Primerdesign™ Ltd

Avian Influenza A Virus Subtype H9N1

Hemagglutinin H9 & Neuramidase N1

genesig® Advanced Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to 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 Contents

- **Hemagglutinin (HA) H9 gene primer/probe mix (150 reactions BROWN)**
  FAM labelled

- **Neuramidase (NA) N1 gene primer/probe mix (150 reactions BROWN)**
  FAM labelled

- **Hemagglutinin (HA) H9 gene positive control template (for Standard curve RED)**

- **Neuramidase (NA) N1 gene 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

- **H9 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 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 with minimal PCR inhibitors.

oasig™ Lyophlised OneStep or Precision™ OneStepPLUS 2x qRT-PCR Mastermix

Contains complete one step qRT-PCR Mastermix

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 -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® 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.

Trademarks

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Principles of the test

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.

Real-time PCR

A H9N1 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 H9N1 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.

One Step vs. Two step real-time PCR

When detecting/quantifying the presence of a target with an RNA genome Primerdesign recommend the use of a one step qRT-PCR protocol. One step qRT-PCR combines the reverse transcription and real-time PCR reaction in a simple closed tube protocol. This saves significant bench time but also reduces errors. The sensitivity of a one step protocol is also greater than a two step because the entire biological sample is available to the PCR without dilution. This kit will also work well with a two step approach (Precision™ nanoScript2 reverse transcription kit and PrecisionPLUS™ Mastermix) if required but the use of oasig™ Onestep or Precision™ OneStepPLUS Mastermix is the preferred method.

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 H9N1 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 H9N1 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 H9N1 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 H9N1 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. Primerdesign 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	
H9 primer/probe mix (BROWN)	165 µl
N1 primer/probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
H9N1 RT primer mix (GREEN)	165 µl
Endogenous control primer/probe mix (BROWN)	165 µl
Pre-PCR heat-sealed foil	
Internal extraction control RNA (BLUE)	600 µl

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	
H9 Positive Control Template (RED) *	500 µl
N1 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 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 4µl 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 RT-PCR detection protocol

A one step approach combining the reverse transcription and amplification in a single closed tube is the preferred method. If, however, a two step approach is required see page 11.

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
oasig™ OneStep or Precision™ OneStepPLUS 2x qRT-PCR Mastermix	10 µl
H9 or N1 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 Precision™ OneStepPLUS 2x qRT-PCR Mastermix	10 µl
Endogenous control primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final Volume	**15 µl**

3. **Pipette 15µl of these mixes into each well according to your real-time PCR experimental plate set up.**

4. **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.

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

Component	Volume
oasig™ OneStep or Precision™ OneStepPLUS 2x qRT-PCR Mastermix	10 µl
H9 or N1 primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final Volume	**15 µl**

6. **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 10 µl 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

7. **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.

One Step Amplification Protocol

Amplification conditions using oasig™ OneStep or Precision™ OneStepPLUS 2x RT-qPCR Mastermix.

	Step	Time	Temp
	Reverse Transcription	10 mins	55 °C
	Enzyme activation	2 mins	95 °C
Cycling x50	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

Alternative two step reverse transcription/real-time PCR protocol

Reverse Transcription

If you need to perform separate reverse transcription and amplification (two step Real Time PCR) then we recommend the Primerdesign Precision™ nanoScript2 Reverse Transcription kit. A reverse transcription primer (GREEN) is included and is designed for use with the Precision™ nanoScript2 reverse transcription kit. A protocol for this product is available at www.primerdesign.co.uk

1. **After reverse transcription, prepare a reaction mix according to the table below for each cDNA sample**

Component	Volume
PrecisionPLUS™ 2x qPCR Mastermix	10 µl
H9 or N1 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. **Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.**

3. **Prepare sample cDNA templates for each of your samples by diluting the RT reaction mix 1:5 in RNAse/DNAse free water.**

4. **Pipette 5µl of cDNA template into each well, according to your experimental plate setup.**
The final volume in each well is 20µl. For negative control wells use 5µl of RNAse/DNAse free water.

Alternative two step amplification protocol

Amplification conditions using PrecisionPLUS™ 2x qPCR Mastermix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37 °C
	Enzyme activation	2 mins	95 °C
Cycling x50	Denaturation	10 secs	95 °C
	DATA COLLECTION *	60 secs	60 °C

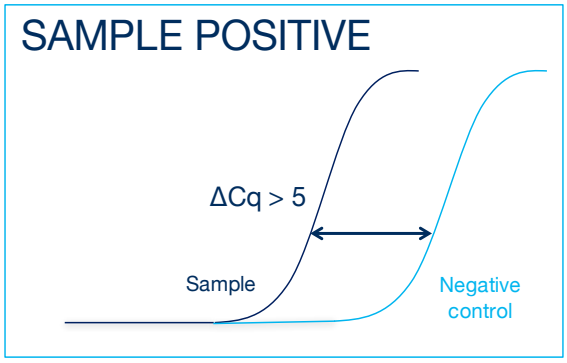
* Fluorogenic data should be collected during this step through the FAM and VIC channels
** Required if your Mastermix includes UNG to prevent PCR carryover contamination

Interpretation of Results

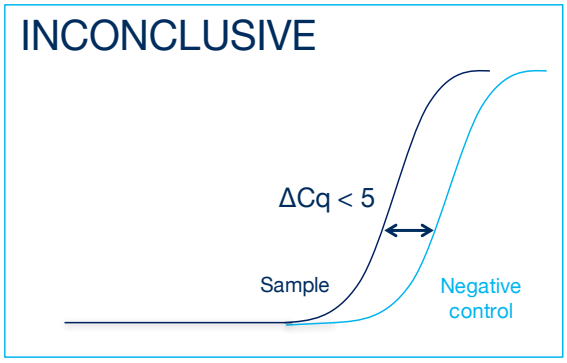
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	-	+	-	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may 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 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 are within the normal range. When amplifying a H9N1 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.