Facilitating Detection of SARS-CoV-2 Directly from Patient Samples: Precursor Studies with RT-qPCR and Colorimetric RT-LAMP Reagents

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Introduction

This Application Note is presented in the context of the COVID-19 global pandemic caused by the SARS-CoV-2 coronavirus. At the time of writing, over two million COVID-19 cases with over 150,000 deaths have been confirmed globally. The number of individuals infected with SARS-CoV-2 is likely much higher, however, owing in part to limited testing. It has been widely acknowledged that broader testing is critically needed.

In the United States, most diagnostics approved to date under FDA Emergency Use Authorization (EUA) have employed RT-qPCR for hydrolysis probe-based (e.g., TaqMan) detection of viral targets in RNA purified from patient samples. The inclusion of an up-front RNA purification step, however, adds to protocol time and cost per sample, and overall can reduce testing throughput. In addition, the scale of demand for testing materials has led to supply shortages for some RNA purification reagents. Diagnostics that are capable of robust detection directly from patient samples would therefore be highly advantageous, potentially enabling testing that is faster, cheaper, higher-throughput, and better positioned to scale for anticipated demand.

To help accelerate diagnostics development efforts, here we demonstrate detection of synthetic SARS-CoV-2 viral RNA targets using NEB’s RT-qPCR and colorimetric RT-LAMP reagents. The colorimetric RT-LAMP work is a follow-up on a recent publication by researchers at NEB, in collaboration with researchers at the Wuhan Institute of Virology in China, demonstrating colorimetric RT-LAMP detection of SARS-CoV-2 viral RNA purified from patient samples. Further, we evaluate two requisite features for direct detection from patient samples: tolerance to Universal Transport Medium and resistance to inhibition and/or RNA degradation in the presence of human cell lysates.

Results

Reagent tolerance to Universal Transport Medium (UTM)

Nasopharyngeal (NP) swabs are the predominant patient sample type recommended for SARS-CoV-2 diagnostic testing and are most commonly stored in Universal Transport Medium (UTM) prior to processing. UTM tolerance is thus a critical reagent feature for direct detection of SARS-CoV-2 in patient samples. We therefore tested the effects of UTM on candidate NEB reagents for viral RNA detection, including the Luna Universal Probe One-Step RT-qPCR Kit (for hydrolysis probe-based detection of RNA), LunaScript RT SuperMix Kit (for first-strand cDNA synthesis in two-step RT-qPCR workflows), and WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA) (for rapid reverse transcription and isothermal amplification of RNA targets, with a simple visible detection).

Both the Luna one-step and two-step RT-qPCR reagents were highly tolerant of UTM, with no detectible effect on detection and quantification with 5 μl UTM per 20 μl reaction (25% v/v) (Figure 1A-D). Sensitive detection and linear quantitation were observed for both a human mRNA target (actin) from human total RNA (Figures 1A,C) and a SARS-CoV-2 viral RNA target (N-gene) from synthetic viral RNA (Figures 1B,D).

Materials

Luna Universal Probe One-Step RT-qPCR Kit (NEB #E3006)
Luna Universal Probe One-Step RT-qPCR Kit (ROX-free) (NEB #E3007)*
Luna Cell Ready Probe One-Step RT-qPCR Kit (NEB #E3031)
Luna Cell Ready Lysis Module (NEB #E3032)
LunaScript RT SuperMix Kit (NEB #E3010)
WarmStart Colorimetric LAMP 2X Master Mix (NEB #M1800)

* Note: This is a new product released to meet recent increased demand. Performance is equivalent to NEB #E3006 for real-time instruments that do not use ROX signal-based reference dye normalization.
FIGURE 1: Tolerance of Luna RT-qPCR reagents to Universal Transport Medium

A-B. Simultaneous quantitation of SARS-CoV-2 N-gene (A) and human actin mRNA (B) from human total RNA (5 pg to 50 ng) spiked with synthetic SARS-CoV-2 viral RNA (50 to 500,000 copies), in the presence (darker color) vs absence (lighter color) of 5 μl UTM per reaction (25% v/v), as determined by multiplex RT-qPCR using the Luna Universal One-Step Probe RT-qPCR Kit. Synthetic viral RNA was generated by in vitro transcription of RNA corresponding a portion of N-gene from the SARS-CoV-2 genome (GenBank Accession Number MN908947). C-D. Quantitation of actin (C) or N-gene (D) by Two-Step RT-qPCR using the LunaScript RT SuperMix Kit for first-strand cDNA synthesis and Luna Universal Probe qPCR Master Mix for subsequent amplification and quantitation.

In the case of the more rapid colorimetric RT-LAMP workflow, a color change is observed in the presence of amplification (pink to yellow). This color change is pH-dependent, relying on the production of protons and a subsequent drop in pH that results from extensive DNA polymerase activity in a positive LAMP reaction. Thus, we also tested whether additional buffer present in UTM may inhibit color change, and if the color of UTM itself (pink) may mask RT-LAMP color change at higher UTM volumes. The results indicated that color change was still visible at up to 2 μl UTM per 25 μl reaction (8% v/v) (Figure 2A). Amplification itself remained robust at higher sample volumes, up to 5 μl per 25 μl reaction (20% v/v) (Figure 2B), but required an alternative detection method (fluorescent dye-based detection of double-stranded DNA).
Direct Detection of SARS-CoV-2 RNA Targets in Cell Lysates

Direct detection of SARS-CoV-2 in patient samples will require that reagents be resistant to inhibition by cell lysates, and that viral RNA be protected from degradation by cellular RNases prior to detection. To assess both these factors, we lysed cells in the presence of synthetic SARS-CoV-2 RNA (vRNA) and then assessed detection and quantitation via both one-step RT-qPCR and colorimetric RT-LAMP, using purified vRNA alone as a control. Cell lysis was carried out using the Luna Universal Probe One-Step RT-qPCR Kit, which includes an RNA Protection Reagent to prevent RNA degradation. It should be noted that the synthetic vRNA used here should only be regarded as a partial proxy for targets within the full-length, capsid-enclosed viral RNA genome (see Discussion Section).

Using the Luna Universal Probe One-Step RT-qPCR Kit, SARS-CoV-2 vRNA targets were detected with high sensitivity (consistent detection at approximately 50 copies per reaction; lower titers not yet tested), in both the absence and presence of cell lysates (Figures 3A-C). Additionally, linear quantitation was observed over a broad range of vRNA titers (50 to 500,000 copies, efficiency = 93.8% for Orf1a quantitation, 99.1% for N-gene quantitation) (Figures 3A,B). Cell lysates ranged in density from 0.2 to 2,000 cell equivalents per RT-qPCR reaction, and equivalent tolerance was observed over this range.
Using the WarmStart Colorimetric LAMP 2X Master Mix for colorimetric RT-LAMP, SARS-CoV-2 vRNA targets were also detected with good sensitivity (consistent detection at approximately 480 copies per reaction) (Figure 4), with up to 200 cell equivalents per reaction. Of note, 1:10 dilution of Cell Ready lysates was required for colorimetric detection, as the cell lysis buffer would prevent pH change at higher concentrations.

FIGURE 4:
Direct detection of SARS-CoV-2 RNA targets in cell lysates by RT-LAMP

WarmStart Colorimetric LAMP 2X Master Mix was used to detect SARS-CoV-2 RNA targets in cell lysates. Human (HeLa) cells were lysed in the presence of synthetic vRNA using the Luna Cell Ready Lysis Module. Lysates were then diluted 1:10, and 1 μl then added to each RT-LAMP reaction, giving 48 to 4,800 vRNA copies as indicated and 2 to 200 cell equivalents per reaction.
Discussion
The data presented here is intended to serve as a starting point, which we hope will enable further studies and test development. Additional questions remain, and development of direct diagnostics will ultimately require testing with COVID-19 patient samples. NP swab samples contain not only UTM, nasal epithelial cells (and/or lysed cell contents) and viral particles, but also mucus and other nasal mucosal secretions; direct testing of samples will be required to determine whether these additional factors affect reagent performance and/or viral RNA stability during testing workflows. Additionally, while naked synthetic RNA was used in our testing as a proxy, most viral RNA in patient samples is sequestered in proteinaceous capsids within enveloped virion particles. This protects viral RNA from RNases secreted by nasal mucosa, and thus helps prevent RNA degradation prior to testing; however, the virion envelope and enclosed capsid must ultimately be disrupted to enable viral RNA detection.

Despite these challenges, a recent study demonstrated direct detection of SARS-CoV-2 RNA in patient samples using Luna One-Step RT-qPCR reagents. While a drop in sensitivity was observed for direct detection (C$_{q}$ = 23) compared to quantitation from purified RNA for the same samples (C$_{q}$ = 18.7), this was still far above apparent detection limits for the assay, suggesting that direct detection could be viable even at much lower viral titers than those of the patient samples tested. The observed C$_{q}$ delay may result from incomplete viral RNA release from encapsidation, reagent inhibition, and/or viral RNA degradation; evaluating and mitigating these factors may further improve assay performance.

While RT-qPCR has been the dominant form of COVID-19 diagnostic testing thus far, a test employing colorimetric RT-LAMP could provide several critical advantages. First, it allows broad accessibility as a field or point-of-care diagnostic, as it only requires incubation at a single reaction temperature (for isothermal amplification) and employs visual detection. In contrast, RT-qPCR requires more expensive real-time PCR instrumentation to allow thermocycling and fluorescence detection. Second, RT-LAMP is rapid, with results after ≤ 30 minutes of incubation. Colorimetric RT-LAMP thus offers the potential for a simple, rapid, inexpensive and broadly deployable test allowing in-visit diagnosis in both lab and point-of-care settings, and even testing in the field at points of need to aid surveillance and prevention.

NEB has and will continue to supply and support customers who are working diligently to develop better diagnostic tools for the COVID-19 virus. We hope that the work described here may contribute to these efforts.

References

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Colorimetric RT-LAMP
• UTM at up to 8% (v/v) total reaction volume is tolerated for color change detection using the WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA), and at up to 20% (v/v) for amplification using alternative detection methods (e.g., fluorescence).

• Detection and quantitation of viral RNA targets using colorimetric RT-LAMP can be achieved with cell lysates, at up to 200 cell equivalents per 25 μl reaction.

• For color change detection, Luna Cell Ready lysates and other buffered lysates should be diluted prior to addition to colorimetric RT-LAMP reactions. We recommend adding 1 μl of a 1:10 dilution per 25 μl reaction as a starting point.