



T3-Uptake (T3U) Product Code: 525-300

Intended Use: The Measurement of the Total Amount of Binding Sites Available for the Thyroid Hormones in Human Serum or Plasma by a Microplate Enzyme Immunoassay

SUMMARY AND EXPLANATION OF THE TEST

The thyroid gland under the regulatory control of thyrotropin hormone secretes thyroxine (T4) and triiodothyronine (T3) into the general circulation. The released hormones do not circulate as free molecules but are almost entirely (99.9%) bound to specific serum proteins.

Three protein fractions with varying affinities and capacities for interaction with T3 and T4 have been identified by reverse flow paper electrophoresis (1). Thyroxine binding globulin (TBG) carries 65–75% of the total circulating concentration. Thyroxine binding pre-albumin (TBPA) has an intermediate avidity for thyroxine (carries approx.15–25%) but little if any avidity for triiodothyronine. Albumin with a low affinity but high capacity carries 10% of thyroxine and 30% of the available triiodothyronine (1, 2, 3).

Since the metabolic processes are regulated entirely by the concentration of the free thyroid hormones, which are inversely related to the levels of the binding proteins, an assessment of the binding capacity of human serum was developed in 1957 by Hamolsky (4). In this early method, radioactive T3 was added to a specimen of whole blood. After an incubation period, the mixture was centrifuged and the red cells washed. The radioactivity uptake of the red cells was inversely related to the binding capacity of the serum. Although this method had severe limitations, it proved to be a valuable diagnostic tool.

Further technical improvements in the assay methodology of the T3-uptake test resulted as various separation agents such as coated charcoal (5), ion-exchange resins (6), denatured albumin (7), silicates (8), antibodies and organic polymers were employed in place of the red cells.

This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-T3 conjugate and thyroxine (T4) are added, and then the reactants are mixed. The endogenous binding proteins of the sample react with the thyroxine, **but not with the enzyme conjugate**. This leads to a higher binding of the enzyme conjugate to the antibody combining sites, reactive for triiodothyronine and thyroxine, immobilized on the well as the binding capacity of the specimen increases.

After the completion of the required incubation period, the antibody bound enzyme- triiodothyronine conjugate is separated from the unbound enzyme- triiodothyronine 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.

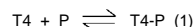
The employment of several serum references of known unsaturated thyroid hormone binding capacity permits construction of a graph of absorbance and concentration. From

comparison to the dose response curve, an unknown specimen's absorbance can be correlated with thyroid hormone binding capacity

PRINCIPLE

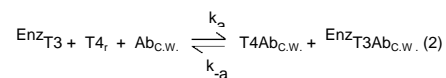
Competitive Enzyme Immunoassay (TYPE 5):

The required components for assessing the binding capacity of human serum are enzyme-T3 conjugate, thyroxine, binding protein (P), and immobilized thyroxine antibody (Ab). Upon mixing the enzyme-conjugate and thyroxine with the specimen, a binding reaction results between the patient's binding proteins and the added thyroxine **but not with the enzyme conjugate**. This interaction is represented below:



T4 = Thyroxine added (constant quantity)
P = Specific binding proteins (varying quantity)

The added thyroxine (T4) not consumed in reaction 1 then competes with the enzyme-T3 conjugate for a limited number of insolubilized binding sites. The interaction is illustrated by the following equation:



Ab_{C.W.} = Immobilized Antibody (Constant Quantity)

T4_r = Added T4 unreacted in reaction (1) (Variable Quantity)

EnzT3 = Enzyme-antigen Conjugate (Constant Quantity)

T4Ab_{C.W.} = Thyroxine-Antibody Complex

EnzT3 Ab_{C.W.} = Enzyme-T3 Conjugate -Antibody Complex

k_a = Rate Constant of Association

k_a = Rate Constant of Disassociation

K = k_a / k_a = Equilibrium Constant

After equilibrium is attained, the antibody-bound fraction is separated from unbound enzyme-antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the binding capacity of the specimen. Thus, in hypothyroidism, the binding proteins are relatively unsaturated (due to the low level of thyroid hormones) resulting in higher consumption of the added thyroxine than a euthyroid specimen. This leads to higher binding of the enzyme-triiodothyronine conjugate caused by the reduced concentration of the available thyroxine. In hyperthyroidism, the reverse is true. The binding proteins are relatively saturated with thyroxine (due to the high level of thyroid hormone) resulting in lower consumption of the added thyroxine. The remaining thyroxine is relatively much higher than a euthyroid specimen resulting in lower enzyme-antigen antibody binding due to the increased competition of the thyroxine for the limited antibody sites.

REAGENTS

Materials Provided.

- Human Serum References -- 1ml/vial - Icons A-D**
Four (4) vials of serum reference of unsaturated thyroid hormone binding capacity at **approximate*** levels of 18 (A), 25 (B), 35 (C), and 47 (D) %U. Store at 2-8°C. A preservative has been added.
* Exact levels are given on the labels on a lot specific basis.
- T3 Uptake Enzyme Reagent -- 1.5ml/vial - Icon E**
One (1) vial of triiodothyronine-horseradish peroxidase (HRP) conjugate and thyroxine in an albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

- T3-Uptake Conjugate Buffer-- 13 ml - Icon B**
One (1) bottle reagent containing buffer, orange dye and preservative. Store at 2-8°C.
- T4 Antibody Coated Plate -- 96 wells - Icon F**
One 96-well microplate coated with sheep anti-thyroxine serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.
- Wash Solution -- 20ml - Icon G**
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.
- Substrate A -- 7ml/vial - Icon S^A**
One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Substrate B -- 7ml/vial - Icon S^B**
One (1) bottle containing hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.
- Stop Solution -- 8ml/vial - Icon H**
One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.
- Product Instructions.**

Required But Not Provided:

- Pipette capable of delivering 25µl volumes with a precision of better than 1.5%.
- Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
- Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate and substrate dilutions.
- Microplate washers or a squeeze bottle (optional).
- Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- Test tube(s) for dilution of enzyme conjugate and substrate A and B.
- Absorbent Paper for blotting the microplate wells.
- Plastic wrap or microplate cover for incubation steps.
- Vacuum aspirator (optional) for wash steps.
- Timer.
- Quality control materials.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.

Note 3: Above reagents are for a single 96-well microplate.

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 Publication No. (CDC) 88-8395.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood; serum or plasma 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 anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin.. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

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 repetitive freezing and thawing. When assayed in duplicate, 0.05ml of the diluted specimen is required.

REAGENT PREPARATION:

- Working Reagent A - T3U-Enzyme Reagent Solution**
Dilute the T3U-enzyme reagent 1:11 with T3 Uptake conjugate buffer in a suitable container. For example, dilute 160µl of conjugate with 1.6ml of buffer for 16 wells (A slight excess of solution is made). This reagent should be used within twenty-four hours for maximum performance of the assay. Store at 2-8°C.
General Formula:
Amount of Buffer required = Number of wells * 0.1
Quantity of T3-Enzyme necessary = # of wells * 0.01
i.e. = 16 x 0.1 = 1.6ml for Total T3/T4 conjugate buffer
16 x 0.01 = 0.16ml (160µl) for T3 enzyme conjugate
- Wash Buffer**
Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature 20-27°C for up to 60 days.
- Working Substrate Solution**
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.

Note: Do not use the working substrate if it looks blue.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).

- Format the microplates' wells for each serum reference, 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.
- Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.
- Add 0.100 ml (100µl) of Working Reagent A, T3U-enzyme solution to all wells.
- Swirl the microplate gently for 20-30 seconds to mix and cover.
- Incubate 60 minutes at room temperature.
- Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
- Add 300µl 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.**
- 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

- Incubate at room temperature for fifteen (15) minutes.
- Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
- 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.**

CALCULATION OF RESULTS

A dose response curve is used to ascertain the unsaturated thyroid binding capacity in unknown specimens.

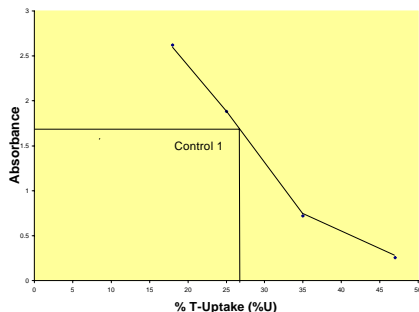
- Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
- Plot the absorbance for each duplicate serum reference versus the corresponding %T3-Uptake (%U) on linear graph paper (do not average the duplicates of the serum references before plotting).
- Connect the points with the best-fit curve.
- To determine the %T3-uptake 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 reference response, and read the %T-uptake (%U) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.690) intersects the reference curve at 26.6%U (See Figure 1).

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value %U)
Cal A	A1	2.644	2.622	18
	B1	2.600		
Cal B	C1	1.888	1.880	25
	D1	1.872		
Cal C	E1	0.710	0.718	35
	F1	0.726		
Cal D	G1	0.265	0.256	47
	H1	0.247		
Ctrl 1	A2	1.701	1.690	26.6
	B2	1.680		
Ctrl 2	C2	0.330	0.314	45.5
	D2	0.298		

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

Figure 1



The T-Uptake can also be expressed as a T-uptake Ratio. Divide the %U by 30% to convert into a T-Uptake ratio. See Example 2:

$$\text{EXAMPLE 2} \\ 27.3\%U / 30\% = 0.910$$

Q.C. PARAMETERS

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

- The absorbance (OD) of calibrator A should be ≥ 1.3 .
- Four out of six quality control pools should be within the established ranges.

LIMITATIONS OF PROCEDURE

- It is important that the time of reaction in each well is held constant for reproducible results.
- Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
- If more than one (1) plate is used, it is recommended to repeat the dose response curve.
- Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time-deviation during reaction.
- Plate readers measure vertically. Do not touch the bottom of the wells.
- Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
- Use components from the same lot. No intermixing of reagents from different batches.

B. Interpretation

- The T3-Uptake Test is dependent upon a multiplicity of factors: thyroid gland and its regulation, thyroxine binding globulin (TBG) concentration, and the binding of the thyroid hormones to TBG. Thus, the T3-Uptake Test alone is not sufficient to assess clinical status.
- The free thyroxine index (FTI), which is the product of the T-Uptake Ratio and the total thyroxine concentration, has gained wide clinical acceptance as a more accurate assessment of thyroid status (9). The FTI value compensates for any condition or drug, such as pregnancy or estrogens, which alters the TBG and the T4 levels but does not change the thyrometabolic status. A table of interfering drugs and conditions, which affect the T3-Uptake Test, has been compiled by the Journal of the American Association of Clinical Chemists (10).

QUALITY CONTROL

Each laboratory should assay controls at levels in the hypothyroid, euthyroid and hyperthyroid 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. 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.

EXPECTED RANGES OF VALUES

A study of an euthyroid adult population (85 specimens) was undertaken to determine expected values for the T-Uptake and are presented in Table 1.

TABLE 1
Expected Values for the T-Uptake EIA Test System

Thyroid Status	% T-Uptake	T- Ratio
Euthyroid	25 - 35	0.83-1.17
[Hypothyroid or TBG excess binding]	< 25	< 0.83
[Hyperthyroid or TBG saturation]	>35	> 1.17

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.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precisions of the T-Uptake AccuBind™ test system were determined by analyses on three different levels of pool control sera. The number (N), mean values (X), 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 %U)

Sample	N	X	σ	C.V.
Low	24	28.7	0.39	1.37%
Normal	24	37.8	0.51	1.36%
High	24	45.4	0.33	0.73%

TABLE 3
Between Assay Precision (Values in %U)

Sample	N	X	σ	C.V.
Low	10	28.4	0.45	1.6%
Normal	10	37.1	0.65	1.8%
High	10	45.7	0.52	1.1%

*As measured in ten experiments in duplicate over a ten day period.

B. Accuracy (Method Comparison)

The T-Uptake AccuBind™ test method was compared with a T3 Uptake radioassay method. Biological specimens from hypothyroid, euthyroid and hyperthyroid and pregnancy populations were used (The values ranged from 14% – 48%U). The total number of such specimens was 120. The least square regression equation and the correlation coefficient were computed for this T-Uptake AccuBind™ test method in comparison with the reference method. The data obtained is displayed in Table 4.

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method	29.3	$y = 1.56 + 0.956(x)$	0.972
Reference	29.0		

Only slight amounts of bias between this 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.

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Size	96(A)	192(B)
Reagent (fill)	A) 1ml set	1ml set
	B) 1 (1.5ml)	2 (1.5ml)
	C) 1 (13ml)	2 (13ml)
	D) 1 plate	2 plates
	E) 1 (20ml)	1 (20ml)
	F) 1 (7ml)	2 (7ml)
	G) 1 (7ml)	2 (7ml)
	H) 1 (8ml)	2 (8ml)

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