Primer Design Ltd

R01291

Avian Influenza A Virus Subtype H9N1

Kit version: 3

H9 - Hemagglutinin (HA) gene
N1 - Neuramidase (N) gene

genesig® Advanced Kit

150 tests

GENESIG
Kits by Primerdesign

For general laboratory and research use only

Quantification of Avian Influenza A virus subtype H9N1 genomes v3
genesig® Advanced kit handbook
Published Date: 14 October 2024

Product Description

This genesig® Advanced qPCR detection kit targets the hemagglutinin (HA) gene and neuraminidase (N) gene from avian influenza A virus subtype H9N1 which is commonly known as H9N1. H9N1 is an RNA virus which primarily infects poultry but may also infect wild birds and in rare cases, humans. Infection can lead to avian influenza. Influenza A is divided into subtypes based on two surface proteins: hemagglutinin (H) and neuraminidase (N). There are 18 different hemagglutinin subtypes and 11 neuraminidase subtypes, of which only H9N1 subtype will be detected by this qPCR kit.

Specificity

The kit is designed for the in vitro quantification of avian influenza A virus subtype H9N1 genomes and to have a broad detection profile. Specifically, the primers will detect over 95% of sequences available on the GISAID EpiFlu database at the time of last review.

Sequences from the prior five-year period and ten-year period were used for the H9 and N1 assay reviews respectively. Due to the sequence evolution observed in the Influenza genome over time, this is considered to be an adequate time period for analysis. Due to the limited sequence information for N1, a greater time period was used.

The H9 primer and probe set is designed to detect H9 sequences and therefore may detect H9 sequences from non-H9N1 subtypes.

The N1 primer and probe set is designed to detect N1 sequences from the H9N1 lineage but may detect N1 sequences from other subtypes.

A positive result for both H9 and N1, indicates the presence of H9N1 specifically

The dynamics of genetic variation means that new sequence information may become available after the initial design. If you require further information or have a specific question about the detection profile of this kit, then please send an e-mail to techsupport@primerdesign.co.uk and our team will answer your question.

Kit contents

Quantity	Component	Tube	Cap Colour
1	**H9_v2.0 primer/probe mix (150 reactions)** FAM labelled		**BROWN**
1	**N1(H7N1&H9N1) primer/probe mix (150 reactions)** FAM labelled		**BROWN**
1	**H9_v2.0 positive control template**		**RED** (in silver foil wrapper)
1	**N1(H7N1&H9N1) positive control template**		**RED** (in silver foil wrapper)
2	**Internal extraction control primer/probe mix (150 reactions)** VIC labelled as standard		**BROWN**
1	**Internal extraction control RNA (150 reactions)**		**BLUE** (in gold foil wrapper)
1	**Endogenous control primer/probe mix (150 reactions)** FAM labelled, Target: chicken ACTB as standard		**BROWN**
3	**Template preparation buffer** for resuspension of internal control template, positive control template and standard curve preparation		**YELLOW**
1	**RNase/DNase free water** for resuspension of primer/probe mixes		**WHITE**

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 nucleic acid with minimal PCR inhibitors.

oasig®PLUS OneStep Lyophilised qPCR Master Mix
This kit is intended for use with oasig®PLUS OneStep Lyophilised qPCR Master Mix.

Pipettors and filter tips

Vortex and centrifuge

1.5 ml microtubes

qPCR plates or 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.

Primer Design Ltd does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

This kit can be used with all types of samples from various origins. Please ensure that the extracted nucleic acid samples are suitable in terms of purity, concentration, and RNA integrity. An internal extraction control is provided to test for non-specific PCR inhibitors.

Dynamic range of test

Under optimal PCR conditions the kit can achieve priming efficiencies between 90-110% and detect less than 100 copies of target template. If running a positive control standard curve for a quantitative result, and an efficiency of between 90% to 110% is not achieved, then the run should be repeated with a freshly prepared standard curve.

Principles of the test

Real-time PCR

Target specific primer/probe mixes are provided, and these can be detected through the FAM channel.

The primer/probe mixes provided exploit the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridise to the target 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 qPCR platforms.

Positive control

For copy number determination and as a positive control for the PCR set-up, the kit contains positive control templates. This can be used to generate standard curves of the target copy number/Cq value. Alternatively, the positive controls 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/probes for detecting the target genes 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 controls do 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 positive controls to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive controls into the positive control wells.

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. It is also known as a No Template Control or NTC.

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 indicates 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 detection of the 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

A primer/probe mix for detection of the endogenous control gene is included in the kit, which allows confirmation of a valid biological sample from this host. Detection of the endogenous control is through the FAM channel, and it is therefore NOT possible to perform a multiplex reaction with the target specific primer/probe mix. Amplification of the endogenous control may depend on the sample type used. Please note that if samples from a different species are used, the endogenous control may not be appropriate, but the internal extraction control is advised to be used

Resuspension protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting is 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 the lyophilised primer/probe mix or template is in the base of the tube and is not lost 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
Pre-PCR pack	
H9_v2.0 primer/probe mix (BROWN)	165 µl
N1(H7N1&H9N1) 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
Pre-PCR heat-sealed foil	
Internal extraction control RNA template (BLUE)	600 µl
Post-PCR heat-sealed foil	
H9_v2.0 Positive Control Template (RED) *	500 µl
N1(H7N1&H9N1) 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 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 prepared proceed immediately to the OneStep 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®PLUS OneStep Lyophilised qPCR Master Mix	10 µl
H9_v2.0 or N1(H7N1&H9N1) 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 useful information regarding the quality of the biological sample.

Component	Volume
oasig®PLUS OneStep Lyophilised 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 15µl of these mixes into each well according to your qPCR 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®PLUS OneStep Lyophilised qPCR Master Mix	10 µl
H9_v2.0 or N1(H7N1&H9N1) 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 90µl of template preparation buffer into 5 tubes and label 2-6**
   b) **Pipette 10µl of Positive Control Template (RED) into tube 2**
   c) **Vortex thoroughly**
   d) **Change pipette tip and pipette 10µl 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)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 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.

OneStep RT-qPCR Amplification Protocol

Amplification conditions using oasig®PLUS OneStep Lyophilised 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

*H9_v2.0 (FAM)	*N1 (H7N1&H9N1) (FAM)	Internal control (VIC)	**Positive control	Negative control	***Interpretation
+	-	+/-	+	-	**POSITIVE RESULT FOR H9**
-	+	+/-	+	-	**POSITIVE RESULT FOR N1**
+	+	+/-	+	-	**POSITIVE RESULT FOR H9N1**
-	-	+	+	-	**NEGATIVE RESULT**
+ / -	+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+ / -	+ / -	+	> 35	****
-	-	-	+	-	**SAMPLE PREPARATION FAILED**
+ / -	+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

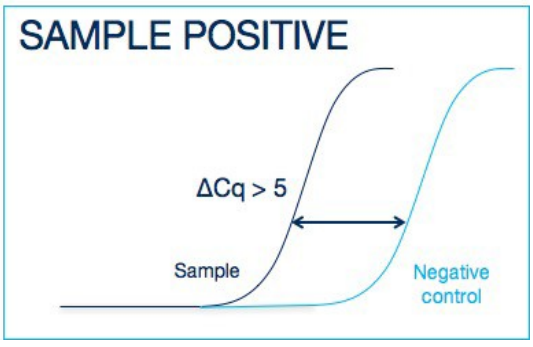
* The Cq value for the targets (FAM) should either be ≤30 with positive or negative internal control (IC), or >30 with a positive internal control (IC), to use the result for **quantitation.** In this case, the copy number can be calculated.

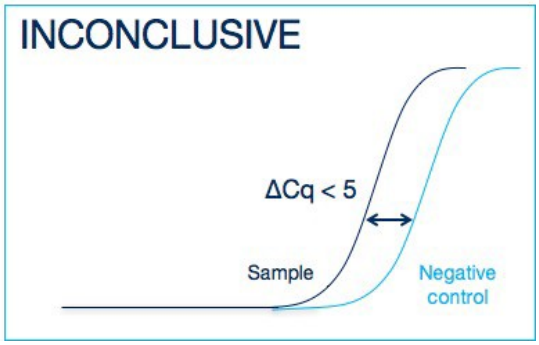
If the target Cq is >30 and the IC is negative, this is a **qualitative** result. Do not report the copy number as this may be due to poor sample extraction.

** 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.

*** If a sample is positive for H9 but negative for N1, this could be due to the presence of one of the other H9 subtypes. Conversely if a sample is positive for N1 but negative for H9, this could be due to the presence of one of the other N1 subtypes. A sample is confirmed as H9N1 if both targets (FAM) are positive.

**** 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 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.

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, Primer Design Ltd genesig® detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained in violation of the general GLP guidelines and the manufacturer's recommendations, the right to claim under guarantee is expired.

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