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Development of high-affinity monoclonal antibodies specific to human thyroid stimulating hormone (TSH) that recognize the TSH variant R55G

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INTRODUCTION

High-sensitivity TSH immunoassays are critical for the accurate determination of TSH concentrations, particularly in patients with hyperthyroidism and those undergoing post-surgical management for differentiated thyroid cancer. Such assays must also detect TSH variants, including the novel R55G β-subunit mutation described by Drees *et al.* This R55G mutation alters an epitope on TSH, preventing its detection by antibodies in four widely used FDA-approved immunoassays. Therefore, for accurate TSH measurements, assays capable of detecting both wild-type (WT) TSH and the R55G variant are needed.

The aim of this study was to develop high-affinity monoclonal antibodies (MAbs) for TSH assays with a functional sensitivity of 0.001 μIU/ml, which are specifically designed to recognize the TSH R55G variant.

METHODS

- WT human TSH and TSH R55G variant were expressed in a mammalian cell line, and purified by affinity chromatography to >95% homogeneity (**Fig. 1**). Neither protein contained any tags.
- Sheep MAbs specific to the human TSH were developed. Chimeric antibodies were constructed by fusing sheep IgG variable domains with human IgG1 constant domains.
- In sandwich chemiluminescent immunoassays, detection antibodies were labeled with alkaline phosphatase, while solid-phase antibodies were immobilized on magnetic beads. AMPPD served as the chemiluminescent substrate.
- The Limit of Detection (LoD) was determined using human native TSH (WHO International Standard NIBSC code 81/565) that was diluted in an assay buffer to concentrations of 0.00059 – 0.0024 μIU/ml. Twenty replicates of each TSH dilution and assay buffer were tested in a single experiment. The sample volume was 50 μl; the incubation time was 16 min. LoD was calculated as follows:

$$LoB = \text{mean blank} + 1.645\ SD_{\text{blank}}$$

$$LoD = LoB + 1.645\ SD_{\text{low concentration sample}}$$

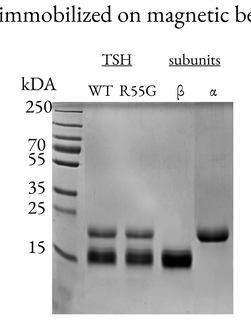
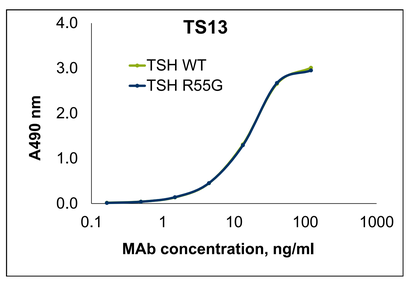
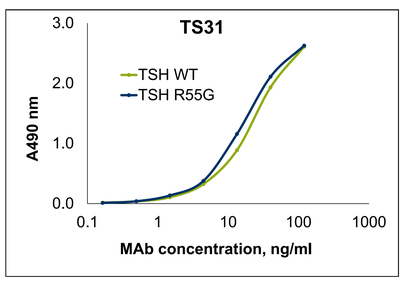


Figure 1. SDS-PAGE analysis of WT TSH and TSH R55G in reducing conditions. Proteins (5 μg per lane) were analyzed on 10-20% polyacrylamide gel.

RESULTS

We developed twenty-nine sheep antibodies that recognized both the WT TSH and the TSH R55G variant. Representative graphs are presented in **Fig. 2**. Two antibody groups were identified based on specificity analysis. One group recognized both the TSH dimer and free β-subunit, which suggests their epitope is on the β-subunit. The second group poorly recognized the free β-subunit compared to the TSH dimer. This suggests that their epitope is conformational, located on the intact TSH dimer.





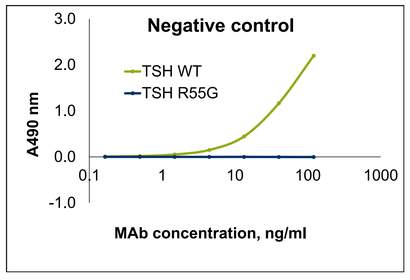
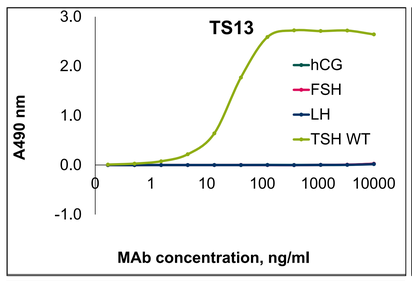
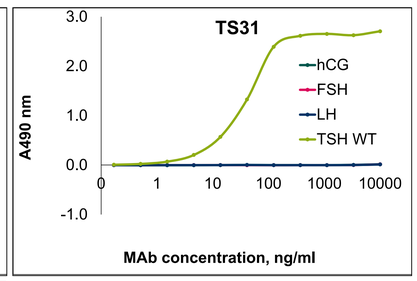


Figure 2. Detection of the TSH WT and the TSH R55G variant in ELISA. An antibody that doesn't recognize the TSH R55G variant was used as a negative control.

All of the antibodies demonstrated high specificity and showed no recognition of recombinant luteinizing hormone (LH), follicle-stimulating hormone (FSH), or chorionic gonadotropin (hCG). Representative results are presented in **Fig. 3**.





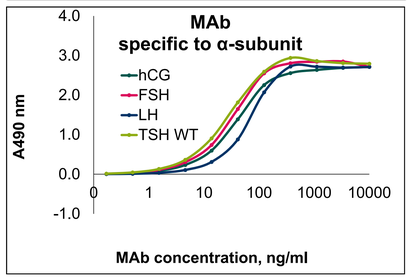


Figure 3. The detection of recombinant WT TSH, LH, FSH, and hCG in ELISA. The MAb specific to common glycoprotein hormones α-subunit was used as a positive control.

Equilibrium dissociation constants (K_D) were determined using the biolayer interferometry-based Octet RED96 instrument. The observed K_D values, ranging from 10^{-10} to 10^{-11} M, indicated a strong affinity for TSH. Results for the 6 best MAbs are provided in **Table 1**.

Table 1. Kinetic and affinity constants of MAbs.

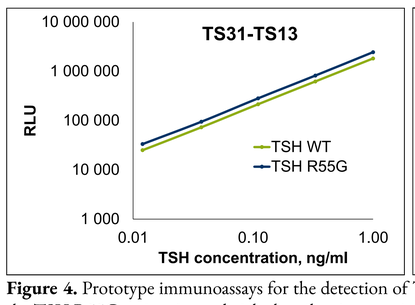
	Association rate constant ka (1/Ms)	Dissociation rate constant kdis (1/s)	Equilibrium dissociation constant K_D (M)	Specificity
TS13	3.73E+05	3.09E-05	8.27E-11	β-subunit
TS18	3.10E+05	2.98E-05	9.58E-11	β-subunit
TS21	2.36E+05	1.08E-05	4.58E-11	TSH dimer
TS25	2.89E+05	1.83E-05	6.31E-11	β-subunit
TS31	2.57E+05	6.32E-05	2.46E-10	TSH dimer
TS32	2.57E+05	1.05E-05	4.08E-11	TSH dimer

Antibodies were tested as capture and detection using a chemiluminescent sandwich immunoassay, and those exhibiting the highest signal-to-noise ratios were selected to develop five prototype immunoassays (**Table 2**). Each of these assays utilized one antibody that was specific to the TSH dimer and another that was specific to the β-subunit.

Table 2. Prototype immunoassays for the detection of TSH.

Capture MAb	Specificity	Detection MAb	Specificity
TS21	TSH dimer	**TS18**	β-subunit
TS21		**TS13**	
TS21		**TS25**	
TS32		**TS13**	
TS31		**TS13**	

All of the prototype immunoassays recognized the TSH R55G variant along with TSH WT. Representative results are presented in **Fig. 4**.



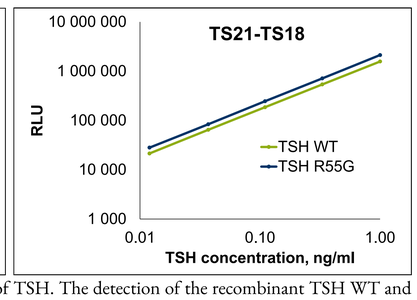
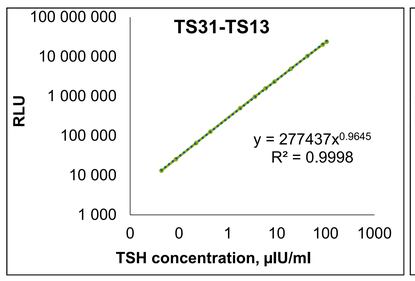


Figure 4. Prototype immunoassays for the detection of TSH. The detection of the recombinant TSH WT and the TSH R55G variant in sandwich chemiluminescent immunoassays.
Sample volume: 20 μl
Incubation time: 8 min
Incubation temperature: +37°C

Typical calibration curves in the range 0.04 – 105 μIU/ml for prototype immunoassays are presented in **Fig. 5**. This range covers the full clinical spectrum of TSH concentrations, from hyperthyroidism to hypothyroidism.



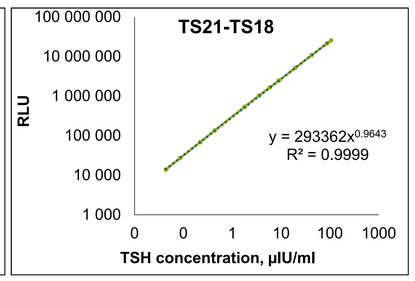
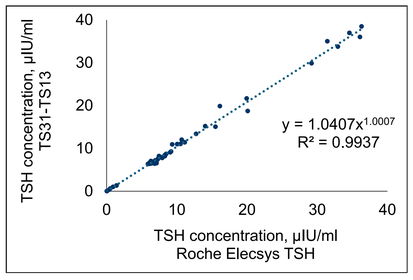


Figure 5. Typical calibration curves for the prototype immunoassays.
Sample volume: 30 μl
Incubation time: 19 min
Incubation temperature: +37°C

TSH concentrations in 50 human serum samples were determined using five prototype immunoassays and the comparison method Roche Elecsys TSH assay. A high correlation (Pearson's correlation coefficient > 0.99) was observed between TSH concentrations determined using prototype immunoassays and the Roche Elecsys TSH assay. Representative results are presented in **Fig. 6**.



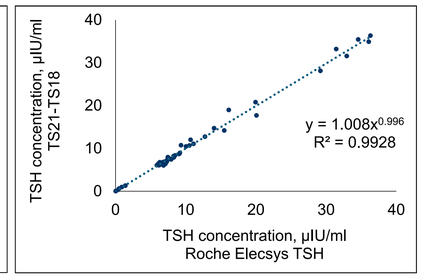


Figure 6. TSH concentrations were determined in human serum samples using prototype assays and the comparison method Roche Elecsys TSH.
Sample volume: 30 μl
Incubation time: 19 min
Incubation temperature: +37°C

For all prototype assays, the LoD for TSH in an assay buffer was 0.00077 μIU/ml. Given that the coefficient of variation (CV) at this concentration was below 2% for all assays, this LoD also represents the assays' functional sensitivity.

CONCLUSIONS

- **We developed novel sheep monoclonal antibodies which enable the quantification of extremely low TSH concentrations in human serum. Prototype immunoassays utilizing these antibodies demonstrated a functional sensitivity of less than 0.001 μIU/ml.**
- **The antibodies recognized the TSH R55G variant as well as the wild type TSH, which will allow accurate TSH detection in serum samples from individuals carrying R55G mutation.**
- **The antibodies exhibited no cross-reactivity with other glycoprotein hormones.**

REFERENCES

Drees J *et al.* Falsely undetectable TSH in a cohort of South Asian euthyroid patients. J Clin Endocrinol Metab. 2014 Apr;99(4):1171-9