

## Apta-PCR

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### Abstract

Real-time Apta-PCR is a methodology that can be used for a wide variety of applications ranging from food quality control to clinical diagnostics. This method takes advantage of the combination of the sensitivity of nucleic acid amplification with the selectivity of aptamers. Ultra-low detection of target analyte can potentially be achieved, or, improved detection limits can be achieved with aptamers of low-medium affinity. Herein, we describe a generic methodology coined real-time Apta-PCR, using a model target ( $\beta$ -conglutin) and a competitive format, which can be adapted for the detection of any target which an aptamer has been selected for.

**Key words** Apta-PCR, Aptamer, Protein quantification

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## 1 Introduction

The quantitative polymerase chain reaction (qPCR) facilitates the specific and sensitive real-time detection of nucleic acid molecules, whilst enzyme-linked immunosorbent assays (ELISA) or radioimmunoassays (RIA) are used for the quantitative detection of a wide range of protein targets. In the early 1990s, Cantor's group reported the exploitation of the quantitative amplification achievable with qPCR for the detection of a protein target in a technique he called Immuno-PCR [1], where specific antibody receptors were conjugated to DNA tags, that following formation of a sandwich-type immunocomplex served as a template for the qPCR reaction. Immuno-PCR offers great sensitivity, enhancing the sensitivity of a common immunoassay by up to 100,000-fold [2–5]; the technique suffers from some drawbacks related to the coupling of the antibody with the DNA reporter moieties. Such coupling often results in an uneven numbers of reporters per antibody, thus hindering true quantification [6, 7]. Additionally, following the immuno-recognition step, the DNA needs to be eluted from the antibody for subsequent amplification.

The real-time Apta-PCR described herein represents a further advancement and simplification of Immuno-PCR, where a single aptamer molecule replaces the cumbersome antibody-DNA complex. Recently, the aptamer specificity has been combined with qPCR sensitivity in various approaches for the ultrasensitive detection of proteins, including a proximity ligation assay [8–12], nuclease protection assay [13], capillary electrophoresis (CE) [14], and the use of target-modified magnetic microparticles [15]. In contrast to these reported approaches, real-time Apta-PCR does not rely on previous knowledge of the aptamer receptor and the instruments and methods used are widely available. The only requirement of the method is that the reporter aptamer to be employed is flanked with primer regions, and as the vast majority of reported aptamers have been selected by SELEX, they originally were flanked by primer-binding sites and this does not represent an issue—even in the scenario where part or all of the primer is involved in target binding, the aptamer will be released from the aptamer-target complex prior to amplification [16, 17]. Similar to ELISA, different types of real-time Apta-PCR can be performed, including direct or indirect sandwich and competitive assays.

In sandwich real-time Apta-PCR a capture affinity molecule, such as an aptamer or antibody is immobilized to capture the analyte of interest, which is effectively sandwiched between a capture biomolecule and a reporter aptamer. This format can provide better specificity as each of the capture and reporter biomolecules bind to a specific region on the analyte of interest. Competitive real-time Apta-PCR exploits competition of the analyte of interest with an immobilized standard amount of analyte for binding to the aptamer [18–20].

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## 2 Materials

### 2.1 Plate Preparation

1. Immobilization buffer: 50 mM Carbonate/bicarbonate buffer: 44 mM sodium bicarbonate, 6 mM sodium carbonate, pH 9.6 (Sigma-Aldrich). Store at 4 °C.
2. Blocking buffer (PBS-Tween): 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05 % v/v Tween-2<sup>®</sup> (*see Note 1*). Store at 4 °C.
3. Model target protein:  $\beta$ -Conglutin (Extracted, isolated and characterized as previously described [21]). Store at –20 °C.
4. Flat-bottomed microtiter plate.

### 2.2 Competitive Apta-PCR Assay

1. SELEX binding buffer (*see Note 2*): Phosphate-buffered saline (PBS)+1.5 mM MgCl<sub>2</sub>. Adjust to pH 7.4 with HCl and NaOH. Store at room temperature.

2. Model aptamer sequence:  $\beta$ -Conglutinin binding aptamer ( $\beta$ -CBA) 5'-agc tga cac agc agg ttg gtg ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa cca cga gtc gag caa tct cga aat-3'. Store at  $-20\text{ }^{\circ}\text{C}$ .
3. Salmon sperm DNA. Store at  $-20\text{ }^{\circ}\text{C}$ .
4. MilliQ water ( $18.2\text{ M}\Omega\cdot\text{cm}$ ).

### 2.3 Real-Time PCR

1.  $10\times$  PCR buffer: 200 mM Tris-HCl, 500 mM KCl. Adjust to pH 8.4 with HCl and NaOH. Store at  $-20\text{ }^{\circ}\text{C}$ .
2. 50 mM  $\text{MgCl}_2$ . Store at  $-20\text{ }^{\circ}\text{C}$ .
3. 2 mM dNTPs. Store at  $-20\text{ }^{\circ}\text{C}$ .
4. 10,000-fold concentrate SYBR Green I in DMSO (*see Note 3*). Store at  $-20\text{ }^{\circ}\text{C}$ .
5. 1  $\mu\text{M}$  ROX (*see Note 4*). Store at  $-20\text{ }^{\circ}\text{C}$ .
6. 10  $\mu\text{M}$  forward primer: 5'agc tga cac agc agg ttg gtg 3'. Store at  $-20\text{ }^{\circ}\text{C}$ .
7. 10  $\mu\text{M}$  reverse primer: 5'att tcg aga ttc ctc gac tcg tg 3'. Store at  $-20\text{ }^{\circ}\text{C}$ .
8. Taq DNA polymerase. Store at  $-20\text{ }^{\circ}\text{C}$ .
9. 96/384-well plates for qPCR.
10. Sealing film.

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## 3 Methods

### 3.1 Plate Preparation

1. Dissolve the protein in immobilization buffer to a final concentration of 20  $\mu\text{g}/\text{mL}$ .
2. Incubate 50  $\mu\text{L}/\text{well}$  of the protein solution on each well of the microtiter plate for 30 min. at  $37\text{ }^{\circ}\text{C}$  under shaking conditions.
3. Remove the supernatant.
4. Wash three times with 200  $\mu\text{L}$  of PBS-Tween buffer.
5. Incubate 200  $\mu\text{L}/\text{well}$  of blocking buffer within the microtiter plate for 30 min. at  $37\text{ }^{\circ}\text{C}$  under shaking conditions.
6. Discharge the supernatant.
7. Wash three times with 200  $\mu\text{L}$  of PBS-Tween buffer (*see Note 5*).

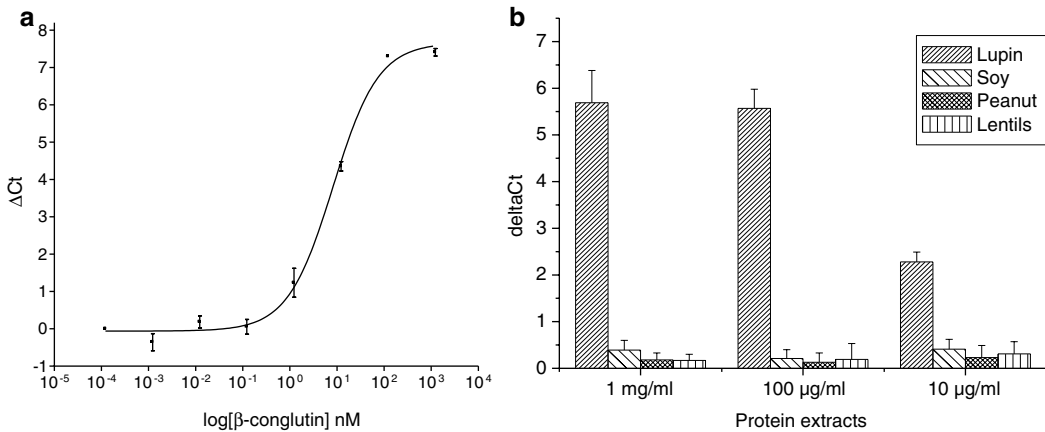
### 3.2 Competitive Apta-PCR Assay

1. In individual Eppendorf tubes, prepare a series of dilutions (0–1  $\mu\text{M}$ ) of the protein of interest (*see Note 6*) with 1 nM of aptamer (*see Note 7*), in the presence of 3  $\mu\text{g}/\text{mL}$  salmon sperm DNA from baker's yeast in SELEX binding buffer. All samples including standards are carried out in triplicate.

2. Incubate this mixture solution for 30 min at 37 °C under moderate shaking condition (*see* **Note 8**).
3. Carefully transfer the 50  $\mu\text{L}$  of this solution into individual wells of the previously coated microtiter plate.
4. Incubate the plate for 30 min at 37 °C under shaking conditions.
5. Carefully remove the supernatant.
6. Wash the plate three times with 200  $\mu\text{L}$  of SELEX binding buffer and remove the supernatant.
7. Add 50  $\mu\text{L}$  of MilliQ water.
8. Incubate the plate at 95 °C for 5 min.
9. Recover the supernatant containing the eluted aptamer.

### 3.3 Real-Time PCR

1. Prepare 1 $\times$  PCR Master Mix on ice for desired amount of samples (*see* **Note 9**):  
Master Mix solutions for ten samples
  - (a) 10  $\mu\text{L}$  10 $\times$  PCR buffer
  - (b) 10  $\mu\text{L}$   $\text{MgCl}_2$
  - (c) 10  $\mu\text{L}$  0.2 dNTPs
  - (d) 1  $\mu\text{L}$  of 1  $\mu\text{M}$  ROX dye
  - (e) 1  $\mu\text{L}$  of forward primer
  - (f) 1  $\mu\text{L}$  of reverse primer
  - (g) 1  $\mu\text{L}$  of water solution of SYBR Green I 1–100
  - (h) Taq enzyme 1  $\mu\text{L}$
  - (i) Water to 100  $\mu\text{L}$
2. Transfer 1  $\mu\text{L}$  of the eluted aptamer into individual wells of a 384 qPCR plate (*see* **Note 10**). Triplicate repeats are recommended. Three non-template controls (NTC) consisting of 1  $\mu\text{L}$  of MilliQ water should also be included.
3. Using a multichannel pipette, transfer 9  $\mu\text{L}$  1 $\times$  Master Mix (on ice) to each well.
4. Carefully seal the plate with the sealing film.
5. Place the plate in a qPCR instrument, previously programmed (*see* **Note 11**):
  - (a) Denaturation:  
95 °C for 2 min.
  - (b) 40 $\times$  PCR cycles:  
95 °C for 10 s  
58 °C for 30 s  
72 °C for 30 s.



**Fig. 1** Real-time Apta-PCR detection of  $\beta$ -conglutinin. (a) Raw dates obtained from real-time PCR experiment. The numbers (1–8) indicate Ct values of different  $\beta$ -conglutinin concentration from 0–365  $\mu\text{g}/\text{mL}$ , 1:10 dilutions. (b) Calibration curve obtained for  $\beta$ -conglutinin via real-time Apta-PCR competitive assay. Calibration curve was obtained by plotting the  $\Delta\text{Ct}$  values versus the concentration of  $\beta$ -conglutinin, obtaining a linear range from 1.3 (0.382  $\mu\text{g}/\text{mL}$ ) to 68.4 nM (20.1  $\mu\text{g}/\text{mL}$ ) of  $\beta$ -conglutinin with an  $\text{EC}_{50}$  value of 8.38 (2.5  $\mu\text{g}/\text{mL}$ ) and a L.O.D. of 85 pM (25 ng/mL). Inset: Cross-reactivity studies with soy, peanut and lentils. The error bars represent the standard deviation of 5 repetitions [18]

(c) Melting curve analysis:

95 °C for 15 s.

60 °C for 15 s.

95 °C for 15 s.

- Analyze results using real-time PCR software to calculate the amount of aptamer in the sample, which, in turn, is proportional to the amount of analyte present.
- Calculate  $\Delta\text{Ct}$ , which is the difference between the cycle threshold of the sample and the blank (sample containing aptamer not exposed to the target) (Fig. 1).

## 4 Notes

- Buffers should be chosen according to their compatibility with the protein of interest. All solutions are prepared with deionized water purified with a resistivity of 18.2  $\text{M}\Omega\cdot\text{cm}$  at 25 °C.
- To achieve better performances, the buffer normally used is the one in which the aptamer was selected.
- Pre-formulated Master Mix containing all the components (except template, primers and water) are commercially available.
- ROX is a passive reference dye specific for the qPCR instrument used. Some other brands might use different dyes and the last generation of qPCR instruments do not need this internal reference.

5. Normally, plates filled with 200  $\mu\text{L}$  of PBS-Tween can be stored at 4  $^{\circ}\text{C}$  up to 1 week, but this might vary according to the protein of interest.
6. From this step on, filtered tips should be used as they minimize the risk of contamination.
7. We have noticed that this particular concentration works consistently for different targets, giving controls, where the protein is absent, with an amplification profile equal to the one from the nontemplate control (the NTC and the no-protein control should have the same Ct, threshold Cycle). If the negative control differs from the NTC having a lower Ct, the concentration of the aptamer needs to be lowered.
8. If pre-prepared plates are used, during this step the microtiter plate should be equilibrated at 37  $^{\circ}\text{C}$  during this step. In case the microtiter plate are freshly prepared, this step is carried out at the same time as the **step 5** of the microtiter plate preparation.
9. Without the enzyme the buffer can be safely stored for a couple of weeks in freezer once prepared. The primer and the magnesium concentrations of this master mix have been optimised for  $\beta$ -conglutin detection but may vary for other targets. Other formulations containing DMSO, BSA, betaine, or other reagents can be used to improve the performance of the reaction.
10. In case of low reproducibility, higher amount of eluted DNA can be used for amplification: 10  $\mu\text{L}$  of eluted DNA is transferred to individual wells of 96 qPCR plate with 10  $\mu\text{L}$  of 2 $\times$  PCR Master Mix to obtain a final volume of 20  $\mu\text{L}$  of PCR product.
11. The PCR protocol should be selected according to the enzyme used.

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