PrimerDesign ™ Ltd

Quantification of Human Influenza A Virus subtype H9 genomes

Haemoglutinin H9 gene

For general laboratory and research use only

Advanced Kit

Contents

Introduction to Human Influenza Virus	**3**
Specificity	**3**
Kit Contents	**4**
Reagents and equipment to be supplied by the user	**4**
Kit storage and stability	**4**
Suitable sample material	**6**
Dynamic range of test	**6**
Principles of the test	**7**
Bench side Protocol	**9**
Amplification Protocol	**13**
Interpretation of Results	**14**
Notices and disclaimers	**15**
Trademarks	**15**

Introduction to Human Influenza 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.[1] 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, feces 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. 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 that 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™ Quantification Kit for Influenza A Virus (H9)* Genomes is designed for the *in vitro* quantification of all current H9 isolates. The primers have very high homology with all reference sequences submitted since 2000 and therefore have a very broadest detection profile. These primers will also detect most historical sequences for this subtype. However, due to the inherent instability of RNA viral genomes, it is not possible guarantee quantification of all clinical isolates.

Kit Contents

- **Pathogen specific primer/probe mix (150 reactions BROWN)**
- **Pathogen positive control template (for Standard curve RED)**
- **Internal extraction control RNA (150 reactions BLUE)**
- **Internal extraction control primer/probe mix (150 reactions BROWN)**
- **Endogenous ACTB primer/probe mix (150 reactions BROWN)**
- **Internal extraction control/Pathogen/ACTB RT primer mix (150 reactions GREEN)**
- **RNAse/DNAse free water**

Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
- **Mastermix or Mastermix components**
  This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x *Precision*™ 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. Once the lyophilised components have been re-suspended the kit should be stored at -20°C. Unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.

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 PrimerDesign pathogen detection kits have very high priming efficiencies of >95% and can detect between $1X10^8$ and $1X10^2$ copies of target template.

Principles of the test

Real-time PCR

A pathogen specific primer and probe mix is provided and the target sequence 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 pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5`-reporter dye and a downstream, 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.

Positive control

For copy number determination, and as a positive control for the PCR set up, the kit contains positive control template. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in your 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.

Negative control

To confirm absence of contamination, 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. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.

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 pathogen target cDNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a CT value of 26+/-3.

Endogenous ACTB control

To confirm extraction of a valid biological template a primer and probe mix is included to detect the Actin Beta (ACTB) gene. Detection of ACTB is through the FAM channel and it is **NOT** therefore possible to perform a multiplex for ACTB and the pathogen primers. A poor ACTB signal may indicate that the sample did not contain sufficient human material.

Carry-over prevention using UNG (optional)

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 0.2 U 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 (95°C for 10 minutes).

Bench side 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 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 according to the table below**
   To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
Pre-PCR box	
Pathogen Primer/Probe mix (**BROWN**)	165 µl
Internal extraction control RNA (**BLUE**)	600 µl
Internal extraction control primer/probe mix (**BROWN**)	165 µl
Internal extraction control/Pathogen/ACTB RT primer mix (**GREEN)**	165 µl
Endogenous ACTB primer/probe mix (**BROWN**)	165 µl
Post PCR bottle	
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 biological sample as this will lead to degradation and a loss in signal strength.

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 manufactures protocols**

Reverse Transcription

The protocols below are for PrimerDesign *Precision*™ Reverse Transcription kit. If you are using an alternative RT kit then follow the manufacturer's instruction.

1. **For each RNA sample prepare a reaction mix according to the table below:**

Component	1 reaction
RNA template	X µl
Internal extraction control/Pathogen/ACTB RT primer mix (**GREEN)**	1.0 µl
dNTP mix 10mM of each (**ORANGE**)	1.0 µl
RNAse/DNAse free water (**WHITE**)	X µl
Final volume	**10 µl**

2. **Heat samples to 65°C for 5 minutes.**
   This can be achieved using a thermostatically controlled "hot block" or heated water bath. Do not heat the RNA to a higher temperature as this can lead to RNA degradation.

3. **Immediately cool the tubes in an ice water bath.**
   It is important that the samples are transferred directly from 65°C to the ice. Do not allow the samples to cool prior to cooling on ice.

4. **For each RNA sample prepare a reaction mix according to the table below:**

Component	1 reaction
MMLV 5Xbuffer (**CLEAR**)	4.0 µl
RNA/DNAse free water (**WHITE**)	5.2 µl
MMLV enzyme (**CLEAR**)	0.8 µl
Final volume	**10 µl**

5. **Add 10µl of this mix to each of the samples on ice.**

6. **Apply a lid to each sample, mix tubes by briefly vortexing and incubate at 42°C for 60 minutes.**
   This can be achieved using a thermostatically controlled "hot-block" or heated water bath

7. **Store cDNA samples at -20°C until use.**

Real-time PCR detection

1. **Prepare a reaction mix according to the tables below:**
   Include sufficient reactions for the standard curve wells (8 samples in duplicate) and also the negative control.

   Pathogen detection mix

Component	1 reaction
2X Precision™ Mastermix	10 µl
Pathogen primer/probe mix (**BROWN**)	1.0 µl
Internal extraction control primer/probe mix (**BROWN**)	1.0 µl
RNAse/DNAse Free water	3.0 µl
Final volume	**15 µl**

   Endogenous ACTB detection mix (Optional)

Component	1 reaction
2X Precision™ Mastermix	10 µl
Endogenous ACTB primer/probe mix (**BROWN**)	1.0 µl
RNAse/DNAse Free water	4.0 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 (suggested concentration 5ng/µl) in RNAse/DNAse free water.**
   If the concentration of RNA that was used to make the cDNA is not known, then dilute your RT reaction mix 1:20 (10µl of sample DNA and 90µl of water)

4. **Pipette 5µl of diluted cDNA 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. Preparation of standard curve dilution series

1) Pipette 900µl of RNAse/DNAse free water into 7 tubes and label 2-8
2) Pipette 100µl of Positive Control Template (**RED**) into tube 2
3) Vortex thoroughly
4) Change pipette tip and pipette 100µl 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**)	$2X10^7$ per µl
Tube 2	$2X10^6$ per µl
Tube 3	$2X10^5$ per µl
Tube 4	$2X10^4$ per µl
Tube 5	$2X10^3$ per µl
Tube 6	$2X10^2$ per µl
Tube 7	20 per µl
Tube 8	2 per µl

6. Pipette 5µl of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20µl

Amplification Protocol

Amplification conditions using PrimerDesign 2X *Precision*™ MasterMix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37°C
	Enzyme Activation (if required)***	10 mins	95°C
50 cycles	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C

* Fluorogenic data for the control DNA should be collected during this step through the FAM and VIC channels
** Required if your mastermix includes UNG to prevent PCR carryover contamination
*** Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.

Interpretation of Results

Pathogen	Internal control	Negative control	Positive control	Interpretation
+ve	+ve	-ve	+ve	+ve
+ve	-ve	-ve	+ve	+ve
-ve	+ve	-ve	+ve	-ve
-ve	-ve	-ve	-ve	Exp Fail
+ve	+ve	+ve	+ve	Exp Fail

Internal PCR control

When used according to the above protocols, assuming a 100% extraction efficiency, that 1:4 of extracted RNA is used in the RT reaction and that 1:20 of the RT reaction is used in an individual well, then a CT value of 26 is expected. However this can vary significantly depending on the extraction efficiency, the quantity of RNA added to the RT and PCR reaction and the individual machine settings. CT values of 26±3 are within the normal range. When amplifying a pathogen sample with 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 ACTB control

The signal obtained from the ACTB primer and probe set will vary according to the amount of biological material present in a given sample.

Notices and disclaimers

During the warranty period PrimerDesign pathogen 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.

Black Hole Quencher", "BHQ", "CAL Fluor, "Quasar" and "Pulsar" are registered trademarks of Biosearch Technologies, Inc., Novato, CA. This technology is protected by U.S. and World-wide patents either issued or in application and is licensed and sold under agreement with Biosearch Technologies, Inc. These products are sold exclusively for R&D use by the purchaser. They may not be used for human or veterinary *in vitro* diagnostic (IVD) applications and they may not be re-sold, distributed or re-packaged without express written authorization from Biosearch

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. The purchase of Biosearch Technologies products does not, either expressly or by implication, provide a license to use this or other patented technology. Licensing information can be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404 or the Licensing Department at Roche Molecular Systems Inc., 1145 Atlantic Avenue, Alameda, CA 94501."

Trademarks

PrimerDesign ™ is a trademark of PrimerDesign Ltd.
The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG.
ABI, 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.