Molecular assay development

Evaluation of detection methods for air-dried LAMP reagents: turbidity, calcein, SYBR Green I stain, and agarose gel electrophoresis

Introduction

Effective disease control heavily relies on rapid testing, particularly in resource-scarce environments such as the field or simple clinics [1]. The global spread of SARS-CoV-2 highlighted the need for simplified testing methods, leading to a significant increase in attempts to adapt isothermal amplification techniques for molecular testing [2]. Compared to PCR, these methods can often decrease assay time and eliminate the need for expensive equipment without sacrificing specificity [3]. Moreover, nucleic acid amplification assays may be dried for increased stability at room temperature and then easily reconstituted for use at the point of testing [4]. This flexibility is beneficial if environmental temperature control is an issue. Currently, the most common way to produce dried reagents is lyophilization, but this consumes considerable energy and can take up to 2 days. To address these limitations, we developed reagents for loop-mediated isothermal amplification (LAMP) and reverse transcription LAMP (RT-LAMP) that can be dried in as little as 3 hours using a ventilated oven set to 50°C. When sealed, such assays can be stable at room temperature for up to a year. These assays may then be reconstituted and used for viral or bacterial nucleic acid detection.

In this application note, we describe the use of reagents from the Invitrogen™ Dry-Ready™ RT-LAMP Kit to prepare assays for detection of SARS-CoV-2 RNA, measles virus RNA, human adenovirus 41 (HAdV41) DNA, and *Mycoplasma pneumoniae* DNA. To further demonstrate the flexibility of the assays, commonly applied detection methods were tested including calcein dye, reaction turbidity, Invitrogen™ SYBR™ Green I stain, and agarose gel electrophoresis.

Materials and methods

Laminar flow hood cleaning

All reactions were assembled in a laminar flow hood that was cleaned using 70% ethanol and Invitrogen™ DNA*Zap*™ PCR DNA Degradation Solutions.

Air-drying of reaction mixes

Prior to air-drying, RT-LAMP and LAMP reaction mixes (10 µL) were assembled on ice in 0.2 mL PCR strip tubes (Table 1) in a laminar flow hood. All reactions with Dry-Ready reagents incorporated HPLC-purified unmodified primers targeting specific genomic regions (Table 2).

Table 1. Reaction mix setup for air-drying.

Component	Final concentration	Supplier
5X Dry-Ready Reaction Buffer for RT-LAMP	2.5X	Thermo Fisher Scientific
Dry-Ready dNTP Mix, 100 mM each	3.5 mM each	Thermo Fisher Scientific
FIP/BIP primers	4.0 µM each	Metabion
F3/B3 primers	0.5 µM each	Metabion
LoopF/LoopB primers*	1.0 µM each	Metabion
Calcein**	192.5 µM	MilliporeSigma
$MnCl_2$**	625 µM	Thermo Fisher Scientific
Dry-Ready *Bst* DNA Polymerase	0.6 U/µL	Thermo Fisher Scientific
Dry-Ready SuperScript IV Reverse Transcriptase†	2.5 U/µL	Thermo Fisher Scientific
Nuclease-free water	To 10 µL	Thermo Fisher Scientific

* Not added to the reaction mix for *M. pneumoniae* detection.

** Calcein and $MnCl_2$ were only added to reaction mixes in which they were used as a detection method.

† Dry-Ready SuperScript IV Reverse Transcriptase was only added to reaction mixes that amplified an RNA target.

Table 2. Nucleic acid targets and primer targets used.

Organism	Nucleic acid material supplier	Region targeted by primers
SARS-CoV-2	Twist Bioscience	*ORF1ab*
Measles virus	Twist Bioscience	*F* gene
HAdV41	Vircell	*E1B* gene
***Mycoplasma pneumoniae* (FH strain of Eaton Agent)**	ATCC	*gyrB* gene

After the reaction mixes were assembled, they were carried to a ventilated oven (Memmert) preheated to 50°C. The PCR tubes were placed in the oven for 3 hours to air-dry. After drying, the PCR tubes were closed and kept at room temperature for up to 24 hours before the LAMP reaction. An additional 8-tube strip for calcein-based detection was prepared. This strip was stored on the benchtop for 4 weeks in a tube rack before reconstitution and testing.

Control and air-dried assays

Control RT-LAMP and LAMP reaction mixes were assembled on ice (Table 3) in a laminar flow hood. Air-dried RT-LAMP and LAMP reaction mixes were reconstituted at room temperature using 25 µL of reconstitution solution containing a template (Table 4). Then, the reconstituted reaction mixes were incubated at room temperature for approximately 2 min, vortexed, centrifuged, and then placed on ice. All reactions had two technical replicates, including a no-template control (NTC).

Table 3. RT-LAMP and LAMP control reaction mixes.

Component	Final concentration	Supplier
5X Dry-Ready Reaction Buffer for RT-LAMP	1X	Thermo Fisher Scientific
$MgCl_2$	8 mM	Thermo Fisher Scientific
Dry-Ready dNTP Mix, 100 mM each	1.4 mM each	Thermo Fisher Scientific
FIP/BIP primers	1.6 µM each	Metabion
F3/B3 primers	0.2 µM each	Metabion
LoopF/LoopB primers*	0.4 µM each	Metabion
Calcein**	77 µM	Sigma Aldrich
$MnCl_2$**	250 µM	Thermo Fisher Scientific
Dry-Ready *Bst* DNA Polymerase	0.24 U/µL	Thermo Fisher Scientific
Dry-Ready SuperScript IV Reverse Transcriptase†	1 U/µL	Thermo Fisher Scientific
Template	4 or 40 copies/µL	Various
Nuclease-free water	To 25 µL	Thermo Fisher Scientific

* Not added to the reaction mix for *M. pneumoniae* detection.

** Calcein and $MnCl_2$ were only added to reaction mixes in which they were used as a detection method.

† Dry-Ready SuperScript IV Reverse Transcriptase was only added to reaction mixes that amplified an RNA target.

After the reactions were assembled, they were incubated at 65°C for 15–25 min. Incubations were initially carried out for 20 min and later optimized (increased or decreased in 5 min increments if detection was not reliable). All reactions were inactivated at 95°C for 5 min to facilitate sample storage prior to evaluation. Inactivation is not necessary if detection is performed immediately.

Table 4. Reconstitution solution for air-dried reaction mix.

Component	Final concentration	Supplier
$MgCl_2$	8 mM	Thermo Fisher Scientific
Template	4 or 40 copies/µL	Various
Nuclease-free water	To 25 µL	Thermo Fisher Scientific

Endpoint detection

RT-LAMP and LAMP products were detected using multiple methods that are listed in Table 5.

Calcein-based detection

Calcein was dissolved in DMSO to prepare a 7.7 mM solution. The solution was stored at –20°C. For RT-LAMP and LAMP reactions, the calcein and $MnCl_2$ were added to the reaction mixes with Dry-Ready reagents prior to drying, or immediately into reaction mixes that had not been dried. Results were photographed for detection under normal or UV light.

Turbidity assessment

Following LAMP, the samples were kept overnight at –20°C. Then, the samples were thawed at room temperature and vortexed. After photographing, these samples were used for detection with SYBR Green I stain where appropriate.

Detection with SYBR Green I stain

Invitrogen™ SYBR™ Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO, was diluted 10-fold using nuclease-free water; then 1 µL of the solution was added to each completed LAMP reaction.

Agarose gel electrophoresis

For visualization with gel electrophoresis, 10 µL of each LAMP reaction was combined with 2 µL of 6X Thermo Scientific™ TriTrack™ DNA Loading Dye; 10 µL of the mixture was loaded onto a 1% agarose gel prepared with 1X TAE buffer containing ethidium bromide, with 7 µL of Thermo Scientific™ GeneRuler™ 1 kb Plus DNA Ladder used for the molecular weight marker. Samples were run on a gel after detection with calcein or turbidity and SYBR Green I stain.

Table 5. Detection types used.

Detection type	Indication of positive sample	Time of addition
Calcein	Orange to yellow color change	During reaction setup
Turbidity	Cloudy appearance	No additives, consequence of reaction
SYBR Green I stain	Orange to yellow color change	Added after reaction incubation
Agarose gel electrophoresis	Ladder-like pattern on gel	No additives, gel is run after reaction has occurred

Results

The Dry-Ready RT-LAMP Kit may be used to prepare dried, room temperature–stable assays to detect various DNA and RNA targets. Various options for endpoint detection are essential for resource-scarce environments. Advantages and disadvantages of the detection methods tested are summarized in Table 6.

Table 6. Advantages and disadvantages of detection types.

Detection type	Advantages	Disadvantages
Calcein	Can be added to the reaction mix during setup	Requires $MnCl_2$
	Can be dried with the assay	Color difference can be unclear between positive and negative samples
	Indicator of LAMP reaction, not nucleic acid concentration	
	Naked-eye and UV light–based detection possible	
Turbidity	No additional reagents required	Requires sample cooling and good lighting
	Indicator of positive reaction, not presence of nucleic acid	
SYBR Green I stain	Clear difference between positive and negative sample	Can only be added at the end of the LAMP reaction incubation
		Higher risk of contamination
		Intercalating dye, will bind to nontarget dsDNA if present (e.g., contaminants)
Agarose gel electrophoresis	Clear difference between positive and negative sample	Needs extra reagents and laboratory equipment
	Can be more sensitive or specific than other methods	Prolongs time to detection by about 1 hr

Measles virus and SARS-CoV-2

Measles virus and SARS-CoV-2 RNA detection via RT-LAMP used primers that produced comparatively early signals in real-time detection. Thus, incubation of the RT-LAMP reaction at 65°C was carried out for 20 and 15 minutes. In all cases, the sample was detected at 1,000 copies and 100 copies per reaction (40 and 4 copies/µL), and no positive signal with the NTC was observed (Figure 1, Figure 2). Turbidity was observed in the RT-LAMP reaction containing measles virus RNA only after 20 minutes of incubation, while all other detection methods provided clear signals after 15 minutes. This difference could reflect the inherent lower sensitivity of the turbidity method as a means of detection compared to other methods.

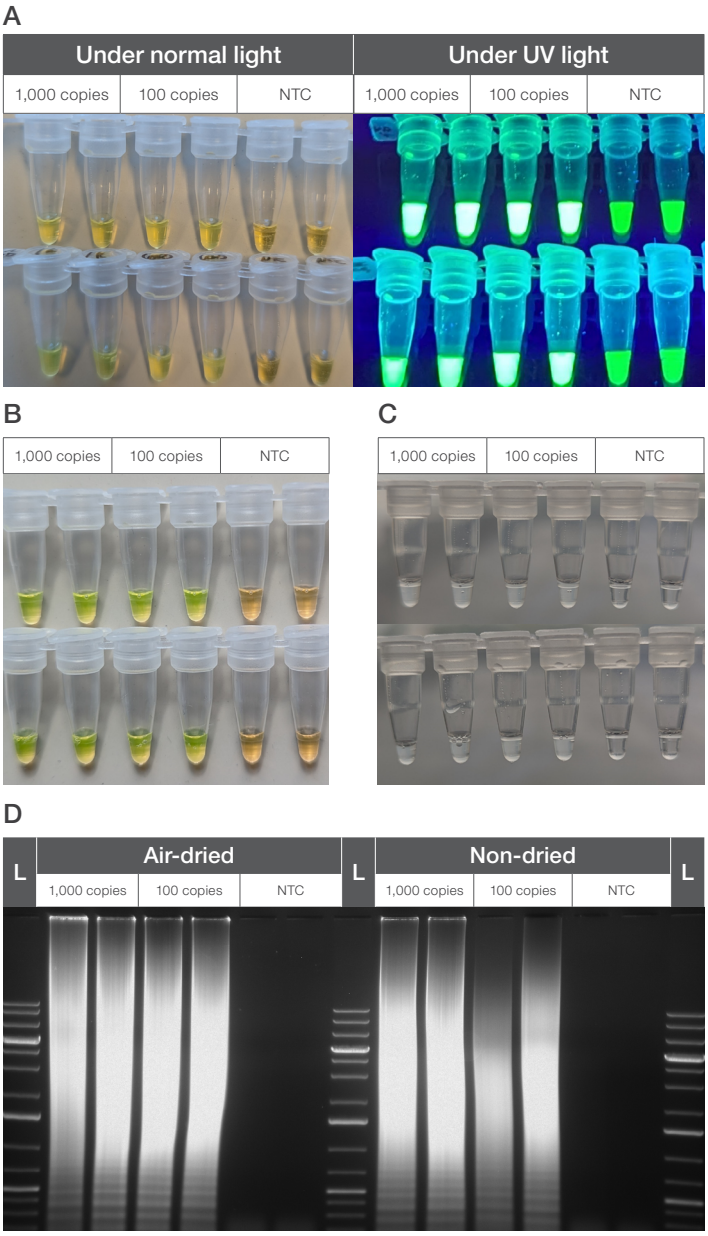

Figure 1. Measles virus RNA detection by RT-LAMP after incubation at 65°C. Detection was performed using the following methods: **(A)** calcein after 15 min incubation, **(B)** SYBR Green I stain after 15 min incubation, **(C)** reaction turbidity after 20 min incubation, and **(D)** agarose gel electrophoresis after 15 min incubation. In all photographs of tube strips, air-dried assays are on top, and the control non-dried assays are on the bottom. L = GeneRuler 1 kb Plus DNA Ladder.

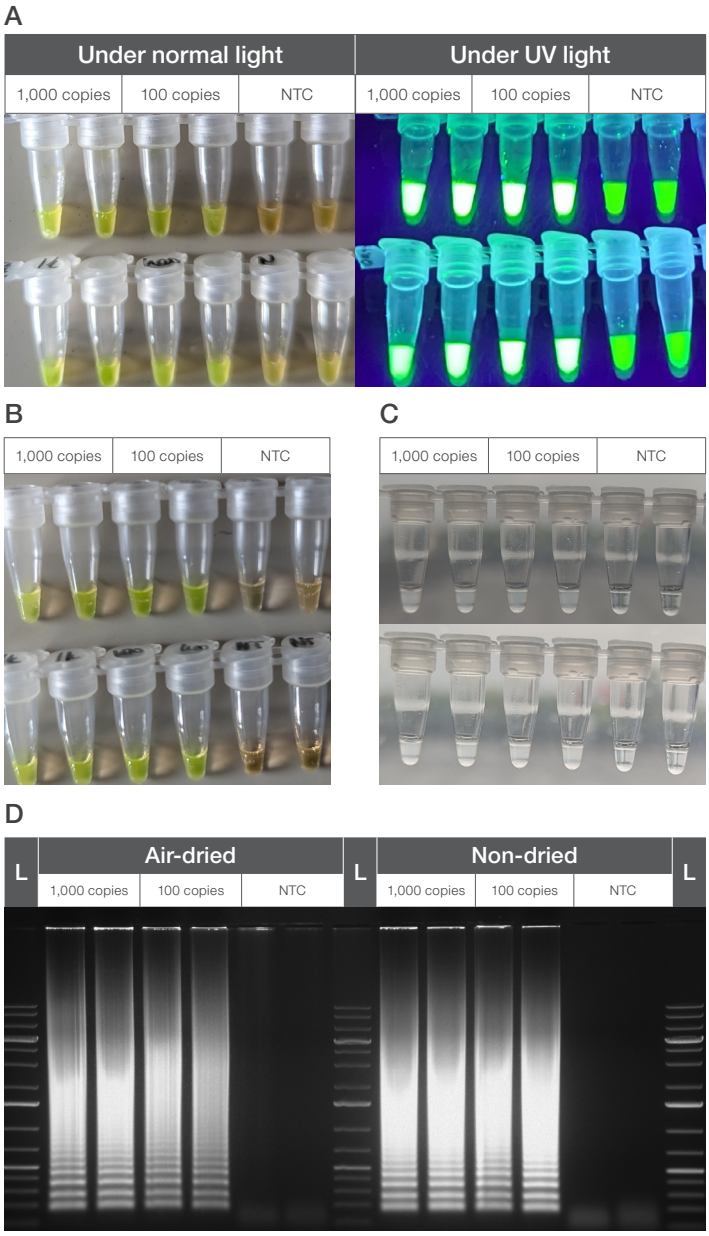

Figure 2. SARS-CoV-2 RNA detection by RT-LAMP after 20 min incubation at 65°C. Detection was performed using the following methods: **(A)** calcein, **(B)** SYBR Green I stain, **(C)** reaction turbidity, and **(D)** agarose gel electrophoresis. In all photographs of tube strips, air-dried assays are on top, and the control non-dried assays are on the bottom. L = GeneRuler 1 kb Plus DNA Ladder.

Additionally, assays that were dried and then stored on the benchtop for 4 weeks were tested with SARS-CoV-2 RNA (Figure 3). They showed no significant differences compared to no storage, suggesting that calcein does not have a destabilizing effect on the dried reaction for at least 4 weeks.

HAdV41 and M. *pneumoniae*

LAMP for human adenovirus 41 (HAdV41) and *M. pneumoniae* detection used two specific sets of primers. Primers targeting HAdV41 were optimized for speed of detection, while primers targeting *M. pneumoniae* did not contain the loop primers, resulting in a slower LAMP reaction. HAdV41 showed results after as little as 15 min with colorimetric methods, whereas other methods needed longer incubation (Figure 4, Figure 5). Overall, with slight variations in sample incubation, all endpoint detection was successful with both 1,000 and 100 copies of the template.

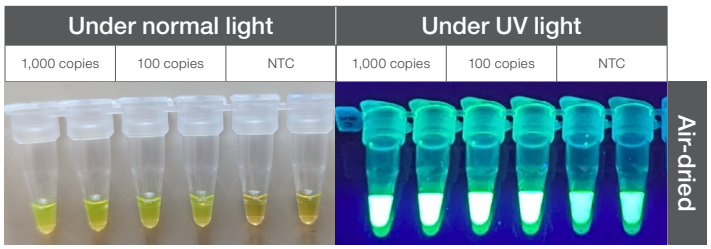

Figure 3. SARS-CoV-2 RNA detection by RT-LAMP assay containing calcein after 4-week air-dried storage at room temperature. Samples were incubated for 20 min at 65°C.

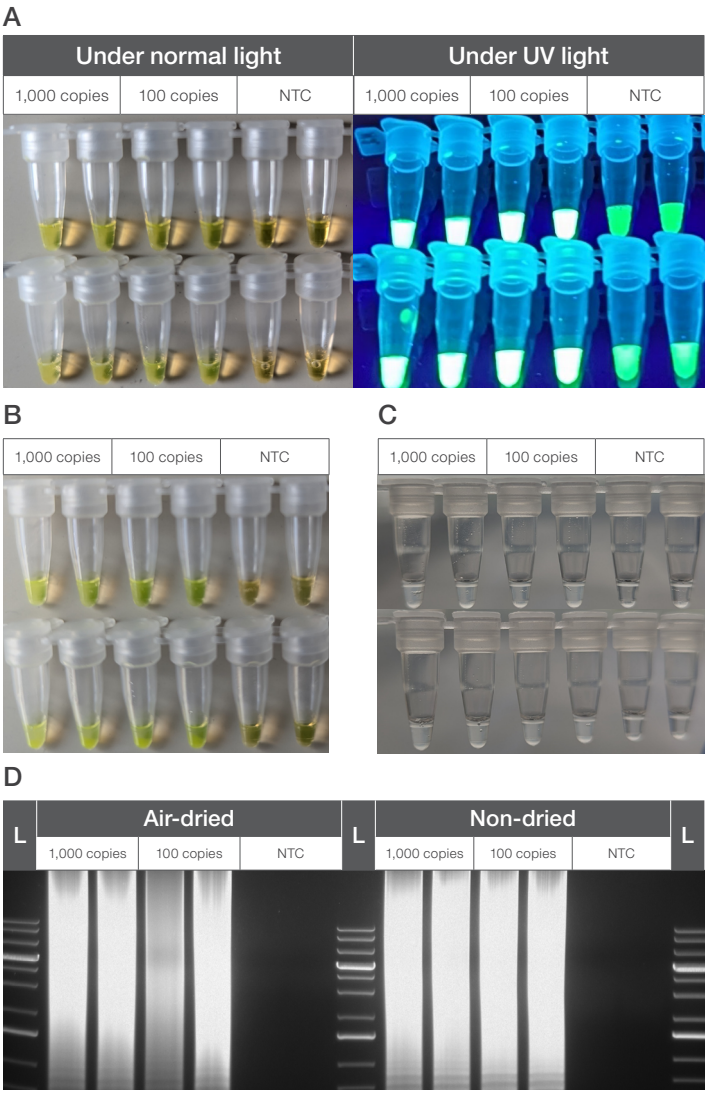

Figure 4. HAdV41 DNA detection by LAMP after incubation at 65°C. Detection was performed using the following methods: **(A)** calcein after 15 min incubation, **(B)** SYBR Green I stain after 15 min incubation, **(C)** reaction turbidity after 20 min incubation, and **(D)** agarose gel electrophoresis after 20 min. In all photographs of tube strips, air-dried assays are on top, and the control non-dried assays are on the bottom.

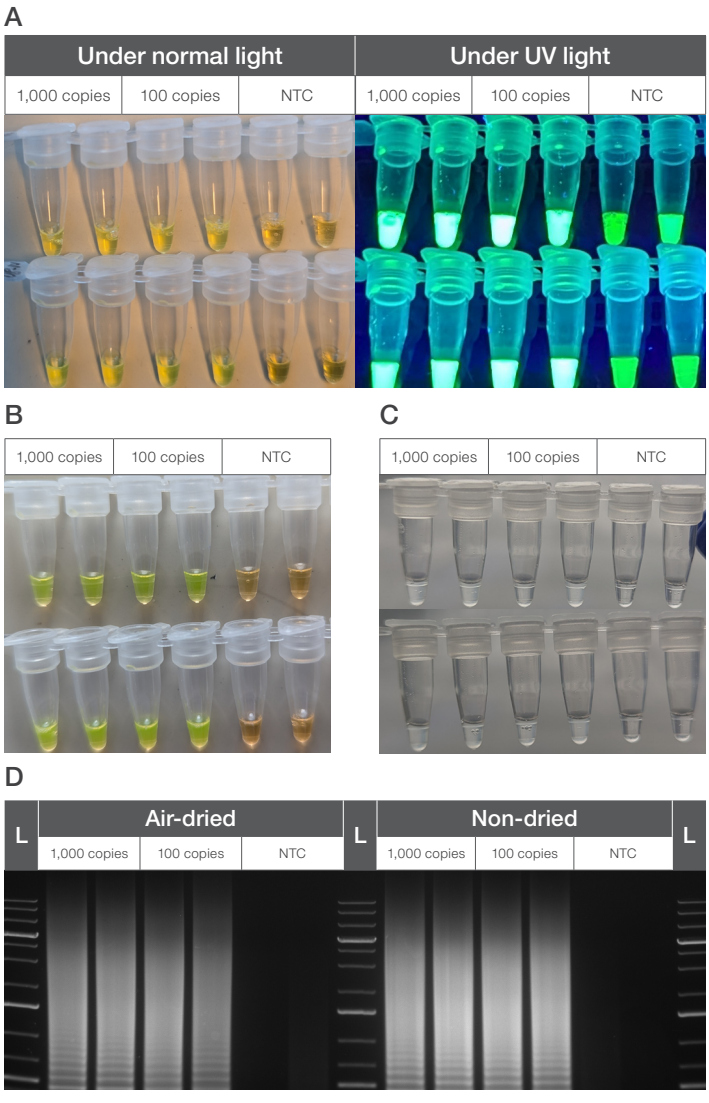

Figure 5. *M. pneumoniae* DNA detection by LAMP after incubation at 65°C. Detection was performed using the following methods: **(A)** calcein after 25 min incubation, **(B)** SYBR Green I stain after 20 min incubation, **(C)** reaction turbidity after 20 min incubation, and **(D)** agarose gel electrophoresis after 20 min. In all photographs of tube strips, air-dried assays are on top, and the control non-dried assays are on the bottom.

Conclusions

The Dry-Ready RT-LAMP Kit can be successfully used with a variety of detection methods for both DNA and RNA targets. Each method of detection offers distinct advantages for specific practical applications. Calcein enables simple visual detection and may be dried with the assay, but the color change indicating a positive sample may be difficult to differentiate. This is particularly true with primer sets prone to dimer formation. The turbidity method is less costly in terms of reagents but requires significant sample chilling, extending time-to-results and requiring extra equipment. SYBR Green I stain offers high sensitivity but necessitates opening of the tubes post-amplification, increasing the risk of contamination. Agarose gel electrophoresis has high sensitivity with clear visual confirmation but demands additional equipment and prolongs time required for detection. Thus, when choosing a detection method to use with an assay assembled with the Dry-Ready RT-LAMP Kit, the most important consideration should be given to the resources available to the end user. Our results demonstrate that the Dry-Ready RT-LAMP Kit may be used with a variety of detection methods across different templates and primer designs.

References

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4. Song X, Coulter FJ, Yang M et al. (2022) A lyophilized colorimetric RT-LAMP test kit for rapid, low-cost, at-home molecular testing of SARS-CoV-2 and other pathogens. *Sci Rep* 12(1):7043.

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