

PCRmax Ltd [™] qPCR test

Influenza A virus subtype H5N1 (avian influenza)

Hemagglutinin H5
&
Neuramidase N1

150 tests

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.[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, Estoepongastie 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 PCRmax qPCR Kit for Influenza A virus subtype H5N1 (avian influenza) (H5N1) genomes is designed for the in vitro quantification of H5N1 genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the H5N1 genome.

The primers and probe sequences in this kit have 100% homology with a broad range of H5N1 sequences based on a comprehensive bioinformatics analysis.

The primers have 100% homology with all avian H5 and N1 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. The quantification of both subtyping genes ensures accurate determination of the H5N1 genotype in a single experiment.

If you require further information, or have a specific question about the detection profile of this kit then please send an e.mail to help@pcrmax.com and our bioinformatics team will answer your question.

Kit Contents

- **Hemagglutinin H5 primer/probe mix (150 reactions BROWN)**
FAM labelled
- **Neuramidase N1 primer/probe mix (150 reactions BROWN)**
FAM labelled
- **Hemagglutinin H5 positive control template (for Standard curve RED)**
- **Neuramidase N1 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
- **H5 and N1/Internal extraction control/endogenous control RT primer mix (150 reactions GREEN)**
Required for two step protocol only
- **RNAse/DNAse free water (WHITE)**
for resuspension of primer/probe mixes and internal extraction control RNA
- **Template preparation buffer (YELLOW)**
for resuspension of positive control templates and standard curve preparation

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

RNA extraction kit

This kit is designed to work well with all processes that yield high quality RNA with minimal PCR inhibitors.

Lyophilised 2x RT-qPCR MasterMix

This kit is designed to be compatible with all commercially available OneStep Mastermixes that run with standard cycling conditions.

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 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.

PCRmax 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 PCRmax H5N1 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 PCRmax 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 Applied Biosystems 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

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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 Applied Biosystems (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 PCRmax 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

The kit contains two primer and probe sets. The H5 primer and probe set are designed to detect all influenza H5 sequences from the avian H5N1 lineage. The N1 primer and probe set detect all N1 sequences regardless of the species of origin. Results from both primer sets can be used to make a positive determination of the H5N1 genotype. Samples that test negative for H5 and positive for N1 represent alternative circulating subtypes that contain the N1 gene .

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 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 real-time PCR 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 real-time PCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration.

A separate RT primer mix and a real-time PCR primer/probe mix are supplied with this kit to detect the exogenous RNA using real-time PCR. 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 H5N1 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 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.

Carry-over prevention using UNG (unsuitable for onestep procedure and optional for two step procedure)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PCRmax recommend the application of 0.2U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step.

Reconstitution 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. Reconstitute 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 primer/probe mix (BROWN) | 165 μ l |
| N1 primer/probe mix (BROWN) | 165 μ l |
| Internal extraction control primer/probe mix (BROWN) | 165 μ l |
| RT primer mix (GREEN) | 165 μ l |
| Endogenous control primer/probe mix (BROWN) | 165 μ l |
| Pre-PCR heat-sealed foil | |
| Internal extraction control RNA (BLUE) | 600 μ l |

- 3. Reconstitute the positive control templates 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 Positive Control Template (RED) * | 500 μ l |
| N1 Positive Control Template (RED) * | 500 μ l |

* 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 4 μ l of the Internal extraction control RNA (BLUE) to each sample in RNA lysis/extraction buffer per sample.**
- 2. Complete RNA extraction according to the manufacturers protocols.**

One Step qRT-PCR detection protocol

A one step approach combining the reverse transcription and amplification in a single closed tube is the preferred method.

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 One Step 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 |
|--|-----------------------------|
| Lyophilised OneStep 2x RT-qPCR MasterMix | 10 μ l |
| H5 or N1 primer/probe mix (BROWN) | 1 μ l |
| Internal extraction control primer/probe mix (BROWN) | 1 μ l |
| RNAse/DNAse free water (WHITE) | 3 μ l |
| 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 |
|---|-----------------------------|
| Lyophilised OneStep 2x RT-qPCR MasterMix | 10 μ l |
| Endogenous control primer/probe mix (BROWN) | 1 μ l |
| RNAse/DNAse free water (WHITE) | 4 μ l |
| Final Volume | 15 μl |

- 3. Pipette 15 μ l of these mixes into each well according to your real-time PCR experimental plate set up.**
- 4. Pipette 5 μ l of RNA template into each well, according to your experimental plate set up.**
For negative control wells use 5 μ l of RNAse/DNAse free water. The final volume in each well is 20 μ l.

5. If a standard curve is included for quantitative analysis prepare a reaction mix according to the table below:

| Component | Volume |
|--|-----------------------------|
| Lyophilised OneStep 2x RT-qPCR MasterMix | 10 μ l |
| H5 or N1 primer/probe mix (BROWN) | 1 μ l |
| RNAse/DNAse free water (WHITE) | 4 μ l |
| Final Volume | 15 μl |

6. Preparation of standard curve dilution series.

- 1) Pipette 90 μ l of template preparation buffer into 5 tubes and label 2-6
- 2) Pipette 10 μ l of Positive Control Template (RED) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 10 μ l 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 μ l |
| Tube 2 | 2 x 10 ⁴ per μ l |
| Tube 3 | 2 x 10 ³ per μ l |
| Tube 4 | 2 x 10 ² per μ l |
| Tube 5 | 20 per μ l |
| Tube 6 | 2 per μ l |

7. Pipette 5 μ l of standard template into each well for the standard curve according to your plate set-up
The final volume in each well is 20 μ l.

One Step Amplification Protocol

Amplification conditions using Lyophilised OneStep 2x RT-qPCR MasterMix.

| | Step | Time | Temp |
|--------------|--------------------------|---------|-------|
| | Reverse Transcription | 10 mins | 55 °C |
| | Enzyme activation | 2 mins | 95 °C |
| Cycling x 50 | Denaturation | 10 secs | 95 °C |
| | DATA COLLECTION * | 60 secs | 60 °C |

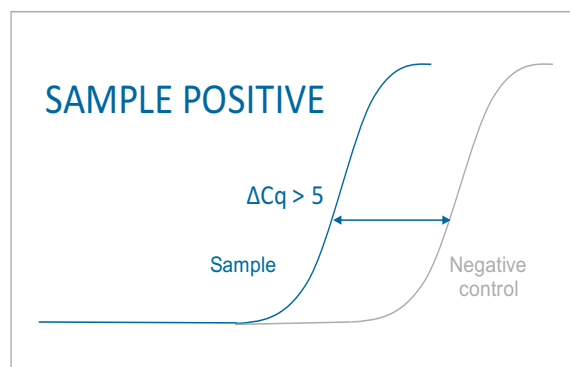
* Fluorogenic data should be collected during this step through the FAM and VIC channels

Interpretation of Results

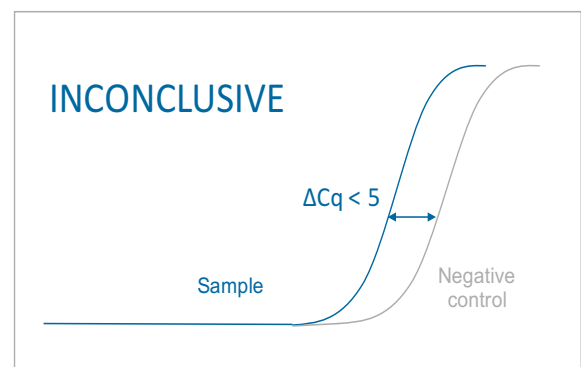
| Target (FAM) | Internal control (VIC) | Positive control | Negative control | Interpretation |
|--------------|------------------------|------------------|------------------|--|
| ≤ 30 | + / - | + | - | POSITIVE QUANTITATIVE RESULT calculate copy number |
| > 30 | + | + | - | POSITIVE QUANTITATIVE 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 | * |
| - | - | + | - | 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 CT 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. CT values of 28 ± 3 are 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.