Japanese encephalitis virus real-time RT-PCR Version 3

Judy Northill,Mitchell Finger,Michael Lyon,Ian Mackay

Abstract

A real-time RT-PCR using an MGB probe, this assay detects Japanese encephalitis virus (JEV) from human and mosquito samples.

The assay targets the 3'UTR region of known JEV strains.

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Guidelines

The concentration of ROX in this method is for the ABI7500 real-time thermocycler. It is not necessary for a Rotor-gene, however we run the assay with it in the recipe on a Rotor-gene machine. Concentration should be adjusted if using a different machine. Check your manufacturers' manual for guidance.

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’
JEVMGBTAQ_For_10486	GTGCTGYCTGCGTCTCAGT
JEVMGB-Rev2017	GAGACGGTTYTGAGGGCTTTC
JEVMGB-PROBE_10514	6FAM- ACTGGGTTAACAAATCTGACA-MGB

Reagents

Step 2.

REAGENTS

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

Technologies

Reaction Set-up

Step 3.

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

Prepare sufficient for number of reactions plus a 'dead volume' usually 2 extra. Adjust as necessary if using a robotic dispenser.

MIX PREPARATION

Reagent	Vol (µL) X1	Final reaction concentration
Nuclease-free water	4.43	
JEVMGBTAQ_For_10486 (200pmol/µL)	0.07	700nM
JEVMGB-Rev2017 (200pmol/µL)	0.05	500nM
JEVMGB-Probe_10514 (100pmol/µL)	0.01	50nM
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

NOTES

Version 3: Concentration of the oligonucleotides has been modified and validated.

Amplification

Step 4.

RT-PCR

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

*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) 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

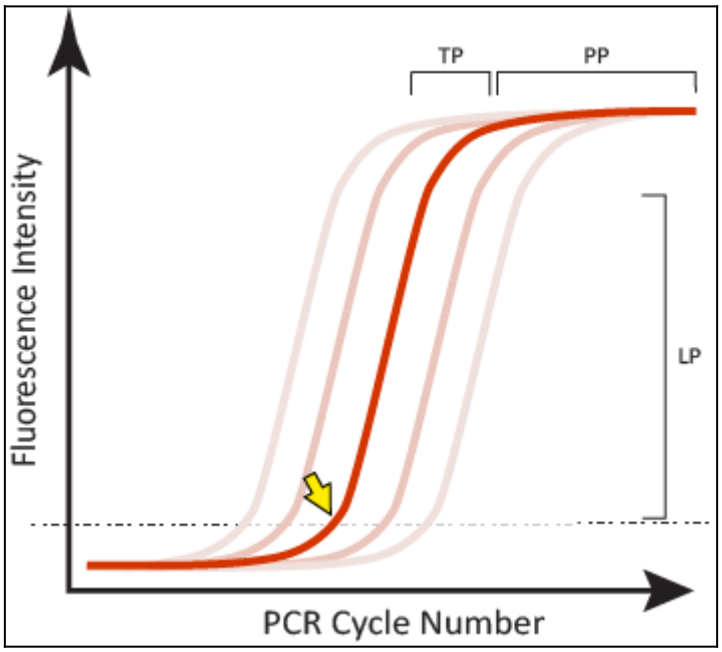

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary)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