Development of a multiplexed RT-qPCR for the surveillance of SARS-CoV-2 variants of world-wide concern V.1

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1 *Works for me* This protocol is published without a DOI.

Coronavirus Method Development Community

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ABSTRACT

Multiple SARS-CoV-2 variants have evolved that increase the transmissibility of the virus and/or cause its escape from immune response. Whole genome sequencing remains the gold standard and is implemented into the national surveillance programs worldwide to detect these novel variants of concern. However, this technique cannot immediately be scaled up when a fast action is needed to limit the spread of SARS-CoV-2 variants every time a new outbreak occurs, as this can be laborious and time-consuming.
Two key mutations with multiple reassurance in the SARS-CoV-2 spike glycoprotein have been identified to have a distinct signature enabling to detect five SARS-CoV-2 variants (B1.351, B1.1.7, P1 and N439K/Y453F variants). Three of them are SARS-CoV-2 variants of major concern worldwide. We developed a specific multiplexed RT-qPCR to detect these key mutations, the H69/V70deletion and the N501Y mutation and increased the sensitivity of the PCR by using LNA probes targeting the N501Y mutation carrying a single amino acid exchange. Additionally, we can exclude target failure and secure SARS-CoV-2 detection by a primer/probe pair detecting the Wt sequence (SARS-CoV-2; Wuhan lineage) at aa position 501. Our multiplexed RT-qPCR assay offers an alternative strategy for SARS-CoV-2 variant surveillance detecting five SARS-CoV-2 variants, which can build up on to detect new emerging SARS-CoV-2 variants.

PROTOCOL CITATION

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KEYWORDS

Pandemic, mutation, viral lineages, SARS-CoV-2 variants, Cluster 5, Danish mink variants, B1.351, B1.1.7, P1 and N439K/Y453F variants

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GUIDELINES

The intention of this assay is to screen for the probable presence of the B1.351, B1.1.7, P1 and N439K/Y453F variants. Variant detection should be confirmed by sequencing.

MATERIALS TEXT

Material

Luna®Universal Probe One-step RT-qPCR Kit

Primer and probes

Primer/probe name	primer/probe	TM	primer/probe
SARS-CoV-2_deletion	forward primer	58.5	5´-ACATTCAACTCAGGACTTGTTCT – 3`
SARS-CoV-2_deletion	reverse primer	58.0	5´-TCATTAAATGGTAGGACAGGGTT– 3`
SARSCoV2_deletion*	probe	61.2	5`-HEX-TTCCATGCTATCTCTGGGACCA-BHQ2-3`
SNP_501	forward primer	57.7	5'-TGTTACTTTCCTTTACAATCATATGGT-3'
SNP_501	reverse primer	58.9	5'-TGCTGGTGCATGTAGAAGTTCA-3'
Probe4_501_Y	LNA probe	63.2	5'-FAM -CCC AC+T +T+AT GG+T GTT GGT-BHQ1-3'
Probe4_N_501_mutant	LNA probe	62.6	5'-Quasar 670-CCC AC+T +A+AT GG+T GTT GGT-BHQ2-3'

*Please be aware that it is more common to use BHQ1 quencher together with HEX, but this system works fine with a BHQ2 quencher.

Controls:
TWIST synthetic RNA controls: TWIST control 1, part number 102019, Australian/VIC01/2020 and TWIST control 14, part number 103907, England 205041766/2020

Bio-Rad CFX96 touch real-time PCR detection system

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Protocol

- Work on ice
- Vortex and centrifuge reagents before use, except the Luna WarmStart® RT Enzyme Mix here only mix carefully by pipetting or short vortexing
- Dilute the primer and probe stock (100µM) 1:5 with nuclease free water to a working concentration of 20µM
- Prepare mastermix on ice containing the following:

Luna® Universal Probe One-Step RT-qPCR Kit	*per reaction*
Luna® Universal Probe One-Step RT-qPCR Kit 2x	12.5 µl
Luna WarmStart® RT Enzyme Mix (20X)	1.25 µl
SNP_501_F_2 (20µM)	0,5 µl
SNP_501_R_2 (20µM)	0,5 µl
Probe4_Wt_n_501_LNA (20µM)	0,25 µl
Probe4_B117_y_LNA (20µM)	0,25 µl
SARS-CoV-2_deletion_F (20µM)	0.5 µl
SARS-CoV-2_deletion_R (20µM)	0.5 µl
SARSCoV2_deletion_P (20µM)	0,25 µl
H2O	3.5 µl
Total	20 µl
Sample	5µl
Total	25µl

- Add 20µL of mastermix to each well/ PCR tube
- Add 5µl of the control (positive controls such as virus cultures, sequenced patient samples with known information about the SARS-CoV-2 variant or TWIST synthetic RNA controls and negative controls such as negative tested patient samples and nuclease free water) to the corresponding wells containing the mastermix and mix by pipetting
- Add 5µl of extracted RNA (unknown sample) to the designated wells and mix by pipetting
- Seal the plates/tubes
- Centrifuge to remove air bubbles
- Run the PCR with the following cycler conditions and set the thermocycler to read the **HEX**, **FAM** and **Cy5** channels:

Cycler protocol

Cycle	**Time**	**Temperature**
1 cycle	10min	550C
1 cycle	3min	950C
45 cycles	15s	950C
	30s	580C

Data evaluation

H69/70 deletion

- HEX channel: no Ct value = no detection of the H69/70 deletion, Ct ≤ 38 H69/70 deletion detected

N501Y mutation

- Mark negative control (NTC) as NTC in plate set-up, otherwise the wild type and the N501Y mutant will not be grouped correctly.
- Correct undershooting curves (often for weak samples) by adjusting ”Cycles to analyze”. This also helps the software to group wildtype and the N501Y mutant correctly.
- In the tab Allelic discrimination: Set Selected Fluorophores (right low corner) to X: FAM, Y: Cy5
- Allele 1 –FAM probe detects the N501Y mutant. Allele 2 – Cy5 detects the SARS-CoV-2 (Wuhan lineage). Sometimes the Wt (Cy5) is marked as Heterozygote and not Allele 2.

Interpreting results

Result	69/70del (HEX)	501 mutant (FAM)	501 wildtype (Cy5)
Potentially B.1.1.7 (UK)	Ct <45	Grouped as allele 1 (RFU FAM > RFU Cy5 at Ct 45)	
Potentially B.1.351 (South Africa) or P.1 (Brazil)	Ct Undetected	Grouped as allele 1 (RFU FAM > RFU Cy5 at Ct 45)	
Potentially B.1.258 (N439K mutation), other lineages	Ct <45		Grouped as allele 2 or heterozygote (RFU Cy5 > RFU FAM at Ct 45)
Wildtype, other lineages	Ct Undetected		Grouped as allele 2 or heterozygote (RFU Cy5 > RFU FAM at Ct 45)