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Research article

Production and evaluation of egg derived hot start antibodies

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ABSTRACT

Background: Hot start can greatly improve specificity, sensitivity and yield of PCR. Non-specific amplification can occur in PCR when reaction mixture is prepared at room temperature, because Taq DNA polymerase is active and the primers can hybridize non-specifically. Hot start Taq DNA polymerases remain inactive at room temperature and are activated after heating at 95°C preventing non-specific amplification. Monoclonal antibodies against Taq DNA polymerase is the first line of reagents used for turn on regular Taq DNA polymerase into Hot start one. The goal of this research was to produce and evaluate Hot Start antibodies derived from chicken eggs.
Results: We performed affinity purification of yolk immunoglobulin (IgY) and obtained polyclonal Hot Start antibodies. The yield of specific antibodies was 0.36 mg per egg or 0.2% of total yolk antibodies. The protocol for real time measurement and Hot start IgY activity assessment was developed. We found that Hot start IgY can reversibly block Taq DNA polymerase activity at 50°C and have no negative impact neither on the Taq DNA polymerase activity after denaturation nor on the reverse transcriptase. We estimated that 1.0 μg of Hot start IgY effectively blocks 5 U activity of Taq DNA polymerase.
Conclusions: Egg derived Hot Start polyclonal antibodies are the cheapest source of Hot start antibodies, from one immune egg we can isolate 0.36 mg IgY, this quantity is enough for producing 1800 U activity of Hot start Taq DNA Polymerase.
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1. Introduction

Egg yolk derived antibodies (IgY) are well known as the cheapest source and equal in quality to mammalian IgG antibodies and with a few outstanding advantages over the last. These advantages associated with the development and usage of IgY include better immune responsiveness to mammalian antigens, higher affinity with persistent titers, non-invasive sampling, simple and low cost isolation process, large yield and scalable production [1]. Since 1996, IgY technology has become the internationally accepted term for describing the production and use of IgY [2,3]. European Centre for the Validation of Alternative Methods (ECVAM) workshop recommended the use of IgY instead of mammalian IgG, in order to minimize pain caused by invasive antibodies sampling, and promoted a wider implementation of chicken IgY, since this method satisfies scientific and commercial interests as well as a concern for animal welfare [3].

The monoclonal antibody to Taq DNA polymerase which is the core enzyme in polymerase chain reaction (PCR) deactivates the polymerase at ambient temperature. In the following, heating a reaction mixture causes activation of the polymerase, and the amplification proceeds in a specific and efficient manner [4,5,6]. This is the so called Hot start PCR mediated by antibodies. It can be particularly useful for the reliable detection of low copy number templates in the complex DNA backgrounds. But the recent research has shown that many commercial Taq enzyme preparations marketed as “Hot start” exhibit polymerase activity prior to thermal activation. The failure of many Hot start enzymes to perform as expected has profound implications [7]. We address this problem mainly by using the monoclonal antibodies as Hot start reagent and propose to investigate the feasibility of using polyclonal antibodies. The chosen model is polyclonal antibodies IgY derived from chicken egg.

The goal of this research was to produce and evaluate Hot start antibodies derived from chicken eggs, develop the protocol for real

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time measurement of antibody blocking activity, formulate Hot start Master mix and evaluate the influence of IgY on Taq polymerase and Reverse Transcriptase activity in Real time PCR.

2. Materials and methods

2.1. Hen immunization and IgY purification

Two twenty-week-old Brown hens were kept in cage with food and water *ad libitum*, following EVCAM recommendation [2]. All animals were acclimatized to the lab environment for at least one week before starting the experiment. Immunization schedule was approved by the Institutional Ethical Committee (protocol No 8, from 2.09.2018).

Hens were immunized intramuscularly (*Musculus pectoralis*) with Taq DNA Polymerase (Jiaxiang Biotech, Zhuji China) 0.2 mg in 0.5 mL PBS emulsified in the same volume of Freund's complete adjuvant (FCA) for the first injection. Two more booster immunizations in the same conditions were made using Freund's incomplete adjuvant (FIA). Three immunizations were administered in 10-d intervals. The following immunizations with FIA were made monthly during 3 months. Eggs were collected daily, marked and stored at 4°C for further processing.

Total IgY were isolated using 0.1% pear pectin as delipidation solution followed by ammonium sulfate precipitation 35% (*w*/*v*), according to Tong et al. [8]. IgY concentration was calculated by measuring spectra at 280 nm Nano-100 UV–Vis spectrophotometer (Allsheng Instruments, Hangzhou, China) and using extinction coefficient 1.36. Purity of isolated IgY was confirmed by reducing 10% PAAG-SDS electrophoresis.

2.2. ELISA

Specific activity of isolated total IgY against Taq polymerase was confirmed by indirect ELISA. High binding polystyrene Stripwell Microplate (Costar, USA) wells were coated with 100 μL of Taq polymerase (5 μg/mL) in 50 mM Sodium bicarbonate buffer (pH 9.6), and incubated at 4°C overnight. After washing (HydroFlex microplate washer, Tecan, Switzerland) three times with PBS containing 0.05% (*v*/*v*) Tween 20 (PBS-T), the non-specific binding sites were blocked with 5% (*w*/*v*) nonfat milk powder in PBS-T for 1 h at 37°C. After three times washing, 100 μL of total IgY (10 μg/mL) or Taq DNA polymerase specific IgY (2.5 μg/mL) in PBS-T were added to the wells as a primary antibody and then incubated for 1 h at 37°C. The plate was washed again and incubated with 100 μL of HRP labeled goat anti-chicken IgG (1:6000) as the secondary antibody for 0.5 h at 37°C. Finally, the HRP activity was detected with 100 μL of 3,3′,5,5′-tetramethylbenzidine (1% TMB, *w*/*v*) as substrate for 15 min at room temperature. The reaction was stopped by adding 50 μL of 2 mol/L H_2SO_4 to the wells. Optical density (OD) at 450 nm was determined on micro titer plate reader (Infinite F50, Tecan, Switzerland).

2.3. Affinity purification of Taq polymerase specific IgY

Affinity resin was prepared by standard method [9] of coupling Taq DNA polymerase large subunit, KlenTaq (Jiaxiang Biotech, Zhuji, China) with in-house synthesized NHS-activated Sepharose 4B (GE, Life Science).

Total IgY fraction was diluted to 1.0 mg/mL in PBS and applied to affinity resin column C10/10, (GE, Life Science) equilibrated in PBS, using "ACTA Prime plus" chromatography purification system with flow rate of 0.1 mL/min, until optical densities of flow trough A_{280} reached basic level. Elution of bound specific antibodies was carried out by 0.1 M Glycine buffer (pH 2.3) and fractioned in 5.0 mL. Eluted antibodies were neutralized by addition of the same volume of 0.2 M Sodium Phosphate buffer (pH 8.0). Eluted fractions were combined and dialyzed overnight against 100 volumes PBS.

Table 1
The used oligonucleotide sequences list.

Name	Sequence 5′-'3'	Target
Act-F	ACCACACCTTCTACAATGAGC	Human β-actin gene
Act-R	AGGTCTCAAACATGATCTGCG	
Act-P	FAM-CAACCCCAAGGCCAACCGC-TAMRA	
CSFV-F	TGAGTACAGGACAGTCGTCAGTAGTTC	Classical swine virus 5′ UTR RNA
CSFV-B	TGCCCTCGTCCACATAGCA	
CSFV-P	FAM-ACGTGAGCAGAAGCCCACCTC GA-TAMRA	

2.4. Taq polymerase activity IgY blocking assay

For testing of the IgY blocking activity on Taq polymerase, we have applied the "EvaEZ™ Fluorometric Polymerase Activity Assay Kit" (Biotum, USA) using the instruction manual with our modifications. Different quantities of IgY (0.25, 0.5 and 1 μg) were pre-incubated for 15 min with 5 U Taq DNA Polymerase in 10 μL of 2× Taq buffer (40 mM Tris–HCl, pH 8.8, 100 mM KCl, 0.2 mM EDTA, 2 mM DTT) at room temperature. Reaction mixtures were diluted ten-fold with 0.5× Taq buffer (10 mM Tris–HCl, pH 8.8, 25 mM KCl, 0.05 mM EDTA, 0.5 mM DTT) to final Taq polymerase concentration 0.5 U/μL, and 1 μL (0.5 U Taq DNA polymerase) were used in Fluorometric Polymerase Activity Assay using Step One Real Time PCR system (Applied Biosystem). Thermocycling was performed at 50°C for 10 min followed by 95°C for 2 min and finally 72°C for 10 min. Data were collected at 50°C and 72°C on FAM channel (Emission 520 nm).

2.5. Hot start master mixes formulation and real time PCR assays

We have formulated two Master Mixes (2X): *Hot Start IgY PCR Master Mix* and *One tube Reverse Transcription IgY PCR Master Mix*. Each Master Mix contains Taq DNA polymerase buffer 2× (100 mM Tris–HCl, pH 9.9, 100 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 2 mM EDTA), 0.4 mM each dNTP, and 1.5 U IgY Hot start Taq DNA polymerase. For One tube RT IgY PCR Master Mix, 500 U MMLV (RNAse H minus) Reverse Transcriptase (Jiaxiang Biotech, Zhuji, China) was added. Two Master Mixes were lyophilized with 5% Trehalose and 0.5% PEG 6000 as cryoprotection additives.

To test *Hot start IgY PCR Master Mix*, we have applied human genomic DNA amplification, using actin specific primer/probe detection system (Table 1). Serial dilutions of the human DNA: 6.0, 0.6 and 0.06 ng to reach 2000, 200 and 20 gene copies, respectively were applied to PCR using following cycling conditions: 3 min/95°C and 45 cycles at 5 s/95°C and 10 s/58°C, fluorescent signal (FAM) was measured at

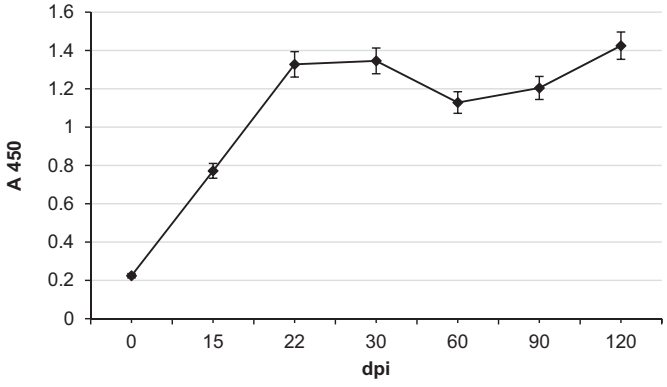

Fig. 1. ELISA kinetic of IgY (1.0 μg) titers in eggs from laying chickens, immunized by Taq DNA polymerase. The results are expressed as the mean OD_{450} (n = 3), values obtained after normalization to the background absorbance, dpi: days past immunization.

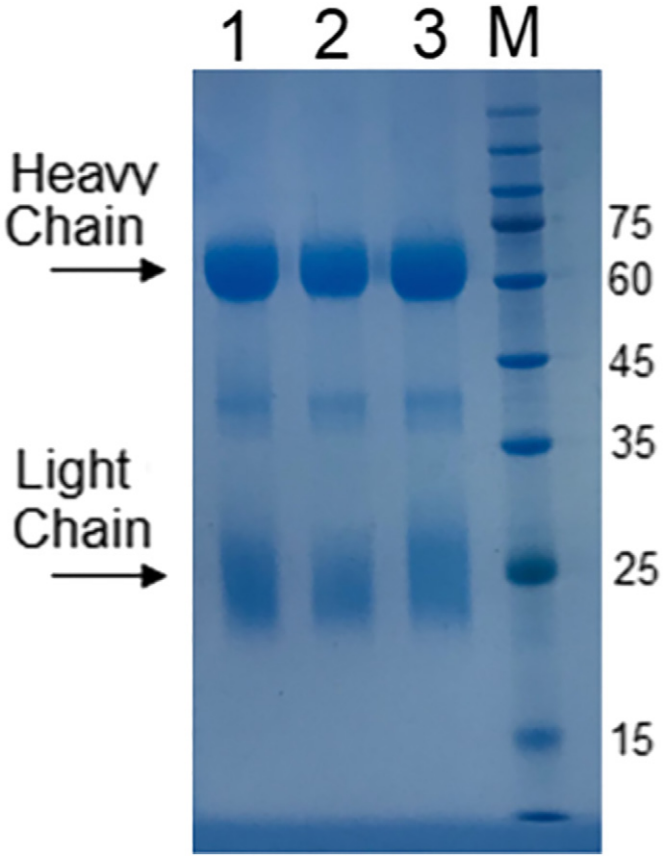

Fig. 2. Representative analysis of raw IgY purity by 10% SDS PAGE, isolated from eggs collected at different dpi: 1–20 dpi; 2–30 dpi; 3–90 dpi.

58°C. Each samples analyzed in duplicate. Calibration curve was generated to assess PCR efficiency.

To test *One tube Reverse Transcription IgY PCR Master Mix*, we have applied Classical swine fever virus (vaccine strain) RNA amplification, using 5′ UTR specific primer/probe detection system (Table 1). Serial dilutions of virus RNA: 2.0, 1.0 and 0.5 ng were applied to RT-PCR using following cycling conditions: 10 min/50°C; 3 min/95°C and 45 cycles at 5 s/95°C and 10 s/58°C, fluorescent signal (FAM) was measured at 58°C. Each samples analyzed in duplicate. Calibration curve was generated to assess RT PCR efficiency.

3. Results

The development of specific IgY antibodies against Taq DNA polymerase was observed fifteen days after the first immunization (15 dpi) and reached maximum level at 22 dpi. The presence of specific antibodies was detected in the egg yolk by indirect ELISA during all course of immunization (Fig. 1). Hens tolerated well immunization; eggs were produced up to 4 month after the first immunization, and no decline of eggs productivity was observed, confirming that Taq DNA polymerase is not toxic as antigen and not affect the eggs productivity.

The first step of the IgY isolation from egg yolk involves the extraction of soluble proteins from lipids and lipoproteins. This protocol includes the precipitation of lipids by 5 fold dilution egg yolk in 0.1% (*w*/*v*) water solution of pear pectin at slightly acid conditions [8]. The second step of the IgY isolation includes precipitation of water soluble proteins, including IgY fraction, by saturation with 35% (*w*/*v*) ammonium sulfate. This simple two-step IgY purification protocol allow us to isolate IgY from egg yolk with high yield and purity. Average yield of total isolated IgY was up to 180.0 ± 1.4 mg per egg. The IgY purity was estimated by 10% SDS-PAGE under reducing conditions (Fig. 2). It showed that IgY antibodies contained two major bands of 27 kDa and 65 kDa proteins, corresponding to the light and heavy chains, respectively.

For affinity purification of isolated IgY, we have synthesized affinity resin based on the Sepharose 4B as matrices and exonuclease deficient version of Taq polymerase (large subunit, KlenTaq DNA polymerase) as the ligand. We have chosen KlenTaq DNA polymerase for affinity resin synthesis to sequester only the polymerase domain specific IgY and thus increase possibility of blocking the active center of enzyme. During synthesis of affinity matrices, we have estimated the content of amino groups after the amination of epoxy-activated Sepharose, which composed near 24 μM/g of moist weight gel. We have found that 2.5 mL of affinity matrices (50% slurry) allow us to isolate near 2.0 mg Taq DNA polymerase specific antibodies, specificity of which has been confirmed by ELISA (Fig. 3).

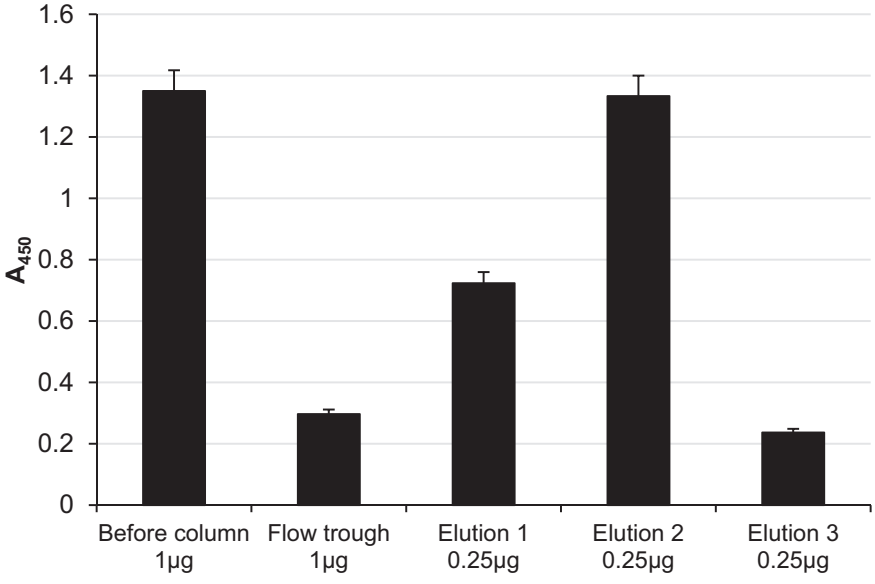

Fig. 3. Affinity purification of IgY: The results are expressed as the mean OD_{450} (n = 3), values obtained after normalization to the background absorbance.

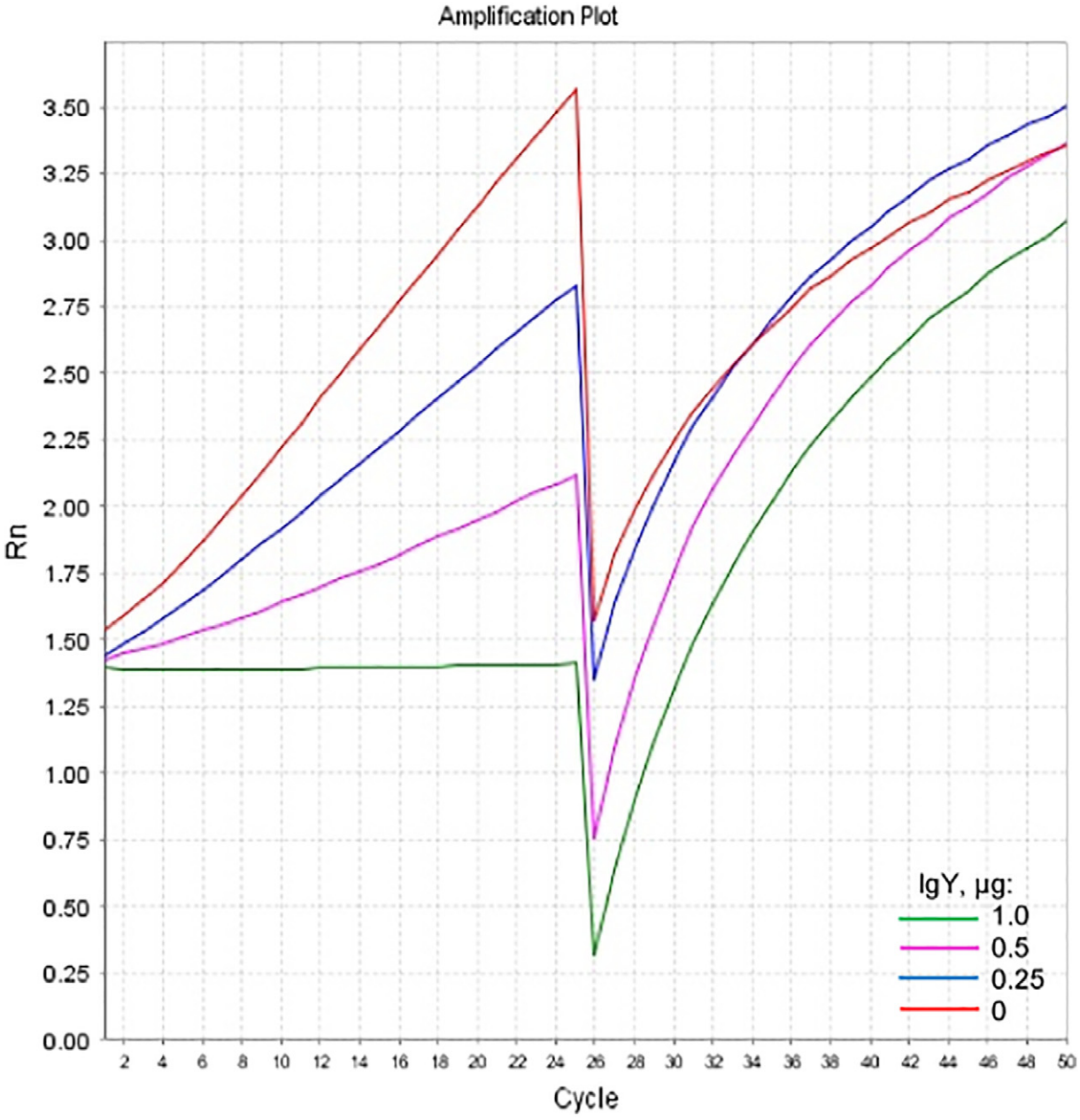

Fig. 4. Kinetic curves of reversible Taq DNA Polymerase (5 U) blocking by different quantity of Hot start IgYs. Fluorescence was monitored continuously on FAM channel at 50°C (cycles 1–25) and 72°C (cycles 26–50), using "EvaEZ™ Fluorometric Polymerase Activity Assay Kit" (Biotum, USA) and 0.5 U of blocked and unblocked Taq DNA polymerases.

For functional antibodies testing, we have developed a simple protocol based on the "EvaEZ™ Fluorometric Polymerase Activity Assay Kit" with our modification; the main feature composed in real time monitoring of the Taq DNA polymerase activity while blocked with affinity purified IgY at not permissive temperature 50°C during first 10 min followed by "Hot start" denaturation at 95°C for 2 min, and finally polymerase activity was measured at an acceptable temperature 72°C for 10 min. We have titrated different quantities of IgY completely and partially blocking Taq DNA polymerase activity at 50°C without affecting polymerase activity after Hot start activation. It was found that 1.0 μg of Hot start IgY effectively blocks 5 U activity of Taq polymerase, while 0.5 and 0.25 μg of Hot start IgY partially blocks Taq polymerase (Fig. 4) in comparison with unblocked counterpart. According to the kit manual, the rate of increase of fluorescence is positively correlated with activity of polymerase. It can be seen at Fig. 4 that unblocked Taq polymerase (red curve) has the highest slope in the first part of kinetic curve (0% of blocking), confirming the activity of Taq polymerase at 50°C. In contrast, the curve generated by Taq DNA polymerase incubated with 1.0 μg of IgY (green curve) looks like a flat line (100% of blocking), confirming the lack of polymerase activity at 50°C. In the same time 0.5 and 0.25 μg of Hot start IgY only partially blocked Taq polymerase activity (violet and blue curves, respectively), the curves sloping down with increasing IgY quantity, consistently with the expected values (80% and 40% blocking efficiency, respectively), showing dose depended inhibition manner. However, after denaturation at 95°C for 2 min reflected as sharp decreasing fluorescence in the middle of the chart, polymerase activity was restored during incubation at 72°C as seen at the last part of curves in Fig. 5. Taq DNA polymerase blocked with 1.0 μg of Hot start IgY shows similar activity as its unblocked counterpart (same curves slope). Hence, we conclude that Hot start IgY do not inhibit polymerase activity at 1 μg per 5 U Taq DNA Polymerase, after denaturation step, but rather reversibly block it.

Similar blocking effect has been achieved at 50°C using mouse monoclonal Hot start antibodies (TCP-101, Toyobo, Osaka, Japan). At

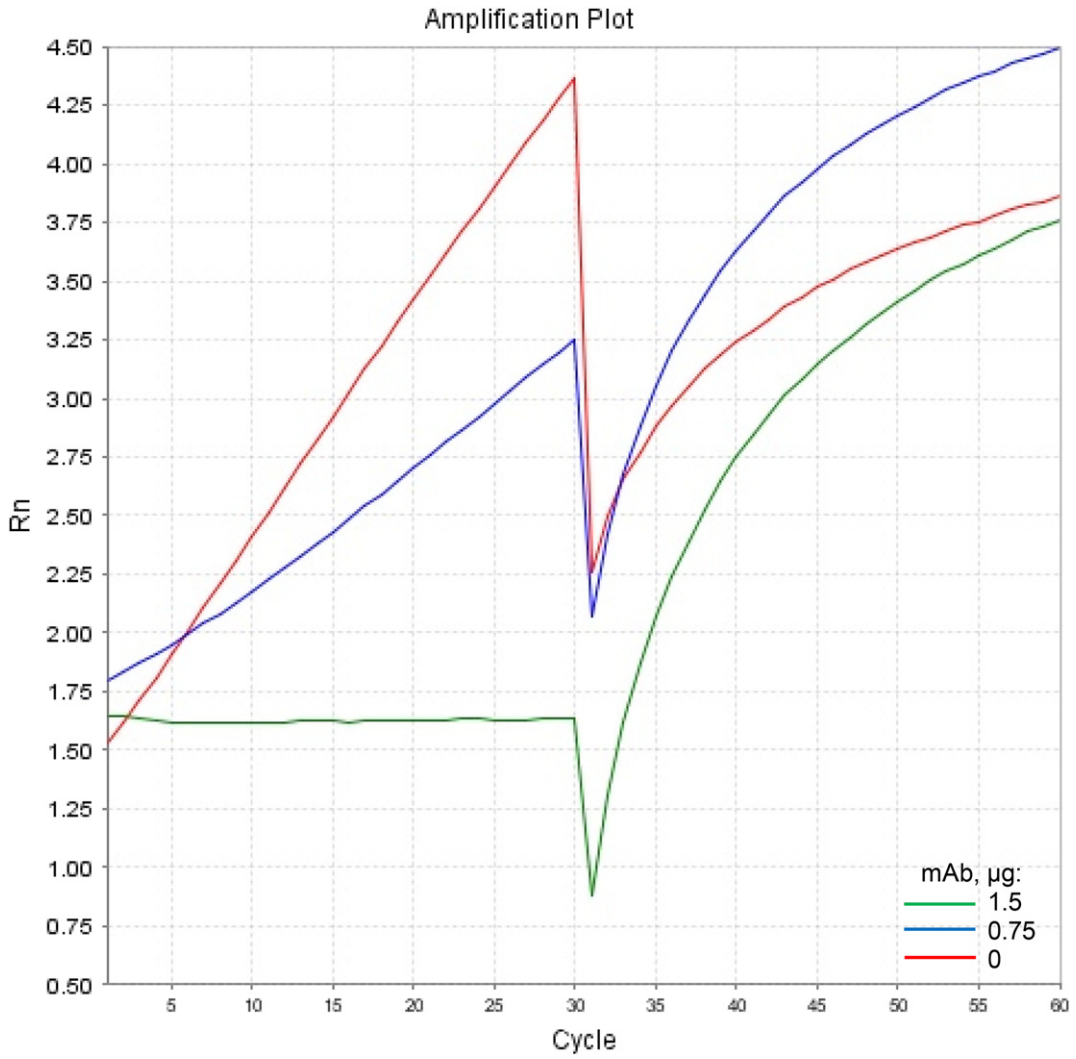

Fig. 5. Kinetic curves of reversible Taq DNA Polymerase (5 U) blocking by different quantity of Hot start monoclonal antibodies (TCP-101, Toyobo, Osaka, Japan). Fluorescence was monitored continuously on FAM channel at 50°C (cycles 1–25) and 72°C (cycles 26–50), using "EvaEZ™ Fluorometric Polymerase Activity Assay Kit" (Biotum, USA) and 0.5 U of blocked and unblocked Taq DNA polymerases.

least 1.5 µg of the antibodies completely reversibly blocks 5 U of Taq DNA polymerase (green curve, Fig. 5), while 0.75 µg was not enough to completely blocks polymerase activity at 50°C (blue curve, Fig. 5). According to the instructional manual 1 µg of monoclonal Hot start antibodies blocks 5 U of Taq DNA polymerase with 99.3% efficiency, which is in concordance with our findings.

To proof the concept of applied protocol for real time monitoring of Hot start Taq DNA polymerase evaluation, we have tested commercially available Hot start polymerase – Dream Taq Hot Start DNA Polymerase (Thermo Scientific) along with IgY blocked Taq DNA Polymerase, and found similar profiles of kinetic curves, generated using the "EvaEZ™ Fluorometric Polymerase Activity Assay Kit" (Fig. 6) and 0.5 U of each Hot Start DNA Polymerases.

To assess the effect of eggs derived Hot start antibodies on the PCR and RT-PCR performance, we have formulated, dried and tested two kinds of PCR Master Mixes (2 X): Hot start IgY PCR Master Mix and One tube Reverse Transcription IgY PCR Master Mix. Serial dilutions of human genomic DNAs and Classical swine fever virus RNAs has been used for PCR efficiency assessments. We have found no negative impact of IgY neither on the PCR efficiency (R^2 0.996; Efficiency 93%) nor on the RT PCR efficiency (R^2 0.995; Efficiency 91%), Fig. 7 and Fig. 8, respectively. We have performed our blocking experiments at 50°C especially to avoid interferences between Taq DNA polymerase and MMLV Reverse transcriptase during reverse transcription in one tube reaction, because as previously shown the Taq DNA Polymerase is highly active at 50°C (Fig. 4, red curve). One tube RT PCR results indicate that the virus RNA can be easily reverse transcribed and amplified in the presence of Hot start IgY blocked Taq polymerase, and IgY did not inhibit the activity of MMLV RT (Fig. 8).

4. Discussion

Hot start can greatly improve specificity, sensitivity and yield of PCR. Non-specific amplification and primer dimers formation during PCR are still the most serious problems if reaction mixture is prepared at room temperature, since Taq polymerase is active and the primers can hybridize non-specifically [4]. Hot start Taq polymerases remain inactive at room temperature and are activated after heating at 95°C, preventing non-specific amplification.

The original approach for Hot start PCR was implemented by spatial separation Taq polymerase from primers and target DNA using a heat-labile wax or jelly barrier that melts and permits mixing of aqueous components only at an elevated temperature. Drawbacks arising from the use of these "hot start" methods include the increased probability of crossover contamination whenever the reaction tubes must be

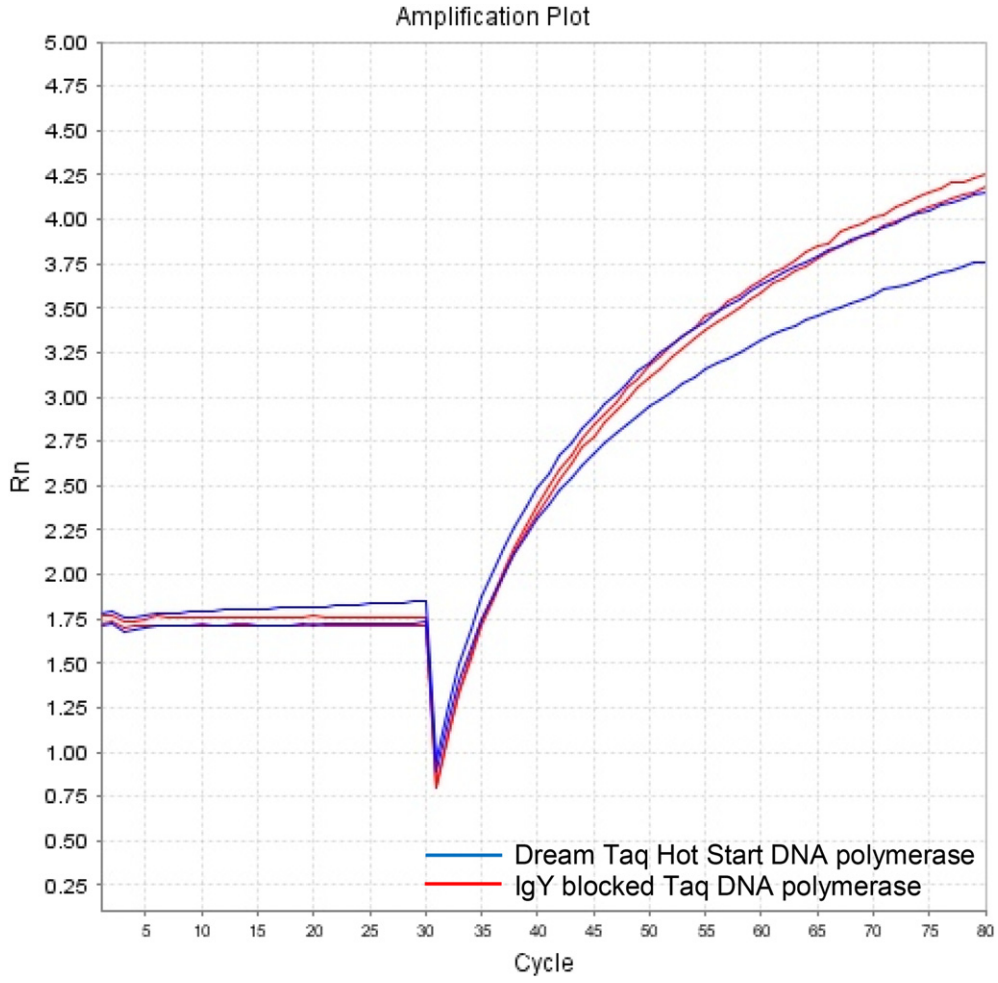

Fig. 6. Kinetic curves of Hot start Taq DNA polymerase activity evaluations: Dream Taq Hot Start DNA Polymerase (Thermo Scientific) – blue curves and IgY blocked Taq DNA polymerase (Jiaxiang Biotech) – red curves. Fluorescence was monitored continuously on FAM channel at 50°C (cycles 1–30) and 72°C (cycles 31–80), using "EvaEZ™ Fluorometric Polymerase Activity Assay Kit" (Biotum, USA) and 0.5 U of Hot start Taq DNA polymerases. Samples were tested in duplicate.

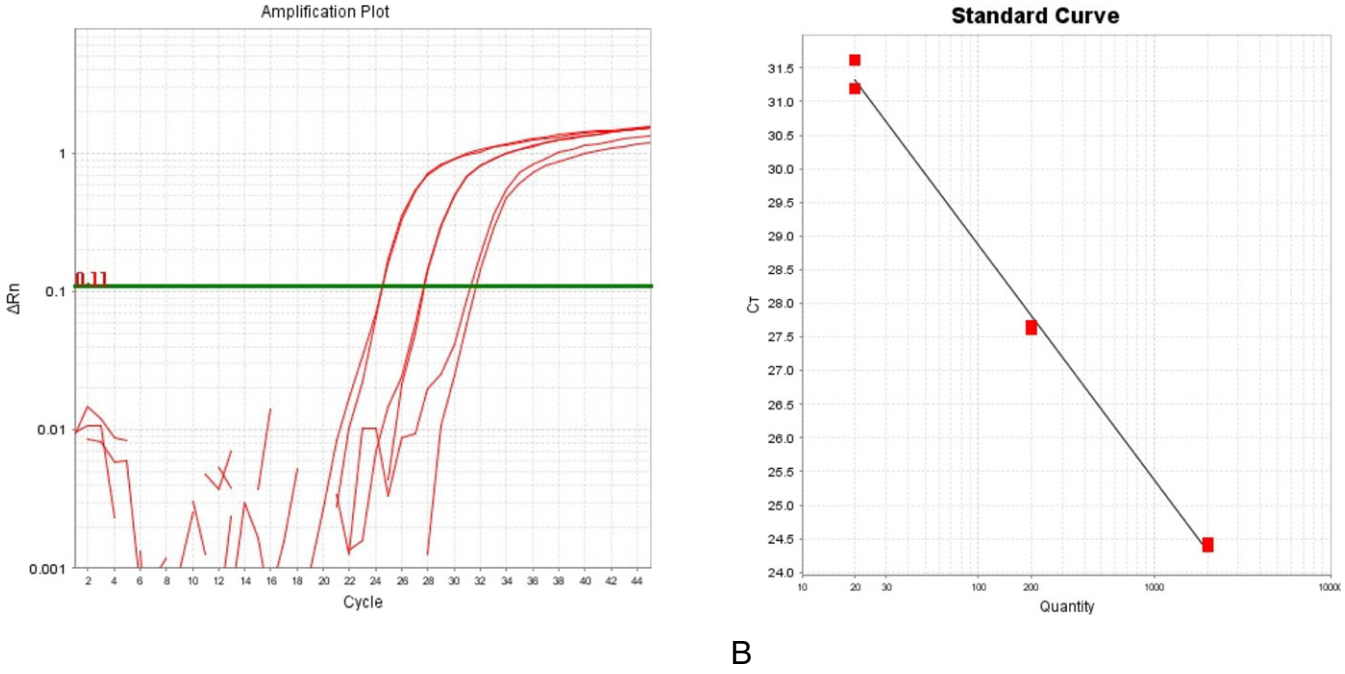

Fig. 7. PCR efficiency estimation (A) Amplification plot of serial dilutions human DNA: 6.0, 0.6 and 0.06 ng by Hot start IgY PCR Master Mix and actin specific primer set (B) Standard curve (Slope −3.5; Y-intercept 35.9; R^2 0.996; Efficiency 93%). Samples were tested in duplicate.

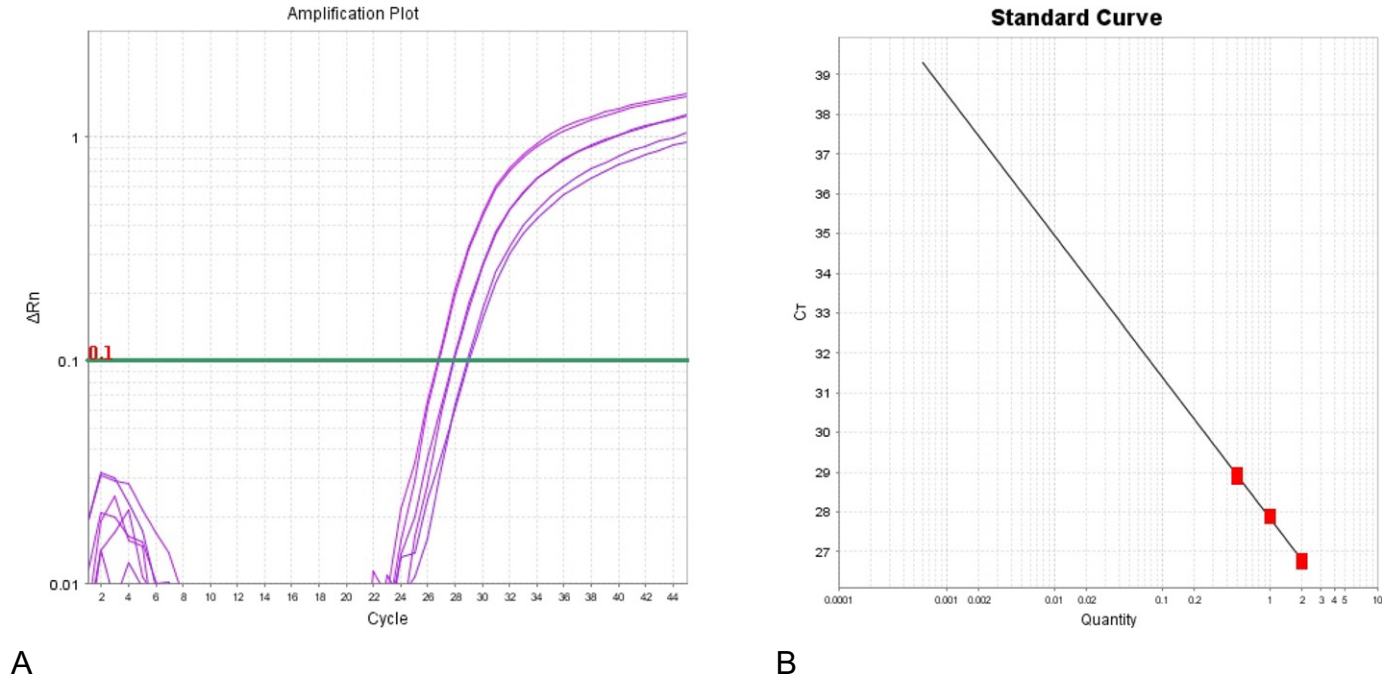

Fig. 8. One step RT-PCR efficiency estimation (A) Amplification plot of serial dilutions CSF virus RNA: 2.0, 1.0, and 0.5 ng by One tube Reverse Transcription IgY PCR Master Mix (B) Standard curve (Slope − 3.5; Y-intercept 27.8; R^2 0.995; Efficiency 91%). Samples were tested in duplicate.

reopened, and time-consuming when working with multiple samples compared with the conventional PCR techniques [4].

Nowadays two main strategies exist in the production of Hot start Taq polymerase: internal modifications and external modifications. Internal modifications include chemical [10,11] or cold sensitive mutations [12] improving the enzyme features, while the external modifications involve the addition of antibodies [4,5,6], oligonucleotide aptamers [13,14,15] or more recently, quantum dots [16]. All these methods have some advantages and drawbacks over each other. In any case, hot start antibodies are the most common modification method. Still, the recent research has shown that many commercial enzyme preparations marketed as “hot start” exhibit polymerase activity prior to thermal activation [7].

We addressed this problem mainly by using the monoclonal antibodies as Hot start reagent. During storage, monoclonal antibodies can dissociate from Taq polymerase which leads to the reduced blocking efficiency. Antigenic epitope mapping of surface Taq polymerase revealed at least nine epitopes, seven of which are located at polymerase domain and responsible for blocking polymerase activity [17]. Using polyclonal antibodies, probably, can solve this problem; blocking efficiency may be supplemented by polyclonal antibodies interacting with different epitopes on the Taq polymerase surface.

We also proposed and adjusted protocol for real time monitoring of blocking efficiency of Hot start antibodies using commercially available the “EvaEZ™ Fluorometric Polymerase Activity Assay Kit.” That is different from the end point measurement methods used before, such as hairpin extension [12], radioisotope labeling extended DNA [11] and primer dimers amplification systems [7]. Proposed methods allow us to visually determine the percentage of inhibition in a simplest and fastest way. The simplicity of this procedure makes this technique very attractive for quality control of blocking activity commercial preparation of Hot start Taq polymerases and avoiding the undesirable consequences associated with incomplete blocking of Taq enzymes. Moreover, we have found that polyclonal Hot start IgY can also reversibly block activity of Pfu DNA Polymerase as well as Taq DNA Polymerase (data not showed) confirming the utility of using polyclonal IgY as Hot start reagent. We have found that Hot start IgY has no negative effect neither on the Taq DNA polymerase (after initial denaturation step) nor on the MMLV reverse transcriptase activity, during One tube RT PCR protocol.

In conclusions, we produced and characterized the functionality of the eggs derived Hot start IgY antibodies. It was calculated that one immunized egg allows us producing 0.36 mg of Hot Start IgY's for blocking at least 1800 U of Taq DNA polymerase, that enough for producing 1200 tests (1.5 U per test). Egg derived Hot start polyclonal antibodies are the cheapest source of Hot start antibodies and have potential to replace mouse monoclonal antibodies on the market for production of Hot start Taq DNA polymerases.

Conflict of interest

The authors declare no competing interests.

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