Primerdesign™ Ltd

R01012

Influenza A virus subtype H5N1 (avian influenza)

Version Number: 2

Haemagglutinin gene

Neuraminidase gene

genesig® Standard Kit

150 tests

GENESIG

Kits by Primerdesign

Specificity of primers and probes last reviewed on:
24th February 2022

For general laboratory and research use only

Introduction to Influenza A virus subtype H5N1

Influenza A virus subtype H5N1, also known as A(H5N1) or H5N1, is a subtype of the Influenza A virus that is capable of causing illness in many animal species, including humans. [1] A bird-adapted strain of H5N1, called HPAI A (H5N1) for "highly pathogenic avian influenza virus of type A of subtype H5N1", is the causative agent of H5N1 flu, commonly known as "avian influenza" or simply "bird flu" and is endemic in many bird populations, especially in Southeast Asia. One strain of HPAI A (H5N1) of Asian lineage is spreading globally. It is epizootic (an epidemic in non-humans) and panzootic (a disease affecting animals of many species, especially over a wide area), killing tens of millions of birds and spurring the culling of hundreds of millions of other birds in an attempt to control its spread. Most references in the media to "bird flu" and to H5N1 are about this specific strain.[2]

HPAI A(H5N1) is an avian disease, and there is no evidence of efficient human-to-human transmission or of airborne transmission of HPAI A (H5N1) to humans. In almost all cases, those infected with H5N1 have had extensive physical contact with infected birds. However, around 50% of humans known to have been infected with the current Asian strain of HPAI A (H5N1) have died from H5N1 flu, and H5N1 has the potential to mutate or re-assort into a strain capable of efficient human-to-human transmission. On September 29, 2005, David Nabarro, the newly appointed Senior United Nations System Coordinator for Avian and Human Influenza, warned the world that an outbreak of avian influenza could kill anywhere between 5 million and 150 million people.[3] Experts have identified key events (creating new clades, infecting new species, spreading to new areas) marking the progression of an avian flu virus towards becoming pandemic, and many of those key events have occurred more rapidly than expected.

References

1. International Committee on Taxonomy of Viruses (2002). 46.0.1. Influenzavirus A. Retrieved on 2006-04-17.
2. a b Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepangestie AT, Chaisingh A, Auewarakul P, Long HT, Hanh NT, Webby RJ, Poon LL, Chen H, Shortridge KF, Yuen KY, Webster RG, Peiris JS. (2004). "Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia". Nature 430 (6996): 209-213. PubMedDOI:10.1038/nature02746.
3. United Nations. "Press Conference by UN System Senior Coordinator for Avian, Human Influenza", UN News and Media Division, Department of Public Information, New York, 2005 -09-29. Retrieved on 2006-04-17.

Specificity

The Primerdesign genesig Kit for Influenza A virus subtype H5N1 (avian influenza) (H5N1) is designed for the in vitro quantification of H5N1 genomes. The kit is designed to have a broad detection profile. Specifically, the primers will detect over 95% of the sequences on the GISAID EpiFlu database, identified as H5N1 from avian origin and collected within the three years to the time of the most recent review.

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

- **H5(H5N1)_V2 specific primer/probe mix (150 reactions BROWN)**
  FAM labelled

- **N1(H5N1)_V2 specific primer/probe mix (150 reactions BROWN)**
  FAM labelled

- **H5(H5N1)_V2 positive control template (for Standard curve RED)**

- **N1(H5N1)_V2 positive control template (for Standard curve RED)**

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

- **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 or PrecisionPLUS 2X qPCR Master Mix

Pipettors and Tips

Vortex and centrifuge

1.5 ml PCR reaction tubes

PCR 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 -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 (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 genesig® H5N1 detection kits have very high priming efficiencies of >90% and can detect less than 100 copies of target template.

Notices and disclaimers

Trademarks

Principles of the test

Real-time PCR

An H5N1 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 H5N1 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 a positive control template. This can be used to generate a standard curve of H5N1 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 H5N1 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 know as a No Template Control or NTC.

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 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	
H5(H5N1)_V2 primer/probe mix (BROWN)	165 µl
N1(H5N1)_V2 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
Post-PCR heat-sealed foil	
H5(H5N1)_V2 Positive Control Template (RED) *	500 µl
N1(H5N1)_V2 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.

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 PrecisionPLUS OneStep 2X RT-qPCR Master Mix	10 µl
H5(H5N1)_V2 or N1(H5N1)_V2 primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

2. **Pipette 15µl of these mixes 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:**

Component	Volume
oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix	10 µl
H5(H5N1)_V2 or N1(H5N1)_V2 primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

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	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

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.

	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

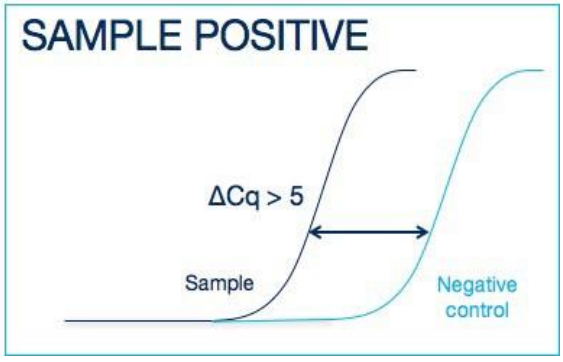
Interpretation of results

H5 (H5N1) (FAM)	N1 (H5N1) (FAM)	*Positive control	Negative control	**Interpretation
+	-	+	-	**POSITIVE RESULT FOR H5**
-	+	+	-	**POSITIVE RESULT FOR N1**
+	+	+	-	**POSITIVE RESULT FOR H5N1**
-	-	+	-	**NEGATIVE RESULT**
+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+ / -	+	> 35	***
-	-	+	-	**SAMPLE PREPARATION FAILED**
+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

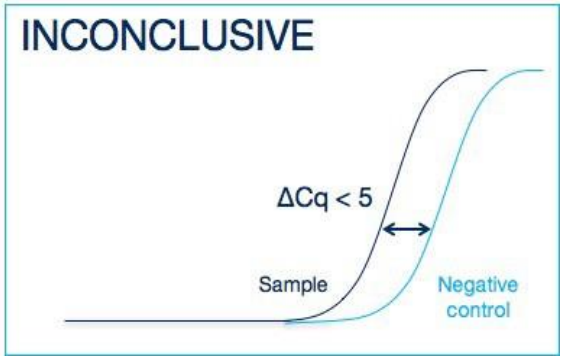
*Positive control template (**RED**) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

**If a sample is positive for H5 but negative for N1, this could be due to the presence of one of the other H5 subtypes. Conversely if a sample is positive for N1 but negative for H5, this could be due to the presence of one of the other N1 subtypes. A sample is confirmed as H5N1 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.

Disposal Considerations

Dispose of tested samples according to any local, national or regional regulations.

Product: Offer surplus and non-recyclable solutions to a licensed disposal company.

Contaminated packaging: Dispose of as unused product.