RespiDetect™

RespiDetect™ Respiratory Panel 1 RT-qPCR Assay

In vitro Diagnostic Assay for detection of SARS-CoV-2, Influenza A/B & RSV A/B

INSTRUCTIONS FOR USE

PentaBase

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REFERENCE NUMBERS

Dispense Ready (DR)
8200 (200 reactions)

Ready-to-Use (RTU)
8201 (96 reactions)

Version 1.2
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CE IVD

Table of Contents

1 Intended purpose

RespiDetect™ Respiratory Panel 1 RT-qPCR Assay is a semi-quantitative real-time RT (Reverse Transcriptase) Polymerase Chain Reaction (PCR) assay intended for semi-quantitative detection of RNA from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Influenza (Inf) A, Inf B, and respiratory syncytial virus (RSV) A/B viruses. The assay is used with real-time PCR systems. Viral RNA can be found in the upper or lower respiratory tracts of infected individuals. Samples can be obtained by nasopharyngeal swabs, oropharyngeal swabs, and/or saliva. Samples can be purified on automated platforms or in manual workflows.

1.1 Intended user

The RespiDetect™ Respiratory Panel 1 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.

2 Test principle

Influenza (Flu) and respiratory syncytial virus (RSV) are common respiratory pathogens that can cause seasonal epidemics. Influenza is often associated with less severe symptoms as uncomplicated upper respiratory tract illness but can in rare cases result in complicated disease with severe viral pneumonia. RSV infection can present itself as the common cold-like symptoms but can lead to severe lower respiratory infection.

Since the clinical presentation of SARS-CoV-2, influenza and RSV infections have similar symptoms, diagnosis of these is challenging, especially, rapid diagnosis of SARS-CoV-2 is important in individuals suspected of a respiratory infection. The RespiDetect™ Respiratory Panel 1 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, influenza, and RSV 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.

2.1 Explanation of the assay

The RespiDetect™ Respiratory Panel 1 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 EasyBeacons, 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. Oligos modified with pentabases 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 RespiDetect™ Respiratory Panel 1 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®[1] probe) 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 RespiDetect™ Respiratory Panel 1 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.

2.1.3 EasyBeacon™

An EasyBeacon™ probe is similar to a molecular beacon but is based on oligonucleotides modified with pentabases, which keep the probe quenched at all temperatures, without the need of an internal stem structure. This effect is due to hydrophobic interactions between the pentabases in the unbound probe. Another feature introduced by the Pentabase-modifications is nuclease resistance. These features result in a good signal-to-noise ratio.

2.2 Product variants

The RespiDetect™ Respiratory Panel 1 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.

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

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2.3 Principle of the procedure

RespiDetect™ Respiratory Panel 1 RT-qPCR Assay is designed for use with Real-Time PCR Instrument for nucleic acid amplification and detection of the target sequence in biological samples.

The RespiDetect™ Respiratory Panel 1 RT-qPCR Assay targets two viral sequences of the SARS-CoV-2 genome (named IP2 and E), two targets of the Influenza A H1N1, Influenza A H3N2, Influenza B Victoria and Influenza B Yamagata genome (named PB1 and PA) and two targets of both the Human orthopneumovirus A and Human orthopneumovirus B genome (named N and P). Selective amplification of the viral sequences is achieved by using sequence-specific forward and reverse primers with EasyBeacons™ (only SARS-CoV-2) or HydrolEasy™ probes labelled with FAM, Cal Fluor Red 610 or Quasar®670. 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 PentaYellow™-labelled HydrolEasy® probe which share no homology with the virus genomes. Amplified target is detected by a fluorescently labelled oligonucleotide probe specifically targeting the viral or human sequence of interest, all listed in **Table 1**. A heat- and inhibitor-resistant RT enzyme combined with a thermostable DNA polymerase enzyme is used for reverse transcription and subsequent amplification.

The RespiDetect™ Respiratory Panel 1 RT-qPCR Assay includes a SARS-CoV-2 RNA positive control and a negative control sample, which should be included in each RNA extraction procedure.

Table 1. List of detected regions in the RespiDetect™ Respiratory Panel 1 RT-qPCR diagnostic assay.

Targeted Regions	Gene	Fluorophore
Influenza A H1N1 PB1	RNA-directed RNA polymerase catalytic subunit gene marker	FAM
Influenza A H3N2 PB1	RNA-directed RNA polymerase catalytic subunit gene marker	FAM
Influenza A H1N1 PA	Polymerase acidic protein gene marker	FAM
Influenza A H3N2 PA	Polymerase acidic protein gene marker	FAM
Influenza B Victoria PB1	RNA-directed RNA polymerase catalytic subunit gene marker	Quasar®670
Influenza B Yamagata PB1	RNA-directed RNA polymerase catalytic subunit gene marker	Quasar®670
Influenza B Victoria PA	Polymerase acidic protein gene marker	Quasar®670
Influenza B Yamagata PA	Polymerase acidic protein gene marker	Quasar®670
SARS-CoV-2 IP2	RNA dependent RNA polymerase gene marker	Quasar®670
SARS-CoV-2 E	Envelope protein gene marker	Quasar®670
Respiratory syncytial virus A	Nucleoprotein gene marker	CAL Fluor Red 610
Respiratory syncytial virus B	Nucleoprotein gene marker	CAL Fluor Red 610
Respiratory syncytial virus A	Phosphoprotein gene marker	CAL Fluor Red 610
Respiratory syncytial virus B	Phosphoprotein gene marker	CAL Fluor Red 610
RNP	Human RNase P (Internal Control)	PentaYellow™

3 Reagents and materials

The materials provided with the RespiDetect™ Respiratory Panel 1 RT-qPCR Assay can be found in **Table 2**. Materials required, but not provided, can be found in **Table 3**.

3.1 Storage

This assay should be stored at -20°C. The RespiDetect™ Respiratory Panel 1 RT-qPCR Assay is shipped on dry ice or frozen ice bricks. Repeated thawing and freezing should be kept to a minimum. 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.

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 Ready-to-Use PCR tubes and dispensed Primer-Probe and Master Mix should be disposed following your local guidelines on disposal of biological waste. The reagents included in the RespiDetect™ Respiratory Panel 1 RT-qPCR Assay are not for reuse.

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3.2 Materials provided

Table 2. List of materials provided for the RespiDetect™ Respiratory Panel 1 RT-qPCR Assay.

Dispense Ready (DR)	
Kit components	**Reagent ingredients**
RespiDetect™ Respiratory Panel 1 RT-qPCR Primer/Probe mix	Synthetic DNA.
AmpliSmaRT™ One Step RT-qPCR Master Mix	Enzymes and buffer for reverse transcription and qPCR.
RespiDetect™ Respiratory Panel 1 RT-qPCR Positive Extraction Control	Buffer solution including inactivated SARS-CoV-2 RNA and human DNA.
RespiDetect™ Respiratory Panel 1 RT-qPCR Negative Extraction Control	Buffer solution free of SARS-CoV-2 RNA
Ready-to-Use (RTU)	
Kit components	**Reagent ingredients**
RespiDetect™ Respiratory Panel 1 RT-qPCR Assay	Synthetic DNA. Enzymes and buffer for reverse transcription and qPCR
RespiDetect™ Respiratory Panel 1 RT-qPCR Positive Extraction Control	Buffer solution including inactivated SARS-CoV-2 RNA and human DNA
RespiDetect™ Respiratory Panel 1 RT-qPCR Negative Extraction Control	Buffer solution free of SARS-CoV-2 RNA

3.3 Materials and instruments required but not provided

RespiDetect™ Respiratory Panel 1 RT-qPCR Assay is designed to run on open platforms and has currently been validated using samples purified with the BasePurifier™ Nucleic Acid Extraction System (PentaBase ref, no. 715) and analysed with the BaseTyper™ 48.4 Quiet HRM Real-time PCR instrument (PentaBase, ref. no. 754). 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 with the ability to measure fluorescence at two temperatures, that are not compatible with the RespiDetect™ Respiratory Panel 1 RT-qPCR Assay. However, when running RespiDetect™ Respiratory Panel 1 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 A/S or your local distributor for support.

Table 3. Material 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
Nasopharyngeal swab
Saliva collector
RNA extraction method or instrument (e.g., BasePurifier™, PentaBase A/S)
RNA Extraction kit (e.g., Viral DNA and RNA Extraction Kit, PentaBase A/S)
Real-time qPCR
Real-time PCR instrument (e.g.,BaseTyper™,PentaBase A/S)

[2] Only when using Dispense Ready version

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4 Warnings and precautions

- For in vitro diagnostic use.
- Treat all biological specimens, including used RespiDetect™ Respiratory Panel 1 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 RespiDetect™ Respiratory Panel 1 RT-qPCR assay tubes.
- Do not use a RespiDetect™ Respiratory Panel 1 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.I
- 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 RespiDetect™ Respiratory Panel 1 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.
- RespiDetect™ Respiratory Panel 1 RT-qPCR Assay should be protected from light due to the presence of HydrolEasy® and EasyBeacons™ 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.
- Inadequate or 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.

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 specimen should be nasopharyngeal or oropharyngeal swabs 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 RespiDetect™ Respiratory Panel 1 RT-qPCR Assay, using suitable RNA purification methods, such as the BasePurifier™ 32 (ref. no. 715, PentaBase A/S) and the Viral DNA and RNA Extraction Kit (PentaBase A/S), according to the manufacturer's instructions. Be aware that the outcome from the purification method may influence the results of the RespiDetect™ Respiratory Panel 1 RT-qPCR Assay.

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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.

The Positive extraction control contains 200 SARS-CoV-2 RNA copies and 0.5 ng human genomic DNA per microliter, while the Negative extraction control contains nuclease free water.

NOTE: The Positive extraction control cannot be added directly to the RespiDetect™ Respiratory Panel 1 RT-qPCR Assay but must be subjected to a nucleotide extraction procedure first. Use the maximum amount (up to 200 µl) of Positive and Negative Extraction Control recommended by the supplier of the RNA purification kit that you use.

6 Procedure

6.1 Dispense Ready

1. Add 10 µl 2x AmpliSmaRT™ One-Step RT-qPCR Master Mix to each PCR tube or well.
2. Add 5 µl 4x primer/probe multiplex mix to the PCR tubes or wells.
3. Add 5 µl of template (sample, positive control or negative control) pr. Reaction.
4. Seal all tubes.
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**).

6.3 RT-qPCR program

Table 4. RT-qPCR protocol for running RespiDetect™ Respiratory Panel 1 RT-qPCR Assay.

Protocol	Temperature [°C]	Time [sec]	Cycles	Ramping [°C/sec]	Channels
Stage 1					
Reverse Transcription	52	300	1	2	
	95	10		2	
Stage 2 (Cycle 1-45)					
3-step amplification*	94	5	45	2	
	56	15		2	FAM
	72	30		3	HEX Cy5 Texas Red

***Note:** The PCR program should be set up to measure fluorescence at both 56°C and 72°C.

7 Data Analysis

The RespiDetect™ Respiratory Panel 1 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 2 are compared to predefined cutoff values to determine if the individual samples are positive or negative for SARS-CoV-2, influenza, or RSV (Section 7.2).

7.1 Baseline and threshold settings

Results from the RespiDetect™ Respiratory Panel 1 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 15 to cycle 20 depending on the amplification curve of the sample. When setting the threshold manually, the threshold should be set to 10% of the Relative Fluorescence Unit (RFU) on the relative dye channel.

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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 33 for SARS-CoV-2 (Cy5), and below 34 for the RNase P internal control (fluorophore HEX) at 56°C. No template (NTC) negative control should produce no Ct values. Ct cutoff values at 56°C for the RespiDetect™ Respiratory Panel 1 RT-qPCR assay are shown in **Table 6**.

Table 5. Ct cutoff values at 56°C for the RespiDetect™ Respiratory Panel 1 RT-qPCR Assay.

Case	Channel	Ct (56°C)	Ct (72°C)	Conclusion	Comments
Positive Case 1	HEX	<34	-	Influenza A positive	The sample is positive for Influenza A if FAM and HEX are positive.
	FAM	<34	-		
	Texas Red	≥34	-		
	Cy5	≥33	-		
Positive Case 2	HEX	<34	-	RSV positive	The sample is positive for RSV if Texas Red and HEX are positive.
	FAM	≥34	-		
	Texas Red	<34	-		
	Cy5	≥33	-		
Positive Case 3	HEX	<34	-	SARS-CoV-2 positive	The sample is positive for SARS-CoV-2 if Cy5 and HEX are positive. Cy5 shall only be positive at 56°C.
	FAM	≥34	-		
	Texas Red	≥34	-		
	Cy5	<33	-		
Positive Case 4	HEX	<34	-	Influenza B positive	The sample is positive for Influenza B if Cy5 and HEX are positive. Cy5 shall be positive both at 56°C and 72°C.
	FAM	≥34	-		
	Texas Red	≥34	-		
	Cy5	<33	(<42)		
Co-infection Case 5	HEX	<34	-	Influenza A and RSV positive	The sample is positive for co-infection of Influenza A and RSV if FAM, HEX and Texas Red are positive.
	FAM	<34	-		
	Texas Red	<34	-		
	Cy5	≥33	-		
Co-infection Case 6	HEX	<34	-	Influenza A and Influenza B positive	The sample is co-positive for Influenza A and B if Cy5, FAM and HEX are positive. Cy5 shall be positive both at 56°C and 72°C.
	FAM	<34	-		
	Texas Red	≥34	-		
	Cy5	<33	(<42)		
Co-infection Case 7	HEX	<34	-	Influenza A and SARS-CoV-2 positive	The sample is co-positive for Influenza A and SARS-CoV-2 if Cy5, FAM and HEX are positive. Cy5 shall only be positive at 56°C.
	FAM	<34	-		
	Texas Red	≥34	-		
	Cy5	<33	-		
Co-infection Case 8	HEX	<34	-	RSV and SARS-CoV-2	The sample is co-positive for RSV and SARS-CoV-2 if Cy5, Texas Red and HEX are positive. Cy5 shall only be positive at 56°C.
	FAM	≥34	-		
	Texas Red	<34	-		
	Cy5	<33	-		
Co-infection Case 9	HEX	<34	-	RSV and Influenza B positive	The sample is co-positive for RSV and Influenza B if Cy5, Texas Red and HEX are positive. Cy5 shall be positive both at 56°C and 72°C.
	FAM	≥34	-		
	Texas Red	<34	-		
	Cy5	<33	(<42)		
Co-infection Case 10	HEX	<34	-	Influenza A, RSV and Influenza B positive	The sample is co-positive for RSV, Influenza A and Influenza B if Cy5, Texas Red, FAM and HEX are positive. Cy5 shall be positive both at 56°C and 72°C.
	FAM	<34	-		
	Texas Red	<34	-		
	Cy5	<33	(<42)		
Co-infection Case 11	HEX	<34	-	Influenza A, RSV and SARS-CoV-2 positive	The sample is co-positive for RSV, Influenza A and Influenza B if Cy5, Texas Red, FAM and HEX are positive. Cy5 shall only be positive at 56°C.
	FAM	<34	-		
	Texas Red	<34	-		
	Cy5	<33	-		
Negative Case 12	HEX	<34	-	Negative	Positive HEX signal is required for sample to be negative.
	FAM	≥34	-		
	Texas Red	≥34	-		
	Cy5	≥33	-		
Invalid Case 13	HEX	≥34	-	Invalid	The sample does not contain enough material for analysis if no signals show. Take a new specimen if possible.
	FAM	≥34	-		
	Texas Red	≥34	-		
	Cy5	≥33	-		

7.2.1 Positive samples

The sample is positive when Ct values for Cy5 is below 33 and/or one or both of FAM, Texas Red or are below 34 and the Ct value for the HEX signal is below 34. It is possible that a sample may be positive for more than one virus.

To discriminate between the two targets on Cy5 channel (influenza B and SARS-CoV-2), an extra measurement is implemented at 72°C. In the case that a sample is only positive in the Cy5 channel at 56°C and not 72°C, the sample would be determined as SARS-CoV-2 positive. In the case that a sample is positive in the Cy5 channel at both 56°C and

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72°C the sample would be determined as a positive case of Influenza B. However, due to the way that the assay is constructed it would not be possible to rule out a co-infection with SARS-CoV-2 and influenza B. As such it is advised that the influenza B positive sample is tested for SARS-CoV-2 using a separate assay.

7.2.2 Negative samples

The sample is considered negative for the detection of the respiratory viruses tested if the sample is positive for HEX but negative in FAM, Texas Red and Cy5.

7.2.3 Invalid samples

In the case of no or late amplification of HEX (Ct≥34). 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.

7.2.3.1 No sample signal

In case of no signal in any channel, check if you have added the sample to the well, and that the sample has the required concentration. If more specimen is available, repeat the extraction and run the test again. If all markers remain negative after repeated testing, no diagnosis can be concluded, and if possible, a new specimen should be collected for testing.
In the case of no signals for any sample, check if you have run the correct program. The correct program is found in **Table 4**. If the correct program has been used and there is no signal in any of the samples, contact PentaBase A/S for support.

7.2.3.2 No positive control signal

In case of no signals for the positive control, check if you have run the correct program and if you have added the positive control to the well. Check that the instrument is acquiring on FAM, HEX, Texas Red and Cy5 channels in Step 2 and step 3 of Stage 2. The correct program is found in table **Table 4**. 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 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.

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8 Performance evaluation

8.1 Analytical sensitivity – Limit of Detection

The limit of detection (LOD) of the RespiDetect™ Respiratory Panel 1 RT-qPCR assay has been evaluated on synthetic Influenza A H1N1, influenza A H3N2, influenza B Victoria, influenza B Yamagata, RSV A, RSV B and SARS-CoV-2 DNA template (Twist Bioscience) using BaseTyper™ Real-Time PCR instrument, diluted in 25ng wild type human genomic DNA background. The LOD of the assay has been determined to be 75 copies/reaction.

Table 6. Limit of detection (LOD) of the RespiDetect™ Respiratory Panel 1 RT-qPCR assay using H1N1, H3N2, Victoria, Yamagata, RSV A, RSV B and SARS-CoV-2 RNA spiked into wild type human DNA. RT-qPCR was performed using the BaseTyper™ instrument.

RNA (copies per reaction)	Observations (n)	Assay	Positives 56°C	Positives (%)	Positives 72°C	Positives (%)
50	20	H1N1	20	100	-	-
		H3N2	20	100	-	-
		VIC® [3]	20	100	20	100
		YAM	19	95	20	100
		RSV	20	100	-	-
		SARS-COV-2	18	90	-	-
75	20	H1N1	20	100	-	-
		H3N2	20	100	-	-
		VIC®	20	100	100	100
		YAM	20	100	100	100
		RSV	20	100	-	-
		SARS-COV-2	20	100	-	-

8.2 Inclusivity

RespiDetect™ Respiratory Panel 1 RT-qPCR assay were blasted against the 6 most common SARS-CoV-2 strains, influenza A H1N1 and H3N2, influenza B Victoria and Yamagata and RSV A and B (**Figure 1**).

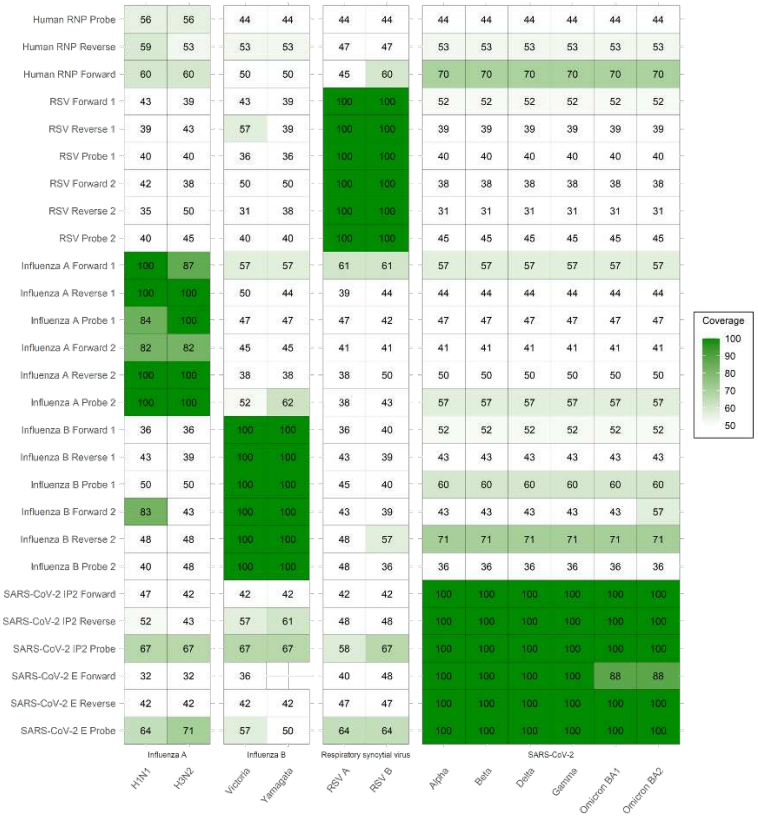

Figure 1. RespiDetect™ Respiratory Panel 1 RT-qPCR Assay targets specific oligonucleotides blasted against influenza A H1N1 and H3N2, Influenza B Victoria and Yamagata, RSV A and B and the different SARS-CoV-2 variants.

[3]VIC is a registered tradename of Applied Biosystems

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All oligonucleotides find the analyzed sequences with 100% agreements, except influenza Probe 2, influenza Forward 1 and influenza Forward 2 for influenza A. Primers and probes for influenza A is designed as PAN and is able to detect both H1N1 and H3N2. Mismatches was included to make the assay specific towards both strains, and the mismatches are not expected to have any significant impact on the performance.

All sequences analyzed for SARS-CoV-2 showed 100% agreement with the oligonucleotides except SARS-CoV-2 E Forward. The discrepancy of the primer is due to single mismatches in the 5'-end of the primers and is not expected to have any significant impact on the performance (**Figure 2**).

```
E.Fw1
Omicron BA.1     cgaacttatgtactcattcgtttcggaagagataggtacgttaatagttaatagcgtact
Omicron BA.2     cgaacttatgtactcattcgtttcggaagagataggtacgttaatagttaatagcgtact
E.Fw1            ------------------------------gacaggtacgttaatagttaatagc-----
                                               **.**********************
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Figure 2. RespiDetect™ Respiratory Panel 1 RT-qPCR Assay SARS-CoV-2 specific oligonucleotides blasted against the different SARS-CoV-2 variants.

8.3 Analytical specificity

8.3.1 In-silico analysis

RespiDetect™ Respiratory Panel 1 RT-qPCR assay oligo sequences were aligned with common respiratory pathogens (**Figure 3**).

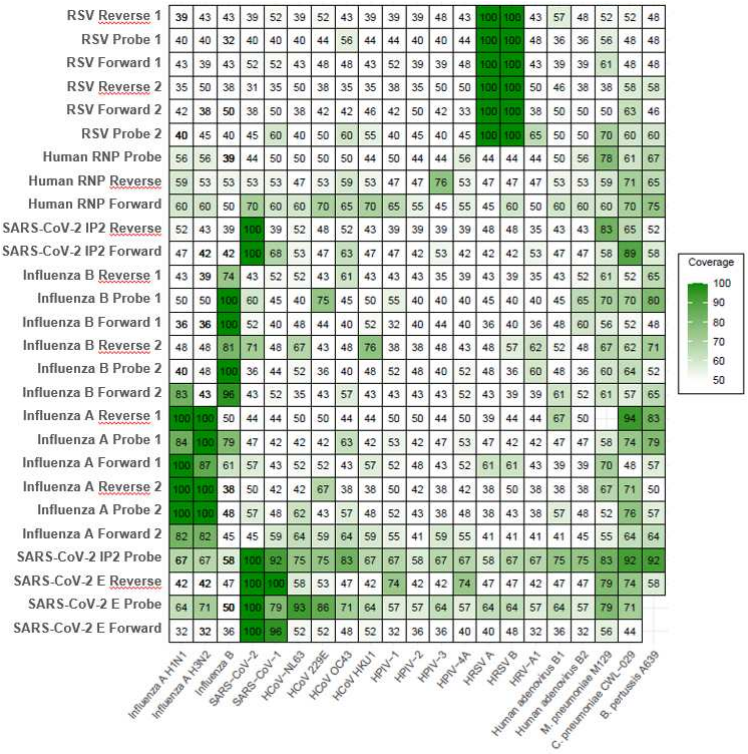

Figure 3. In silico analysis of RespiDetect™ Respiratory Panel 1 RT-qPCR Assay oligo sequences and alignment with common respiratory pathogens.

There is a high agreement with the SARS-CoV-2 E forward , SARS-CoV-2 E reverse , SARS-CoV-2 E probe and SARS-CoV-2 IP2 probe and SARS-CoV-1. This could indicate that there is a possibility of a false positive result for SARS-CoV-2 when it is SARS-CoV-1. According to CDC[4] has no cases of SARS-CoV-1 been observed since 2004.

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

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9 Limitations

- Performance of the the RespiDetect™ Respiratory Panel 1 RT-qPCR assay has only been tested on specimens from nasopharyngeal or oropharyngeal swabs or saliva.
- A negative test result does not exclude infection 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 viruses, it may affect the sensitivity of the test and may result in false-negative results. Two targets for each virus are included to increase resistance toward false-negative results due to mutation.
- A mutation in influenza B segment 3, may result in a false-positive result for SARS-CoV-2, as it will affect the signal at 72°C.
- The test cannot exclude that the patient is infected with other viruses or bacteria.
- The test cannot exclude that the patient has a co-infection with SARS-CoV-2 when tested positive for influenza B.

10 Symbols

The following symbols are used in labelling of RespiDetect™ Respiratory Panel 1 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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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.