

Primerdesign™ Ltd

# Hand, Foot and Mouth Disease

polyprotein gene

genesig® Standard Kit

150 tests

G E N E S I G

Kits by Primerdesign

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 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. Attachment 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 Primerdesign genesig 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 a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences available at the time of design.

The dynamics of genetic variation means that new sequence information may become available after the initial design. Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

The Primerdesign genesig kit for Hand, Foot and Mouth Disease (HFMD) contains two primer/probe kits 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 HEV-C primers and probe sequences have 100% homology with a broad range of HEV-C sequences based on a comprehensive bioinformatics analysis. This kit will also detect Coxsackievirus which is associated with HFMD infection.

The EV-71 primers and probe sequences have 100% homology with a broad range of EV-71 sequences based on a comprehensive bioinformatics analysis. This kit will also detect Coxsackievirus and Echovirus. Both of which have been associated with HFMD infection.

In silico analysis predicts that this kit shows cross-reactivity with Human poliovirus 1 isolate 13688 and Swine vesicular disease.

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If you require further information or have a specific question about the detection profile of this kit then please send an email to [enquiry@primerdesign.co.uk](mailto:enquiry@primerdesign.co.uk) and our bioinformatics team will answer your question.

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# 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**)
- RNase/DNase free water (**WHITE**)  
for resuspension of primer/probe mixes
- 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

### Extraction kit

This kit is recommended for use with genesig Easy DNA/RNA Extraction kit. However, it is designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

### oasisig™ lyophilised OneStep or Precision®PLUS OneStep 2X RT-qPCR Master Mix

Contains complete OneStep RT-qPCR master mix

### 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 lyophilised components have been resuspended they should not be exposed to temperatures above -20°C for longer than 30 minutes at a time 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.

Primerdesign 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. 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 genesig HFMD detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

## Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period Primerdesign genesig 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. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

## Trademarks

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# 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 DNA/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 qPCR 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.

# Resuspension 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. Resuspend 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
<b>Pre-PCR pack</b>	
HEV-C primer/probe mix (BROWN)	165 µl
EV-71 primer/probe mix (BROWN)	165 µl

## 3. Resuspend 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
<b>Post-PCR heat-sealed foil</b>	
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.

# OneStep RT-qPCR detection protocol

## 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 OneStep 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
oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix	10 µl
HEV-C or EV-71 primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
<b>Final Volume</b>	<b>15 µl</b>

### 2. Pipette 15µl of this mix into each well according to your qPCR experimental plate set up.

### 3. 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.

### 4. If a standard curve is included for quantitative analysis prepare a reaction mix according to the table below:

Component	Volume
oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix	10 µl
HEV-C or EV-71 primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
<b>Final Volume</b>	<b>15 µl</b>

## 5. Preparation of standard curve dilution series

- 1) Pipette 90 $\mu$ l of template preparation buffer into 5 tubes and label 2-6
- 2) Pipette 10 $\mu$ l of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 10 $\mu$ l 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 ( <b>RED</b> )	$2 \times 10^5$ per $\mu$ l
Tube 2	$2 \times 10^4$ per $\mu$ l
Tube 3	$2 \times 10^3$ per $\mu$ l
Tube 4	$2 \times 10^2$ per $\mu$ l
Tube 5	20 per $\mu$ l
Tube 6	2 per $\mu$ l

## 6. Pipette 5 $\mu$ l of standard template into each well for the standard curve according to your plate set up

The final volume in each well is 20 $\mu$ l.

## OneStep RT-qPCR amplification protocol

Amplification conditions using oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix.

	Step	Time	Temp
	Reverse Transcription	10 min	55 °C
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	<b>DATA COLLECTION *</b>	60 s	60 °C

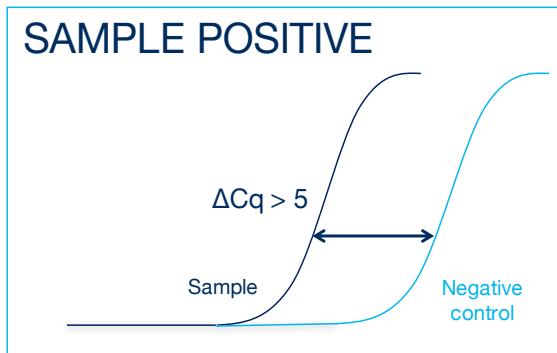
\* Fluorogenic data should be collected during this step through the FAM channel

# Interpretation of results

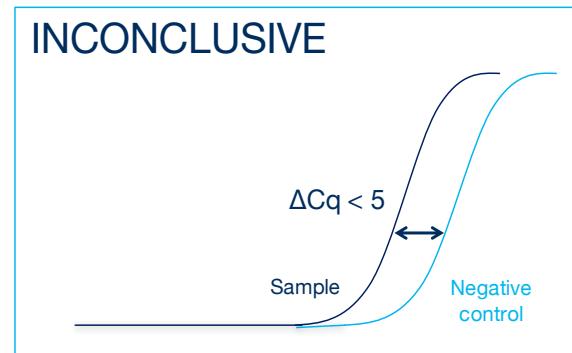
Target	Positive control	Negative control	Interpretation
+	+	-	<b>POSITIVE QUANTITATIVE RESULT</b> calculate copy number
-	+	-	<b>NEGATIVE RESULT</b>
+ / -	+	$\leq 35$	<b>EXPERIMENT FAILED</b> due to test contamination
+ / -	+	$> 35$	*
+ / -	-	+ / -	<b>EXPERIMENT FAILED</b>

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.