Parechovirus real-time RT-PCR Version 2

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Abstract

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Protocol

Oligonucleotide sequences...

Step 1.

AN345_panHPeV/LV (sense primer)

GTAACASWWGCCTCTGGGSCCAAAAG

AN344_panHPeV/LV (antisense probe)

GGCCCCWGRTCAGATCCAYAGT

AN257_HPeV/LV

FAM-CCTRYGGGTACCTYCWGGGCATCCTTC-BHQ1

Reaction setup...

Step 2.

Below is the reaction setup for a single RT-PCR reaction.

Ideally, this work is conduct in a laboratory separate to any space used to perform PCR, molecular cloning or the analysis or high concentration DNA.

This volume has been used in 0.1-0.2ml tubes or various other connected tube configurations such as 100-place rings.

Multiply this according to the number of reactions you will need, remembering to include a positive control and at least two non-template controls (NTCs)

You may also need to allow some extra volume, depending on the method used to pipette mix into tubes for the run. For example, some robot-loaded tubes can require two reaction 'dead volumes'.

Reagent (stock concentration)	***Vol (µL) / reaction***	***Final concentration***
Nuclease free water	4.47	N/A
AN345_panHPeV/LV (200pmol/ul)	0.03	300nM
AN344_panHPeV/LV (200pmol/ul)	0.03	300nM
AN257_HPeV/LV FAM-BHQ1 (100pmol/ul)	0.03	150nM
2X Reaction Mix[1]	10	1X
Rox Reference Dye 25mM[1]	0.04	50nM
SuperScript® III/Platinum® *Taq* Mix[1]	0.4	1X
Template extract RNA	5	N/A
Final volume	20µl	

[1]SuperScript® III Platinum® One-Step qRT-PCR Kit, Cat No. 11732088

Amplification...

Step 3.

This assay has been optimized and validated for use with a Rotor-Gene 6000 or Rotor-Gene Q thermal cycler.

The cycling conditions are as follows:

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

*Florescence acquisition step

Result calling...

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 controls using (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
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

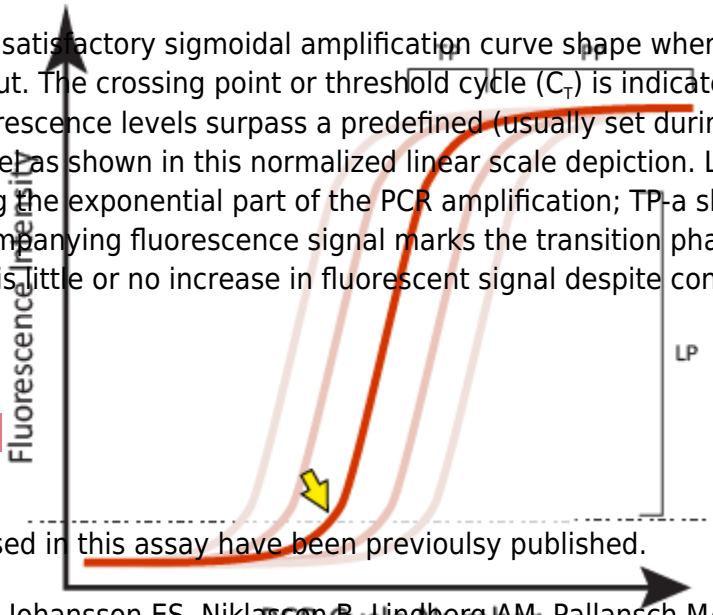

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary)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

References...

Step 5.

The oligonucleotides used in this assay have been previoulsy published.

1. Nix WA, Maher K, Johansson ES, Niklasson B, Lindberg AM, Pallansch MA, Oberste MS. Detection of all known parechoviruses by real-time PCR. J Clin Microbiol.46(8):2519-24.