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Multiplexed RT-qPCR to screen for SARS-COV-2 B.1.1.7 variants

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Coronavirus Method Development Community

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ABSTRACT

As the B.1.1.7 "UK" variant continues to spread, there is a need for labs to screen COVID-19 samples for these viruses. While sequencing is the gold standard, it cannot always be scaled or implemented in some settings. The Applied Biosystems TaqPath COVID-19 assay (ThermoFisher), a PCR test, was discovered to have a distinct signature (spike gene target failure, [SGTF]) when testing viruses containing the Δ69/70 HV deletion, like the B.1.1.7 variant. Detecting the Δ69/70 HV deletion alone is not definitive for the B.1.1.7 variant, but tracking the frequencies of SGTFs helped the UK track the B.1.1.7 variant. In the US and other countries, screening samples for the SGTF helped to identify potential B.1.1.7 variants for sequencing prioritization. ThermoFisher, however, has not released their spike probe sequence, so the assay needs to be recreated to be used more broadly. Here, we designed a multiplexed PCR assay that recreates the SGTF signature using primer/probe set that targets the Δ69/70 HV deletion, the CDC N1 set as a positive control, and the CDC RNase P set as an extraction/sample control. Samples positive for the CDC N1 set but negative for the Δ69/70 HV deletion set can be tracked and prioritized for sequencing.

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Disclaimer and intended use: This multiplexed protocol is still under development and is for research purposes only. It should not be used for clinical diagnosis. The intention of this assay is to screen for the probable presence of the B.1.1.7 (aka “501Y.V1 or “UK”) variant lineage of SARS-CoV-2.

MATERIALS TEXT

- **NEB Luna® Universal Probe One-Step RT-qPCR Kit**
  > MM; Luna Universal Probe One-Step Reaction Mix, 2X
  > RT; Luna WarmStart® RT Enzyme Mix (20X)
  > Nuclease-free water
- **Primers/probes**
  > CDC_N1; Forward Primer (100 µM), Reverse primer (100 µM), probe (100 µM)
  > Yale_69/70del; Forward Primer (100 µM), Reverse primer (100 µM), probe (100 µM)
  > CDC_RP; Forward Primer (100 µM), Reverse primer (100 µM), probe (100 µM)

A	B	C	D	E
Set name	**Nt positions**	**TM**	**Primer/probe**	**Sequence**
CDC_N1	28,287	53.6	Forward primer	GACCCCAAAATCAGCGAAAT
	28,335	57.7	Reverse primer	TCTGGTTACTGCCAGTTGAATCTG
	28,309	63.3	Probe	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
Yale_69/70del	21,710-21,733	59.3	Forward primer	TCAACTCAGGACTTGTTCTTACCT
	21,796-21,817	57.4	Reverse primer	TGGTAGGACAGGGTTATCAAAC
	21,755-21,779	61.2	Probe (drop-out)	HEX-TTCCATGCTATACATGTCTCTGGGA-BHQ1
CDC_RP			Forward primer	AGATTTGGACCTGCGAGCG
			Reverse primer	GAGCGGCTGTCTCCACAAGT
			Probe	Cy5-TTCTGACCTGAAGGCTCTGCGCG-BHQ2

- **Positive control**; Twist synthetic SARS-CoV-2 RNA controls at 100 copies/uL

DISCLAIMER:

ABSTRACT

As the B.1.1.7 "UK" variant continues to spread, there is a need for labs to screen COVID-19 samples for these viruses. While sequencing is the gold standard, it cannot always be scaled or implemented in some settings. The Applied Biosystems TaqPath COVID-19 assay (ThermoFisher), a PCR test, was discovered to have a distinct signature (spike gene target failure, [SGTF]) when testing viruses containing the Δ69/70 HV deletion, like the B.1.1.7 variant. Detecting the Δ69/70 HV deletion alone is not definitive for the B.1.1.7 variant, but tracking the frequencies of SGTFs helped the UK track the B.1.1.7 variant. In the US and other countries, screening samples for the SGTF helped to identify potential B.1.1.7 variants for sequencing prioritization. ThermoFisher, however, has not released their spike probe sequence, so the assay needs to be recreated to be used more broadly. Here, we designed a multiplexed PCR assay that recreates the SGTF signature using primer/probe set that targets the Δ69/70 HV deletion, the CDC N1 set as a positive control, and the CDC RNase P set as an extraction/sample control. Samples positive for the CDC N1 set but negative for the Δ69/70 HV deletion set can be tracked and prioritized for sequencing.

RT-qPCR Protocol

1 Briefly vortex and centrifuge reagents before use.

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2 Prepare 20 µM working stocks of the primers and probes, by adding 20 µL of 100 µM stock to 80 µL nuclease-free water.

3 Use the 20 uM working stocks to prepare **primer-probe-water mix** containing the following:

A	B	C
Component	**Volume (1 reaction)**	**Volume (100 reactions)**
CDC_N1_F (400 nM/reaction)	0.4 µL	40 µL
CDC_N1_R (400 nM/reaction)	0.4 µL	40 µL
CDC_N1_P (200 nM/reaction)	0.2 µL	20 µL
Yale_69/70del_F (400 nM/reaction)	0.4 µL	40 µL
Yale_69/70del_R (400 nM/reaction)	0.4 µL	40 µL
Yale_69/70del_P (200 nM/reaction)	0.2 µL	20 µL
CDC_RP_F (200 nM/reaction)	0.2 µL	20 µL
CDC_RP_R (200 nM/reaction)	0.2 µL	20 µL
CDC_RP_P (100 nM/reaction)	0.1 µL	10 µL
Nuclease-free water	1.5 µL	150 µL

NOTE: a larger volume of primer-probe-water mix can be prepared in advance, aliquoted in LightSafe microcentrifuge tubes, and stored at -20°C.

4 Diagram sample, standard, and control positions on a 96-well plate map.

5 1. On ice, prepare a master mix containing the following (account for 10% extra lost during pipetting), except RNA:

A	B
Component	**Volume in 20 µL reaction**
Tube label = MM	10 µL
Tube label = RT	1 µL
Tube label = primer-probe-water mix	4 µL
Viral RNA, positive control, or negative control	5 µL (do not add to master mix)

6 Add 15 µL of mastermix to each well (on ice).

7 Add 5 µL of positive control (Twist 100 copies/uL) and no-template control (NTC - water) to the designated wells (on ice). Mix by pipetting (avoid bubbles).

8 Add 5 µL of viral RNA to the designated wells (on ice). Mix by pipetting (avoid bubbles).

Cover with plate sealer. Centrifuge to remove bubbles, if present.

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10 Set the thermocycler to read **FAM, HEX, and Cy5** fluorophores.

11 Run the following thermocycler conditions:

A	B	C
Step	**Temperature**	**Time**
1	55°C	10 min
2	95°C	1 min
3	95°C	10 sec
4	55°C	30 sec
5	Read plate	
Repeat steps 3-5 for 44 cycles.		

12 **Interpreting results:**

A	B	C	D
Result	**CDC_N1**	**Yale_69/70del**	**CDC_RP**
Potentially B.1.1.7 (or other B.1 variant)	CT ≤ 40	CT > 40 or undetected	Any value
Not B.1.1.7	CT ≤ 40	CT ≤ 40	Any value
Invalid	CT > 40 or undetected	CT > 40 or undetected	CT > 40 or undetected
Negative	CT > 40 or undetected	CT > 40 or undetected	CT < 40

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