Primerdesign™ Ltd

Swine H1N1 Influenza Human Pandemic Strain

M1 - global Influenza A
&
N1- specific for Swine H1N1 Influenza – Human Pandemic Strain

genesig® Advanced Kit

150 tests

GENESIG
Kits by Primerdesign

For general laboratory and research use only

Introduction to Swine H1N1 Influenza Human Pandemic Strain

Influenza, commonly known as the flu, is an infectious disease of birds and mammals caused by an RNA virus of the family Orthomyxoviridae (the influenza viruses). In people, common symptoms of influenza are fever, sore throat, muscle pains, severe headache, coughing, weakness and fatigue. In more serious cases, influenza causes pneumonia, which can be fatal, particularly in young children and the elderly. Although the common cold is sometimes confused with influenza, it is a much less severe disease and caused by a different virus. Similarly, gastroenteritis is sometimes called "stomach flu" or "24-hour flu", but is unrelated to influenza.

Typically, influenza is transmitted from infected mammals through the air by coughs or sneezes creating aerosols containing the virus, and from infected birds through their droppings. Influenza can also be transmitted by saliva, nasal secretions, faeces and blood. Infections either occur through direct contact with these bodily fluids, o by contact with contaminated surfaces. Flu viruses can remain infectious for over 30 days at 0°C (32°F) and about one week at human body temperature, although they are rapidly inactivated by disinfectants and detergents.

Flu spreads around the world in seasonal epidemics, killing millions of people in pandemic years and hundreds of thousands in non-pandemic years. Three influenza pandemics occurred in the 20th century – each following a major genetic change in the virus – and killed tens of millions of people. Often, these pandemics result from the spread of a flu virus between animal species. Since it first killed humans in Asia in the 1990s a deadly avian strain of H5N1 has posed the greatest influenza pandemic threat. However, this virus has not yet mutated to spread easily between people.

Vaccinations against influenza are most common in high-risk humans in industrialised countries and farmed poultry. The most common human vaccine is the trivalent flu vaccine which contains purified and inactivated material from three viral strains. Typically this vaccine includes material from two influenza A virus subtypes and one influenza B virus strain. A vaccine formulated for one year may be ineffective in the following year since the Influenza A Virus changes every year and different strains become dominant. Antiviral drugs can be used to treat influenza, with neuraminidase inhibitors being particularly effective.

Specificity

The Primerdesign genesig Kit for Swine H1N1 Influenza Human Pandemic Strain (H1N1- swine) genomes is designed for the in vitro quantification of H1N1-swine genomes. The kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences available at the time of design.

The dynamics of genetic variation mean that new sequence information may become available after the initial design. Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

The kit has 2 primer and probe sets. The M1 primers and probe detect ALL influenza A isolates including those originally isolated from both human and swine. The primers have 100% homology with over 95% of pandemic H1N1 sequences.

The N1 primer and probe set detects ONLY the pandemic swine flu H1N1. The primers and probe have 100% homology with over 95% of pandemic strains. The primers will not detect N1 from any seasonal endemic human or swine N1 strains.

If you require further information or have a specific question about the detection profile of this kit then please send an email to enquiry@primerdesign.co.uk and our bioinformatics team will answer your question.

Kit contents

- **M1 global influenza A primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **N1 swine H1N1 influenza specific primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **M1 global influenza A positive control template (for Standard curve RED)**
- **N1 swine H1N1 influenza specific 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
- **RNase/DNase-free water (WHITE)**
  for resuspension of primer/probe mixes
- **Template preparation buffer (YELLOW)**
  for resuspension of internal control template, positive control template and standard curve preparation

Reagents and equipment to be supplied by the user

Real-time PCR Instrument

Extraction kit
This kit is recommended for use with the genesig Easy DNA/RNA Extraction kit. However, it is designed to work well with all processes that yield high-quality RNA and DNA 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 0.1 ml PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20°C 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 H1N1-swine detection kits have very high priming efficiencies of >90% and can detect less than 100 copies of the target template.

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Principles of the test

The kit contains two Influenza A primers and probe sets. The M1 primer and probe set are designed to detect all influenza A sequences regardless of the species of origin. Samples that test positive with the M1 primer set contain an influenza A infection of unknown serotype. The N1 primer and probes are specific to the swine H1N1 pandemic strain. Samples that test positive with the N1 primer contain the pandemic swine H1N1 Influenza. Samples that test positive for M1 and negative for N1 are indicative of a naturally circulating influenza A infection which is not swine flu.

Real-time PCR

The primer and probe mix provided exploits with the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the target DNA/cDNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labelled 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 a standard curve for each target for 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 for each target must be included in the run. A positive result indicates that the primers and probes for detecting the H1N1-swine gene worked well 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 the 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 qPCR for the control RNA also indicate 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 the detection of the H1N1-swine target cDNA even when present at a 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 the 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 target primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Resuspension Protocol

To minimise 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 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
M1 primer/probe mix (BROWN)	165 µl
N1 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
Internal extraction control RNA (BLUE)	600 µl
M1 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 4 µl 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 setup 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 artefacts 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
M1 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 PrecisionPLUS OneStep 2X RT-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 15 µl of these mixes into each well according to your qPCR experimental plate set-up.

4. Pipette 5 µl of RNA template into each well, according to your experimental plate set-up.
For negative control wells, use 5 µl of RNase/DNase-free water. The final volume in each well is 20 µl.

5. 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
M1 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.

a. Pipette 90 µl of template preparation buffer into 5 tubes and label 2-6
b. Pipette 10 µl of Positive Control Template (RED) into tube 2
c. Vortex thoroughly
d. Change pipette tip and pipette 10 µl 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 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 5 µl of the standard template into each well for the standard curve according to your plate set-up
The final volume in each well is 20 µl.

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	95ºC
Cycling x50	Denaturation	10 s	95ºC
	DATA COLLECTION *	60 s	60ºC

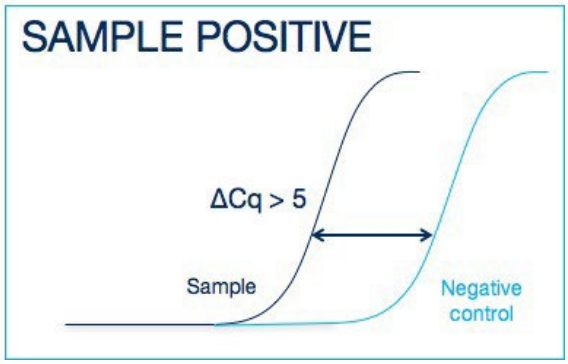
* Fluorogenic data should be collected during this step through the FAM and VIC channels

Interpretation of results

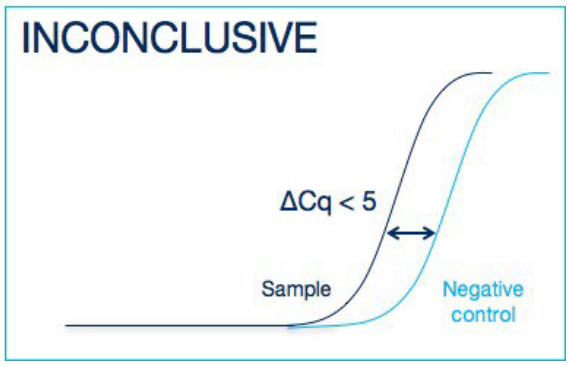
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
> 30	+	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
> 30	-	+	-	POSITIVE QUALITATIVE RESULT do not report the copy number as this may be due to poor sample extraction
-	+	+	-	NEGATIVE RESULT
+ / -	+ / -	+	≤ 35	EXPERIMENT FAILED due to test contamination
+ / -	+ / -	+	> 35	*
-	-	+	-	SAMPLE PREPARATION FAILED
+ / -	+ / -	-	+ / -	EXPERIMENT FAILED

A 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 an H1N1-swine 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.