Ross River virus E2 TaqMan 2017 (RRV-E2-TM2017)

Ian Mackay,Alyssa Pyke,Judy Northill

Abstract

The protocol aims explicitly to amplify RRV viruses and not other viruses.
Alyssa Pyke designed the original assay which was published in 2011 (see below). Subsequently, Alyssa Pyke modified the reverse primer, and it is this most recent, revised version, described here.
The assay targets the E2 gene region and is designed as a qualitative test for investigating RRV infection of humans and arthropods.

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Before start

- 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.

Protocol

Oligonucleotide sequences

Step 1.

Name	Sequence 5'-3'
RRV E2 F	ACGGAAGAAGGGATTGAGTACCA
RRV E2 R3*	TCGTCAGTTGCGCCCA**Y**A
RRV E2 FAM	FAM - CAACAACCCGCCGGTCCGC - BHQ1

*Modified after original publication (Hall RA, Prow NA, Pyke AT. Ross River virus. In: Liu, D ed. Molecular Detection of Human Viral Pathogens. Boca Raton, FL: CRC Press, 2011:349-359)

Reagents

Step 2.

READAGENTS

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	Volume (µl) x1	Final reaction concentration
Nuclease-free water	4.35	N/A
RRV E2 F 200pmol/µl	0.09	900nM
RRV E2 R3 200pmol/µl	0.09	900nM
BFV-FAM 100pmol/µl	0.03	150nM
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 well.
- Add 5µL of template (extracted RNA, controls or NTC [nuclease-free water]).
- Total reaction volume is 20µL

Amplification

Step 4.

CYCLING CONDITIONS

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

[1]Fluorescence 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), 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 >40 cycles is considered a negative result.
5. NTCs should not produce a curve

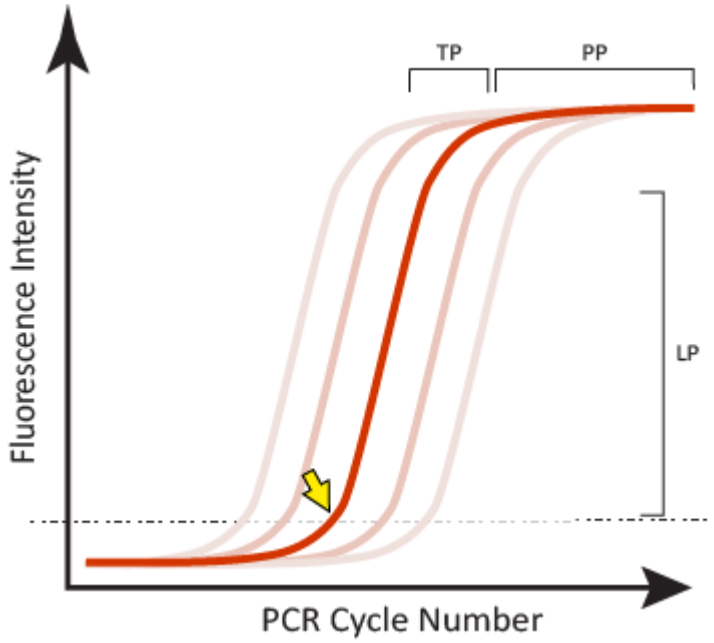

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary)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