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Extraction and PCR for animal samples using the Thermofisher Scientific TaqPath COVID-19 Combo Kit

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We use this protocol and it's working

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Abstract

To describe the steps and materials needed to perform a multiplex real-time reverse transcriptase PCR test intended for the qualitative detection of nucleic acid from SARS-CoV-2, the causative agent of COVID-19, in animal respiratory swab samples.

This document includes the information and procedures to comply with the ThermoFisher Scientific TaqPath™ COVID-19 Combo Kit under the Food and Drug Administration's (FDA) Emergency Use Authorization (EUA). The TaqPath™ COVID-19 Combo Kit includes a Real Time PCR assay multiplex that contains three primer/probe sets specific to the SARS-CoV-2 and a primer probe set for bacteriophage MS2 as an internal extraction control.

Sections of this procedure are taken directly from the TaqPath™ COVID-19 Combo Kit Instructions for Use, Publication MAN0019181, Version G.0. For additional information, please refer to these instructions.

Guidelines

1. The TaqPath™ RT-PCR COVID-19 Kit workflow should be performed by qualified and trained staff to avoid the risk of erroneous results. Use separate areas for the preparation of patient samples and controls to prevent false positive results. Samples and reagents must be handled in a biological safety cabinet.

2. The assay is for *in vitro* diagnostic use under the FDA Emergency Use Authorization Only.

3. This test has not been FDA cleared or approved.

4. This test has been authorized only for the detection of nucleic acid fromSARS-CoV-2, not for any other viruses or pathogens.

5. This test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of *in vitro* diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Act, 21
U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.

6. Samples and controls should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.

7. Follow necessary precautions when handling specimens. Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.

8. Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNases.

9. Do not eat, drink, smoke, or apply cosmetic products in the work areas.

10. Reagents must be stored and handled as specified. See manufacture instructions.

11. Components of reagent kits of different lot numbers must not be interchanged.

12. Do not use the kit after the indicated expiry date.

13. Dispose of waste in compliance with local, state, and federal regulations.

Positive results are indicative of the presence of SARS-CoV-2 RNA

Materials

Reagents:

- 0.1 ml PCR reaction tubes and caps or PCR plate
- 1.7 ml microcentrifuge tubes, nuclease-free
- 50 ml conical tubes or large bottles for bead/lysis mixture
- 70% ethanol (cleaning)
- 100% ethanol Absolute, Molecular Biology Grade or 80% Molecular Biology Grade ethanol
- 96-well Real-time PCR reaction plate and optical clear film
- Bleach (~10% solution for cleaning)
- Biohazard bags
- Disposable RNase/DNase-free pipette tips
- KingFisher™ 96 tip comb for DW magnets (or equivalent)
- KingFisher™ 96 KF microplate (or equivalent)
- KingFisher™ Deepwell 96 plate (or equivalent)
- Latex/Nitrile gloves
- MagMAX Viral/Pathogen Nucleic Acid Isolation Kit
- MicroAmp™ Clear Adhesive Film or equivalent
- MicroAmp™ Optical Adhesive Film
- MicroAmp™ Fast Optical 96-Well Reaction Plate
- Nuclease-free water
- Positive and negative extraction and/or amplification controls
- RNase eliminator like RNaseZAP or Eliminase
- TaqPath™ COVID-19 Combo Kit (Catalog #A47814 or equivalent)

Equipment:

- Applied Biosystems™ 7500 Fast Real-Time PCR Instrument (thermal cycler) with SDS software v1.5.1 or 7500 Software v2.3 or equivalent.
- Applied Biosystems™ COVID-19 Interpretive Software v1.3
- Freezer: approximately -20°C and -80°C
- KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well head
- Microcentrifuge
- N95 respirator or CAPR/PAPR
- Biological Safety Cabinet (BSC)
- Refrigerator
- Vortex

Troubleshooting

Safety warnings

Proper PPE including double gloves, disposable or autoclavable gown, N95 with face shield or CAPR unit is required and must be used when working in the Biosecure Necropsy Suite and BSL-3. Gloves and lab coats are required for extraction and PCR setup. Gloves must be worn at any time when touching extraction or PCR plates or reagents.

SARS-CoV-2 RNA Extraction

1 **Prepare working areas by cleaning with ~10% bleach or ~70% alcohol, followed by a rinse and use of another solution to reduce the presence of RNases such as RNaseZAP or Eliminase.**

2 **Prepare Binding Beads/Lysis mix on each day of use.**

2.1 Vortex the Total Nucleic Acid Magnetic Beads to ensure that the bead mixture is homogeneous.

2.2 For the number of required reactions, prepare the Binding Bead/Lysis Mix according to Table 1

Table 1: Binding Bead/Lysis Mix

	A	B	C
	Component	Volume per RNA Sample or Control	Volume for 150 rxns
	Binding Solution	530 µL	79.5 mL
	Nucleic Acid Magnetic Beads	20 µL	3.0 mL
	Proteinase K	10 µL	1.5 mL
	MS2 Phage Control	10 µL	1.5 mL
	Total Reaction Mix volume	**570 µL**	**85.5 mL**

***Highly recommend more than a 10% overage.

Note

Human protocol does not allow for combining PK, phage, beads and binding solution all together; however, it worked for animal samples. Saves a significant amount of time and tips.

2.3 Mix well by inversion, then store at room temperature.

3 **Prepare Plates for extraction following table 2.**

Table 2: Plate setup.

	A	B	C	D	E
	Plate Position	Plate ID	Plate Type	Reagent	Volume per Well
	1	Sample Plate	KingFisher™ Deepwell 96 Plate or Equivalent	400 µLSample* + 570 µL Bead/lysis mix	
	2	Wash 1		Wash Buffer	1000 µL
	3	Wash 2		80% Ethanol	1000 µL
	4	Elution Plate		Elution Solution	50 µL
	5	Tip Comb	Place a KingFisher™ 96 tip comb for DW magnets in a KingFisher™ 96 KF microplate		

*Add sample to plate in a biological safety cabinet.

Safety information

Handle samples according to institutional biosafety standards

Note

According to manufacterer, avoid DEPC water.

It is recommend to cover filled wash and elution plates with a seal to avoid evaporation if it takes a while to load the sample plate.

A new tip is recommended for each well when pipetting Bead/lysis mix due to foaming.

3.1 Invert the Binding bead Mix (from 2.2) five times gently to mix, then add 570 µL (binding solution + beads + PK + MS2 phage control) to each sample well and negative control well in the sample plate.

Note

Remix the Binding Bead Mix by inversion frequently during pipetting to ensure even distribution of beads to all samples or wells. The Binding Bead Mix is viscous, so pipet slowly to ensure that the correct amount is added. DO NOT reuse pipette tips to add Binding Bead Mix to the samples, as the high viscosity will cause variations in the volumes added.

3.2 Add samples and controls to the appropriate wells:
Samples: 400 µL of sample to a well.
Negative Control: 400 µL **L of Nuclease-free Water** (not DEPC-Treated) to the Negative Control

Note

If use a 96 well plate: up to 94 samples can be on ran, two wells are reserved for controls, one for the negative extraction control and one for the positive amplification control that is not added until the PCR step (step 6).

3.3 Seal sample plate with film. Wipe outside of plate with disinfectant and place in transport container. Wipe/disinfect transport container and carefully bring to BSC in Extraction Room.

4 Select the program **MVP_2Wash_400_Flex** on the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head

4.1 Start the run and load prepared plates in positions when prompted by the instrument.

4.2 Immediately remove the elution plate from the instrument and cover with film/foil. Run lasts ~24 minutes.

Seal immediately to avoid evaporation and store up to 48hrs at 4°C, -20°C, or -70°C.

SARS-CoV-2 PCR

5 If frozen, thaw the reagents on ice. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tubes.

6 Prepare a Master Mix of the PCR reagents containing enough for all samples, controls, plus extra. Master Mix is prepared manually using the volumes in Table 3.

Table 3. PCR Reagent Volumes.

	A	B
	Nuclease-free Water	12.5 µL
	4X TaqPath 1-Step Multiplex MMX (No ROX)	6.25 µL
	COVID-19 Real Time PCR Assay Multiplex	1.25 µL
	20 µL MMX per rxn	

6.1 Pipette 20 µL of master mix into each well.

6.2 Add 5 µL of extraction RNA or negative control to corresponding well. Total reaction volume will be 25 µL

Note

Sample plate can be vortexed and centrifuged briefly to collect liquid to the bottom of the plate.

6.3 Add 2 µL of diluted positive control + 3 µL of nuclease-free water for positive control well.

6.4 Diluted positive control is made by:

Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly

6.5 Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the dilution created in substep 13.1. Mix well, then centrifuge briefly.

Note

The positive control does not contain the MS2 phage.

6.6 Once sample and controls have been added, seal plate thoroughly.

6.7 Vortex plate at high speed for 10-30 seconds. Recommend a plate holder attachment to avoid damaging wells.

6.8 Centrifuge the reaction plate for 2 minutes (~600 x g) to remove bubbles and collect liquid to the bottom of the reaction plate.

Note

Speed may vary based on plate spinner. Manufacturer recommended ≥ 650 x g.

7 **Setting up Thermal Cycler**

This protocol used the ABI 7500 Fast or ABI 7500 DX thermal cycler.

Set up thermal cycler according to manufacture instruction manuals along with table 4 and 5.
Refer to https://www.fda.gov/media/136112/download in reference sections for more information

7.1 **Table 4. Target, dyes, and quencher.**

	A	B	C
	Target	Reporter dye	Quencher
	ORF1ab	FAM	None
	N gene	VIC	None
	S gene	ABY	None
	MS2	JUN	None

7.2 **Table 5. Thermal cycler conditions.**

	A	B	C	D
	Step	Temperature	Time	Number of cycles
	UNG incubation	25 C	2 min	1
	Reverse transcription	53 C	10 min	1
	Activation	95 C	2 min	1
	Denaturation	95 C	3 sec	40
	Anneal/extension	60 C	30 sec	

8 Interpretation of Results

8.1 A minimum of one Negative Control and one Positive Control must be present for each run. Additional Negative Control wells must be run for each extraction that is represented on a real-time RT-PCR plate. All control wells must pass for the real-time RT-PCR plate to be considered valid.

8.2 Validation of results is performed automatically by the Applied Biosystems™ COVID-19 Interpretive Software based on performance of the Positive, Negative, and Internal Controls. See Table 6.

Table 6. Interpretation of Results

ORF1ab	N gene	S gene	MS2	Status	Result	Action
NEG	NEG	NEG	NEG	INVALID	NA	Repeat test by re-extracting the original sample and repeating the RT-PCR. If the repeat result remains invalid, consider collecting a new specimen.
NEG	NEG	NEG	POS	VALID	SARS-CoV-2 Not Detected	Report results to the healthcare provider and appropriate public health authorities. Consider testing for other viruses.
Only one SARS-CoV-2 target = POS			POS or NEG	VALID	SARS-CoV-2 Inconclusive	1. Repeat test by re-extracting the original sample and repeating the RT-PCR. 2. After retesting one time, report results to the healthcare provider and appropriate public health authorities. IMPORTANT! Samples with a result of SARS-CoV-2 Inconclusive shall be retested one time. If the repeat result remains inconclusive, the healthcare provider should conduct additional confirmation testing with a new specimen, if clinically indicated.
Two or more SARS-CoV-2 targets = POS			POS or NEG	VALID	Positive SARS-CoV-2	Report results to the healthcare provider and appropriate public health authorities.

Note

Inconclusive sample should be re-extracted and retested one time.

Protocol references

https://www.fda.gov/media/136112/download