Primerdesign™ Ltd

R01008

genesig™PLEX Viral Meningitis Real-Time PCR Multiplex Kit

Kit version: 01

Enterovirus (5’non coding region)

HHV3 (IE62 gene)

HSV 1 and 2 (DNA polymerase (UL30) gene)

genesig™PLEX kit

100 tests

GENESIG

Kits by Primerdesign

Specificity of primers and probes last reviewed on:
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For general laboratory and research use only

Introduction

Enterovirus

The Enterovirus genus is a member of the Picornaviridae family and consists of eight single-stranded RNA virus species; of these eight species, the following five infect humans: Poliovirus; Human Enterovirus A (HEV-A); Human Enterovirus B (HEV-B); Human Enterovirus C (HEV-C) and Human Enterovirus D (HEV-D). These viruses are non-enveloped with an icosahedrally symmetric capsid of approximately 27-30 nm in diameter containing 12 capsomers. The non-segmented, positive-sense RNA genome is up to 8.5 kilobases 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 faecal-oral route, with viral replication initially occurring in the cells of the digestive tract.

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 six viruses that can also cause aseptic meningitis as well as infectious myocarditis, infectious pericarditis and pleurodynia.

Enteroviruses are typically found in the gastrointestinal tract and can spread through different routes, including faecal-oral transmission, respiratory droplets, and direct contact with contaminated surfaces. Once the viruses enter the bloodstream, they can travel to the meninges, the protective membranes surrounding the brain and spinal cord. The presence of enteroviruses in the meninges triggers an immune response, leading to inflammation and the characteristic symptoms of viral meningitis. Enteroviral meningitis often manifests with flu-like symptoms such as fever, headache, nausea, vomiting, and muscle aches. In some cases, patients may also experience a stiff neck, sensitivity to light (photophobia), and a rash.

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

HHV3

Herpes zoster, colloquially known as shingles, is the reactivation (from the general area of the spinal cord) of varicella zoster virus (VZV, primary infection of which leads to chickenpox), one of the Herpesviridae group, leading to a crop of painful blisters over the area of a dermatome. Shingles, or herpes zoster, is a neurological disease affecting the nervous system, with or without the appearance of a rash on the skin.

The causative agent for herpes zoster is varicella zoster virus (VZV). Most people are infected with this virus as a child, as it causes chickenpox. The body eliminates the virus from

the system, but it remains dormant in the ganglia adjacent to the spinal cord (called the dorsal root ganglion) or the ganglion semilunare (ganglion Gasseri) in the cranial base.

Generally, the immune system suppresses reactivation of the virus. In the elderly, whose immune response generally tends to deteriorate, as well as in those patients whose immune system is being suppressed; this process fails. The virus starts replicating in the nerve cells, and newly formed viruses are carried down the axons to the area of skin served by that ganglion (a dermatome). Here, the virus causes local inflammation in the skin, with the formation of blisters.

While VZV primarily affects the skin, it can also lead to complications involving the central nervous system, such as viral meningitis. VZV-associated meningitis typically occurs as a rare complication of primary VZV infection or reactivation of the virus. The virus can enter the bloodstream and reach the meninges, causing inflammation and subsequent symptoms of meningitis. VZV-related meningitis may present with symptoms like fever, headache, neck stiffness, and sensitivity to light. In addition to meningitis, VZV can also lead to more severe neurological complications, including encephalitis.

HSV-1&2

The herpes simplex virus (HSV) is a virus that manifests itself in two common viral infections (HSV1 and HSV 2), each marked by painful, watery blisters on the skin, mucous membranes (such as the mouth or lips) or on the genitals. The double-stranded DNA virus belongs to the family Herpesviridae, subfamily Alpha Herpesviridae and has a circular genome of 155 kb. HSV-1 and HSV-2 share the same overall structure, as well as very high similarity in most genes.

Both HSV-1 and HSV-2 have two unique regions, named UL and US surrounded and defined by three sets of inverted repeats, named LTRa, LTRb and LTRc. The UL and US regions are found in both forward and inverted directions and wild type HSV occurs equally in all four possible arrangements (isomers) of the UL and US regions. The coordinate system is defined to begin at the first nucleotide of the shortest terminal repeat, LTRa, followed by LTRb, and the long unique region, UL.

The disease is contagious, particularly during an outbreak, and is currently incurable. HSV-1 and HSV-2 are closely related but differ in epidemiology. HSV-1 is traditionally associated with facial disease while HSV-2 is traditionally associated with genital disease. HSV is transmitted by close personal contact, and infection occurs via inoculation of virus into susceptible mucosal surfaces (eg, oropharynx, cervix, conjunctiva) or through small cracks in the skin. The virus is readily inactivated at room temperature and by drying; hence, aerosol and fomitic spread are rare.

The clinical course of herpes simplex infection depends on the age and immune status of the host, the anatomic site of involvement, and the antigenic virus type. Primary herpes simplex virus HSV–1 and HSV-2 infections are accompanied by systemic signs, longer duration of symptoms, and higher rate of complications. Recurrent episodes are milder and shorter.

HSV-1&2 viruses can also cause viral meningitis. When HSV infects the central nervous system, it can lead to meningitis, resulting in inflammation of the meninges. HSV-associated meningitis often occurs during primary infection or viral reactivation and may present with symptoms similar to other forms of viral meningitis, such as fever, headache, neck stiffness, and photophobia. In addition to meningitis, HSV can also cause encephalitis, a more severe form of central nervous system infection. Antiviral medications, such as acyclovir, are typically prescribed to manage HSV-associated meningitis and minimize potential complications. Proper education about safe sexual practices and the use of barrier methods can help reduce the risk of HSV transmission and subsequent meningitis.

Specificity

The genesig™PLEX Viral Meningitis kit is designed for the in vitro detection of Enterovirus, HHV3 and HSV1&2.

The HSV1&2 and HHV3 assays within this kit are predicted to detect over 95% of sequences available on the NCBI database at the time of design. For the Enterovirus assay, 95% of sequences from the last three years are predicted to be detected. Due to the sequence evolution observed in the genomes of RNA viruses, a period of three years is considered to be an adequate time period for analysis, depending on the number of sequences on the NCBI database.

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.

This kit is predicted to cross react with Macacine alphaherpesvirus 1, Chimpanzee alpha-1 herpes virus, Papiine herpesvirus 2, Cercopithecine herpesvirus 16, Cercopithecine herpesvirus 2, Ateline alphaherpesvirus 1 and Saimiriine herpesvirus 1 which would give a signal in the ROX channel.

If you require further information or have a specific question about the detection profile of this kit, then please send an email to techsupport@primerdesign.co.uk and our team will answer your question.

Kit contents

- **Multiplex primer/probe mix (2x 50 reactions BROWN)**
  FAM, VIC, ROX and Cy5 labelled (see table below)

Target	Fluorophore
Enterovirus	FAM
HHV3	VIC
HSV1&2	ROX
Internal extraction control	Cy5

- **Multiplex positive control template (RED)**
- **Internal extraction control RNA (BLUE)**
- **2x Lyophilised OneStep Master Mix (GOLD)**
- **2x oasig™ resuspension buffer (BLUE)**
  for resuspension of the lyophilised master mix
- **Template preparation buffer (YELLOW)**
  for resuspension of the positive control template
- **RNase/DNase free water (WHITE)**
  for resuspension of the primer/probe mix

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 or exsig®Mag however, it is designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

Pipettors and filter tips

Vortex and centrifuge

1.5 ml tubes

qPCR plates or 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 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. 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 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 kits have very high priming efficiencies of >90% and can detect between $1X10^6$ and $1X10^2$ copies of target template.

Notices and disclaimers

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Principles of the test

Real-time PCR

Individual primer and probes designed for each pathogen have been combined into a single reaction and these can be detected through the different fluorescent channels as described in the kit contents.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the target DNA. Fluorogenic probes are included in the same reaction mixture which consists of a DNA probe labelled 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 a positive control, the kit contains a single positive control that contains templates for the Enterovirus, HHV3 and HSV1&2 targets in the test. The kit positive control will give an Enterovirus signal through the FAM channel, a HHV3 signal through the VIC channel and a HSV1&2 signal through the ROX channel. 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 each virus are working properly in that particular run. 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 these components 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 confirm the 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.

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 qPCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration.

A separate qPCR primer/probe mix are supplied with this kit to detect the exogenous RNA using qPCR. 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 target cDNA even when present at low copy number. The Internal control is detected through the Cy5 channel and gives a Cq value of 28+/-3 depending on the level of sample dilution.

Resuspension protocol

To minimise the risk of contamination with foreign RNA, 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 lost upon opening the tube.

2. **Resuspend the primer/probe mix in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, vortex the tube thoroughly.

Component – resuspend in water	Volume
Pre-PCR pack	
Multiplex primer/probe mix (**BROWN**) (volume each tube)	55µl

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

Component – resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control RNA (**BLUE**)	500µl
Post-PCR heat-sealed foil	
Positive control template (**RED**)*	500µl

*** 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.**

4. **Resuspend the lyophilised OneStep Master Mix in oasig resuspension buffer, according to the table below:**

Component – resuspend in oasig resuspension buffer	Volume
Lyophilised OneStep Master Mix (**GOLD**)	525µl

RNA extraction

The internal extraction control RNA can be added to either lysis/extraction buffer or to the 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 manufacturer's recommended protocols.

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 prepared, 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
Lyophilised OneStep Master Mix (**GOLD**)	10µl
Multiplex primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

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

3. **Pipette 5µl of RNA sample 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. **Pipette 5µl of positive control template into each well according to your plate set up.**
   The positive control contains templates for Enterovirus, HHV3 and HSV1&2. The final volume in each well is 20µl.

OneStep RT-qPCR amplification protocol

Amplification conditions using lyophilised OneStep Master Mix

	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, VIC, ROX and Cy5 channels.

Interpretation of results

Positive control

The positive control well should give an amplification plot through the FAM channel (Enterovirus), the VIC channel (HHV3) and the ROX channel (HSV1&2). There is no internal extraction control template within the positive control so the Cy5 channel should give no signal (flat amplification plot). The positive control signals indicate that the kit is working correctly to detect each virus.

No template control (NTC)

The NTC should give a flat line (flat amplification plots) through all channels. Signals in the NTC indicate cross contamination during plate set up.

Internal RNA extraction control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of RNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a target 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.

Sample data

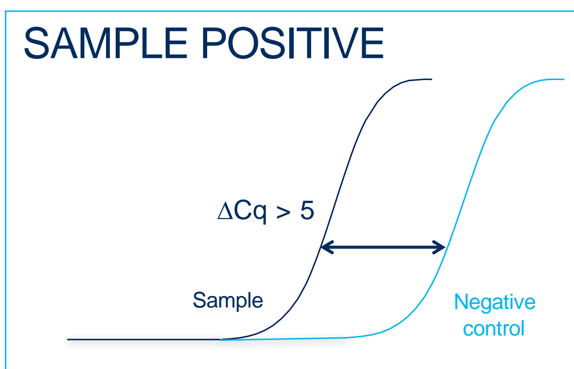
Presence of the viruses are detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those viruses. It may be possible for samples to contain multiple viruses, therefore positive results in the FAM, VIC and ROX channels may be present.

Summary of data interpretation

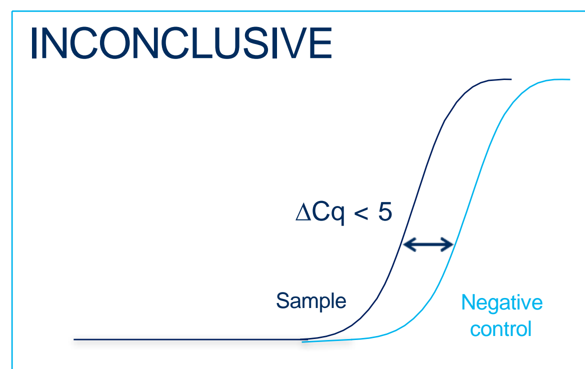
Target (FAM/VIC/ROX)	Internal extraction control (Cy5)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**ENTEROVIRUS POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**HHV3 POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**HSV1&2 POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE 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.