

Optimized conditions for the CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay using Luna® One-Step RT-qPCR Reagents

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INTRODUCTION

Widespread implementation of rapid, reliable diagnostic COVID-19 tests has been key to limiting the spread of the SARS-CoV-2 virus within communities and providing healthcare to infected individuals. Multiplex versions of molecular diagnostic tests have also been developed to accurately differentiate common respiratory viral infections that present with similar symptoms, such as influenza A (FluA), influenza B (FluB), and SARS-CoV-2 (SC2). In July 2020, the U.S. Food and Drug Administration (FDA) granted an Emergency Use Authorization (EUA) for a multiplex assay developed by the U.S. Centers for Disease Control and Prevention (CDC) that could simultaneously detect and distinguish these three viruses ([cdc.gov/coronavirus/2019-ncov/lab/testing.html](https://www.cdc.gov/coronavirus/2019-ncov/lab/testing.html)). This Influenza SARS-CoV-2 (Flu SC2) assay detects viral RNA using quantitative reverse-transcription polymerase chain reaction (RT-qPCR), which is the gold standard for RNA detection (1). Importantly, the Flu SC2 multiplex assay minimizes the use of test reagents and allows higher throughput than single-plex testing, lessening the burden on testing facilities.

In this application note, we evaluated the use of the Luna Probe One-Step RT-qPCR 4X Mix with UDG (Luna Mix – NEB #M3019) and an identical 4X mix without ROX (Luna Mix (No ROX) – NEB #M3029) with the EUA-approved Flu SC2 Multiplex assay. The Luna 4X mixes can detect up to five targets per reaction and are enabled with carryover prevention (dUTP/Thermolabile UDG). The Luna Mix (NEB #M3019) is currently used in the EUA-approved SalivaDirect™ protocol from Yale for SARS-CoV-2 detection (2) and has also been employed in multiplex SARS-CoV-2 reactions with clinical swabs (3) and wastewater samples (4). The reagent also powers our Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit (NEB #E3019) in a 3-plex reaction, detecting

the original CDC N1 and N2 SARS-CoV-2 targets along with a modified RNase P control in a single reaction. As part of the Flu SC2 assay evaluation, we found reaction conditions that improved the consistency and sensitivity of FluA, FluB, and SARS-CoV-2 multiplexed reactions using shorter run times across different qPCR instruments. Direct detection of viral RNAs from heat-inactivated virus and swab samples is demonstrated. We also evaluated a lyophilized version of the Luna Mix, LyoPrime Luna™ Probe One-Step RT-qPCR Mix with UDG (NEB #L4001), and present initial limit of detection (LOD) findings for the three viral targets.

MATERIALS

- Luna® Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019) or Luna Probe One-Step RT-qPCR 4X Mix with UDG (No ROX) (NEB #M3029)
- Oligos/probes (Integrated DNA Technologies)
- Monarch® RNA Miniprep Kit (NEB #T2010)

 TABLE 1: EUA/CDC specifications for influenza A, influenza B and SARS-CoV-2 detection

Cycling conditions: 25°C for 2 min., 50°C for 15 min., 95°C for 2 min., 45 cycles of 95°C for 15 sec., 55°C for 30 sec.

Instrument specification: Applied Biosystems® 7500 Fast Dx Real-Time PCR System, standard ramp speed

Passive reference: None

PRIMERS/PROBES	DESCRIPTION	OLIGONUCLEOTIDE SEQUENCE (5'→3')	FINAL CONC. (µM)
FluA-Forward	InfA For1	CAA GAC CAA TCY TGT CAC CTC TGA C	0.4
	InfA For2	CAA GAC CAA TYC TGT CAC CTY TGA C	0.4
FluA-Reverse	InfA Rev1	GCA TTY TGG ACA AAV CGT CTA CG	0.6
	InfA Rev2	GCA TTT TGG ATA AAG CGT CTA CG	0.2
FluB-Forward	InfB For	TCC TCA AYT CAC TCT TCG AGC G	0.8
FluB-Reverse	InfB Rev	CGG TGC TCT TGA CCA AAT TGG	0.8
SC2-Forward	SC2 For	CTG CAG ATT TGG ATG ATT TCT CC	0.8
SC2-Reverse	SC2 Rev	CCT TGT GTG GTC TGC ATG AGT TTA G	0.8
RNaseP-Forward	RNase P For	AGA TTT GGA CCT GCG AGC G	0.8
RNaseP-Reverse	RNase P Rev	GAG CGG CTG TCT CCA CAA GT	0.8
FluA-Probe	InfA Probe	5'-/FAM/TGC AGT CCT /ZEN/ CGC TCA CTG GGC ACG/3IABkFQ/-3'	0.2
FluB-Probe	InfB Probe	5'-/YakYeI/CCA ATT CGA/ZEN/ GCA GCT GAA ACT GCG GTG/3IABkFQ/-3'	0.2
SC2-Probe	SC2 Probe	5'-/TexRd-XN/ATT GCA ACA/TAO/ ATC CAT GAG CAG TGC TGA CTC/3IABRQSp/-3'	0.2
RNaseP-Probe	RNase P Probe	5'-/CY5/TTC TGA CCT /TAO/ GAA GGC TCT GCG CG/3IABRQSp/-3'	0.2

RESULTS

Detecting SARS-CoV-2, influenza A, and influenza B using CDC recommendations

The CDC's Flu SC2 multiplex assay was first evaluated using EUA/CDC-authorized reaction conditions, including the TaqPath® 1-Step Multiplex Master Mix (No ROX), which served as a benchmark for the assessment of the Luna mixes. The Flu SC2 assay is a 4-plex reaction including ten primers and four probes at concentrations ranging from 0.2–0.8 μM (Table 1, page 1). Because the assay uses a Texas Red probe, which is detected in the ROX channel, ROX normalization is not possible on Applied Biosystems (ABI) instruments, and therefore, the CDC assay was developed using mixes without ROX.

The TaqPath Multiplex Mix detected all three targets from 10^5 down to 10 copies of purified viral genomic RNA on the ABI 7500 Fast Real-Time PCR System in standard mode, which determines the temperature ramp rate (Figure 1A).

With some PCR instruments, overall experimental run times can be decreased by accelerating the temperature ramp rate, speeding up the transition between temperatures. A faster ramp speed provides several benefits, including less instrument time per test and higher sample throughput per instrument. The ABI real-time PCR instrument used here includes a built-in function to increase the ramp rate during the PCR cycling protocol (fast mode). When the fast ramp speed was used instead of the standard ramp speed, the PCR duration decreased from ~95 min to ~75 min. However, the detection of FluA and FluB by the TaqPath mix deteriorated when using the faster ramp speed (Figure 1A). To understand whether the decrease in efficiency and sensitivity was instrument-specific, we also evaluated the assay using the default ramp rate on the Bio-Rad® CFX96 Touch Real-Time PCR Detection System. Undesirable performance was also observed for FluA with this instrument, which was typically prone to failure when the viral RNA concentration was low.



FIGURE 1: Detection of influenza A, influenza B and SARS-CoV-2 following the EUA CDC-recommended setup

The 4-plex RT-qPCR assay contained primers and probes recommended in the EUA/CDC specification. Each reaction contained viral RNA templates from 10^5 to 10 copies in the presence of 15 ng/ μl Jurkat total RNA. The real-time PCR runs were carried out on the Applied Biosystems 7500 Fast Real-Time PCR System or the Bio-Rad CFX96 Touch Real-Time PCR Detection System using cycling conditions of 25°C, 2 min.; 50°C, 15 min.; 95°C, 2 min.; 45 cycles of 95°C, 15 sec. and 55°C, 30 sec.

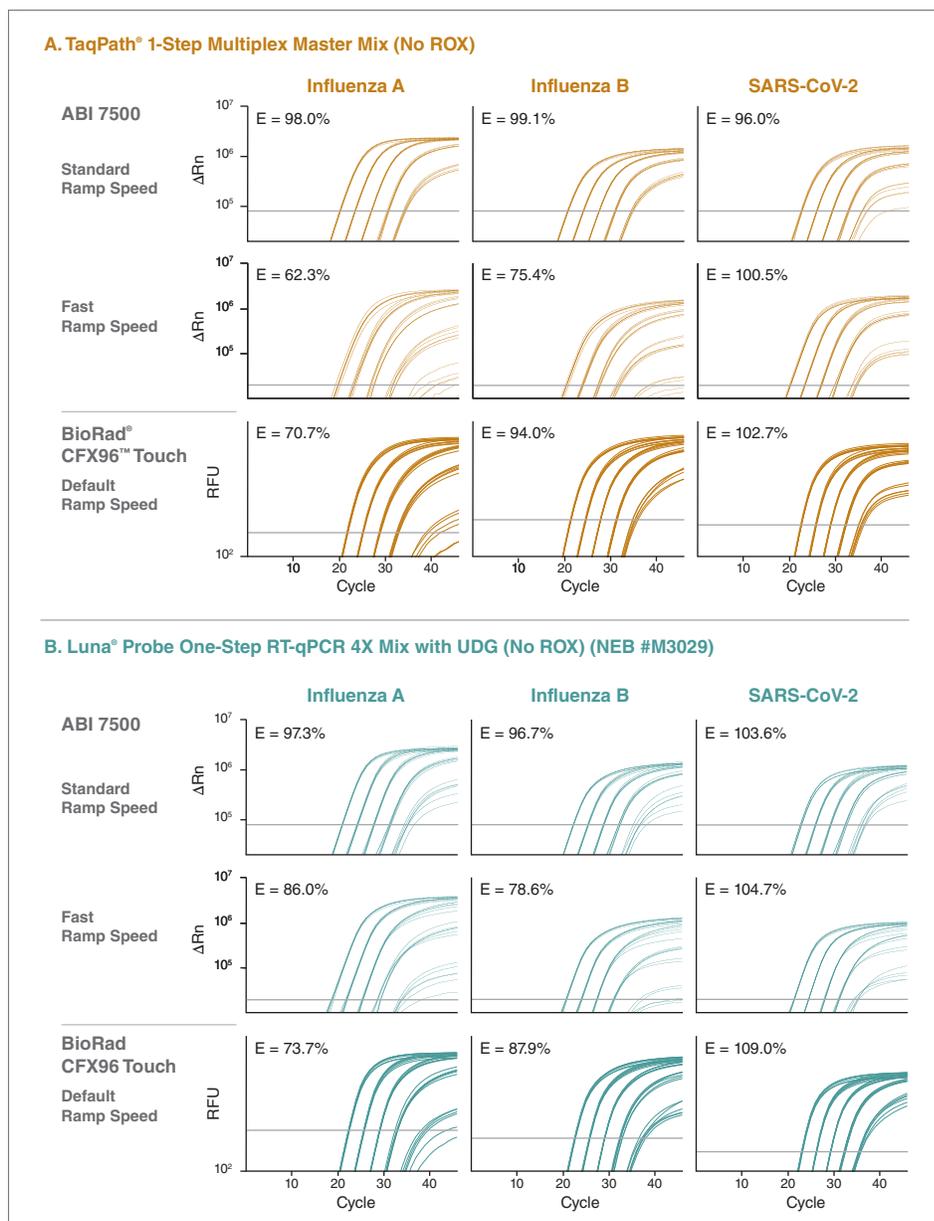
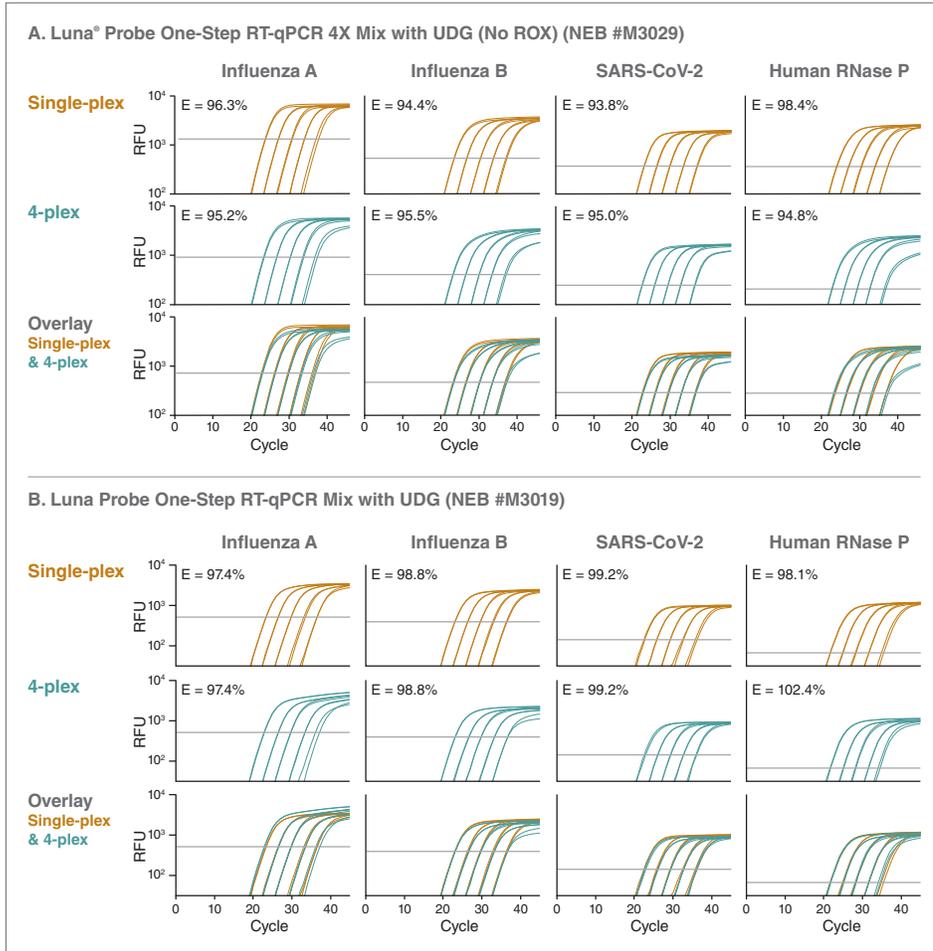




FIGURE 2: Optimization of influenza A, influenza B and SARS-CoV-2 detection using the Luna 4X mixes

Detection of FluA, FluB, SARS-CoV-2, and human RNase P (RP) using A) Luna Mix (No ROX) or B) Luna Mix on the Bio-Rad CFX96 Touch Real-Time PCR Detection System using cycling conditions of 25°C 30 sec; 55°C 10 min; 95°C 1 min; 45 cycles of 95°C 10 sec, 60°C 30 sec. The single-plex RT-qPCR assay contained each primer and probe at 0.2 µM in the presence of the viral RNA templates from 10⁵ to 10 copies or human total RNA from 100 ng to 10 pg. The 4-plex RT-qPCR assay contained all primers and probes at 0.2 µM in the presence of three viral RNA templates from 10⁵ to 10 copies and human total RNA from 100 ng to 10 pg.



Similar results were observed with the Luna Mix (No ROX) when using the CDC assay conditions outlined in Table 1. When the Flu SC2 assay was performed with the Luna Mix (No ROX) on the ABI 7500 instrument using the standard ramp rate, all three targets were detected with desirable efficiencies (Figure 1B, page 2), but detection of FluA and FluB was not ideal using fast mode. On the Bio-Rad instrument, detection at low input was inconsistent for FluA.

Our evaluation found that the original Flu SC2 multiplex assay conditions worked reasonably well in standard mode as specified on the ABI instrument, whereas achieving shorter PCR run times and broader instrument compatibility required additional optimization.

Optimizing detection of SARS-CoV-2, influenza A and influenza B

To optimize the 4-plex reaction conditions for the Luna mixes, we first examined the individual single-plex assays on the Bio-Rad CFX. Assay conditions were simplified by fixing all primer and probe concentrations at 0.2 µM while using recommended cycling conditions. Under these conditions, the Luna mixes detected all targets with ideal linearity over a 5-log template dilution series (Figure 2, single-plex). Moving to the more complex 4-plex assay with these same assay conditions (Table 2), we observed that each viral RNA target was detected with similar linearity down to ~10 copies (Figure 2, 4-plex). In addition, the human RNase P target was also detected linearly over 5-logs of input in the 4-plex reaction. Figure 2 also shows the overlay of the amplification curves from the single- and 4-plex reactions, highlighting successful multiplexing as detection of each target remained similar between the two assay formats.



TABLE 2: Optimized conditions for influenza A, influenza B and SARS-CoV-2 detection using the Luna 4X mixes

RECOMMENDATIONS FOR LUNA MIXES	
Reaction setup	FluA, FluB, SC2, RP primers (10 primers, final 0.2 µM each) FluA, FluB, SC2, RP probes (4 probes, final 0.2 µM each)
Cycling conditions	25°C for 30 sec. 55°C for 10 min. 95°C for 1 min. 45 cycles of 95°C for 10 sec.; 60°C for 30 sec.
Instrument compatibility	Luna Probe One-Step RT-qPCR 4X Mix with UDG (No ROX) (NEB #M3029) Applied Biosystems 7500 Fast Real-Time PCR System QuantStudio 6 Flex Real-Time PCR System Bio-Rad CFX Real-Time PCR Detection Systems Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019) Bio-Rad CFX Real-Time PCR Detection Systems

Furthermore, we evaluated both Luna Mix and Luna Mix (No ROX) using the Luna 4-plex Flu SC2 assay conditions (Table 2) under the maximum ramp rate (5°C per second) and a slow ramp rate (1.5°C per second) on the Bio-Rad instrument. Importantly, both Luna mixes displayed excellent linearity and sensitivity with the faster ramp rate under these conditions (Figure 3).

Detection of SARS-CoV-2, influenza A and influenza B on two ABI instruments

The 4-plex Flu SC2 assay using the Luna Mix (No ROX) was then evaluated on the ABI 7500 Fast Real-Time System. All three targets, FluA, FluB, and SARS-CoV-2, were detected with ideal linearity using the standard mode (Figure 4A). By combining the optimized Luna reaction conditions with the faster ramp speed, the RT-qPCR run time was reduced to approximately 65 min from the original protocol (95 min.). All three viral targets were detected with similar linearity under the fast ramp mode. Robust performance was also achieved on the QuantStudio® 6 Flex Real-Time PCR System using both the standard and fast ramp modes (Figure 4B).

Detection of various Influenza and SARS-CoV-2 viral samples

To further examine the robustness of detection with Luna, the optimized assay conditions were evaluated with three different Influenza A strains and two SARS-CoV-2 templates. These samples were mixed in different combinations, and 4-plex detection was conducted using either Luna Mix or Luna Mix (No ROX). As shown in Figure 5 (page 5), the three Influenza A strains (H1N1/1934, H1N1/2009, H3N2/2009) were consistently detected over a 5-log range of viral RNA input in the six different template mixes for Luna Mix (No ROX). Additionally, heat-inactivated SARS-CoV-2 virus was detected with similar linearity as the synthetic RNAs. Linear detection of all six viral templates was also observed using the Luna Mix (data not shown).



FIGURE 3: Robust performance of the Luna Flu SC2 multiplex assay on the Bio-Rad instrument

Both Luna Mix and Luna Mix (No ROX) displayed strong performance in the Flu SC2 multiplex assay on the Bio-Rad CFX96 Touch Real-Time PCR Detection System under two ramp rates using cycling conditions of 25°C, 30 sec.; 55°C, 10 min.; 95°C, 1 min.; 45 cycles of 95°C, 10 sec. and 60°C 30 sec. (ramp rate of 5°C/sec. or 1.5°C/sec.).

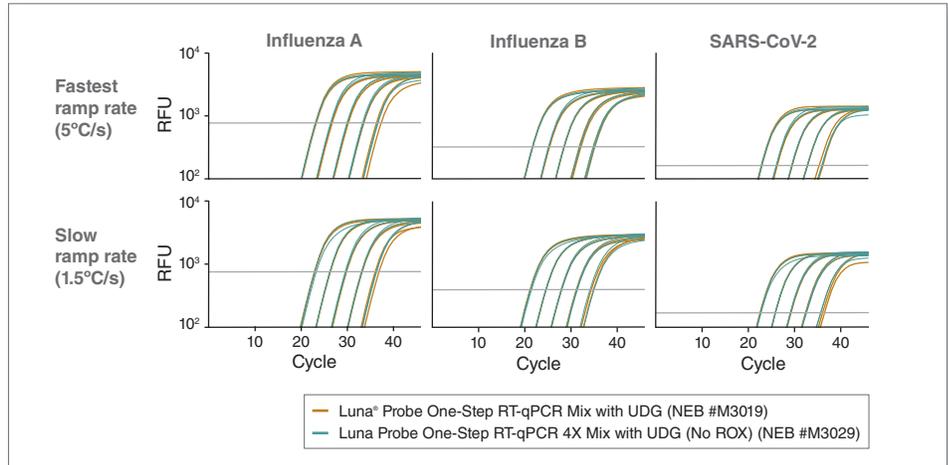


FIGURE 4: Luna Detection of influenza A, influenza B and SARS-CoV-2 using Luna conditions on the Applied Biosystems 7500 Fast Real-Time PCR System and QuantStudio 6 Flex Real-Time PCR System

The 4-plex RT-qPCR reactions contained each primer and probe at 0.2 μM in the presence of three viral RNA templates from 10⁵ to 10 copies. Real-time PCR runs were carried out on Applied Biosystem 7500 or QuantStudio 6 using cycling conditions of 25°C, 30 sec.; 55°C, 10 min.; 95°C, 1 min.; 45 cycles of 95°C, 10 sec. and 60°C 30 sec. in fast mode or standard mode, as noted.

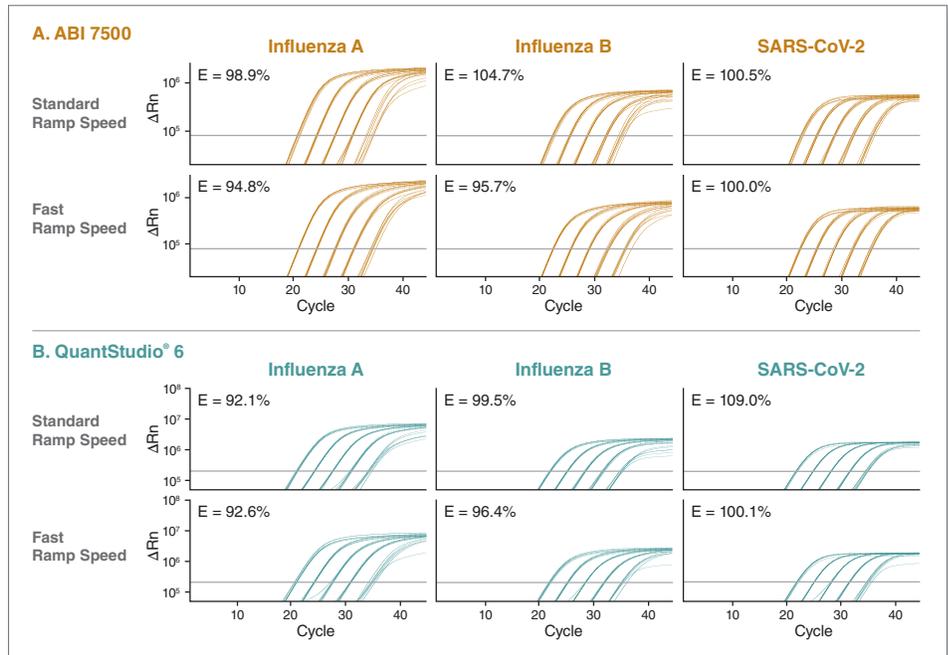
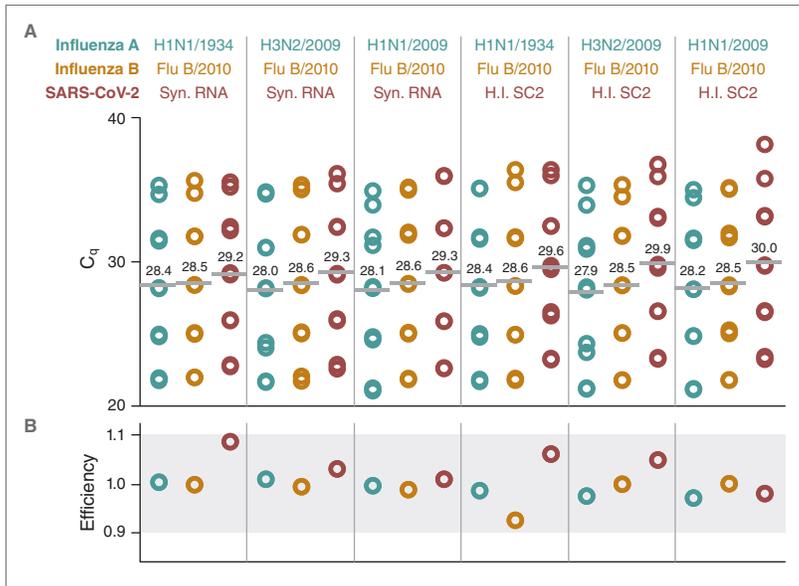


FIGURE 5: Detection of various Influenza and SARS-CoV-2 viral samples

Six different templates were mixed by combining one of three Influenza A viral RNAs, heat-inactivated (H.I.) SARS-CoV-2 virus or Twist synthetic SARS-CoV-2 RNA (Syn. RNA), and Influenza B viral RNAs. Luna Mix (No ROX) detected Flu A, Flu B, SC2 linearly across 5-log inputs on the Bio-Rad Real-Time instrument using cycling conditions of 25°C, 30 sec.; 55°C, 10 min.; 95°C, 1 min.; 45 cycles of 95°C, 10 sec and 60°C, 30 sec.

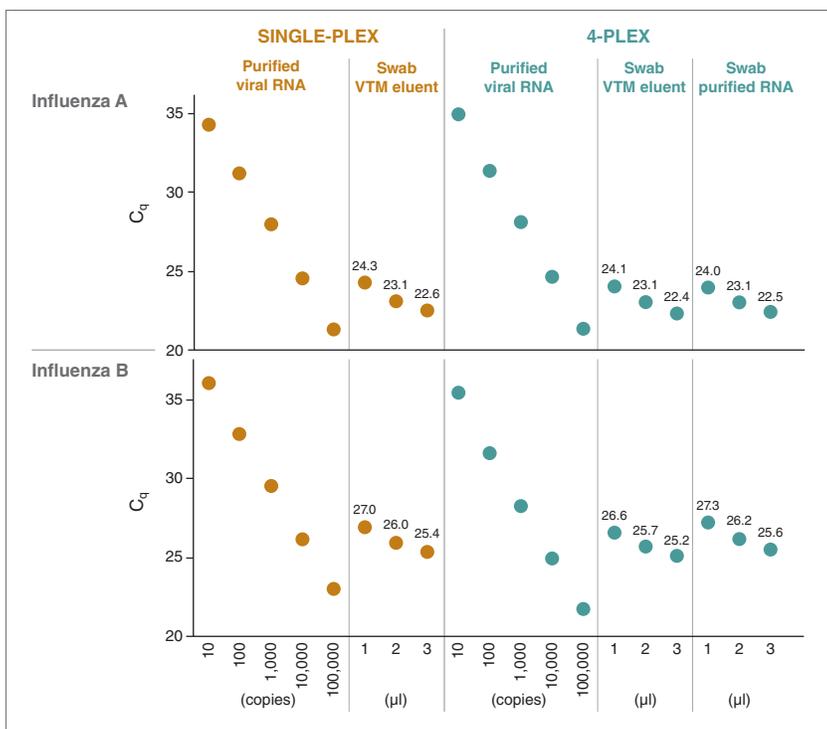


Direct detection of viral RNAs from swabs

Direct detection of viral RNAs from clinical specimens can streamline the diagnostic workflow, shorten the turnaround time, and mitigate reagent shortages (5). Clinical samples involving respiratory infections are often collected on swabs, including nasopharyngeal or anterior nares swabs. We evaluated the Luna mixes in direct viral detection from mock swab samples containing inactivated Influenza A or Influenza B (Microbix®, Toronto). Viral templates were first eluted from swabs in 0.5 ml of viral transport media (VTM) by incubating at room temperature for 30 minutes. The eluent was then used directly in RT-qPCR. One to three µl of VTM elution could be used to detect FluA or FluB with the Luna Mix (No ROX) (Figure 6). Comparable C_t values were obtained for FluA and FluB using either a single-plex or 4-plex reaction. The C_t values correspond proportionately to the amount of sample tested, indicating that up to 15% of VTM is tolerated in a 20 µl Luna reaction. To estimate the effectiveness of direct detection, RNA was purified from 200 µl of the swab eluent using the Monarch® RNA Miniprep kit (NEB #T2010) and eluted into 200 µl water. When one to 3 µl of purified RNAs were analyzed using the 4-plex reaction, the C_t values were comparable to the values of the direct VTM sample, indicating reliable FluA and Flu B detection from VTM eluents.

FIGURE 6: Direct detection from viral swab and liquid samples

Luna Mix (No ROX) was used to detect viral targets from swab VTM eluents or purified RNAs on the Bio-Rad Real-Time instrument using cycling conditions of 25°C, 30 sec.; 55°C, 10 min.; 95°C, 1 min.; 45 cycles of 95°C, 10 sec. and 60°C, 30 sec. In single-plex reactions, the FluA primers and probe were present at 0.2 µM in the detection of the 5-log dilutions of FluA RNA or swab samples. In 4-plex reactions, all ten primers and four probes were present at 0.2 µM in the detection of the 5-log dilutions of FluA RNA or swab samples. In single-plex reactions, the FluB primers and probe were present at 0.2 µM in the detection of the 5-log dilutions of FluB RNA or swab samples. In 4-plex reactions, all ten primers and four probes were present at 0.2 µM in the detection of the 5-log dilutions of FluB RNA or swab samples.



Limit of Detection (LOD) on viral RNAs

To determine the LOD of each target (the lowest input that results in a 95% detection rate), two users evaluated the detection of FluA, FluB, and SARS-CoV-2 targets in the 4-plex reactions containing ~10 copies of all three viral templates. The LODs were established using 40 replicates at 10-copy input and 16 non-template controls. In the 4-plex reaction using Luna Mix (No ROX), each viral RNA had a positive detection rate of ≥ 97% on both the QuantStudio Flex 6 and the ABI 7500 instruments using either standard or fast mode (Table 3, page 6). Similar results were achieved using a lyophilized RT-qPCR reagent based on the Luna Mix, Lyo-Prime Luna Probe One-Step RT-qPCR Mix with UDG (NEB #L4001). These results highlight robust and sensitive detection of all three respiratory viruses using both liquid and lyophilized Luna RT-qPCR reagents.

CONCLUSION

Respiratory viral infections caused by influenza and SARS-CoV-2 share many symptoms, and thus healthcare providers may turn to diagnostic tests to accurately determine the nature of these infections. The CDC's Flu SC2 multiplex assay relies on RT-qPCR to determine if FluA, FluB, or SARS-CoV-2 viral RNA are present in a specimen. Using purified control materials, our evaluation concluded that the Flu SC2 assay conditions specified in the CDC/EUA performed well on the ABI 7500 Fast instrument using the standard ramp rate. However, the sensitivity and linearity decreased under the fast ramp rate or on the Bio-Rad CFX instrument. Thus, optimizing the Flu SC2 multiplex assay reagents and conditions to decrease turnaround time and compatibility could help facilitate timely detection.

Flu SC2 detection using the Luna mixes with optimized assay conditions provides several advantages over the original protocol (Table 4). First, the modified assay uses a simplified reaction setup by keeping all the primers and probes at 0.2 µM while maintaining a robust 5-log detection of viral RNAs from 10⁵ to ~10 copies. Furthermore, the optimized assay performs well not only on the ABI instruments under both standard and fast modes using Luna Mix (No ROX), but also on the Bio-Rad instruments under different ramp rates using either Luna Mix or Luna Mix (No ROX). The Luna conditions also support direct detection from heat-inactivated SARS-CoV-2 virus as well as Influenza A and Influenza B from swab samples.

LOD detection of ~10 copies was observed using both liquid Luna 4X mixes and the lyophilized LyoPrime Luna mix (NEB #L4001) on either the QuantStudio Flex 6 instrument or the ABI 7500 Fast thermal cycler. Taken together, the Luna RT-qPCR reagents offer a fast and robust system for simultaneous detection of FluA, FluB, and SARS-CoV-2.

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TABLE 3: Sensitive Detection of Influenza A, Influenza B, SARS-CoV-2 using Luna Mix (No ROX) or LyoPrime Luna

Forty reaction replicates at 10-copy input of all three viral RNA templates were tested in the 4-plex assay on both the ABI 7500 and QuantStudio Flex by two independent users using standard or fast mode. Each viral RNA had a positive detection rate of ≥ 97%.

	LUNA PROBE ONE-STEP RT-qPCR 4X MIX WITH UDG (NO ROX)						LYOPRIME LUNA PROBE ONE-STEP RT-qPCR MIX WITH UDG					
	FLU A		FLU B		SARS-COV-2		FLU A		FLU B		SARS-COV-2	
Analyst	1	2	1	2	1	2	1	2	1	2	1	2
ABI 7500												
Standard mode	40/40	40/40	40/40	40/40	40/40	39/40	40/40	40/40	40/40	40/40	40/40	40/40
Fast mode	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40
QuantStudio Flex 6												
Standard mode	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40	40/40
Fast mode	40/40	40/40	40/40	40/40	40/40	40/40	40/40	39/40	40/40	40/40	40/40	40/40

TABLE 4: Benefits of the Luna Flu SC2 assay

LUNA REACTION SETUP & CYCLING SPECIFICATION	
Primer/probe concentrations	Single concentration
Run time	Fast run times enabled
Cost per reaction	Reduced cost (due to reagents and decreased primer/probe concentration) and shorter instrument time)
Performance	Linear 5-log detection compatible on Applied Biosystems 7500 Fast Real-Time PCR System, QuantStudio 6 Flex Real-Time PCR System and Bio-Rad CFX Real-Time PCR Detection System

We hope these data and recommendations are helpful to labs that are developing new multiplex assays or making adjustments to existing assays to meet their own needs.

Acknowledgments

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References

- Shu, B., et al. (2021) *Emerging Infectious Diseases*. 27(7), 1821–1830.
- Vogels, C.B.F., et al. (2021) *Med* 2(3), 263-280.e6.
- Stahl, E.C., et al. (2021) *PLoS One* 16(11), e0258263.
- Wong, J.C.C., et al. (2021) *Sci. Total Environ.* 786, 147419.
- Coonrod, J.D., et al. *The Direct Detection of Microorganisms in Clinical Samples*. Academic Press, 2012.