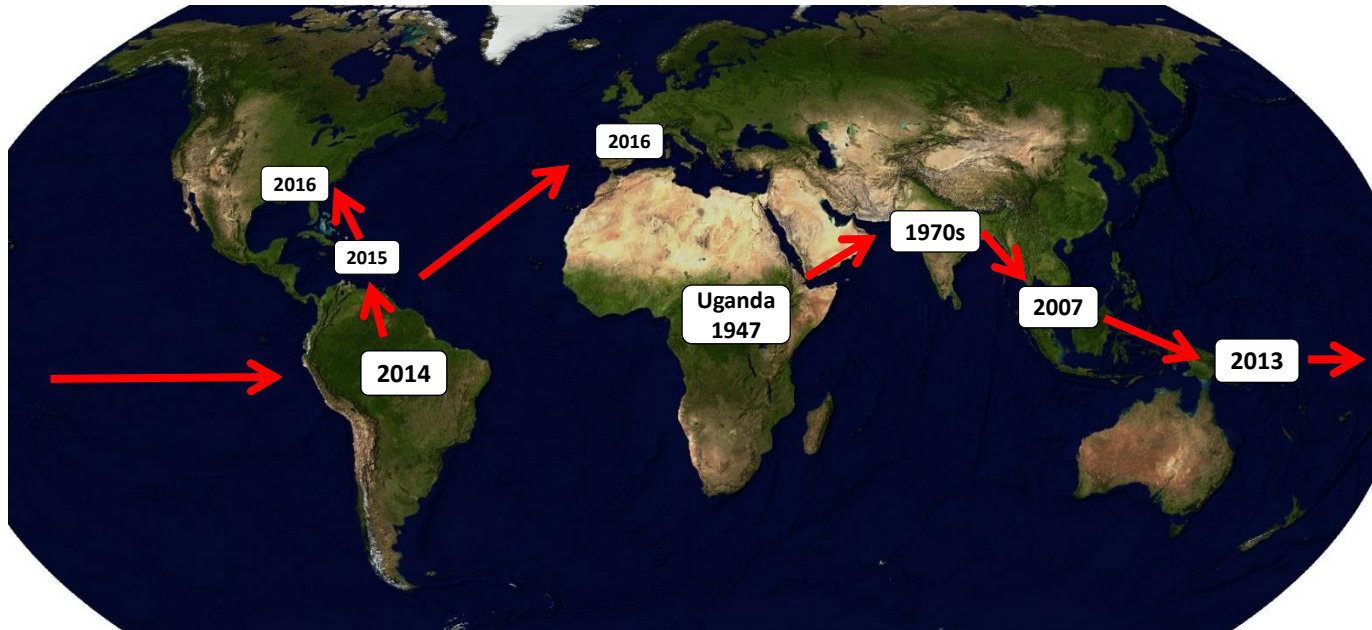
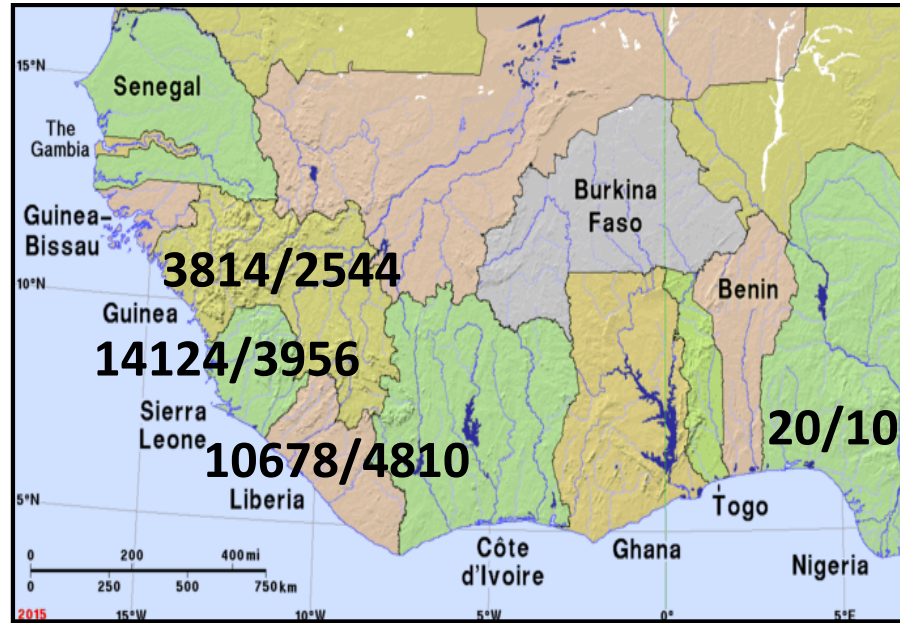




Figure 33



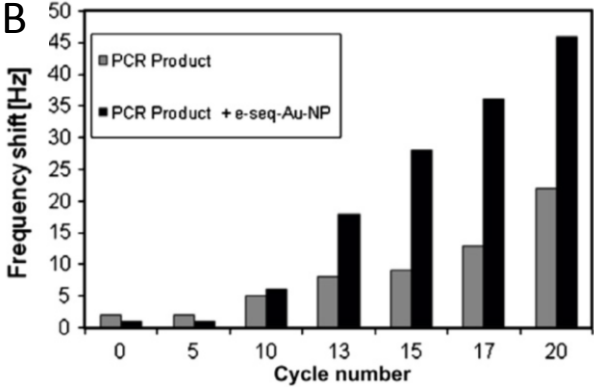
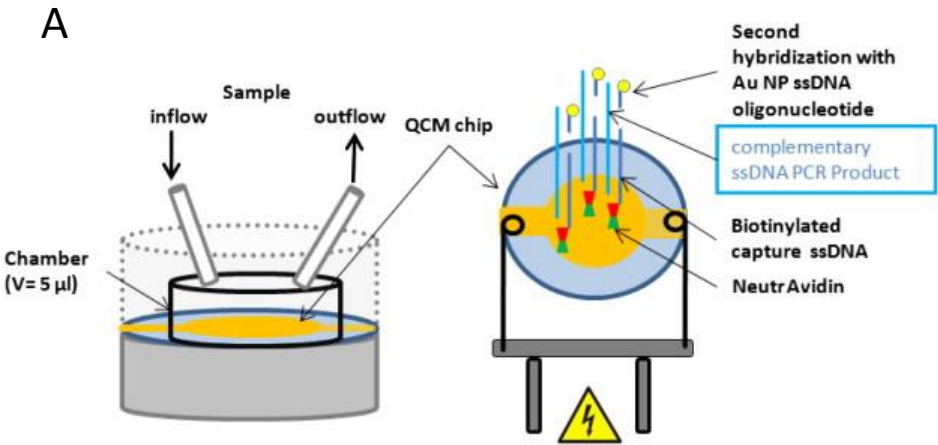
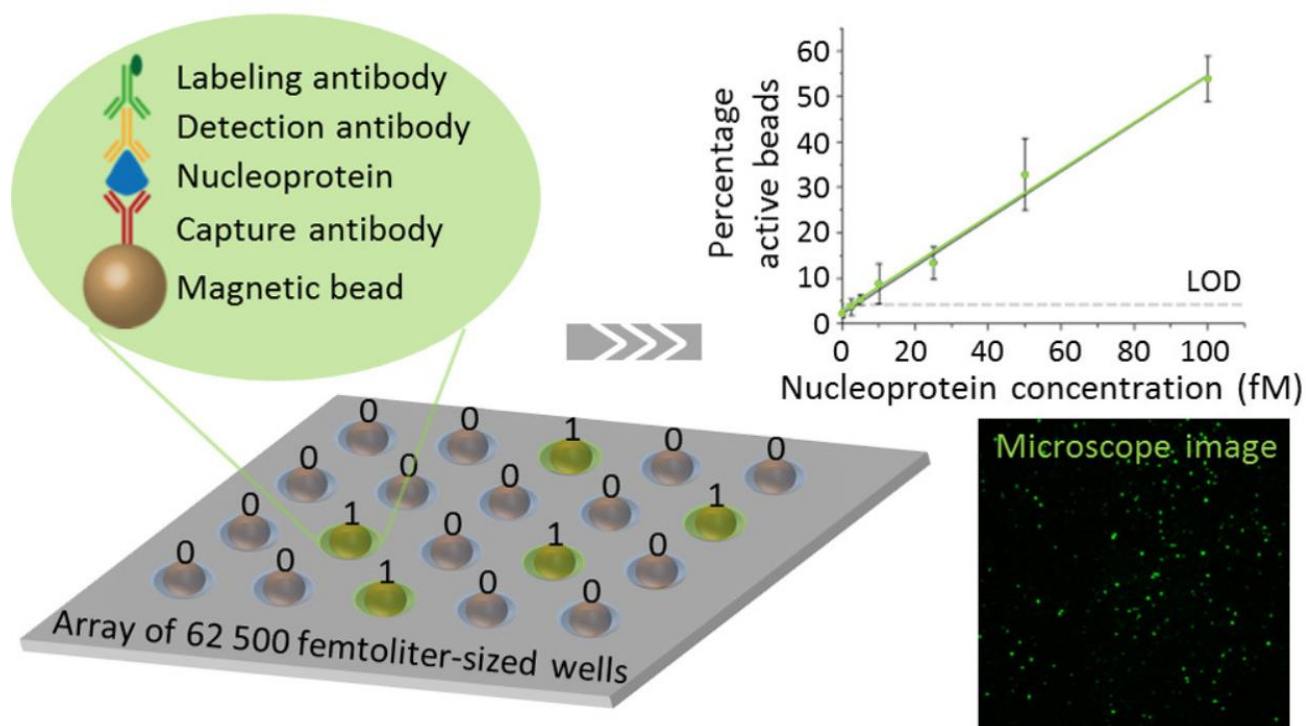


Figure 35



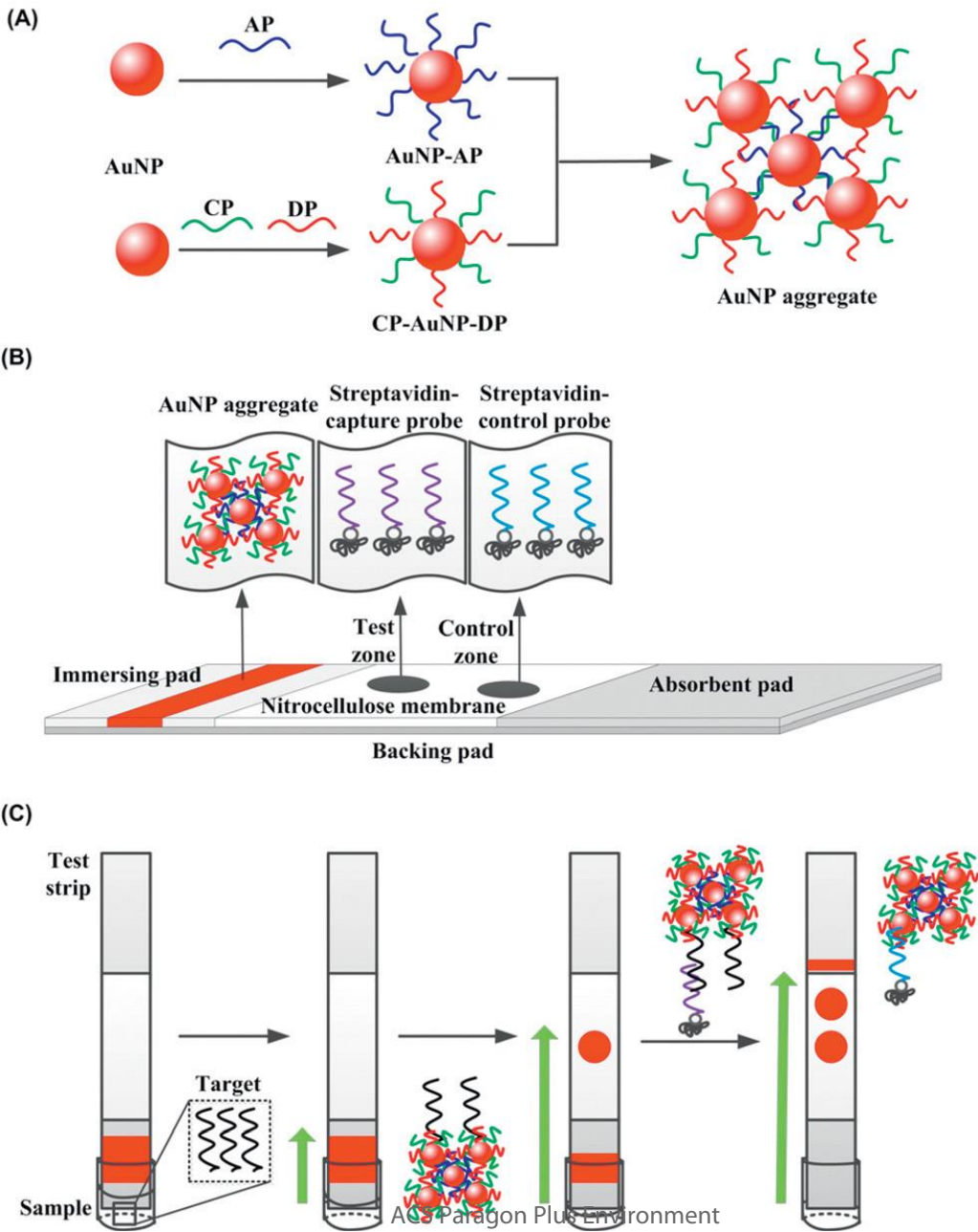
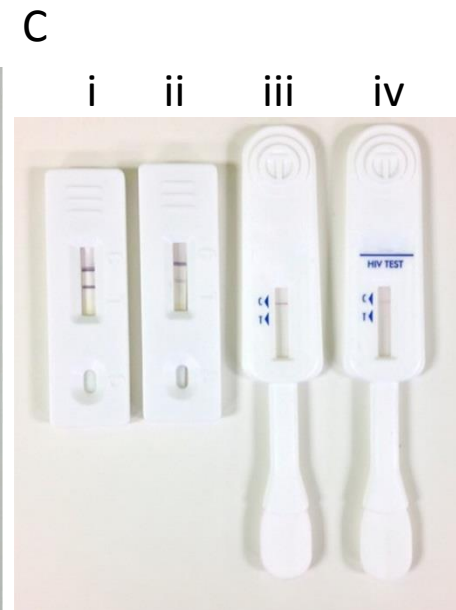
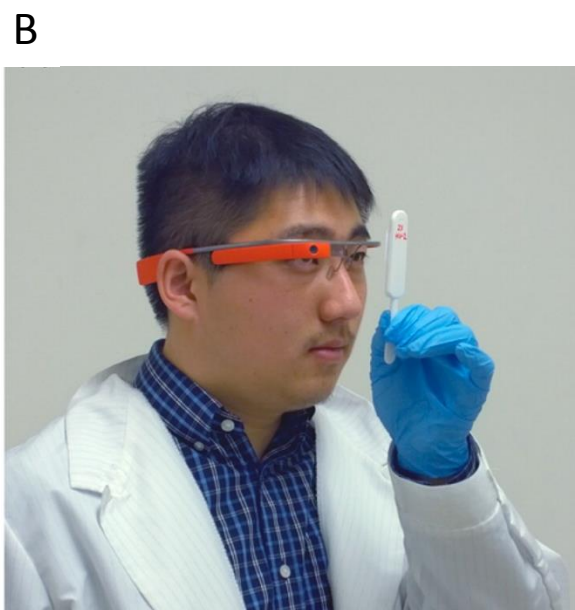
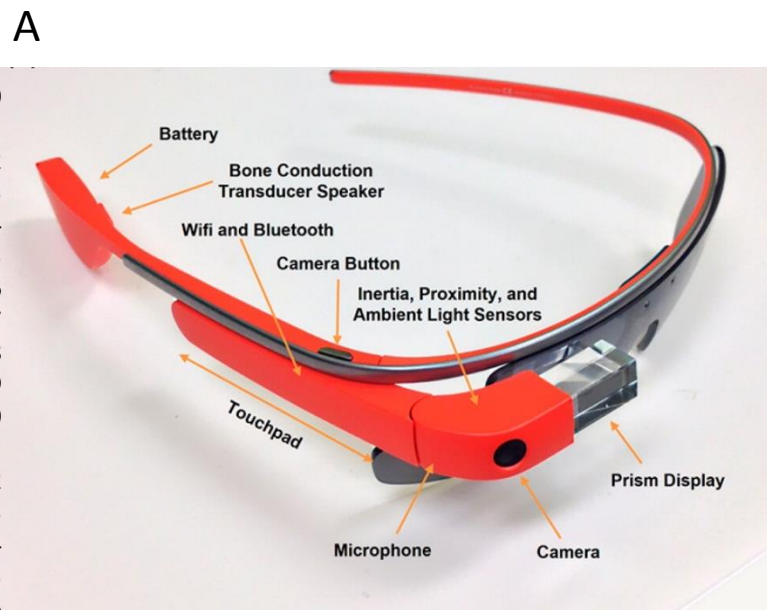


Figure 37



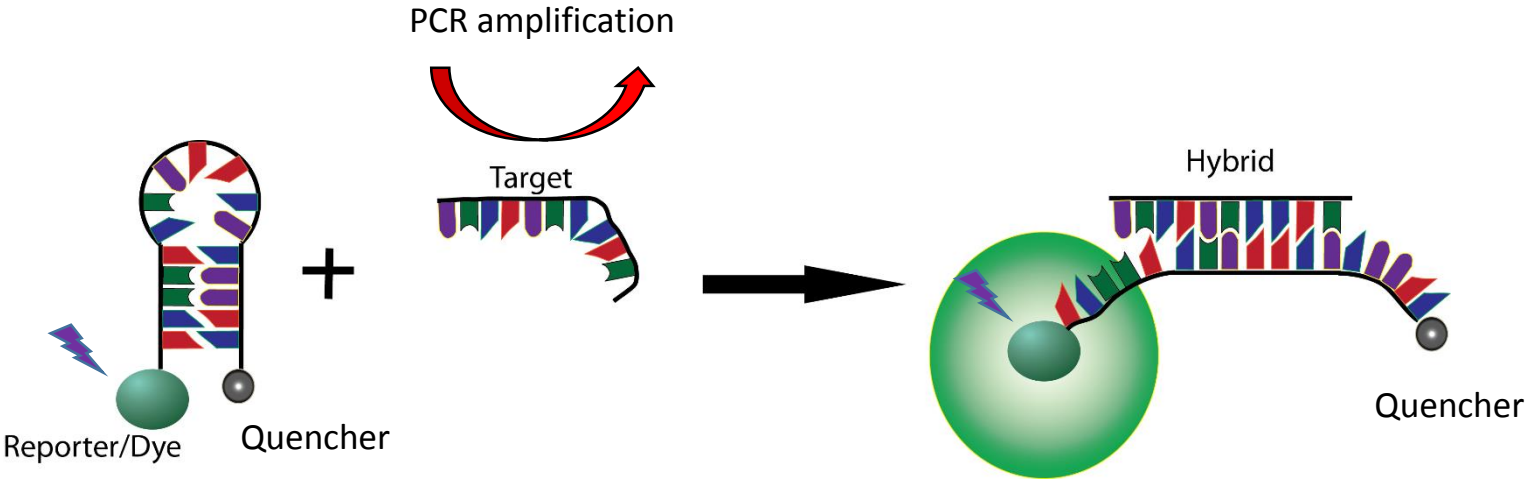
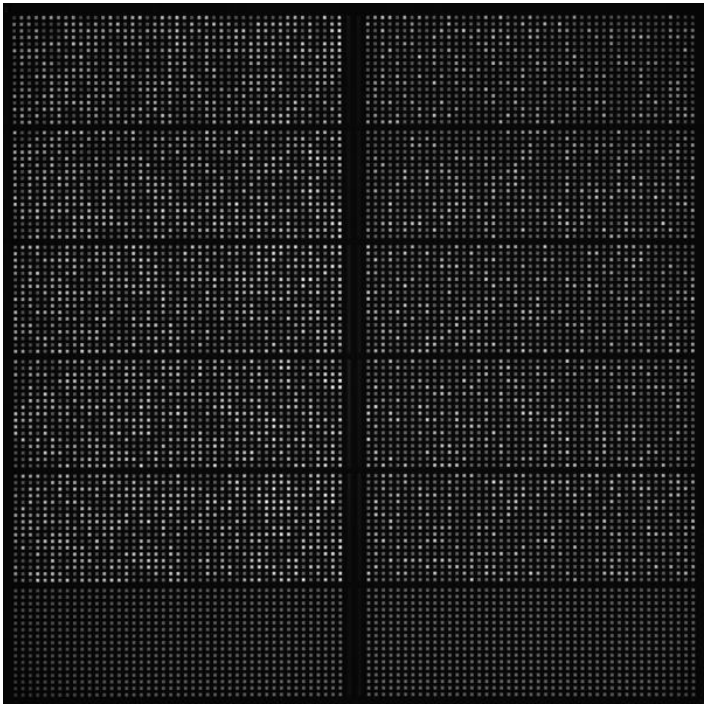


Figure 39

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A



B

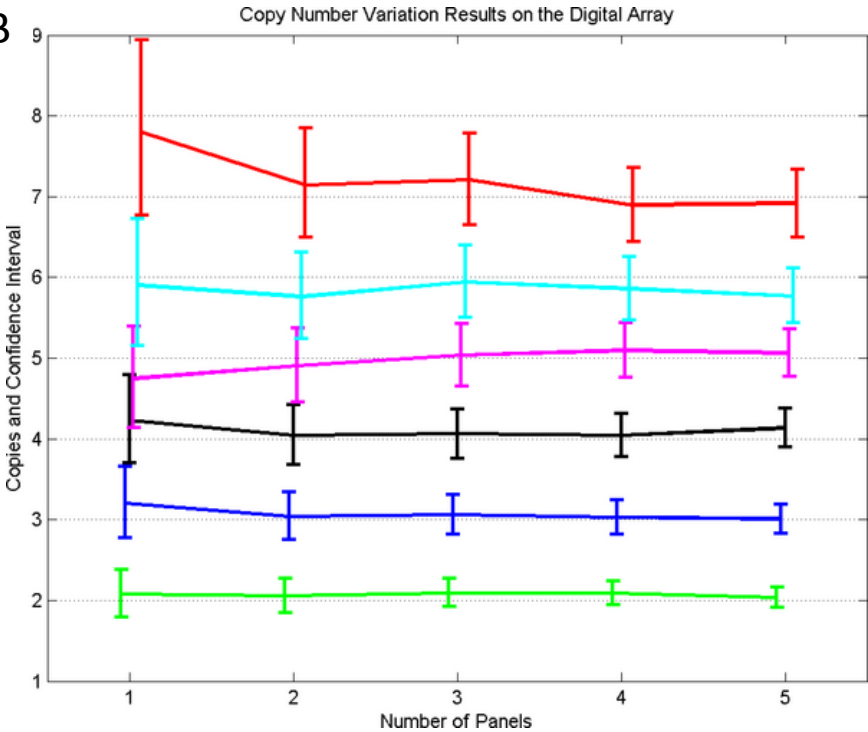


Figure 40

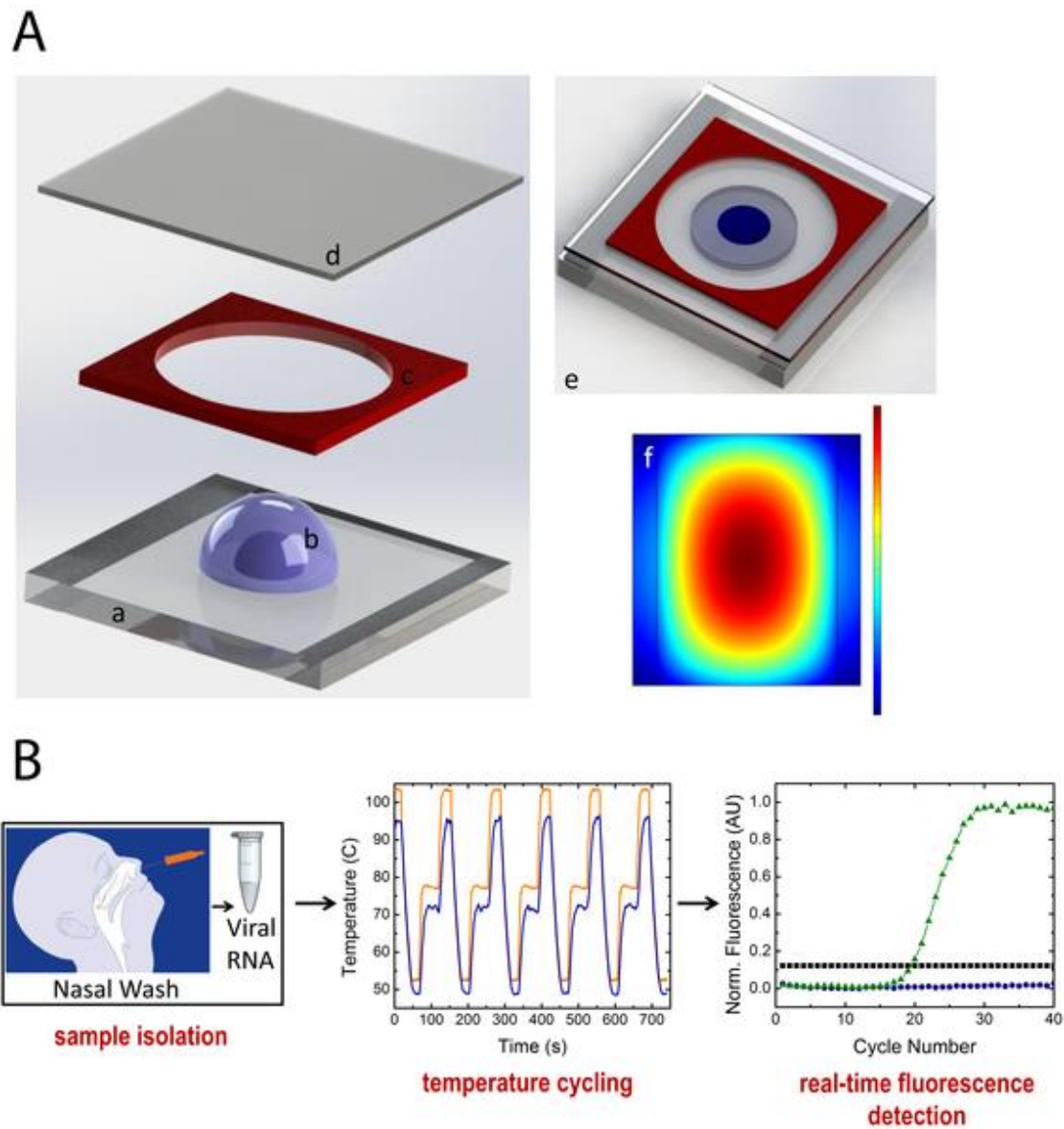
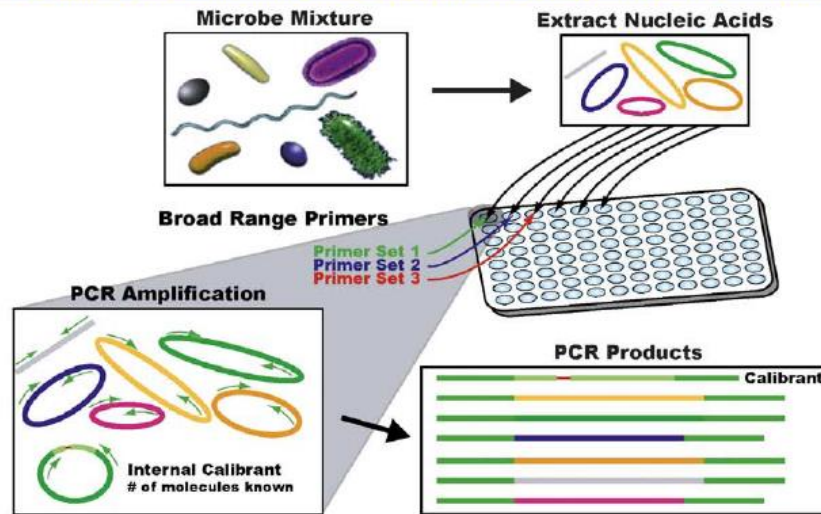
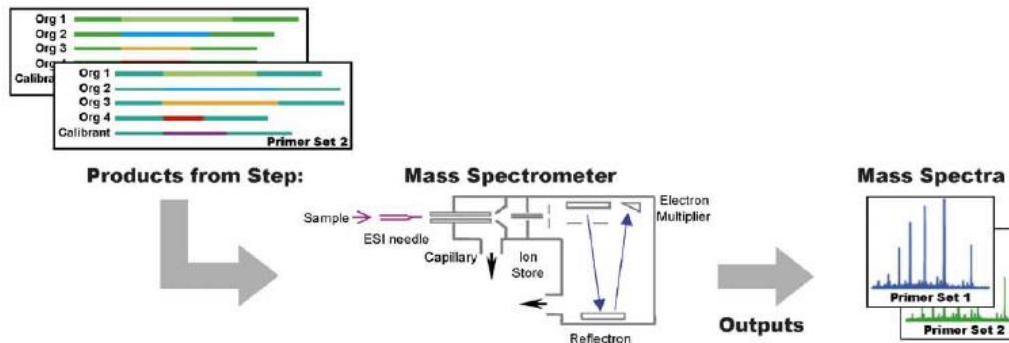


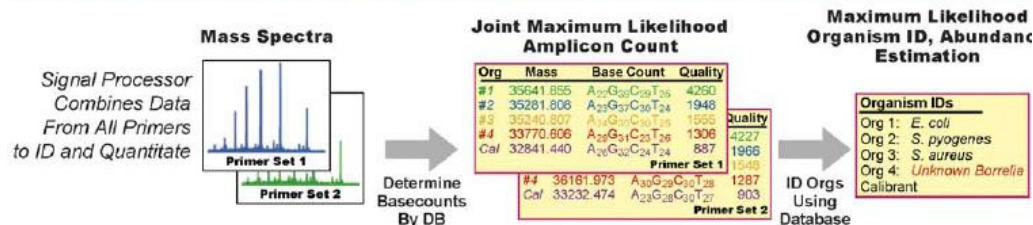
Figure 41



STEP #2 - Electrospray products into mass spectrometer



STEP #3 - Signal Processing - ID organism using Mass Spec data from multiple primers



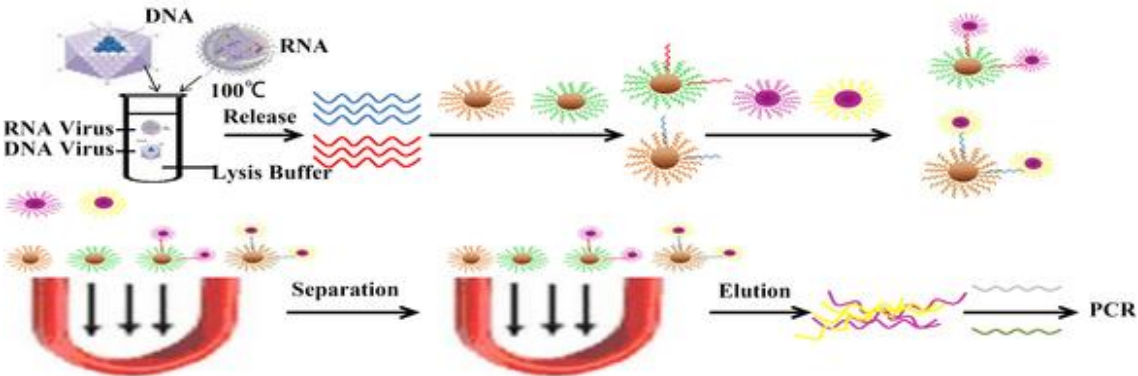
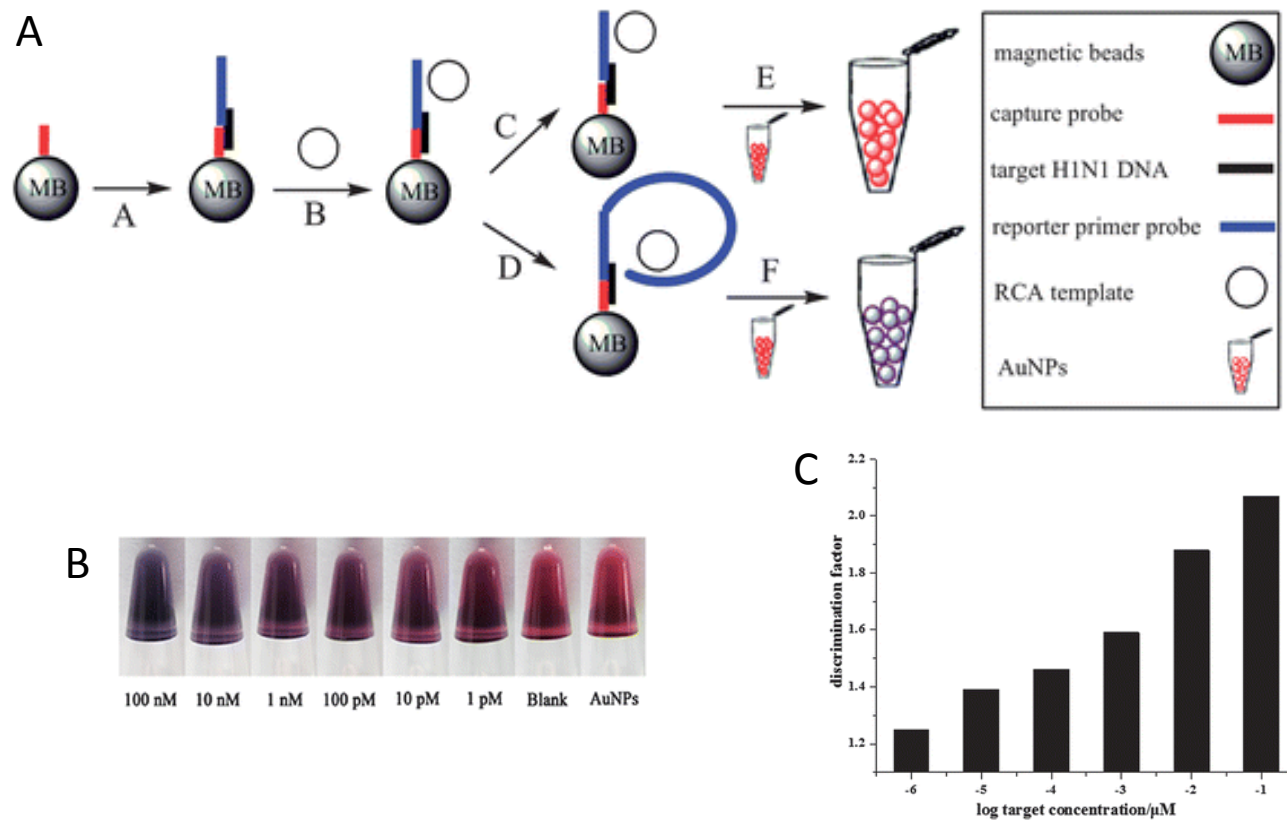


Figure 43



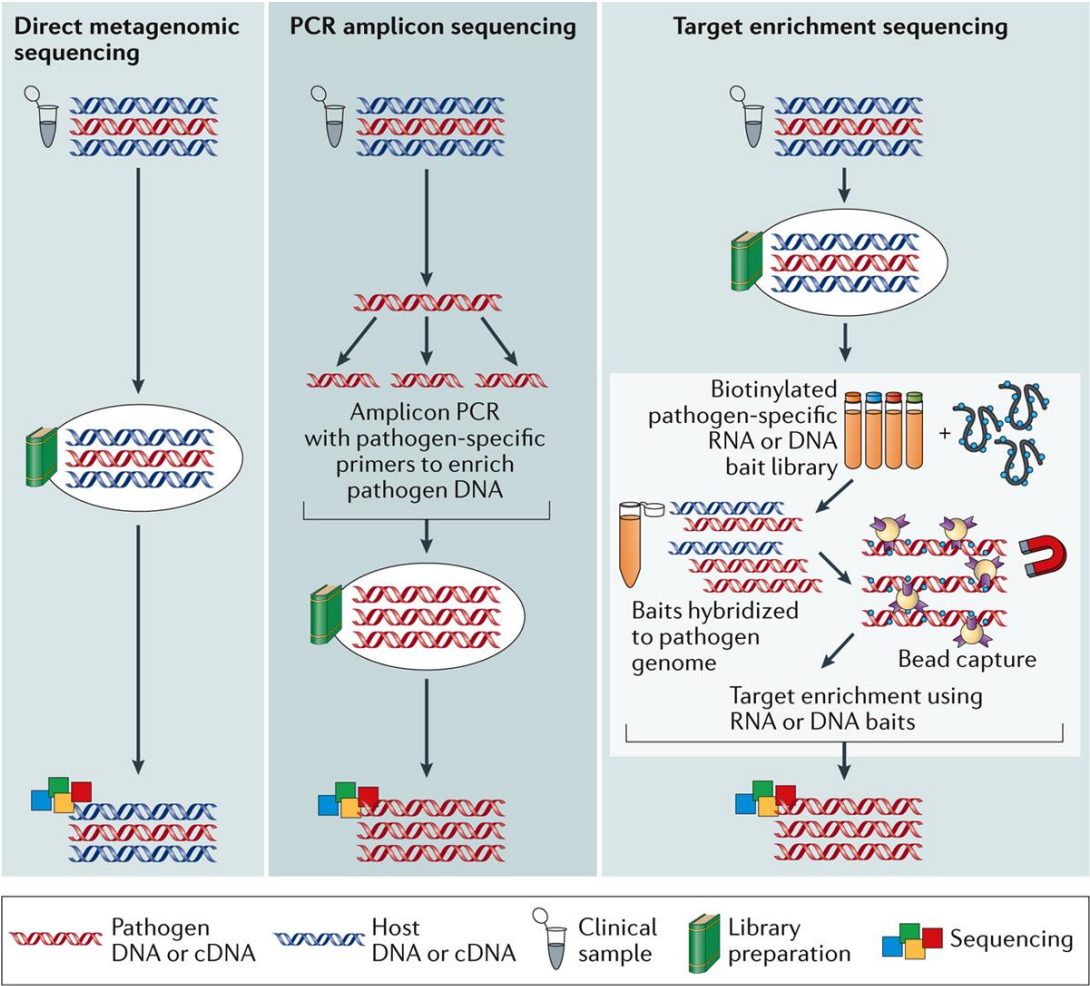


Figure 45

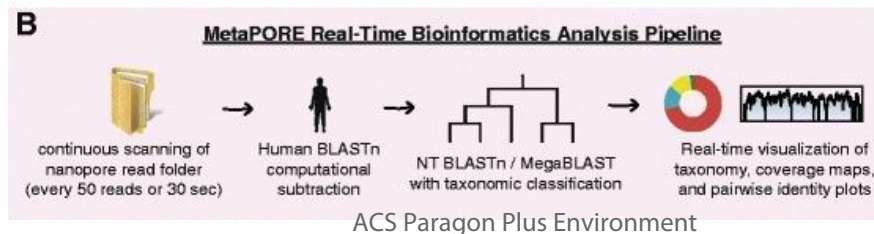
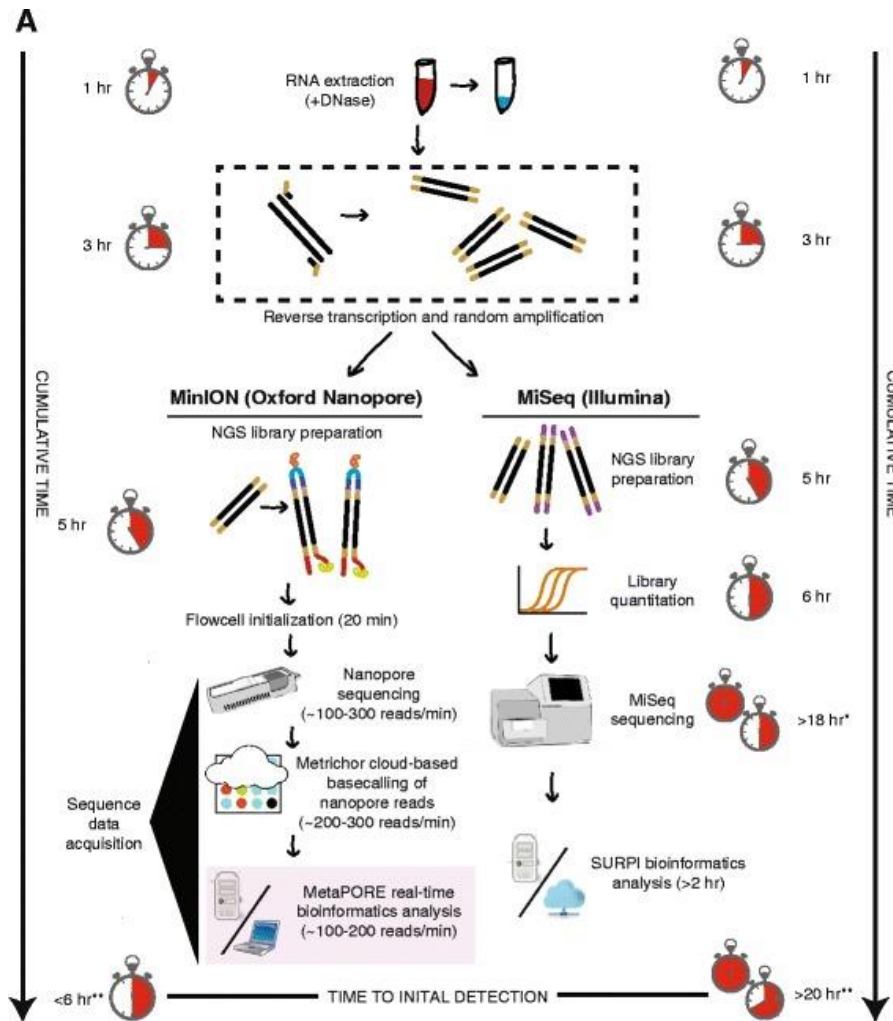


Figure 46

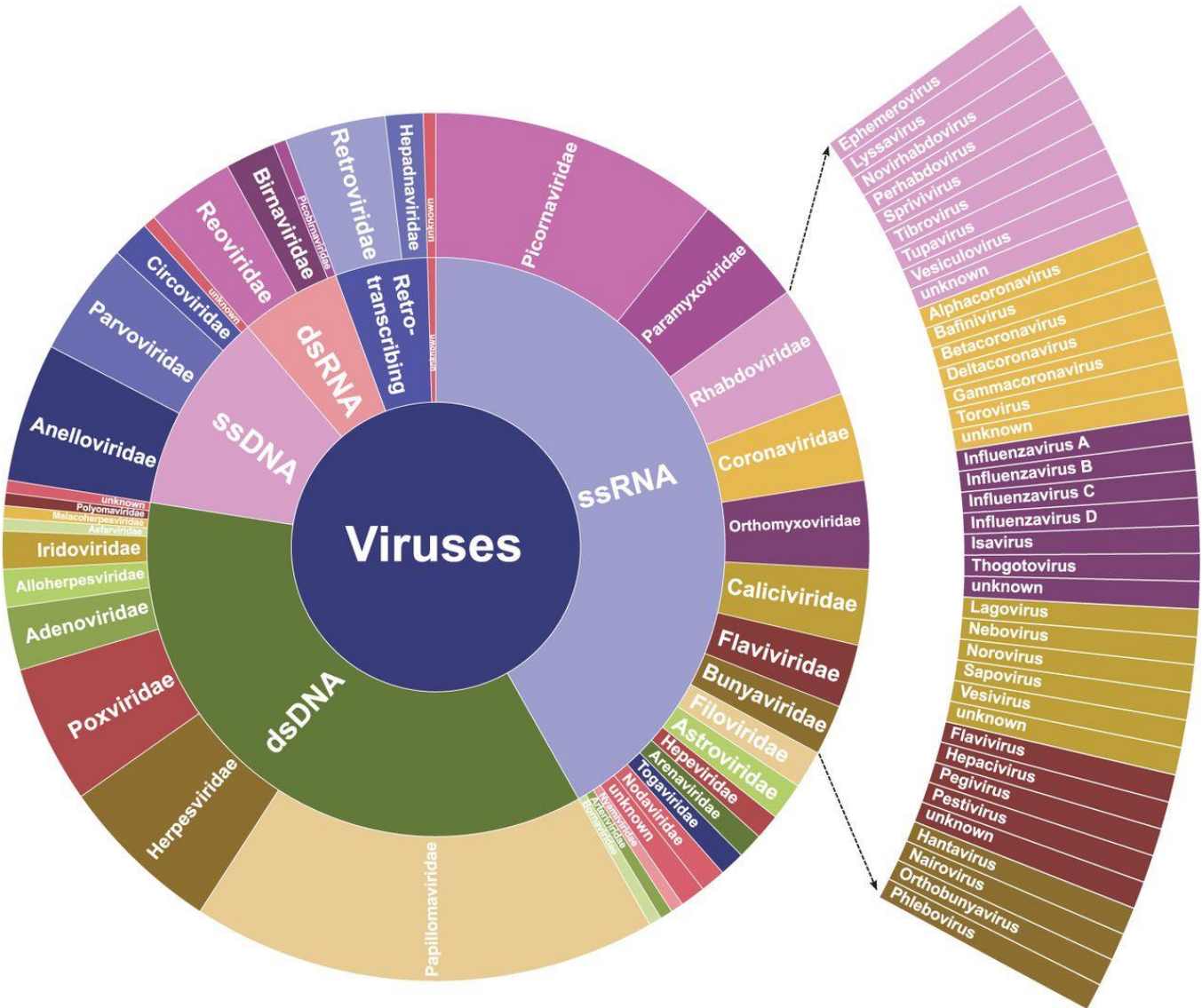
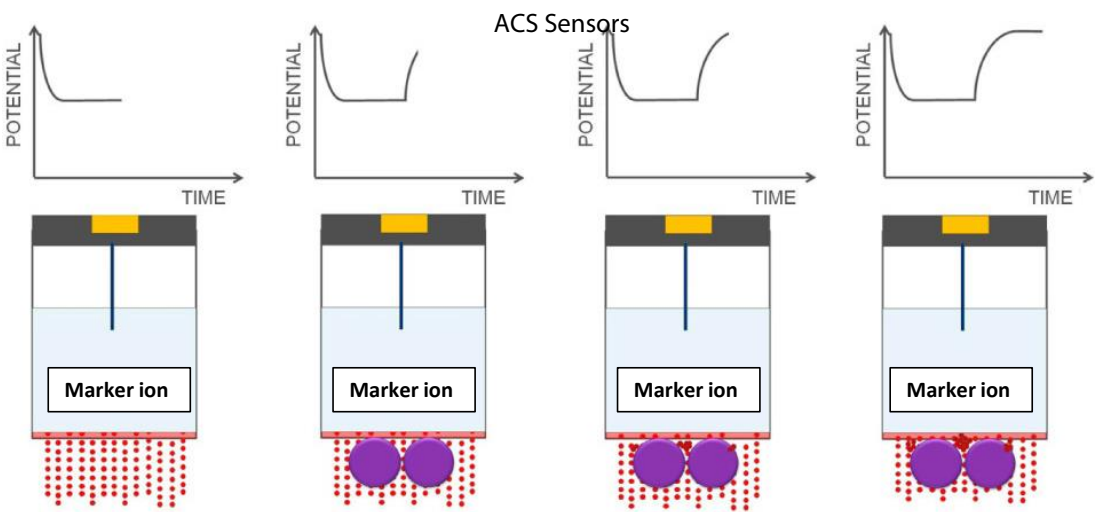
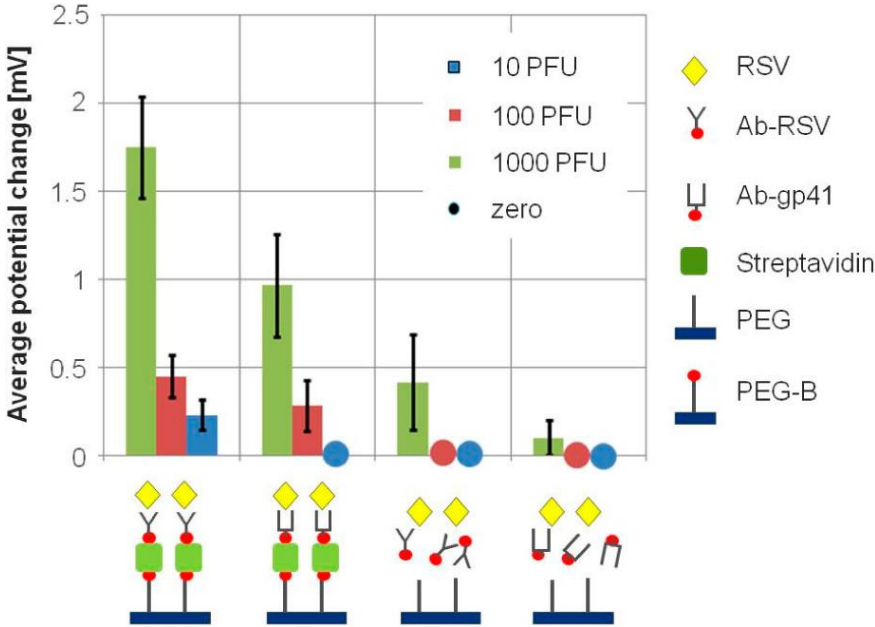


Figure 47

A



B



ACS Paragon Plus Environment

Figure 48

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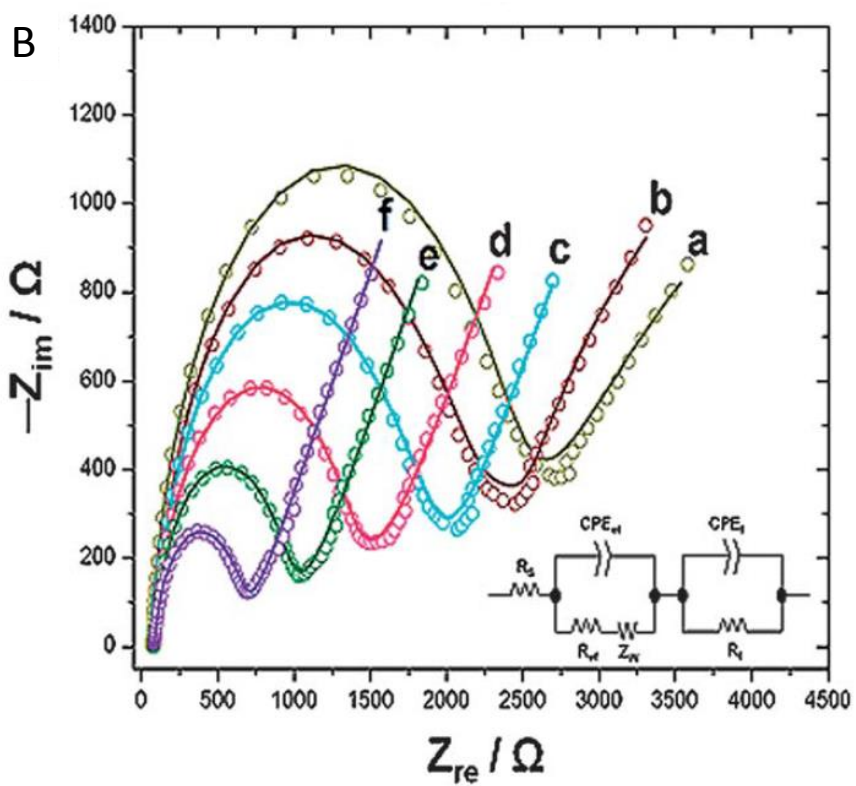
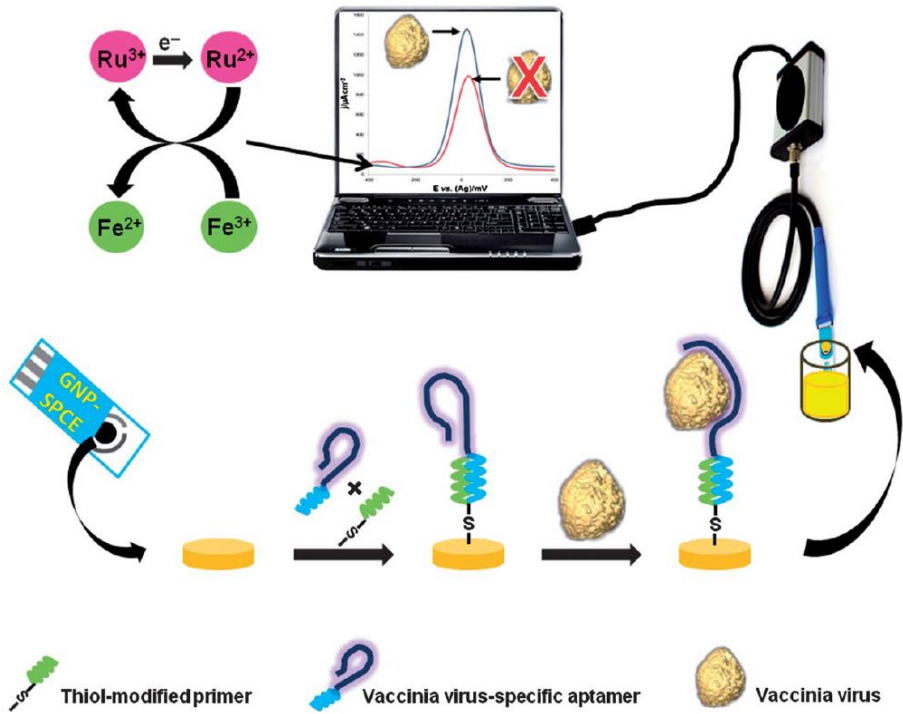
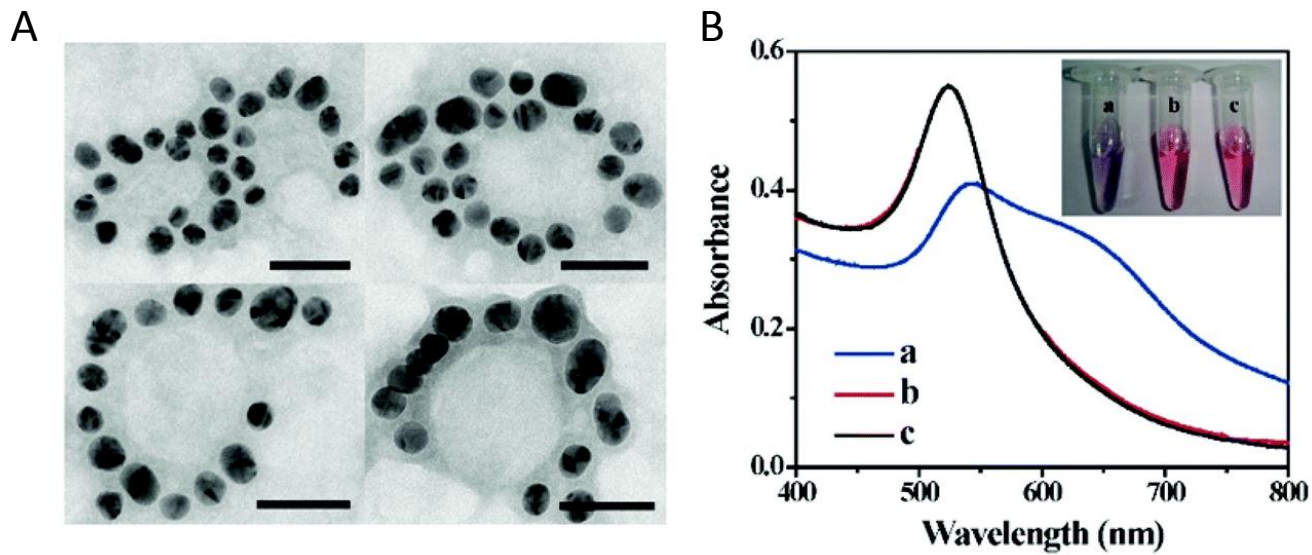


Figure 49



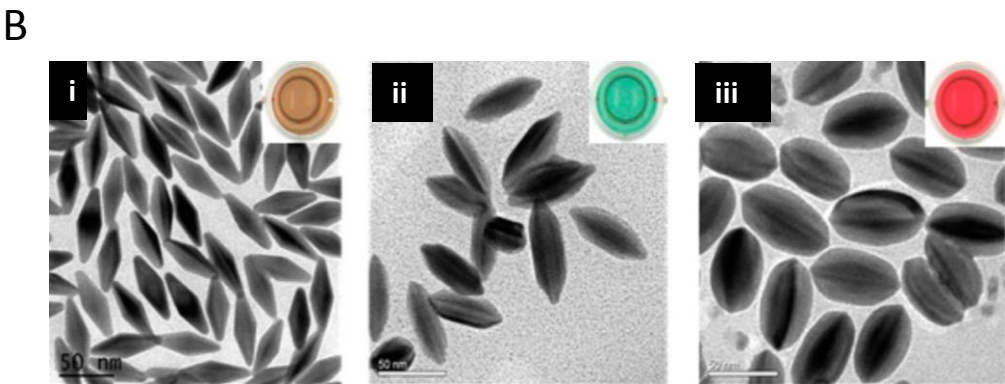
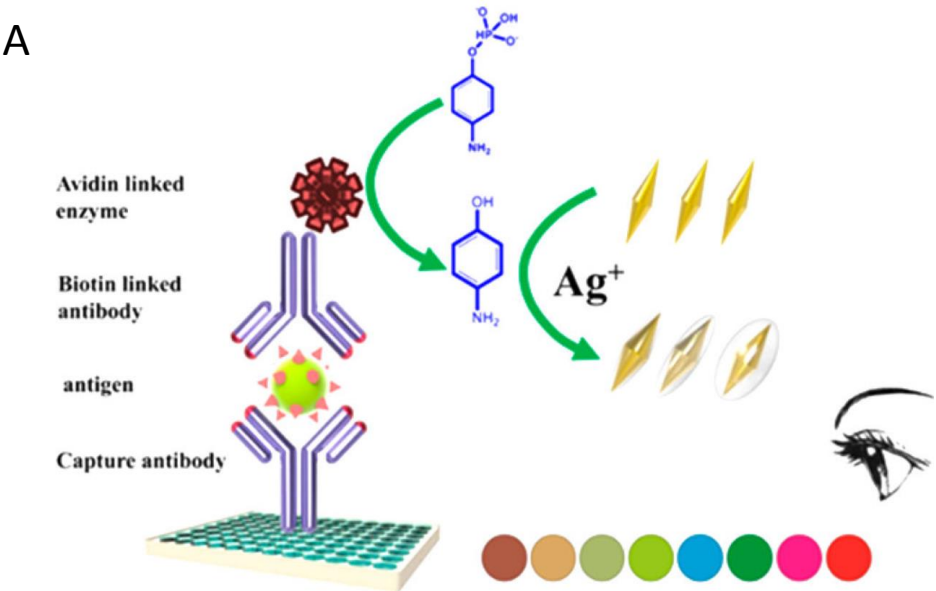


Figure 51

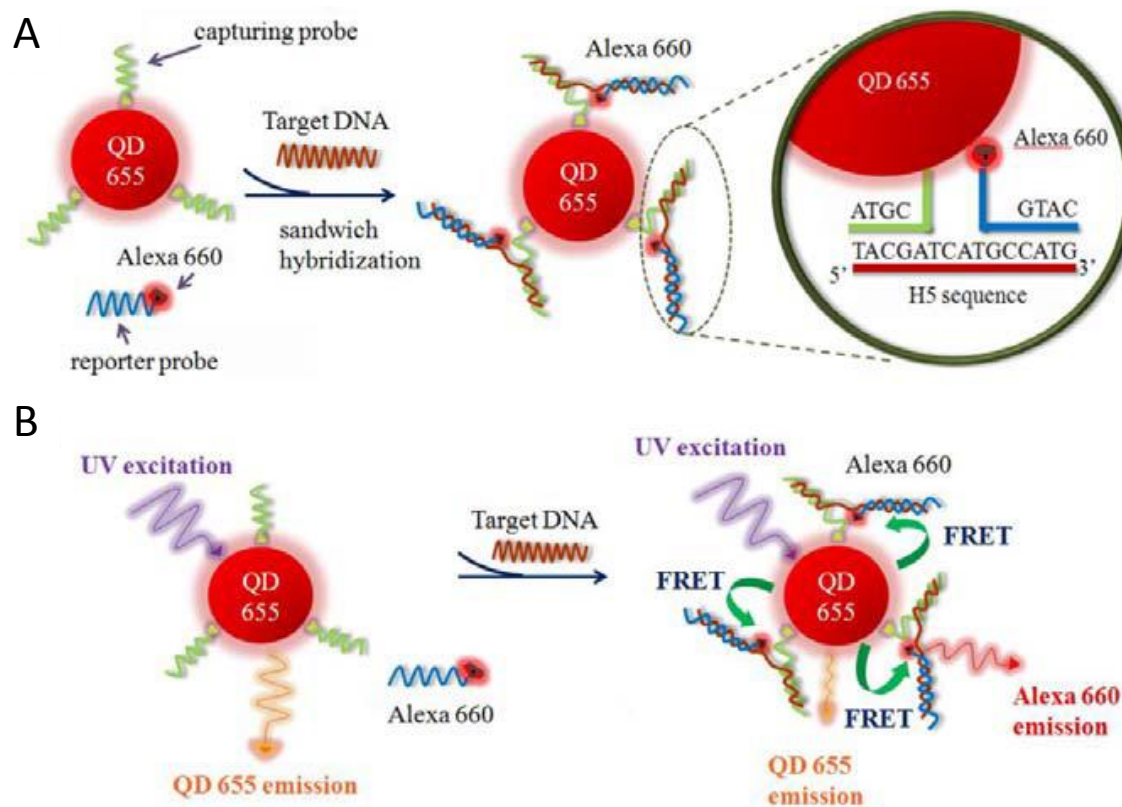
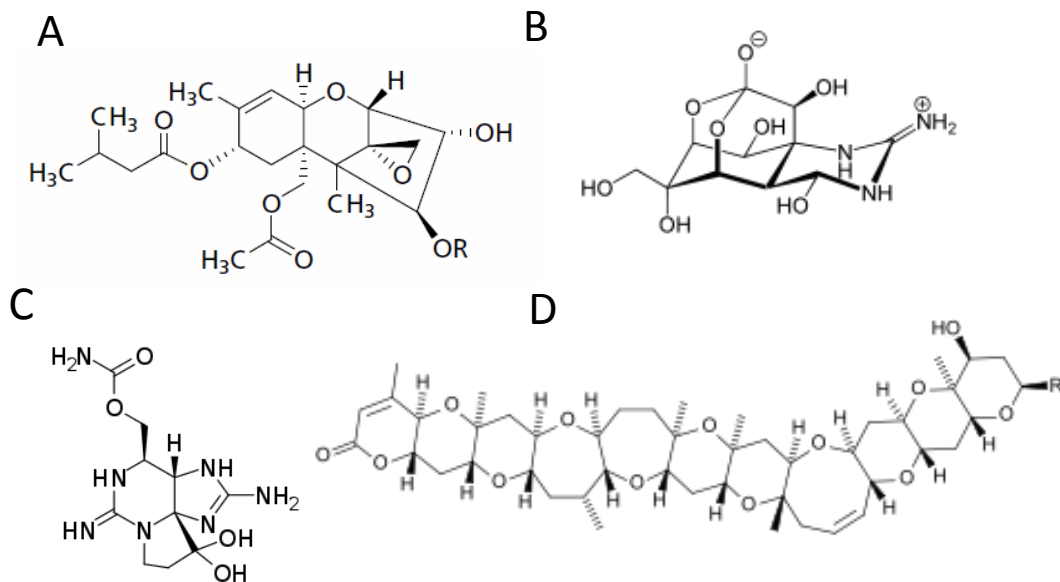




Figure 53



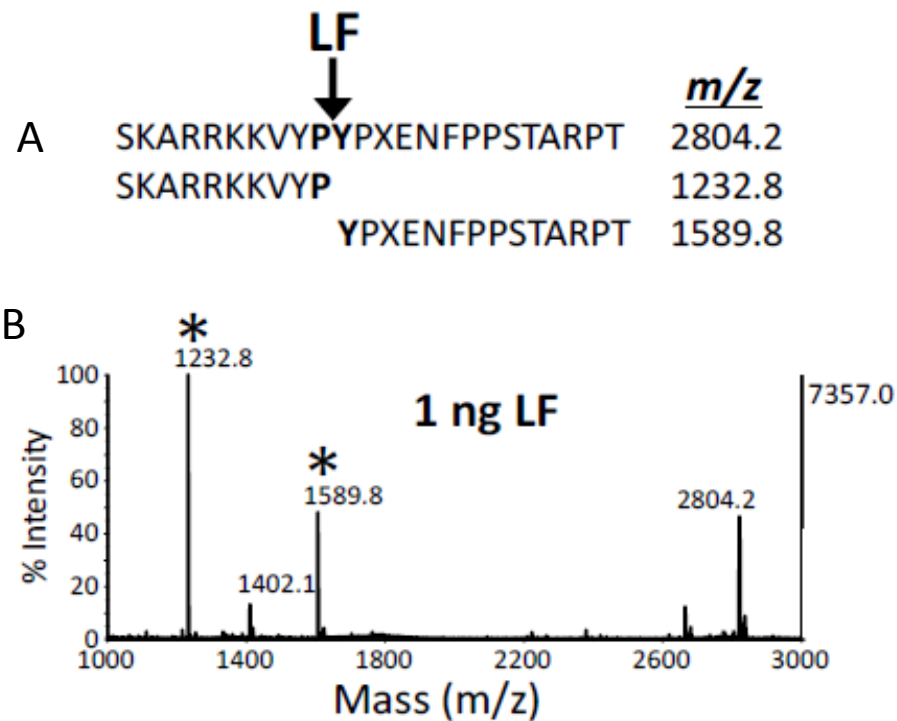
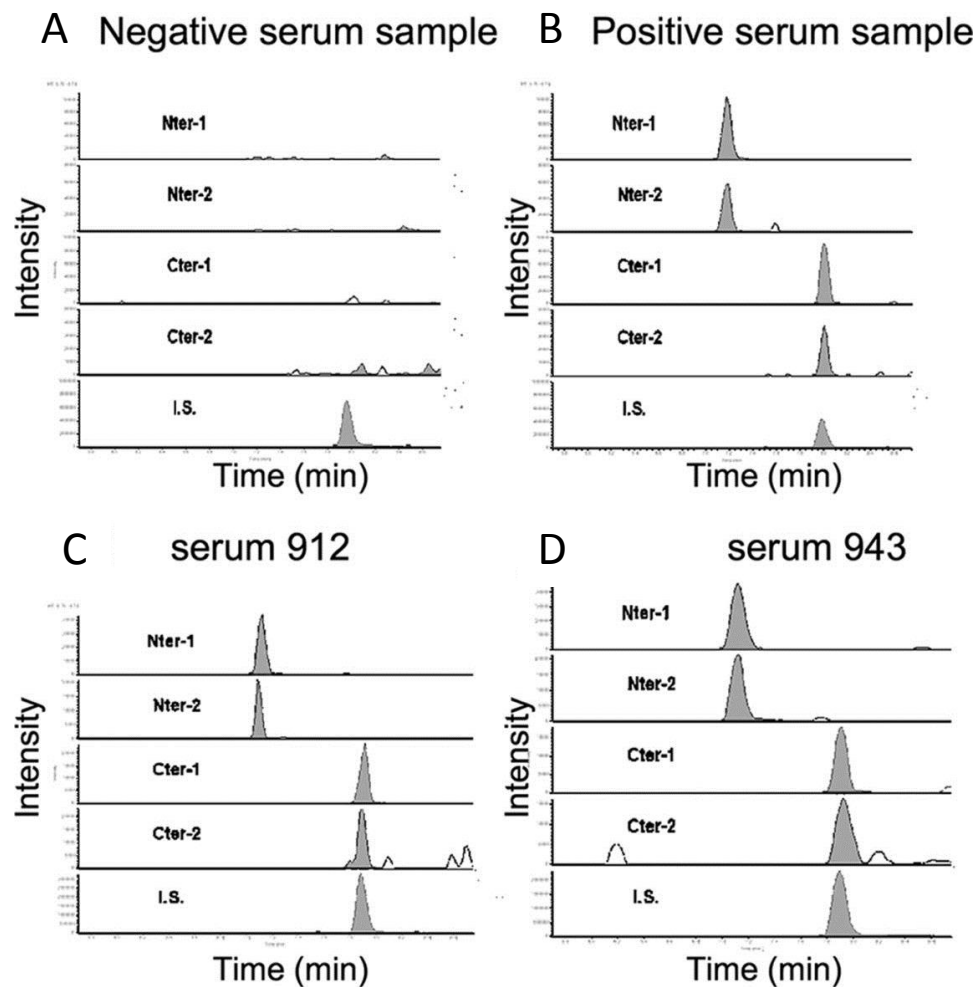


Figure 55



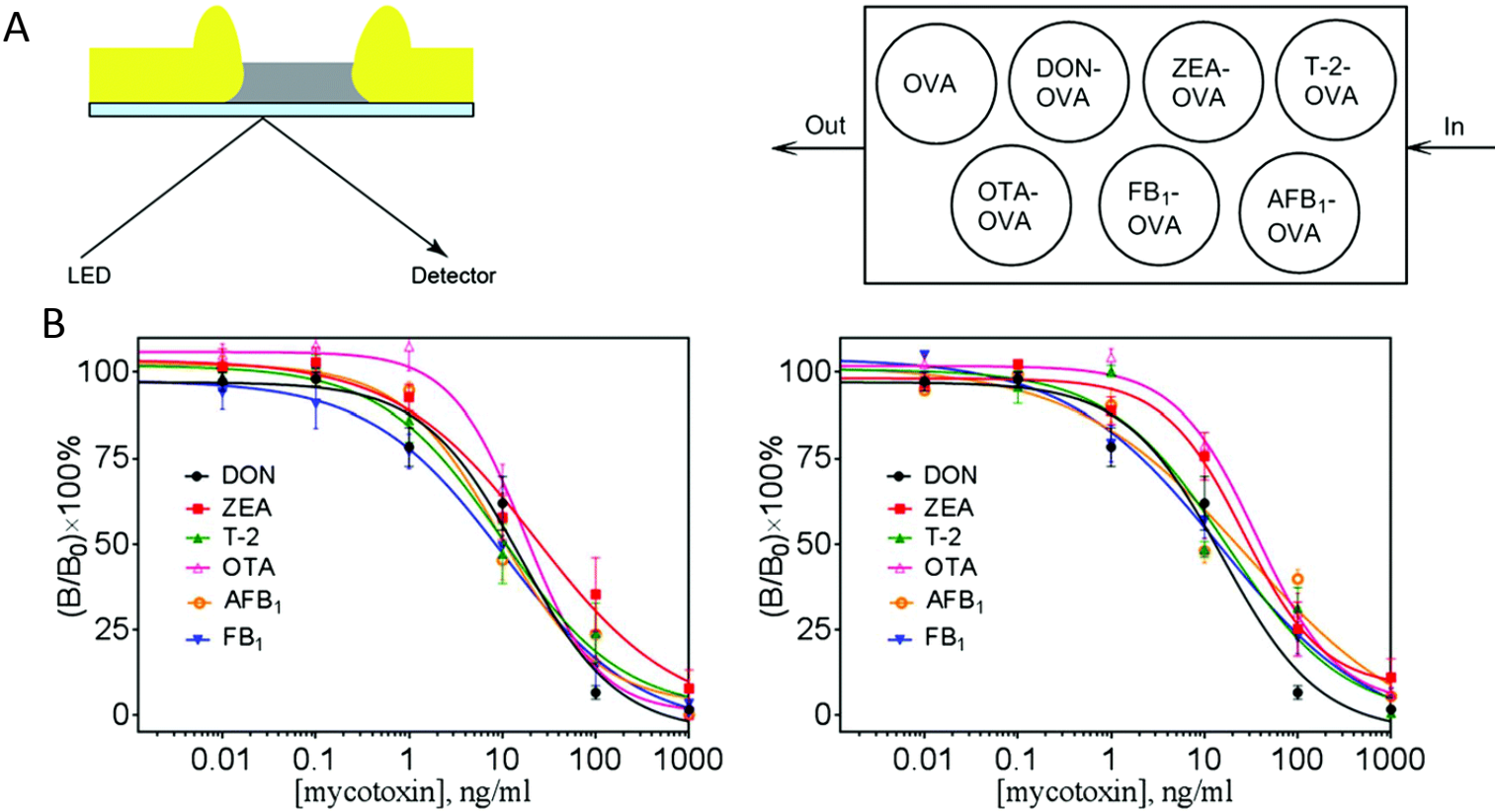
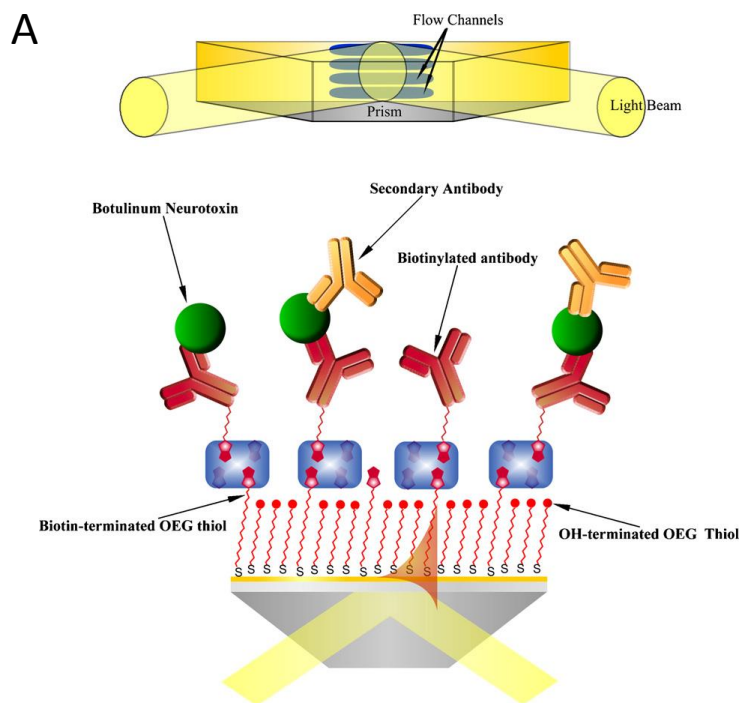


Figure 57



B



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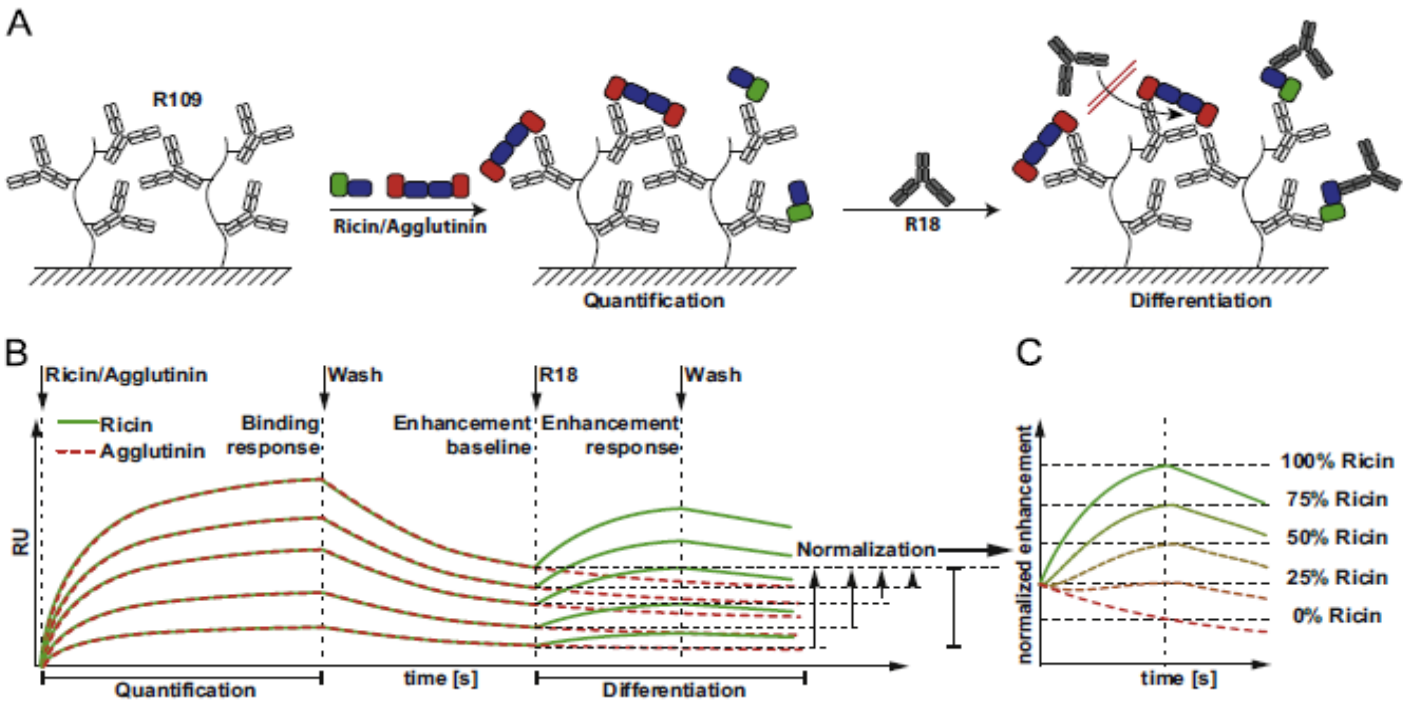
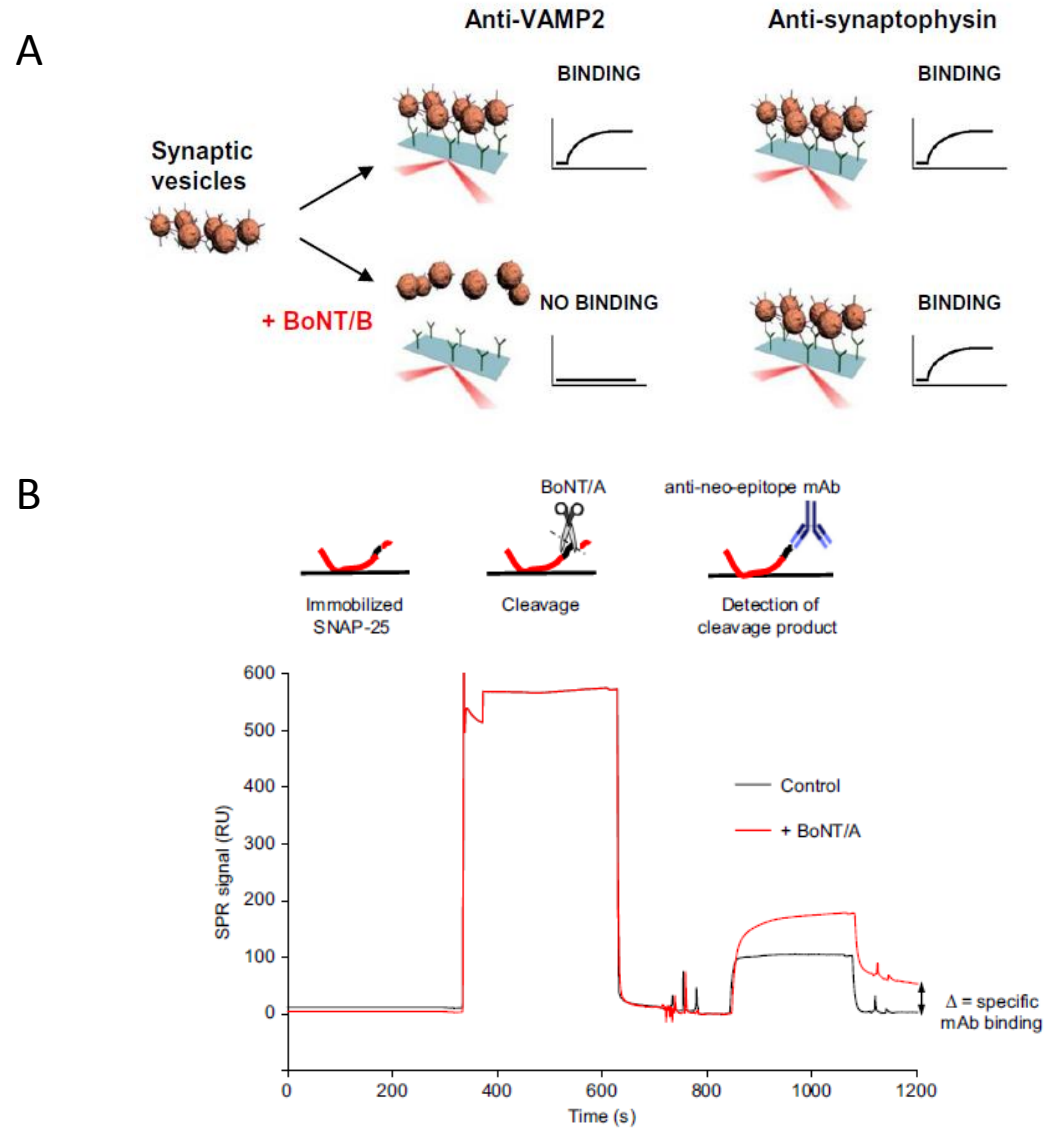


Figure 59



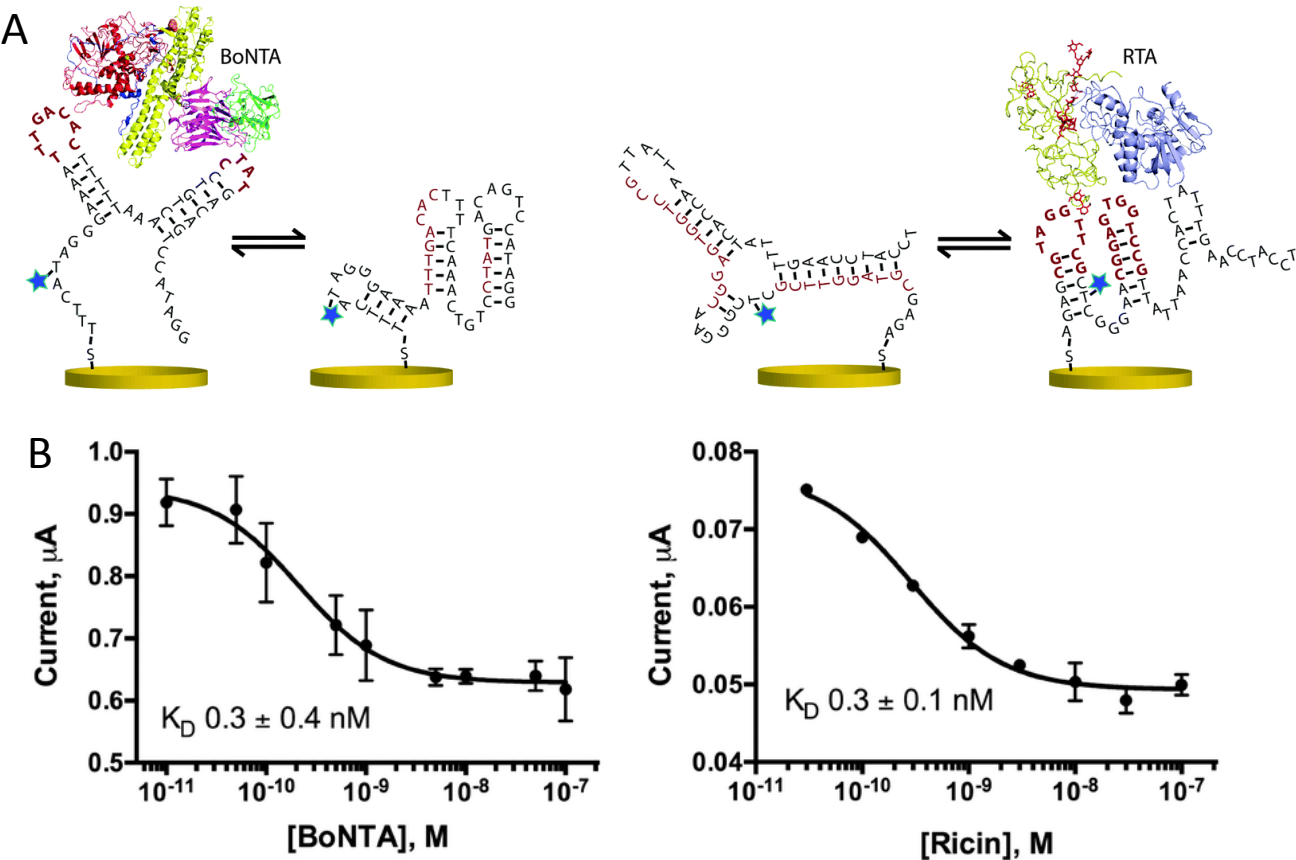
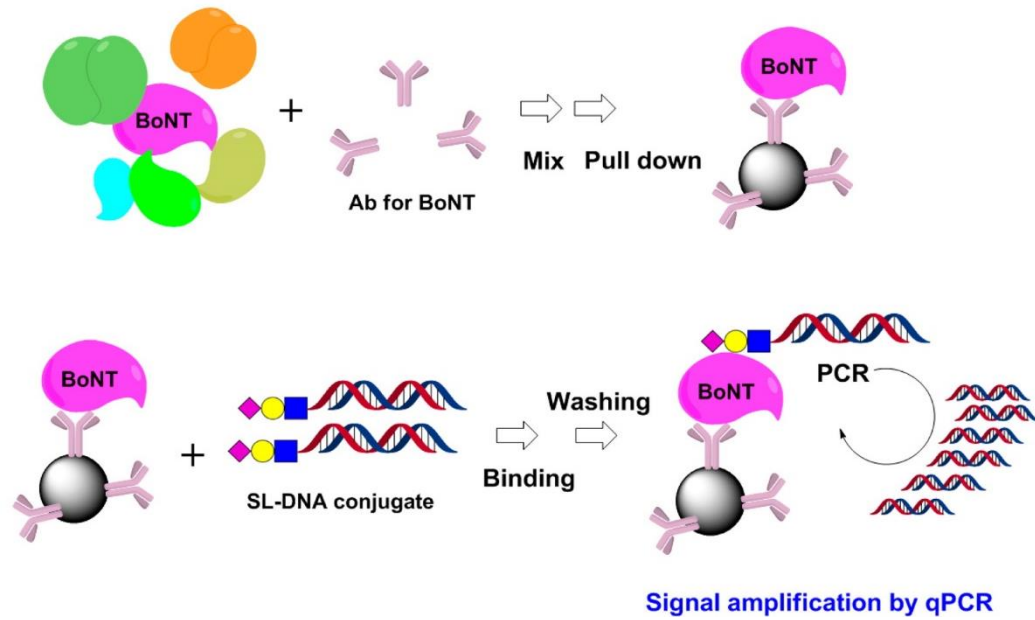


Figure 61



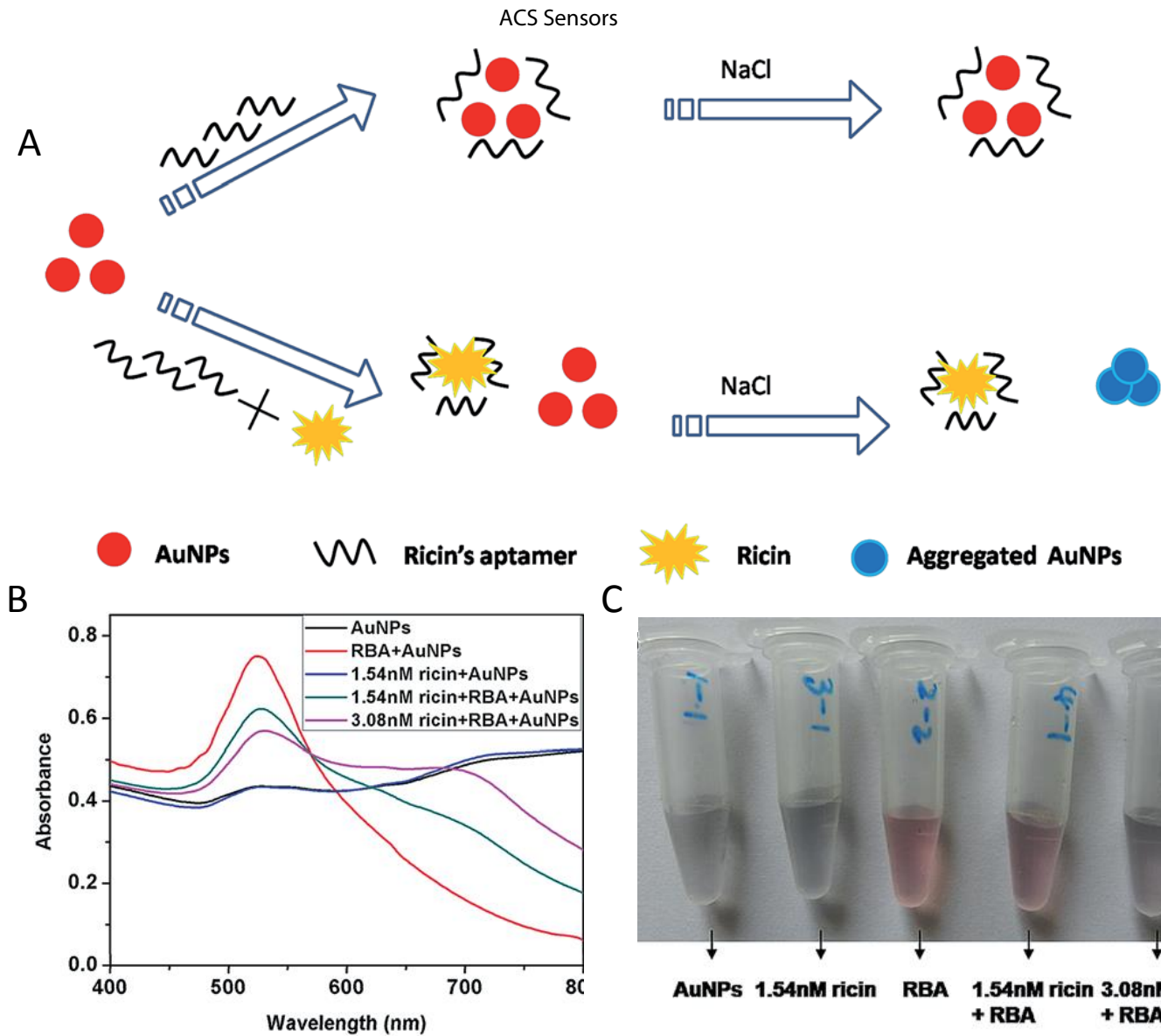
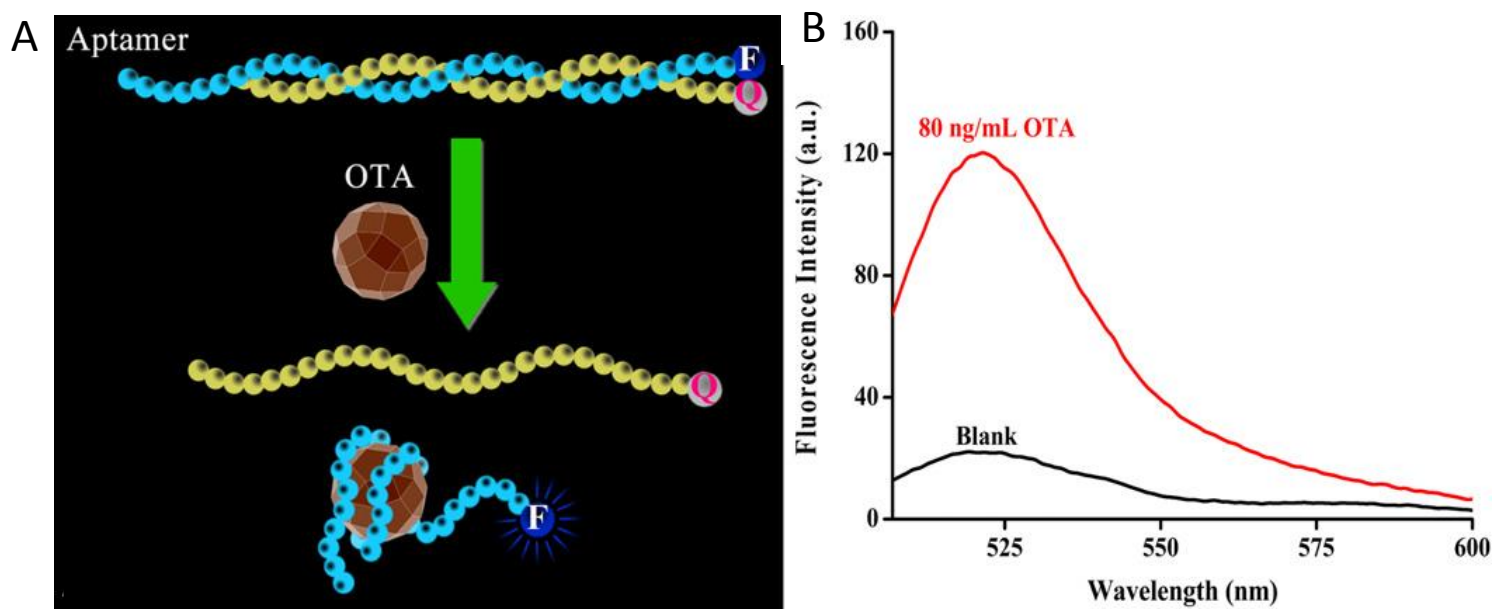


Figure 63



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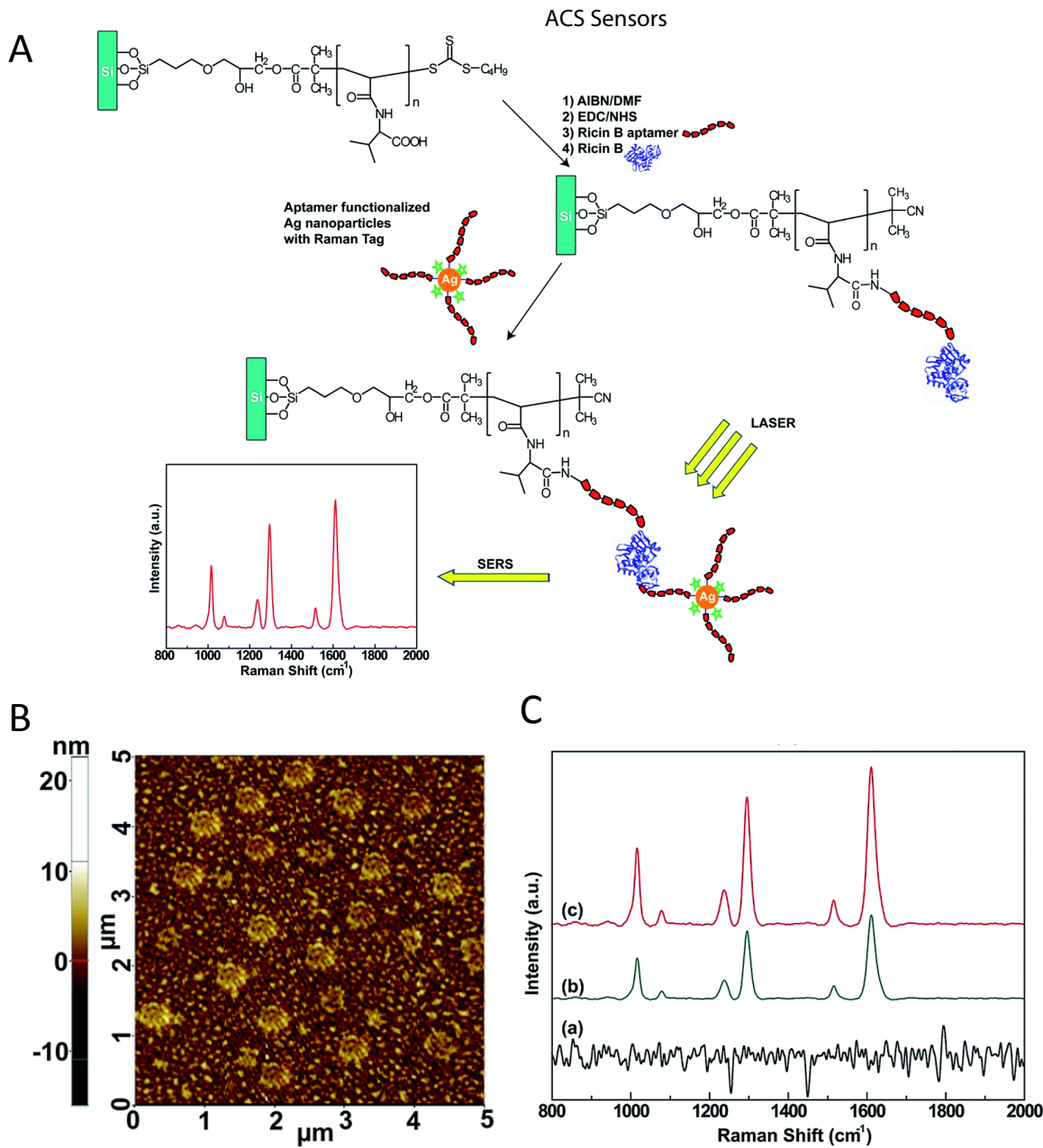
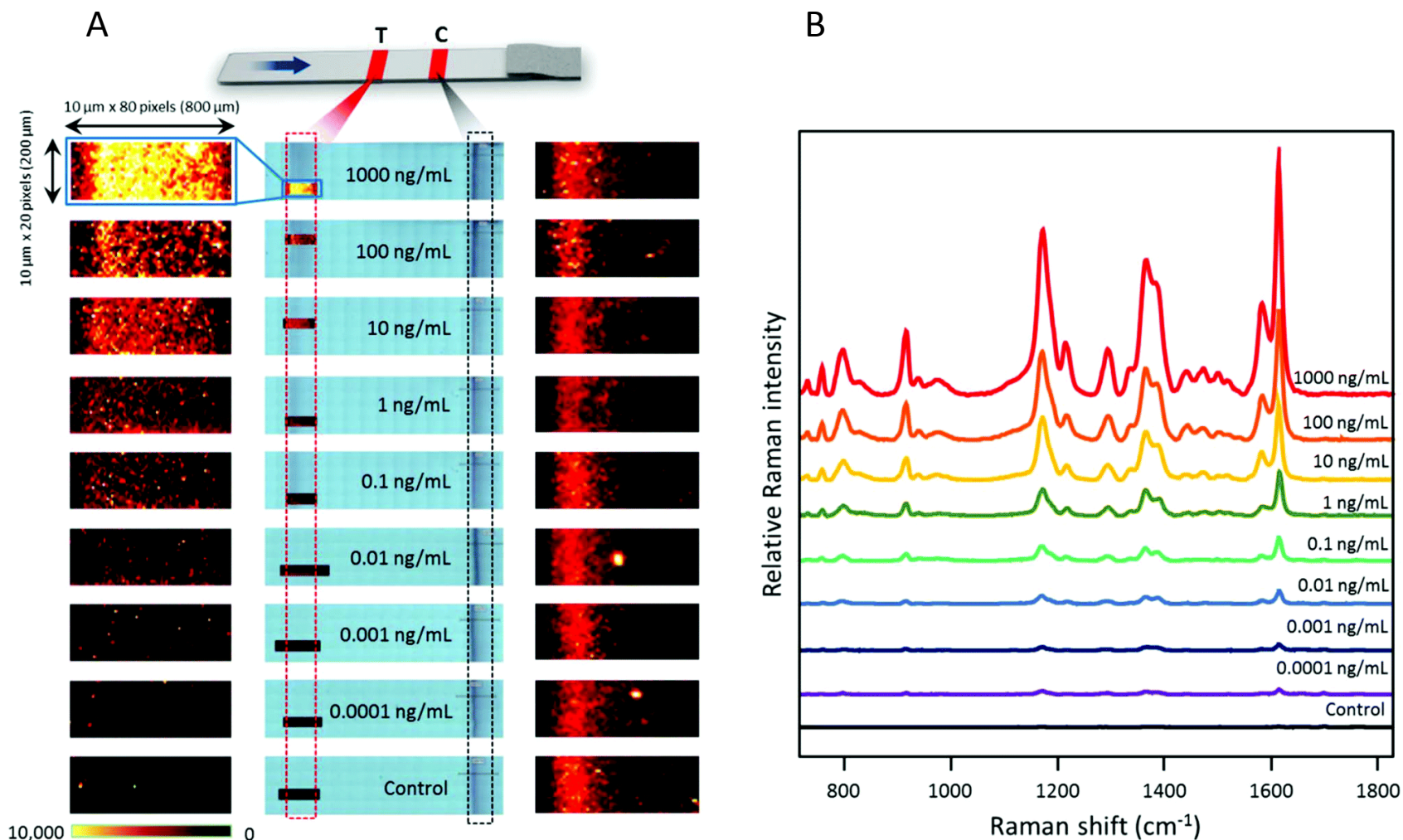


Figure 65



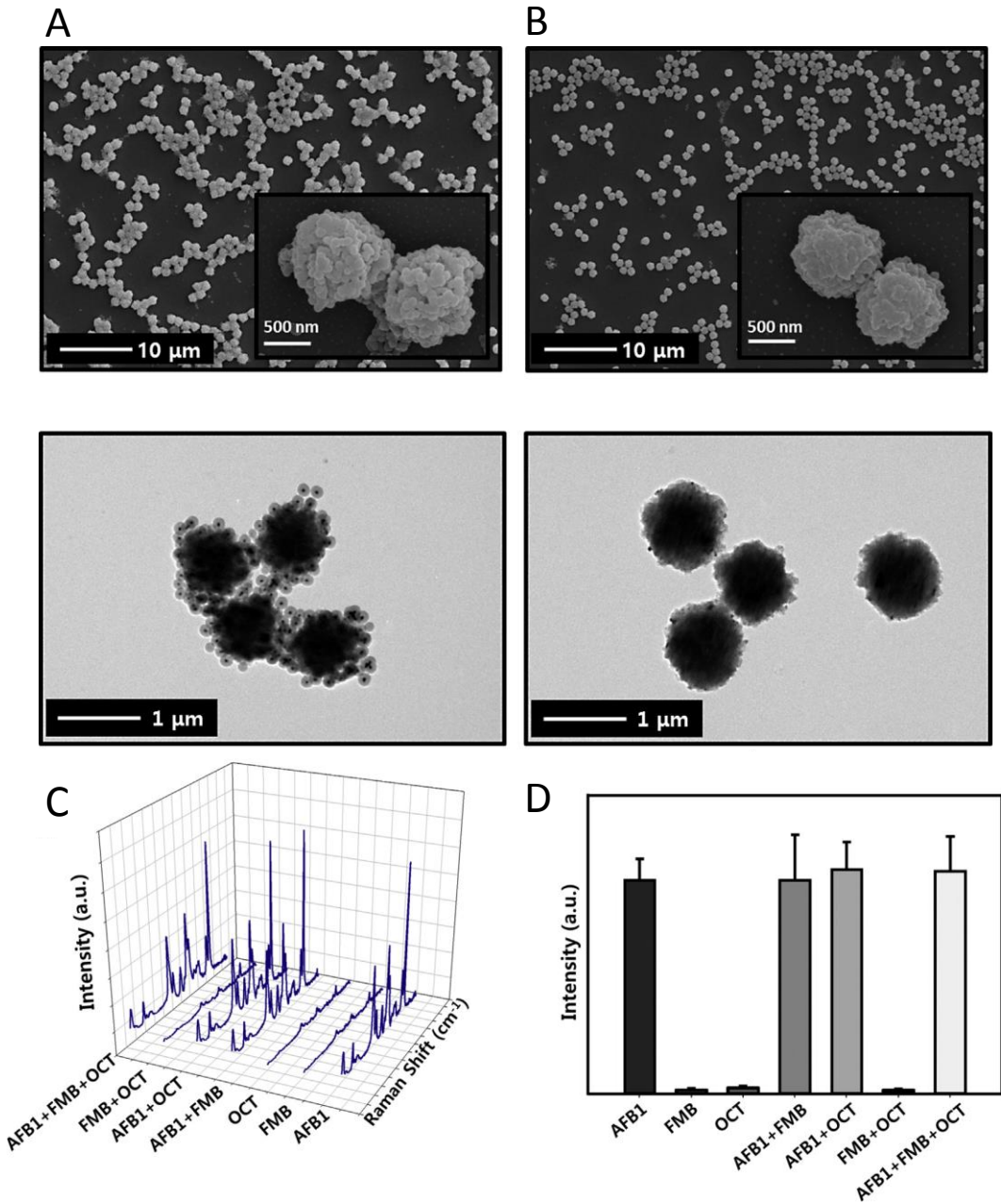
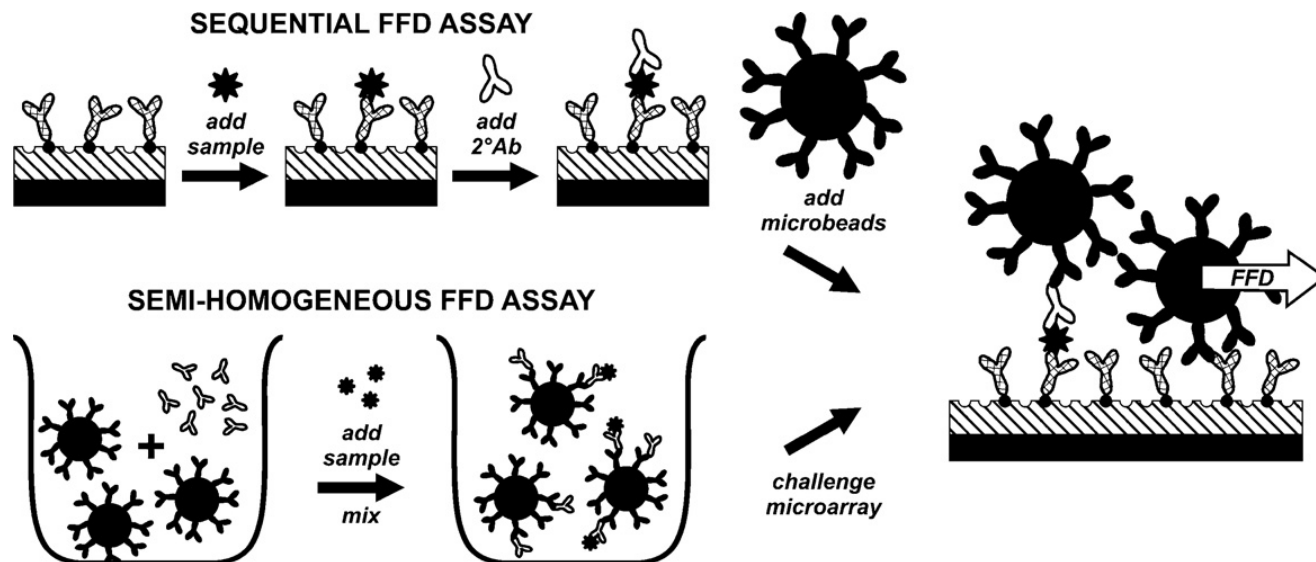
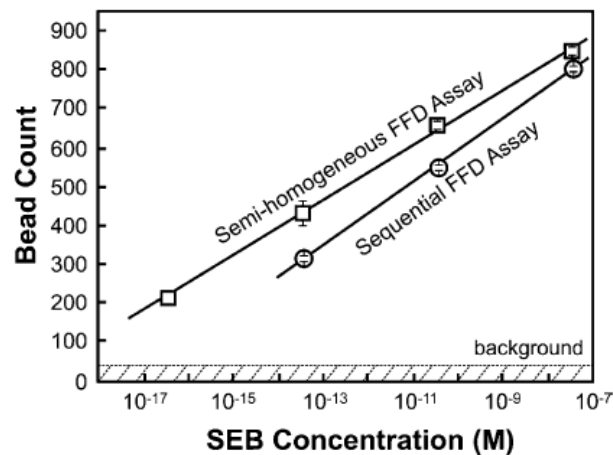


Figure 67

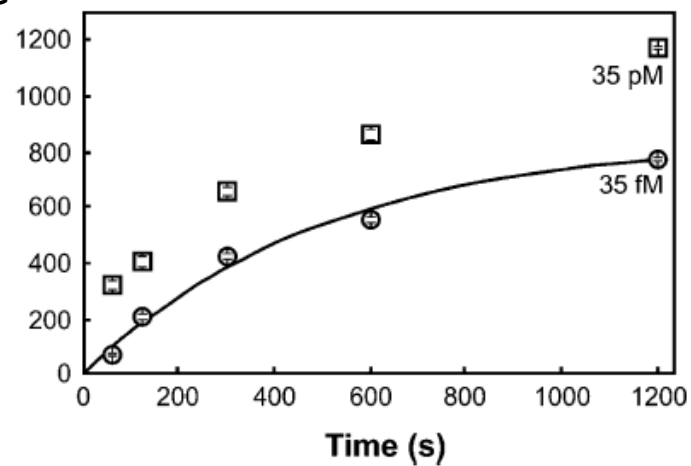
A



B



C



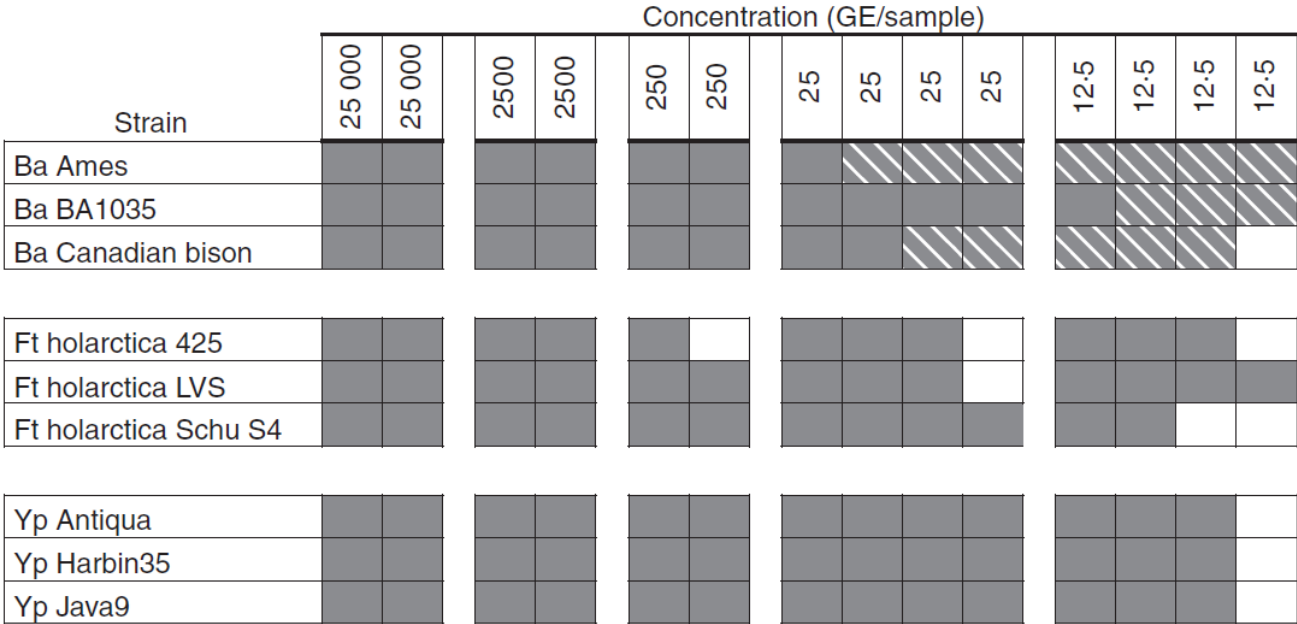
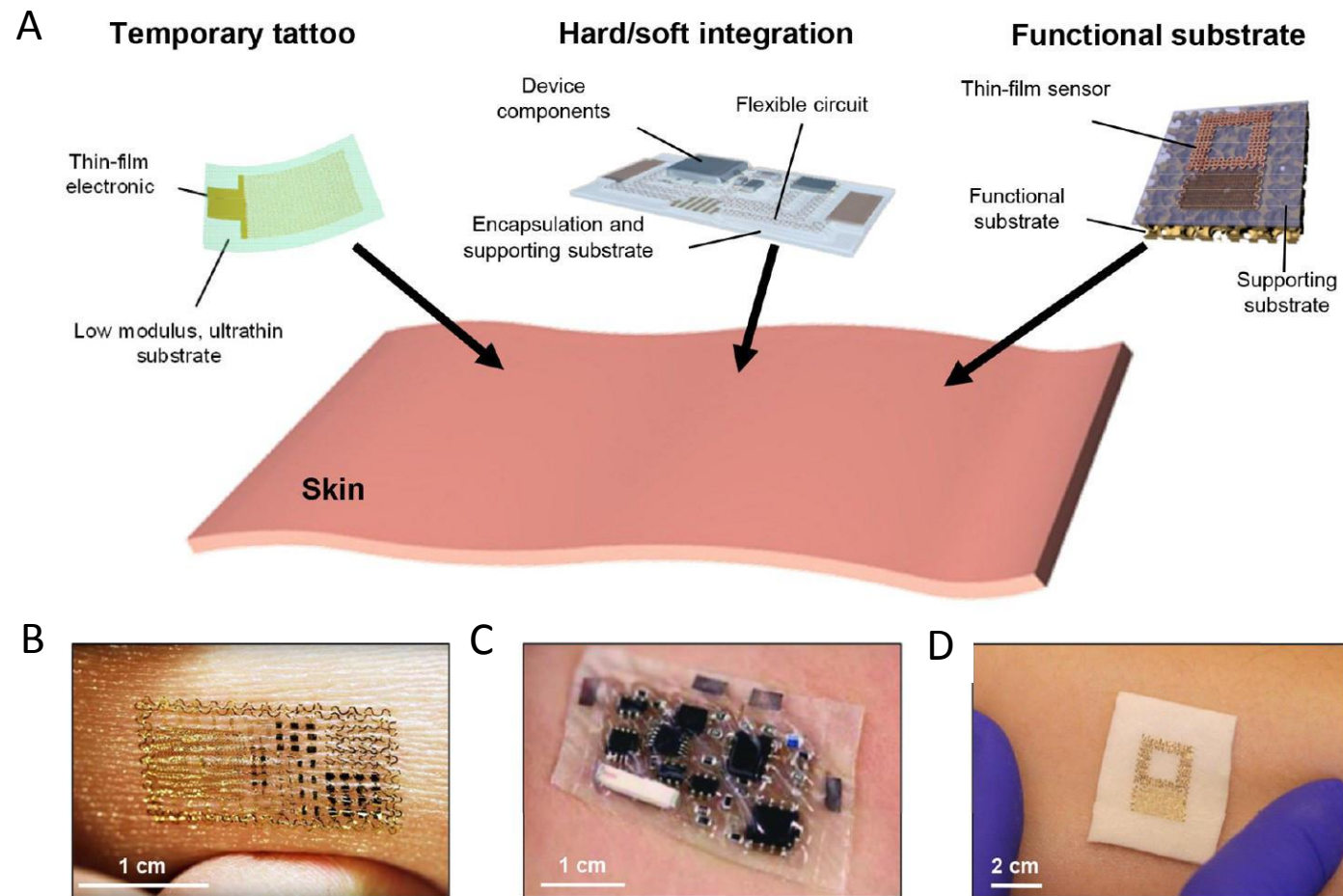


Figure 69



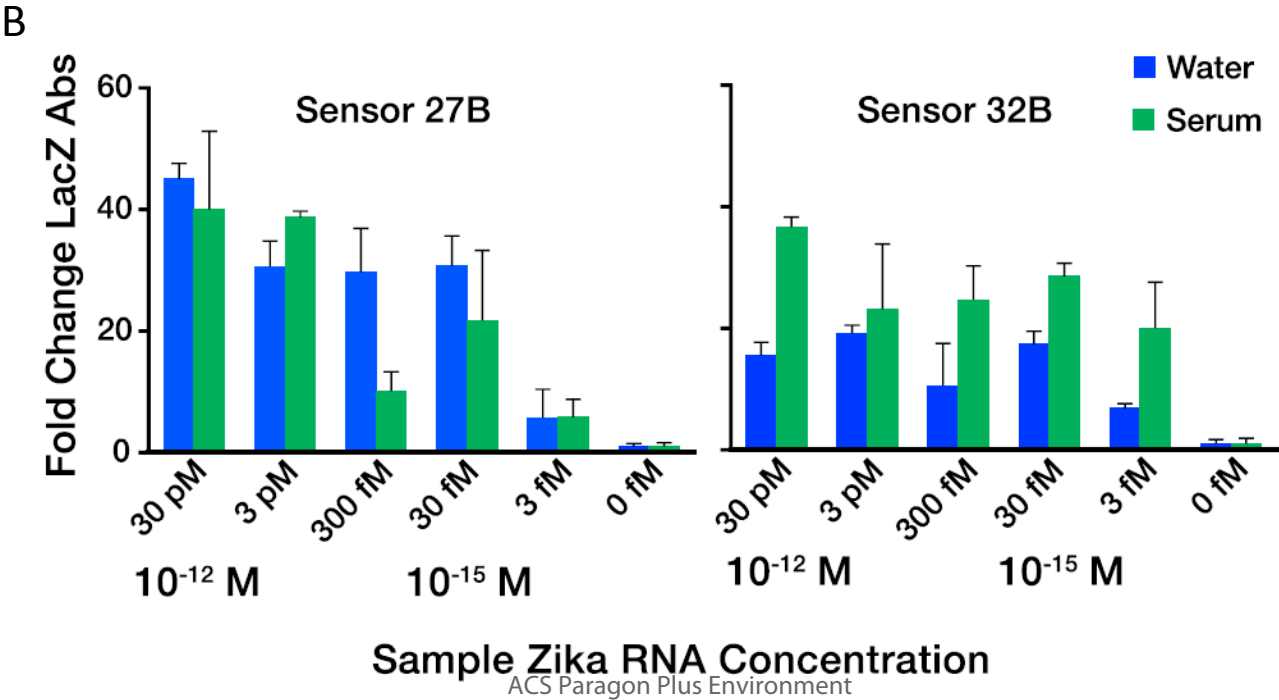
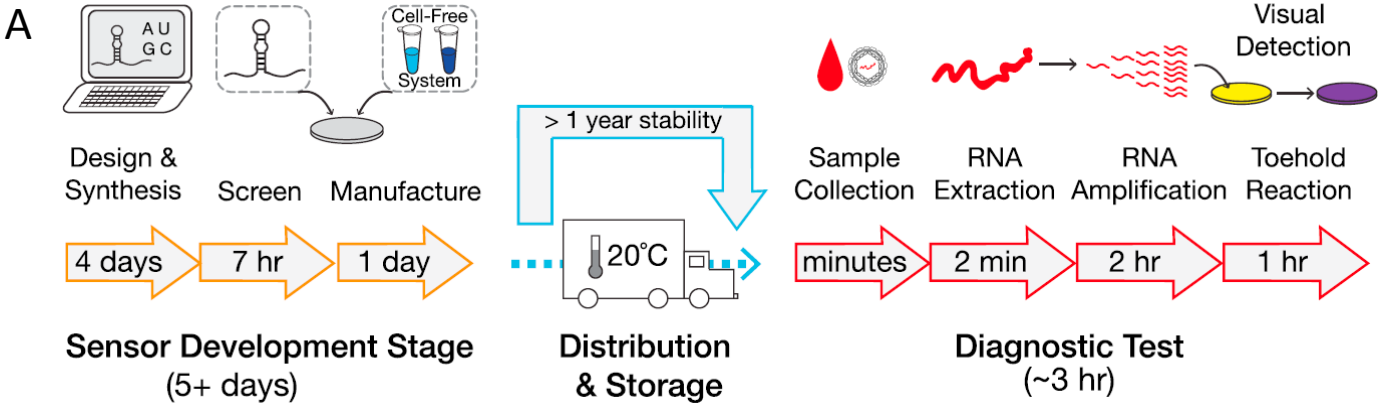


Figure 71

