

PrimerDesign™ Ltd

Quantification of Human *Enterovirus* Genomes

5'non coding region

For general laboratory and research use only

Standard Kit





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Introduction to Human Enterovirus

The *Enterovirus* genus is a member of the *Picornaviridae* family and consists of 8 single-stranded RNA virus species, of these 8 species the following 5 infect humans: Poliovirus; Human Enterovirus A (HEV-A); HEV-B; HEV-C and HEV-D. These viruses are non-enveloped with an icosahedrally symmetric capsid of approximately 27-30nm in diameter containing 12 capsomers. The non-segmented, positive sense RNA genome is up to 8.5kbases in length with an untranslated region at the C-terminus which is important in translation and virulence.

Poliovirus is the causative agent of poliomyelitis, a disease of the central nervous system. Infection with Poliovirus is via the fecal-oral route with viral replication initially occurring in the cells of the digestive tract. After replication, viral particles are shed in the faeces of infected individuals resulting in transmission to a new host. From the digestive tract the virus can enter the blood stream causing viremia which is usually asymptomatic, or can infect sites in the immune system or muscle where secondary viremia occurs after sustained viral replication causing fever. When the virus enters the central nervous system it targets motor neurons, where massive viral replication causes cell death resulting in paralysis.

Other viruses within the *Enterovirus* genus include Coxsackie viruses, Enteroviruses and Echoviruses.

Coxsackie viruses have two subtypes, A and B. Coxsackie A viruses are a group of 23 viruses that can cause Hand, Foot and Mouth Disease (HFMD), as well as conjunctivitis, aseptic meningitis or high fever and blisters in the mouth or throat, on the palms of the hands and soles of the feet. Coxsackie B viruses are a group of 6 viruses that can also cause aseptic meningitis as well as infectious myocarditis, infectious pericarditis and pleurodynia.

Enteroviruses can cause HFMD, a disease that usually affects small children and can be spread by contact with infected mucus, saliva or faeces. The disease presents with fever, headache, malaise, vomiting and diarrhea and has an incubation period of up to one week. There are no vaccines or antiviral drugs available to prevent this disease.

Echoviruses cause acute febrile illnesses in small children and can also cause aseptic meningitis. Echovirus infection is biased toward males and infection during the first two weeks of a babies life can cause a fatal disease that causes liver failure and myocarditis.

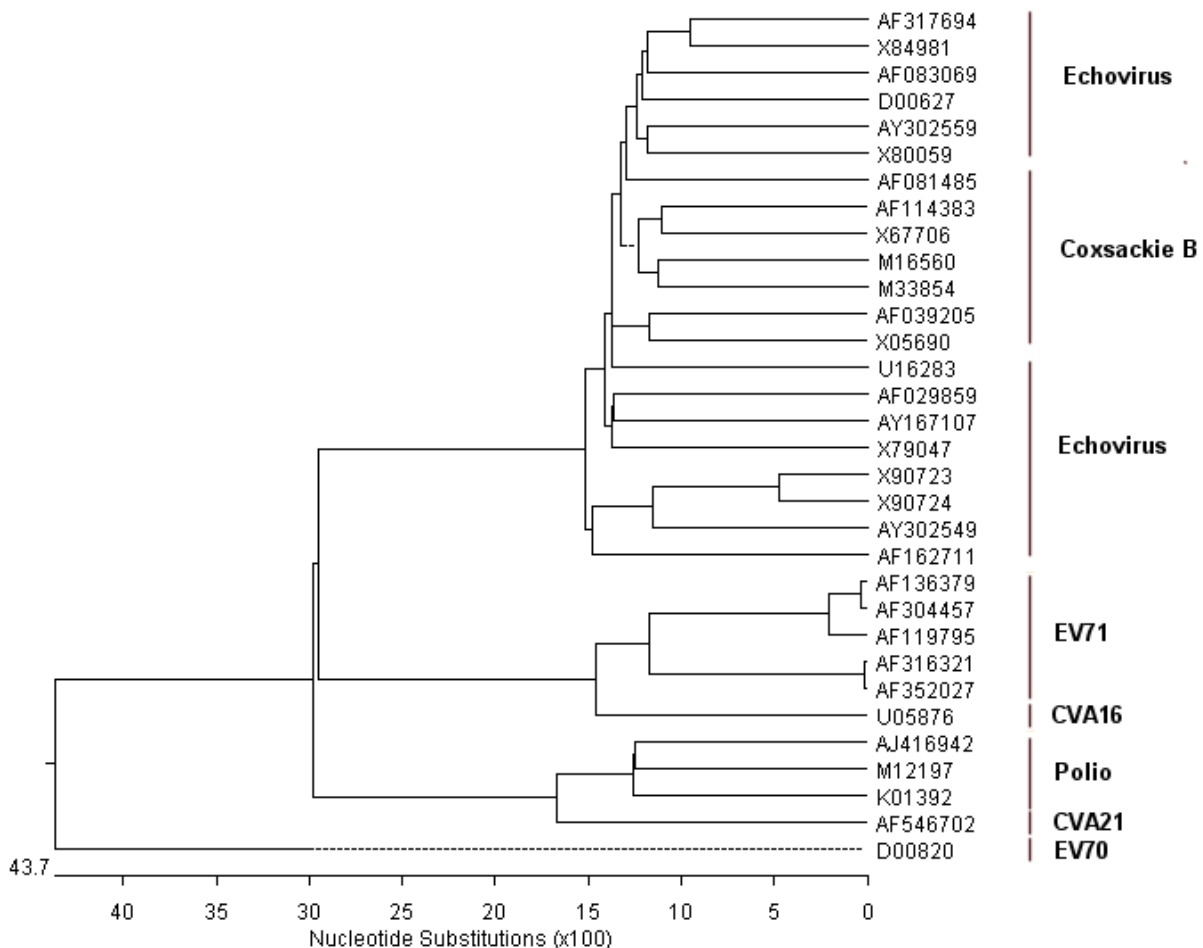
All of the viruses within this genus that infect humans do so via close contact with infected individuals, mainly via the fecal-oral route although some viruses have been shown to be transmitted by respiratory droplets.



Specificity

The *PrimerDesign™ Quantification Kit human enterovirus* genomes is designed for the *in vitro* quantification of all enterovirus isolates. The primers are based on the alignments of Uta Dierssen 2007 *et.al.* and therefore have very high homology with reference sequences from a wide variety of strains. The primers are predicted to detect all the sequences in the phylogenetic tree below (fig.1) and therefore have a very broad quantification profile. However, due to the inherent instability of RNA viral genomes, it is not possible to guarantee quantification of all clinical isolates.

Fig.1 Enterovirus strains detected





Kit Contents

- Pathogen specific primer/probe mix (150 reactions **BROWN**)
- Pathogen positive control template (for Standard curve **RED**)
- Pathogen RT primer mix (150 reactions **GREEN**)
- RNase/DNase free water

Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
- **Mastermix or Mastermix components**
This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x *Precision*[™] Mastermix.
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Thin walled 1.5 ml PCR reaction tubes**

Kit storage and stability

This kit is stable at room temperature but should be stored at -20°C on arrival. Once the lyophilized components have been re-suspended unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.



Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity. Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions PrimerDesign pathogen detection kits have very high priming efficiencies of >95% and can detect between 1×10^8 and 1×10^2 copies of target template.



Principles of the test

Real-time PCR

A pathogen specific primer and probe mix is provided and the target sequence can be detected through the **FAM** channel.

The primer and probe mix provided exploits the so-called TaqMan[®] principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

Positive control

For copy number determination, and as a positive control for the PCR set up, the kit contains positive control template. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in your particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment.

Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.



Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PrimerDesign recommend the application of 0.2 U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).



Bench side Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps.

1. Pulse-spin each tube in a centrifuge before opening.

This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. Reconstitute the kit components according to the table below

To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

| Component | Volume |
|--|--------|
| Pre-PCR box | |
| Pathogen Primer/Probe mix (BROWN) | 165 µl |
| Pathogen RT primer mix (GREEN) | 165 µl |
| Post PCR bottle | |
| Positive Control Template (RED) * | 500 µl |

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.



Reverse Transcription

The protocols below are for PrimerDesign *Precision*[™] Reverse Transcription kit. If you are using an alternative RT kit then follow the manufacturer's instruction.

1. For each RNA sample prepare a reaction mix according to the table below:

| Component | 1 reaction |
|---|-----------------------------|
| RNA template | X μ l |
| Pathogen RT primer mix (GREEN) | 1.0 μ l |
| dNTP mix 10mM of each (ORANGE) | 1.0 μ l |
| RNAse/DNAse free water (WHITE) | X μ l |
| Final volume | 10 μl |

2. Heat samples to 65°C for 5 minutes.

This can be achieved using a thermostatically controlled “hot block” or heated water bath. Do not heat the RNA to a higher temperature as this can lead to RNA degradation.

3. Immediately cool the tubes in an ice water bath.

It is important that the samples are transferred directly from 65°C to the ice. Do not allow the samples to cool prior to cooling on ice.

4. For each RNA sample prepare a reaction mix according to the table below:

| Component | 1 reaction |
|---------------------------------------|-----------------------------|
| MMLV 5Xbuffer (CLEAR) | 4.0 μ l |
| RNA/DNAse free water (WHITE) | 5.2 μ l |
| MMLV enzyme (CLEAR) | 0.8 μ l |
| Final volume | 10 μl |

5. Add 10 μ l of this mix to each of the samples on ice.

6. Apply a lid to each sample, mix tubes by briefly vortexing and incubate at 42°C for 60 minutes.

This can be achieved using a thermostatically controlled “hot-block” or heated water bath

7. Store cDNA samples at -20°C until use.



Real-time PCR detection

1. Prepare a reaction mix according to the tables below:

Include sufficient reactions for the standard curve wells (8 samples in duplicate) and also the negative control.

Pathogen detection mix

| Component | 1 reaction |
|--|--------------|
| 2X Precision™ Mastermix | 10 µl |
| Pathogen primer/probe mix (BROWN) | 1.0 µl |
| RNAse/DNAse Free water | 4.0 µl |
| Final volume | 15 µl |

2. Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.

3. Prepare sample cDNA templates for each of your samples (suggested concentration 5ng/µl) in RNAse/DNAse free water.

If the concentration of RNA that was used to make the cDNA is not known, then dilute your RT reaction mix 1:20 (10µl of sample DNA and 90µl of water)

4. Pipette 5µl of diluted cDNA template into each well, according to your experimental plate set up.

For negative control wells use 5µl of RNAse/DNAse free water. The final volume in each well is 20µl



5. Preparation of standard curve dilution series

- 1) Pipette 900µl of RNase/DNase free water into 7 tubes and label 2-8
- 2) Pipette 100µl of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 100µl from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

| Standard Curve | Copy Number |
|--|------------------------|
| Tube 1 Positive control (RED) | 2×10^7 per µl |
| Tube 2 | 2×10^6 per µl |
| Tube 3 | 2×10^5 per µl |
| Tube 4 | 2×10^4 per µl |
| Tube 5 | 2×10^3 per µl |
| Tube 6 | 2×10^2 per µl |
| Tube 7 | 20 per µl |
| Tube 8 | 2 per µl |

6. Pipette 5µl of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20µl



Amplification Protocol

Amplification conditions using PrimerDesign 2X Precision™ MasterMix.

| | Step | Time | Temp |
|-----------|------------------------------------|---------|------|
| | UNG treatment (if required) ** | 15 mins | 37°C |
| | Enzyme Activation (if required)*** | 10 mins | 95°C |
| 50 cycles | Denaturation | 10s | 95°C |
| | DATA COLLECTION* | 60s | 60°C |

* Fluorogenic data for the control DNA should be collected during this step through the FAM channel

** Required if your mastermix includes UNG to prevent PCR carryover contamination

*** Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.



Notices and disclaimers

During the warranty period PrimerDesign pathogen detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired.

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