Primerdesign™ Ltd

Avian Influenza A Virus Subtype H9N1

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	
H9 primer/probe mix	5 µl		
Your RNA sample	5 µl		
oasig OneStep Mastermix	10 µl		

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

For each positive control

Component	Volume	Lab-in-a-box pipette	
H9 primer/probe mix	5 µl		
Positive control template	5 µl		
oasig OneStep Mastermix	10 µl		

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

For each negative control

Component	Volume	Lab-in-a-box pipette	
H9 primer/probe mix	5 µl		
Water	5 µl		
oasig OneStep Mastermix	10 µl		

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

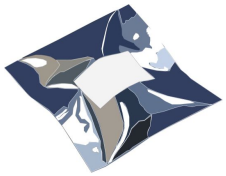
Kit Contents

- **H9 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 Mastermix**

- **Lyophilised oasig OneStep Mastermix resuspension buffer (BLUE lid)**

- **H9 positive control template (RED lid)**
- **N1 positive control template (RED lid)**



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

- **RNAse/DNAse free water (WHITE lid)**

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

Reagents and equipment to be supplied by the user

genesig® q16 instrument

genesig® Easy DNA/RNA Extraction Kit

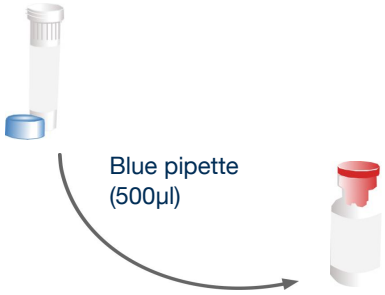
This kit is designed to work well with all processes that yield high quality RNA but the genesig® Easy extraction method is recommended for ease of use.

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

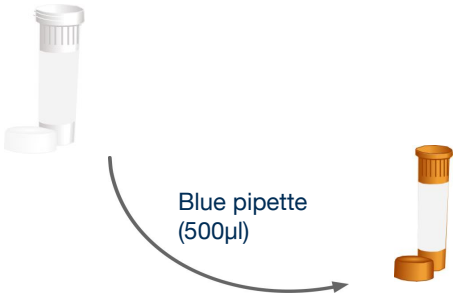
1. Resuspend the test components



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

Use the blue pipette to transfer 500µl* of the oasig OneStep mastermix resuspension buffer into the tube of lyophilised oasig OneStep mastermix and mix well by inversion.

*Transfering 525µl of the oasig OneStep mastermix resuspension buffer to your oasig OneStep mastermix (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 H9 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.**

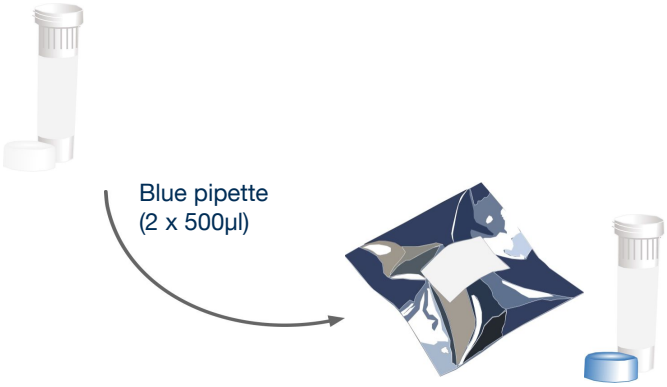
These components are now ready to use.

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



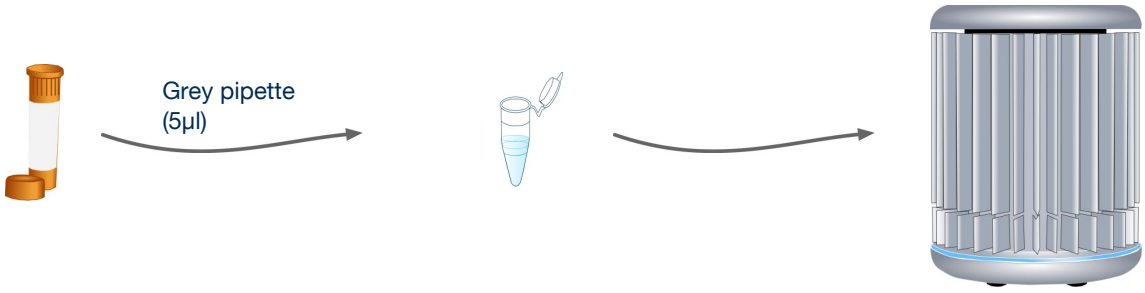
Use the blue pipette to transfer 1000µl (2 x 500µl) of water into the Internal Extraction Control RNA tube. Cap and shake tube to mix.

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

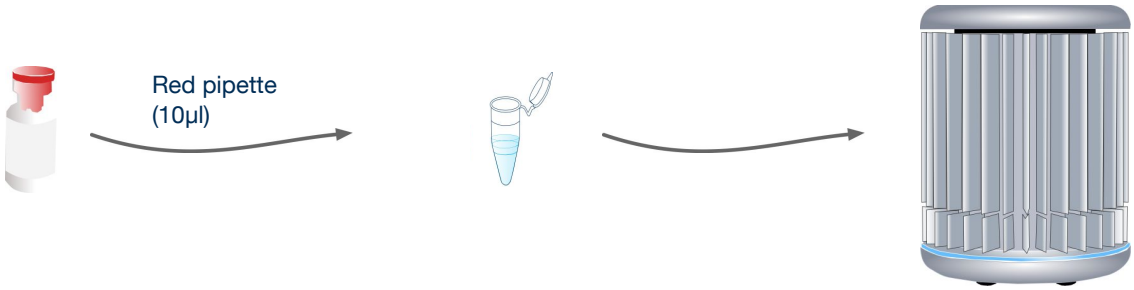


For each reaction to be run, use the grey pipette to add 5µl of your H9 or N1 primers/probe mix to every tube.

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 mastermix to all reaction tubes



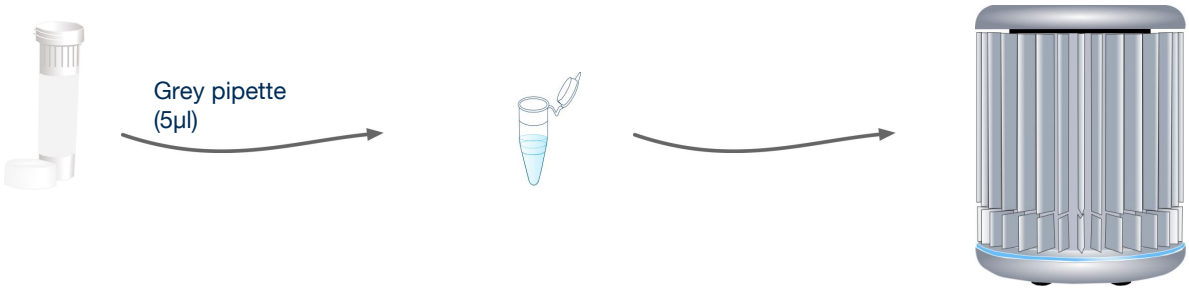
For reaction to be run, use the red pipette to add 10µl of the oasig OneStep mastermix to the tubes containing primer/probe mix.

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

Top tip

- Always add the oasig OneStep mastermix 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.

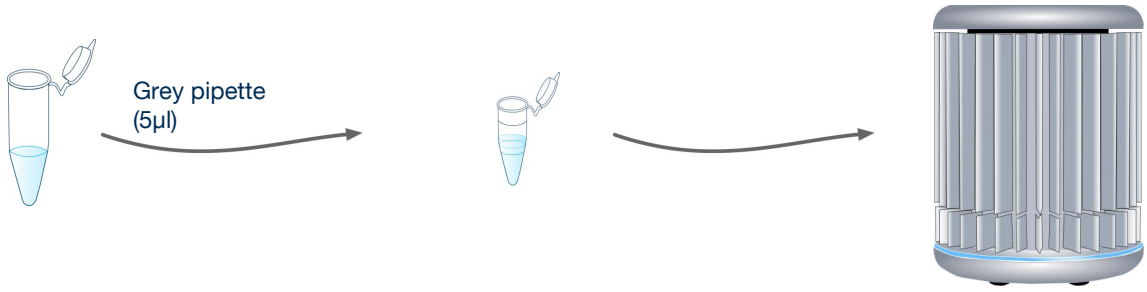
To create a negative control reaction simply use the grey pipette to add 5µl of the water to the required reaction tubes. Close these tubes after adding the water.

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

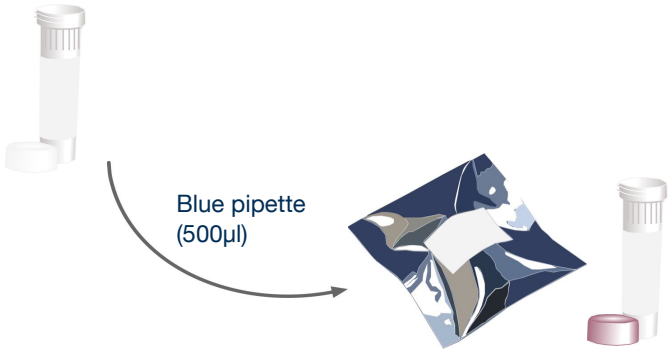


For each sample you wish to analyse, use the grey pipette to add 5µl of your RNA sample to the required reaction tubes. Close these tubes after adding the sample. Always change pipette tips between samples.

Top tip

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

7. Positive control

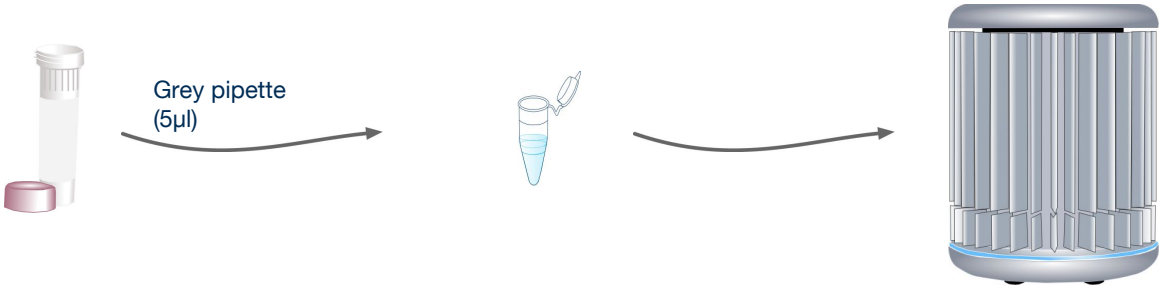


Use the blue pipette to transfer 500µl of water into each of the positive control template tubes. Cap and shake tube to mix.

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.



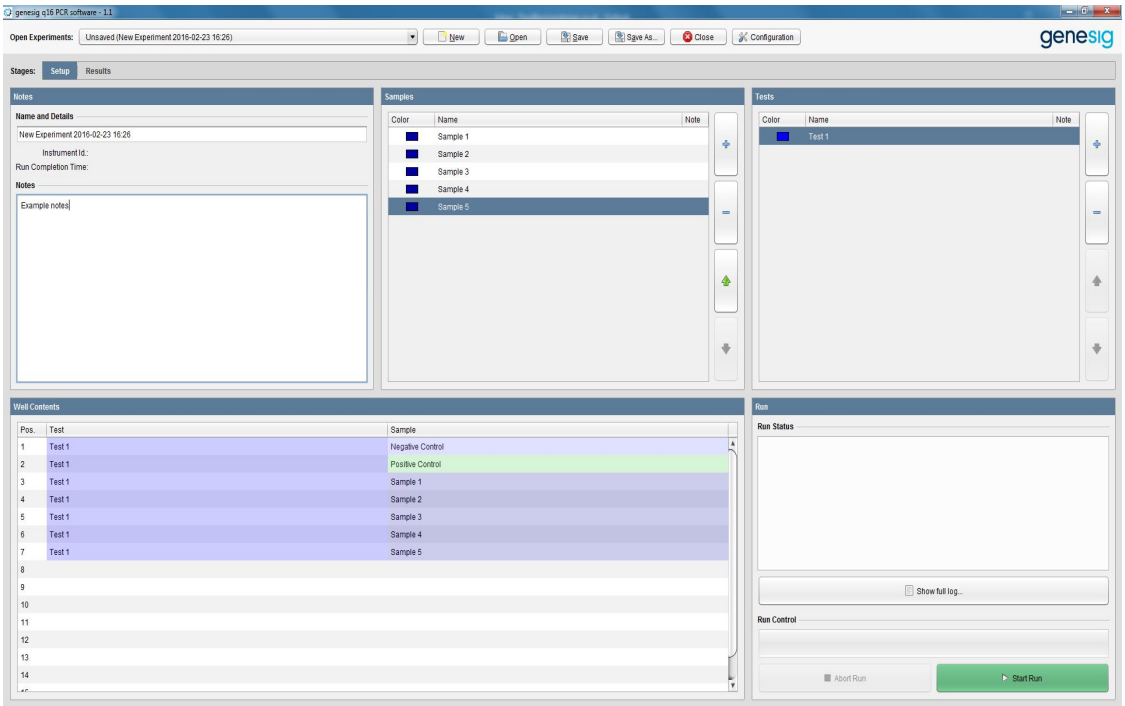
Take great care when setting up your positive control. The positive control template has the potential to give you a false positive signal in your other samples. Set positive controls up last after all other sample tubes are closed. Always change your pipette tip between samples. You may even choose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



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 H9 primer and probe set is designed to detect relevant avian influenza H9 sequences from a range of subtypes. Likewise the N1 primer and probe set will detect N1 sequences from a range of avian subtypes. Results from both primer sets taken together can therefore be used to make a positive determination for both the H9 and N1 segments of the H9N1 subtype. Samples that test negative for either the H9 or N1 alone indicates that an alternative subtype is present in the sample.

“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 solution such as “DNA remover” 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 experiment 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 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. The clean area should be decontaminated regularly with DNA remover to keep it clean.

"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 or if the sample is of low quality. This error message means that this quality control test has failed and the sample is not fit for analysis.

Solutions
1. Check the DNA/RNA extraction protocol for any user errors during preparation and repeat the DNA/RNA extraction.
2. Poor samples can result from overloading the DNA/RNA extraction with too much starting material. Try reducing the amount of starting material and repeat the DNA/RNA extraction.
3. Failing to add the Internal Extraction Control RNA to your sample during the DNA/RNA extraction process 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 secure as a positive test. 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. The exact copy number of DNA/RNA present cannot be accurately calculated in this instance. If you require quantitative information for your sample then proceed with the solutions below.

Solutions
1. Check the DNA/RNA extraction protocol for any user errors during preparation and repeat the DNA/RNA extraction.
2. Poor samples can result from overloading the DNA/RNA extraction with too much starting material. Try reducing the amount of starting material and repeat the DNA/RNA extraction.
3. Failing to add the Internal extraction Control RNA to your sample during the DNA/RNA extraction process can also lead to a reported result of "positive result, poor quality sample". 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.

“Test failed”

Explanation
The Positive Control is present to show that all aspects of the test are working correctly together. This error message shows that the quality control test has failed and the test as a whole is invalidated. This finding indicates that a problem has occurred in the test set-up part of the experiment and has nothing to do with DNA/RNA extraction.

Solutions

1. Check the entire workflow to look for any user errors during test set-up and repeat the test e.g. have the right colour pipettes and solutions been used with the correct tubes?
2. A component of the test may have ‘gone off’ due to handing errors, incorrect storage or exceeding the shelf life. Open a new kit and run a simplified 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. If the Positive Control works, the mock sample will now be called as a negative result indicating that all the components of this kit are working correctly.

“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.

Avian Influenza A Virus Subtype H9N1

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 some strains of avian influenza are pathogenic in humans typically H5N1, H7N3, H7N7 and H7N9. H10N8 has proven fatal in China as of Dec 2013.
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.
Influenza type A viruses are 80–120 nanometers 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 hemagglutinin (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.

Specificity

The Primerdesign™ genesig® Kit for Avian Influenza A Virus Subtype H9N1 (H9N1) genomes is designed for the in vitro quantification of H9N1 genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the H9N1 genome.

The primers and probe sequences in this kit have 100% homology with a broad range of H9N1 sequences based on a comprehensive bioinformatics analysis.

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® H9N1 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.

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