CoviDetect™

COVID-19 4-plex β γ δ Mutation RT-PCR Assay

In Vitro Diagnostic Assay for Detection of Mutations found in SARS-CoV-2 B.1.617., B.1.617.2, B.1.617.3, B1.1.617.2.1, B.1.351 and P.1

Instructions for use

Please read these instructions carefully before using PentaBase's CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay. It is recommended to save the *Instructions for use* for future use. Purchasers of PentaBase's CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay is only granted the right to use, but no general licensing or patent rights.

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Table of Contents

1. Intended use

The CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR diagnostic assay is a real-time RT (reverse transcription) PCR assay intended for detection of genetic variations from positive SARS-CoV-2 samples. Results are for the detection of genetic variations L452R, P681H, E484K and K417N. Genetic variants of SARS-CoV-2 RNA can be found in the liquid from upper or lower respiratory tracts of infected individuals. Samples can be obtained by nasopharyngeal or oropharyngeal swabs or from sputum or saliva. Note that infection with any variant of SARS-CoV-2 can occur without showing any symptoms.

Negative RT-PCR results do not exclude present or hinder future infection with SARS-CoV-2 virus, or any genetic variations and the result should always be combined with clinical observations, patient history, and epidemiological information.

The CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay is intended for use by health professionals or qualified laboratory personnel specifically instructed and trained in the techniques of real-time PCR as well as proficient in handling biological samples. Medical interventions based on results from this product requires medical authorization.

The *Instructions for Use* or *Quick guide* is also available for download on our webpage: www.pentabase.com.

2. Summary and explanation of the assay

2.1 Indications for use

On December 31, 2019, China alerted the World Health Organization to several cases of unusual pneumonia in Wuhan. This infection has since been identified to be caused by the novel coronavirus, named SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2). COVID-19 is the first pandemic caused by coronavirus with a fast spread rate and potentially fatal infection that resulted in significant worldwide morbidity and mortality.

Viruses constantly change through mutation, which makes the emergence of new variants an expected occurrence, and accurate diagnosis of positive SARS-CoV-2 samples is important as risk related to the spread of new SARS-CoV-2 variants is of current concern. In recent months, a diversification of SARS-CoV-2 due to evolution and adaptable processes has been observed globally.

The CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay is a molecular *in vitro* diagnostic assay based on melting curve analysis for detection and differentiation of SARS-CoV-2 variants in individuals. The Assay is provided as a 4-plex configuration enabling detection of L452R, P681H, E484K and K417N in only one analysis.

2.2. Explanation of the assay

The CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay combines real-time PCR with PentaBase's novel and selective technologies comprising both standard synthetic oligonucleotides as well as proprietary modified synthetic oligonucleotides such as EasyBeacon™ probes and SuPrimers™ for specific and sensitive amplification. The technology applies to several well dispersed real-time PCR instruments as well as PentaBase's own portfolio of instruments using standard procedures. Pentabase-modified oligos contain synthetic DNA analogues comprising a flat heteroaromatic, hydrophobic molecule and a linker. These modifications are inserted into the oligonucleotides at fixed positions during synthesis. With the use of CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assays, detection of four genetics variations from positive SARS-CoV-2 samples can be detected quickly, sensitively, and selectively by real-time RT-PCR followed by a DNA melt analysis in one run.

An EasyBeacon™ probe is similar to a molecular beacon but is based on pentabase-modified oligonucleotides, which keep the probe quenched at all temperatures, without the need of an internal stem structure. This effect is due to hydrophobic interactions between the "pentabases" in the unbound probe. Another feature introduced by the pentabase-modifications is nuclease resistance. These features result in a good signal-to-noise ratio as well as a nuclease resistant probe intact for an affinity study (DNA melt analysis) after the RT PCR reaction.

SuPrimers™ are standard DNA primers modified with one or more PentaBases. Pentabases provide increased specificity and sensitivity and reduce primer-dimer formation.

2.3 Principles of the procedure

The CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay is performed on a Real-Time PCR Instrument for nucleic acid amplification and DNA melting curve analysis for detection and differentiation of SARS-CoV-2 variants in individuals.

CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay is supplied as either Dispense Ready (DR) or Ready-To-Use (RTU) versions. The Dispense Ready version includes Primer-Probe Mix and Master Mix in separate tubes to be dispensed in own plasticware before the addition of RNA. The Ready-To-Use version is pre-dispensed in 8-tube regular profile (0.2 ml) or low profile (0.1 ml) Real-Time PCR strips and needs only the addition of RNA before amplification and DNA melting curve analysis.

The CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay targets four viral sequence of the SARS-CoV-2 (Table 1). Amplification of SARS-CoV-2 sequence, which comprises the site of variation from the sample is achieved by using specific forward and reverse primers surrounding the site of potential variation and an EasyBeacon labelled either with FAM™, HEX™, Cy5™ or Texas Red™ spanning the site of potential variation. A heat- and inhibitor-resistant RT enzyme combined with a thermostable DNA polymerase enzyme is used for reverse transcription and subsequent amplification, and detection of amplified mutation target is achieved by melt analysis.

Table 1. List of detected regions in the CoviDetect™ COVID-19 Mutation RT-PCR Assays.

Targeted Regions	Gene	Fluorophore
L452R	Spike Protein	HEX™
P681H	Spike Protein	Cy5™
E484K	Spike Protein	FAM™
K417N	Spike Protein	Texas Red™

3. Reagens and materials

The materials provided for CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay can be found in Table 2. Materials required, but not provided can be found in Table 4 and 5. Reagent handling and storage can be found in Table 3.

Refer to the section of **Reagent and materials** and **Precautions and handling requirements** for the hazard information for the products.

3.1 CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay reagents

All unopened assay tubes and Master Mix must be stored as recommended in Table 3.

Table 2. List of materials provided for CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay.

CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay Dispense Ready (DR)		
Kit components	**Reagent ingredients**	**Safety symbol and warning**
COVID-19 4-plex β γ δ Mutation RT-PCR Assay	Synthetic DNA	Not applicable
AmpliSmaRT™ One Step RT-qPCR Master Mix	Not applicable	EUH210 Safety data sheet available on request.
CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay Ready-To-Use (RTU)		
Kit components	**Reagent ingredients**	**Safety symbol and warning**
COVID-19 4-plex β γ δ Mutation RT-PCR Assay	Synthetic DNA	Not applicable

3.2 Reagent storage and handling

The CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay is shipped with dry ice or frozen ice bricks. Reagents must be stored and handled as specified in Table 3 immediately upon arrival. The CoviDetect™ 4-plex β γ δ Mutation RT-PCR Assay should be stored in the original packaging and is stable at -20°C until the expiration date. Reagents should not be used past any expiration date indicated on the assay packaging. If the assay's protective packaging is damaged upon receipt or has been shipped at incorrect temperature, please contact PentaBase for instructions. Attention should be paid to the expiration date specified on the pack label. The reagents should be discarded following the disposal instructions in Section 9.

When in use, the assay components should be returned to the freezer promptly after use to minimize the time at room temperature. Repeated thawing and freezing should be kept to a minimum.

Table 3. Reagent storage and reagent expiry conditions.

Reagent	Storage Temperature	Storage Time
CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay **(DR)**	-20°C to -80°C	Stable until expiration date indicated
AmpliSmaRT™ One Step RT-qPCR Master Mix	-20°C to -80°C	Stable until expiration date indicated
CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay **(RTU)**	-20°C to -80°C	Stable until expiration date indicated

3.3 Additional materials required

Table 4. Materials and consumables required but not provided.

Material
Plasticware compatible with the PCR instrument[1]
Pipette Tips
Centrifuge for spinning tubes or plate
Nuclease free H_2O
Collection Kits
Nasopharyngeal Swab
Nasal Swab
Extraction Kit
Viral DNA/RNA Extraction Kit

3.4 Instrumentation required

Table 5. Instrumentation.

Equipment
Nucleic Acid Extraction System
Real-Time PCR instrument (four channels)

4. Precautions and handling requirements

Warnings and precautions

- For *in vitro* diagnostic use.
- Treat all biological specimens, including used CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay tubes and transfer pipettes, as if capable of transmitting infections agents. All biological specimens should be treated with universal precautions, as it is often impossible to know which specimens might be infectious.
- Follow your institution's safety procedures for working with chemicals and handling biological samples.
- Good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary. Wear laboratory coats, laboratory gloves and eye protection when handling biological samples and reagents. Laboratory gloves must be changed between handling different biological samples to avoid contamination of reagents.
- Remove gloves and wash hands thoroughly after handling samples and reagents.
- Do not use damaged CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay tube.
- Do not use a CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay pre-dispensed in a Ready-To-Use PCR tube that has been dropped while open.
- Do not open the tubes or unseal wells during or after amplification following the completion of the PCR program.
- For additional warnings, precautions, and procedures to reduce the risk of contamination for the Nucleic Acid Extraction System or Real-Time PCR Instrument consult the respective System User Guides.
- Dispose of used CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR tube, pipette, and specimen tube according to local, state, and federal regulations for hazardous material.

[1] Only when using Dispense Ready version

- Safety Data Sheets (SDS) are available on request from your local PentaBase representative.
- Due to the high sensitivity of the assays, contamination of the work area with previous samples might cause false results. Therefore, use extreme caution not to contaminate reagents and handle samples according to standard laboratory practice.
- The reagents should not be diluted to a lower concentration than stated in the protocol. This may affect the performance of the assay.
- Do not substitute the reagents with others as it may affect the performance of the assay.
- Specimen collection must be performed using the appropriate swab types as recommended in Table 4. Inadequate or inappropriate sample collection, storage, and transport may yield incorrect or invalid results. DO NOT use cotton or calcium alginate swab, or swabs with wood shafts.
- Ensure there is no sign of leakage from the collection tube prior to running the analysis.

5. Sample collection, transport, and storage

Note: Handle all biological samples and controls as if they are capable of transmitting infectious agents.

5.1 Sample collection

The specimen should be nasopharyngeal or oropharyngeal swabs, saliva, or sputum. Preferentially, use the same sample from which SARS-CoV-2 was detected, to genotype it for mutations. Ineffective or inappropriate sample collection can result in false test results. Training in specimen collection is therefore recommended to ensure the best quality.

5.2 Transport and storage

- Transportation of collected specimens must comply with all applicable regulations for the transport of biological agents.
- Specimens can be stored in suitable buffers at 2-8°C for up to 72 hours after collection.
- If delivery and processing exceed 72 hours, the specimen should be transported or store specimens at -70°C or lower.
- Extracted RNA should always be stored at -70°C or lower in an RNase free environment.

6. Instructions for use

6.1 Procedural notes

- Do not use CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay or AmpliSmaRT™ One-Step RT-qPCR Master Mix after expiry dates.
- Do not reuse consumables. They are for one-time use only.

6.2 Reagent Preperation

6.2.1 Dispense Ready

a. Add **10 µl** 2x AmpliSmaRT™ One-Step RT-qPCR Master Mix to each PCR tube or well.
b. Add **5 µl** 4x multiplex primer/probe mix to the PCR tubes or wells
c. Add **5 µl** of the template to each PCR tube or well. One patient is analyzed for four different mutations in a single PCR tube or well.
d. Close all PCR tube or seal plates.

6.2.2 Ready-To-Use

a. Spin down the PCR strips or plates before the adding of the template to ensure that all reagents are collected at the bottom.
b. Add **5 µl** of the template to each PCR tube or well in the plate. One patient is analyzed in a single PCR tube or well.
c. Close all PCR tube or seal plates.

6.2.3 SARS CoV-2 RNA control

When running the melting analysis, we recommend a SARS CoV-2 wild-type RNA control to be included. 200 µl (20 copies/µl) should be added during the RNA extraction procedure.

6.3 Running CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay

a. Spin down the PCR strips or plates (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the strips or wells and to eliminate air bubbles in the mixes.
b. Place the PCR strips or plate in the Real-Time PCR instrument and run the program listed in Table 6.

Table 6. RT-PCR protocol for running CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay.

Protocol	Temperature [°C]	Time	Cycles	Channel
Reverse transcription	52	300 sec	1	-
Hold	95	10 sec	1	-
3-step amplification	95 58 72	5 sec 30 sec 60 sec	45	HEX™ (538nm/551nm) Cy5™ (647nm/665nm) FAM™ (495nm/516nm) Texas Red™ (596nm/615nm) Acquire on FAM™, HEX™, Cy5™ and Texas Red™ in second step
Continuous Melt	95 45	60 sec	1	HEX™ (538nm/551nm) Cy5™ (647nm/665nm) FAM™ (495nm/516nm) Texas Red™ (596nm/615nm)
	Up to 75	10 readings/°C ***Note**		
***Note**: For CFX use 2 readings/°C.				

7. Data Analysis

The melting temperature and graphs for the CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay, and how to analyze the data are listed in the following sections. It is not possible to determine the genotype in case there is no melting curve of the sample. If more specimen is available, repeat the extraction and run the test again. If all markers remain negative after repeating the test, no diagnosis can be concluded, and if possible, a new specimen should be collected for testing or the sample should be sent for sequencing.

7.1 Interpretation of results

7.1.1 Score Chart

An overview of the possible outcomes of the different CoviDetect™ COVID-19 4-plex β γ δ Mutation Assays are shown in Table 7.

Table 7. Analysis outcomes based on target melting curves. Conclusions are based on target melting curve compared to an WT SARS-Co-V-2 RNA control. R=P681R, Q=E484Q, T=K417T.

	B.1.351	P.1	B.1.617	B.1.617.2	B.1.617.3	B.1.617.2.1
L452R	-	-	+	+	+	+
P681H	-	-	R	R	R	R
E484K	+	+	Q	-	Q	-
K417N	+	T	-	-	-	+

7.1.2 BaseTyper™

7.1.2.1 L452R

Table 8. Analysis outcomes based on target melting curves on BaseTyper™. Genotype is based on target melting curve compared to a WT SARS-Co-V-2 RNA control.

Melting peak [°C]	Channel	Conclusion
>58.0	HEX™	The sample is positive for the L452R mutation
51.5-55.5	HEX™	The sample is negative for the L452R mutation
<51.5	HEX™	The sample is negative for the L452R mutation, but most likely contain a different mutation in the probe area.
No peaks	HEX™	Not possible to determine genotype

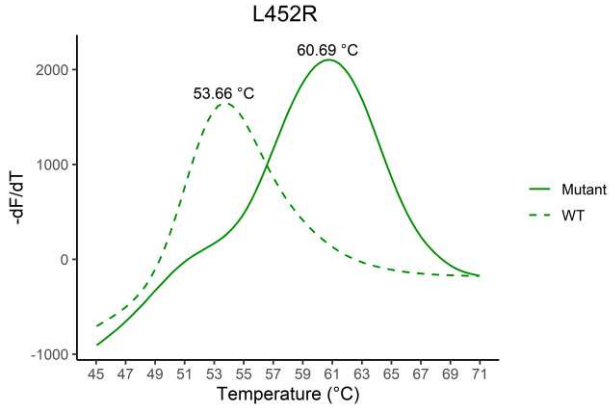

Figure 1. The melting curve of a sample positive for L452R analysed on the BaseTyper™. The L452R mutation has a higher affinity for the probe and will melt at a higher temperature.

7.1.2.2 P681H

Table 9. Analysis outcomes based on target melting curves on BaseTyper™. Conclusions are based on target melting curve compared to an WT SARS-Co-V-2 RNA control.

Melting peak [°C]	Channel	Conclusion
>66.0	Cy5™	The sample is positive for the P681H mutation
61.5-64.5	Cy5™	The sample is negative for the P681H mutation
57.5-60	Cy5™	The sample is most likely positive for the P681R mutation
<57.5	Cy5™	The sample is negative for the P681H mutation, but most likely contain a different mutation in the probe area.
No peaks	Cy5™	Not possible to determine genotype

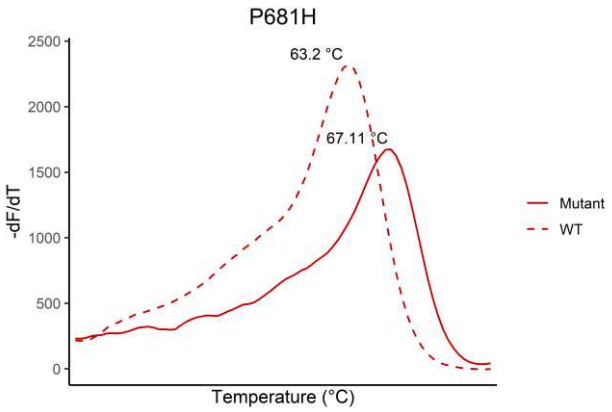

Figure 2. The melting curve of a sample positive for P681H analysed on the BaseTyper™. The P681H mutation has a higher affinity for the probe and will melt at a higher temperature.

7.1.2.3 E484K

Table 10. Analysis outcomes based on target melting curves on BaseTyper™. Conclusions are based on target melting curve compared to an WT SARS-Co-V-2 RNA control.

Melting peak [°C]	Channel	Conclusion
>60.0	FAM™	The sample is positive for the E484K mutation
56.0-59.0	FAM™	The sample is most likely positive for the E484Q mutation
52.0-55.0	FAM™	The sample is negative for the E484K mutation
<52.0	FAM™	The sample is negative for the E484K mutation, but most likely contain a different mutation in the probe area.
No peaks	FAM™	Not possible to determine genotype

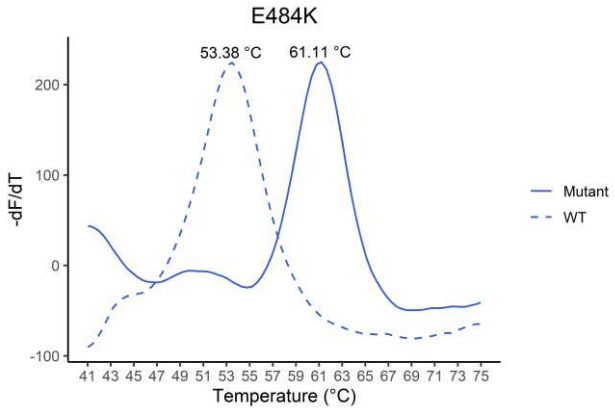

Figure 3. The melting curve of a sample positive for E484K analysed on the BaseTyper™. The E484K mutation has a higher affinity for the probe and will melt at a higher temperature.

7.1.2.3 K417N

Table 11. Analysis outcomes based on target melting curves on BaseTyper™. Conclusions are based on target melting curve compared to an WT SARS-Co-V-2 RNA control.

Melting peak [°C]	Channel	Conclusion
>61.5	Texas Red™	The sample is positive for the K417N mutation
55.0-57.5	Texas Red™	The sample is negative for the K417N mutation
52.0-54.0	Texas Red™	The sample is most likely positive for K417T mutation
<52.0	Texas Red™	The sample is negative for the K417N or K417T mutation, but most likely contain a different mutation in the probe area.
No peaks	Texas Red™	Not possible to determine genotype

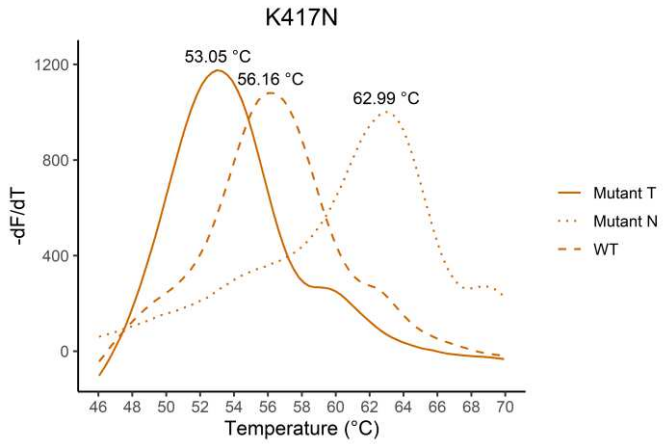

Figure 4. The melting curve of a sample positive for K417N analysed on the BaseTyper™. The K417N mutation has a higher affinity for the probe and will melt at a higher temperature.

8. Limitations

- A negative test result does not exclude infection with SARS-CoV-2, and treatment of a patient should not exclusively be based on the test result. Multiple specimens collected at different times from the same patient may be necessary to detect the virus since it is unknown when the viral levels in the body will peak.
- Incorrect collection, transportation, or handling of the sample could cause false-negative test results. Also, a very low amount of virus RNA in the specimen or amplification inhibitors could give false-negative test results.
- Do not use reagents that have expired.
- The assays cannot exclude that the patient is infected with other viruses or bacteria.

9. Disposal

The disposal of unused kit reagents, biological samples and post-amplified PCR tubes or plates according to local, state and federal regulations.

10. Symbols

The following symbols are used in labeling for CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay products.

Table 11. Symbols used in labelling for CoviDetect™ COVID-19 4-plex β γ δ Mutation RT-PCR Assay products.

	Date of manufacture		In vitro diagnostic medical device
	Use-by date		Do not reuse


Contains sufficient for <*n*>


Manufacture


Temperature limit


CE marking of conformity; this device is in conformity with the applicable requirements for CRof an *in vitro* diagnostic medical device


Consult instructions for use

11. Manufacturer and distributors

For technical assistance in Denmark please contact PentaBase ApS:
Petersmindevej 1A
DK-5000 Odense, Denmark

Telephone: (+45) 36 96 94 96
Email: support@pentabase.com
Webpage: www.pentabase.com
For technical assistance in all other countries, contact your local distributor. A complete list of distributors is available at www.pentabase.com.