BeneSphera™

T4 Microwell ELISA

Enzyme Immunoassay for quantitative determination of Thyroxine (T4) concentration in human serum and plasma, (for *in vitro* diagnostic use only)

SUMMARY:

Thyroxine hormone is secreted in the thyroid gland which is the most important component of the endocrine system. Over 99% thyroxine secreted in the blood is bound to Thyroxine binding globulin (TBG), albumin and prealbumin. The total Thyroxine(T4) content in blood is important in detecting thyroid disorders. In hyperthyroidism, like in those with Grave's disease, the T4 level is increased in blood, while in hypothyroidism, like in those with myxedema, theT4 level is decreased.

TEST PRINCIPLE:

Competitive EIA (Quantitative)

In a competitive EIA, enzyme linked antigen competes with antigen from the specimen for a limited number of binding sites on the immobilized antibody coated on the micro wells. Unbound antigen fraction is then washed away. The enzyme activity in the antibody bound function which is inversely proportional to the native antigen concentration, is measured by addition of the substrate. By utilizing calibrators of known antigen values, a dose response curve may be generated from which the antigen concentration of an unknown can be obtained.

KIT CONTENTS:

1. **T4 Reaction Coated Microplate (96 wells)-** 1
   One 96-well microplate coated with sheep antithyroxine serum and packaged in an aluminum bag with a dessicant. Store at 2-8°C.
2. **T4-Enzyme Conjugate (1.5ml/vial) -** 2
   One vial of thyroxine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.
3. **T4 Conjugate Buffer (13 ml)- Ready to use.** 3
   One bottle reagent containing buffer, red dye, preservative, and binding protein inhibitors. Store at 2-8°C.
4. **T4 Calibrators (1 ml/vial)-** 4A - 4F
   Six vials of serum reference for thyroxine at concentrations of 0 4A, 2.0 4B, 5.0 4C, 10.0 4D, 15.0 4E and 25.0 4F µg/dl. Store at 2-8°C. A preservative has been added.
5. **Substrate A (7ml/vial)-** 5
   One bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
6. **Substrate B (7ml/vial)-** 6
   One bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.
7. **Wash Solution Concentrate (20ml) -** 7
   One vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.
8. **Stop Solution (8ml/vial) -** 8
   One bottle containing a strong acid (1N HCl). Store at 2-30°C.
9. **Product Insert** - 01 No.

PRECAUTIONS: ⚠

1. All Reagents are for in vitro diagnostic use only.
2. Please handle all reagents and controls provided in the kit as potentially infectious although they are non reactive for HIV 1 and 2, HBsAg and HCV by FDA approved tests.
3. The stop solution provided in the kit is a strong acid (1N HCl). Please wear gloves and face mask to avoid contact with the skin.
4. Please use a disposable container or acid washed tubes for preparing the substrate (Washed with 1N HCl or 1N H_2SO_4 rinsed well with deionised water and dried before use.)

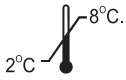
MATERIAL, TOOLS AND EQUIPMENT REQUIRED:

I. Micro plate reader with 450 nm and 620 nm wave length absorbance capability (the 620 nm filter is optional).
2. Micro plate washer or a squeeze bottle (optional).
3. Quality control material.
4. Timer.
5. Micro plate cover for incubation steps.
6. Storage container for storage of wash buffer.
7. Vacuum aspirator (optional) for wash steps.
8. Deionized water.
9. Pipettes capable of delivering 25 µl to 100 µl.
10. Dispenser(s) for repetitive deliveries of 0.1 ml and 0.3 ml. volumes with a precision of better than 1.5% (optional).
11. Containers for mixing reagents.

SAMPLE COLLECTION AND PREPARATION :

Serum or plasma may be used for the test. Serum or plasma should be prepared from a whole blood specimen obtained by approved aseptic technique. If testing cannot be done within an hour after sample collection, refrigerate (upto maximum of 48 hours) the specimen immediately.

PREPARATION OF REAGENTS AND STORAGE:



1. **Working Conjugate Solution**
   Prepare the working conjugate solution by mixing 160 µl of conjugate concentrate with 1.6 ml of the conjugate buffer (1:11 ratio). This working solution is sufficient for two strips. The prepared reagents should be stored at 28°C and must be used within 24 hrs of preparation.
2. **Wash Solution**
   Prepare a 1:50 dilution of Washing Solution by mixing 1 ml of concentrated wash solution with 49 ml of distilled or deionised water. The prepared diluted wash solution can be preserved at 21-25°C for 60 days.
   When stored at 2-8°C the wash solution concentrate may get crystalized, use that only after thawing properly by keeping at 37°C for some time.
3. **Working Substrate Solution**
   Determine the amount of reagent needed and prepare by mixing equal portions of Substrate A and Substrate B in a suitable container. For example, add 1ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made. Discard the unused portion. Do not expose it to direct light).
   Note: Do not use the working substrate if it looks blue.

ASSAY PROTOCOL:

Before starting the assay, allow all the reagents, serum references & controls to reach room temperature.

1. Format the micro plate wells for each calibrator and patient specimen to be assayed. Replace the unused micro well strips back into the aluminum foil, seal and store at 2-8°C.
2. Add 25 µl of the calibrator and the patient specimen to the assigned wells.

3. Add 100 µl of the prepared enzyme conjugate solution to each micro well.
4. Care should be taken to dispense the entire reagent to the bottom of the coated well.
5. Shake the microplate gently for 20-30 seconds & cover.
6. Incubate for 60 min. at controlled room temperature (21-25°C).
7. Aspirate the contents of the wells & fill them completely (approximately 300 µl) with diluted washing solution. Repeat the aspiration and washing procedure two more times. After the last wash, blot the micro plate on absorbent tissue to remove excess liquid from wells.
8. Add 100 µl of Working Substrate Solution to all the wells. Always add reagent in the same order to minimize reaction time difference between wells.
9. Incubate at controlled room temperature (21-25°C) for fifteen minutes.
10. Add 50 µl of Stop Solution to each well and mixwell for 15-20 seconds.
11. Read the absorbance in each well at 450 nm (using a reference wave length of 620-630 nm to minimize well imperfections) in a micro plate reader.

QUALITY PARAMETERS:

Every control with known concentration in hypothyroid, euthyroid and hyperthyroid range must be included in every run. Each laboratory must establish its own acceptable assay performance limits. Run to run reproducibility must be observed in a batch. If there is any deviation from the established data, it could be due to degradation in the kit components or change in the experimental conditions.

The absorbance of the calibrator 4A (0.0 µg/dl) should be > 1.3 for an assay to be valid.

CALCULATION OF RESULTS:

A. Calculate the absorbance value of calibrators & samples at 450 nm. (Use 620 nm filter as reference filter, if available).
B. Plot a point to point curve by plotting the absorbance of each calibrator on y axis against concentration of each calibrator on the x axis.
C. Using the absorbance value for each sample determine the corresponding concentration of thyroxine in µg/dl. from the standard curve obtained.

GUIDELINES:

A. Performance of the assay

1. Same sequence of reagent addition should be maintained throughout the run so that assay drift can be avoided.
2. Do not touch the bottom of the wells.
3. Improper washing may lead to faulty results.
4. Lipemic, hemolyzed and contaminated specimen should not be used.

B. Interpretation of results:

1. The concentration obtained of the calibrators should be within ± 10% of the assigned values in µg/dl.
2. Total serum thyroxine concentration obtained is regulated by many factors like the thyroid gland function and its regulation, thyroxine binding globulin (TBG) concentration and the binding of thyroxine to TBG. Thus total thyroxine concentration alone is not sufficient to assess clinical status.
3. It has been observed that a decrease in total triiodothyronine is found in protein wasting diseases, certain lever diseases and administration of testosterone, diphenylhydantoin or salicylates.
4. It has been observed that an increase in total thyroxine is found in conditions such as pregnancy or administration of oral contraceptives.

RANGE:

A euthyhroid adult population was studied and the generalized range obtained is as given below.

Expected ranges (male) 4.4-10.8 µg/dl

Expected ranges (female) 4.8-11.6 µg/dl

PRECISION & ACCURACY:

BeneSphera™ T4 was studied for intra assay & inter assay reproducibility by analysis on pooled control sera. The number of sample, mean value, standard deviation.

Intra Assay Reproducibility:
(Value in µg/dl)

3 sets pooled samples (24 in each) were studied for reproducibility and gave a coefficient of variation of 6.7% for low range samples and <5% for normal and high sampels.

Inter Assay Reproducibility:
(Values in µg/dl)

3 sets of pooled samples were studied tor inter assay reproducibility and gave a coefficient of variation of 8.3% for low value samples and <5% for normal and high samples.

Accuracy:

BeneSphera™ T4 results were compared with a coated tube immunometric assay. Total 131 samples from hypothyroid, euthyroid and hyperthyroid samples were used (Range 0.8 µg/dl-25 µg/dl)

The correlation coefficient and least square regression equation were completed for **BeneSphera™ T4** in comparison with the reference method.

A very high degree of correlation was found between **BeneSphera™ T4** and the reference method.

SENSITIVITY AND SPECIFICITY:

Sensitivity

80 pg. Equivalent to a sample containing a concentration of 0.4 µg/dl.

Specificity

The extent of cross reactivity of **BeneSphera™ T4** with interfering substances was found to be as follows.

I-Thyroxine - 1.00
d-Thyroxine - 0.98
d-Triiodothyronine- 1.5% increase in O.D.
I - Triiodothyronine - 3% increase in O.D. at 100 µg/dl.
Diiodothyronine 0.01% increase in O.D. at 100 µg/dl.

BIBLIOGRAPHY:

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