A Nuclease Protection Assay for Colorimetric and Electrochemical Detection of Nucleic Acid

Jessie Filer[1,2], Rob Channon[3], Charles Henry[3,4], Brian Geiss[1,2,4]

[1]*Cell and Molecular Biology Graduate Program, Colorado State University, Fort Collins, CO*

[2]*Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO*

[3]*Department of Chemistry, Colorado State University, Fort Collins, CO*

[4]*School of Biomedical Engineering*

Abstract

Nucleic acid testing (NAT) assays like quantitative polymerase chain reaction (qPCR) are the preferred method used to diagnose viral diseases like Zika virus (ZIKV). And while qPCR offers excellent sensitivity and specificity, its complexity and instrumentation requirements limit its clinical use to specialized diagnostic laboratories. Because ZIKV circulates in regions with few diagnostic laboratories, sample volume is often overwhelming, increasing the turn-around time. Reducing assay complexity and cost increases the accessibility of NAT for diagnostic use in regions where laboratory resources are limited. S1 nuclease protection has been used for over forty years to map genomic and messenger RNA elements, detect cellular and viral microRNAs, and even to monitor algal species in the environment. Although it has not seen much use in a clinical context, the technique demonstrates high specificity and has served as an effective replacement for Northern blotting and PCR techniques in laboratory research. We have modified the nuclease protection assay to replace gel visualization with an enzymatic readout. Briefly, the nuclease protection ELISA (NP-ELISA) mixes and hybridizes probe oligos with a nucleic acid sample. Because S1 nuclease is specific for single stranded nucleic acids, only hybridized double-stranded products remain after digestion. Remaining protected probe is immobilized to a neutravidin plate via a 3' biotin linkage and HRP-conjugated antibody is added to bind to the 5' digoxigenin linkage. Although HRP reactions are typically visualized colorimetrically, the enzymatic two electron oxidation of its 3,3',5,5'-Tetramethylbenzidine (TMB) substrate can also be detected electrochemically to enhance sensitivity. Using complementary oligos as a model system, the electrochemical assay demonstrates high specificity and detection ranges comparable to authorized qPCR assays. The NP-ELISA is a good candidate for an alternative NAT tool with fewer reagents, inexpensive equipment, and potential for automation.

Nuclease protection-ELISA combines S1 nuclease protection with an enzymatic readout

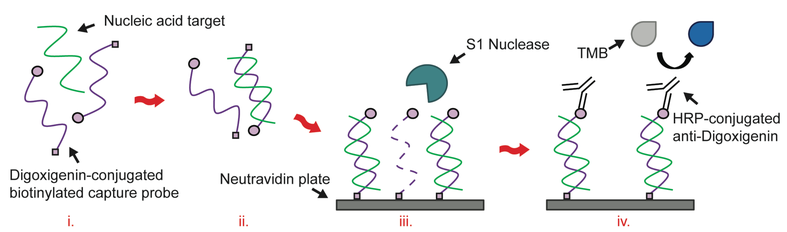

Figure 1. Conceptualization of NP-ELISA. Oligo capture probes specific for ZIKV (BG992) or KUNV (BG975) are mixed with target nucleic acid (**i**) and are allowed to hybridize (**ii**). The hybridized probe is immobilized to a neutravidin plate via a 5' biotin molecule (**iii**). S1 nuclease degrades any unbound probe, leaving only the hybridized probe behind (**iii**). An HRP-conjugated antibody binds to the 3' Digoxigenin molecule on the probe and catalyzes the oxidation of TMB to produce a colorimetric or electrochemical signal (**iv**).

Assay Optimization

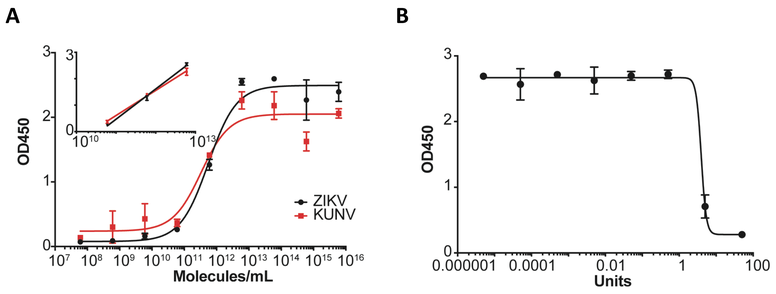

Figure 2. A) The effect of probe concentration on absorbance signal was examined. A sigmoidal response was observed with a linear range of 6×10^{10}- 6×10^{12} molecules mL^{-1}. Probe sequence had no effect on signal response. 50 fmol of probe produced maximum signal and was chosen for assay use. **B)** The effect of S1 nuclease concentration on absorbance signal was investigated. Dilutions lower than 5U had no effect on the signal. 50U of enzyme caused total loss of signal, indicating complete digestion of the probe .

Spectrophotometric target detection is highly specific but not sensitive

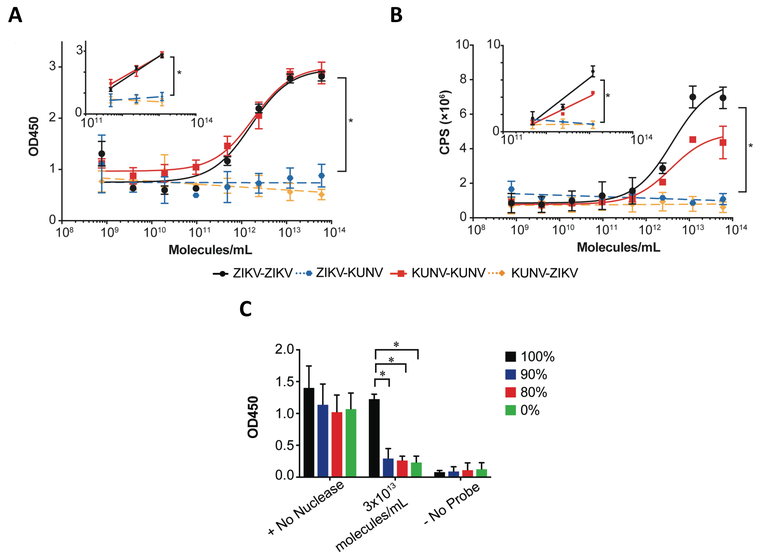

Figure 3. A) Absorbance readout. A sigmoidal signal response was obtained with a linear dynamic range of 9.64×10^{10} – 1.20×10^{13} molecules mL^{-1}. The limit of detection was calculated as *9.80×10^{10} molecules mL^{-1}*. **B)** Chemiluminescence readout. A similar sigmoidal signal was observed with a linear range of 4.82×10^{11} – 1.20×10^{13} molecules mL^{-1}, which is smaller than the range determined for absorbance detection. **C)** Mutations were added to target oligos and tested for protection capacity. Even at 90% complementarity, the low absorbance signal indicates that mutated targets were unable to protect probe from digestion.

Electrochemical Characterization of TMB and Hydroquinone/Benzoquinone

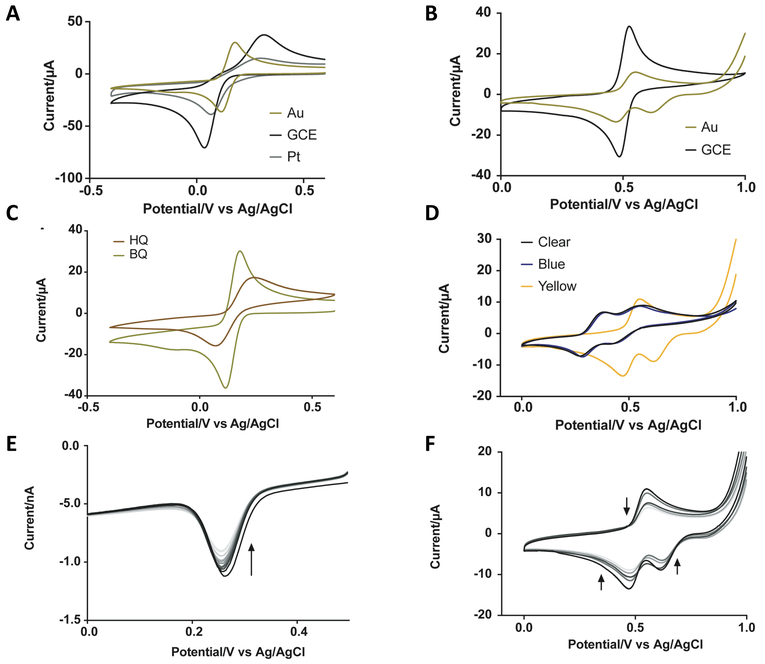

Figure 4. Different electrodes were compared to optimize the electrochemical analysis for **A)** benzoquinone (oxidation product of hydroquinone) and **B)** ox2-TMB (oxidation product of TMB). Gold electrodes were then used for cyclic voltammetry characterization of **C)** hydroquinone and benzoquinone and **D)** TMB and its oxidation states (ox1-TMB and ox2-TMB). Fouling of the electrode was observed for both **E)** benzoquinone and **F)** ox2-TMB and was mitigated by polishing between measurements.

Evaluation of TMB and Hydroquinone as electrochemical HRP substrates

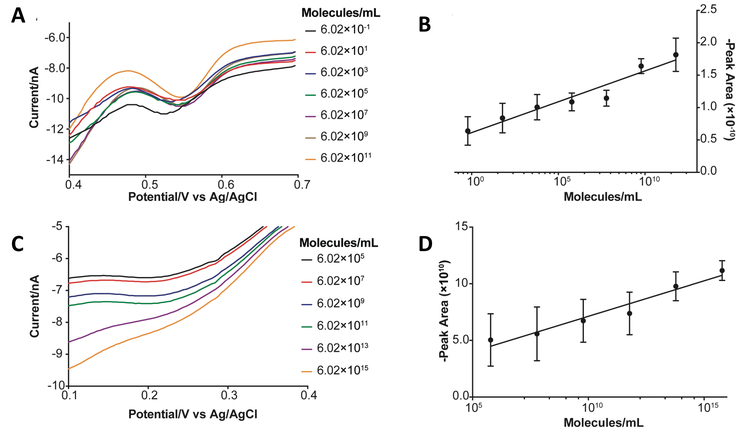

Figure 5. Square wave voltammetry was used to evaluate the signal response from a serial dilution of probe oligo using **A)** TMB and **C)** hydroquinone. Peaks were integrated and the area was plotted as a function of probe concentration for both **B)** TMB and **D)** hydroquinone. For TMB, a linear range of 0 – 6×10^{11} molecules mL^{-1} was determined and a range of 6.02×10^{5} – 6.02×10^{15} molecules mL-1 was determined for hydroquinone. With its wider dynamic range, TMB was chosen for assay analysis.

Electrochemical Target Detection with TMB

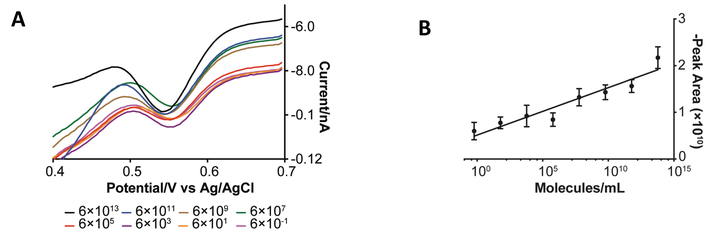

Figure 5. A) Square wave voltammetry was used to evaluate a serial dilution of target oligo. **B)** Peak area was integrated and plotted as a function of target concentration. A linear curve was obtained from 0 - 6×10^{13} molecules mL^{-1} with a limit of detection of 3.72×10^{3} molecules mL^{-1} . This limit of detection compares well to comparable PCR assays like the CDC's Trioplex assay (which has a detection limit of 2.45×10^{3} genome copy equivalents (GCE) mL^{-1}

Conclusions and Future Work

- Nuclease protection can be combined with enzymatic readout (NP-ELISA) to detect nucleic acid target in a concentration-dependent manner.
- TMB performs better as an electrochemical HRP substrate compared to hydroquinone.
- The assay works with absorbance, chemiluminescence, and electrochemistry, but electrochemistry has a detection limit seven orders of magnitude better than spectrophotometric methods.
- Collaborative work is currently being done to adapt the assay to a disposable paper device.

Acknowledgements

The authors would like to thank Erin Lynch, Joe Russo and Kristen Bullard-Feibelman for technical assistance and support. We would also like to thank Kelly DuPont for assistance with poster design. This work was partially funded by supported by a National Science Foundation grant (DGE-1450032). Any opinions, findings, conclusions or recommendations expressed are those of the authors and do not necessarily reflect the views of the National Science Foundation.