Primer Design Ltd

R01011

Influenza A virus subtype H5 (avian influenza)

Kit version: 2

Target region:

Haemagglutinin gene

genesig® Standard Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Influenza A virus subtype H5

Influenza type A viruses are 80–120 nanometres 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. Of particular concern to humans are ones which are infectious to both humans and birds. Specific strains such as H1N1 have been the subject of much media concern and speculation over pandemics and its widespread transmission globally. Only two influenza A virus subtypes A(H1N1) pdm09, and A(H3N2), are currently circulating among people.

Since 2016, H5N8 has been spreading rapidly through migration of wild birds in Europe and Asia, causing deaths in the wild bird population and domestic poultry. An outbreak of H5N8 in Russia was confirmed by WHO in 2020, viruses were also detected in poultry or wild birds in Bulgaria, the Czech Republic, Egypt, Germany, Hungary, Iraq, Japan, Kazakhstan, the Netherlands, Poland, Romania, and the United Kingdom. There is a low possibility of human infection with H5N8, however, it should not be excluded as infections in 7 humans were reported by Russia in 2021, the first confirmed cases of H5N8 in humans because of the 2020 outbreak. It was also reported in January 2023, that human infection by H5N1 was identified by PCR in Ecuador.

Specificity

The genesig® Standard Kit for Influenza A virus subtype H5 (avian influenza) is designed for the in vitro quantification of Influenza A virus subtype H5 genomes. The kit is designed to have a broad detection profile. Specifically, the primers will detect over 95 % of sequences available on the GISAID database at the time of last review.

When designing this Influenza A H5 kit, sequences from a three-year period were analysed from the GISAID EpiFlu database. Due to the sequence evolution observed in the influenza A genome over time, this is considered to be an adequate time period for analysis.

The dynamics of genetic variation mean that new sequence information may become available after the most recent review. 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 H5_v2.0 primer/probe mix (150 reactions, BROWN)**
  FAM labelled
- **1x H5_v2.0 positive control template (for Standard curve, RED)**
- **1x RNase/DNase-free water (WHITE)**
  for resuspension of primer/probe mixes
- **2x 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

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 OneStep or PrecisionPLUS® OneStep 2X RT-qPCR Master Mix
This kit is intended for use with oasig® lyophilised OneStep or PrecisionPLUS® 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 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 RNA integrity.

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/probe mix is provided, and this can be detected through the FAM channel.

The primer/probe mix provided exploits with the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the target RNA. 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 the 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/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, RNase/DNase-free water should be used instead of the 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 RNA/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 that 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 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
Pre-PCR pack	
H5_v2.0 primer/probe mix (**BROWN**)	165 µl

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
Post-PCR heat-sealed foil	
H5_v2.0 Positive Control Template (**RED**)	500 µl

* This component contains a 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 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® lyophilised OneStep or PrecisionPLUS® OneStep 2X RT-qPCR Master Mix	10 µl
H5_v2.0 primer/probe mix (**BROWN**)	1 µl
RNase/DNase-free water (**WHITE**)	4 µl
Final Volume	**15 µl**

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

3. **Pipette 5 µl of RNA template into each well, according to your experimental plate set up.**
   For negative control wells us 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.

4. (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.

4.1 Reaction mix preparation for the standard curve.

Include sufficient reactions for each dilution of the standard curve.

Component	Volume
oasig® lyophilised OneStep or PrecisionPLUS® OneStep 2X RT-qPCR Master Mix	10 µl
H5_v2.0 primer/probe mix (**BROWN**)	1 µl
RNase/DNase-free water (WHITE)	4 µl
Final Volume	**15 µl**

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

a. pipette 90 µl of template preparation buffer (YELLOW) into 5 tubes and label them 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** across the tubes 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

4.3 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 20 µl.

OneStep RT-qPCR Amplification Protocol

Amplification conditions using oasig® lyophilised 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
	DATA COLLECTION *	60 s	60 °C

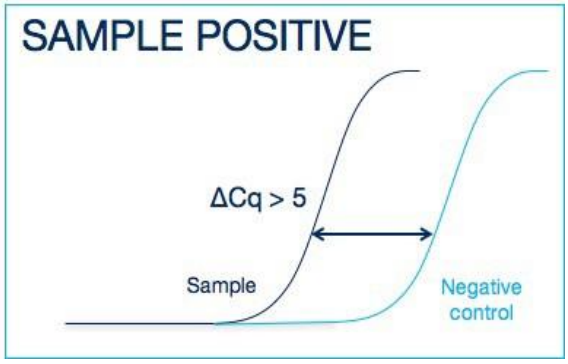
* Fluorogenic data should be collected during this step through the FAM channels

Interpretation of results

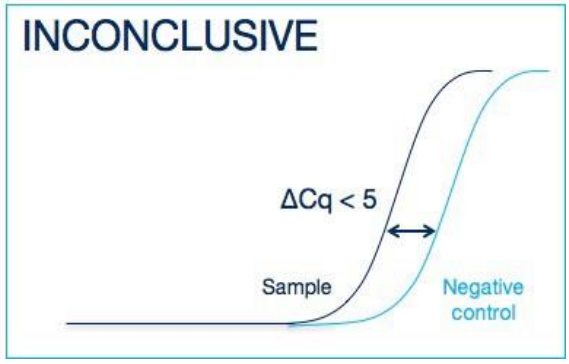
Target	Positive control	Negative control	Interpretation
+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
-	+	-	**NEGATIVE RESULT**
+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+	> 35	*
+ / -	-	+ / -	**EXPERIMENT FAILED**

A positive control template 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.

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