CoviDetect™

COVID-19 Multiplex RT-qPCR Assay

In vitro Diagnostic Assay for Detection of SARS-CoV-2

INSTRUCTIONS FOR USE

REFERENCE NUMBERS

Dispense Ready (DR)
8010 (200 reactions)
8015 (500 reactions)

Ready-to-Use (RTU)
8079 (96 reactions)

Version 5.0
Last revised: January 2023

CE IVD

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1 Intended purpose

CoviDetect™ COVID-19 Multiplex RT-qPCR Assay is a semi-quantitative RT (Reverse Transcriptase) real-time Polymerase Chain Reaction (PCR) assay intended for the detection of nucleic acids from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. The assay is used with real-time PCR systems. SARS-CoV-2 RNA can be found in the upper or lower respiratory tracts of infected individuals. Samples can be obtained either by nasopharyngeal swabs, oropharyngeal swabs, and/or saliva. Samples can be purified on automated platforms or in manual workflows. The results of the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay may aid in the diagnosis of coronavirus disease 2019 (COVID-19). The assay is provided in a multiplex format analysing two SARS-CoV-2 viral targets and one human target sampling control in the same PCR reaction tube.

1.1 Intended user

CoviDetect™ COVID-19 Multiplex RT-qPCR Assay is intended for use by healthcare professionals or qualified laboratory personnel 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 authorisation.

2 Test principle

2.1 Explanation of the assay

The CoviDetect™ COVID-19 Multiplex RT-qPCR Assay combines real-time qPCR with PentaBase's novel and selective technologies comprising both standard synthetic oligonucleotides as well as proprietary modified synthetic oligonucleotides such as HydrolEasy® probes and SuPrimers™ for specific and sensitive amplification. The technology applies to several common real-time PCR instruments as well as PentaBase's own portfolio of instruments using standard procedures. Pentabase-modified oligonucleotides contain at least one synthetic DNA analogue comprising a flat heteroaromatic, hydrophobic molecule and a linker. These modifications are inserted into the oligonucleotides at fixed positions during synthesis. Using the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay, the presence of virus RNA in a sample can be detected quickly, sensitively, and selectively by real-time RT-PCR analysis.

2.1.1 HydrolEasy® probe

A HydrolEasy® probe is similar to a standard hydrolysis probe (also referred to as a TaqMan® probe[1]) labelled with a fluorophore at the 5' end and a quencher at the 3' end, but with the addition of pentabases. HydrolEasy® probes are based on oligonucleotides modified with pentabases, giving the probe a significantly improved signal-to-noise ratio, higher specificity, and higher sensitivity compared to conventional hydrolysis probes. HydrolEasy® probes in the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay are labelled with either FAM, HEX, or Cy5.

2.1.2 SuPrimers™

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

2.2 Product variants

CoviDetect™ COVID-19 Multiplex RT-qPCR Assay is supplied as either Dispense Ready (DR) or Ready-to-Use (RTU). The DR version includes Primer-Probe Mix and Master Mix in separate tubes, which need to be dispensed into suitable plasticware before the addition of template. The RTU version is pre-dispensed and only need the addition of RNA before RT-qPCR.

2.3 Principle of the procedure

The CoviDetect™ COVID-19 Multiplex RT-qPCR Assay is designed for use with real-time PCR instruments for nucleic acid amplification and detection of the target sequence in biological samples.

The CoviDetect™ COVID-19 Multiplex RT-qPCR Assay targets two viral sequences of the SARS-CoV-2 nucleocapsid protein gene (named N1 and N2) (**Table 1**). Selective amplification of N1 and N2 sequences are achieved by using sequence-specific forward and reverse primers together with sequence specific HydrolEasy™ probes labelled with FAM or HEX, respectively. Selective amplification of a region within the human Ribonuclease P gene (*RNase P*) is used as sampling control and is achieved by combining non-competitive sequence-specific forward and reverse primers with a sequence specific Cy5-labelled HydrolEasy™ probe which has no homology with the coronavirus genome. The amplified target is detected by measuring the increased fluorescence generated by release of the fluorophore from the fluorescently labelled oligonucleotide probe specifically targeting either of the SARS-CoV-2 sequences or the human sequence of interest. A heat- and inhibitor-resistant RT enzyme combined with a thermostable DNA polymerase enzyme is used for reverse transcription and subsequent amplification.

[1]Taqman is a registered tradename of Roche Molecular Systems, Inc

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Table 1. Amplified genomic regions by the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay.

Targeted Regions	Gene	Fluorophore
N1	Nucleocapsid protein gene marker	FAM
N2	Nucleocapsid protein gene marker	HEX
RNase P	Human RNase P	Cy5

3 Reagents and materials

The materials provided with the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay can be found in **Table 2**. Materials and instruments required, but not provided can be found in **Table 3**.

3.1 Storage and stability

Refer to the label for expiry date. This assay should be stored at -20°C. Repeated thawing and freezing should be kept to a minimum and should not exceed 12 freeze-thaw cycles.

3.1.1 In-use stability

When in use, the assay components should be returned to the freezer promptly after use to minimise 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™ COVID-19 Multiplex RT-qPCR Assay are not for reuse.

3.2 Materials provided

Table 2. List of materials provided with the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay as either Dispense Ready (DR) or Ready-to-Use (RTU).

Dispense Ready (DR)	
CoviDetect™ COVID-19 Multiplex RT-qPCR Assay	
Kit components	**Content**
COVID-19 Multiplex RT-qPCR 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)	
CoviDetect™ COVID-19 Multiplex RT-qPCR Assay	
Kit components	**Content**
COVID-19 Multiplex RT-qPCR 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 3**. CoviDetect™ COVID-19 Multiplex RT-qPCR Assay is designed to run on open platforms and has been validated using samples purified with the BasePurifier™ 32 Nucleic Acid Extraction Oscillating System (BasePurifier™, PentaBase ref, no. 715) and analysed with the BaseTyper™ 48 and BaseTyper™ 96 (PentaBase, ref. no. 758-759), CFX384 (Bio-Rad, ref. no. 1855484) or LightCycler® 480 II (Roche, ref. no. 05 015 278 001) 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 three-channel real-time qPCR instruments that are not compatible with the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay. However, when running CoviDetect™ COVID-19 Multiplex RT-qPCR 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. Contact PentaBase or your local distributor for support.

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Table 3. Materials and instruments required but not provided.

Materials
Plasticware compatible with the used real-time PCR instrument[2]
Pipettes (1-10 µL, 10-100 µL)
Pipette Tips
Centrifuge for spinning PCR tubes, strips or plates
Collection Kits (one of the following)
Nasopharyngeal swab
Oropharyngeal swab
Saliva collector
RNA extraction method or instrument
RNA Extraction kit (E.g. Viral DNA and RNA Extraction Kit, PentaBase A/S, ref. no. 727)
Extraction instrument (E.g. BasePurifier™, PentaBase A/S, ref. no. 715)
Real-time qPCR
Real-time PCR instrument like: BaseTyper™ (PentaBase A/S), CFX96/384™ (Bio-Rad), LightCycler® 480 (Roche)

4 Warnings and precautions

- For *in vitro* diagnostic use.
- 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™ COVID-19 Multiplex RT-qPCR Assay tubes.
- Do not use a CoviDetect™ COVID-19 Multiplex RT-qPCR 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.
- Be aware of the placement and orientation of the PCR tubes in the PCR machine in relation to how the samples are named in the PCR software.
- 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 7.1 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™ COVID-19 Multiplex RT-qPCR tubes, pipette tips and specimen tubes according to local, state and federal regulations for biological 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.
- Minimise the exposure of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay to light due to the presence of light sensitive HydrolEasy® 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.
- Inappropriate sample collection, storage, and transport may yield incorrect or invalid results. 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.

[2] 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 either be nasopharyngeal, oropharyngeal, 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 Sample transport and storage

Transportation of collected specimens must comply with all applicable regulations for the transport of biological agents. Specimens should be stored in suitable buffers, such as viral transport media. Please follow the specific instructions for use of the transport vial.

5.3 Sample purification

Specimens should be subjected to RNA purification prior to analysis by CoviDetect™ COVID-19 RT-qPCR Assay using suitable RNA purification methods such as the BasePurifier™ 32 Nucleic Acid Extraction System (BasePurifier™) (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. Be aware that the outcome from the purification method may influence the results of the CoviDetect™ COVID-19 RT-qPCR Assay.

5.4 Positive and Negative Extraction Control

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. 8039).

The Positive Extraction control contains 20 SARS-CoV-2 RNA copies and 0.5 ng human genomic DNA per microliter.

NOTE: The Extraction Controls cannot be added directly to the CoviDetect™ COVID-19 RT-qPCR 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 AmpliSmaRT™ RT-qPCR Master Mix to each PCR tube (vial, strip or plate).
2. Add 5 µL Primer-Probe Multiplex Mix to the PCR tubes.
3. Add 5 µL of template (sample, positive, or negative control) to the needed PCR tubes.
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 4**).

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 tubes in the real-time PCR instrument and run the RT-qPCR program (**Table 4**).

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6.3 RT-qPCR program

Table 4. RT-qPCR program for running CoviDetect™ COVID-19 Multiplex RT-qPCR Assay.

Protocol	Temperature [°C]	Time [sec]	Cycles	Ramping [°C/sec]	Channel
Stage 1					
Hold	52	300	1	2	
Stage 2					
Hold	95	10	1	2	
Stage 3 (Cycle 1-7)					
2-step amplification	95	5	7	2	
	66	30			
Stage 4 (Cycle 1-38)					
2-step amplification	95	5	38	2	
	60	30			FAM/SYBR (green) HEX/VIC® (yellow) Cy5 (red)

7 Data Analysis

For the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay analysis, determining the cycle threshold (Ct) is a central part of the data analysis procedure. The Ct is defined as the cycle in which the fluorescence signal of a given assay exceeds the threshold value, which is set as part of the analysis procedure. The Ct values of the PCR program (**Table 4**, stage 4) are compared to predefined cut-off values to determine if the individual samples are positive or negative for SARS-CoV-2 (Section 7.2).

7.1 Baseline and threshold settings

Results from CoviDetect™ COVID-19 Multiplex RT-qPCR 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

An overview of the possible outcomes of the analysis is shown in **Table 5**. The results are only valid if the included Positive control Ct values are below 35 for N1 and N2, and below 28 for the RNase P sample control. No template control (NTC) should produce no Ct values for the N1 and N2 SARS-CoV-2-specific targets. NTC Ct values above cycle 31 for the RNase P sample control are acceptable.

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Table 5. Analysis outcomes and Ct threshold based on target amplification curves for CoviDetect™ COVID-19 Multiplex RT-qPCR Assay. Conclusions are based on target Ct values compared to the cut-off values.

Sample status	Target	Outcome	Ct	Conclusion	Comments
Positive case 1	N1	+	<35	SARS-CoV-2 positive	When both N1 and N2 Ct values are below 35, and the Ct value of RNase P is below 28, the sample is positive.
	N2	+	<35		
	RNase P	+	<28		
Positive case 2	N1	-	≥35		The sample is positive if only either N1 or N2 is positive, and RNase P is positive in two separate runs.
	N2	+	<35		
	RNase P	+	<28		
Positive case 3	N1	+	<35		
	N2	-	≥35		
	RNase P	+	<28		
Positive case 4	N1	+	<35		The RNase P signal may be suppressed when N1 and N2 are positive, especially in samples with high amounts of viral RNA.
	N2	+	<35		
	RNase P	-	≥28		
Negative case 5	N1	-	≥35	SARS-CoV-2 negative	A positive RNase P signal is required for a sample to be considered negative.
	N2	-	≥35		
	RNase P	+	<28		
Invalid case 6	N1	-	≥35	Invalid	The sample does not contain enough material for the analysis. Take a new specimen if possible.
	N2	-	≥35		
	RNase P	-	≥28		

7.2.1 Positive samples

The sample is positive for SARS-CoV-2 when Ct values for both viral N1 and N2 assays are below 35 (**Table 5**, positive case 1). Please notice that the RNase P signal may be suppressed in some samples and particularly when a sample is containing large amounts of viral RNA. These samples are considered valid if the Ct values of both N1 and N2 are below 35 even when RNase P is negative (**Table 5**, positive case 4).

If a sample is only positive for one of the two viral genes, the sample should be rerun in order to call it positive or negative (**Table 5**, positive case 2-3). If the sample is again positive for at least one of the viral genes, the sample is considered positive. If it is not positive for any of the viral genes in the second run, but positive for the RNP sample control, the sample is deemed negative. The lack of signal in either N1 or N2 may be due to mutations present in the target regions of the assay.

7.2.2 Negative samples

The sample is considered negative for the detection of SARS-CoV-2 if the sample is positive for RNase P but negative for N1 and N2 (**Table 5**, negative case 5).

7.2.3 Invalid samples

7.2.3.1 No sample signals

In the case of no or late amplification of RNase P (Ct ≥ 28), the test is invalid unless both N1 and N2 are positive (Ct < 35) (**Table 5**, invalid case 6). The concentration or the quality of the RNA in the sample is too low. Add more sample if possible or collect a new specimen and 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.

7.2.3.2 No positive control signals

If no signal occurs from the positive control samples, make sure that the RT-qPCR program has been defined correctly and that the instrument is acquiring on FAM, HEX and Cy5 channels in Step 2 of Stage 4 (**Table 5**). Results from a run where the control signals are not present should not be used. The control reagents might be degraded, the RNA extraction not successful, or the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay not functional. If you cannot locate the root cause of the problem, please contact PentaBase A/S or your local distributor for support.

7.2.3.3 Signal in NTC

NTC Ct values above cycle 31 for the RNase P sample control are acceptable. Make sure that the threshold has been set correctly above any background fluorescence. If this is the case, the reagents may be contaminated. 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.

7.2.4 Estimation of SARS-CoV-2 RNA concentration

CoviDetect™ COVID-19 Multiplex RT-qPCR Assay is a semi-quantitative test that can be used to estimate the concentration of SARS-CoV-2 RNA (C_{RNA}) in a sample as described in Equation 1. Equation 1 is based on linear regression analysis of N1 and N2 Ct values as a function of the logarithm of controlled sample concentrations of SARS-CoV-2 RNA:

$$C_{RNA\ (copies/\mu L)} = 10^{\left(\frac{\overline{Ct}-33.478}{-4.1721}\right)} \qquad (1)$$

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where $\overline{Ct}$ represents the average Ct in stage 4 of the program shown in **Table 4** of N1 and N2 (FAM and HEX). An example is shown below:

Example:

1. Calculate $\overline{Ct}$ when $Ct_{N1} = 30.28$ and $Ct_{N2} = 29.76$

$$\overline{Ct} = \frac{(Ct_{N1} + Ct_{N2})}{2} = \frac{(30.28 + 29.76)}{2} = 30.02$$

2. Calculate concentration of SARS-CoV-2 RNA in the sample

$$C_{RNA\ (copies/\mu L)} = 10^{\left(\frac{\overline{Ct} - 33.478}{-4.1721}\right)} = 10^{\left(\frac{30.02 - 33.478}{-4.1721}\right)} = 6.7\ \text{copies/}\mu\text{L}$$

NOTE: Equation 1 is only valid for concentrations at or above the limit of detection as described in **Table 6**. Samples were purified and analysed using the BasePurifier™ with the Viral DNA and RNA Extraction Kit. The assay was run on the BaseTyper™ Real-Time PCR Instrument - 48 well block with 4 channel detection (BaseTyper™) (ref. no. 750). The use of other purification methods and/or real-time PCR instruments may affect the calculation of estimated sample concentrations. To verify the calculations when using other instrument combinations, a standard curve should be made using relevant clinical matrix.

8 Performance evaluation

8.1 Analytical sensitivity – Limit of Detection

The limit of detection (LOD) of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay has been evaluated by spiking synthetic SARS-CoV-2 RNA (Twist Bioscience, ref. no. 102015) into a negative clinical oropharyngeal matrix. Based on an initial dilution series, 7000 and 3500 copies of SARS-CoV-2 RNA were each spiked into 3 mL of 20 oropharyngeal specimens. RNA was extracted using the Viral DNA and RNA Extraction Kit and the BasePurifier™ 32 Nucleic Acid Extraction System. RT-qPCR amplification by CoviDetect™ COVID-19 Multiplex RT-qPCR Assay was performed using three different qPCR instruments: BaseTyper™ 48 Real-Time PCR Instrument (PentaBase A/S, ref. no. 750), CFX384™ C1000™ Touch Real-Time System (Bio-Rad, ref. no. 1855484) and LightCycler® 480 II (Roche, ref. no. 05 015 278 001). The LOD was found to be 250 SARS-CoV-2 RNA copies per extraction (1.25 copies/µL) when using the BaseTyper™ and CFX384™ instruments, and 500 copies per extraction (2.5 copies/µL) when using the LightCycler® 480 instrument (**Table 6**).

Table 6. Limit of detection (LOD) of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay using SARS-CoV-2 RNA spiked into oropharyngeal matrix. RNA was extracted using the BasePurifier™.

Target	SARS-CoV-2 RNA Copies/µL	Positive/Total		
		BaseTyper™ (PentaBase)	CFX384™ (Bio-Rad)	LightCycler® 480 (Roche)
N1	2.5	20/20	20/20	20/20
	1.25	18/20	16/20	16/20
N2	2.5	20/20	20/20	19/20
	1.25	18/20	18/20	15/20
N1 or N2	2.5	20/20	20/20	20/20
	1.25	19/20	19/20	16/20

8.2 Analytical precision

Within-laboratory repeatability was determined for CoviDetect™ COVID-19 Multiplex RT-qPCR Assay at the limit of detection using the BaseTyper™ 48 Real-Time PCR Instrument (PentaBase), CFX96 (Bio-Rad), and LightCycler® 480 real-time qPCR instruments. Data for repeatability standard deviations and precision as determined by the coefficient of variation (%CV) are summarised in **Table 7**. The %CV was found to be below the 10% acceptance limit and thus is acceptable for all targets and instruments.

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Table 7. Within-laboratory repeatability data for CoviDetect™ COVID-19 Multiplex RT-qPCR Assay when using the BaseTyper™ (PentaBase), CFX96 (Bio-Rad) and LightCycler® 480 real-time qPCR Instruments. *One outlier removed following Grubbs' test.

Instrument	SARS-CoV-2 RNA Copies/reaction	Target	Positives/ Total	Positives (%)	Mean Ct	Standard deviation (SD)	Precision (%CV)
BaseTyper™ (PentaBase)	250	N1	19/20	95	31.11	1.71	5.50
		N2	18/20	90	29.24	1.91	6.54
		RNP	20/20	100	22.67	1.24	5.47
CFX384™ (Bio-Rad)	250	N1	16/20	85	28.73	1.18	4.11
		N2	18/20	90	27.84	1.20	3.58
		RNP	20/20	100	22.40	1.12	5.01
LightCycler® 480 (Roche)	500	N1	19/19*	100	28.43	2.07	7.20
		N2	19/20	95	29.94	1.20	4.02
		RNP	20/20	100	22.63	1.15	5.07

Between-laboratory precision of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay was evaluated at four different PentaBase laboratory sites in Denmark (Billund, Aalborg, Aarhus, and Odense) using CoviDetect™ positive control samples (100 SARS-CoV-2 RNA copies (Twist Bioscience, ref. no. 102015) and 25 ng human Genomic DNA (Promega ref. no. G3041)) per reaction. Samples were purified using the BasePurifier™ and the Viral DNA and RNA Extraction Kit and amplified using the BaseTyper™ 48 Real-Time PCR Instrument. Experiments were performed between March 3 and October 1, 2021. Each site used its own BasePurifier™ 32 Nucleic Acid Extraction System and BaseTyper™ instruments and operators. Data are summarised in **Table 8**.

Table 8. Between-laboratory precision of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay.

Target	Mean (Ct)	Reproducibility	
		SD	%CV
N1	24.01	0.99	4.11
N2	23.56	0.74	3.15
RNP	20.78	1.20	5.80

8.3 Inclusivity

8.3.1 In-silico analysis

CoviDetect™ COVID-19 Multiplex Assay oligonucleotides were blasted against the 6 most common SARS-CoV-2 strains (**Figure 1A and B**). All oligonucleotides except the N1 probe were found to show 100% agreement with the analysed SARS-CoV-2 strains. The discrepancy of the N1 probe is due to a single mismatch in the 5'-end of the probe and is not expected to have any significant impact on the performance of the assay due to the presence of several pentabases in the probe (**Figure 1C** and data not shown).

Inclusivity was further evaluated by analysing selected clinical oropharyngeal swab samples, found to be positive for SARS-CoV-2 with the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay, with the CoviDetect™ Variants Assays. The mutational status of the evaluated subjects and the corresponding SARS-CoV-2 variants are summarised in **Table 9**. The analysed samples include at least four cases of each of the SARS-CoV-2 strains Alpha, Delta, Omicron BA.1 and Omicron BA.2.

Table 9. Clinical oropharyngeal swab samples used for inclusivity testing of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay.

N	SARS-CoV-2 variant	Mutations detected
7/7 positive	Alpha	N501Y+P681H N501Y+Del69-70 N501Y+P681H+Del69-70
6/6 positive	Delta	P681R+L452R+T478K
4/4 positive	Omicron BA.1	K417N+H655Y+S371F+S373P+S375F
4/4 positive	Omicron BA.2	S371L+S373P+S375F

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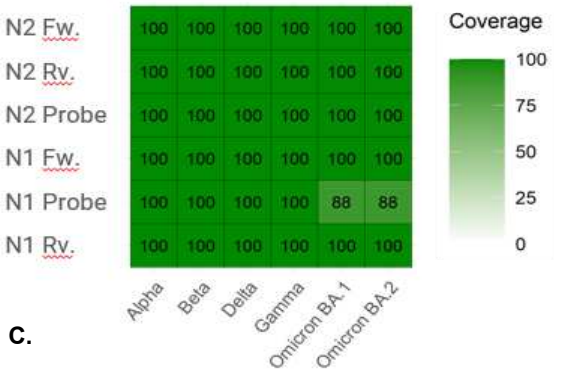

B.

SARS-CoV-2 strain	GenBank ID
Alpha	MZ344997.1
Beta	MW598419.1
Delta	MZ359841.1
Gamma	MZ169911.1
Omicron BA.1	OL672836.1
Omicron BA.2	OM296922.1

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N1.Probe                        5'------ACCCCGCATTACGTTTGGTGGACC------3'
OL672836.1|Omicron BA.1         5'-AATGCACTCCGCATTACGTTTGGTGGACCCTCAG-3'
OM296922.1|Omicrom BA.2         5'-AATGCACTCCGCATTACGTTTGGTGGACCCTCAG-3'
                                  **.********************
```

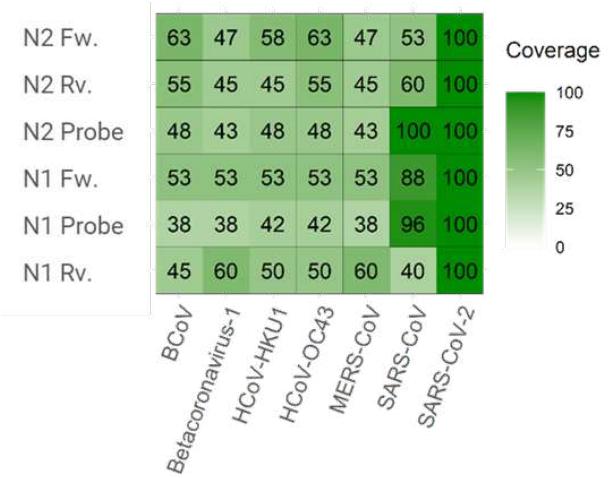
Figure 1. A. SARS-CoV-2-specific oligonucleotides of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay blasted against the most common SARS-CoV-2 variants. **B.** SARS-CoV-2 strains used for analysis of agreement with CoviDetect™ COVID-19 Multiplex RT-qPCR Assay SARS-CoV-2 specific oligonucleotide sequences. **C.** Alignment of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay N1 Probe oligonucleotide sequence with SARS-CoV-2 Omicron BA.1 and BA.2 variants.

8.4 Analytical specificity

8.4.1 In-silico analysis

CoviDetect™ COVID-19 Multiplex Assay oligonucleotide sequences were aligned with common Beta coronaviruses (**Figure 2A**). There was no significant agreement with the investigated coronaviruses except in the case of the SARS-CoV-1 (SARS-CoV) coronavirus and the N2 forward primer (N2 Fw.), N1 reverse primer (N1 Rv.) and N1 probe. Further analysis showed that the N2 forward and N1 reverse primers are priming amplification in the opposite directions of the other primer and therefore cannot lead to the generation of an amplicon in SARS-coronavirus (**Figure 2B**). Furthermore, experimental laboratory analysis found no amplification of a synthetic SARS-CoV-1 template (Section 8.4.2).

A.



B.

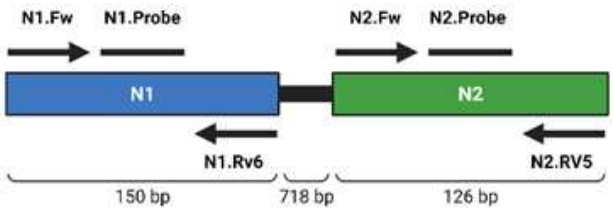


Figure 2. A. In silico analysis of CoviDetect™ COVID-19 Multiplex Assay oligonucleotide sequences and alignment with common coronaviruses. **B.** Alignment of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay N1 forward, N2 reverse and N1 probe oligonucleotide with SARS-CoV-1.

8.4.2 Experimental analysis

The specificity of the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay was evaluated using common microorganism panels, specific respiratory verification panels and relevant synthetic templates, including other coronaviruses than SARS-CoV-2, influenza, parainfluenza, Rhinovirus and RSV. No samples gave rise to amplification by the CoviDetect™ COVID-19 Multiplex RT-qPCR SARS-CoV-2-specific assays except *S. pyogenes* where there was amplification by the SARS-CoV-2 specific N2 assay (data not shown).

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8.5 Clinical evaluation

8.5.1 Cohort 1

Nasopharyngeal swab specimens from patients suspected of SARS-CoV-2 were extracted using Viral DNA and RNA Extraction Kit (ref. no. 727) and BasePurifier™ (ref. no. 715). RT-qPCR was performed using CoviDetect™ COVID-19 Multiplex RT-qPCR Assay and Comparator Method 1 on the BaseTyper™ Real-Time PCR Instrument (**Table 10**). The analysis was performed using automatic baseline and threshold settings. Correlation of Ct values between CoviDetect™ and Comparator Method 1 is illustrated in **Figure 3**.

Table 10. Comparison of clinical performance of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay to Comparator Method 1 using Cohort 1 samples.

Clinical performance Cohort 1 Oropharyngeal swabs		CoviDetect™ COVID-19 RT-qPCR Assay		
		Positive	**Negative**	**Total**
Comparator Method 1	**Positive**	27	0	27
	Negative	0	62	62
	Total	27	62	89
Positive Percent Agreement: 27/27 or 100% (CL95: 87.5-100%)				
Negative Percent Agreement: 62/62 or 100% (CL95: 94.2-100%)				

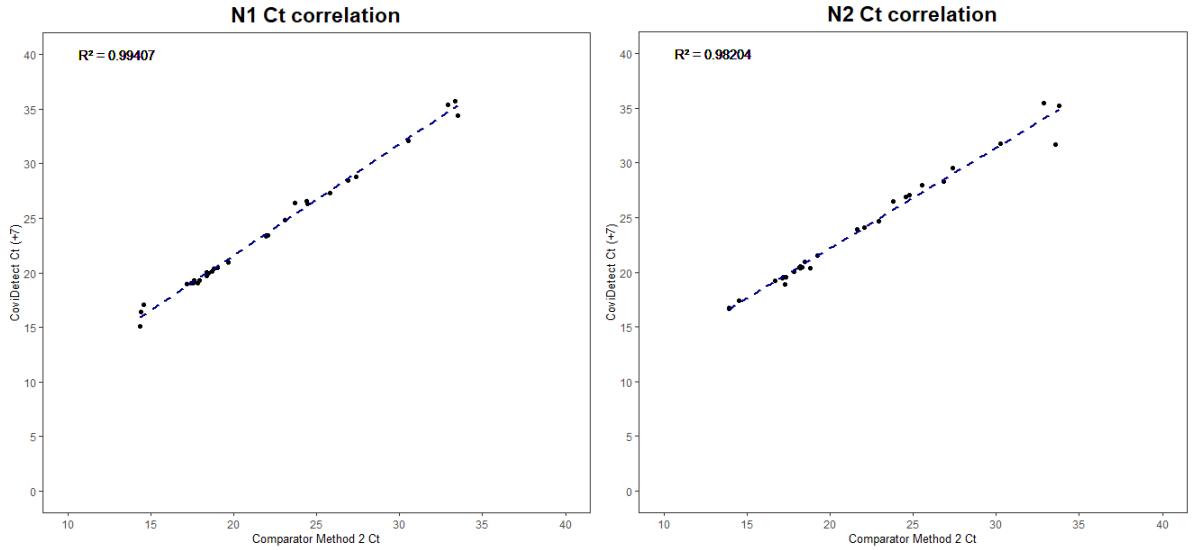

Figure 3. Correlation of N1 and N2 assay Ct values between CoviDetect™ and Comparator Method 1 using SARS-CoV-2 positive leftover nasopharyngeal swab specimens (Cohort 1).

8.5.2 Cohort 2

The clinical performance of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay was evaluated with the use of oropharyngeal swabs clinical specimens from patients suspected of COVID-19. Specimens were previously analysed for the presence of SARS-CoV-2 using the comparator RT-qPCR method at a clinical laboratory in Denmark. Stored samples were collected for subsequent analysis by CoviDetect™ COVID-19 Multiplex RT-qPCR Assay. Extraction of RNA was performed using the Viral DNA and RNA Extraction Kit for the BasePurifier™ 32 Nucleic Acid Extraction System. RT-qPCR was performed using the CFX96 real-time PCR Detection System (BioRad) and data analysis was performed using software version 3.1. Standard analysis settings were used except that the threshold for the FAM channel was set to 100 RFU. Data are summarised in **Table 11**.

Table 11. Summary of clinical evaluation of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay using cohort 2. *Due to limited sample material, it was not possible to re-run the 4 discrepant cases or the 4 cases where only one of the CoviDetect™ COVID-19 Multiplex RT-qPCR SARS-CoV-2-specific assay (N1 or N2) was positive.

Clinical performance Cohort 2 Oropharyngeal swabs		CoviDetect™ COVID-19 RT-qPCR Assay		
		Positive	**Negative**	**Total**
Comparator Method 2	**Positive**	26	4*	30
	Negative	0	51	51
	Total	26	55	81
Positive Percent Agreement: 26/30 or 100% (CL95: 70.3-94.7%)				
Negative Percent Agreement: 51/51 or 100% (CL95: 93-100%)				

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The Ct values of CoviDetect™ (Ct + 7) compared to Comparator Method 2 of agreed positive and discrepant samples are illustrated in **Figure 4**. The lowest Ct value of each sub-assay (N1 or N2 of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay and E, RdRp or N gene of Comparator Method 2) was used for the comparison. Discrepant samples are shown as data points with CoviDetect™ Ct values of 0.

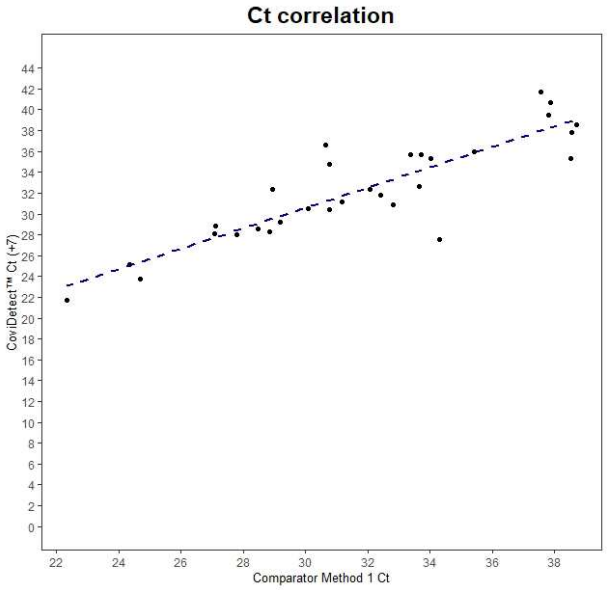

Figure 4. Correlation of Ct values between CoviDetect™ and Comparator Method 2 using leftover oropharyngeal swabs (Cohort 2). Discrepant cases are illustrated as datapoints with CoviDetect™ Ct values of 0.

9 Limitations

- Performance of the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay has only been tested on specimens from nasopharyngeal swabs, oropharyngeal swabs and 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.
- Please note that an estimate of 1 out of 40,000 samples carry a mutation in the RNase P gene, that make them test negative for the internal control. For such cases use a different assay.
- A positive test result following SARS-CoV-2 infection is possible at least up to 37 days after the date of the first positive test result.
- A positive test result following SARS-CoV-2 infection may arise from detection of both active and inactive SARS-CoV-2 virus even in asymptomatic or post-symptomatic individuals that are no longer able to transmit COVID-19.
- 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 (N1 and N2 markers) 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.
- Infections with *S. pyogenes* may produce false positive results.

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10 Symbols

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

 Date/country of manufacture

 In vitro diagnostic medical device

 Use-by date

 Do not reuse

 Contains sufficient for <*n*>

 Manufacturer

 Temperature limit

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

 Consult electronic available instructions for use

11 Manufacturer

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Email: info@pentabase.com
Webpage: www.pentabase.com

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.