Primerdesign™ Ltd

H10N8

Hemagglutinin (HA) gene & Neuraminidase (NA) gene

genesig® Standard Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to H10N8

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 but now H10N8 has proven fatal in China as of Dec 2013.

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 H10N8 (H10N8) genomes is designed for the in vitro quantification of H10N8 genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the H10N8 genome.
The primers and probe sequences in this kit have 100% homology with a broad range of H10N8 sequences based on a comprehensive bioinformatics analysis.

The H10 primer and probe set is designed to detect all relevant H10 sequences. The N8 primer and probe set is designed to detect all relevant N8 sequences from the avian H10N8 lineage and may detect N8 from other subtypes.

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) gene primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **Neuraminidase (NA) gene primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **Hemagglutinin (HA) gene positive control template (for Standard curve RED)**
- **Neuraminidase (NA) gene positive control template (for Standard curve RED)**
- **H10 and N8(H10) RT primer mix (150 reactions GREEN)**
  Required for two step protocol only
- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **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™ lyophilised OneStep or Precision®PLUS OneStep 2X RT-qPCR Master Mix

Contains complete OneStep RT-qPCR master mix

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. 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 H10N8 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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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 Applera Genomics (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 Primerdesign 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

This kit contains two primer and probe sets. The h10 primer and probe set has been designed to detect all relevant avian H10 sequences from a range of avian subtypes. The N8 primer and probe set has been designed to detect all relevant avian N8 sequences from a range of subtypes. In order to determine whether the H10N8 subtype is present in a sample, both primer and probe sets must be tested and both be positive. Negative results from either the H10 or N8 primer and probe assays are indicative of the presence of alternative subtypes.

Real-time PCR

A H10N8 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 H10N8 DNA/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.

OneStep vs. Two step qPCR

When detecting/quantifying the presence of a target with an RNA genome Primerdesign recommend the use of a OneStep RT-qPCR protocol. OneStep RT-qPCR combines the reverse transcription and qPCR reaction in a simple closed tube protocol. This saves significant bench time but also reduces errors. The sensitivity of a OneStep 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 Master Mix) if required but the use of oasig OneStep or PrecisionPLUS OneStep Master Mix 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 H10N8 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 H10N8 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.

Resuspension 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. **Resuspend 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	
H10 primer/probe mix (BROWN)	165 µl
N8(H10) primer/probe mix (BROWN)	165 µl
H10N8 RT primer mix (GREEN)	165 µl

3. **Resuspend 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	
H10 Positive Control Template (RED) *	500 µl
N8(H10) 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.

OneStep RT-qPCR detection protocol

A OneStep 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 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 OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix	10 µl
H10 or N8(H10) primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

2. **Pipette 15µl of this mix into each well according to your qPCR experimental plate set up.**

3. **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.

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

Component	Volume
oasig OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix	10 µl
H10 or N8(H10) primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

5. **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 10µl 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

6. **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 OneStep or PrecisionPLUS OneStep 2X RT-qPCR Master Mix.

	Step	Time	Temp
	Reverse Transcription	10 min	55 °C
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

* Fluorogenic data should be collected during this step through the FAM channel

Alternative two step detection protocol

Reverse Transcription

If you need to perform separate reverse transcription and amplification (two step qPCR) 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 Master Mix	10 µl
H10 or N8(H10) primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

2. **Pipette 15µl of this mix into each well according to your qPCR 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 Master Mix.

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

* Fluorogenic data should be collected during this step through the FAM channel

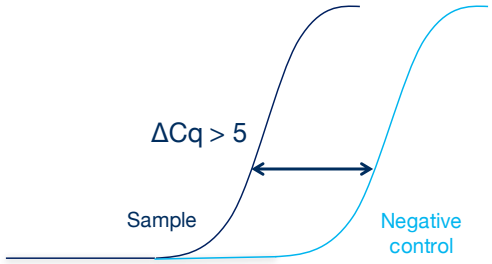
Interpretation of results

Target	Positive control	Negative control	Interpretation
+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
-	+	-	**NEGATIVE RESULT**
+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+	> 35	*
+ / -	-	+ / -	**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

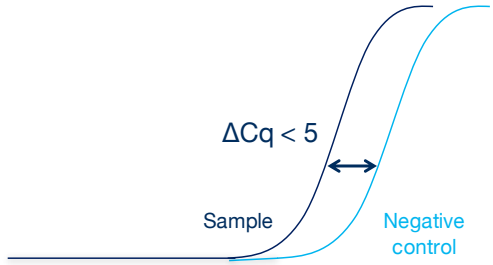
*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:

SAMPLE POSITIVE



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.

INCONCLUSIVE



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.