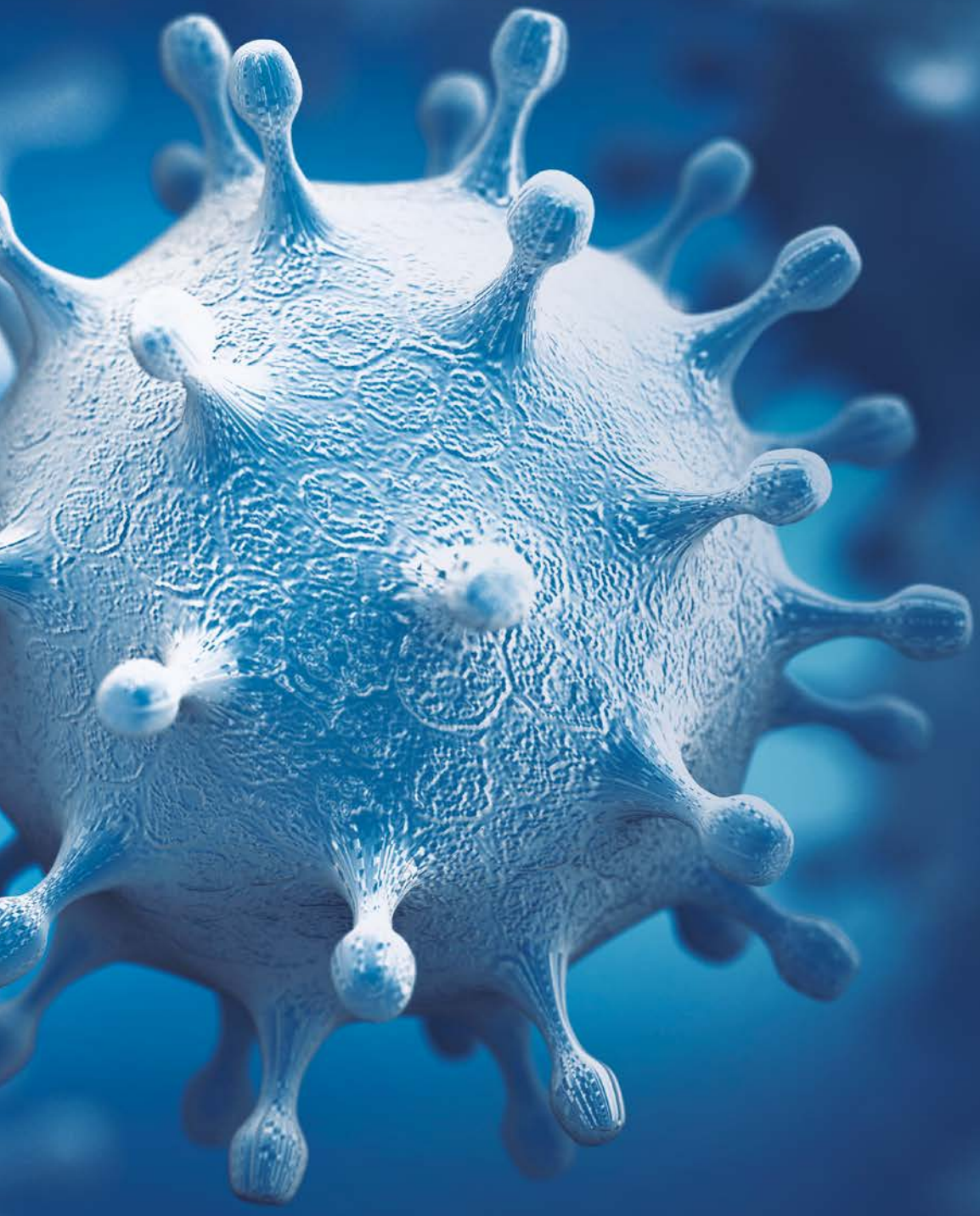
Fall/Winter 2020

NEB expressions

a scientific update



IN THIS ISSUE

NEW ENGLAND BioLabs®
be INSPIRED drive DISCOVERY stay GENUINE

Loop-mediated isothermal amplification (LAMP) – a fast-growing technology with a wide range of applications

By Joanne Gibson, Ph.D., New England Biolabs, Inc.

Polymerase Chain Reaction – PCR

The polymerase chain reaction (PCR) is an indispensable tool in many molecular biology laboratories, and has been transforming research and medical diagnoses for decades. Over the years, this technology has seen many advances. In the early days before thermocyclers became available, researchers sat diligently by multiple water baths set at different temperatures, timer in hand, ready to swap the tubes from one water bath to the next for each temperature-specific step – denaturation, annealing and extension. Before the use of heated thermocycler lids, mineral oil was layered on top of the reaction mixture to prevent evaporation. Then, *Taq* DNA Polymerase (NEB #M0273), the first heat-stable polymerase, was introduced. Prior to this discovery, a heat-labile polymerase was laboriously added at the start of every cycle because it was destroyed by the high temperatures required for the denaturation step. Scientists could now add polymerase for the entire amplification at the beginning of the experiment. Reaction setup became even more user-friendly with the introduction of hot start polymerases, in which dissociation from an inhibitor is required for activity, allowing for increased specificity and room temperature reaction set up.

Now, PCR is much more efficient and a mainstream laboratory technique for a wide range of applications. It requires equipment such as a thermocycler and gel electrophoresis or real-time instrumentation, which require an electricity source. These are readily available in well-equipped laboratories – along with the knowledge required to design and carry out a PCR. However, in some instances there are time-sensitive, or location-specific aspects to a study that make transport of samples to a laboratory the limiting factor in acquiring prompt, accurate results. In these cases, the need for a simpler form of nucleic acid amplification became apparent – not in place of traditional PCR, but as an option for use in a broader range of settings.

Loop-Mediated Isothermal Amplification – LAMP

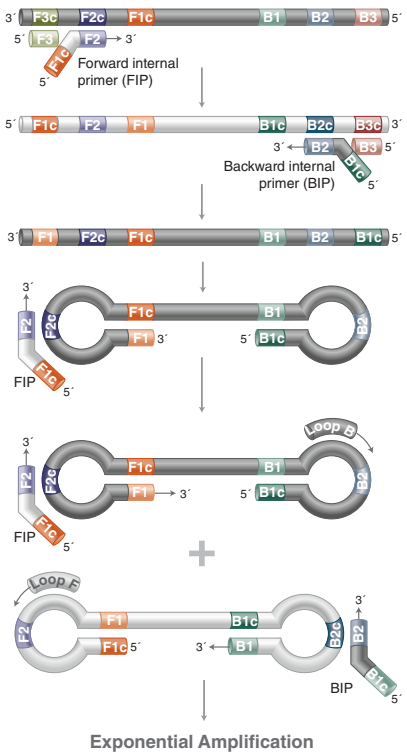
In response to this need, researchers developed a new method of nucleic acid amplification: loop-mediated isothermal amplification (LAMP) (1). In a nutshell, LAMP is a powerful method for amplifying a target sequence in a single tube, at a single temperature using a variety of simple detection methods.

LAMP uses 4 to 6 primers that recognize 6 to 8 regions of the target DNA or RNA sequence (Figure 1). The design of two of the primers intentionally results in self-hybridizing loop structures that form a dumbbell, providing numerous sites for synthesis initiation. *Bst* DNA Polymerase, Large Fragment (NEB #M0275), which is active at elevated temperatures and ideal for this application, displaces downstream DNA that is encountered during strand synthesis. These features of LAMP – the hyper priming of the target sequence, coupled with the use of a strand-displacing DNA Polymerase – lead to exponential amplification at a rate that can be detected by a variety of methods in approximately 30 minutes. NEB-engineered versions of the *Bst* enzyme have expanded LAMP utility by reducing inhibition by common sample contaminants and dUTP. Increased dUTP tolerance enabled carryover prevention, once common only in PCR-based techniques, to be adapted to LAMP. Additionally, robust RNA-based amplification can be accomplished by the simple addition of the NEB WarmStart RTx enzyme, which is also included in NEB LAMP master mixes. Therefore, amplification of RNA or DNA substrates can be performed outside the walls of a laboratory; in fact, the entire reaction takes place at 65°C and could be carried out in a cup of hot water.

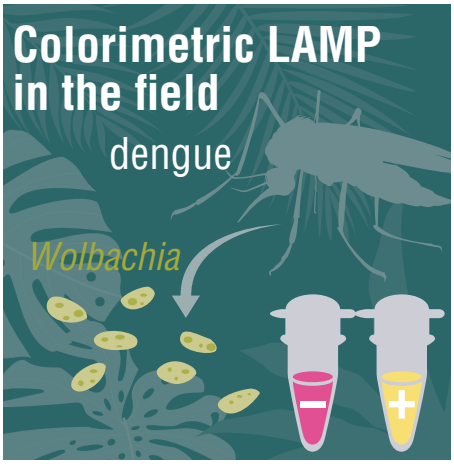
One of the advantages of LAMP is its broad utility. LAMP reaction progress can be tracked in real time by adding a fluorescent dye that binds to dsDNA as it amplifies. For high throughput applications, LAMP can be incorporated into automated workflows, where endpoint detection is measured via an absorbance plate reader (2). It can also be adapted for performance outside of a traditional lab, where analysis "in the field", requires a simplified output that shows amplification success. Various detection methods have been tested, including visualizing the precipitation of magnesium pyrophosphate or the color change of a metal-sensitive indicator, but both of these methods are difficult to detect by eye and require approximately 60 minutes before detection is possible.

NEB scientists developed a solution to this – a very visible, pH-based colorimetric detection (3). This method utilizes a pink-to-yellow color change that is visible to the naked eye, resulting from a change in pH. During amplification, a proton is released with every dNTP that is incorporated into the growing DNA strand; in the presence of a lightly buffered solution, this leads to a decrease in pH of 2-3 units, which is attributed to the large amount of DNA made in LAMP reactions. Incorporation of a pH indicator directly into the reaction mixture enables visual detection of amplification. This eliminates the need for specialized detection methods, such as gel electrophoresis, which is not practical in remote settings where point-of-care diagnostics might be desirable or for high-throughput analysis in field studies. As a result, LAMP lends itself well to a variety of applications.

Figure 1:



Loop-mediated isothermal amplification (LAMP) uses 4-6 primers recognizing 6-8 distinct regions of target DNA. A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification.



There is no better example of just how beneficial amplification in the field is than in the work conducted by the World Mosquito Program (WMP) (worldmosquitoprogram.org), whose research aims to eradicate vector-borne illnesses.

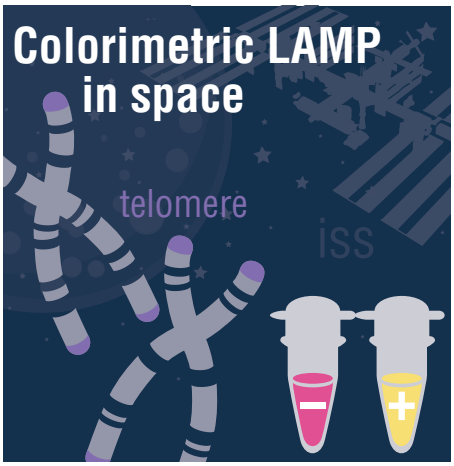
Dengue, chikungunya and Zika viruses are transmitted by arthropods that acquire the diseases by feeding on infected human blood and subsequently passing it to the next human they bite. The mosquito (*Aedes aegypti*), is one of these arboviral disease-disseminating organisms. The World Health Organization estimates that 2.5 billion people live in dengue transmission areas; it is one of the top ten global health threats and the most rapidly spreading mosquito-borne disease.

Interestingly, 40-60% of all the different species of insects worldwide (including butterflies and dragonflies) contain a maternally-transmitted, endosymbiotic Gram-negative bacterium called *Wolbachia pipientis* in their reproductive cells; *Aedes aegypti* mosquitoes are one of the few insects that do not normally carry *Wolbachia*. Once a virus (e.g., dengue) enters cells, the presence of *Wolbachia* in those cells prevents viral growth by slowing replication and rapidly degrading viral RNA (4), preventing downstream infections in humans.

This potential biocontrol strategy was recognized by the WMP and researchers embarked on a massive effort to stop transmission by disseminating *Wolbachia*-containing mosquitoes in dengue-endemic countries in Central and South America and into Asia and the Pacific regions. Researchers injected the *w*Mel strain of *Wolbachia* from fruit flies into *Aedes aegypti* eggs and the *Wolbachia*-infected mosquitoes were released in Northern Australia on a controlled schedule. Within a few months, close to 100% of mosquitoes contained *Wolbachia*, an infection rate that has been retained years later (5).

The WMP adapted LAMP for field analysis and compared it with the established TaqMan qPCR assay. LAMP primers were designed to detect the *wsp* gene from two *Wolbachia* strains. Using the WarmStart Colorimetric LAMP 2X Master Mix (NEB #M1800), primers and target DNA, they found that in just 30 minutes at 65°C, a simple color change can reliably determine if the mosquitoes are infected with *Wolbachia*: pink = negative (mosquito does not contain *Wolbachia* DNA), yellow = positive (mosquito contains *Wolbachia* DNA) (6).

Screening mosquitoes in the field using colorimetric LAMP is inexpensive compared to the TaqMan qPCR assay, and it avoids the lengthy process of transporting samples back to a specialized laboratory for analysis. Results are in real time, and this improves accuracy regarding the geographical localization of the *Wolbachia*-containing mosquitoes.



The field of space biology is still in its infancy but an ever-expanding list of biological experiments is being conducted on the International Space Station (ISS) each year, some with the help of students, who design these experiments as part of the Genes in Space contest (www.genesinspace.com). In early experiments, reactions to be conducted on the ISS were typically prepared on Earth before being executed in space, and then transported back to Earth where they could be analyzed and interpreted. In this way, the first PCR was conducted in space in 2016 aboard the ISS (7). But with the goal of extended space travel there remained a need to enable astronauts to analyze reactions that could be conducted on station without having to send samples back down to Earth. In March 2017, colorimetric LAMP experiments were conducted on the ISS to detect a specific repetitive telomeric DNA sequence and observe the colorimetric results (8). Telomeres are DNA-protein structures at the ends of chromosomes that support chromosomal stability by protecting them from degradation; abnormal telomere shortening is associated with human disease. The colorimetric LAMP assays that were designed to assess telomere dynamics were prepared on the ground but after amplification on station using a portable miniPCR™ instrument, the results (a simple pink to yellow color change enabled by the WarmStart Colorimetric LAMP Master Mix) were easily visualized, indicating successful amplification. Traditional PCR using both *Taq* and Q5 DNA polymerases (NEB #M0494) were run in parallel and all reactions were compared to those run on Earth to demonstrate the study's success. Analysis of the LAMP results on the ISS was made possible by the fact that colorimetric LAMP required only visual inspection – photographing the tubes against a white piece of paper highlighted the simplicity of the methodology and its suitability for even one of the most extreme settings.

continued on page 4...

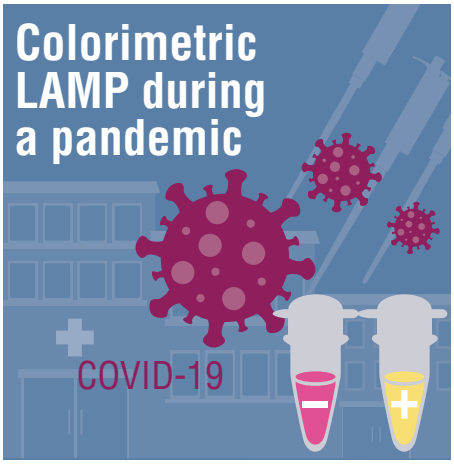
Product Tips:

NEB offers convenient WarmStart LAMP Kits and Master Mixes for robust & sensitive DNA/RNA detection!

Ordering information:

Product	NEB #	Size
WarmStart LAMP Kit (DNA & RNA)	E1700S/L	100/500 rnxs
WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA)	M1800S/L	100/500 rnxs
WarmStart Colorimetric LAMP 2X Master Mix with UDG *(for carryover prevention)*	M1804S/L	100/500 rnxs

Visit **www.neb.com/IsoAmp** to learn more, or contact your local distributor!

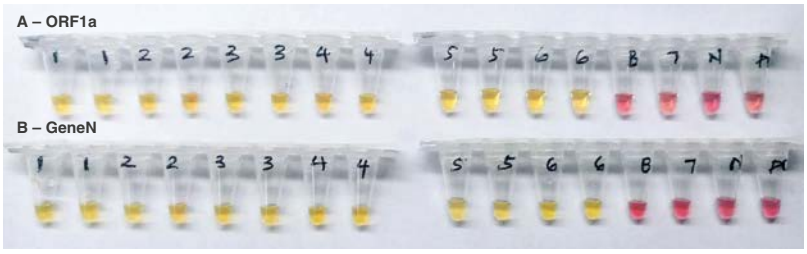


During a global pandemic, rapid, widespread testing of the infectious agent is critical – not just for testing symptomatic cases, but also as a screening method as people return to work, and to generally monitor infection levels in a community.

The SARS-CoV-2 virus causes symptoms that resemble the flu, so molecular diagnosis is essential for confirmation of infection and epidemiological monitoring. RT-qPCR is the standard for the diagnosis of acute infections from upper and lower respiratory tract specimens, and testing is typically conducted in authorized labs that can perform high complexity tests. However, the scientific community is collaborating to develop faster, alternative approaches to detect this pathogen.

Scientists at NEB demonstrated the ability of a colorimetric LAMP assay to detect RNA from the SARS-CoV-2 virus that causes COVID-19 (9). The assay was evaluated using samples from respiratory swabs from confirmed infections from patients in Wuhan, China (Figure 2) where isothermal amplification of SARS-CoV-2 RNA leads to a color change from pink to yellow (Figure 3). Additional studies have examined colorimetric LAMP in comparison to commercial RT-qPCR tests (10). LAMP can be used with samples that do not require RNA isolation (11), including those using human saliva as a sample input (12). NEB recently released a research-use only (RUO) product based on this simple technique, the SARS CoV-2 Rapid Colorimetric LAMP Assay Kit (NEB #E2019). For more details, see page 5. Colorimetric LAMP has huge potential as a disease screening method during a pandemic because of the low cost, rapid results and ease of use in a broad range of settings. The quick sample preparation and portability of this assay represent the next-generation method of enabling our customers' point-of-need diagnostics that will aid in disease prevention or containment and lead to improved outcomes.

Figure 2:

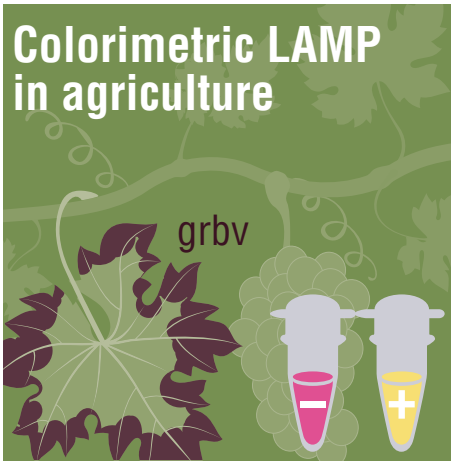


SARS-CoV-2 detection from COVID-19 patient samples in Wuhan, China. Samples testing positive (1-6) or negative (7) with commercial RT-qPCR tests were assayed using colorimetric LAMP assay with primer set targeting ORF1a (A) and GeneN (B). Yellow indicates a positive detection after 30 min incubation, and pink a negative reaction with results compared to the negative control (N). B, Blank control without template. P, samples containing a plasmid used as positive control for qPCR.

Figure 3: **Rapid, simple, color-based detection of SARS-CoV-2 RNA**

NTC = Non-Template Control
PC = Positive Control
IC = Internal Control

The SARS-CoV-2 Rapid Colorimetric LAMP Assay, targeting the nucleocapsid (N) and envelope (E) genes, was carried out using the indicated controls and either positive sample (human total RNA + synthetic SARS-CoV-2 RNA) or negative sample (human total RNA alone). Valid results for Non-Template Control (NTC, pink), Positive Control (PC, yellow) and Internal Control (IC, yellow) are shown. In the positive test sample, isothermal amplification of SARS-CoV-2 RNA leads to a color change from pink to yellow.



The widespread use of colorimetric LAMP doesn't end there. Due to its sensitivity and low cost (under a dollar per sample) it is also being applied in agriculture. For example, Grapevine red blotch virus (GRBV) is spread by the three-cornered alfalfa leafhopper and it affects the viticulture industry in North America. It causes a reduction in both yield and quality, and while characteristic leaf changes are observed, they are not reliable and so diagnostic testing is required. The sample collection is as simple as inserting a sterile pipette into the leaf petiole and then incubating the tip in sterile water. Sample testing using a colorimetric LAMP assay takes place onsite (13). Compare this with the time and cost of traditional methods for screening for GRBV – sample collection, transport to a specialist lab, a 2-hour DNA extraction method and PCR analysis. Additionally, the sensitivity of the LAMP assay was reported to be 2 orders of magnitude lower than traditional PCR and qPCR methods.

The value of colorimetric LAMP lies in its simple and inexpensive application in study settings that have not previously been PCR-friendly. Colorimetric LAMP opens up opportunities for human health and medical diagnosis that until recently, have not been possible due to the specialized requirements of traditional PCR. Any study environment whereby samples currently need to be transported from the field to a specialized lab is an opportunity for the development of a colorimetric LAMP assay.

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Supporting COVID-19 Research

As the SARS-CoV-2 virus continues to impact our communities, our manufacturing and distribution teams continue to be fully operational, and we are working closely with our suppliers and distribution partners to ensure uninterrupted access to our products and technical support.

NEB's products are available for research purposes only. However, we are supplying and supporting customers who are working diligently to develop better diagnostic tools and vaccines for the SARS-CoV-2 virus. By now, more than 650 publications, preprints and EUA protocols have been published using NEB products.

We are ready to supply our customers with the reagents needed to develop and validate them as diagnostic tools for their lab-based or point-of-care settings.

For a list of NEB products, **selected citations and EUA protocols** used in COVID-19 research, please visit:

www.neb.com/COVID19

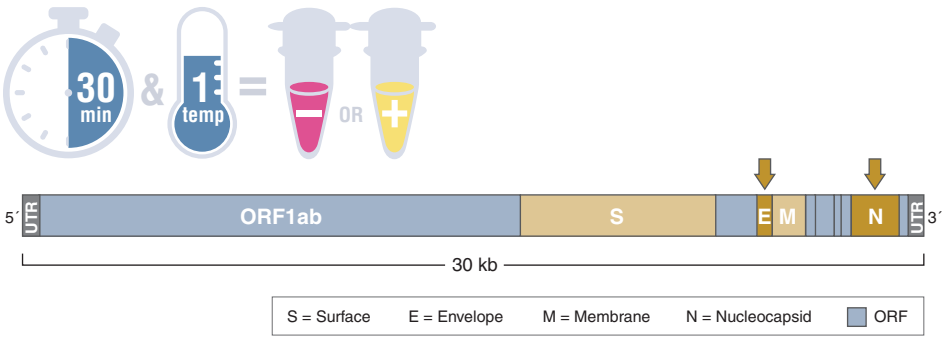
Learn more about the products available for these applications:

- **RNA Extraction**
- **Virus Detection**
- **Sequencing & Epidemiology**
- **Vaccine Development**

SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit

The SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit utilizes loop-mediated isothermal amplification for use in the analysis of SARS-CoV-2, the novel coronavirus that causes COVID-19.

The kit includes WarmStart Colorimetric LAMP 2X Master Mix with uracil-DNA glycosylase (UDG) and a primer mix targeting the N and E regions of the viral genome. Controls are provided to verify assay performance and include an internal control primer set and a positive control template. Guanidine hydrochloride has been shown to increase the speed and sensitivity of the RT-LAMP reaction and is also included.



SARS-CoV-2 genome. Arrows indicate regions targeted by assay.

For **selected citations and EUA protocols on RT-LAMP** using NEB products in SARS-CoV-2 detection, visit:

www.neb.com/covid-LAMP

Advantages:

- Colorimetric LAMP enables simple, visual detection (pink-to-yellow) of amplification of SARS-CoV-2 nucleic acid in only 30 min
- Set up reactions quickly and easily, using a simple heat source and unique WarmStart technology
- Reduce risk of carryover contamination, with UDG and dUTP included in the master mix
- Assay targets N and E regions of the SARS-CoV-2 genome, for optimized sensitivity and specificity
- Bring confidence to your results using the provided controls

Ordering information:

Product	NEB #	Size
SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit	E2019S	96 rxns

Supporting COVID-19 Research

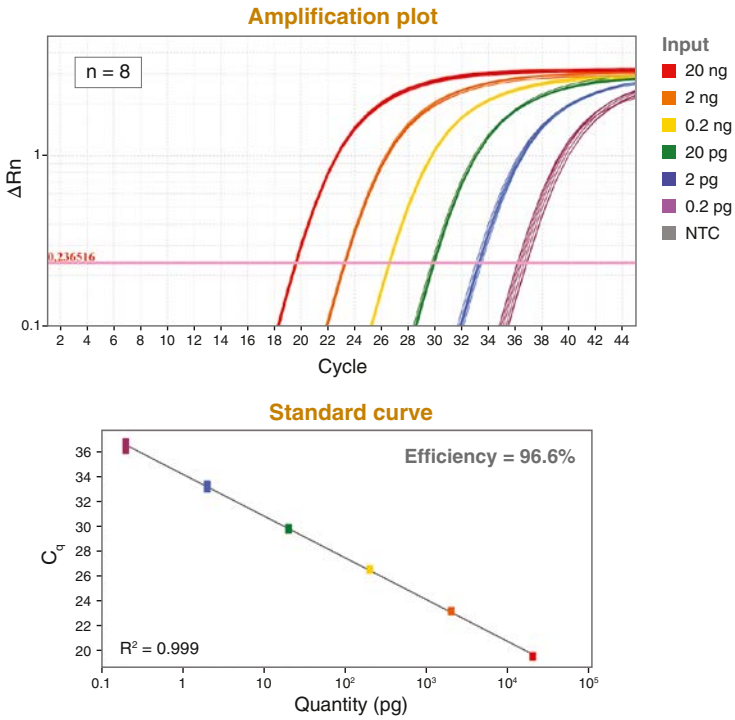
Visit www.neb.com/COVID19 to learn more

Luna® Universal Probe One-Step RT-qPCR Kit and Luna Probe One-Step RT-qPCR Kit (No ROX)

For sensitive, fast and extremely reliable RNA quantitation, we recommend the NEB Luna Universal Probe One-Step RT-qPCR Kit (NEB #E3006) and, for instruments that do not require ROX normalization, the corresponding Luna Probe One-Step RT-qPCR Kit (No ROX) (NEB #E3007).

Both kits are optimized for real-time quantification of target RNA sequences using hydrolysis probes and emply a novel WarmStart-activated reverse transcriptase, allowing control of enzyme activity via reversible, aptamer-based inhibition for improved performance. This unique WarmStart RTase is also available separately as LunaScript RT SuperMix Kit (NEB #E3010) optimized for Two-Step protocols (see back cover for more).

NEB's Luna Universal Probe One-Step RT-qPCR Kit offers exceptional sensitivity, reproducibility and RT-qPCR performance



RT-qPCR targeting human GAPDH was performed using the Luna Universal Probe One-Step RT-qPCR Kit over an 8-log range of input template concentrations (1 µg – 0.1 pg Jurkat total RNA) with 8 replicates at each concentration.

For **selected citations and EUA protocols** on **RT-qPCR** using Luna Universal products in SARS-CoV-2 detection, visit:

www.neb.com/covid-qPCR

and scroll down to the RT-qPCR section.

Advantages:

- Optimize your RT-qPCR with Luna WarmStart Reverse Transcriptase
  - – Novel, thermostable reverse transcriptase (RT) improves performance
  - – WarmStart RT paired with Hot Start *Taq* increases reaction specificity and robustness
- Experience best-in-class performance
  - – All Luna products have undergone rigorous testing to optimize specificity, sensitivity, accuracy and reproducibility
  - – Products perform consistently across a wide variety of sample sources
  - – A comprehensive evaluation of commercially-available qPCR and RT-qPCR reagents demonstrates superior performance of Luna products
- Make a simpler choice
  - – Convenient master mix formats and user-friendly protocols simplify reaction setup
  - – Non-interfering, visible tracking dye helps to eliminate pipetting errors

> The choice of One-step kit is critical for sensitivity and at the same time a major cost factor. We recommend the NEB Luna Universal Probe One-Step RT-qPCR Kit (E3007), which is attractively priced and performs among the most sensitive One-step kits we tested.
>
> VIENNA COVID-19 DETECTION INITIATIVE (VCDI)

Ordering information:

Product	NEB #	Size
Luna Probe One-Step RT-qPCR Kit	E3006S/L/X/E	200/500/1000/2500 rxns
Luna Probe One-Step RT-qPCR Kit w/o ROX	E3007E	2500 rxns
LunaScript RT SuperMix Kit	E3010S/L	25/100 rxns

RNA Extraction made easy – Monarch® Nucleic Acid Purification Kits

Extraction of RNA is often the first step in viral detection assays. The Monarch Total RNA Miniprep Kit can be used to successfully extract viral RNA from various samples including saliva, buccal swabs, and nasopharyngeal samples*. Extraction can be completed in under an hour with our new simplified workflow. Additionally, the Monarch RNA Cleanup Kits, which were originally developed for RNA cleanup applications, have been shown to successfully extract viral RNA from saliva and swabs when used in conjunction with the Monarch DNA/RNA Protection Reagent (NEB #T2011). This extended utility of the RNA cleanup kits enables purification of viral RNA in under 10 minutes. Both the Monarch Total RNA Miniprep Kit and RNA Cleanup Kit workflows are automatable with minor protocol modifications on the QIACube and the KingFisher™ Flex platforms, to further facilitate efficient extraction. RNA purified with these kits is ready for downstream detection by RT-qPCR, RT-LAMP or other methods.

**NEB has confirmed viral RNA extraction on simulated nasopharyngeal samples.*

Advantages:

- Efficient and fast extraction of viral RNA from saliva, buccal swabs, and nasopharyngeal samples*
- Enables sensitive detection by RT-qPCR, RT-LAMP and other methods
- Automatable on the QIAcube and KingFisher Flex

For **protocols on viral RNA extraction** using Monarch RNA kits, visit:

www.neb.com/COVID19

Ordering information:

Product	NEB #	Size
Monarch Total RNA Miniprep Kit	T2010S	50 preps
Monarch RNA Cleanup Kit (10 µg)	T2030S/L	10/100 preps
Monarch RNA Cleanup Kit (50 µg)	T2040S/L	10/100 preps

Products for Oxford Nanopore Technologies® and Illumina® Sequencing

The following reagents are being recommended in a number of third party COVID-19 sequencing protocols, including “PCR tiling of COVID-19 virus” in Oxford Nanopore Technologies’ Nanopore Community, and the "ARTIC-NEB: SARS-CoV-2 Library Prep" protocol for Illumina platforms.

Ordering information:

Product	NEB #	Size
LunaScript RT SuperMix Kit	E3010S/L	25/100 rxns
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/X/L	100/500/500 rxns
NEBNext Ultra II End Repair/dA-Tailing Module	E7546S/L	24/96 rxns
NEBNext Ultra II Ligation Module	E7595S/L	24/96 rxns
NEBNext Quick Ligation Module	E6056S/L	20/100 rxns
Blunt/TA Ligase Master Mix	M0367S/L	50/250 rxns
NEBNext Ultra II FS DNA Library Prep Kit for Illumina	E7805S/L	24/96 rxns

For **selected citations and protocols** using **NEBNext** products in SARS-CoV-2 sequencing on ONT and Illumina platforms, please visit:

www.neb.com/covid-seq

Supporting COVID-19 Research

GMP-grade reagents for RNA synthesis – Tools to take you from template to transcript

For over 45 years, NEB has been a world leader in the discovery and production of reagents for the life science industry. Our enzymology expertise effectively positions us to supply reagents for the synthesis of high-quality RNA – from template generation and transcription, to capping, tailing and cleanup after synthesis. These products are designed and manufactured based on decades of molecular biology experience, so that you can be confident they will work for your application.

NEB's portfolio of research-grade and GMP-grade* reagents enables bench-scale to commercial-scale mRNA manufacturing. Our optimized HiScribe™ kits enable convenient *in vitro* transcription (IVT) workflows. When it is time to scale up and optimize reaction components, our standalone reagents are readily available in formats matching our GMP-grade offering, enabling a seamless transition to large-scale therapeutic mRNA manufacturing.

Not sure whether you need research-grade or GMP-grade materials?

Learn more about the difference between GMP-grade and research-grade reagents by visiting:

www.neb.com/GMP

TEMPLATE GENERATION	*IN VITRO* TRANSCRIPTION	RNA CAPPING	POLY(A) TAILING	RNA PURIFICATION
Q5 High-Fidelity DNA Polymerase	HiScribe™ T7 ARCA mRNA Synthesis Kit (with tailing)			Monarch RNA Cleanup Kit (10 µg)
dNTP solution mixes	HiScribe T7 ARCA mRNA Synthesis Kit		*E. coli* Poly(A) Polymerase	Monarch RNA Cleanup Kit (50 µg)
BspQI, XbaI, HindIII, other restriction enzymes & cloning reagents. Inquire about custom restriction enzyme formulations with Recombinant Albumin.	GMP COMING SOON HiScribe T7 High Yield RNA Synthesis Kit	GMP Vaccinia Capping System		Monarch RNA Cleanup Kit (500 µg)
DNA Assembly: NEBuilder HiFi DNA Assembly Golden Gate Assembly	HiScribe T7 Quick High Yield RNA Synthesis Kit	GMP mRNA Cap 2´-O-Methyltranferase		Lithium Chloride
	HiScribe SP6 High Yield RNA Synthesis Kit	ARCA and other mRNA cap analogs		
	T3 & SP6 RNA Polymerases			
	GMP T7 RNA Polymerase			
	GMP COMING SOON Hi-T7 RNA Polymerase			
	Companion Products			**Companion Products**
	GMP RNase inhibitor (Murine)			Monarch RNA Cleanup Binding Buffer
	RNase Inhibitor (Human Placental)			Monarch RNA Cleanup Wash Buffer
	GMP Pyrophosphatase, Inorganic (*E. coli*)			Nuclease-free Water
	Pyrophosphatase, Inorganic (Yeast)			
	GMP DNase I (RNase-free)			
	NTPs			

GMP COMING SOON Polymerases, restriction endonucleases, ligases

GMP available in GMP-grade

* "GMP-grade" is a branding term NEB uses to describe reagents manufactured at NEB's Rowley facility. The Rowley facility was designed to manufacture reagents under more rigorous infrastructure and process controls to achieve more stringent product specifications and customer requirements. Reagents manufactured at NEB's Rowley facility are manufactured in compliance with ISO 9001 and ISO 13485 quality management system standards. However, at this time, NEB does not manufacture or sell products known as Active Pharmaceutical Ingredients (APIs), nor does NEB manufacture its products in compliance with all of the Current Good Manufacturing Practice regulations.

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

by Andrew N. Gray, Ph.D., Guoping Ren, Ph.D., Yinhua Zhang, Ph,D., Nathan Tanner, Ph.D. and Nicole M. Nichols, Ph.D., New England Biolabs, Inc.

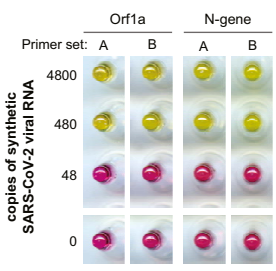
Introduction

The following is an excerpt from an application note, presented in the context of the COVID-19 global pandemic caused by the SARS-CoV-2 coronavirus. To view the full application note, visit www.neb.com/SARS-CoV-2appnote.

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. 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 (1). 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.

Figure 1: **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.

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, LunaScript RT SuperMix Kit and WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA).

In the case of the more rapid colorimetric RT-LAMP workflow, a color change is observed in the presence of amplification (pink to yellow). 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.

Direct Detection of SARS-CoV-2 RNA Targets in Cell Lysates

Direct detection of SARS-CoV-2 in patient samples requires 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 (Figure 1), using purified vRNA alone as a control. Cell lysis was carried out using the Luna Cell Ready Lysis Module, which includes an RNA Protection Reagent to prevent RNA degradation.

Summary of Results

RT-qPCR

- UTM at up to 25% (v/v) total reaction volume does not affect performance of either the Luna Universal Probe One-Step RT-qPCR Kit (for One-Step viral RNA detection and quantitation) or the LunaScript RT SuperMix Kit (for first-strand cDNA synthesis from viral RNA in Two-Step RT-qPCR workflows).
- Synthetic SARS-CoV-2 viral RNA is protected from degradation by cellular RNases during Luna Cell Ready lysis protocols, and during subsequent detection and quantitation by Luna One-Step RT-qPCR.
- Detection and quantitation of viral RNA targets using the Luna Universal Probe One-Step RT-qPCR Kit is achievable with cell lysates, at up to 2,000 cell equivalents per 20 µl reaction.

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.

Discussion

The data presented in this application note 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 (2).

References:
1. Zhang, Y. et al. Rapid Molecular Detection of SARS-CoV-2 (COVID-19) Virus RNA Using Colorimetric LAMP. medRxiv 2020.02.26.20028373 (2020) (preprint) doi:10.1101/2020.02.26.20028373.
2. Bruce, E.A. et al. RT-qPCR Detection of SARS-CoV-2 RNA from Patient Nasopharyngeal Swab using Qiagen RNEasy Kits or Directly via Omission of an RNA Extraction Step. bioRxiv 2020.03.20.001008 (2020) (preprint) doi:10.1101/2020.03.20.001008

The **full note** including performance data can be downloaded at:

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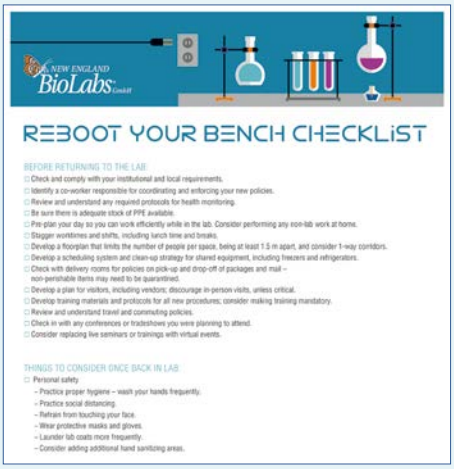

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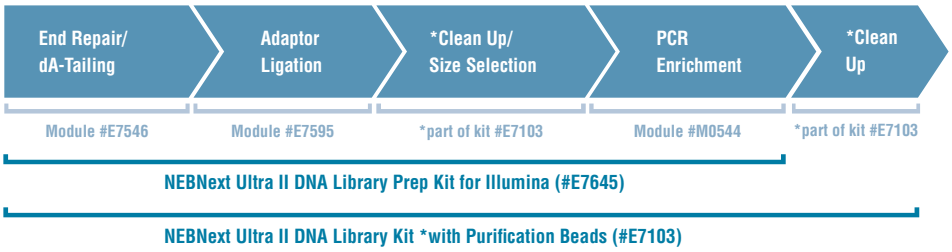

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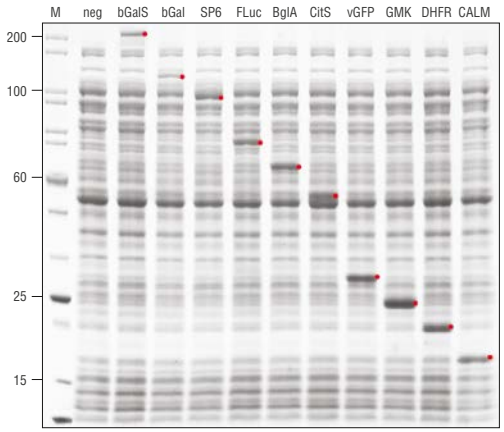

SDS-PAGE Analysis of Protein synthesis using the NEBExpress Cell-free *E. coli* Protein Synthesis System

50 µl reactions containing 250 ng template DNA were incubated at 37°C for 3 hours. The red dot indicates the protein of interest. M = Unstained Protein Standard, Broad Range (NEB #P7717); "neg" = negative control, no DNA.

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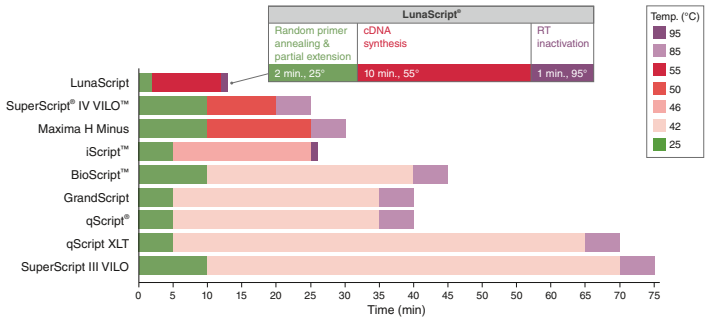

Comparison of recommended protocols for cDNA synthesis. The LunaScript RT SuperMix Kit requires the shortest reaction time and tolerates elevated temperatures, reducing complications from RNA secondary structure.

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