

## The Establishment of Real-time Fluorescent Quantitative Polymerase chain reaction (PCR) for Detection of Highly Pathogenic Avian Influenza Virus Subtype H5N1

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### Abstract

Highly pathogenic strains of avian influenza virus (AIV), which are influenza A viruses, cause severe disease in domestic poultry and humans. The objective of this study was to establish a fluorescent quantitative RT-PCR assay for detection of highly pathogenic avian influenza virus (AIV) subtype H5N1. The H5 and N1 subtype-specific probe sets were developed based on avian influenza virus sequences detected in China. Two pairs of primers and two fluorescent probes were strictly designed and optimized in a reaction system. According to the amount of plasmid RNA extracted from H5N1 strains, the standard curve DWQBGWDWQBGW of fluorescent quantitative PCR was drawn and all of the specimens were then tested by means of Real-time PCR. The test of highly pathogenic AIV subtype H5N1 was identified to be specific and its sensitivity level was  $10^2\sim 10^3$  copies/reaction. The standard curve was accomplished at  $10^9\sim 10^5$  DNA copies/reaction. It took only three hours from viral RNA extraction through to completion of the test. The assay was easy to carry out and highly reproducible. In conclusion, fluorescent quantitative PCR, described here, provides a rapid, specific and sensitive method to detect not only the H5 but N1 genes as well.

### Keywords

Real-time quantitative PCR; Avian influenza virus; H5N1

### Introduction

Avian influenza (bird flu) is a disease or rather a syndrome that inflicts birds and human beings caused by influenza A virus. Hong Kong has witnessed its first outbreak caused by subtype H5N1, which has led to substantial economic loss in the poultry industry since 1997. Over the past few years, it has caused about 30 patient deaths in China. The overall fatality rate among hospitalized patients with avian influenza A (H5N1) infection has amounted to 57% [1]. Although bird influenza in Asia only infected people sporadically in some areas, human beings as a whole are vulnerable and non-immune to H5N1

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strains. The high mortality rate was registered in people infected. As a result, several studies have focused on the methods for detecting the avian influenza virus in a quick and accurate manner.

The detection of avian influenza virus can be fulfilled mainly in two ways: the traditional AIV detecting methods and molecular biology methods. The inoculation of the virus in embryonated eggs, a traditional AIV detecting method, is classic, accurate and sensitive, but time-consuming and not conducive to the rapid diagnosis of AIV. In recent years, PCR as a modern diagnostic technique of molecular biology has emerged and it constitutes a specific, sensitive, simple and quick means, through which we can detect trace pathogens within a few hours. The techniques, such as RT-PCR, RT-PCR-ELISA, and other methods on the basis of PCR were thus used to detect AIV, with considerable sensitivity and specificity compared to traditional virus isolation and culture. However, because of operational contamination or other unavoidable lab procedures, it was found that these methods produced high false positive rate. With the in-depth exploration of molecular biology, real-time fluorescent quantitative PCR technology has been developed and applied [2]; it can improve processing efficiency and reduce the risk of carryover contamination. However, it can only identify the virus subtype H5 [3-6]. In all AIV H5 type, H5N1 is the most pathogenic and is of widespread prevalence in Asia, but there were few reports about the proper methods for simultaneously detecting both the H5 and N1 genes [7,8]. In this study, we established the real-time quantitative fluorescent PCR method. The H5 and N1 subtype-specific probe sets were developed based on avian influenza virus sequences obtained in China. The results brought us the expectation for detecting highly pathogenic H5N1 strain of avian influenza virus.

### Materials

#### Samples and Virus

**Samples:** Throat swab samples were collected from 11 sick or dying chickens presenting with abnormal neurological signs and diarrhoea, and 65 staffs' throat swab samples also were collected who worked as sellers of chickens in the bird markets in Guangdong province, China, during the H5N1 outbreak in 2007. The swabs were kept at 4°C and transported to the laboratory within 24 h in PBS at 4°C supplemented with 200 mg streptomycin ml<sup>-1</sup>, 100 U penicillin ml<sup>-1</sup> and 10 µg amphotericin B ml<sup>-1</sup>. On receipt of the specimen, prior to any manipulation of the specimen, some aliquots were removed in a type III biological safety cabinet for molecular analysis and virus isolation.

**Virus:** The standard avian influenza A virus/H5N1 panel were conserved and supplied by the open laboratories of the Ministry of Agriculture poultry and poultry disease prevention and treatment of the South China Agricultural University. It contained different isolates of influenza virus: A/H5N1:A/duck/wushan/B/03(the duck-origin avian influenza virus strains) and A/chicken/Guangdong/C/03(the chicken-origin avian influenza virus strains) which were separated from embryonated chicken, a total of 15; chicken- origin of the avian flu virus A/chicken/Neimenggu/ZH/02, and duck-origin avian influenza virus A / duck / Hong Kong/Y439/97 strains were AIV H9N2 , H1N1 and H3N2 subtype virus isolated embryonated

chicken, a total of 15; Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), Egg drop syndrome virus (EDSV), a total of 15. All strains of the virus were inactivated by 0.5~1.0 g / L  $\beta$  C lactone.

**Main reagents and equipment:** 5×FQ MIX, BC-tag from Guangzhou DA AN genetic testing center, MMLV, dNTPs from Promega, trizol from Gibco, chloroform, DEPC, and other from sigma; fluorescent quantitative PCR 7300 apparatus, probe and primer design software from the United States ABI, frozen centrifuges from sigma, gel analysis system from the United Kingdom UVP companies.

## Methods

### Primer and probe design and synthesis

The specific RNA gene fragment of AIV subtype H5N1 was obtained from NCBI Genbank database and our unpublished H5N1 genome sequences, and chose a conservative region to design the primers and probe. As to the origin, there were downloaded from the NCBI that hemagglutinin gene sequences of subtype H5 representative strain and neuraminidase gene sequences on subtype N1 representative strain from birds, which had the same sequence with people. Two pairs of specific primer and two fluorescent probes were designed by ABI Primer Express 2 software. The synthesis of primers and probes was completed by Guangzhou branch of Shanghai Yingjun Company. The sequences were showed as follows: (Table 1).

### RNA extraction

① 0.5 ml inactivated samples and 1ml trizol were mixed, then were fully blended and put at room temperature for three minutes; 0.2 ml chloroform were added in, then fully blended and put for five minutes;

② The mixture was given to the low-temperature centrifuge at 12,000 per minute for 10 minutes;

③ 2/3 supernatant was taken into another cleansing EP tube (the DEPC treatment), and by being added the same volume of isopropanol; Fully mixed, to low-temperature centrifuge at 12,000 per minute for 10 minutes;

④ Supernatant was disposed, and then 1 ml 70% ethanol (0.1% DEPC Water preparation) was added in for precipitation;

⑤ Pentrifugated at 12,000 per minute for five minutes; medium was disposed, and step ④ was repeated for one time (it need to be absorbed fully; If a little liquid linked to the wall, we could move it with transferpettor after instantaneous centrifugating);

⑥ The EP tube was put in the air about 10 minutes, then waiting for volatilizing of ethanol;

⑦ Precipitation would be handled by 0.1% DEPC and 50  $\mu$ l solute were get and reserved at -80°C.

### Reverse transcription

5×RT 5  $\mu$ l buffer, 2.5  $\mu$ l 10 mm dNTPS, 200U MMLV, 50 U RNA enzyme inhibitors, 10 pmol random primer, 8  $\mu$ l RNA template were added into the EP tube respectively, and RNA removal of ultra-pure water would be added to the total volume of 25  $\mu$ l, at 37°C for one hour, at 95°C role for five minutes.

### Reaction system and condition optimizing

On the condition of templates in the same concentration with reaction system, the best concentration of primer and probe was chosen. Meanwhile, in the TaqMan fluorescence quantitative RT-PCR reaction system, the Tm value (45°C~65°C) was adjusted, and the same concentration of positive nucleic acid template was tested. Throughout the screening process, a minimum of CT value and a maximum of fluorescence intensity were chosen and optimized to add  $\Delta$  Rn value as the criterion.

### The establishment of standard curve and the sensitivity of real-time PCR

The plasmid DNA from the H5N1 strain was extracted as external standard, then diluted at a series of 10 times ultimately 5 dilute strength of DNA plasmid( $10^9$ ~ $10^5$  copies /reaction)were chosen and the standard curve was drawn. Through the standard curve, unknown samples could be quantitated. Then the cDNA reverse product of H5N1 was diluted at  $10^7$  ~  $10^1$ copies / reaction, and 5  $\mu$ l each dilution was taken to be added H5 and N1 of the primers and probes respectively. Therefore, the sensitivity of fluorescence quantitative PCR method was tested.

### The specificity of real-time PCR

Optimizing fluorescence quantitative PCR method was tested to detect the collection of other avian viruses: AIV H9N2, H1N1, H3N2 subtype, Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious bursa disease virus (IBDV), egg drop syndrome virus (EDSV),and was identified the specificity.

### The repeatability of real-time PCR

The cDNA reverse product of H5N1 were diluted at  $10^7$ ~ $10^1$ copies / reaction, and 5  $\mu$ l each dilution were made to three parallel. The three parallel of each dilution were compared with the CT value and the position of positive dynamic curve.

## Results

### The establishment of reaction system and optimization of condition

For enhancing the efficiency and sensitivity, a matrix was applied to optimize the concentration of primers and probes. Tm value was chosen from 45°C to 65°C by using the gradient function of fluorescence quantitative PCR instrument. The optimizing reaction system was: 10  $\mu$ l 5 × FQ MIXI, 3 U BC-tag, 1ul 10 mmol dNTPs, 10 pmol amphi-primers, 5 pmol probe, 5  $\mu$ l cDNA, then ultra-pure water was added in to the total reaction volume 50  $\mu$ l. After instantaneous centrifugalization, they were put into ABI7300 fluorescence quantitative PCR instrument carefully, and the placing order was recorded. The seized samples were filled in the template document with negative and positive control. According to the following

Table 1: PCR primers and probe used for AIV H5N1 subtype detection.

Primers/probe	Primer sequences (5'→3')
H5-178F	5'-TGGCTCCTCGAAACCCTAT-3'
H5-252R	5'-ACTGGCCTTCTCCACTATGTAAGAC-3'
H5-199T	5'-FAM-TGTGACGAATTCATCAATGTGCCGGA-3'-tam
N1-500F	5'-TGAGGCTCCTTCCCACATACA-3'
N1-572R	5'-AGTGCCATCATGACAAGCACTT-3'
N1-521T	5'-Fam-CTCGAGGTTTGTAGTCTGTTGCTTGGTCG-3'tam

response parameters: the first phase, 94°C 180 s; second stage, 94°C 45 s, 55°C 60 s for 10 cycle, respectively; third phase, 94°C 30 s, 55°C 45s for 30 cycle, respectively.

**The establishment of standard curve:** H5N1 DNA plasmid ( $10^9$ ~ $10^5$ copies / reaction) was a template through being diluted by a series of 10 times. After the fluorescence quantitative PCR amplification, the return curves were drawn by the logarithm of opening template as x-axis, Ct value as the y-axis, that is, in the following equation:  $y = -3.3226x + 46.0602$ ,  $R^2 = 0.99467$ .

**The sensitivity of real-time PCR:** The cDNA reverse product of H5N1 was diluted at  $10^7$  ~  $10^1$ copies / reaction, and 5  $\mu$ l each dilution was taken and added H5 and N1 of the primers and probes respectively, which was tested through fluorescence quantitative PCR. The ceiling of H5 detection was 103copies / Reaction, it means, as long as 1 copies in 5  $\mu$ l reaction solution, H5 virus could be detected (Figures 1 and 2). The ceiling of N1 detection was 102 copies / reaction, that is, as

long as 1 copies in 50  $\mu$ l reaction solution, N1 virus could be detected (Figures 3 and 4). To sum up, it could be believed that the sensitivity of real-time quantitative PCR about H5N1 detection was  $10^3$  copies / reaction.

**The specificity of real-time PCR:** With the establishment of the fluorescence quantitative PCR method, all the samples collected were detected. In addition to the avian flu virus H5N1 samples, and The standard avian influenza A virus/H5N1 panel appeared positive(15/15,100%), which demonstrated by virus isolation(VI). 11 sick or dying chickens, which were diagnosed AIV, appeared positive(10/11,90.9%); H9N2, H1N1, H3N2, Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), egg drop syndrome virus (EDSV), and the blank control were all negative(30/30,100%); 65 staffs' throat swab samples also were collected who worked as sellers of chickens in the bird markets were all negative(65/65,100%).

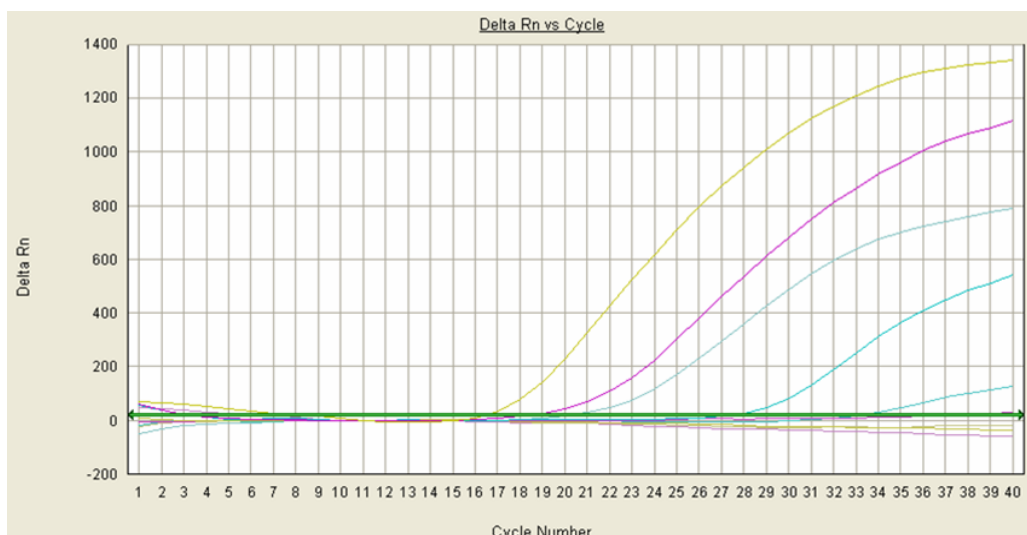


Figure 1: The dilution gradient of H5 positive samples: a maximum for  $10^7$  copies / response, the minimum for  $10^3$  copies / reaction.

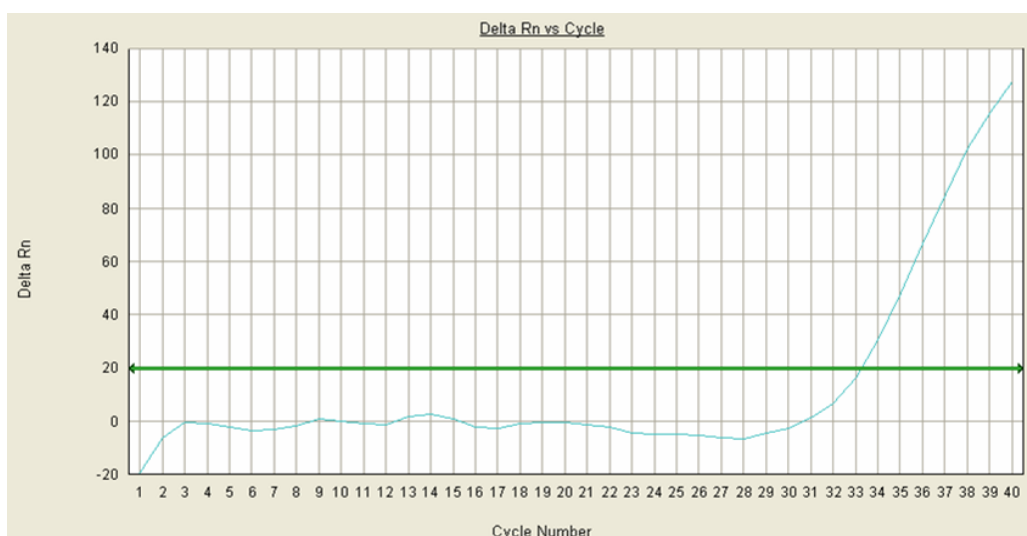


Figure 2: The dynamics curve of H5 positive samples at  $10^3$  copies / reaction.

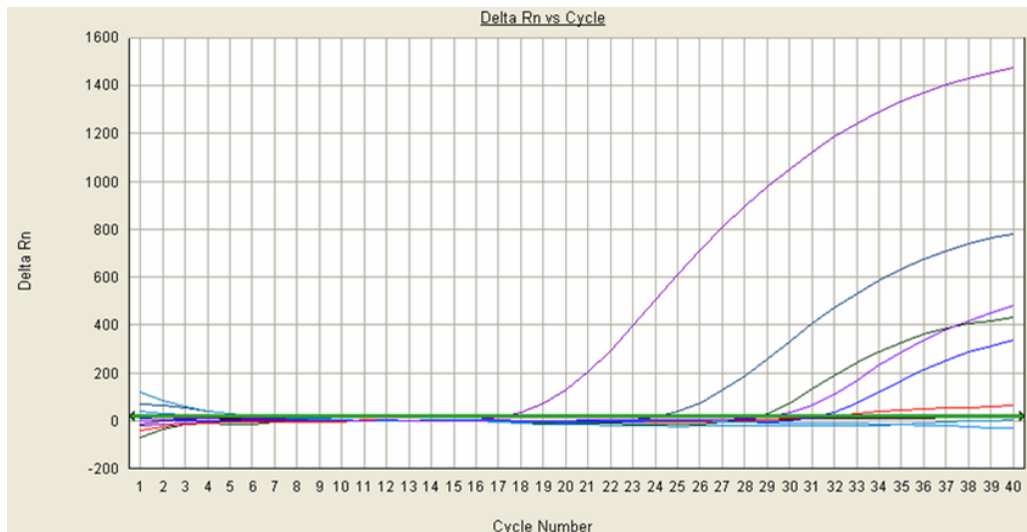


Figure 3: The dilution gradient of N1 positive samples: a maximum for  $10^7$  copies / response, the minimum for  $10^2$ copies / reaction.

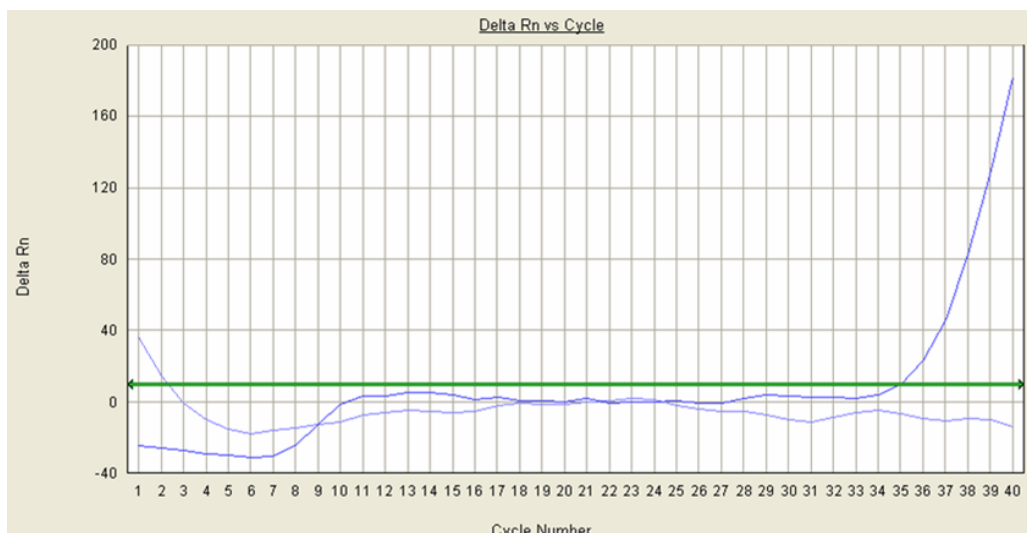


Figure 4: The dynamics curve of N5 positive samples at  $10^2$  copies / reaction.

### The repeatability of real-time PCR

Three different concentrations of external standard were chosen to be tested with fluorescence quantitative PCR. Each sample was repeated for three times, the accuracy was verified by calculating the different CT value of the same sample. Tables 2 and 3 showed that the coefficient of variation (CV) were all below 5 percent. It indicated that the detection system was of well stability and repeatability.

### Discussion

There were some reports in China about fluorescent PCR detection method for subtype H5, and some about fluorescent PCR detecting avian influenza virus subtypes H5, H7 [9,10]. However, the main subtype of avian influenza virus is H5N1, which causing high mortality and distributing widely in Asia. Commercially available rapid antigen tests for influenza A virus are rapid and simple, but subtyping of viruses shows cross-reactivity, and as a result, there was

a certain rate of misdiagnosis [5]. In our work, two pairs of specific primers and two fluorescent probes were designed according to the specific region of AIV H5N1 H and N genes respectively, and it was ensured that this method can be applied for the rapid diagnosis of influenza virus subtype H5N1 from various sources of poultry, and the method was well specific through testing different strain of virus, particularly without existing cross-reaction among H5N1 and the other subtypes as H9N2, H1N1 and H3N2.

We also found that the single-step RT-PCR affected the sensitivity of the assay, our method detection limitation could reach  $10^2$ - $10^3$  copies / reaction, and the sensitivity was higher than  $10^3$ - $10^4$  copies / reaction reported by Erica and Zhang Xia ohe [11], with the one-step AIV H5N1 fluorescent PCR detection. Although this one-step method is reported to be less sensitive than the two-step RT-PCR procedure, the method is enough sensitive, it did not need to separate and proliferate pathogens from clinical samples.

**Table 2:** The repeatability of the detection of H5 avian influenza virus with fluorescence quantitative PCR.

Sample	Ct			Average	SD	CV
	1	2	3			
1	16.45	16.68	16.56	16.56	0.115	0.69%
2	21.04	21.23	21.75	21.33	0.368	1.73%
3	28.30	28.44	28.24	28.33	0.103	0.36%

**Table 3:** The repeatability of the detection of N1 avian influenza virus with fluorescence quantitative PCR.

Sample	Ct			Average	SD	CV
	1	2	3			
1	17.56	17.68	18.12	17.79	0.295	1.66%
2	24.66	24.51	24.03	24.4	0.329	1.35%
3	28.10	28.36	28.54	28.33	0.221	0.78%

In addition, we made use of standard known to chart standard curve and then determine the amount of unknown samples in this study, according to the copy number of plasmid template (copy number = (DNA quality / DNA molecular weight) × 6.02 × 10<sup>23</sup>). Namely, Standard curve was drawn to quantitate clinical specimens. The negative and positive internal and external control were set up; pollution and possible false positive and false negative phenomenon were controlled, and sharply reduced the risks of cross contamination because the tubes are not reopened once the template is added. So that the quantitative method was more stable and accurate.

In conclusion, fluorescent quantitative PCR testing platform, described here provides a rapid, specific and sensitive method to simultaneously detect both the H5 and N1 genes from clinical poultry specimens, particularly at times of H5N1 influenza A virus outbreaks. It is helpful for the establishment of real-time fluorescent quantitative polymerase chain reaction (PCR) for detection of highly pathogenic avian influenza virus subtype H7N9 during outbreaks of H7N9 influenza virus nowadays.

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