Monobind Inc.
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AccuBind
ELISA Microwells

Thyrotropin (TSH) Test System
Product Code: 325-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Thyrotropin Concentration in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric

2.0 SUMMARY AND EXPLANATION OF THE TEST

Measurement of the serum concentration of thyrotropin (TSH), a glycoprotein with a molecular weight of 28,000 Daltons and secreted from the anterior pituitary, is generally regarded as the most sensitive indicator available for the diagnosis of primary and secondary (pituitary) hypothyroidism.[1,2] The structure of human TSH is similar to that of the pituitary and placental gonadotropins, consisting of an 89-amino acid α-subunit which is similar or identical between these hormones and a 115-amino acid β-subunit, which apparently confers hormonal specificity. The production of the 2 subunits is separately regulated with apparent excess production of the α-subunit. The TSH molecule has a linear structure consisting of the protein core with carbohydrate side chains; the latter accounts for 16% of the molecular weight.

TSH measurements are equally useful in differentiating secondary and tertiary (hypothalamic) hypothyroidism from the primary thyroid disease. TSH release from the pituitary is regulated by thyrotropin releasing factor (TRH), which is secreted by the hypothalamus, and by direct action of T4 and triiodothyronine (T3), the thyroid hormones, at the pituitary. Increase levels of T3 and T4 reduces the response of the pituitary to the stimulatory effects of TRH. In secondary and tertiary hypothyroidism, concentrations of T4 are usually low and TSH levels are generally low or normal. Either pituitary TSH deficiency (secondary hypothyroidism) or insufficiency of stimulation of the pituitary by TRH (tertiary hypothyroidism) causes this. The TRH stimulation test differentiates these conditions. In secondary hypothyroidism, TSH response to TRH is blunted while a normal or delayed response is obtained in tertiary hypothyroidism.

Further, the advent of immunoenzymometric assays has provided the laboratory with sufficient sensitivity to enable the differentiating of hyperthyroidism from euthyroid population and extending the usefulness of TSH measurements. This method is a second-generation assay, which provides the means for discrimination in the hyperthyroid-euthyroid range. The functional sensitivity (<20% between assay CV) of the one-hour procedure is 0.195 µIU/ml while the two-hour procedure has a functional sensitivity of 0.095µIU/ml.[3]

In this method, TSH calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies are added and the reactants mixed. Reaction between the various TSH antibodies and native TSH forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the antibody bound enzyme-thyrotropin conjugate is separated from the unbound enzyme-thyrotropin conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known thyrotropin levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with thyrotropin concentration.

3.0 PRINCIPLE

Immunoenzymometric assay (TYPE 3):
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, **in excess,** and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-TSH antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(p)} + Ag_{TSH} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(p)} - Ag_{TSH} - {}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)
Ag_{TSH} = Native Antigen (Variable Quantity)
$^{Enz}Ab_{(p)}$ = Enzyme -Polyclonal Antibody (Excess Quantity)
$^{Enz}Ab_{(p)} - Ag_{TSH} - {}^{Btn}Ab_{(m)}$ = Antigen-Antibodies Sandwich Complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:
$^{Enz}Ab_{(p)} - Ag_{TSH} - {}^{Btn}Ab_{(m)} + Streptavidin_{C.W.} \Rightarrow$ immobilized complex
$Streptavidin_{C.W.}$ = Streptavidin immobolized on well
Immobilized complex = sandwich complex bound to the well surface

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

Materials Provided:

A. TSH Calibrators – 1ml/vial - Icons A-G
Seven (7) vials of references for TSH Antigen at levels of 0(**A**), 0.5(**B**), 2.5(**C**), 5.0(**D**), 10(**E**), 20(**F**) and 40(**G**) µIU/ml. Store at 2-8°C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the WHO 2nd IRP 80/558.

B. TSH Enzyme Reagent – 13ml/vial - Icon (E)
One (1) vial containing enzyme labeled affinity purified polyclonal goat antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Streptavidin Coated Plate – 96 wells – Icon ⇓
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate – 20 ml/ml - Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

E. Substrate A – 7ml/vial - Icon S^A
One (1) vial containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

F. Substrate B – 7ml/vial - Icon S^B
One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

G. Stop Solution – 8ml/vial - Icon (STOP)
One (1) vial containing a strong acid (1N HCl). Store at 2-8°C.

H. Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. **Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.**
Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided:
1. Pipette(s) capable of delivering 0.050ml (50µl) and 0.100ml (100µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5% (optional).
3. Microplate washer or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Storage container for storage of wash buffer.
10. Distilled or deionized water.
11. Quality Control Materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface antigen, HIV 1&2 and HCV antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS.

Safe disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum in type, and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or gel barrier. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, (100µl) 0.100 ml of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal, and elevated range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the dose response curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. **Wash Buffer**
Dilute contents of wash concentrate to 1000ml with distilled or de-ionized water in a suitable storage container. Store at 2-30°C for up to 60 days.
2. **Working Substrate Solution –** Stable for one year
Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note1: Do not use the working substrate if it looks blue.
Note 2: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).
*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.050 ml (50µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of the TSH Enzyme Reagent to each well. **It is very important to dispense all reagents close to the bottom of the coated well.**
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 60 minutes at room temperature. ******
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section) decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). **Always add reagents in the same order to minimize reaction time differences between wells.**

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9. Incubate at room temperature for fifteen (15) minutes.
10. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
11. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within thirty (30) minutes of adding the stop solution.**

** For better low-end sensitivity (< 0.5µIU/ml), incubate 120 minutes at room temperature. The 40µIU/ml calibrator should be excluded since absorbance over 3.0 units will be experienced. Follow the remaining steps.

Note: Dilute samples reading over 40 µIU/ml by 1:5 and 1:10 with TSH '0' Calibrator. Multiply the results by the dilution factor to obtain accurate results.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of thyrotropin in unknown specimens.
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1
2. Plot the absorbance for each duplicate serum reference versus the corresponding TSH concentration in µIU/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

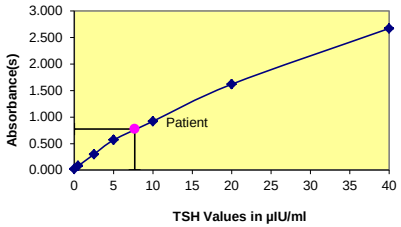
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of TSH for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µIU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.775) intersects the dose response curve at (7.66 µIU/ml) TSH concentration (See Figure 1).

Note: Computer data reduction software designed for ELISA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Number	Abs	Mean Abs	Value (µIU/ml)
Cal A	A1	0.018	0.019	0
	B1	0.021		
Cal B	C1	0.076	0.079	0.5
	D1	0.082		
Cal C	E1	0.302	0.298	2.5
	F1	0.293		
Cal D	G1	0.556	0.567	5.0
	H1	0.577		
Cal E	A2	0.926	0.921	10
	B2	0.916		
Cal F	C2	1.610	1.619	20
	D2	1.629		
Cal G	E2	2.694	2.671	40
	F2	2.647		
Control	G2	0.800	0.775	**7.66**
	H2	0.751		
Patient	A3	1. 391	1.383	**16.65**
	B3	1.375		

Figure 1



*The data presented in Example 1 and Figure 1 are for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay.

11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance of calibrator 'G' (40 µIU/ml) should be ≥ 1.3.
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product is available on request from Monobind Inc.

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind IFU may yield inaccurate results.
10. Patient specimens with TSH concentrations over 40µIU/ml may be diluted (1:5 or 1:10) with the '0' calibrator and re-assayed. The sample's concentration is obtained by multiplying the result by the dilution factor.
11. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
12. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
13. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

1. ***Measurement and interpretation of results must be performed by a skilled individual or trained professional.***
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays (Boscato LM, Stuart MC. 'Heterophilic antibodies: a problem for all immunoassays' Clin. Chem. 1988:3427-33). For diagnostic purposes, the results from this assay should be in combination with clinical examination, patient history and all other clinical findings.For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
5. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
6. Serum TSH concentration is dependent upon a multiplicity of factors: hypothalamus gland function, thyroid gland function, and the responsiveness of pituitary to TRH. **Thus, thyrotropin concentration alone is not sufficient to assess clinical status.**
7. Serum TSH values may be elevated by pharmacological intervention. Domperiodone, amiodazon, iodide, phenobarbital, and phenytoin have been reported to increase TSH levels.
8. A decrease in thyrotropin values has been reported with the administration of propranolol, methimazol, dopamine and d-thyroxine.[4]
9. Genetic variations or degradation of intact TSH into subunits may affect the binding characteristics of the antibodies and influence the final result. Such samples normally exhibit different results among various assay systems due to the reactivity of the antibodies involved.

"NOT INTENDED FOR NEWBORN SCREENING"

13.0 EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values for the TSH AccuBind® ELISA Test System. The number and determined range are given in Table 1. A nonparametric method (95% Percentile Estimate) was used.

TABLE I
Expected Values for the TSH ELISA Test System (in µIU/ml)

Number	139	2.5 Percentile–70% Conf Int	
Low Normal	0.39	Low Range	0.28 – 0.53
High Normal	6.16	High Range	5.60 – 6.82

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal"-persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precisions of the TSH AccuBind® test system were determined by analyses on three different levels of pool control sera. The number (N), mean (X) value, standard deviation (σ) and coefficient of variation (C.V.) for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in µIU/ml)

Sample	N	X	σ	C.V.
Pool 1	24	0.37	0.03	8.1%
Pool 2	24	6.75	0.43	6.4%
Pool 3	24	29.30	1.94	6.6%

TABLE 3
Between Assay Precision* (Values in µIU/ml)

Sample	N	X	σ	C.V.
Pool 1	10	0.43	0.04	9.3%
Pool 2	10	6.80	0.54	7.9%
Pool 3	10	28.40	1.67	5.9%

*As measured in ten experiments in duplicate over seven days.

14.2 Sensitivity

The sensitivity (detection limit) was ascertained by determining the variability of the 0 µIU/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose:

For I hr incubation = 0.078 µIU/ml
For 2 hr incubation = 0.027 µIU/ml

14.3 Accuracy

The TSH AccuBind® ELISA test system was compared with a reference immunochemiluminescence assay. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.01µIU/ml – 61µIU/ml). The total number of such specimens was 241. The least square regression equation and the correlation coefficient were computed for the TSH AccuBind® ELISA method in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
Monobind	4.54	y = 0.47 + 0.968 (x)	0.995
Reference	4.21		

Only slight amounts of bias between the TSH AccuBind® ELISA method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

14.4 Specificity

The cross-reactivity of the TSH AccuBind® ELISA test system to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of thyrotropin needed to produce the same absorbance.

Substance	Cross Reactivity	Concentration
Thyrotropin (hTSH)	1.0000	-
Follitropin (hFSH)	< 0.0001	1000ng/ml
Lutropin Hormone (hLH)	< 0.0001	1000ng/ml
Chorionic Gonadotropin(hCG)	< 0.0001	1000ng/ml

14.5 Correlation between 1 hr and 2 hr incubation

The one- (1) hr and two (2) hr (optional) incubation procedures were compared. Thirty (30) biological specimens (ranging from 0.1 – 18.5 µIU/ml) were used The least square regression equation and the correlation coefficient were computed for the 2 hr procedure (y) in comparison with the 1 hr method (x). Excellent agreement is evidenced by the correlation coefficient, slope and intercept: Y = 0.986 (x) + 0.119 Regression Correlation =0.998

15.0 REFERENCES

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MP325 Product Code: *325-300*

Size		96(A)	192(B)	480(D)	960(E)
Reagent (fill)	A)	1ml set	1ml set	2ml set	2ml set x2
	B)	1 (13ml)	2 (13ml)	1(60ml)	2 (60ml)
	C)	1 plate	2 plates	5 plates	10 plates
	D)	1 (20ml)	1 (20ml)	1 (60ml)	2 (60ml)
	E)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)
	F)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)
	G)	1 (8ml)	2 (8ml)	1 (30ml)	2 (30ml)

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Glossary of Symbols
(EN 980/ISO 15223)