Primer Design Ltd

Z-Path-EHV-1

Equid alphaherpesvirus 1

Kit version: 1

Target region:

ORF-63

genesig® Advanced Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Equid alphaherpesvirus 1

Equid alphaherpesvirus 1 (EHV-1) is a double stranded DNA virus which is responsible for acute febrile respiratory disease in horses upon primary infection and is characterized by rhinopharyngitis and tracheobronchitis. It is a member of the genus Varicellovirus, which belongs to the subfamily Alphaherpesvirinae.

The genome of EHV-1 is approximately 150kb long. The linear DNA is composed of two regions, L (Long) and S(Short). The region S is flanked by two inverted repeat regions: an internal repeat sequence (IRS) and a terminal repeat sequence (TRS) which allow the generation of two equimolar isomers. Region L consists of a unique sequence flanked by small, inverted repeats (IRS and TRS).

Transmission occurs by direct or indirect contact with infective nasal discharges, aborted foetuses, and placental fluids. It is notable for its ability to target and attack three separate organ systems of the horse. Primary infection of the virus occurs at the vascular epithelium especially the nasal mucosa and lungs. It gains access to peripheral tissues via cell-associated viremia, which may manifest as abortion or neurologic disease. The infection damages the microvasculature of the central nervous system due to initiation of an inflammatory cascade, vasculitis, microthrombosis and extravasation of mononuclear cells resulting in haemorrhage. After initial infection, EHV-1 can establish a latent infection in the horse, meaning the virus can remain dormant in the horse's body and may reactivate later, especially during times of stress.

Specificity

The genesig® Advanced Kit for Equid alphaherpesvirus 1 (EHV-1) is designed for the in vitro quantification of EHV-1 genomes. The kit is designed to have a broad detection profile. Specifically, the primers will detect over 95% of sequences available on the NCBI database at the time of last review.

The dynamics of genetic variation means that new sequence information may become available after the initial design. If you require further information or have a specific question about the detection profile of this kit then please send an e-mail to techsupport@primerdesign.co.uk and our team will answer your question.

Kit contents

- **1x EHV-1 primer/probe mix (150 reactions, BROWN)**
  FAM labelled
- **1x EHV-1 positive control template (RED)**
- **1x Internal extraction control primer/probe mix (150 reactions, BROWN)**
  VIC labelled as standard
- **1x Internal extraction control DNA (150 reactions, BLUE)**
- **1x Endogenous control primer/probe mix (150 reactions, BROWN)**
  FAM labelled, Target: E.caballus ACTB gene as standard
- **1x RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **3x Template preparation buffer (YELLOW)**
  for resuspension of internal control template, positive control template 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 or exsig®Mag. However, it is designed to work well with all processes that yield high-quality nucleic acid with minimal PCR inhibitors.

oasig® lyophilised or PrecisionPLUS® 2X qPCR Master Mix
This kit is intended for use with oasig® lyophilised or PrecisionPLUS® 2X qPCR Master Mix.

Pipettors and filter tips

Vortex and centrifuge

1.5 ml microtubes

qPCR plates or reaction tubes

Kit storage and stability

This kit is stable for shipping at ambient temperature but should be stored at -20°C upon 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.

Primer Design Ltd does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

This kit can be used with all types of samples from various origins. Please ensure that the extracted nucleic acid samples are suitable in terms of purity, concentration, and DNA integrity. An internal extraction control is provided to test for non-specific PCR inhibitors.

Dynamic range of test

Under optimal PCR conditions the kit can achieve priming efficiencies between 90-110% and detect less than 100 copies of target template. If running a positive control standard curve for a quantitative result, and an efficiency of between 90% to 110% is not achieved, then the run should be repeated with a freshly prepared standard curve.

Principles of the test

Real-time PCR

A target 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 target DNA. A fluorogenic probe is 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 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 target 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 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. It is also known as a No Template Control or NTC.

Internal DNA extraction control

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

A separate primer and probe mix is supplied with this kit to detect the exogenous DNA using qPCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the target DNA 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

A primer/probe mix for detection of the endogenous control gene is included in the kit, which allows confirmation of a valid biological sample from the host. Detection of the endogenous control is through the FAM channel, and it is therefore NOT possible to perform a multiplex reaction with the target specific primer/probe mix. Amplification of the endogenous control may depend on the sample type used. Please note that if samples from a different species are used, the endogenous control may not be appropriate, but the internal extraction control is advised to be use.

Resuspension protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting is 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/probe mix or template is in the base of the tube and is not lost upon opening the tube.

2. **Resuspend the primer/probe mixes 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	
EHV-1 primer/probe mix (**BROWN**)	165 µl
Internal extraction control primer/probe mix (**BROWN**)	165 µl
Endogenous control primer/probe mix (**BROWN**)	165 µl

3. **Resuspend the internal control template and positive control template 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
Pre-PCR heat-sealed foil	
Internal extraction control DNA (**BLUE**)	600 µl
Post-PCR heat-sealed foil	
EHV-1 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.

DNA extraction

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

DO NOT add the internal extraction control DNA 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 DNA (BLUE) to each sample in DNA lysis/extraction buffer.**

2. **Complete DNA extraction according to the manufacturer's protocols.**

qPCR detection protocol

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

Component	Volume
oasig® lyophilised or PrecisionPLUS® 2X qPCR Master Mix	10 µl
EHV-1 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. **(Optional) For each DNA sample prepare an endogenous control reaction according to the table below.**
   This control reaction will provide useful information regarding the quality of the biological sample.

Component	Volume
oasig® lyophilised or PrecisionPLUS® 2X qPCR Master Mix	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 experimental plate set up.**

4. **Pipette 5µl of DNA template into each well, according to your experimental plate set up.**
   For negative control wells use 5 µl of RNase/DNase free water (WHITE). For positive control wells use 5 µl of the positive control template (**RED**). The final volume in each well is 20 µl.

5. (Optional) Standard curve preparation for quantitative analysis.

For quantitative analysis of the samples a standard curve dilution series can be prepared using the positive control template (**RED**). This is not required for qualitative analysis.

5.1 Reaction mix preparation for the standard curve.

Include sufficient reactions for each dilution of the standard curve.

Component	Volume
oasig® lyophilised or PrecisionPLUS® 2X qPCR Master Mix	10 µl
EHV-1 primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (**WHITE**)	4 µl
Final Volume	**15 µl**

5.2 Preparation of a 10-fold standard curve dilution series.

a. Pipette 90 µl of template preparation buffer (**YELLOW**) into 5 tubes and label them tube 2-6. The neat positive control tube (**RED**) is considered tube 1.

b. Pipette 10 µl of positive control template (**RED**) into tube 2.

c. Vortex thoroughly.

d. Change pipette tip and pipette 10 µl from tube 2 into tube 3.

e. Vortex thoroughly

Repeat steps d and e 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

5.3. Pipette 15 µl of reaction mix and 5 µl of the respective standard into each well for the standard curve according to your plate set up.

The final volume in each well is 20µl.

qPCR amplification protocol

Recommended amplification conditions when using oasig® lyophilised or PrecisionPLUS® 2X qPCR Master Mix.

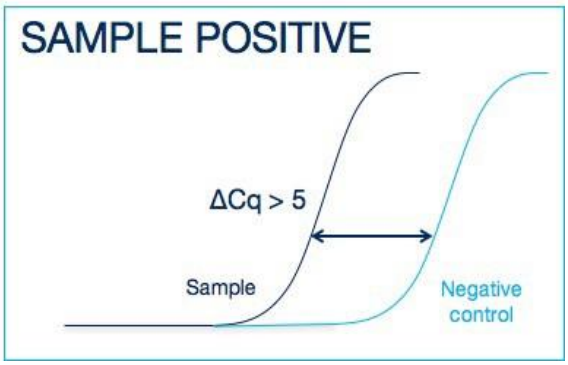
	Step	Time	Temp
Cycling x50	Enzyme activation	2 min	95 °C
	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

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

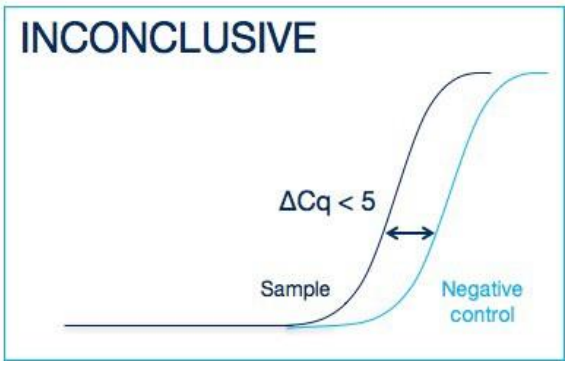
Interpretation of results

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**

*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.

Positive Control

The positive control template is expected to amplify between Cq 16 – 23 in the FAM channel. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised and should be repeated.

Internal PCR control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA 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.

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.

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