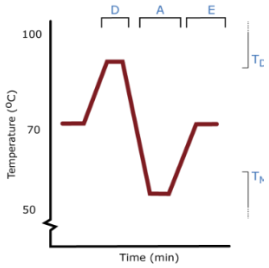
Nov 11, 2017 Version 3

Human Parechovirus A real-time RT-PCR ["Nix assay"; 2008-2015] V.3



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Manuscript citation:
The oligonucleotides used in this assay have been previoulsy published.
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):2519-2524.

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Protocol status: Working
We used this protocol in our group and it worked.

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Keywords: parechovirus, respiratory virus, RT-PCR, real-time PCR, rtPCR , human parechovirus, assay comparison, nix assay, period of assay comparison, benschop assay, olignucleotide, assay, pcr, least hpev, cycle improvements to ct value, aft least hpev, silico sequence alignment

Abstract

I and my team 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 olignucleotides could theoretically detect aft least HPeV 1-7, 17 and 18. 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.

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

Materials

STEP MATERIALS

SuperScript™ III Platinum™ One-Step qRT-PCR Kit **Life Technologies Catalog #**11732088

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Protocol materials

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Troubleshooting

Oligonucleotides...

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

2

SuperScript™ III Platinum™ One-Step qRT-PCR Kit **Life Technologies Catalog #**11732088

Reaction setup...

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...

4 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 95°C	5min 2min	
	95°C 60°C	3s 30s*	40X

*Florescence acquisition step

Result calling...

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

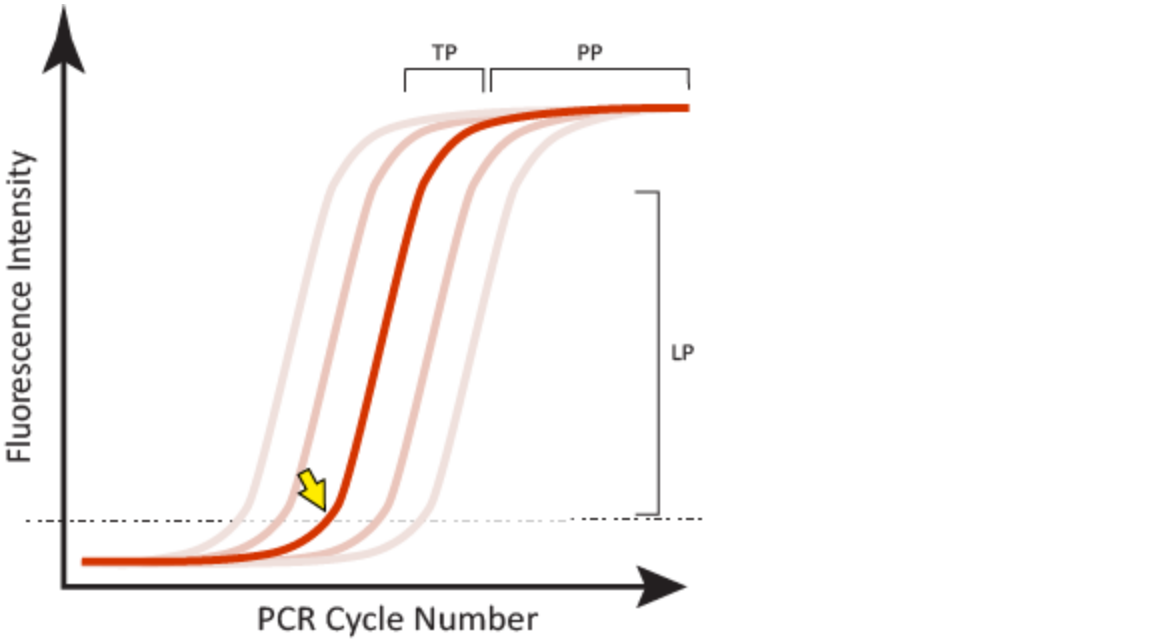

Figure 1. Examples of satisfactory sigmoidal amplification curve shape when considering an assay's fluorescent signal output. The crossing point or threshold cycle (C_T) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary)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