CoviDetect™ Variants

COVID-19 RT-PCR Assay

Assays for Detection of Mutations found in SARS-CoV-2 for Research use Purposes

INSTRUCTIONS FOR USE

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REFERENCE NUMBERS

For reference numbers on available mutation detection assays, see appendix A, or contact PentaBase A/S or your local distributor

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RUO

For Research Use Only. Not to be used for diagnostic purposes.

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1 Intended purpose

CoviDetect™ Variants COVID-19 RT-PCR Assay is a device for closed tube RT (Reverse Transcription) Polymerase Chain Reaction (PCR) with end-point melt-analysis intended for identification of genetic variations in positive severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) samples for research use purposes. The assay is used with real-time PCR systems. Genetic variants of SARS-CoV-2 RNA can be found in samples from the upper or lower respiratory tracts of infected individuals. Samples can be obtained by nasopharyngeal swabs, oropharyngeal swabs, and/or saliva. Samples can be purified on automated platforms or in manual workflows. This assay is not for in vitro diagnostic.

1.1 Intended user

CoviDetect™ Variants COVID-19 RT-PCR Assay is intended for use by healthcare professionals or qualified laboratory personnel specifically instructed and trained in the techniques of real-time PCR as well as proficient in handling biological samples.

2 Test principle

Accurate detection of mutations within the genome of SARS-CoV-2 for surveillance of variants in individuals infected with SARS-CoV-2 to prevent spread of Variants of Concern (VOC).
To meet the need for faster testing of different SARS-CoV-2 variants, the CoviDetect™ Variants COVID-19 RT-PCR Assay has been developed as a tool for significant reduction of the answer time compared to common alternative procedures.

2.1 Explanation of the assay

The CoviDetect™ Variants COVID-19 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 Mutation RT-PCR Assays, detection of genetic 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.
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.

2.2 Product variants

CoviDetect™ Variants COVID-19 RT-PCR Assay is supplied as either Dispense Ready (DR) or Ready-to-Use (RTU) versions. The DR 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 qPCR strips and needs only the addition of RNA before amplification.

2.3 Principle of the procedure

CoviDetect™ Variants COVID-19 RT-PCR Assay targets the spike amino acid change in the SARS-CoV-2 genome. Amplification of the SARS-CoV-2 sequence, which comprises the site of the variation from the sample, is achieved by using specific forward and reverse primers surrounding the site of variation in the spike region and an EasyBeacon labelled either with PentaYellow™ 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.

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3 Reagents and materials

The materials provided for CoviDetect™ Variants COVID-19 RT-PCR Assay can be found in **Table 1**. Materials required, but not provided can be found in **Table 2**.

3.1 Storage

Refer to the label for expiry date. This assay should be stored at -20°. Repeated thawing and freezing should be kept to a minimum.

3.1.1 In-use stability

When in use, the assay components should be returned to the freezer promptly after use to minimize the time at room temperature and exposure to light.
Used Ready-to-Use PCR tubes and dispensed Primer-Probe and Master Mix should be disposed following your local guidelines on disposal of biological waste. The reagents included in CoviDetect™ Variants COVID-19 RT-PCR Assay are not for reuse.

3.2 Materials provided

Table 1. List of materials provided with the CoviDetect™ Variants COVID-19 RT-PCR Assay as either Dispense Ready (DR) or Ready-to-Use (RTU).

Dispense Ready (DR)	
Kit components	**Reagent ingredients**
CoviDetect™ Variants COVID-19 RT-PCR Assay Primer/Probe mix	Synthetic DNA.
AmpliSmaRT™ One Step RT-qPCR Master Mix	Enzymes and buffer for reverse transcription and qPCR.
COVID-19 RT-qPCR Positive Extraction Control	Buffer solution including inactivated SARS-CoV-2 RNA and human DNA.
COVID-19 RT-qPCR Negative Extraction Control	Buffer solution free of SARS-CoV-2 RNA
Ready-to-Use (RTU)	
Kit components	**Reagent ingredients**
CoviDetect™ Variants COVID-19 RT-PCR Assay	Synthetic DNA. Enzymes and buffer for reverse transcription and qPCR
COVID-19 RT-qPCR Positive Extraction Control	Buffer solution including inactivated SARS-CoV-2 RNA and human DNA
COVID-19 RT-qPCR Negative Extraction Control	Buffer solution free of SARS-CoV-2 RNA

3.3 Materials and instruments required but not provided

Materials and instruments required but not provided are listed in **Table 2**. CoviDetect™ Variants COVID-19 RT-PCR Assay is designed to run on open platforms and has currently been validated using samples purified with the BasePurifier™ Nucleic Acid Extraction System (PentaBase ref, no. 715) and analysed with the BaseTyper™ (PentaBase, ref. no. 750-751) or CFX96/384 (Bio-Rad, ref. no. 1855484) real-time PCR instruments. There is currently no evidence available to PentaBase suggesting that there are certain relevant commercially available nucleotide purification methods and instruments or four-channel real-time qPCR instruments that are not compatible with the CoviDetect™ Variants COVID-19 RT-PCR Assay. However, when running CoviDetect™ Variants COVID-19 RT-PCR Assay on instruments not validated by PentaBase, it is highly recommended that a specific validation is performed, using clinical samples and reference controls, to verify cycle thresholds and cut-offs. Please contact PentaBase or your local distributor for support.

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Table 2. Materials and consumables required but not provided.

Materials
Plasticware compatible with the used real-time PCR instrument[1]
Pipettes (1-10 µl, 10-100 µl)
Pipette Tips
Centrifuge for spinning PCR tubes, strips or plates
Collection Kits (one of the following)
Oropharyngeal swab
Saliva collector
RNA extraction method or instrument (E.g. BasePurifier™*, PentaBase A/S, Ref. no. 715)
RNA Extraction kit (E.g. BasePurifier™ Viral DNA and RNA Extraction Kit, PentaBase A/S, Ref. No. 727)
Real-time qPCR
Real-time PCR instrument with 4 channels such as: BaseTyper™ (PentaBase A/S, Ref. No. 750), CFX96/384™ (Bio-Rad), or QuantStudio™ 5 (Applied biosystems)

4 Warnings and precautions

- For research use only.
- Treat all biological specimens as if capable of transmitting infectious 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 reagents that have expired.
- Do not use damaged CoviDetect™ Variants COVID-19 RT-PCR Assay tubes.
- Do not use a CoviDetect™ Variants COVID-19 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 completing the PCR program.
- Baseline drift, a slowly rising signal in the amplification plot with no or late exponential phase, may lead to false positive results if not corrected. Refer to section 8.1 for more information.
- Consult relevant nucleic acid extraction and real-time qPCR Instrument User Guides for additional warnings, precautions, and procedures to reduce the risk of contamination.
- Dispose of used CoviDetect™ Variants COVID-19 RT-PCR Assay, pipette tips, and specimen tubes according to local, state, and federal regulations for hazardous material.
- Due to the high sensitivity of the assays, contamination of the work area with previous positive samples might cause false-positive results. Therefore, use extreme caution not to contaminate reagents and handle samples according to good laboratory practice.
- Minimize the exposure of CoviDetect™ Variants COVID-19 RT-PCR Assay to light due to the presence of light sensitive EasyBeacon™ probes.
- 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 appropriate swab types. Inadequate or inappropriate sample collection, storage, and transport may yield incorrect or invalid results. Note: Cotton or calcium alginate swabs, or swabs with wood shafts have not been thoroughly tested with CoviDetect™ Variants COVID-19 RT-PCR Assay.
- Specimen collection should be performed at least 30 minutes after tooth brushing, eating, or drinking to decrease the risk of inaccurate results.
- Ensure there is no sign of leakage from the collection tube before running the analysis.

[1] Only when using Dispense Ready version

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5 Sample handling

Handle all biological samples and controls as if they are capable of transmitting infectious agents. Please follow your local guidelines for handling and disposal of the sample material.

5.1 Sample collection

The specimens should be collected from nasopharyngeal swab, oropharyngeal swab or saliva. 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, such as viral transport media. Please follow the specific instructions for use of the transport vial

5.3 Purification

Specimens should be subjected to RNA purification prior to analysis by CoviDetect™ Variants COVID-19 RT-PCR Assay using suitable RNA purification methods such as the BasePurifier™ Nucleic Acid Extraction System (ref. no. 715, PentaBase A/S) and the *Viral DNA and RNA Extraction Kit* (Ref. No. 727, PentaBase A/S) according to the manufacturer's instructions.
Please be aware that the outcome from the purification method may influence the results of the CoviDetect™ Variants COVID-19 RT-PCR Assay.

5.4 Positive and Negative extraction controls

At least one Positive and one Negative extraction control should be included in each purification and subsequent RT-qPCR run. There are enough positive and negative control samples included in the kit to purify an average of four samples per run. If less than four samples are purified on average per run, additional controls can be ordered from PentaBase or your local distributor (ref. no. IVDS-42 and IVD-43).

The Positive extraction control contains 20 SARS-CoV-2 RNA copies and 0.5 ng human genomic DNA per microliter, while the Negative extraction control contains nuclease free water.

NOTE: The Positive extraction control cannot be added directly to the CoviDetect™ Variants COVID-19 RT-PCR Assay but must be subjected to a nucleotide extraction procedure first. Use the maximum amount (up to 200 µl) of Positive and Negative Extraction Control recommended by the supplier of the RNA purification kit that you use.

6 Procedure

6.1 Dispense Ready

1. Add 10 µL 2x AmpliSmaRT™ One-Step RT-qPCR Master Mix to each PCR tube (vial, strip or plate).
2. Add 5 µL 4x primer/probe multiplex mix to the PCR tubes or wells.
3. Add 5 µL of the template (sample, positive control or negative control) per reaction.
4. Seal all tubes.
   'Optional step: Briefly vortex PCR tubes (2-3 sec.) to enhance elimination of air bubbles'
5. Spin down the PCR tubes (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the tubes and to eliminate air bubbles.
6. Place the PCR tubes in the real-time PCR instrument and run the RT-qPCR program (**Table 3**).

6.2 Ready-to-Use

1. Spin down the PCR strips (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the strips to eliminate air bubbles.
2. Open PCR tubes and add 5 µL of template (sample, positive, or negative control). Continue with different templates and tubes until all templates are added to individual tubes.
3. Seal all tubes.
   'Optional step: Briefly vortex PCR strips (2-3 sec.) to enhance elimination of air bubbles'
4. Spin down the PCR strips (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the strips and to eliminate air bubbles.
5. Place the PCR strips in the real-time qPCR instrument and run the RT-qPCR program (**Table 3**).

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6.3 RT-qPCR program

Table 3. RT-PCR protocol for running CoviDetect™ Variants N501Y COVID-19 RT-PCR Assay.

Protocol	Temperature [°C]	Time [sec]	Cycles	Channel
Stage 1				
Reverse transcription	52	300	1	-
Hold	95	30	1	-
Stage 2				
Cycling	94 60	15 45	45	HEX™ (538nm/551nm) Cy5™ (647nm/665nm) FAM™ (495nm/516nm) Texas Red™ (596nm/615nm) Measured fluorescence intensity during annealing/elongation (60°C)
Stage 3				
Hold	95	60	1	-
Continuous Melt	40 40 up to 80	60 10 readings/°C*	1	HEX™ (538nm/551nm) Cy5™ (647nm/665nm) FAM™ (495nm/516nm) Texas Red™ (596nm/615nm) Measured fluorescence intensity during melting
***Note:** For CFX use 2 readings/°C				

7 Data Analysis

The melting temperature and graphs for the CoviDetect™ Variants COVID-19 RT-PCR Assay, and how to analyse 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 result can be concluded, and if possible, a new specimen should be collected for testing, or the sample should be sent for sequencing.

7.1 Baseline and threshold settings

Results from CoviDetect™ Variants COVID-19 RT-PCR Assay can be analysed using both automatic and manual baseline and threshold settings. If automatic baseline and threshold settings are used, it is recommended to also perform a visual inspection of the amplification curves since some cases might need manual adjustment of baseline and/or threshold due to baseline drift and/or incorrect baselining. When setting the baseline manually, it is recommended to use 5 cycle intervals such as from cycle 10 to cycle 15 depending on the amplification curve of the sample. When setting the threshold manually, the threshold should be set to cross at the beginning of the exponential PCR phase and above any background or baseline fluorescence. If there is significant background or baseline fluorescence, adjust the baseline interval.

7.2 Interpretation of results

Results from CoviDetect™ Variants COVID-19 RT-PCR Assay are evaluated in the continuous melt analysis (stage 3 in **Table 3**). The assay the melting temperature of the probe when bound to a sample. The sample can be evaluated for the mutation, using the respective table and figure under each of the mutations sections. In case of no melting peak, the sample is invalid and cannot be subtyped.

7.2.1 N501Y

Table 4. 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
>62.0	HEX™	The sample is positive for the N501Y mutation
56.0-60.5	HEX™	The sample is negative for the N501Y mutation
<56.0	HEX™	The sample is negative for the N501Y mutation, but most likely contain a different mutation in the probe area
No peaks	HEX™	Not possible to determine genotype

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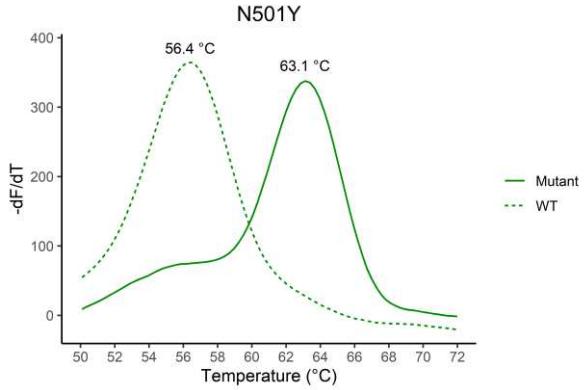

Figure 1. The melting curve of a sample positive for N501Y analysed on the BaseTyper™. The N501Y mutation has a higher affinity for the probe and will melt at a higher temperature.

7.2.2 P681H

Table 5. 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
>64.0	Cy5™	The sample is positive for the P681H mutation
59.5-62.0	Cy5™	The sample is most likely positive for the P681R mutation
55.0-58.5	Cy5™	The sample is negative for the P681H mutation
<55.0	Cy5™	The sample is negative for the P681H mutation, but most likely contain a different mutation in the probe area

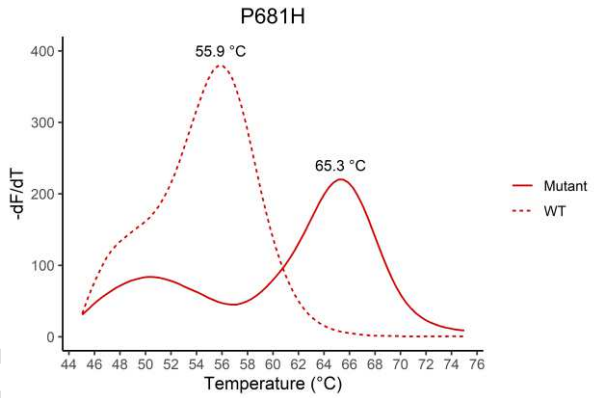

Figure 2. The melting curve of a sample positive for P681H analysed on the BaseTyper™. The P681H mutation has a higher affinity for the probe and will melt at a higher temperature.

7.3 Negative samples

The sample is negative for N501Y when the melting temperature is <62.0°C.
If the sample has a lower melting point than <56.0°C, then the sample will be negative for N501Y but have another mutation in the binding site of the probe. For monitoring of new variants, it is recommended to send the sample for sequencing. (See table 6)

7.4 Invalid samples

In case of no melting peak, the sample is considered invalid. For invalid results, if more specimen is available, repeat the extraction and run the test again. If it remains negative after repeating the test, the test is inconclusive, and if possible, a new specimen should be collected for a new test.
In case of no signals in any of the samples in a run, check that the correct PCR program has been used (Section 6.3).

7.4.1.1 No positive control signals

In case of no signals for the positive control, check if the correct PCR program has been used (Section 6.3) and that a positive control has been included in the run tube. The correct program is found in section 6.3. If the correct program has

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been used and there is no signal in any of the samples repeat the extraction and run the test again. If it remains negative after repeating the test, contact PentaBase A/S or your local distributor for support.

7.4.1.2 Signal in NTC

Signals in the NTC sample(s) indicate contamination of the reagents and thus all positive samples in the run should be considered invalid. Make sure that the threshold for the melting curve has been set correctly and/or repeat the extraction of all samples and run the test again. If the problem persists, find the cause of contamination by checking or replacing all potential sources of the contamination such as pipettes and instruments. If the contamination cannot be located contact PentaBase A/S or your local distributor for support.

8 Performance evaluation

8.1 Analytical sensitivity – Limit of Detection

The limit of detection of CoviDetect™ Variants N501Y COVID-19 RT-PCR Assay using BaseTyper™ Real-Time PCR instrument independent of extraction method was determined using SARS-CoV-2 RNA (Twist Bioscience, Cat. no. 102015) diluted in a 25ng wild type human genomic DNA background. The Limit of Detection of CoviDetect™ Variants N501Y COVID-19 RT-PCR Assay is 50 copies.

Table 6. Limit of detection (LOD) of CoviDetect™ Variants N501Y COVID-19 RT-PCR Assay using SARS-CoV-2 RNA spiked into wild-type human DNA. RT-qPCR of SARS-CoV-2 RNA was performed using the BaseTyper™ Real-Time PCR instrument.

Copies	Observations (n)	Positives	Positives (%)
0	22	0	**0**
20	20	15	**75**
50	20	20	**100**
100	20	20	**100**
250	20	20	**100**

8.2 Inclusivity

CoviDetect™ Variants N501Y COVID-19 RT-PCR Assay oligonucleotides were blasted against the 6 most common SARS-CoV-2 strains (Figure 2)

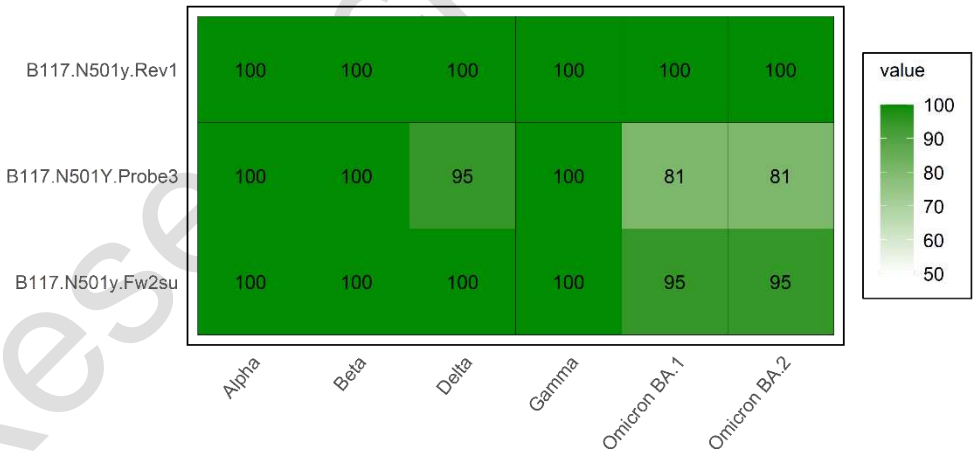

Figure 3. CoviDetect™ Variants N501Y COVID-19 RT-PCR Assay SARS-SoV-2 specific oligonucleotides blasted against the different SARS-CoV-2 variants. *The score of the probe against the BA.1 and BA.2 are lower than reality due to the sequences being trimmed.

The N501Y probe did not fit with the Omicron BA.1 and BA.2. This results in the assay not being able to detect the mutation in an Omicron patient. The melting temperature of the assay will end up being lower even when the N501Y mutation is in the sample. The probe cannot find the Delta as well, but it was expected due to no mutation in the spike N501 amino acid spot.

The B117.N501Y.Fw2su primer has a mismatch in the middle of the primer in the BA.1 and BA.2 variants. This should not have an impact on the assay.

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8.3 Clinical evaluation

The clinical performance was evaluated from 82 positive SARS-CoV-2 leftover nasopharyngeal, oropharyngeal swabs and saliva samples were analysed CoviDetect™ Variants N501Y COVID-19 RT-PCR Assay and Sanger sequencing of the SARS-CoV-2 Spike region. The extraction of RNA was performed using the Viral DNA and RNA Extraction Kit for the BasePurifier™ Nucleic Acid Extraction Instrument. RT-qPCR was performed using BaseTyper™ Real-Time PCR Instrument. The analysis was performed using automatic baseline and threshold settings. Evaluation summary is shown in table 8.

Table 8. Summary of clinical evaluation of CoviDetect™ Variants N501Y COVID-19 RT-PCR Assay. The assay is found more sensitive than Sanger sequencing. *A few of the positive samples did not agree. The quality of the Sanger sequencing was low in these cases resulting in a risk of a false positive N501Y result.

Positive SARS-CoV-2 samples	Assay	Sanger sequencing	Agreement
Positive	43	36	92.3% (PPA)*
Negative	37	20	100% (NPA)
Invalid	2	26	

9 Limitations

- Performance of the CoviDetect™ Variants COVID-19 RT-PCR Assay has only been tested on the specimens from nasopharyngeal swabs, oropharyngeal swabs, or saliva.
- 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.
- If mutations occur in the targeted region of the virus, it may affect the sensitivity of the test and may result in false, negative results.
- The test cannot exclude that the patient is infected with other viruses or bacteria.

10 Symbols

The following symbols are used in labelling of CoviDetect™ Variants COVID-19 RT-PCR Assay.


Date/country of manufacture

For Research Use Only. Not for Diagnostic Purposes.


Use-by date


Do not reuse

Contains sufficient for <*n*>


Manufacturer


Temperature limit


Consult electronic available instructions for use

11 Manufacturer

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For technical assistance please contact your local distributor or PentaBase A/S. A complete list of distributors is available at www.pentabase.com.
NOTICE TO USERS: any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Appendix A

Below is a table with all available CoviDetect™ Variants COVID-19 RT-PCR Assay. Contact PentaBase A/S (section 11) or your local distributor for guidance.

REFERENCE NUMBERS

N501Y

Dispense Ready (DR)

8084 (200 reactions)

Ready-to-Use (RTU)

8053 (96 reactions)

P681H

Dispense Ready (DR)

8056 (200 reactions)

Ready-to-Use (RTU)

8055 (96 reactions)

K417N

Dispense Ready (DR)

10001 (200 reactions)

Ready-to-Use (RTU)

10002 (96 reactions)

N439K

Dispense Ready (DR)

8072 (200 reactions)

Ready-to-Use (RTU)

8086 (96 reactions)

E484K

Dispense Ready (DR)

8063 (200 reactions)

Ready-to-Use (RTU)

8062 (96 reactions)

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