CoviDetect™ FAST

COVID-19 Multiplex RT-qPCR Assay

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

INSTRUCTIONS FOR USE

REFERENCE NUMBERS

Dispense Ready (DR)
8018 (200 reactions)
8016 (500 reactions)

Ready-to-Use (RTU)
8022 (96 reactions)

Version 3.1
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CE IVD

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1 Intended purpose

CoviDetect™ FAST 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 from saliva. Samples can be purified on automated platforms or in manual workflows. The results of the CoviDetect™ FAST 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 three SARS-CoV-2 viral targets and one human target sampling control in the same PCR reaction tube.

1.1 Intended user

CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay is intended for use by healthcare professionals or qualified laboratory personnel specifically instructed and trained in the techniques of real-time qPCR as well as proficient in handling biological samples. Medical interventions based on results from this product requires medical authorisation.

2 Test principle

Accurate and fast detection of SARS-CoV-2 is important in individuals suspected of a respiratory infection to prevent spreading of COVID-19 and to initiate treatment when relevant.

To meet the need for faster diagnostic testing for SARS-CoV-2, the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay has been developed as a fast and highly sensitive assay reducing answering time significantly compared to common alternative procedures.

2.1 Explanation of the assay

The CoviDetect™ FAST 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 oligos 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™ FAST 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-qPCR 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 oligos 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™ FAST COVID-19 Multiplex RT-qPCR Assay are labelled with either FAM, HEX, CAL Flour Red 610, 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™ FAST 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 needs the addition of RNA before RT-qPCR.

2.3 Principle of the procedure

The CoviDetect™ FAST 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™ FAST COVID-19 Multiplex RT-qPCR Assay targets three viral sequences of the SARS-CoV-2 genome, two regions from the RNA dependent RNA polymerase (named IP2 and IP4) gene and one region from the envelope protein gene (named E gene) (**Table 1**). Selective amplification of IP2, IP4 and E sequences is achieved by using sequence-specific forward and reverse primers with HydrolEasy® probes labelled with FAM, HEX, and CAL Flour Red 610, 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

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

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detected by measuring the increased fluorescence generated by cleavage of the probe, releasing 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.

Table 1. List of amplified genomic regions by the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay.

Targeted Regions	Gene	Fluorophore
IP2	RNA dependent RNA polymerase gene marker	FAM
IP4	RNA dependent RNA polymerase gene marker	HEX
E	Envelope protein gene marker	CAL Flour Red 610
RNase P	Human RNase P	Cy5

3 Reagents and materials

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

3.1 Storage

Refer to the label for expiry date. This assay should be stored at -20°C. Keep the DR version freeze-thaw cycles to a minimum and do not exceed 10 cycles. Do not repeat freeze-thawing of the RTU version.

3.1.1 In-use stability

The assay components should be returned to the freezer promptly after use (DR) to minimise the time at room temperature and exposure to light.
Used RTU 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™ FAST COVID-19 Multiplex RT-qPCR Assay are not for reuse.

3.2 Materials provided

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

Dispense Ready (DR)	
CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay	
Kit components	**Content**
COVID-19 FAST 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™ FAST COVID-19 Multiplex RT-qPCR Assay	
Kit components	**Content**
COVID-19 FAST 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 CoviDetect™ FAST 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 System 32 oscillating rods and analysed with the BaseTyper™ Real-Time PCR Instrument - 48 well block with 4 channel detection (BaseTyper™) (PentaBase A/S, ref. no. 750) CFX96 (Bio-Rad, ref. no. 1845097) or QuantStudio™ 5 (Applied Biosystems™ ref. no. A28574) 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™ FAST COVID-19 Multiplex RT-qPCR Assay. However, when running CoviDetect™ FAST 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. Please contact PentaBase or your local distributor for support.

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Table 3. Materials and consumables 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)
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)
Real-time qPCR
Real-time PCR instrument like: BaseTyper™ (PentaBase A/S)

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™ FAST COVID-19 Multiplex RT-qPCR Assay tubes.
- Do not use a CoviDetect™ FAST 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™ FAST 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™ FAST 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.
- Infections with SARS-CoV-1 may produce false positive results.

[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 be collected from 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 Sample purification

Specimens should be subjected to RNA purification prior to analysis by CoviDetect™ FAST COVID-19 RT-qPCR Assay using suitable RNA purification methods such as the BasePurifier™ 32 and the Viral DNA and RNA Extraction Kit (see **Table 3**) according to the manufacturer's instructions. Be aware that the outcome from the purification method may influence the results of the CoviDetect™ COVID-19 FAST COVID-19 RT-qPCR 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 A/S or your local distributor.

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™ FAST 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 6 µL AmpliSmaRT™ One-Step RT-qPCR Master Mix to each PCR tube (vial, strip or plate).
2. Add 1 µL Primer/probe multiplex mix to the PCR tubes or wells.
3. Add 5 µL of the template (sample, positive control 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 strips in the real-time qPCR instrument and run the RT-qPCR program (**Table 4**).

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6.3 RT-qPCR program

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

Protocol	Temperature [°C]	Time [sec]	Cycles	Ramping [°C/sec]	Channel
Stage 1					
Hold	52	180	1	8	
Stage 2					
Hold	95	30	1	8	
Stage 3 (Cycle 1-45)					
2-step amplification	90	1	45	8	
	60	12		8	FAM (green) HEX/VIC® (yellow) Texas Red (orange) Cy5 (red)

7 Data Analysis

For the CoviDetect™ FAST 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 3) 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™ FAST COVID-19 Multiplex RT-qPCR 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

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 32 for IP2, IP4 and E, and below 29 for the RNase P sample control. No template control (NTC) should produce no Ct values for the IP2, IP4 and E SARS-CoV-2-specific targets. NTC Ct values above cycle 38 for the RNase P sample control are acceptable.

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Table 5. Ct threshold values for CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay.

Sample status	Target	Outcome	Ct	Conclusion	Comments
Positive case 1	IP2	+	≤42	SARS-CoV-2 positive	The sample is considered SARS-CoV-2 positive when two or three SARS-CoV-2 specific targets are positive, even if the RNase P target is negative
	IP4	+	≤42		
	E	+	≤42		
	RNase P	+/-	(≤34)		
Positive case 2	IP2	-	>42		The sample is considered SARS-CoV-2 positive when two of the SARS-CoV-2-specific targets are positive. Even if the RNase P target is negative.
	IP4	+	≤42		
	E	+	≤42		
	RNase P	+/-	≤34		
Positive case 3	IP2	+	≤42		The sample is considered SARS-CoV-2 positive when two of the SARS-CoV-2-specific targets are positive. Even if the RNase P target is negative.
	IP4	-	>42		
	E	+	≤42		
	RNase P	+/-	≤34		
Positive case 4	IP2	+	≤42		The sample is considered SARS-CoV-2 positive when two of the SARS-CoV-2-specific targets are positive. Even if the RNase P target is negative.
	IP4	+	≤42		
	E	-	>42		
	RNase P	+/-	≤34		
Positive case 5	IP2	+	≤42		The sample is considered SARS-CoV-2 positive when one of the SARS-CoV-2-specific targets are positive in two independent runs.
	IP4	-	>42		
	E	-	>42		
	RNase P	+	≤34		
Positive case 6	IP2	-	>42		The sample is considered SARS-CoV-2 positive when one of the SARS-CoV-2-specific targets are positive in two independent runs.
	IP4	+	≤42		
	E	-	>42		
	RNase P	+	≤34		
Positive case 7	IP2	-	>42		The sample is considered SARS-CoV-2 positive when one of the SARS-CoV-2-specific targets are positive in two independent runs. Please note that if E is below Ct 30 this might be due to presence of SARS-CoV-1.
	IP4	-	>42		
	E	+	≤42		
	RNase P	+	≤34		
Negative case 8	IP2	-	>42	SARS-CoV-2 negative	A positive RNase P signal is required for a sample to be considered negative.
	IP4	-	>42		
	E	-	>42		
	RNase P	+	≤34		
Invalid case 9	IP2	-	>42	Invalid	The sample is invalid if no signal is present in any of the targets.
	IP4	-	>42		
	E	-	>42		
	RNase P	-	>34		
Invalid case 10	IP2	+	≤42		The sample is invalid if only in one of the SARS-CoV-2-specific targets is positive and the human control (RNase P) is negative.
	IP4	-	>42		
	E	-	>42		
	RNase P	-	>34		
Invalid case 11	IP2	-	>42		The sample is invalid if only in one of the SARS-CoV-2-specific targets is positive and the human control (RNase P) is negative.
	IP4	+	≤42		
	E	-	>42		
	RNase P	-	>34		
Invalid case 12	IP2	-	>42		The sample is invalid if only in one of the SARS-CoV-2-specific targets is positive and the human control (RNase P) is negative.
	IP4	-	>42		
	E	+	≤42		
	RNase P	-	>34		

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7.2.1 Positive samples

The sample is positive for SARS-CoV-2 when at least two Ct values for viral IP2, IP4 and E gene targets are below 42, even when RNase P is negative (**Table 5**, cases 1-4). Please notice that the RNase P signal may be suppressed in some samples and particularly when containing large amounts of viral RNA. Furthermore, a sample is also considered SARS-CoV-2 positive if only one of the three SARS-CoV-2-specific targets are positive in two independent runs, when assuming that the RNase P sample control, positive control, and negative control are all valid (**Table 5**, cases 5-7). **Note** that a sample is considered SARS-CoV-2 positive even in cases where the positive SARS-Cov-2-specific target in the re-run is different from the first run. The lack of signal in either IP2, IP4 or E may be due to very limited amount of virus in the sample or the presence of mutations in the target regions of the assay.

Note: The E gene target can also detect SARS-CoV-1, and followingly, if the E gene target has a Ct below 30 and the other SARS-CoV-2-specific target are negative, it can be due to the presence of SARS-CoV-1 in the sample. Thus, in case of a confirmed positive sample where only the SARS-CpV-2-specific E gene target is positive and with a Ct below 30 in two independent runs, it is recommended to send the sample for sequencing to clarify whether the case is SARS-CoV-1 or SARS-CoV-2 positive.

7.2.2 Negative samples

The sample is considered negative for detection of SARS-CoV-2 if the sample is positive for RNase P but negative for IP2, IP4 and E (**Table 5**, case 8).

7.2.3 Invalid samples

In case of no or late amplification of RNase P, (Ct ≥ 34), the test is invalid (**Table 5**, cases 9-12). For invalid results, if more specimen is available, repeat the extraction and run the test again. If all markers remain negative after repeating the test, the test is inconclusive, and if possible, a new specimen should be collected for a new test.

7.2.3.1 No sample signals

In case of no signal in any of the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay targets (**Table 5**, case 9), check if template has been added to the tube. If more specimen is available, repeat the extraction and run the test again. If all targets remain negative after repeating the test, no diagnosis can be concluded, and if possible, a new specimen should be collected for testing. 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).

7.2.3.2 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. If the correct program has been used and there is no signal in any of the samples repeat the extraction and run the test again. If all markers remain negative after repeating the test, contact PentaBase A/S or your local distributor for support.

7.2.3.3 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 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.

7.2.4 Estimation of SARS-CoV-2 RNA concentration

CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay is a semi-quantitative test that can be used to estimate the concentration of SARS-CoV-2 RNA in a sample as described in Equation 1. Equation 1 is based on linear regression analysis of IP2 and IP4 Ct values as a function of the logarithm of controlled sample concentrations of SARS-CoV-2 RNA:

$$C_{RNA\,(copies/\mu L)} = 10^{\left(\frac{\overline{Ct}-36.412}{-2.995}\right)} \qquad (1)$$

where $\overline{Ct}$ represents the average Ct in stage 3 of the program shown in **Table 4** of IP2 and IP4 (FAM and HEX). An example is shown below:

Example:

1. Calculate $\overline{Ct}$ when $Ct_{IP2} = 29.11$ and $Ct_{IP4} = 29.01$

$$\overline{Ct} = \frac{(Ct_{IP2} + Ct_{IP4})}{2} = \frac{(29.11 + 29.01)}{2} = 29.06$$

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

$$C_{RNA\,(copies/\mu L)} = 10^{\left(\frac{\overline{Ct}-36.412}{-2.995}\right)} = 10^{\left(\frac{29.06-36.412}{-2.995}\right)} = 282\ \text{copies}/\mu\text{L}$$

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NOTE: Equation 1 is only valid for concentrations at or above the limit of detection as described in **Table 6** and **Table 7**. Samples were purified and analysed using the BasePurifier™ 32 (ref. no. 715, PentaBase A/S) with the Viral DNA and RNA Extraction Kit, and the BaseTyper™ Instrument. 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

8.1.1 Oropharyngeal matrix

The limit of detection (LOD) of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay was found to be 1 copy of SARS-CoV-2 RNA per µL. Synthetic SARS-CoV-2 RNA (Twist Bioscience, cat. no. 102015) was spiked into a negative clinical oropharyngeal matrix. RNA was extracted using the BasePurifier™ 32 and Viral DNA and RNA Extraction Kit and RT-qPCR was performed using the BaseTyper™ Instrument (**Table 6**).

Table 6. Limit of detection (LOD) of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay using SARS-CoV-2 RNA spiked into oropharyngeal matrix. RNA was extracted using the BasePurifier™ 32 and RT-qPCR was performed using the BaseTyper™ Instrument.

Target	SARS-CoV-2 RNA (Copy/µL)	Positives/Total	Positives (%)
IP2	0.5	14/20	70
IP4		2/20	10
E		11/20	55
IP2 or IP4 or E		16/20	80
IP2	1.0	18/20	90
IP4		4/20	20
E		17/20	85
IP2 or IP4 or E		20/20	100

The LOD might be higher when using other PCR instruments such as QuantStudio™ and CFX96.

8.1.2 Saliva matrix

The limit of detection (LOD) of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay has been evaluated by spiking synthetic SARS-CoV-2 RNA (Twist Bioscience, cat. no. 102015) into a negative clinical saliva matrix. The LOD was found to be 1 copy per µL. Based on an initial dilution series, 6000 and 3000 copies of SARS-CoV-2 RNA were each spiked into 6 mL of 20 oropharyngeal specimens. RNA was extracted using the BasePurifier™ 32 and Viral DNA and RNA Extraction Kit (**Table 7**).

Table 7. Limit of detection (LOD) of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay using SARS-CoV-2 RNA spiked into wild type human DNA. RT-qPCR was performed using the BaseTyper™ Instrument.

Target	SARS-CoV-2 RNA (Copy/µL)	Positives/Total	Positives (%)
IP2	0.5	8/20	40
IP4		0/20	0
E		9/20	45
IP2 or IP4 or E		14/20	70
IP2	1.0	14/20	70
IP4		2/20	10
E		13/20	65
IP2 or IP4 or E		19/20	95

8.1.3 False-positive rate of clinical saliva samples

The false-positive rate of saliva sampling was evaluated on 120 saliva samples from non-infected individuals. RNA was extracted using the BasePurifier™ 32 and Viral DNA and RNA Extraction Kit. RT-qPCR was performed using the BaseTyper™ Instrument. All 120 samples were found to be SARS-CoV-2 negative using the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay. RNase P sample control Ct values were found to be lower than 34 in all cases (data not shown).

8.2 Analytical precision

The analytical precision when using the CoviDetect™ FAST for three different lots and with different operators. The reproducibility had low standard deviation and high precision. The standard deviation was below the acceptance criteria ≤2.5 and the %coefficient of variation (%CV) was ≤10%. The assay showed great reproducibility with a standard deviation below 1 and a %CV below 3.

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Table 8. Analytical precision of the assay. Three different lots produced at different time by different operators.

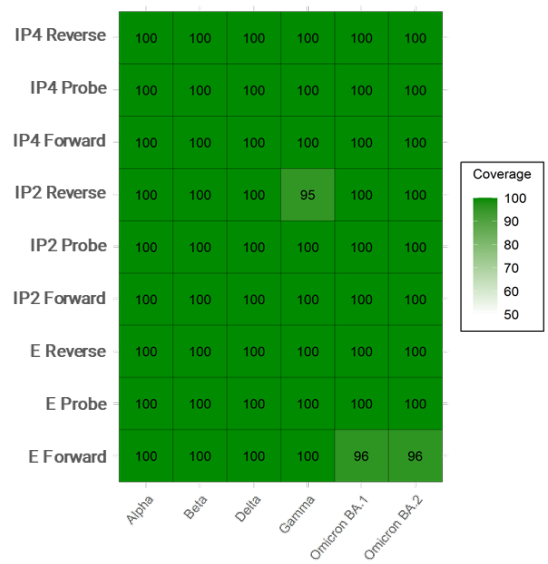
Analytical precision	Gene	LOT A	LOT B	LOT C	LOT A+B+C
Samples (N)	NA	10	10	10	30
Ct mean	IP2	30.41	30.02	29.90	30.11
	IP4	29.68	29.83	30.20	29.90
	E	29.61	29.21	29.25	29.36
	RNase P	26.24	26.52	26.51	26.43
Standard deviation (SD)	IP2	0.25	0.25	0.11	0.31
	IP4	0.54	0.76	0.47	0.64
	E	0.59	0.59	0.55	0.60
	RNase P	0.19	0.24	0.37	0.30
Precision (%CV)	IP2	0.83	0.84	0.35	1.01
	IP4	1.83	2.54	1.54	2.14
	E	1.99	2.02	1.89	2.06
	RNase P	0.72	0.89	1.39	1.13

8.3 Inclusivity

8.3.1 In sillico analysis

CoviDetect™ FAST COVID-19 Multiplex Assay oligonucleotides were blasted against the 6 most common SARS-CoV-2 strains (**Figure 1A and B**). All oligonucleotides except the IP2 Reverse and E Forward primer were found to show 100% agreement with the analysed SARS-CoV-2 strains. The discrepancy of the two primers is due to single mismatches in the 5'-end of the primers (**Figure 2**).

A.



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

Figure 1. A. SARS-SoV-2 specific oligonucleotides of CoviDetect™ FAST 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™ FAST COVID-19 Multiplex RT-qPCR Assay SARS-CoV-2 specific oligonucleotide sequences.

Current data available to PentaBase indicate that the E-assay Ct values (relative to IP2 and IP4) for the omicron variant are slightly higher (0.99 Ct) compared to the other variants, whereas there is no data supporting generally higher Ct values of the IP2 assay (relative to UP4 and E) for the gamma variant compared to other variants. Since the sample is considered SARS-CoV-2 positive when only one of the SARS-CoV-2-specific targets is positive in two independent runs (when the RNase P target is also positive) this higher relative Ct value of the E assay relative to IP2 and IP4 for the omicron variants is not expected to have any significant impact on the overall performance of the assay.

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```
E.Fw1
Omicron BA.1    cgaacttatgtactcattcgtttcggaagagataggtacgttaatagttaatagcgtact
Omicron BA.2    cgaacttatgtactcattcgtttcggaagagataggtacgttaatagttaatagcgtact
E.Fw1           ------------------------------gacaggtacgttaatagttaatagc-----
                                              **.**********************

Gamma     tcttgtgctgccggtactacacaaactgcttgcactgatgacaatgcgttagcttattac
IP2.Rv3   ------------------------------------tgacaatgcgttagcttactac
                                               ******************.***
```

Figure 2. CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay SARS-SoV-2 specific oligonucleotides blasted against the different SARS-CoV-2 variants.

Inclusivity was further evaluated by analysing selected clinical oropharyngeal swab samples previously found to be positive with the CoviDetect™ FAST 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, Gamma, Delta, Omicron BA.1 and Omicron BA.2.

Table 99. Positive samples used for inclusivity testing of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay.

N	SARS-CoV-2 variant	Mutations detected
10/10 positive	Alpha	N501Y, P681H, Del69-70
4/4 positive	Gamma	E484K (absence of other mutations for Beta)
5/5 positive	Delta	P681R, L452R, T478K
5/5 positive	Omicron BA.1	S371L+S373P+S375F
5/5 positive	Omicron BA.2	S371F, S373P, S375F, T376A

8.4 Analytical specificity

8.4.1 In-silico analysis

CoviDetect™ FAST COVID-19 Multiplex Assay oligo sequences were aligned with common Beta coronaviruses (**Figure 3**). There was significant agreement with the investigated coronaviruses in the case of the SARS-CoV-1 (SARS-CoV) coronavirus and the E forward primer (E.Fw1.), E reverse primer (E.Rv1) and E probe (E.Probe2).

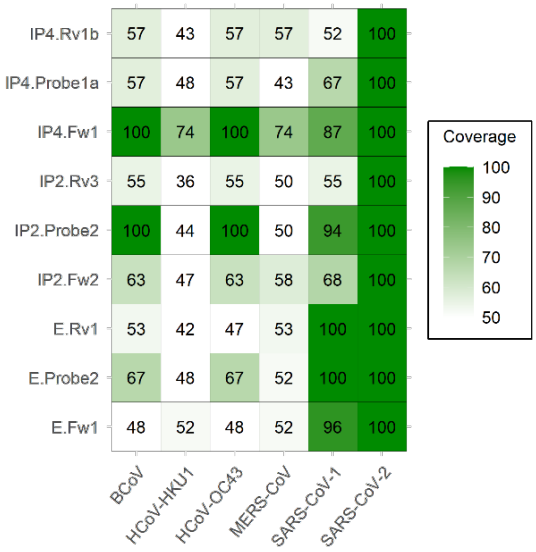

Figure 3. In silico analysis of CoviDetect™ FAST COVID-19 Multiplex Assay oligo sequences and alignment with common coronaviruses.

Experimental evaluation of this cross-reactivity did confirm amplification of a synthetic SARS-CoV-1 template. Therefore, while there have been no cases of people transmitted with SARS-CoV-1 since the outbreak in 2002-2004[3], and the probability of a false positive SARS-CoV-2 case due to a positive SARS-CoV-1 sample therefore is negligible, samples with positive results the E assay (below Ct 30) but negative for IP2 and IP4 may be the result of SARS-CoV-1 infection.

[3] https://www.cdc.gov/dotw/sars/index.html

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8.5 Clinical evaluation

8.5.1 Oropharyngeal sampling

The clinical performance of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay was evaluated using leftover material from oropharyngeal swabs. The specimens were analysed for the presence of SARS-CoV-2 using the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay (PentaBase A/S, Denmark, Ref. No. 8079). Samples were in parallel subjected to analysis with the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay. The extraction of RNA was performed using the Viral DNA and RNA Extraction Kit for the BasePurifier™ 32. RT-qPCR was performed using BaseTyper™ Instrument. The analysis was performed using automatic baseline and threshold settings. Evaluation summary is shown in **Table 10**. The RNase P Ct values of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay compared to CoviDetect™ COVID-19 Multiplex RT-qPCR Assay (Ct + 7) of agreed positive and negative samples are illustrated in **Figure 4A**. Correlation of averaged IP2, IP4 and E sequence Ct values of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay and CoviDetect™ COVID-19 Multiplex RT-qPCR Assay averaged N1 and N2 sequence Ct (+7) values is illustrated in **Figure 4B**.

Table 10. Summary of clinical evaluation of CoviDetect™ COVID-19 Multiplex RT-qPCR versus CoviDetect™ FAST COVID-19 Multiplex RT-qPCR.

Clinical performance Oropharyngeal swabs		CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay		
		Positive	**Negative**	**Total**
CoviDetect™ COVID-19 Multiplex RT-qPCR	**Positive**	20	0	20
	Negative	0	75	75
	Total	20	75	95
Positive Percent Agreement: 20/20 or 100% (CL95: 83.9-100%)				
Negative Percent Agreement: 75/75 or 100% (CL95: 95.1-100%)				

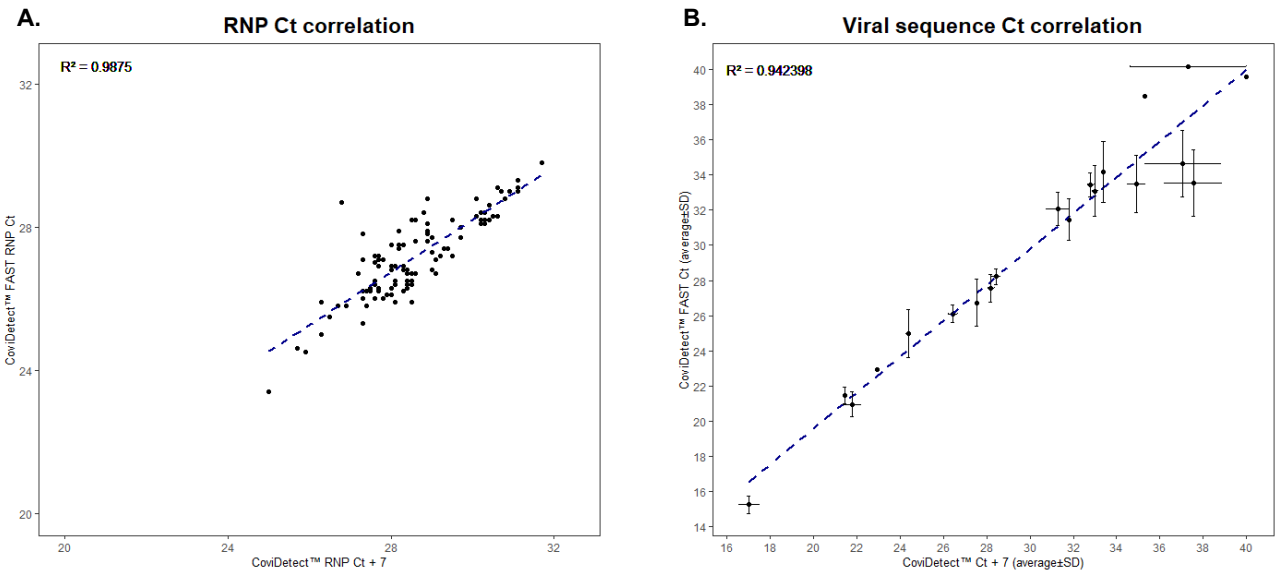

Figure 4. A. Correlation of human RNase P sample control Ct values between CoviDetect™ COVID-19 Multiplex RT-qPCR and CoviDetect™ FAST COVID-19 Multiplex RT-qPCR using oropharyngeal swab clinical samples. **B.** Correlation of averaged Ct values (±SD) for viral sequences between CoviDetect™ COVID-19 Multiplex RT-qPCR and CoviDetect™ FAST COVID-19 Multiplex RT-qPCR using oropharyngeal swab clinical samples.

8.5.2 Saliva sampling

Clinical performance of saliva sampling versus oropharyngeal swab was analysed for the presence of SARS-CoV-2 in 361 individuals using the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay. The extraction of RNA was performed using the Viral DNA and RNA Extraction Kit for the BasePurifier™ 32 Instrument. RT-qPCR was performed using BaseTyper™ Instrument. The analysis was performed using automatic baseline and threshold settings. Evaluation summary is shown in **Table 11**. Of the 358 samples, 26 were found positive for SARS-CoV-2 and 332 were found negative using oropharyngeal swab. In comparison was 28 samples positive using saliva samples. The two methods both found 25 samples positive which gave a positive agreement between the two methods of 25/26 or 96.2% (CL95: 81.1-99.3%, using Wilson score). Both methods detected 329 samples as negative, giving a negative agreement of 329/332 or 99.1% (CL95: 97.4-99.7%, using Wilson score).

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Table 11. SARS-CoV-2 positive and negative samples for oropharyngeal swab and saliva sampling.

CoviDetect™ FAST performance: Oropharyngeal swab compared with saliva		Saliva samples		
		Positive	**Negative**	**Total**
Oropharyngeal swabs	**Positive**	25	1	26
	Negative	3	329	332
	Total	28	330	358
Positive Percent Agreement: 25/26 or 96.2% (CL95: 81.1-99.3%)				
Negative Percent Agreement: 329/332 or 99.1% (CL95: 97.4-99.7)				

9 Limitations

- Performance of the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR 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.

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10 Symbols

The following symbols are used in labelling of CoviDetect™ FAST 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.