Human Thyroid-Stimulating Hormone (TSH)

Thyroid-stimulating hormone (TSH) is produced by the pituitary gland at the base of the brain. It has a molecular mass of approximately 28 000 Da. TSH is a member of the glycoprotein hormones family, which consists of TSH, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and chorionic gonadotropin (CG). Glycoprotein hormones are heterodimers that consist of α- and β-subunits. The α-subunit is the same for all glycoprotein hormones – TSH, LH, FSH, and CG. Meanwhile, the β-subunit is specific for each hormone. However, a high degree of homology exists between human TSH, LH, FSH, and CG β-subunit sequences.

"Gold standard" for thyroid dysfunction

Measuring the TSH level in the blood is the most sensitive and specific test for diagnosing thyroid dysfunction, including hypothyroidism and hyperthyroidism (1). The normal range of TSH in serum is 0.58 – 4.1 μIU/ml (2). The TSH concentration in the blood increases in patients with hypothyroidism and decreases in patients with hyperthyroidism.

Sensitive TSH assays

The sandwich immunoassay method is used for the determination of TSH concentration in human serum samples. In the sandwich immunoassay method, one antibody is immobilized on a solid phase, while the second antibody is conjugated to a label. Most of the current TSH assays are third-generation assays with a functional sensitivity of 0.01-0.02 μIU/mL or less. These assays reliably distinguish patients with hyperthyroidism from those with euthyroidism.

The fourth generation TSH assays provide a 10-fold increase in the functional sensitivity (0.001-0.002 μIU/mL) and, therefore, ensure a more accurate measurement of TSH in patients with low TSH levels. The sensitivity of the assay is determined by the affinity of the antibodies used and the sensitivity that the detection system can provide. For the fourth generation TSH assays, high-affinity antibodies are needed as they can provide such a high level of sensitivity.

High specificity

In order to accurately determine the concentration of TSH in serum samples, it is required that the pair of antibodies used in the assay doesn't recognize other glycoprotein hormones (LH, FSH, and CG). Furthermore, for the one-step TSH sandwich immunoassay in which capture and detection antibodies are incubated with serum samples simultaneously, both antibodies that are used in the immunoassay should not have cross-reaction with LH, FSH, and CG.

CLINICAL UTILITY

- **Thyroid dysfunction**
- **Thyroid management and screening during pregnancy, postpartum, and for newborns**

TSH variant R55G

In order to accurately measure TSH concentration in serum, the assay must be able to measure all forms of TSH in the blood. In 2014, a novel point mutation in the TSH β-subunit that resulted in the replacement of arginine with glycine at position 55 (R55G) was reported (3). This mutation altered an epitope on TSH, which prevented antibodies that were specific to that epitope from binding. In patients, homozygous for R55G mutation TSH was undetectable by four widely used immunoassays that had been approved by the US Food and Drug Administration (FDA). When using such assays, patients carrying the TSH R55G variant cannot be distinguished from patients with subclinical hyperthyroidism. Patients with falsely undetectable TSH levels have been incorrectly diagnosed with hyperthyroidism and treated. For accurate measurements of TSH in patients homozygous for the R55G mutation, TSH assays should be able to detect the TSH variant R55G. This is possible if both antibodies used in the assay do not recognize the epitope on the TSH β-subunit containing arginine at position 55 (R55G).

Antibody pairs recommendations

Hytest offers six new monoclonal antibodies (MAbs) for the development of sensitive and specific TSH immunoassays. These MAbs have been developed with the aim of obtaining antibodies that are suitable for fourth generation TSH assays, which do not have cross-reactivity with other glycoprotein hormones and are able to recognize the TSH R55G variant. The MAbs TS13, TS18, and TS25 are specific to one epitope on the TSH molecule (group A), while the MAbs TS21, TS31, and TS32 are specific to another epitope (group B). MAbs do not cross-react with the free human glycoprotein hormones common α-subunit (data not shown), human LH, human FSH, and human CG (see Figure 1).

The performance of the MAbs was evaluated using a chemiluminescent sandwich immunoassay. The detection antibodies were labelled with alkaline phosphatase (ALP). Meanwhile, the capture antibodies were biotinylated and conjugated with streptavidin-coated magnetic beads. The recommended capture-detection pairs for TSH sandwich immunoassays are shown in Table 1.

Table 1.
Recommended MAb combinations for the detection of TSH.

Capture	Detection
TS21	TS18
TS21	TS13
TS21	TS25
TS32	TS13
TS31	TS13

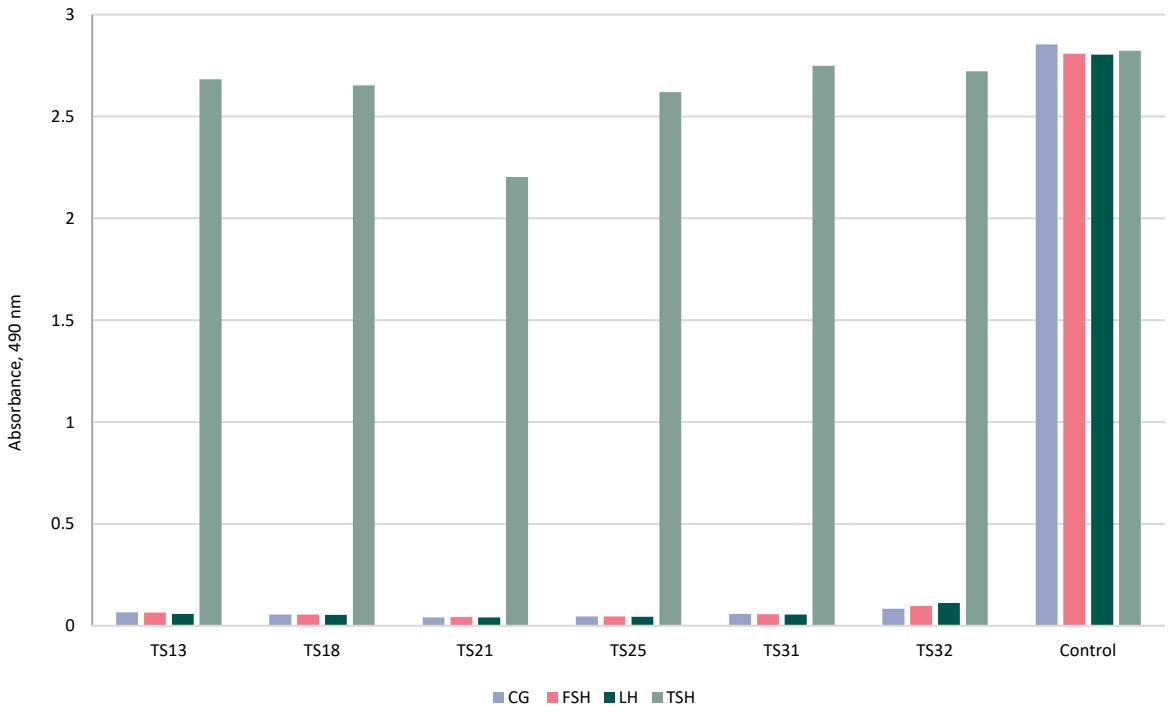

Figure 1.
Cross-reactivity testing of MAbs with human glycoprotein hormones TSH, FSH, LH, and CG. The specificity of MAbs was determined by ELISA. An antibody specific to glycoprotein hormones common α-subunit was used as a positive control for the immunoreactivity of FSH, LH, and CG.

The typical calibration curves for recommended combinations are presented in Figure 2.

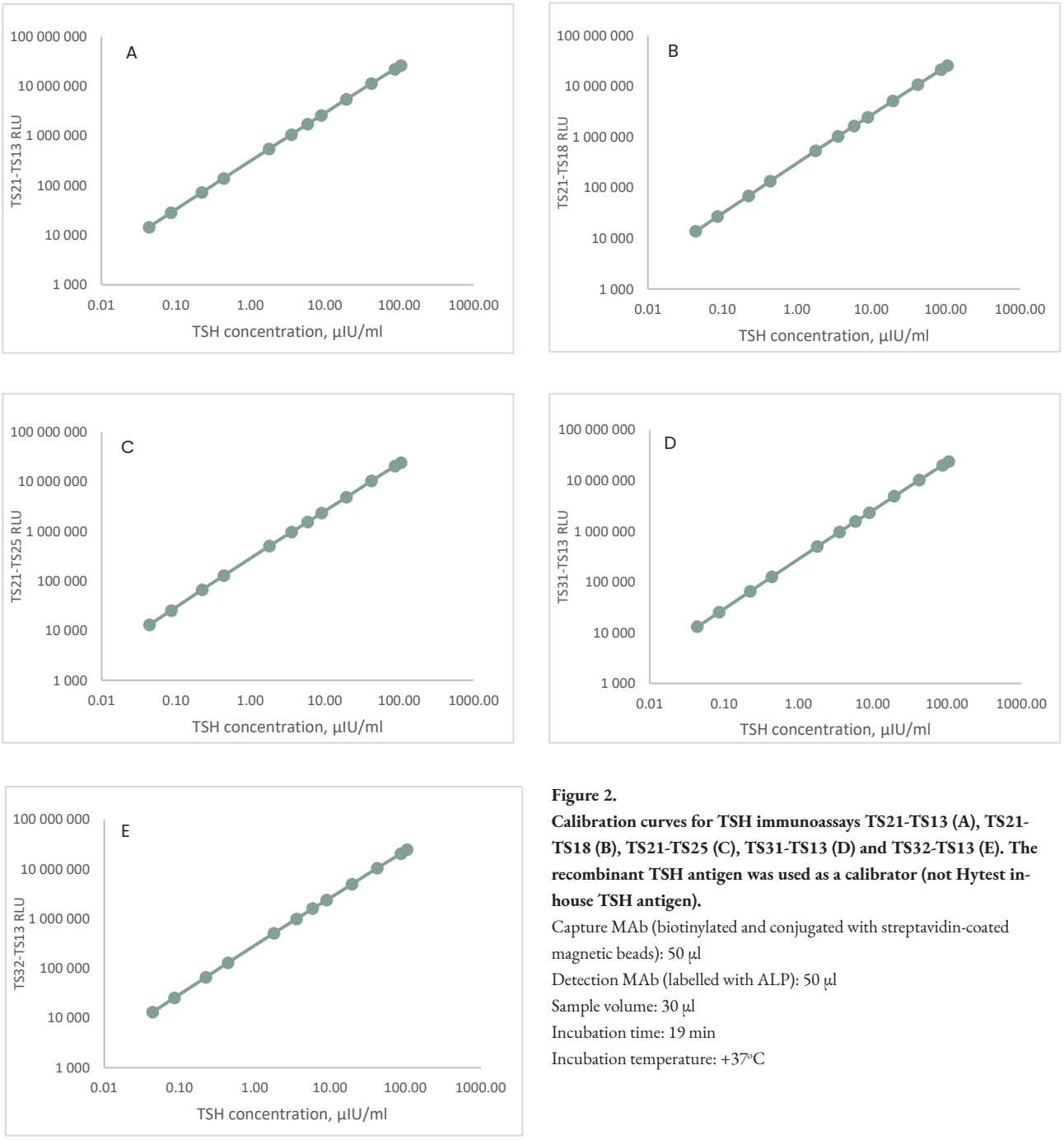

Figure 2.
Calibration curves for TSH immunoassays TS21-TS13 (A), TS21-TS18 (B), TS21-TS25 (C), TS31-TS13 (D) and TS32-TS13 (E). The recombinant TSH antigen was used as a calibrator (not Hytest in-house TSH antigen).
Capture MAb (biotinylated and conjugated with streptavidin-coated magnetic beads): 50 µl
Detection MAb (labelled with ALP): 50 µl
Sample volume: 30 µl
Incubation time: 19 min
Incubation temperature: +37°C

Limit of detection (LoD) determination

We used human native TSH (WHO International Standard NIBSC code 81/565) diluted in an assay buffer to concentrations of 0.0024, 0.0018, 0.0014, 0.001, 0.00077, and 0.00059 µIU/ml in order to determine LoD for selected antibody pairs. Twenty replicates of assay buffer and TSH dilutions were tested in one experiment in chemiluminescent sandwich immunoassays. Limit of Blank (LoB) and Limit of Detection (LoD) were calculated using the formulas provided below:

LoB = mean blank + 1.645(SD blank)
LoD = LoB + 1.645(SD low concentration sample)

The experiments were performed under the immunoassay conditions:

Capture MAb (biotinylated and conjugated with streptavidin-coated magnetic beads): 50 µl
Detection MAb (labelled with ALP): 50 µl
Sample volume: 50 µl
Incubation time: 16 min
Incubation temperature: +37°C

The LoD for TSH diluted in the buffer for all MAb combinations was the same and amounted to 0.00077 µIU/ml. CV was < 2.16% for all tested TSH dilutions. This data shows that selected antibody pairs demonstrate sensitivity that is equivalent to the fourth generation TSH assays.

The correlation of serum TSH concentrations

TSH concentration in human serum samples (n = 50) was determined using recommended MAb combinations and compared with TSH concentrations determined by the reference assay Roche Elecsys TSH. We observed a good correlation between TSH concentrations determined using recommended combinations and the reference assay (Figure 3).

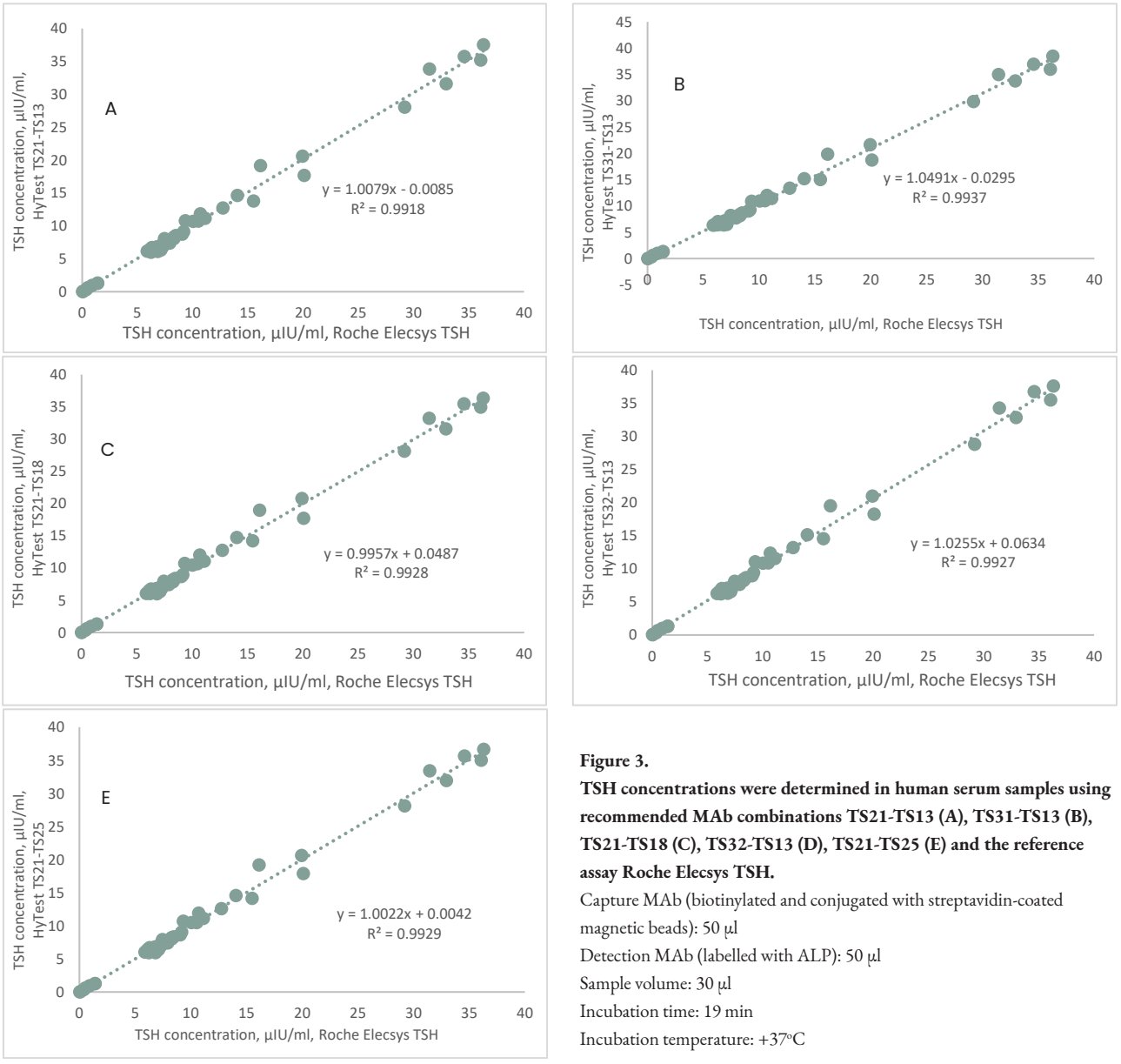

Figure 3.
TSH concentrations were determined in human serum samples using recommended MAb combinations TS21-TS13 (A), TS31-TS13 (B), TS21-TS18 (C), TS32-TS13 (D), TS21-TS25 (E) and the reference assay Roche Elecsys TSH.
Capture MAb (biotinylated and conjugated with streptavidin-coated magnetic beads): 50 µl
Detection MAb (labelled with ALP): 50 µl
Sample volume: 30 µl
Incubation time: 19 min
Incubation temperature: +37°C

Cross-reactivity testing with the TSH variant R55G

For in-house testing purpose, we developed recombinant human TSH containing arginine instead of glycine at position 55 (TSH variant R55G). Additionally, we produced a recombinant wild-type TSH in-house using the same protocol. All selected antibodies recognized the TSH variant R55G similarly to wild-type TSH in ELISA. The detection of wild-type TSH and the TSH variant R55G using recommended combinations is shown in Figure 4.

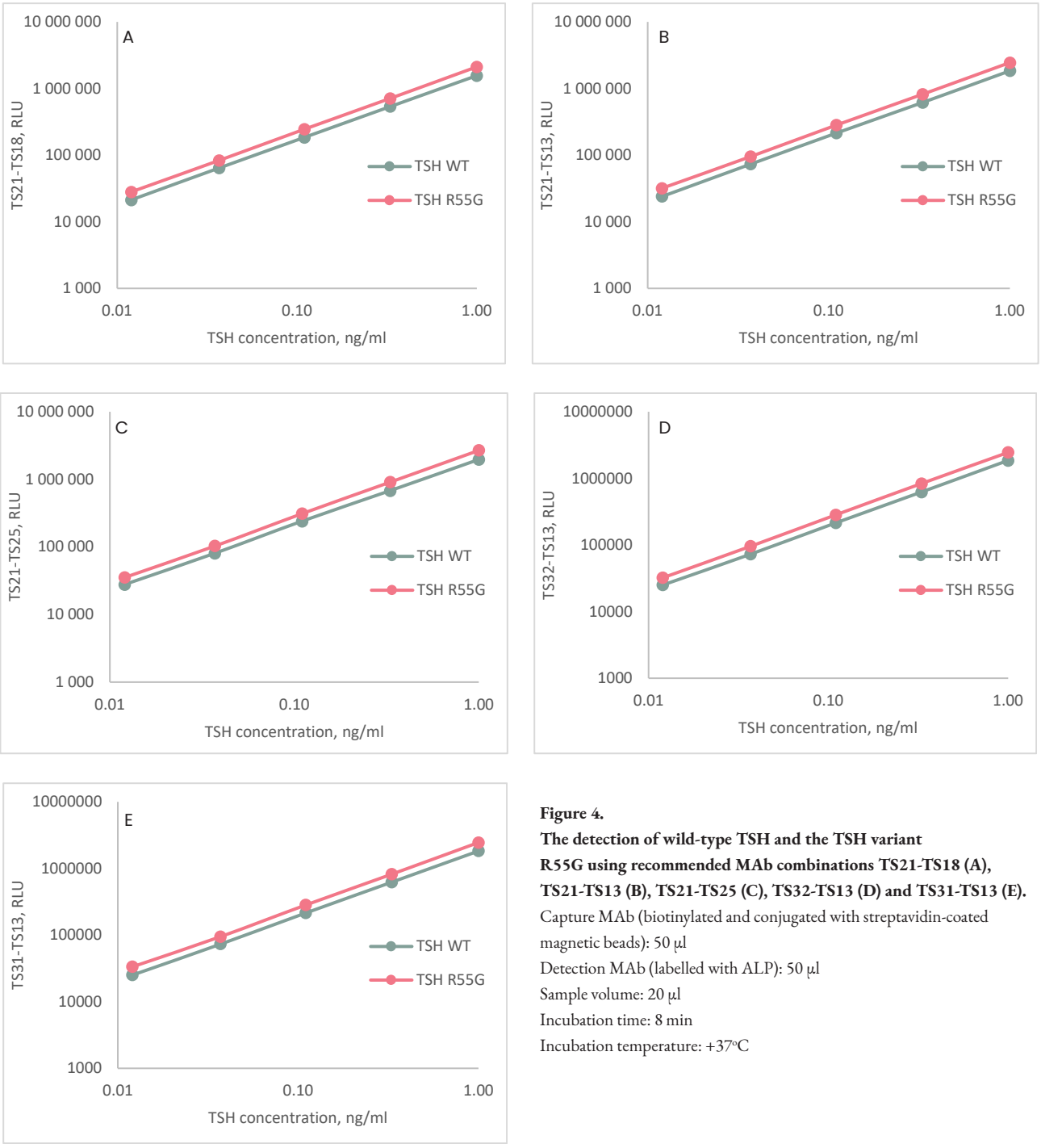

Figure 4.
The detection of wild-type TSH and the TSH variant R55G using recommended MAb combinations TS21-TS18 (A), TS21-TS13 (B), TS21-TS25 (C), TS32-TS13 (D) and TS31-TS13 (E).
Capture MAb (biotinylated and conjugated with streptavidin-coated magnetic beads): 50 µl
Detection MAb (labelled with ALP): 50 µl
Sample volume: 20 µl
Incubation time: 8 min
Incubation temperature: +37°C

Cross-reactivity testing with human hormones LH, FSH, CG

Human LH, FSH, and CG were diluted in an assay buffer to a concentration of 10 IU/ml (LH, FSH) or 1000 IU/ml (CG) and tested in chemiluminescent sandwich immunoassays. Cross-reactivity was < 0.000% for all recommended combinations with LH, FSH, and CG.

Measurement of the affinity of antibodies

Association constant (Ka), dissociation constant (Kd), and equilibrium dissociation constant (KD) were determined using the Octet Bio-Layer Interferometry platform (Table 2).

Table 2.
Affinity constants of antibodies.

Antibody	ka (1/Ms)	kdis (1/s)	KD (M)
TS13	3.73E+05	3.09E-05	8.27E-11
TS18	3.10E+05	2.98E-05	9.58E-11
TS21	2.36E+05	1.08E-05	4.58E-11
TS25	2.89E+05	1.83E-05	6.31E-11
TS31	2.57E+05	6.32E-05	2.46E-10
TS32	2.57E+05	1.05E-05	4.08E-11

Recombinant human TSH

We provide recombinant human TSH (Cat# 8HTS7) that can be used as a calibrator in immunoassays. Recombinant human TSH is produced by the co-expression of the alpha and beta subunits in a mammalian cell line. Synthetic DNA fragments encoding the alpha (92 aar; UniProtKB P01215) and the beta (118 aar; UniProtKB P01222) subunits of TSH were used for expression.

While the cDNA encodes the beta subunit of 118 aar, native TSH isolated from the human pituitary gland contained a beta subunit of 112 aar. It has been demonstrated that 6 C-terminal residues (113–118 aar) of the beta subunit are missing in native TSH. These residues are presumably cleaved post-translationally.

The function of the 6 C-terminal aar of the beta subunit is unknown. It has been demonstrated that they are not necessary for TSH synthesis, including the association with the alpha subunit, glycosylation, and secretion (4). They are also not needed for the biological activity of TSH. Preparations of recombinant TSH containing the beta subunits of 112 aar and 118 aar showed similar biological activity (5).

Neither of the subunits contains additional tags.
The purity of the recombinant TSH product is ≥ 95% and it might contain the free beta subunit. The degree of recombinant TSH purity was analyzed by SDS gel electrophoresis in non-reducing conditions. The upper band on the gel corresponds to the alpha and beta subunit and the lower band corresponds to the beta subunit. Two beta subunit bands are always present, and the ratio between upper and lower beta bands may vary in different batches. The electrophoretic mobility and relative positions of TSH subunit bands in the gel differ for electrophoresis in reducing and non-reducing conditions. In reducing conditions, the upper band corresponds to an alpha subunit while the lower band corresponds to a beta subunit (see Figure 5).

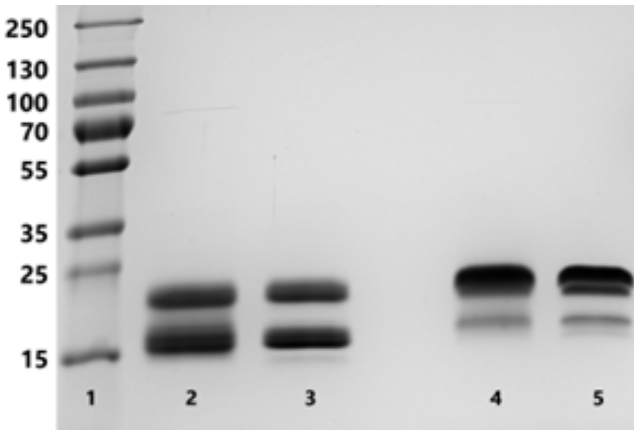

Figure 5.
SDS-PAGE of recombinant TSH in reducing (lanes 2, 3) and non-reducing conditions (lanes 4, 5) in a gradient gel (10-20%). Lane 1 – protein standards, lanes 2 and 4 – 4µg of recombinant TSH (Cat. # 8HTS7), lanes 3 and 5 – 4µg of recombinant TSH Thyrogen (Genzyme)

TSH subunits were identified by Western blotting using a monoclonal antibody specific to the beta subunit (Cat.# 2TS11/2TS11cc, MAb 11E4cc) and a monoclonal antibody specific to glycoprotein hormones common alpha subunit human hCG (Cat.# 2H8, MAb 77F12) (see Figure 6).

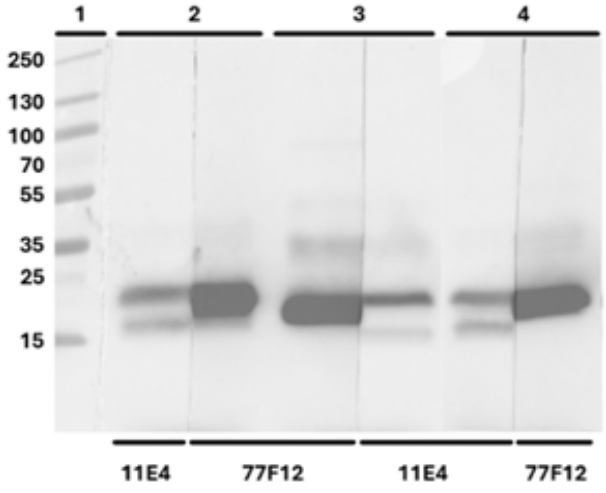

Figure 6.
Western blotting of TSH samples using a monoclonal antibody specific to the beta subunit (Cat.# 2TS11/2TS11cc, the MAb 11E4cc) and a monoclonal antibody specific to glycoprotein hormones common alpha subunit human hCG (Cat.# 2H8, the MAb 77F12). Lane 1 – protein standards, Lane 2 – recombinant TSH (Cat. # 8HTS7), Lane 3 – native TSH (Scripps), Lane 4 – recombinant TSH Thyrogen (Genzyme). SDS-PAGE was performed in a gradient gel (10-20%) in non-reducing conditions.

Freeze-thaw stability of Recombinant human TSH

We tested the immunochemical activity of three individual TSH batches with concentrations of 0.78 mg/ml, 0.5 mg/ml, and 0.58 mg/ml in a storage buffer (PBS, 0.1M D-mannitol, 0.1% CHAPS), both non-lyophilized and reconstituted after lyophilization, following repeated freeze-thaw cycles. The immunochemical activity was measured using the 11E4cc–77F12 prototype assay specific to the TSH dimer. The monoclonal antibody 11E4cc is specific to the TSH beta subunit and a monoclonal antibody 77F12 is specific to the alpha subunit. Immunochemical activity did not change significantly after the samples were subjected to ten freeze-thaw cycles (see Figure 7). Thus, recombinant TSH can withstand up to 10 freezing cycles in the recommended storage buffer.

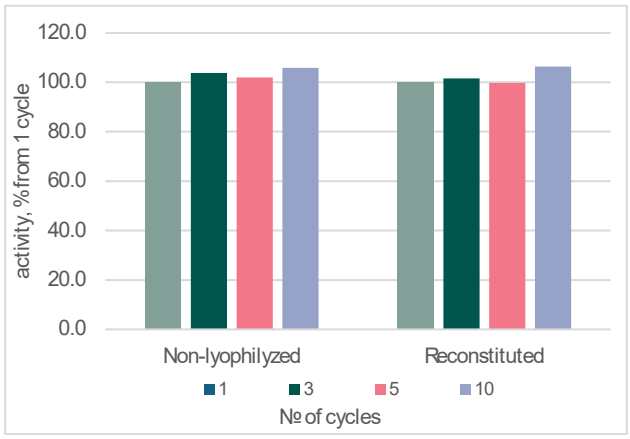

Figure 7.

Freeze-thaw stability of recombinant TSH. Aliquots of recombinant TSH, both liquid and reconstituted, were subjected to ten freeze-thaw cycles. Immunoreactivity after the first, third, fifth, and tenth cycles was measured using a sandwich immunoassay (11E4cc-77F12). The immunochemical activity of the sample after one freeze-thaw cycle was taken as 100%.

Stability of Recombinant human TSH at different temperatures

Non-lyophilized, lyophilized, reconstituted after lyophilization aliquots of three individual TSH batches with concentrations of 0.78 mg/ml, 0.5 mg/ml, and 0.58 mg/ml in a storage buffer (PBS, 0.1M D-mannitol, 0.1% CHAPS) were incubated at different temperatures for two weeks, and compared with the aliquots stored at -70°C using the 11E4cc–77F12 prototype assay. The immunochemical activity of TSH in all samples did not decrease after two weeks of incubation at +4ºC, +25ºC, and +37ºC. Lyophilized TSH was stable after two weeks of incubation at +45ºC. The immunochemical activity in a non-lyophilized TSH solution and TSH reconstituted after lyophilization decreased by 10% after two weeks of incubation at +45ºC. The results are presented in Figure 8.

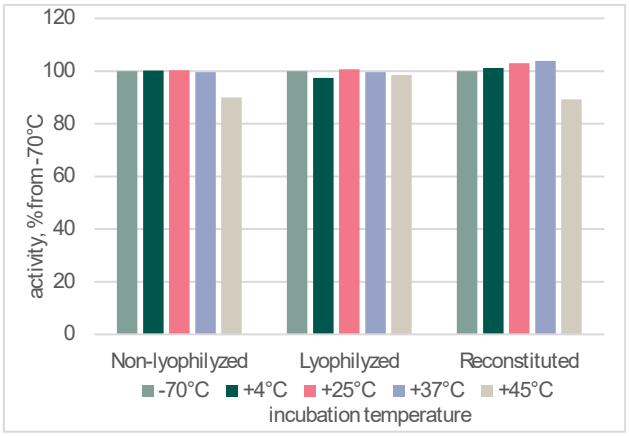

Figure 8.

Stability of recombinant TSH incubated at different temperatures for a period of two weeks. Immunoreactivity was measured using a sandwich immunoassay (11E4cc-77F12). The immunochemical activity of the sample stored at -70ºC was taken as 100%.

In addition, we tested lyophilized recombinant TSH stability at different temperatures at a low concentration (0.1 mg/ml in a storage solution) and in a working solution (100 ng/ml in Assay Buffer - 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.5% BSA, 0.01% Tween 40, 0.05% NaN3).The immunochemical activity of TSH in samples with a concentration of 0.1 mg/ml remained unchanged after two weeks of incubation at +4ºC, +25ºC, and +37ºC (see Figure 9). The immunochemical activity of TSH in samples with a concentration of 100 ng/ml decreased by 5-8% after two weeks of incubation at +4ºC, +25ºC, and +37ºC. When incubated at +45ºC, immunochemical activity decreased by 13% for the sample with a concentration of 0.1 mg/ml, while it decreased by 20% for the sample with a concentration of 100 ng/ml activity (see Figure 9).

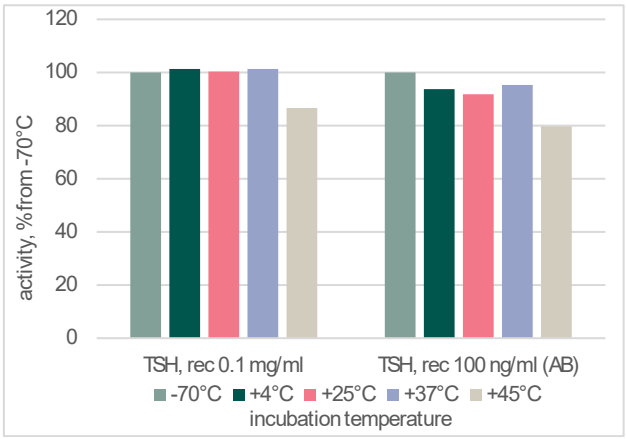

Figure 9.

Freeze-thaw stability of recombinant TSH. Aliquots of recombinant TSH, both liquid and reconstituted, were subjected to ten freeze-thaw cycles. Immunoreactivity after the first, third, fifth, and tenth cycles was measured using a sandwich immunoassay (11E4cc-77F12). The immunochemical activity of the sample after one freeze-thaw cycle was taken as 100%.

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ORDERING INFORMATION

MONOCLONAL ANTIBODIES

Product name	Cat. #	MAb	Subclass	Remarks
Thyroid-Stimulating Hormone (TSH)	2TS11cc	TS13	IgG1	CLIA, beta-subunit, recombinant chimeric antibody, N/cr with human LH, FSH, CG
		TS18	IgG1	CLIA, beta-subunit, recombinant chimeric antibody, N/cr with human LH, FSH, CG
		TS21	IgG1	CLIA, whole molecule, recombinant chimeric antibody, N/cr with human LH, FSH, CG
		TS25	IgG1	CLIA, beta-subunit, recombinant chimeric antibody, N/cr with human LH, FSH, CG
		TS31	IgG1	CLIA, whole molecule, recombinant chimeric antibody, N/cr with human LH, FSH, CG
		TS32	IgG1	CLIA, whole molecule, recombinant chimeric antibody, N/cr with human LH, FSH, CG
		10C7cc	IgG1	*In vitro*, EIA, whole molecule, N/cr with human LH, FSH, CG
		11E4cc	IgG1	*In vitro*, EIA, beta-subunit, N/cr with human LH, FSH, CG
		1CT1cc	IgG1	*In vitro*, EIA, WB in non-reducing conditions, beta-subunit, N/cr with human LH, FSH, CG
		7G12cc	IgG1	*In vitro*, EIA, whole molecule, N/cr with human LH, FSH, CG
	2TS11	7CT8	IgG1	EIA, beta-subunit, N/cr with human LH, FSH, CG

ANTIGENS

Product name	Cat. #	Purity	Source
Thyroid stimulating hormone (TSH), human, recombinant	8HTS7	>95%	Recombinant

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