Primerdesign™ Ltd

Influenza A virus subtype H5N1 (avian influenza)

Hemagglutinin H5 & Neuramidase N1

genesig® Advanced Kit

150 reactions

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For general laboratory and research use only

Introduction to Influenza A virus subtype H5N1 (avian influenza)

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 [1].

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. In 2005, The World Health Organization (WHO) and influenza experts worldwide urged all countries to develop or update their influenza pandemic preparedness plans to respond to the potential outbreak of avian influenza [2,3], with the pandemic estimated to cause potentially 2 to 7 million human deaths [2]. 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. Li, K., Guan, Y., Wang, J., Smith, G., Xu, K., Duan, L., Rahardjo, A., Puthavathana, P., Buranathai, C., Nguyen, T., Estoepangestie, A., Chaisingh, A., Auewarakul, P., Long, H., Hanh, N., Webby, R., Poon, L., Chen, H., Shortridge, K., Yuen, K., Webster, R. and Peiris, J., 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature, 430(6996), pp.209-213.
2. Idris F. M. (2005). The Bird Flu Threat - Why Aren’t We Worrying?. The Malaysian journal of medical sciences : MJMS, 12(1), 1–2.
3. Monto A. S. (2005). The Threat of an Avian Influenza Pandemic. New England Journal of Medicine, 352(4), 323–325. doi:10.1056/NEJMp048343

Specificity

The Primerdesign genesig Kit for Influenza A virus subtype H5N1 (avian influenza) (H5N1) genomes are designed for the *in vitro* quantification of H5N1 genomes. The kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology, with over 95% of the NCBI database reference sequences available at the time of design.

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

The primers have 100% homology with over 95% of avian H5N1 isolates globally that have been entered into the influenza sequence database in the NCBI database since 2001. The primers have low sequence homology to other influenza subtypes, and the quantification of both subtyping genes ensures an accurate determination of the H5N1 genotype in a single experiment. However, due to the inherent instability of RNA viral genomes, novel emerging sequences may not be detected. Please contact us if you would like us to check the homology of our primers to specific strains you are working with.

If you require further information or have a specific question about the detection profile of this kit, then please send an email to enquiry@primerdesign.co.uk, and our bioinformatics team will answer your question.

Kit contents

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

H5N1 positive control template (for Standard curve RED)

Internal extraction control primer/probe mix (150 reactions BROWN)
VIC-labelled as standard

Internal extraction control RNA (150 reactions BLUE)

Endogenous control primer/probe mix (150 reactions BROWN)
FAM-labelled

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
Contains complete OneStep RT-qPCR master mix

Pipettors and tips

Vortex and centrifuge

Thin-walled 0.1 ml PCR 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. 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 the 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 practise PCR. Additional information on purchasing licenses to practise 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 US Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by US Patent 5,538,848, owned by The Perkin-Elmer Corporation.

Trademarks

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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). BIOMEK® 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 authorisation or implicit license to practise PCR under any patents held by Hoffmann-LaRoche Inc.

Principles of the test

Real-time PCR
A 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 hybridise to the H5N1 cDNA. 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 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.

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 indicate 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 the detection of the H5N1 target cDNA even when present at a 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 and 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 H5N1 primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Resuspension protocol

To minimise 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 that lyophilised primer and probe mix is in the base of the tube and is not spilt 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
H5N1 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
Internal extraction control RNA (BLUE)	600 µl
H5N1 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 the 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 4 µl 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 poured proceed immediately to the OneStep amplification protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artefacts 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
H5N1 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 crucial information regarding the quality of the biological sample.

Component	Volume
oasig OneStep or PrecisionPLUS 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 15 µl of these mixes into each well according to your qPCR experimental plate set-up.**

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

5. **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
H5N1 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 90 µl of template preparation buffer into 5 tubes and label 2-6**
   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

7. **Pipette 5 µl of the standard template 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 OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix.

	Step	Time	Temp
	Reverse Transcription	10 min	55 ºC
	Enzyme activation	2 min	95 ºC
Cycling x50	Denaturation	10 s	95 ºC
	DATA COLLECTION *	60 s	60 ºC

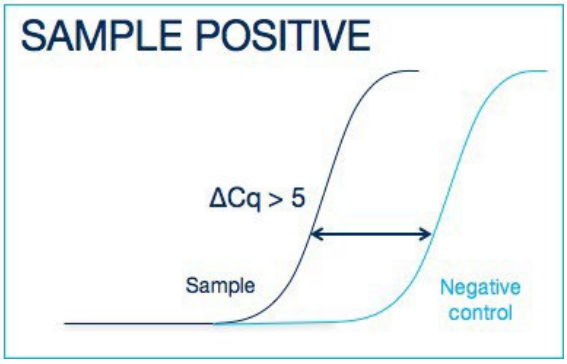
* Fluorogenic data should be collected during this step through the FAM and VIC channels

Interpretation of results

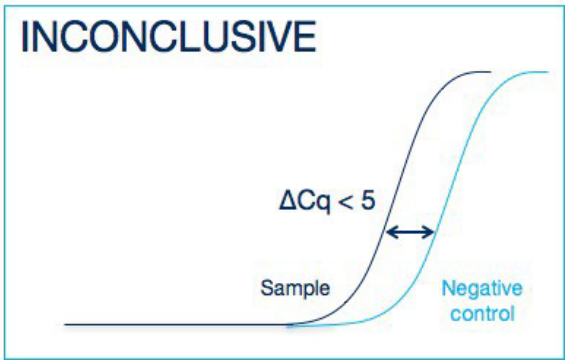
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
> 30	+	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
> 30	-	+	-	POSITIVE QUALITATIVE RESULT Do not report copy number as this may be due to poor sample extraction
-	+	+	-	NEGATIVE RESULT
+ / -	+ / -	+	≤ 35	EXPERIMENT FAILED due to test contamination
+ / -	+ / -	+	≤ 35	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.

*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 is within the normal range. When amplifying a H5N1 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.