JAN 23, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.n2bvj81xxgk5/v1

Protocol Citation: Tracy Lee, frankie.tsang, Kathleen Kolehmainen, Natalie Prystajecky, Agatha N. Jassem, John R. Tyson, Michael Chan 2024. Detection of Influenza A viruses and Avian H5 Subtype using a triplex qRT-PCR assay on the ABI Quantstudio 7 PCR system. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n2bvj81xxgk5/v1

Detection of Influenza A viruses and Avian H5 Subtype using a triplex qRT-PCR assay on the ABI Quantstudio 7 PCR system

Tracy Lee[1], frankie.tsang[1], Kathleen Kolehmainen[1], Natalie Prystajecky[1,2], Agatha N. Jassem[1,2], John R. Tyson[1,2], Michael Chan[1]

[1]BCCDC Public Health Laboratory; [2]University of British Columbia

BCCDC Public Health Laboratory

BCCDC Public Health Laboratory

ABSTRACT

This procedure provides instructions on how to perform a real-time PCR (qPCR) for the detection of Influenza A viruses and avian H5 subtypes from nucleic acid extracted from respiratory samples. This assay targets the avian influenza hemagglutinin (HA) gene of Influenza A subtype H5, primers and probes sequences are updated to enable detection of recent outbreak of Influenza A subtype H5N1. The matrix (M) gene of Influenza A virus is included in this triplex for detection of suspect avian influenza virus (AIV). This assay is not validated and does not contain an endogenous control for the human or avian samples.

MANUSCRIPT CITATION:
Tracy
D. Lee, Frankie Tsang, Kathleen Kolehmainen, Natalie
A. Prystajecky, Agatha
N. Jassem, John R. Tyson. 2023. A multiplex qRT-PCR assay for detection of Influenza A and H5 subtype targeting new SNPs present in high pathogenicity avian influenza Canadian 2022 outbreak strains. medRxiv 2023.12.13.23298992; doi: https://doi.org/10.1101/2023.12.13.23298992

Protocol status: Working
We use this protocol and it's working

Created: Mar 17, 2023

Last Modified: Jan 23, 2024

PROTOCOL integer ID: 78987

Funders Acknowledgement:
This study was funded through British Columbia Centre for Disease Control operational funding sources.

GUIDELINES

This assay is not validated and does not contain an endogenous control for the human or avian samples.

Nucleic acid extracted from avian samples or patient respiratory samples according to validated procedures using the MagMax Express. All human samples for non-human Influenza A subtyping should be Influenza A positive by another method and be considered non-typable for H3 and pH1N1.

Quality Control:

A.Positive Extraction Control
Influenza A culture with expected Ct range at Ct 25 - 27

B.Negative Extraction Control
MEM or UltraPure DNase/RNase-Free Distilled water extracted alongside samples.

C.Positive PCR control (Synthetic)
Prepare synthetic control to act as a positive control for hemagglutinin H5, and the matrix gene (all subtypes). Examples can include gBlocks, GeneArt Strings, commerically prepared nucleic acids, or previous positive sample.

D.Negative PCR control (no template)
UltraPure DNase/RNase-Free Distilled water in PCR mastermix.

MATERIALS

A	B	C
Reagents	**Equipment**	**Supplies**
TaqMan™ Fast Virus 1-Step Master Mix (ThermoFisher Scientific, 444434)	ABI Quantstudio 7 Pro real-time PCR system	MicroAmp Optical Adhesive Film and Applicator
IDTE 1x TE Buffer pH 8.0	MicroAmp 96-Well Plate Holder	Applied Biosystems Fast Optical 96-Well Plates
UltraPure™ DNase/RNase-Free Distilled Water	Biological safety cabinet- 6ft, Nuaire, Class II Type A/B3	Combi tips
Custom primers and probes from ThermoFisher Scientific and IDT	Appropriate volume dispensing pipettes (single and multi-channel)	Pipet tips; various sizes. Filter plugged and nuclease free
DNA AWAY	Plate centrifuge	1.7 ml microcentrifuge tube
	Vortex Mixer	Ziploc bags
	Repeater pipet	Disposable Powder-free Gloves and Gowns
		Discard pail
		Waste bags

Preparation of 20x Primer/Probe Mix

1 In the reagent preparation clean room, take out the primers and probes listed in table 1 from the freezer and thaw in the refrigerator.

A	B	C	D
Gene Target	Oligo Name	Final Concentration (nM)	Sequence 5’ - 3’
H5-P3	H5_22_F3	450	GTTTATAGAGGGAGGATGGCAG
	H5_22_R3	450	ATGATTGAGTTGACCTTATTGGTAACTC
	H5_22_FAM_MGB_P3	150	ATGGTTGATGGTTGGTATG
H5-P4	H5_22_F4	450	GACGTATGACTACCCTCAGTATTCAGA

	A	B	C	D
		H5_22_R4	450	CATTGGAGCACATCCATAAAGATAGA
		H5_22_VIC_MGB_P4	150	AGGAACTTACCAGATACTGTCAA
	Influenza A (M Fouchier) (1)	FluA-M253R	400	AGGGCATTTTGGACAAAKCGTCTA
		FluA-M52C	400	CTTCTAACCGAGGTCGAAACG
		FluA-M96C_taq ABY-QSY	200	CCGTCAGGCCCCCTCAAAGC

Table 1. Primers and Probes used in this assay.

2 Once thawed, vortex well and centrifuge down all components

3 In a clean BSC, prepare the 20x primer/probe mix according to the recipe in Table 2 (Adjust volume accordingly based on the amount needed):

	A	B	C	D	E	F
	20x H5 Triplex Fochier M Region, H5_P3, H5_P4					
	Stock Reagent	Stock conc. (uM)	Final conc./rxn (nM)	**100 rxn (µl)**	**500 rxn (µl)**	**1000 rxn (µl)**
	M52C primer	100	400	8	40	80
	M253R primer	100	400	8	40	80
	M96C (ABY QSY)	100	200	4	20	40
	H5_22_P4 Forward	100	450	9	45	90
	H5_22_P4 Forward	100	450	9	45	90
	H5_22_P4 (VIC MGB)	100	150	3	15	30
	H5_22_P3 Forward	100	450	9	45	90
	H5_22_P3 Forward	100	450	9	45	90
	H5_22_P3 (FAM MGB)	100	150	3	15	30
	IDTE 1x TE Buffer pH 8.0	1x	1x	38	190	380

Table 2. 20X Primer/Probe mix Recipe. "Rxn": reaction.

Note: Note: Store the 20x mixes at -20°C in the dark. Excessive exposure to light may affect the fluorescent probes. Do not perform more than 10 freeze-thaw cycles. If you expect to freeze-thaw the 20x mix more than five times, consider aliquoting the 20x into smaller volume to minimize the number of freeze-thaw cycles

Setting up and Running qPCR on the ABI Quantstudio 7 Pro

4 Prepare the qRT-PCR cocktail using the TaqMan® Fast Virus 1-Step Master Mix Kit, following the recipe. Calculate the volume needed based on number of reactions per run. Record lot number of reagents used.

	A	B
	Reagent	Vol./reaction (µl)
	PCR grade Water	9
	TaqMan® Fast Virus 1-Step Master Mix (4x)	5
	20x primer/probe mix	1

Table 3. Master Mix Cocktail Recipe

Vortex and spin down the mastermix cocktails.

5 Remove an Applied Biosystems Fast Optical 96-Well Plate from the box, inside the BSC, and place it in a MicroAmp 96-Well Plate Holder to ensure that the bottom of the plate has no contact with anything that could fluoresce.

Note: *Do not use permanent marker on optical plates, the ink will fluoresce.*

6 Aliquot 15µL of each cocktail to the wells being used for the assays in the ABI Fast Optical 96-Well plate. Seal with an adhesive film aseptically.

7 Place the plate in a clean plastic bag and transport it to the genomic preparation room. Place the plate in a MicroAmp 96-Well Plate Holder.

8 In the genomic room, add 5µL of sample template and controls to the appropriate wells of the 96-well plate.

9 Seal the plate with a MicroAmp optical plate film using the plate film applicator.

Note: Be careful not to contaminate the plate film optical surface with anything that could fluoresce such as ink, dust or fingerprints.

10 Place the sealed 96-well plate back in the clean plastic bag. Centrifuge in a plate centrifuge and ensure there are no bubbles in the wells before proceeding. Transport the plate to the ABI QuantStudio 7 Pro.

Note: *Centrifugation and transportation in the clean plastic bag prevents contamination with dust prior to loading on the instrument.*

11 Load the PCR plate into the ABI Quantstudio 7 Pro.

12 Load the following run method on the ABI Quantstudio 7 Pro:

Thermalcycling conditions:

	A	B	C	D
	Step	Temperature (°C)	Time (min:sec)	Cycles
	Reverse Transcription	50	5:00	1
	Initial Denaturation	95	0:20	1
	Denaturation	95	0:03	40
	Annealing/Extension	60	0:30	

Table 4. ABI Quantstudio 7 Pro Thermalcycling Program

Experimental Properties:

- Analysis Module: Presence Absence
- Run Mode: Fast
- Heat Cover Temperature: 105.0 °C
- Reaction Vol. per Well: 20µL
- Template: 5µL

- Passive Reference: ROX

Confirm that the cycling conditions are correct before starting the instrument.

13 Enter the samples under "Plate Set-up" with the following Target properties:

Target	Reporter	Quencher
H5-P3	FAM	NFQ-MGB
H5-P4	VIC	NFQ-MGB
Flu A-M96C	ABY	QSY

Table 5. Target properties used in the Plate Set-up

14 Save then start the run. Confirm that the instrument is running before you leave.

Results Analysis

15 Remove your plate from the QS 7, place it back in the clean plastic bag, seal, and discard in biohazardous bin.

16 Ensure the following thresholds are set for each target:

A	B
Target	**Threshold**
Influenza A (M-Fouchier)	0.05
H5-P3	0.06
H5-P4	0.1

Table 6. Threshold settings

17 Analyze the qRT-PCR results of the controls. Verify that the results fall within the acceptable range.