Orientia tsutsugamushi real-time PCR

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Abstract

A real-time PCR to detect Orientia tsutsugamushi DNA. This method has been adapted from a publication by Jiang et al 2004, and the oligonucleotides have been modified and a different PCR kit used.

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Guidelines

- 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.

Materials

SensiFAST™ Probe Lo-ROX Kit BIO-84002 by Bioline

Protocol

Oligonucleotide sequences

Step 1.

Name	5'-3'
OtsuFP630	AACTGATTTTATTCAAACTAATGCTGCT
OtsuRP747	TATGCCTGAGTAAGATACRTGAATRGAATT
Ot2017-P	6FAM-TGGTGGVCCGATGTTTAATCTTGAAGGA-TAMRA

Reaction set-up

Step 2.

- Assay has been used on both a Rotor-Gene 6000 / Rotor-Gene Q 5-plex using 100-place rotor discs.
- Total reaction volume is 20µL.
- Prepare sufficient for number of reaction plus a 'dead volume' usually 2 extra. Adjust as necessary if using a robotic dispenser.

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Reagent	Vol (µL) x1	Final reaction concentration
Nuclease free water	4.85	-
OtsuFP630 200pmol/µl	0.07	700nM
OtsuRP747 200pmol/µl	0.05	500nM
Ot2017-P 100pmol/µl	0.03	150nM
SensiFAST probe Lo-ROX mix (2X)[1]	10	1X
TOTAL VOLUME	**15**	

[1] SensiFAST probe Lo-ROX mix 2X, Bioline, BIO-84020

Dispense 15µL to each reaction well.
Add 5µL of template, extracted DNA, controls or NTC (nuclease-free water).
Total reaction volume is 20µL

Amplification

Step 3.

The assay has been optimised and validated for the Rotor-Gene 6000 and Rotor-Gene Q thermocyclers.

PCR

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

*Florescence acquisition step

Result Analysis

Step 4.

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

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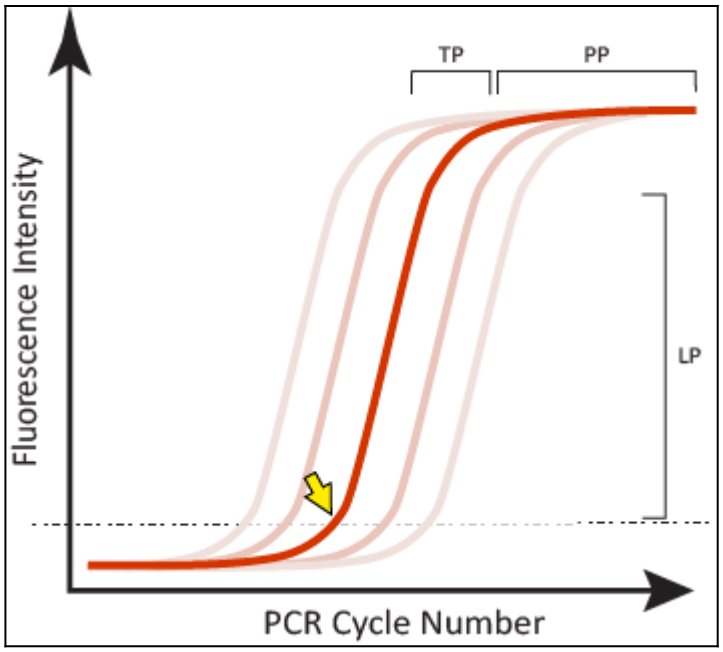

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary)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