Journal of Immunological Methods 501 (2022) 113199

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

A rapid homogenous bioassay for detection of thyroid-stimulating antibodies based on a luminescent cyclic AMP biosensor

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ARTICLE INFO

Keywords:
Autoimmune thyroid disease
Homogeneous cAMP biosensor
Thyroid blocking immunoglobulins or antibodies
Thyroid-stimulating hormone receptor
Thyroid stimulating immunoglobulins or antibodies

ABSTRACT

Graves' disease (GD) is an autoimmune disease caused by antibodies to the thyroid stimulating hormone receptor (TSHR). The FDA-cleared Thyretain™ TSI bioassay is a highly specific method to detect thyroid stimulating antibodies (TSAb/TSI) in the blood of patients with autoimmune thyroid disease (AITD), particularly GD. To simplify the workflow of this bioassay and to support a semi-quantitative result, we have generated a stable CHO-K1 cell line expressing both a chimeric TSH receptor (TSHR-Mc4) and a luciferase-based homogeneous cAMP biosensor (GS luciferase). Here, we describe a rapid, real-time, homogenous bioassay (Turbo™ TSI Bioassay) to directly assess the functional activity of TSI and produce results in International Units of IU/L. The Turbo™ TSI bioassay works by measuring changes in the intracellular cAMP level induced by a G-protein coupled receptor (G-PCR) signaling cascade which is triggered by the binding of TSI to the TSHR. Upon binding to cAMP, the GS luciferase reporter is activated through conformational changes and generates light that can be measured in intact cells with a luminometer. The LoD and LoQ of the assay were determined to be 0.016 IU/L and 0.03 IU/L, respectively and the preliminary assay cutoff was determined to be 0.024 IU/L by ROC analysis using the Thyretain™ TSI bioassay results as reference. The analytical performance of the Turbo™ TSI bioassay is comparable to the Thyretain™ TSI bioassay as evidenced by similar EC_{50} values for a TSHR stimulating monoclonal antibody (M22). The specificity of the Turbo™ TSI bioassay was demonstrated by showing no response to a high concentration of a human monoclonal TSHR blocking antibody (K1–70). The precision of the assay was excellent with an overall within-laboratory precision <15% CV. When testing 198 clinical samples, the positive and negative percent agreement between the Turbo™ TSI and the Thyretain™ TSI bioassays were 98.7% and 93.5%, respectively. While both bioassays yield equivalent analytical and clinical performances, the Turbo™ TSI bioassay is much simpler to perform. It does not require cell culture, sample dilution, washing or cell lysis steps, resulting in a dramatically reduced turnaround time from about 21 h to 60 min. In addition, the same cell line showed its capability of detecting thyroid blocking antibodies (TBAb/TBI) in a competitive format. The Turbo™ TSI bioassay is user-friendly and is a very promising advancement to aid the diagnosis of autoimmune thyroid disease (AITD).

1. Introduction

Autoantibodies to the thyroid stimulating hormone receptor (TSHR) are uniquely able to affect thyroid function. Thyroid-stimulating antibodies/immunoglobulins (TSAb/TSI) are the cause of Graves' disease, an autoimmune hyperthyroid condition (Smith and Hall, 1974). Thyroid-blocking antibodies/immunoglobulins (TBAb/TBI) can often be found in serum of patients with autoimmune hypothyroidism. Measurement of the TSHR autoantibodies has become increasingly important for diagnosis and management of autoimmune thyroid disease.

Abbreviations: AITD, autoimmune thyroid disease; cAMP, cyclic adenosine monophosphate; GS, GloSensor; HCBS, homogeneous cAMP biosensor; RLU, relative light units; TSHR, thyroid-stimulating hormone receptor; TSI, thyroid stimulating immunoglobulins; TSAb, thyroid stimulating antibody; TBI, thyroid blocking immunoglobulins; TBAb, thyroid blocking antibody.

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https://doi.org/10.1016/j.jim.2021.113199
Received 19 November 2019; Received in revised form 25 June 2021; Accepted 29 November 2021
Available online 4 December 2021

Whereas many automated binding assays are available to detect anti-TSHR, only cell-based bioassays are capable of determining the functionality of these antibodies and specifically, distinguishing between TSI and TBI in patient sera (Allelein et al., 2019; Lytton et al., 2010; Diana et al., 2016; Lytton et al., 2018).

The TSHR found on thyrocytes is a member of the G protein-coupled receptors (GPCRs), the largest known superfamily of cell surface receptors (Fredriksson et al., 2003). As with all GPCRs, conformation changes in TSHR upon binding of agonists such as thyroid-stimulating hormone (TSH), initiates a signaling cascade in the cytoplasm via second messengers such as cyclic adenosine monophosphate (cAMP) and other effector molecules (Rall and Sutherland, 1958; Strasser et al., 1986). Measurement of cytoplasmic cAMP levels is often used to detect TSHR activation and historically, bioassays for TSH were developed based on cAMP. Using the same principles, many cell-based TSI bioassays have been described (Kasagi et al., 1982; Morgenthaler et al., 1998). cAMP-based assays were originally radiometric and later replaced by various immunoassays using a colorimetric, fluorescent or chemiluminescent readout (Diana et al., 2020; Kasagi et al., 1982; Rapoport et al., 1982; Pierce et al., 2012). Despite these advances, one of the limitations of these methods is that they must be performed on cell lysates. This adds to the complexity of the bioassays which is problematic for clinical laboratories. Another method used to measure TSHR activation is to engineer cells to express cAMP-dependent reporter genes such as luciferases (Lytton et al., 2010). These methods also require lysis of cells before measuring luciferase. In addition, these methods take more time than direct measurement of cAMP because signal transduction, translocation to the nucleus, transcription, and translation must occur before luciferase activity can be measured. The entire process can take hours which lengthens the duration of the bioassay and again, reduces the feasibility of TSI bioassays for clinical laboratories.

Recently, novel biosensors have been developed that allow for real-time measurement of cAMP dynamics inside live cells which can provide a quantitative measurement of GPCR activation (Dupriez et al., 2002; Mithofer and Mazars, 2002; Binkowski et al., 2009; Kumar et al., 2017). These biosensors allow for simple and homogeneous live, cell-based, high-throughput screening assays and thus are excellent tools in both research and drug discovery laboratories. A live-cell TSI bioassay that uses a cyclic nucleotide-gated calcium channel and aequorin was recently reported (Araki et al., 2015). This aequorin TSI assay does not require sterile cell culture, but requires 4 h to obtain test resutls of clinical samples.

The one FDA-cleared commercially-available TSI bioassay (Thyretain™ TSI, Quidel, San Diego, CA) utilizes engineered CHO-K1 cells and cyclic AMP-dependent luciferase expression (Lytton et al., 2010). This bioassay is a highly specific method to detect TSI in the bloodstream but requires an overnight cell culture step as well as a 3-h luciferase induction time, leading to an assay turnaround time of 21–22 h. We sought to improve the Thyretain™ TSI bioassay to address these shortcomings. Toward this end we employed a cAMP biosensor (GloSensor, Promega, Madison, WI) that is based on a mutant form of firefly luciferase in which a cAMP-binding domain of protein kinase A (PKA) is fused between the N- and C-termini such that the luciferase is inactive until cAMP binds to the cAMP binding site and thus luciferase activity is proportional to intracellular cAMP levels (Binkowski et al., 2009). This fusion protein which we refer to as the homogenous cAMP biosensor (HCBS), enables near real-time monitoring of cAMP dynamics in living cells (Buccioni et al., 2011; Kumar et al., 2017). To develop an HCBS-based TSI bioassay, we generated a CHO-K1 stable cell line that expresses both the same chimeric TSHR (Mc4) used in the Thyretain™ bioassay and the GloSensor™ cAMP biosensor.

The HCBS-TSHR-Mc4 stable cell line enabled the development of a rapid, real-time, homogenous bioassay (Turbo™ TSI bioassay) for the detection of TSI in patient serum. Briefly, serum samples are added to the wells of a white 96-well plate and mixed with freshly thawed HCBS-TSHR-Mc4 cells in Reaction Buffer containing luciferase substrate (6:100). The TSI, if present in the patient serum, binds to TSHR on the cell surface and induces a signaling cascade resulting in increased production of intra-cellular cAMP. This increased production of cAMP is evidenced by increased biosensor luciferase activity which can be measured as relative light units (RLU) by a luminometer in intact cells. This new assay platform allows for the detection of TSI in serum samples at room temperature in a real-time homogeneous format which delivers results at 60 min, without requiring cell culture, sample dilution, or washing and cell lysis steps.

In this report we describe the generation of stable cell lines co-expressing the HCBS and TSHR-Mc4; development of the Turbo™ TSI bioassay using the reporter cell line; and performance of the new bioassay in comparison with the industry standard Thyretain™ TSI bioassay.

2. Materials and methods

2.1. Serum samples and monoclonal antibodies

TSI-positive serum samples were a kind gift of Dr. George Kahaly. Anti-thyroid peroxidase (TPO) antibody-positive human serum samples and nonthyroidal autoimmune disease state human serum samples were obtained from Discovery Life Science (Los Osos, CA). All of the serum samples were de-identified, aliquoted, and frozen at -80 °C. The samples had gone through at least two freeze-thaw cycles before being used in this study. The TSHR stimulating monoclonal antibody M22 and the TSHR blocking monoclonal antibody K1-70 were purchased from Kronus (Star, ID). ThyroS monoclonal antibody (ThyroS MAb) which has TSHR stimulatory activity was developed by Quidel Inc. Bovine TSH (bTSH) was purchased from Sigma (St. Louis, MO).

2.2. Standards and controls

Five levels of the ThyroS MAb that had been value-assigned against the WHO International Standard for TSI (08/204) were used to generate a standard curve during the assay development. The target concentrations of five standards were 0.01, 0.06, 0.3, 3.0 and 11 IU/L. Three levels of positive controls (LPC, MPC and HPC) were prepared with ThyroS MAb ranging 0.04–0.06 IU/L for LPC, 2.0–3.0 IU/L for MPC, and 7.0–9.0 IU/L for HPC. The negative control (NC) was prepared with human normal serum. The assay results would be acceptable when all PC results fell within the determined concentration range for each standard while NC was negative.

2.3. Transient transfection of CHO-K1 cells with pGloSensor™-22F plasmid along with either TSHR-Mc4 or wild type TSHR plasmid

The pGloSensor™-22F plasmid (GS-22F; Promega, Madison, WI) was linearized by restriction enzyme digestion with *Pst*I. TSHR-Mc4 and wild type TSHR (TSHR-Wt) plasmids containing either the chimeric TSHR gene or wild type TSHR gene (Quidel, Athens, OH) were both linearized by restriction enzyme digestion with *Pci*I. To perform transient transfection, Chinese hamster ovary cells (CHO-K1) (ATCC, Manassas, VA) at 10×10^5/mL were seeded in a 96-well tissue culture plate at 100 μL/well. After a 24-h incubation at 37 °C with 5% CO_2, one side of the plate (48 wells) was co-transfected with GS-22F and TSHR-Mc4 DNA fragments following the technical manual provided by Promega. The other half of the plate (48 wells) was co-transfected with GS-22F and TSHR-Wt DNA fragments following the same procedure.

2.4. Stable CHO-K1 cell line expressing HCBS and TSHR-Mc4

CHO-K1 cells were co-transfected with the linearized GS-22F plasmid and TSHR-Mc4 plasmid (Fig. 1) in two wells of a 24-well plate using FuGENE HD transfection reagent (Promega, Madison, WI). After a 24-h incubation with the transfection mixture, the cells were pooled from the

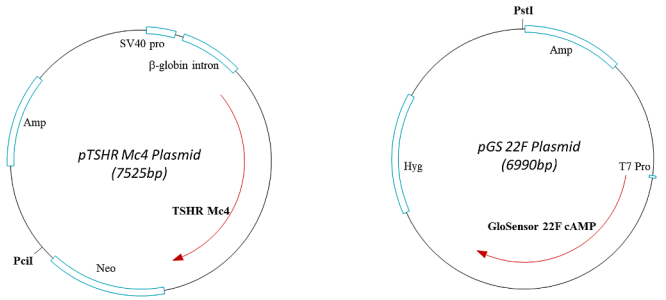


Fig. 1. Schematic diagram of the TSHR-Mc4 plasmid (Quidel) and GS-22F plasmid (Promega, Madison, WI) used for generating the HCBS-TSHR-Mc4 stable CHO-K1 cell line (2C1E3). The two plasmids were linearized by restriction enzyme digestion with either *Pci*I (TSHR-Mc4 plasmid) or *Pst*I (GS-22F plasmid) before being used for stable transfection of CHO-K1 cells.

two wells and serially diluted in a 96-well plate with CHO Growth Medium (Quidel) containing 250 μg/mL of Hygromycin B (Invitrogen, Carlsbad, CA). The plate was incubated at 37 °C with 5% CO_2 for 1 week to select the cells that express the GS reporter. The medium was then replaced with CHO growth medium containing 600 μg/mL of G418 (Sigma, St. Louis, MO) to select the cells that co-express TSHR-Mc4 receptor for an additional 5 days. The cell isolates that grew in the selection medium were trypsinized and plated in duplicate in a new black 96-well plate (Costar, Corning, NY), and screened following the GS assay procedure (GloSensor™ cAMP Assay Technical Manual) using 1 μg/mL or 0.01 μg/mL M22 antibody as the TSHR stimulator. Several positive cell isolates were identified based on both signal response to M22 stimulation and cell density. These selected cell isolates were further cloned by limiting dilution using growth medium containing both hygromycin B (125 μg/mL) and G418 (300 μg/mL). During the screening of clones (*2.6*), one clone (2C1E3) that outperformed the other five clones was chosen and expanded for developing the Turbo™ TSI bioassay. All subsequent experimental data were generated with the 2C1E3 clone or subclones and referred to as the HCBS-TSHR-Mc4 cells.

2.5. DNA sequence analysis of TSHR-Mc4 receptor in the CHO-K1 cell line

To confirm that the cell line (2C1E3) indeed incorporated the TSHR-Mc4 gene, genomic DNA was extracted from the stable cell line and from the original CHO-K1 cells via bioMérieux EasyMag instrument (Marcy-l'Étoile, France) following the manufacturer's instructions. End-point PCR was performed using each genomic DNA as the template on the Eppendorf Mastercycler (Hamburg, Germany). The TSHR-Mc4 gene was amplified with a primer pair that spans the full length of TSHR (5'-*AGGCCGGCGGACTTGCTG*-3′; and 3'-*CAAAACCGTTTGC*-5′) using Invitrogen Platinum Taq DNA Polymerase High Fidelity reagents (Thermofisher, Waltham, MA). The size of the PCR product was analyzed by agarose gel electrophoresis followed by purification of the PCR product from the gel for DNA sequencing confirmation (Genewiz, South Plainfield, NJ).

2.6. Screening of stable CHO-K1 cell lines for TSI-induced biosensor luciferase activity

The CHO-K1 cells transfected with GS-22F and TSHR-Mc4 (HCBS-TSHR-Mc4 cells) were seeded at 1-2 × 10^4 /well in CHO growth medium in either a black or a white 96-well plate (Costar, Corning, NY) and incubated for 24 h at 37 °C with 5% CO_2. The medium was removed from the plate and 100 μL of 6% (*v*/v) GloSensor™ luciferase substrate (Promega, Madison, WI) diluted in Reaction Buffer (Quidel) was added to each well. Without pre-equilibration of the cells with the luciferase substrate, 10 μL of 1.0 μg/mL of M22 antibody was immediately added to each clone in the plate in duplicate. The plate was read every 10 min up to 60 min at room temperature in a GloMax Navigator luminometer (Promega, Madison, WI).The data from the 60-min read time were used for clone selection.

2.7. Turbo ™ TSI bioassay

The optimum cell density for the Turbo ™ TSI bioassay was determined by testing three low TSI positive samples with nine different concentrations of the HCBS-TSHR-Mc4 cell line (2C1E3). The cells with the best concentration were aliquoted and kept frozen at -80 °C for a long term storage. To perform the Turbo ™ TSI bioassay, one freezer vial of the HCBS-TSHR-Mc4 cell line (2C1E3) was thawed in a 37 °C water bath for 2–5 min. The cells were transferred into 5 mL of luciferase substrate prepared in Reaction Buffer (Quidel) and placed in a reagent reservoir. TSI standards, positive and negative controls, and samples to be tested were added into a white 96-well plate at 5 μL per well. The cell suspension was then dispensed into the wells of the plate at 50 μL/well. The plate was read every 10 min up to 90 min at room temperature by GloMax Navigator luminometer. Results were reported as IU/L calculated from a standard curve generated using the method of 5-parameter logistic (5-PL) regression. Multiple lots of assay reagents including cells were used for assay development. All samples and controls were tested in duplicate or triplicate.

2.8. LoD of Turbo ™ TSI bioassay

The Turbo TSI bioassay LoD was determined by a two-step process. First, repeated measurements of four low-level clinical samples were conducted. Thirty-six replicates of each serum were tested by two operators using the same lot of reagents. A total of 144 data points were obtained for the four sera. These measurements were used to provide an estimate of the standard deviation expected from low-level samples. This standard deviation was used in combination with the previously determined LoB value (obtained from 156 measurements of four normal human serum samples) to calculate the Tentative LoD using the following formula:

$$\text{Tentative LoD} = \text{LoB} + c\beta\text{*SD low positive samples}$$

The second step of the process was to verify the assay LoD by spiking a negative serum with five different concentrations of the WHO International Standard for TSI (WHO IS) to produce the concentration in IU/L values around the Tentative LoD. Twenty-six replicates of each serum were tested by two operators. The LoD was reported as the lowest concentration of spiked WHO IS (IU/L) at which at least 95% of replicates produced the TSI concentration (IU/L) above the Turbo TSI LoB.

2.9. LoQ of Turbo ™ TSI bioassay

Based on the Turbo TSI LoD study result, a normal serum was individually spiked with the WHO IS at four different concentrations (0.02, 0.03, 0.04, and 0.05 IU/L). A total of 60 replicates of each WHO IS-spiked sample were tested by two operators using the same lot of reagents. The TSI concentration of each measurement was calculated based on the TSI standard curve (5-PL) and results were analyzed for Precision (%CV), Bias (% Change), and Total Error (TE) using the formulas below. The LoQ of the Turbo™ TSI bioassay was identified as the lowest TSI concentration that met the acceptance criteria (%CV $\leq$ 15% and TE $\leq$45%).

$$Bias(\%Change) = \frac{Calculated\ Conc. - Spiked\ Conc}{Spiked\ Conc.} \times 100\%$$

$$Total\ Error = Bias + 1.96 \times Precision\ (CV)$$

2.10. Testing potential cross-reactants in Turbo ™ TSI bioassay

Twenty-two potential cross-reactants including the TSHR blocking antibody K1–70 were individually spiked into a normal serum and tested directly by the Turbo™ TSI bioassay. The concentrations of the cross-reactants tested in the study are much higher than their physiological concentrations. The same set of the samples were also prepared at their 2× testing concentrations and then combined with an equal volume of a low TSI positive serum (0.3 IU/L) individually. A control sample was prepared for each individual interferent by combining each appropriate sample diluent with the same TSI positive sample using the same method. This low positive sample mixed with each individual interferent and the same sample mixed with each individual interfering substance diluent were tested in the Turbo™ TSI bioassay following the standard procedure. Percent change between the low TSI positive sample spiked with the interferent and the same sample spiked with interferent diluent were evaluated.

2.11. Thyretain™ bioassay

The assay was performed following the manufacture's instruction (Quidel, San Diego, CA). Briefly, the inner 48 wells of a black 96-well plate were treated with Attachment Solution. One vial of frozen Thyretain™ cells was thawed and added to 5 mL of CHO Growth Medium in a reagent reservoir. The cell suspension was dispensed into the plate at 100 µL/well. After 18-h incubation at 37 °C with 5% CO_2, the medium was removed from the plate. The plate was washed once with Reaction Buffer and then refilled with 100 µL of Reaction Buffer. Samples were added to the plate at 100 µL per well in duplicate after being diluted 1:11 in Reaction Buffer. The plate was incubated at 37 °C with 5% CO_2 for 3 h to allow luciferase induction. After decanting the contents of the plate, 75 µL of substrate and lysis reagent (Promega, Madison, WI) was added to each well and allowed to stand at room temperature for 10 min. The luciferase expression levels (RLU signals) were measured by the GloMax Navigator luminometer. Results were reported as signal over reference ratio (%SRR) using the formula below.

$$\%SRR = \frac{Sample\ RLU}{Reference\ RLU} \times 100\%$$

3. Results

3.1. Selection of TSHR gene (TSHR-Mc4 vs. TSHR-Wt) for stable transfection of CHO-K1 cells

We first evaluated the performance of the wild type (Wt) and chimeric (Mc4) TSH receptors side-by-side by a modified Promega Glosensor assay in transient transfection experiments. The same quantity of the TSHR-Mc4 and TSHR-Wt plasmids were transiently transfected into CHO-K1 cells individually along with the same amount of the GS reporter plasmid GS-22F. The samples consisted of bTSH (2 mIU/mL), a TSI positive serum, and a normal serum sample. The TSI-positive sample was selected based on the Thyretain™ bioassay results, which showed a %SRR value of 501% (Thyretain™ Cutoff is 140% SRR). Reaction Buffer with luciferase substrate (6:100) was used as the assay blank and results were reported as signal over blank (S/B) ratios. As shown in Fig. 2, cells co-transfected with GS-22F/TSHR-Mc4 plasmids exhibited a lower biological response to bTSH stimulation but retained higher reactivity to the TSI positive sample stimulation (red bars) when compared to the cells transfected with GS-22F/TSHR-Wt (blue bars). This experiment was repeated by a different operator and similar results were obtained.

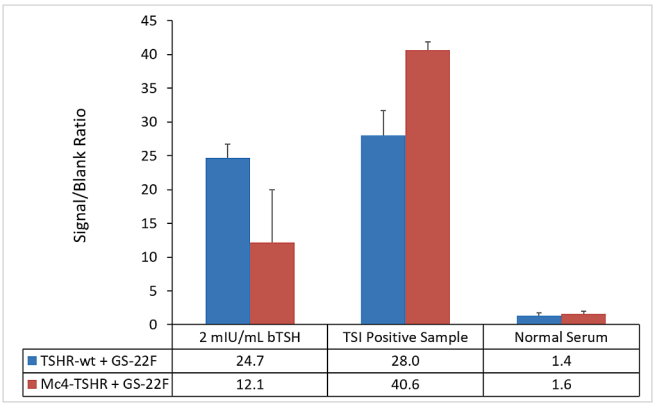


Fig. 2. Comparison of TSHR-Mc4 and TSHR-Wt by the modified GloSensor assay using transiently transfected CHO-K1cells. Bars represent Mean + Standard Deviation (SD) of Signal/Blank ratios obtained from two transient transfection wells for each sample.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Stable CHO-K1 cell line generation with GS-22F and TSHR-Mc4 plasmids

Based on the transient transfection results which are in agreement with the previously published results by other groups (Tahara et al., 1997; Li et al., 2013), the TSHR-Mc4 plasmid was chosen for generating stable CHO-K1 cell lines to be used in the Turbo™ TSI bioassay. The TSHR-Mc4 plasmid and GS-22F plasmid were linearized and co-transfected into CHO-K1 cells. Following antibiotic selection, a total of 372 clones were isolated and screened by the modified GloSensor assay following stimulation with the M22 antibody. Six clones that showed high signal over blank ratios were selected for further cloning by limiting dilution. The cloned cell lines were seeded at the same density and tested again with the M22 antibody. Results showed that clone 2C1E3 outperformed the other five clones by producing the highest signal response (RLU) with a Signal/Blank ratio of 39 (Fig. 3). This cell line (2C1E3) was propagated, and the optimum cell density was further determined by testing three low TSI positive samples on nine different cell concentrations (Data not shown). Base on the results, the cells were aliquoted at 4×10^6 /mL/vial so that the final concentration of the cells would be 6.7×10^5/mL after being added to 5 mL of luciferase substrate when performing the assay. The cell aliquots were frozen at -80 °C for the Turbo™ TSI bioassay development, designated as the HCBS-TSHR-Mc4 cell line.

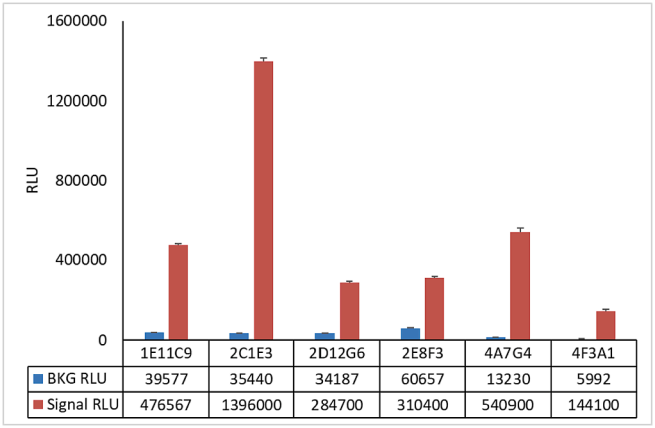


Fig. 3. Screening of candidate HCBS-TSHR-Mc4 cell lines in the modified GloSensor assay. Bars represent Mean + Standard Deviation (SD) of RLU obtained from three assay wells for each sample.

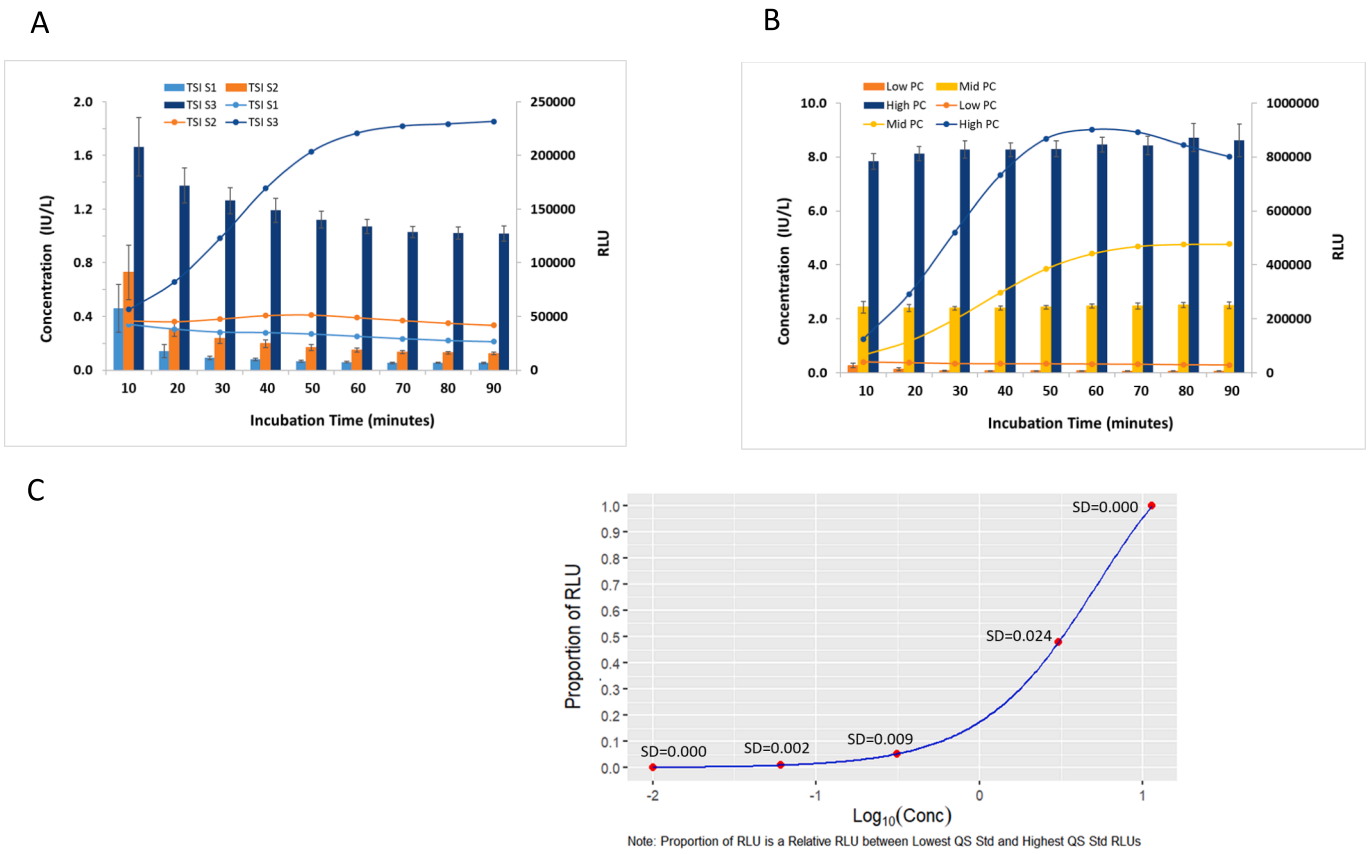


Fig. 4. Real time measurement of TSI positive samples and controls by Turbo™ TSI bioassay over 12 days.
A. Real time measurement of three TSI positive samples by Turbo ™ TSI bioassay (mean of 24 measurements of each sample obtained by two operators over 12 days). Calculated mean TSI concentrations (IU/L) for the samples are presented in the bar graph with standard deviation (SD) obtained from 12 days results. Average sample RLU values are presented in the line graph.
B. Real time measurement of three TSI positive controls by Turbo ™ TSI bioassay (mean of 24 measurements of each control obtained by two operators over 12 days). Calculated mean TSI concentrations (IU/L) for the controls are presented in the bar graph with standard deviation (SD) obtained from 12 days results. Average control RLU values are presented in the line graph.
C. Turbo™ TSI bioassay standard curve generated with the data from 60 min incubation. Plotted as proportion of RLU vs. Log_{10} concentration of TSI standards (IU/L). Standard deviation (SD) of each standard measured by two operators over 12 days is shown in the graph.

Both black 96-well plates with clear bottom and white solid 96-well plates were used to screen the cell lines. We found that the assay performed in white solid plates produced significantly higher RLU in general when compared to the black plates with clear bottom. Therefore, white solid plates were used for further assay development.

3.3. *Confirmation of TSHR-Mc4 gene integration in the stable CHO-K1 cell line*

The Thyretain™ bioassay uses a cell line expressing the chimeric TSHR (Mc4) on the cell surface in which TSHR residues 262–368 are substituted by residues 262–334 from the rat LH-CGR (luteinizing hormone/choriogonadotropin receptor) (Nunez Miguel et al., 2012). To comfirm that the HCBS-TSHR-Mc4 cell line (2C1E3) indeed expresses TSHR-Mc4, genomic DNA was isolated from the cell line as well as non transfected CHO-K1 cells (negative control). PCR was performed with TSHR (Mc4) gene specific primers using the same amount of each genomic DNA as the template. A PCR product was only obtained from DNA of the TSHR-Mc4 stable cell line with the expected size of 2.1 kb (data not shown). To verify the receptor sequence, the PCR product was sequenced then analyzed using the Clone Manager software (Sci Ed Software, Westminster, CO). Deduced amino acid sequence of the PCR product was aligned against the published predicted TSHR-Mc4 protein sequence (Lytton et al., 2010) showing a 100% mtach between the two sequences (data not shown). The results demostrate integration of the TSHR-Mc4 gene into the genome of the HCBS-TSHR-Mc4 cell line (2C1E3).

3.4. *Real-time measurement of Turbo™ TSI bioassay results*

The Turbo™ TSI bioassay provides real-time detection of a signal transduction event initiated by binding of TSI to the TSHR. The optimal assay time for obtaining the final result was studied. Three TSI serum samples, the assay standards and controls were tested by two operators for 12 days. The assay results were measured at room temperature every 10 min up to 90 min. Fig. 4A shows that sample RLU values and its calculated TSI concentrations (IU/L) become stabilized with a decreased standard deviation when the 60-min incubation time is reached. The results from testing three levels of positive controls are similar to the samples (Fig. 4B). The incubation time of 60 min was therefore used for further studies. The negative control data was not included in the figure because its TSI concentration was calculated to be 0 IU/L. Fig. 4C shows the TSI standard curve generated with five different levels of ThyroS MAb using a 5-parameter logistic regression.

3.5. *Analytical sensitivity of Turbo™ TSI bioassay*

Following CLSI guideline (EP-17A), the tentative LoD of the Turbo™ TSI bioassay was calculated to be 0.014 IU/L using the formular "Tentative LoD = LoB + cβ*SD low positive samples" (data not shown).

Table 1
LoD of Turbo™ TSI bioassay.

Parameter	Concentration of WHO IS for TSI in Normal Serum					
	0.010 IU/L	0.012 IU/L	0.014 IU/L	0.016 IU/L	0.018 IU/L	0.020 IU/L
Replicate Number	26	26	26	26	26	26
Average (IU/L)	0.0052	0.0095	0.0176	0.0167	0.0220	0.0255
St Dev	0.0063	0.0066	0.0059	0.0051	0.0047	0.0042
%CV	123%	69%	34%	31%	21%	16%
% results above LoB	35%	65%	92%	100%	100%	100%

Table 2
LoQ of Turbo™ TSI bioassay.

Parameter	Concentration of WHO IS for TSI in Normal Serum			
	0.02 IU/L	0.03 IU/L	0.04 IU/L	0.05 IU/L
Replicate Number	60	60	60	60
Bias	8%	2%	15%	20%
%CV	21%	15%	11%	12%
Total Error	49%	33%	37%	43%

To verify the LoD, a negative serum was spiked with six different concentrations of the WHO International Standard for TSI (WHO IS) to produce the TSI concentrations (IU/L) around the Tentative LoD. Thirteen replicates of each serum sample were tested by each of two operators using the same lot of reagents. As shown in Table 1, the LoD of the Turbo TSI™ bioassay was confirmed to be 0.016 IU/L as it was the lowest concentration of spiked WHO IS (IU/L) at which great than 95% of replicates produced the TSI concentration (IU/L) above the assay LoB.

The assay LoQ was determined following CLSI guideline (EP-17A) as well. Based on the Turbo TSI LoD study results, a normal serum was individually spiked with the WHO IS at four different concentrations (0.02, 0.03, 0.04, and 0.05 IU/L). A total of 60 replicates of each WHO IS-spiked sample were tested by two operators using the same lot of Turbo TSI reagents. The LoQ of the Turbo TSI bioassay ™ was determined to be 0.03 IU/L as it was the lowest concentration that yielded %CV and TE that met the acceptance criteria (CV $\leq$ 15%, TE $\leq$ 45%) (Table 2).

The analytical performance of the assay was further compared with the Thyretain™ TSI bioassay by measuring EC_{50} of the M22 antibody in each assay. The M22 antibody (100 ng/mL) was serially diluted into 7 concentrations and tested concurrently in both assays. Although RLU values generated by the Turbo™ TSI bioassay were about 10-fold higher than the Thyretain™ assay for the same concentration of the M22 antibody, the antibody dose response curves from the two assays were very similar. The EC_{50} value was 4.7 ng/mL for the Turbo™ TSI bioassay and 5.5 ng/mL for the Thyretain™ bioassay (Fig. 5A and B). These results show that the Turbo™ TSI bioassay is comparable to the Thyretain™ TSI bioassay for the detection of human TSHR stimulating antibody M22.

3.6. Precision of Turbo™ TSI bioassay

The Turbo™ TSI bioassay precision was determined by two operators over 12 days with five TSI-positive samples and four assay controls. An average RLU of three replicates for each sample and control was used to obtain results. The TSI concentrations of each sample and assay control were calculated based on the standard curve generated in the same plate each day and a total of 24 data points was generated for each analyte. The overall results as summarized in Table 3 show that %CV values are less than 11% for the calculated concentrations of all samples and positive controls.

3.7. Cutoff and clinical sensitivity of Turbo™ TSI bioassay

130 TPO antibody positive serum samples and 68 serum samples from either clinically normal donors or clinically Graves' disease patients were tested in the Turbo™ TSI bioassay to determine the assay cutoff. All of these samples were also tested by the Thyretain™ TSI bioassay to classify the samples as true positive or true negative for TSI. A ROC curve was generated with the TSI concentration values (IU/L) determined by the Turbo™ TSI bioassay. Results as illustrated in Fig. 6A indicate the high accuracy of the Turbo™ TSI bioassay compared to the Thyretain™ TSI assay. The most appropriate Turbo™ TSI bioassay cutoff was 0.024 IU/L at which the highest true positive rate together with the lowest false positive rate against the Thyretain™ TSI assay results were achieved. The Turbo™ TSI bioassay showed sensitivity at 98.7% and specificity at 93.5% when using 0.024 IU/L as the assay cutoff (Fig. 6B).

3.8. Specificity of Turbo™ TSI bioassay

To exclude cross-reactivity with blood components and therapeutic drugs, 22 potential cross-reactants including the TSHR blocking antibody K1–70 were individually spiked into a normal serum and tested directly by the Turbo™ TSI bioassay. None of these substances at the concentration that was well above a normal physiological concentration gave a result above the assay cutoff meaning no cross-reactivity could be detected (Table 4). The same set of samples were tested by mixing their 2× testing concentrations with an equal volume of a low TSI positive serum (0.3 IU/L) individually. Percent change between the low TSI positive sample spiked with the interferent and the same sample spiked with interferent diluent were evaluated. As shown in Table 4, Percent Change is $\leq$13% for all of the potential interfering substances meaning that the substances at tested concentrations do not interfere with the assay results. The TSHR blocking antibody K1–70 at 1000 ng/mL did not show any cross reactivity in the study demonstrating high specificity of the Turbo™ TSI bioassay to TSI. When spiking the K1–70 antibody in the low TSI positive sample, the antibody did not interfere with the result until its concentration was above 7.8 ng/mL.

3.9. Detection of TSHR blocking antibody (TBAb/TBI) using the same cell line

Previous work has shown that the TSHR-Mc4-Luc cell line used in the Thyretain™ TSI bioassay can also be used to detect TSHR blocking antibody (Li et al., 2013). Therefore, we also evaluated the possibility of developing a rapid, homogenous bioassay in a competitive assay format (Turbo™ TBI bioassay). An optimal concentration of bovine TSH (bTSH) was determined and used to mix with serially diluted human TSHR blocking antibody K1–70 and two mouse TSHR blocking antibodies, 3H10 (DSMZ, Braunschweig, Germany) and 4C1 (Santa Cruz BioTech, Dallas, Texas) individually to investigate if these blocking antibodies also react with the TSHR on the HCBS-TSHR-Mc4 cell line under the same assay conditions with a 60-min turnaround time. Results showed all the three blocking antibodies had an inhibitory effect on bTSH-induced activity, and the blocking activity of K1–70 antibody was significantly more potent compared to the other two mouse blocking antibodies (Fig. 7). This result supports the conclusion drawn by other groups that the TSHR antibodies with different functional activities have overlapping binding sites on the concave surface of TSHR (Nunez Miguel et al., 2012; Furmaniak et al., 2013). Because a TSHR blocking antibody

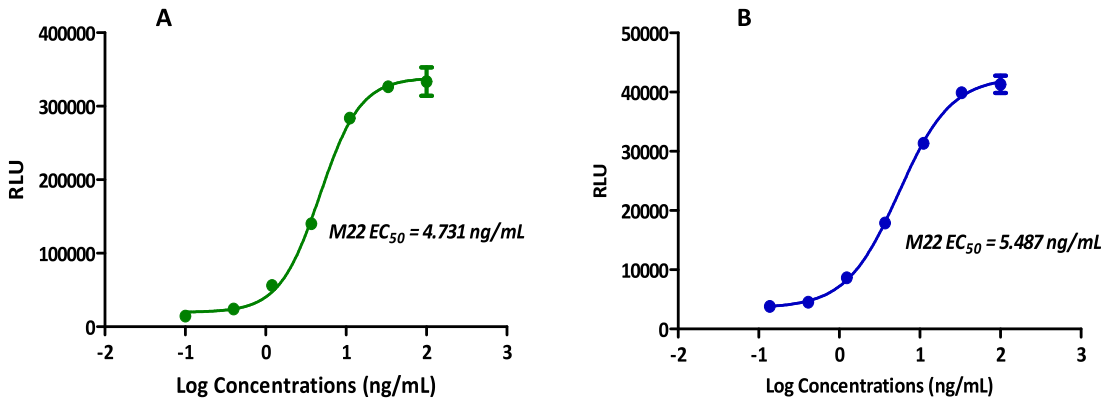


Fig. 5. M22 antibody dose response curve and EC_{50} generated by Turbo™ TSI bioassay and Thyretain™ TSI bioassays. Bars represent Mean + Standard Deviation (SD) of RLU obtained from six assay wells for each sample.
A: M22 antibody dose response curve generated by the Turbo™ TSI bioassay.
B: M22 antibody dose response curve generated by the Thyretain™ TSI bioassay.

Table 3
Precision of Turbo™ TSI bioassay determined using serum samples from five Grave's disease patients.

Sample ID	Days	Operators	Data Points	Average TSI Conc. (IU/L)	SD	%CV[a]
TSI Sample #1	12	2	24	0.060	0.006	10%
TSI Sample #2	12	2	24	0.148	0.015	10%
TSI Sample #3	12	2	24	1.070	0.053	5%
TSI Sample #4	12	2	24	3.670	0.321	9%
TSI Sample #5	12	2	24	6.171	0.708	11%
Positive Control-High	12	2	24	8.461	0.265	3%
Positive Control-Mid	12	2	24	2.476	0.075	3%
Positive Control-Low	12	2	24	0.067	0.007	10%
Normal Serum	12	2	24	0.000	n/a	n/a

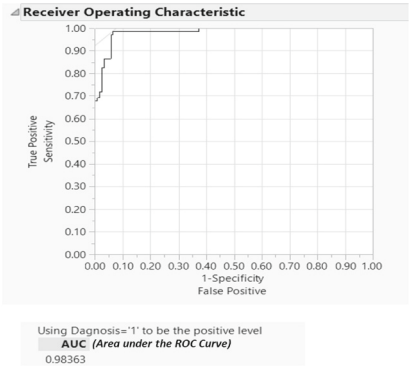
[a] Acceptable %CV for the assay is ≤15%. Mean RLU of three analyte replicates was used for TSI concentration (IU/L) calculation.

does not cause a change in the intracellular concentration of cAMP, the Turbo™ TSI bioassay can only detect TSI. However, this study result demonstrates that the HCBS-TSHR-Mc4 cell line can be used to develop a rapid homogeneous TBI bioassay (Turbo™ TBI bioassay) to complement the Turbo™ TSI bioassay. The paired bioassays will be an important advancement in the long-term effort to develop a simplified set of bioassays that can differentiate anti-TSHR antibodies with stimulating or blocking activity.

4. Discussion

Autoantibodies to the TSHR are unique in their ability to act as agonist or antagonists of the TSHR and as a result affect the function of the thyroid gland. Thyroid stimulating antibodies are directly involved in the pathophysiology of Graves' disease, the most common cause of autoimmune hyperthyroidism (Weetman, 2000). Thyroid blocking antibodies are found in certain patients with autoimmune hypothyroidism such as Hashimoto's thyroiditis. Numerous test kits including antibody binding assays and cell-based bioassays are commercially available for measuring TSHR autoantibodies (TRAb), but only cell-based bioassays

A



B

		Thyretain™ TSI Bioassay	
		Pos	Neg
Turbo™ TSI Bioassay	Pos	74	8
	Neg	1	115
Sensitivity: 98.7% (95 CI: 0.9283-0.9976)			
Specificity: 93.5% (95 CI: 0.8769-0.9667			

Fig. 6. Determination of Turbo™ TSI bioassay cutoff by ROC analysis.
A: ROC-analysis of the Turbo™ TSI bioassay results showing high diagnostic accuracy.
B: Sensitivity and specificity of the Turbo™ TSI bioassay analyzed by a 2 × 2 table.

Table 4
Cross-reactivity and interference testing by Turbo™ TSI bioassay.

Sample	Cross reactivity		Inference testing	
	Passing concentration	Results (IU/L)	Passing concentration	Result (% Change)
Hemoglobin	200 mg/dL	0.000	200 mg/dL	7.3%
Bilirubin	40 mg/dL	0.000	40 mg/dL	2.2%
LH	60 IU/L	0.005	60 IU/L	11%
TSH	5 mIU/L	0.000	5 mIU/L	1%
FSH	300 IU/L	0.000	300 IU/L	7.7%
hCG	50,000 IU/L	0.000	50,000 IU/L	1.6%
Estrogen	4.5 ng/mL	0.000	4.5 ng/mL	0.1%
Progesterone	2.2 μg/mL	0.000	2.2 μg/mL	5.7%
Prolactin	3.9 μg/mL	0.002	3.9 μg/mL	8.6%
Levothyroxine (artificial T4)	1.6 μg/mL	0.003	0.2 μg/mL	12%
Liothyronine (artificial T3)	20 μg/mL	0.000	20 μg/mL	5.2%
Selenium Supplement	2 μg/mL	0.000	2 μg/mL	5%
6-propyl-2-thiouracil	91 μg/mL	0.000	91 μg/mL	13%
Lithium	75 μg/mL	0.000	75 μg/mL	2.2%
Interferon α 2B	4.4 ng/mL	0.000	4.4 ng/mL	0.8%
Interleukin-2 human	14 μg/mL	0.001	14 μg/mL	4.1%
Iodine	1 μg/mL	0.000	1 μg/mL	8.6%
Sunitinib malate	200 μg/mL	0.000	200 μg/mL	12%
Imatinib mesylate	26 μg/mL	0.000	26 μg/mL	2.8%
Ipilimumab	580 μg/mL	0.006	290 μg/mL	0.4%
Alemtuzumab	264 μg/mL	0.006	264 μg/mL	0.8%
K1–70 TSHR blocking antibody	1000 ng/mL	0.000	7.8 ng/mL	13%

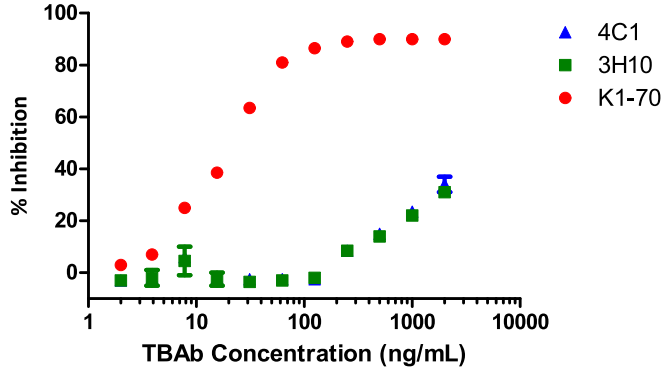


Fig. 7. Inhibitory effect of TBAbs on bTSH-induced luminescence in the Turbo™ TBI bioassay. Dots represent Mean + Standard Deviation (SD) of % Inhibition obtained from two assay wells for each sample concentration.

such as the Thyretain™ TSI (TSAb) and TBI (TBAb) bioassays are able to distinguish between stimulating and blocking antibodies (Lytton et al., 2018). However, bioassays usually require cell culture and a longer turnaround time than binding assays which make them more difficult for clinical laboratories to adapt.

In order to improve the workflow of the Thyretain™ TSI bioassay, we developed a rapid anti-TSHR bioassay based on a homogenous real-time assay biosensor for intracellular concentrations of cAMP. We hypothesized that a cell line that expresses both the cAMP biosensor and TSHR could be generated and used to detect TSI and TBI with a rapid and simple-to-perform protocol.

We chose to use the chimeric TSHR for generating HCBS-TSHR-Mc4 cell line based on data that showed it was less responsive to bTSH stimulation while exhibiting a higher reactivity to TSI positive samples when compared to the wild type of TSHR. This data was very similar to the TSHR-Mc4 cell line used in the Thyretain™ bioassay (Tahara et al., 1997; Li et al., 2013). The stable CHO-K1 cell line that was generated using the GS-22F and TSHR-Mc4 plasmids enabled the development of a rapid, homogenous real-time bioassay (Turbo™ TSI bioassay) for the detection of TSI based on changes in the intracellular cAMP levels. The Turbo™ TSI bioassay is very simple to perform and requires only four steps: samples and controls are added to the wells of a white 96-well plate, an aliquot of a freshly-thawed cells are mixed with reaction buffer containing luciferase substrate, the cell suspension is dispensed into each well and maintained at room temperature for 60 min, and finally the plate is read in a luminometer and the test result is pasted to a calculation software and reported in IU/L. Total hands-on time is less than 20 min and time from initiation to result is less than 90 min. The assay is fully compatible with automation and, in fact, its work-flow is less complex than the existing immunoassays for the detection of TSH receptor antibodies (TRAb).

The Turbo™ TSI bioassay delivers a very similar analytical performance as the Thyretain™ bioassay demonstrated by the results of the M22 antibody dose-response study. The preliminary clinical sensitivity of the Turbo™ TSI bioassay was also evaluated by testing 130 anti-TPO positive serum samples and 68 serum samples from either clinically normal donors or clinically Graves' disease patients in comparison with the Thyretain™ TSI bioassay. The presence of anti-TPO antibodies in patient blood suggests an AITD, such as Hashimoto's disease or Graves' disease (Hollowell et al., 2002; Prummel and Wiersinga, 2005). According to the testing results, the positive and negative percent agreement (PPA, NPA) between the two bioassays were 98.7% and 93.5%, respectively.

Human TSHR blocking monoclonal antibody K1–70 at a high concentration of 1000 ng/mL did not show false positive results in the Turbo™ TSI bioassay because the TSHR blocking antibody does not activate the TSHR and thus does not increase the intracellular concentration of cAMP. Therefore, the Turbo™ TSI bioassay only detects TSI, or a net stimulatory effect of TSI when both types of the antibodies coexist in the samples. The reduction of TSI activity by K1–70 is only observed at concentration above 7.8 ng/mL. The ability K1–70 to interfere with TSI activity supports the conclusion drawn by other groups that the TSHR antibodies with either stimulating or blocking activities have overlapping binding sites on the concave surface of TSHR (Nunez Miguel et al., 2012; Furmaniak et al., 2013). However, the same cell line can be used to develop a companion assay (Turbo™ TBI bioassay) for the detection of TSHR blocking antibodies by measuring their inhibitory effects on bTSH-induced stimulatory activity with the same turnaround time as the Turbo™ TSI bioassay.

In conclusion, using a CHO-K1 cell line expressing a cAMP biosensor and TSHR-Mc4, we have developed a rapid, homogenous and real-time bioassay for the detection of TSI in patient serum samples. The new assay reduces the total incubation time required for a conventional bioassay from 21–22 h to 60 min minutes while retaining similar analytical and clinical performance when compared to the current Thyretain™ TSI bioassay. The data presented in this report demonstrates that the newly developed Turbo™ TSI bioassay is a promising advancement as an aid in differential diagnosis of autoimmune thyroid disease (AITD) and has the potential to enable more widespread clinical use of TSI and TBI bioassays.

Funding

The work was funded by Quidel Corporation, San Diego, CA, USA.

Acknowledgements

We wish to thank Ron Lollar and Jennifer Halliday for providing and testing the 130 anti-TPO positive serum samples by the Thyretain™ TSI assay. We wish to thank Dr. George Kahaly for kindly providing the clinical TSI positive serum samples. We wish to thank Dr. Jason Sun for providing ThyroS monoclonal antibodies. We wish to thank Dr.

Yunsheng Li for constructing the TSHR-Mc4 plasmid.

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