Primer Design Ltd

R00977

Influenza A virus subtype H7N1 (avian influenza)

Kit version: 2

Haemagglutinin gene

Neuraminidase gene

genesig® Advanced Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Influenza A virus subtype H7N1

Influenza type A viruses are 80–120 nanometers in diameter and usually roughly spherical, made up of a viral envelope containing two main types of proteins, wrapped around a central core. The two large proteins found on the outside of viral particles are haemagglutinin (HA) and neuraminidase (NA). HA is a protein that mediates binding of the virion to target cells and entry of the viral genome into the target cell, while NA is involved in the release of progeny virions from infected cells. Influenza type A viruses are categorized into subtypes based on the type of these two proteins on the surface of the viral envelope. The central core of a virion contains the viral genome and other viral proteins that package and protect the genetic material. The entire Influenza A virus genome is ~13,588 bases long and is contained on 8 RNA segments that code for 11 proteins.

Avian Influenza (‘avian flu’ or ‘bird flu’) strains all belong to the Influenza A virus which are generally adapted to birds but not exclusive to them. From 1999 to 2000 a H7N1 outbreak resulted in the death of 13 million chickens and caused extensive economic loss. A serial passage test has also shown that H7N1 can be adapted to become capable of airborne transmission in mammals.

Specificity

The genesig® Advanced Kit for Influenza A virus subtype H7N1 (avian influenza) is designed for the in vitro quantification of H7N1 genomes. The kit is designed to have a broad detection profile. Specifically, the H7 primers will detect 85% of H7 sequences and the N1 primers will detect over 95% of H7N1 sequences on the GISAID EpiFlu database, collected within the three years to the time of the most recent review. Due to the sequence evolution observed in the genomes of RNA viruses, a period of three years is considered an adequate time period for analysis.

The H7 primer and probe set is designed to detect H7 sequences and therefore may detect H7 sequences from non-H7N1 subtypes.

The N1 primer and probe set is designed to detect N1 sequences from the H7N1 lineage but may detect N1 sequences from other subtypes.

A positive result for both, H7 and N1, indicates the presence of H7N1 specifically.

The dynamics of genetic variation mean that new sequence information may become available after the most recent review. Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

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 H7 primer/probe mix (150 reactions BROWN)
  FAM labelled.
- 1x N1(H7N1&H9N1) primer/probe mix (150 reactions BROWN)
  FAM labelled.
- 1x H7 positive control template (RED)
- 1x N1(H7N1&H9N1) positive control template (RED)
- 2x Internal extraction control primer/probe mix (150 reactions BROWN)
  VIC labelled as standard.
- 1x Internal extraction control RNA template (150 reactions BLUE)
- 1x Endogenous control primer/probe mix (150 reactions BROWN)
  FAM labelled.
- 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. However, it is designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

oasig® lyophilised OneStep or Precision®PLUS OneStep 2X RT-qPCR Master Mix
This kit is intended for use with oasig® lyophilised OneStep or Precision®PLUS OneStep 2X RT-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 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 -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

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 (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 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.

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

PrecisionPLUS® is a registered trademark of Primer Design Ltd.

genesig® is a registered trademark of Primer Design Ltd.

oasig® is a registered trademark of Primer Design Ltd.

exsig®Mag is a registered trademark of Primer Design 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. TaqMan® is a registered trademark of Roche Molecular Systems, Inc., The purchase of the Primer Design Ltd 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

H7N1 specific primer/probe mixes are provided, and these can be detected through the FAM channel.

The primer/probe mixes provided exploit the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the H7N1 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 positive control templates. This can be used to generate standard curves of H7N1 copy number/Cq value. Alternatively, the positive controls 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 H7N1 genes 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 controls do 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 wells.

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 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 is 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 H7N1 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/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 H7N1 primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

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 the 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	
H7 primer/probe mix (BROWN)	165 µl
N1(H7N1&H9N1) 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 RNA template (BLUE)	600 µl
Post-PCR heat-sealed foil	
H7 Positive Control Template (RED) *	500 µl
N1(H7N1&H9N1) 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 manufacturer's 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
oasig® OneStep or Precision®PLUS OneStep 2X RT-qPCR Master Mix	10 µl
H7or N1(H7N1&H9N1) 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 useful information regarding the quality of the biological sample.

Component	Volume
oasig® OneStep or Precision®PLUS OneStep 2X RT-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 qPCR 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
oasig® OneStep or Precision®PLUS OneStep 2X RT-qPCR Master Mix	10 µl
H7 or N1(H7N1&H9N1) primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

6. **Preparation of standard curve dilution series.**
   a) **Pipette 90µl of template preparation buffer into 5 tubes and label 2-6**
   b) **Pipette 10µl of Positive Control Template (RED) into tube 2**
   c) **Vortex thoroughly**
   d) **Change pipette tip and pipette 10µl 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)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 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.

OneStep RT-qPCR Amplification Protocol

Amplification conditions using oasig® OneStep or Precision®PLUS 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
	DATA COLLECTION *	60 s	60 °C

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

Interpretation of results

*H7 (FAM)	*N1 (H7N1&H9N1) (FAM)	Internal control (VIC)	**Positive control	Negative control	***Interpretation
+	-	+/-	+	-	**POSITIVE RESULT FOR H7**
-	+	+/-	+	-	**POSITIVE RESULT FOR N1**
+	+	+/-	+	-	**POSITIVE RESULT FOR H7N1**
-	-	+	+	-	**NEGATIVE RESULT**
+ / -	+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+ / -	+ / -	+	> 35	****
-	-	-	+	-	**SAMPLE PREPARATION FAILED**
+ / -	+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

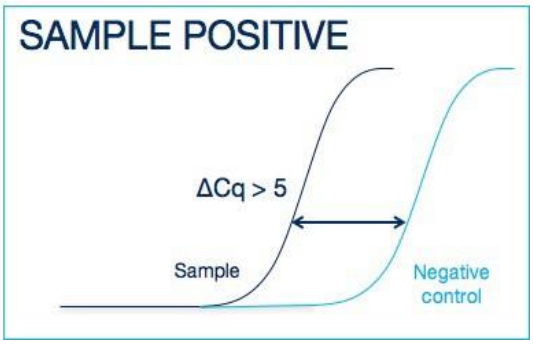
* The Cq value for the targets (FAM) should either be ≤30 with either positive or negative internal control or >30 with a positive internal control (IC) to use the result for **quantitation.** In this case, the copy number can be calculated.

If the target Cq is >30 and the IC is negative, this is a **qualitative** result. Do not report the copy number as this may be due to poor sample extraction.

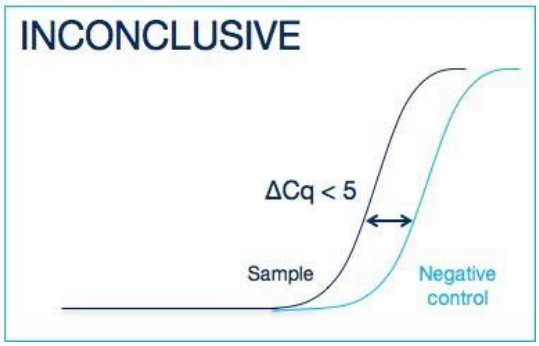
** Positive control template (**RED**) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

*** If a sample is positive for H7 but negative for N1, this could be due to the presence of one of the other H7 subtypes. Conversely if a sample is positive for N1 but negative for H7, this could be due to the presence of one of the other N1 subtypes. A sample is confirmed as H7N1 if both targets (FAM) are positive.

**** 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 Cq 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. Cq values of 28±3 are within the normal range. When amplifying a 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.