DIAGNOVITAL

Influenza A/B SARS-CoV-2 Multiplex

Qualitative RT-PCR-based detection of Influenza A/B and SARS-CoV-2

For *in-vitro* diagnostic use

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Intended Use

This document describes the use of real-time RT-PCR assay intended for the simultaneous qualitative detection and differentiation of SARS-CoV-2, influenza A virus, and influenza B virus nucleic acid in upper or lower respiratory specimens (such as nasopharyngeal, oropharyngeal and nasal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals suspected of respiratory viral infection consistent with COVID-19 by a healthcare provider and is not intended to detect influenza C. Symptoms of respiratory viral infection due to SARS-CoV-2 and influenza can be similar.

RNA from SARS-CoV-2, influenza A, and influenza B is generally detectable in respiratory specimens during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2, influenza A, and/or influenza B RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease.

Diagnovital® Influenza A/B SARS-CoV-2 Multiplex Real-Time PCR Kit is an *in vitro* nucleic acid amplification assay for qualitative detection of Novel Coronavirus SARS-CoV-2 and/or Influenza A/B in respiratory specimens using RTA Viral Nucleic Acid Isolation Kit and BIO-RAD CFX96-IVD or Rotor-Gene 3000/6000 or Himedia Insta Q96 or Applied Biosystems 7500 or QuantStudio 5 Real-Time PCR Detection Systems for amplification, detection and analysis.

The kits follow CDC's and WHO's latest testing guidelines.

Product Description

Diagnovital® Influenza A/B SARS-CoV-2 Multiplex detects a highly specific gene sequence of SARS-CoV-2 at **Texas Red** channel, Influenza A/B gene sequences at **FAM** channel and a sequence specific for human RNA serving as a human extraction control (HEC, RNAseP) at the **HEX** channel.

The oligonucleotide primers and probe for detection of SARS-CoV-2 were selected from an evolutionarily conserved region of the 3' terminus of SARS-CoV-2 genome and include part or the carboxy-terminal portion of the nucleocapsid (N) gene. Primers and probes for the detection of influenza A viruses were selected from an evolutionarily well conserved region of the matrix (M1) gene. The primers and probe selected for detection of influenza B viruses were selected from a conserved region of the nonstructural 2 gene (NS2).

Additionally, a non-infectious positive control and a negative human extraction control are included. Human Extraction Control (HEC) is needed to ensure appropriate RNA extraction, purification and reverse transcription and all reagents involved in reaction. The master mix contains primers and probe for an endogenous human target, the Human Extraction Control (HEC), which is extracted from the swab during the extraction step. Therefore, there is no need to put an external DNA or RNA template as extraction control. The positive control is used to confirm functionality of the assays and overall PCR performance, the human extraction control is included to evaluate the quality of sampling and the RNA isolation independently from the SARS-CoV-2 and Influenza A/B targets.

REAL TIME PCR-BASED DETECTION OF SARS-CoV-2 AND INFLUENZA A/B

The first step in the detection of SARS-CoV-2 and Influenza A/B is the conversion of viral RNA into cDNA. Afterwards, the target sequences unique for SARS-CoV-2, Influenza A/B and HEC are specifically amplified with amplification monitored in real time through the use of fluorescently labelled probes: upon incorporation into the newly amplified DNA strands, the fluorophore is released and an increase in fluorescence signal can be observed.

Due to the intrinsic mutation rate of RNA viruses, it is possible that mutations in the target sequences occur and accumulate over time. This can lead to false-negative results with a PCR-based detection approach. **Diagnovital® Influenza A/B SARS-CoV-2 Multiplex** addresses this issue by using highly conserved viral target sequences to minimize the chance of false-negative results caused by an altered target sequence.

The original target sequences for SARS-CoV-2 and Influenza A/B are included as a non-infectious target positive control (TPC) to check the integrity of the detection assays.

Diagnovital® Influenza A/B SARS-CoV-2 Multiplex is compatible with every qPCR with calibrated **FAM™, Texas Red** and **HEX/VIC** channel, whereas normalization with the **ROX** channel is optional.

Materials Provided

	Reagents	Quantity and Volume (25 tests)	Quantity and Volume (50 tests)	Quantity and Volume (100 tests)
1	Diagnovital® Influenza A/B SARS-CoV-2 **Multiplex Mix**	1 × 375 µl	1 × 750 µl	1 × 1500 µl
2	Influenza A/B SARS-CoV-2 **Target Positive Control (TPC)**	1 × 45 µl	1 × 75 µl	1 × 150 µl
3	Nuclease-free dH_2O (NTC)	1 × 1000 µl	1 × 1000 µl	1 × 1000 µl

Additional Materials Required

- Suitable means & equipment for nucleic acid extraction
- Real-time PCR detection system equipped for FAM™, Texas Red and HEX detection
- Adjustable pipettes & fitting filtered pipette tips
- Appropriate personal protective equipment & workspaces for working with potentially infectious samples
- Surface decontaminants such as DNAZap™ (Life Technologies), DNA Away™ (Fisher Scientific), RNAse Away™ (Fisher Scientific), 10% bleach (1:10 dilution of commercial 5.25-6.0% sodium hypochlorite)
- Nuclease-free tubes / strips / plates to prepare dilutions, master mixes etc.
- Nuclease-free tubes / strips / plates corresponding to the PCR device
- Suitable storage options for reagents and specimen (4°C, -20°C, -70°C)

Storage

- Store all components at -20°C and avoid repeated freeze and thaw cycles.
- Protect the 2X qPCR master mixes from light as prolonged exposure can diminish the performance of the fluorophores.
- If the kit components have been damaged during transport, contact RTA Laboratories. Do not use as performance may be compromised.
- Keep reagents separate from sample material to avoid contamination.
- Do not use after the designated expiry date.

Considerations Before Starting

BIOSAFETY

- Wear appropriate personal protective equipment (e.g. gowns, powder-free gloves, eye protection) when working with clinical specimen.
- Specimen processing should be performed in a certified class II biological safety cabinet following biosafety level 2 or higher guidelines.
- For more information, refer to:
  Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (SARS-CoV-2) https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html
- Biosafety in Microbiological and Biomedical Laboratories 5th Edition available at http://www.cdc.gov/biosafety/publications
- The use of **Diagnovital® Influenza A/B SARS-CoV-2 Multiplex** is restricted to trained laboratory personnel only.

SPECIMENS

Only use appropriate specimens for testing, such as:

- Respiratory specimens including nasopharyngeal / oropharyngeal swabs and bronchoalveolar lavage.
- Swab specimens should be collected only on swabs with a synthetic tip (such as polyester or Dacron®) with plastic shafts. Swabs with calcium alginate or cotton tips with wooden shafts are not acceptable

SPECIMENS - HANDLING AND STORAGE

- Specimens can be stored at 4°C for up to 72 hours after collection.
- If a delay in extraction is expected, store specimens at -70°C or lower.
- Extracted nucleic acids should be stored at -70°C or lower.

Do not use specimens if

- they were not kept at 2-4°C (≤ 3 days) or frozen at -70°C or below.
- they are insufficiently labelled or lack documentation.
- they are not suitable for this purpose (see above for suitable sample material).
- the specimen volume is insufficient.

Sample Preparation

- The performance of RT-PCR assays strongly depends on the amount and quality of sample template RNA. It is strongly recommended to qualify and validate RNA extraction procedures for recovery and purity before testing specimens.
- Suitable nucleic acid extraction systems successfully used in combination with **DIAGNOVITAL® DETECTION KITS** include: RTA Viral NA Isolation Kit from Swabs, bioMérieux NucliSens® systems, QIAamp® Viral RNA Mini Kit, QIAamp® MinElute Virus Spin Kit or RNeasy® Mini Kit (QIAGEN), EZ1 DSP Virus Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, Roche MagNA Pure Compact Nucleic Acid Isolation Kit, and Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit, and Invitrogen ChargeSwitch® Total RNA Cell Kit.
- **Diagnovital® Influenza A/B SARS-CoV-2 Multiplex** is also validated with DIAGNOVITAL® MagicPrep Fast RNA Isolation Kit.
- Store and keep residual specimens and extracted nucleic acids at -70°C.
- Only thaw the number of specimen extracts that will be tested in a single day.
- Do not freeze/thaw extracts more than once before testing as each freeze/thaw cycle will decrease the RNA quality.

Reaction Setup

1. Make sure that all necessary equipment and devices are suitable, calibrated and functional before starting the experiments.

2. Decontaminate equipment and workspace and prepare everything needed for the following experiment to keep the workflow short and repeatable.

3. Switch on the PCR detection system and program it to avoid delays after setting up the reactions.

4. Thaw all components of **Diagnovital® Influenza A/B SARS-CoV-2 Multiplex** on ice and mix gently but thoroughly to ensure even distribution of components. Collect liquid at the bottom of the tube with a quick spin (via microcentrifuge).

5. Set up your **Master mix Plate**:

    a. Always prepare control reactions with nuclease-free dH_2O (**NTC**) instead of sample material to detect contamination in your reagents.

    b. When using the provided target positive control (**TPC**), use **5 µl / reaction** (max volume of the reaction should be 20 µ).

Component	Volume
Diagnovital® Influenza A/B SARS-CoV-2 Multiplex Mix	15 µl
Isolated sample RNA / TPC / NTC	5µl / 5µ / 5µ dH_2O

c. The master mix is ready to use. Distribute the master mix to your strips/plate. An example setup is given in **Fig 1**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**	**Master Mix**
B												
C												
D												
E												
F												
G												
H												

Figure 1: Example pipetting scheme for the distribution of master mixes

6. Transfer the Master mix Plate to a separate workspace to add the sample material. Preparing reagents and final reaction setup in separate workspaces helps to avoid contamination of equipment and reagents with sample material.

   a. Prepare negative reactions first and seal them before handling positive samples. It is recommended to only bring potentially positive sample material and the included target positive control to the workspace once the NTC is prepared and sealed.

   b. Add your samples to the Master mix Plate. An example setup is given in **Fig 2**:

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	TPC
B												
C												
D												
E												
F												
G												
H												

Figure 2: Example pipetting scheme for the addition of samples.

c. Keep reactions on ice until transferring them to the PCR device.

7. Transfer the reactions to the PCR device, then proceed according to these guidelines:

Step	Cycles	Temperature	Duration
Reverse Transcription	**1**	**45°C**	**10 minutes**
Initial Denaturation	**1**	**95°C**	**2 minutes**
Amplification	**40**	**95°C**	**10 seconds**
		55°C*	**30 seconds**

*Enable Data Collection for **FAM™** (for Influenza A/B), **Texas Red** (for SARS-CoV-2) and **HEX** (for human extraction control).

8. Once the run is finished, do not open the reaction tubes to avoid contamination and discard according to local guidelines and regulations. Do not autoclave as this may contaminate laboratory equipment with amplicons.

Analysis & Troubleshooting

EXEMPLARY RESULT

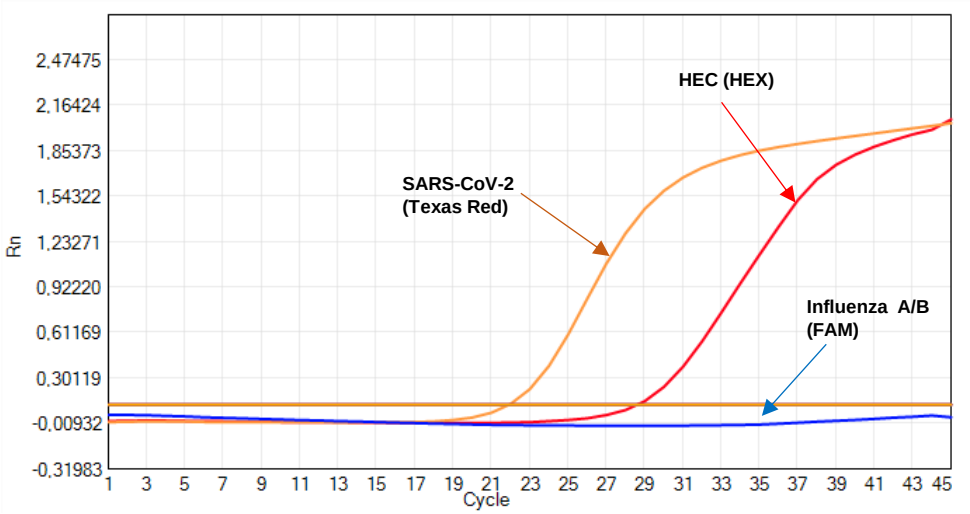

Figure 3: Amplification curves of a sample positive for SARS-CoV-2

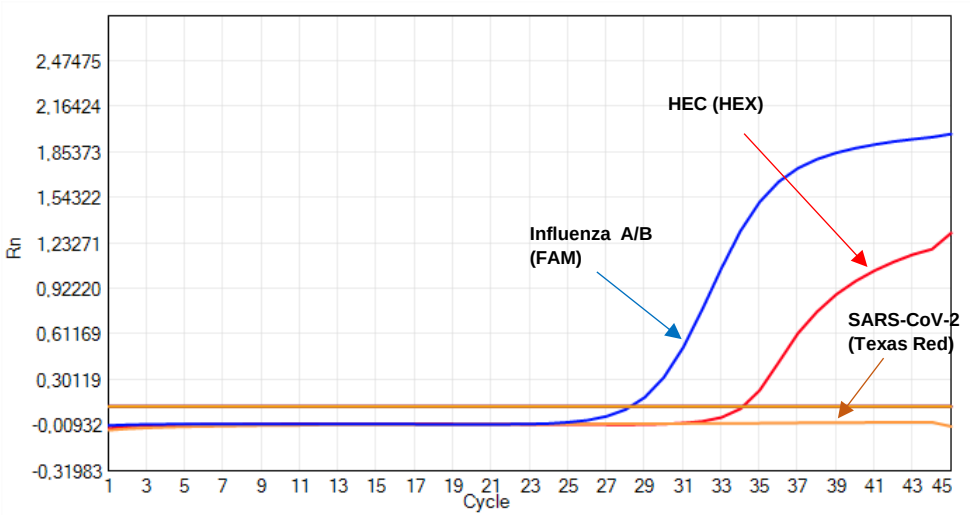

Figure 4: Amplification curves of a sample positive for Influenza A/B

- **dH_2O controls (NTC) must not give a positive Ct for any assay**. If they do, the reaction was contaminated with sample RNA / DNA. Decontaminate equipment and workspace and repeat the reactions.

- **For a sample to be considered positive for SARS-CoV-2, the Texas Red channel must give a positive Ct value**. Amplification of the **HEC** in the **HEX** channel is expected around Ct < 35 cycles. If the **HEC** fails to amplify, the sample must still be considered positive.

- **For a sample to be considered negative for SARS-COV-2, the SARS-CoV-2 assays in the Texas Red channel must not give a positive Ct value.** The HEC must give a positive Ct value (< 35 cycles) in the **HEX** channel or Influenza A/B in the **FAM** channel for these samples to ensure that sample material of suitable quality was present.

- **For a sample to be considered positive for Influenza A/B, the Influenza A/B assays in the FAM channel must give a positive Ct value.** Amplification of the **HEC** in the **HEX** channel is expected around Ct < 35 cycles. If the **HEC** fails to amplify, the sample must still be considered positive.

- **For a sample to be considered negative for Influenza A/B, the Influenza A/B assays in the FAM channel must not give a positive Ct value.** The HEC must give a positive Ct value (< 35 cycles) in the **HEX** channel or SARS-CoV-2 in the **Texas Red** channel for these samples to ensure that sample material of suitable quality was present.

- **All reactions negative for both Influenza A/B and SARS-CoV-2 isolate must give positive Ct values for the HEC assay. The Ct values should be < 35 cycles**. Failure to amplify the negative human extraction control indicates a flawed RNA extraction or loss of RNA isolate due to RNase contamination. The sample is not sufficient, results cannot be interpreted.

- **When using the TPC, a positive Ct in the FAM, Texas Red and HEX channels must be observed**. **The Ct value for the TPC should be < 35 cycles in all channels**. If the Ct value does not correspond to the expected value or not all assays are tested positive, PCR was compromised. Check the reaction setup and PCR device settings and repeat the reactions. Repeated freeze and thaw cycles of the TPC can compromise its quality resulting in late Ct values.

- **If no amplification signal is observed for any assay, PCR was inhibited**. Check reaction setup and device settings and repeat the RNA extraction if necessary. Results are invalid and cannot be interpreted.

Influenza A/B	SARS-CoV-2	HEC	Interpretation
+	-	+ or -	Influenza A/B target sequences were detected. The sample is considered positive for Influenza A/B.
-	+	+ or -	SARS-CoV-2 target sequence was detected. The sample is considered positive for SARS-CoV-2.
+	+	+ or -	Influenza A/B and SARS-CoV-2 target sequences were detected. The sample is considered positive for Influenza A/B and SARS-CoV-2.
-	-	+ (<35 Ct)	Only internal control target sequence was detected. The sample is considered negative for Influenza A/B and SARS-COV-2.
-	-	- (≥ 35 Ct)	Invalid result. Consider repeat of extraction and/or qPCR or collecting a new specimen.

Limitations

- For reliable results, it is essential to adhere to the guidelines given in this manual. Changes in reaction setup or cycling protocol may lead to failed experiments.
- Depending on the sample matrix, inhibitors may be present in the isolated RNA and disable reverse transcription and/or PCR amplification. If this is the case, another sample type or isolation method may be beneficial.
- Spontaneous mutations within the target sequence may result in failure to detect the target sequence.
- Results must always be interpreted in consideration of all other data gathered from a sample. Interpretation must be performed by personnel trained and experienced with this kind of experiment.

Trademarks

DIAGNOVITAL®®, NucliSens® (bioMérieux), QIAamp®, RNeasy® (QIAGEN), ChargeSwitch® (Invitrogen), ROX™, FAM™ (Life Technologies), DNAZap™, DNA Away™, RNAse Away™

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Symbols


Expiry Date

Lot/Batch

Catalog number


Temperature limitation


Caution; consult accompanying documents


Manufacturer

In Vitro Diagnostic Medical Device


Consult instructions for use


Contains sufficient for (n) amount tests

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