Influenza A H3 virus TaqMan assay

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Abstract

This assay is a modification to the World Health Organization's influenza A H3 TaqMan documented in 'WHO information for molecular diagnosis of influenza virus - update 1' (see file below). New primers were added and the WHO primers discarded.

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Before start

- If using a different brand or model of real-time thermocycler, check that the concentration of ROX is adequate.
- Method assumes the user is familar with the thermocycler and software used to run the protocol.

Protocol

Oligonucleotides

Step 1.

Name	5'-3' SEQUENCE
H3hFor1	GGTACGG**Y**TTCAGGCAT
H3hRev1	TCAATCTGATGGAATTTCTCGTTG
H3h-1144dProbe	6FAM-CTGCTGCTTGTCCTCTTCCCT-BHQ1

The oligoprobe is from the World Health Orgnization protocol linked below.

New primers were designed to improve assay performance.

LINK:
http://www.who.int/entity/influenza/gisrs_laboratory/molecular_diagnosis_influenza_virus_humans_update_201403rev201505.pdf?ua=1

Step 2.

REAGENTS
SuperScript™ III Platinum™ One-Step qRT-PCR Kit 11732088 by Life Technologies

Reaction Set-up

Step 3.

The assay has been used on both a Rotor-Gene 6000 and a Rotor-Gene Q real-time thermocycler

Prepare sufficient mix for the number of reactions.

Include a suitable 'dead volume' as necessary if using a robotic dispenser.

MIX PREPARATION

Reagent	Vol (µL) X1	Final reaction concentration
Nuclease-free water	4.43	
H3hFor1 (200pmol/µL)	0.05	500nM
H3hRev1 (200pmol/µL)	0.05	500nM
H3h-1144dProbe (100pmol/µL)	0.03	150nM
2X Reaction Mix[1]	10.0	1X
ROX reference Dye (25µM)[1,2]	0.04	50nM
SuperScript™ III/Platinum™ Taq Mix[1]	0.4	
TOTAL	**15**	

[1]Superscript™III Platinum™ One-step qRT-PCR kit; [2]See Guidelines

Dispense 15µL to each reaction well.

Add 5µL of template (extracted RNA, controls or NTC [nuclease-free water]).

Total reaction volume is 20µL

Amplification

Step 4.

RT-PCR

50°C	5min	
95°C	2min	
95°C	3s	40X
60°C	30s*	

*Florescence acquisition step

Result analysis

Step 5.

The definition used for a satisfactory positive result from a real-time fluorogenic PCR should include each of the following:

1. A **sigmoidal curve** – the trace travels horizontally, curves upward, continues in an exponential rise and followed by a curve towards a horizontal plateau phase
2. A **suitable level of fluorescence** intensity as measured in comparison to a positive control (y-axis)
3. A defined threshold (C_T) value which the fluorescent curve has clearly exceeded (Fig.1 arrow) and which sits early in the log-linear phase and is <40 cycles
4. A flat or non-sigmoidal curve or a curve that crosses the threshold with a C_T value >40 cycles is considered a negative result
5. NTCs should not produce a curve

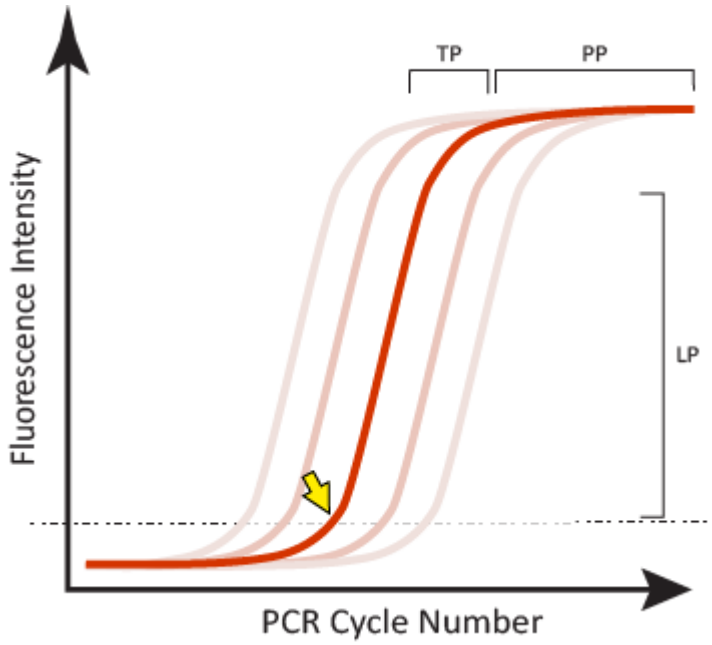

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary) threshold level as shown in this normalized linear scale depiction. LP-log-linear phase of signal generated during the exponential part of the PCR amplification; TP-a slowing of the amplification and accompanying fluorescence signal marks the transition phase; PP-the plateau phase is reached when there is little or no increase in fluorescent signal despite continued cycling.