Primerdesign Ltd

SNPsig® SARS- CoV-2 (L452R)

Identification of L452R mutation

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 L452R genotyping primer/probe mix (96 reactions BROWN)**
  FAM and HEX/VIC labelled
- **Wild-type positive control template (RED)**
- **Mutant positive control template (RED)**
- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mix
- **Template preparation buffer (YELLOW)**
  for resuspension of positive control templates
- **Onestep Lyophilised Mastermix (RED)**
  contains complete Onestep RT-qPCR Mastermix
- **Mastermix resuspension buffer (BLUE)**
  for resuspension of the lyophilised Mastermix

Reagents and equipment to be supplied by the user

Real-time PCR Instrument
Must be able to read fluorescence through FAM and HEX/VIC channels (plus Cy5 if using optional internal control)

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 L452R 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 alter 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 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.

One of the recent clinically significant mutations identified in the spike protein is L452R. L452R appeared as part of the set of mutations identified in the Variant of Concern 20C.S.452R, first identified in California. This mutation has been considered to confer the ability to increase infectivity (8) and to evade antibodies (8-9).

The increased rapid transmission of SARS-CoV-2 has implications for health sectors, as more community transmission will result in a higher hospital burden. This test will enable identification of the significant L452R mutation on multiple platforms.

Principles of the test

Genotyping by real-time PCR using hydrolysis probes
Each genotyping primer/probe mix contains two labelled probes homologous to the two genotypes under investigation. During Real-Time PCR amplification of the reverse-transcribed target RNA, the probes will compete for binding across the variant region. The probe that is 100% homologous to the binding site will preferentially bind and give a fluorescent signal as PCR proceeds. It follows that the wild-type sequence will give a strong amplification plot through one channel whilst giving a very weak signal through the alternative channel. Variant samples will give an exactly inverse result. Most hardware platforms can perform this analysis automatically. The SNPsig® assays are compatible with all Real-Time PCR instruments capable of detecting fluorescence in FAM and HEX/VIC (plus Cy5 if using internal control) emission channels, including selected genesig® family instruments.

Positive controls
The kit contains positive control templates for each of the two genotypes. These can be run as parallel samples to give control signals for each genotype. In order to provide good positive control data that is directly relevant to the samples under test, the positive control template should be used at a similar copy number to the sample RNA. 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 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 and HEX/VIC 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.

Mastermix compatibility

Onestep Lyophilised Mastermix 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 Mastermix 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 and probe mix is in the base of the tube and is not spilt 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 **L452R** genotyping primer/probe mix (BROWN)	110 µl

3. **Resuspend the Mastermix in resuspension buffer supplied, according to the table below.**

Component - Resuspend in resuspension buffer	Volume
Onestep Lyophilised Mastermix (RED)	525 µl

4. **Resuspend the positive control templates 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
Wild-type Positive Control Template (RED) *	500 µl
Mutant Positive Control Template (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.

Real-Time PCR detection protocol

If using optional internal control, refer to page 11 for protocol amendments.

1. **Prepare a complete genotyping reaction mix for the primer/probe mix according to the table below:**

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

Component	Volume
Onestep Lyophilised Mastermix	10 µl
SARS-CoV-2 **L452R** genotyping primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

2. **Pipette 15 µl of this mix into each well according to your Real-Time PCR experimental plate set up.**

3. **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.

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

   For negative control wells use 5 µl of RNase/DNase free water. The final volume in each well is 20 µl.

5. **Pipette 5 µl of each positive control RNA according to your experimental plate set-up.**

Real-Time PCR amplification protocol

The following protocol is recommended for optimum resolution between genotypes:

Protocol for Onestep Lyophilised Mastermix

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

* Fluorogenic data should be collected during this step through the **FAM** and **HEX/VIC** channels

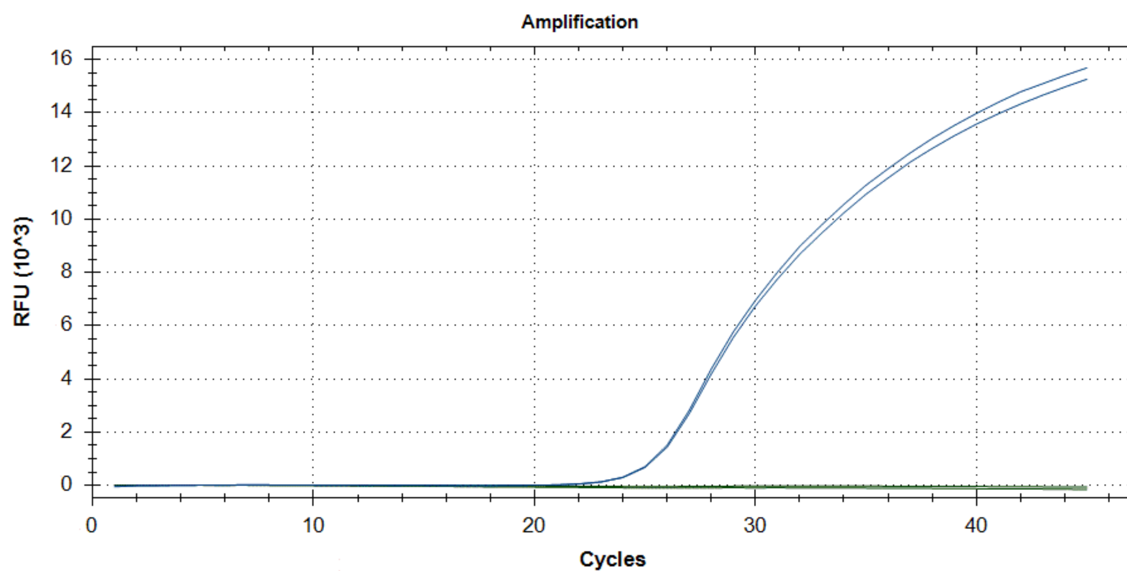
Interpretation of results

The wild-type probe is labelled to read through the FAM channel whilst the mutant probe is labelled to read through the HEX/VIC channel. On wild-type sequences the FAM channel will give a strong amplification plot and the HEX/VIC channel none or very low detection. The signals are reversed on mutant samples. Cq values >40 should be disregarded and counted as inconclusive.

If using an internal control (see protocol below), then the Cy5 channel should be consulted where the test is negative in both the FAM and HEX/VIC channels. If neither HEX/VIC or FAM amplify, but Cy5 channel is observed, then the reaction worked, and the test is a true negative. If no Cy5 amplification is observed, then the test failed.

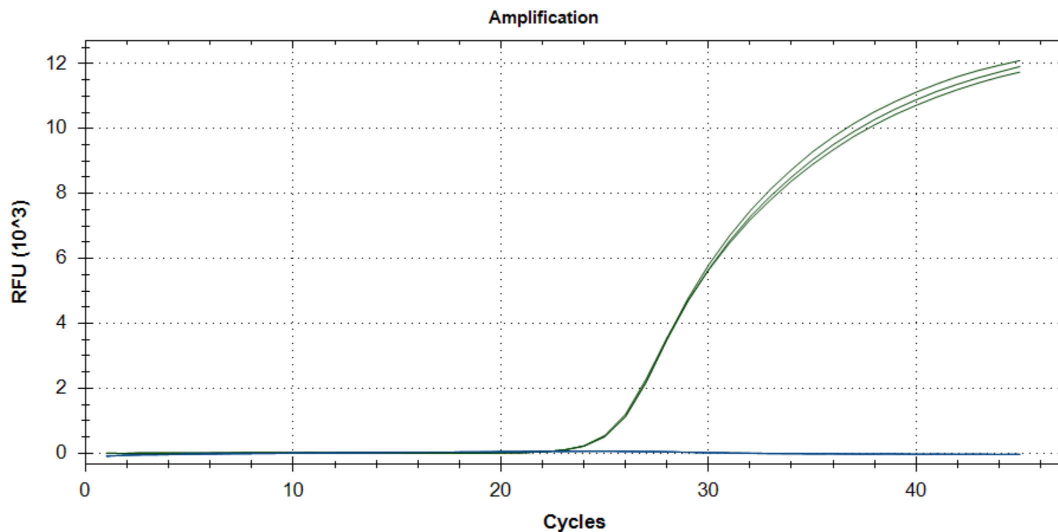
Sample data

Wild Type sample (**WT signal**, **Mutant signal**)



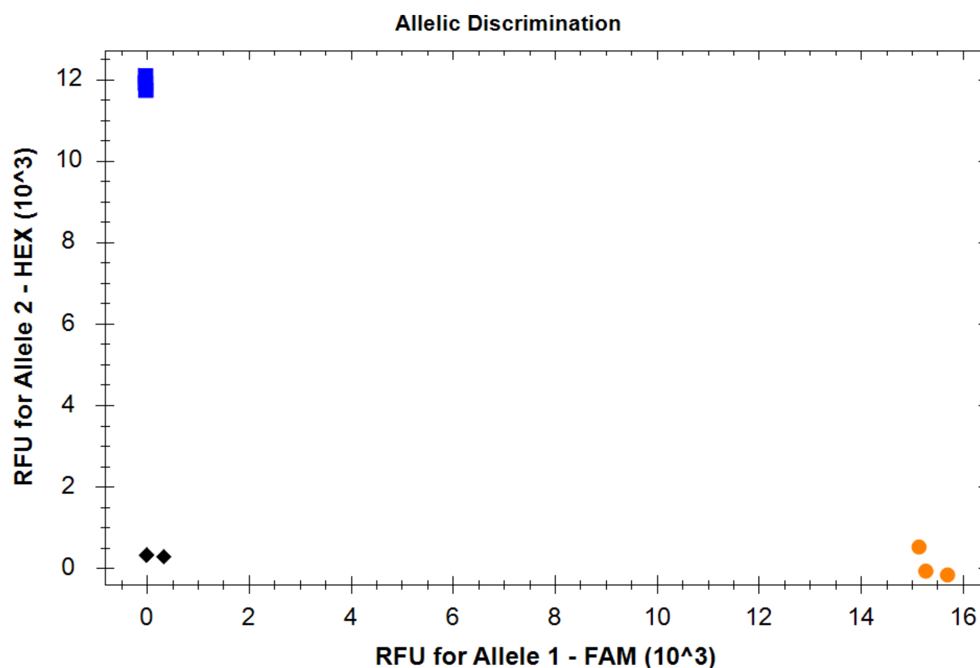
Amplification in the FAM (Blue) channel with no amplification in the HEX/VIC (green) channel indicates the presence of wild type template only.

Variant DNA sample (**WT signal**, **Mutant signal**)



Amplification in the HEX/VIC (green) channel with no amplification in the FAM (blue) channel indicates the presence of mutant template only.

The raw data above can also be visualised by using a cluster analysis, plotting the end point fluorescence data from the FAM channel on one axis and the end point fluorescence data from the HEX/VIC channel on the other axis. Most Real-Time PCR software platforms will perform this analysis automatically so follow the manufacturer's instructions for your software. The data are quickly resolved into clusters corresponding to the wild-type, and mutant variant samples.



Orange dots are indicative of WT presence (amplification only on FAM channel), **blue** dots are indicative of MUT presence (amplification only on HEX channel) and **black** dots indicates residual non-specific amplification.

Optional: Use of Internal Control (CY5)

Internal RNA control (CY5) is available as a separate product, compatible with this kit (Product Code: Z-INT-RNA-CY5-LL).

To confirm the occurrence of PCR, an internal control reaction can be used. Successful Real-Time PCR amplification of the internal control indicates that PCR inhibitors are not present at a high concentration. An internal control primer/probe mix labelled with the CY5 fluorophore can be used with this kit to detect the exogenous RNA template following cDNA synthesis using Real-Time PCR. The primers and CY5 probe allow detection of the control cDNA alongside the target cDNA in a multiplex reaction. Amplification of the control cDNA does not interfere with detection of the target gene even when the target gene is present at low copy number. If a positive result is obtained, this confirms that the reaction was successful and that any negative result for the primary reaction is a true negative.

Resuspension Protocol

To minimize the risk of contamination with foreign RNA, 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.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure that the lyophilised primer and 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
Internal Control primer/probe mix CY5 (BROWN)	165 µl

3. **Resuspend internal control in 500 µl Template Preparation Buffer.**

Component - Resuspend in template preparation buffer	Volume
Internal Control RNA template (BLUE)*	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.

4. **Prepare a complete genotyping reaction mix including the internal control primer/probe mix and the internal control template according to the table below:** Include sufficient reactions for all samples, positive and negative controls.

Component	Volume
Onestep Lyophilised Mastermix	10 µl
SARS-CoV-2 **L452R** primer/probe mix (BROWN)	1 µl
Internal Control primer/probe mix (BROWN)	1 µl
Internal Control RNA template	1 µl
RNase/DNAse free water (WHITE)	2 µl
Final Volume	**15 µl**

5. **Follow Real-Time PCR amplification directions on page 8 with the exception that fluorogenic data should be collected through the FAM, HEX/VIC and Cy5 channels.**

References

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