Primerdesign Ltd

SNPsig® SARS-CoV-2 (EscapePLEX)

Identification of clinically significant mutations E484K, K417N, K417T and P681R

SNPsig® Real-Time PCR
SARS-CoV-2 mutation detection/allelic discrimination kit

96 tests

For general laboratory and research use only

Kits by Primerdesign

Kit contents

- **SARS-CoV-2 EscapePLEX genotyping primer/probe mix tube 1 (96 reactions BROWN)**

  FAM, HEX/VIC, ROX and Cy5 labelled
- **SARS-CoV-2 EscapePLEX genotyping primer/probe mix tube 2 (96 reactions BROWN)**

  FAM, HEX/VIC and ROX labelled
- **Positive control templates tube 1 (RED)**
- **Positive control templates tube 2 (RED)**
- **Internal control RNA (BLUE)**
- **RNase/DNase free water (WHITE)**

  for resuspension of primer/probe mix
- **2 tubes of template preparation buffer (YELLOW)**

  for resuspension of positive control templates
- **4 vials of Onestep Lyophilised Master Mix (GOLD)**

  contains complete Onestep RT-qPCR Master Mix
- **4 tubes of Master Mix resuspension buffer (BLUE)**

  for resuspension of the lyophilised Master Mix

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument
Must be able to read fluorescence through FAM, HEX/VIC, ROX and Cy5 channels

Pipettes and Tips

Vortex

Centrifuge

Suitable Real-Time PCR 96W plates or Real-Time PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20ºC on arrival. Primerdesign does not recommend using the kit after the expiry date stated on the pack. Once the lyophilised components have been resuspended, unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

Suitable sample material

SNPsig® SARS-CoV-2 (EscapePLEX) is intended for use as a reflex test only. Thus, a primary confirmation test for SARS-CoV-2 would be carried out using suitable methodology, and the extracted RNA from patient samples (or any material suited for PCR amplification) thereafter applied to this test. Please ensure that the samples are suitable in terms of purity, concentration, and RNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the template RNA sample with RNase/DNAse free water.

Notices and disclaimers

This product is developed, designed, and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period Primerdesign SNPsig® detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

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Introduction

The novel coronavirus disease 2019 (COVID-19) pandemic, caused by SARS-CoV-2 virus, represents a major threat to health. SARS-CoV-2 has resulted in widespread morbidity and mortality. COVID-19 is known to have infected more than 100 million people (1). As the virus has spread across the world, mutations have arisen resulting in divergent clusters (clades) with different prevalence in different geographic regions (2,3).

Since the beginning of the pandemic, governments and scientists have applied sequencing technologies to identify mutations which modify the characteristics of the SARS-CoV-2, including mutations which alter transmissibility (4) or that are associated with a reduced affinity for key neutralising antibodies (5-7). These mutations mostly occur within the spike protein and are predicted to alter the way that SARS-CoV-2 interacts with Angiotensin I converting enzyme 2 (ACE2) receptor.

Recently, four clinically significant mutations have been identified as “high risk” due to the concerning increase in numbers of active cases carrying them.

E484K has been detected in a wide variety of Variants of Concern (VOC) and Variants Under Investigation (VUI), including Beta (B.1.351) Gamma (P.1), VOC-21FEB-02 (Alpha (B.1.1.7) with E484K), Zeta (P.2) and Theta (P.3) (8-11). Multiple studies on E484K have associated this change in amino acid with the ability to evade antibodies (12-14), increase disease severity and its potential impact on vaccines (15-18) with enormous implications for public health approaches in combatting COVID-19 cases.

The site K417 in the spike protein corresponds with an area in the protein that is considered a hotspot for antibody recognition (19). Therefore, changes in this position would have a potential role in antibody and vaccine escape. At present, two mutations on this site have been recognised and associated with reduced antibody protection. K417T was firstly identified in the VOC P.1 (20) and K417N was firstly identified as part of the VOC Beta (3.1.351) (19-20). In addition, K417N has been demonstrated to provide the virus with a moderate increase in transmissibility rates due to the very specific change induced by the K to N amino acid replacement (21).

P681R has been found in the recently identified VOC and VUI Kappa (B.1.617.1), Delta (B.1.617.2) and B.1.617.3 related variants (22). This mutation has been associated with a potential increase in transmissibility rate based on mechanistic studies on the capacity of the virus to identify and infect human cells (22).

Identifying variants that carry any of these four mutations in a timely manner may be important for making public health decisions. The product will be developed in a standard SNPsig format. It will enable the identification of these mutations (E484K, K417N/T and P681R) on previously identified SARS-CoV-2 positive samples.

Principles of the test

Genotyping by Real-Time PCR using hydrolysis probes

Each genotyping primers/probe mix contain four (tube 1) and three (tube 2) labelled probes homologous to the genotypes under investigation and the Internal Control. The SNPsig® assays are compatible with all Real-Time PCR instruments capable of detecting fluorescence in FAM, HEX/VIC, ROX and Cy5 emission channels, including selected genesig® family instruments.

Reagent Label	FAM	HEX/VIC	ROX	Cy5
SNPsig® SARS-CoV-2 (EscapePLEX) Tube 1	E484K	K417N	K417T	P681R
SNPsig® SARS-CoV-2 (EscapePLEX) Tube 2	Orf1ab region	Internal Control	M region	N/A

Positive control Template

The kit contains two positive control templates tubes. Tube 1 contains the four synthetic templates homologous to the clinically significant mutations E484K, K417N, K417N and P681R. Tube 2 contains synthetic templates homologous to the Orf1ab and M gene target regions. These should be run with the appropriate primer/probe tube to obtain control signals for each target. Positive control templates are a potential contamination risk to subsequent tests so must be handled carefully.

Negative control

To confirm the absence of contamination a negative control reaction should be included for each primer/probe tube, every time the kit is used. In this instance, the RNase/DNAse free water should be used instead of template RNA. A negative result in FAM, HEX/VIC, ROX and Cy5 channels indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained, this indicates contamination, therefore the sample results will be invalid, and the run should be repeated.

Possible sources of contamination should first be explored and removed.

Master mix compatibility

Onestep Lyophilised Master Mix contains the enzyme, nucleotides, buffers, and salts at precisely the correct concentration for this application. The annealing temperatures of the primers and probes have been carefully calibrated and any change in the reaction buffer can significantly alter the performance of the assay. For this reason, Primerdesign can only guarantee accurate genotyping results when Onestep Lyophilised Master Mix is used.

Resuspension Protocol

To minimize the risk of contamination with foreign RNA/DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps. The positive control template is a significant contamination risk and should therefore be pipetted after negative control and sample wells.

1. **Pulse-spin each tube in a centrifuge before opening.**

This will ensure that the lyophilised primer/probe mix is in the base of the tube and is not lost upon opening the tube.

2. **Resuspend the primer/probe mix in the RNase/DNase free water supplied, according to the table below.**

To ensure complete resuspension, vortex the tube thoroughly.

Component - Resuspend in water	Volume
SARS-CoV-2 **(EscapePLEX)** genotyping primer/probe mix **tube 1** (BROWN)	110 µl
SARS-CoV-2 **(EscapePLEX)** genotyping primer/probe mix **tube 2** (BROWN)	110 µl

3. **Resuspend the Master Mix in Resuspension Buffer supplied, according to the table below.**

Component - Resuspend in Resuspension Buffer	Volume
Onestep Lyophilised Master Mix (GOLD)	525 µl

4. **Resuspend the Positive Control Templates tube in the Template Preparation Buffer supplied, according to the table below.**

   To ensure complete resuspension, vortex each tube thoroughly.

Component - Resuspend in Template Preparation Buffer	Volume
Positive Control Template tube 1 (RED) *	500 µl
Positive Control Template tube 2 (RED) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

5. **Resuspend the Internal Control Templates tube in the Template Preparation Buffer supplied, according to the table below.**

Component - Resuspend in Template Preparation Buffer	Volume
Internal Control Template (BLUE)	500 µl

Real-Time PCR detection protocol

1. **Prepare 2 reaction mixes, one for each primers/probes mix.**

2. **Include sufficient reactions for all samples, positive and negative controls.**

Reaction mix for Tube 1:

Component	Volume
Onestep Lyophilised Master Mix	10 µl
SARS-CoV-2 **(EscapePLEX)** genotyping primer/probe mix Tube 1	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

Reaction mix for Tube 2:

Component	Volume
Onestep Lyophilised Master Mix	10 µl
SARS-CoV-2 **(EscapePLEX)** genotyping primer/probe mix Tube 2	1 µl
Internal Control	1 µl
RNase/DNase free water (WHITE)	3 µl
Final Volume	**15 µl**

3. **Pipette 15 µl of the appropriate reaction mix into each well according to your Real-Time PCR experimental plate set up, to enable each sample to be tested with each reaction mix.**

4. **Prepare RNA templates for each of your samples.**

   As this is a reflex test, the RNA obtained from the confirmatory primary assay for SARS- CoV-2 will be used.

5. **Pipette 5 µl of RNA template into each well, according to your experimental plate set up.**

6. **For negative control wells use 5 µl of RNase/DNase free water.**

7. **Pipette 5 µl of each positive control RNA according to your experimental plate set-up. The final volume in each well is 20 µl.**

Real-Time PCR amplification protocol

The following protocol is recommended for optimum resolution between genotypes:

Protocol for Onestep Lyophilised Master Mix

	Step	Time	Temp
	Reverse transcription	10 min	55 °C
	Enzyme activation	2 min	95 °C
Cycling x45	Denaturation	10 s	95 °C
	Annealing and extension*	60 s	60 °C

* Fluorogenic data should be collected during this step through the **FAM, HEX/VIC, ROX** and **Cy5** channels for tube 1 and **FAM, HEX/VIC** and **ROX** for tube 2.

Interpretation of results

The SNPsig® SARS-CoV-2 (EscapePLEX) allows simultaneous discriminatory identification of the four clinically significant mutations, E484K, K417N, K417T and P681R in tube 1. Tube 2 allows the confirmation of a SARS-CoV-2 positive sample.

In tube 1:

- E484K should be producing a fluorescence signal on FAM channel.
- K417N should be producing a fluorescence signal on HEX/VIC channel.
- K417T should be producing a fluorescence signal on ROX channel.
- P681R should be producing a fluorescence signal on Cy5 channel (see table below).

FAM (E484K)	HEX/VIC (K417N)	ROX (K417T)	Cy5 (P681R)	Results	Variant detected
Cq (+)	Cq (-)	Cq (-)	Cq (-)	Positive for E484K	VOC-21FEB-02 (Alpha (B.1.1.7) + E484K) (Bristol, UK)
Cq (+)	Cq (+)	Cq (-)	Cq (-)	Positive for E484K + K417N	Beta. (B.1.351) (South Africa)
Cq (-)	Cq (+)	Cq (-)	Cq (-)	Positive for K417N	*
Cq (-)	Cq (-)	Cq (+)	Cq (-)	Positive for K417T	*
Cq (+)	Cq (-)	Cq (+)	Cq (-)	Positive for E484K + K417T	Gamma (P.1.) (Japan (previously Manaus, Brazil))
Cq (-)	Cq (-)	Cq (-)	Cq (+)	Positive for P681R	Kappa/Delta (B.1.617+) (India)

Lack of fluorescence in a channel could either indicate a sample with Wild-type sequence at that site or a sample with a different mutation at the target site. Combination of mutation (Cq (+) in more than one channel) is possible. For any additional fluorescence profiles, we suggest sending the sample for sequencing.

* At the moment, there is no known variant that only has K417N or K417T mutations independently without E484K mutation.

In tube 2:

- Orf1ab region should be producing a fluorescence signal on FAM channel.
- IC should be producing a fluorescence signal on HEX/VIC channel.
- M region should be producing a fluorescence signal on ROX channel (see table below).

FAM (Orf1a region)	HEX/VIC (IC)	ROX (M region)	Cy5 (N/A)	Results
Cq (+)	Cq (+)	Cq (+)	N/A	SARS-CoV-2 Positive
Cq (+)	Cq (+)	Cq (-)	N/A	SARS-CoV-2 Positive
Cq (-)	Cq (+)	Cq (+)	N/A	SARS-CoV-2 Positive
Cq (-)	Cq (+)	Cq (-)	N/A	SARS-CoV-2 Negative

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