ALGERIAN JOURNAL OF ENGINEERING AND TECHNOLOGY

Algerian Journal of Engineering and Technology
ISSN: 2716-9227 (Print)
EISSN: 2716-9278 (Online)
DOI:
Hamdi et al / vol. 10, n°2 - 2025

Original Article Open Access

Radiobinding Assay: A Sensitive Approach for Selecting Anti-TSH Antibodies in Thyroid Disorder Detection

Hamdi Nejla* and Asselah Doria

Department of Nuclear Applications, Nuclear Research Center of Draria' , B.P.43 Sebala-Draria; Algiers; Algeria.

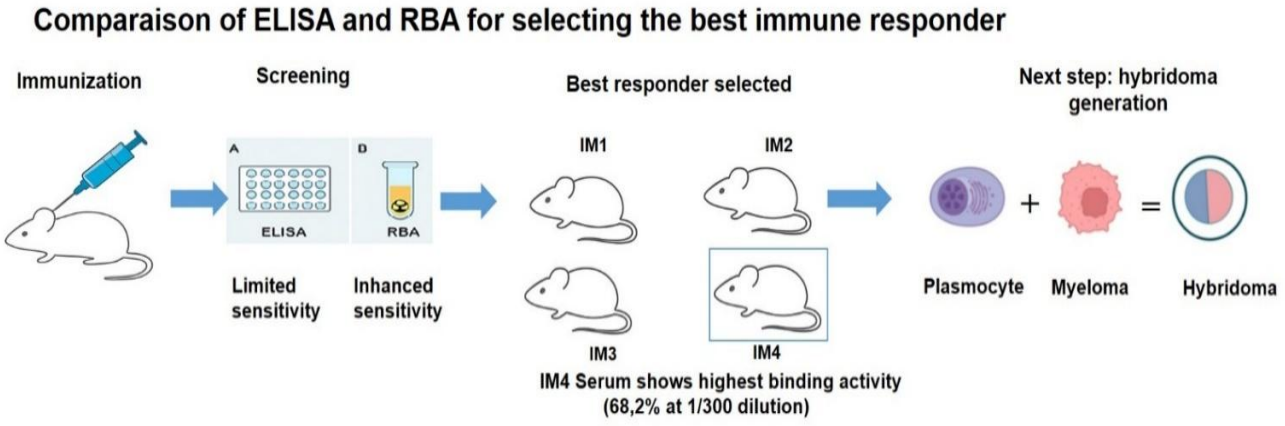
* *Corresponding author.* E-mail address: n-hamdi@crnd.dz
Article history: Received 01 September 2025, Revised 29 September 2025, Accepted 05 October 2025

ABSTRACT

The study aimed to evaluate the Radiobinding Assay (RBA) as a sensitive method for assessing antibody responses following immunization, and to compare its performance with the conventional enzyme-linked immunosorbent assay (ELISA). BALB/c mice were immunized with purified human thyroid-stimulating hormone (hTSH) using Freund's adjuvants, and antisera were collected after successive booster injections. Antibody production was initially screened by indirect ELISA and subsequently re-evaluated by RBA employing ^{125}I-labeled hTSH to measure antigen–antibody binding with higher precision. RBA analysis revealed notable inter-individual variability and demonstrated superior sensitivity in differentiating strong from weak immune responders. Among the immunized mice, serum from IM4 exhibited the highest binding activity, reaching 68.2% at 1:300 dilution. These findings highlight the value of RBA as a robust and highly sensitive approach for identifying optimal antibody producers. This approach provides a strong foundation for subsequent monoclonal antibody generation and the development of advanced immunoassays for the clinical detection of thyroid disorders.

Keywords: Radiobinding Assay; ELISA; Iodine 125; Antibody detection; Immunoassay; Thyroid disorders.

Graphical abstract



Recommended Citation

Hamdi N., Asselah D. Radiobinding Assay: A Sensitive Approach for selecting anti-TSH antibodies in Thyroid Disorder Detection. . *Alger. J. Eng. Technol. 2025*, 10(2): 25-31.

1. Introduction

Monoclonal antibodies (mAbs) are indispensable tools in biomedical research, diagnostics, and therapeutics due to their high specificity and reproducibility compared with polyclonal antibodies. Over the past decades, their development has significantly advanced the design of sensitive and standardized immunoassays for clinically relevant biomarkers [1]. Moreover, Thyroid-Stimulating Hormone (TSH) is a key regulator of thyroid gland function, and its accurate quantification is essential for the diagnosis and management of thyroid disorders [2]. Consequently, the production of antibodies against TSH is a crucial step in the development of reliable diagnostic reagents., particularly for radioimmunometric and enzyme-linked assays.

A pivotal stage in antibody development is the identification of the most effective immune responders following immunization. The enzyme-linked immunosorbent assay (ELISA) remains a widely used and convenient preliminary screening method; however, it may lack the sensitivity required to distinguish between strong and weak responders. The Radiobinding Assay (RBA), which employs ^{125}I-labeled TSH as a tracer, offers superior analytical sensitivity and reproducibility in detecting antigen–antibody interactions [3,4]. To our knowledge, although both ELISA and radiobinding assays (RBA) have been previously employed for antibody screening and comparative analyses, no study has clearly reported the sequential application of these two methods to identify the best immune responder prior to hybridoma generation against human TSH. Therefore, the present study aimed to compare the performance of RBA and indirect ELISA for evaluating antisera from immunized mice, with the purpose of identifying the most suitable immune responders for subsequent monoclonal antibody production and developing a sensitive diagnostic assay for thyroid disorder detection.

2. Materials and Methods

2.1. Material

Purified human thyroid-stimulating hormone (*h*TSH) was obtained from Fitzgerald International (USA) and resuspended in phosphate-buffered saline (PBS) to prepare antigen stock solutions. Freund's Complete Adjuvant (FCA) was used for the primary immunization, and Freund's Incomplete Adjuvant (FIA) was used for booster injections. Sodium radioiodide ($Na^{125}I$) for tracer labeling was purchased from Izotop (Hungary). Four female BALB/c mice (8 weeks old) were obtained from the Pasteur Institute (Algiers, Algeria). The animals were maintained under standard laboratory conditions (22 ± 2 °C, 12 h light/dark cycle) with free access to food and water. Special attention was given to animal welfare: injections were carefully administrated to minimize stress, and all mice were monitored daily for general health and behavior. All other chemical reagents were supplied by Sigma-Fluka (Germany). The equipment used included a multi-well gamma counter (Perkin Elmer, Wizard-2 with data processing and IRMA software), a sealed, ventilated glove box and an ELISA plate reader (Thermo Scientific).

2.2. Preparation of antigens emulsion and administration

To enhance the immune response, 50 µg of purified *h*TSH was diluted in 180 µL of 0.9% NaCl and emulsified with 200 µL of adjuvant. The emulsion was prepared by mixing thoroughly the antigen and adjuvant using two interconnected glass syringes joined by a Luer-lock connector, until a stable water-in-oil emulsion was obtained. For the primary immunization, Freund's Complete Adjuvant (FCA) was used, and the emulsion was administered intraperitoneally. For the booster injections, Freund's Incomplete Adjuvant (FIA) was employed, and the injections were also performed intraperitoneally. For the final immunization, the antigen was administered intravenously via the tail vein without adjuvant.

2.3. Protocol for antibody induction

Four 8-week-old female BALB/c mice, weighing between 21 and 23 g, were immunized with purified human thyroid-stimulating hormone (*h*TSH) as a preliminary step for monoclonal antibody production. The animals were housed in an isolation system with ad libitum access to food and water under standard laboratory conditions (12 h light/dark cycle) with ad libitum access to food and water. The immunization schedule consisted of three injections:

- **Day 0:** intraperitoneal injection of *h*TSH emulsified in Freund's complete adjuvant (FCA),
- **Day 21:** intraperitoneal injection of *h*TSH emulsified in Freund's incomplete adjuvant (FIA),
- **Day 42**: intravenous booster injection via the tail vein using purified *h*TSH diluted in 0.9% NaCl (without adjuvant).

Each injection was administered in a volume of 0.2 mL per mouse. Blood samples were collected 15 days after the second and three days after the final one via a small tail incision. Drops of blood were absorbed onto Whatman filter paper disks, incubated in 300 µL of phosphate-buffered saline (PBS) at 37 °C for 1 h, and stored at 4 °C for 30 min before aliquoting.

These eluates were analyzed by indirect ELISA to assess polyclonal antibody response [5]. A control serum was obtained from a non-immunized mouse and stored frozen until use. All sera were thawed and processed under identical ELISA conditions to ensure comparability.

2.4. Screening Assay

2.4.1. Indirect ELISA for serum antibody detection

Polystyrene 96-well microtiter plates were coated with 100 µL per well of purified human thyroid-stimulating hormone (*h*TSH, 5 µg/mL) prepared in 0.05 M sodium bicarbonate buffer (pH 9.5). The plates were incubated for 1 h at 37 °C, followed by overnight incubation at 4 °C to allow antigen adsorption. To reduce non-specific binding, wells were washed and blocked with 100 µL of 0.2% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. Serial dilutions of mouse sera (1:5, 1:50, 1:100, 1:500, 1:1000, and 1:2000) were prepared in PBS containing 3% BSA. Duplicate aliquots (100 µL per well) of each dilution were added to the coated plates and incubated for 2 h at 37 °C. After washing, 100 µL of horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (1:2000 in sample buffer) were added and incubated for 1 h at 37 °C. Color development was achieved by adding 100 µL of O-phenylenediamine dihydrochloride (OPD) substrate per well and incubating for 30 min at room temperature in the dark. The enzymatic reaction was stopped with 50 µL of 1 M sulfuric acid (H_2 SO_4). Absorbance was measured at 492 nm using a microplate reader.

All measurements were performed in duplicate under identical conditions to ensure reproducibility. The mean absorbance of blank wells (containing buffer and substrate only) was subtracted from all readings to correct for background signal. The absorbance values for each serum dilution were then compared with those obtained for the non-immunized (NI) control. The cut-off value for positivity was defined as the mean absorbance of the negative control plus three standard deviations (mean + 3SD).

2.4.2. RBA for serum antibody detection

Antisera obtained from immunized mice, along with serum from a non-immunized mouse used as a negative control, were diluted in assay buffer consisting of 0.05 M phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide. The following serial dilutions were prepared: 1:300, 1:600, 1:900, 1:1200, 1:2400, 1:4800, 1:9600, and 1:19200. Aliquots of 100 µL from each dilution were dispensed into polystyrene tubes. The radiolabeled tracer (^{125}I-*h*TSH) was prepared in-house using a standard iodination procedure with Na^{125}I [6] Each batch underwent quality control testing to determine labeling efficiency and radiochemical purity (% PRC), ensuring suitability for RBA analysis. To each tube, 100 µL of the ^{125}I-*h*TSH tracer and 100 µL of assay buffer were added. Two additional tubes containing tracer only were included to determine total binding. All tubes were gently mixed and incubated for 2 h in a water bath at 37 °C.

After incubation, 1 mL of 22% polyethylene glycol (PEG) solution and 25 µL of cold γ-globulin (1 mg/mL) were added to each tube—except those containing tracer alone—to precipitate antigen–antibody complexes. Tubes were mixed and centrifuged at 3000 rpm for 25 min at 4 °C. The supernatant was carefully removed, and the radioactivity of each pellet was measured in a gamma counter for 10 s.

Non-Specific Binding (NSB) was determined in parallel tubes containing all reagents except specific anti- *h*TSH antibodies. Specific binding was calculated using the following formula:

$$Binding\ \% = \frac{\bar{X}CPM\ of\ pellet - \bar{X}CPM\ NSB}{\bar{X}\ Total\ CPM} \times 100$$

NSB values were subtracted from all raw counts to correct for background signal, ensuring accurate quantification of specific antibody binding. The percentage of bound tracer was compared to that of the non-immunized control to assess specific immune responses. The cut-off value for positivity was defined as the mean of the negative control plus three standard deviations (mean + 3SD).

Serum dilutions used in ELISA and RBA were not identical because each assay has distinct sensitivity ranges and dynamic detection limits. Dilutions were optimized empirically to achieve measurable signals within the linear range of each method

3. Results and Discussion

The indirect ELISA provided a preliminary quantitative assessment of antibody binding, expressed as optical density (OD) values measured at 492 nm.

Among the tested serum dilutions (1:5 –1:2000), Mouse IM4 consistently showed the highest absorbance values (Fig. 1), indicating a stronger antibody response and a higher relative titer compared with the other immunized mice (IM1, IM2, and IM3). As expected, OD readings progressively decreased with increasing dilution, while the non-immunized control serum exhibited minimal reactivity, confirming the specificity of the immune response.

The radiobinding assay (RBA), employing the ^{125}I-labeled *h*TSH tracer, confirmed the ELISA findings and demonstrated superior sensitivity and discrimination power. Among the tested sera, mouse IM4 again exhibited the highest binding percentages across all dilutions (1:300 to 1:19200) (Fig. 2), reaching 68.2% at 1:300. These results indicate a strong and persistent antibody response, suggestive of high-affinity antibodies. In contrast, the non-immunized control showed negligible binding (< 5%), confirming assay specificity and the success of immunization.

A clear and consistent correlation was observed between the results obtained by ELISA and RBA, despite differences in employing analytical principles and serum dilution ranges. Both techniques effectively monitored the presence and relative levels of polyclonal anti-*h*TSH antibodies in mouse sera, providing a reliable basis for identifying the most responsive animal mAbs production. The strong agreement between the two methods validates the overall reliability of the immunization and screening process and aligns with previous studies demonstrating that both solid-phase and liquid-phase assays can reliably rank antibody responders [7]. These complementary assays thus offer a robust framework for evaluating immune reactivity prior to hybridoma generation.

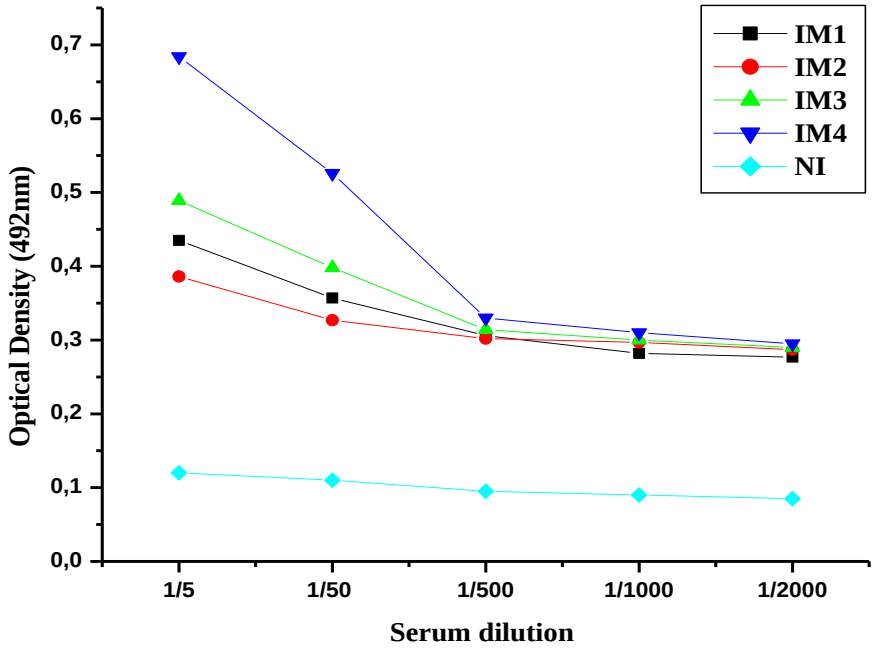


Fig 1. Optical density (OD_{492}) values of sera from immunized and non-immunized BALB/c mice measured by indirect ELISA.

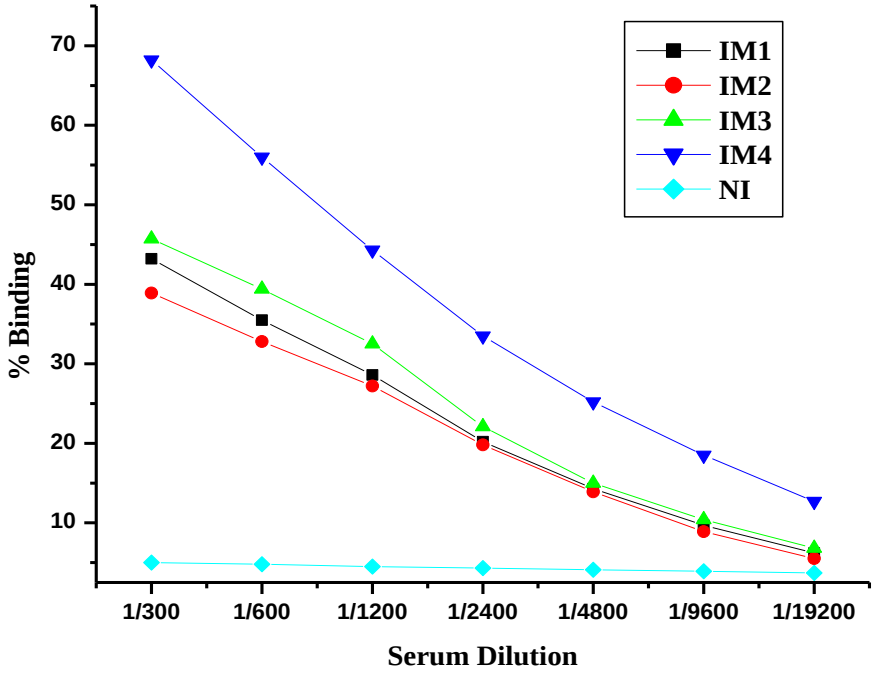


Fig 2. Percentage of antigen-antibody binding measured by RBA using ^{125}I-*h*TSH tracer

Although ELISA and RBA yielded concordant overall trends, the conformation and accessibility of epitopes differ markedly between these two assay formats. In ELISA, *h*TSH is immobilized on a solid surface, which can partially alter its native conformation and predominantly expose linear or surface-accessible epitopes. As a result, antibodies recognizing conformational determinants may exhibit reduced binding under these conditions. In contrast, RBA employs radiolabeled *h*TSH in liquid phase, preserving the native tertiary structure of the antigen and allowing recognition of both linear and conformational epitopes. This fundamental structural distinction accounts for the higher apparent sensitivity frequently observed in RBA and supports its continued use as a reference method in antibody standardization programs [7,8]. Overall, RBA better reflects antigen–antibody interactions occurring under physiological conditions, whereas ELISA primarily detects antibodies directed against immobilized or partially denatured antigenic sites [9].

Consequently, these conformational differences translate into distinct performances. While ELISA remains a practical and cost-effective method for initial screening, RBA offers superior analytical sensitivity and specificity, particularly at high serum dilutions. Its ability to detect measurable binding even at a 1:19200 dilution highlights the presence of high-affinity antibodies, enabling more precise discrimination between strong and weak immune responders. The identification of IM4 as the most responsive mouse underscores the value of combining complementary assay systems to ensure the reliable selection of optimal candidates for monoclonal antibody production — a critical step toward the development of sensitive and specific radioimmunoassays for diagnostic applications [10,11].

Conclusion

The combined use of ELISA and RBA provided a comprehensive evaluation of the humoral immune response to human thyroid-stimulating hormone (*h*TSH) in immunized mice. Despite minor quantitative differences between the two assays—mainly resulting from distinct antigen presentation formats (solid-phase for ELISA versus liquid-phase for RBA) and concentration ranges—the strong concordance in binding profiles underscores their complementarity and reliability in antibody evaluation. RBA, through its superior analytical sensitivity and preservation of the native conformation of *h*TSH, proved particularly effective in detecting high-affinity antibody interactions, even at very low serum concentrations. This dual advantage—precise quantification of low antibody levels and maintenance of antigen structural integrity—confirms RBA as a reference method for high-precision antibody characterization and selection. Identification of Mouse IM4 as the optimal immune responder validates the robustness of the immunization strategy and provides a solid basis for the next experimental phase. The forthcoming fusion of IM4 splenocytes with myeloma cells will enable the generation of hybridomas producing anti-TSH monoclonal antibodies. These monoclonal antibodies will constitute essential reagents for the development of standardized, high-performance immunoassays and radioimmunoassays, ultimately contributing to more sensitive and accurate diagnostic tools for thyroid disorders.

Ethical Statement

All animal experiments were conducted in accordance with internationally accepted principles for the care and a use of laboratory animals. This study does not contain any studies with human subjects performed by any of the authors.

Conflict of Interest

The authors declare that they have no conflict of interest.

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