CoviDetect™

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™ COVID-19 Multiplex RT-qPCR Assay. It is recommended to save the *Instructions for use* for future use. Purchasers of PentaBase's CoviDetect™ 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™ COVID-19 Multiplex RT-qPCR diagnostic assay is a Real-Time RT (reverse transcriptase) PCR assay intended for the detection of nucleic acids from the SARS-CoV-2 virus. SARS-CoV-2 RNA can 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 sputum.

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™ 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.

2.2. Explanation of the assay

The CoviDetect™ 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™ 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.

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™ 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™ 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™ 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™ COVID-19 Multiplex RT-qPCR Assay targets two viral sequences of the SARS-CoV-2 nucleocapsid protein gene (named N1 and N2). Selective amplification of N1 and N2 sequences are achieved by using sequence-specific forward and reverse primers with HydrolEasy™ probes labelled with FAM™ or HEX™, respectively. Selective amplification of RNA Internal Control is achieved by using 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™ 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 in each RT-qPCR run for validation of the complete workflow.

Table 1. List of detected regions in the CoviDetect™ COVID-19 Multiplex RT-qPCR Assay

Targeted Regions	Gene	Fluorophore
N1	Nucleocapsid protein gene marker	FAM™
N2	Nucleocapsid protein gene marker	HEX™
RNP	Human RNase P (Extraction Control)	Cy5™

3. Reagent and materials

The materials provided for CoviDetect™ 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™ 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™ COVID-19 Multiplex RT-qPCR Assay.

CoviDetect™ COVID-19 Multiplex RT-qPCR Assay Dispense Ready (DR)		
Kit components	**Reagent ingredients**	**Safety symbol and warning**
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™ COVID-19 Multiplex RT-qPCR Assay Ready-To-Use (RTU)		
Kit components	**Reagent ingredients**	**Safety symbol and warning**
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™ 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™ 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.
When in use, the assay components should be returned to the freezer promptly after use to minimize the time at room temperature. Repeated thawing and freezing should be kept to a minimum and should not exceed 12 freeze-thaw cycles.

Table 3. Reagent storage and reagent expiry conditions.

Reagent	Storage Temperature	Storage Time
CoviDetect™ 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™ COVID-19 Multiplex RT-qPCR Assay **(RTU)**	-20°C to -80°C	Stable until expiration date indicated
CoviDetect™ SARS CoV-2 RNA Positive control	-20°C	Stable until expiration date indicated
CoviDetect™ 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
Nasopharyngeal swab
Nasal swab
Extraction Kit
Viral DNA/RNA Extraction kit

3.4 Instrumentation required

Table 5. Instumentation.

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

4. Precautions and handling requirements

Warnings and precautions

- For *in vitro* diagnostic use.
- Treat all biological specimens, including used CoviDetect™ 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™ COVID-19 Multiplex RT-qPCR Assay tube.

[2] Only when using Dispense Ready version

- 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.
- For additional warnings, precautions, and procedures to reduce the risk of contamination for the Nucleic Acid Extraction System or Real-Time PCR Instrument consult the respective System User Guides.
- Dispose of used CoviDetect™ COVID-19 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.
- 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 standard laboratory practice.
- CoviDetect™ 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 before running the analysis.

5. Sample collection, transport, and storage

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

5.1 Sample collection

The specimen should be nasopharyngeal or oropharyngeal swabs or saliva or sputum. Ineffective or inappropriate sample collection can result in false test results. Training in specimen collection is therefore recommended to ensure the best quality.

5.2 Transport and storage

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

6. Instructions for use

6.1 Procedural notes

- Do not use CoviDetect™ 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 **10 µl** 2x AmpliSmaRT™ One-Step RT-qPCR Master Mix to each PCR tube or well.
b. Add **5 µl** 4x primer/probe multiplex mix to the PCR tubes or wells.
c. Add **5 µl** of template to each PCR tube. One patient is analyzed in a single PCR tube.
d. Seal all tubes.

6.2.2 Ready-To-Use

a. Spin down the PCR strips or plates prior to addition of template to ensure that all reagents are collected at the bottom.

b. Add **5 µl** of 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™ 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™ COVID-19 RT-qPCR Assay but must be subjected to a nucleotide extraction procedure first.

6.3 Running CoviDetect™ 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. 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™ COVID-19 Multiplex RT-qPCR Assay.

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

7. Data Analysis

The CoviDetect™ 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 4 are compared to predefined cutoff values to determine if the individual samples are positive or negative for SARS-CoV-2 (Section 8.3).

7.1 Instruments

CoviDetect™ COVID-19 Multiplex RT-qPCR assay is designed to run on open platforms and has currently been validated on BaseTyper™ (PentaBase), CFX96 (BioRad) and LightCycler® 480 II (Roche) 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™ 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™ 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

An overview of the possible outcomes of the analysis is shown in Table 7. The results are only valid if the included positive control Ct values are below 35 for N1 and N2, and below 28 for RNase P internal control. No template (NTC) negative control should produce no Ct values. Ct cutoff values for CoviDetect™ COVID-19 Multiplex RT-qPCR Assay are shown in Table 8.

Table 7. Analysis outcomes based on target amplification curves. Conclusions are based on target Ct values compared to the cutoffs found in Table 8.

Target	Positive Case 1	Positive Case 2[3]	Positive Case 3	Positive Case 4	Negative	Invalid
RNase P	+	-	+	+	+	-
N1	+	+	-	+	-	-
N2	+	+	+	-	-	-

Table 8. Ct cutoff values for CoviDetect™ COVID-19 Multiplex RT-qPCR Assay.

Interpretation of RT-qPCR results (Stage 4)			
Assay	**Ct**	**Conclusion**	**Comments**
N1	<35	SARS-CoV-2 positive	Ct values should be below 35 for both N1 and N2 for samples to be positive for SARS-CoV-2.
N2	<35		
RNase P	<28	SARS-CoV-2 positive	The sample is positive if only N1 is positive and RNAse P is positive.
N1	<35		
N2	≥35		
RNase P	<28	SARS-CoV-2 positive	The sample is positive if only N2 is positive and RNAse P is positive.
N1	≥35		
N2	<35		
RNase P	<28	SARS-CoV-2 negative	A positive RNase P signal is required for a sample to be considered negative.
N1	≥35		
N2	≥35		
RNase P	≥28	Invalid	The sample does not contain enough material for the analysis. Take a new specimen if possible.
N1	≥35		
N2	≥35		

7.3.1 Positive samples

The sample is positive for SARS-CoV-2 when Ct values for both viral N1 and N2 assays are below 35. Please notice that the RNase P signal may be repressed in some samples and particularly when 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.

The sample is also considered positive if either N1 or N2 is positive when RNase P is positive. The lack of signal in either N1 or N2 may be due to mutations present in the target regions of the assay. In case of a confirmed positive sample where there is only signal in either N1 or N2, 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 the detection of SARS-CoV-2 if the sample is positive for RNase P but negative N1 and N2.

7.3.3 Invalid samples

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). 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.

[3]The RNase P signal may be suppressed when N1 and N2 are positive, especially in samples with high amounts of viral RNA.

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, Cat. 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 BasePurifier™ Nucleic Acid Extraction Instrument and viral DNA/RNA extraction kit (Table 7).

Table 7. 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™ nucleic acid extraction instrument.

Instrument	Measurement	N1		N2		N1 or N2	
BaseTyper™	RNA (copies per extraction)	500	250	500	250	500	250
	RNA concentration (copies/µl)	2.5	1.25	2.5	1.25	2.5	1.25
	Positives/Total	20/20	18/20	20/20	18/20	20/20	19/20
	Mean Ct (Stage 4)	30.0	31.1	28.5	29.2	NA	NA
	Standard Deviation (Ct)	1.2	1.7	1.1	1.9	NA	NA
CFX96	RNA (copies per extraction)	500	250	500	250	500	250
	RNA concentration (copies/µl)	2.5	1.25	2.5	1.25	2.5	1.25
	Positives/Total	20/20	17/20	20/20	18/20	20/20	19/20
	Mean Ct (Stage 4)	28.3	28.7	27.2	27.8	NA	NA
	Standard Deviation (Ct)	1.0	1.7	0.9	1.9	NA	NA
LightCycler® 480	RNA (copies per extraction)	500	250	500	250	500	250
	RNA concentration (copies/µl)	2.5	1.25	2.5	1.25	2.5	1.25
	Positives/Total	20/20	16/20	19/20	15/20	20/20	16/20
	Mean Ct (Stage 4)	29.2	30.5	30.0	29.7	NA	NA
	Standard Deviation (Ct)	2.6	1.4	1.2	0.7	NA	NA

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

RNA (copies per reaction)	Observations (n)	Assay	Positives	Positives (%)
0	18	N1	0	0
		N2	0	0
		N1 or N2	0	0
1	12	N1	2	17
		N2	2	17
		N1 or N2	2	17
5	30	N1	19	63
		N2	19	63
		N1 or N2	21	70
10	36	N1	27	75
		N2	31	86
		N1 or N2	32	89
20	28	N1	21	75
		N2	26	93
		N1 or N2	27	96
50	10	N1	10	100
		N2	10	100
		N1 or N2	10	100
100	8	N1	8	100
		N2	8	100
		N1 or N2	8	100

Table 9. 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 QuantStudio™ 5 Real-Time PCR System.

RNA (copies per reaction)	Observations (n)	Assay	Positives	Positives (%)
0	24	N1	0	0
		N2	0	0
		N1 or N2	0	0
2	20	N1	15	75
		N2	14	70
		N1 or N2	15	75
5	20	N1	19	95
		N2	19	95
		N1 or N2	20	100
10	20	N1	20	100
		N2	15	75
		N1 or N2	20	100
50	20	N1	20	100
		N2	20	100
		N1 or N2	20	100
100	38	N1	38	100
		N2	38	100
		N1 or N2	38	100

Table 10. 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)	Assay	Positives	Positives (%)
0	16	N1	0	0
		N2	0	0
		N1 or N2	0	0
2	20	N1	10	50
		N2	0	0
		N1 or N2	10	**50**
5	20	N1	15	75
		N2	0	0
		N1 or N2	15	**75**
10	20	N1	20	100
		N2	14	70
		N1 or N2	20	**100**
25	20	N1	20	100
		N2	19	95
		N1 or N2	20	**100**
50	20	N1	20	100
		N2	15	75
		N1 or N2	20	**100**
100	20	N1	4	100
		N2	4	100
		N1 or N2	4	**100**

8.2 Inclusivity

CoviDetect™ COVID-19 Multiplex assay oligo sequences have been aligned with Global SARS-CoV-2 sequences from GISAID (excluding Denmark, see section 9.2.2). Mismatch frequencies were found to be less than 5%.

N1 Forward Primer

```
GACCCCAAAATCAGCGAAAT
|||||||||||| ||||||  >hCoV-19/USA/WA-UW112/2020|EPI_ISL_416650|2020-03-10
||||| |||||||||||||  >hCoV-19/pangolin/Guangdong/1/2019|EPI_ISL_410721|2019
|||||||||||| ||||||  >hCoV-19/Netherlands/ZuidHolland_28/2020|EPI_ISL_415532|2020-03-09
||||||||||| |||||||||  >hCoV-19/Senegal/026/2020|EPI_ISL_418209|2020-03-03
||||| |||||||||||||||  >hCoV-19/Foshan/20SF207/2020|EPI_ISL_406534|2020-01-22
||||||||||| || ||||||  >hCoV-19/bat/Yunnan/RaTG13/2013|EPI_ISL_402131|2013-07-24
|||||||||||| ||||||  >hCoV-19/USA/WA-UW112/2020|EPI_ISL_416650|2020-03-10
||||| |||||||||||||||  >hCoV-19/pangolin/Guangdong/1/2019|EPI_ISL_410721|2019
|||||||||||| ||||||  >hCoV-19/Netherlands/ZuidHolland_28/2020|EPI_ISL_415532|2020-03-09
||||||||||| |||||||||  >hCoV-19/Senegal/026/2020|EPI_ISL_418209|2020-03-03
||||| |||||||||||||||  >hCoV-19/Foshan/20SF207/2020|EPI_ISL_406534|2020-01-22
||||||||||| || ||||||  >hCoV-19/bat/Yunnan/RaTG13/2013|EPI_ISL_402131|2013-07-24
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N1 Reverse Primer

```
CGCAGTATTATTGGGTAAACC
```

No mismatches found

N1 Probe

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ACCCCGCATTACGTTTGGTGGACC
|||||| ||||||||||||||||||  >hCoV-19/USA/TX_2967/2020|EPI_ISL_420798|2020-03-01
|||||||||||||||| |||||||  >hCoV-19/France/ARA11997/2020|EPI_ISL_419176|2020-03-21
|||||| ||||||||||||||||||  >hCoV-19/Tianmen/HBCDC-HB-07/2020|EPI_ISL_412983|2020-02-08
|||||| ||||||||||||||||||  >hCoV-19/Australia/VIC41/2020|EPI_ISL_419760|2020-03-11
|||||| ||||||||||||||||||  >hCoV-19/USA/TX_2967/2020|EPI_ISL_420798|2020-03-01
|||||||||||||||| |||||||  >hCoV-19/France/ARA11997/2020|EPI_ISL_419176|2020-03-21
|||||| ||||||||||||||||||  >hCoV-19/Tianmen/HBCDC-HB-07/2020|EPI_ISL_412983|2020-02-08
|||||| ||||||||||||||||||  >hCoV-19/Australia/VIC41/2020|EPI_ISL_419760|2020-03-11
```

N2 Forward primer

```
AGGAACTGATTACAAACATTGGC
|||||||||||||||||| ||||   >hCoV-19/Estonia/ChVir1985/2020|EPI_ISL_420067|2020-03
|||||||||||||||||| ||||   >hCoV-19/pangolin/Guangdong/1/2019|EPI_ISL_410721|2019
```

N2 Reverse Primer

```
TGTAGGTCAACCACGTTCCC
|||||||||||| ||||||||      >hCoV-19/USA/WI-43/2020|EPI_ISL_421301|2020-03-19
||||||| ||||||||||||      >hCoV-19/Hungary/2/2020|EPI_ISL_418183|2020-03-17
```

N2 Probe

```
TGCACAATTTGCCCCCAGCG
|||||||||||||||| ||||      >hCoV-19/Wales/PHWC-255AC/2020|EPI_ISL_421008|2020-03-23
||||||||||| |||||||||      >hCoV-19/Iceland/30/2020|EPI_ISL_417773|2020-03-03
|||||||||||||||| ||||      >hCoV-19/Chongqing/YC01/2020|EPI_ISL_408478|2020-01-21
||||||||| || |||||||      >hCoV-19/bat/Yunnan/RaTG13/2013|EPI_ISL_402131|2013-07-24
```

8.3 Analytical specificity

CoviDetect™ COVID-19 Multiplex assay oligo sequences have been aligned with common Betacoronaviruses. There is only potential binding of the N1 assay to the original SARS coronavirus (SARS-CoV) when less than 5 mismatches are included. Subsequent wet test of cross-reactivity to SARS-CoV was negative.

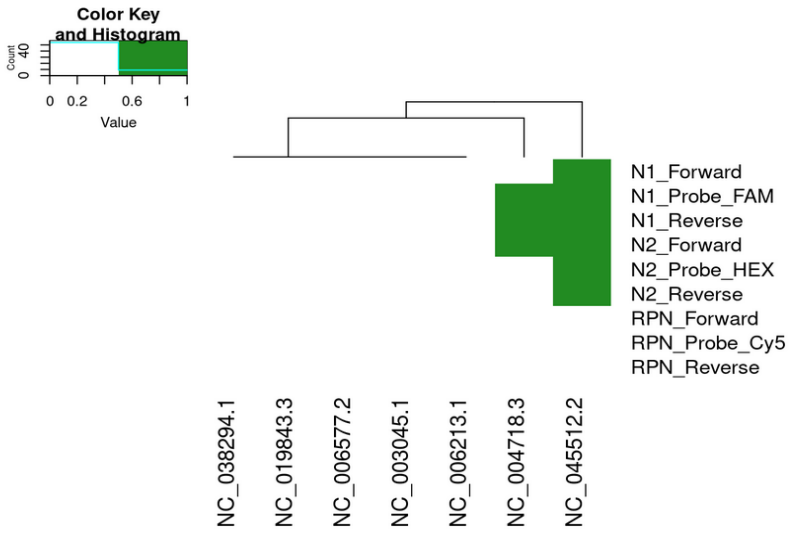

Figure 1. In silico analysis of cross-reactivity to betacoronaviruses including the common cold. NC_038294.1: Betacoronavirus England 1, NC_019843.3: Middle East Respiratory syndrome coronavirus, NC_006577.2: Human coronavirus HKU1, NC_003045.1: Bovine coronavirus, NC_006213.1: Human coronavirus OC43 strain ATCC VR-759, NC_004718.3: SARS coronavirus, NC_045512.2: SARS-Coronavirus 2 (SARS-CoV-2).

8.3.1 Danish cases

CoviDetect™ COVID-19 Multiplex assay oligo sequences have been aligned with +300 Danish SARS-CoV-2 sequences from GISAID. Two and one mutation(s) were identified in the target sequences of the N1 and N2 probes, respectively.

N1 Forward Primer

GACCCCAAAATCAGCGAAAT

No mismatches found

N1 Reverse Primer

CGCAGTATTATTGGGTAAACC

No mismatches found

N1 Probe

ACCCCGCATTACGTTTGGTGGACC

One Danish strain has been identified with an insertion of a T at position 23:

ACCCCGCATTACGTTTGGTGGACC | N1_Probe_FAM
ACCCCGCATTACGTTTGGTGGA<u>T</u>CC | hCoV-19/Denmark/ALAB-SSI480/2020|EPI_ISL_429559|2020-03-25

Two Danish strains have been identified with a G -> T mutation at position 22:

ACCCCGCATTACGTTTGGTG<u>T</u>ACC | hCoV-19/Denmark/ALAB-SSI209/2020|EPI_ISL_429405|2020-03-10
ACCCCGCATTACGTTTGGTG<u>T</u>ACC | hCoV-19/Denmark/ALAB-SSI201/2020|EPI_ISL_429399|2020-03-09

The effect of the identified mutations on N1 probe affinity has been investigated using synthetic DNA (Figure 2). The T23Ins and G22T mutations reduce the melting temperature from 78.5°C (Yellow line) to 76°C (Blue) and 72.5°C (Purple), respectively. Based on these findings the effect on the performance of the assay is considered to be low.

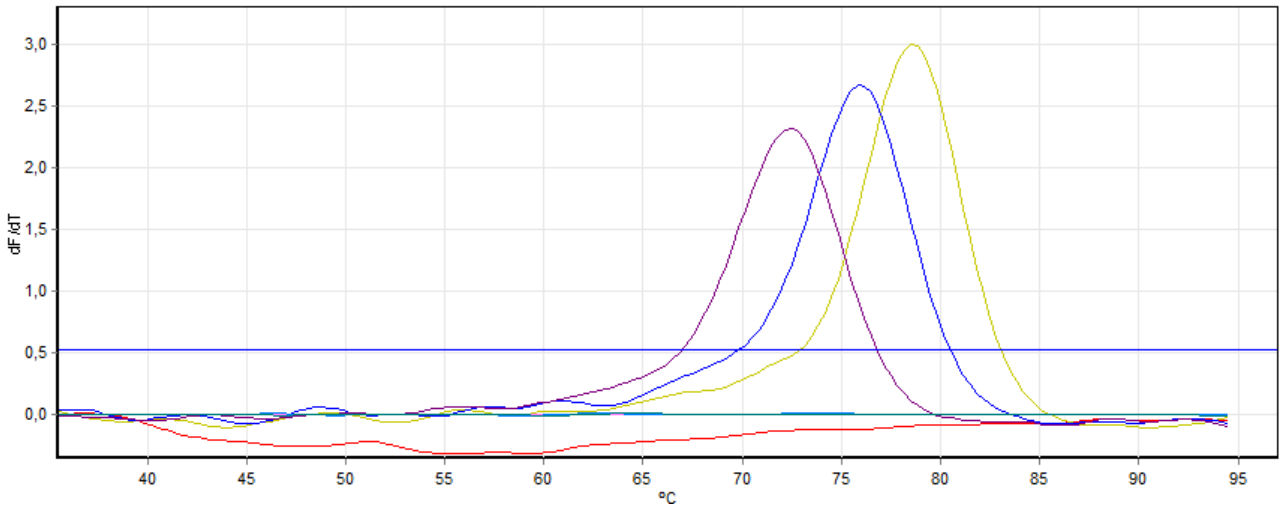

Figure 2. Melt studies using CoviDetect™ COVID-19 Multiplex assay N1 probe and synthetic DNA strands containing the T23Ins and G22T mutations identified in Danish SARS-CoV-2 strains.

N2 Forward primer
AGGAACTGATTACAAACATTGGC

No mismatches found

N2 Reverse Primer
TGTAGGTCAACCACGTTCCC

No mismatches found

N2 Probe
TGCACAATTTGCCCCCAGCG

Four Danish strains have been identified with a C -> T mutation at position 16:
TGCACAATTTGCCCCTAGCG hCoV-19/Denmark/alab-hh89/2020|epi_isl_429329|2020-03-15
TGCACAATTTGCCCCTAGCG hCoV-19/Denmark/alab-ssi414a/2020|epi_isl_429512|2020-03-23
TGCACAATTTGCCCCTAGCG hCoV-19/Denmark/alab-ssi413a/2020|epi_isl_429510|2020-03-23
TGCACAATTTGCCCCTAGCG hCoV-19/Denmark/alab-ssi595/2020|epi_isl_429590|2020-03-28

The impact of this mutation on probe affinity is currently under investigation.

8.4 Clinical evaluation

The clinical performance of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay was evaluated with the use of leftover oropharyngeal swabs and expectorate clinical specimens from patients suspected of COVID-19. Specimens were previously analyzed 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™ Nucleic Acid Extraction Instrument. 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.

Table 11. Summary of clinical evaluation of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay using cohort 1.

	Assay	CoviDetect™	Comparator Method 1	Agreement
Oropharyngeal swabs	SARS-CoV-2 positive	26	30	87% (PPA)
	SARS-CoV-2 negative	55	51	100% (NPA)
Expectorates	SARS-CoV-2 positive	4	5	80% (PPA)

	SARS-CoV-2 negative	0	0	Not applicable

The Ct values of CoviDetect™ (Ct + 7) compared to Comparator Method 1 of agreed positive and discrepant samples is illustrated in Figure 3. Discrepant samples are shown as data points with CoviDetect™ Ct values of 0. Due to lack of material, it has not been possible to confirm the results by a third method. Correlation of Ct values between CoviDetect™ and Comparator Method 1 of the shared positive samples is illustrated in Figure 4.

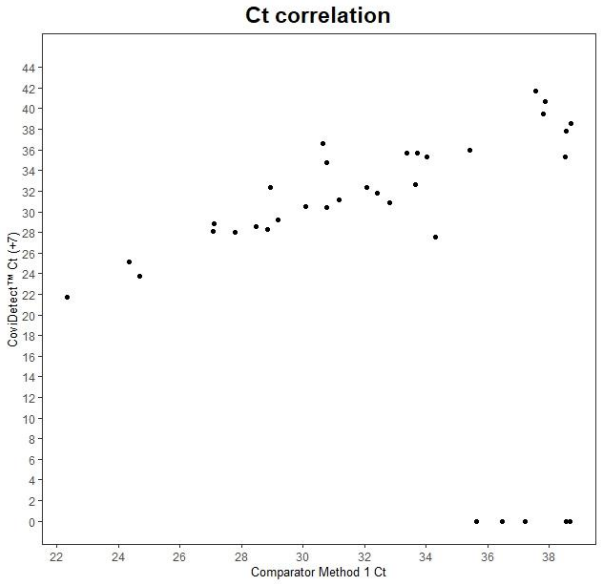

Figure 3. Correlation of Ct values between CoviDetect™ and Comparator Method 1 using oropharyngeal swabs and expectorate clinical samples (Cohort 1). Discrepant cases are illustrated as datapoints with CoviDetect™ Ct values of 0.

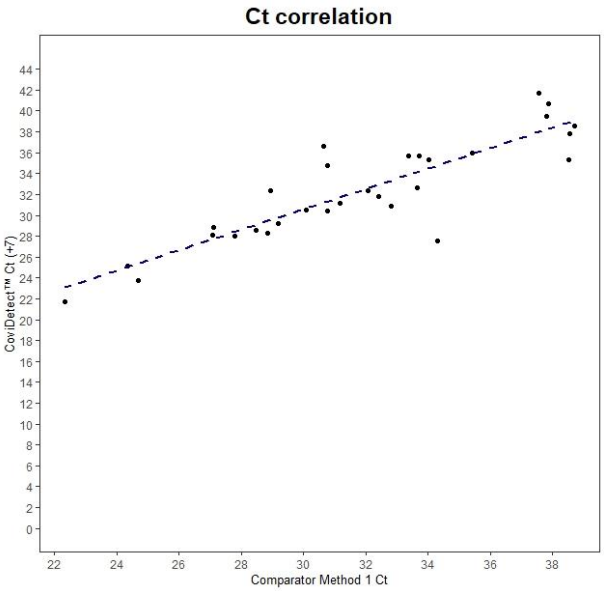

Figure 4. Correlation of Ct values between CoviDetect™ and Comparator Method 1 using leftover oropharyngeal swabs and expectorate clinical samples (Cohort 1).

8.4.1 Cohort 2

In cohort 2, leftover nasopharyngeal swab specimens from patients suspected of COVID-19 were extracted using the Viral DNA and RNA Extraction Kit for the BasePurifier™ Nucleic Acid Extraction Instrument. RT-qPCR was performed using CoviDetect™ COVID-19 Multiplex RT-qPCR Assay and Comparator Method 2 on the BaseTyper™ Real-Time PCR Instrument. The analysis was performed using automatic baseline and threshold settings. Evaluation summary is shown in Table 8. Correlation of Ct values between CoviDetect™ and Comparator Method 2 is illustrated in Figures 5-7.

Table 12. Comparison of clinical performance of CoviDetect™ COVID-19 Multiplex RT-qPCR Assay to IDT 2019-nCoV CDC EUA Kit using Cohort 2 samples.

	Result	CoviDetect™	IDT 2019-nCoV CDC EUA Kit	Agreement
Nasopharyngeal swab specimens	SARS-CoV-2 positive	27	27	100% (PPA)
	SARS-CoV-2 negative	62	62	100% (NPA)

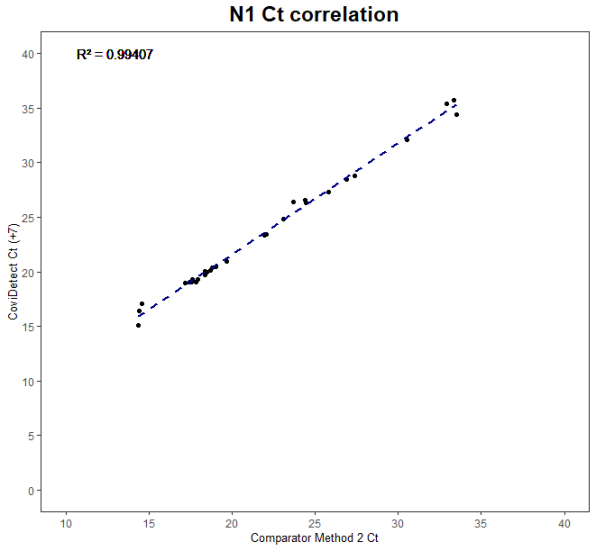

Figure 5. Correlation of N1 assay Ct values between CoviDetect™ and Comparator Method 2 using SARS-CoV-2 positive leftover oropharyngeal swab specimens (Cohort 2).

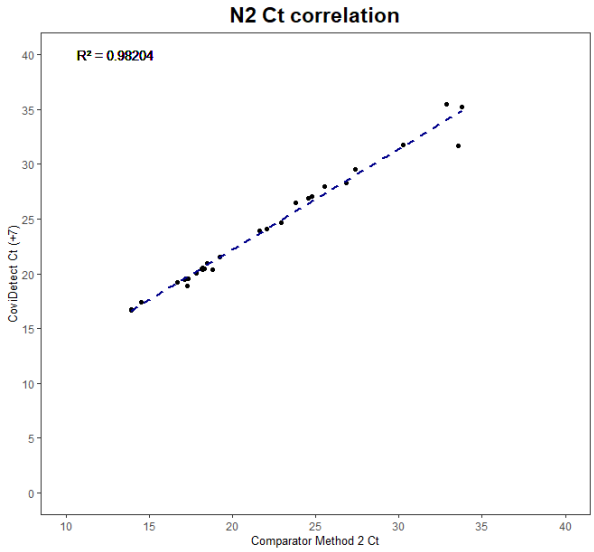

Figure 6. Correlation of N2 assay Ct values between CoviDetect™ and Comparator Method 2 using SARS-CoV-2 positive leftover oropharyngeal swab specimens (Cohort 2).

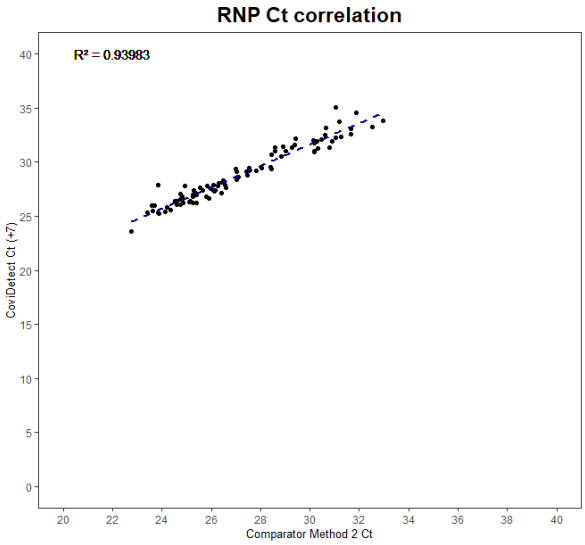

Figure 7. Correlation of RNP assay Ct values between CoviDetect™ and Comparator Method 2 using leftover oropharyngeal swab specimens (Cohort 2).

9. Limitations

- Performance of the CoviDetect™ COVID-19 Multiplex RT-qPCR assay has only been tested on specimens from nasopharyngeal or oropharyngeal swabs or sputum.
- A negative test result does not exclude infection with SARS-CoV-2 and treatment of a patient should not exclusively be based on the test result. Multiple specimens collected at different times from the same patient may be necessary to detect the virus since it is unknown when the viral levels in the body will peak.
- Incorrect collection, transportation or handling of the sample could cause false-negative test results. Also, a very low amount of virus RNA in the specimen or amplification inhibitors could give false-negative test results.
- Do not use reagents that have expired.
- 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.

10. Troubleshooting

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

Table 11. 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/VIC and Cy5 channels in Step 2 of Stage 4.
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 ApS 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™ COVID-19 RT-qPCR products.

Table 12. Symbols used in labelling for CoviDetect™ COVID-19 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 CR of an *in vitro* diagnostic medical device
 Consult instructions for use	

13. Manufacturer and distributors

For technical assistance in Denmark please contact PentaBase ApS:

Petersmindevej 1A
DK-5000 Odense, Denmark

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

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