Primer Design Ltd

R00953

Human Influenza A Virus (M2)

Kit version: v2

Target region:

M1/M2 genes

genesig® Advanced Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Human Influenza A Virus

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, and 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, or 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. The most recent influenza pandemic was the swine flu pandemic of 2009 with an estimated death toll between 150,000-500,000 people. Often, these pandemics result from the spread of a flu virus between animal species. Influenza virus A includes only one species: Influenza A virus which causes influenza in birds and some mammals. Strains of all subtypes of influenza A virus have been isolated from wild birds, although disease is uncommon. Some isolates of influenza A virus cause severe disease both in domestic poultry and, rarely, in humans. Occasionally viruses are transmitted from wild aquatic birds to domestic poultry, and this may cause an outbreak or give rise to human influenza pandemics.

Specificity

The genesig® Advanced Kit for Human Influenza A Virus (M2) (FluA_M2) is designed for the in vitro detection and quantification of Human Influenza A Virus genomes. The kit is designed 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.

This is intended as a human seasonal influenza kit. Specifically, this kit was designed to detect 95% of sequences on the GISAID EpiFlu database of the two subtypes of Influenza A virus, H1N1 and H3N2 from the last 5 years and human vaccine strains from 2012 onwards. There will also be some detection/cross reactivity with H1N1/H3N2 subtypes from animal origin and other subtypes of Influenza A. Due to the sequence evolution observed in the influenza A genome over successive flu seasons, 5 years is considered to be an adequate time period for analysis.

The dynamics of genetic variation mean that new sequence information may become available after the most recent review. 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

- **1x FluA_M2_v2.0 primer/probe mix (150 reactions, BROWN)**
  FAM labelled
- **1x FluA_M2_v2.0 positive control template (for Standard curve, RED)**
- **1x Internal extraction control primer/probe mix (150 reactions, BROWN)**
  VIC labelled as standard
- **1x Internal extraction control RNA (150 reactions, BLUE)**
- **1x Endogenous control primer/probe mix (150 reactions, BROWN)**
  FAM labelled, Target: Human ACTB gene as standard
- **1x RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **3x 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 genesig® Easy DNA/RNA Extraction kit or exsig®Mag. However, it is designed to work well with all processes that yield high-quality nucleic acid with minimal PCR inhibitors.

oasig® lyophilised OneStep or Precision Plus® OneStep 2X RT-qPCR Master Mix

This kit is intended for use with oasig® lyophilised OneStep or PrecisionPLUS® OneStep 2X RT-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 for shipping at ambient temperature but should be stored at -20°C upon 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

A target 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 target 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 a positive control template. This can be used to generate a standard curve of target 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 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. 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 RNA does not interfere with detection of the target RNA 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 minimize the risk of contamination with foreign RNA/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 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	
FluA_M2_v2.0 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 (**BLUE**)	600 µl
Post-PCR heat-sealed foil	
FluA_M2_v2.0 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.**

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® OneStep or Precision®PLUS OneStep 2X RT-qPCR Master Mix	10 µl
FluA_M2_v2.0 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. **(Optional) For each RNA sample prepare an endogenous control reaction according to the table below.**
   This control reaction will provide useful information regarding the quality of the biological sample.

Component	Volume
oasig® OneStep or Precision®PLUS 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 15µl of these mixes into each well according to your experimental qPCR 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 (**WHITE**). For positive control wells use 5µl of the positive control template (**RED**). The final volume in each well is 20µl.

5. (Optional) Standard curve preparation for quantitative analysis.

For quantitative analysis of the samples a standard curve dilution series can be prepared using the positive control template (**RED**). This is not required for qualitative analysis.

5.1 Reaction mix preparation for the standard curve.

Include sufficient reactions for each dilution of the standard curve:

Component	Volume
oasig® OneStep or Precision®PLUS OneStep 2X RT-qPCR Master Mix	10 µl
FluA_M2_v2.0 primer/probe mix (**BROWN**)	1 µl
RNase/DNase free water (**WHITE**)	4 µl
Final Volume	**15 µl**

5.2 Preparation of a 10-fold standard curve dilution series.

a. Pipette 90 µl of template preparation buffer (**YELLOW**) into 5 tubes and label them tube 2-6. The neat positive control tube (**RED**) is considered tube 1.

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** across the tubes 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

5.3 Pipette 15µl of reaction mix and 5µl of the respective standard 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

Recommended amplification conditions when using oasig® OneStep or Precision®PLUS OneStep 2X RT-qPCR Master Mix.

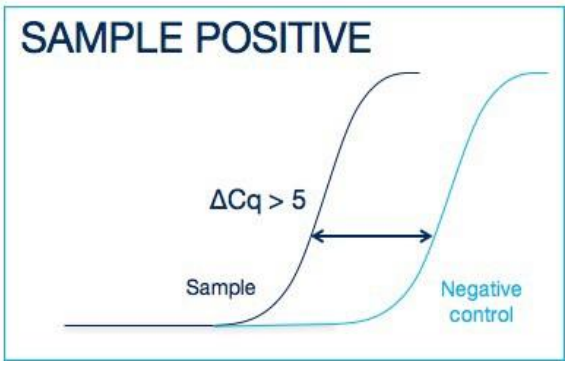
	Step	Time	Temp
	Reverse Transcription	10 min	55 °C
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

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

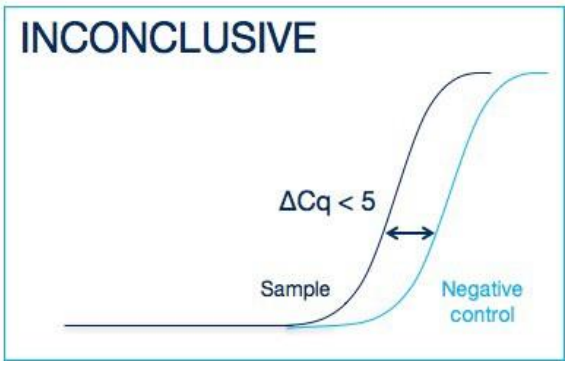
Interpretation of results

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 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**

*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.

Positive Control

The positive control template is expected to amplify between Cq 16 – 23 in the FAM channel. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised and 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.

Please note that if samples from a different species or environmental samples are used, the endogenous control may not be appropriate and would not result in amplification.

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