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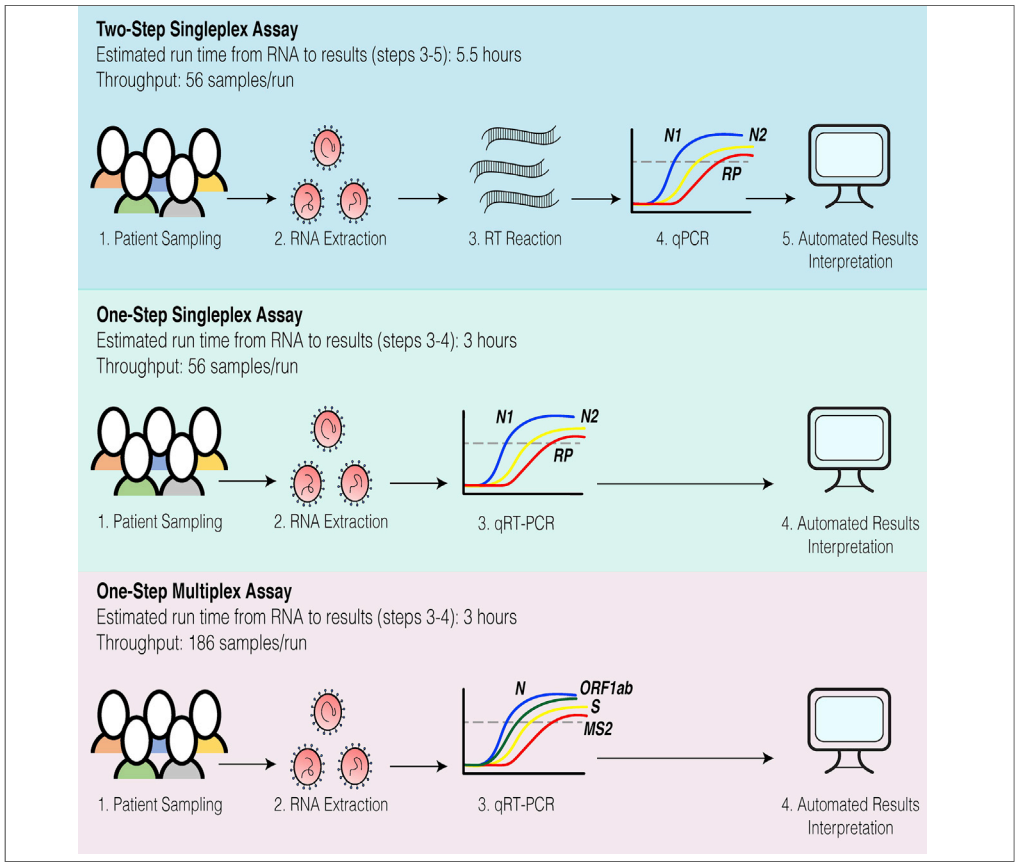
qRT-PCR Platforms for Diagnosing and Reporting SARS-CoV-2 Infection in Human Samples



The protocols herein outline the use of qRT-PCR to detect the presence of SARS-CoV-2 genomic RNA in patient samples. In order to cope with potential fluctuations in supply chain and testing demands, and to enable expedient adaptation of reagents and assays on hand, we include details for three parallel methodologies (one- and two-step singleplex and one-step multiplex assays). The diagnostic platforms described can be easily adapted by basic science research laboratories for SARS-CoV-2 diagnostic testing with relatively short turnaround time.

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HIGHLIGHTS

Single- and multiplex qRT-PCR protocols for detecting SARS-CoV-2 viral RNA

FDA EUA certified ready-to-use protocols for use in CLIA-certified laboratories

Protocols utilize reagents commonplace to basic science research laboratories

Included R script allows for automated interpretations of results

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qRT-PCR Platforms for Diagnosing and Reporting SARS-CoV-2 Infection in Human Samples

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SUMMARY

The protocols herein outline the use of qRT-PCR to detect the presence of SARS-CoV-2 genomic RNA in patient samples. In order to cope with potential fluctuations in supply chain and testing demands and to enable expedient adaptation of reagents and assays on hand, we include details for three parallel methodologies (one- and two-step singleplex and one-step multiplex assays). The diagnostic platforms described can be easily adapted by basic science research laboratories for SARS-CoV-2 diagnostic testing with relatively short turnaround time.
For complete details on the use and execution of this protocol, please refer to Vanuytsel et al. (2020).

BEFORE YOU BEGIN

The assays described herein are commonplace in molecular and cellular biology laboratories across the world. To this end, several academic, basic science research laboratories have already contributed to COVID-19 testing during the height of the pandemic (Vanuytsel et al., 2020, IGI Testing Consortium 2020). As demonstrated, it is conceivable for university laboratories to implement the included protocols in an effort to aid in community surveillance testing to enable deep contact tracing as well as to assist local hospitals in keeping up with testing demands with relatively short turnaround times in the event of a second wave of infections. As stated above, as supply chains and testing requirements fluctuate, utilizing several assays (e.g., single- and multiplex assays) will enable laboratories to remain flexible and simultaneously meet demand.

To ensure best practices in the implementation of these in-house platforms, it is crucial to perform appropriate validation studies as outlined by the United States Centers for Disease Control and Prevention (CDC) and Food and Drug and Administration (FDA). As outlined in Vanuytsel et al., 2020, it is possible for basic science research laboratories to obtain CLIA licensure – necessary for diagnostic reporting of patient samples – as extensions of affiliated, relevant hospital departments.

⚠ CRITICAL: As stated in our laboratory's FDA approved emergency use authorization (EUA) application for utilizing the protocols described below for diagnosing SARS-CoV-

2 infection in patient samples (see File S1), positive results are indicative of the presence of SARS-CoV-2 nucleic acid; however, clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses. Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

⚠ CRITICAL: The single- and multiplex protocols outlined below are based on FDA approved EUA applications filed by Boston Medical Center (BMC) and the Center for Regenerative Medicine (CReM) of BMC and Boston University as well as ThermoFisher for use in diagnosing patient samples with SARS-CoV-2 infection. Significant changes to said assays may require users to file for an additional EUA application requiring independent validation experiments to be performed. With this being said however, depending on the stability of existing supply chains, it may be necessary to utilize slightly different reagents or equipment. We recommend contacting the FDA to determine if proposed deviations from said protocols will be covered under their original EUA, or if they require further bridging and/or validation studies before they can be used for diagnostic purposes. While small changes to the listed protocols are likely covered under their associated EUA, it is best practice to perform optimization and validation experiments to ensure equivalent outcomes when running these protocols via different methodologies/reagents.

Inactivation of Patient Samples

Timing: 15 min per patient sample

Note: Healthcare providers will take respiratory sample and insert swab into viral transport media utilizing BD Universal Transport Media (UTM) 3-mL collection kit with flexible minitip flocked swab (BD, Cat. No. 220531).

1. Transfer 300 µL of patient sample (e.g., UTM or saliva) to an RNase free microcentrifuge tube.

Pause Point: Prior to sample inactivation and RNA extraction, patient samples can be stored at 4°C for short-term storage (e.g., 16–20 h) or −20°C for long-term storage (e.g., 48–72 h).

Note: If testing saliva samples for the presence of SARS-CoV-2, add 20 µL proteinase K (20 mg/mL stock) to each sample and vortex for 30 s prior to buffer RLT addition as described previously (Ott et al., 2020; Wyllie et al., 2020).

2. Add 300 µL of buffer RLT from Qiagen RNeasy Minikit (Qiagen, Cat. No. 74106) and mix by pipetting 5–10 times.
3. Incubate at 20°C for 10 min.
4. Add equal volume (600 µL) 100% molecular biology grade EtOH (Millipore Sigma, Cat. No. E7023) and mix by pipetting 5–10 times or briefly vortexing.

⚠ CRITICAL: If utilizing the one-step multiplex assay, prior to RNA extraction, add 15 µL MS2 bacteriophage RNA provided in the TaqPath COVID-19 Combo Kit (ThermoFisher, Cat. No. A47814) to inactivated patient sample (total volume of 1.2 mL), proceed to RNA isolation. As discussed below, this artificial RNA serves as an internal control for RNA extraction and sample stability.

RNA Extraction

Timing: 3 h for 46 samples assuming one operator

5. Extract RNA via the Qiagen RNeasy Mini Kit (Qiagen, Cat. No. 74106) per manufacturer's protocol. Elute samples in 30 μL nuclease free H_2O (Fisher Scientific, Cat. No. BP2484100).

Note: To control for the extraction process and sample stability, it is best practice to receive previously confirmed positive and negative patient samples and repeat the entire protocol (from sample inactivation to running the qRT-PCR assay of choice).

Note: Total volume of inactivated patient sample (1.2 mL) is larger than the maximum capacity of Qiagen RNeasy Mini Kit spin column (700 μL). Add 600 μL of sample, perform the first spin, discard flowthrough, add the remaining 600 μL to the same spin column, and proceed as written in the manufacturer's protocol.

Note: During extraction of RNA from saliva samples, due to sample viscosity, it may be necessary to perform additional spin steps of the spin column.

Pause Point: Extracted/purified RNA samples can be stored at −80°C for long-term storage (e.g., 2 weeks).

Generation of Viral Standards for Two-Step Singleplex Assay

Timing: 30 min hands-on time, 3 h PCR program

6. To generate synthetic SARS-CoV-2 RNA standards, thaw commercially available synthetically engineered stock RNA (ATCC, Cat No. VR-3276SD) on ice.

Note: Different viral standards are used for each assay described (two- and one-step singleplex assays and one-step multiplex assay).

Note: The dilutions outlined below can be included in the qRT-PCR plate to generate a standard curve (i.e., C_T value per concentration of positive control). If patient results are to be reported, per CDC and FDA, it is recommended that each run of the assays in question includes a positive control at maximum 5× the limit of detection (LoD) for each run of the assay. Please see CDC and FDA guidelines on how to determine the LoD for the given patient sample and/or molecular assay utilized. After identifying the LoD for a given assay, it may be necessary to adjust the concentrations included within the standard curves of each plate.

⚠ CRITICAL: Confirm stock solution concentration with ATCC via catalog and lot numbers prior to cDNA generation.

7. Generate cDNA using 10^6 genome copies of stock RNA via reverse transcription (RT) reaction. See "RT master mix for cDNA generation" and "cDNA generation PCR steps" for RT reaction master mix recipe and PCR program, respectively.
8. After generating cDNA, make serial 1:10 dilutions of the 10^6 genome copies/μL solution in nuclease-free H_2O until you have the following concentrations: 10^4, 10^3, 10^2, 10^1, 10^0 copies/μL.

Pause Point: cDNA standards can be stored at −20°C and used for 1 week.

RT Master Mix for cDNA Generation	
Reagent	Volume per reaction (μl)
10× RT Buffer	2.0
25× dNTP Mix	0.8
10× RT Random Primers	2.0
RT	1.0
Total Volume	5.8

cDNA generation PCR steps			
Step	Cycles	Temp	Time
1	1	25°C	10 min
2	1	37°C	2 h
3	1	85°C	5 min
4		4°C	Infinity

Generation of Viral Standards for One-Step Singleplex Assay

Timing: 30 min

9. To generate synthetic SARS-CoV-2 RNA standards, thaw commercially available synthetically engineered stock RNA (ATCC, Cat No. VR-3276SD) on ice.

⚠ CRITICAL: Confirm stock solution concentration with ATCC via lot number prior to one-step singleplex assay.

10. From the stock solution of RNA, make a solution at 10^6 gc/μL in nuclease-free H_2O.
11. From the 10^6 gc/μL solution, make serial 1:10 dilutions in nuclease-free H_2O until you have the following dilutions: 10^4, 10^3, 10^2, 10^1, 10^0 copies/μL.

Note: As the one-step singleplex assay has an RT reaction built into the qRT-PCR step, RNA is added directly to the qPCR plate and loaded master mix (i.e., no cDNA reaction is needed). Diluted RNA can be kept at −80°C for long-term storage.

Generation of Viral Standards for One-Step Multiplex Assay

Timing: 30 min

12. To generate SARS-CoV-2 RNA standards, thaw TaqPath COVID-19 Combo Kit (ThermoFisher, Cat. No. A47814) positive control on ice.
13. From the stock solution of 10^4 gc/μL RNA, make serial 1:10 dilutions in nuclease-free H_2O until you have the following dilutions: 10^4, 10^3, 10^2, 10^1, 10^0 copies/μL.

Note: Similar to the one-step singleplex assay above, the one-step multiplex assay has an RT reaction included in the qRT-PCR step. As such, RNA is added directly to the qPCR plate and master mix (i.e., no RT reaction is needed). Diluted RNA can be kept at −80°C for long-term storage.

⚠ CRITICAL: The two- and one-step singleplex assays detect the presence of N1 and N2 viral genes. The one-step multiplex assay detects the presence of N, S, and ORF1ab. It is

important to note that the ATCC RNA standard referenced contains regions of the ORF1ab, N, and Env viral genes. The primer/probe sets utilized in the two- and one-step singleplex assays span regions of the N1 and N2 genes present in the ATCC RNA positive control, while the one-step multiplex assay does not. Utilizing this ATCC RNA standard with the one-step multiplex assay will result in insufficient detection of the ORF1ab, N, and S genes. Please note however, that it is possible to obtain full-length SARS-CoV-2 genomic RNA from ATCC (Cat. No. VR-1986D) to use in lieu of or addition to the TaqPath COVID-19 Combo Kit positive control.

Aliquoting Primer/Probe Sets for Two- and One-Step Singleplex Assays

Timing: 15 min

14. Thaw N1, N2, and RP primer/probe sets on ice, protected from light.
15. Label individual nuclease-free microcentrifuge tubes with primer/probe set name.
16. Transfer required volume to appropriate tubes.

Note: The two- and one-step singleplex assays both use the same primer/probe sets (IDT, Cat. No. 110006606). As a result, this step is the same for the two assays. The one-step multiplex assay has primer/probe sets within one reagent. While excessive freeze/thaw cycles (10 at most) should be limited, making individual aliquots is not necessary.

Note: Primer/probe sets can be kept at −20°C for long-term storage. Determine the appropriate volume for each aliquot to minimize freeze/thaw cycles (e.g., one aliquot contains enough primer/probe set for one full qRT-PCR plate).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples	Healthcare providers	N/A
Critical Commercial Assays		
Universal Transport Media (UTM) 3-mL collection kit with flexible minitip flocked swab	BD	220531
MicroAmp™ Optical 384-Well Reaction Plate with Barcode	Fisher Scientific	43-098-49
*High Capacity cDNA Reverse Transcription Kit 1000 rxn	Fisher Scientific	43-688-13
Synthetic SARS-CoV-2 RNA	ATCC	SD-3276
RNeasy Mini Kit (250)	Qiagen	74106
*TaqMan™ Fast Advanced Master Mix	ThermoFisher	4444557
2019-nCoV CDC Emergency Use Authorization Kits	IDT	110006606
MicroAmp Optical Adhesive Film	ThermoFisher	4311971
†Taqpath qPCR Master Mix, CG	ThermoFisher	A47814
^TaqPath 1-Step Multiplex Master Mix (No ROX)	ThermoFisher	A28522
*0.2 mL PCR 8-strip with indiv. attached dome caps	USA Scientific	1402-2900
Proteinase K	Roche	3115836001
	USA Scientific	1615-5510

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Seal-Rite 1.5 mL graduated microcentrifuge tube, (500 tubes)		
RNase away	ThermoFisher	21-402-178
Nuclease-Free H_2O	Fisher Scientific	BP2484100
Molecular Biology Grade Ethanol	Millipore Sigma	E7023
Software and Algorithms		
GraphPad Prism version 7	GraphPad Software, La Jolla, CA	www.graphpad.com
RStudio	RStudio, Inc., Boston, MA	www.rstudio.com
Script for automated analysis, reporting, and uploading of qRT-PCR data	This paper	https://github.com/TaylorMatte/Quant6-Covid_Analysis
Other		
FDA approved BMC-CReM EUA submission	This paper	See File S1
QuantStudio 6 Flex Real-Time PCR System, 384-well, desktop	ThermoFisher	4485701
*Mastercycler Nexus X2 (software version 3.5.1)	Eppendorf	6336000023

Note: Reagents labeled with "*" denote those specific to the two-step singleplex assay, reagents labeled with "†" denote those specific to the one-step singleplex assay, reagents with "^" denote those specific to the one-step multiplex assay. All other listed reagents are common to all protocols.

MATERIALS AND EQUIPMENT

The *BMC-CReM COVID-19* test is to be used with:

Two-step singleplex assay:
QuantStudio 6 Flex Real-Time PCR System, 384-well, desktop (Cat. No. 4485701) (software version 1.3)
Mastercycler Nexus X2 (Cat. No. 6336000023) (software version 3.5.1)
One-step singleplex and one-step multiplex assay:
QuantStudio 6 Flex Real-Time PCR System, 384-well, desktop (4485701) (software version 1.3)

Alternatives: **RNA extraction:** The protocols described throughout this text first rely on the extraction of RNA from patient samples via the Qiagen RNeasy Minikit procedure. As demands for testing are likely to fluctuate in the future, this kit may not be available to researchers. To combat this, an alternative approach would be to utilize a phenol/chloroform-based RNA extraction methodology (e.g., TRIzol, ThermoFisher, Cat. No. 15596026). Although cheap and potentially yielding larger quantities of RNA, it is important to note that this technique generates hazardous chemical waste requiring appropriate storage – a potentially limiting factor if processing large sample numbers.

Alternatives: **Thermocycler and qPCR machines:** Here, we utilized the QuantStudio 6 Flex Real-Time PCR System, 384-well, desktop (Cat. No. 4485701) (software version 1.3) and Mastercycler Nexus X2 (Cat. No. 6336000023) (software version 3.5.1) for RT reaction and qPCR, respectively. Other thermocyclers and/or qRT-PCR platforms can be utilized for these steps as long as the appropriate cycling parameters are set into the machine prior to running. We recommend validating new thermocycler and/or qPCR machines by running either an appropriate standard curve and/or previously confirmed positive and negative patient samples through the entire assay protocol.

STEP-BY-STEP METHOD DETAILS

Two-Step Singleplex Assay

Timing: 5.5 h post RNA extraction to results

The two-step singleplex assay first generates cDNA from RNA standards or RNA extracted from patient samples via an independent RT reaction. RT-generated cDNA is subsequently loaded onto a qRT-PCR machine and interrogated for the presence of SARS-CoV-2 viral genes (here, nucleocapsid N1 and N2) and human RNase P (RP) as an internal control for extraction and sample stability. C_T values for N1, N2, and RP are in turn used to make diagnostic decisions regarding SARS-CoV-2 infection.

1. Generation of cDNA from RNA (standards or patient samples)
   a. Prepare master mix for reverse transcriptase reaction in a microcentrifuge tube.

RT Master Mix for cDNA Generation	
Reagent	Volume per Reaction (µl)
10× RT Buffer	2.0
25× dNTP Mix	0.8
10× RT Random Primers	2.0
RT	1.0
Total Volume	5.8

   b. Mix by briefly pipetting or gently vortexing.
   c. Pipette 5.8 µL of master mix into PCR strip tubes.
   d. Add 14.2 µL RNA to respective PCR strip tube and gently mix by pipetting 5–10 times.

Note: In order to control for potential contamination occurring during cDNA generation and eventual qPCR plating, a no template control (NTC) should be used. Here, the NTC is defined as nuclease-free H_2O at 14.2 µL in place of RNA in the RT reaction.

   e. Spin PCR strip tubes briefly in a mini-centrifuge (e.g., Spectrafuge™ mini-centrifuge, Millipore Sigma, Cat. No. S7816) at 2,000 × *g* for approximately 10 s.
   f. Insert tubes into Mastercycler Nexus GX2 thermocycler and run the following program:

cDNA generation PCR steps			
Step	Cycles	Temp	Time
1	1	25°C	10 min
2	1	37°C	2 h
3	1	85°C	5 min
4		4°C	Infinity

Pause Point: RT (reaction can be left at 4°C step in PCR machine 16–20 h). Moreover, cDNA can be kept at −20°C for long-term storage (e.g., months) or 4°C for short-term storage (e.g., 1 week).

2. Newly generated cDNA is subsequently diluted by adding 40 µL nuclease-free H_2O for a final total volume of 60 µL.

   Note: This volume allows for a patient sample's cDNA to be re-run in the event of an inconclusive result.

3. Preparation of master mix for qPCR and respective plate setup
   a. Thaw 2× TaqMan Fast Advanced Master Mix on ice.

   Note: Once thawed, master mix can be stored at 4°C.

   b. Thaw primer/probe set on ice, protected from light.
   c. Mix Master Mix and primer/probes by inversion 5 times, then centrifuge for 5–10 s.
   d. Label one microcentrifuge tube for each primer/probe set (i.e., three tubes total: N1, N2, and RP).
   e. Determine the number of reactions (N) to set up per assay. For a standard curve of 5 concentrations with two replicates each, you will need 48 wells. Account for margin of error.

Two-Step Singleplex qPCR Master Mix Recipe	
Reagent	**Volume per Reaction (µl)**
Nuclease-free H_2O	2.33
Combined primer/probe mix	1
TaqMan Fast Advanced Master Mix 2×	6.66
Final total RXN volume	10

   Note: Technical duplicates are used for each sample and primer/probe set. For research purposes, as N1 and N2 transcripts are being probed for in separate wells, it may not be necessary to utilize technical duplicates – saving time and reagents while at the same time increasing sample throughput.

   f. Prepare the above mixture and mix by pipetting slowly up and down.
   g. If mixed via gentle vortexing, centrifuge each reaction mix for 5–10 s.
   h. Dispense 10 µL each master mix reaction into a 384-well plate.
   i. Gently vortex and centrifuge all standard and any samples.
   j. Add 5 µL each standard or sample to the 384-well plate containing the reaction mixture. In the two-step singleplex assay, 56 patient samples can be run per plate. Figure 1 depicts a sample plate layout for a full 384-well plate including standard curve, NTC, and positive and negative controls with technical duplicates.

   Note: Use of an automated liquid handling robot (e.g., Biomek platforms, Beckman Coulter, Cat. No. A31841) or multichannel pipette (e.g., ThermoFisher, Cat. No. 4672100BT) can dramatically improve the efficiency and speed of this step.

   k. Cover the loaded 384-well plate with adhesive cover and centrifuge the 384-well plate in a mini plate spinner (e.g., Labnet MPS 1000 Mini plate spinner, Millipore Sigma, Cat. No. Z723533) at 500 × *g* for approximately 20 s.

4. qPCR
   a. Turn on the machine and allow the block to equilibrate.
   b. Set up the following program:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	10^4	10^4	10^4	10^4	10^4	10^4	pt09	pt09	pt09	pt09	pt09	pt09	pt25	pt25	pt25	pt25	pt25	pt25	pt41	pt41	pt41	pt41	pt41	pt41
B	10^3	10^3	10^3	10^3	10^3	10^3	pt10	pt10	pt10	pt10	pt10	pt10	pt26	pt26	pt26	pt26	pt26	pt26	pt42	pt42	pt42	pt42	pt42	pt42
C	10^2	10^2	10^2	10^2	10^2	10^2	pt11	pt11	pt11	pt11	pt11	pt11	pt27	pt27	pt27	pt27	pt27	pt27	pt43	pt43	pt43	pt43	pt43	pt43
D	10^1	10^1	10^1	10^1	10^1	10^1	pt12	pt12	pt12	pt12	pt12	pt12	pt28	pt28	pt28	pt28	pt28	pt28	pt44	pt44	pt44	pt44	pt44	pt44
E	10^0	10^0	10^0	10^0	10^0	10^0	pt13	pt13	pt13	pt13	pt13	pt13	pt29	pt29	pt29	pt29	pt29	pt29	pt45	pt45	pt45	pt45	pt45	pt45
F	NTC	NTC	NTC	NTC	NTC	NTC	pt14	pt14	pt14	pt14	pt14	pt14	pt30	pt30	pt30	pt30	pt30	pt30	pt46	pt46	pt46	pt46	pt46	pt46
G	POS	POS	POS	POS	POS	POS	pt15	pt15	pt15	pt15	pt15	pt15	pt31	pt31	pt31	pt31	pt31	pt31	pt47	pt47	pt47	pt47	pt47	pt47
H	NEG	NEG	NEG	NEG	NEG	NEG	pt16	pt16	pt16	pt16	pt16	pt16	pt32	pt32	pt32	pt32	pt32	pt32	pt48	pt48	pt48	pt48	pt48	pt48
I	pt01	pt01	pt01	pt01	pt01	pt01	pt17	pt17	pt17	pt17	pt17	pt17	pt33	pt33	pt33	pt33	pt33	pt33	pt49	pt49	pt49	pt49	pt49	pt49
J	pt02	pt02	pt02	pt02	pt02	pt02	pt18	pt18	pt18	pt18	pt18	pt18	pt34	pt34	pt34	pt34	pt34	pt34	pt50	pt50	pt50	pt50	pt50	pt50
K	pt03	pt03	pt03	pt03	pt03	pt03	pt19	pt19	pt19	pt19	pt19	pt19	pt35	pt35	pt35	pt35	pt35	pt35	pt51	pt51	pt51	pt51	pt51	pt51
L	pt04	pt04	pt04	pt04	pt04	pt04	pt20	pt20	pt20	pt20	pt20	pt20	pt36	pt36	pt36	pt36	pt36	pt36	pt52	pt52	pt52	pt52	pt52	pt52
M	pt05	pt05	pt05	pt05	pt05	pt05	pt21	pt21	pt21	pt21	pt21	pt21	pt37	pt37	pt37	pt37	pt37	pt37	pt53	pt53	pt53	pt53	pt53	pt53
N	pt06	pt06	pt06	pt06	pt06	pt06	pt22	pt22	pt22	pt22	pt22	pt22	pt38	pt38	pt38	pt38	pt38	pt38	pt54	pt54	pt54	pt54	pt54	pt54
O	pt07	pt07	pt07	pt07	pt07	pt07	pt23	pt23	pt23	pt23	pt23	pt23	pt39	pt39	pt39	pt39	pt39	pt39	pt55	pt55	pt55	pt55	pt55	pt55
P	pt08	pt08	pt08	pt08	pt08	pt08	pt24	pt24	pt24	pt24	pt24	pt24	pt40	pt40	pt40	pt40	pt40	pt40	pt56	pt56	pt56	pt56	pt56	pt56

N1 probe N2 probe RP

Figure 1. Sample Plate Layout for a Fully Loaded 384-Well qRT-PCR Plate Using Two- and One-Step Singleplex Assays

Sample plate layout for a full 384-well qRT-PCR plate. Green shading denotes wells containing N1 primer/probe sets, orange shading denotes wells containing N2 primer/probe sets, and blue shading denotes wells containing RP primer/probe sets. The two- and one-step singleplex assays are able to run 56 patients samples on each 384-well qRT-PCR plate. Each sample and primer/probe set is ran in technical duplicate. Wells labeled 10^4–10^0 represent the standard curve samples, ptXX denotes individual patient samples (here, 01–56), NTC refers to no template control (H_2O in the place of RNA in the RT reaction), POS and NEG represent previously identified positive and negative patient samples, respectively.

Total run time: ~ 1 h 15 min

Two-Step Singleplex qPCR Program			
Step	**Cycles**	**Temp**	**Time**
Preamplification (UNG inactivation)	1	25°C	120 s
Preamplification (polymerase activation)	1	95°C	120 s
Amplification	45	95°C	3 s
		55°C	30 s

c. Place the 384-well plate in the machine and start the run

5. Results interpretation

a. Per the US CDC panel, recommended interpretations are:

Note: Reactions reading a C_T value of ≥ 40 are taken as negative. C_T values below 40 are classified as positive. Be advised that in the assays described herein, a C_T value <40 for RP is considered positive. Other FDA approved EUAs, utilizing similar qRT-PCR-based methodologies for diagnosing SARS-CoV-2 infection, utilize a more stringent cutoff for this internal housekeeping gene (e.g., positive values are only considered if C_T values are <35). If employing this cutoff, it is important to adjust the respective results interpretation table.

Note: In an effort to limit the potential for errors in interpreting qRT-PCR results, the use of an automated results reporting script is highly recommended. To this end, please see our recently developed R script capable of importing and interpreting C_T values from qRT-PCR of patient samples in an unbiased manner (available at https://github.com/TaylorMatte/Quant6-Covid_Analysis). The R scripts included within this link contain comments which provide step-by-step instructions. Video S1 describes how to use this script to interpret singleplex assay results based on the diagnostic matrix outlined above in Table 1. Sample input and output files can be seen in Figure 2.

Table 1. Interpretation of Results for Patient Samples Based on Data from Either Singleplex Assay

N1	N2	RP	Results Interpretation	Report	Follow-Up Action
+	+	–/+	SARS-CoV-2 detected	POSITIVE	report into electronic medical record (EMR)
If only one of these targets is detected		–/+	SARS-CoV-2 detected	POSITIVE	report into EMR
If signal is detected, but no target reaches 2/2 detected technical repeats		+	inconclusive	INCONCLUSIVE	sample is repeated at qRT-PCR step once more. If sample is still inconclusive, the result is reported into the EMR as such and it is recommended that a new sample is obtained from the patient
–	–	+	SARS-CoV-2 not detected	NEGATIVE	report into EMR
–	–	–	Invalid	INVALID	the result is reported into the EMR as such and it is recommended that a new sample is obtained from the patient

One-Step Singleplex Assay

Timing: 3 h post RNA extraction to results

The one-step singleplex assay relies on a RT reaction programmed within the qPCR step. As with the two-step singleplex assay, the one-step platform assesses for the presence of SARS-CoV-2 viral genes N1 and N2, with RP as an internal control for extraction and sample stability. C_T values for N1, N2, and RP are subsequently interpreted using the identical matrix utilized for the two-step singleplex assay (Table 1).

6. Reaction Master Mix and Plate Setup for qRT-PCR
   a. Thaw 4× TaqPath 1-Step RT-qPCR Master Mix and primer/probe sets on ice, protected from light.
   b. Mix master mix reagent and primer/probes by inversion 5 times, then centrifuge for 5–10 s.
   c. Label one microcentrifuge tube for each primer/probe set (i.e., three tubes in total, for N1, N2, and RP).
   d. Determine the number of reactions (N) to set up per assay. For a standard curve with two replicates, you will need 48 wells. Account for margin of error.

One-Step Singleplex qPCR Master Mix Recipe	
Reagent	Volume per reaction (µl)
4× TaqPath 1-Step RT-qPCR Master Mix	5
Combined primer/probe mix	1
Nuclease-free H_2O	11.50
Final total RXN volume	20

Note: Technical duplicates are used for each sample and primer/probe set. As stated previously, for research purposes, as N1 and N2 transcripts are being probed for in separate wells, it may not be necessary to utilize technical duplicates – saving on time and reagents while at the same time increasing sample throughput.

   e. Prepare the above mixture and mix by pipetting slowly up and down.
   f. If mixed via gentle vortexing, centrifuge each reaction mix for 5–10 s.

A

	Well	Well Positic	Omit	Sample Na	Target Nan	Task	Reporter	Quencher	CT
1	A1		FALSE	10^6	N1	UNKNOWN	FAM	NFQ-MGB	18.516
2	A2		FALSE	10^6	N1	UNKNOWN	FAM	NFQ-MGB	18.818
3	A3		FALSE	10^6	N2	UNKNOWN	FAM	NFQ-MGB	18.138
4	A4		FALSE	10^6	N2	UNKNOWN	FAM	NFQ-MGB	18.203
5	A5		FALSE	10^6	RP	UNKNOWN	FAM	NFQ-MGB	Undetermir
6	A6		FALSE	10^6	RP	UNKNOWN	FAM	NFQ-MGB	Undetermir
7	A7		FALSE	pt_00040	N1	UNKNOWN	FAM	NFQ-MGB	Undetermir
8	A8		FALSE	pt_00040	N1	UNKNOWN	FAM	NFQ-MGB	Undetermir
9	A9		FALSE	pt_00040	N2	UNKNOWN	FAM	NFQ-MGB	Undetermir
10	A10		FALSE	pt_00040	N2	UNKNOWN	FAM	NFQ-MGB	Undetermir
11	A11		FALSE	pt_00040	RP	UNKNOWN	FAM	NFQ-MGB	30.395
12	A12		FALSE	pt_00040	RP	UNKNOWN	FAM	NFQ-MGB	30.817
13	A13		FALSE	7521 NEG	N1	UNKNOWN	FAM	NFQ-MGB	Undetermir
14	A14		FALSE	7521 NEG	N1	UNKNOWN	FAM	NFQ-MGB	Undetermir
15	A15		FALSE	7521 NEG	N2	UNKNOWN	FAM	NFQ-MGB	Undetermir
16	A16		FALSE	7521 NEG	N2	UNKNOWN	FAM	NFQ-MGB	Undetermir
17	A17		FALSE	7521 NEG	RP	UNKNOWN	FAM	NFQ-MGB	31.563
18	A18		FALSE	7521 NEG	RP	UNKNOWN	FAM	NFQ-MGB	31.910
31	B7		FALSE	pt_00048	N1	UNKNOWN	FAM	NFQ-MGB	30.349
32	B8		FALSE	pt_00048	N1	UNKNOWN	FAM	NFQ-MGB	30.565
33	B9		FALSE	pt_00048	N2	UNKNOWN	FAM	NFQ-MGB	31.638
34	B10		FALSE	pt_00048	N2	UNKNOWN	FAM	NFQ-MGB	31.495
35	B11		FALSE	pt_00048	RP	UNKNOWN	FAM	NFQ-MGB	29.655
36	B12		FALSE	pt_00048	RP	UNKNOWN	FAM	NFQ-MGB	30.123
49	C1		FALSE	10^5	N1	UNKNOWN	FAM	NFQ-MGB	21.054
50	C2		FALSE	10^5	N1	UNKNOWN	FAM	NFQ-MGB	21.239
51	C3		FALSE	10^5	N2	UNKNOWN	FAM	NFQ-MGB	20.853
52	C4		FALSE	10^5	N2	UNKNOWN	FAM	NFQ-MGB	20.920
53	C5		FALSE	10^5	RP	UNKNOWN	FAM	NFQ-MGB	Undetermir
54	C6		FALSE	10^5	RP	UNKNOWN	FAM	NFQ-MGB	Undetermir
55	C7		FALSE	pt_00041	N1	UNKNOWN	FAM	NFQ-MGB	Undetermir
56	C8		FALSE	pt_00041	N1	UNKNOWN	FAM	NFQ-MGB	Undetermir
57	C9		FALSE	pt_00041	N2	UNKNOWN	FAM	NFQ-MGB	Undetermir
58	C10		FALSE	pt_00041	N2	UNKNOWN	FAM	NFQ-MGB	Undetermir

B

EXAMPLE_EXTERNAL

Sample	Well	TestID	Result	Instrument	Date
NEGATIVE_CONTROL1	13	CReM_qPCR	NEGATIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE1	7	CReM_qPCR	NEGATIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE2	55	CReM_qPCR	NEGATIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE3	103	CReM_qPCR	POSITIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE4	151	CReM_qPCR	NEGATIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE6	247	CReM_qPCR	POSITIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE7	295	CReM_qPCR	POSITIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE9	31	CReM_qPCR	POSITIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE10	79	CReM_qPCR	POSITIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE11	127	CReM_qPCR	POSITIVE	278861101	2020-03-23 16:44:54 PM EDT
SAMPLE12	175	CReM_qPCR	POSITIVE	278861101	2020-03-23 16:44:54 PM EDT

C

EXAMPLE_SUMMARY

Total	Positive	Negative	Inconclusive	Invalid
13	7	4	2	0

Figure 2. Example Input and Output Files for Automated Results Interpretation and Reporting R Script

(A) Raw data (i.e., C_T values) exported from QuantStudio platform in.xls format. File contains identifiers, gene target assayed for, and corresponding C_T values for all patient samples, controls, and standards. This file is directly imported into R script.

(B) Output file generated from R script interpretation of raw data. Data table consists of sample identifier, instrument/test identifiers (applicable if using multiple platforms), date and time result was called, and interpretation of result for sample based on C_T values imported from QuantStudio device.

(C) Summary of interpretations for all results obtained from a particular run.

g. Dispense 17.5 μL each master mix reaction into a 384-well plate.

h. Gently vortex and centrifuge each standard and any samples.

i. Add 2.5 μL each standard or samples to the 384-well plate containing the reaction mixture. In the one-step singleplex assay, 56 patient samples can be run per plate. Figure 1 depicts a sample plate layout for a full 384-well plate including standard curve, NTC, and positive and negative controls with technical duplicates – this is the same layout as the two-step singleplex assay, described above.

Note: Use of an automated liquid handling robot (e.g., Biomek platforms, Beckman Coulter, Cat. No. A31841) or multichannel pipette (e.g., ThermoFisher, Cat. No. 4672100BT) can dramatically improve the efficiency and speed of this step.

j. Cover the loaded 384-well plate with adhesive cover and centrifuge the 384-well plate in a mini plate spinner (e.g., Labnet MPS 1000 Mini plate spinner, Millipore Sigma, Cat. No. Z723533) at 500 × *g* for approximately 20 s.

7. Running qPCR

a. Turn on the machine and allow the block to equilibrate

b. Set up the following program:

Total run time: ~1 h 15 min

One-step singleplex qPCR program		
Cycles	Temp	Time
1	25°C	2 min
1	50°C	15 min
1	95°C	2 min
40 (including)	95°C	3 s
	60°C	30 s

Table 2. Interpretation of Results for Patient Samples Based on Data from the One-Step Multiplex Assay

N	S	ORF1ab	MS2	Results Interpretation	Report	Follow-Up Action
–	–	–	–	Invalid	INVALID	the result is reported into the EMR as such and it is recommended that a new sample is obtained from the patient
If two or more targets are detected (2/2 technical replicates detected for each)			+/–	SARS-CoV-2 detected	POSITIVE	report into EMR
–	–	–	+	SARS-CoV-2 not detected	NEGATIVE	report into EMR
If signal is detected but only one target reaches 2/2 detected technical replicates			+/–	inconclusive	INCONCLUSIVE	sample is repeated at qRT-PCR step once more. If sample is still inconclusive, the result is reported into the EMR as such and it is recommended that a new sample is obtained from the patient
If signal is detected in >1 disparate targets, but none reaches 2/2 detected technical replicates			+/–	SARS-CoV-2 detected	POSITIVE	report into EMR

c. Load the 384-well plate and start the run.
d. Generate a standard curve for N1, N2. In the standard curve, RP assays should not show significant amplification.
e. Please see Table 1 for US CDC panel recommended interpretations for diagnostic reporting for the one-step singleplex assay.

Note: As with the two-step singleplex assay, in this platform, reactions reading a C_T value of ≥ 40 are taken as negative. C_T values below 40 are classified as positive. As stated above, here we classify a C_T value <40 for RP as positive. Other FDA approved EUAs utilizing similar qRT-PCR-based methodologies for diagnosing SARS-CoV-2 infection utilize a more stringent cut-off for this internal housekeeping gene (e.g., positive values are only considered for C_T values <35). If employing this cutoff, it is important to adjust the respective results interpretation table.

One-Step Multiplex Assay

Timing: 3 h post RNA extraction to results

The one-step multiplex assay relies on a RT reaction programmed within the qPCR step. Unlike the previous assays, the one-step multiplex assay tests patient samples for the presence of SARS-CoV-2 S, N, and ORF1ab genes. In place of RP as a control for RNA extraction and sample stability, this platform assesses the presence of MS2 transcript – a bacteriophage transcript not present in human or SARS-CoV-2 genomes. This depends on spiking in MS2 RNA into patient sample prior to RNA extraction (described in Before You Begin section above). C_T values for S, N, ORF1ab, and MS2 are subsequently interpreted using the diagnostic decision matrix in Table 2.

8. Reaction Master Mix and Plate Setup for qRT-PCR
   a. Thaw TaqPath 1-Step RT-qPCR Master Mix (no ROX) (4×) and COVID-19 Real-Time PCR Assay Multiplex on ice, protected from light.
   b. Mix master mix and COVID-19 Real-Time PCR Assay Multiplex reagents by inversion 5 times, then centrifuge for 5–10 s.
   c. Determine the number of reactions (N) to set up per assay. For a standard curve with two replicates, you will need 14 wells. Account for margin of error.
   d. See "One-step multiplex qPCR master mix recipe" below.

One-Step Multiplex qPCR Master Mix Recipe	
Reagent	Volume per Reaction (µl)
TaqPath 1-Step Multiplex Master Mix (no ROX) (4×)	6.25
COVID-19 Real-Time PCR Assay Multiplex	1.25
Nuclease-free H_2O	12.50
Final total RXN volume	20

Note: Technical duplicates are used for each sample.

⚠ CRITICAL: For the one-step multiplex assay, utilize the RNA standards provided within the TaqPath COVID-19 Combo Kit. Different synthetic RNAs may contain partial regions of the SARS-CoV-2 genome and therefore might not include the N, S, and ORF1ab sequences assayed for in the COVID-19 Real-Time PCR Assay Multiplex system.

e. Prepare the above mixture and mix by pipetting slowly up and down.
f. If mixed via gentle vortexing, centrifuge each reaction mix for 5–10 s.
g. Dispense 20 µL master mix reaction into a 384-well plate.
h. Gently vortex and centrifuge each standard and any sample.
i. Add 5 µL each standard or sample to the 384-well plate containing the reaction mixture. In the one-step multiplex assay, 186 patient samples can be ran per plate. Figure 3 depicts a sample plate layout for a full 384-well plate including standard curve and negative control, with technical duplicates.

j. Centrifuge the 384-well plate for 30 s using plate spinner.

9. qPCR
a. Turn on the machine and allow the block to equilibrate.
b. Set up the following program:
Total run time: ~1 h 15 min

One-Step Multiplex qPCR Program		
Cycles	Temp	Time
1	25°C	2 min
1	53°C	10 min
1	95°C	2 min
40 (including)	95°C	3 s
	60°C	30 s

⚠ CRITICAL: Prior to initializing the run, designate each gene target with the appropriate fluorophore (N=VIC, S=ABY, ORF1ab=FAM, MS2=JUN).

c. Load the 384-well plate and start the run
d. Generate a standard curve for N, ORF1ab, and S. MS2 should be present at roughly equivalent amounts throughout all samples (not including standard curve) ran.
e. Please see Table 2 for US CDC panel recommended interpretations for diagnostic reporting for the one-step multiplex assay adapted from the TaqPath COVID-19 Combo Kit manual.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	10^4	10^4	pt11	pt11	pt27	pt27	pt43	pt43	pt59	pt59	pt75	pt75	pt91	pt91	pt107	pt107	pt123	pt123	pt139	pt139	pt155	pt155	pt171	pt171
B	10^3	10^3	pt12	pt12	pt28	pt28	pt44	pt44	pt60	pt60	pt76	pt76	pt92	pt92	pt108	pt108	pt124	pt124	pt140	pt140	pt156	pt156	pt172	pt172
C	10^2	10^2	pt13	pt13	pt29	pt29	pt45	pt45	pt61	pt61	pt77	pt77	pt93	pt93	pt109	pt109	pt125	pt125	pt141	pt141	pt157	pt157	pt173	pt173
D	10^1	10^1	pt14	pt14	pt30	pt30	pt46	pt46	pt62	pt62	pt78	pt78	pt94	pt94	pt110	pt110	pt126	pt126	pt142	pt142	pt158	pt158	pt174	pt174
E	10^0	10^0	pt15	pt15	pt31	pt31	pt47	pt47	pt63	pt63	pt79	pt79	pt95	pt95	pt111	pt111	pt127	pt127	pt143	pt143	pt159	pt159	pt175	pt175
F	NEG	NEG	pt16	pt16	pt32	pt32	pt48	pt48	pt64	pt64	pt80	pt80	pt96	pt96	pt112	pt112	pt128	pt128	pt144	pt144	pt160	pt160	pt176	pt176
G	pt01	pt01	pt17	pt17	pt33	pt33	pt49	pt49	pt65	pt65	pt81	pt81	pt97	pt97	pt113	pt113	pt129	pt129	pt145	pt145	pt161	pt161	pt177	pt177
H	pt02	pt02	pt18	pt18	pt34	pt34	pt50	pt50	pt66	pt66	pt82	pt82	pt98	pt98	pt114	pt114	pt130	pt130	pt146	pt146	pt162	pt162	pt178	pt178
I	pt03	pt03	pt19	pt19	pt35	pt35	pt51	pt51	pt67	pt67	pt83	pt83	pt99	pt99	pt115	pt115	pt131	pt131	pt147	pt147	pt163	pt163	pt179	pt179
J	pt04	pt04	pt20	pt20	pt36	pt36	pt52	pt52	pt68	pt68	pt84	pt84	pt100	pt100	pt116	pt116	pt132	pt132	pt148	pt148	pt164	pt164	pt180	pt180
K	pt05	pt05	pt21	pt21	pt37	pt37	pt53	pt53	pt69	pt69	pt85	pt85	pt101	pt101	pt117	pt117	pt133	pt133	pt149	pt149	pt165	pt165	pt181	pt181
L	pt06	pt06	pt22	pt22	pt38	pt38	pt54	pt54	pt70	pt70	pt86	pt86	pt102	pt102	pt118	pt118	pt134	pt134	pt150	pt150	pt166	pt166	pt182	pt182
M	pt07	pt07	pt23	pt23	pt39	pt39	pt55	pt55	pt71	pt71	pt87	pt87	pt103	pt103	pt119	pt119	pt135	pt135	pt151	pt151	pt167	pt167	pt183	pt183
N	pt08	pt08	pt24	pt24	pt40	pt40	pt56	pt56	pt72	pt72	pt88	pt88	pt104	pt104	pt120	pt120	pt136	pt136	pt152	pt152	pt168	pt168	pt184	pt184
O	pt09	pt09	pt25	pt25	pt41	pt41	pt57	pt57	pt73	pt73	pt89	pt89	pt105	pt105	pt121	pt121	pt137	pt137	pt153	pt153	pt169	pt169	pt185	pt185
P	pt10	pt10	pt26	pt26	pt42	pt42	pt58	pt58	pt74	pt74	pt90	pt90	pt106	pt106	pt122	pt122	pt138	pt138	pt154	pt154	pt170	pt170	pt186	pt186

Figure 3. Sample Plate Layout for a Fully Loaded 384-Well qRT-PCR Using One-Step Multiplex Assay

Sample plate layout for a full 384-well qRT-PCR plate utilizing the one-step multiplex assay. Yellow shading denotes wells containing standard curve samples, non-shaded boxes label patient samples. The one-step multiplex assay is able to run 186 patients samples on each 384-well qRT-PCR plate. All samples and standards are run in technical duplicate. Wells labeled 10^4–10^0 represent the standard curve samples, ptXX denotes individual patient samples (here, pt01–pt186), NEG refers to no template control (H_2O in the place of RNA in the RT reaction).

Note: Reactions reading a C_T value of ≥ 40 are taken as negative. C_T values below 40 are classified as positive.

EXPECTED OUTCOMES

Two- and One-Step Singleplex Assay

No Template Control (NTC)

There should be minimal fluorescence emitted from NTC wells via qRT-PCR. If NTC wells have a C_T value <40, this implies contamination of the qRT-PCR reaction and therefore the plate/run is not used for diagnostic decisions. In this case, the assay will be repeated from the RT-PCR step using residual extraction material. If the repeat test result is positive for these targets, all samples will need to be re-extracted and re-tested.

SARS-CoV-2 Positive Control cDNA

The positive control cDNA must have detectible C_T values (<40 C_T) for N1 and N2 (not RP) for concentrations 5× (and above) the determined LoD of the assay for the plate to be valid. If the positive control cDNA fails to yield N1 and N2 C_T values (i.e., undetected) for concentrations a minimum of 5× the LoD of the assay, the plate/run is not used for diagnostic decisions. In this case the qRT-PCR reaction needs to be repeated for all samples using residual extraction material. If the repeat test result for N1 and N2 is negative again, all samples will need to be re-extracted and re-tested. Figure 4 depicts expected results for a sample standard curve composed of positive control cDNA of various concentrations.

Human RP Gene Internal Control

If a sample shows no amplification for N1 and N2 and also fails to show detectable levels for RP, the sample is deemed invalid and needs to be re-extracted and re-tested.

Positive and Negative Extraction Controls (POS, NEG)

If N1 or N2 are not detected in the positive extraction control sample, this suggests sample degradation and/or faulty RNA extraction – the plate/run should not be used for diagnosis. If N1 or N2 are detected in the negative extraction control, this suggests contamination – the plate/run should not be used for diagnosis. In this case, all samples included on the plate need to be re-extracted and re-tested. Figure 4 depicts sample C_T values for N1, N2, and RP probes in positive patient samples.

⚠ CRITICAL: If results from control samples deviate from what is expected, do not generate any diagnostic reports from the respective qRT-PCR run. Discard the plate and re-run

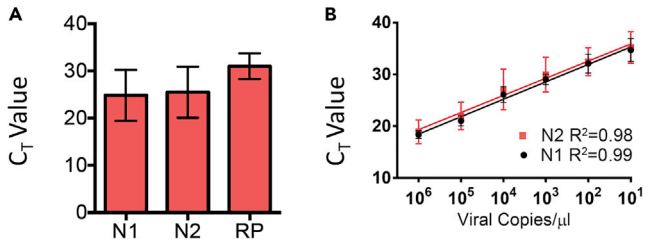

Figure 4. Expected C_T Values for the Two-Step Singleplex Assay Target Genes

(A) Representation of SARS-CoV-2 target genes detected in positive patient samples using the two-step singleplex assay (N = 96). Data are represented as mean ± standard deviation.

(B) Average C_T values for N1 and N2 targets for standard curves run on two-step singleplex assay (N = 6). Data are represented as mean ± standard deviation. Graphs modified from Vanuytsel et al. (2020) (https://doi.org/10.1016/j.medj.2020.05.001).

samples depending on which control failed. See "Troubleshooting" section below for interpretation of controls.

One-Step Multiplex Assay

No Template Control (NTC)

There should be minimal fluorescence emitted from NTC wells via qRT-PCR. If NTC wells have a C_T value <40, this implies contamination of the qRT-PCR reaction and therefore the plate/run is not used for diagnostic decisions. In this case, the assay will be repeated from the RT-PCR step using residual extraction material. If the repeat test result is positive for these targets, all samples will need to be re-extracted and re-tested.

TaqPath Combo Kit Positive Control

The positive control cDNA must have detectible C_T values (<40 C_T) for N, S, and ORF1ab for concentrations 5× and above the determined LoD of the assay for the plate to be valid. If the positive control cDNA fails to yield N, S, or ORF1ab C_T values (i.e., undetected) for concentrations at a minimum of 5× the LoD, the plate/run is not used for diagnostic decisions. In this case, the qRT-PCR reaction needs to be repeated for all samples using residual extraction material. If the repeat test result for N, S, and ORF1ab is negative again for all concentrations of this control, all samples will need to be re-extracted and re-tested.

Bacteriophage MS2 Gene Internal Control

If a sample shows no amplification for any target tested while also failing to show detectable levels for MS2, the sample is deemed invalid and needs to be re-processed and re-tested.

Positive and Negative Extraction Controls (POS, NEG)

If N, S, or ORF1ab are not detected in the positive extraction control sample, this suggests sample degradation and/or faulty RNA extraction – the plate/run should not be used for diagnosis. If N, S, or ORF1ab are detected in the negative extraction control, this suggests contamination – the plate/run should not be used for diagnosis. In this case, all samples included on the plate need to be re-extracted and re-tested

⚠ CRITICAL: If results from control samples deviate from what is expected, do not generate any diagnostic reports from the respective qRT-PCR run. Discard the plate and re-run samples depending on which control failed. See "Troubleshooting" section below for interpretation of controls.

Table 3. Interpretation and Purpose of Controls for Two- and One-Step Singleplex Assays

Control Type	Control Name	Used to Monitor/Where to Repeat Protocol	Expected Results			Expected C_T Values
			N1	N2	RP	
Negative	NTC	contamination during qRT-PCR process	–	–	–	undetected
Positive	SARS-CoV-2 RNA	amplification/primer-probe integrity	+	+	–	<40 (N1, N2,) and undetected (RP)
Extraction	POS EC	extraction/sample stability	+	+	+	<40 (N1, N2, RP)
	NEG EC	extraction cross-contamination	–	–	+	undetected (N1, N2) and <40 (RP)
	human RP gene	extraction/amplification/sample stability	NA	NA	+	<40 (RP)

LIMITATIONS

Pending CLIA certification and/or FDA approval, the protocols herein can be utilized to diagnose patient samples with the presence of SARS-CoV-2 genomic RNA. With this said, these assays are only capable of identifying viral RNA present at a singular timepoint (namely, the time of sample collection). It is possible that immediately after a sample is obtained from an individual that she or he can become infected with SARS-CoV-2. Similarly, it is possible that levels of viral RNA (and therefore the probability of detection) may fluctuate throughout the course of infection and/or disease. Furthermore, it is unclear how symptomatology correlates with C_T values of viral targets. If there is an inverse correlation with disease severity/progression and said C_T values (i.e., a person at the earliest stages of infection or an asymptomatic carrier has a high N1/N2/ORF1ab/S C_T value), it is possible that infected individuals may be reported as negative by these assays. It is important to consider obtaining multiple samples from an individual/patient throughout her or his treatment. Additionally, as stated above, negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

TROUBLESHOOTING

Problem 1

Inappropriate Results from Controls

As noted above, in the event that results from control samples deviate from what is expected, this indicates a step in the accompanying protocol failed.

Potential Solution

To ensure accurate reporting, all data retrieved from the respective run is discarded and the assay is repeated at the earliest failed step (as determined by the failed control). For example, in the event of contamination during the cDNA generation step, repeat the protocol at this stage. Tables 3 and 4 represent interpretations of controls for the singleplex and multiplex assays, respectively.

Table 4. Interpretation and Purpose of Controls for the One-Step Multiplex Assay

Control Type	Control Name	Used to Monitor/Where to Repeat Protocol	Expected Results				Expected C_T Values
			N	S	ORF1ab	MS2	
Negative	NTC	contamination during qRT-PCR process	-	-	-	-	Undetected
Positive	TaqPath Combo Kit positive control	amplification/primer-probe integrity	+	+	+	-	<40 (N, S, ORF1ab) and undetected (MS2)
Extraction	POS EC	extraction/sample stability	+	+	+	+	<40 (N, S, ORF1ab, MS2)
	NEG EC	extraction cross-contamination	-	-	-	+	undetected (N, S, ORF1ab) and <40 (MS2)
	phage MS2 gene	extraction/amplification/sample stability	NA	NA	NA	+	<40 (MS2)

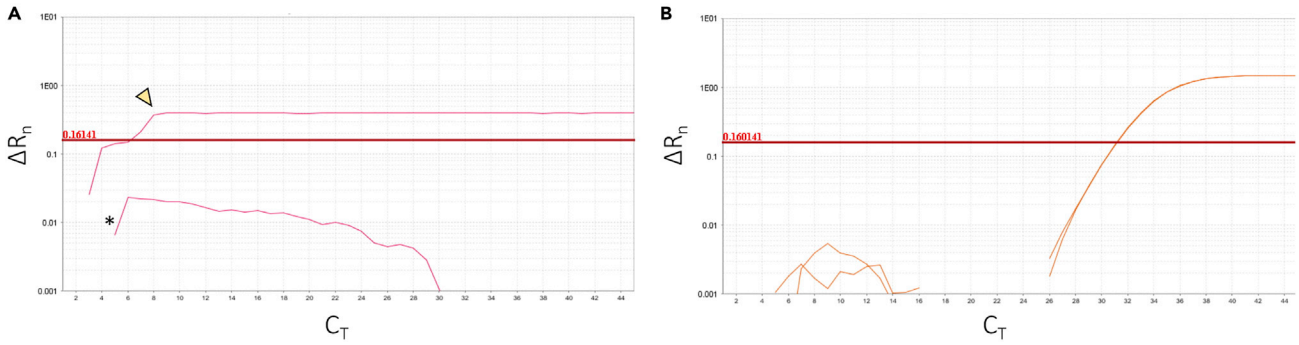

Figure 5. Appropriate and Inappropriate qRT-PCR Curves for Samples

(A) Representation of curves for N1 gene in one patient sample. Each curve represents one technical replicate (i.e., one well) of the qRT-PCR run. The curve labeled with an asterisk (*) denotes what one would expect if N1 transcript is not present in the sample. The curve labeled with yellow arrow head represents a spurious curve with a C_T value of 5.594 likely due to technical artifact.

(B) Technical duplicate curves generated for N1 gene in one positive patient sample. These curves, with C_T value of 31.131, are what one would expect if the qRT-PCR run identified SARS-CoV-2 viral genomic RNA present in patient samples.

Note: NTC in the two-step singleplex assay refers to nuclease-free H_2O ran in the place of RNA in the cDNA generation step (i.e., RT reaction). In the one-step singleplex assay, NTC refers to nuclease-free H_2O ran in the place of RNA sample in the qRT-PCR machine. All other controls listed are common in both platforms.

Problem 2

Technical Artifact Curves

It is sometimes possible to find spurious curves on the qRT-PCR results for specific SARS-CoV-2 target genes (Figure 5). Unfortunately, the qRT-PCR software does not necessarily identify these curves as technical artifacts. As a result, the machine reports the C_T value for the sample with said curve as if it were appropriate. Frequently, these C_T values are low (e.g., ~5 vs. ~30).

Potential Solution

While the reporting algorithm should discard samples with one spurious curve and one undetected replicate as inconclusive, as a quality control step, it is worthwhile to briefly survey all curves generated from the run to preemptively identify artifactual curves which may yield unreliable C_T values. (Please note that despite the low C_T value for the spurious curve described above, it is possible to get such a curve at a later value with abnormal shape upon analyzing on the qRT-PCR machine.)

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Kim Vanuytsel (kimvan@bu.edu).

Materials Availability

There were no unique reagents generated in this study nor are there any restrictions to availability.

Data and Code Availability

To access the R script enabling automated reporting into hospital EMR, please visit: https://github.com/TaylorMatte/Quant6-Covid_Analysis. Video S1 demonstrates all of the steps required in utilizing said script for automated result reporting.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100102.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.M.G., K.V., N.S.M., C.D.A., and G.J.M.; Writing – Original Draft, R.M.G.; Writing – Review & Editing, R.M.G., K.V., and G.J.M.; Methodology, R.M.G., A.M., K.V., G.J.M., T.M.M., A.K.Y., T.W.D., and R.B.W.; Funding Acquisition, G.J.M. and C.D.A.; Resources, G.J.M., G.J.M., C.D.A., and N.S.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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