# Avian influenza virus, serotype H5N6 - Real Time RNA

BioinGentech Biotechnology

50 /100 /150 tests (Ready to use kit)

H5N6 Real Time PCR Kit is a screening assay for rapid and accurate detection of Avian influenza virus, serotype H5N6.

## Principles of the test:

One Step Bioingentech® qRT-PCR Kits provide components for onestep reverse transcription and quantitative PCR (qRT-PCR) in a convenient format that is compatible with both rapid and standard qPCR cycling conditions. Both cDNA synthesis and PCR are performed in a single tube using gene-specific primers and either total RNA or mRNA. These one step qRT-PCR kits have been formulated for use with fluorogenic probe-based 5' nuclease technology probes.

The One qPCR Enzyme Mix includes Reverse Transcriptase, Recombinant Ribonuclease Inhibitor in an optimized formulation, also include Bioingentech® Taq DNA polymerase and all reagents for an optimized qRTPCR.

The Avian influenza virus, serotype H5N6 specific primer and probe mix are provided in the kit and these can be detected through your real time platform by the 5' nuclease PCR detection method. During PCR amplification, forward and reverse primers hybridize to the Avian influenza virus, serotype H5N6 target cDNA generated. Fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5-reporter FAM and 3-quencher which can be detected through green channel **(see Table 8 and 9).**

To confirm extraction of a valid biological template an Internal control primer and probe mix is included, consists of a DNA probe labeled with a 5-reporter HEX and a 3-quencher which hybridize inside a specific housekeeping endogenous target gene. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. As a result, a fluorescence increase can be detected on a range of real time PCR platforms through yellow channel **(see Table 8 and 9).**

Also our RNA Kits include an exogenous source of RNA template that is spiked into the lysis buffer. A separate qPCR primer/probe mix are supplied with this kit to detect an exogenous RNA using qPCR. The PCR primers are present at PCR limiting concentrations which consists of a DNA probe labeled with a 5-dye HEX and a 3-quencher that during PCR amplification a fluorescence increase can be detected through yellow channel of real time PCR platforms. Successful co-purification and qPCR for the control RNA indicates that PCR inhibitors are not present at a high concentration **(see Table 8 and 9).**

Our kits also include Positive and Negative Control which are details in FAQ section.

## Principle and use:

This amplification kit has been manufactured by Bioingentech Ltd. Chile to detect Avian influenza virus, serotype H5N6 in real time PCR. This is a possibility relative/absolute quantification or qualitative assay.

Real time PCR is based on fluorogenic dyes. Ct value between 0 – 36 should be taken positive. Value between 36-40 Ct should be taken as marginal positive. Ct above 40 must be considered as negative **(for more details see Table 5).** This kit needs RNA as a template which can be isolated from blood, serum, faeces, respiratory fluid, cerebrospinal fluid, digestive system, tissue, Heptopancreas, Gills, Pleopods, Cloacal, Egg Yolk, Milk, swabs, Lee, bacterial cultures, cell lines and others.

## Table 1. Kit Components:

| Reactions Tubes | 50 test | 100 test | 150 test |
|---|---|---|---|
| Universal qPCR Master Mix | (1 unit) | (1 unit) | (1 unit) |
| Primer, Probes and Internal Control Mix | (1 unit) | (1 unit) | (1 unit) |
| One qRTPCR Enzyme Mix | (1 unit) | (1 unit) | (1 unit) |
| H5N6 Positive Control | (1 unit) | (1 unit) | (1 unit) |
| H5N6 Negative Control | (1 unit) | (1 unit) | (1 unit) |
| PCR grade Water | (1 unit) | (1 unit) | (1 unit) |
| Exogenous RNA control | (1 unit) | (1 unit) | (1 unit) |
| Exogenous RNA control Primer and Probes Mix | (1 unit) | (1 unit) | (1 unit) |

* Remember that all our OneHumqPCR-realtime™ H5N6 RNA. Real Time PCR Kits include reagents and procedures. Also we always can offer you a complete technical support at info@kitpcr.com

## Table 2. Instrument Compatibility:

- ABI 7300
- ABI 7500FAST
- ABI 7900
- AB Step One
- AB Step One Plus
- Agilent Mx3005P
- CFX96 & CFX384
- ExiCyclerTM 96
- iQ5 & MyiQ Cycler
- Illumina Eco
- LightCycler Nano
- CFX Opus
- Bioer Quant gene 9600
- Mastercycler® ep realplex
- LightCycler 2.0 and 480
- Mx3000P QPCR System
- Mx3005P QPCR System
- RotorGene 3000
- RotorGene 6000
- RorotGeneQ
- SLAN® Real-Time PCR
- Smartcycles II
- CFX Opus 96
- CFX Opus 384
- Quantstudio 3
- Quantstudio 5

## Procedure:

Please read through the entire procedure before starting.

## Before Starting

- Pulse-spin each tube in a centrifuge before opening.
- Homogenize the solutions for 5 seconds prior to pipetting.
- You must consider use different tips in order to avoid cross contamination.
- Use only sterile, RNAses, DNAases and pyrogens free tips.
- Add Exogenous RNA Control to the lysis/binding solution concentrate that is used for the RNA isolation. You must add 2 µL of undiluted Exogenous RNA Control (20,000 copies) per desirable isolation.

## Step 1

Prepare a Master mix according to the reaction table 3.

### Table 3. Reaction components for PCR

| Tubes | Sample and Internal Control | Positive Control | Negative Control | Exogenous RNA Control |
|---|---|---|---|---|
| Universal qPCR Master Mix | 7,5 µL | 7,5 µL | 7,5 µL | 7,5 µL |
| Primer, Probes and Internal Control Mix | 0,6 µL | 0,6 µL | 0,6 µL | |
| One qRTPCR Enzyme Mix | 0,5 µL | | | 0,5 µL |
| Exogenous RNA control Primer and Probes Mix | | | | 0,6 µL |
| PCR grade Water | 2,9 µL | 3,4 µL | 3,4 µL | 2,9 µL |
| Exogenous RNA Control | | | | 3,5 µL |
| H5N6 Positive Control | | 3,5 µL | | |
| H5N6 Negative Control | | | 3,5 µL | |
| RNA Sample | 3,5 µL | | | |
| Total Volume | 15 µL | 15 µL | 15 µL | 15 µL |

## Step 2

Place the tubes in a thermal cycler and perform One Step qPCR according to the program outlined in **Table 4.**

### Table 4. Recommended PCR Cycling table

| Cycles | Steps | Time | Temp. (°C) |
|---|---|---|---|
| 1 Cycle | Reverse Transcription | 20 min | 45 °C |
| 1 Cycle | Denaturation Step | 2 min | 95 °C |
| 40 Cycles | Denaturation | 10 seg | 95 °C |
| | Annealing | 1 min | 60 °C |
| | Hold | ---- | 4 °C |

Note 1: Colect data in 60 °C
Note 2: Hold Step is optional.

## Interpretation of the test

## 1) Qualitative analysis:

Ct (Threshold cycle) value of each sample can be read as follows.

### Table 5. Ct value result

| Ct value | Result |
|---|---|
| 0 - 36 | Positive |
| 36 - 40 | Marginal Positive |
| > 40 | Negative |

1.1.-Ct values over 40 are considered Negative. If the Ct value is in the 0 - 36 range, it must be considered as Positive. This is depending of the sample initial concentration use d for each reaction. Note that the sample real concentration could be altered by the sample purity when it is quantified.

1.2.- For more technical information you must request the **QUALITY CONTROL** for each kits. Also you canrequest more information writing to our email **info@kitpcr.com**

## 2) Quantitative analysis:

### Table 6. Preparation of standard curve dilution series. H5N6 positive control:

| Average Positive Control Concentration | |
|---|---|
| H5N6 | See Quality Control |

| Standar curve | Preparation series a fresh dilution | Concentration | Copy Number |
|---|---|---|---|
| Tube N°1: | 2uL H5N6 Positive Control (2 ng/µL) + 18 µL de PCR grade Water | See quality control | See quality control |
| Tube N°2: | 2uL Tube N°1 + 18 µL de PCR grade Water | See quality control | See quality control |
| Tube N°3: | 2uL Tube N°2 + 18 µL de PCR grade Water | See quality control | See quality control |
| Tube N°4: | 2uL Tube N°3 + 18 µL de PCR grade Water | See quality control | See quality control |
| Tube N°5: | 2uL Tube N°4 + 18 µL de PCR grade Water | See quality control | See quality control |
| Tube N°6: | 2uL Tube N°5 + 18 µL de PCR grade Water | See quality control | See quality control |
| Tube N°7: | 2uL Tube N°6 + 18 µL de PCR grade Water | See quality control | See quality control |

2.1. Important Note: Don't forget Homogenize the tubes.
2.2. We will send a Quality Control report for each purchase.
2.3. If you want to obtain less DNA copies you must include a new dilution tube **(Tube N°8).**
2.4. Final DNA copy number will depend of the DNA concentration **(you can see it in Quality Control Report).**

## Table 7. Standard curve set up

For each of the tubes (tube 1 to tube 7), take 3,5 µl of the final 15 µl mixture.

| | Tube A | Tube B | Tube C | Tube D | Tube E | Tube F | Tube G |
|---|---|---|---|---|---|---|---|
| Universal qPCR Master Mix | 7,5 µL | 7,5 µL | 7,5 µL | 7,5 µL | 7,5 µL | 7,5 µL | 7,5 µL |
| Primer, Probes and Internal Control Mix | 0,6 µL | 0,6 µL | 0,6 µL | 0,6 µL | 0,6 µL | 0,6 µL | 0,6 µL |
| PCR grade Water | 3,4 µL | 3,4 µL | 3,4 µL | 3,4 µL | 3,4 µL | 3,4 µL | 3,4 µL |
| Tube N° 1 (Positive Control) | 3,5 µL | | | | | | |
| Tube N° 2 | | 3,5 µL | | | | | |
| Tube N° 3 | | | 3,5 µL | | | | |
| Tube N° 4 | | | | 3,5 µL | | | |
| Tube N° 5 | | | | | 3,5 µL | | |
| Tube N° 6 | | | | | | 3,5 µL | |
| Tube N° 7 | | | | | | | 3,5 µL |
| Total Volume | 15 µL | 15 µL | 15 µL | 15 µL | 15 µL | 15 µL | 15 µL |

Take table N°6

2.5.- Assess the Ct value when the amplification curve of Standard tubes 1, 2, 3, 4, 5, 6, 7 passes the threshold line. Four tubes are sufficient for a standard curve (tubes 1 to 4).
2.6.- Calculate the quantitative value in order to compare the Ct value of the unknown samples with the standard curve.
2.7.- When visualizing the result for the Positive Control in the Real Time thermal cycler, there will be only one amplification curve for the target gene. It will not have an amplification curve for the Internal Control.

## 3) Test validation:

3.1.- Each Ct value standard should be as follows. Standard 1 < Standard 2 < Standard 3 < Standard 4 < Standard 5 < Standard 6 < Standard 7.
3.2.- R-value of standard curve should be 0.900 - 0.999. R-value represent how well the experimental data fit the regression line. A significant difference in observed Ct values between replicates will lower the R-value.
3.3.- The standard curve slope result should be all negative.
3.4.- The desired amplification efficiencies vary from 90% to 110%. The the oretical maximum of 100% indicates that the polymerase enzyme is functioning at its maximum capacity. Low reaction efficiencies may be caused by poor primer design or by suboptimal reaction conditions. Reaction efficiencies >110 may indicate pipetting error in your serial dilutions or coamplification of non specific products, such as primer-dimers.

## Visual explanation FAQ:

### 1.-Positive control:

The Positive control assay uses a FAM dye and should be detected through the Green channel of your real time PCR instrument. 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 Avian influenza virus, serotype H5N6 copy number / Ct value.

Alternatively, the positive control can be used at a single dilution Avian influenza virus, serotype H5N6 on where full quantitative analysis of the sample is not required. Each time the kit is used, at least one positive control reaction must be included in the run. Particularly, due to amount of this reagent, you should run a positive control for each 12 samples. A positive result indicates that the primer and probes for detecting the target Avian influenza virus, serotype H5N6 gene worked properly in that particular experimental scenario. If a negative result is obtained the test results should be invalid and must be repeated **(see Table 11)**. Sealing all other samples and negative controls before pipetting the positive control into the positive control well tube.

### 2.-Internal Control:

The internal control is included in Primer, Probes and Internal Control Mix along to the target pathogen detection. In order to interpreted results, read the yellow channel. The internal control assay uses a HEX dye and should be detected through the Yellow channel of your real time PCR instrument and gives a Ct value of 28 (+/-5) depending on the level of sample dilution and concentration. A positive result through the Yellow channel the refore indicates that PCR conditions are suitable for detection of the target pathogen gene. If a negative result is obtained through the Yellow channel the results should be analyzed by combination of result, follow the **Table 11 data.**

### 3.-Negative control:

To confirm absence of contamination a negative control reaction should be included every time the kit is used. Particularly, due to amount of this reagent, you should run a negative control for each 12 samples. In this instance the Avian influenza virus, serotype H5N6 Negative Control should be used in place of template. A negative result indicates that the reagents have not become contaminated. If a positive result and Ct value less than 36 is obtained, the results should be analyzed and check if a correct amplification curve was obtained. When you obtain a clear amplification curve you should consider repeat your assay due to probably the sample was contaminated **(see Table 11).**

* Remember: We recomment running a Positive control and Negative control for each 12 samples. For reaction mix you must use Universal qPCR Master Mix.

## 4.-Internal RNA control:

An exogenous source of RNA template 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 te extraction process. Amplification of this control cDNA does not interfere with detection of the target cDNA even when present at low copy number. Previous of PCR setup you must add 2µl (20.000 copies) of undiluted Exogenous RNA Control to the lysis/binding solution concentrate that is used for the RNA isolation.

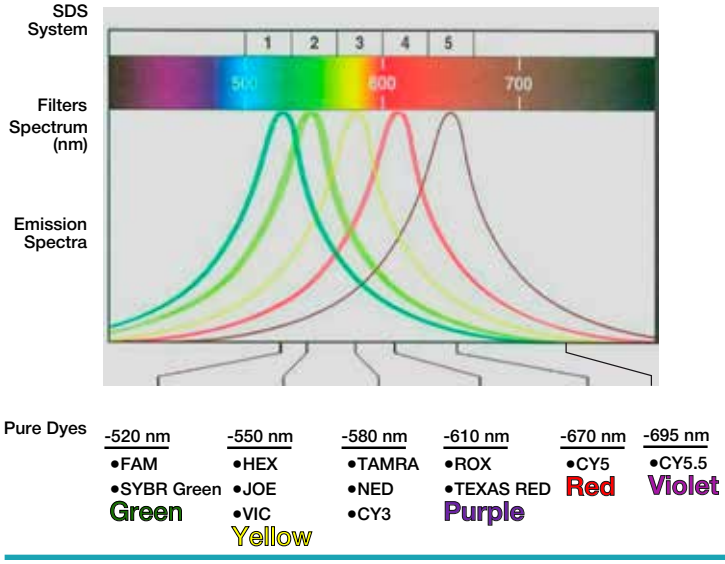
The Internal control is detected through the Yellow channel and gives a Ct value up to 30 depending on the level of sample dilution. Succesful co-purification and qPCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration. To confirm this, Internal RNA control should be included every time the kit is used. **Particularly, due to amount of this reagent, we recommend including and running one Internal RNA control for each 12 samples.**

* Exogenous RNA control is just referential and its negative result should not interpreted as a failed assay when the sample and internal control give a positive result **(see Table 11).**

## Table 8. Fluorogenic probes, Channels and Dyes

* In order to setup your software to read the probes, **(see Table 8 and 9)** and run the corresponding channel:

| Channel | Source | Detector | Dyes |
|---|---|---|---|
| Green | 470(Nm) | 520(Nm) | **FAM**, Sybr green1, Fluorescein, Eva green, Alexa fluor 488 |
| Yellow | 530(Nm) | 550(Nm) | Joe, Vic,**HEX**, Tet, Cal Fluorgold 540, YaKima Yellow |
| Orange | 585(Nm) | 610(Nm) | Rox, Cal Fluor Red 610, Cy3.5, Texas Red, Alexa Fluor 568 |
| Red | 625(Nm) | 660(Nm) | Cy5, Quasar 670, Lightcycler, Red 640, Alexa Fluor 633, Aeon Zw™. |
| Crimson | 680(Nm) | 710(Nm) | Quasar 705, Lightcycler Red 705, Alexa Fluor 680 |




## Table 9. Similitary of our fluorophores with HEX and FAM dyes.

| | Channel | Dyes |
|---|---|---|
| 1.- Positive Control | Green | FAM |
| 2.- Internal Control | Yellow | HEX |
| 3.- Sample Target | Green | FAM |
| 4.- Exogenous RNA control | Yellow | HEX |

## Important Note:

Request a Certificate of Analysis when you purchase one of our PCR Kits.

Is important mentioned that we can develop special request for other pathogens or multiplex detection according client's specifications.

We strongly recommend don't use or combine our products with reagents from another kits or unknown provenance. We can't assure good result if incompatibility problems occur.

## Temperature

All our reagents are made through protein engineering and are stable at room temperature, the label temperature is just a recommendation after the product is open.

## Table 10. Store Temperature Kits

| | | |
|---|---|---|
| | Store temperature | The label temperature |
| | Shipping temperature | At room temperature |

## Table 11. Interpretation of Results

| Sample | I.C | N.C | P.C | eRNA Control | Result |
|---|---|---|---|---|---|
| + | + | - | + | + | POSITIVE |
| + | - | - | + | + | POSITIVE |
| + | + | - | + | - | POSITIVE |
| + | - | - | + | - | POSITIVE |
| + | + | + | + | + | Check Ct and Consider repeat assay* |
| + | - | + | + | + | Check Ct and Consider repeat assay* |
| + | + | + | + | - | Check Ct and Consider repeat assay* |
| + | + | - | - | - | Check Ct and Consider repeat assay* |
| + | + | + | - | - | Check Ct and Consider repeat assay* |
| + | + | - | - | + | Exp. Fail |
| + | + | + | - | + | Exp. Fail |
| + | - | - | - | - | Exp. Fail |
| + | - | - | - | + | Exp. Fail |
| + | - | + | - | - | Exp. Fail |
| - | + / - | + / - | + / - | + / - | NEGATIVE |

* Sometimes amplification curves for Negative or Internal control with Ct < 30 can be seen, however it might not be a Positive result. If the amplification curve is not sigmoid, you should consider it as a Negative result.

## Table 12. Other Products

| Products |
|---|
| OneHumqPCR-realtime™ H5N6<br>Avian influenza virus, serotype H5N6 Real Time<br>50 tests (Ready to use kit) / Cat. No: Oneq-VH756-50R |
| OneHumqPCR-realtime™ H5N6<br>Avian influenza virus, serotype H5N6 Real Time<br>100 tests (Ready to use kit) / Cat. No: Oneq-VH756-100R |
| OneHumqPCR-realtime™ H5N6<br>Avian influenza virus, serotype H5N6 Real Time<br>150 tests (Ready to use kit) / Cat. No: Oneq-VH756-150R |

## Table 13. Products Specifications

| | |
|---|---|
| **Technology** | 5’ nuclease probe based real time PCR assay |
| **Type of nucleic acid Kit** | RNA |
| **Kit storage** | Shipped at room temperature, the label temperature is just a recommendation after the product is open. |
| **Detection Limit** | **See Quality Control file. Request it!** |
| **Sensitivity & Specify** | Ct value between 0 – 36 should be taken positive. Value between 36-40 Ct should be taken as marginal positive. Ct values above 40 must be considered as negative. |
| **Controls included** | Internal control, Positive control and Negative control included. |
| **Channels** | **FAM Green channel detect pathogen aplicons** |
| | **HEX Yellow channel detect internal control amplicons.** |
| | **FAM Green channel detect Positive Control.** |
| | **HEX Yellow channel detect Exogenous RNA control.** |

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