PCRmax Ltd ™ qPCR test

Hand, Foot and Mouth Disease

polyprotein gene

150 tests

For general laboratory and research use only

Introduction to Hand, Foot and Mouth Disease

Hand, foot and mouth disease (HFMD) is a human syndrome caused by intestinal viruses Human enterovirus C and enterovirus 71 (EV-71). It is moderately contagious and is spread through direct contact with the mucus, saliva, or feces of an infected person.

Aside from HFMD, EV71 is sometimes associated with severe central nervous system diseases. It is a non-enveloped, spherical virion, about 30 nm in diameter with a capsid surrounding the naked RNA genome. The linear genome is single stranded sense RNA of 7.4 kb and is composed of a single ORF encoding a polyprotein.

Human enterovirus C belongs to the genus Enterovirus. The isometric capsid has a diameter of 28-30 nm and appears round. The genome is not segmented and contains a single molecule of linear positive-sense, single-stranded RNA consisting of 7400 nucleotides. They are also capable of causing causing herpangina and acute hemorrhagic conjunctivitis (AHC).

HFMD typically affects children under 10 years of age. Adults and older children with HFMD tend to develop a milder form of the illness compared with younger children. The incubation period is 3-5 days. The disease generally presents as a fever, malaise, sore mouth and development of a rash. Mouth lesions appear on the inside surfaces of the cheeks, gums and on the sides of the tongue. Raised pink spots that develop into blisters, which may persist for seven to ten days, can also occur as a rash, especially on the palms, fingers, soles and occasionally on the buttocks. Attachement of the virus to host receptors mediates endocytosis of the virus into the host cell. The capsid then undergoes a conformational change and releases VP4 that opens a pore in the host endosomal membrane and the viral genomic RNA penetrates into the host cell cytoplasm (the empty capsid remains intact). VPg is removed from the viral RNA, which is then translated into a processed polyprotein. Replication occurs in viral factories made of membrane vesicles derived from the ER. A dsRNA genome is synthesized from the genomic ssRNA(+) and packaged into preassembled capsids which are then released by cell lysis.

Most cases are passed on by coughing and sneezing which transmits the virus into the air and outbreaks are reported in nurseries and schools where children are in close proximity to each other. The virus is also spread by direct contact with nasal and throat secretions or faeces of the infected person. Infected individuals are often contagious several weeks after the symptoms have disappeared.

Specificity

The PCRmax qPCR Kit for Hand, Foot and Mouth Disease (HFMD) genomes is designed for the in vitro quantification of HFMD genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the HFMD genome.
The primers and probe sequences in this kit have 100% homology with a broad range of HFMD sequences based on a comprehensive bioinformatics analysis.

If you require further information, or have a specific question about the detection profile of this kit then please send an e.mail to help@pcrmax.com and our bioinformatics team will answer your question.

Kit Contents

- **Human enterovirus C primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **Human enterovirus 71 primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **Human enterovirus C positive control template (for Standard curve RED)**
- **Human enterovirus 71 positive control template (for Standard curve RED)**
- **Internal extraction control primer/probe mix (150 reactions BROWN)**
  VIC labelled as standard
- **Internal extraction control RNA (150 reactions BLUE)**
- **Endogenous control primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **HEV-C and EV-71/Internal extraction control/endogenous control RT primer mix (150 reactions GREEN)**
  Required for two step protocol only
- **RNAse/DNAse free water (WHITE)**
  for resuspension of primer/probe mixes and internal extraction control RNA
- **Template preparation buffer (YELLOW)**
  for resuspension of positive control templates and standard curve preparation

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

RNA extraction kit
This kit is designed to work well with all processes that yield high quality RNA with minimal PCR inhibitors.

Lyophlised 2x RT-qPCR MasterMix
This kit is designed to be compatible with all commercially available OneStep Mastermixes that run with standard cycling conditions.

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 -20ºC on arrival. Once the lyophilised components have been resuspended they should not be exposed to temperatures above -20ºC for longer than 30 minutes and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

If a standard curve dilution series is prepared this can be stored frozen for an extended period. If you see any degradation in this serial dilution a fresh standard curve can be prepared from the positive control.

PCRmax does not recommend using the kit after the expiry date stated on the pack.

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 (An internal PCR control is supplied to test for non specific PCR inhibitors). 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 PCRmax HFMD detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

Notices and disclaimers

Trademarks

Principles of the test

The kit contains two sets of primers and probes. Each of the polyprotein gene primer and probe sets are designed to detect the two pathogens known to be the causative agents of Hand, Foot and Mouth disease, namely Human enterovirus C and Human enterovirus 71.

Real-time PCR

A HFMD specific primer and probe mix is provided and this 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 HFMD cDNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 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 a positive control template. This can be used to generate a standard curve of HFMD copy number / Cq value. Alternatively the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target HFMD gene worked properly in that 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. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To validate any positive findings 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.

Internal RNA extraction control

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This control RNA is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration.

A separate RT primer mix and a real-time PCR primer/probe mix are supplied with this kit to detect the exogenous RNA using real-time PCR. The PCR primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control cDNA does not interfere with detection of the HFMD target cDNA even when present at low copy number. The Internal control is detected through the VIC channel and gives a Cq value of 28+/-3 depending on the level of sample dilution.

Endogenous control

To confirm extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is NOT therefore possible to perform a multiplex with the HFMD primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Carry-over prevention using UNG (unsuitable for onestep procedure and optional for two step procedure)

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. PCRmax recommend the application of 0.2U 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.

Reconstitution 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 lab or 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 in the RNAse/DNAse free water supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
Pre-PCR pack	
HEV-C primer/probe mix (BROWN)	165 µl
EV-71 primer/probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
RT primer mix (GREEN)	165 µl
Endogenous control primer/probe mix (BROWN)	165 µl
Pre-PCR heat-sealed foil	
Internal extraction control RNA (BLUE	600 µl

3. **Reconstitute the positive control templates in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Post-PCR heat-sealed foil	
HEV-C Positive Control Template (RED) *	500 µl
EV-71 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.**

RNA extraction

The internal extraction control RNA can be added either to the RNA lysis/extraction buffer or to the RNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control RNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

1. **Add 4µl of the Internal extraction control RNA (BLUE) to each sample in RNA lysis/extraction buffer per sample.**

2. **Complete RNA extraction according to the manufacturers protocols.**

One Step qRT-PCR detection protocol

A one step approach combining the reverse transcription and amplification in a single closed tube is the preferred method.

For optimum performance and sensitivity.

All pipetting steps and experimental plate set up should be performed on ice. After the plate is poured proceed immediately to the One Step amplification protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artifacts that reduce the sensitivity of detection.

1. **For each RNA sample prepare a reaction mix according to the table below:**
   Include sufficient reactions for positive and negative controls.

Component	Volume
Lyophilised OneStep 2x RT-qPCR MasterMix	10 µl
HEV-C or EV-71 primer/probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	3 µl
Final Volume	**15 µl**

2. **For each RNA sample prepare an endogenous control reaction according to the table below (optional):**
   This control reaction will provide crucial information regarding the quality of the biological sample.

Component	Volume
Lyophilised OneStep 2x RT-qPCR MasterMix	10 µl
Endogenous control primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final Volume	**15 µl**

3. **Pipette 15µl of these mixes into each well according to your real-time PCR experimental plate set up.**

4. **Pipette 5µl of RNA 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. **If a standard curve is included for quantitative analysis prepare a reaction mix according to the table below:**

Component	Volume
Lyophilised OneStep 2x RT-qPCR MasterMix	10 µl
HEV-C or EV-71 primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final Volume	**15 µl**

6. **Preparation of standard curve dilution series.**

1) Pipette 90µl of template preparation buffer into 5 tubes and label 2-6
2) Pipette 10µl of Positive Control Template (RED) into tube 2
3) Vortex thoroughly
4) Change pipette tip and pipette 10 µl 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 x 10^5 per µl
Tube 2	2 x 10^4 per µl
Tube 3	2 x 10^3 per µl
Tube 4	2 x 10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

7. **Pipette 5µl of standard template into each well for the standard curve according to your plate set-up**
The final volume in each well is 20µl.

One Step Amplification Protocol

Amplification conditions using Lyophilised OneStep 2x RT-qPCR MasterMix.

	Step	Time	Temp
	Reverse Transcription	10 mins	55 °C
	Enzyme activation	2 mins	95 °C
Cycling x 50	Denaturation	10 secs	95 °C
	DATA COLLECTION *	60 secs	60 °C

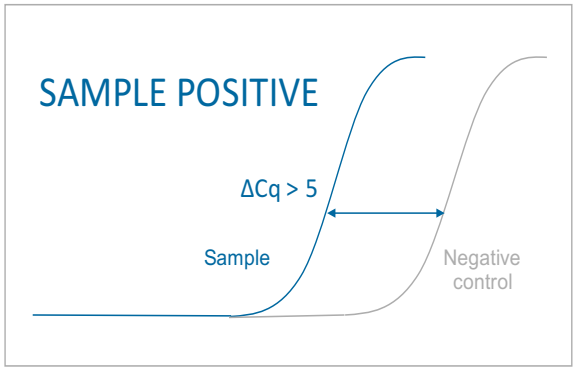
* Fluorogenic data should be collected during this step through the FAM and VIC channels

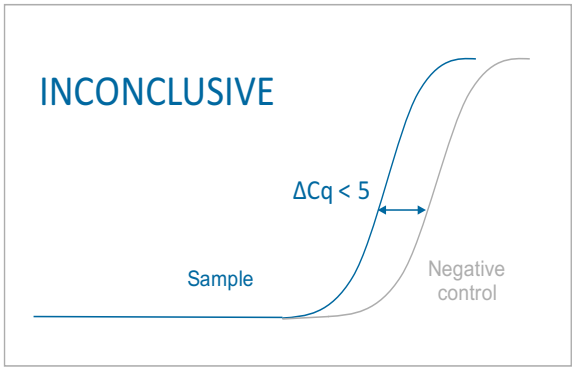
Interpretation of Results

Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	-	+	-	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may be due to poor sample extraction
-	+	+	-	**NEGATIVE RESULT**
+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+ / -	+	> 35	*
-	-	+	-	**SAMPLE PREPARATION FAILED**
+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:





If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.

If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Internal PCR control

The CT value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of RNA added to the RT and PCR reaction and the individual machine settings. CT values of 28±3 are within the normal range. When amplifying a HFMD sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous control

The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.