Version 2
Jan 22, 2021

Multiplexed RT-qPCR to screen for SARS-COV-2 B.1.1.7, B.1.351, and P.1 variants of concern V.2

Chantal Vogels[1], Joseph Fauver[1], Nathan Grubaugh[1]
[1]Department of Epidemiology of Microbial Diseases, Yale School of Public Health

In Development dx.doi.org/10.17504/protocols.io.brrhm536

Coronavirus Method Development Community

Nathan Grubaugh
Department of Epidemiology of Microbial Diseases, Yale Schoo...

ABSTRACT

With the emergence of SARS-CoV-2 variants that may increase transmissibility and/or cause escape from immune responses, there is an urgent need for targeted molecular surveillance methods. While sequencing is the gold standard, it cannot always be immediately scaled or implemented in some settings to detect variants when their frequencies are low. 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 first detected in the UK. However, a sample with a SGTF is not definitive for B.1.1.7, and cannot detect other variants of concern that lack the Δ69/70 HV deletion, such as B.1.351 detected in South Africa and P.1 recently detected in Brazil. We developed a multiplexed RT-qPCR assay that can detect all three variants by targeting the Δ3675-3677 SGF deletion in the ORF1a gene, which has not yet been widely detected in other SARS-CoV-2 lineages. Furthermore, by also targeting the Δ69/70 HV deletion in the spike gene, our assay can differentiate B.1.1.7 from B.1.351 and P.1. Finally, we include the CDC N1 primer and probe set in our multiplexed assay as a control to ensure that target failures are likely due to the presence of the ORF1a and/or spike deletions and that there is sufficient virus RNA for sequencing confirmation. Our multiplexed RT-qPCR assay can be rapidly scaled to support SARS-CoV-2 variant surveillance.

DOI

dx.doi.org/10.17504/protocols.io.brrhm536

PROTOCOL CITATION

Chantal Vogels, Joseph Fauver, Nathan Grubaugh 2021. Multiplexed RT-qPCR to screen for SARS-COV-2 B.1.1.7, B.1.351, and P.1 variants of concern. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.brrhm536
Version created by Nathan Grubaugh

CREATED

Jan 22, 2021

LAST MODIFIED

Jan 22, 2021

PROTOCOL INTEGER ID

46601

GUIDELINES

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, B.1.351, and P.1 variants. Variant detection should be confirmed by sequencing.

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)
  > Yale_ORF1a-del; Forward Primer (100 µM), Reverse primer (100 µM), probe (100 µM)
- **Bio-Rad CFX96 touch real-time PCR detection system**

A	B	C	D	E
Set name	**Nt positions**	**TM**	**Primer/probe**	**Sequence**
CDC_N1	28,287	53.6	Fwd primer	GACCCCAAAATCAGCGAAAT
	28,335	57.7	Rev primer	TCTGGTTACTGCCAGTTGAATCTG
	28,309	63.3	Probe	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
Yale_69/70del	21,710-21,733	59.3	Fwd primer	TCAACTCAGGACTTGTTCTTACCT
	21,796-21,817	57.4	Rev primer	TGGTAGGACAGGGTTATCAAAC
	21,755-21,779	61.2	Probe	HEX-TTCCATGCTATACATGTCTCTGGGA-BHQ1
Yale_ORF1a-del	11,229-11,248	60.0	Fwd primer	TGCCTGCTAGTTGGGTGATG
	11,332-11,356	57.8	Rev primer	TGCTGTCATAAGGATTAGTAACACT
	11,283-11,312	61.9	Probe	Cy5-GTTTGTCTGGTTTTAAGCTAAAAGACTGTG-BHQ2

- **Positive control**; Twist synthetic SARS-CoV-2 RNA controls at 100 copies/uL (control 2 = reference strain, control 14 = B.1.1.7)

DISCLAIMER:

RT-qPCR Protocol

1 Briefly vortex and centrifuge reagents before use.

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 µM working stocks to prepare **primer-probe-water mix** containing the following:

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A	B	C	D	E
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		
Yale_ORF1a-del_F (400 nM/reaction)	0.4 µL	40 µL		
Yale-ORF1a-del_R (400 nM/reaction)	0.4 µL	40 µL		
Yale-ORF1a-del_P (200 nM/reaction)	0.2 µL	20 µL		
Nuclease-free water	1.0 µL	100 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 synthetic RNA control at 100 copies/µL) 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).

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

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

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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 39 cycles.		

12 **Interpreting results:**

A	B	C	D
Result	**CDC_N1**	**Yale_69/70del**	**Yale_Orf1a-del**
Potentially B.1.1.7	CT ≤ 35	Undetected	Undetected
Potentially B.1.351 or P.1	CT ≤ 35	CT ≤ 35	Undetected
Potentially B.1.375	CT ≤ 35	Undetected	CT ≤ 35
Other lineages	CT ≤ 35	CT ≤ 35	CT ≤ 35
Inconclusive	CT > 35 or undetected	Any value	Any value

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