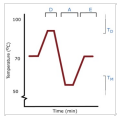

Enterovirus (EV) D68 TaqMan 2018 (EV-D68-TM2018)

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ABSTRACT

This protocol aims to amplify enterovirus (EV) D68 viruses and not other viruses.
This protocol is modified from a previously published method cited below. Details of the modification are included in the method.
The oligonucleotides target the 5'UTR noncoding region. This is a qualitative test for investigating EV-D68 infection of humans.
The test has identified both the original EV-D68 strains and contemporary strains which has been confirmed by 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-D68 direct from extracted clinical samples.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

This assay was modified from published version: The emergence of enterovirus D68 in a Dutch University Medical Center and the necessity for routinely screening for respiratory viruses. J Clin Virol. 2015 Jan;62:1-5. doi: 10.1016/j.jcv.2014.11.011. Epub 2014 Nov 15. https://www.ncbi.nlm.nih.gov/pubmed/25542461

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'
EV-D68-For1	TGTTYCCACGGTTGAAAAYAA
EV-D68-For2	TTCCCACGGTTGAAARYRAC
EV-D68-Rev	CAAGCTACACACGGGTTAGT
EV-D68-FAM-TM2018	FAM - CCGTTAWCCGCTATAGTACTTCGAGAAACC - BHQ1

*Modified from publication: Poelman R, Schölvinck EH, Borger R, Niesters HG, van Leer-Buter C.The emergence of enterovirus D68 in a Dutch University Medical Center and the necessity for routinely screening for respiratory viruses. J Clin Virol. 2015 Jan;62:1-5. doi: 10.1016/j.jcv.2014.11.011. Epub 2014 Nov 15.

MODIFICATIONS TO THE PUBLISHED ASSAY:

1. Two modified forward primers, targeting the same region as the original assays single primer, included different configurations of degenerate bases
2. An entirely new reverse primer design and location
3. One new FAM-labelled, degenerate, extended exonuclease or "TaqMan" oligoprobe replacing the 2x original assay's VIC-labelled TaqMan-MGB oligoprobes
4. Conditions, concentrations and reagents used all differ from those originally published
5. Addition of in vitro transcribed synthetic template oligonucleotide controls (PRIMER and PROBE controls)

Some of the design philosophies behind these modifications included ensuring that our assay could detect old and new variants of EV-D68 and reducing 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 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.

Reagent	Volume (µl) x1	Final reaction concentration
Nuclease-free water	4.28	N/A
EV-D68-For1 200pmol/µl	0.09	900nM
EV-D68-For2 200pmol/µl	0.09	900nM
EV-D68-Rev	0.09	900nM
EV-D68-FAM-TM2018 100pmol/µl	0.01	50nM
2X Reaction Mix[1]	10	1X
SuperScript® III/Platinum® *Taq* Mix[1]	0.4	1X
ROX Reference Dye (25µM)	0.04	0.05µM
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:

- 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

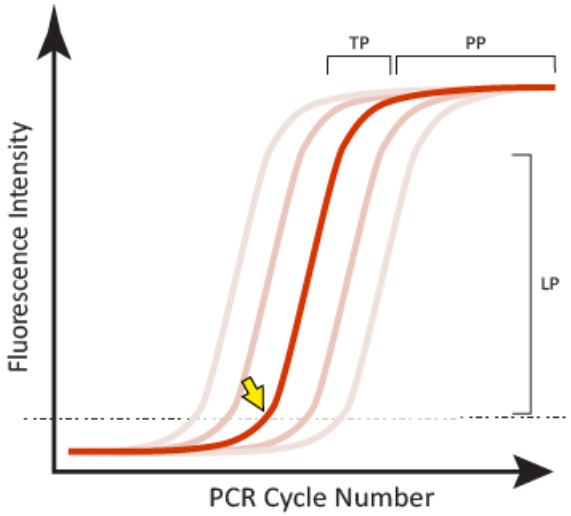

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary)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