FluV19 RT-PCR (RUO) Multiplex Kit

Reference: G210221
Kit: 1,000 Reaction Kit
Revision: 210810.v2

Intended Use

The following kit is being distributed for research use only (RUO) detection of Influenza A, Influenza B, and SARS-CoV-2 by multiplex RT-PCR assay (FluV19). This kit contains enough reagents for amplification of 1,000 samples. The following outline specifies the kit components, storage requirements, and recommendations for use by real-time reverse transcriptase polymerase chain reaction. This kit is a laboratory developed test (LDT) and is for research use only (RUO) and not for use in clinical or *in vitro* diagnostics.

1. COMPONENTS OF THE FLUV19 RT-PCR (RUO) MULTIPLEX KIT

1.1. The FLUV19 RT-PCR (RUO) Kit contains the following components:

Reagent Tube	Storage Conditions	Description	# Tubes/Kit (Volume/Tube)	# Reactions/Tube
Component A	≤ -20°C	TaqMan Human Control RNA (50 ng/µl)	**4** (100 µl/tube)	Sufficient for n=250, 200 µl aliquots
Component B	2-8 °C	Primer cocktail mix	**2** (1,300 µl/tube)	Sufficient for n=1,000 reactions
Component C	2-8 °C	Probe cocktail mix	**2** (1,300 µl/tube)	Sufficient for n=1,000 reactions
Component D	-20 ± 5°C	Enzyme Mix (+ROX)	**4** (1,250 µl/tube)	Sufficient for n=250, reactions.
Component E	2-8 °C	Positive Template Control (PTC)	**4** (1,250 µl/tube)	Sufficient for n=250 control reactions

1.2. **Component A: 50x Non-Viral Extraction Control (NVC).**

1.2.1. Contains: Human Control RNA

1.2.2. Use: Functions as a Non-Viral Extraction Control (NVC) for human ribonuclease protein (RNase P).

1.2.3. Expectations: The efficiency of nucleic acid extraction of human samples is evaluated by extraction of the NVC alongside human samples.

1.2.4. Preparation: Thaw 1 tube of Component A and centrifuge briefly. Add 900 µl of 10 mM Tris (pH 8.0) to the 100 µl of Human Control RNA per tube of Component A. Triturate (pipette) several times and transfer the entire contents (1 ml) into 4.9 ml of 10 mM Tris (pH 8.0). Vortex thoroughly to mix. Prepare 200 µl single-use aliquots.

1.2.5. Storage: Store unopened/unprepared 50 ng/µl NVC (100 µl) at ≤ -70°C. Store single use aliquots of NVC at ≤ -20°C.

1.3. **Component B: Primer cocktail mix.**

1.3.1. Contains: Multiplex primers for Influenza A (InfA), Influenza B (InfB), SARS-CoV-2 (SARS2), and human ribonuclease protein (RP).

1.3.2. Use: Amplification of Influenza A, Influenza B, and SARS-CoV-2 pathogens. Amplification of human RNase P present in human samples.

1.3.3. Expectations: Amplification of RNase P in human samples indicates that the sample contains human cells which may or may not harbor virus. Amplification of Influenza A, Influenza B, and/or SARS-CoV-2 indicates the presence of RNA from the respective pathogen(s). Capable of amplifying RNA from any combination of viral pathogens for which the primers target.

1.4. **Component C: Probe cocktail mix.**

1.4.1. Contains: Multiplex probes for Influenza A (InfA), Influenza B (InfB), SARS-CoV-2 (SARS2), and human ribonuclease protein (RP).

1.4.2. Use: Detection of Influenza A, Influenza B, and SARS-CoV-2 pathogens. Amplification of human RNase P present in human samples.

1.4.3. Expectations: Detection of RNase P in human samples indicates that a valid clinical specimen has been collected. Detection of Influenza A, Influenza B, and/or SARS-CoV-2 indicates the presence of RNA from the respective pathogen(s). Capable of detecting RNA from any combination of viral pathogens for which the probes target.

1.5. **Component D: Enzyme mix with ROX passive reference.**

1.5.1. Contains: RT-PCR Enzyme mix

1.5.2. Use: One-step RT-PCR mix for cDNA synthesis and amplification of sequences target by primers and probes in Component A and Component B. Reverse Transcriptase Quality Control (RT-QC) is verified by cDNA synthesis and amplification of human RNase P in the NVC (Component A).

1.5.3. Expectations: Detection of target sequences through amplification on real-time instruments.

1.6. **Component E: 10x Positive Template Control (PTC)**

1.6.1. Contains: Plasmid template sequences of Influenza A, Influenza B, SARS-CoV-2, and human RNase P.

1.6.2. Caution: Plasmid DNA is stable on surfaces and can easily be transported on contaminated gloves and equipment. Use proper molecular techniques to avoid contamination of samples and reagents. Store PTC in areas separate from extraction controls and samples.

1.6.3. Use: PCR positive template control for verifying the amplification efficiency of Component A, Component B, Component C, and Component D.

1.6.4. Expectations: Amplification and detection of Influenza A, Influenza B, SARS-CoV-2, and RNase P in the PTC.

1.6.5. Preparation: In an area separate from sample extraction and PCR preparation, centrifuge Component E at full speed for 30-seconds to remove droplets from the lid and sides. Prepare 30 µl aliquots (enough for n=6 PCR plates). Label as "PTC".

1.6.6. Storage: Store 30 µl aliquots at 2-8°C for up to 2-months. For long-term storage, store aliquots of PTC at ≤ -70°C.

2. EXTRACTION OF COMPONENT A (NVC) ALONGSIDE SAMPLES

*Component A has been assessed on KingFisher FLEX Magnetic Particle Processors using the MVP II MagMax Viral/Pathogen Nucleic Acid Isolation Kit with 50 µl elution volume.
*Component A has been assessed with manual extraction methods using Qiagen QIAmp Viral RNA Mini Kit with 50 µl elution.

2.1. Extraction

2.1.1. Extract 200 µl of Component A alongside samples (MVP II Low Volume protocol).

2.1.2. Extract 400 µl of Component A alongside samples (MVP I or MVP II 400 µl protocol)

2.2. Elution

2.2.1. KingFisher Flex

2.2.1.1. Elute in 50 µl of KingFisher Elution Buffer

2.2.2. Manual Extraction

2.2.2.1. Elute in 50 µl of 10 mM Tris (pH 7.0-8.0)

3. PREPARE PCR REACTION MIX

3.1. Prepare the PCR Reaction Mix in a clean area under a dedicated laminar flow hood as follows:

	†Number of Reactions					
Reagent	**1x**	**5x**	**10x**	**20x**	**50x**	**100x**
Component B	2.4 µl	12 µl	24 µl	48 µl	120 µl	240 µl
Component C	2.4 µl	12 µl	24 µl	48 µl	120 µl	240 µl
Component D	5.0 µl	25 µl	50 µl	100 µl	250 µl	500 µl
*Molecular Grade Water	5.2 µl	26 µl	52 µl	104 µl	260 µl	520 µl
Total Reaction Mix	15 µl	75 µl	150 µl	300 µl	750 µl	1500 µl

†Reaction Mix volumes are calculated with no overages to compensate for error. Suggested: calculate one more reaction than is needed to compensate for errors in pipetting.

* Do Not use DEPC-treated water in PCR reaction mixes as they can inhibit PCR reactions.

3.2. Triturate the PCR Reaction Mix well by pipetting multiple times. Avoid vortexing as Component D contains albumin and is prone to frothing resulting in loss of volume in the reaction mix.

3.3. Transfer 15 µl of reaction mix into each well of a 96-well or 384-well optical plate to receive specimen elution.

3.4. Transfer 5 µl of extracted sample elution, 5 µl or extracted NVC, and 5 µl of PTC (Component D) into the respective wells of the 96-well or 384-well optical plate containing PCR reaction mix.

3.5. Seal the PCR plate with optical film and centrifuge PCR reaction plate at 1,000 xg for 1-minute. Inspect wells for the presence of bubbles. If bubbles are present, tap the PCR reaction plate gently and re-centrifuge at 1,000 xg for an additional 1-minute.

4. THERMAL CYCLING CONDITIONS

Ensure that your real-time instrument has current spectral calibrations performed for the following detectors: HEX, FAM, TAMRA, CY5, ROX.

4.1. Set the thermal cycling conditions on your real-time instrument as follows:

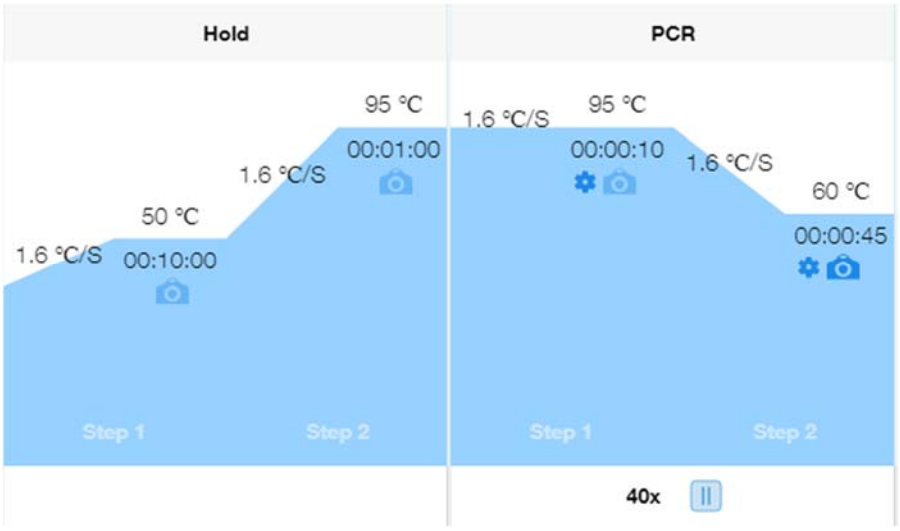
4.1.1. Passive Reference: ROX

4.1.2. Cycling Conditions:

4.1.2.1. Step 1 (1 cycle): Reverse Transcription: 50°C for 10 minutes

4.1.2.2. Step 2 (1 cycle): RT Inactivation: 95°C for 1 minute

4.1.2.3. Step 3 (40 cycles): PCR: 95°C for 10 seconds; 60°C for 45 seconds (Data Capture)



4.2. Fluorophore Detection Settings

4.2.1. Influenza A (InfA)

4.2.1.1. Detector: CY5

4.2.1.2. Quencher: NONE

4.2.2. Influenza B (InfB)

4.2.2.1. Detector: HEX

4.2.2.2. Quencher: NONE

4.2.3. SARS-2 (SARS2)

4.2.3.1. Detector: FAM

4.2.3.2. Quencher: NONE

4.2.4. RNase P (RP)

4.2.4.1. Detector: TAMRA

4.2.4.2. Quencher: NONE

5. SUGGESTED ANALYSIS OF AMPLIFICATION CURVES

*Suggested Analysis Software: Thermo Fisher Scientific Design & Analysis Software. This software can be downloaded from: https://www.thermofisher.com/us/en/home/global/forms/life-science/quantstudio-6-7-pro-software.html. If not using the Design & Analysis Software, and complete analysis using your instrument's analysis software.

6. EXAMPLE FOR INTERPRETATION OF RESULTS

6.1. The suggested cutoff of the assay is Cq/Ct ≤ 38.00 but this cutoff should be independently established based on each laboratories extraction methodologies and molecular analysis platforms.

6.2. Samples with suspicious amplification curves between 38.00-40.00 cycles should be evaluated further.

6.3. Refer to the following table for an example for interpretation of results:

	INFA	INFB	SARS-2	RNP	Result Interpretation
NVC	Ct > 38	Ct > 38	Ct > 38	Ct 26-30	Extraction Successful Reverse Transcriptase QC Valid
PTC	Ct 24-28	Ct 24-28	Ct 24-28	Ct 24-28	PCR Reagents QC Valid
Sample	Ct > 38	Ct > 38	Ct > 38	**Ct < 38**	Influenza A, Influenza B, SARS-CoV-2 RNA Not Detected
Sample	Ct > 38	Ct > 38	Ct > 38	**Ct > 38**	Invalid Sample or PCR Inhibitors Present in Sample
Sample	**Ct < 38**	Ct > 38	Ct > 38	**Ct < 38**	Influenza A RNA Detected
Sample	Ct > 38	**Ct < 38**	Ct > 38	**Ct < 38**	Influenza B RNA Detected
Sample	Ct > 38	Ct > 38	**Ct < 38**	**Ct < 38**	SARS-CoV-2 RNA Detected
Sample	**Ct < 38**	**Ct < 38**	Ct > 38	**Ct < 38**	Influenza A & Influenza B RNA Detected
Sample	**Ct < 38**	Ct > 38	**Ct < 38**	**Ct < 38**	Influenza A & SARS-CoV-2 RNA Detected
Sample	Ct > 38	**Ct < 38**	**Ct < 38**	**Ct < 38**	Influenza B & SARS-CoV-2 RNA Detected
Sample	**If any Viral Gene Target or combination thereof displays amplification < 38**			Ct > 38 or Undetected	Respective Viral RNA Detected

* Samples with suspicious amplification curves between 38.00-40.00 cycles should be evaluated further.

7. LIMITATIONS OF THE ASSAY

7.1. This assay has not been approved by the Food and Drug Administration (FDA) and does not have EUA approval for the clinical detection of Influenza A, Influenza B, or SARS-CoV-2. This multiplex assay is for Research Use Only (RUO) and is not intended for use in clinical or *in vitro* diagnostics.

7.2. The limits of detection of this assay are based on detection of single gene copies of Influenza A, Influenza B, and SARS-CoV-2 and are estimated at 100 gene equivalents of Influenza A Matrix Protein (M2), 100 gene equivalents of Influenza B Non-Structural Protein 2 (NS2), and 60 gene equivalents of SARS-CoV-2 Nucleoprotein (N). However, laboratories using this assay should independently verify the limits of detection using their extraction methods and molecular analysis platforms.

8. DISCLAIMER

The contents of this kit including the Primer and Probe sets and Positive Template Control are proprietary and are not to be reverse engineered or misused. The contents of this kit are not to distributed to any third party without the expressed written consent from GRD.