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Large scale screening of SARS-CoV-2 variants of worldwide concern by RT-qPCR

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Coronavirus Method Development Community

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ABSTRACT

Multiple SARS-CoV-2 variants have evolved increasing the transmissibility of the virus and/or cause its escape from immune response. Whole genome sequencing remains the gold standard and is implemented into national surveillance programs worldwide to detect these novel variants of concern. However, this technique can have a high turnaround time and it might not be possible to immediately be scaled up when fast action is required to limit the spread of SARS-CoV-2 variants every time a new local outbreak occurs.

Four key mutations H69/V70 deletion, L452R, E484K and N501Y with multiple reassurance in the SARS-CoV-2 spike (S) glycoprotein were identified to have a distinct signature enabling to detect SARS-CoV-2 variants of worldwide concern (Alpha [B.1.1.7], Alpha [B.1.17 + E484K], Beta [B.1.351], Gamma [P1], Delta [B.1.617,2]) as well as variants of interest/notes. We developed RT-qPCRs to detect these key mutations and increased the sensitivity of the PCRs by using modified probes targeting the 452R, 484K and 501Y mutations, with a single nucleotide exchange present, leading to an amino acid substitution. Large scale screening of the SARS-CoV2 variants of concern was included into the national surveillance program in Denmark, where the RT-qPCRs run as multiplexed (H69/V70 and N501Y mutation) - or as a single reactions detecting all four key mutations in parallel in a 384-well format.

Our RT-qPCR systems offer a fast and flexible testing strategy to detect SARS-CoV-2 variants of concern and of interest/note, especially needed in countries where whole genome sequencing in large scale is too cost- and labour intensive.

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KEYWORDS

SARS-CoV-2, PCR, variant, N501Y, E484K, L452R, H69/V70, Allelic discrimination

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GUIDELINES

The intention of this PCR assay is to screen for the presence of deletion H69/V70 and SNPs N501Y, E484K and L452R in SARS-Cov-2 through allelic discrimination. The assays can be run on a 96 well PCR format or a 384 well PCR format. Variant detection should be confirmed by sequencing.

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MATERIALS TEXT

Enzyme

Luna®Universal Probe One-step RT-qPCR Kit (New England Biolabs Inc.)

Primers and probes

Primers and probes can be synthesized by Biosearch Technologies, Denmark, except for the MGB-probes that can be synthesized by Eurogentec, Belgium.

A	B	C	D
Target	**Primer/Probe name**	**Tm**	**Primer/Probe sequence 5' – 3'**
H69/V70Δ	SARS-CoV-2_H69/V70 F	58.5	ACATTCAACTCAGGACTTGTTCT
	SARS-CoV-2_H69/V70 R	58.0	TCATTAAATGGTAGGACAGGGTT
	SARS-CoV-2_H69/V70 P (1)	61.2	HEX-TTCCATGCTATCTCTGGGACCA-BHQ2
N501Y	SARS-CoV-2 N501Y F	57.7	TGTTACTTTCCTTTACAATCATATGGT
	SARS-CoV-2 N501Y R	58.9	TGCTGGTGCATGTAGAAGTTCA
	SARS-CoV-2 501Y_mutant MGB P	64.8	FAM-CCCACTTATGGTGTTGGT-MGB
	SARS-CoV-2 N501 WT MGB P	64.8	Cy5-CCCACTAATGGTGTTGGT-MGB
E484K	SARS-CoV-2_E484K F	58.5	AGGAAGTCTAATCTCAAACCTTTTGA
	SARS-CoV-2_E484K R	60.2	GTCCACAAACAGTTGCTGGTG
	SARS-CoV-2_484K_mutant MGB FAM P	64.6	FAM-TGGTGTTAAAGGTTTTAAT-MGB
	SARS-CoV-2_E484K_WT MGB P	63.5	Texas Red-TGGTGTTGAAGGTTTTAA-MGB
L452R	SARS-CoV-2_L452R F	60.5	CAGGCTGCGTTATAGCTTGGA
	SARS-CoV-2_L452R R	57.1	CCGGCCTGATAGATTTCAGT
	SARS-CoV-2_452R_mutant BHQ+ P	58.2	HEX-TATAATTACCGGTATAGATTGTT-BHQ1
	SARS-CoV-2_L452_WT BHQ+ P	58.0	Cal Fluor Red 610-TATAATTACCTGTATAGATTGTTTA-BHQ2

Primer and probe sequences. MGB = Minor Groove Binder, BHQ+ = BHQplus modified probe.
(1)Please be aware that it is more common to use BHQ1 quencher together with HEX, but this system works fine with a BHQ2 quencher.

PCR cycler 96 well format

Bio-Rad CFX96 touch real-time PCR detection system

PCR cycler 384 well format

Bio-Rad CFX384 touch real-time PCR detection system

Positive controls

Cultivated virus originating from patient samples, TWIST Synthetic SARS-CoV-2 RNA controls (MT007544.1/Australia/VIC01/2020), (MT103907 England/205041766/2020), (MT104043 South African/KRISP-EC-K005299/2020) and (MT104044 Japan(IC-0564/2021) from TWIST bioscience.

Negative controls

Negative tested patient samples and nuclease free water.

1 Protocol for 96 and 384 well PCR format

- Prepare three different mastermixes.
- Make sure to vortex all reagents before use.

- Mastermix 1, for detection of H69/V70 and N501Y, contains the following:

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A	B	C
Luna® Universal Probe One-Step RT-qPCR Kit	*Volume per reaction (µL) 96 well format*	*Volume per reaction (µL) 384 well format*
Luna® Universal Probe One-Step RT-qPCR Kit 2x	12.5	7.5
Luna WarmStart® RT Enzyme Mix (20X)	1.25	0.75
SARS-CoV-2 N501Y F (100µM)	0.1	0.1
SARS-CoV-2 N501Y R (100µM)	0.1	0.1
SARS-CoV-2 501Y_mutant MGB P (100µM)	0.05	0.05
SARS-CoV-2 N501 WT MGB P (100µM)	0.05	0.05
SARS-CoV-2_H69/V70 F (100µM)	0.1	0.1
SARS-CoV-2_H69/V70 R (100µM)	0.1	0.1
SARS-CoV-2_H69/V70 P (100µM)	0.05	0.05
H2O	5.7	1.2
Total	20	10
Sample	5	5
Total	25	15

- Mastermix 2, for detection of E484K, contains the following:

A	B	C
Luna® Universal Probe One-Step RT-qPCR Kit	*Volume per reaction (µL) 96 well format*	*Volume per reaction (µL) 384 well format*
Luna® Universal Probe One-Step RT-qPCR Kit 2x	12.5	7.5
Luna WarmStart® RT Enzyme Mix (20X)	1.25	0.75
SARS-CoV-2_E484K F (100µM)	0.1	0.1
SARS-CoV-2_E484K R (100µM)	0.1	0.1
SARS-CoV-2_484K_mutant MGB FAM P (100µM)	0.15	0.15
SARS-CoV-2_E484K_WT MGB P (100µM)	0.05	0.05
H2O	5.85	1.35
Total	20	10
Sample	5	5
Total	25	15

- Mastermix 3, for detection of L452R, contains the following:

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A	B	C
Luna® Universal Probe One-Step RT-qPCR Kit	*Volume per reaction (µL) 96 well format*	*Volume per reaction (µL) 384 well format*
Luna® Universal Probe One-Step RT-qPCR Kit 2x	12.5	7.5
Luna WarmStart® RT Enzyme Mix (20X)	1.25	0.75
SARS-CoV-2_L452R F (100µM)	0.1	0.1
SARS-CoV-2_L452R R (100µM)	0.1	0.1
SARS-CoV-2_452R_mutant BHQ+ P (100µM)	0.05	0.05
SARS-CoV-2_L452_WT BHQ+ P (100µM)	0.05	0.05
H2O	5.95	1.45
Total	20	10
Sample	5	5
Total	25	15

- Add 20µL (96 well) or 10µL (386 well) of mastermix to each well.
- Add 5µl of extracted RNA (unknown sample) to each of the three mastermixes.
- Add 5µl of 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 or nuclease free water) to the corresponding mastermixes.
- Seal the plates/tubes
- Centrifuge to remove air bubbles

Cycler protocol

Run the PCR with the following cycler conditions and set the thermocycler to read the FAM, HEX, ROX and Cy5 channels:

A	B	C
Cycle	**Time**	**Temperature**
1 cycle	10min	55°C
1 cycle	3min	95°C
45 cycles	15s	95°C
	30s	58°C

Data evaluation

- Mark negative control (NTC) as NTC in plate set-up, otherwise the wild type and the mutant will not be grouped correctly.
- Correct undershooting curves (often for weak samples). This also helps the software to group wildtype and mutant correctly.
- Set correct fluorophores (depending on which mastermix is being analyzed) in allelic discrimination chart.
- Use a threshold RFU>200 for all fluorophores in case one of the probes in the allelic discrimination pair fails.
- Perform allelic discrimination at Ct = 45 for all SNPs.

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A	B	C	D	E
SARS-CoV-2 variant	**H69/V70Δ HEX**	**501Y FAM vs. N501 Cy5**	**484K FAM vs. E484 ROX**	**452R HEX vs. L452 ROX**
WILDTYPE	Ct ≥ 38	RFU Cy5 > RFU FAM	RFU ROX > RFU FAM	RFU ROX > RFU HEX
Alpha	***Ct < 38***	***RFU FAM > RFU Cy5***	RFU ROX > RFU FAM	RFU ROX > RFU HEX
Beta	Ct ≥ 38	***RFU FAM > RFU Cy5***	***RFU FAM > RFU ROX***	RFU ROX > RFU HEX
Gamma	Ct ≥ 38	***RFU FAM > RFU Cy5***	***RFU FAM > RFU ROX***	RFU ROX > RFU HEX
Delta	Ct ≥ 38	RFU Cy5 > RFU FAM	RFU ROX > RFU FAM	***RFU HEX > RFU ROX***

Mutations are marked in bold

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