CoviDetect™ FAST

COVID-19 Multiplex RT-qPCR Assay

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

Instructions for use

Please read these instructions carefully before using PentaBase's CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay. It is recommended to save the *Instructions for use* for future use. Purchasers of PentaBase's CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay are only granted the right to use, but no general licensing or patent rights.

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CE IVD

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1. Intended use

CoviDetect™ FAST COVID-19 Multiplex RT-qPCR diagnostic Assay is a Real-Time RT (reverse transcriptase) PCR assay intended for the quantitative detection of nucleic acids from SARS-CoV-2. SARS-CoV-2 RNA can for example be found in the liquid from the upper or lower respiratory tracts of infected individuals. Samples can be obtained by nasopharyngeal or oropharyngeal swabs or from saliva.

Results are for the detection of SARS-CoV-2 RNA. Positive results indicate an infection with SARS-CoV-2 virus, but do not eliminate the possibilities of co-infections with other viruses or bacteria. Note that infection with SARS-CoV-2 can occur without showing any symptoms.

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

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

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

2. Summary and explanation of the assay

2.1 Indications for use

On December 31, 2019, China alerted the World Health Organization to several cases of unusual pneumonia in Wuhan. The virus was unknown. This infection has since been identified to be caused by the novel coronavirus, named SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2). Coronavirus disease 2019 (COVID-19) has been declared a public health emergency of international concern and caused millions of confirmed human infections. COVID-19 is the first pandemic caused by coronavirus with a fast spread rate and potentially fatal infection that resulted in significant worldwide morbidity and mortality.

Accurate diagnosis of SARS-CoV-2 is important in individuals suspected of a respiratory infection. The CoviDetect™ COVID-19 Multiplex RT-qPCR Assay is a molecular *in vitro* diagnostic assay based on PentaBase's highly sensitive technology to identify the presence of SARS-CoV-2 RNA in individuals. The assay is provided in a multiplex format, which means that one sample from a patient can be analyzed in one tube.

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.

2.2. Explanation of the assay

The CoviDetect™ FAST COVID-19 Multiplex RT-qPCR 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 HydrolEasy™ 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. 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.

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, a quencher at the 3' end, but is based on Pentabase-modified oligos 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™, Texas Red™ or Cy5™.

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

[1]Roche Molecular Systems, Inc

2.3 Principles of the procedure

The CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay is performed on Real-Time PCR Instrument for nucleic acid amplification and detection of the target sequence in biological samples.

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

The CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay targets three viral sequences of the SARS-CoV-2. The assay targets two regions from the RNA dependent RNA polymerase (named IP2 and IP4) gene and one region from the envelope protein gene (named E gene). 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 Texas Red™, respectively. Selective amplification of RNA Internal Control is achieved with the use of non-competitive sequence-specific forward and reverse primers with a Cy5™-labelled HydrolEasy™ probe which have no homology with the coronavirus genome. Amplified target is detected by cleavage of fluorescently labelled oligonucleotide probes specifically targeting the SARS-CoV-2 or 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.

The CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay includes SARS-CoV-2 RNA positive and negative control samples, which should be included in the RNA extraction procedure and each RT-qPCR runs for validation of the complete workflow.

Table 1. List of detected regions in 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	Texas Red™
RNP	Human RNase P (Swab and Extraction Control)	Cy5™

3. Reagent and materials

The materials provided for CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay can be found in Table 2. Materials required, but not provided can be found in Table 4 and 5. Reagent handling and storage can be found in Table 3.

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

3.1 CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay reagents and controls

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

Table 2. List of materials provided for CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay.

CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay Dispense ready (DR)		
Kit components	**Reagent ingredients**	**Safety symbol and warning**
FAST COVID-19 Multiplex RT-qPCR Assay	Synthetic DNA	Not applicable
AmpliSmaRT™ One Step RT-qPCR Master Mix	Not applicable	EUH210 Safety data sheet available on request.
SARS CoV-2 RNA Positive control	Tris buffer, EDTA, Guanidinium Thiocyanate, 0.125% SDS	EUH210 Safety data sheet available on request.  **DANGER**

		H302+H332 Harmful if swallowed or if inhaled. H314 Harmful to aquatic life with long lasting effects EUH032 Contact with acids liberates very toxic gas. P280 Wear protective gloves/protective clothing/eye protection/face protection. P304+P340+P312 IF INHALED Remove person to fresh air and keep at rest in position comfortable for breathing. Immediately call a POISON CENTER/doctor IF IN EYES Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER/ doctor. 593-84-0 Guanidinium Thiocyanate
SARS CoV-2 RNA Negative control	DNase/RNase free media	Not applicable
CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay Ready-To-Use (RTU)		
Kit components	**Reagent ingredients**	**Safety symbol and warning**
FAST COVID-19 Multiplex RT-qPCR Assay	Synthetic DNA	Not applicable
SARS CoV-2 RNA Positive control	Tris buffer, EDTA, Guanidinium Thiocyanate, 0.125% SDS	EUH210 Safety data sheet available on request.  **DANGER** H302+H332 Harmful if swallowed or if inhaled. H314 Harmful to aquatic life with long lasting effects EUH032 Contact with acids liberates very toxic gas. P280 Wear protective gloves/protective clothing/eye protection/face protection. P304+P340+P312 IF INHALED Remove person to fresh air and keep at rest in position comfortable for breathing. Immediately call a POISON CENTER/doctor IF IN EYES Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER/ doctor. 593-84-0 Guanidinium Thiocyanate
SARS CoV-2 RNA Negative control	DNase/RNase free media	Not applicable

3.2 Reagent storage and handling

The CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay is shipped on dry ice or frozen ice bricks. Reagents must be stored and handled as specified in Table 3 immediately upon arrival. The CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay should be stored in the original packaging and is stable for up to 7 months stored at -20°C. Reagents should not be used past any expiration date indicated on the assay packaging. If the assay's protective packaging is damaged upon receipt or has been shipped at the incorrect temperature, please contact PentaBase for instructions. Attention should be paid to the expiration date specified on the pack label. The reagents should be discarded following the disposal instructions in Section 11.

The assay components should be returned to the freezer promptly after use to minimize the time at room temperature. Repeated thawing and freezing should be kept to a minimum and should not exceed 12 freeze-thaw cycles.

Table 3. Reagent storage and reagent expiry conditions.

Reagent	Storage Temperature	Storage Time
CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay **(DR)**	-20°C to -80°C	Stable until expiration date indicated
AmpliSmaRT™ One Step RT-qPCR Master Mix	-20°C to -80°C	Stable until expiration date indicated
CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay **(RTU)**	-20°C to -80°C	Stable until expiration date indicated
CoviDetect™ FAST SARS CoV-2 RNA Positive control	-20°C	Stable until expiration date indicated
CoviDetect™ FAST SARS CoV-2 RNA Negative control	-20°C	Stable until expiration date indicated

3.3 Additional materials required

Table 4. Material and consumables required but not provided.

Material
Plasticware compatible with the PCR instrument[2]
Pipette Tips
Centrifuge for spinning tubes or plate
Nuclease free H_2O
Collection Kits
Oropharyngeal Swab
Nasopharyngeal Swab
Saliva Collector
Extraction Kit
Viral DNA/RNA Extraction kit

3.4 Instrumentation required

Table 5. Instrumentation.

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

4. Precautions and handling requirements

Warnings and precautions

- For *in vitro* diagnostic use.
- Treat all biological specimens, including used CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay tubes and transfer pipettes, as if capable of transmitting infections agents. All biological specimens should be treated with universal precautions, as it is often impossible to know which specimens might be infectious.
- Follow your institution's safety procedures for working with chemicals and handling biological samples.
- Good laboratory practices and careful adherence to the procedures specified in these Instructions for use are necessary. Wear laboratory coats, laboratory gloves and eye protection when handling biological samples and reagents. Laboratory gloves must be changed between handling different biological samples to avoid contamination of reagents.
- Remove gloves and wash hands thoroughly after handling samples and reagents.
- Do not use damaged CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay tube.
- Do not use a CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay pre-dispense 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.
- For additional warnings, precautions, and procedures to reduce the risk of contamination for the Nucleic Acid Extraction System or Real-Time PCR Instrument consult the respective System User Guides.
- Dispose of used CoviDetect™ FAST COVID-19 Multiplex RT-qPCR tube, pipette, and specimen tube according to local, state, and federal regulations for hazardous material.
- Safety Data Sheets (SDS) are available on request from your local PentaBase representative.

[2] Only when using Dispense Ready version

- Due to the high sensitivity of the assays, contamination of the work area with previous samples might cause false-positive results. Therefore, use extreme caution not to contaminate reagents and handle samples according to standard laboratory practice.
- CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay should be protected from light due to the presence of 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.
- Specimen collection must be performed using the appropriate swab types as recommended in Table 4. Inadequate or inappropriate sample collection, storage, and transport may yield incorrect or invalid results. DO NOT use cotton or calcium alginate swab, or swabs with wood shafts.
- Ensure there is no sign of leakage from the collection tube prior to running the analysis.

5. Sample collection, transport, and storage

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

5.1 Sample collection

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

5.2 Transport and storage

- Transportation of collected specimens must comply with all applicable regulations for the transport of biological agents.
- Specimens can be stored in suitable buffers at 2-8°C for up to 72 hours after collection.
- Extracted RNA should always be stored at -70°C or lower.

6. Instructions for use

6.1 Procedural notes

- Do not use CoviDetect™ FAST COVID-19 RT-qPCR Assay, AmpliSmaRT™ One-Step RT-qPCR Master Mix, SARS CoV-2 RNA Positive control or SARS CoV-2 RNA Negative control after expiry dates.
- Do not reuse consumables. They are for one-time use only.

6.2 Reagent Preparation

6.2.1 Dispense-Ready

a. Add **6 µl** 2x AmpliSmaRT™ One-Step RT-qPCR Master Mix to each PCR tube or well.
b. Add **1 µl** 12x primer/probe multiplex mix to the PCR tubes or wells.
c. Add **5 µl** of the template to each PCR tube. One patient is analyzed in a single PCR tube.
d. Close all PCR strips or seal plates.

6.2.2 Ready-To-Use

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

6.2.3 Positive and negative controls

Positive controls (20 copies/µl) and negative controls are provided with the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay and 200 µl should be added during the RNA extraction procedure.

Note: The positive control contains Guanidine thiocyanate and SDS and cannot be added directly to the CoviDetect™ FAST COVID-19 RT-qPCR Assay but must be subjected to a nucleotide extraction procedure first.

6.3 Running CoviDetect™ FAST COVID-19 RT-qPCR Assay

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

Table 6. RT-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 60	1 12	45	8 8	FAM™ (green) HEX™/VIC (yellow) Texas Red™ (orange) Cy5™ (red)

7. Data Analysis

The CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay, which determines 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 stage 3 are compared to predefined cutoff values to determine if the individual samples are positive or negative for SARS-CoV-2 (Section 8.3). A sample is valid if the Ct value for the internal control is lower than 34 or if at least two out of the three viral targets have Ct values below 41.

7.1 Instruments

CoviDetect™ FAST COVID-19 Multiplex RT-qPCR assay is designed to run on open platforms and, currently, has been validated on BaseTyper™ (PentaBase), and CFX96 (BioRad) Real-Time PCR instruments. Optimal PCR profiles are developed for each validated instrument. Please write to info@pentabase.com for current instrument-specific instructions for use available. To run CoviDetect™ FAST COVID-19 Multiplex RT-qPCR assay on other instruments, you must validate settings yourself. It is recommended to perform a specific validation using patient samples and synthetic controls to set a cycle threshold and cutoffs correctly. Please contact PentaBase or your local distributor for support.

7.2 Baseline and threshold settings

Results from CoviDetect™ FAST COVID-19 Multiplex RT-qPCR can be analyzed 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. Please refer to the troubleshooting section (Section 10) for more guidance on correcting improper analysis settings.

7.3 Interpretation of results

A sample can either be positive, negative, or invalid. The results are only valid if the included positive control Ct values are ≤34 for IP2, ≤33 for IP4 and E, and ≤28 for RNase P internal control. No template (NTC) negative control should produce no Ct values.

7.3.1 Positive samples

The sample is positive for SARS-CoV-2 when at least two Ct values for viral IP2, IP4 and E assays are below 41 even when RNAse P is negative. Please notice that the RNase P signal may be repressed in some samples and particularly when containing large amounts of viral RNA. The sample is also considered positive if at least two out of three of IP2, IP4 and/or E are positive when RNase P is positive. Furthermore, a sample is also positive if only one out of the three viral-specific assays come up in two independent runs, anticipating that internal, positive, and negative controls are all valid. The lack of signal in either IP2, IP4 or E may be due to very limited amount of virus or the presence of mutations present in the target regions of the assay (or reflect the presence of a very low amount of viral template). In case of a confirmed

positive sample where there is only signal in either one or two of the markers IP2, IP4 and E, it is recommended to send the sample for sequencing if possible and report the mutated strain to support@pentabase.com.

7.3.2 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.

7.3.3 Invalid samples

In case of no or late amplification of RNase P (Ct ≥ 34), the test is invalid unless at least two out of three of IP2, IP4 and E are positive (Ct < 41). If more specimen is available, repeat the extraction and run the test again. If all markers remain negative after repeating the test, no diagnosis can be concluded, and if possible, a new specimen should be collected for testing.

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 has been evaluated by spiking synthetic SARS-CoV-2 RNA (Twist Bioscience, Cat. no. 102015) into a negative clinical oropharyngeal matrix. Based on an initial dilution series, 3000, 1500 and 750 copies of SARS-CoV-2 RNA were each spiked into 3 ml of 20-24 oropharyngeal specimens. RNA was extracted using the BasePurifier™ Nucleic Acid Extraction Instrument and viral DNA/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 oropharyngeal matrix. RNA was extracted using the BasePurifier™ Nucleic Acid Extraction System.

RNA (copies/ml)	Observations (n)	Sequence	Positives	Positives (%)
0	20	IP2	0	0
		IP4	0	0
		E	0	0
		IP2 or IP4 or E	0	0
0.25	24	IP2	11	46
		IP4	2	8.3
		E	13	54
		IP2 or IP4 or E	18	75
0.5	20	IP2	14	70
		IP4	2	10
		E	11	55
		IP2 or IP4 or E	16	80
1.0	20	IP2	18	90
		IP4	4	20
		E	17	85
		IP2 or IP4 or E	20	100

The limit of detection of CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay using BaseTyper™ (Table 8), CFX (Table 9), QuantStudio™ 5 (Table 10) or Rotor-Gene® (Table 11) 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.

Table 8. 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 of SARS-CoV-2 RNA was performed using the BaseTyper™ Real-Time PCR instrument.

RNA (copies per reaction)	Observations (n)	Sequence	Positives	Positives (%)
0	26	IP2	0	0
		IP4	0	0
		E	0	0
		IP2 or IP4 or E	0	0
2	22	IP2	11	50
		IP4	1	4.5
		E	6	27.3
		IP2 or IP4 or E	16	72.7
5	22	IP2	18	81.8
		IP4	8	36.4
		E	22	100
		IP2 or IP4 or E	22	100
10	23	IP2	18	78.3
		IP4	20	87.0
		E	23	100
		IP2 or IP4 or E	23	100
20	24	IP2	24	100
		IP4	24	100
		E	24	100
		IP2 or IP4 or E	24	100

Table 9. 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 of SARS-CoV-2 RNA was performed using the CFX96 instrument.

RNA (copies per reaction)	Observations (n)	Sequence	Positives	Positives (%)
0	20	IP2	0	0
		IP4	0	0
		E	0	0
		IP2 or IP4 or E	0	0
2	20	IP2	10	50
		IP4	2	10
		E	10	50
		IP2 or IP4 or E	13	65
5	20	IP2	16	80
		IP4	3	15
		E	19	95
		IP2 or IP4 or E	20	100
10	20	IP2	19	95
		IP4	19	95
		E	19	95
		IP2 or IP4 or E	19	95

Table 10. 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 of SARS-CoV-2 RNA was performed using the QuantStudio™ 5 Real-Time PCR System.

RNA (copies per reaction)	Observations (n)	Sequence	Positives	Positives (%)
0	20	IP2	0	0
		IP4	0	0
		E	0	0
		IP2 or IP4 or E	0	0
1	20	IP2	10	50
		IP4	0	0
		E	1	5
		IP2 or IP4 or E	11	55
2	26	IP2	21	81
		IP4	5	19
		E	16	61.5
		IP2 or IP4 or E	25	96
5	26	IP2	25	96
		IP4	10	38.5
		E	25	96
		IP2 or IP4 or E	26	100
10	26	IP2	26	100
		IP4	12	46
		E	26	100
		IP2 or IP4 or E	26	100
50	20	IP2	20	100
		IP4	20	100
		E	20	100
		IP2 or IP4 or E	20	100
100	20	IP2	20	100
		IP4	20	100
		E	20	100
		IP2 or IP4 or E	20	100

Table 11. Limit of detection (LOD) of CoviDetect™ COVID-19 Multiplex Assay using SARS-CoV-2 RNA spiked into wild type human DNA. RT-qPCR of SARS-CoV-2 RNA was performed using the Rotor-Gene® Real-Time PCR Cycler.

RNA (copies per reaction)	Observations (n)	Sequence	Positives	Positives (%)
0 (NTC)	12	IP2	0	0
		IP4	0	0
		E	0	0
		IP2 or IP4 or E	0	0
5	20	IP2	5	25
		IP4	6	30
		E	5	25
		IP2 or IP4 or E	12	60
10	20	IP2	9	45
		IP4	6	30
		E	16	80
		IP2 or IP4 or E	18	90
50	20	IP2	20	100
		IP4	20	100
		E	20	100
		IP2 or IP4 or E	20	100

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. Based on an initial dilution series, 6000, 3000 and 1500 copies of SARS-CoV-2 RNA were each spiked into 6 ml of 20-24 oropharyngeal specimens. RNA was extracted using the BasePurifier™ Nucleic Acid Extraction Instrument and viral DNA/RNA extraction kit (Table 12).

Table 12. 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 of SARS-CoV-2 RNA was performed using the BaseTyper™ instrument.

Copies/µl media	Observations (n)	Sequence	Positives	Positives (%)
0	20	IP2	0	0
		IP4	0	0
		E	0	0
		IP2 or IP4 or E	**0**	**0**
0.25	20	IP2	5	25
		IP4	0	0
		E	7	35
		IP2 or IP4 or E	**10**	**50**
0.5	20	IP2	8	40
		IP4	0	0
		E	9	45
		IP2 or IP4 or E	**14**	**70**
1.0	20	IP2	14	70
		IP4	2	10
		E	13	65
		IP2 or IP4 or E	**19**	**95**

8.1.2.2 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™ Nucleic Acid Extraction Instrument and viral DNA/RNA extraction kit. All 120 samples were found SARS-CoV-2 negative using the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay. RNP signal in the internal control was found to Ct<34.

8.2 Inclusivity

CoviDetect™ FAST COVID-19 Multiplex RT-qPCR oligonucleotide sequences have been aligned with Global SARS-CoV-2 sequences from GISAID (total of 229.993 sequences). Mismatch frequencies were found to be less than 5% (1.7%).

IP2 Forward primer

```
CCTGTTGCACTACGACAGA
|||||||| |||||||||  >hCoV-19/India/GJ-GBRC-379/2020|EPI_ISL_524738|
||||  |||||||||||||  >hCoV-19/Wuhan/HB-WH4-200/2020|EPI_ISL_454952|
|||||||  ||||||||||  >hCoV-19/Japan/PG-0010/2020|EPI_ISL_479797|
|||||||||||| |||||  >hCoV-19/Australia/VIC16095/2020|EPI_ISL_640440|
|||||||  ||||||||||  >hCoV-19/England/ALDP-B1A0BD/2020|EPI_ISL_645731|
||||||||||  |||||||  >hCoV-19/England/LIVE-DB2BA5/2020|EPI_ISL_651355|
||||||||  ||||||||||  >hCoV-19/England/ALDP-B1A545/2020|EPI_ISL_647546|
```

IP2 Reverse Primer

```
GTAGTAAGCTAACGCATTGTCA
|||||||||||| |||||||||  >hCoV-19/South_Africa/KRISP-K002815/2020|EPI_ISL_602636|
||||||||||| |||||||||  >hCoV-19/South_Africa/KRISP-0574/2020|EPI_ISL_498112|
|||||  ||||||||||||||  >hCoV-19/Singapore/739/2020|EPI_ISL_524446|
|||||||| ||||||||||||  >hCoV-19/Bangladesh/BCSIR-NILMRC-374/2020|EPI_ISL_514250|
||||||||||||| ||||||||  >hCoV-19/Lebanon/LAU4-53460/2020|EPI_ISL_637113|
||||||||||| |||||||||  >hCoV-19/Ecuador/USFQ-520/2020|EPI_ISL_660539|
||||||||||| |||||||||  >hCoV-19/Italy/APU-IZSPB_297PT/2020|EPI_ISL_653791|
```

IP2 Probe

```
CTTGTGCTGCCGGTACTA
|||  ||||||||||||||  >hCoV-19/India/MH-NIV-6285/2020|EPI_ISL_454541|
||||||||||  |||||||  >hCoV-19/India/MH-GA97/2020|EPI_ISL_511940|
||||||||||  |||||||  >hCoV-19/Japan/TKYT22342/2020|EPI_ISL_649126|
||||||||||||  |||||  >hCoV-19/India/DL-5504375-CSIR-IGIB/2020|EPI_ISL_636787|
||||||||||  |||||||  > hCoV-19/Japan/TKYT22342/2020|EPI_ISL_649126|
|||||||  ||||||||||  >hCoV-19/Brazil/PE-IAM1468/2020|EPI_ISL_572396|
||||  ||||||||||||||  >hCoV-19/Colombia/COR-GVI-97527/2020|EPI_ISL_447817|
||||||||||||||  ||||  >hCoV-19/Australia/VIC4465/2020|EPI_ISL_519761|
```

IP4 Forward primer

```
GGTAACTGGTATGATTTCGGTGA
||||||||||||||||||  |||||  >hCoV-19/South_Africa/KRISP-K002609/2020|EPI_ISL_535473|
||||||  ||||||||||||||||||  >hCoV-19/South_Korea/CNUHV03/2020|EPI_ISL_479662|
||||||||||||  ||||||||||||  > hCoV-19/Bangladesh/BCSIR-NILMRC-364/2020|EPI_ISL_514237|
||||||||||||||||||  |||||  > hCoV-19/India/KA-nimh-14834/2020|EPI_ISL_515958|
||||  ||||||||||||||||||  >hCoV-19/Australia/VIC4989/2020|EPI_ISL_519793|
||||  ||||||||||||||||||  >hCoV-19/Australia/VIC5603/2020|EPI_ISL_518418|
||||  ||||||||||||||||||  >hCoV-19/Australia/VIC17519/2020|EPI_ISL_663319|
```

IP4 Reverse Primer

```
CCTGGTCAAGGTTAATATAGGCA
||||||||||||||||||  |||||  >hCoV-19/South_Africa/R05475/2020|EPI_ISL_435059|
|||||||||  ||||||||||||||  > hCoV-19/India/OR-ILSCV32652/2020|EPI_ISL_481160|
||||  ||||||||||||||||||  >hCoV-19/Singapore/794/2020|EPI_ISL_512830|
||||  ||||||||||||||||||  >hCoV-19/Singapore/841/2020|EPI_ISL_518005|
||||||||||  ||||||||||||  >hCoV-19/Ecuador/52255/2020|EPI_ISL_491952|
||||  ||||||||||||||||||  >hCoV-19/Australia/VIC2134/2020|EPI_ISL_480732|
||||||||||||||||  ||||||  >hCoV-19/Australia/NSW1130/2020|EPI_ISL_593743|
||||||||||||||||  ||||||  >hCoV-19/Australia/VIC7844/2020|EPI_ISL_564695|
```

IP4 Probe

```
CATACAAACCACGCCAGGTAG
||||||||||||  ||||||||  >hCoV-19/South_Africa/KRISP-K003360/2020|EPI_ISL_602751|
||||||||||||||  ||||||  >hCoV-19/Gambia/GC192789/2020|EPI_ISL_561145|
||||||||||||  ||||||||  >hCoV-19/bat/Yunnan/RaTG13/2013|EPI_ISL_402131|
|||||||||  ||||||||||  >hCoV-19/Indonesia/JI-NIHRD-PME2054/2020|EPI_ISL_538499|
||||||||||||||  |||||||  >hCoV-19/United_Arab_Emirates/H9/2020|EPI_ISL_528721|
||||||||||||  ||||||||  >hCoV-19/Suriname/SR-62/2020|EPI_ISL_518811|
||||||||||||||  ||||||  >hCoV-19/Brazil/MG-0216/2020|EPI_ISL_470582|
||||||||||||||  ||||||  >hCoV-19/Australia/VIC1365/2020|EPI_ISL_456411|
||||||||||||||||  ||||  >hCoV-19/Australia/NSW145/2020|EPI_ISL_427708
||||||||||  |||||||||  >hCoV-19/Australia/SAP380/2020|EPI_ISL_492145|
```

E Forward Primer

```
GACAGGTACGTTAATAGTTAATAGC
|||||||||||||||||||| |||||  >hCoV-19/DRC/4012/2020|EPI_ISL_471402|
|||||||||| ||||||||||||||  >hCoV-19/India/UT-50464-CSIR-IGIB/2020|EPI_ISL_636759|
|||||||||||||||||| |||||||  >hCoV-19/India/TG-NIV-906/2020|EPI_ISL_547877|
|||||||||||||||||| ||||||  >hCoV-19/Brazil/RJ-2669/2020|EPI_ISL_467353|
||||||| ||||||||||||||||||  > hCoV-19/England/CAMB-1BA49F/2020|EPI_ISL_664983|
||||||| ||||||||||||||||||  > hCoV-19/England/CAMB-1BB65C/2020|EPI_ISL_665016|
|||||||||||||||||||| |||  > hCoV-19/England/CAMC-B21596/2020|EPI_ISL_647538|
|||||||||||||||||||| |||||  > hCoV-19/England/QEUH-AAEADF/2020|EPI_ISL_652982|
```

E Reverse Primer

```
CAGCAGTACGCACACAATC
||||||||| |||||||||  >hCoV-19/Mali/M002659/2020|EPI_ISL_487451|
||||| |||||||||||||  >hCoV-19/DRC/299/2020|EPI_ISL_420840|
||||| |||||||||||||  >hCoV-19/Thailand/Bangkok_323/2020|EPI_ISL_447916|
|||||||| ||||||||||  >hCoV-19/Singapore/1383/2020|EPI_ISL_648737|
||||||||| |||||||||  >hCoV-19/Argentina/argenTAG-12CF5045/2020|EPI_ISL_648212|
||||| |||||||||||||  >hCoV-19/Australia/VIC6036/2020|EPI_ISL_518077|
|||||||| ||||||||||  >hCoV-19/Australia/VIC15000/2020|EPI_ISL_593099|
||||| |||||||||||||  >hCoV-19/USA/NY-NYUMC259/2020|EPI_ISL_428773|
||||||||| |||||||||  >hCoV-19/USA/WA-S296/2020|EPI_ISL_430150|
```

E Probe

```
CTTTCGTGGTATTCTTGCTAG
||| ||||||||||||||||  >hCoV-19/South_Africa/KRISP-K005335/2020|EPI_ISL_678623|
||| ||||||||||||||||  >hCoV-19/South_Africa/KRISP-K005332/2020|EPI_ISL_678620|
|||||||||| ||||||||||  >hCoV-19/India/TG-CCMB-K600B/2020|EPI_ISL_447581|
|||||||||||||||| ||||  >hCoV-19/Singapore/1283/2020|EPI_ISL_648768|
|||||||||| ||||||||||  >hCoV-19/India/UT-AR8/2020|EPI_ISL_508205|
|||||| |||||||||||||  >hCoV-19/Brazil/PE-IAM1126/2020|EPI_ISL_572379|
|||| ||||||||||||||||  >hCoV-19/Brazil/PE-IAM109/2020|EPI_ISL_572334|
|||| ||||||||||||||||  >hCoV-19/Peru/LIM-UPCH-0126/2020|EPI_ISL_568522|
|||||||||| ||||||||||  >hCoV-19/Australia/NSW335/2020|EPI_ISL_451597|
||||||||||||||||||||   >hCoV-19/Australia/VIC8638/2020|EPI_ISL_565125|
```

The following entries were found to contain mismatch mutations at more than one primer/probe sequence. As no entries with variation in more than two CoviDetect™ FAST COVID-19 Multiplex RT-qPCR oligonucleotides covered areas among close to 200000 published SARS-CoV2 sequences where encountered, we consider it highly unlikely that CoviDetect™ FAST COVID-19 Multiplex Rt-qPCR Assay will be unable to recognize current subjects due to sequence mismatch.

Mismatch mutation in IP4.Rv1b and IP2.Fw2:

hCoV-19/India/GJ-GBRC-379/2020|EPI_ISL_524738|2020-06-18

Mismatch mutation in IP4.Probe1a and E.Probe2:

hCoV-19/USA/CA-ALSR-4459/2020|EPI_ISL_649042|

hCoV-19/England/ALDP-AA3C2F/2020|EPI_ISL_624440|

hCoV-19/England/ALDP-AA3C98/2020|EPI_ISL_624524|

hCoV-19/England/QEUH-B0E8B4/2020|EPI_ISL_642081|

hCoV-19/England/QEUH-B0F983/2020|EPI_ISL_641977|

hCoV-19/England/QEUH-B10BD4/2020|EPI_ISL_642584|

hCoV-19/Scotland/CVR5812/2020|EPI_ISL_666030|

Mismatch mutation in IP4.Rv1b and E.Probe2:

hCoV-19/England/QEUH-9F3395/2020|EPI_ISL_588625|

Mismatch mutation in IP2.Probe2 and IP2.Fw2:

hCoV-19/England/ALDP-B1A545/2020|EPI_ISL_647546|

Mismatch mutation IP2.Probe2 and E.Probe2:

hCoV-19/Czech_Republic/NRL-10208/2020|EPI_ISL_660583|
hCoV-19/Czech_Republic/NRL-10207/2020|EPI_ISL_660573|

8.3 Clinical evaluation

8.3.1 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 analyzed for the presence of SARS-CoV-2 using the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay. 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™ 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 12.

Table 12. Summary of clinical evaluation of CoviDetect™ COVID-19 Multiplex RT-qPCR versus CoviDetect™ FAST COVID-19 Multiplex RT-qPCR.

	Assay	CoviDetect™	CoviDetect™ FAST	Agreement
Oropharyngeal swabs	SARS-CoV-2 positive	20	20	100% (PPA)
	SARS-CoV-2 negative	44	44	100% (NPA)

The RNP 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 1.

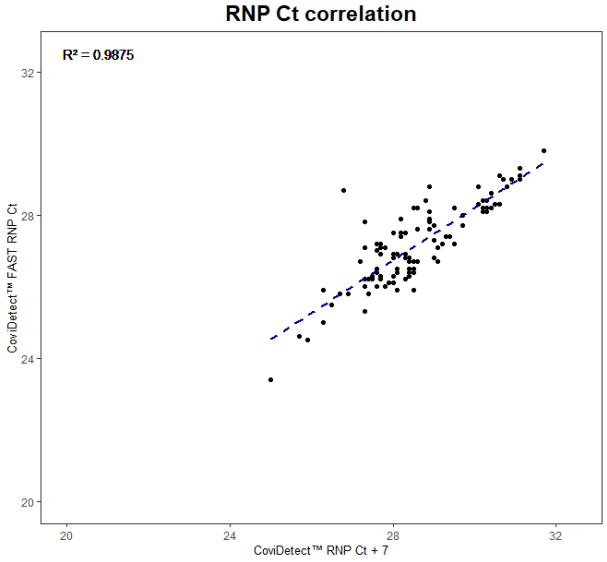

Figure 1. Correlation of internal control human RNP Ct values between CoviDetect™ COVID-19 Multiplex RT-qPCR and CoviDetect™ FAST COVID-19 Multiplex RT-qPCR using oropharyngeal swab clinical samples.

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 2.

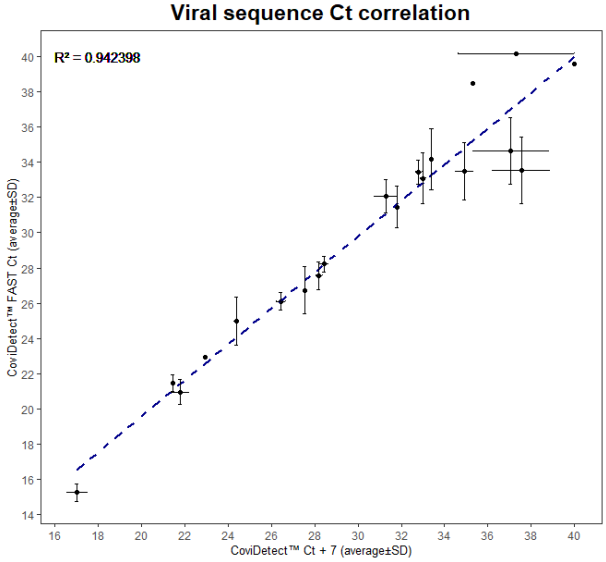

Figure 2. Correlation of averaged Ct values (±SD) for viral sequences between CoviDetect™ COVID-19 Multiplex RT-qPCR and CoviDetect™ FAST COVID-19 Multiplex RT-qPCR using oropharyngeal swab clinical samples.

8.3.2 Saliva sampling

Clinical performance of saliva sampling versus oropharyngeal swab was analyzed 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™ 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 13.

Table 13. SARS-CoV-2 positive and negative samples for oropharyngeal swab and saliva sampling.

SARS-CoV-2 positive and negative samples for oropharyngeal swab and saliva			
	Oropharyngeal swab	**Saliva**	**Agreement**
SARS-CoV-2 positive	26	28	25
SARS-CoV-2 negative	332	329	329
Invalid samples	0	3*	-

* Three SARS-CoV-2 negative samples were invalid in saliva.

Of the 361 tested persons, 29 were found positive for SARS-CoV-2 and 332 were found negative on either the oropharyngeal swab, saliva sampling or both sampling methods.

One sample was found SARS-CoV-2 positive using the oropharyngeal swabs and negative by saliva sampling. The saliva sampling found three samples SARS-CoV-2 positive which was found negative using the oropharyngeal swabs. The specificity was 100% for both sampling methods. The sensitivity was higher using saliva samples (96.6%) compared to sampling using oropharyngeal swabs (89.7%).

The boxplot (Figure 3) illustrates that the mean Ct values of the three SARS-CoV-2 specific genes are very similar for both sampling methods. The mean Ct value for the saliva samples is observed to be lower on the internal control gene (IC)

compared to oropharyngeal swab. In addition, a higher standard deviation is observed on the oropharyngeal swab samples for the SARS-CoV-2 specific genes.

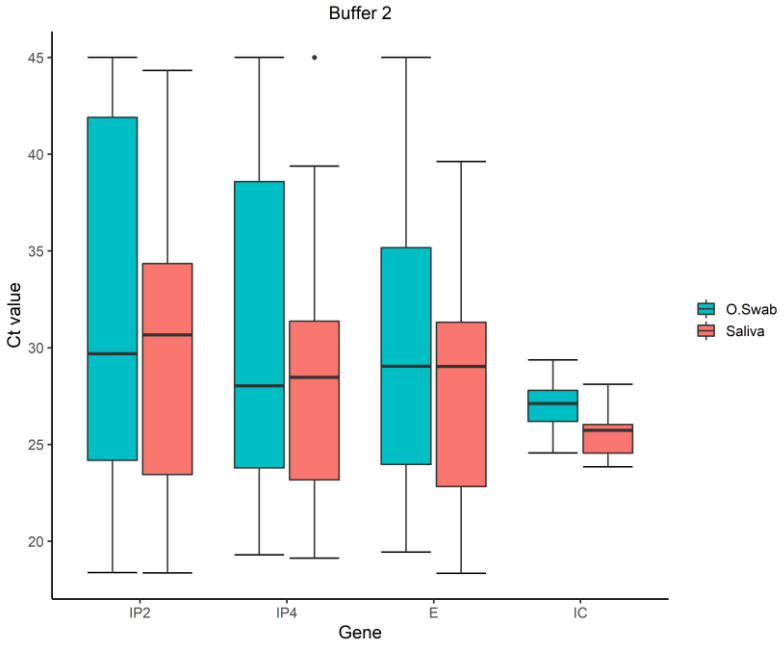

Figure 3. Boxplot for Ct values of three SARS-CoV-2 specific genes (IP2, IP4 and E) and an internal control (IC) for n=29 SARS-CoV-2 positive samples. The data is grouped as samples taken with oropharyngeal swab (O. Swab) and saliva. Mean Ct values are similar for oropharyngeal swab and saliva samples for the virus specific genes.

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.

10. Troubleshooting

The troubleshooting guide covers some of the most frequent questions and problems that can occur when using the CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay and how these may be solved.

Table 14. Troubleshooting guide

Problem	Solution
No extraction control signal	Make sure that the PCR program has been defined correctly and that the instrument is acquiring on FAM™, HEX™, Texas Red™ and Cy5™ channels in Step 2 of Stage 3.
No sample signal	The concentration or the quality of the RNA in the sample is too low. Add more sample if possible or collect a new specimen.
Signal in NTC	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 or your local distributor.
Baseline drift	Baseline drift is a slowly rising signal in the amplification plot with no or late exponential phase. Baseline drift can occur when baselining has not been done properly. Baseline drift can be corrected by adjusting the baseline interval manually or applying baseline drift correction as part of the analysis settings. In both cases the amplification curve should be aligned at or close to the baseline but should not go below before any subsequent exponential phase. If baseline drift cannot be corrected and/or there is any doubt about the quality of the amplification curve, the sample should be rerun.

11. Disposal

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

12. Symbols

The following symbols are used in labeling for CoviDetect™ FAST COVID-19 Multiplex RT-qPCR products.

Table 12. Symbols used in labelling for CoviDetect™ FAST COVID-19 Multiplex RT-qPCR products.


Date of manufacture

In vitro diagnostic medical device


Use-by date


Do not reuse


Contains sufficient for <*n*>


Manufacture


Temperature limit


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


Consult instructions for use

13. Manufacturer and distributors

For technical assistance in Denmark please contact Manufacturer PentaBase A/S:

Petersmindevej 1A
DK-5000 Odense, Denmark

Telephone: (+45) 36 96 94 96
Email**:** support@pentabase.com
Webpage**:** www.pentabase.com

For technical assistance in all other countries, contact your local distributor. A complete list of distributors is available at www.pentabase.com.