Human Parechovirus A conventional nested genotyping RT-PCR ["Harvala assay"; 2008-2015]

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Abstract

I and my team used this assay between 2008-2015; we dubbed it the "Harvala assay". It produces an amplicon that includes the 3' end of VP3 and the 5' end of VP1, spanning the junction.

In silico sequence alignments indicated the highly degenerate oligonucleotides could theoretically detect af least HPeV 1-7, 17 and 18.

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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.

Round	Name	5'-3' oligonucleotide sequence
Round 1. RT-PCR	HPeV_VP3/1_OS	GAYAATGCYATMTAYCAWATYTGTGA
Round 1. RT-PCR	HPeV_VP3/1_OAS	ACWGTRAARATRTCHACATTSATDG
Round 2. nPCR	HPeV_VP3/1_IS	TTYTCMACHTGGATGMGGAARAC
Round 2. nPCR	HPeV_VP3/1_IAS	DGGYCCATCATCYTGWGCTGA

OS-outer sense; OAS-outer antisense; IS-inner sense; IAS-inner antisense

Reagents...

Step 2.

REAGENTS

SensiFAST Probe no ROX one-step kit BIO-76005 by Bioline

MyTaq HS DNA Polymerase BIO-21113 by Bioline

Reaction setup...

Step 3.

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

Ideally, this work is conducted 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'.

Round 1: RT-PCR

Reagent (stock concentration)	***Vol (µL) / reaction***	***Final concentration***
Nuclease free water	7.28	
AN345_panHPeV/LV (200pmol/ul [200uM])	0.06	600nM
AN344_panHPeV/LV (200pmol/ul [200uM])	0.06	600nM
SensiFast OneStep Mix(2x)	10	1X
RNase inhibitor	0.4	
RT/Taq (6U/mL)	0.2	1X
Template extract RNA	2	
Final volume	20µl	

Round 2: nPCR

Reagent (stock concentration)	*Vol (µL) / reaction*	*Final concentration*
Nuclease free water	12.424	
AN345_panHPeV/LV (200pmol/ul [200uM])	0.038	380nM
AN344_panHPeV/LV (200pmol/ul [200uM])	0.038	380nM
MyTaq Reaction Buffer (5X)	4	1X
MgCl2 (25mM)	1.4	
MyTaq HS DNA Polymerase (5U/uL)	0.1	1X
Round 1 amplicon	2	
Final volume	20µl	

Cycling conditions...

Step 4.

This assay has been optimized and validated for use with a RotorGene 6000 or RotorGene Q thermal cyclers.

The cycling conditions for the one-step RT-PCR and the nested PCR (nPCR) are as follow:

Round 1: RT-PCR		
45°C	20min	
94°C	2min	
95°C	30s	
60°C	30s	40X
72°C	105s	
72°C	7min	
4°C	∞	

Round 2: nPCR		
94°C	1min	
94°C	30s	
50°C	30s	40X
72°C	105s	
72°C	7min	
4°C	∞	

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

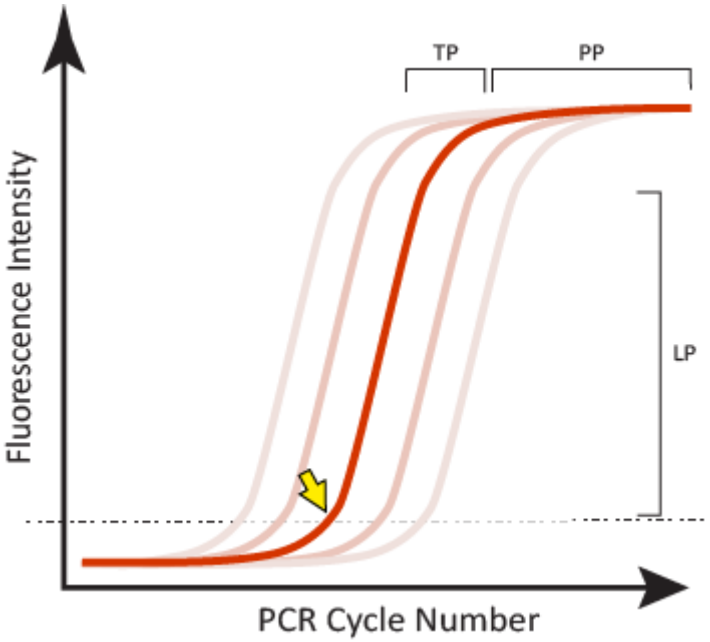

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary)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