EXPERT REVIEWS

Antibodies specific for nucleic acids and applications in genomic detection and clinical diagnostics

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Detection of nucleic acids using antibodies is uncommon. This is in part because nucleic acids are poor immunogens and it is difficult to elicit antibodies having high affinity to each type of nucleic acid while lacking cross-reactivity to others. We describe the origins and applications of a variety of anti-nucleic acid antibodies, including ones reacting with modified nucleosides and nucleotides, single-stranded DNA, double-stranded DNA, RNA, DNA:RNA hybrids, locked-nucleic acids or peptide nucleic acid:nucleic acid hybrids. Carefully selected antibodies can be excellent reagents for detecting bacteria, viruses, small RNAs, microRNAs, R-loops, cancer cells, stem cells, apoptotic cells and so on. The detection may be sensitive, simple, rapid, specific, reproducible, quantitative and cost-effective. Current microarray and diagnostic methods that depend on cDNA or cRNA can be replaced by using antibody detection of nucleic acids. Therefore, development should be encouraged to explore new utilities and create a robust arsenal of new anti-nucleic acid antibodies.

Keywords: anti-nucleic acids antibodies • apoptotic cells detection • biosensor • cancer detection • hybrid capture • metakaryotic stem cells • microarray • miRNA • R-loop detection • sRNA

Immunoassays measure the presence and/or concentration of an antigen in a solution through the use of an antibody for specific noncovalent binding. Immunoassays for the detection of nucleic acids as antigens are much less frequently used, probably because PCR methods have high sensitivity and specificity. Nucleic acids are poor immunogens, making it difficult to elicit antibodies specific to different types of nucleic acids. Furthermore, the anti-nucleic acid antibodies created decades ago are largely neglected today. However, antibodies of this type, together with modern technologies, can allow expanding or upgrading of methods currently widely used in clinical labs and biological research – the potential for antibody utilizations has not been reached yet.

Here, we summarize important anti-nucleic acid antibodies isolated in the past, suggest new ones to be produced, and present and propose their use in clinical and genomic detection and diagnostics. Readers interested in recent progress regarding antibodies against nucleic acids for their roles in autoimmune diseases, or recognition of nucleic acids by the innate immune system, can read other recent reviews [1–3].

Anti-nucleic acid antibodies

Animals exhibit tolerance to native DNA. For example, injecting calf thymus native DNA into rabbits usually fails to induce an antibody response [4]. On the other hand, synthetic homopolymer nucleic acids are immunogenic, and the antibodies induced show relatively little dependence on base sequence and are specific for the helix conformation [5]. Anti-nucleic acid antibodies can also be isolated from human and animals with autoimmune diseases. Table 1 summarizes those original antibodies for use as possible research tools (most of them have not been tested extensively). Table 2 summarizes the applications of nucleic acid detection by antibodies. Readers can track the labs for possible antibody sharing or produce their own antibodies based on the demonstrated successful methods.

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Antibodies binding modified nucleosides & nucleotides

Antibodies to various modified nucleosides or nucleotides have been obtained, usually by coupling them as haptens to protein carriers such as methylated bovine serum albumin (MBSA). These antibodies can be used for the detection of unusual bases in DNA, tRNA or rRNA, as well as the cap structure of mRNA. For example, polyclonal antibody specific for $m^7G(5')$ pppGm was elicited in rabbits by using the chemical conjugated to BSA plus complete Freund's adjuvant [6]. The antibody can be used to detect the 5′ terminus of eukaryotic cellular and viral RNAs [6]. Therefore, this antibody can be used to isolate capped intact RNA or 5′ fragments of RNA (complementary to isolating mRNA by using its poly(A) tail), or to view mRNA *in vivo*. Monoclonal antibody (mAb) specific for 5-bromodeoxyuridine (BrdU) (B44) was produced from mice immunized with a conjugate of iodouridine and ovalbumin [7]. It is highly specific for BrdU and iododeoxyuridine (IdU) (anti-IdU), but binds chlorodeoxyuridine (CldU) weakly. It neither cross-reacts with thymidine (an analog of BrdU) nor with unmodified uridine, cytidine or purine nucleosides. This antibody binds BrdU in single-stranded (ss) DNA, BrdU attached to a protein carrier, or free BrdU. Immunocytochemical detection of BrdU incorporated into DNA in place of thymidine is a powerful tool to study cytokinetics of normal cells and cancer cells, as well as cell proliferation changes caused by cytotoxic drugs. This technique is as accurate and sensitive as that of autoradiography after incorporation of ^3H-labeled nucleotide [8]. Because anti-BrdU antibody was recognized for its significant applications, polyclonal and monoclonal anti-BrdU antibodies with subtle differences are also produced in rat, sheep, rabbit and chicken cells in addition to multiple murine cell lines [8–11] (Table 1). For example, rat anti-BrdU mAb (anti-CldU) reacts with CldU stronger than does B44 [9] and does not cross-react with IdU. A different mouse anti-BrdU mAb (BU-1) does not require DNA denaturation when staining [10]. Additional antibodies against modified nucleosides and nucleotides are discussed in these reviews [12–14].

Anti-ssDNA antibodies

Antibodies against ssDNA were isolated from animals and patients with autoimmune diseases [15], or induced with synthetic DNA [16]. The widely used anti-ssDNA antibodies Mab3034 (clone 16–19, Chemicon) and BV04–01 were isolated from autoimmune New Zealand mice [17], although both are not fully specific to ssDNA (~90% of reactivity to ssDNA and ~10% to dsDNA). Antibodies that react only with DNA, such as AK-30–10 (anti-single and double-stranded(ds) DNA, but not RNA, Chemicon CBL186), are valuable DNA reagents since the epitopes are virtually universal [18]. Mouse mAb C11 binds ssDNA and also interacts with dsDNA and RNA weakly [19]. Murine mAb MRss-1 (D2D4) [20] is an anti-ssDNA antibody with guanine as its antigenic determinant, but it cross-reacts to some extent with dsDNA and RNA [21]. Therefore, C11 and MRss-1 may be useful as reagents to detect any nucleic acid. DNA-1 is a recombinant anti-ssDNA Fab with a weak binding to RNA [22]. Mouse mAb F7–26 specifically reacts with deoxycytidine-containing ssDNA of at least 25–30 bases in length, but not with native dsDNA, RNA, nucleosides and deoxyribohomopolymers [23]. It is not known why F7–26 does not bind dsDNA, presumably the antigen is buried in the dsDNA. Perhaps a crystal structure of the antibody bound to ssDNA would shed light on this mystery. Mouse mAb AP-13, generated the same way as F7–26, is an anti-ssDNA antibody with deoxythymidine as its antigenic determinant in a larger stretch of ssDNA [24]. Mouse mAb ADP-1 binds specifically to Poly(ADP-ribose), which may play a role in cell cycle control or DNA repair [25]. Increased synthesis of poly(ADP-ribose) was found in the nuclei of autoimmune mice.

Antibodies to duplex, triplex & quadruplex DNA

Antibodies to Z-form dsDNA, triplex DNA & quadruplex DNA

The double-helical structures of nucleic acids are generally grouped into the three categories of A, B and Z forms. Both A and B helices are in the right-handed conformation while the Z form is left-handed. Native dsDNA exists primarily in the right-handed B helical form. Although native B DNA is not a good immunogen, other forms of nucleic acids coupled to positively charged proteins have been used successfully for antibody production. These nucleic acids include denatured DNA, synthetic polyribonucleotides and polydeoxyribonucleotides, dsRNA (A-form), DNA:RNA hybrids, Z DNA, triple-helical polynucleotides, cruciform structures and chemically modified DNA [13,26,27]. Polyclonal and monoclonal anti-Z DNA immunoglobulins [27] were produced when poly[d(GC)], poly[d(AC):d(GT)] and related variants were used to immunize rabbits. These antibodies were used to detect Z DNA determinants on plasmid pBR322 and simian virus 40 [28]. Similarly, murine anti-Z DNA mAbs, including Jel150, were created and applied for the detection of Z DNA in the chromosomes of *Drosophila melanogaster* [29–31]. Of these, Z22 has proved to be an effective anti-Z DNA mAb, possessing a K_d approximately10^{-8} M [29,31–33]. Mouse mAb Jel 318 and *me*^{r}IIB4 are specific for triplex [34] and quadruplex DNA [35], which the antibodies could detect in eukaryotic chromosomes.

Antibodies against native dsDNA

Almost all anti-B DNA mAbs were isolated from humans or mice with autoimmune diseases [36–39]. Mouse mAb Jel 274 and Jel 241 are autoantibodies binding to dsDNA, with at least 100-fold lower affinity to ssDNA and no binding to RNA [40,41]. Jel 274 has little sequence specificity while Jel 241 may recognize methylation of pyrimidines in alternating sequences. Mouse mAb 27–14-D9 reacts with dsDNA but not with ssDNA [42,43]. It was used extensively for the detection of DNA:DNA hybrids. Mouse mAb CH26–1352 was produced by immunizing mice with poly[d(AT)] and poly[d(IC)], and boosting with EcoR1-digested plasmid pBR322. This antibody has exclusive specificity for native dsDNA in a sequence-independent manner [44].

Anti-RNA antibodies

Anti-RNA antibodies were induced in animals by immunizing with ssRNA complexed with MBSA [45]. Sera from rabbits immunized with complexes of MBSA and poly (rA):Poly (rU) react with naturally occurring dsRNA, and to a lesser extent with DNA:RNA hybrids; they did not cross-react with either ssDNA or dsDNA [46]. These sera were used as effective reagents for detection of dsRNA to distinguish uninfected from arbovirus-infected mammalian cells [47]. The sera were also used to show that dsRNA is responsible for interferon induction in eukaryotic cells [48]. This was an early example that anti-RNA antibodies can be used for studying the function of extracellular RNAs (exRNAs) [49] and may provide therapeutic tools targeting diseases involving extracellular nucleic acids. Anti-dsRNA sera were also induced with poly (rI):poly (rC) in rabbits [50] and in guinea pigs [51]. The rabbit sera were used to detect viral dsRNA of the Fiji disease virus in sugar cane leaves, mycovirus infections and in extracts of tobacco plants infected with tobacco mosaic virus [50,52,53]. Four murine anti-dsRNA mAbs (J2, J5, K1 and K2) were induced with a 4.3-kb dsRNA as antigen [54]. These mAbs, specific to dsRNA without sequence discrimination, can be tools to detect RNA:RNA hybrids. The antibodies were used to detect various plant viruses [55], and J2 only recognizes dsRNA with at least 40 bp [56]. Mouse mAb Jel 103, induced with synthetic poly(rI):poly(dC) [57,58], is highly specific to poly(rI). Recombinant anti-RNA antibodies (Fabs) were generated by using phage display technology [59,60].

Since RNA is usually single-stranded, anti-ssRNA antibodies would be crucial tools for RNA biology. As diagnostic reagents, such antibodies are also currently difficult to find. Anti-ssRNA antibodies may be induced if uracil or its analogs are used as haptens, based on suggestions of antigenic determinants for induction of antibodies to ssDNA as well as modified nucleosides and nucleotides, as presented above. Uracil specific, anti-ssRNA antibodies [61], anti-(U1) small nuclear RNA antibodies [62] and anti-tRNA antibodies [63] exist in autoimmune patients [3]. Anti-RNA mAbs D4, D44 and D444 were isolated from mice with autoimmune diseases, which did not bind to ssDNA and dsDNA [64]. D44 was used to purify different sized RNA and RNA-binding proteins from *in vitro* reactions with high sensitivity (pg) and efficiency (1 min) [65].

Anti-DNA:RNA hybrid antibodies

Polyclonal anti-DNA:RNA hybrid antibodies

DNA:RNA hybrid duplexes consist of a strand of ribonucleic acid hybridized to a complementary strand of deoxyribonucleic acid. Anti-DNA:RNA hybrid antibodies were described in patients with systemic lupus erythematosus [66] and have been induced in animals by immunizing with poly (rA):Poly (rU) [46], poly (rI):poly(dC) [57,67,68], poly(rA):poly(dT) [4,67,69,70] and a phage øX174 DNA:RNA heteroduplex [71–73]. Sera from rabbits immunized with poly (rA):poly (dT) reacted strongly with T4 phage DNA:RNA hybrid [4]. The sera was also used for high-resolution detection of DNA:RNA hybrids *in situ* by indirect immunofluorescence for certain genes in chromosomes of *D. melanogaster* [74,75], and a similar technique was used for the identification of R-loops (described later). The first preparation of a polyclonal antibody induced with natural heteropolymer DNA:RNA hybrids was made by Nakazato [73,76]. The antibodies were fractionated from rabbit sera: 15% were specific for ssDNA, 71% was specific for both DNA:RNA hybrids and dsRNA, and 13% were specific for DNA:RNA hybrids only. The anti-hybrid fraction was highly selective and displayed higher affinity for a mixed sequence hybrid.

Monoclonal anti-DNA:RNA hybrid antibodies

The mAb Jel 99 [57,68] was shown to be specific for DNA:RNA hybrids [77]. G10H4, G11K3 and A6P3 are mAbs produced by hybridomas cell lines ATCC HB-8076, HB-8077 and HB-8078 [69,78]. They react with natural hybrid duplex sequences and are capable of distinguishing them from ssDNA, ssRNA, dsDNA or dsRNA. mAb 20D3 [72] had Kds of 9.52×10^{-13}, 4.72×10^{-11} and 5.95×10^{-8} for øX174 DNA:RNA, cDNA:mRNA of the rabbit globin gene and poly (rA):poly (dT), respectively. It had no cross-reaction to single- or double-stranded DNA. However, significant binding to ssRNA and dsRNA was observed. The level of inhibition by ssRNA was no more than 40%, indicating that the avidity of 20D3 for DNA:RNA is higher than that for ssRNA.

Murine mAb S9.6 (ATCC HB-8730) [71,79] has a high affinity for DNA:RNA duplex (Kd = ~1.18×10^{-11} M). As determined by competitive binding measurements, S9.6 binds single- or double-stranded DNA or RNA less than about 1/1000–1/10,000 as strongly. The S9.6 scFv was recently cloned, sequenced and produced in *Escherichia coli* [80]. It recognized a hybrid of at least 6-bp and its affinity is not strongly influenced by various buffer conditions or by ionic strength below 500 mM NaCl [80,81]. It was confirmed that nucleotide composition does not strongly influence S9.6 scFv affinity for its primary target DNA:RNA hybrids, nor for GC-rich RNA:RNA hybrids. However, S9.6 scFv bound AU-rich RNA:RNA, although with a weaker affinity (~five-fold) than to the DNA:RNA hybrids. It appears that antibodies binding to dsRNA and to DNA:RNA hybrids frequently cross-react with each other [4,46,71,76,82]. Immunizing with the helical A conformation DNA:RNA or RNA:RNA may create backbone-specific antibodies with little or no discrimination of base composition [67,83]. However, it may still be possible to make antibodies specific to DNA:RNA hybrids without cross-reaction to dsRNA [76] since the structures of dsRNA and DNA:RNA hybrids have subtle differences [84]. S9.6 was recently produced at NIH [85] and other laboratories [77,81,86–91]. It may be available for use in academic research upon request. We suggest that locally produced S9.6 should be validated before its application as can be done with an inexpensive mini-array chip [85].

Antibodies to hybrids containing LNA or PNA

A DNA oligonucleotide containing locked-nucleic acids (LNAs) binds complementary RNA strands with considerably higher affinity and increasing specificity than those of the

DNA-only counterpart [92]. Peptide nucleic acids (PNA) oligomers also have a surprisingly high affinity and high specificity for complementary DNA or RNA sequences [93]. The destabilizing effect of base mismatches in PNA- or LNA-containing heterodimers is much higher than in dsDNA or dsRNA. Therefore, LNA and PNA are versatile tools in genetic diagnostics and molecular biology. Interestingly, S9.6 also recognizes LNA-containing DNA:RNA heteroduplexes, although with a lower affinity compared to native DNA:RNA hybrids [81]. Additional polyclonal and monoclonal antibodies to LNA-containing nucleic acids may be induced by coupling LNA as haptens to MBSA as immunogens, or be made by using hybrids containing LNA as immunogens. Polyclonal, monoclonal and recombinant antibodies to different PNA:nucleic acid complexes are available [94]. The rabbit polyclonal antibodies in this patent reacted strongly with PNA:DNA and PNA:RNA without sequence discrimination, and not with dsDNA, DNA:RNA, ssDNA or ssPNA. Murine mAb 1G12 is specific to PNA:DNA hybrids irrespective of base sequence, whereas mAb 1B11 is specific to the sequence of the particular PNA:DNA hybrid used for immunization. Antibodies specific to hybrids containing LNA or PNA may potentially replace antibodies specific to DNA:DNA or DNA:RNA hybrids in some of the diagnostic and detection applications described below.

Detection of bacteria & viruses

Hybrid Capture (HC) is a universal, signal-amplified, solution-based hybridization assay for detecting DNA or RNA targets [95–97]. Its advantages include the higher rate of hybrid formation in liquid (as opposed to reactions using a reactant immobilized to membranes, tubes, beads, etc.) and the ease with which nonspecific materials can be washed away. Anti-nucleic acid antibodies (and/or streptavidin-biotin reagents) are frequently coated onto the surface of solid supports to capture the resulting soluble DNA:DNA or DNA:RNA hybrids [72,82,98,99]. The hybrids can then be detected by a specific anti-hybrid mAb conjugated to an enzyme with appropriate chromogenic substrate [42,96,99–101].

Coutlee *et al.* used plasmid pSP65 as a model system for the investigation of an S9.6-based HC assay [95,96]. The detection limit for the complementary RNA of the plasmid was 0.16 pg in a 50-µl assay. A 3-kb pSP65 RNA probe formed hybrids with its target plasmid DNA in 100 min, with the majority completed in the first 32 min. Five pg of pSP65 DNA could be detected in the presence of 10^6 pg of irrelevant nucleic acid from mammalian Hela cells. The detection limit was directly proportional to the length of DNA:RNA hybrid. With a 25, 55, 238 or 1427-bp hybrid, the limit was 312, 10, 10 and 3.2 pg/ml respectively. In other experiments, the sensitivity of detection of eukaryotic mRNA of IL-1 with a 1.3-kb DNA probe and IL-2 with a 1.1-kb DNA probe [102] was comparable to methods using P^{32}-labeled probes. mRNA of IL-1 could be detected in as little as 50 ng of total RNA.

Antibody-based nucleic acid detection systems have been used for detection of various bacteria, including *E. coli* [101], *Bacillus subtilis* [100], *Campylobacter jejuni* [98], *Chlamydia trachomatis* (CT) [103–107], *Neisseria gonorrhoeae* (GC) [103,104,106], *Listeria monocytogenes* [108,109], *Yersinia enterocolitica* [110] and *Helicobacter pylori* [111]. Similar methods were applied for detection of viruses, including rotaviruses [82], HIV [112,113], enteroviruses [114], HPV [97,115–117], cytomegalovirus [118], HSV [119], hepatitis A, B, C, D viruses [42,120–122] and plant viruses [55,123]. The enzyme-linked immunoassays (EIA) with antibodies for nucleic acid detection were sensitive, simple, rapid, specific, reproducible and inexpensive (Table 2).

The most notable applications are the US FDA-approved Qiagen's Digene HC technologies for *in vitro* diagnostic tests of HPV, CT and GC, which are based on polyclonal antibodies capturing and later mAb detecting DNA:RNA hybrids. More than 10 million tests for HPV are done using this test each year [124]. A pool of full-length genomic RNA probes of different oncogenic HPV types is used for HPV detection. The long ssRNA probes are employed so as to hybridize efficiently with the entire 8-kb HPV genome [97]. The RNA probes for GC are complementary to approximately 15,000 bases of its genome (0.7%) [125]. The RNA probes for CT are complementary to the entire chlamydia cryptic plasmid sequence (7500 bases) and to approximately 39,300 bases of its genome (4%) [105]. Probe length is critical to signal amplification [71,81,85,86,95], and the detecting mAb to the nucleic acid hybrid is conjugated to multiple molecules of alkaline phosphatase for maximum signal amplification [97]. Qiagen hybrid capture II HPV DNA Tests are extremely specific when used in conjunction with the Papanicolau test and yield quantitation of HPV DNA in the range of 1×10^3–5×10^7 genomes in a single assay [97]. Although the detection sensitivity is not as good as PCR, it reaches clinical endpoints [126].

HC and other immunoassays can be more sensitive if a target and/or probe are first amplified by PCR. Such a PCR-EIA for nucleic acid detection is more sensitive than PCR alone due to EIA signal amplification, and more specific than PCR due to nucleic acid hybridization. For example, 3–6 copies of HPV-16 or HPV-18 genome could be detected by PCR-EIA with S9.6 in several hours [115,117]. One hundred copies or less was demonstrated for HPV DNA genotype detection by using Luminex XMAP, genome amplification and anti-DNA:RNA hybrid mAb [127]. GEN-ETIK-DEIA (DiaSorin, Saluggia, Italy), a PCR-EIA kit for detection of DNA:DNA hybrid with 27–14-D9, was available in the 1990s [42,120–122].

Detection of non-coding RNAs & R-loops & analysis of transcriptomes with microarrays

Antibody-based microarray detection of bacterial sRNAs & yeast transcriptome

Current microarray detection limitations

Microarray systems have been widely used in almost every area of biological research. For analysis of RNA transcript levels, microarray analyses typically begin with synthesis of fluorescently labeled cDNA from the RNA samples in a reaction catalyzed by

Table 1. Anti-nucleic acids antibodies to be reagents.

Name	Immunogen[a]	Animal	Type	Reactivities[‡]	Ref.
Antibodies binding modified nucleosides and nucleotides					
Anti- 5'cap	m7G(5')pppGm	Rabbit	Polyclonal	5'capped mRNA, weak to G(5') pppGm(1/180), G(5')pppG(1/430)	[6]
Anti-IdU (B44)[†]	Iodouridine	Mouse	Monoclonal, IgG1	BrdU, IdU, weak to CldU, not thymidine, uridine, cytidine or purine	[7]
BU1/75(ICR1)[†]	BrdU	Rat	Monoclonal, IgG2a	Specific to BrdU, CldU (anti-CldU); not IdU, thymidine	[9]; Abcam (ab6326)
BU-1[†]	Iodouridine	Mouse	Monoclonal, IgG2a	BrdU, IdU, CldU, not FrdU, thymine; not require denatured DNA	[10]
Anti-BrdU	BrdU	Sheep	Polyclonal, IgG	BrdU	Abcam (ab1893)
Anti-BrdU	BrdU	Chicken	Polyclonal, IgY	BrdU	Abcam (ab92837)
Anti-BrdU	BrdU analog(s)	Rabbit	Polyclonal, IgG	BrdU	Abcam (ab152095)
CA1USR	Modified uridine	Mouse	Monoclonal, IgG1	2'-deoxy-2'-C-allyl uridine containing nucleic acids	[163]
Anti-5-MeCyd[†]	5-methylcytidine	Mouse	Monoclonal, IgG1	specific to 5-methylcytidine	[164]
Anti-4-AcCyd	4-acetylcytidine	Mouse	Monoclonal, IgG1	Specific to 4-acetylcytidine	[164]
Anti-1-MeIno	1-methylinosine	mouse	Monoclonal, IgG1	Specific to 1-methylinosine	[164]
Anti-1-MeAdo	1-methyladenosine	Mouse	Monoclonal, IgG1	Specific to 1-methyladenosine	[164]
Anti-7-MeGuo	7-methylguanosine	Mouse	Monoclonal, IgG1	Specific to 7-methylguanosine	[164]
Anti-ψ-Urd	Pseudouridine	Mouse	Monoclonal, IgG2a	Specific to pseudouridine	[164]
Antibodies to single-stranded DNA					
dC1	poly(dC)	Mouse	Monoclonal, IgM	ssDNA, poly(dC); not poly(dT), poly (dU)	[165]
dC7	poly(dC)	Mouse	Monoclonal, IgG	ssDNA, poly(dC)	[166]
Mab3034, BV04–01	Autoimmune	Mouse	Monoclonal, IgG2a	ssDNA (~90%), poly(dT), weak to dsDNA (~10%)	[17]
C11	Autoimmune	Mouse	Monoclonal, IgG	ssDNA, dsDNA, RNA	[19]
H43, H44	Autoimmune	Mouse	Monoclonal, IgG, IgM	ssDNA, poly I; both AD: guanine or hypoxanthine	[167]
F7–26[†]	Calf thymus ssDNA	Mouse	Monoclonal, IgM	ssDNA, not dsDNA, RNA; AD: deoxycytidine with >25 bases	[23]
AP-13	Calf thymus ssDNA	Mouse	Monoclonal, IgM	ssDNA, not dsDNA, RNA; AD: deoxythymidine	[24]
D2D4 (MRss-1)	Autoimmune	Mouse	Monoclonal, IgG	ssDNA, weak to dsDNA, RNA; AD: guanine	[21]; ATCC HB69
ADP-1	poly(ADP-ribose)	Mouse	Monoclonal, IgG	poly(ADP-ribose), not ssDNA, dsDNA	[25]
DNA-1	Autoimmune	Mouse	Phage display, Fab	ssDNA, weak RNA, AD: oligo (dT)	[22]

[†]Indicates the antibody has been more documented; a: protein carriers, adjuvants and other chemical treatments not mentioned.
[‡]AD: Antigenic determinant; BrdU: 5-Bromodeoxyuridine; CldU: Chlorodeoxyuridine; FrdU: 5-Fluoro-2-deoxyuridine; IdU: Iododeoxyuridine; LNA: Locked-nucleic acid; PNA: Peptide nucleic acid.

Table 1. Anti-nucleic acids antibodies to be reagents (cont.).

Name	Immunogen[a]	Animal	Type	Reactivities[‡]	Ref.
Antibodies to duplex, triplex or quadruplex DNA					
Anti-Z DNA	Multiple	Rabbits Mouse Goat	Polyclonal Monoclonal IgG	Z DNA helices, sequence specific or not	[28,29]
Jel150	Brominated poly[d(GC)]	Mouse	Monoclonal, IgG	Z-DNA, not native dsDNA	[30]
Z22[†]	Brominated poly[d(GC)]	Mouse	Monoclonal, IgG	Z-DNA, not native dsDNA, ssDNA	[31,33]
AK 30–10	Autoimmune?	Mouse	Monoclonal, IgM	ssDNA, dsDNA; not RNA	[18]; Chemicon CBL186
2–27–2	Autoimmune	Mouse	Monoclonal, IgG	dsDNA, weak to ssDNA	[168]
Jel 274, Jel 241	Autoimmune	Mouse	Monoclonal, IgG	dsDNA, Jel 274: sequence independent; Jel 241: methylation of pyrimidines	[40,41]
Ch26–1352	poly[d(AT)], poly[d(IC)], pBR322 DNA	Mouse	Monoclonal, IgM	Native dsDNA, not ssDNA, RNA	ATCC HB-8329
27–14-D9[†]	Autoimmune	Mouse	Monoclonal, IgG	dsDNA; not ssDNA	[42]
HYB331–01	Autoimmune	Mouse	Monoclonal, IgG2a	dsDNA, weak to ssDNA(1/10), very weak to RNA	[38]; Abcam (ab27156)
11B6	Unknown	Mouse	Mouse monoclonal, IgM	dsDNA, weak to ssDNA(1/20), not RNA	Abcam (ab418)
dsDNA-mAb32 dsDNA-mAb33	Autoimmune	Human	Monoclonal, IgG	dsDNA, ssDNA, not RNA	[39]; DIARECT AG
Anti-d(G:C) polymer	poly(dG):poly(dC)	Rabbit	Antisera	Specific for poly(dG):poly(dC); not RNA or DNA	[4]
Jel318	poly[d(Tm5C):poly[d(GA)]:poly[d(m+5CT)]	Mouse	Monoclonal, IgG	Specific for AT-rich triplexes, not ssDNA, dsDNA, RNA	[34]
Jel466	poly[d(Tm5C): poly[d(GA)]	Mouse	Monoclonal, IgG2a	Specific for GC-rich triplexes, not ssDNA, dsDNA, RNA	[169]
Me^{V}IIB4	Autoimmune	Mouse	Monoclonal, IgG3	Specific to quadruplex structures	[35]
Anti-RNA antibodies					
Anti-dsRNA	poly (rA):Poly (rU)	Rabbit	Antisera	dsRNA, less DNA:RNA; not ssDNA, dsDNA, ssRNA, poly(rA), poly(rU)	[46]
Anti-dsRNA	poly (rI):Poly (rC)	Rabbit	Antisera	dsRNA, not DNA, ssRNA	[50]
Anti-dsRNA	poly (rI):Poly (rC)	Guinea pig	Polyclonal, IgG	dsRNA	[51]
J2[†]	L-dsRNA (4.3 kb)	Mouse	Monoclonal, IgG2a	dsRNA, not DNA, ssRNA, DNA:RNA; AD: A2N9A3N9A2	[56]; Scicons Hungary
J5, K1, K2[†]	L-dsRNA (4.3kb)	Mouse	Monoclonal,Ig2b, IgG2a,IgM	dsRNA, not DNA, ssRNA, DNA:RNA	[54]; Scicons; biolinks
D4, D44, D444	Autoimmune	Mouse	Monoclonal, IgG3	ssRNA, not DNA, AD: a small (3–4 nt) RNA sequence rich in G, C	[64]

[†]Indicates the antibody has been more documented; a: protein carriers, adjuvants and other chemical treatments not mentioned.
[‡]AD: Antigenic determinant; BrdU: 5-Bromodeoxyuridine; CldU: Chlorodeoxyuridine; FrdU: 5-Fluoro-2-deoxyuridine; IdU: Iododeoxyuridine; LNA: Locked-nucleic acid; PNA: Peptide nucleic acid.

Table 1. Anti-nucleic acids antibodies to be reagents (cont.).

Name	Immunogen[a]	Animal	Type	Reactivities[‡]	Ref.
Anti-RNA antibodies					
Anti-tRNA	Autoimmune	Human	Antisera	tRNA, AD: D and the TψC loops	[63]
Jel 103	poly(rI):poly(dC)	Mouse	Monoclonal, IgG	Specific for poly(rI)	[57]
S9.6	ϕX174 DNA:RNA	Mouse	Monoclonal, IgG2a	AU-rich dsRNA	[80]
Synthetic	Phage display		Fabs	RNA	[59,60]
Antibodies to DNA:RNA hybrids					
Anti-DNA:RNA	poly(rA):poly(dT)	Rabbit	Antisera	DNA:RNA hybrid	[4]
DNA:RNA hybrid	poly(rA):poly(dT)	Rabbit	Polyclonal, IgG	DNA:RNA hybrid	[70]
Anti-DNA:RNA	poly(rA):poly(dT)	Rabbit, goat	Polyclonal, IgG	DNA:RNA hybrid, less poly(rA):poly (rU)	[67]
Anti-DNA:RNA	ϕX174 DNA:RNA	Rabbit	Polyclonal, IgG	71% DNA:RNA and dsRNA, 13% specific for DNA:RNA, 15% ssDNA	[76]
Jel 99	poly(rI):poly(dC)	Mouse	Monoclonal, IgM	DNA:RNA hybrid, weak to poly(rA): poly(dT), not dsDNA, rRNA	[57,68]
G10H4, G11K3, A6P3	poly(rA):poly(dT)	Mouse	Monoclonal, IgG, IgM	DNA:RNA, not ssDNA, dsDNA, RNA	ATCC HB 8076, HB 8077, HB 8078
S9.6[†]	ϕX174 DNA:RNA	Mouse	Monoclonal, IgG2a	DNA:RNA, not ssDNA, dsDNA, ssRNA, GC-rich dsRNA	[71,80]
20D3	ϕX174 DNA:RNA	Mouse	Monoclonal, IgM	DNA:RNA, weak (40%) to ssRNA, dsRNA, not ssDNA, dsDNA	[72]
6B5	globin cDNA:mRNA	Mouse	monoclonal, IgM	poly(rA):poly(dT),ssRNA, weak to cDNA:mRNA, ssDNA, dsDNA	[72]
Antibodies to LNA or PNA:nucleic acid hybrids					
S9.6	ϕX174 DNA:RNA	Mouse	Monoclonal, IgG2a	LNA:RNA	[81]
Anti-PNA hybrids	PNA:DNA	Mouse	Antisera	PNA:DNA, PNA:RNA, not ssDNA, dsDNA, ssPNA, DNA:RNA, RNA not tested	[94]
1G12	PNA:DNA	Mouse	Monoclonal	PNA:DNA, not ssDNA, dsDNA, ssPNA, not tested to DNA:RNA, PNA:RNA, RNA	[94]
1B11	PNA:DNA	PNA:DNA mouse	Monoclonal	certain PNA:DNA, not PNA:RNA, ssDNA, dsDNA, ssPNA, DNA:RNA	[94]
Other anti-nucleic acids antibodies					
Other antibodies[†]	Various	Various	Various	Various	[3,12–14,27,170,171]

[†]Indicates the antibody has been more documented; a: protein carriers, adjuvants and other chemical treatments not mentioned.
[‡]AD: Antigenic determinant; BrdU: 5-Bromodeoxyuridine; ClDU: Chlorodeoxyuridine; FrdU: 5-Fluoro-2-deoxyuridine; IdU: Iododeoxyuridine; LNA: Locked-nucleic acid; PNA: Peptide nucleic acid.

the enzymes reverse transcriptase and DNA polymerase. RNA can also be amplified and labeled for the generation of cRNA prior to microarray hybridization. In either case, due to the multiple steps involved in labeling, accurate representation of labile RNAs may be difficult, as well as being time consuming, labor intensive and expensive. In addition, labeled nucleic acids, with increased hydrophobicity, may exhibit decreased hybridization kinetics, resulting in elevated assay background. Detection of small, non-coding regulatory RNAs (sRNAs, 50–300 nt) in bacteria, as well as eukaryotic microRNAs, is even more challenging due to their small size, low concentration, short lives and complex secondary structures [128,129].

The traditional microarray technology was used to play a confirmatory role in identifying novel sRNAs in *E. coli* [130]. Apparently it was not efficient, as evidence that well-studied sRNA OxyS (110 nt) [131] and DsrA (85 nt) [132] were not detected under the conditions where their expressions were at a low level. Interestingly, OxyS and DsrA were also not detected by Northern blot under the same growth conditions. The Northern analysis was supposed to be sensitive since the probes were PCR generated, radioactive-labeled and the amount of total RNA used was 5 μg [130].

Antibody-based microarrays for the detection of bacterial sRNAs

Ideally, microarrays could be used to detect and quantitate unmodified DNA or RNA. In practice, the difficulty in detecting the resulting hybrids has required that nucleic acid samples be amplified and labeled prior to their hybridization to a microarray. However, antibody-based methods have been successfully used to detect unmodified bacterial sRNAs. For example, sRNAs of *E. coli* that co-immunoprecipitate with the RNA chaperone Hfq protein were successfully identified using components of the previously available Digene ExpressArray kit [133]. Hfq-enriched sRNAs [133] were hybridized directly to the 25-mer DNA probes of an Affymetrix microarray that covers the entire *E. coli* genome; goat polyclonal anti-DNA:RNA hybrid antibody from the ExpressArray kit was added to detect the hybrids on the arrays. The polyclonal antibody was recognized by biotin-labeled rabbit anti-goat IgG, followed by incubation with streptavidin-phycoerythrin. The arrays were very sensitive. OxyS and DsrA both gave strong signals in the microarray experiments. The possible reasons for this are 1) OxyS and DsrA were enriched by co-immunoprecipitation with the Hfq protein; 2) antibody-based microarray detection system has a high sensitivity, perhaps exceeding that of traditional, probe-labeled microarrays [130]; and 3) both 1 and 2.

The same group later developed a similar antibody-based microarray assay for sRNA detection [85], which does not depend on the Digene reagents that are no longer available. Instead of a polyclonal antibody to DNA:RNA hybrids, this assay uses the S9.6 mAb. The advantage of this microarray was evident when detection of sRNAs was done with small custom spotted microarrays. Identical 8-μg samples of total *E. coli* RNA grown at 37° C were processed both 1) in a traditional, fluorescent-labeled cDNA method and 2) without labeling. All eight sRNAs examined, including OxyS and DsrA, were detected at levels well above the background by the antibody method but only two (neither OxyS nor DsrA) were detected by the cDNA microarray method. OxyS and DsrA were not enriched by Hfq protein, nor induced by growth in hydrogen peroxide (OxyS) [131], or in the low temperature (DsrA) [132]. OxyS and DsrA could be identified with 200 ng of total RNA without RNA fragmentation, and with 2 μg of total RNA, OxyS and DsrA could be well identified at signal/noise ratios equaling those previously reported [Hu Z, Zhang A, Storz G, Gottesman S, Leppla SH, Unpublished Data]. This also demonstrated that this antibody-based microarray is superior to Northern blot in the ability to detect OxyS under these conditions. Significant signals were detectable for as little as 250 attomoles of OxyS RNA or rRNA, which was done in a 25-μl volume (10 pM) [85].

The microarray was designed to include OxyS oligonucleotide probes of lengths of 15, 20, 25, 30, 35 and 40 nt in addition to the 50-mers, the size for most probes in the array. The signals for the OxyS RNA were highly dependent on probe length, with signals detected only on probes of 20 nt and longer, with the signal for 25-mers being only 1/10 that of 50-mers [85]. This is consistent with the report that the signal for HPV detection is directly related to probe length [71,81,85,86,95]. The gain from longer probes suggests that the simultaneous binding of both antigen-binding sites of the antibody to neighboring DNA:RNA duplex regions greatly increases retention of antibody on the array element. Qavi *et al.* also suggested that the S9.6 binding epitope is on the order of 6 bp in size, and the footprint established by the first bound S9.6 allows greater access for subsequent interactions on the same DNA:RNA duplex [80,81]. The effective detection of the OxyS RNA with a probe of 20-nt hints that S9.6 can be used to detect microRNAs, small interfering RNAs, piwi-interacting RNA and the like in eukaryotes [129].

The S9.6-based microarray with the Affymetrix platform was used to study *E. coli* Hfq mutants association with sRNAs and their target mRNAs *in vivo* [134]. Upon closer examination of the publication, the major reason for strong signals for OxyS and DsrA is due to the sRNAs enrichment by Hfq. Here, the antibody-based microarray plays a moderate role in the Affymetrix microarray data with 25-mer probes. Therefore, the amount of RNA [134] and probe lengths [85] both play an important role in the detection of RNA expression. A protocol for identifying sRNAs as well as for quantifying global RNA expression by using antibody-based whole-genome tiling arrays was recently published [135]. The identification of sRNAs would be unbiased since the bioinformatic conservation-based searches and any information about promoter and terminators may not be needed [135].

Yeast transcriptome mapping

The S9.6-based microarray was adapted to map the dynamic transcriptome of *Schizosaccharomyces pombe* under multiple growth conditions [86]. An Agilent high-density 60-mer DNA oligonucleotide tiling microarray was used. The arrays yielded abundant, highly reproducible RNA transcripts, resolving strand-specific coding, non-coding, antisense and structural RNAs with high resolution. Many of these RNA transcripts were previously unknown. The RNA transcripts were credible since many were confirmed by qPCR and RNA-Seq [86,136]. The non-coding RNAs dhrev and dhfor, transcribed from both strands of the same locus, were further verified recently [137].

Antibody-based microarray discussion

Although 25-mer probes work in the Affymetrix platform, probes for antibody-based microarray should probably have lengths of at least 50–60 nt to generate good data [85,86]. Since

a probe with a length of 250–350 nt was reported having maximal sensitivity for the HC assays [95,102], the optimum length of a microarray probe for a given format needs to be evaluated experimentally in each case.

We now know that the detection limits for bacteria or viruses with long probes are thousands of equivalent genomic DNA per assay by the HC assays [97,108,119]. PCR-EIA pushed the limits to a few cells or CFU [107,109,115,117]. When combining long oligonucleotide probes with target amplification, sensitivity and specificity of antibody-based microarray may be as good as qPCR. In the Supplementary Figure 6, the signal intensity ratios (sense/antisense) of the antibody-based microarray were comparable to those of qPCR even without target amplification [86]. Hundreds of attomoles of OxyS [85], 3.3×10^{-2} attomoles of fusion transcript [87] or less than 10 fM [138] of target molecules can be detected even without target amplification, which are already very sensitive.

A critical step for successful antibody-based microarray analysis is to destroy target RNA complex structure by heating and fragmentation. Since S9.6 is a protein, its nonspecific interaction with an array surface may cause a major problem. However, anti-streptavidin antibody, involved in other types of microarray systems, seems to have little problem with nonspecific binding [139]. Careful controls [86,87] should help to solve this issue. Other methods may include conjugating S9.6 with Alexa 647 [138] or Cy3 to avoid the second antibody detection of S9.6, and testing various formats of immunological assays for best detection of the hybrids [85,108,139]. F7–26 (or other anti-ssDNA antibodies, following) can be used as a quantitative negative control. In this case, the antibody only binds unhybridized ssDNA probes on the array, leaving the hybrids undetected. Antibody-based microarray specific to DNA:DNA hybrids should be developed by using 27–14-D9 (or other anti-dsDNA antibodies, afterward) in a way similar to S9.6 for DNA:RNA. Such arrays may be simple yet effective to perform for DNA:DNA detection, and replacing RNA targets with DNA reduces microarray cross-hybridization [140].

Antibody-based microarrays containing thousands of appropriate nucleic acid probes, processed with or without sample amplification, would be sufficient to screen or verify the most common cause(s) of a microbial foodborne disease outbreak. Those probes should be able to detect typical DNA or RNA sequences of common pathogenic bacteria, viruses and parasites that frequently contaminate food or water. The probes presented in 'Detection of Bacteria and Viruses' may be used as good references. Another possible application is to incorporate thousands of human disease biomarkers into the array system, to quickly identify one's physiological states – such as if the person has breast cancer [138] or Ewing sarcoma [87]. This next-generation microarray may be simple and rapid to perform, and it may be quantitative, specific, reproducible, unbiased and cost–effective.

MicroRNA detection

Qavi *et al.* reported the application of arrays of silicon photonic microring resonators (biosensors) for the multiplexed detection and label-free quantitation of miRNAs [141]. By using capture ssDNA probes, approximately 150 fmol of miRNA could be detected in 10 min. While such sensitivity is sufficient for many miRNA applications, after addition of S9.6 in the reaction in 40 min, the detection limit was further lowered by approximately three orders of magnitude down to approximately 350 attomoles or 10 pM in 35-µl volume (Figure 1A) [81]. The S9.6 detection of miRNAs was specific, allowing simultaneous multiplexed miRNA analysis [81]. The technology was applied for detection of four different miRNAs extracted from mouse brain total RNA, and the results correlated well with published reports that used other detection methods.

Sipova *et al.* [142] independently reported a novel method for rapid, sensitive and label-free detection of miRNAs with a portable six-channel surface plasmon resonance (SPR) biosensor. Binding of miR-122 to its ssDNA capture probes, covalently attached to the surface of the biosensor chip, was detectable at 0.2 nM. However, addition of S9.6 increased the sensitivity 100-fold (Figure 1B), giving a detection limit of 2 pM, or 900 attomoles in 440 µl. The method was applied for detection of miR-122 levels in total RNA extracted from livers of mice treated with or without acetaminophen, which regulates miR-122 levels. The concentrations measured by the SPR biosensor were in excellent agreement with those obtained by qPCR. One can imagine that the technology, complementary to qPCR or other detection tools, can be applied for rapid investigation of a foodborne disease outbreak or criminal clinical investigation by field scientists using a portable biosensor.

Pri-miRNAs (~80 nt) and pre-miRNAs (~70 nt) should be effectively detected by the antibody-based microarray since the size of the precursors of miRNAs falls in the range of sRNAs in bacteria [129]. The size of miRNAs (~20–25 nt), siRNAs (~19–25 nt) and piRNAs (~23–32 nt) [129] is close to that of Affymetrix DNA probes (25-mer), and the 20-mer [85] or 25-mer probes [133,134,143] have been proven to have the capability to detect the DNA:RNA hybrids as long as sufficient enriched RNAs are provided. For example, 2 µg of size-fractionated total RNA with depletion of 16S rRNA, 23S rRNA and most mRNAs was enough to identify 27 sRNAs in *Caulobacter crescentus* [135,143]. 40 µg of total RNA of *Bacillus anthracis* did not cause any nonspecific binding to DNA probes or array surface in the Affymetrix system [Hu Z, Ravel J, Storz G, Gottesman S, Leppla SH, Unpublished Data]. miRNAs, siRNAs, piRNAs and their precursors can be enriched by isolation kits or by co-immunoprecipitation with their binding proteins. Another method to increase sensitivity of detection is to attach a 20-nt universal tag sequence to all miRNAs (22-nt) – it is now easier to detect miRNAs with 42-nt probes in the antibody-based microarray than with shorter probes. Oligonucleotide spacers may be needed to add at the ends of the short miRNA probes closest to the array surface anyway, to overcome steric interference of the surface and electrostatic surface rejection [144].

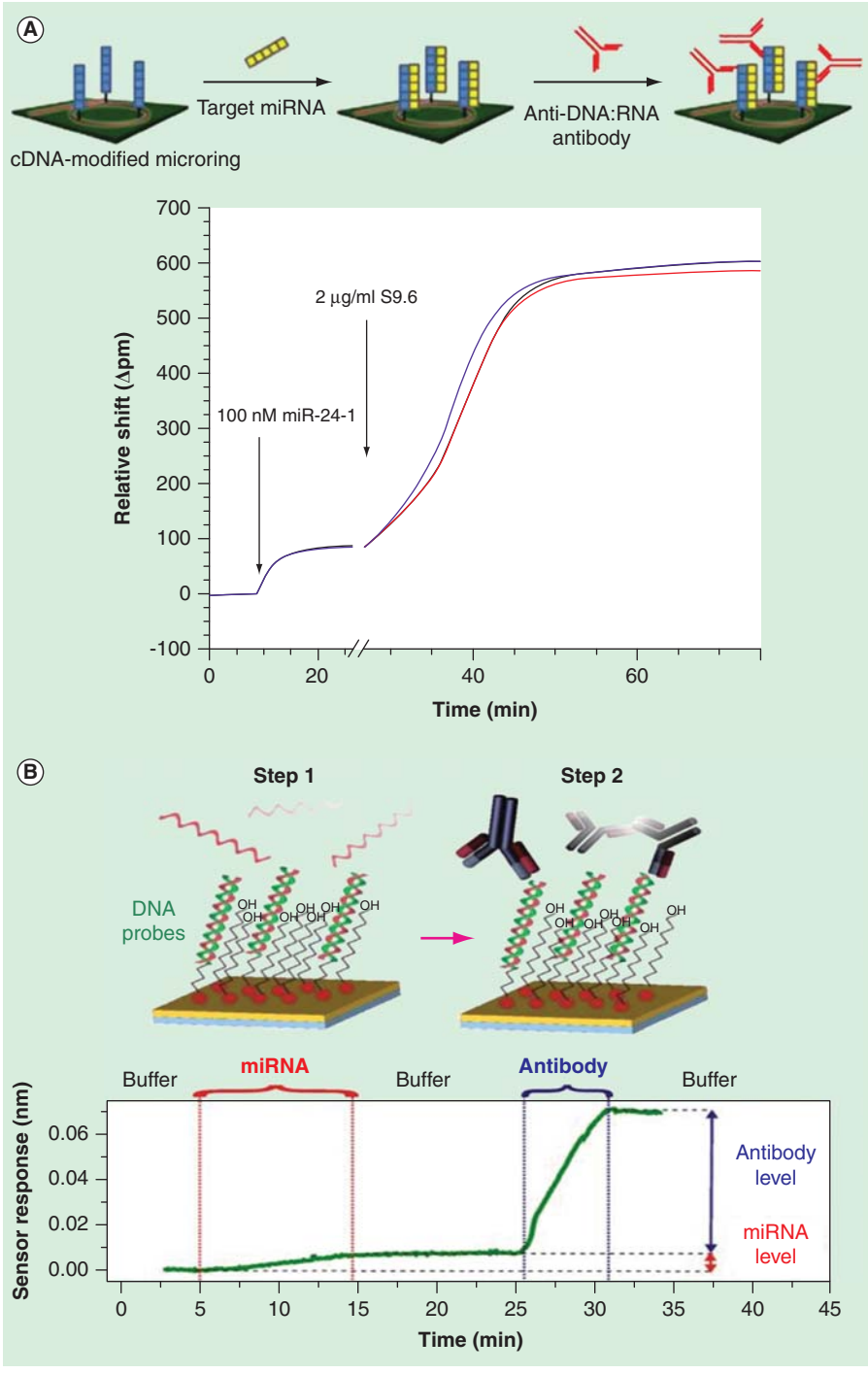

Figure 1. MicroRNA detection by biosensors and S9.6. (A) miRNA detected by silicon photonic microring resonators with S9.6. **(B)** miRNA detected by surface plasmon resonance with S9.6. In both cases, S9.6 significantly enhances the detection signals. Reproduced with permission from [81] © American Chemical Society (2011). Reproduced from [142].

R-loop detection

An R-loop (*R* for *R*NA:DNA hybrid) is a molecular structure where an RNA molecule is partially or completely hybridized with one strand of a dsDNA, and the displaced single unpaired DNA strand forms a loop [145]. Recent reports found that native R-loops are abundant throughout mitochondrial, *E. coli*, yeast and human genomic DNA [146,147]. R-loops can lead to genomic instability since they impede replication fork progress, transcription elongation, as well as exposing the non-template single strand to DNA damage and rearrangements. Cells have developed multiple protection systems to avoid the accumulation of harmful R-loops. R-loops may be also beneficial. Ginno *et al.* proposed that the formation of R-loops prevents CpG islands from being methylated in humans [77]. In *Arabidopsis,* R-loop formation in the promoter region of a long non-coding antisense RNA suppresses its expression and regulates subsequent gene expression [148]. In bacteria, R-loops are formed during the targeting of foreign DNA by the CRISPR immune defense system [149].

Detection of R-loops *in vivo* can be difficult [146]. Three decades ago, antibodies to DNA:RNA hybrids were used for localization of hybrids via *in situ* hybridization [69,74,75]. In the past few years, S9.6 has been used to detect and isolate the DNA:RNA hybrids of R-loops *in vivo* and *in vitro* [77,88–91,137,148,150]. Theoretically, an R-loop location can be detected by anti-hybrid and anti-ssDNA antibodies simultaneously. When cytological immunofluorescent detection of DNA:RNA hybrids was conducted with S9.6 [77,137,150], numerous, sometimes very bright spots with high backgrounds were seen. Chicken anti-DNA:RNA hybrid antibody (IgY) [151], as well as anti-LNA or PNA antibody, may increase detection sensitivity as well as reduce nonspecific background.

Purified polyclonal antibodies specific to DNA:RNA hybrids were used to isolate rDNA (coding for ribosomal RNA) of *Dictyostelium discoideum* by using the R-loop formation in 1978 [70]. Recently, chromatin/DNA:RNA immunoprecipitation (ChIP/DRIP) [88–91,148] has been used to isolate/purify R-loops via the binding of S9.6 to the DNA:RNA hybrids of R-loops. Jel 99 was used for the DNA:RNA hybrid binding for detection of stable episomal R-loops *in vivo* [77]. Genome-wide search for R-loops was done by immunoprecipitation of S9.6 with whole nucleic acid extracted from cells to identify the DNA:RNA hybrids,

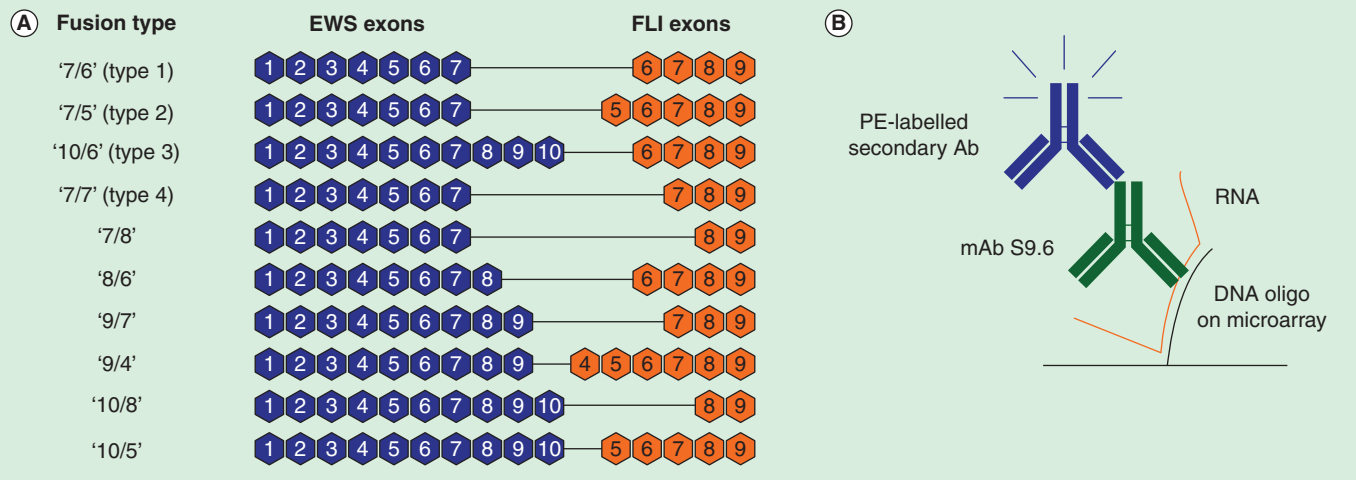

Figure 2. Detection of Ewing sarcoma. (A) Ten different EWS/FLI isoforms are represented by fusions between different EWS and FLI exons. **(B)** DNA probes of the microarray were designed for all possible exon-exon fusions between each of the potential partners. RNA transcripts from samples form DNA:RNA hybrids with the DNA oligonucleotides on the microarray. These DNA:RNA hybrids are recognized by S9.6, which is then detected by PE(R-Phycoerythrin)-labeled secondary antibody.
Reproduced from [87].

followed by high-throughput DNA sequencing. The data strongly suggest that R-loops are formed in thousands of human CGIs (CpG islands) promoters [77].

Detection of eukaryotic cells

Cancer detection

FFPE breast cancer

Formalin-fixed, paraffin-embedded (FFPE) tissue samples are routinely processed and collected in hospitals. Total RNA extracted from FFPE specimens may be severely degraded and cross-linked during the fixation and embedding process. The RNA may not be suitable for subsequent RNA analyses such as RT-qPCR and microarray assays. Schwers *et al.* developed a successful spotted, planar waveguide microarray system for gene expression analysis of FFPE breast cancer samples [138]. S9.6 antibody was conjugated with Alexa 647 directly, an assay format that may explain the high sensitivity achieved (less than 10 fmol/L RNA transcripts relevant to breast cancer). The sensitivity and precision of this microarray correlated well with those of RT-qPCR [138]. We suggest that the degraded RNA from the FFPE tissue is equivalent to the RNA from other samples that is intentionally fragmented to facilitate better RNA:DNA hybridization to microarrays.

Human chronic myeloid leukemia

PCR-EIA was used to detect reciprocal translocations between chromosomes 9 and 22, which generate chimeric *bar-abl* mRNA transcripts, leading to human chronic myeloid leukemia [42,152]. Previous research identified the specific breakpoints for the translocations, allowing design of an RT-PCR to amplify the region containing the translocation breakpoints. EIA with anti-dsDNA mAb 27–14-D9 was used to confirm the specific translocation of the RT-PCR products and to increase the detection sensitivity. The method was as good as ^{32}P-labelled probes and it could detect one positive cell out of 10^5 negative cells in cell culture. It identified six translocation positive cases out of 10 clinical patients; five had the b3/a2 splice, and one had a b2/a2 splice [152].

Ewing sarcoma

Luo *et al.* developed an S9.6-based microarray for the detection of genomic translocations in Ewing sarcoma samples [87]. Ewing sarcoma frequently includes genomic breakpoints in introns of Ewing sarcoma breakpoint region1 (EWSR1) and Friend leukemia virus integration 1 (FLI1). EWS/FLI has at least 10 different transcript fusions between EWS and FLI exons (Figure 2A). Detection of translocations at the exon level has been difficult. DNA microarray probes were designed for all possible exon-exon fusions (Figure 2B). RNA was hybridized to the microarray and hybrids were detected with antibody S9.6. The test was very sensitive, with the EWS/FLI 7/6 translocation event detected in as little as 0.2 μg of total RNA (~3.3×10^{-2} attomoles of fusion transcript). The test was capable of detecting translocation transcripts not only from fresh tumor specimens and cell lines, but also from frozen tumors and FFPE samples.

Detection of metakaryotic stem cells

Certain stem cells found in the primordial organs and tumors have large, open-mouthed, bell-shaped nuclei without obvious nuclear membranes [153,154]. The nuclei divide by symmetrical and/or asymmetric amitoses. They were named 'metakaryotes' because their nuclear fission behaviors lie between the prokaryotic division without chromatin condensation and the eukaryotic complete condensation of chromatin in mitosis. Interestingly, during duplicating a dsDNA helix to form two identical copies of sister dsDNA helices, nuclei of metakaryotic stem cells

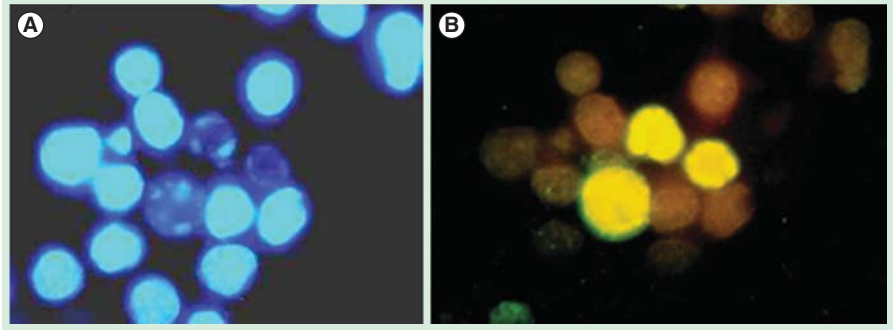

Figure 3. Detection of apoptotic cells. Fluorescence microphotographs of MB-MDA-468 cells treated with staurosporine, heated in formamide, stained with mAb F7–26 and counterstained with DNA fluorochrome DAPI. The same field is shown **(A)** after UV excitation for DAPI and **(B)** visible light excitation for fluorescein-labeled antibody. Note that only the three apoptotic cells having chromatin condensed at the nuclear periphery are stained with the mAb.
Images and legends are reproduced with permission from [156] © Apostain Inc.

undergo genomic replication and segregation employing a substantial fraction (~10–100%) of a double-stranded DNA:RNA hybrid intermediate(s), an unorthodox mechanism for most mitotic eukaryotic cell divisions. The hybrids were detected using purified labeled polyclonal anti-hybrid antibody. Following treatment with RNase A, antibody F7–26 or acridine orange could detect the ssDNA.

Detection of apoptotic cells (Apostain & TUNEL)

Apoptosis is the natural, beneficial process of programmed cell death that may occur in multicellular organisms, while necrosis is the premature death of cells and living tissue caused by external factors such as infection, toxins or trauma. mAb F7–26 was used to detect apoptosis cells but not necrotic cells, nor cells with DNA breaks in the absence of apoptosis [155]. Apostain Inc. (Surfside, FL) was founded on the basis of this antibody staining method. Condensed chromatin of apoptotic cells is treated with heat and formamide, inducing DNA denaturation *in situ* only in apoptotic nuclei (Figure 3) [156]. Apoptotic cells can be detected in sections of frozen or FFPE tissues by immunohistochemistry, and in cell suspensions by flow cytometry or fluorescence microscopy. When combined with ELISA for high-throughput screening of anticancer drugs in microtiter plates, the method can discriminate anticancer drugs from toxic chemicals, predict selective toxicity to cancer cells and detect drug synergism [23,24,155]. It seems that Apostain is more specific than the other popular apoptosis detection method, TUNEL (the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling), and it can detect apoptosis at an earlier stage [157]. Another apoptosis TUNEL assay utilizes Br-dUTP (bromodeoxyuridine triphosphate), which is more readily incorporated into DNA strand breaks than other larger ligands such as fluorescein, biotin or digoxigenin. The Br-dUTP sites are identified by a red fluorescence labeled anti-BrdU mAb (Abcam, ab66110), resulting in brighter signals.

Expert commentary

What's old is new again. We have highlighted certain applications of anti-nucleic acid antibodies in clinical microbiology and genomic diagnostics. Because nucleic acids are the most important, essential biological macromolecules and are abundant in all living things, perhaps every life scientist might have some interest in antibody-based nucleic acids analyses. We hope this review will encourage others to revisit anti-nucleic acid antibodies offer in research and clinical applications, and to consider generating and improving additional antibodies for various types of nucleic acids.

Five-year view

An S9.6-based tiling microarray was used to identify candidate sRNAs and riboswitches in *Bacillus anthracis* [Hu Z, Ravel J, Storz G, Gottesman S, Leppla SH, Unpublished Data]. Despite a relatively low efficiency due to use of 25-mer probes on an Affymetrix microarray, good evidence was obtained for sRNAs, riboswitches and T-boxes. Hfq-associated sRNAs were identified by comparison of sRNAs in wildtype strains with those in Hfq mutant strains. Today, many bioinformatics programs for prediction of non-coding RNAs are available on the web; genome-wide searches for non-coding RNAs by the antibody-based microarray should provide an unbiased, independent experimental tool to test these predictions. For example, long non-coding RNAs should be easily and effectively identified by the antibody-based microarray.

Combining anti-nucleic acid antibodies with chemically modified nucleic acids may yield especially effective analytical tools. For example, LNA-modified oligonucleotide probes have been used in miRNA Northern blot and *in situ* hybridization in plants and animals [158,159]. One can imagine that signals from the LNA:RNA or PNA:RNA hybrids in Northern blot (and *in situ* hybridization) can be further amplified by binding specific anti-hybrid antibody, conjugated with enzymes, nanoparticles [160] or other detectable labels, resulting in higher sensitivity and specificity ('NorthWestern blot'). Additional mAbs to LNA:RNA, LNA:DNA, PNA:RNA and PNA:DNA may be needed. Since PNA and LNA are unnatural polymers, these antigens may be more immunogenic than natural nucleic acids. Another avenue for optimizing these antibody reagents would be used to determine crystal structures for complexes of antibodies S9.6, 27–14-D9, F7–27, D44, J2 and others with their nucleic acid ligands. Such structures would facilitate computer-aided design of improved antibodies and would guide focused mutagenic screens, including phage display, for variant antibodies with increased affinities and improved specificities [160,161].

An alternative method to enhance assay sensitivity is to label antibodies such as F7–27, 27–14-D9, S9.6, D44 and J2 with

Table 2. Applications of anti-nucleic acids antibodies in genomic detection and clinical diagnostics since 1987.

Detection targets	Antibodies	Techniques involved	Sensitivity	Specificity	Note	Year	Ref.
Bacillus subtilis 23S rRNA	S9.6 F(ab') detecting	Hybrid capture	1000 cells/assay	Very low cross-hybridization of leukocyte lysate in solution	Hybrid formations completed within 5 min	1987	[100]
Campylobacter jejuni 16S rRNA	Anti-DNA:RNA pAb capturing	Hybrid capture	70,000 cells/sample; 98.7%	98.20%	Rapid (2.5 h) and simple	1987	[98]
E. coli 23S rRNA	S9.6 F(ab') detecting	Antibody detection	500 cells/assay; 96.2%	Negative predictive value 91.2%	DNA probes on latex particles formed hybrids in 10–15 min	1988	[101]
Plasmid pSP65	S9.6 F(ab') detecting	Hybrid capture	0.16 pg cRNA in 50-µl assay	5 pg DNA in 1 µg foreign DNA	Hybrid formed in 32 min; probe length important	1989	[95,96]
Rotaviruses dsRNA	S9.6 capturing and detecting	Sandwich immunoassay	10 PFU of standard strains of group A rotaviruses	Other rotaviruses can be detected, dsRNA specific	Using S9.6 anti-dsRNA property	1989	[82]
HIV RNA	S9.6 F(ab') detecting	Hybrid capture	0.05 pg RNA in 50-µl assay, 93.5%	94.60%	Comparable to a ^{32}P-labeled probe in a dot hybridization	1989	[112]
Enteroviruses RNA	S9.6 F(ab') detecting	Hybrid capture	500 pfu/ml of coxsackievirus B3	Also coxsackie B1, A1–6, 15, poliovirus types 1–3, enteroviruses 7, 11, 71	Probes length 1100–3520 base; hybridization done in 100 min	1989	[114]
IL-1, 2 mRNA	S9.6 F(ab') detecting	Hybrid capture	IL-1 mRNA detectable in 50 ng of total RNA	High	Comparable to a ^{32}P-labeled probe	1990	[102]
Chlamydia trachomatis MOMP gene	S9.6 F(ab') detecting	PCR-EIA	All 46 culture-positive specimens tested positive	From 58 culture-negative specimens, 56 negative and 2 positive	Sensitive assay	1990	[107]
HIV *gag*	S9.6 F(ab') detecting	PCR-EIA	10 copies of HIV genome, 97%	100.00%	Assays can be done in 2–4 h	1990	[113]
RNA transcripts	D44 capturing and detecting	RNA capture	A few picogram, if submicrogram of D44 used	Specific to RNA or RNA-binding proteins	RNA size of 100 – thousands nt tested and worked; rapid (1 min)	1991	[65]
HPV type 16 E7-E1 DNA	S9.6 F(ab') detecting	PCR-EIA	3 copies of HPV 16 genome	21 of 81 specimens test positive for HPV 16	Streptavidin binding biotinylated hybrids efficiency variable	1992	[117]
Human chronic myeloid leukemia	27–14-D9 detecting	PCR-EIA	Detect one positive cell out of 10^5 negative cells	Identified six translocation positive cases out of 10 clinical patients	Prototype of GEN-ETIK-DEIA kit;as good as ^{32}P-labelled probes	1993	[152]

Table 2. Applications of anti-nucleic acids antibodies in genomic detection and clinical diagnostics since 1987 (cont.).

Detection targets	Antibodies	Techniques involved	Sensitivity	Specificity	Note	Year	Ref.
Listeria monocytogenes hlyA	Anti-DNA:RNA pAb detecting	PCR-EIA	Less than 5 cells/assay	*L. monocytogenes*-specific	An enhancement of 200-fold in sensitivity over regular PCR	1994	[109]
Plant viruses ssRNA	J2,K2 capturing and detecting	ELISA,immunoblot	21/25 positive by ELISA; 18/25 positive by immunoblot	2–20 fold difference between infected and control samples by ELISA	Multiplexed, rapid, simple, sensitive; good for cryptic viruses	1994	[55]
Listeria genus rRNA	6B5 capturing, anti-DNA:RNA pAb detecting	Hybrid capture	2.5 pg rRNA, 500 cells from inoculated meat homogenate	Listeria spp genus-specific	Biotin-streptavidin detecting gave high background signals	1995	[108]
Yersinia enterocolitica 4 genes	20D3 or 6B5 detecting	PCR-EIA	0.5–62.5 ng of target DNA	Specific to pathogenic *Yersinia enterocolitica*	Multiplexed, rapid, simple, efficient assay, dot blotting	1995	[110]
HPV type 18 L1 DNA	S9.6 F(ab') detecting	PCR-EIA	6 copies of HPV 18 genome	97.70%	Low-level cross-reactivity to some HPV types	1995	[115]
Helicobacter pylori ureC	27–14-D9 detecting	PCR-EIA	89.3–94.4%	81.0–90.4%	GEN-ETIK-DEIA kit used	1997	[111]
HSV genome	Anti-DNA:RNA, pAb capturing, mAb detecting	hybrid capture	5000–10000 copies of HSV genome/assay, 93.2%	100.00%	Better than culture method	1997	[119]
Hepatitis A, B, C, D viruses genes	27–14-D9 detecting	PCR-EIA	10–400 genome copies/ assay	Specific	DiaSorin GEN-ETIK-DEIA built on these experiments	1999	[42,120–122]
C. trachomatis genome	Anti-DNA:RNA, pAb capturing, mAb detecting	Hybrid capture	95.4–97.7%	98.2–99.8%	US FDA cleared HC in 2001, on market today	2002	[103–106]
Neisseria gonorrhoeae genome	Anti-DNA:RNA, pAb capturing, mAb detecting	Hybrid capture	92.2–98.9%	98.5–99.4%	FDA cleared HC in 2001, on market today	2002	[103,104,106]
HPV genome	Anti-DNA:RNA, pAb capturing, mAb detecting	Hybrid capture	1×10^3–5×10^7 genomes in a single assay	With Papanicolau test, almost 100% negative predictive value	FDA approved HC in 2003, 10 millions doses/ year today	2003	[97,116]
E. coli sRNAs	Anti-DNA:RNA, pAb detecting	Microarray	More sensitive than cDNA- or cRNA-based microarray	sRNAs need be confirmed by other techniques, such as Northern blot	Digene ExpressArray kit	2003	[133]
Human bcl-2 Mbr triplex	Jel318, Jel466, detecting	Gel shift, EM	Triplexes detected, matched with other experiments	Peak III and peak I detected	Jel 318 and Jel 466 detect different triplexes	2005	[172]

Table 2. Applications of anti-nucleic acids antibodies in genomic detection and clinical diagnostics since 1987 (cont.).

Detection targets	Antibodies	Techniques involved	Sensitivity	Specificity	Note	Year	Ref.
Human genomic methylation DNA	Anti-5-MeCyd (α-5mC)	MeDIP, microarray	High-resolution analysis of DNA methylation sites	Difference detected among different cells	Unbiased detection of methylated DNA	2005	[173]
Pine cryptic virus	J2 detecting	Immunoblot	Existence of a new putative cryptovirus in pine tree	1.5 and 1.58 kb dsRNAs identified	Cryptic virus identification example	2006	[123]
E. coli sRNAs	S9.6 detecting	Microarray	250 amol, 10 pM in a 25-μl reaction	sRNAs need be confirmed by other techniques	Sensitive, simple, rapid, quantitative, reproducible, inexpensive	2006	[85]
Apoptotic cells	F7–26, AP-13 detecting	Apostain	Sensitive, can detect apoptosis at an earlier stage	Specific	More specific than TUNEL with dUTP-digoxigenin	2006	[157]
Cytomegalovirus (CMV) genome	Anti-DNA:RNA, pAb capturing, mAb detecting	Hybrid capture	2600 copies of CMV genome per ml	Lower than qPCR	qPCR replaced HC CMV owing to speed, sensitivity, specificity	2007	[118]
Eukaryotic R-loops	S9.6 detecting	Immunofluorescence	A crowd of fluorescent speckles represening DNA:RNA	Comparable to the number (10^5) detected by other methods	R-loop detection *in vivo*	2007	[150]
S. pombe transcriptome	S9.6 detecting	Microarray	Abundant, reproducible RNA transcripts detected	Some verified by qPCR	60mer probes used, data unbiased	2008	[86]
Breast cancer genes	S9.6 detecting	Microarray	Less than 10 fmol/L	Correlation coefficient of 0.87 to state-of-the-art RT-qPCR technology	Precision comparable to RT-qPCR; worked for FFPE samples	2009	[138]
microRNA	S9.6 detecting	Biosensor	Approximately 900 amol or 2 pM in 440-μl sample volume	Specific in surface plasmon resonance biosensor	S9.6 lowered detection limit from 200 pM to 2 pM	2010	[142]
HPV genotype	mAb anti-DNA:RNA, capturing and detecting	Luminex XMAP	100 copies or less	No cross-reactivity among the HPV types up to 1 million copies	Cohen's kappa = 0.72 with PCR and HC2	2010	[127]
microRNAs	S9.6 detecting	biosensor	Approximately 350 amol or 10 pM in 35-μl sample volume	Specific in the silicon photonic microring resonators	S9.6 lowered approximately 3 orders of magnitude for detection limit	2011	[81]
Yeast R-loops	S9.6 detecting	Chromatin/ DNA:RNA IP	R-loops IP with S9.6 and amplified by qPCR	Control low signals	R-loop detection *in vitro*	2011	[89]
Apoptotic cells	Anti-BrdU mAb	TUNEL	Better than TUNEL with dUTP-digoxigenin	Use bromolated deoxyuridine triphosphate nucleotides	More readily incorporated than fluorescein, biotin, digoxigenin	2011	abcam, ab66110

Table 2. Applications of anti-nucleic acids antibodies in genomic detection and clinical diagnostics since 1987 (cont.).

Detection targets	Antibodies	Techniques involved	Sensitivity	Specificity	Note	Year	Ref.
Caulobacter crescentus sRNAs	Anti-DNA:RNA, pAb or S9.6 detecting	Microarray	27 novel sRNAs identified	Verified by Northern blot	Polyclonal or monoclonal anti-DNA:RNA hybrid antibodies used	2012	[135,143]
Human R-loops	S9.6, Jel 99 detecting	IP, sequencing	Thousands in CGIs promoters and gene 3′-ends	Helped with computational predictions with software program	Genome-wide search for human R-loops	2012	[77,174]
Human Ewing sarcoma	S9.6 detecting	Microarray	Approximately 3.3×10^{-2} amol of fusion transcript	Specific, confirmed by qPCR and sequencing	Also worked for FFPE specimens	2012	[87]
Human DNA G-quadruplex	BG4 detecting	Immunofluorescence	K_d = 0.5–1.6 and 2 nM, intra- and inter-molecular quadruplexes	Specific to G-quadruplex, no particular conformation preference	BG4 generated by phage display	2013	[175]
Human RNA G-quadruplex	BG4 detecting	Immunofluorescence	K_d = approximately 5.5–18 nM, RNA G-quadruplexes	RNA G-quadruplexes reported in human cells	BG4 binds both DNA and RNA G-quadruplexes	2014	[176]
Human, murine G-quadruplex	1H6 detecting	Immunofluorescence	Affinity for many, but not all quadruplexes	Specific to G-quadruplexes, no cross-reaction to other nucleic acids	Murine mAb 1H6 generated with quadruplex as immunogen	2014	[177]
Metakaryotic stem cells	Anti-DNA:RNA pAb and F7–26 detecting	Immunofluorescence	Detecting pangenomic DNA:RNA intermediates	Need confirmed by bell-shaped nuclei	Significant for diagnostic, prognostic, treatment for diseases	2014	[154]

dsDNA tags, which are then used for signal generation (real-time) by quantitative immune-PCR (qiPCR) [162]. Such (real-time) qiPCR would be a powerful tool for detecting ultra-low quantities of ssDNA, dsDNA, DNA:RNA, ssRNA and dsRNA. For example, qiPCR may be a simple and effective method to detect very short RNAs, such as miRNA, via miRNA:DNA hybrids. HC techniques can be applied for capturing specific hybrids. qiPCR may be able to detect exRNAs [49] and intracellular dsRNAs by using different anti-RNA antibodies. exRNAs and intracellular dsRNAs can also be identified by immunoprecipitation with anti-RNA or anti-DNA:RNA antibodies, following by qPCR, antibody-based microarray or sequencing [77].

HC and microarray have demonstrated that labeled immunoassay is a sensitive and specific method for nucleic acid detection. In many cases, it may be not necessary to make cDNA or cRNA, as is currently required by many assays, since anti-nucleic acid antibodies are available for direct detection. mAbs F7–27, 27–14-D9, S9.6, D44 and J2 can be coupled to beads or columns (or just immunoprecipitation), which will be excellent tools for specific purification of ssDNA, dsDNA, DNA:RNA, ssRNA or dsRNA, as well as proteins binding to specific types of nucleic acids, from nucleic acid mixtures [65]. Antibodies J2 and K2 may also be able to detect certain human RNA viruses *in vivo*. Modified antibodies may be the basis of therapeutic agents targeting specific extracellular or intracellular nucleic acids (anti-protein antibodies are already widely used in clinical applications).

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Disclaimer

Some antibodies and other research tools described here are available from commercial sources. We are not

recommending a particular product, but only providing an example of where it might be available. The authors do not stand for official FDA and NIH opinions of the biologic, drug or device products mentioned in this article.

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Key issues

- Immunoassays for detection of nucleic acids as antigens are not frequently used. This is most likely because:
  - PCR and sequencing methods have high sensitivity and specificity.
  - Nucleic acids are poor immunogens, making it difficult to elicit antibodies specific to different types of nucleic acids.
  - The anti-nucleic acid antibodies created decades ago are now largely neglected.
- Anti-nucleic acid antibodies include types that react with modified nucleosides and nucleotides, single-stranded DNA, double-stranded DNA, single-stranded RNA, double-stranded RNA, DNA:RNA hybrids, locked-nucleic acids or peptide nucleic acid:nucleic acid hybrids and others.
- Anti-hybrid antibodies, combined with hybrid capture and PCR-EIA, are used for simple and rapid detection of bacteria and viruses.
  - The most notable applications are the US FDA-approved Qiagen's Digene Hybrid Capture technologies for *in vitro* diagnostic tests of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and HPV.
- Current microarray systems have problems detecting low concentrated small non-coding RNAs due to the multiple steps involved in labeling and synthesis of cDNA or cRNA.
  - Antibody-based microarray detection is sensitive and unbiased since synthesis of cDNA or cRNA is avoided. It was successfully applied for bacterial sRNA detection, yeast transcriptome mapping and cancer detections from FFPE samples.
- Anti-nucleic acids antibodies, combined with biosensors, ChIP/DRIP, sequencing and immunofluorescence, are used for successful detections of miRNAs, R-loops, metakaryotic stem cells and apoptotic cells.
- Anti-nucleic acid antibodies, together with modern technologies, can lead to the expanding or upgrading of current methods widely used in clinical labs and biological research.

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