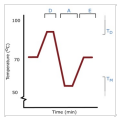

Enterovirus (EV) A71 real-time RT-PCR (EV-A71-TM2018)

Version 2

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[1]Public Health Virology, Forensic and Scientific Services

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ABSTRACT

This protocol aims to amplify enterovirus (EV) A71 viruses but not other viruses.
This protocol was designed by us.
The oligonucleotides target the 5'UTR noncoding region. This is a qualitative test for investigating EV-A71 infection of humans.
The test has identified both historical EV-A71 strains and contemporary strains and has confirmed these using subgenomic sequencing of partial 5'UTR-VP2 and partial VP1 sequencing. Culture of the virus is not required as this assay is capable of detecting EV-A71 direct from extracted clinical samples.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

STEPS MATERIALS

NAME	CATALOG #	VENDOR
SuperScript™ III Platinum™ One-Step qRT-PCR Kit	11732088	Life Technologies

BEFORE STARTING

If using a different brand or model of real-time thermocycler, check the concentration of ROX is adequate.

Method assumes the user is familiar with the thermocycler and software used to run the protocol and with PCR in general.

Oligonucleotide sequences

1

Name	Sequence 5'-3'
EVA71-VP4-For1	TAYTAYAAAGAYTCBTATGCYG
EVA71-VP4-Rev1	CCTTRACAGGRTTWGCRAACTT
EVA71-VP4-Rev2	CTTTRACAGGRTTWGCAAATTT
EVA71-VP4-Rev3	CCTTCACAGGGTTCGCAAACTT
EVA71-VP4-P1	FAM - ACAGCVGGCAAGCAGAGYCTCAA - BHQ1
EVA71-VP4-P2	FAM - ACAGCRGGYAAACAGAGYCTCAA - BHQ1
EVA71-VP4-P3	FAM - ACTGCTGGAAAGCAAAGTCTCAA - BHQ1

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The design philosophies.

- to ensure our assay detects old and new variants of EV-D68
- to reduce the total number of degenerate positions in any single primer

Reagents

2

SuperScript™ III Platinum™ One-Step qRT-PCR Kit
by Life Technologies
Catalog #: 11732088

Reaction set-up

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

- Prepare sufficient mix for the number of reactions.
- Include a suitable 'dead volume' as necessary if using a robotic dispenser.

Reagent	Volume (µl) x1	Final reaction concentration
Nuclease-free water	2.91	N/A
EVA71-VP4-For1 200pmol/µl	0.09	900nM
EVA71-VP4-Rev1 200pmol/µl	0.09	900nM
EVA71-VP4-Rev2 200pmol/µl	0.09	900nM
EVA71-VP4-Rev2 200pmol/µl	0.09	
EVA71-VP4-P1 100pmol/µl	0.03	150nM
EVA71-VP4-P2 100pmol/µl	0.03	150nM
EVA71-VP4-P3 100pmol/µl	0.03	150nM
MgSO4 (50mM)	1.2	6mM
2X Reaction Mix[1]	10	1X
SuperScript® III/Platinum® *Taq* Mix[1]	0.4	1X
ROX Reference Dye (25µM)	0.04	50nM
Template	5	N/A
TOTAL	20	

1-Superscript™ III Platinum™ One-step qRT-PCR kit

- Dispense 15µL to each reaction vessel.
- Add 5µL of template (extracted RNA, controls or no-template control [NTC; nuclease-free water]).
- Total reaction volume is 20µL

Amplification

4

50°C	5min	1X
95°C	2min	1X
95°C	3sec	|40X
60°C	30sec[1]	|

1-Fluorescence acquisition step

Result Analysis

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

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

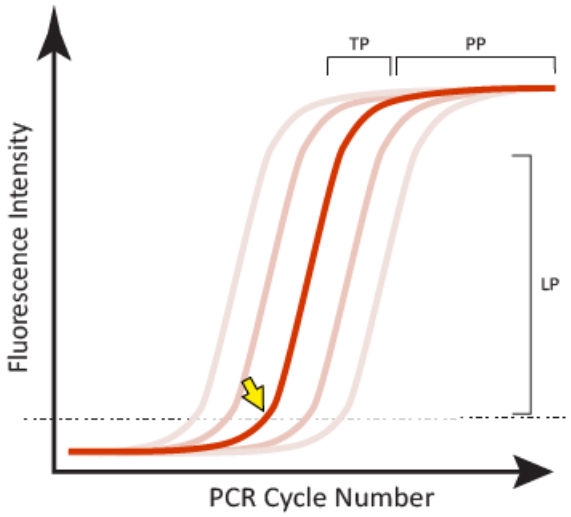

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary) 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.

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