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A single electrochemical biosensor designed to detect any virus

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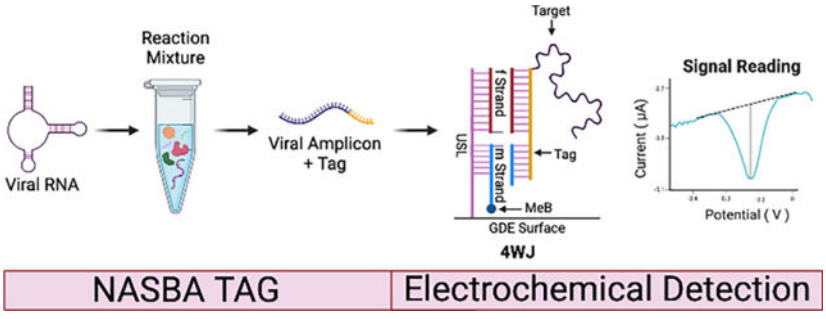
Abstract

Viruses are the primary cause of many infectious diseases in both humans and animals. Various testing methods require an amplification step of the viral RNA sample before detection, with Quantitative reverse transcription polymerase chain reaction (RT-qPCR) being one of the most widely used, along with lesser-known methods like Nucleic Acid Sequence-Based Amplification (NASBA). NASBA offers several advantages, such as isothermal amplification and high selectivity for specific sequences, making it an attractive option for low-income facilities. In this research, we employed a single Electrochemical biosensor (E-Biosensor) designed for potentially detecting any virus by modifying the NASBA protocol. In this modified protocol, a reverse primer is designed with an additional 22-nucleotide sequence (tag region) at the 5'-end, which is added to the NASBA process. This tag region becomes part of the final amplicon generated by NASBA. It can hybridize with a single specific E-Biosensor probe set, enabling subsequent virus detection. Using this approach, we successfully detected three different viruses with a single E-Biosensor design, demonstrating the platform's potential for virus detection.

Graphical Abstract



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Author Contributions

K.C.T., F.T.S., J.O. conceived and planned the work. F.T.S. implemented the NASBA protocol. F.T.S., J.O., C.C. conducted experiments and analyzed the results. Y.G., K.C.T. supervised the project. J.O. wrote the initial draft of the manuscript with contributions from all authors. J.O., F.T.S., C.C., K.C.T., Y.G. edited.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website.

Materials and reagents, E-biosensor preparation and characterization, sequences of all nucleotides used in this study and agarose gel electrophoresis analysis of NASBA amplicons for HIV, Zika and Influenza. (PDF)

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Keywords

Nucleic Acid Sequence Based Amplification; biosensors; HIV; Zika Virus; Influenza A Virus

Introduction

Currently, the challenge of disease tracing remains a global issue. Different organizations promote the development of new techniques that could be applied at point-of-care (POC) testing centers as a way to get testing closer to the patients, especially in low-income countries, where there is limited access to sophisticated clinical infrastructure.[1,2]

One of the most employed nucleic acid amplification techniques (NAATs), and currently the gold standard for RNA amplification, is reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR converts RNA into cDNA through the reverse transcriptase enzyme, which is then used as a template for amplification through repeated cycles of heating and cooling in a reaction mixture containing DNA template, DNA polymerase, primers, and nucleotides.[3–5] Despite the numerous advantages of RT-PCR, several studies have indicated that, in the case of SARS-CoV-2, it is necessary to use both RT-PCR and antibody techniques to accurately confirm the results,[6,7] owing to the inconsistency of RT-PCR assays.[8] Furthermore, the high associated costs have prompted the proposal of various alternative NAATs.

NAATs have already demonstrated the capability to detect viral RNA at early stages.[9,10] They exhibit advantages over antibody-based techniques, which have certain limitations. For instance, immunoglobulins might not appear until the 5th or 14th days after infection,[11] and the false-positive rate is significantly smaller with NAATs.[12] Thus offering the advantage of and accurate early disease detection.[10]

Many studies have made efforts to provide different cycling conditions to enhance the NAATs amplification step.[13] Example alternatives include the use of Loop-Mediated Isothermal Amplification (LAMP) for SARS-CoV-2 and Influenza viruses,[14] the use of Rolling Circle Amplification (RCA) for Dengue,[15] and the use of Nucleic Acid Sequence-Based Amplification (NASBA) for Zika and SARS-CoV-2.[16,17]

NASBA is particularly interesting because it can operate at temperatures ranging from 37 to 42°C, eliminating the need for specialized thermocycling equipment. Additionally, the primer design for NASBA is relatively straightforward, involving the addition of a T7 RNA polymerase recognition sequence to one of the primer pairs.[18,19]

Currently, NASBA has been successfully coupled with an electrochemical biosensor (E-Biosensor) as a readout system.[16,17] However one significant drawback is the need to design a new E-biosensor DNA probe for each virus that needs to be analyzed, requiring different designs and protocols for each virus. To address this challenge, we have introduced a 22-nucleotide sequence (tag) on the NASBA reverse primer. This modification generates a tag RNA section at the 5’ end of the RNA amplicon. Consequently, only one E-Biosensor design can be used to detect multiple viruses, simplifying the process, and making it more versatile.

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Experimental Section

Material and Reagents

Tris(2-carboxyethyl phosphine hydrochloride (TCEP), Trizma hydrochloride (Tris-HCl), 6-Mercaptohexanol (MCH), and magnesium chloride ($MgCl_2$) were purchased from Sigma Aldrich (St. Louis, USA). TE buffer, NASBA Primers, m-MeB strand, f-strand were obtained from Integrated DNA Technologies (Coralville, USA). The universal stem-loop (USL) was purchased from Biosearch Technologies, Inc. (Petaluma, USA). All oligonucleotide sequences used are shown in Table S1. NASBA kits (including NASBA buffer, ribonucleotides, and enzyme cocktail) were purchased from Life Sciences Advanced Technologies (St. Petersburg, USA). Ultrapure Agarose-1000 and SYBR Safe dye were obtained from Thermo Fisher Scientific (Waltham, MA). Purified HIV-1 RNA, group O (HIV-O) and group M subtype B (HIV-B) with GenBank number AF447819 and AY173952, respectively, were obtained from SeraCare Life Sciences (Milford, MA). Genomic RNA from Influenza A Virus (InfA), A/Wisconsin/67/05 (HA, NA) x A/Puerto Rico/8/34 (H3N2), Reassortant X-161B-NR-10045; Influenza B Virus (InfB), B/Ohio/01/2005 (Victoria Lineage), NR-43753; Zika Virus, strain PRVABC59, NR- 50244 isolated from the blood of a human in Puerto Rico with the complete genomic sequence GenBank: KU501215; Dengue Virus Type 1, Hawaii, NR-4287 and GenBank: EU848545, were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID (Manassas, VA). ssRNA ladder and RNA loading dye were obtained from New England Biolabs (Ipswich, MA). NaCl, NaOH, and H_2SO_4 were purchased from Fisher Scientific (Pittsburgh, USA). Gold disc electrodes (GDEs) were purchased from CH Instruments (Austin, USA). Alumina slurry (1.0, 0.3, and 0.05 μm) was obtained from Buehler (Lake Bluff, USA). All aqueous solutions were prepared with deionized water (18.2 MΩ cm) using a Siemens PURELAB Ultra system (Lowell, USA).

Immobilization buffer (IB) was prepared as follows: 50 mM Tris-HCl and 250 mM NaCl. The hybridization buffer (HB) was prepared using 50 mM Tris-HCl, 100 mM NaCl, and 50 mM $MgCl_2$. The pH of both buffers were adjusted to pH 7.40 using a 1.0 M NaOH solution.

Electrochemical Measurement

Square-wave voltammetry (SWV) was performed using a CHI660D Electrochemical Workstation. A three-electrode system was used where the modified GDE, a platinum wire, and Ag/AgCl (1 M KCl) were used as working, counter, and reference electrodes, respectively. SWV was performed in HB from 0.0 to −0.5 V, at a frequency of 100 Hz, amplitude of 70 mV, and step potential of 3 mV. Before measurements were taken, nitrogen was bubbled into the HB solution of the electrochemical cell for 15 minutes to remove dissolved oxygen. Measurements were taken, in triplicate, after immobilization with MCH to obtain a baseline signal and after 4WJ formation at 25 °C.

E-biosensor preparation

The GDEs were initially cleaned via immersion in piranha solution (1:3 ratio of 30% H_2O_2 and concentrated H_2SO_4. Caution: The piranha solution releases heat and should be handled with care) for 10 minutes. Next, the electrodes were manually polished using a microcloth

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and alumina slurry (1.0 μm, then 0.5 μm, then 0.03 μm) and sonicated in ethanol and deionized water to remove any residual alumina from the electrode surface, for 2 minutes each. Lastly, the GDEs were activated via cyclic voltammetry (CV) from 1.6 to −0.1 V at a scan rate of 100 mV/s in 0.5 M H_2SO_4. The electrochemically active area of each GDE was calculated using the reduction gold peak from CV.

To prepare the USL for immobilization onto the GDE, the disulfide bond was reduced by adding 25 μL of 1.0 mM TCEP in IB to 10 μL of 1.0 μM USL in TE buffer. This solution was then shaken for 1 hour at 25 °C. After shaking, the solution was further diluted using 65 μL IB to a final concentration of 0.1 μM of USL. 15 μL of the USL solution was then drop-casted onto the surface of each GDE and incubated for 30 minutes. The electrodes were then rinsed with IB and dried with nitrogen, and then drop-casted with 15 μL of 2 mM MCH in IB and allowed to incubate for 30 minutes.

E-Biosensor Characterization

The target was diluted to the appropriate concentration in HB and then mixed with 0.25 μM m-MeB strand and 0.50 μM f strand. 15 μL of this solution was then drop casted onto the electrode surface and incubated for 60 minutes. SWV was used to obtain signal measurements, as described above. The signal, expressed as the current density (jp), was measured for three electrodes, after the 4WJ formation, and the baseline signal was subtracted. These values were then averaged for each concentration. A calibration curve for Zika amplicons was constructed ranging from 2×10^1 to 2×10^4 copies/μL.

NASBA tagging Reaction.

The purified RNAs were used as templates for NASBA. The reaction was carried out using a NASBA Kit (Life Sciences Advanced Technologies, Inc., St Petersburg, FL) with slight modifications to the manufacturer’s instructions. The total volume of each reaction mixture was 12 μl, and the reaction time conditions were set to 90 minutes.

The samples were prepared by mixing 4 μl of 3x NASBA Reaction Buffer, 2 μl of 6x Nucleotide Mix, 2 μl of 1 μM primer mix (which include our designed Reverse primer with the tag fragment), and 1 μl of RNase-free water (for the no-target control, NTC) or RNA template. The samples were incubated at 65 °C for 2 minutes, followed by cooling to 41 °C for 10 minutes. Subsequently, 3 μl of 4x NASBA enzyme cocktail were added, and the samples were incubated at 41 °C for 90 minutes.

The samples were analyzed by electrophoresis in a 2% agarose gel containing SYBR Safe DNA Gel Stain and visualized using a Bio-Rad Gel Doc XR+ with Image Lab software. The amplicon, which served as the target, was purified from the other components of the amplification reaction mixture using an RNA Clean & Concentrator-5 RNA clean-up kit (Zymo Research, Corp., Irvine, CA).

Results and Discussion

A crucial capability that conventional RT-PCR lacks is the ability to quantify viral load, which has been proven important for estimating transmission probability and

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understanding disease transmission across multiple variants.[20] To provide a platform capable of quantification, Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) incorporates a fluorescent dye into the common RT-PCR protocol. This dye interacts with double-stranded cDNA, enabling real-time detection, as illustrated in Scheme 1.[4] This protocol follows the same denaturation, annealing, and extension steps in thermocycling conditions as regular PCR.

Our proposed methodology, which also possesses the ability to quantify viral load through an electrochemical detection readout system, involves an initial amplification step using a modified NASBA process. Beyond its benefits as an isothermal technique operating at 41–65 °C, this process incorporates a tag-designed reverse primer that adds a specific 22-nt sequence to the RNA amplicon (see Scheme 1, yellow sequence). This primer also includes the promoter region, enabling the reverse transcriptase enzyme, similar to RT-qPCR, to convert RNA into cDNA. Subsequently, a combination of RNase and RNA polymerase produces multiple tagged RNA amplicons (see Scheme 1).

The tagged fragments of the amplicons now function as a hybridized region which allows binding to our E-biosensor design. With this protocol, selectivity relies on primer design, requiring only a single E-Biosensor design as a readout system. For this purpose, a 4WJ-based E-biosensor was selected. The 4WJ (see Scheme 2) consists of a universal stem-loop strand (USL) attached to the electrode's surface through a thiol bond, along with two auxiliary strands (m and f). These auxiliary strands contain complementary fragments to both USL and the tag region of the target. This design follows the concept of multicomponent hybridization probes,[21] where the 4WJ forms only if both target-binding fragments of the m- and f-strands fully complement. The m strand is covalently modified with a methylene blue (MeB) dye, enabling the electrochemical signal once the 4WJ structure is formed. By being designed to hybridize with the specific tag sequence, a single E-biosensor design may be suitable for potentially detecting any virus (Scheme 2).

As a proof of concept, we evaluated the detection of three viruses (see Scheme 2). Different sets of primers were developed for tagging and amplifying HIV-1 group O (HIV-O), Zika virus (Zika), and Influenza A (InfA) virus. To test the selectivity of the primers, each viral RNA target template has a negative control (NC): HIV-1 group M, subtype B (HIV-B) would be used as a negative control for HIV-O primers, Dengue RNA would be used as a negative control for Zika primers, and Influenza B (InfB) would be used as a negative control for InfA primers.

In our primer design, the 22-nucleotide tag sequence was incorporated into each reverse primer adjacent to the promoter region (AATTCTAATACGACTCACTATAG sequence on Scheme 1 in yellow). The NASBA primer design followed the protocol established by Deiman et al.,[22] with minor adjustments. In our case, the sequence tag was included in the reverse primer rather than the forward primer, and the melting temperatures for the hybridizing sequences were slightly higher than the recommended 48–51°C range.[22] This decision is based on our experience, where easy access to the hybridizing region, guided by its secondary structure, is more relevant than slightly higher melting temperatures. Additionally, even though the 65°C annealing step can be omitted to streamline the

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protocol,[23] we opted to retain this step due to the additional time needed for the creation of the amplicons.

After obtaining the RNA tag amplicons, a cleanup procedure was performed to remove any potential remaining reverse primer. Figure 1 displays the current density signal of each set of amplicons. In all cases, both the NTC and the NC exhibited signals that were lower or equal to the blank (compared using a t-test). In contrast, the Zika, HIV-O, and InfA RNA tag amplicons displayed robust signals, confirming that the same biosensor design effectively and selectively detected all three tag RNA samples. Gel electrophoresis results, presented in Figure S1, confirms the amplicon sizes of 151 nts for Zika, 180 nts for HIV-O, and 209 nts for Influenza A.

Additionally, this platform incorporates the capability to quantify viral load using the calibration curve method (refer to Figure 2). A calibration curve for the Zika tag amplicon was constructed with concentrations ranging from 2×10^1 to 2×10^4 copies/μL (Square wave voltammetry response illustrated in Figure S2). These curves demonstrated an R^2 value of 1.00, signifying a positive relationship between the logarithm of the concentration and the current density signal. Notably, a concentration as low as 2×10^2 copies/μL was successfully distinguished from the NTC control. Similar to our previous work where we detected 115 copies/μL for a specific SARS-CoV-2 gene S RNA using the 4WJ system with a target-specific 4WJ design,[16] our current approach, which appears to reach similar limits of detection, may be associated with the design itself and its ability to accommodate sequences of this length.

Other electrochemical approaches, such as RCA, have demonstrated the ability to detect both N and S genes of SARS-CoV-2 up to 1 copy/μL each, utilizing a specifically designed probe for each electrode.[24] Additionally, amplification methods like recombinase polymerase amplification (RPA) have been successfully integrated with electrochemical platforms for pathogen detection. For instance, in the case of *Pseudomonas syringae*, a common crop pathogen, gold nanoparticles functionalized with specific probes have exhibited a sensitivity 10×10^3 times greater than traditional PCR techniques.[25]

In conclusion, a single E-biosensor design, based on the 4WJ system, demonstrated the capability to detect multiple RNA targets (HIV-O, Zika, and InfA) using the same protocol. This was achieved through a modified NASBA, which selectively tags and amplifies RNA, thereby creating a standardized protocol with the potential to detect a broad range of viruses. While other approaches, utilizing various amplification techniques and electrochemical materials, have shown greater sensitivities, our approach addresses the challenge of designing different probes for each target to be analyzed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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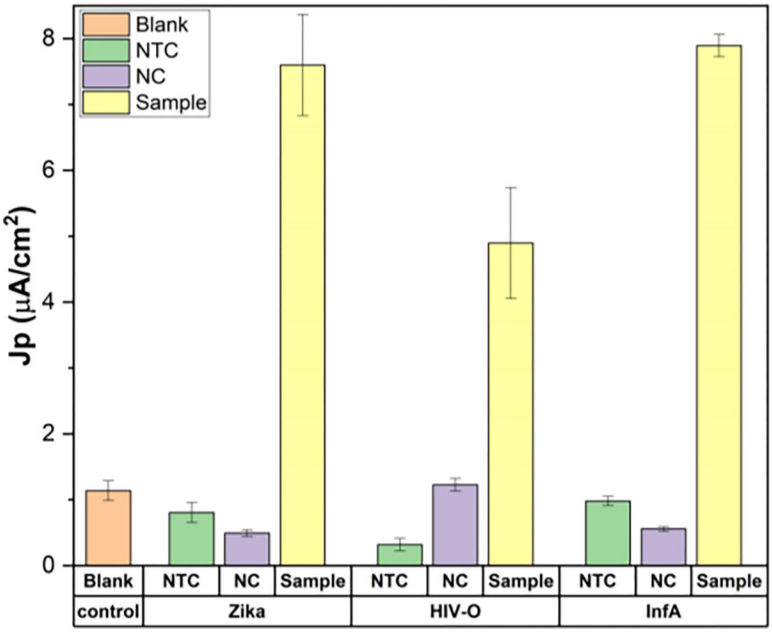

Figure 1.

Response of the E-biosensor to the amplicons obtained from NASBA at 60 min hybridization time on gold disk electrodes. NTC: NASBA No-template control, NC: Negative control, Sample: 2% v/v for Zika and InfA amplicons and 10% v/v for HIV-O amplicons.

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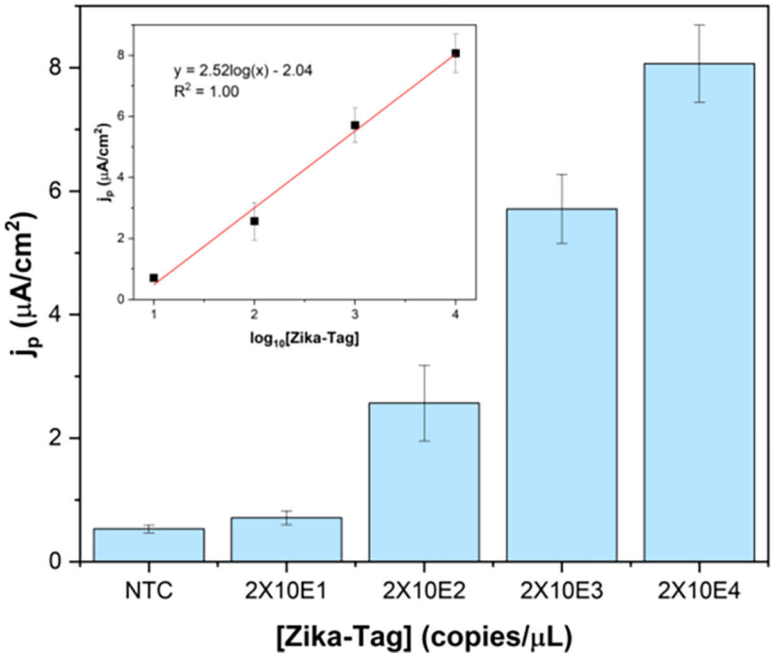

Figure 2.

Response of the E-biosensor to the Zika NASBA amplicons after the clean-up procedure at 60 minutes hybridization time on GDEs at different concentrations. NTC: non-target control. Inset: Dependence of the E-biosensor response on the logarithm of Zika RNA concentration.

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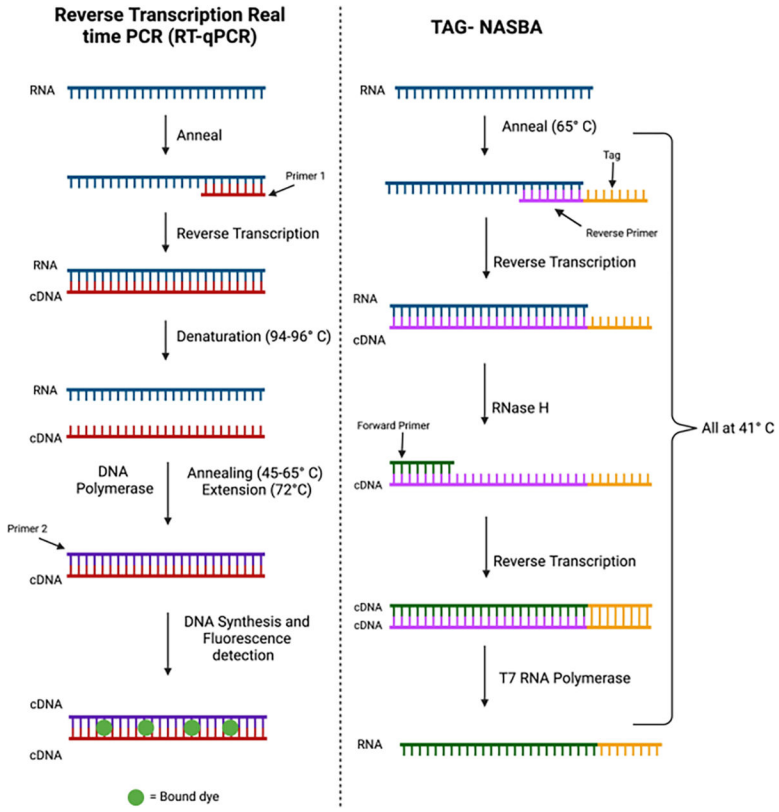

Scheme 1.

Schematic comparison of RT-qPCR protocol and our proposed modified Tag-NASBA protocol.

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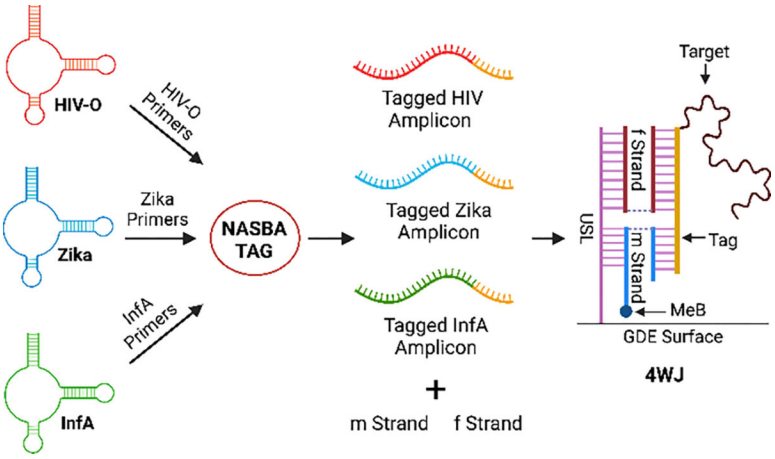

Scheme 2.

Schematic representation of the coupling of the NASBA Tag protocol with the 4WJ based E-Biosensors for detection of HIV, Zika and Influenza with the same E-Biosensors design. GDE: Gold disk electrode.