A high-throughput workflow for SARS-CoV-2 detection using the Colorimetric ReadiLAMP Kit

Introduction

The Invitrogen™ Colorimetric ReadiLAMP™ Kit enables rapid, robust, and specific identification of SARS-related coronavirus 2 (SARS-CoV-2). SARS-CoV-2 screening continues to be of paramount importance [1,2], especially given the proportion of asymptomatic individuals, emergence of viral variants, and desire to return to work and school. To address the scale and urgency of SARS-CoV-2 screening, we combined automated viral nucleic acid isolation on the existing Thermo Scientific™ KingFisher™ Flex system with a novel high-throughput Colorimetric ReadiLAMP Kit workflow. Here we describe the Colorimetric ReadiLAMP Kit modifications for plate reader compatibility, outline a spectrophotometric absorbance ratio for interpreting SARS-CoV-2 presence or absence, and validate the superior performance of this approach in a high-throughput pilot assay. This integrated workflow combining automated sample preparation with a high-throughput SARS-CoV-2 detection assay will reduce sample turnaround time and cost, yielding high-confidence viral detection throughout a screened population.

Materials and methods

RNA isolation

Contrived experimental samples were created by combining 200 µL of raw saliva with γ-irradiated SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, Cat. No. NR-52287). Nucleic acid was isolated using the Applied Biosystems™ MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit and the Thermo Scientific™ KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head.

Colorimetric ReadiLAMP Kit protocol for plate reader–based data collection and interpretation

1. Assemble the Colorimetric ReadiLAMP assay mix as outlined in Table 1. Master mixes may be used when appropriate.
2. Add purified RNA samples or controls to individual wells. Mix by repeated pipetting.
3. Overlay sample with mineral oil. Remove any residual mineral oil from the top of the assay plate using a lint-free laboratory tissue (e.g., Kimwipes™ wipe).
4. Firmly seal the plate with a clear adhesive film to reduce contamination from loop-mediated isothermal amplification (LAMP) amplicons.
5. Centrifuge the assay plate at 500 x *g* for 2 min. The LAMP reaction mixture (purple) and mineral oil (transparent) should form two distinct layers.
6. Incubate the assay plate at 65°C for 30 min. The use of a uniform heat block–based instrument, such as the Eppendorf™ Thermomixer™ R, is recommended to reduce any plate-based effects created by uneven heating rates.
7. Incubate the assay plate at room temperature for 2 min. If there is any condensate present, collect the solution by centrifuging the assay plate at 500 x *g* for 5 min.

Table 1. Comparison of reaction components for ReadiLAMP colorimetric assays, for visual or spectrophotometric interpretation.

	Interpreted visually			Interpreted by spectrophotometry					
	Strip tube format			96-well plate			384-well plate		
Component	Positive control	Negative control	Sample	Positive control	Negative control	Sample	Positive control	Negative control	Sample
Nuclease-free water	10.5 µL	12.5 µL	7.5 µL	15.75 µL	18.75 µL	11.25 µL	6.3 µL	7.5 µL	4.5 µL
5X LAMP Buffer	5.0 µL			7.5 µL			3.0 µL		
5X SARS-CoV-2 Primer Mix	5.0 µL			7.5 µL			3.0 µL		
10X Enzyme Mix	2.5 µL			3.75 µL			1.5 µL		
SARS-CoV-2 Control RNA	2.0 µL	0 µL	0 µL	3.0 µL	0 µL	0 µL	1.2 µL	0 µL	0 µL
Experimental sample	0 µL	0 µL	5.0 µL	0 µL	0 µL	7.5 µL	0 µL	0 µL	3.0 µL
Mineral oil	0 µL			25 µL			10 µL		
Total volume	25 µL 1.0X LAMP reaction			62.5 µL 1.5X LAMP reaction			25 µL 0.6X LAMP reaction		

8. Measure and record A_{650} and A_{540} values of all reactions. Data reported here were measured on a FLUOstar™ Omega system using multi-user MARS Data Analysis Software (BMG Labtech).

9. Calculate A_{650}/A_{540} values. Using this ratio from positive and negative controls, interpret experimental sample reactions as positive or negative for SARS-CoV-2.

10. Dispose of all used consumables in a biohazardous waste container. Do not open the reaction plates, as they may contain high concentrations of amplicons.

Calculation of high-throughput screening metrics

Accuracy of the Colorimetric ReadiLAMP assay was calculated as the number of true positives, while specificity was calculated as the number of true negatives at the completion of the LAMP workflow based on visual observation. Z′ factors [3] were calculated as:

$$Z' = 1 - \frac{3 \times (\sigma_{\text{SARS-CoV-2 positive}} + \sigma_{\text{SARS-CoV-2 negative}})}{|\mu_{\text{SARS-CoV-2 positive}} - \mu_{\text{SARS-CoV-2 negative}}|}$$

where µ is the sample average and σ is the sample standard deviation. Additionally, due to the inherent nature of false positives and false negatives arising in LAMP-based assays, robust Z′ factors [4] were applied to appropriately evaluate high-throughput assays described herein as:

$$Robust\ Z' = 1 - \frac{3 \times (\text{MAD}_{\text{SARS-CoV-2 positive}} + \text{MAD}_{\text{SARS-CoV-2 negative}})}{|\tilde{x}_{\text{SARS-CoV-2 positive}} - \tilde{x}_{\text{SARS-CoV-2 negative}}|}$$

where $\tilde{x}$ is the median and MAD is the median absolute deviation of the sample.

Results

The Colorimetric ReadiLAMP Kit allows the user to visually interpret LAMP reactions as positive (blue) or negative (purple) for the presence or absence of SARS-CoV-2, respectively. However, some users may prefer to record and interpret results using a quantitative measurement, such as monitoring spectral intensity changes. To address this need, we developed a robust workflow, transitioning the LAMP assay from a strip tube format to a 96- or 384-well assay plate format that is compatible with plate reader–based spectral or single-wavelength monitoring (Figure 1). Three modifications from the Colorimetric ReadiLAMP Kit User Guide are outlined. First, LAMP reaction volumes are modified to suit the working volume ranges of consumables, and spectrophotometer dynamic linear ranges defined for absorption. Secondly, mineral oil overlays are applied to LAMP reactions before isothermal incubation to reduce evaporation. Finally, an engineered barrier is added to assay plates by use of an optically clear seal. The combination of the mineral overlay and plate seal reduces contamination of instruments and lab spaces by LAMP amplicons.

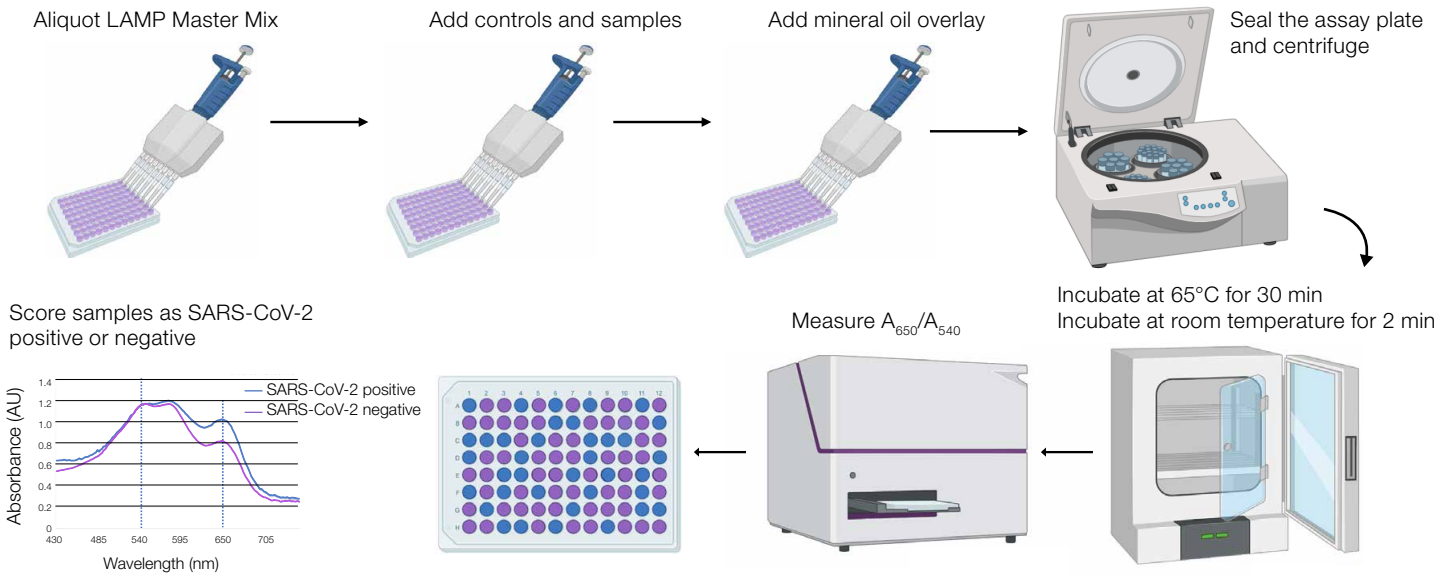

Figure 1. High-throughput Colorimetric ReadiLAMP Kit workflow. Schematic description of the high-throughput screening assay with representative results spectra for true positive and true negative reactions.

The performance of the Colorimetric ReadiLAMP Kit using strip tube and 96-well plate workflows were directly compared. In both formats, the provided SARS-CoV-2 positive control and non-template negative control performed as expected, with only the positive controls transitioning to blue reactions (Figure 2). Additionally, both workflows correctly distinguished the 12 saliva samples lacking SARS-CoV-2 virions and 12 contrived saliva samples spiked with 20 virions/µL. Overall, these workflows performed exceedingly well, with 100% accurate and 100% specific detection of SARS-CoV-2.

To score samples as positive or negative for the SARS-CoV-2 virus based on UV/Vis spectrophotometry, we propose measuring the absorbance from the Colorimetric ReadiLAMP Kit at two defined peaks and calculating the ratio of these values (A_{650}/A_{540}). As reactions transition from initial purple to positive blue, the highest increase in absorbance occurs at a 650 nm peak, while there is a slight decrease in absorbance at a second 540 nm peak (see representative spectra in Figure 1). There are several advantages to interpreting LAMP results using A_{650}/A_{540}, including data collection at a single time point, internal normalization of all reactions, ease of interpretation by comparing results to control reactions on a single plate, and strong statistical metrics for an assay (validated in the following screen).

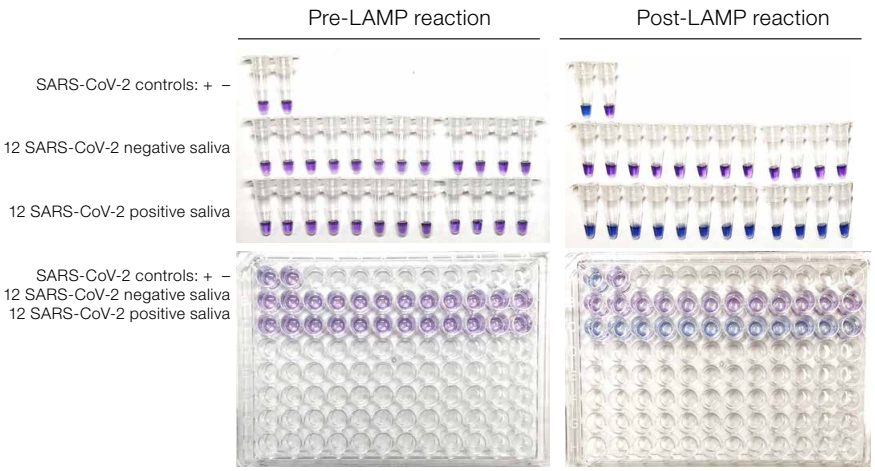

Figure 2. Compatibility of the Colorimetric ReadiLAMP Kit with strip tube and plate consumable formats. Both workflows resulted in 100% accuracy and 100% specificity for SARS-CoV-2 detection.

The robustness of the Colorimetric ReadiLAMP Kit workflow with spectrophotometric detection was evaluated using a high-throughput pilot screen. This screen was divided into three 384-well plates, each evaluating RNA samples isolated from contrived saliva samples spiked with 20 SARS-CoV-2 virions/µL (n = 192) or unspiked, no-template controls (n = 192). Upon completing this workflow, exceptional LAMP performance metrics of 100% accuracy and 97.4 ± 0.05% specificity, determined based on visually scoring, were recorded across the pilot assay (Figure 3 and Table 2). This screen resulted in robust signal (signal-to-noise ratio ≥15), clear distinction of SARS-CoV-2–containing samples (assay window ≥1.5), and overall superior performance (robust Z′ factor of 0.72 ± 0.03; normalized score of 0–1.0 where values of ≥0.5 are indicative of an excellent assay). Ultimately, these strong assay statistics validate the proposed modifications of the Colorimetric ReadiLAMP Kit for high-throughput screening applications.

Conclusions

The workflow described here outlines the adaptation of the Colorimetric ReadiLAMP Kit from a moderate-throughput visual assay to a high-throughput absorbance assay. This adapted workflow incorporates modifications in consumables and physical barriers for plate reader compatibility and reduced contamination by LAMP amplicons. Furthermore, a pilot-screen assay of >1,100 contrived saliva samples validated the approach and supported the superior assay performance. When integrated with existing KingFisher automation for viral nucleic acid isolation, this assay may allow rapid, inexpensive, and accurate screening for SARS-CoV-2.

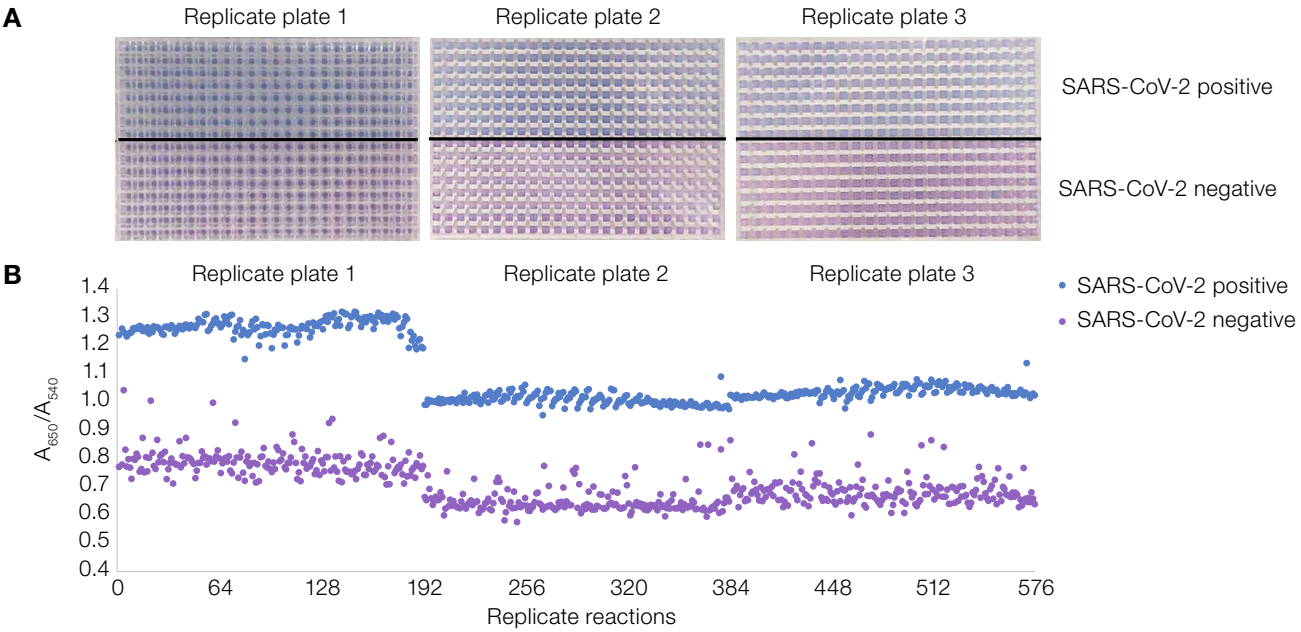

Figure 3. Colorimetric ReadiLAMP high-throughput pilot screen of contrived saliva samples for SARS-CoV-2 detection. Visual inspection of the 384-well assay plates **(A)** and spectral ratio results **(B)** demonstrate that the presence of SARS-CoV-2 can be clearly identified. Positive amplification results in blue reactions with ≥1.5x the spectral ratio of no-template control reactions, which remain purple.

Table 2. Performance of high-throughput pilot screening assay using the Colorimetric ReadiLAMP Kit. Absorbance values, LAMP performance metrics, and high-throughput assay statistics fully support the use of the outlined workflow for SARS-CoV-2 detection.

	Replicate plate 1		Replicate plate 2		Replicate plate 3	
Attribute	**Positive control**	**Negative control**	**Positive control**	**Negative control**	**Positive control**	**Negative control**
A_{650}/A_{540}	1.27 ± 0.0303	0.789 ± 0.0517	1.01 ± 0.0194	0.649 ± 0.0426	1.04 ± 0.0189	0.684 ± 0.0464
Signal-to-noise ratio (µ/σ)	42	15	52	15	55	15
Assay window	1.6		1.6		1.5	
Accuracy	100%		100%		100%	
Specificity	97.9%		96.9%		97.4%	
Robust Z (Z′)	0.75 (0.49)		0.72 (0.48)		0.69 (0.47)	

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References

1. Mercer TR, Salit M (2021) Testing at scale during the COVID-19 pandemic. *Nat Rev Genet* 22:415–426.
2. Centers for Disease Control and Prevention. CDC Science Agenda for COVID-19, 2020–2023 Building the Evidence Base for Ongoing COVID-19 Response. Retrieved July 26, 2021, from cdc.gov/coronavirus/2019-ncov/science/science-agenda-covid19.html
3. Zhang JH, Chung TDY, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 4:67–73.
4. Birmingham A et al. (2009). Statistical methods for analysis of high-throughput RNA interference screens. *Nat Methods* 6:569–575.

Ordering information

Product	Cat. No.
Recommended materials for colorimetric ReadiLAMP assay	
MagMAX Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit	A48383
Fisherbrand 96-Well Plates, clear, flat bottom	12-565-501
Fisherbrand 384-Well Polystyrene Plates, clear, rounded square well	12-565-506
Alfa Aesar Mineral Oil, high purity	AAJ62592AE
MicroAmp Clear Adhesive Film	4306311
Eppendorf Thermomixer R	05-400-205
Eppendorf Microplate Thermoblock	05-400-207

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