CoviFLU™

COVID-19 and Influenza RT-qPCR Assay

In vitro Diagnostic Assay for Detection of SARS-CoV-2 and 4 Common Influenza Strains

INSTRUCTIONS FOR USE

REFERENCE NUMBERS

Dispense Ready (DR)
8177 (200 reactions)

Ready-to-Use (RTU)
8180 (96 reactions)

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CE IVD

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1 Intended purpose

CoviFLU™ COVID-19 and Influenza RT-qPCR Assay is a semi-quantitative real-time RT (Reverse Transcriptase) Polymerase Chain Reaction (PCR) assay with end-point melt-analysis intended for the quantitative detection and identification of RNA from SARS-CoV-2 and four common strains of influenza (B Yamagata, B Victoria, A H1N1, and A H3N2) 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

CoviFLU™ COVID-19 & Influenza RT-qPCR Assay is intended for use by healthcare 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 authorisation.

2 Test principle

Accurate and fast detection of SARS-CoV-2 and the four most common strains of influenza is important in individuals suspected of a respiratory infection to prevent transmission of COVID-19 and influenza.
To meet the need for faster diagnostic testing of SARS-CoV-2, Influenza A (H1N1 and H3N2) and Influenza B (Victoria and Yamagata), viruses with potentially fatal consequences for people with weak immune systems, reduced lung capacity and/or elder. The CoviFLU™ COVID-19 and Influenza RT-qPCR Assay has been developed as a fast and highly sensitive assay, reducing answering time significantly compared to common alternative procedures.

2.1 Explanation of the assay

The CoviFLU™ COVID-19 and Influenza 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, EasyBeacon™ 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 oligonucleotides contain at least one 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 CoviFLU™ SARS-CoV-2 and influenza RT-PCR Assay, the presence of SARS-CoV-2 or Influenza RNA in patients can be detected quickly, sensitively, and selectively by real-time RT-PCR followed by a DNA melt analysis in one run.

2.1.1 HydrolEasy® probe

A HydrolEasy® probe is similar to a standard hydrolysis probe (also referred to as a TaqMan® probe[1]) labelled with a fluorophore at the 5' end a quencher at the 3'end, but with the addition of pentabases. HydrolEasy® probes are based on oligonucleotides modified with pentabases, giving the probe a significantly improved signal-to-noise ratio, higher specificity, and higher sensitivity compared to conventional hydrolysis probes. HydrolEasy® probes in the CoviFLU™ SARS-CoV-2 and influenza RT-PCR Assay are labelled with Cy5.

2.1.2 SuPrimers™

SuPrimers™ are standard DNA primers modified with one or more pentabases. Pentabases in primers normally 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 as well as a nuclease-resistant probe intact for an affinity study (DNA melt analysis) after the RT-PCR reaction. EasyBeacon™ probes in the CoviFLU™ SARS-CoV-2 and influenza RT-PCR Assay are labelled with either FAM, PentaYellow™ or CAL Flour Red 610.

2.2 Product variants

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

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

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2.3 Principle of the procedure

The CoviFLU™ COVID-19 and Influenza RT-qPCR Assay is designed for use with real-time PCR instrument for nucleic acid amplification and DNA melting curve analysis for detection of the target sequences in biological samples.

The CoviFLU™ COVID-19 and Influenza RT-qPCR Assay targets two viral sequences of the SARS-CoV-2, two viral sequences of Influenza A, two viral sequences of Influenza B, and one human genomic DNA sequence for extraction control (**Table 1**). Selective amplification of SARS-CoV-2 sequences are achieved by using sequence-specific forward and reverse primers surrounding the site of potential targeted regions and an HydrolEasy® probe labelled with FAM spanning the site of the targeted regions. Amplification of Influenza A and Influenza B sequences are achieved by using sequence-specific forward and reverse primers, surrounding the site of potential targeted regions and an EasyBeacon™ probe labelled with either PentaYellow™ or CAL Flour® Red 610, respectively, spanning the site of the targeted regions. Amplification of a human genomic DNA sequence is achieved by using specific forward and reverse primers surrounding human RNase P and a HydrolEasy® probe labelled with Cy5. A heat- and inhibitor-resistant RT enzyme combined with a thermostable DNA polymerase enzyme is used for reverse transcription and subsequent amplification. Discrimination of influenza subtypes is achieved by melt analysis.

Table 1. List of amplified regions by the CoviFLU™ SARS-CoV-2 and influenza RT-PCR Assay.

Targeted Regions	Description	Fluorophore
SARS-CoV-2 IP2	RNA dependent RNA polymerase gene marker	FAM
SARS-CoV-2 E	Envelope protein gene marker	FAM
Influenza A H1N1	Hemagglutinin gene marker	PentaYellow™
Influenza A H3N2	Hemagglutinin gene marker	PentaYellow™
Influenza B Victoria	Hemagglutinin gene marker	CAL Flour® Red 610
Influenza B Yamagata	Hemagglutinin gene marker	CAL Flour® Red 610
RNase P	Human RNase P (Extraction Control)	Cy5

3 Reagents and materials

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

3.1 Storage and stability

Refer to the label for expiry date. This assay should be stored at -20°C. Repeated thawing and freezing should be kept to a minimum.

3.1.1 In-use stability

When in use, the assay components should be returned to the freezer promptly after use to minimise the time at room temperature and exposure to light.
Used Ready-to-Use PCR tubes and dispensed Primer-Probe and Master Mix should be disposed following your local guidelines on disposal of biological waste. The reagents included in CoviFLU™ COVID-19 and Influenza RT-qPCR Assay are not for reuse.

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3.2 Materials provided

Table 2. List of materials provided for CoviFLU™ COVID-19 and Influenza RT-qPCR Assay as either Dispense Ready (DR) or Ready-to-Use (RTU).

Dispense Ready (DR)	
CoviFLU™ COVID-19 and Influenza RT-qPCR Assay	
Kit components	**Content**
CoviFLU™ COVID-19 and Influenza RT-qPCR Primer/Probe mix	Synthetic DNA.
AmpliSmaRT™ One Step RT-qPCR Master Mix	Enzymes and buffer for reverse transcription and qPCR.
COVID-19 and Influenza RT-qPCR Positive Extraction Control 1	Buffer solution including inactivated SARS-CoV-2 and Influenza RNA and human DNA.
COVID-19 and Influenza RT-qPCR Positive Extraction Control 2	Buffer solution including inactivated SARS-CoV-2 and Influenza RNA and human DNA.
COVID-19 and Influenza RT-qPCR Negative Extraction Control	Buffer solution free of SARS-CoV-2 and Influenza RNA.
Ready-to-Use (RTU)	
CoviFLU™ COVID-19 and Influenza RT-qPCR Assay	
Kit components	**Reagent ingredients**
CoviFLU™ COVID-19 and Influenza RT-qPCR Assay	Synthetic DNA. Enzymes and buffer for reverse transcription and qPCR
COVID-19 and Influenza RT-qPCR Positive Extraction Control 1	Buffer solution including inactivated SARS-CoV-2 and Influenza RNA and human DNA.
COVID-19 and Influenza RT-qPCR Positive Extraction Control 2	Buffer solution including inactivated SARS-CoV-2 and Influenza RNA and human DNA.
COVID-19 and Influenza RT-qPCR Negative Extraction Control	Buffer solution free of SARS-CoV-2 and Influenza RNA.

3.3 Materials and instruments required but not provided

Materials and instruments required but not provided are listed in **Table 3**. CoviFLU™ COVID-19 and Influenza RT-qPCR Assay is designed to run on open platforms and has currently been validated using samples purified with the BasePurifier™ 32 Nucleic Acid Extraction System 32 oscillating rods (BasePurifier™ 32) and analysed with the BaseTyper™ Real-Time PCR Instrument - 48 well block with 4 channel detection and BaseTyper™ 48.4 Quiet HRM Real-Time PCR System. There is currently no evidence available to PentaBase suggesting that there are certain relevant commercially available nucleotide purification methods and instruments or four-channel real-time qPCR instruments that are not compatible with the CoviFLU™ COVID-19 and Influenza RT-qPCR Assay. However, when running CoviFLU™ COVID-19 and Influenza 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 is performed to verify cycle thresholds and cut-offs. Please contact PentaBase or your local distributor for support.

Table 3. Materials and consumables required but not provided.

Materials
Plasticware compatible with the used real-time PCR instrument[2]
Pipettes (1-10 µL, 10-100 µL)
Pipette Tips
Centrifuge for spinning PCR tubes, strips or plates
Collection Kits (one of the following)
Oropharyngeal swab
Saliva collector
RNA extraction method or instrument
RNA Extraction kit (e.g., BasePurifier™ Viral DNA and RNA Extraction Kit, PentaBase A/S, ref. no. 727)
Extraction instrument (e.g., BasePurifier™, PentaBase A/S)
Real-time qPCR
Real-time PCR instrument (e.g., BaseTyper™, PentaBase A/S), CFX96/384 Touch Real-Time PCR Detection Systems (Bio-Rad), QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™)

[2]Only when using Dispense Ready version

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4 Warnings and precautions

- For *in vitro* diagnostic use.
- Treat all biological specimens as if capable of transmitting infectious agents. All biological specimens should be treated with universal precautions, as it is often impossible to know which specimens might be infectious.
- Follow your institution's safety procedures for working with chemicals and handling biological samples.
- Good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary. Wear laboratory coats, laboratory gloves and eye protection when handling biological samples and reagents. Laboratory gloves must be changed between handling different biological samples to avoid contamination of reagents.
- Remove gloves and wash hands thoroughly after handling samples and reagents.
- Do not use reagents that have expired.
- Do not use damaged CoviFLU™ COVID-19 and Influenza RT-qPCR Assay tubes.
- Do not use a CoviFLU™ COVID-19 and Influenza 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 the completion of the PCR program.
- Be aware of the placement and orientation of the PCR tubes in the PCR machine in relation to how the samples are named in the PCR software.
- Baseline drift, a slowly rising signal in the amplification plot with no or late exponential phase, may lead to false positive results if not corrected. Refer to section 7.1 for more information.
- Consult relevant nucleic acid extraction and real-time qPCR Instrument User Guides for additional warnings, precautions, and procedures to reduce the risk of contamination.
- Dispose of used CoviFLU™ COVID-19 and Influenza RT-qPCR Assay tube, pipette, and specimen tube according to local, state, and federal regulations for biological material.
- Due to the high sensitivity of the assays, contamination of the work area with previous samples might cause false results. Therefore, use extreme caution not to contaminate reagents and handle samples according to standard laboratory practice.
- Minimise the exposure of CoviFLU™ COVID-19 and Influenza RT-qPCR Assay to light due to the presence of light sensitive HydrolEasy® probes.
- The reagents should not be diluted to a lower concentration than stated in the protocol. This may affect the performance of the assay.
- Do not substitute the reagents with others as it may affect the performance of the assay.
- Inappropriate sample collection, storage and transport may yield incorrect or invalid results. Specimen collection should be performed at least 30 minutes after tooth brushing, eating or drinking to decrease the risk of inaccurate results.
- Performance of the CoviFLU™ Assay has only been tested on specimens from nasopharyngeal swabs, oropharyngeal swabs, and saliva.
- Ensure there is no sign of leakage from the collection tube prior to running the analysis.
- Infections with SARS-CoV-1 may produce false positive results.

5 Sample handling

Handle all biological samples and controls as if they are capable of transmitting infectious agents. Please follow your local guidelines for handling and disposal of the sample material.

5.1 Sample collection

The specimens should be oropharyngeal swabs, nasopharyngeal 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 CoviFLU™ COVID-19 and Influenza RT-qPCR Assay using suitable RNA purification methods such as the BasePurifier™ 32 and the Viral DNA and RNA Extraction Kit (see **Table 3**), according to the manufacturer's instructions. Be aware that the quality and amount of RNA from the purification method may influence the results of the CoviFLU™ COVID-19 and Influenza RT-qPCR Assay.

5.4 Positive and Negative extraction controls

At least two Positive and one Negative Extraction Control should be included in each purification and subsequent RT-qPCR run. The Positive Extraction Control 1 contains SARS-CoV-2, Influenza B Victoria and Influenza A H1N1, while the

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Positive Extraction Control 2 contains SARS-CoV-2, Influenza B Yamagata and influenza A H3N2. There are enough positive and negative control samples included in the kit to purify an average of four samples per run. If less than four samples are purified on average per run, additional controls can be ordered from PentaBase or your local distributor (ref. no. 8182).

NOTE: The Extraction Controls cannot be added directly to the CoviFLU™ COVID-19 and Influenza RT-qPCR Assay but must be subjected to a nucleotide extraction procedure first. Use the maximum amount (up to 200 µL) of Positive and Negative Extraction Control recommended by the supplier of the RNA purification kit that you use.

6 Procedure

6.1 Dispense Ready

1. Add 10 µL AmpliSmaRT™ RT-qPCR Master Mix to each PCR tube (vial, strip or plate).
2. Add 5 µL Primer-Probe Multiplex Mix to the PCR tubes.
3. Add 5 µL of template (sample, positive control or negative control) to needed PCR tubes.
4. Seal all tubes.
   'Optional step: Briefly vortex PCR tubes (2-3 sec.) to enhance elimination of air bubbles'
5. Spin down the PCR tubes (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the tubes and to eliminate air bubbles.
6. Place the PCR tubes in the real-time PCR instrument and run the RT-qPCR program (**Table 4**).

6.2 Ready-to-Use

1. Spin down the PCR strips (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the strips to eliminate air bubbles.
2. Open PCR tubes and add 5 µL of template (sample, positive, or negative control). Continue with different templates and tubes until all templates are added to individual tubes.
3. Seal all tubes.
   'Optional step: Briefly vortex PCR strips (2-3 sec.) to enhance elimination of air bubbles'
4. Spin down the PCR strips (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the strips and to eliminate air bubbles.
5. Place the PCR strips in the real-time qPCR instrument and run the RT-qPCR program (**Table 4**).

6.3 RT-qPCR program

Table 4. RT-PCR protocol for running CoviFLU™ SARS-CoV-2 and influenza RT-PCR Assay.

Protocol	Temperature [°C]	Time [sec]	Cycles	Ramping [°C/sec]	Channel
Stage 1					
Reverse transcriptase	52	300	1	2	
Stage 2					
Hold	95	10	1	2	
Stage 3					
3-step amplification	90	5	45	2	
	56*	10			FAM (green) HEX (yellow) Cy5 (red) Texas Red (orange)
	72	20			
Stage 4					
Continuous Melt	95 40	60 60	1	2	FAM (green) HEX (yellow) Cy5 (red) Texas Red (orange)
	Up to 75	10 readings/°C **Note			

***Fluorescent measurement:** Measure the fluorescent at 56°C.
****NOTE:** For CFX use 2 readings/°C

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7 Data Analysis

For the CoviFLU™ SARS-CoV-2 and influenza RT-PCR Assay, determining the cycle threshold (Ct) is a central part of the data analysis procedure. The Ct is defined as the cycle in which the fluorescence signal of a given assay exceeds the threshold value, which is set as part of the analysis procedure. The Ct values of the PCR program (**Table 4**, stage 3) are compared to predefined cut-off values to determine if the individual samples are positive or negative for Influenza A, Influenza B, or SARS-CoV-2. For analysis of Influenza A and B subtypes melt-curve peaks in the PCR program (**Table 4**, stage 4) are evaluated.

7.1 Baseline and threshold settings

Results from CoviFLU™ COVID-19 and Influenza RT-qPCR Assay can be analysed using both automatic and manual baseline and threshold settings. If automatic baseline and threshold settings are used, it is recommended to also perform a visual inspection of the amplification curves since some cases might need manual adjustment of baseline and/or threshold, due to baseline drift and/or incorrect baselining. When setting the baseline manually, it is recommended to use 5 cycle intervals such as from cycle 10 to cycle 15 depending on the amplification curve of the sample. When setting the threshold manually, the threshold should be set to cross in the beginning of the exponential PCR phase and above any background or baseline fluorescence. If there is significant background or baseline fluorescence, adjust the baseline interval.

7.2 Interpretation of results

An overview of the possible outcomes of the analysis is shown in **Table 6**. The results are only valid if the included positive control Ct values for the SARS-CoV-2 are below 32 and the curves of the four influenza subtypes are visually represented in the melting curve. No template control (NTC) should produce no Ct values. Ct cut-off values for CoviFLU™ COVID-19 and Influenza RT-qPCR Assay are shown in section 7.2.1 positive samples.

For analysis of Influenza A and B subtypes, the melt curve peaks in the PCR program stage 4 are evaluated. PentaYellow™ channel peaks define Influenza A subtypes and Cal Fluor Red 610 channel peaks discriminate Influenza B subtypes as illustrated in **Table 5**.

Table 5. Analysis outcomes based on the melt curves. The Influenza A and B subtypes can be discriminated on the basis of the melting temperature. Be aware that melting temperature can vary a little due to machine variation.

Influenza Strain	Channel	Melting temperature [°C]	Genetic variation Melting temperature [°C]
Influenza A, H1N1	HEX	53.0-59.0	48.0-53.0
Influenza A, H3N2	HEX	63.0-67.0	59.5-63.0
Influenza B, Victoria	Texas Red	57.5-61.5 (Extra peak at 54)	50.0-57.5
Influenza B, Yamagata	Texas Red	66.0-69.0	62.0-66.0

7.2.1 Positive samples

The sample is positive for SARS-CoV-2 when the Ct value of the FAM channel is at or below 42 (**Table 6**, case 1). 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 the FAM channel are below 42 even when RNase P is negative.

The sample is positive for Influenza A when the Ct value of the HEX channel is at or below 42 (**Table 6**, case 2). 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 the HEX channel are below 42 even when RNase P is negative.

The subtype of Influenza A is determined using **Table 5**. NOTE: If a melt peak within the indicated temperature range in **Table 5** is present but no qPCR curve is, the sample is still considered positive for one of the subtypes.

The sample is positive for Influenza B when the Ct value of the Texas red channel is at or below 42 (**Table 6**, case 3). 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 the Texas red channel are below 42 even when RNase P is negative.

The subtype of Influenza B is determined using **Table 5**. NOTE: If a melt peak is present within the indicated temperature range in **Table 5** but no qPCR curve is, the sample are still considered positive for one of the subtypes.

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Table 6. Ct threshold values for CoviFLU™ COVID-19 and Influenza RT-qPCR Assay.

Sample status	Target	Outcome	Ct	Conclusion	Comments
Positive case 1	SARS-CoV-2	+	≤42	SARS-CoV-2 positive	The sample is considered SARS-CoV-2 positive when the specific target is positive even the RNase P target is negative
	Influenza A	-	>42		
	Influenza B	-	>42		
	RNase P	+/-	(≤34)		
Positive case 2	SARS-CoV-2	-	>42	Influenza A positive	The sample is considered influenza A positive when the specific target is positive even the RNase P target is negative
	Influenza A	+	≤42		
	Influenza B	-	>42		
	RNase P	+/-	(≤34)		
Positive case 3	SARS-CoV-2	-	>42	Influenza B positive	The sample is considered influenza B positive when the specific target is positive even the RNase P target is negative
	Influenza A	-	>42		
	Influenza B	+	≤42		
	RNase P	+/-	(≤34)		
Negative case 4	SARS-CoV-2	-	>42	Negative	A positive RNase P signal is required for a sample to be considered negative. *__Note__: The sample is positive for Influenza A or B if a peak is observed in the melt-curve (**Table 5**).
	Influenza A	-	>42*		
	Influenza B	-	>42*		
	RNase P	+	≤34		
Invalid case 5	SARS-CoV-2	-	>42	Invalid	The sample is invalid if no signal is present in any of the targets. Reperform the nucleotide extraction and subsequent RT-qPCR analysis of the sample or take a new specimen if possible.
	Influenza A	-	>42		
	Influenza B	-	>42		
	RNase P	-	>34		

7.2.2 Negative samples

The sample is considered negative for the detection of SARS-CoV-2, Influenza A and Influenza B if the sample is positive for RNase P (Cy5), but negative for the SARS-CoV-2, Influenza A and B (**Table 6**, negative case 4).

7.2.3 Invalid samples

In the case of no or late amplification of RNase P (Ct≥34), the test is invalid unless either SARS-CoV-2, influenza A or Influenza B are positive (Ct < 42) (**Table 6**, case 5). 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 signals

In case of no amplification in any of the CoviFLU™ COVID-19 and Influenza RT-qPCR Assay, check if template has been added to the tube. If more specimen is available, repeat the extraction and run the test again. If all targets remain negative after repeating the test, no diagnosis can be concluded, and if possible, a new specimen should be collected for testing.
In case of no signals in any of the samples in a run, check that the correct PCR program has been used (Section 6.3).

7.2.3.2 No positive control signals

In case of no signals for the positive control, check if the correct PCR program has been used (Section 6.3) and that a positive control has been included in the run tube. The correct program is found in section 6.3. If the correct program has been used and there is no signal in any of the samples repeat the extraction and run the test again. If all markers remain negative after repeating the test, contact PentaBase A/S or your local distributor for support.

7.2.3.3 Signal in NTC

Signals in the NTC sample(s) indicate contamination of the reagents and thus all positive samples in the run should be considered invalid. Make sure that the threshold has been set correctly and/or repeat the extraction of all samples and run the test again. If the problem persists, find the cause of contamination by checking or replacing all potential sources of the contamination such as pipettes and instruments. If the contamination cannot be located contact PentaBase A/S or your local distributor for support.

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8 Performance evaluation

8.1 Analytical sensitivity – Limit of Detection

The limit of detection (LOD) of CoviFLU™ COVID-19 and Influenza RT-qPCR Assay has been evaluated by spiking synthetic SARS-CoV-2 RNA (Twist Bioscience, cat. no. 102015) or RNA from inactivated Influenza A (H1N1 and H3N2) or inactivated Influenza B (Yamagata and Victoria) into wild-type human genomic DNA. RT-qPCR was performed using the BaseTyper™ Real-Time PCR Instrument.

Data for samples spiked with SARS-CoV-2 RNA is shown in **Table 8** and the LOD when using synthetic SARS-CoV-2 RNA was found to be 4 copies/µL. Data for samples spiked with influenza including the specific subtypes can be found in **Table 9**. The assay was found less sensitive when subtyping the Influenza A and B samples with LODs for influenza A H1N1, H3N2, and influenza B Victoria and Yamagata found to be 10, 100, 10, and 100 copies, respectively.

Table 7. Limit of detection (LOD) of CoviFLU™ 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™ Real-Time PCR Instrument.

SARS-CoV-2 RNA (copies/µL)	Sequence	Positives/Total	Positives (%)
0 (NTC)	E and/or IP2	0/16	0
1	E and/or IP2	14/20	70
2	E and/or IP2	17/20	85
4	E and/or IP2	20/20	100
10	E and/or IP2	20/20	100

Table 8. Limit of detection (LOD) of CoviFLU™ COVID-19 and Influenza RT-qPCR Assay. RT-qPCR of influenza RNA was performed using the BaseTyper™ Instrument.

Influenza RNA (copies/µL)	Sequence	Melt-curve Positives	qPCR Positive	Melt-curve sensitivity	qPCR sensitivity
0 (NTC)	A H1N1	0/16	0/16	0%	0%
	A H3N2	0/16	0/16	0%	0%
	B Victoria	0/16	0/16	0%	0%
	B Yamagata	0/16	0/16	0%	0%
2	A H1N1	2/20	10/20	10%	50%
	A H3N2	0/20	0/20	0%	0%
	B Victoria	0/20	0/20	0%	0%
	B Yamagata	0/20	1/20	0%	5%
4	A H1N1	7/20	18/20	35%	90%
	A H3N2	0/20	0/20	0%	0%
	B Victoria	0/20	0/20	0%	0%
	B Yamagata	0/20	1/20	0%	5%
10	**A H1N1**	**19/20**	**20/20**	**95%**	**100%**
	A H3N2	0/20	6/20	0%	30%
	B Victoria	**19/20**	**3/20**	**95%**	**15%**
	B Yamagata	0/20	18/20	0%	90%
20	A H1N1	19/20	20/20	95%	100%
	A H3N2	0/20	15/20	0%	75%
	B Victoria	20/20	8/20	100%	40%
	B Yamagata	**0/20**	**20/20**	**0%**	**100%**
100	A H1N1	N/A	N/A	N/A	N/A
	A H3N2	**0/20**	**20/20**	**0%**	**100%**
	B Victoria	N/A	N/A	N/A	N/A
	B Yamagata	3/20	20/20	15%	100%
200	A H1N1	N/A	N/A	N/A	N/A
	A H3N2	0/20	20/20	0%	100%
	B Victoria	N/A	N/A	N/A	N/A
	B Yamagata	20/20	20/20	100%	100%
400	A H1N1	N/A	N/A	N/A	N/A
	A H3N2	20/20	20/20	100%	100%
	B Victoria	No data	No data	N/A	N/A
	B Yamagata	No data	No data	N/A	N/A

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8.2 Inclusivity

CoviFLU™ COVID-19 and Influenza RT-qPCR Assay oligonucleotide sequences were aligned with the different variants of influenza and SARS-CoV-2 outlined in **Table 10** and the result of the alignment is depicted in **Figure 1**.

Table 9. SARS-CoV-2 and influenza variants used for analysis of agreement with CoviFLU™ COVID-19 and Influenza RT-qPCR Assay specific oligonucleotide sequences.

Viral variants	GenBank ID
Influenza A – H1N1	GCA_001343785.1
Influenza A – H3N2	GCA_000865085.1
Influenza B - Victoria	EPI_ISL_407969 (GISAID)
Influenza B - Yamagata	EPI_ISL_299488 (GISAID)
SARS-CoV-2 - Alpha	MZ344997.1
SARS-CoV-2 - Beta	MW598419.1
SARS-CoV-2 - Delta	MZ359841.1
SARS-CoV-2 - Gamma	MZ169911.1
SARS-CoV-2 - Omicron BA.1	OL672836.1
SARS-CoV-2 - Omicron BA.2	OM296922.1

All SARS-CoV-2 oligonucleotides except the IP2 Reverse primer and E forward primer were found to show 100% agreement with the analysed SARS-CoV-2 strains (**Figure 1** top left).

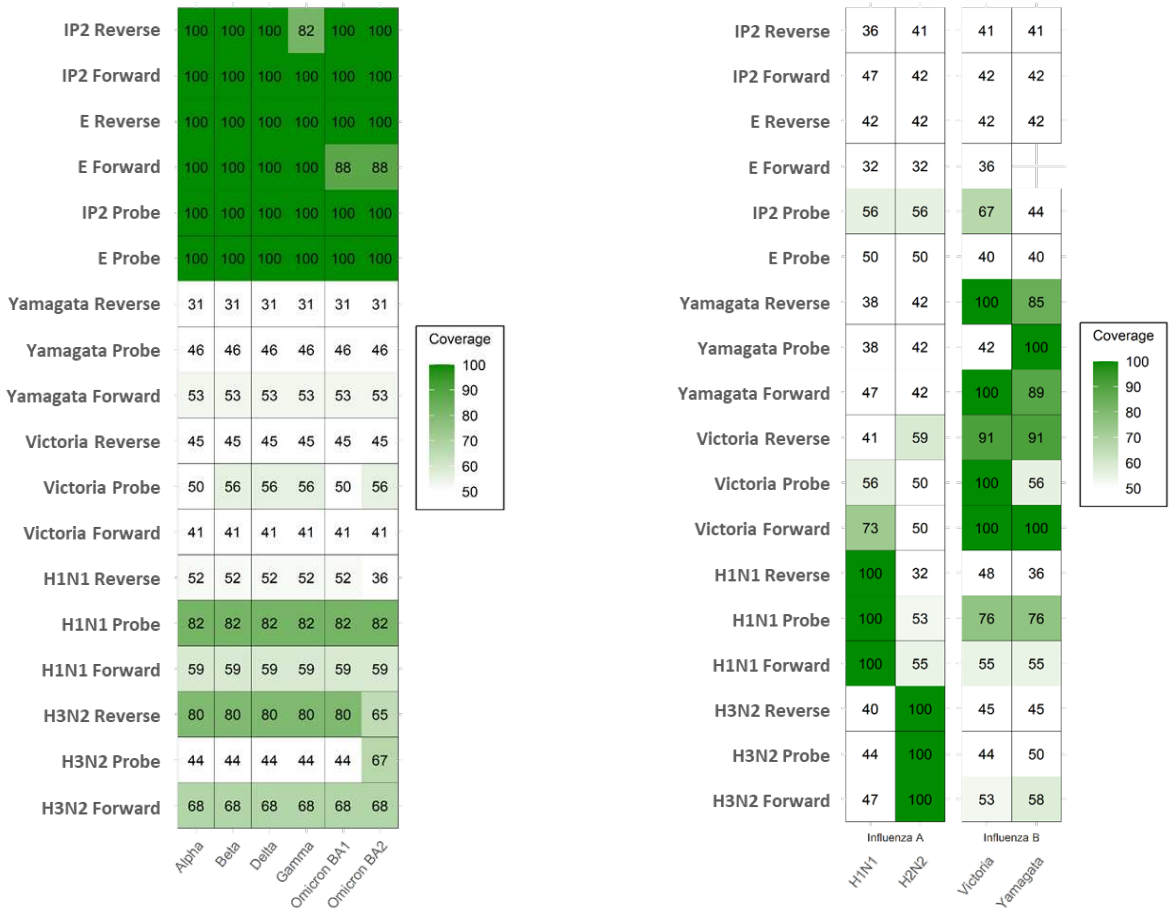

Figure 1. In-silico analysis of inclusivity of different variants of influenza and SARS-CoV-2. Sequence identity of CoviFLU™ oligonucleotides was evaluated using BLAST and displayed as the Query Coverage Per HSP (qcovhsp).

The discrepancy of the two primers 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**).

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```
E.Fw1
Omicron BA.1     3′-cgaacttatgtactcattcgtttcggaagagataggtacgttaatagttaatagcgtact-5′
Omicron BA.2     3′-cgaacttatgtactcattcgtttcggaagagataggtacgttaatagttaatagcgtact-5′
E Forward        5′----------------------------gacaggtacgttaatagttaatagc---------3′
                                              **.**********************
Gamma          tcttgtgctgccggtactacacaaactgcttgcactgatgacaatgcgttagcttattac-3′
IP2 Reverse     ------------------------------------tgacaatgcgttagcttactac-5′
                                                    ******************.***
```

Figure 2. CoviFLU™ COVID-19 and Influenza RT-qPCR Assay SARS-SoV-2 specific oligonucleotides blasted against the different SARS-CoV-2 variants.

All influenza oligonucleotides show 100% agreement with the blasted influenza sequences, except the Victoria Reverse primer, due to a single mismatch intentionally included in the primer design (**Figure 1**)

8.2.1 Analytical specificity

CoviFLU™ COVID-19 and Influenza RT-qPCR Assay oligonucleotide sequences were aligned with common viruses (**Figure 3**).

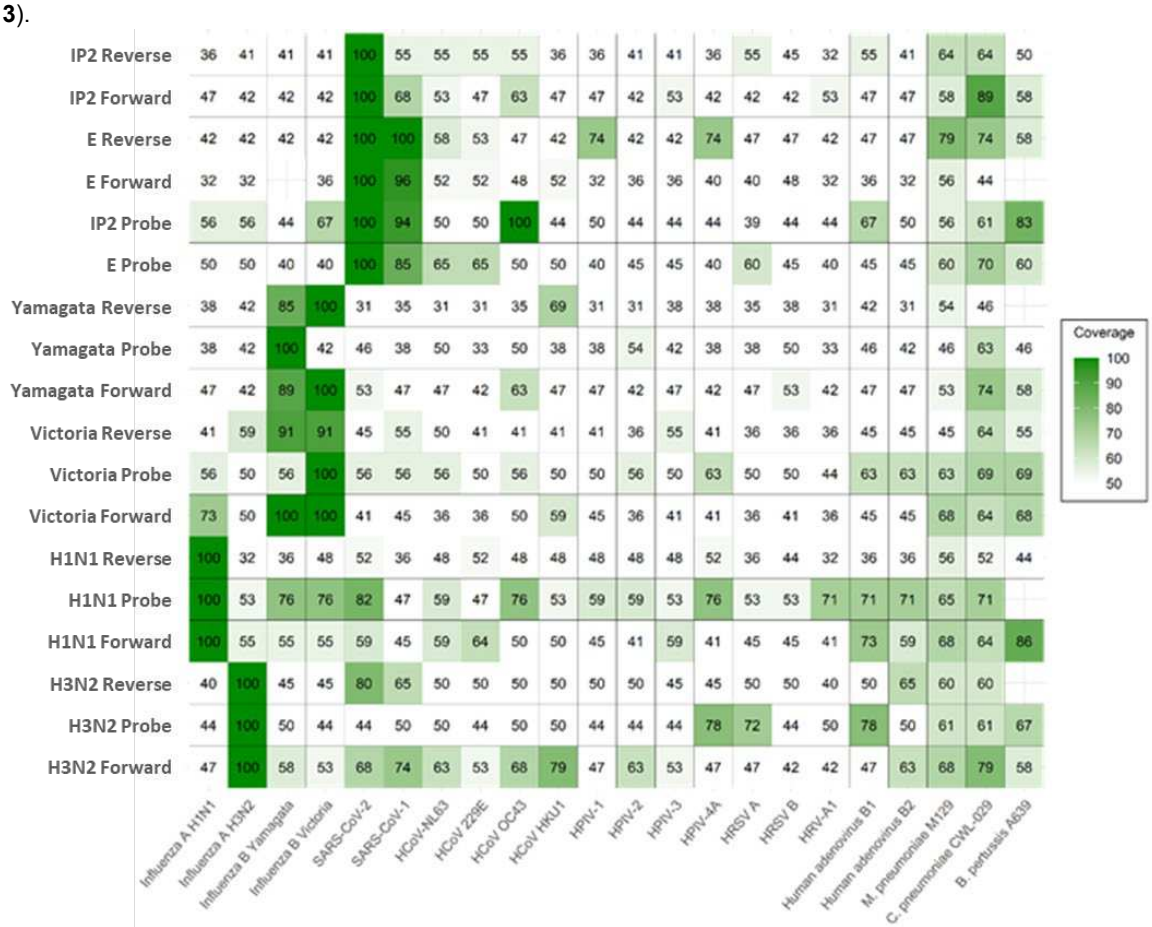

Figure 3. In silico analysis of CoviFLU™ COVID-19 and Influenza RT-qPCR Assay oligonucleotide sequences and alignment with common viruses.

There was significant agreement with the investigated coronaviruses in the case of the SARS-CoV-1 (SARS-CoV) coronavirus and the E forward primer, E reverse primer and E probe. This is an indication of the capability of the E gene to detect SARS-CoV-1. There have been no cases of people transmitted with SARS-CoV-1 since the outbreak in 2002-2004.[3] The probability of a false positive SARS-CoV-2 case due to a positive SARS-CoV-1 sample is negligible.

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

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8.3 Clinical evaluation

8.3.1 Cohort 1 – Influenza specimens

The clinical performance of CoviFLU™ COVID-19 and Influenza RT-qPCR Assay was evaluated with the use 38 retrospective oropharyngeal swab samples for the different influenza types. Specimens were previously analysed for the presence of Influenza using the comparator method at a clinical laboratory in Denmark. Stored samples were collected for subsequent analysis by CoviFLU™ SARS-CoV-2 and influenza RT-PCR Assay. Extraction of RNA was performed using the Viral DNA and RNA Extraction Kit for the BasePurifier™ 32 Instrument. RT-qPCR was performed using the BaseTyper™ Instrument and data analysis was performed using software version 1.0.231. Standard analysis settings were used.

Table 10. Summary of clinical evaluation of influenza samples tested on CoviFLU™ COVID-19 and Influenza RT-qPCR Assay and a comparator method.

Clinical performance Influenza A positive samples		CoviFLU™ COVID-19 and Influenza RT-qPCR Assay		
		Positive	**Negative**	**Total**
Comparator method	**Positive**	21	0	21
	Negative	0	0	0
	Total	21	0	21
Percent positive agreements was 21/21 or 100% (CI 95: 85-100%) for Influenza A				

Clinical performance Influenza B positive samples		CoviFLU™ COVID-19 and Influenza RT-qPCR Assay		
		Positive	**Negative**	**Total**
Comparator method	**Positive**	17	0	17
	Negative	0	0	0
	Total	17	0	17
Percent positive agreements was 17/17 or 100% (CI 95: 82-100%) for Influenza B				

8.3.2 Cohort 2 – SARS-CoV-2 specimens

The clinical performance of CoviFLU™ COVID-19 and Influenza RT-qPCR Assay was evaluated for SARS-CoV-2 using 78 samples collected by either oropharyngeal swab (56 samples) or saliva (22 samples). Specimens were previously analysed for the presence of SARS-CoV-2 using CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay. Extraction of RNA was performed using the Viral DNA and RNA Extraction Kit for the BasePurifier™ 32 Instrument. RT-qPCR was performed using the BaseTyper™ and data analysis was performed using software version 1.0.231. Standard analysis settings were used.

Table 11. Summary of clinical evaluation of patient samples suspected of SARS-CoV-2 tested on CoviFLU™ COVID-19 and Influenza RT-qPCR Assay and CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay.

Clinical performance SARS-CoV-2 Saliva samples		CoviFLU™ COVID-19 and Influenza RT-qPCR Assay		
		Positive	**Negative**	**Total**
CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay	**Positive**	14	0	14
	Negative	0	8	8
	Total	14	8	22
Positive Percent Agreement: 14/14 or 100% (CL95: 78.5-100%)				
Negative Percent Agreement: 8/8 or 100% (CL95: 67.6-100%)				

Clinical performance SARS-CoV-2 Oropharyngeal swab samples		CoviFLU™ COVID-19 and Influenza RT-qPCR Assay		
		Positive	**Negative**	**Total**
CoviDetect™ FAST COVID-19 Multiplex RT-qPCR Assay	**Positive**	21	1	22
	Negative	0	34	34
	Total	21	35	56
Positive Percent Agreement: 21/22 or 95.5% (CL95: 78.2-99.2%)				
Negative Percent Agreement: 34/34 or 100% (CL95: 89.9-100%)				

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9 Limitations

- Performance of the CoviFLU™ COVID-19 and Influenza RT-qPCR Assay has only been tested on specimens from oropharyngeal swabs, nasopharyngeal swabs or saliva
- A negative test result does not exclude infection with SARS-CoV-2 or Influenza A and B 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.
- A positive test result following SARS-CoV-2 infection is possible at least up to 37 days after the date of the first positive test result. A positive test result following SARS-CoV-2 infection may arise from detection of both active and inactive SARS-CoV-2 virus even in asymptomatic or post-symptomatic individuals that are no longer able to transmit COVID-19. This may also apply for influenza.
- 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, 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 Symbols

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


Date/country of manufacture

In vitro diagnostic medical device


Use-by date
Do not reuse

Contains sufficient for <*n*>


Manufacturer


Temperature limit


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


Consult electronically 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.