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Production of monoclonal antibodies and development of a quantitative immuno-polymerase chain reaction assay to detect and quantify recombinant Glutathione S-transferase

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ABSTRACT

GST-tagged proteins are important tools for the production of recombinant proteins. Removal of GST tag from its fusion protein, frequently by harsh chemical treatments or proteolytic methods, is often required. Thus, the monitoring of the proteins in tag-free form requires a significant effort to determine the remnants of GST during purification process. In the present study, we developed both a conventional enzyme-linked immunosorbent assay (ELISA) and an immuno-polymerase chain reaction (IPCR) assay, both specific for detection of recombinant GST (rGST). rGST was expressed in *Escherichia coli JM109*, using a pGEX4T-3 vector, and several anti-rGST monoclonal antibodies were generated using hybridoma technology. Two of these were rationally selected as capture and detection antibodies, allowing the development of a sandwich ELISA with a limit of detection (LOD) of 0.01 μg/ml. To develop the rGST-IPCR assay, we selected "Universal-IPCR" format, comprising the biotin-avidin binding as the coupling system. In addition, the rGST-IPCR was developed in standard PCR tubes, and the surface adsorption of antibodies on PCR tubes, the optimal neutravidin concentrations, the generation of a reporter DNA and the concentration effect were studied and determined. Under optimized assay conditions, the rGST-IPCR assay provided a 100-fold increase in the LOD as well as an expanded working range, in comparison with rGST-ELISA. The proposed method exhibited great potentiality for application in several fields in which measurement of very low levels of GST is necessary, and might provide a model for other IPCR assays.

1. Introduction

Affinity tags have become essential tools for the production of recombinant proteins in a wide variety of settings, from academic research to high-throughput structural biology as well as in industrial applications [1]. One of the most used tags is Glutathione S-transferase (GST), a naturally occurring 26 kDa protein found in eukaryotic cells. The GST gene from the parasitic helminth *Schistosoma japonicum* was used in the development of the pGEX vectors, becoming a versatile system for the expression, detection and purification of GST-tagged proteins produced in bacteria, yeast, insect cells, and mammalian expression system [2,3].

Removal of the GST tag is often necessary to obtain a pure protein, for instance to perform biophysical studies and structure determinations of the target protein. The tag is usually removed by harsh chemical treatments or proteolytic methods (e.g., thrombin and factor Xa) [4,5]. Regardless of the method used to remove the tag, a high level of purity is usually required. Thus, it becomes essential to determine the amount of GST that remains in the fraction of purified protein [6–8]. A variety of methods have been designed to estimate both the relative level of expression and purity of the protein purified, such as SDS-PAGE, Western Blot, enzyme immunoassay (EIA) and enzymatic activity methods among others. For instance, the enzyme-linked immunosorbent assay (ELISA) is a class of immunoassay able to detect the presence of a target substance using an antibody, suitable for rapid and simple analysis of a target protein [9]. Although ELISA techniques have displayed adequate sensitivity in several applications, it is highly valuable to develop methods that contribute with a higher sensitivity and remains operationally simple [10].

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In 1992, Sano et al. [11] created a new technique called the immuno-polymerase chain reaction (commonly referred to as immuno-PCR or IPCR), in which they replaced the detection enzyme in ELISA with a biotinylated reporter DNA [12]. This reporter DNA is then amplified by PCR for signal generation, being the number of PCR amplicons produced proportional to the initial quantity of antigen to be detected [13]. Since Sano et al. created this technique, different strategies have been applied to link antibodies with DNA templates [14–16] and several IPCR methods have been developed for the detection of several antigens [12,17,18], including viral antigens [19–21], bacterial antigens [22], prions [23,24], toxins [25,26], tumor markers [27], cytokines [28], polycyclic aromatic hydrocarbons [29–31], and hormones [32,33] as well as for immunogenicity testing [34]. Together, these and other studies have shown that the IPCR is able to reach a 10- to 10000-fold higher detection limit than an ELISA. Here, we developed an IPCR assay for the detection of recombinant GST (rGST) expressed from pGEX-transformed *Escherichia coli*. We showed that IPCR permits a robust detection of low concentrations of rGST, with a higher sensitivity than ELISA. The IPCR assay described here is based on what is known as a "Universal" format of IPCR, and was performed in standard PCR tubes without pretreatment. These characteristics support its potential usefulness for target protein purification monitoring.

2. Materials and methods

2.1. Expression and purification of rGST

The GST gene from the parasitic helminth *Schistosoma japonicum* was used to develop pGEX vectors [2]. rGST was expressed in *E. coli JM109* (Stratagene) using a pGEX4T-3 vector (GE Healthcare; GenBank accession no. U13855). The protein was obtained according to protocols previously described [35]. Briefly, *E. coli JM109* transformed with pGEX4T-3 were cultured overnight and then diluted 1: 20 in 400 ml of fresh medium and grown for 2 h at 37 °C before adding 80 μl of IPTG 0.5 M. After a further 3 h of growth, cells were pelleted and resuspended in PBS. Cells were lysed on ice by mild sonication and after adding Triton 20% (Sigma-Aldrich) were subjected to centrifugation at 14000 rpm for 10 min at 4 °C. Then, supernatant containing rGST was purified by affinity chromatography using prepacked columns with Glutathione Sepharose (GSTrap™ HP; GE Healthcare), in accordance with the manufacturer's protocols. Briefly, the column was equilibrated with 5 column volumes of binding buffer (PBS, pH 7.3). 20 ml of supernatant containing rGST was applied to a flow rate of 0.2–0.5 ml/min. Elution was performed with 5–10 column volumes of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) at a flow rate of 0.5 ml/min. Finally, the purified recombinant antigen was evaluated by SDS-PAGE analysis.

2.2. Generation of hybridoma cells and purification of monoclonal antibodies (MAbs)

Hybridomas were established from spleen cells from BALB/c mice, following immunization with *E. coli*-derived rGST, using a standard fusion protocol [36]. Two BALB/c mice (8 weeks old) were immunized with pure rGST by subcutaneous injection. For the first injection, 50 μg of the immunogen was emulsified with complete Freund's adjuvant (Sigma–Aldrich). Two subcutaneous booster injections consisting of 50 μg of the immunogen in incomplete Freund's adjuvant (Sigma–Aldrich) were given every 3 weeks. One week after the last immunization, blood was collected by a vertical incision of the tail vein and antibody levels were measured by indirect specific ELISA, using 96-well microtiter plates (Greiner Bio-one) coated with rGST. The rGST antigen was first diluted (1 μg/ml) in coating buffer (0.05 M $NaHCO_3$ and 0.05 M Na_2CO_3, pH 9.6). Then, 100 μl of antigen solution was pipetted into each well on the plate. Plates were incubated overnight at 4 °C to accomplish the immobilization. After washing with PBS containing 0.05% Tween 20 (PBS-T), 200 μl of the blocking buffer PBS containing 1% (w/v) bovine serum albumin (PBS-BSA) was added into each well and the plate was left at 37 °C for 1:30 h to block the free sites on the plate. Subsequently, plates were washed 5 times with PBS-T and serial dilutions of mouse sera (starting from 1:500) in PBS-T containing 0.1% (w/v) BSA (PBS-T-BSA) were added and incubated for 1 h at 37 °C. After washing, peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako) diluted 1:2000 were added to the wells and incubated for 1 h at 37 °C. After washing, 100 μl of tetramethylbenzidine (TMB, Sigma- Aldrich) substrate was added to each well and the plates were incubated at room temperature (RT) in the dark. After 10 min, the reaction was stopped with 100 μl of stop solution (12% H_2SO_4) added to each well. The Optical Density (OD) was measured at 450 nm in a microplate reader (Thermo Scientific Multiskan EX).

Three days before the cell fusion, mice were intraperitoneally boosted with 20 μg of rGST without any adjuvant [37,38]. Stimulated spleen cells were fused with NS0 myeloma cells at a 10:1 ratio in 50% (w/v) polyethylene glycol 3350 (Sigma-Aldrich). Hybrids resulting from hypoxanthine/aminopterin/thymidine selection were screened by indirect specific ELISA in 96-well microplates coated with 100 ng of rGST. Positive hybridomas were cloned by the limiting dilution method and the selected clones were expanded in 25- and 75-cm^2 flasks (Greiner Bio-One), using 10% (v/v) fetal bovine serum (FBS)-supplemented culture media (DMEM, high glucose, pyruvate; Gibco). The clones were then harvested and cryopreserved in FBS 90% and dimethylsulfoxide (DMSO, Sigma-Aldrich) 10%. The MAbs were purified from culture supernatants using prepacked columns with protein G (GE Healthcare), following the manufacturer's protocols. The concentration of the purified MAbs was calculated by absorbance at 280 nm and immunoreactivity was examined by ELISA, using serial dilutions of purified MAbs by indirect specific ELISA in 96-well microplates coated with 100 ng of rGST.

2.3. Determination of the isotype and the affinity constant (Kaff)

The isotypes of the MAbs were determined by ELISA, using 96-well microtiter plates coated with goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM (Sigma-Aldrich) at 1:1000. The supernatant of the selected clones (100 μl) was added to each well, and ELISA was performed according to the protocol described above. On the other hand, the affinity constant (Kaff) of MAbs was determined by competitive ELISA [39,40]. Briefly, different concentrations of rGST (10 μg/ml – 100 ng/ml) were preincubated overnight at RT with a constant amount of each MAb. Then, an aliquot of each mixture was added in microtiter plates previously coated with 100 ng/well of rGST. The plates were incubated for 2 h at 37 °C and, after washing, further incubated for 1 h at 37 °C with peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako) diluted 1:2000 with PBS-T-BSA. The plates were washed and incubated in the dark with TMB. The assay was performed in triplicate.

2.4. Biotinylation of MAbs and rGST

rGST and selected MAbs were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) according to the technical manual. Briefly, 2 mg of protein (antibody or rGST) was dissolved in 1 ml of 50 mM bicarbonate buffer, pH 7.5. Next, 2 mg of EZ-Link Sulfo-NHS-LC-Biotin was added to 360 μl of distilled water, and

then 13.5, 27 or 54 μl of solution was added to the antibody solution. The reaction mixture was incubated for 30 min at RT. After centrifuging the product at 1000 × g for 30 min using Centricon-10 (Amicon, Inc.), the sample was completed with 0.1 M sodium phosphate, pH 7.5, until its original volume. The concentrations of antibodies were determined by absorbance at 280 nm and stored at 4 °C. The immunoreactivity of the biotinylated antibodies was examined by indirect specific ELISA.

2.5. Mapping of rGST-derived epitopes by competitive binding assay

A solid-phase ELISA using biotin-labeled rGST and non-biotinylated MAbs was used, according to the method described by Oggero and colleagues [41]. Briefly, microtiter plates coated (100 μl/well) with each purified MAb (1 μg/ml diluted in coating buffer) were incubated for 1:30 h at 37 °C with PBS-BSA to block non-specific binding. After washing with PBS-T, the plates were incubated with a constant amount of biotinylated rGST (50 ng/ml) in the presence of increasing amounts of a soluble homologous or heterologous competitor MAb (from 0.1 to 100 μg/ml) diluted in PBS-T-BSA. In this way, homologous antibodies (the same antibody used as immobilized and soluble reagent) completely competed for the probe while heterologous MAbs showed different competition patterns. After incubating for 2 h at 37 °C and washing with PBS-T, the plates were incubated with peroxidase-labeled streptavidin (Sigma) diluted 1:1000 with PBS-T-BSA (1 h at 37 °C). The plates were washed and incubated with the substrate solution as described above.

2.6. Sandwich ELISA: calculation of the limit of detection (LOD)

The capture MAb was diluted in coating buffer [0.05 M carbonate buffer (pH 9.6), 0.01 M phosphate (pH 7.4), or 0.01 M citric buffer (pH 4.8)] and incubated in microtiter plates (100 μl/well) overnight at 4 °C or for 2 h at RT. After washing with PBS-T, non-specific binding sites were saturated with PBS-BSA 1% or PBS-milk 1%, for 2 h at 37 °C or overnight at 4 °C. After washing with PBS-T, different concentrations of rGST antigen (500−0.01 ng/ml) diluted in PBS-T-BSA were added to each well. After incubation for 1 h at 37 °C, the microplate was washed with PBS-T. The biotinylated detection antibody was pipetted into each well and incubated for 1 h at 37 °C. The wells were washed again with PBS-T, and peroxidase-conjugated streptavidin (Sigma) diluted 1:2000 in PBS-T-BSA was added and incubated for 30 min at 37 °C. Peroxidase activity was measured adding TMB. The reaction was left to develop for 10 min in the dark at RT, and stopped by the addition of 12% H_2SO_4. The absorbance was determined at 450 nm in a microplate reader (Thermo Scientific Multiskan EX). All blanks, negative controls, and standards were loaded in triplicate. Linear regressions of the standard curve and correlation equations were analyzed. The precision of the assay was tested by determination of the intra- and inter-assay coefficients of variation (CVs) with different concentrations of rGST [42].

The LOD, expressed as the concentration, C_L, or the quantity, Q_L, is derived from the smallest measure, X_L, that can be detected with reasonable certainty for a given analytical procedure. The value of X_L is given by the equation $X_{L\,=}\, B_{bi} + KS_{bi}$, where B_{bi} is the mean of the blank measures, S_{bi} is the standard deviation (SD) of the blank measures, and K is a numerical factor chosen according to the confidence level desired [43]. Thus, we calculated the LOD as equal to the negative control (NC) + 3SD.

2.7. IPCR

The sensitivity of an IPCR assay depends on both the method itself and a series of experimental parameters. The experiments designed to optimize these parameters are discussed below.

2.8. Adsorption of capture antibodies onto standard PCR tubes

The adsorption of the capture antibody was evaluated in standard 0.2-ml PCR polypropylene tubes (Nest Biotechnology). Both the antibody concentration (1 μg/ml − 20 μg/ml) and different incubation buffers (bicarbonate pH 9.6, PBS pH 7.5, and borate pH 8.2) were evaluated. The capture MAb was diluted in the different coating buffers and incubated (50 μl/tube) in standard PCR single tubes and PCR 8-strip tubes (Nest Biotechnology) overnight at 4 °C. After washing with 150 μl of PBS-T, non-specific binding sites were saturated with PBS-BSA (100 μl/tube) and incubated for 2 h at RT. The wells were washed again and peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako) diluted 1:2000 in PBS-T-BSA was added to each tube and incubated for 30 min at RT. After five washes with PBS-T, peroxidase activity was measured by chromogenic reaction with TMB. The reaction was left to develop for 10 min in the dark at RT, and was stopped by the addition of 12% H_2SO_4. Results were analyzed by visual inspection. Controls containing no capture MAb were included (negative control). In addition, the efficiency of capture of the antigen was qualitatively evaluated by performing sandwich ELISAs in PCR 8-strip tubes in comparison with conventional ELISA microplates, using the same capture and detection antibodies. The antigen was assayed at concentrations ranging from 0.01 to 100 μg/ml.

2.8.1. Determination of the optimal neutravidin concentration for IPCR

To determine the optimal neutravidin concentration, we next studied the inhibitory effect of different neutravidin concentrations on sandwich ELISAs performed on microtiter plates. The assay was carried out following the same steps described for the indirect specific ELISA until the addition of the biotinylated detection antibody. Next, the plate was washed to remove the unbound biotinylated detection antibody, serial dilutions of neutravidin (2 mg/ml) in PBS-T-BSA were added, and plates were incubated at RT for 30 min. After washing with PBS-T, peroxidase-conjugated streptavidin (Sigma) diluted 1:2000 in PBS-T-BSA was added to each well and incubated for 30 min at RT. Then, peroxidase activity was measured by chromogenic reaction (TMB). The reaction was left to develop for 10 min in the dark at RT and then stopped with 12% H_2SO_4. The absorbance was determined at 450 nm in a microplate reader.

2.8.2. Concentration of reporter DNA

Reporter DNA was generated as a biotinylated double-strand DNA by PCR amplification of plasmid Bluescript SK- (Stratagene) with a 5′-biotinylated M13 (−20) forward primer (biotin-5′-GTAAAACGACGGCCAGT-3′) and a non-biotinylated M13 reverse primer (5′ GGAAACAGCTATGACCATG-3′). PCR was performed in a real-time PCR cycler (Rotor-Gene, Qiagen) under the following reaction conditions: Eva Green qPCR Mix Plus 20% (Solis BioDyne, Biocientífica), 20 pM of each primer, 5 pg of pBluescript and water in a total volume of 20 μl. The temperature profile was as follows: initial denaturation at 95 °C for 15 min and 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, extension at 72 °C for 15 s, and final extension at 72 °C for 5 min. The product purity was confirmed by dissociation curves, and the 227-bp PCR product was checked by electrophoresis on a 1.5% agarose gel with DNA gel stain (SYBR Safe; Thermo Fisher Scientific). The 227-bp PCR products were purified with the QIAquick PCR purification kit (Qiagen).

To determine the optimal concentration of reporter DNA and reduce the background signal, we studied serial dilutions of DNA

probes. We performed sandwich IPCR pilot assays with the capture antibody immobilized on standard PCR tubes (10 μg/ml), detection MAb (1 mg/ml) and rGST (1 μg/ml). The working concentration of neutravidin was 1 μg/ml and the serial dilutions of DNA probes evaluated were: 1:500, 1:1000, 1:2000, 1:5000 and 1:10000.

2.8.3. Sandwich IPCR assay. Calculation of the LOD for rGST-IPCR

The IPCR assays for rGST were performed with the same capture and detection antibodies (MAb 8G6 and biotinylated MAb 3E12, respectively), based on the general method described by Niemeyer et al. (2007) but with some modifications. The procedure was carried out in standard polypropylene PCR tubes (Nest Biotechnology). PCR tubes were coated overnight at 4 °C, using 50 μl/well of the capture MAb (10 μg/ml) in bicarbonate pH 9.6. After washing with 150 μl PBS-T, non-specific binding sites were saturated with PBS-BSA, for 2 h at RT. After washing with PBS-T, solutions covering a working range of $1–1.10^{-6}$ μg/ml of the antigen (rGST) were prepared in PBS-BSA-T and incubated in PCR tubes for 1 h at RT. Then, the tubes were washed and the biotinylated detection antibody was incubated for 1 h at RT. The tubes were washed to remove the unbound biotinylated detection antibody, and 1 μg/ml neutravidin in PBS-BSA-T was added and incubated at RT for 30 min. The tubes were washed and then the biotinylated reporter DNA diluted in PBS-BSA-T was bound with neutravidin and incubated at RT for 30 min. At each step, the incubation volume was 50 μl. The plate was washed five times with PBS-T and 10 times with distilled water, and then subjected to PCR using a real-time PCR cycler (Rotor-Gene, Qiagen). PCR was carried out under the following reaction conditions: 20% Eva Green qPCR Mix Plus (Solis BioDyne, Biocientífica), 20 pM of detection forward primer (5′-TATAGGGCGAATTGGGTA-3′) and detection reverse primer (5′- GCTATGACCATGATTACGC-3′), 75% water in a total volume of 50 μl. The temperature profile was as follows: initial denaturation at 95 °C for 15 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s, and extension at 72 °C for 30 s.

Amplification curves were analyzed with sequence detection system software (Qiagen). The product purity was confirmed by dissociation curves, and the PCR products were analyzed by electrophoresis on a 1.5% agarose gel with DNA gel stain (SYBR Safe; Thermo Fisher Scientific). Distilled water and biotinylated reporter DNA were used as negative and positive controls, respectively, for the PCR.

The cycle threshold (C_t) values obtained in real-time PCR were inversely proportional to the antigen concentrations. Thus, to allow comparison of the data obtained from the IPCR and conventional ELISA, we calculated delta C_t (ΔC_t) values by subtracting the C_t values obtained for individual samples from the total number of cycles carried out in the experiment ($\Delta C_t = 40 - C_t$), according to Niemeyer and colleagues [16]. This mathematical conversion allowed us to compare the data of the IPCR and the ELISA and thus estimate the performance of the IPCR. Then, we calculated the LOD as explained (NC + 3 SD).

2.9. Reconstitution experiment

In order to test the usefulness of the rGST-IPCR assay, we measured rGST in presence of a highy purified protein of pharmacological interest. To that end, we prepared solutions of rGST and recombinant human growth hormone (rhGH). rhGH is a single polypeptide chain of 191 amino acids with a molecular weight of 22 kDa, mostly produced in *E. coli* expression system [44,45]. The ultrapure rhGH, used for biotherapy, was generously provided for Zelltek S.A (Lot: 16-6HOR-146-099). Different quantities of rGST were added to a constant amount of rhGH. Briefly, solutions covering a working range of 0.1–100 ng/ml of the antigen (rGST) were prepared in PBS or PBS-rhGH (1 mg/ml) and incubated in PCR tubes for 1 h at RT. rGST was quantified following the protocol described for the Sandwich rGST-IPCR assay (see above). All samples, blanks and negative controls were loaded in triplicate. PBS was used as negative controls for the IPCR. The product purity was confirmed by dissociation curves.

3. Results and discussion

3.1. Production and characterization of the anti-rGST MAb

As described in the Materials and Methods section, rGST was expressed in *E. coli JM109* using a pGEX4T-3 vector, following protocols previously described [35]. After immunization, the levels of anti-rGST antibodies in the mouse sera were measured by indirect specific ELISA. Due to the higher titer of rGST-specific antibody observed in mouse M1 (see Supplementary data, Fig. 1), this mouse was selected for hybridoma production.

After fusion between spleen cells from mouse M1 and NS0 cells, supernatants of growing hybridoma cells were screened based on reactivity with rGST by indirect ELISA. Ten positive hybridomas were selected and cloned by limiting dilution. Taking into account the capacity to bind to rGST coated to plastic plates and the IgG immunoglobulin isotype, six MAbs were selected after the cloning procedure. These six MAbs belonged to the IgG_1 subclass and had an affinity constant of at least 10^8 M^{-1} (Fig. 2 in Supplementary data show the immunoreactivity of the selected antibodies).

On the other hand, the epitope specificity of each MAb in terms of analogous or overlapped epitopes (identity) or different epitopes (non-identity) was analyzed by a solid-phase mutual competition assay [41,46]. Biotin-labeled rGST was used as a probe to detect the ability of immobilized and soluble antibodies to compete for antigen binding. The labeling with biotin was previously evaluated and did not modify the antigen–antibody binding properties (data not shown). As described in M&M, an increasing amount of competitor antibody (soluble) was assayed with respect to a fixed amount of coated MAb and the probe [41]. Competition experiments between antibodies were useful to delineate two non-overlapping rGST epitopes (Table 1), designated as A and B.

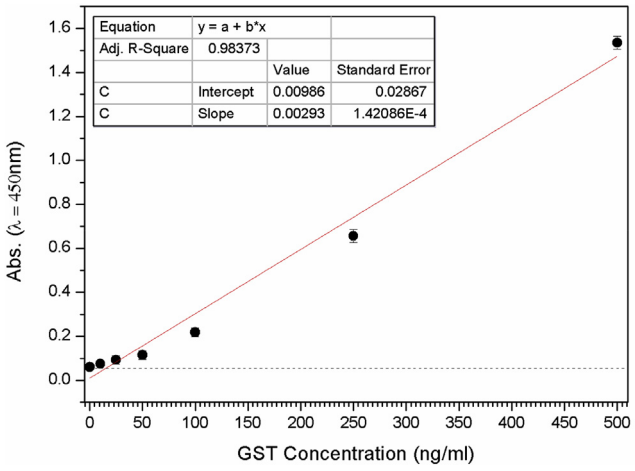

Fig. 1. Detection of rGST by sandwich ELISA and calculation of LOD. The antigen was assayed at concentrations ranging from 0.1 to 500 ng/ml. The absorbance, measured at 450 nm, is plotted on the ordinate and the antigen concentrations (0.01–500 ng/ml) are plotted on the abscissa.

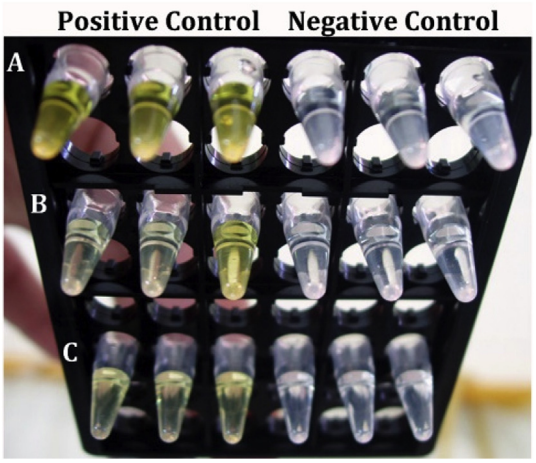

Fig. 2. Evaluation of the adsorption of the capture antibody to the surface of polypropylene PCR tubes, using different sensitization buffers. [A]: bicarbonate pH 9.6, [B]: PBS pH 7.5 and [C]: borate pH 8.2. Positive control: tubes coated with MAb 8G6 10 μg/ml. Negative control: tubes sensitized with the corresponding coating buffer in the absence of antibody.

3.2. Optimization of the sandwich ELISA and LOD

Sandwich ELISA requires two specific antibodies: the capture and the detection antibody. Based on the mapping of rGST-derived epitope by competitive binding assay, three MAbs (3E12, 1D12 and 1H7) were labeled with biotin to select a MAb as detection antibody. It is known that excess biotinylation may inactivate a biological molecule and that, in contrast, insufficient biotinylation may lead to low amplification effect. Hence, we studied the effect of biotinylation on the conjugate activity. Biotin solution (27 μl, with an amount of 20-fold molar excess against the MAb concentration) was selected and used in the subsequent optimization tests. The immunoreactivity of the biotinylated MAbs was compared by specific ELISA. This showed that MAb 3E12 had the highest immunoreactivity (see Supplementary data, Fig. 3).

To select the capture antibody, conditions for adsorption of antibodies as well as some experimental procedures were evaluated. These parameters are discussed below.

The solid phase condition determines the sensitivity of an assay to a great extent. The buffers frequently used for coating are 0.05 M carbonate buffer (pH 9.6), 0.01 M phosphate (pH 7.4), and 0.01 M citric buffer (pH 4.8). The results of the reciprocal competitive binding assay showed a non-competition pattern between MAbs 3E12 and 8G6. Thus, MAb 8G6 was selected as potential capture antibody. Then, 8G6 was diluted with different buffers to determine the effect of the coating buffer on immobilization. When 0.05 M carbonate buffer was used, absorbance was the highest among the three aforementioned coating buffers with the same MAb concentration, exhibiting maximum amount of MAb immobilization (data not shown).

Since PBS-BSA and PBS-milk are commonly used as reagents to block the free sites on a solid phase, they were evaluated as blocking buffers. PBS-milk showed a higher background signal, resulting in a poor sensitivity of the assay. Hence, PBS-BSA was chosen as the suitable blocking buffer. The optimum time and temperature for both the sensitization of microplates with capture MAb and the blocking of free sites were also studied. Finally, the microplate was coated at 4 °C overnight and blocked for 2 h at 37 °C with PBS-BSA 1%. Based on the assays of optimization described above, MAb 8G6 and biotin-labeled MAb 3E12 were selected as the capture and detection antibodies, respectively, and used in the subsequent optimization tests.

LOD, which is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit [43], was determined using serial dilutions of rGST comprising a working range of 0.01–500 ng/ml (Fig. 1). The calculated value of LOD was 10 ng/ml (0.01 μg/ml).

A validated assay implies provision of valid test results. It follows that evaluation of repeatability and reproducibility are essential to ensure that the assay generates valid test results [9]. To estimate the precision and reproducibility of sandwich ELISA, we measured both the intra- and inter-assay CVs (see Supplementary data, Table 1), and based on the values obtained, we concluded that the assay is operating within acceptable limits [9].

3.3. IPCR

As described in the introduction, IPCR involves the use of nucleic acid molecules as markers to be amplified by PCR for signal generation. For the two-sided (sandwich) immunoassay, a specific

Table 1
Reciprocal competitive binding assays using biotinylated rGST. Three competition patterns were defined: + + + complete (0%–20% of probe binding); ++ partial (21%–80% of probe binding); and + no competition (more than 80% of probe binding). Two different epitopes were identified: A and B.

	MAb inmobilized on ELISA plate					
Competitor MAb	1D12	1H7	3E12	4F5	6C7	8G6
1D12	+++	++	++	+	++	+
1H7	+++	+++	++	+	+	++
3E12	+++	++	+++	++	++	+
4F5	++	++	++	+++	+++	++
6C7	++	+	++	++	+++	+++
8G6	+	++	+	++	+++	+++
Epitope	A			B		

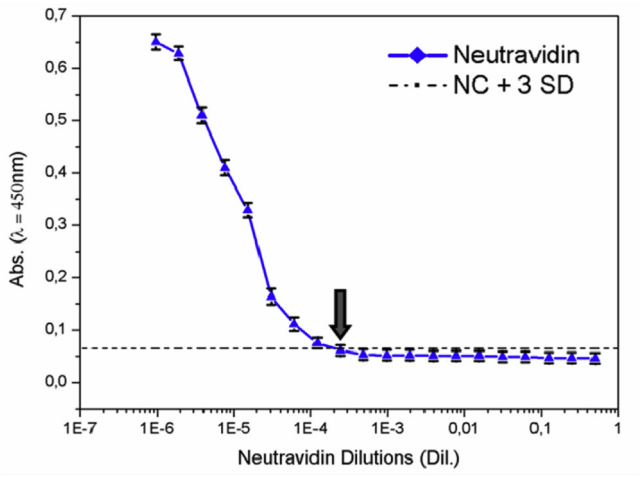

Fig. 3. Analysis of optimal neutravidin concentration for the IPCR. The absorbance, measured at 450 nm, is plotted on the ordinate and the neutravidin dilutions (2 mg/ml) are plotted on the abscissa. NC + 3SD refers to the mean of negative control +3 SD, displayed as a dotted line. The optimal working concentration selected was 1 μg/ml neutravidin.

linkage between the detection antibody and the DNA marker is necessary to avoid binding of the reagent to the capture antibody. Owing to the versatility and high affinity of the biotin–avidin binding, many groups have explored this coupling system to generate IPCR reagents. In fact, the assembly of signal-generating complexes in situ based on conjugates of biotinylated DNA, biotinylated IgG and avidin has been referred to by Zhou et al. as "Universal IPCR", and is still one of the most commonly used formats applied in most IPCR applications published so far [12]. However, because the assembly of signal-generating complexes in situ comprises successive incubation steps, different parameters must be optimized. Since our present study involves the conversion of the sandwich ELISA into an IPCR assay, we assessed several typical parameters of IPCR procedures. This process of optimization is described below.

3.4. Adsorption of capture antibodies onto standard PCR tubes

Since one of our goals was to develop the rGST-IPCR assay in standard PCR tubes, we firstly studied the capability of adsorption of the capture antibody to the surface of our PCR tubes. To that end, we evaluated the adsorption of MAb 8G6 (10 μg/ml) in different incubation buffers (bicarbonate pH 9.6, PBS pH 7.5 and borate pH 8.2) and found that binding was considerably greater in bicarbonate pH 9.6 than in PBS pH 7.5 or borate pH 8.2 (Fig. 2).

Then, sandwich rGST-ELISA was carried out in PCR tubes simultaneously with ELISA in microplates. The protocol of ELISA was modified when it was run in PCR tubes, to obtain the same LOD as in microplates. Results showed a strong similarity between the sandwich rGST-ELISA performed in ELISA microplates and that performed in PCR tubes (see Supplementary data, Fig. 4). Besides, the rGST-ELISA in PCR tubes and the ELISA in microplates showed the same LOD (0.05 μg/ml).

3.4.1. Optimal neutravidin concentration

As previously described, the universal format of IPCR, comprising the biotin-avidin binding as coupling system, was selected. In this approach, sequential coupling of a biotinylated detection antibody, neutravidin, and a biotinylated DNA marker assembles a signal-generating immunocomplex [13,47]. To determine the optimal concentration of neutravidin, different concentrations of neutravidin by inhibition ELISAs were tested. Based on the results displayed in Fig. 3, 1 μg/ml neutravidin was selected as the working concentration.

3.4.2. Effect of reporter DNA concentration on amplification by IPCR

The concentration of reporter DNA may have a marked influence on the background signal [48]. Thus, serial dilutions of reporter DNA were evaluated. Based on the results, the DNA probe dilution 1:2000 was selected as the appropriate working dilution of reporter DNA. In summary, optimal concentrations of reporter DNA and neutravidin were determined to be 1:2000 and 1 μg/ml, respectively.

3.4.3. LOD of the rGST-IPCR

The LOD of the rGST-IPCR assay, determined using serial dilutions of *Schistosoma japonicum* rGST covering a working range of 1.10^{-6} – 1 μg/ml rGST (Fig. 4), was 1.10^{-4} μg/ml (0.1 ng/ml or 100 pg/ml).

Based on these results, we concluded that the LOD of the rGST-IPCR was 100-fold lower than that of the sandwich rGST-ELISA. Fig. 5 shows a graphical comparison of both LODs.

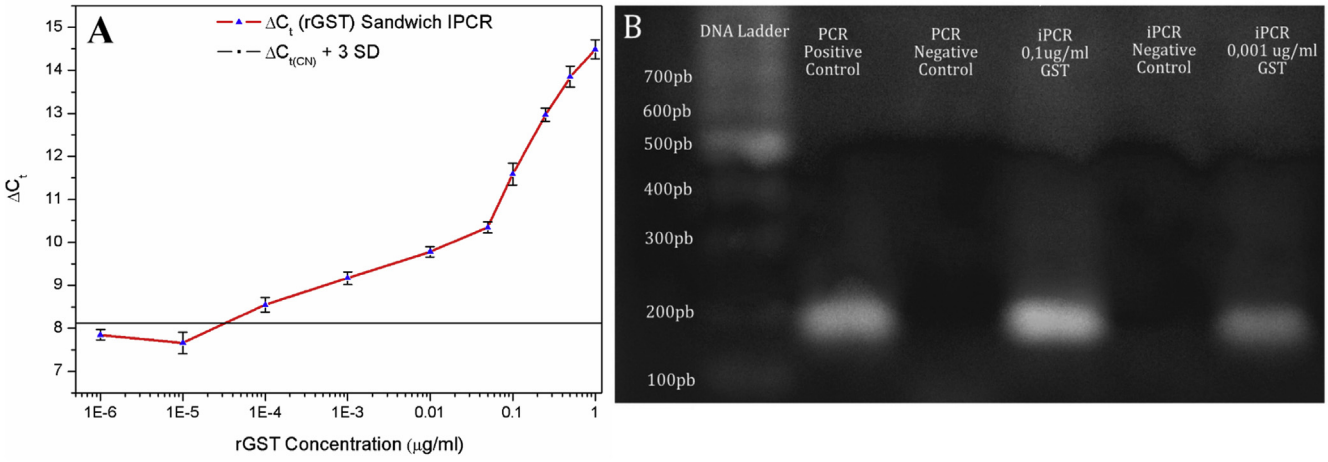

Fig. 4. Detection of rGST by IPCR. (A) The delta Ct (ΔCt) is plotted on the ordinate and the antigen concentrations (1.10^{-6} – 1 μg/ml) are plotted on the abscissa. ΔCt(NC) + 3SD refers to the mean of negative control + 3 SD, displayed as a continuous line. [B] represents the analysis of PCR products by agarose gel electrophoresis. rGST was detected using serial dilutions of rGST in the range of 1 μg/ml to 1 pg/ml.

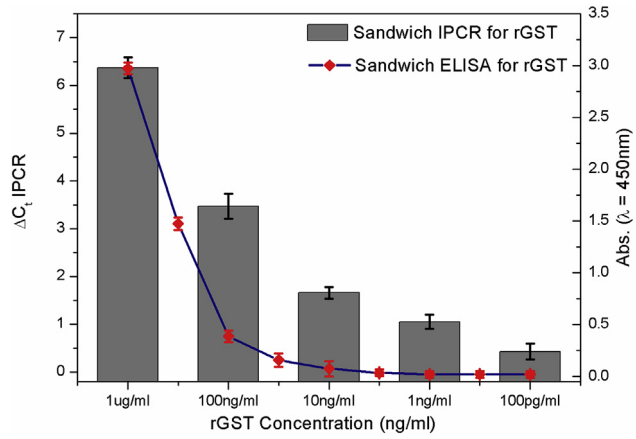

Fig. 5. Comparison of the LOD of the IPCR and sandwich ELISA specific of rGST antigen, using the same capture and detection antibodies. The data points represent the relative signal intensities for increasing amounts of antigen. ΔCt IPCR is plotted on the left ordinate, the absorbance measured at 450 nm is plotted on the right ordinate, and the antigen concentrations (1 μg/ml – 100 pg/ml) are plotted on the abscissa.

3.5. Reconstitution experiment

The results displayed absence of interference to quantify rGST in presence of rhGH. Recoveries in the range of 98.5–99.32% were obtained in all cases (Fig. 6).

Downstream processes include all steps required to purify a biological product from cell culture broth to final pure product. The level of purity required in the biopharmaceutical industry, sometimes exceeding 99%, is not easily achieved by traditional methods. Commonly, to reach the desired purity level, multiple chromatographic steps are required [49,50]. The post chromatography steps are termed polishing steps and their function is to remove the remaining impurities such as host cell protein impurities (HCP) or leached proteins introduced during the purification process [51]. In general, industry and regulatory authorities set 1–100 ppm (1–100 ng HCP or leachables per milligram biotherapeutic protein) as the desirable range limit of impurities in the final product [52,53]. Whilst there isn't a precise specification about the adequate level of total impurities content in final therapeutic preparations, the biological products are judged on case-by-case, considering immunogenicity as the major potential risk of impurities [54,55].

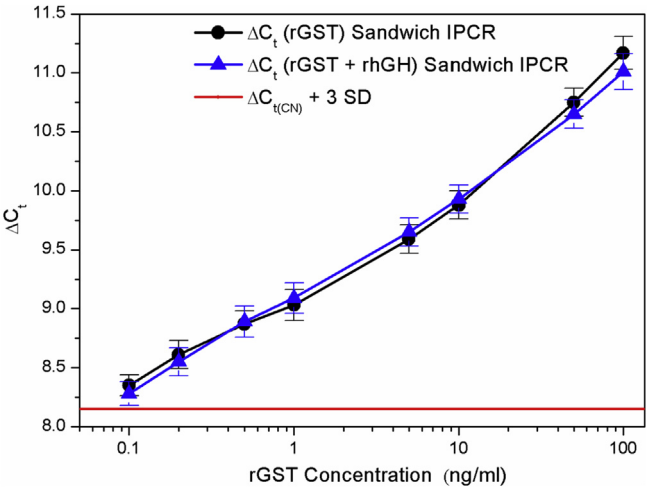

Fig. 6. Comparison of detection by IPCR of rGST in PBS or in rhGH solution (1 mg/ml). The delta Ct (ΔCt) is plotted on the ordinate and the antigen concentrations (0.1–100 ng/ml) are plotted on the abscissa. ΔCt(NC) + 3SD refers to the mean of negative control + 3 SD, displayed as a continuous line.

4. Conclusions

GST-tagged proteins are important tools for the production of recombinant proteins. Removal of the GST is often necessary to produce a pure protein and, accordingly, different methods have been applied to measure the remnant amount of GST. The objective of this work was to demonstrate the suitability of IPCR to detect low concentrations of rGST. Thus, we developed an IPCR assay to measure rGST and compared its analytical performance with that of an rGST-ELISA. The method described comprises MAb immobilized solid phase, the biotinylated MAb, neutravidin and biotinylated reporter DNA as detection system. Several features make this assay suitable to measure GST. The sensitivity, easiness to use, and low cost of IPCR support this technique as a potential platform for the detection of GST in tag separation. Mainly, the LOD of the method proposed is 100-fold higher than the LOD of the ELISAs reported by us or others. In addition, the IPCR method proposed involves a universal format of IPCR performed in standard PCR tubes. In contrast to other reports using standard PCR tubes, in our case it wasn't necessary to perform a pretreatment of PCR tubes. Therefore, we consider that the present method has great potentiality for application in several fields in which measurement of GST levels has demonstrated to be an important tool of analysis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2017.04.014.

References

[1] A.S. Pina, C.R. Lowe, A.C. Roque, Challenges and opportunities in the purification of recombinant tagged proteins, Biotechnol. Adv. 32 (2014) 366–381.
[2] D.B. Smith, K.S. Johnson, Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with Glutathione S-Transferase, Gene 67 (1988) 31–40.
[3] D.B. Smith, Generating fusions to glutathione S-transferase for protein studies, Methods Enzym. 326 (2000) 254–270.
[4] J. Arnau, C. Lauritzen, G.E. Petersen, J. Pedersen, Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins, Protein Expr. Purif. 48 (2006) 1–13.
[5] D.S. Waugh, An overview of enzymatic reagents for the removal of affinity tags, Protein Expr. Purif. 80 (2011) 283–293.
[6] P.T. Wingfield, Overview of the purification of recombinant proteins, Curr. Protoc. protein Sci. 80 (2015) 1–35, 6 1.
[7] D.S. Waugh, Making the most of affinity tags, Trends Biotechnol. 23 (2005) 316–320.
[8] S. Costa, A. Almeida, A. Castro, L. Domingues, Fusion tags for protein solubility, purification and immunogenicity in *Escherichia coli*: the novel Fh8 system, Front. Microbiol. 5 (2014) 63.
[9] J.R. Crowther, The ELISA Guidebook, Humana Press, Totowa, NJ, 2001.
[10] K.P. Janssen, K. Knez, D. Spasic, J. Lammertyn, Nucleic Acids for Ultra-sensitive Protein Detection, vol. 13, Sensors, Basel, 2013, pp. 1353–1384.

[11] T. Sano, C.L. Smith, C.R. Cantor, Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates, Science 258 (1992) 120–122.
[12] L. Chang, J. Li, L. Wang, Immuno-PCR: an ultrasensitive immunoassay for biomolecular detection, Anal. Chim. acta 910 (2016) 12–24.
[13] C.M. Niemeyer, M. Adler, R. Wacker, Immuno-PCR: high sensitivity detection of proteins by nucleic acid amplification, Trends Biotechnol. 23 (2005) 208–216.
[14] M. Adler, R. Wacker, C.M. Niemeyer, A real-time immuno-PCR assay for routine ultrasensitive quantification of proteins, Biochem. biophysical Res. Commun. 308 (2003) 240–250.
[15] K. Lind, M. Kubista, Development and evaluation of three real-time immuno-PCR assemblages for quantification of PSA, J. Immunol. methods 304 (2005) 107–116.
[16] C.M. Niemeyer, M. Adler, R. Wacker, Detecting antigens by quantitative immuno-PCR, Nat. Protoc. 2 (2007) 1918–1930.
[17] P.K. Mehta, A. Raj, N.P. Singh, G.K. Khuller, Detection of potential microbial antigens by immuno-PCR (PCR-amplified immunoassay), J. Med. Microbiol. 63 (2014) 627–641.
[18] N. Malou, D. Raoult, Immuno-PCR: a promising ultrasensitive diagnostic method to detect antigens and antibodies, Trends Microbiol. 19 (2011) 295–302.
[19] M.J. Deng, X.Z. Xiao, Y.M. Zhang, X.H. Wu, L.H. Zhu, X.Q. Xin, D.L. Wu, A highly sensitive immuno-PCR assay for detection of H5N1 avian influenza virus, Mol. Biol. Rep. 38 (2011) 1941–1948.
[20] M. Adler, S. Schulz, R. Fischer, C.M. Niemeyer, Detection of Rotavirus from stool samples using a standardized immuno-PCR ("Imperacer") method with end-point and real-time detection, Biochem. Biophysical Res. Commun. 333 (2005) 1289–1294.
[21] J. Barletta, A. Bartolome, N.T. Constantine, Immunomagnetic quantitative immuno-PCR for detection of less than one HIV-1 virion, J. Virological Methods 157 (2009) 122–132.
[22] H. Liang, S.E. Cordova, T.L. Kieft, S. Rogelj, A highly sensitive immuno-PCR assay for detecting Group A *Streptococcus*, J. Immunol. methods 279 (2003) 101–110.
[23] S. Gofflot, M.B. El, D. Zorzi, L. Melen, S. Roels, D. Quatpers, J. Grassi, E. Vanopdenbosch, E. Heinen, W. Zorzi, Immuno-quantitative polymerase chain reaction for detection and quantitation of prion protein, J. Immunoass. Immunochem. 25 (2004) 241–258.
[24] J.M. Barletta, D.C. Edelman, W.E. Highsmith, N.T. Constantine, Detection of ultra-low levels of pathologic prion protein in scrapie infected hamster brain homogenates using real-time immuno-PCR, J. Virological Methods 127 (2005) 154–164.
[25] D. Babu, P.M. Muriana, Immunomagnetic bead-based recovery and real time quantitative PCR (RT iq-PCR) for sensitive quantification of aflatoxin B(1), J. Microbiol. Methods 86 (2011) 188–194.
[26] W. Zhang, M. Bielaszewska, M. Pulz, K. Becker, A.W. Friedrich, H. Karch, T. Kuczius, New immuno-PCR assay for detection of low concentrations of shiga toxin 2 and its variants, J. Clin. Microbiol. 46 (2008) 1292–1297.
[27] A.H. Khan, E. Sadroddiny, Application of immuno-PCR for the detection of early stage cancer, Mol. Cell. Probes 30 (2016) 106–112.
[28] L. Potuckova, F. Franko, M. Bambouskova, P. Draber, Rapid and sensitive detection of cytokines using functionalized gold nanoparticle-based immuno-PCR, comparison with immuno-PCR and ELISA, J. Immunol. Methods 371 (2011) 38–47.
[29] Q. Ye, H. Zhuang, C. Zhou, Detection of naphthalene by real-time immuno-PCR using molecular beacon, Mol. Cell. Probes 23 (2009) 29–34.
[30] H.S. Zhuang, C. Zhou, Determination of anthracene by real-time immuno-polymerase chain reaction assay, Anal. Chim. acta 633 (2009) 278–282.
[31] X.Y. Meng, Y.S. Li, Y. Zhou, Y.Y. Zhang, B. Qiao, Y. Sun, L. Yang, P. Hu, S.Y. Lu, H.L. Ren, J.H. Zhang, X.R. Wang, Z.S. Liu, Real-time immuno-PCR for ultra-sensitive detection of pyrene and other homologous PAHs, Biosens. Bioelectron. 70 (2015) 42–47.
[32] E.R. Hendrickson, T.M. Truby, R.D. Joerger, W.R. Majarian, R.C. Ebersole, High sensitivity multianalyte immunoassay using covalent DNA-labeled antibodies and polymerase chain reaction, Nucleic Acids Res. 23 (1995) 522–529.
[33] D. Gaudet, D. Nilsson, T. Lohr, C. Sheedy, Development of a real-time immuno-PCR assay for the quantification of 17beta-estradiol in water, J. Environ. Sci. Health Part B, Pestic. Food Contam. Agric. Wastes 50 (2015) 683–690.
[34] M. Spengler, M. Adler, A. Jonas, C.M. Niemeyer, Immuno-PCR assays for immunogenicity testing, Biochem. Biophysical Res. Commun. 387 (2009) 278–282.
[35] J. Varayoud, L. Monje, G.S. Moreno-Piovano, G.H. Galoppo, E.H. Luque, M. Munoz-de-Toro, J.G. Ramos, Sexually dimorphic expression of receptor-alpha in the cerebral cortex of neonatal *Caiman latirostris (Crocodylia: Alligatoridae)*, General Comp. Endocrinol. 179 (2012) 205–213.
[36] G. Galfre, C. Milstein, Preparation of monoclonal antibodies: strategies and procedures, Methods Enzym. 73 (1981) 3–46.
[37] J.W. Goding, Antibody production by hybridomas, J. Immunol. methods 39 (1980) 285–308.
[38] W.M. Yokoyama, Appendix 3, in: J. Paul Robinson, managing editor, et al. (Eds.), Production of Monoclonal Antibodies. Current Protocols in Cytometry/Editorial Board, 2006. Appendix 3J.
[39] B. Friguet, A.F. Chaffotte, L. Djavadi-Ohaniance, M.E. Goldberg, Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay, J. Immunol. Methods 77 (1985) 305–319.
[40] F.J. Stevens, Modification of an ELISA-based procedure for affinity determination: correction necessary for use with bivalent antibody, Mol. Immunol. 24 (1987) 1055–1060.
[41] M. Oggero, G. Forno, R. Kratje, M. Etcheverrigaray, Rational selection of an antibody probe to detect the heterogeneous collection of CHO-derived rhGM-CSF glycoforms, Biotechnol. Lett. 28 (2006) 2049–2056.
[42] C. Santamaria, M. Durando, M. Munoz de Toro, E.H. Luque, H.A. Rodriguez, Ovarian dysfunctions in adult female rat offspring born to mothers perinatally exposed to low doses of Bisphenol A, J. steroid Biochem. Mol. Biol. 158 (2016) 220–230.
[43] IUPAC, Compendium of Chemical Terminology. Gold Book, 2014. Version 2.3.3: p839.
[44] M. Savari, S.H. Zarkesh Esfahani, M. Edalati, D. Biria, Optimizing conditions for production of high levels of soluble recombinant human growth hormone using Taguchi method, Protein Expr. Purif. 114 (2015) 128–135.
[45] M. Rezaei, S.H. Zarkesh-Esfahani, Optimization of production of recombinant human growth hormone in *Escherichia coli*, J. Res. in Med. Sci. Off. J. Isfahan Univ. Med. Sci. vol. 17 (2012) 681–685.
[46] M. Kuroki, A simple solid-phase competition assay with labeled antigen, Methods Mol. Biol. 66 (1996) 47–53.
[47] C.M. Niemeyer, M. Adler, D. Blohm, Fluorometric polymerase chain reaction (PCR) enzyme-linked immunosorbent assay for quantification of immuno-PCR products in microplates, Anal. Biochem. 246 (1997) 140–145.
[48] K. Sugawara, D. Kobayashi, K. Saito, D. Furuya, H. Araake, A. Yagihashi, T. Yajima, K. Hosoda, T. Kamimura, N. Watanabe, A highly sensitive immuno-polymerase chain reaction assay for human angiotensinogen using the identical first and second polyclonal antibodies. Clinica chimica acta, Int. J. Clin. Chem. 299 (2000) 45–54.
[49] C. Fields, P. Li, J.J. O'Mahony, G.U. Lee, Advances in affinity ligand-functionalized nanomaterials for biomagnetic separation, Biotechnol. Bioeng. 113 (2016) 11–25.
[50] M. Kalyanpur, Downstream processing in the biotechnology industry, Mol. Biotechnol. 22 (2002) 87–98.
[51] J.H. Chon, G. Zarbis-Papastoitsis, Advances in the production and downstream processing of antibodies, New Biotechnol. 28 (2011) 458–463.
[52] J. Zhu-Shimoni, C. Yu, J. Nishihara, R.M. Wong, F. Gunawan, M. Lin, D. Krawitz, P. Liu, W. Sandoval, M. Vanderlaan, Host cell protein testing by ELISAs and the use of orthogonal methods, Biotechnol. Bioeng. 111 (2014) 2367–2379.
[53] P. Knight, Downstream processing, Bio/Technology 7 (1989) 777.
[54] A.L. Tscheliessnig, J. Konrath, R. Bates, A. Jungbauer, Host cell protein analysis in therapeutic protein bioprocessing - methods and applications, Biotechnol. J. 8 (2013) 655–670.
[55] L.M. Chiverton, C. Evans, J. Pandhal, A.R. Landels, B.J. Rees, P.R. Levison, P.C. Wright, C.M. Smales, Quantitative definition and monitoring of the host cell protein proteome using iTRAQ - a study of an industrial mAb producing CHO-S cell line, Biotechnol. J. 11 (2016) 1014–1024.