

Accurate and sensitive high-throughput multiplex RT-qPCR for SARS-CoV-2 viral RNA detection using the Luna® SARS-CoV-2 RT-qPCR Multiplex Assay Kit

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INTRODUCTION

The coronavirus disease COVID-19, caused by the severe acute respiratory syndrome coronavirus (SARS-CoV-2), has deeply impacted public health, societal relationships and daily life across the globe. As of March 04, 2021, the virus has spread to 192 countries and regions, caused more than 115 million confirmed cases, and claimed more than 2.5 million lives world-wide (<https://coronavirus.jhu.edu>). Due to its prevalence, infection rate and high incidence of asymptomatic infections, comprehensive and timely testing is essential to help curb spread of the disease. The gold standard and most common method for SARS-CoV-2 detection has been hydrolysis probe-based (e.g., TaqMan®) RT-qPCR, owing to its high accuracy and sensitivity. However, unprecedented demand for RT-qPCR diagnostics has led to reagent supply shortages, testing bottlenecks and long sam-

ple-to-result delays. Advances are being made in rapid and at-home testing modalities, but there is still an urgent need for RT-qPCR reagents and workflows that provide fast, accurate detection of SARS-CoV-2 while simultaneously enabling high sample throughput.

From New England Biolabs, the recently developed Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit (Figure 1, <https://www.neb.com/e3019>) offers highly accurate and sensitive SARS-CoV-2 detection, with a limit of detection (LOD) as low as 5 copies per reaction (as assessed using synthetic viral RNA). Further, the kit employs simultaneous multiplex detection of two viral RNA targets and one human RNA internal control target to increase accuracy and throughput. The targets are based on those designed by the Centers for Disease Control and

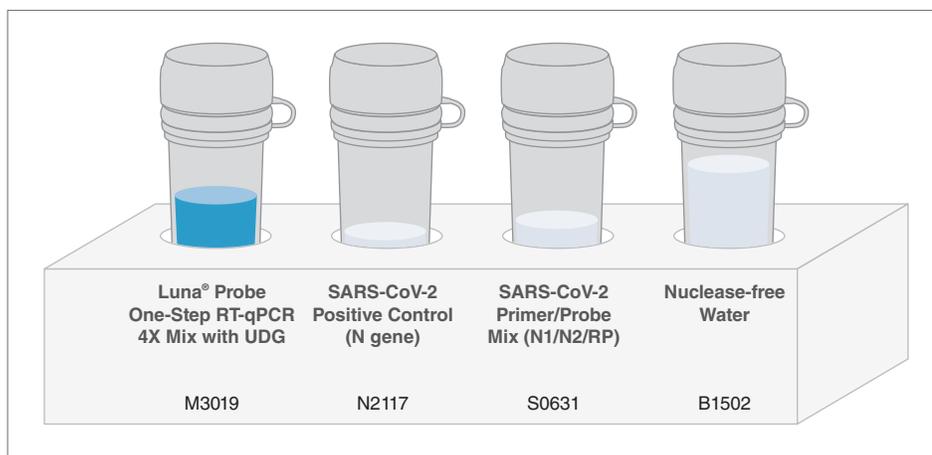
MATERIALS

- Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit (NEB #E3019)
- Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019)

Prevention (CDC) for SARS-CoV-2 detection (<https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf>), and consist of two unique fragments of the viral N gene (2019_nCoV_N1 and 2019_nCoV_N2) and human RNase P. Multiplex detection of N1 (HEX), N2 (FAM) and RNase P (Cy5) increases throughput three-fold over the original singleplex CDC assay. The Luna kit also employs a redesigned RNase P reverse primer that anneals across an exon-exon boundary to reduce the possibility of amplifying genomic DNA. Lastly, the kit includes the Luna Probe One-Step RT-qPCR 4X Mix with UDG (<https://www.neb.com/m3019>, referred to from here as the Luna RT-qPCR 4X Mix) for simplified reaction setup and enabling increased sample input volume.

In this application note, we additionally demonstrate features of the Luna kit that enable strong performance in automated, high-throughput SARS-CoV-2 detection workflows. These features include use of a thermolabile UDG enzyme to reduce carryover contamination, room temperature stability before and after reaction setup, and compatibility with sample pooling and automation platforms. Compatibility with automation opens the door to high-throughput workflows, reaction miniaturization, and 384-well real-time instrument formats. Together, these features allow rapid and robust automated reaction setup and high-throughput multiplex detection for up to 382 samples per RT-qPCR run, with the option to further increase throughput using sample pooling.

 **FIGURE 1: Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit components**



RESULTS

Thermolabile UDG enables efficient carryover prevention and is rapidly inactivated

In situations where the same target is amplified repeatedly, as is the case for SARS-CoV-2 and other diagnostic qPCR/RT-qPCR workflows, there is an increased potential for PCR products from previous rounds of testing to contaminate reactions in subsequent rounds and produce false positives. Such “carryover” contamination can be prevented by incorporating dUTP during amplification, which allows contaminating, dU-containing PCR products from previous reactions to be eliminated during an initial incubation step with Uracil DNA Glycosylase (UDG) (1). However, this strategy can face challenges in one-step RT-qPCR, as UDG from traditional bacterial sources like *E. coli* can also degrade newly synthesized cDNA during the RT step if it remains active (2,3). To address this, the Luna RT-qPCR 4X Mix incorporates Antarctic Thermolabile UDG, which is active during setup and up-front incubations at 4°C and/or room temperature (Figure 2B) but is completely inactivated during the RT step at 55°C (Figure 2C). This efficient yet thermosensitive activity profile enables carry-over prevention without compromising one-step RT-qPCR efficiency or sensitivity (Figure 3).

Activity control enhances stability at room temperature, enabling greater workflow flexibility

In addition to reagent and consumable shortages, the demand for SARS-CoV-2 diagnostic testing has produced logistical challenges and workflow bottlenecks, including instrument and personnel availability. These challenges can lead to numerous delays, including those between reaction setup and thermocycling. In addition, because most automation platforms operate at or near room temperature, use of these platforms necessitates that reagents and reactions remain stable during room-temperature setup. To address this, the Luna RT-qPCR 4X Mix features Hot Start *Taq* DNA Polymerase combined with Luna WarmStart® Reverse Transcriptase, allowing temperature-dependent control of both enzyme activities via reversible, aptamer-based inhibition. This capability helps to prevent non-specific



FIGURE 2: Antarctic Thermolabile UDG is active during reaction setup and pre-incubation, and is rapidly inactivated in RT-qPCR

- A. Schematic illustration of a capillary electrophoresis (CE) assay for UDG activity. 5' FAM-labeled dsDNA containing a dU residue on the labeled strand is used as substrate. UDG converts dU into an abasic site, which is susceptible to hydrolytic cleavage at elevated temperature at high pH. The extent of substrate cleavage thus indicates UDG activity, and can be quantitated by CE analysis.
- B. Flexibility of Antarctic Thermolabile UDG reaction temperature. 20 pmol (1.2×10^{13} molecules) of dU-containing substrate was incubated with 2.5 μ l Luna RT-qPCR 4X Mix for 30 sec at the indicated temperatures. UDG was then inactivated and reaction products were analyzed by CE. Significant UDG activity was observed even at 4°C.
- C. Comparison of Antarctic Thermolabile UDG and *E. coli* UDG inactivation at different temperatures. 0.5 U of enzyme was subjected to inactivation under the indicated conditions, and then incubated with substrate for 30 min at 25°C to assure detection of residual UDG activity. At 55°C (the temperature recommended for Luna RT), Antarctic Thermolabile UDG was completely inactivated after 5 min, whereas significant *E. coli* UDG activity remained.

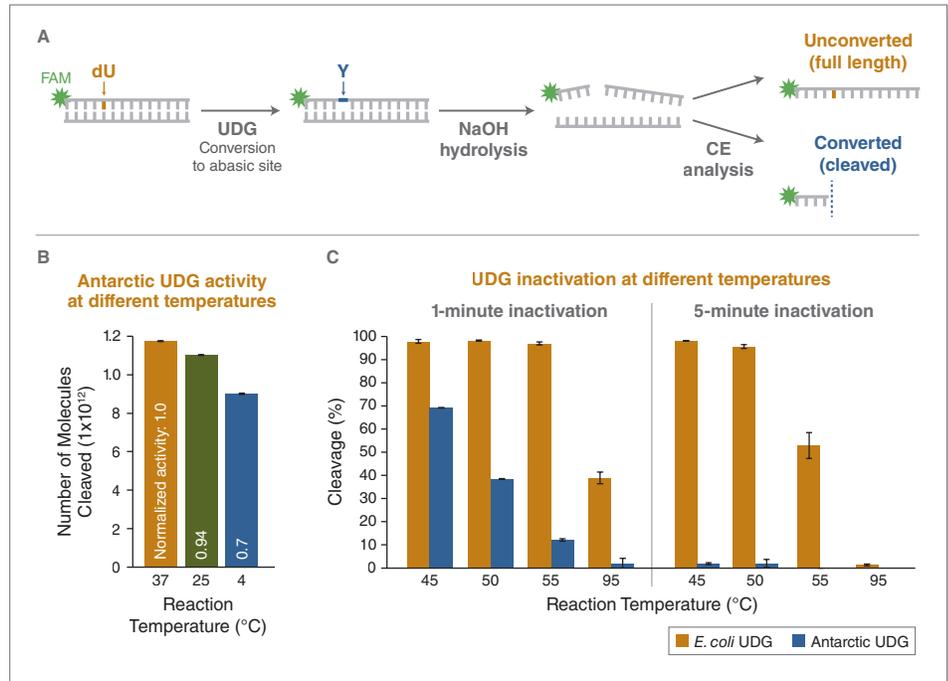
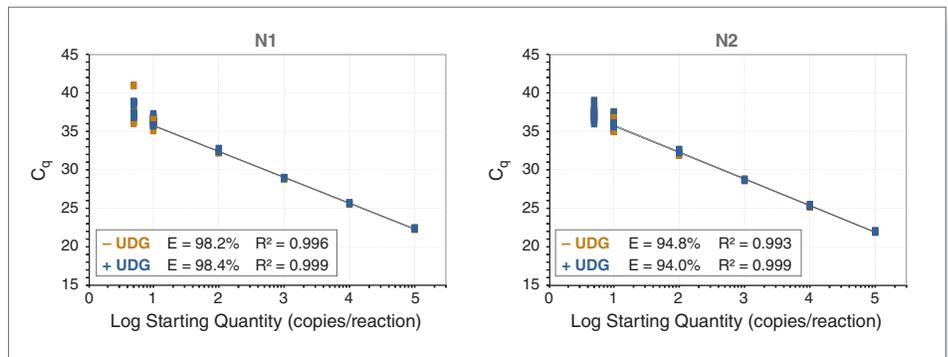


FIGURE 3: Antarctic Thermolabile UDG does not interfere with SARS-CoV-2 RNA detection and quantitation

Standard curves for N1 and N2 viral targets from multiplex SARS-CoV-2 assays, conducted using the Luna RT-qPCR 4X Mix (+ UDG, blue) or an equivalent mix lacking UDG (– UDG, gold). A 5-log dilution series of SARS-CoV-2 synthetic RNA was used to evaluate efficiency (E) and linearity (100,000 to 10 copies/reaction, in triplicate), with additional replicates at low copy to evaluate sensitivity (10 and 5 copies/reaction, n = 12 each). All reactions contained human (Jurkat) total RNA (10 ng/reaction) to mimic clinical samples. Linear amplification and sensitive detection were observed for both mixes (12/12 at 10 copies; 11/12 – UDG and 12/12 + UDG at 5 copies). Data shown were collected using an ABI® 7500Fast real-time instrument.



priming and extension at room temperature, and additionally prevents amplification prior to inactivation of thermolabile UDG. To evaluate the benefit of this dual activity control in SARS-CoV-2 detection assays, we examined the performance of pre-assembled SARS-CoV-2 multiplex RT-qPCR reactions held at room temperature for extended periods, compared to reactions that were run immediately after set-up. Even after 24 hours, pre-assembled reactions maintained linear amplification for the SARS-CoV-2 N1 and N2 targets without significant C_q delays, for reactions in both 96-well and 384-well formats (Figure 4). An LOD of 5 copies/reaction was maintained for the pre-assembled reactions after up to 8 hours at room temperature; and even after 24 hours, an LOD of 10 copies/reaction was observed for both targets (Figure 5). Lower reaction volumes (e.g., 5 μ l) can be used for 384-well reactions (Figure 8), but may introduce additional variability with extended room temperature incubation due to evaporation, condensation and other factors. Other specimen types and/or assay conditions should also be empirically evaluated in new workflows. However, similar room temperature stability with



FIGURE 4: Stability of preassembled Luna SARS-CoV-2 assays at room temperature enables workflow flexibility

Standard curves for multiplex SARS-CoV-2 assays, assembled in 96-well (20 μ l reaction) or 384-well (10 μ l reaction) formats and run either immediately (blue) or after incubation at room temperature for 24 hours (gold). Equivalent C_q values and linear quantification were observed under both conditions. Linearity was assessed for a 5-log input range of synthetic SARS-CoV-2 RNA (100,000 to 10 copies/reaction, in triplicate), and sensitivity assessed by percent detection at 5 and 10 copies/reaction. Data shown were collected using Bio-Rad[®] CFX96 and CFX384 real-time instruments.

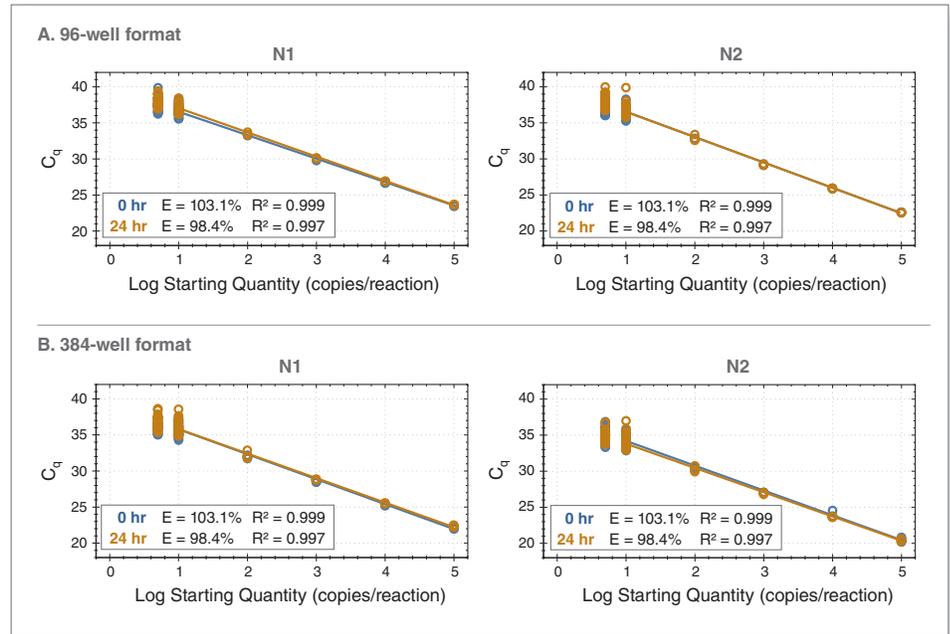
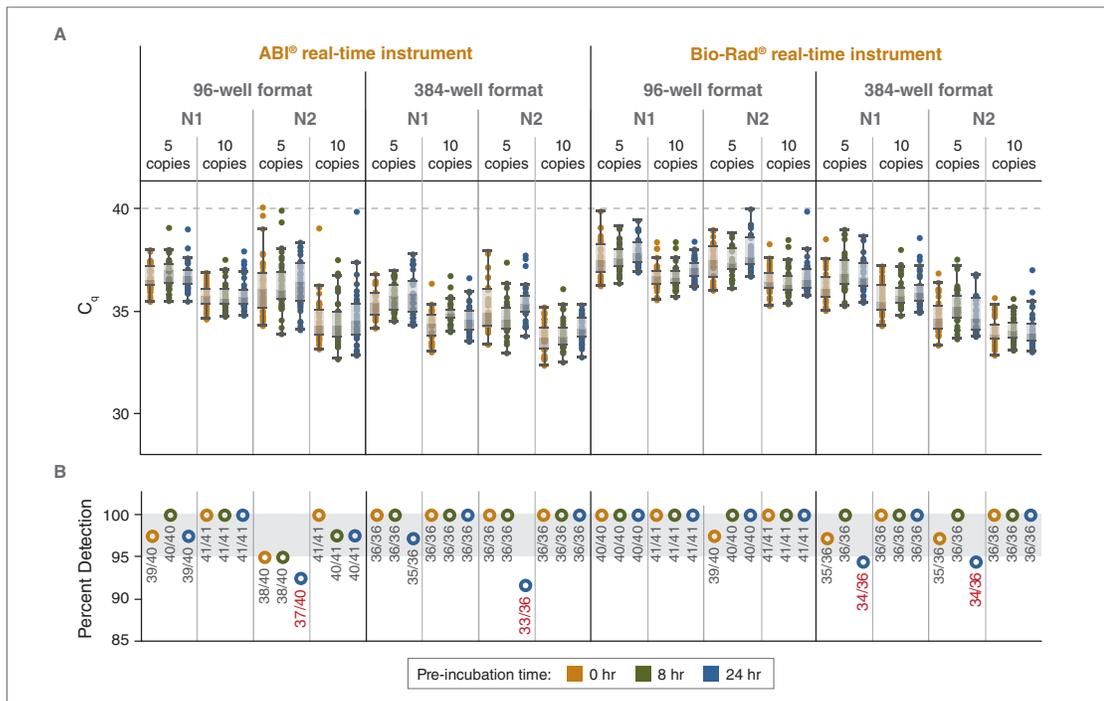


FIGURE 5: Low input replicate testing demonstrates retention of Luna SARS-CoV-2 assay sensitivity after extended incubation at room temperature

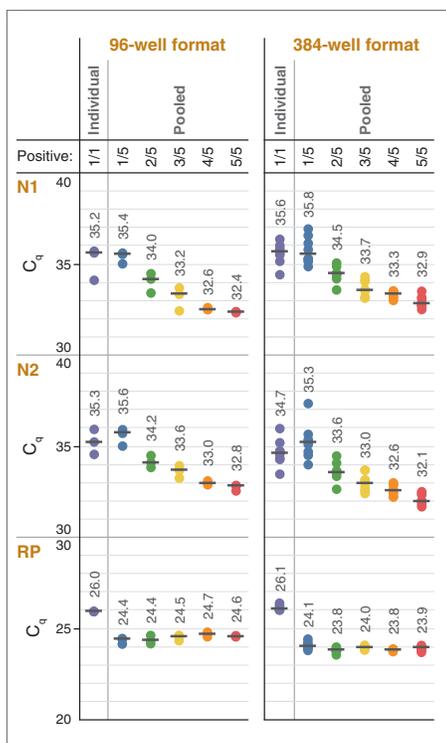
C_q values (A) and percent detection (B) for multiplex SARS-CoV-2 assays, assembled in 96-well (20 μ l reaction) or 384-well (10 μ l reaction) formats and run either immediately (blue) or after incubation at room temperature for 8 hours (orange) or 24 hours (red). Shaded boxes denote middle 50% (two quartiles) of data; error bars denote 1.5X interquartile range (IQR). Dotted line indicates $C_q \leq 40$ cut-off for detection. Reactions contained either 5 or 10 copies of synthetic SARS-CoV-2 RNA. Detection sensitivity was maintained with an LOD ($\geq 95\%$ detection) of 5 copies/reaction after incubation for 8 hours at room temperature. After a 24-hour incubation at room temperature, a slight loss of sensitivity was observed (red text in B), but an LOD of 10 copies/reaction was still achieved. Data shown were collected using ABI 7500Fast (96-well), ABI QuantStudio[™] 6 Flex (384-well), Bio-Rad CFX96 (96-well), and Bio-Rad CFX384 (384-well) real-time instruments.



pre-assembled reactions has also been observed for other human RNA targets using the Luna RT-qPCR 4X Mix (data not shown). Dual activity control can thus provide workflow flexibility for room temperature setup and hold steps.

FIGURE 6: Luna RT-qPCR 4X Mix supports sampling pooling with high sensitivity and accuracy

C_q values for multiplex SARS-CoV-2 assays with individual and pooled mock samples, carried out in 96-well (20 μ l reaction) and 384-well (10 μ l reaction) formats as indicated. Line and number indicate average C_q for each condition. Each mock sample contained 10 ng human (Jurkat) total RNA; mock positives also contained 10 copies of SARS-CoV-2 synthetic RNA. For pooled samples, the number of positive samples in the pool is indicated. For all mock positive samples, both viral targets (N1 and N2) targets were detected, and C_q values were proportional to the number of positive samples in a pool. For RNase P (RP), C_q values were proportional to human total RNA input (10 ng for individual samples, 50 ng total for pooled samples). Data shown were collected using ABI 7500Fast (96-well) and ABI QuantStudio 6 Flex (384-well) real-time instruments.



Sample pooling without compromising sensitivity

Sample pooling – that is, combining samples to test multiple individuals with a single reaction – is a strategy that can reduce the total number of tests needed to screen a population (4), thereby increasing throughput, decreasing costs, and improving average turn-around time by alleviating testing backlogs. This strategy has already been applied in some COVID-19 testing workflows, with pooling conducted at stages of either specimen collection, sample processing or RT-qPCR (5). The inclusion of a 4X mix within the Luna SARS-CoV-2 Assay Kit enables the use of up to 13 μ l of sample input, making it an attractive option for sample pooling at the RT-qPCR step. Additionally, an important criteria for testing pooled samples is that the employed assay be sufficiently sensitive, such that dilution of positive samples upon pooling does not lead to false negative results. As a proof-of-principle assessment, we therefore tested the effect of mock sample pooling on the sensitivity of the Luna SARS-CoV-2 Assay

Kit. We assembled pools containing 1 to 5 mock positive samples, each containing 10 copies of synthetic SARS-CoV-2 RNA, and assessed sensitivity and C_q values in both 96-well and 384-well formats, with comparisons to an unpooled control. Sensitive detection was fully maintained following sample pooling, and C_q values appropriately reflected the total number of copies in the pooled sample (Figure 6), demonstrating continued high sensitivity and accuracy of the Luna SARS-CoV-2 Assay Kit within a sample pooling workflow.

Higher throughput enabled by robust compatibility with automated reaction assembly, 384-well formats and reaction miniaturization

Increased assay throughput is required to meet the ongoing demand for SARS-CoV-2 diagnostic testing. As described above, this can be accomplished in part by using multiplex detection and/or sample pooling. Throughput

FIGURE 7: Luna RT-qPCR 4X Mix and SARS-CoV-2 Assay Kit are compatible with automated reaction assembly and 384-well formats

Standard curves for multiplex SARS-CoV-2 assays, assembled either manually or with the indicated automated liquid handling platform in either 96-well or 384-well formats are shown, as indicated. Linearity was assessed for a 5-log input range of synthetic SARS-CoV-2 RNA (100,000 to 10 copies/reaction, in triplicate), and sensitivity by percent detection at low copy (5 and 10 copies/reaction; quantitation in Figure 8). Log starting quantity indicates SARS-CoV-2 synthetic RNA copies/reaction for viral N1 and N2 targets, and human (Jurkat) total RNA ng/ μ l for RNase P (RP) target. Linear quantitation was observed under all tested conditions (see also Table 3), and equivalent C_q values were observed for manual and automated reaction assembly in 384-well format. Data shown were collected using Bio-Rad CFX96 and CFX384 real-time instruments. Similar linearity and LOD results were also observed using ABI 7500 Fast (96-well) and ABI QuantStudio 6 Flex (96- and 384-well) real-time instruments (data not shown).

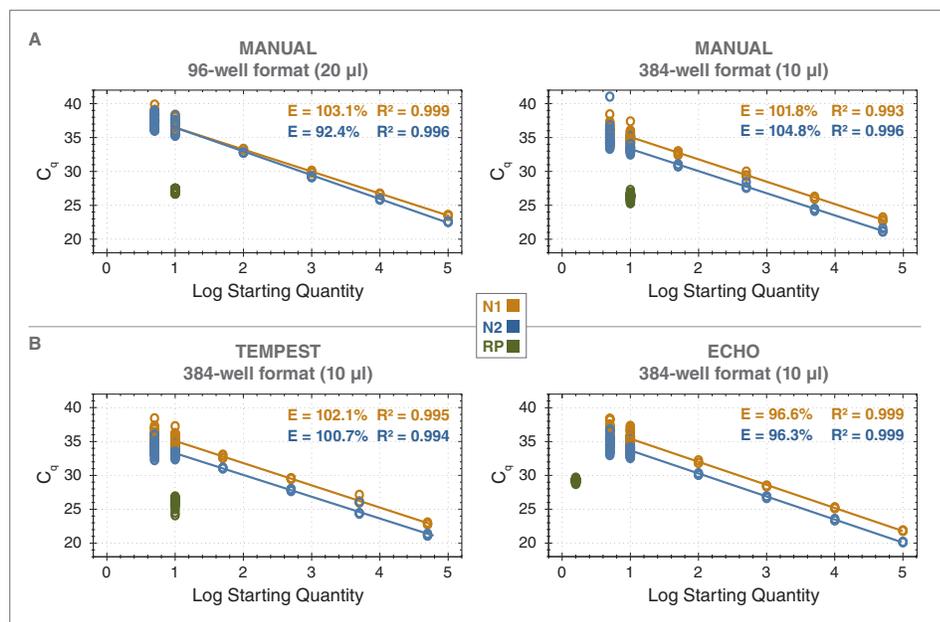


TABLE 1: Liquid class settings for automated dispensing

REAGENT	ECHO 525			TEMPEST
	RESERVOIR	384PP 2.0	384LDV OR 384LDV PLUS	HIGHVOLUME
	Working volume: 250–2800 µl	Working volume: 15–65 µl	Working volume: 4.5–14 µl	
LUNA SARS-COV-2 RT-QPCR MULTIPLEX ASSAY KIT (NEB #E3019) COMPONENTS				
Individual components	Liquid Class			Liquid Class
Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019)	GPSB	GPSB	(N.R.*)	Medium
SARS-CoV-2 Primer/Probe Mix (N1/N2/RP) (NEB #S0631)	BP	BP	(N.R.*)	Normal
SARS-CoV-2 Positive Control (N gene) (NEB #N2117)	BP	BP	GP	Medium
Assay mix (4X Mix + Primer/Probe Mix + water)	GPSB	GPSB	(N.R.*)	
SAMPLES				
In water, TE or other detergent-free buffers	(N.R.*)	BP	GP	(Not tested)
In detergent-containing buffers**	(N.R.*)	SP	GP	(Not tested)

* Not recommended (due to working volume range). 384LDV Plus plates are optimal for small volume (< 1 µl) sample transfers. Reservoir plates are optimal for large volume reagent transfers.

** For detergent-containing buffers, verify compatibility and liquid class before dispensing samples.

can be further increased by automating reaction setup and testing in 384-well rather than 96-well format. Automated liquid handling can also reduce setup time, error, and variability. We therefore examined Luna SARS-CoV-2 assay performance and sensitivity after automated reaction setup using two common liquid handling platforms: the Beckman Coulter Echo 525 Acoustic Liquid Handler and the Formulatrix® Tempest® Microfluidic Liquid Handler (Table 1). With both platforms, we also examined the feasibility of reducing reagent costs via reaction miniaturization, decreasing reaction volumes from the standard 20 µl in 96-well format to either 10 µl or 5 µl in 384-well format (Table 2). Under all setup conditions, linear quantitation was observed for both the SARS-CoV-2 N1 and N2 targets over a 5-log input range of synthetic SARS-CoV-2 RNA (100,000 to 10 copies/reaction) (Figure 7). High sensitivity was also maintained, with an LOD of 5 copies/reaction for all conditions tested (Figure 8). No amplification was observed in negative control (NTC) reactions under any of the conditions tested (n = 3 for 96-well data shown; n = 8 or 12 for 384-well data shown; n > 1000 total across all experiments), demonstrating high specificity under all tested conditions. Thus, the Luna SARS-CoV-2 Assay Kit offers a versatile option to meet demand for high-throughput SARS-CoV-2 testing.

TABLE 2: Luna multiplex SARS-CoV-2 assay reaction setup for 10 µl and 5 µl reactions

COMPONENT	10 µl REACTION	5 µl REACTION	FINAL CONCENTRATION
Luna Probe One-Step RT-qPCR 4X Mix with UDG	2.5 µl	1.25 µl	1X
10X SARS-CoV-2 Primer Mix	1 µl	0.5 µl	1X
Sample*	Up to 5 µl	Up to 2 µl	Varies
Nuclease-free Water	To 10 µl	To 5 µl	

* For SARS-CoV-2 Positive Control, use 1 µl or 0.5 µl per reaction for 10 µl or 5 µl reactions, respectively

TABLE 3: Luna multiplex SARS-CoV-2 assays maintain consistent efficiency and linearity for a range of reaction formats, volumes and automated assembly methods

FORMAT	REACTION VOLUME	TARGET	SETUP METHOD	EFFICIENCY	R ²
96-well	20 µl	N1	Manual	103.1	0.999
		N2	Manual	92.4	0.996
384-well	5 µl	N1	Manual	100.7	0.993
			Tempest	105.3	0.997
			Echo	98.7	0.998
		N2	Manual	97.2	0.996
			Tempest	101.8	0.997
			Echo	98.5	0.996
	10 µl	N1	Manual	101.8	0.993
			Tempest	102.1	0.995
		N2	Manual	104.8	0.996
			Tempest	100.7	0.994
			Echo	96.3	0.999

CONCLUSION

COVID-19 has tested both the capabilities and limitations of modern molecular diagnostics like no crisis before it. RT-qPCR assays for viral detection have been a critical tool in fighting spread of the disease; however, the scale of the global pandemic has strained testing capacity, and made clear the need for methods to increase efficiency and throughput to both curb the current pandemic and better prepare for potential future public health crises. In this work, we have demonstrated features of the Luna RT-qPCR 4X Mix and SARS-CoV-2 Assay Kit that enable higher assay throughput while maintaining high accuracy and sensitivity. These features include multiplex detection, sample pooling, and robust automation compatibility. Inclusion of Antarctic Thermolabile UDG in the Luna RT-qPCR 4X Mix provides convenient carryover prevention without impairing performance, a critical feature to help prevent false positives in high-volume testing. The combination of Luna WarmStart Reverse Transcriptase and Hot Start *Taq* provides dual activity control to prevent spurious amplification, enabling robust room temperature stability before and after reaction setup. This stability affords workflow flexibility that is particularly important in automated workflows. Finally, we directly demonstrate kit compatibility with common automated liquid handling platforms, including both microfluidic and acoustic modes of transfer. Taken together, these features make the Luna RT-qPCR 4X Mix and SARS-CoV-2 Assay Kit strong choices for efficient, high-throughput detection workflows.

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FIGURE 8: Automation-compatible, high-throughput multiplex RT-qPCR for detection of SARS-CoV-2 RNA with high sensitivity using Luna reagents

C_t values (A) and percent detection (B) for multiplex SARS-CoV-2 assays assembled in 96-well or 384-well format either manually or using the indicated automated liquid handling platform. Reactions contained either 5 or 10 copies of synthetic SARS-CoV-2 RNA. An LOD of 5 copies/reaction was maintained for all assembly workflows. Data shown were collected using Bio-Rad CFX96 and CFX384 real-time instruments.

