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## Paper-Based RNA Extraction, *in Situ* Isothermal Amplification, and Lateral Flow Detection for Low-Cost, Rapid Diagnosis of Influenza A (H1N1) from Clinical Specimens

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### Abstract

The 2009 Influenza A (H1N1) pandemic disproportionately affected the developing world and high-lighted the key inadequacies of traditional diagnostic methods that make them unsuitable for use in resource-limited settings, from expensive equipment and infrastructure requirements to unacceptably long turnaround times. While rapid immunoassay diagnostic tests were much less costly and more context-appropriate, they suffered from drastically low sensitivities and high false negative rates. An accurate, sensitive, and specific molecular diagnostic that is also rapid, low-cost, and independent of laboratory infrastructure is needed for effective point-of-care detection and epidemiological control in these developing regions. We developed a paper-based assay that allows for the extraction and purification of RNA directly from human clinical nasopharyngeal specimens through a poly(ether sulfone) paper matrix, H1N1-specific *in situ* isothermal amplification directly within the same paper matrix, and immediate visual detection on lateral flow strips. The complete sample-to-answer assay can be performed at the point-of-care in just 45 min, without the need for expensive equipment or laboratory infrastructure, and it has a clinically relevant viral load detection limit of 10<sup>6</sup> copies/mL, offering a 10-fold improvement over current rapid immunoassays.

### Graphical Abstract

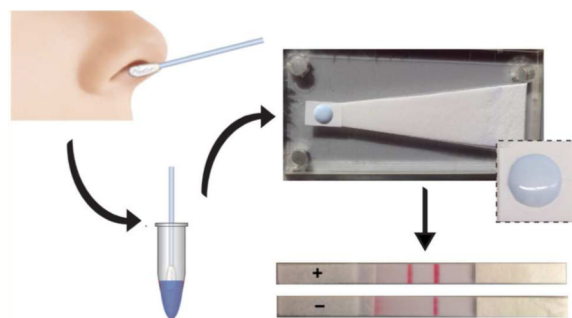
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Supporting Information

Primer sequences, extraction setup images, extraction times and flow rates, RT-LAMP product restriction enzyme digests, glycogen effects, and negative clinical samples. The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.analchem.5b01594](https://doi.org/10.1021/acs.analchem.5b01594).

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The 2009 Influenza A (H1N1) pandemic caused an estimated global mortality of 284,400 deaths worldwide, more than half of which occurred in developing countries in Southeast Asia and Africa.<sup>1</sup> This disproportionate number of deaths suggests that efforts to prevent future pandemics need to more effectively target these developing regions. The standard molecular diagnostic approach for H1N1 virus infection is currently laboratory sample preparation followed by nucleic acid amplification and detection by real-time RT-PCR.<sup>2</sup> However, expensive equipment, highly skilled technicians, and established laboratory and transportation infrastructure requirements make this method unsuitable for use in resource-limited settings. Immunoassay-based rapid diagnostic tests (RDTs) offer a faster, lower-cost solution for resource-limited settings, but they suffer from low test sensitivities and specificities, commonly resulting in false negative and/or false positive detection.<sup>3</sup> Thus, strategies that combine the high sensitivity and specificity of nucleic acid amplification with the rapid, portable, and low-cost nature of RDTs are needed to facilitate clinical care, infection control, and epidemiological investigations in these settings.<sup>4</sup> Paper-based microfluidic technologies have garnered much attention for RDT applications because they are inexpensive, portable, disposable, and most importantly, they eliminate the need for pumps or fluid flow instrumentation due to paper's natural ability to wick fluids through capillary action.<sup>5</sup> Nucleic acid testing involves three main steps, all of which are achievable in paper matrices: (i) sample preparation consisting of sample lysis and nucleic acid extraction, (ii) amplification of the extracted nucleic acids to detectable copy numbers, and (iii) detection of the amplified products. To date, no disposable paper-based devices have been developed that successfully combine the extraction, amplification, and detection steps required for a fully integrated nucleic acid detection system, however a number of advances have been made in each of these individual steps.

Dried-blood spot (DBS) testing, the first form of paper-based DNA capture employed in remote settings, has been used for decades to store HIV DNA on Whatman FTA filter paper for downstream HIV testing in remote settings.<sup>6</sup> More recently, DNA extraction and purification via filtration through a paper matrix has also been reported.<sup>7,8</sup> While these studies have demonstrated the efficacy of paper-based sample preparation systems that allow for elution of purified nucleic acids for downstream PCR analysis, two major challenges remain: (i) how to integrate these extraction modules with the downstream amplification and detection steps required for a "sample-to-answer" total analysis system, and (ii) how to successfully adapt these processes to RNA targets. The typical bind-wash-elute strategy used in DBS and paper-based extraction systems requires off-chip elution and also means that the

extracted nucleic acid will be diluted prior to analysis, which may be undesirable in situations where low concentrations result in suboptimal detection sensitivities. Furthermore, while great strides have been made in paper-based DNA extraction and amplification methods, RNA targets have been largely neglected due to the additional challenges specific to RNA in point-of-care diagnostics. In contrast to DNA, RNA is much less stable, has a high risk of degradation by ubiquitous RNases in biological samples and the environment, and typically involves a reverse-transcription step by an additional enzyme in most amplification methods, adding to the complexity of the assay. Nonetheless, the need for RNA point-of-care diagnostics is becoming increasingly apparent following the 2009 Influenza pandemic, and the very recent Ebola epidemic in resource-limited settings in Africa.<sup>9</sup>

To address these challenges, we sought to develop a paper-based RNA extraction method that allows for an *in situ* RNA amplification reaction to occur directly in the same paper extraction matrix without the need for off-chip elution. We use a rapid, isothermal, reverse-transcription loop-mediated amplification assay (RT-LAMP), which eliminates the need for a thermal cycler.<sup>10</sup> Additionally, we designed the RT-LAMP assay to incorporate probes for immediate downstream visual detection on an immunochromatographic, or a lateral flow detection (LFD) test strip, like those used in common pregnancy tests, further eliminating the need for detection equipment.

Our group and others have shown that molecular amplification of nucleic acids is achievable in a paper-based format.<sup>11–15</sup> Our group has previously reported the capture of *Chlamydia trachomatis* bacterial cells onto paper supports followed by *in situ* isothermal helicase dependent amplification (tHDA) and lateral flow detection of DNA in a pressure-driven system.<sup>11</sup> Ali et al. and Rohrman et al. separately demonstrated isothermal amplification of DNA in a paper matrix followed by fluorescent imaging or lateral flow detection, respectively.<sup>12,13</sup> Both studies, however, required traditional benchtop nucleic acid sample preparation prior to amplification. Gan et al. developed a paper-based DNA extraction chip with the capability to perform PCR directly on-chip; however, this method involved substantial off-chip instrumentation including a syringe pump to drive fluid flow, a thermal cycler for PCR, and a separate downstream gel electrophoresis step for detection of PCR products.<sup>14</sup> To our knowledge, only one other study to date has demonstrated combined paper-based capture and amplification of RNA.<sup>15</sup> However, target RNA was spiked into the sample and not directly extracted from clinical specimens, and the detection method required a fluorescence reader interfaced with a computer, limiting utility in the field.

In this study, we sought to develop a paper-based assay that incorporates all three RNA extraction, amplification, and detection steps directly from human clinical specimens without the need for centrifuges or other sample preparation equipment, thermal cyclers, pumps, pressure, or detection instrumentation of any kind. We report a rapid, equipment-free, paper-based assay for the extraction and purification of Influenza A (H1N1) RNA from patient nasopharyngeal specimens, followed by *in situ* isothermal amplification directly within the same paper extraction matrix, and immediate visual detection via paper lateral flow test strips, for point-of-care, sample-to-answer functionality in 45 min.

## MATERIALS AND METHODS

### H1N1 *in Vitro* Transcribed RNA Standards

*In vitro* RNA standards containing the target loci for both RT-LAMP and the qRT-PCR assays were synthesized by cloning the hemagglutinin (HA) gene from a deidentified patient sample that tested positive for influenza A 2009 (H1N1). Genomic RNA from the patient sample was extracted via the QIAamp Viral RNA Mini Kit (Qiagen) and reverse-transcribed with the Superscript III First-Strand Synthesis Kit (Invitrogen) with a gene-specific reverse primer. The target region on the HA gene, from nucleotide 351 to 1735, was PCR amplified with the Phusion High-Fidelity PCR kit (New England BioLabs), purified via a QIAquick Gel Extraction kit (Qiagen), and cloned into the pGEM-T Easy vector (Promega). Plasmids were isolated with a Plasmid Midi Prep Kit (Qiagen), blunt-cut linearized, and served as *in vitro* transcription DNA templates using a Ribomax Transcription kit (Promega). The RNA transcripts were then purified via DNase digestion, acid phenol-chloroform extraction and ethanol precipitation. The concentration of the purified RNA was determined by measuring the OD<sub>260</sub> with the NanoDrop ND-2000c apparatus (Thermo Scientific). The target RNA copy number was calculated, and 50  $\mu$ L aliquots were made and stored at  $-80^{\circ}\text{C}$ .

### Clinical Nasopharyngeal Specimens

Nasopharyngeal swab (NPS) samples were collected during the 2009 influenza A (H1N1) pandemic period from patients at Beth Israel Deaconess Medical Center (BIDMC) during a previously described study that had been reviewed and approved by BIDMC's institutional review board.<sup>16</sup> Briefly, discarded NPS specimens that had been taken during routine clinical care for testing ordered by the patient's clinician were collected and frozen. The NPS specimens were taken using two Copan flocced swabs (COPAN). The first swab was inserted flat and pushed forward with gentle downward pressure on the lower nasal floor to the posterior wall of the nasopharynx, where it was rotated for a few seconds to collect cellular material. The swab was withdrawn and placed into sterile 1X PBS. The collection procedure was repeated using the second flocced swab in the other nostril; the second swab was placed into M4RT (Remel) media for viral culture. The two swabs were then submitted on ice to the BIDMC microbiology laboratory. After routine testing, specimens (approximately 1.0 mL) were stored at  $-80^{\circ}\text{C}$ . The frozen NPS specimens were later deidentified and sent to the Klapperich Laboratory where they were aliquotted and stored at  $-80^{\circ}\text{C}$ .

For gold standard extraction experiments, RNA was extracted from 140  $\mu$ L of each specimen using the QIAamp Viral RNA Mini Kit (Qiagen).

### Paper Extraction Setup

A  $0.8 \times 0.8$  cm ( $0.64$  cm<sup>2</sup>) piece of poly(ether sulfone) (PES) filter paper with a  $0.2$   $\mu$ m pore size (Millipore, cat# GPWP04700) was placed on top of a cellulose (Whatman GB003 blotting paper, cat# 10426972) absorbent pad cut to absorb approximately 400  $\mu$ L of liquid waste. The absorbent pad was shaped as a 6 cm long sector that extended radially from 0.5 cm at the base of the top sheet inlet port to an ultimate width of 2 cm. This sector angle was optimized to achieve the appropriate flow rate as the volume of liquid absorbed by the pad

per unit time has been previously determined to be linearly related to the angle of the sector shape.<sup>17</sup> The PES and absorbent pad were placed into a custom-designed  $0.635 \times 3.5 \times 7.5$  cm acrylic fixture and aligned with the inlet port in the top acrylic sheet (Figure 1b). The fixture was designed in SolidWorks and was cut out of acrylic sheets using a 30W Epilog Zing laser cutter (speed = 5, power = 100, frequency = 5000). The bottom sheet was rastered (speed = 100, power = 60) with the same dimensions as the absorbent pad to ensure its secure placement. The extraction setup was held together using 32 mm binder clips (Figure S1a).

### Paper Extraction Procedure

Based on a single-step lysis, RNA extraction, and alcohol precipitation recipe developed by Cao et al., 25  $\mu$ L of the NPS specimen was mixed with 75  $\mu$ L lysis buffer (2 M GuSCN, 66.7% 2-propanol, 1 $\times$  RNASecure (Ambion))<sup>16</sup> and 3  $\mu$ L of 15 mg/mL Glycoblue coprecipitant (Life Technologies). This mixture was pipetted onto the PES membrane through the extraction setup inlet port. The prevailing capillary forces generated by the absorbent pad quickly wick the liquid phase away from the membrane surface, thus leaving the solid phase behind. As a result, the RNA-Glycoblue precipitate remains on the PES membrane producing a visible blue film (Figure 1a). The PES membrane was then rinsed sequentially with 200  $\mu$ L of 70% ethanol and 100  $\mu$ L of 100% ethanol, which were also wicked away from the inlet port by the absorbent pad. The PES membrane was physically removed from the extraction setup with forceps and placed into a 0.2 mL tube. Tubes were left open for 2 min to allow the PES membrane to dry.

For extraction quantification experiments, the PES membrane was placed into a 0.2 mL tube with 100  $\mu$ L of nuclease-free water and vortexed to dissolve the RNA-Glycoblue complexes and release the RNA into solution. The tube was inverted and a small hole was pierced through the bottom of the tube using a sterile needle (BD Ultra-Fine 30 Gauge Lancets, cat# 325773). The tube was then stacked inside a larger 1.5 mL Eppendorf tube, and centrifuged at 2500 rpm for 1 min to elute the extracted RNA completely out of the PES and into the Eppendorf tube for downstream qRT-PCR analysis.

Paper extractions were compared to traditional centrifugation extraction methods, where instead of capturing the RNA in paper, we precipitate it by centrifugation, and wash and resuspend the pellet. Briefly, 25  $\mu$ L RNA solutions mixed with 75  $\mu$ L lysis buffer were centrifuged at 13,000 rpm for 15 min at room temperature until a blue pellet was visible at the bottom of the tube. The supernatant was removed, and 100  $\mu$ L of 70% ethanol was added to the tube and centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant was removed and 100  $\mu$ L of 100% ethanol was added to the tube and centrifuged a final time at 13,000 rpm for 5 min. All centrifugation steps were performed in an Eppendorf centrifuge model 5424R. The supernatant was removed and tubes were left open for pellets to dry at room temperature on the benchtop for 10 min. The pellets were resuspended in 100  $\mu$ L nuclease-free water and RNA was quantified using qRT-PCR.

## qRT-PCR

To ascertain the RNA extraction yields, the RNA precipitate eluted from the PES into 100  $\mu\text{L}$  of nuclease-free water was amplified via quantitative, reverse-transcription PCR (qRT-PCR). Using the Brilliant II RT-PCR kit (Agilent), PCR was performed on an Applied Biosystems 7500 thermocycler under the following conditions: 60 min at 50  $^{\circ}\text{C}$  for RT, 10 min at 95  $^{\circ}\text{C}$  for polymerase activation, followed by 40 cycles of 30 s at 95  $^{\circ}\text{C}$ , 30 s at 55  $^{\circ}\text{C}$  for primer annealing, and 1 min at 60  $^{\circ}\text{C}$  for amplification. Five microliters of the extracted RNA was used as the template in a 25  $\mu\text{L}$  reaction mixture. The influenza A (H1N1) and SW H1-specific TaqMan loci used in this PCR were identical to the ones recommended by the CDC<sup>18</sup> and are listed in Table S1.

In each qRT-PCR run, a cycle threshold ( $C_T$ ) versus RNA concentration standard curve was generated from a 5-log dilution series of our *in vitro* transcribed H1N1 RNA standards. For each patient sample, the effective viral RNA concentration was quantitated via standard curve interpolations.

## RT-LAMP Assay

A reverse-transcription loop-mediated amplification (RT-LAMP) assay was developed and optimized for rapid, isothermal amplification and detection of the hemagglutinin (HA) gene of the Influenza A (H1N1) 2009 pandemic virus strain. The reaction was carried out in a final volume of 25  $\mu\text{L}$  with 5  $\mu\text{L}$  of the RNA sample, 2 U large fragment Bst 2.0 DNA polymerase and 1 $\times$  Isothermal Amplification Buffer (New England Biolabs), 2 U Thermoscript Reverse Transcriptase (Life Technologies), 0.2 M Betaine, 8 mM  $\text{MgSO}_4$ , 1 mM each dNTP, 3.75 pmol each of forward and reverse outer primers (F3 and B3), 7.5 pmol each of forward and reverse loop primers (LF and LB), 30 pmol each of forward and reverse inner primers (FIP and BIP), and 0.25  $\mu\text{L}$  of 20X EvaGreen and 0.75  $\mu\text{L}$  of 0.002X ROX reference dyes for real-time quantitative analysis. Because we found our RT-LAMP assay was sensitive to reagent freeze-thaw cycles, multiple aliquots of each assay component were made and frozen down initially, and a fresh aliquot was used each time. The reaction was run for 20 min at 65  $^{\circ}\text{C}$ . Primer sequences previously designed by Kubo et al. were used for the specific amplification of segment 4 of the HA gene of the 2009 pandemic strain<sup>19</sup> and are listed in Table S1. Forward and reverse loop primers (LF and LB) were tagged with Fluorescein isothiocyanate (FITC) and biotin, respectively, to enable downstream detection of the amplified products on immunochromatographic, lateral flow detection (LFD) test strips (Ustar Biotechnologies).

Following amplification, the RT-LAMP assay products were analyzed by 2% agarose gel electrophoresis, LFD strips, or both. The LFD strips consist of a sample pad where the sample is loaded, a conjugate pad that contains streptavidin-conjugated gold nanoparticles, a detection strip where the control and test lines are spotted, and an absorbent pad to direct wicking. During amplification, loop primers tagged with FITC and biotin are incorporated into the amplicons. The biotin probe on the amplicon binds to the streptavidin conjugated gold nanoparticles, which then aggregate at the test line (anti-FITC) as the FITC probe gets captured, forming a visible red line to indicate a positive LAMP reaction. The control line

(biotin) binds excess streptavidin coated gold-nanoparticles, creating a visible positive control to show whether the flow strip worked properly.

Additionally, the specificity of the product was confirmed by restriction enzyme digestion with the *Hind*III restriction endonuclease (New England Biolabs) with a cutting site between FIP and BIP.<sup>19</sup> Following digestion at 37 °C overnight, the digested products were analyzed by 2% agarose gel electrophoresis.

LFD strips were imaged using an iPhone 5 camera (Apple). No postprocessing was required for analysis. LFD test line and control line intensities were analyzed using the Gel Analysis feature in ImageJ (National Institutes of Health). For each LFD strip, the intensity of the test line was divided by the intensity of the control line to obtain the percentage of control intensity for each sample. Although control line intensities may vary across strips, normalizing our test line intensity values to the control line ensured a proper reference for each test strip and allowed us to control for any potential variability during manufacturing (amount of gold nanoparticles impregnated in each conjugate pad) or imaging (lighting or distance from strip, which were not controlled for). Unpaired, two-tailed Student's *t* tests were used to determine the significance of each sample compared to the experimental negative control sample.

### Paper Extraction and *in Situ* RT-LAMP Assay

For *in situ* RT-LAMP experiments, the sample was extracted in the paper extraction setup as described above, and the extracted RNA precipitated onto the PES was amplified directly within the PES matrix. Our group previously investigated the effect of different paper materials on isothermal amplification within the paper matrices and has reported on the success of LAMP within PES membranes.<sup>20</sup> The 25  $\mu$ L RT-LAMP reaction mix was pipetted directly onto the RNA-Glycoblue-containing 0.64 cm<sup>2</sup> PES membrane and was fully absorbed by the PES. This size of PES required to completely absorb 25  $\mu$ L of liquid had been calculated using the previously determined water absorbency of PES of 38.82  $\mu$ L/cm<sup>2</sup>.<sup>20</sup> The soaked PES was left in the 0.2 mL tube with the lid closed to prevent evaporation and incubated in a 65 °C heat block for 23 min. Following amplification, the soaked PES containing the entire RT-LAMP reaction volume was placed directly onto the sample pad of the LFD strip using forceps, and the LFD strip and PES were placed between two acrylic sheets aligned with the inlet port (Figure S1b) and the setup was held together with 32 mm binder clips. 50  $\mu$ L of nuclease free water was then pipetted into the inlet port, filtered through the PES, and wicked onto the LFD strip for immediate detection of amplified products.

## RESULTS AND DISCUSSION

### Paper Extraction Yields Are Comparable to Traditional Extraction Methods over 5-log of RNA Concentrations

For initial paper extraction experiments, solutions of influenza A (H1N1) RNA spiked into PBS were prepared at concentrations ranging from 10<sup>10</sup> copies of RNA per mL (cp/mL) down to 10<sup>8</sup> cp/mL, and a negative control containing no RNA. RNA solutions were mixed

with the Glycoblue-containing lysis buffer, pipetted into the inlet port of the extraction setup (Figure 1a,b), and filtered and washed through the PES membrane producing a visible blue film (Figure 1a inset). The complete extraction procedure took approximately 18 min. The flow rates and times for all steps are shown in Figure S2.

The RNA precipitate was eluted from the PES matrix and recovery yields were quantified using qRT-PCR (Figure 1c). Results from triplicate paper extraction experiments show good correlation between input and recovered RNA over 5-log of concentrations ( $r^2 = 0.9989$ ) with yields between 60% and 94%. Quantity values for RNA extracted through PES or via traditional centrifugation methods were compared (Figure 1c). RNA recovery yields through the PES membranes ranged from 66% to 109% of the centrifugation control yields. These results demonstrate that our paper extraction method, which is equipment-free and faster than traditional centrifugation extraction methods (~20 min versus ~35 min including drying times), results in comparable extraction yields over 5-log of RNA concentrations.

### A 5-log Linear, H1N1-Specific RT-LAMP Reaction Can Be Visually Detected within 20 min

Before developing the *in situ* amplification assay, we first optimized a protocol for RT-LAMP amplification of H1N1 RNA in solution. The H1N1 RT-LAMP assay was optimized using our *in vitro* transcribed H1N1 RNA standards. We set a target lower limit of detection of  $10^5$  cp/mL, since the mean pretreatment H1N1 viral load in nasal specimens has been reported to be  $\sim 10^8$  cp/mL with typical viral loads between  $10^6$  and  $10^{10}$  cp/mL, and patients below  $10^5$  cp/mL generally had not yet begun to exhibit symptoms.<sup>16,21,22</sup> The RT-LAMP reaction incubation time at 65 °C required to reach our target lower limit of detection of  $10^5$  cp/mL was determined by amplifying 10-fold serial dilutions of *in vitro* transcribed target RNA (from  $10^{10}$  to  $10^5$  cp/mL) using EvaGreen and ROX reference dyes for real-time quantification of amplification. With our final optimized assay conditions, we were able to amplify  $10^5$  cp/mL (a net 500 copies per sample) to detectable levels within 20 min (Figure 2a). To ensure specificity of the primers for the HA gene of the H1N1 strain, *in vitro* transcribed RNA from a different gene of the influenza A viral genome, the matrix protein-encoding M1 gene, was also tested at a high concentration of  $10^{10}$  cp/mL. There was no amplification of the negative (NTC) control or of the M1 gene, demonstrating H1N1 strain specificity. Amplification results were confirmed by 2% agarose gel electrophoresis (Figure 2b) and lateral flow detection (Figure 2c).

LFD strips produced visible test lines for positive reactions, enabling immediate detection of amplified products with the naked eye. Test line intensities were quantified as a percentage of control line intensities and results from three independent experiments are plotted in Figure 2c. Although lower in intensity, our LFD strips still exhibit a clear, visible test line down to  $10^5$  cp/mL that is statistically different from the negative control.

Additionally, to confirm that RT-LAMP products specifically correspond to the correct H1N1 target sequence, the amplified products were digested with the *Hind*III restriction endonuclease and analyzed by agarose gel electrophoresis (Figure S3). The Influenza A (H1N1) HA gene sequence contains a single *Hind*III cutting site between the FIP and BIP regions, and digested products were in agreement with the expected sizes previously determined by Kubo et al.,<sup>19</sup> confirming the specificity of our product.



### ***In Situ* RT-LAMP within a Paper Matrix Is Detectable across 5-log of RNA Concentrations**

Having previously demonstrated that LAMP reactions are not inhibited in the presence of PES, and can even take place completely within a PES matrix<sup>20</sup> we next asked whether *in situ* amplification of the freshly extracted RNA was possible within the same PES matrix. The optimized protocol for amplification in solution (Figure 2) served as a starting point for optimizing the paper-based RT-LAMP assay. Optimization experiments revealed that a higher MgSO<sub>4</sub> concentration of 11 mM improved RT-LAMP performance *in situ*. All other assay reagent concentrations remained the same as the *in solution* reaction mix. Once the RNA was extracted onto the PES membrane, the 25  $\mu$ L RT-LAMP reaction mix was added directly onto the RNA-containing PES, and the full reaction volume was completely absorbed by the membrane. The soaked PES was then placed inside a 0.2 mL tube to prevent evaporation, and incubated in a 65 °C heat block for 23 min. During preliminary RT-LAMP *in situ* experiments, we found that the Glycoblue that coprecipitates with the RNA onto the PES caused a slight inhibition of the amplification (Figure S4a), and after careful optimization we determined that 23 min was the ideal reaction time for RT-LAMP *in situ* (Figure S4b). After the 65 °C incubation, the soaked PES was then placed directly onto a lateral flow strip and 50  $\mu$ L water was slowly dropped onto the PES to elute the amplified product onto the detection strip. In order to ensure that the water filtered through the PES before reaching the lateral flow strip, the PES and strip were placed between two acrylic sheets aligned with an inlet port, similar to the extraction setup, except the lateral flow strip replaces the absorbent pad (Figure S1b). The complete process from sample to answer took approximately 45 min, including an 18 min average for the paper extraction, followed by 2 min drying, 23 min RT-LAMP reaction, and 2 min for detection on the lateral flow strips.

Solutions of influenza A (H1N1) RNA spiked into PBS at concentrations ranging from 10<sup>10</sup> down to 10<sup>5</sup> cp/mL and a negative control containing no RNA were extracted through the PES membrane just as in Figure 1 and amplified directly within the PES membrane via RT-LAMP *in situ*. The amplified products were eluted directly onto the lateral flow strips and representative lateral flow strips from three individual experiments are shown in Figure 3b and test line intensities from all three sets of strips were quantified and normalized to control line intensity (Figure 3c). Statistical analysis from the three experiments determined that detection of all but the lowest concentration (10<sup>5</sup> cp/mL) were statistically significant when compared to the negative control. As shown in Figure 3c, 10<sup>5</sup> cp/mL was amplified to detectable levels in two of the three experiments, albeit lightly in one of them. From these results, we can conclude that our lower limit of detection for RT-LAMP *in situ* is an order of magnitude higher (10<sup>6</sup> cp/mL) than RT-LAMP in solution, however this would still theoretically cover over 90% of cases given the nasopharyngeal swab sample viral loads previously measured in a large group of patients.<sup>16</sup>

### **Paper Extraction, *In Situ* RT-LAMP, and Lateral Flow Detection of H1N1 RNA from Clinical Nasopharyngeal Specimens**

To ensure compatibility of our paper extraction and *in situ* RT-LAMP assay with clinical specimens, 12 deidentified nasopharyngeal swab samples collected from patients that tested positive for H1N1 during the 2009 pandemic<sup>16</sup> with a range of viral titers were selected for testing and labeled with letters A–L. Additionally, three H1N1-negative samples from

patients exhibiting other respiratory illness at the time of specimen collection were chosen at random and tested by our assay (Figure S5). Prior laboratory testing indicated that one of the samples was Influenza B-positive, and two samples were Respiratory Syncytial Virus (RSV)-positive.

Multiple aliquots were made of each clinical specimen and aliquots were stored at  $-80^{\circ}\text{C}$ . One aliquot from each positive patient sample was extracted via a QIAamp Viral RNA Mini Kit as a gold standard extraction method. We compared our paper extraction method to the Qiagen kit via qRT-PCR (Figure 4a). Our paper extraction yields ranged from 10% to 140% of Qiagen yields, exhibiting some variability that we speculate may be due to slight viral load variations across sample aliquots and possible effects of freeze–thaw cycles. Nonetheless, results show good correlation between recovered RNA quantities from each method.

To be certain that our RT-LAMP assay would correctly amplify these H1N1 positive clinical specimens, we first performed RT-LAMP reactions in solution using purified Qiagen-extracted RNA from each positive sample. All positive samples were amplified to detectable levels as shown by agarose gel electrophoresis (Figure 4b) and lateral flow detection (Figure 4c). Next, a fresh aliquot of each sample was extracted via our paper extraction method and amplified via our *in situ* RT-LAMP assay. A positive ( $10^9$  cp/mL *in vitro* transcribed RNA) and negative (no RNA) sample were also extracted and amplified as controls. After a 23 min incubation at  $65^{\circ}\text{C}$ , the amplified products were eluted from the PES directly onto the lateral flow strips as shown in Figure 3a, and strip test lines were quantified as a percentage of control lines (Figure 4d). None of the negative clinical samples tested positive by our assay, as shown in Figure 5S, confirming our H1N1 strain-specificity. Ten of the 12 samples successfully tested positive via our paper extraction, *in situ* RT-LAMP, and LFD assay. The two samples that were not detected by our assay were the samples with the lowest viral titers ( $\sim 2 \times 10^6$  cp/mL) and also resulted in the lowest test line intensities in the Qiagen-extract RT-LAMP in solution control assay (Figure 4c). This loss in sensitivity with clinical specimens as compared to our *in vitro* transcribed RNA standards tested in Figure 3 could be a result of additional inhibitors in the biological specimens that may have remained in the paper extraction matrix and could potentially have interfered with the LAMP reaction. For example, saline is known to inhibit LAMP amplification in a dose-dependent manner,<sup>23</sup> and it is possible that residual salt from the nasopharyngeal specimens were not entirely rinsed from the PES matrix during ethanol washes. Furthermore, incomplete inactivation of RNases present in the specimens is also possible.

Despite this slight loss in sensitivity with clinical samples, our paper extraction and *in situ* RT-LAMP assay still offers a significant improvement in detection limit over many commercially available rapid influenza diagnostic tests (RIDTs). In a recent study evaluating RIDT performance compared to qRT-PCR, results indicated that while qRT-PCR detected viral loads as low as  $10^3$  cp/mL of influenza A, RIDTs typically showed negative results for viral loads less than  $10^7$  cp/mL.<sup>24</sup> According to the Center for Disease Control (CDC), sensitivities of rapid diagnostic tests for influenza are approximately 50–70% when compared with RT-PCR, and specificities are approximately 90–95%.<sup>3</sup> Our group previously reported a study testing a large group of patient samples ( $n = 119$ ) collected from a clinical

site in Boston during the same time period the samples tested here in Figure 4 were collected, and found that the rapid immunoassays (Xpect Flu and BinaxNOW) were only 49% sensitive and 98% specific, with a positive predictive value of 97% and a negative predictive value of only 60%.<sup>16</sup> Two other independent studies comparing influenza immunoassays have reported sensitivities as low as 39%.<sup>25,26</sup> During the 2009 H1N1 outbreak, a study conducted in New York City found that H1N1-specific rapid diagnostics had sensitivities as low as 9.6% compared to viral culture.<sup>27</sup> Our lower detection limit of  $\sim 10^6$  cp/mL is well within the clinically relevant range, and of the 12 known positive patient samples we tested, 10 (83%) were correctly identified as positive by our assay.

## CONCLUSION

A paper-based assay was developed for the extraction and purification of Influenza A (H1N1) RNA directly from patient nasopharyngeal specimens, *in situ* isothermal RT-LAMP amplification, and immediate lateral flow detection of amplified products. The detection limit of our paper-based assay was  $10^6$  cp/mL, suitable for the vast majority of reported patient viral loads at the onset of symptoms. Our assay required no instrumentation other than a heat block, and the total sample-to-answer assay time was 45 min, making it suitable for a rapid diagnostic. This work can be extended to other pathogen targets simply by changing primer sequences, and it offers a simple and inexpensive platform for point-of-care assay development. While the assay presented here involved three separate modules for extraction, amplification, and detection, future work will include integration of the assay onto a single paperfluidic chip to increase portability and usability in remote settings. In summary, we demonstrate sample preparation, amplification, and detection within a rapid RNA molecular diagnostic made from low-cost materials that brings this technology closer to a completely paper-based molecular RDT for use in resource-limited settings.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

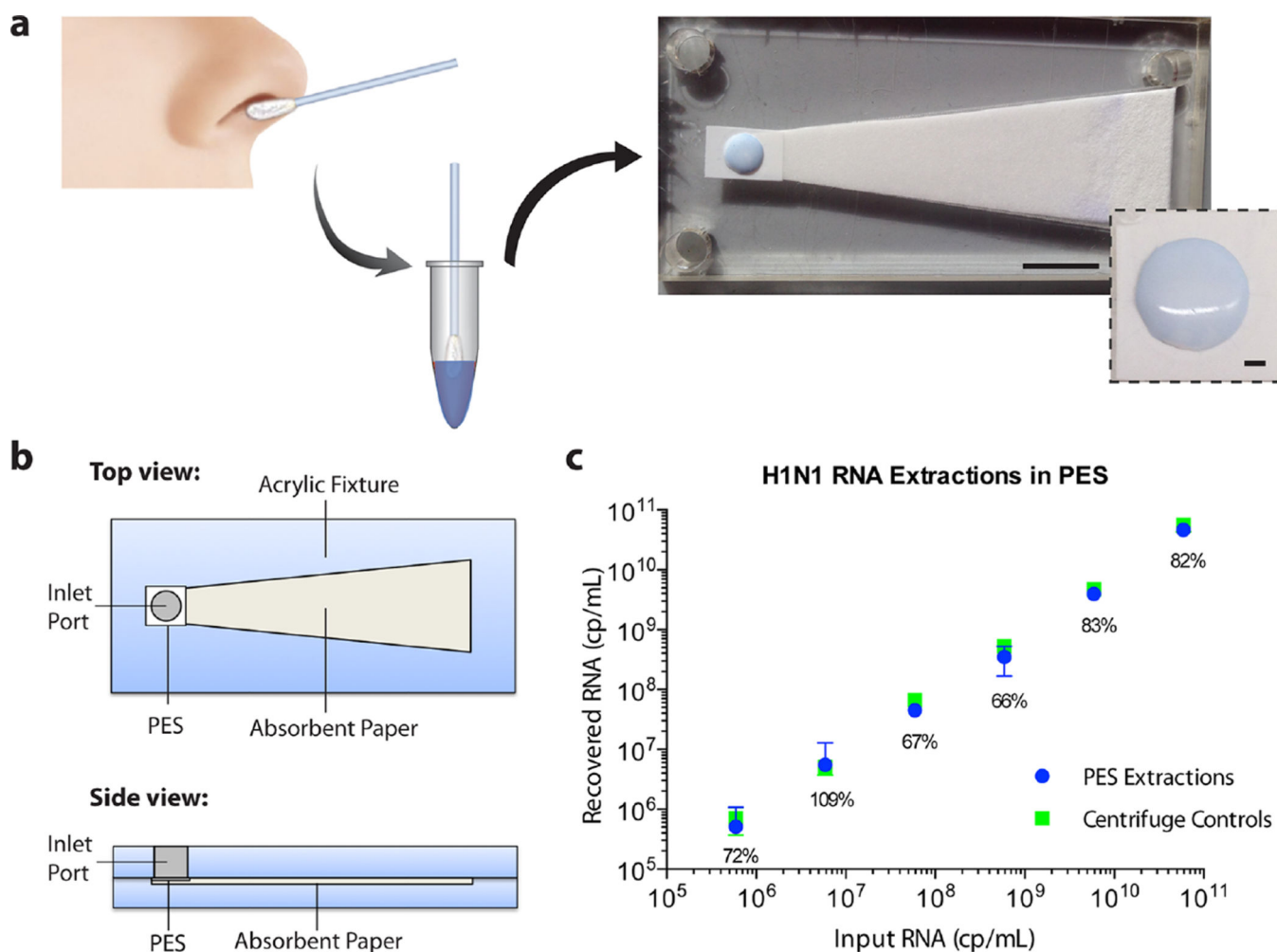
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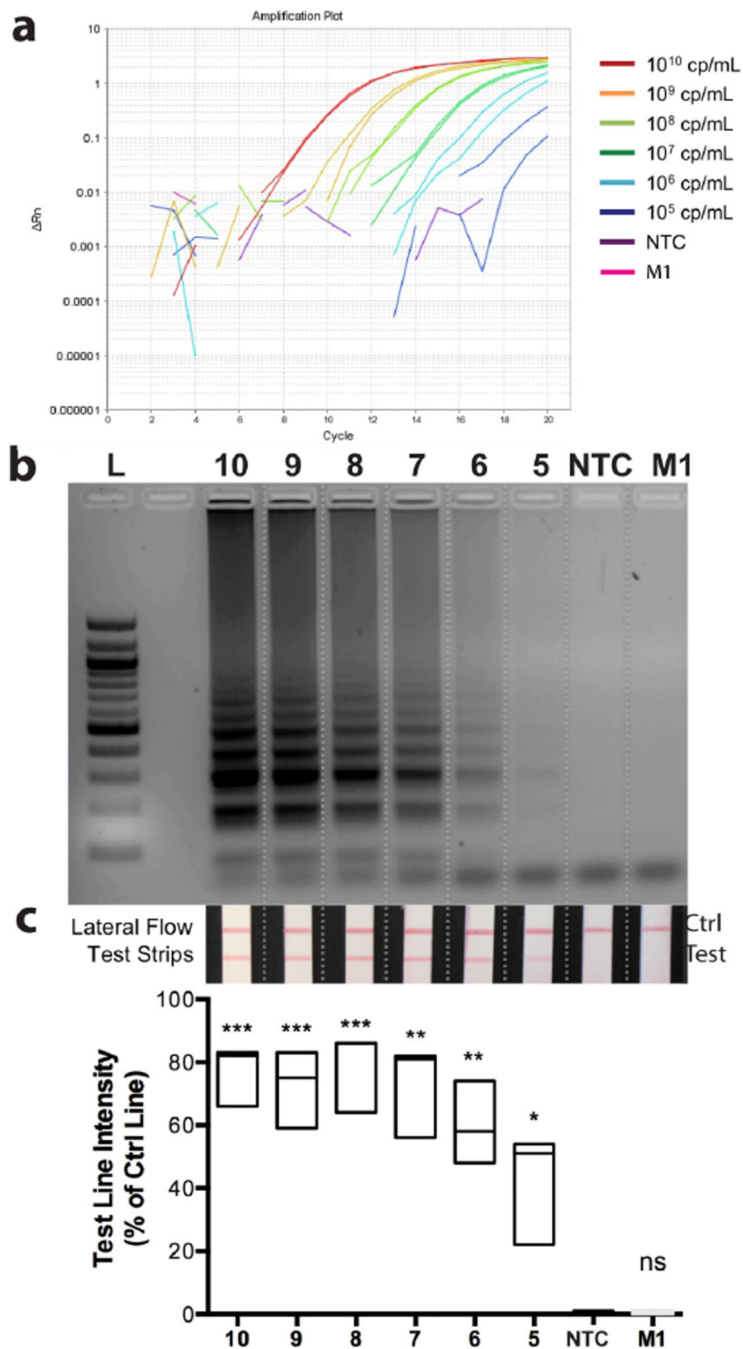
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**Figure 1.**

Paper-based extraction of influenza A (H1N1) RNA. (a) Schematic of the paper RNA extraction method. Nasopharyngeal swab samples are lysed in a Glycoblue-containing lysis buffer and filtered through our paper extraction setup (scale bar = 10 mm). Co-precipitated RNA and Glycoblue result in a visible blue film (inset, scale bar = 1 mm). (b) Extraction set up. (c) Paper extractions of H1N1 RNA standards and centrifuge control extraction yields quantified via qRT-PCR. Error bars: standard deviation,  $n = 3$ . Percentage values indicate paper extraction yields compared to centrifuge control yields.



**Figure 2.** RT-LAMP assay in solution. (a) Real-time RT-LAMP amplification of *in vitro* transcribed H1N1 RNA standards from 10<sup>10</sup> cp/mL down to 10<sup>5</sup> cp/mL. NTC = no template control. (b) 2% Agarose gel electrophoresis of RT-LAMP products. L = 100bp DNA ladder, 10 = 10<sup>10</sup> cp/mL, 9 = 10<sup>9</sup> cp/mL, etc. NTC = no template control. M1 = M1 gene *in vitro* transcribed standards, 10<sup>10</sup> cp/mL. (c) Representative lateral flow strips from three independent experiments show detection of RT-LAMP products. The top line is the flow strip control line; the bottom line is a test line. Test line intensity as percentage of control line intensity

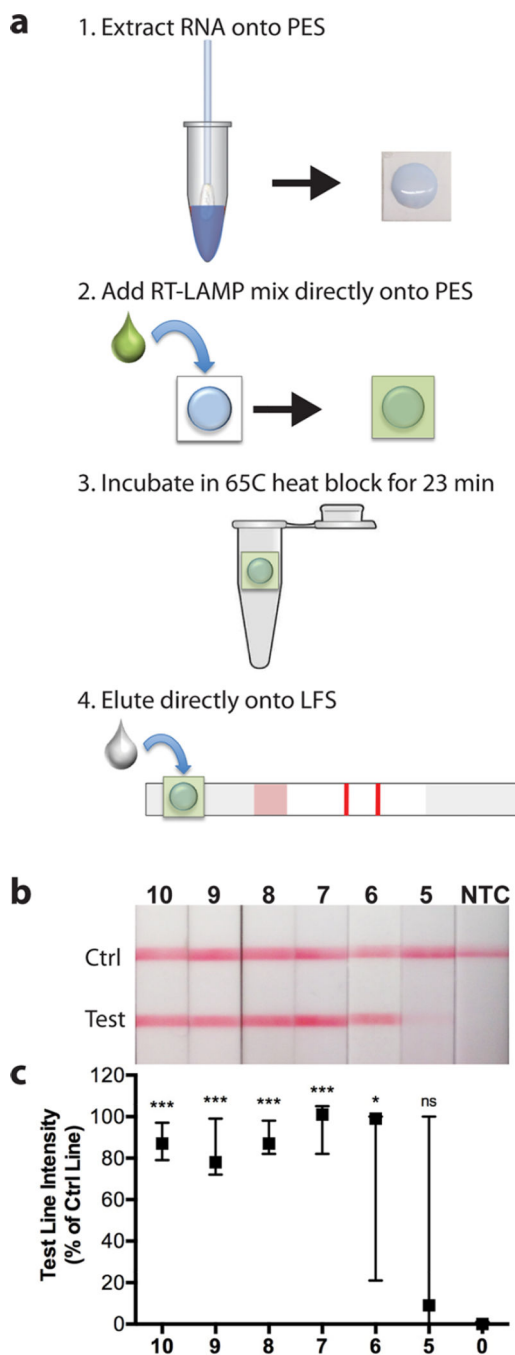
for three experiments is plotted. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns = not significant.)

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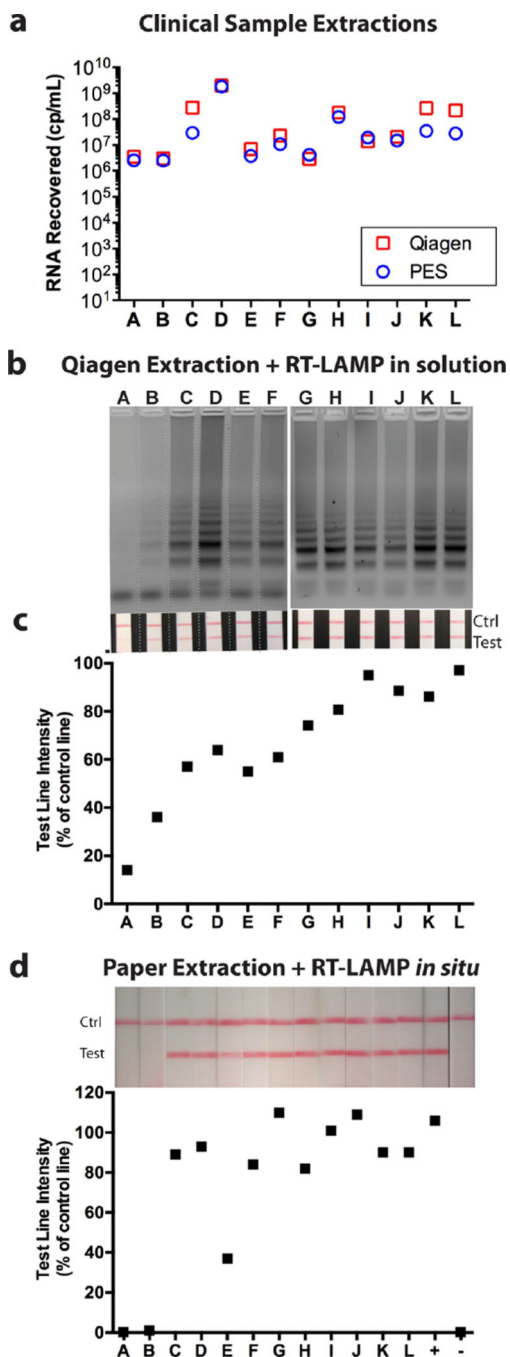
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**Figure 3.** RT-LAMP in situ with *in vitro* transcribed H1N1 RNA standards. (a) Method schematic of paper RNA extraction followed by in situ RT-LAMP and immediate downstream lateral flow detection. (b) Representative lateral flow detection strips. 10 =  $10^{10}$  cp/mL, etc. NTC = no template control. (c) Lateral flow detection strip test line intensities from three independent experiments are plotted as a percentage of control line intensities. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns = not significant.)





**Figure 4.** Clinical nasopharyngeal specimens. (a) Paper extractions and QIAamp kit extractions of clinical specimens A–L. (b) RT-LAMP assay performed in solution with Qiagen-extracted purified RNA from clinical specimens A–L, and gel electrophoresis of products. (c) Lateral flow detection of amplified products; test line intensities plotted as a percentage of control line intensities. (d) Paper extraction of clinical specimens A–L followed by *in situ* RT-

LAMP and lateral flow detection. + = positive control ( $10^9$  cp/mL RNA standard); - = negative control (no RNA).

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