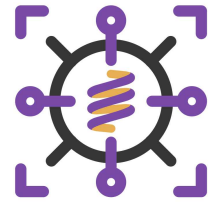


Sep 30, 2025

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

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Chantal Vogels¹, Joseph Fauver¹, Nathan Grubaugh¹

¹Department of Epidemiology of Microbial Diseases, Yale School of Public Health



Andy Sigler

none

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We use this protocol and it's working

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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 $\Delta 69/70$ 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 $\Delta 69/70$ 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 $\Delta 3675-3677$ SGF deletion in the ORF1a gene, which has not yet been widely detected in other SARS-CoV-2 lineages. Furthermore, by also targeting the $\Delta 69/70$ 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.



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.

Version 1: Draft protocol with primer-probe sets targeting the SARS-CoV-2 nucleocapsid (N1), spike 69-70 deletion, and human RNase P control.

Version 2: Updated protocol with primer-probe sets targeting the SARS-CoV-2 nucleocapsid (N1), spike 69-70 deletion, and ORF1a 3675-3677 deletion to differentiate between potential B.1.1.7, B.1.351, and P.1 variants of concern.

Version 3: Updated protocol with reduced N1 concentrations and clarifications on the used channels and conditions under which threshold were determined in the results interpretation section.

Materials

- **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	T M	Primer/pr obe	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_69/70d el	21,710- 21,733	59. 3	Fwd primer	TCAACTCAGGACTTGTCTTACCT
		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)



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:

A	B	C
Component	Volume (1 reaction)	Volume (100 reactions)
CDC_N1_F (200 nM/reaction)	0.2 μL	20 μL
CDC_N1_R (200 nM/reaction)	0.2 μL	20 μL
CDC_N1_P (100 nM/reaction)	0.1 μL	10 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	1.5 μL	150 μL

NB: 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 μ L reaction
Tube label = MM	10 μ L
Tube label = RT	1 μ L
Tube label = primer-probe-water mix	4 μ L
Viral RNA, positive control, or negative control	5 μ L (do not add to master mix)

- 6 Add 15 μ L of mastermix to each well (on ice).
- 7 Add 5 μ L of positive control (Twist synthetic RNA control at 100 copies/ μ L) and no-template control (NTC - water) to the designated wells (on ice). Mix by pipetting (avoid bubbles).
- 8 Add 5 μ L 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.
- 11 Run the following thermocycler conditions:

A	B	C
Step	Temperature	Time
1	55°C	10 min



	A	B	C
	2	95°C	1 min
	3	95°C	10 sec
	4	55°C	30 sec
	5	Read plate	
	<i>Repeat steps 3-5 for 39 cycles.</i>		

12 Interpreting results:

	A	B	C	D
	Result	CDC_N1 (FAM)	Yale_69/70del (HEX)	Yale_Orf1a-del (Cy5)
	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, B.1.258, or other lineages	CT ≤ 35	Undetected	CT ≤ 35
	Other lineages	CT ≤ 35	CT ≤ 35	CT ≤ 35
	Inconclusive	CT > 35 or undetected	Any value	Any value

NB: Thresholds were determined when testing the assay with the NEB Luna Universal Probe One-Step RT-qPCR kit on the Bio-Rad CFX96, and may be different when using other RT-qPCR kits or instruments.