Applications

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Real-time RT-PCR Diagnosis of the avian influenza virus using Mastercycler® ep *realplex* S and AIV RT-PCR kits from PG Biotech

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Abstract

The recent outbreak of avian influenza in different parts of the world not only has caused major economic losses, but also has presented a significant threat to public health due to a potential transmission of the avian influenza virus to humans. The ability to rapidly recognize avian influenza virus (AIV) in biological specimens is of utmost importance to enable fast decision making on appropriate countermeasures to prevent the further spread of the virus. In this study we evaluated the performance of a commercial Kit, AIV RT-PCR Kit (PG Biotech), for the detection of influenza-specific RNA using real-time reverse transcription PCR. The tests were performed on the Mastercycler ep *realplex* S (Eppendorf AG), a 96-well real-time PCR instrument. It could be demonstrated that the fast ramping speed of the Mastercycler ep *realplex* S was able to significantly shorten the run time of the kit as compared to standard real-time PCR instruments. The real-time PCR Kits could be implemented on the instrument using a standard protocol according to manufacturers specifications with no optimization of parameters required. With an average Ct value of 24 obtained for the Influenza A and H5 subtype, the combination of the Mastercycler ep *realplex* S and the AIV RT-PCR Kits outperformed the expected Ct value of 28 stated in the operation manuals of the Kits.

Introduction

The highly pathogenic avian influenza virus threatens to become a worldwide pandemic [1] and as such, very fast screening and detection methods are becoming increasingly important to ensure that appropriate measures can be taken quickly to contain the spread of the virus.

Molecular techniques offer a more rapid approach to detecting and characterizing the viral genome compared to classical methods. Many samples can be analyzed in a shorter time by performing a reverse transcription of the virus RNA followed by an AIV specific PCR or real-time PCR assay.

PCR is a specific and sensitive technique but samples must be analyzed via Gel Electrophoresis following the amplification step. Firstly, this increases the risk of carryover contamination since the tubes need to be opened and secondly, the post PCR handling steps are time consuming and cumbersome. Using real-time PCR offers several advantages over standard PCR when applied for viral screening purposes. Since result monitoring is integrated in the process and can be done in real time, post PCR handling is not required and data can be analyzed while the result is being generated. This shortens the time in obtaining a result considerably. Also, since real-time PCR displays broader dynamic range, it is possible to detect lower copy numbers in a shorter time compared to standard PCR. Real-time PCR assays also offer the added benefit of including several external and internal controls which improves the overall integrity of the assay, thereby reducing the number of false positive, and more importantly, false-negative results. This coupled with the increased sensitivity that real-time PCR offers ads to the overall importance that real-time PCR has in the modern laboratory.

To satisfy the demand of a diagnostic laboratory, real-time PCR offers the possibility to screen large numbers of samples very quickly and obtain accurate results at the same time.
If assays are run on a fast and sensitive real-time PCR platform the time to result can be shortened even more. Eppendorf's Mastercycler® ep *realplex* S 96 well system with heating rates of 6 deg/sec and cooling rates of

4.5 deg/sec allow quick completion of a real-time PCR assay of 96 samples at one go. The use of 96 individual LEDs as an excitation source in combination with minimal moving parts in the optical detection module allow single color fluorescent detection in 8 seconds which is faster than comparable filter based real-time PCR systems.

In this paper, we evaluate the performance of two commercially available real-time RT-PCR avian influenza detection kits, AIV A RT-PCR Kit and AIV H5 RT-PCR Kit (PG Biotech, manufactured for Qiagen) on the Mastercycler® ep *realplex* S.

Overview of Avian Influenza Virus

Influenza viruses have a single-stranded RNA genome and are classified into types A, B or C based on antigenic differences of their nucleo- and matrix proteins. Avian influenza viruses (AIV) belong to type A, which is able to infect a range of species including humans, birds, horses and pigs. The main antigenic determinants of influenza A viruses are the haemagglutinin (H) and the neuraminidase (N) transmembrane glycoproteins. On the basis of the antigenicity of these glycoproteins, influenza A viruses are classified into sixteen H (H1 - H16) and nine N (N1 - N9) subtypes. The subtype H5 of the virus can be transmitted form birds to human causing major health concerns.

Wild aquatic birds are potential carriers and the assumed natural reservoir of all influenza virus A subtypes [2]. These natural hosts normally are not seriously affected by an AIV infection but some domesticated poultry such as chicken or turkey are known to be particularly susceptible to the infection [3].

Materials and Methods

The AIV A RT-PCR Kit and AIV H5 RT-PCR Kit (PG Biotech, manufactured for Qiagen) are kits that are developed for veterinary research. The AIV A RT-PCR kit detects a gene that is common to all subtypes of the avian influenza virus and the AIV H5 RT-PCR kit detects the hemagglutinin gene of the H5 subtype. Both kits use hydrolysis probes that have FAM™ as the reporter dye at the 5‘ end and TAMRA™ as a quencher at the 3‘ end.

These kits were tested on the Mastercycler ep *realplex* S (Eppendorf, Germany) which has a 96 well block format, a gradient function and a heating rate of 6 deg/sec and cooling rate of 4.5 deg/sec. Assays were performed with positive and negative controls which are included in the kits. The reagents were dispensed according to the PG Biotech instruction manual. Assays were run using twin.tec PCR plate 96, skirted (Eppendorf, Germany) in combination with Heat Sealing Film (Eppendorf, Germany).

The investigation process follows a cascade: the presence of influenza A specific RNA is detected through a one-step reverse transcription – real-time polymerase chain reaction (RT – real-time PCR) using primers and probes provided in the AIV A RT-PCR kit that are specific for a gene that is common to all subtypes of avian influenza virus.

When a positive result is obtained, a second one-step RT – real-time PCR assay is set up using primers and probes from the AIV H5 RT-PCR Kit which are specific for a sequence of the hemagglutinin gene that is conserved among avian influenza viruses of subtype H5.

The Mastercycler ep *realplex* S was programmed according to manufacturer’s recommendation for block based systems. See Table 1 for thermal protocols for both kits.

RT-RCR-Thermal Protocol	No. of cycles	Temp (°C)	Time
Reverse transcription		42	30:00
Initial denaturation		92	3:00
3-step cycling: Denaturation Annealing Extension	 5	 92 45 72	 0:10 0:30 1:00
2-step cycling: Denaturation Annealing/extension*	 40	 92 60	 0:10 1:00

Table 1: RT-PCR thermal protocol as per manufacturer’s recommendation

Data acquisition was carried out during the combined annealing/extension step (*).

Results

The positive control with the subtype A primers gave rise to a signal with an average Ct of 24 cycles (Fig. 1). As for the positive control with the type H5 primers, the signal has an average Ct of 24.4 cycles. According to PG Biotech, Ct values of approx. 28 are representative of a good result. The Amplification plots for both AIV A RT-PCR Kit and AIV H5 RT-PCR Kit therefore outperform the expectations stated in the operation manual of the manufacturer. Using the Mastercycler ep *realplex* S Ct values of approx. 24 were obtained. The tightness of the replicates show good reproducibility of the results. As expected the negative controls did not show any amplification.

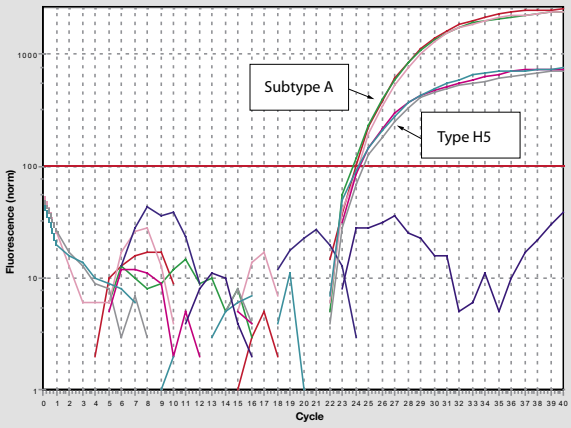

Figure 1: Amplification plots showing positive identification of both subtype A and H5 genes.

Performing the AIV A RT-PCR Kit and AIV H5 RT-PCR Kit on the Mastercycler ep realplex S resulted in a runtime of

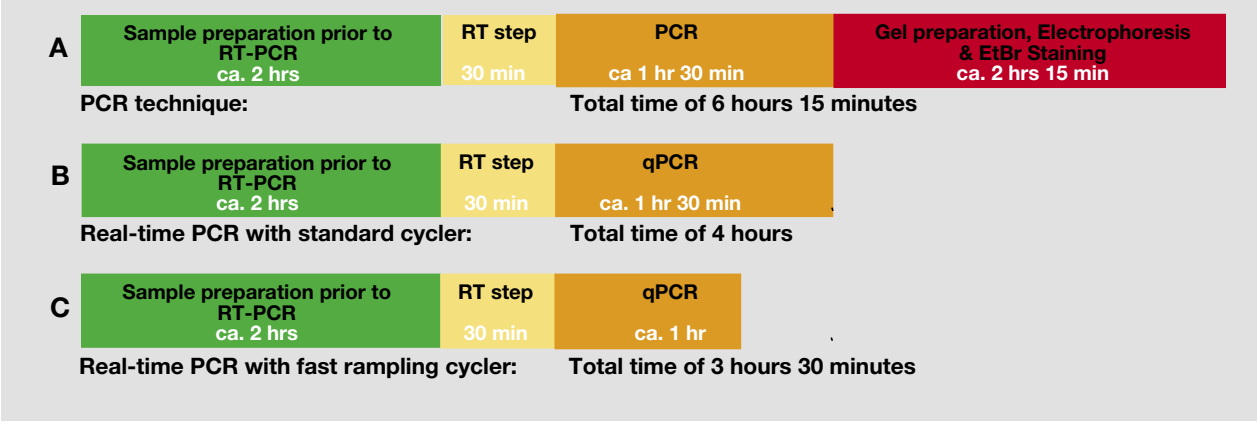

Figure 2: Expected time needed to obtain a result for avian influenza virus detection using PCR or realtime-PCR

only 90 minutes (Fig. 2). In comparison to standard real-time PCR platforms with slower ramp rates and a runtime of 120 min for the same protocol, the run-time was significantly reduced. Looking at time needed starting from sample preparation to the analysis of the results, real-time PCR considerably shortens the time needed in comparison to standard PCR.

Discussion

The AIV A RT-PCR Kit and AIV H5 RT-PCR Kit show optimal performance when used on the Mastercycler ep *realplex* S from Eppendorf. The real-time PCR Kits to detect avian influenza virus were successfully implemented on the platform using a standard protocol according to manufacturers specifications without any optimization of parameters.

With average Ct values of 24 obtained for the Influenza A and H5 subtype, the combination of the Mastercycler ep *realplex* and the AIV RT-PCR Kits outperforms the expectations of 28 Ct stated in the operation manuals of the Kits. The fast ramping speed of the Mastercycler ep *realplex* real-time PCR platform was able to shorten the run time as compared to a standard real-time PCR instrument, which may need at least two hours to complete the same assay.

Reduction of time was achieved without any need for special consumables or reagents. At the same time, having a 96 well block format enables a high number of samples to be processed at one time.

Real-time PCR is a very fast and precise tool that can be used for rapid avian flu virus detection. Results are obtained in real time without having to do any post PCR analysis. Results are obtained without opening the reaction tubes, which eliminates the risk of carryover contamination.

References

[1] Widjaja L, SL Krauss, RJ Webby, X Tao and RG Webster. Matrix gene of influenza A viruses isolated from wild aquatic birds: ecology and emergence of influenza A viruses. J.Virol. 78: 8771-8779.

[2] Kaye D and CR Pringle. Avian influenza viruses and their implication for human health. CID 40: 108-112.

[3] Kamps BS, C Hoffmann and W Preiser. Influenza Report 2006.

Ordering information Eppendorf

Article	Description	Order no. international	Order no. North America
Mastercycler® ep *realplex4* S	with silver block and four emission filters	6302 000.601	950020318
twin.tec PCR Plate 96	skirted, clear, 25 pcs	0030 128.648	960020006
Heat Sealing Film	10 x 10 pcs	0030 127.650	951023060
Heat sealer		5390 000.024	951023078
epDualfilter T.I.P.S.® 0.5 - 10 µl	10 racks of 96	0030 077.520	022491229
epDualfilter T.I.P.S.® 2 - 200 µl	10 racks of 96	0030 077.555	022491296
epDualfilter T.I.P.S.® 50 - 1000 µl	10 racks of 96	0030 077.571	022491253

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