Human Parechovirus A real-time RT-PCR ["Nix assay"; 2017-] Version 4

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Abstract

This version is based on a recipe (see forked version) my team and I used this assay between 2008-2015; we dubbed it the 'Nix assay'. It targets the 5'UTR and employs quite degenerate oligos.

In silico sequence alignments indicated the oligonucleotides could theoretically detect aft least HPeV 1-7, 17 and 18. Further validation of this recipe is ongoing.

However during a period of assay comparison, another assay (see link below), the 'Benschop assay' (J.Clin.Virol. 2008. 41(2):69-74), was found to produce more sigmoidal and higher curves and 1-5 cycle improvements to C_T values when compared among the sample sampe extract set.

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Guidelines

- This protocol assumes the user is familiar with working in a laboratory, with PCR, the thermocycler and software used to run it
- This protocol should be re-evaluated if being used with different reagents, if the oligonucleotide sequences are changed or if the cycling conditions are altered

Protocol

Oligonucleotides...

Step 1.

Name	5'-3' oligonucleotide sequence
AN345_panHPeV/LV (sense primer)	GTAACASWWGCCTCTGGGSCCAAAAG
AN344_panHPeV/LV (antisense probe)	GGCCCCWGRTCAGATCCAYAGT
AN257_HPeV/LV (probe)	FAM-CCTRYGGGTACCTYCWGGGCATCCTTC-BHQ1

Reagents

Step 2.

REAGENTS

Reaction setup...

Step 3.

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 4.

This assay has been optimized 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 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, curves and reaches 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
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. No template contorls (NTCs; water instead of specimen extract) should not produce a curve

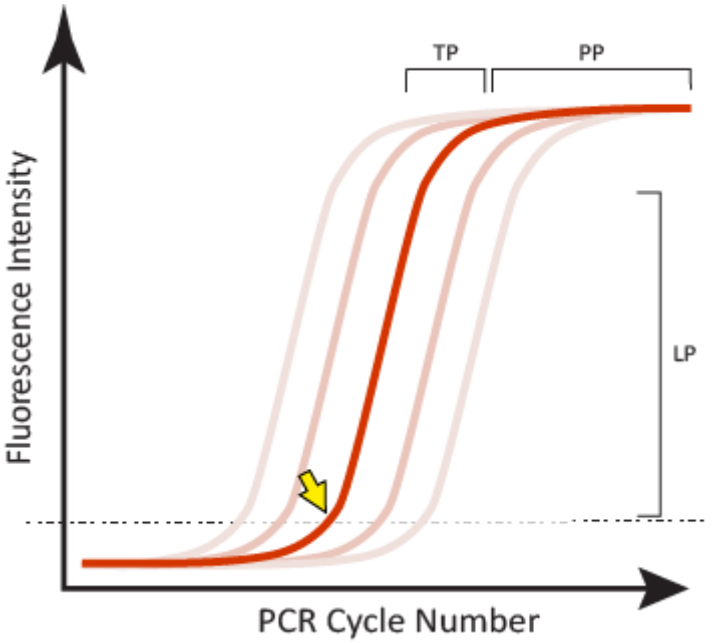

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary)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