Human Parechovirus A conventional nested genotyping RT-PCR ["Harvala assay"; 2008-2015] Version 4

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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, agarose gel electrophoresis, personal protective equipment, and use of a thermocycler
- 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

Nucleospin Extract II 740609.5 by Macherey and Nagel

ExoSAP-IT™ PCR Product Cleanup Reagent 78201.1.ML by Thermo Fisher Scientific Australia

Reaction setup...

Step 3.

Below is the reaction setup for a single conventional 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 0.2ml flip-top tubes.

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).

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 for use with a conventional block thermal cycler.

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.

A positive result is determined by a suitably sized band on an agarose gel after electrophoresis.

Run 2-5ul of both reactions on a 1.5% agarose gel after Round 2 is complete. Only the second round amplicon needs to be sequenced, but if the viral load is high enough, there may be a useful, larger band from Round 1 which can be sequenced instead.

Remaining amplicon is cleaned up following the manufacturer's instructions using either Nucleospin II columns or ExoSAP-IT reagent.