Analytical and Bioanalytical Chemistry (2020) 412:3051–3061
https://doi.org/10.1007/s00216-020-02569-w

PAPER IN FOREFRONT

Paper-based nuclease protection assay with on-chip sample pretreatment for point-of-need nucleic acid detection

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Received: 30 January 2020 / Revised: 22 February 2020 / Accepted: 2 March 2020 / Published online: 19 March 2020

Abstract

Pathogen detection is crucial for human, animal, and environmental health; crop protection; and biosafety. Current culture-based methods have long turnaround times and lack sensitivity. Nucleic acid amplification tests offer high specificity and sensitivity. However, their cost and complexity remain a significant hurdle to their applications in resource-limited settings. Thus, point-of-need molecular diagnostic platforms that can be used by minimally trained personnel are needed. The nuclease protection assay (NPA) is a nucleic acid hybridization–based technique that does not rely on amplification, can be paired with other methods to improve specificity, and has the potential to be developed into a point-of-need device. In traditional NPAs, hybridization of an anti-sense probe to the target sequence is followed by single-strand nuclease digestion. The double-stranded target-probe hybrids are protected from nuclease digestion, precipitated, and visualized using autoradiography or other methods. We have developed a paper-based nuclease protection assay (PB-NPA) that can be implemented in field settings as the detection approach requires limited equipment and technical expertise. The PB-NPA uses a lateral flow format to capture the labeled target-probe hybrids onto a nitrocellulose membrane modified with an anti-label antibody. A colorimetric enzyme-substrate pair is used for signal visualization, producing a test line. The nuclease digestion of non-target and mismatched DNA provides high specificity while signal amplification with the reporter enzyme-substrate provides high sensitivity. We have also developed an on-chip sample pretreatment step utilizing chitosan-modified paper to eliminate possible interferents from the reaction and preconcentrate nucleic acids, thereby significantly reducing the need for auxiliary equipment.

Keywords Nuclease protection assay · Nucleic acid detection · Paper-based device · Lateral flow assay

Introduction

Point-of-need pathogen detection has wide-ranging applications in medical diagnostics [1], food and water safety [2], agriculture [3], air quality and environmental monitoring [4], biosafety [5], and evolutionary biology and epidemiology [6]. Conventional culture-based methods widely employed in pathogen detection have long turnaround times (days to

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00216-020-02569-w) contains supplementary material, which is available to authorized users.

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weeks), relatively high detection limits (300–3000 colony-forming units (CFU) per mL), and a prevalence of false negatives [1]. Moreover, many pathogens cannot be cultured using standard methods or require high-containment culture facilities [7]. Molecular diagnostic methods such as blotting- and hybridization-based techniques, next-generation sequencing (NGS), and nucleic acid amplification tests (NAATs) have become very common for pathogen detection in clinical and research labs across the developed world. NAATs include polymerase chain reaction (PCR) and isothermal amplification techniques, both of which are known to offer low detection limit (~ 3 CFU/mL) because the target of interest is amplified exponentially. However, each step of PCR is performed at a different temperature requiring a thermocycler which adds significant cost and limits portability. To simplify instrumentation needs, newer isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP) [8, 9], template-mediated amplification (TMA) [10], helicase-dependent amplification (HDA) [11], and rolling circle amplification (RCA) [12] have been used for nucleic acid analysis. While these techniques reduce the need for instrumentation by enabling amplification at a fixed temperature, the need for several distinct primers for amplification, significant laboratory infrastructure, and highly trained personnel make their use in the developing world cost prohibitive. Furthermore, NAATs can produce false negative results due to the presence of inhibitors in complex samples and therefore require extensive sample preparation steps [13].

Nuclease protection assays (NPAs) or hybridization protection assays have been in used in RNA mapping and determining gene transcription levels for decades [14]. Unlike NAATs, NPAs do not require nucleic acid amplification, which reduces assay time, cost, and complexity. Traditional NPAs involve hybridizing a labeled probe complementary to the target sequence followed by digestion of unhybridized and non-target nucleic acids using a nuclease which selectively cleaves single-stranded oligomers. Thus, only perfectly matched probe-target hybrids remain intact and can be detected using colorimetric or radiological methods [14–17]. NPAs have been integrated with sandwich hybridization (NPA-SH) to detect harmful algae in aqueous samples [15–18]. Recently, a nuclease protection enzyme-linked immunosorbent assay (NP-ELISA) method has also been developed for the detection of Zika and Kunjin virus sequences with colorimetric, chemiluminescent, and electrochemical detection motifs [14]. However, these NPA strategies require external readers which are expensive and not accessible in resource-limited settings.

Lateral flow assays (LFAs) are frequently used in point-of-care diagnostics, the most ubiquitous of which are the over-the-counter pregnancy tests. LFAs have also been used in direct and indirect detection of pathogens such as *Plasmodium falciparum* [19] and proteins such as Diphtheria Toxin [20]. LFAs can significantly reduce the need for instrumentation and provide user-friendly result readouts, typically with the appearance of a test line when the target is present. This easy-to-read platform offers value as an end-point detection method and has been paired with PCR [21] and isothermal amplification reactions [22–24] to create low-cost nucleic acid sensors. Lateral flow nucleic acid sensors can be divided into two categories, i.e., nucleic acid lateral flow assays (NALFAs) and nucleic acid lateral flow immunoassays (NALFIAs). NALFAs involve direct detection of nucleic acids using oligonucleotide probes immobilized on lateral flow strips while NALFIAs use hapten or other biomolecule-labeled probes and antibodies to capture them in an immunoassay format [25].

We have developed a paper-based nuclease protection assay (PB-NPA) for the rapid detection of nucleic acids at the point-of-need (Fig. 1). The assay involves hybridization of a 5′-digoxigenin- and 3′-biotin-labeled oligonucleotide probe to the target, followed by digestion with P1 nuclease to cleave unhybridized probe, target, and other single-stranded non-target DNA. The protected probe-target hybrids are captured using an anti-digoxigenin antibody immobilized on nitrocellulose membrane and visualized with streptavidin-conjugated horseradish peroxidase (Strep-HRP) and a colorimetric substrate solution (tetramethylbenzidine (TMB) and hydrogen peroxide). A blue line appears in the detection region to indicate the presence of the target DNA. The use of a reporter enzyme allows for signal amplification from the captured protected probes, providing a sensitive method for detection without performing complex nucleic acid amplification reactions. The assay provides simple yes/no readout with the appearance of a test line when the target sequence is present in the sample above the detectable limit. The assay combines the high specificity of the NPA with the user-friendly lateral flow strip platform for end-point detection. When quantification of the target is needed, the colorimetric signal can be captured and analyzed using a cellphone camera and image analysis software.

Materials and methods

Materials

Nitrocellulose FF120HP A4-sized sheets, Whatman grade 1 and 4 filter paper were purchased from GE Healthcare (Chicago, IL). Anti-digoxigenin monoclonal antibody was purchased from Abcam (Cambridge, MA). Trehalose dihydrate and formamide were purchased from EMD Millipore (Burlington, MA). StabilGuard® Immunoassay Stabilizer (BSA-free) was purchased from SurModics, Inc. (Eden Prairie, MN). P1 nuclease (from *Penicillium citrinum*) and 10× P1 nuclease reaction buffer were purchased from New England Biolabs, Inc. (Ipswich, MA). Glycerol was purchased from Mallinckrodt (Staines-upon-Thames, UK).

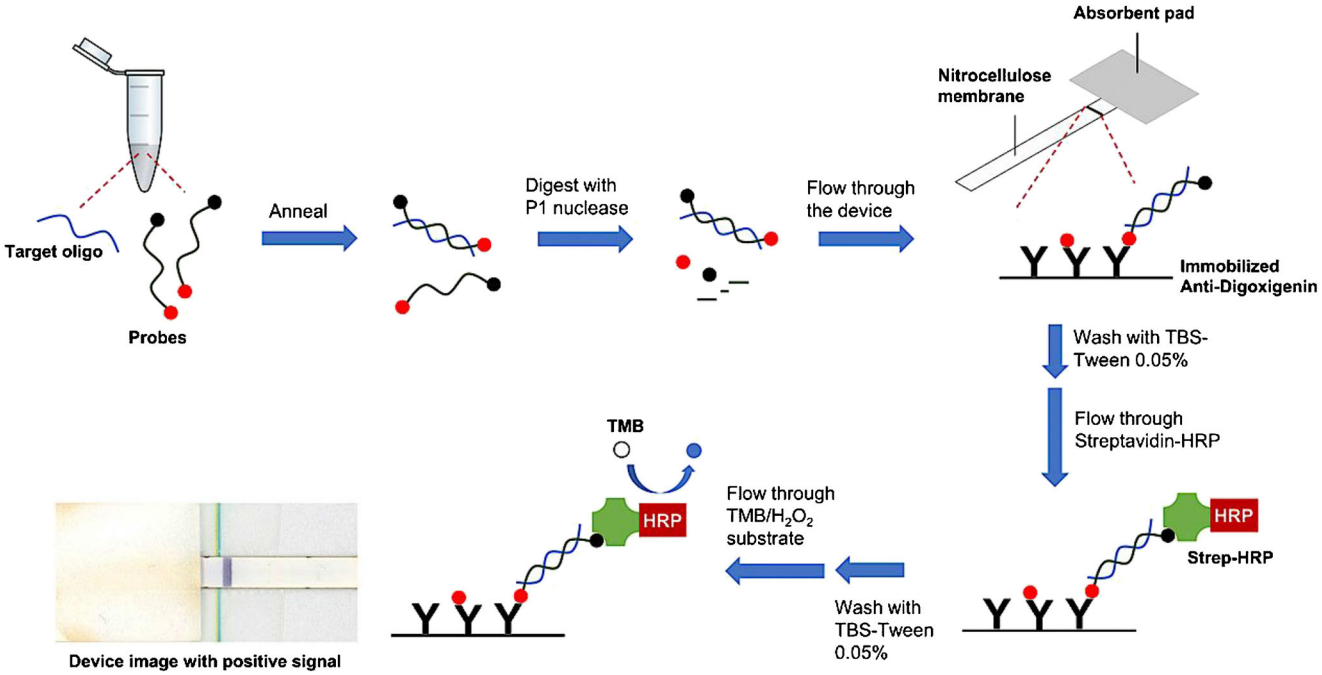

Fig. 1 Schematic representation of the paper-based nuclease protection assay

Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), sodium chloride, ethylenediaminetetraacetic disodium salt acid, 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES), Tris(hydroxymethyl)aminomethane (Tris), Tween®-20, and chitosan oligosaccharide lactate (average M_n 5000) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Streptavidin conjugated to horseradish peroxidase (Strep-HRP) was purchased from R&D Systems, Inc. (Minneapolis, MN). Pierce™ 1-Step Ultra TMB-Blotting Solution was purchased from Thermo Scientific (Waltham, MA). Table 1 gives the NPA probe and DNA oligonucleotide sequences used in the study; all DNA sequences were ordered from Integrated DNA Technologies, Inc. (Coralville, IA).

Device fabrication

A 100 μL anti-digoxigenin Ab solution (1 mg/mL) was mixed with 2.5 μL of 2 M trehalose and 10 μL of 50% glycerol. The solution was then striped twice onto a nitrocellulose (NC) membrane at 0.036 μL/mm deposition rate using an Automated Lateral Flow Reagent Dispenser (ALFRD)™ from ClaremontBio (CA, USA) equipped with a syringe pump (1.5 μL/s flow rate; 42 mm/s striping rate). The membrane was dried at 37 °C for 1 h. To block the surface of the NC membrane from non-specific binding, StabilGuard® Immunoassay Stabilizer solution was added to fully wet the membrane. The membrane was then air-dried at room temperature for 30 min. After that, the membrane can be immediately used to fabricate devices or stored in a closed container for later use.

To construct the device, the Ab-striped NC membrane was cut using a Zing CO_2 laser cutter from Epilog (CO, USA) to create 20 mm × 4 mm strips with the deposited antibody located 5 mm from one edge of the strip (i.e., downstream edge). The NC strip was placed on a transparency sheet (as a backing material) using double-sided adhesive. Two pieces of 20 mm × 15 mm laser cut Whatman no. 4 filter paper were stacked together and placed on the downstream edge of the NC strip

Table 1 NPA probe and DNA sequences

Sequence name	DNA sequences
NPA probe	5′biotin/GTGATTGACGATGGGGCCCAA/3′digoxigenin
Complementary target	5′/TTGGGCCCCATCGTCAATCAC/3′
1-base mismatch	5′/TTGGG**T**CCCATCGTCAATCAC/3′
2-base mismatch	5′/TTGGG**T**CCCAT**G**GTCAATCAC/3′
3-base mismatch	5′/TT**T**GG**T**CCCAT**G**GTCAATCAC/3′
4-base mismatch	5′/TT**T**GG**T**CCCAT**G**GTCAA**A**CAC/3′
Fluorescently labeled oligo	5′ amine modifier C6/AGGTATGTAGAGGCA/3′ 6-FAM (fluorescein)

using a tape such that there was 2- to 3-mm overlapping region between the materials. The Whatman grade 4 qualitative filter paper was used as an absorbent pad.

Paper-based nuclease protection assay

Nuclease protection assays were performed based on a protocol described by Filer et al. [14] with some modifications. For a triplicate experiment, 3 μL probe oligo solution was mixed with 3 μL target oligo solution, 2.4 μL 10× hybridization buffer (0.4 M PIPES pH 6.8, 4 M NaCl, 0.02 M EDTA), and 15.6 μL DI water in a tube. Formamide was added at 0–80% total concentrations by reducing the volume of DI water to accommodate the volume of formamide added without changing the total volume of the solution. The solution was then heated at 95 °C for 2 min and at 53 °C for 10 min. The solution was subsequently placed in ice bath and a 6 μL mixture of P1 nuclease 1 U/μL-10× P1 buffer (1:1 v/v) was added to the solution. To start digestion, the solution was incubated in a water bath at 37 °C for the indicated time. A 10-μL aliquot from nuclease digestion was flowed through the device, followed by an addition of 10 μL TBS-Tween 0.05% wash buffer pH 8.6. The following solutions were then added subsequently to develop the colorimetric signal: 10 μL streptavidin-HRP (1:200 diluted), 10 μL TBS-Tween 0.05% wash buffer, and 2 × 10 μL TMB/H_2O_2 substrate solution.

Testing DNA interaction with chitosan-modified paper

A Xerox® ColorQube 8870 solid-ink printer was used to print wax pattern on the Whatman grade 1 filter paper. Wax-patterned paper were placed on a hot plate set to 150 °C for 1 min to allow the wax to melt and permeate the thickness of the paper, forming a hydrophilic zone surrounded by hydrophobic wax barrier. To study the interaction of DNA with chitosan, fluorescently labeled oligo DNA (fluorescein, Ex_{max} = 495 nm, Em_{max} = 520 nm) was flowed through chitosan-modified wax-patterned filter paper discs (printed diameter of 3 mm) in a vertical flow format with an absorbent pad at the bottom to wick the sample (n = 9 per layer). A fluorescence microscope (Eclipse TE2000-U Inverted Microscope, Nikon Inc., Tokyo, Japan) was used to image the chitosan layer and the absorbent pad. ImageJ image processing software was used to quantify the mean intensity in each of the layers. To determine the optimal conditions for the elution of captured DNA, varying volumes (3–6 μL) of Tris buffer with pH (7.5, 8, 8.5, and 9) were flowed through the chitosan layer. Fluorescence images were captured post-elution for each of the elution buffer conditions (n = 5 per condition) and quantified using ImageJ. After establishing the feasibility of using chitosan to capture and elute DNA, a simple device design was created to perform on-chip sample pretreatment directly on the lateral flow strip.

On-chip sample pretreatment using chitosan-modified paper

Wax-patterned (printed dimensions 3.5mm x 5mm) filter paper was modified with 2 μL of 1% w/v chitosan oligosaccharide lactate prepared in MES buffer pH 5.0; this layer is referred to as the "chitosan layer." The chitosan layer was cut and affixed to a laser cut polymethyl methacrylate (PMMA) support layer. Upon completion of the P1 nuclease digestion step, 10 μL each of the sample was applied to the chitosan layer while in contact with an absorbent pad. The chitosan layer was washed in this position with 40 μL of 100 mM MES buffer pH 5.0 to remove the interfering compounds from the sample while retaining DNA. The membrane was then moved to the lateral flow strip, and the DNA retained in the membrane was eluted on the lateral flow strip using three 10 μL volumes of Tris buffer pH 8.6. The sample pretreatment setup was then removed from the lateral flow strip, and 10 μL of Tris buffer + 0.05% Tween®-20 was applied to the inlet followed by the remaining chromogenic components (Strep-HRP, Tris buffer + 0.05% Tween®-20, and the TMB blotting solution).

Image analysis

After color development, the strips were allowed to dry briefly to enhance the color contrast. These devices were then scanned using a V600 Epson scanner. The resulting image was inverted, and the detection region mean intensity was quantified using ImageJ 1.05i image processing software (open-source software, National Institutes of Health).

Results and discussion

Nucleic acid detection

Colorimetric detection in the lateral flow devices was based on enzymatic conversion of TMB by HRP in the presence of H_2O_2. The use of reporter enzyme allowed for signal amplification to improve detection sensitivity. The binding of digoxigenin/biotin-labeled oligo probe to the capture antibody allowed for accumulation of streptavidin-HRP on the binding sites and subsequent color formation. To assess detection limit of this detection scheme, a series of solutions with varying probe quantities were tested on the flow devices. Colorimetric responses obtained from 64 amol to 5.0 pmol oligo probes are shown in Fig. 2. The colorimetric signal reached a plateau at around 200 fmol of probe, and the detection limit (i.e., mean intensity of blank sample + 3 × standard deviation) was approximately 0.57 fmol (5.7×10^{-16} mol).

Nuclease protection assay

Hybridization between the probes and the target oligos and nuclease digestion of the unhybridized oligos are the two major steps in NPA. For nucleic acid hybridization, stringency/specificity can be manipulated by controlling three parameters: temperature, salt concentration, and formamide concentration [26, 27]. Although cross-reaction among related base sequences can be reduced at a higher temperature in aqueous solution, conditions required for specificity are much more easily adjusted by varying ionic strength and formamide concentration [28]. Thus, to optimize hybridization condition for the NPA, formamide concentrations were varied in the presence of 400 mM sodium salt.

Prior to performing NPA in solutions containing formamide, we assessed the effect of formamide on the binding of labeled probes to the detection elements in the lateral flow devices. This step is important to help distinguish the influence of formamide in different aspects of the PB-NPA including hybridization, nuclease digestion, and detection in the flow devices. Formamide concentrations above 20% reduced binding between the probes and the capture antibodies as shown by linear decrease in colorimetric signal at formamide concentrations ranging from 20 to 50% (Fig. 3a). While antigen-antibody binding activity was reported to increase in the presence of several organic-water miscible solvents such as ethanol and methanol [29], formamide and similar solvents have been shown to disrupt antigen-antibody binding [29, 30] potentially by inducing conformation change of the antibodies or lowering the binding affinity by solvating either or both antigen-labeled oligo probes and capture antibodies. Therefore, dilution of test solution to formamide concentrations equal to 20% or lower was required prior to testing using the lateral flow devices.

The use of a single-strand-specific nuclease is crucial to remove unhybridized labeled probes from the test solution such that any detected signal is only dependent on the quantity of the protected target-probe duplexes. Although more than 30 single-strand-specific nucleases have been identified from various sources, only a few enzymes including S1 nuclease and P1 nuclease have been characterized to a significant extent [31]. S1 nuclease is often employed in NPA [14, 32, 33] due to the well-characterized nature of the enzyme and high selectivity toward single-stranded DNA in the presence of double-stranded DNA [31]. However, we found that the catalytic activity of P1 nuclease in digesting the labeled oligo probes is higher than S1 nuclease as shown in Electronic Supplementary Material (ESM) Fig. S1, and thus selected P1 nuclease for our NPA protocol. To optimize digestion conditions, the amount of P1 nuclease and digestion times were varied in the presence of 10 pmol probes and incubation at 37 °C. As shown in Fig. 3b, significant depletion of colorimetric signal was obtained by using 0.1 U P1 nuclease in a 10 μL solution as compared with control experiment in the absence of nuclease. The background signal was further reduced by increasing nuclease activity to 1 U/10 μL; however, no more discernable reduction of signal was observed at higher nuclease activities. The probes were then digested using 1 U P1 nuclease with digestion time varied from 5 to 30 min. No significant difference was observed in measured colorimetric signals from probes digested for 5 to 30 min (Fig. 3b inset), and thus, digestion with 1 U P1 nuclease for 5 min at 37 °C was selected for the NPA.

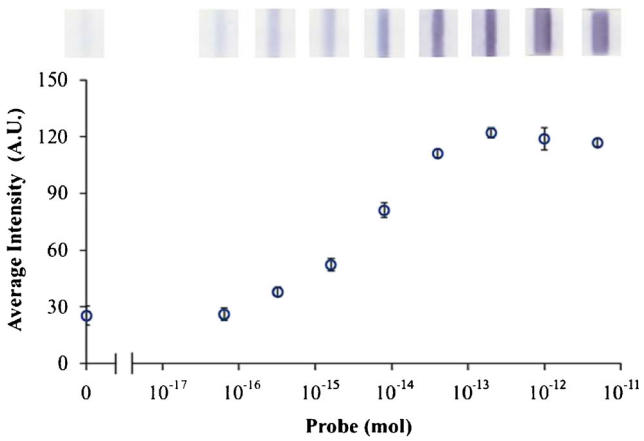

Fig. 2 Dose-response of varying amounts of digoxigenin/biotin-labeled oligo probes. Error bars indicate standard deviations ($n = 4$ devices)

Formamide is commonly used during hybridization in NPA to increase hybridization stringency. The effect of formamide concentrations on the NPA is shown in Fig. 3c. No visible difference in colorimetric signals was observed at 0–80% formamide used in hybridization mixtures, suggesting that formamide neither interferes nor improves duplex formation within the experimental conditions described in the method section. However, increased concentration of formamide did affect the P1 nuclease activity in degrading unhybridized probes. Background signals were observed at 10–20% formamide concentrations in digestion mixtures (equivalent to 40–80% formamide concentration in hybridization mixtures due to 1:4 dilution of solution prior to nuclease digestion). Since no signal improvement was obtained by incorporating formamide into the hybridization mixture and the organic solvent has shown some detrimental effects to nuclease digestion and probe binding to the capture antibody, formamide was omitted from the PB-NPA protocol.

Figure 3d shows the intensities of colorimetric signal as a function of target oligo quantities in PB-NPA using the optimized protocol. Similar to the probe dose-response shown in Fig. 2, NPA with target sequence complementary to the probe also reached saturation at approximately 200 fmol of target oligo, suggesting that the hybridization between target oligo and the probe is highly efficient. The background signal in NPA with target oligo was lower than that in probe-only detection, potentially due to the change in pH and ionic strength in

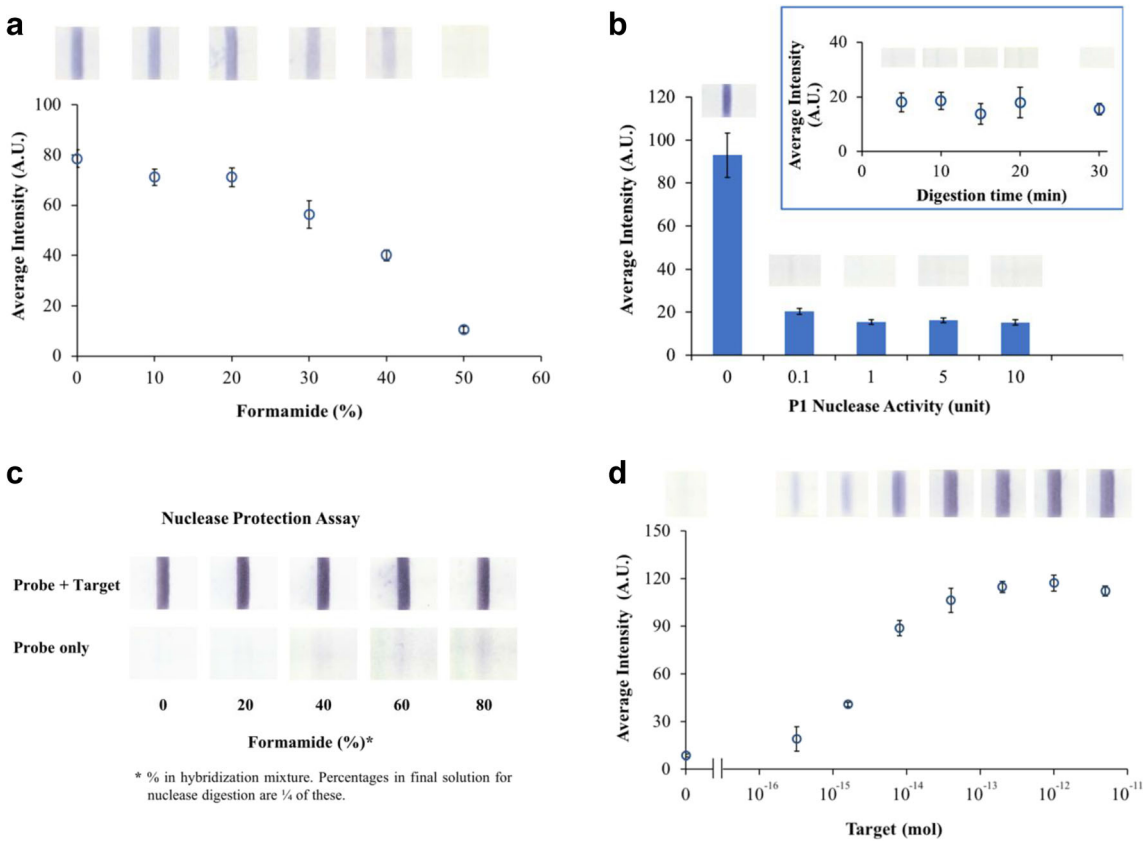

Fig. 3 Optimization of NPA protocol and assay sensitivity: **a** effect of formamide on the detection of 8-fmol oligo probe; **b** effect of P1 nuclease quantity/activity and (digestion time—*right top inset*) on degradation of single-stranded oligo probe. Digestion was carried out for 30 min in experiment shown in the main graph; **c** effect of formamide concentration on NPA; **d** dose-response of varying amounts of target complementary oligos. Error bars indicate standard deviations ($n = 4$ devices)

assay buffer from addition of P1 buffer to the hybridization buffer that was used solely in probe detection. This lower background signal gave slightly improved detection limit for PB-NPA, which was approximately 0.24 fmol (2.4×10^{-16} mol).

Assay specificity

To investigate the specificity of PB-NPA, test oligos with 1, 2, 3, or 4 base mismatches were designed and tested at 1 to 1 ratio and 125:1 ratio to the quantity of the probes. A completely random sequence was also tested at similar ratios. The results are shown in Fig. 4a. Significant reduction of signal was observed for the 1-base mismatch compared with that in complementary oligo, indicating sequence selectivity within the assay. However, the considerably high colorimetric responses obtained in various mismatch sequences (in comparison with the baseline-level signal obtained from the random sequence) suggested the formation of duplexes or secondary structures between the probe and the mismatch oligos that P1 nuclease was not able to degrade. Increasing quantity of either mismatch or random oligo relative to probe quantity did not affect the observed signals. Single-base mismatches are the least accessible to the single-strand-specific nucleases because they present the smallest region for enzyme binding [34]. This difficulty in accessing single-strand binding sites is possibly the reason why considerable amounts of signal were obtained in all mismatch oligo tested by PB-NPA. Although 4 bases were non-complementary in the 4-base mismatch oligo, the location of mismatched bases was designed to be single-base mismatch at 4 different locations within the sequence, posing the same difficulty for the nuclease to bind.

Increasing the amount of P1 nuclease reduced colorimetric responses from 1-base mismatch oligo (Fig. 4b). However, the increased nuclease activity also decreased colorimetric signals in complementary oligo, suggesting that P1 nuclease also degrades double-stranded DNA at high concentrations. Nevertheless, the signal-to-noise ratio (i.e., ratio of colorimetric signal from complementary oligo to that of 1-base mismatch) was improved by increasing the amount of P1 nuclease from 1 U ($S/N = 1.4$) to 50 U ($S/N = 2.2$) despite the lower sensitivity.

On-chip sample pretreatment with chitosan-modified paper

In conventional NPA, extensive sample preparation steps are needed in order to perform detection using colorimetric or

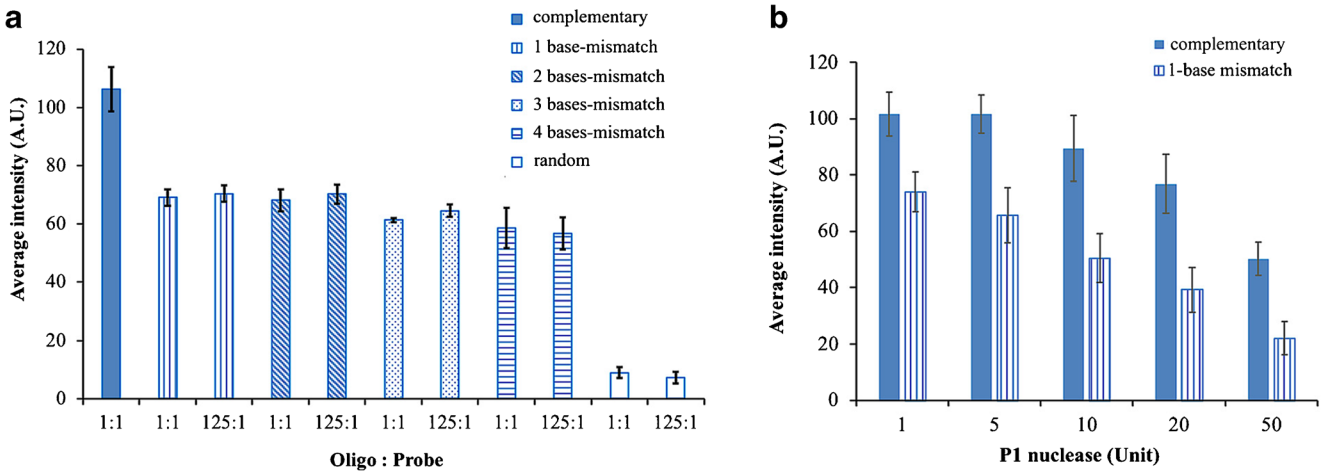

Fig. 4 **a** Colorimetric signals for NPA with complementary, mismatch, and random sequences. 1 U P1 nuclease was used for the digestion. Error bars indicate standard deviations ($n = 4$ devices). **b** NPA signal for complementary and 1-base mismatch at different activities of P1 nuclease. Error bars indicate standard deviations ($n = 3$ devices)

radiological methods. These steps include inactivation of nuclease enzymes, centrifugation to remove free-label resulting from nuclease digestion, and precipitation of protected probe-target hybrids for detection. In PB-NPA, we perform on-chip sample pretreatment utilizing chitosan-modified filter paper. Chitosan (pKa ~ 6.4) is a polycationic polymer that exhibits a pH-dependent affinity for the negatively charged backbone of nucleic acids [35]. This pH-dependent nucleic acid affinity exhibited by chitosan has been exploited to create nanoparticle carriers in gene therapy delivery systems [36, 37], but there are limited reports on the use of chitosan as a means to perform sample pretreatment in lab-on-a-chip or point-of-care nucleic acid sensors [38, 39]. Yager et al. described the use of chitosan to perform preconcentration in a lateral flow format by capturing DNA at a low pH of ~ 5 and eluting the captured DNA into a secondary channel for detection by increasing the pH to ~ 8.5 [40]. Chitosan-modified paper can also be used to preconcentrate DNA from the sample which could further improve the LOD of the PB-NPA device [40, 41]. Schlappi et al. showed that a preconcentration factor of 5000× can be achieved using chitosan-modified porous membranes integrated with in situ PCR [41]. Moreover, chitosan complexed with DNA has been shown to provide protection against nuclease digestion and potentially eliminate the need for nuclease enzyme inactivation in the PB-NPA [35–37].

DNA interaction with chitosan-modified paper

Free digoxigenin generated from the nuclease digestion of unhybridized probe can compete with protected probe-target hybrids for binding sites on the anti-digoxigenin antibody. It is therefore important to remove digoxigenin from the reaction in order to improve the limit of detection of the PB-NPA. We first studied the interaction between chitosan and DNA to determine optimal conditions for the capture and elution of DNA using chitosan-modified paper. Fluorescently labeled oligo DNA and a fluorescence microscope were used to perform these experiments. A two-layered wax-patterned paper device was prepared using the Whatman grade 1 qualitative filter paper; the top layer was treated with 1% w/v chitosan while the bottom layer served as an absorbent pad. DNA solution prepared in MES buffer pH 5.0 was flowed through the paper stack, and a fluorescence image of each layer was recorded ($n = 9$ per layer). Figure 5a shows the mean intensity of each layer including the intensity measured from untreated paper wetted with the same volume of MES buffer to use as background signal. The ratio of background subtracted relative fluorescence intensity in the chitosan layer and the total fluorescence was used to estimate the capture efficiency in chitosan-modified paper. The capture efficiency of filter paper modified with 1% (w/v) chitosan solution was 88.9%, which is comparable to those of previous reports using lateral flow to capture DNA [40]. Next, we studied the effect of elution buffer volume and buffer pH on the elution of DNA from the chitosan-modified filter paper using fluorescently labeled oligos. DNA captured using the method described above was then eluted using TBS buffer with varying volumes (3–6 μL) and pH (ranging from 7.5 to 9.0). Fluorescence intensity of the chitosan layer post-elution under different elution buffer conditions was plotted (Fig. 5b). The data shows that DNA was retained in the chitosan layer when using elution buffer pH 8.0 or less while a significant improvement in elution was observed when using buffer pH ≥ 8.5. Increasing buffer volume above the saturation volume of the chitosan layer (3 μL) improved elution performance, with 6 μL removing nearly all DNA from the chitosan layer and returning the fluorescence to background levels.

On-chip sample pretreatment

We created a simple vertical flow device using 1% chitosan–modified filter paper affixed to a PMMA support layer (Fig.

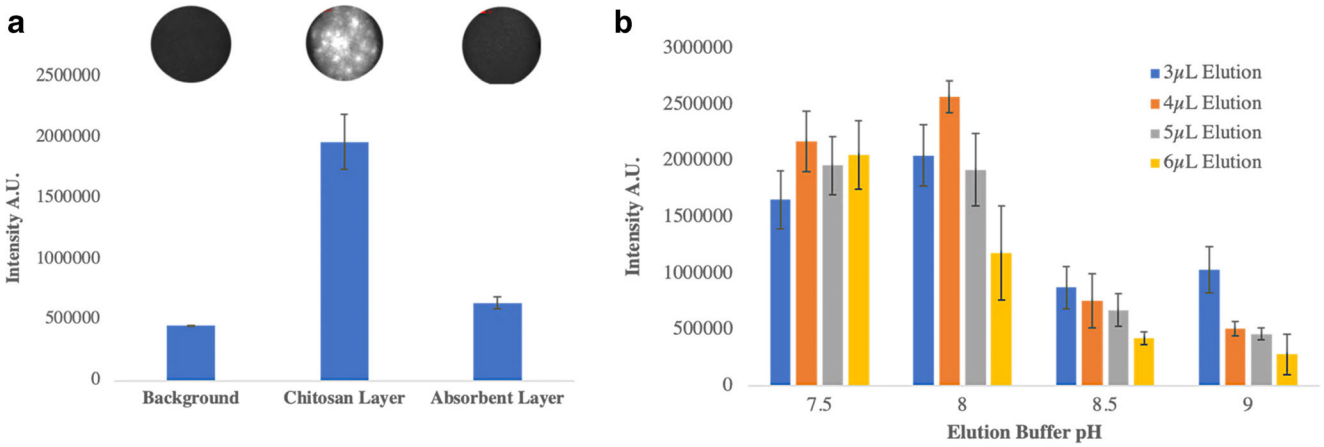

Fig. 5 **a** The capture efficiency of 1% w/v chitosan-modified filter paper in the vertical flow format ($n = 9$ per layer). **b** The captured DNA was eluted using different elution buffer volumes and pH. The fluorescence intensity in the chitosan layer post-elution was measured using a fluorescence microscope

6). We have integrated this sample pretreatment device with the PB-NPA to pretreat nuclease-digested samples and remove digoxigenin and other potential interferents from the reaction. Nuclease-digested samples were flowed through the chitosan-modified filter paper; the nucleic acids from the sample are retained in the chitosan layer through electrostatic interaction while interfering compounds are washed to the absorbent waste pad. The captured DNA in the chitosan layer can then be eluted directly onto the lateral flow strip for detection using an alkaline pH elution buffer (pH ~ 8.6). Wax printing provides a hydrophobic barrier and ensures that all sample flows through the chitosan deposition zone.

Nuclease protection assays were run with varying target oligo amounts and 200 fmol of the NPA probe, and then, the samples were pretreated using the on-chip sample pretreatment method described above (Fig. 7, $n = 3$ devices). Controls were run in triplicates without sample pretreatment; i.e., samples were applied directly to the inlet of the lateral flow strip. Limit of detection was calculated using mean blank + 3.3 × standard deviation. The limit of detection obtained for the control condition was 5.34×10^{-15} mol of target oligo DNA whereas with chitosan sample pretreatment, the LOD was improved to 1.16×10^{-15} mol. The slope of the linear region (sensitivity) of the assay was also improved using chitosan sample pretreatment (avg. intensity = 23.6 × ln(target) + 858) compared with the control condition (avg. intensity = 21.5 × ln(target) + 737). This on-chip pretreatment allowed for removal of free-label and other potential interferents from the reaction, improved the sensitivity and limit of detection of the PB-NPA, and eliminated the need for a nuclease enzyme inactivation step.

While the current format of the assay still relies on multiple manual steps that take a few hours to complete, further optimization by lyophilizing the NPA components for hybridization and nuclease digestion in tubes and storing the detection reagents on the lateral flow strip can provide a rapid, simple, and easy-to-use assay with minimal user interaction. An inexpensive, portable resistive heater can be supplemented to the assay kits to allow for an easy and controlled assay at the point-of-care. To make the PB-NPA suitable for nucleic acid detection in real samples, sample preparation steps need to be incorporated to the test platform. A lysis/extraction buffer can be provided and combined with the chitosan-based sample pretreatment for the extraction and purification of nucleic acid targets from the sample matrices. Depending on the characteristics of the nucleic acid targets (e.g., the lengths, types, origins, and required specificity), the assay components/steps can be adjusted to meet the detection requirement. For example, a longer probe can be used to improve the assay specificity while targeting a class of pathogens with similar, shared traits may benefit from using a shorter probe sequence. Simultaneous detection of multiple pathogens is also possible with the PB-NPA as probes with different labeling molecules and antibodies to the labels can be obtained from commercial vendors. Despite the higher detection limit compared with other conventional techniques including PCR, PB-NPA can serve as a good screening tool for infections in which a high viral load is present in the biological samples such as nasopharyngeal fluids in patients with acute adenovirus infection and patients with chronic infection of hepatitis B virus [42, 43].

Conclusions

These results demonstrate the proof-of-concept for the on-chip sample pretreatment capable lateral flow platform for end-point detection in nuclease protection assays. The PB-NPA can detect sub-femtomole of target DNA with high

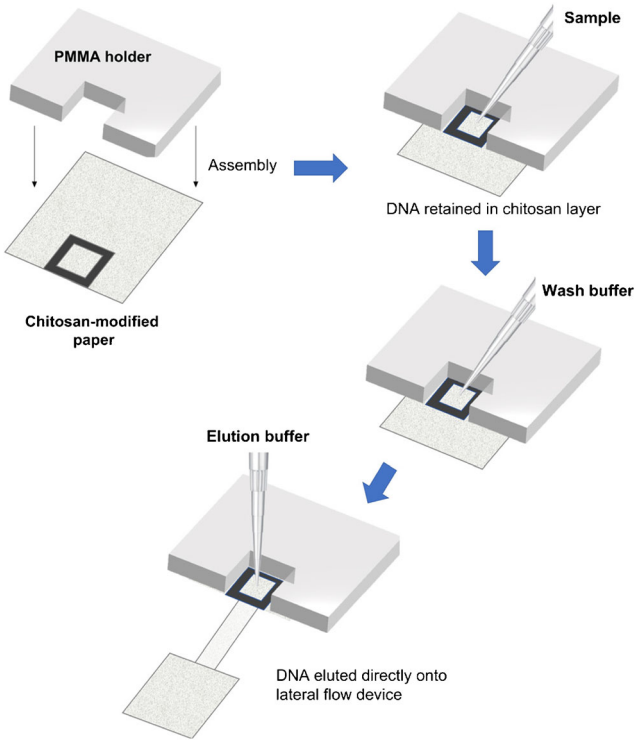

Fig. 6 On-chip sample pretreatment device made of chitosan-modified filter paper affixed to a PMMA template: DNA from nuclease-digested samples is captured in the chitosan layer at an acidic pH (pH ~ 5). The chitosan layer is then washed with a wash buffer (MES pH 5.0), followed by elution of the DNA directly onto the lateral flow device using an alkaline pH buffer (Tris pH 8.6)

specificity. In addition, our vertical flow on-chip sample pretreatment using chitosan-modified paper eliminated the need for a nuclease inactivation step in the assay and further improved the detection limit by ~ 5-fold. The paper-based format allows for simplification of the assay compared with the traditional NPA including the following: (1) easy readout for minimally trained users based on the intensity of the colored lines, (2) lower reagent consumption and less waste generated, and (3) potentially improved assay time for point-of-care applications. The proposed assay only takes around 2–3 h to complete, which is substantially faster than the conventional culture-based method. The assay is also simpler to perform than conventional amplification-based nucleic acid tests, which often require amplification steps, multiple probes, enzymes, and expensive external readers. While the LOD is higher than the amplification-based techniques, PB-NPA can serve a good screening tool for infections in which a high pathogen load is present in the samples.

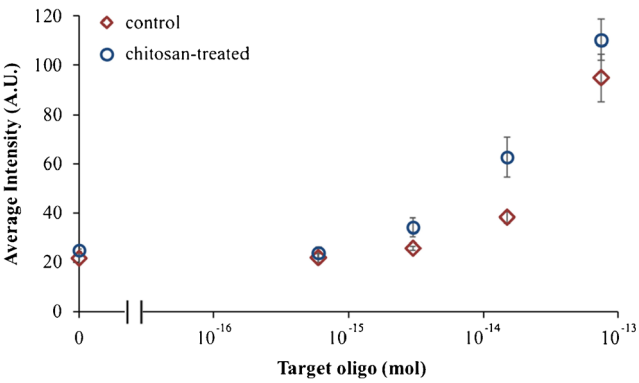

Fig. 7 Colorimetric signals obtained for samples run with chitosan sample pretreatment (blue circle) and untreated controls (red diamond)

Acknowledgments The authors would like to thank Dr. John Wydallis for technical assistance. We also would like to thank the members of the Henry and Geiss labs for the helpful comments and discussions.

Funding information This work was supported by Colorado State University to CSH, DSD, and BJG and the National Institutes of Health (R01 AI132668) to BJG.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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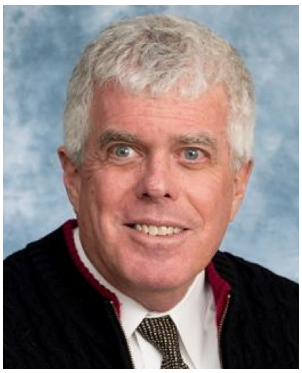

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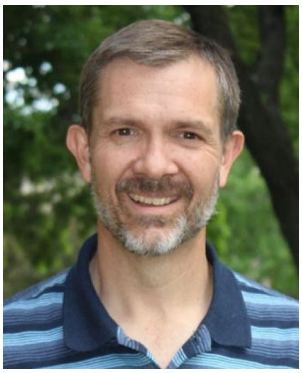
Josephine Hofstetter received her Ph.D. in analytical chemistry from The University of Texas at Austin. She has multiple patents and publications, all directly related to point-of-need diagnostics.



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