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Development of a quantitative immuno-polymerase chain reaction assay to detect and quantify low levels of human thyroid stimulating hormone

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ABSTRACT

In the present study, we developed both a conventional enzyme-linked immunosorbent assay (ELISA) and a highly sensitive immuno-polymerase chain reaction (IPCR) assay specific for detection of human thyroid stimulating hormone (hTSH). Several anti-hTSH monoclonal antibodies (MAbs) were generated using hybridoma technology. Two pairs of MAbs (B-4 and B-9) were rationally selected and the optimal assay conditions of sandwich ELISAs were established. The ELISA prototypes were evaluated with standards calibrated with WHO 2nd International Reference Preparation for hTSH and in comparison with a commercial ELISA Kit. Although the limit of detection (LOD) was 0.1 μIU/ml in all cases, B-9-ELISA showed an analytical performance similar to commercial ELISA Kit. Therefore, we selected the B-9 ELISA to develop a hTSH-IPCR assay applying an "Universal-IPCR" format in standard PCR tubes without pretreatment. The signal amplification was achieved through the interaction between the biotinylated detection MAb and mono-biotinylated DNA probe pre-self-assembled with neutravidin. The hTSH-IPCR assay showed a significant increase in terms of the slope definition of sensitivity in low levels range. Our results support the potential of IPCR technique for being applied in clinical diagnosis of thyroid states.

Introduction

Thyroid gland function is regulated by the thyroid stimulating hormone (TSH), a glycoprotein hormone secreted by the pituitary gland comprising a heterodimer of α- and β-subunits [1]. The glycoprotein α-subunit is common to TSH, luteinizing hormone (LH), follicle stimulating hormone (FSH), and human chorionic gonadotropin (hCG), whereas the β-subunit is unique and confers specificity of action [2]. Gene transcription of both TSH α- and β-subunits is induced by hypothalamic thyrotropin releasing hormone (TRH) [3]. Primarily in response to hypothalamic TRH, the mature TSH (molecular weight, 28 kDa) is released into the circulation. TSH induces thyroid hormone synthesis whereas releases and maintains trophic thyroid cell integrity [4,5]. TSH secretion is in turn regulated by TRH and by a feedback-inhibiting loop in which free hormones triiodothyronine (T3), thyroxine (T4) act at both pituitary and hypothalamic levels [6]. Because feedback control of TSH secretion by peripheral thyroid hormones is sensitive, most thyroid disorders can be diagnosed by measuring basal TSH and thyroid hormone levels. Thus, measurement of TSH in human fluids is utilized as a "first line" thyroid test, in order to assist clinical decision-making [7–11].

Most of the current TSH methods used in clinical laboratories are two-site "sandwich" heterogeneous immunoassays involving (1) enzyme, (2) fluorometric substrate, or (3) chemiluminescent labels. When compared with traditional competitive immunoassays, such as RIA, heterogeneous immunoassays for TSH offer (1) lower limits of detection, (2) rapid turnaround time, and (3) a wider linear measurement range [11]. By virtue of its obvious importance for the clinical diagnosis, there has been an ongoing effort to develop analytical methods with high sensitivity for TSH in human body fluids [10]. However, although the sensitivity and reproducibility of TSH immunoassays have been progressively improved in the last 30 years, some differences in

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IPCR, immuno-polymerase chain reaction; TSH, thyroid stimulating hormone; MAbs, monoclonal antibodies; WHO, World Health Organization; IRP, International Reference Preparation; LOD, limit of detection; NIDDK, National Institute of Diabetes & Digestive & Kidney Diseases; LH, luteinizing hormone; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; TRH, hypothalamic thyrotropin releasing hormone; T3, triiodothyronine; T4, thyroxine; RIA, radioimmunoassay; PCR, polymerase chain reaction; r-hFSH, recombinant human follicle stimulating hormone; PBS, phosphate buffered saline; IP, intraperitoneal; HAT, hypoxanthine; aminopterine, thymidine; FBS, fetal bovine serum; ON, overnight; T, Tween 20; BSA, bovine serum albumin; TMB, tetramethylbenzidine; RT, room temperature; OD, Optical Density; K_{aff}, affinity constant; M, powder milk

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terms of analytical performance and measured TSH concentrations still exist among the commercially available methods [12]. The between-methods variability is the largest at low TSH concentrations (below 0.4 μIU/ml), due to lower analytical sensitivity of some immunoassay methods. However, the use of different calibrators may also cause systematic differences among commercial TSH immunoassays, due to the lack of standardization of TSH methods [13,14].

In 1992, Sano et al. [15] 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 [16]. 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 [17]. Since Sano et al. created this technique, several IPCR methods have been developed for the detection of several antigens [16,18,19], including viral antigens [20–22], bacterial antigens [23], prions [24,25], toxins [26,27], tumor markers [28], cytokines [29], polycyclic aromatic hydrocarbons [30–32], and hormones [33,34] as well as for immunogenicity testing [35]. 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. Recently, we developed an IPCR assay to detect and quantify recombinant Glutathione S-transferase [36]. Here, we developed an IPCR assay for the detection of clinically relevant levels of hTSH, based on what is known as a "Universal" format of IPCR, in standard PCR tubes without pretreatment. We showed that IPCR permits a robust detection of low concentrations of hTSH, with a higher sensitivity than both a commercial and own-made ELISA. These characteristics support its potential usefulness for reliable measurement of low clinically relevant human TSH levels.

Materials and methods

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

The human pituitary hormones, thyroid-stimulating hormone (hTSH) and luteinizing hormone (hLH), and the placental hormone human chorionic gonadotropin (hCG) were obtained through the National Hormone & Peptide Program; provided by National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK). Recombinant human follicle stimulating hormone (r-hFSH), produced in CHO cells and used for biotherapy, was generously provided for Zelltek S.A. (Lot.110623). Eight BALB/c mice (males, 8 weeks old) were immunized with hTSH. For the first injection, 20 μg of the immunogen in 0.2 ml of phosphate buffered saline (PBS) was emulsified 1:1 with complete Freund's adjuvant (Sigma–Aldrich) and given subcutaneously. Three booster injections consisting of 20 μg of the immunogen in 0.2 ml of PBS emulsified 1:1 with incomplete Freund's adjuvant (Sigma–Aldrich) were given intraperitoneally (IP) every 1 or 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 against hTSH. Twelve or twenty four weeks after the last immunization, those mice exhibiting the highest reactivity antibodies by indirect ELISA were chosen as spleen cell donors. Three days before the cell fusion, mice were IP boosted with 10 μg of the antigen in 0.2 ml of PBS without any adjuvant [37,38]. On the third day, these mice were sacrificed to obtain spleen cells for fusion with myeloma cells.

Hybridomas were established using a standard fusion protocol [36,38,39]. Briefly, stimulated spleen cells were fused with NS0 myeloma cells at a 10:1 ratio in 50% (w/v) polyethylene glycol 3350 (Sigma-Aldrich) in 96-well culture plates previously seeded with feeder cells. Hybridomas were selected in HAT medium [DMEM high glucose, pyruvate (Gibco) with 20% (v/v) fetal bovine serum (FBS) supplemented with hypoxanthine – aminopterine - thymidine (Gibco)]. The medium was replaced by fresh HAT medium six days later. The hybrids growth resulting from HAT selection was routinely observed. Supernatants were tested by specific indirect ELISA in 96-well microplates coated with 100 ng hTSH (described below). Positive hybridomas were cloned by limiting dilution method and evaluated by specific indirect ELISA against all the panel of glycoproteins (hTSH, r-hFSH, hLH and hCG) and classified in terms of specificity (described below). The clones were then harvested and cryopreserved in FBS 90% and dimethylsulfoxide (Sigma- Aldrich) 10%. Selected clones were expanded in 175 cm^2 flasks (Greiner Bio-One) using 10% (v/v) fetal bovine serum (FBS)-supplemented culture media (DMEM, high glucose, pyruvate; Gibco). 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 indirect specific ELISA specific for hTSH, r-hFSH, hLH and hCG.

Indirect specific ELISA

Indirect specific ELISA was employed for titration of specific antibodies in the mice sera, screening of supernatants of hibrydomas, classification of MAbs specificity and evaluation of biotinylated MAbs [36]. The antigens, hTSH, r-hFSH, hLH and hCG (100 μg/ml in PBS), were diluted in coating buffer (0.05 M $NaHCO_3$ and 0.05 M Na_2CO_3, pH 9.6). The wells of microtiter plates were coated with 100 μl of antigen solution (0.5 μg/ml) and left overnight (ON) at 4 °C. 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 to block the free sites on the plate. After 2 h incubation, the plates were washed with PBS-T and the samples (100 μl/well) were added and left for 1 h at 37 °C. After washing, peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako) diluted 1:2000 in PBS-T containing 0.1% (w/v) BSA (PBS-T-BSA) were added (100 μl/well). After 1 h incubation and washing, 100 μl of tetramethylbenzidine (TMB, Sigma - Aldrich) substrate was added to each well and the plates were left at room temperature (RT) in the dark. After 5–10 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).

Determination of the isotype and the affinity constant (K_{aff}). Biotinylation of MAbs

The isotypes and the affinity constant (K_{aff}) of MAbs were determined by ELISA and competitive ELISA respectively, according to protocols previously described [36]. On the other hand, selected MAbs were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) according to the technical manual. Briefly, 2 mg of antibody 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 27 μl of solution was added to the antibody solution. The reaction mixture was left for 30 min at RT. After centrifuging the product using Centricon-10 (Amicon, Inc.), the sample was completed with PBS 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 MAbs were examined by indirect specific ELISA.

Study of MAbs pairs able for specific detection of hTSH by sandwich ELISA

Evaluation of MAbs combinations

MAbs were selected based on the K_{aff} and specificity, and then were evaluated as capture antibody as well as detection antibody (labeled with biotin) by performing a hTSH-sandwich ELISA. Microtiter plates coated (100 μl/well) with each purified MAb (from 1 to 10 μg/ml in coating buffer) were blocked with PBS-BSA (200 μl/well) to avoid non-specific binding. After 1:30 h at 37 °C and washing with PBS-T, hTSH diluted 1 μg/ml (100 μl/well) in PBS-T containing 0.1% (w/v) BSA (PBS-T-BSA) were added in each well and left 1 h at 37 °C. After washing with PBS-T, biotinylated detection MAb (from 1 to 10 μg/ml)

diluted in PBS-T-BSA was added. After 1 h incubation at 37 °C and washing with PBS-T, peroxidase-labeled streptavidin (Sigma) diluted 1:2000 (100 µl/well) with PBS-T-BSA were added in each well. After 1 h, the plates were washed and left in the dark with TMB. After 10–15 min, the reaction was stopped with 100 µl of stop solution added to each well. The assays were performed in triplicate.

Evaluation of two-site immunoassays specificity and capture capability in low antigen concentrations

Potential cross-reacting with other glycoprotein hormones (hTSH, r-hFSH, hCG and hLH) was determined for each selected MAbs pairs in comparison with hTSH-ELISA commercial kit (EIAgen U-TSH Kit, Adaltis). Microtiter plates coated (100 µl/well) with each selected purified MAb were left for 1:30 h at 37 °C with PBS-BSA (200 µl/well) to block non-specific binding. After washing with PBS-T, each antigen diluted 1 µg/ml in PBS-T-BSA (100 µl/well) was introduced in the wells. After 1 h incubation and washing with PBS-T, biotinylated detection MAb diluted in PBS-T-BSA was added en the plate. After incubating for 1 h at 37 °C and washing with PBS-T, peroxidase-labeled streptavidin (Sigma) diluted 1:2000 (100 µl/well) with PBS-T-BSA (1 h at 37 °C) was added in each well. The plates were washed and left with the substrate solution as described above. All blanks, negative controls, and standards were loaded in triplicate.

Next, the capacity of the selected MAbs pairs to detect and quantify low hTSH concentrations was evaluated. The selected MAbs combinations, in comparison with TSH-ELISA comercial kit, were tested in response to the presence of low hTSH concentration (0.1 ng/ml – 10 ng/ml) diluted in PBS-T-M (PBS-T containing 0.1% (w/v) powder milk) buffer by sandwich ELISA. The immunoassay scheme was the same protocol described above. All samples, blanks and negative controls were loaded in triplicate.

Evaluation of hTSH-sandwich ELISA in human serum

The ability to detect native hTSH and potential cross-reacting with other serum proteins was investigated in human serum. Pools of human serum (0.45–6.50 µIU/ml) were previously measured as unknown samples by a commercial kit (EIAgen U-TSH Kit, Adaltis). The protocol of sandwich ELISA was the same described above.

Sandwich ELISA specific for hTSH in human serum

We evaluated different blocking reagents (PBS-BSA, PBS-M and no-blocking condition), MAbs concentrations and temperature incubation [37 °C and room temperature (RT)]. On the other hand, we studied two immunoassay schemes: 1-step and 2-step sandwich ELISA. In 1-step scheme, detection MAb (biotinylated antibody) and sample (human serum) were put simultaneously in capture antibody-coated wells. In 2-step sandwich ELISA, detection MAb and sample were added separately at different times. The different incubation temperatures and blocking conditions were assayed with both schemes.

Firstly, we evaluated the blocking buffer conditions. Briefly, the capture MAb was diluted in coating buffer and left in microtiter plates (100 µl/well) overnight at 4 °C. After washing with PBS-T, non-specific binding sites were saturated with PBS-BSA 1% or PBS-M 1% for 2 h at 37 °C or RT. After washing with PBS-T, the antigen diluted in PBS-T-M (10 ng/ml) was added to each well (100 µl). After incubation for 2 h at 37 °C or RT, the microplate was washed with PBS-T. The biotinylated detection MAb diluted (1 µg/ml) in PBS-T-BSA was pipetted into each well (100 µl). In a 1-step assay, detection MAb and the antigen (hTSH) were added together (final volume: 200 µl). After 1.5 h incubation at 37 °C or RT, the wells were washed again with PBS-T, and peroxidase-conjugated streptavidin (Sigma) diluted 1:2000 in PBS-T-BSA was introduced and left for 30 min at 37 °C. Peroxidase activity was measured adding TMB. The reaction was stopped by the addition of 12% H_2SO_4. The absorbance was determined at 450 nm in a microplate reader. All conditions, samples and negative controls, were evaluated using 5-well replicate during three runs. In the 2-steps assay, the protocol is the same described below, except that detection MAb and sample were added separately at different times.

Secondly, we determined optimal concentration for capture and detection antibodies. Capture MAbs were diluted in coating buffer (0.5–50 µg/ml) and left in microtiter plates (100 µl/well) overnight at 4 °C. After washing with PBS-T, the protocols were the same than described above. Next, different concentration of biotinylated MAbs dilutions (0.1–10 µg/ml) on PBS-T-BSA or PBS-T-M were studied. The different immunoassays schemes were the same described and established above.

Finally, we studied the peroxidase-conjugated streptavidin optimal dilution for the sandwich ELISA, evaluating different peroxidase-conjugated streptavidin concentrations (1:500–1:5000), for 30 min incubation at 37 °C and diluted in PBS-T-BSA or PBS-T-M.

Evaluation of optimal protocol on standards calibrator

The capture MAb was diluted in coating buffer and left in microtiter plates (100 µl/well) overnight at 4 °C. After washing with PBS-T, 100 µl of the samples [commercial standards calibrated with WHO 2nd International Reference Preparation (IRP) for hTSH (80/558)] were added to each well together with 100 µl of the detection biotinylated MAb diluted (final volume: 200 µl). After 2 h incubation at RT, the wells were washed again with PBS-T, and peroxidase-conjugated streptavidin (Sigma) diluted 1:1000 in PBS-T-BSA was added and left for 30 min at 37 °C. The plates were washed and the peroxidase activity was measured adding TMB. The reaction was left to develop for 30 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. All negative controls and standards were loaded in triplicate. Linear regressions of the standard curve and correlation equations were analyzed.

Performance characteristics

Sensitivity

Sensitivity is defined as the minimum concentration of TSH which can be statistically distinguished from standard 0 µIU/ml. This value is the smallest dose that is not zero with 95% confidence. The sensitivity was determined by assaying replicates of zero and the standard curve.

Precision

Samples with various TSH concentrations were prepared by spiking different amounts of hTSH to normal human serum pools. Intra-assay variation was calculated from the differences between 5 sets of duplicates. Inter-assay precision was calculated by comparing value for a series of samples included in three assay runs.

Recovery

Recovery test is a useful approach to check the accuracy of the prototypes. Recovery was carried out using euthyroid serum samples with known values of hTSH concentration and then spiked with 0.1, 1.5 and 5 µIU/ml of hTSH. Recovery was calculated by expressing the net hTSH (measured initial hTSH concentration) determined by sandwich ELISA as a percentage of added hTSH.

IPCR

Our present study implies the conversion of the sandwich ELISA into an IPCR assay. The sensitivity of an IPCR assay depends on both the method itself and a series of experimental parameters. Several parameters were established previously by our group [36]. In addition, we specifically study whether the pre-self-assembly of biotinylated DNA-neutravidin would allow us to perform the IPCR assay in a shorter time. The experiments designed to optimize this parameter are discussed below.

DNA probes production (mono-biotinylated DNA)

Reporter DNA was generated as a mono-biotinylated double-strand DNA by PCR amplification of plasmid Bluescript SK- (Stratagene), using a 5′-biotinylated forward primer (biotin-5′- GTAAAACGACGGCCAG-3′) and a non-biotinylated reverse primer (5′ CGGATAACAATTTC ACACAG-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, 1 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 90 °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 240-bp PCR product was checked by electrophoresis on a 1.5% agarose gel with DNA gel stain (SYBR Safe; Thermo Fisher Scientific). The 240-bp PCR products were purified with the QIAquick PCR purification kit (Qiagen).

Pre-self-assembly of mono-biotinylated DNA and neutravidin

Conjugates were prepared by adding biotinylated DNA probes (0.7 µM) to a solution of neutravidin (16 µM) at different proportions. The molar ratio of DNA and neutravidin studied were 1:10, 1:20, 1:50, 1:100, 10:1 and 5:1. The incubation time was 5 min at RT. Next, the samples were analyzed by electrophoresis on 1.5% agarose gel with DNA gel stain (SYBR Safe; Thermo Fisher Scientific).

Sandwich IPCR assay

The IPCR assay for hTSH and the limit of detection (LOD) were performed based on the method previously described by our group [36] and others [40]. The IPCR assay for hTSH was performed with the same capture and detection antibodies used in hTSH-sandwich ELISA. The procedure was carried out in standard 8-strip PCR tubes (MicroAmp®, Thermo Fisher Scientific). PCR strip-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 PBS-T, solutions covering a working range of 100–1.10^{-3} ng/ml and 5–0.1 µIU/ml of the antigen (hTSH) prepared in PBS-T-M or standards commercial calibrators (µIU/ml), were added to each well together with 50 µl of the detection biotinylated MAb (final volume: 100 µl). After 2 h incubation at RT, the tubes were washed and 50 µl of pre-self-assembly biotinylated DNA - neutravidin (1:100) diluted 1:2000 in PBS was added and left at RT for 30 min at 37 °C. 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 (StepOne™, Applied Biosystems™). 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′- TATAGGGCGA-ATTGGGTA-3′) and detection reverse primer (5′- GCTATGACCATGA-TTACGC-3′), 75% water in a total volume of 50 µl. The temperature profile was as follows: initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 90 °C for 10 s, annealing at 57 °C for 15 s, and extension at 72 °C for 15 s. Amplification curves were analyzed with sequence detection system software (StepOne Software). 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 comparison of results between IPCR and ELISA were made following protocols previously published [36]. The limit of quantification (LOQ) was calculated according to IUPAC, Compendium of Chemical Terminology [41].

Evaluation of the specificity in human serum

hTSH, r-FSH, hLH, or hCG (10 ng/ml) was added to a human serum sample, whose endogenous TSH values (2.85 µIU/ml) were previously measured by a commercial kit (EIAgen U-TSH Kit, Adaltis). The protocol of IPCR assay was the same described above. The specificity was further tested by Western blot assays of the pair of antibodies selected (B-9) with human serum. Western blot assay was performed according to protocols previously published [42].

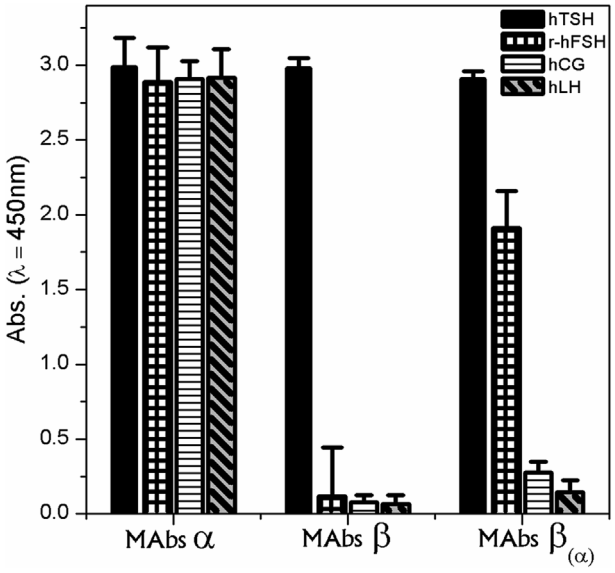

Fig. 1. Specificity study of the MAbs by indirect ELISA against hTSH, r-hFSH, hCG and hLH. The absorbance, measured at λ = 450 nm, for the antigens evaluated are plotted on the ordinate and the different groups on the abscissa. Three groups were identified which bound to distinct specificity against the different glycoproteins: MAbs α; MAbs β; MAbs $\beta_{(\alpha)}$.

Results and discussion

Production and selection of the MAbs specific for hTSH

After immunization, the levels of anti-hTSH antibodies in the mice sera were measured by indirect specific ELISA. Only the mice with highest titer (more than $1{:}5.10^5$) were used for hybridoma production. Five independent fusions yielded over 250 positive supernatants of growing hybridoma cells lines. These positive cell lines were initially screened based on reactivity with hTSH by indirect ELISA. Fifty cell lines were selected based of proliferation and immunoreactivity levels and cloned by limiting dilution and the obtained MAbs were classified in terms of specificity against hTSH, r-hFSH, hCG and hLH (Fig. 1) by indirect ELISA.

Three groups of MAbs were identified based on their different specificity to hTSH, r-hFSH, hCG and hLH: MAbs α; MAbs β; MAbs β(α). MAbs α comprises antibodies showing strong reactivity with the four hormones, recognizing common antigenic regions on the α-subunit of hTSH, r-hFSH, hCG and hLH. Instead, the antibodies belonging to MAbs β showed strong specific reactivity with hTSH, evidencing that recognizes sites only of the hTSH β-subunit. Most of the antibodies displayed characteristics of MAbs β(α) group, showing strong reactivity with both hTSH and rest of glycoprotein hormones (hLH, hCG and r-hFSH). These results suggest that MAbs $\beta_{(\alpha)}$ recognized similar antigenic regions. In fact, β-subunits of glycoprotein hormones have partial homology because they bind to a common α-subunit [43]. Moreover, the conserved sequences in the β-subunits are important for similar global folding [44].

Evaluation of MAbs combination for two-site immunoassay (sandwich ELISA)

Obtaining antibodies able to recognize different regions of hTSH was necessary to design a sandwich ELISA, which requires two specific antibodies: the capture and the detection antibody. MAbs of different

Table 1
hTSH-sandwich ELISA using different combinations of capture and biotinylated detection MAbs. Four antigen capture patterns were defined: + + + complete (100%–80% of antigen capture); + + partial (79%–20% of antigen capture); + minimum (less than 20% of antigen capture); and - negative (no antigen capture).

Solid-phase MAbs		*Soluble-phase MAbs (biotinilyated)*									
		α	β	β	α	β	β(α)	β(α)	β(α)	α	β(α)
		PD.H10	*P5.E7*	*PG.E9*	*P1.G3*	*PH.D4*	*M10.B5*	*9.H8*	*3.F7*	*HA_H2*	*HA_E1*
α	*PD.H10*	●	-	+	+	+	+	+	+	-	+
β	*P5.E7*	-	●	-	-	-	-	-	-	-	-
β	*PG.E9*	+ + +	-	●	+	-	+ + +	+	+	-	+
α	*P1.G3*	+	-	+ + +	●	+ +	+ + +	+	+	-	+
β	*PH.D4*	+ + +	-	-	+	●	+	+	+	-	+
β(α)	*M10.B5*	+ + +	-	+ + +	+ +	+ + +	●	+	+	-	+
β(α)	*9.H8*	+	-	+	+	+	+	●	+	-	+
β(α)	*3.F7*	+	-	+	+	+	+	+	●	-	+
α	*HA_H2*	-	-	-	-	-	-	-	-	●	-
β(α)	*HA_E1*	+	-	+	+	+	+	+	+	-	●

groups (α, β and $\beta_{(\alpha)}$), all of IgG_1 subclass and with a K_{aff} ranging from 5.1×10^8 to 3.6×10^9 M^{-1}, were coupled to biotin and the effect of biotinylation on the immunoreactivity was evaluated by indirect ELISA. Ten MAbs were selected and evaluated as capture as well as detection antibody (labeled with biotin) in a hTSH-sandwich ELISA (Table 1).

Data on Table 1 shows that only eight MAbs pairs displayed at least 80% of hTSH capture (gray boxes). The rest of MAbs combinations evidenced intermediate or minimum hTSH capture. On the other hand,

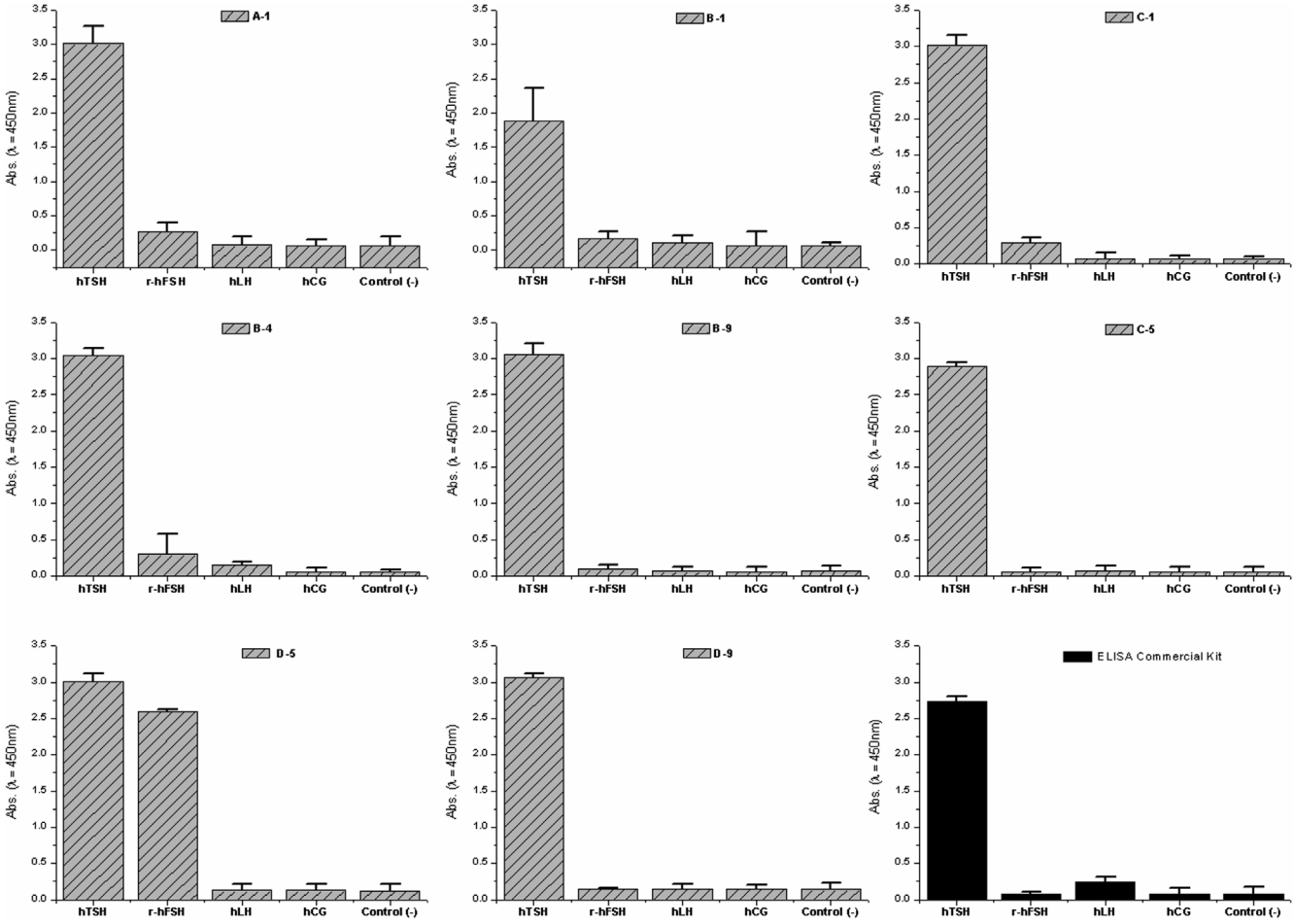

Fig. 2. Detection of hTSH; r-hFSH, hCG and hLH by two-site assay (sandwich ELISA). The antigens were assayed at concentrations 100 ng/ml. The absorbance, measured at λ = 450 nm, is plotted on the ordinate and the antigens are plotted on the abscissa.

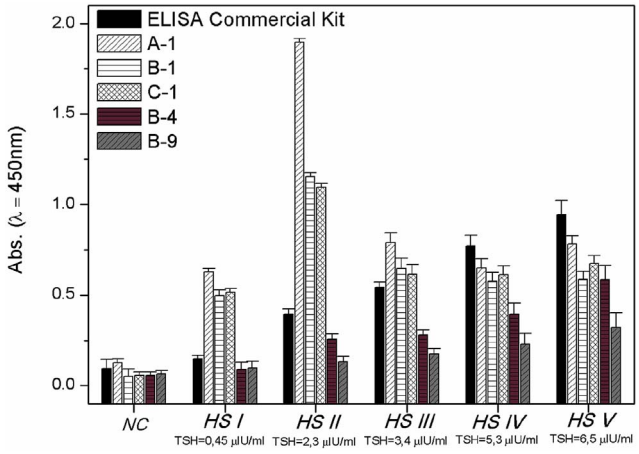

Fig. 3. Detection of hTSH by sandwich ELISA on human serum. The absorbance, measured at λ = 450 nm, is plotted on the ordinate and dilutions of high hTSH sample with human serum are plotted on the abscissa (µIU/ml).

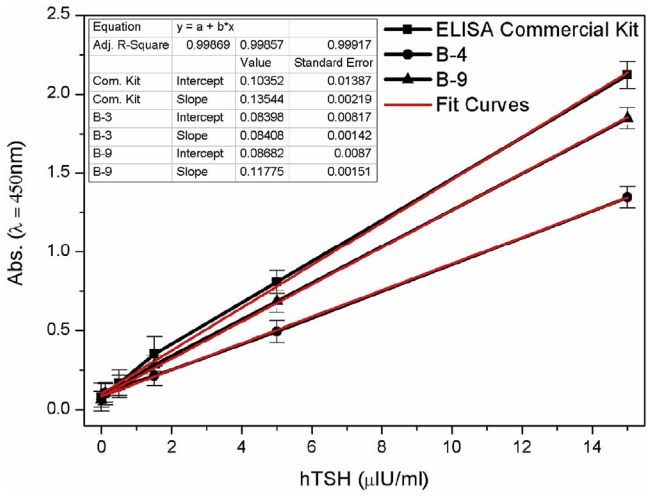

Fig. 4. Detection of hTSH by sandwich ELISA on commercial standards calibrators. The absorbance, measured at λ = 450 nm, is plotted on the ordinate and hTSH (µIU/ml) are plotted on the abscissa.

we observed that P5.E7 or HA_H2 were negative in all cases. We can conclude that both anti-hTSH MAbs P5.E7 and HA_H2 recognize their corresponding epitope on the non native hTSH molecule (indirect ELISA) but not on native antigen (sandwich ELISA). The MAbs pairs that showed antigen capture patterns of at least 80% were renamed as following: A-1 (PG.E9-PD.H10), B-1 (PH.D4-PD.H10), C-1 (M10.B5 – PD.H10), B-4 (M10.B5 – PH.D4), B-9 (M10.B5 – PG.E9), C-5 (PG.E9 – M10.B5), D-5 (P1.G3 – M10.B5) and D-9 (P1.G3 – PG.E9).

Evaluation of selected MAbs combination specificity and the performance in presence of low hTSH concentrations

The potential cross-reacting with hTSH, r-hFSH, hCG and hLH was investigated performing a sandwich ELISA with each one of the eight MAbs combinations (A-1, B-1, C-1, B-4, B-9, C-5, D-5 and D-9). Fig. 2 shows these results in comparison with a hTSH-ELISA commercial kit. All MAbs pairs were highly specific to detect hTSH and showed no specificity to the rest of glycoprotein hormones, except for D-5 which cross-reacted with r-hFSH.

Next, A-1, B-1, C-1, B-4, B-9, C-5 and D-9 were tested to detect and quantify hTSH diluted in PBS-T-M buffer by sandwich ELISA in a range of low concentrations (0.1 ng/ml – 10 ng/ml), in comparison with hTSH-ELISA commercial kit (see Supplementary data, Fig. 1). B-4 showed the highest performance to detect and quantify hTSH, and it was very similar to that of the commercial kit. B-1, C-1, B-9 and A-1 displayed intermediate performances, whereas C-5 and D-9 were ineffective in each concentration analyzed. Therefore, C-5 and D-9 were discarded for the rest of the experiment.

Evaluation of selected MAbs combination performance for hTSH detection on human serum

Then, we tested the ability of the remaining five MAbs combinations (A-1, B-1, C-1, B-4 and B-9) to detect and quantify hTSH in human serum by sandwich ELISA. Pools of human serum with known values of hTSH were prepared as detailed in 2.3.3. section.

In Fig. 3 we can observe that the absorbances obtained with A-1, B-1 and C-1 clearly increased between *NC* to *HS II* standards, but then decreased and remained in low values. Based on these results, we also tested A-1, B-1 and C-1 in a lower range of concentrations comprised by 0.45, 1.0, 1.5 and 2.3 µIU/ml, but results again showed a non linear response (data not shown). Instead, the absorbances reached with B-4 and B-9 MAbs pairs increased gradually along the entire range of concentrations. Thus, we selected B-4 and B-9 MAbs pairs to the next step of optimization of the inherent technical variables of ELISA.

Optimization of the sandwich hTSH-ELISA and comparison with commercial kit

To establish an optimal ELISA protocol conditions for adsorption of

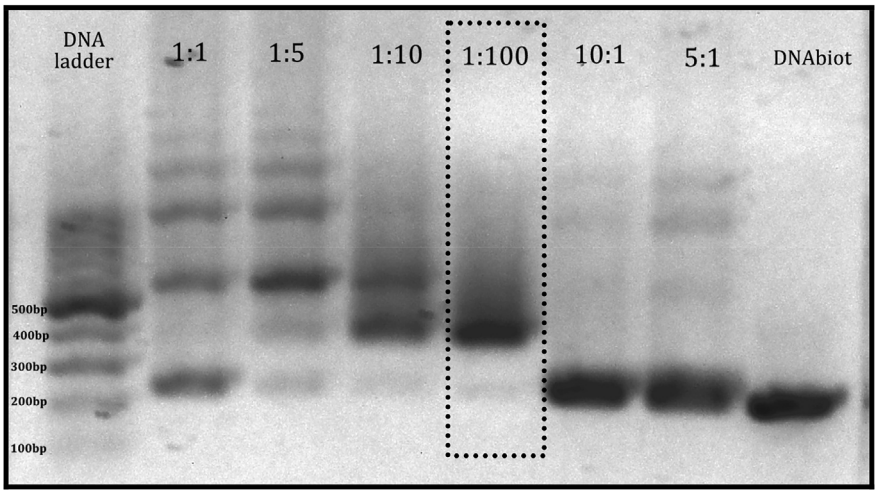

Fig. 5. Self-assembly of mono-biotinylated DNA probe and neutravidin. First lane contains the DNA ladder (CienMarker, Invitrogen) and the next lanes contain mixtures of 240 bp mono-biotininylated DNA probe with different amounts of neutravidin. The relative molar ratios are indicated on the top of the lanes (DNA: Neutravidin). The last lane contains mono-biotinilylated DNA probe without neutravidin.

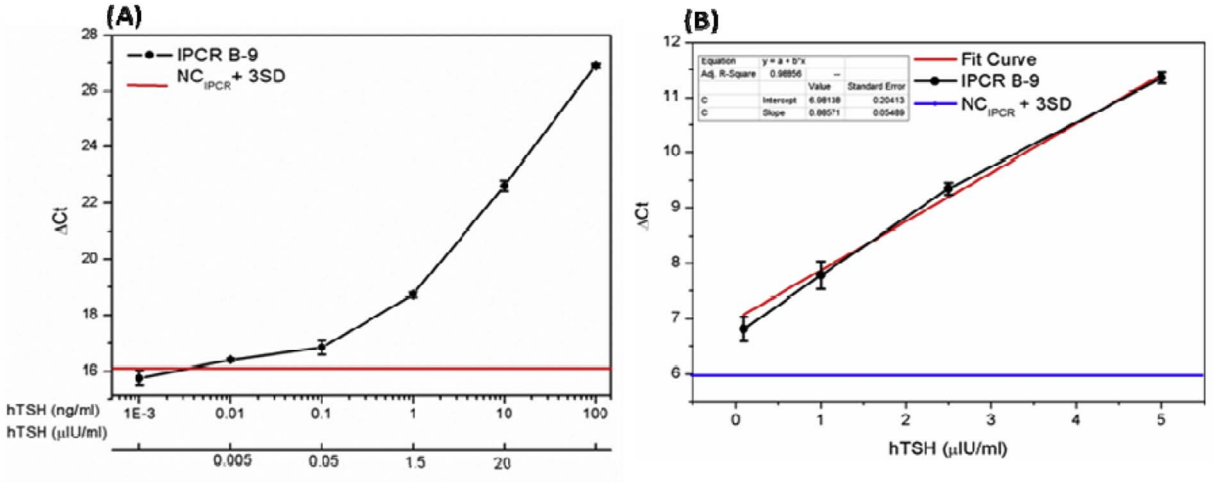

Fig. 6. Detection of hTSH by IPCR. (A) The delta Ct (ΔCt) is plotted on the ordinate and the antigen concentrations (1.10^{-3} – 100 ng/ml) are plotted on the abscissa. ΔCt (NC) + 3SD refers to the mean of negative control + 3 SD, displayed as a continuous red line. (B) The delta Ct (ΔCt) is plotted on the ordinate and the antigen concentrations (5–0.1 μIU/ml) are plotted on the abscissa. ΔCt(NC) + 3SD refers to the mean of negative control + 3 SD, displayed as a continuous blue line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

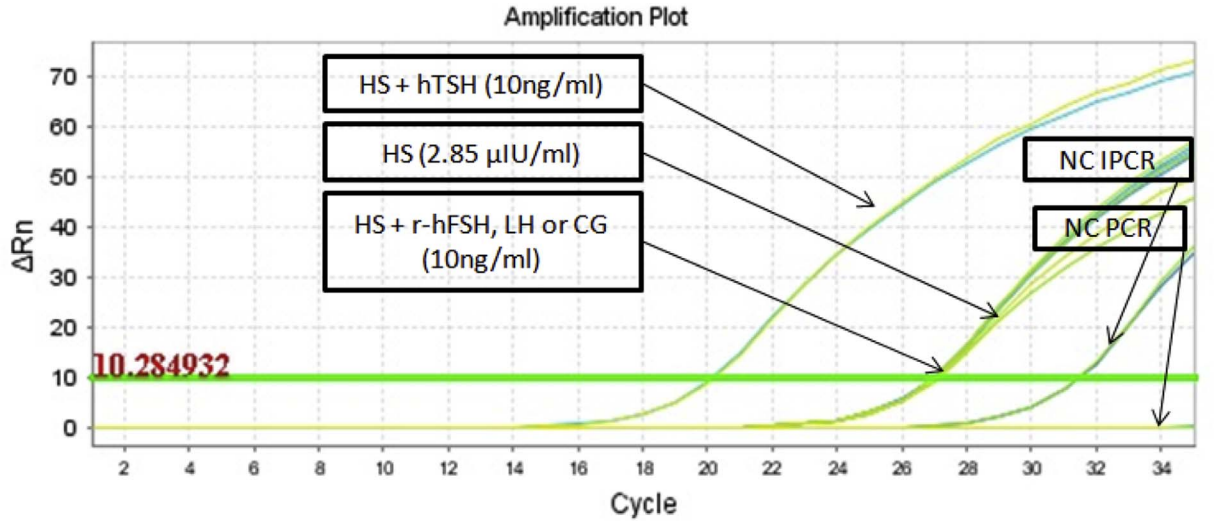

Fig. 7. Real-time amplification curves (log scale) obtained for detection of hTSH by sandwich IPCR (duplicate samples). Glycoproteins hormones (hTSH, r-FSH, LH or CG) were diluted (10 ng/ml) in human serum sample (HS), IPCR background control (NC IPCR) and negative PCR control (NC PCR).

antibodies as well as some experimental procedures were evaluated. These parameters are discussed below.

Since PBS-BSA and PBS-M are commonly used as reagents to block the free sites on ELISA assays, they were evaluated as blocking buffers in comparison with no-blocking condition, at 37 °C and room temperature (RT). We also studied two immunoassay schemes: (1) 1-step sandwich ELISA when detection MAb and the sample were added together in antibody-coated wells; and (2) 2-step sandwich ELISA, when detection MAb and the sample were added separately.

For B-9 prototype, PBS-M and PBS-BSA as blocking reagents showed a higher background signal at both temperatures (37 °C and RT) in two schemes evaluated, resulting in a poor sensitivity of the assay. Instead, background absorbance was lower at RT in no-blocking condition in 1-step assay. On the other hand, B-4 prototype showed the best performance with high specific signal and low background in 1-step assay using PBS-BSA as blocking reagents at RT (see Supplementary data, Table 1). Next, we studied the influence of MAbs concentration. Taking into consideration the signal to background ratio as well as absorbance values, the chosen concentrations of capture antibody were 10 μg/ml for B-9 and 5 μg/ml for B-4, meanwhile the concentrations selected for detection antibodies were 1 μg/ml for B-9 and 5 μg/ml for B-4 (Supplementary data, Figs. 2 and 3).

Finally, the relative molar ratio of the peroxidase-conjugated streptavidin to biotinylated anti-TSH MAb was evaluated, due to excessive peroxidase-conjugated streptavidin or biotinylated anti-TSH MAb may lead to non-specific absorption. Ratio of 1:1000 for peroxidase-conjugated streptavidin was selected based on the signal to background ratio and absorbances observed (data not shown).

The comparison of analytical performance between the commercial kit and our ELISA (B-9 or B-4), were accomplished performing in parallel assays using the standard calibrators of the commercial kit.

Results displayed in Fig. 4 clearly shows that B-9 and B-4 ELISAs had a linear response along the entire range of concentrations. The calculated value of 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 [41], was 0.1 μIU/ml in all cases. However, commercial kit had the highest slope, evidencing a better analytical performance.

A validated assay implies provision of valid test results. Sensitivity, precision and recovery were established (see Supplementary data, Table 2) and thus, based on the values obtained, we concluded that the assay is operating within acceptable limits [45].

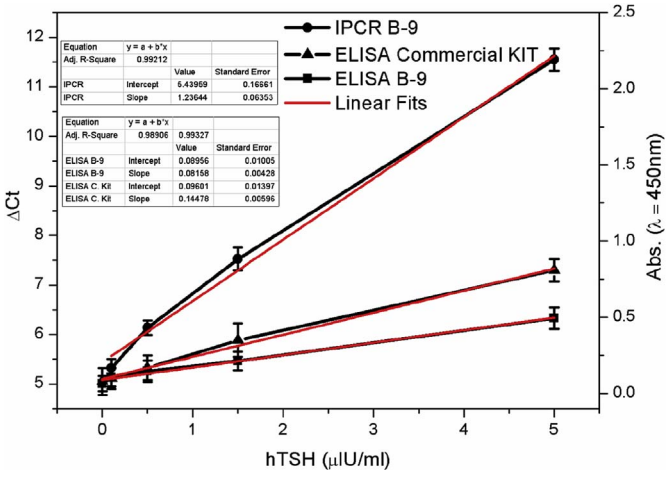

Fig. 8. Comparison of the sensibility of the IPCR assay, sandwich ELISA (using the same capture and detection antibodies) and commercial ELISA Kit on standards calibrators. The data points represent the relative signal intensities for increasing amounts of antigen. ΔCt IPCR is plotted on the left ordinate, the absorbance measured at λ = 450 nm is plotted on the right ordinate, and the antigen concentrations (μIU/ml) are plotted on the abscissa.

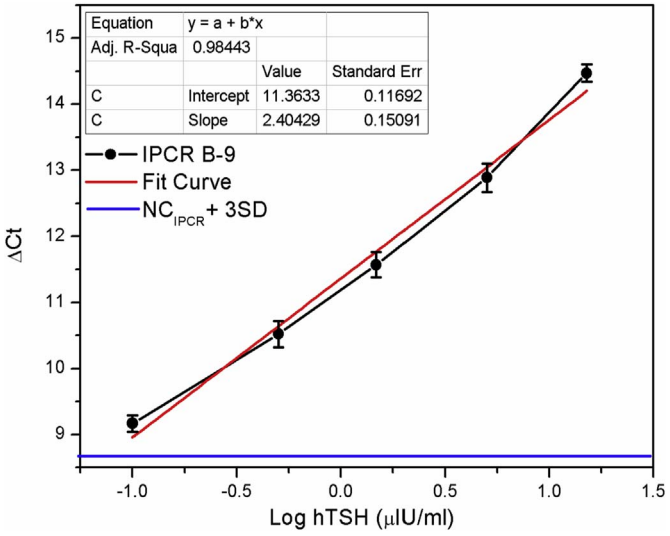

Fig. 9. Calibration curve and LOQ of the IPCR assay. ΔCt IPCR is plotted on the ordinate and Log of antigen concentrations (μIU/ml) are plotted on the abscissa.

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 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” [46], and is still one of the most commonly used formats applied in most IPCR applications published so far [16]. 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 as previously published by our group [36]. This process of optimization is described below.

DNA probes production. Pre-self-assembly of mono-biotinylated DNA and neutravidin

The high affinity of the non-covalent interaction between biotin and streptavidin forms the basis for many diagnostic assays that require the formation of an irreversible and specific linkage between biological macromolecules [47]. On the other hand, the concept of self-assembly using biotinylated DNA and streptavidin, has been experimentally demonstrated by Niemeyer and colleagues [48–50]. Here, the rapid self-assembly of mono-biotinylated DNA probe and tetravalent biotin-binding protein neutravidin have been studied by non-denaturing gel electrophoresis.

A mono-biotinylated double-strand DNA by PCR amplification of plasmid Bluescript SK- (Stratagene), using a 5′-biotinylated forward primer and a non-biotinylated reverse primer, was obtained at 70 ng/ul (Suppl. Data, Fig. 4A, B and C).

Fig. 5 shows the self-assembly between mono-biotinylated DNA probe and different quantities of neutravidin. Lane corresponding to the molar ratio 1:100 shows a unique band, corresponding to two probes of DNA per one neutravidin molecule. Instead, in lanes of 10:1 and 5:1 ratios, was observed only one band of 240 bp, evidencing no formation of supramolecular complexes.

LOD and specificity in human serum of IPCR assay

The LOD of the hTSH-IPCR assay, determined using hTSH diluted in PBS-T-M covering a working range of 100–0.001 ng/ml (Fig. 6A), was 0.01 ng/ml. Due to the most clinically relevant values of TSH are in the range comprised between 0.1 and 5 μIU/ml, we performed the IPCR assay with values comprised between these values (Fig. 6B).

In order to asses the potential cross-reacting with the rest of glycoproteins hormones (r-hFSH, hCG, or hLH) or other human serum proteins, the IPCR assay was performed in a serum sample with r-hFSH, hCG or hLH added to a final concentration of 10 ng/ml. 2.85 uIU/ml was the level of endogenous hTSH that were previously present in the human serum used to dissolve the exogenous r-hFSH, hCG or hLH. The addition of r-hFSH, hCG or hLH didn't increase the signal of IPCR assay (Fig. 7). We also observed that B-9 antibodies didn't react with any unspecific human serum proteins, evidenced by the absence of reaction in Western blot assay (Suppl. Data, Fig. 7). Thus, the assay was highly specific to detect hTSH and showed no detectable cross-reacting with the rest of glycoprotein hormones or with serum proteins.

Evaluation of IPCR assay on commercial standards calibrators and LOQ

Finally, we compared the analytical performance/sensitivity of the hTSH-IPCR assay, our ELISA assay and a commercial ELISA assay. To do that, we performed the assays in parallel using the standards calibrators of the commercial kit (Suppl. Data, Fig. 5C). We observed that the IPCR assay was more sensitive than both ELISAs. Fig. 8 includes the three calibration plots, showing different sensitivities for IPCR B-9, ELISA B-9 and the commercial kit. We observed that IPCR B-9 method is more sensitive than the other two methods, because IPCR gives a larger change in response for a given change in concentration (greater calibration slope) than does both ELISAs. Therefore, hTSH-IPCR assay showed highest sensitivity in terms of the slope definition of sensitivity [51].

Results displayed in Fig. 9 shows the linear response along the entire range of concentrations between 15 and 0.1 μIU/ml. The LOQ of the method is 0.1 μIU/ml.

Conclusions

The objective of the present work was to develop a highly sensitive IPCR assay able to detect low concentrations of hTSH. The method described comprises MAb immobilized solid phase, the biotinylated

MAb, neutravidin and biotinylated reporter DNA as detection system. In addition, we showed the advantages of the usage of pre-self-assembly of mono-biotinylated DNA probe and neutravidin. The hTSH-IPCR assay showed highest sensitivity in terms of the slope definition of sensitivity, providing better quantitative resolution for a given amount of measurement error or, conversely, higher sensitivities can tolerate larger measurement errors for a given amount of quantitative resolution [51]. The sensitivity, ease of use, and low cost of IPCR support this technique as a potential platform for the detection of hTSH hormone in serum samples. In addition, the IPCR method described involves a universal format of IPCR performed in standard PCR tubes. Therefore, we consider that the proposed method has exhibited great potentiality and could act as a good tool for hormone 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.ab.2017.10.023.

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