Equine/Canine influenza (H3N8 & H7N7)
Segment 1 polymerase PB2 (PB2) gene
genesig® Standard Kit
150 tests
Equine influenza (Horse flu) is the disease caused by strains of influenza A that are enzootic in horse species. Equine influenza occurs globally, and is caused by two main strains of virus: equine-1 (H7N7) and equine-2 (H3N8). The disease has a nearly 100% infection rate in an unvaccinated horse population with no prior exposure to the virus. While equine influenza is historically not known to affect humans, impacts of past outbreaks have been devastating due to the economic reliance on horses. The outbreak in North America in 1872 saw 1% of the approximately 7 million horses dying of the disease.

Equine influenza is caused by H7N7 and H3N8. These viruses were first isolated in 1956. They can cross the species barrier to cause an epizootic disease in humans, and recently, in dogs. The equine-1 virus affects heart muscle, while the equine-2 virus is much more severe and systemic. The disease is primarily spread between infected horses but can also survive for up to 48 hours on people’s clothing and other objects such as stools and buckets. Exposure to infected waste materials (urine and manure) in stables leads to rapid spread of the disease.

The symptoms typically include a high temperature lasting for one to five days and a dry, harsh-sounding cough that may linger for several weeks. Coughing is generally worse when eating hay or hard feed. Nearly all affected horses will develop a clear, watery nasal discharge that may turn green or yellow as secondary infections develop. Other common symptoms are: swollen legs and lymph nodes under the jaw; depression; lethargy; and loss of appetite. Pneumonia may develop in the very young and very old. This may be fatal.
Specificity

The Primerdesign genesig Kit for Equine/Canine influenza (H3N8 & H7N7) (H3N8&H7N7) genomes is designed for the in vitro quantification of H3N8&H7N7 genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the H3N8&H7N7 genome.

The primers and probe sequences in this kit have 100% homology with a broad range of H3N8&H7N7 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 enquiry@primerdesign.co.uk and our bioinformatics team will answer your question.
Kit contents

• **H3N8&H7N7 specific primer/probe mix** (150 reactions **BROWN**)  
  FAM labelled

• **H3N8&H7N7 positive control template** (for Standard curve **RED**)

• **H3N8&H7N7 RT primer mix** (150 reactions **GREEN**)  
  Required for two step protocol only

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

• **Template preparation buffer** (**YELLOW**)  
  for resuspension of positive control template and standard curve preparation

Reagents and equipment to be supplied by the user

Real-time PCR Instrument

RNA 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 with minimal PCR inhibitors.

**oasig™ 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 H3N8&H7N7 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

Trademarks
Primerdesign™ is a trademark of Primerdesign Ltd.
genesis® is a registered trademark of Primerdesign Ltd.
The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. BI,
ABI PRISM® GeneAmp® and MicroAmp® are registered trademarks of the Applera Genomics (Applied Biosystems Corporation).
BIOMEX® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-
Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc. GeneAmp®,
TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc., The purchase of the Primerdesign™
reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche
Inc.
Principles of the test

Real-time PCR
A H3N8&H7N7 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 H3N8&H7N7 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.

OneStep vs. Two step qPCR
When detecting/quantifying the presence of a target with an RNA genome Primerdesign recommend the use of a OneStep RT-qPCR protocol. OneStep RT-qPCR combines the reverse transcription and qPCR reaction in a simple closed tube protocol. This saves significant bench time but also reduces errors. The sensitivity of a OneStep protocol is also greater than a two step because the entire biological sample is available to the PCR without dilution. This kit will also work well with a two step approach (Precision nanoScript2 reverse transcription kit and PrecisionPLUS Master Mix) if required but the use of oasig OneStep or PrecisionPLUS OneStep Master Mix is the preferred method.

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 H3N8&H7N7 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 H3N8&H7N7 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.

<table>
<thead>
<tr>
<th>Component - resuspend in water</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-PCR pack</td>
<td></td>
</tr>
<tr>
<td>H3N8&amp;H7N7 primer/probe mix (BROWN)</td>
<td>165 µl</td>
</tr>
<tr>
<td>H3N8&amp;H7N7 RT primer mix (GREEN)</td>
<td>165 µl</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Component - resuspend in template preparation buffer</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-PCR heat-sealed foil</td>
<td></td>
</tr>
<tr>
<td>H3N8&amp;H7N7 Positive Control Template (RED) *</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

* 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

A OneStep approach combining the reverse transcription and amplification in a single closed tube is the preferred method. If, however, a two step approach is required see page 10.

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.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix</td>
<td>10 µl</td>
</tr>
<tr>
<td>H3N8&amp;H7N7 primer/probe mix (BROWN)</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase/DNase free water (WHITE)</td>
<td>4 µl</td>
</tr>
<tr>
<td><strong>Final Volume</strong></td>
<td><strong>15 µl</strong></td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix</td>
<td>10 µl</td>
</tr>
<tr>
<td>H3N8&amp;H7N7 primer/probe mix (BROWN)</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase/DNase free water (WHITE)</td>
<td>4 µl</td>
</tr>
<tr>
<td><strong>Final Volume</strong></td>
<td><strong>15 µl</strong></td>
</tr>
</tbody>
</table>
5. 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 10µl from tube 2 into tube 3
5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

### Standard Curve

<table>
<thead>
<tr>
<th>Standard Curve</th>
<th>Copy Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1 Positive control (RED)</td>
<td>$2 \times 10^9$ per µl</td>
</tr>
<tr>
<td>Tube 2</td>
<td>$2 \times 10^8$ per µl</td>
</tr>
<tr>
<td>Tube 3</td>
<td>$2 \times 10^7$ per µl</td>
</tr>
<tr>
<td>Tube 4</td>
<td>$2 \times 10^6$ per µl</td>
</tr>
<tr>
<td>Tube 5</td>
<td>20 per µl</td>
</tr>
<tr>
<td>Tube 6</td>
<td>2 per µl</td>
</tr>
</tbody>
</table>

6. 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.

**OneStep RT-qPCR amplification protocol**

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>10 min</td>
<td>55 °C</td>
</tr>
<tr>
<td>Enzyme activation</td>
<td>2 min</td>
<td>95 °C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10 s</td>
<td>95 °C</td>
</tr>
</tbody>
</table>

**Cycling x50**

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>DATA COLLECTION *</td>
<td>60 s</td>
<td>60 °C</td>
</tr>
</tbody>
</table>

* Fluorogenic data should be collected during this step through the FAM channel
Alternative two step detection protocol

Reverse Transcription

If you need to perform separate reverse transcription and amplification (two step qPCR) then we recommend the Primerdesign Precision nanoScript2 Reverse Transcription kit. A reverse transcription primer (GREEN) is included and is designed for use with the Precision nanoScript2 Reverse Transcription kit. A protocol for this product is available at www.primerdesign.co.uk

1. **After reverse transcription, prepare a reaction mix according to the table below for each cDNA sample**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrecisionPLUS 2X qPCR Master Mix</td>
<td>10 µl</td>
</tr>
<tr>
<td>H3N8&amp;H7N7 primer/probe mix (BROWN)</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase/DNase free water (WHITE)</td>
<td>4 µl</td>
</tr>
<tr>
<td><strong>Final Volume</strong></td>
<td>15 µl</td>
</tr>
</tbody>
</table>

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

3. **Prepare sample cDNA templates for each of your samples by diluting the RT reaction mix 1:5 in RNase/DNase free water.**

4. **Pipette 5µl of cDNA template into each well, according to your experimental plate setup.**
   The final volume in each well is 20µl. For negative control wells use 5µl of RNase/DNase free water.

Alternative two step amplification protocol

Amplification conditions using PrecisionPLUS 2X qPCR Master Mix.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activation</td>
<td>2 min</td>
<td>95 °C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10 s</td>
<td>95 °C</td>
</tr>
<tr>
<td>DATA COLLECTION *</td>
<td>60 s</td>
<td>60 °C</td>
</tr>
</tbody>
</table>

* Fluorogenic data should be collected during this step through the FAM channel
Interpretation of results

<table>
<thead>
<tr>
<th>Target</th>
<th>Positive control</th>
<th>Negative control</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>POSITIVE QUANTITATIVE RESULT calculate copy number</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>NEGATIVE RESULT</td>
</tr>
<tr>
<td>+ / -</td>
<td>+</td>
<td>≤ 35</td>
<td>EXPERIMENT FAILED due to test contamination</td>
</tr>
<tr>
<td>+ / -</td>
<td>+</td>
<td>&gt; 35</td>
<td>*</td>
</tr>
<tr>
<td>+ / -</td>
<td>-</td>
<td>+ / -</td>
<td>EXPERIMENT FAILED</td>
</tr>
</tbody>
</table>

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:

**SAMPLE POSITIVE**

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.

**INCONCLUSIVE**

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.