Primerdesign™ Ltd

# Influenza A virus subtype H5N1 (avian influenza)

## genesig® Easy Kit
## 2 Target Gene Kit

for use on the genesig® q16

50 reaction

GENESIG

Kits by Primerdesign

For general laboratory and research use only

# genesig® Easy: at a glance guide

### For each RNA test

| Component | Volume | Lab-in-a-box pipette | |
|---|---|---|---|
| H5 primer/probe mix | 5 µl | | |
| Your RNA sample | 5 µl | | |
| oasig OneStep Master Mix | 10 µl | | |

| Component | Volume | Lab-in-a-box pipette | |
|---|---|---|---|
| N1 primer/probe mix | 5 µl | | |
| Your RNA sample | 5 µl | | |
| oasig OneStep Master Mix | 10 µl | | |

### For each positive control

| Component | Volume | Lab-in-a-box pipette | |
|---|---|---|---|
| H5 primer/probe mix | 5 µl | | |
| Positive control template | 5 µl | | |
| oasig OneStep Master Mix | 10 µl | | |

| Component | Volume | Lab-in-a-box pipette | |
|---|---|---|---|
| N1 primer/probe mix | 5 µl | | |
| Positive control template | 5 µl | | |
| oasig OneStep Master Mix | 10 µl | | |

### For each negative control

| Component | Volume | Lab-in-a-box pipette | |
|---|---|---|---|
| H5 primer/probe mix | 5 µl | | |
| Water | 5 µl | | |
| oasig OneStep Master Mix | 10 µl | | |

| Component | Volume | Lab-in-a-box pipette | |
|---|---|---|---|
| N1 primer/probe mix | 5 µl | | |
| Water | 5 µl | | |
| oasig OneStep Master Mix | 10 µl | | |

# Kit Contents

- **H5 specific primer/probe mix (BROWN)**
  Once resuspended the kits should remain at -20ºC until ready to use.
- **N1 specific primer/probe mix (BROWN)**
  Once resuspended the kits should remain at -20ºC until ready to use.

- **Lyophilised oasig™ OneStep Master Mix**

- **Lyophilised oasig™ OneStep Master Mix resuspension buffer (BLUE lid)**

- **H5 positive control template (RED lid)**
- **N1 positive control template (RED lid)**

- **Internal extraction control RNA (BLUE lid)**

- **RNase/DNase free water (WHITE lid)**

- **Template preparation buffer (YELLOW lid)**

- **108 x genesig® q16 reaction tubes**

# Reagents and equipment to be supplied by the user

**genesig® q16 instrument**

**genesig® Easy DNA/RNA Extraction Kit**

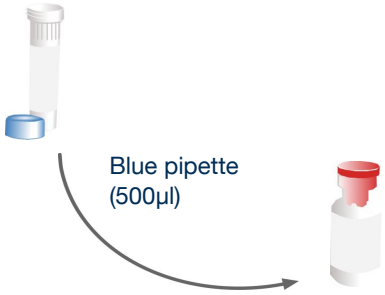
This kit is designed to work well with all processes that yield high quality RNA but the genesig Easy extraction method is recommended for ease of use.

**genesig® Lab-In-A-Box**

The genesig Lab-In-A-Box contains all of the pipettes, tips and racks that you will need to use a genesig Easy kit. Alternatively if you already have these components and equipment these can be used instead.

# Step-by-step guide

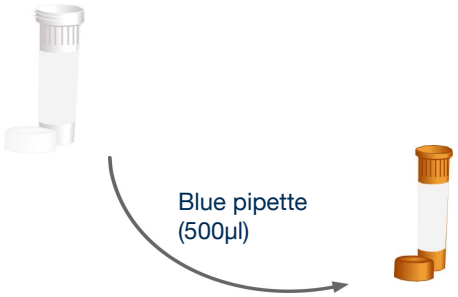
## 1. Resuspend the test components




This step must be performed for both primer/probe mixes.

Use the blue pipette to transfer 500µl* of the oasig OneStep Master Mix resuspension buffer into the tube of lyophilised oasig OneStep Master Mix and mix well by gently swirling.

*Transfering 525µl of the oasig OneStep Master Mix resuspension buffer to your oasig OneStep Master Mix (instead of the 500µl recommended above) will enable you to take full advantage of the 50 reactions by accounting for volume losses during pipetting. In order to do so with the genesig Easy fixed volume pipettes use 1x blue, 2x red and 1x grey pipettes to make the total volume. Please be assured that this will not adversely affect the efficiency of the test.




Then use the blue pipette to transfer 500µl of water into the brown tube labelled H5 or N1 primers/probe. Cap and shake tube to mix. A thorough shake is essential to ensure that all components are resuspended. **Failure to mix well can produce poor kit performance.**

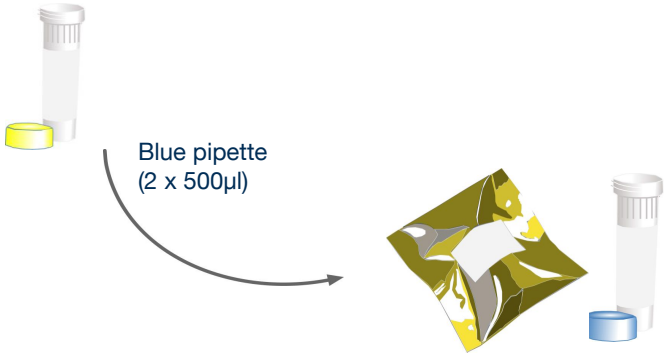
These components are now ready to use.

Store them in the freezer from hereon.

### Top tip

- Ensure that the primer/probe mix is mixed thoroughly before each use by shaking.
- Once resuspended do not expose genesig Easy kit to temperatures above -20°C for longer than 30 minutes at a time.

# 2. Internal extraction control



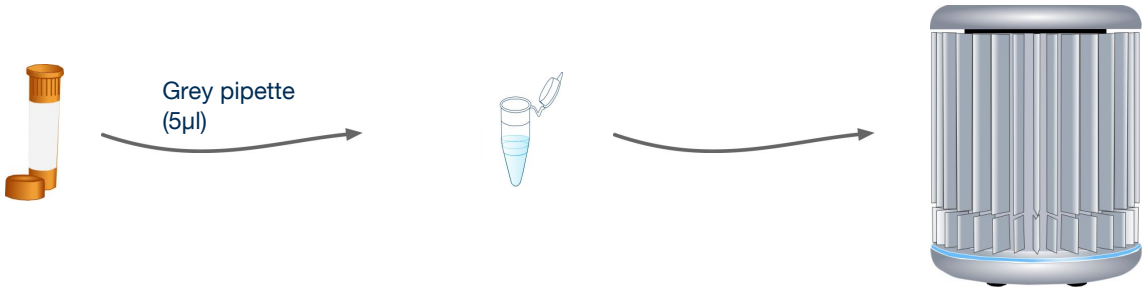

Use the blue pipette to transfer 1000µl (2 x 500µl) of template preparation buffer into the Internal Extraction Control RNA tube. Cap and shake tube to mix.

Your kit contains Internal Extraction Control RNA. This is added to your biological sample at the beginning of the RNA extraction process. It is extracted along with the RNA from your target of interest. The q16 will detect the presence of this Internal Extraction Control RNA at the same time as your target. This is the ideal way to show that your RNA extraction process has been successful.

**If using an alternative extraction kit:**
Use the red pipette to transfer 10µl of Internal Extraction Control RNA to your sample **after** the lysis buffer has been added then follow the rest of the extraction protocol.

**If using samples that have already been extracted:**
Use the grey pipette to transfer 5µl of Internal Extraction Control RNA to your extracted sample.

# 3. Add primer/probe mix to all reaction tubes

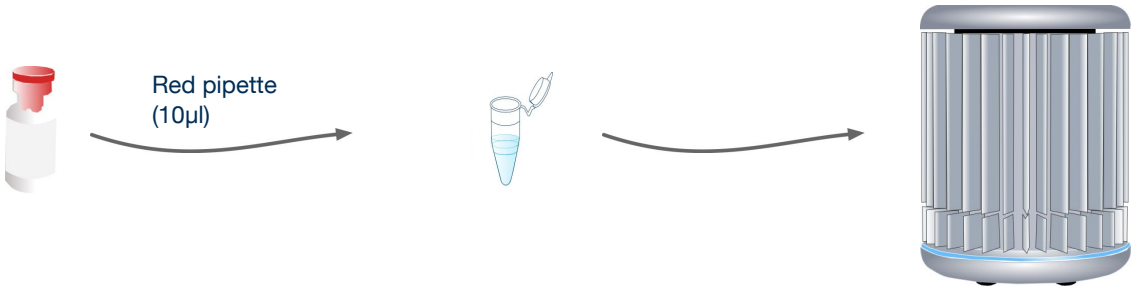



For each reaction to be run, use the grey pipette to add 5µl of your H5 or N1 primers/probe mix to every tube.

**Top tip**

- Always pipette the primer/probe mix directly into the bottom of the tube.
- You can label the tube lids to aid your reaction setup but avoid labelling tube sides.

# 4. Add Master Mix to all reaction tubes



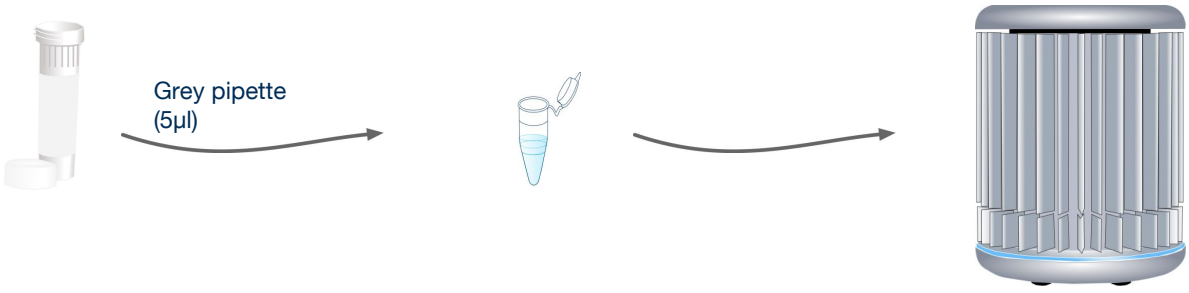

For reaction to be run, use the red pipette to add 10µl of the oasig OneStep Master Mix to the tubes containing primer/probe mix.

Move swiftly to begin your q16 run, as any delay after the oasig OneStep Master Mix has been added can effect the sensitivity of your test.

**Top tip**

- Always add the oasig OneStep Master Mix to the side of the tube to reduce the introduction of bubbles.

# 5. Negative control




For each test you will require a negative control. Instead of RNA, water is used. This sample should typically prove negative thus proving that all of your positive samples really are positive.

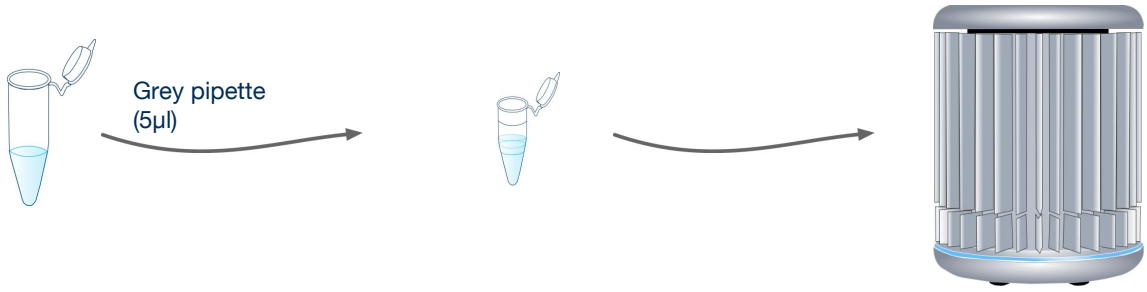
To create a negative control reaction simply use the grey pipette to add 5µl of the water to the required reaction tubes. Close these tubes after adding the water.

Because some genesig kit targets are common in the environment you may occasionally see a “late” signal in the negative control. The q16 software will take this into account accordingly.

**Top tip**

- Always add water to the side of the tube to reduce the introduction of bubbles.

# 6. Set up a test

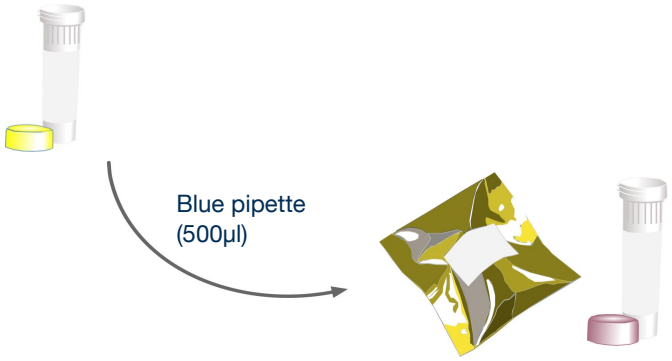



For each sample you wish to analyse, use the grey pipette to add 5µl of your RNA sample to the required reaction tubes. Close these tubes after adding the sample. Always change pipette tips between samples.

**Top tip**

- Always add the RNA sample to the side of the tube to reduce the introduction of bubbles.

# 7. Positive control

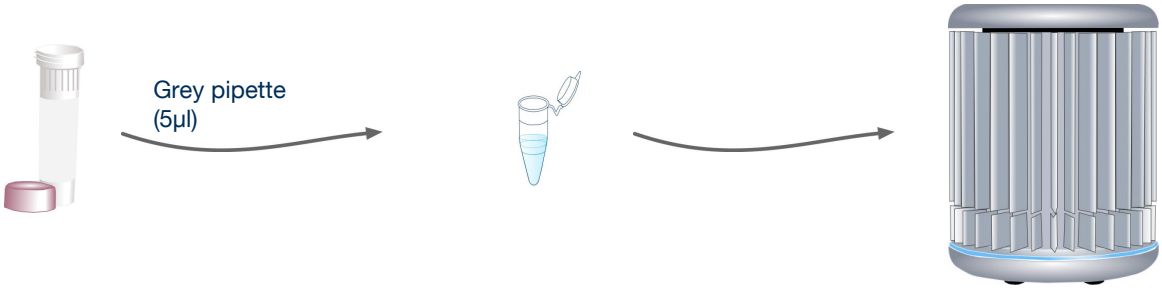



Use the blue pipette to transfer 500µl of template preparation buffer into each of the positive control template tubes. Cap and shake tube to mix.

Each time you run a test you will require a positive control. This is a small portion of RNA from your target of interest. It serves two purposes:

1. It will always test positive so it shows that everything is working as it should be.
2. The q16 software knows how much RNA is present in the positive control. So it can automatically compare your sample of interest with the positive control to calculate the amount of target RNA in your sample.

To create a positive control reaction simply use 5µl of the positive control instead of your RNA sample.



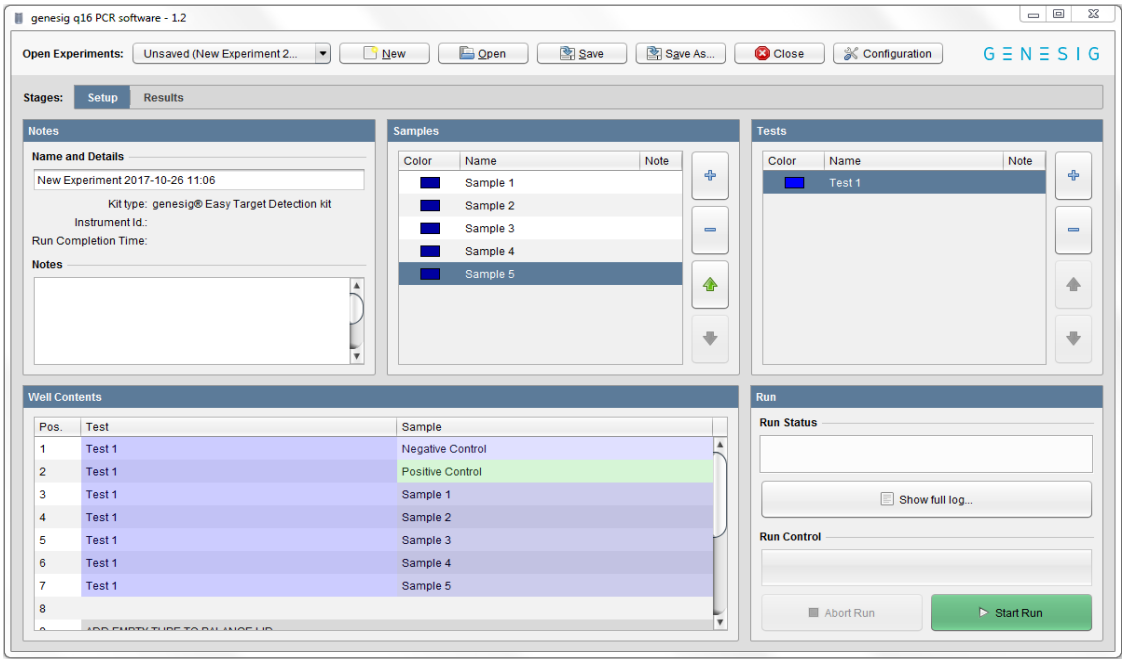

Take great care when setting up your positive control. The positive control template has the potential to give you a false positive signal in your other samples. Set positive controls up last after all other sample tubes are closed. Always change your pipette tip between samples. You may even choose to set up positive controls in a separate room.

**Top tip**

- Always add the positive control template to the side of the tube to reduce the introduction of bubbles.

# 8. Running the test

Place the tubes into the correct positions in your q16 as defined by the software, this may include positioning of empty tubes to ensure that the q16 lid is balanced. The run can then be started.




**Top tip**

- Before loading tubes into the q16, check for bubbles! Flick the bottom of the tubes to remove any bubbles that may have formed during the test setup.
- Apply centrifugal force with a sharp wrist action to ensure all solution is at the bottom of the reaction tube.
- When repeating a test you can use a previous file as a template by clicking ‘open’ then selecting File name > Files of Type > Experiment file as template

# What do my results mean?

Analysis of your data is carried out automatically by the genesig q16. The following information is designed to help you fully understand a result or to troubleshoot:

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 .

## “Positive”

**Explanation**
Your sample has produced a positive result. Your target of interest is present and you can use the reported quantity. As this is a two target gene kit, both target genes must be positive to confirm the test as a genuine positive.

## “Negative”

**Explanation**
Your sample has produced a negative result. The target is not present in your sample.

## “Test contaminated”

**Explanation**
The Negative Control should be completely free of any DNA/RNA. If you see this error message it means that at some point during the setup, the Negative Control has been contaminated with DNA/RNA and has given a positive signal. This contamination has invalidated the test. The Positive Control and your test samples are both possible sources of contaminating DNA/RNA. The genesig q16 reaction tubes from previous runs will also contain very high amounts of DNA so it is important that these are carefully disposed of after the run is completed and NEVER OPENED. It may be the case that your kits have become contaminated which will lead to the same problem occurring repeatedly.

**Solutions**

1. Clean your working area using a commercial DNA remover solution to ensure the area is DNA free at the start of your run and re-run the test.
2. If the problem persists then the kit has become contaminated and it will have to be discarded and replaced with a new kit. When you open the new kit, run a simple test to show that changing the kit has solved the problem. Prepare a test which includes only the Positive Control, the Negative Control and one ‘mock sample’. For the ‘mock sample’ add water instead of any sample RNA. The result for the Negative Control and the mock sample should be negative indicating that contamination is no longer present.

**Preventive action**
An ideal lab set-up has a 'Clean area' where the test reagents are prepared and a 'sample area' where DNA/RNA samples and the Positive Control templates are handled. The best workflow involves setting up all the test components (excluding the positive control template) in the clean area and then moving the tests to the sample area for sample and Positive Control addition. If this method is followed then the kit components are always kept away from possible sources of contamination. For extra security the Negative Control can be completely prepared and sealed in the clean area. All work areas should be decontaminated regularly with DNA remover.

## "Sample preparation failed"

**Explanation**
The test has failed because the quality of the sample was not high enough. The Internal Extraction Control component identifies whether the sample has been prepared correctly and is of suitable quality. This error message means that this quality control test has failed and the sample quality is not high enough for analysis.

**Solutions**
1. Check the sample preparation protocol for any user errors then repeat.
2. Poor quality samples can result from overloading the sample preparation protocol with too much starting material. Try reducing the amount of starting material then repeat.
3. Failing to add the Internal Extraction Control RNA to your sample during the sample preparation protocol can also lead to a reported result of "sample preparation failed". Ensure that this step has not been overlooked or forgotten. If your samples are derived from an archive store or from a process separate from your genesig Easy extraction kit; you must add 5µl of Internal Extraction Control RNA into each 0.5ml of your sample to make it suitable for use on the q16.

## "Positive result, poor quality sample"

**Explanation**
The test is positive so if you are only interested in obtaining a 'present or absent' answer for your sample then your result is reliable. However, the test contains an Internal Extraction Control component that identifies if the sample is of high quality. This quality control test has failed and the sample is not therefore of high enough quality to accurately calculate the exact copy number of RNA present. If you require quantitative information for your sample then proceed with the solutions below.

**Solutions**
1. For appropriate solutions, read the "Sample preparation failed" section of this handbook.

# “Test failed”

**Explanation**
The test has failed because the Positive Control has not worked. The Positive Control is present to show that all aspects of the test are working correctly together. When this control test fails, the test as a whole is invalidated. This finding indicates that a problem has occurred in the reaction set-up part of the experiment and has nothing to do with sample preparation.

**Solutions**

1. Check the entire workflow and test set-up to look for any user errors, then repeat the test e.g. have the right colour pipettes and solutions been used with the correct tubes?
2. Ensure the positive and negative controls are inserted into the correct wells of your q16.
3. A component of the test may have ‘gone off’ due to handing errors, incorrect storage or exceeding the shelf life. When you open a new kit, run a simple test to show that changing the kit has solved the problem. Prepare a test which includes only the Positive Control, the Negative Control and one ‘mock sample’. For the ‘mock sample’ add internal control template instead of any sample RNA. If the Positive Control works, the mock sample will now be called as a negative result.

# “Test failed and is contaminated”

**Explanation**
The Positive Control is indicating test failure, and the Negative Control is indicating test contamination. Please read the “Test Failed” and “Test contamination” sections of this technical support handbook for a further explanation.

**Solution**

1. For appropriate solutions, read both the “Test failed” and “Test contaminated” sections of this handbook.

# 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, 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) 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 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 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 e.mail to enquiry@primerdesign.co.uk and our bioinformatics team will answer your question.

# Kit storage and stability

This lyophilised 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.

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.

# Dynamic range of test

Under optimal PCR conditions genesig 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 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

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