**Draft Protocol: Multiplexed SARS-COV-2 B.1.1.7 variant RT-qPCR**

Version 1.1 (2021.01.01)

Grubaugh Lab, Yale School of Public Health: [grubaughlab.com](http://grubaughlab.com/)

Email questions/comments to: nathan.grubaugh@yale.edu

*Disclaimer and intended use: This multiplexed protocol has not been validated and it is for research purposes only. It should not be used for clinical diagnosis.This assay should only be used with samples that were previously identified to be positive for SARS-CoV-2. Its intention is to screen for the probable presence of the B.1.1.7 (aka “501Y.V1 or “UK”) variant lineage.*

*Development notes:* ***We designed two sets of probes for the B.1.1.7 variant deletions****: one that detects the reference undeleted sequence (“drop out”) and one that directly detects the variant deleted sequence (“detection”). Use only one probe at a time (i.e. both “drop out” or “both detection”). We will test these to determine which set is more sensitive. Protocol updates will be summarized below.*

**Protocol updates**

|  |  |
| --- | --- |
| **Version** | **Update summary** |
| 1.0 | Complete draft protocol |
| 1.1 | Added 2 different probes for each deletion: one that matches the reference genome (positive variant result = “drop out” negative detection) and one that matches the variant (positive result = positive detection). We will try both to see what is more sensitive. |

**Primer/probe sets**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **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) | **Cy5**-TTCCATGCTATACATGTCTCTGGGA-**BHQ2** |
|  | 21,752-21,782 | 65.1 | Probe (detection) | **Cy5**-TGGTTCCATGCTATCTCTGGGACCA-**BHQ2** |
| Yale\_144del | 21,927-21,954 | 57.4 | Forward primer | ACGCTACTAATGTTGTTATTAAAGTCT |
|  | 22,013-22,036 | 59.9 | Reverse primer | TCTGAACTCACTTTCCATCCAACT |
|  | 21,976-22,001 | 60 | Probe (dropout) | **HEX**-TCCATTTTTGGGTGTTTATTACCACA-**BHQ1** |
|  | 21,976-22,001 |  | Probe (detection) | **HEX**-TCCATTTTTGGGTGTTTACCACA-**BHQ1** |

**Reagents**:

* [NEB Luna® Universal Probe One-Step RT-qPCR Kit](https://www.neb.com/products/e3006-luna-universal-probe-one-step-rt-qpcr-kit#Protocols,%20Manuals%20&%20Usage)
	+ 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\_144del**; Forward Primer (100 µM), Reverse primer (100 µM), probe (100 µM)
	+ **\*\*\*NOTE: only use one 69/70del and 144del probe each, either the “drop out” or “detection” set.**
* **1E3 RNA**; Positive control; virus RNA (SARS-COV-2\_USA\_WA1) at 1E3 copies/µL

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

|  |  |  |
| --- | --- | --- |
| **Component** | **Volume (1 reaction)**  | **Volume (100 reactions)** |
| N1-F (400 nM/reaction) | 0.4 µL | 40 µL |
| N1-R (400 nM/reaction) | 0.4 µL | 40 µL |
| 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\_144del\_F (400 nM/reaction) | 0.4 µL | 40 µL |
| Yale\_144del\_R (400 nM/reaction) | 0.4 µL | 40 µL |
| Yale\_144del\_P (200 nM/reaction) | 0.2 µL | 20 µL |
| Nuclease-free water | 1 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*

1. Diagram sample, standard, and control positions on a 96-well plate map.
2. On ice, prepare a master mix containing the following (account for 10% extra lost during pipetting), *except RNA*:

|  |  |
| --- | --- |
| **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)* |

1. Add 15 µL of mastermix to each well (on ice).
2. Add 5 µL of positive control (1E3 RNA) and no-template control (NTC - water) to the designated wells (on ice). Mix by pipetting (avoid bubbles).
3. Add 5 µL of viral RNA to the designated wells (on ice). Mix by pipetting (avoid bubbles).
4. Cover with plate sealer. Centrifuge to remove bubbles, if present.
5. Set the thermocycler to read **FAM, Cy5,** and **Hex** fluorophores.
6. Run the following thermocycler conditions (SARS-COV-2\_qRT-PCR):

|  |  |  |
| --- | --- | --- |
| **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.  |

**Interpreting results with “drop out” probes**

|  |  |  |  |
| --- | --- | --- | --- |
| **Result** | **CDC\_N1 (control)** | **Yale\_69/70del** | **Yale\_144del** |
| Probable B.1.1.7 | CT ≤ 40 | CT > 40 or undetected | CT > 40 or undetected |
| Not B.1.1.7, but has 69/70 deletion | CT ≤ 40 | CT > 40 or undetected | CT ≤ 40 |
| Not B.1.1.7 | CT ≤ 40 | CT ≤ 40 | CT ≤ 40 |
| Invalid | CT > 40 or undetected | Any value | Any value |

**Interpreting results with “detection” probes**

|  |  |  |  |
| --- | --- | --- | --- |
| **Result** | **CDC\_N1 (control)** | **Yale\_69/70del** | **Yale\_144del** |
| Probable B.1.1.7 | CT ≤ 40 | CT ≤ 40 | CT ≤ 40 |
| Not B.1.1.7, but has 69/70 deletion | CT ≤ 40 | CT ≤ 40 | CT > 40 or undetected |
| Not B.1.1.7 | CT ≤ 40 | CT > 40 or undetected | CT > 40 or undetected |
| Invalid | CT > 40 or undetected | Any value | Any value |