Monobind Inc.
Lake Forest, CA 92630, USA

AccuBind
ELISA Microwells

Neonatal Thyroxine (N-T4) Test System

Product Code: 2625-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Total Thyroxine Concentration in Human (Neonates) whole blood by a Microplate Enzyme Immunoassay, Colorimetric

2.0 SUMMARY AND EXPLANATION OF THE TEST

Determination of hypothyroidism within the first few days of birth has been recognized as the single most important diagnostic test in neonates by the American Thyroid Association the need for its early detection and treatment has resulted in the establishment of screening centers by federal and state health departments.

A program of early screening of neonates for congenital hypothyroidism was started in Quebec, Canada in the early seventies. They used dry blood spots on filter paper as the sampling device. Very soon the program was followed by other major public health institutions in Canada and the US. By 1978, almost one million infants had been screened and an incidence rate of congenital hypothyroidism was established to be approximately 1 in 7000 births.

Congenital hypothyroidism is probably the single most common preventable cause of mental retardation. Diagnosis and treatment of congenital hypothyroidism within the first 1-2 months after birth appears to be necessary in order to prevent severe mental retardation.

This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, calibrators, patient specimen, or controls, all made and dried in whole blood are first added to a microplate well. A buffer containing essential ingredients to isolate T4 from blood proteins is added. The blood from the filter paper dots is allowed to elute in the buffer. In the process T4 (Thyroxine) dissociates from the serum (blood) proteins and binds to the antibody that is immobilized on the inside of the microwells. Excess blood is removed using a wash step. Enzyme-T4 conjugate is added. The enzyme labeled T4 then binds to the remaining antibody sites left available by the native T4 that came from the sample. After the completion of the required incubation period, excess enzyme conjugate is removed using a wash step. 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 references, made in whole blood, of known thyroxine concentration permits construction of a dose response curve (DRC-graph) of activity and concentration. An unknown specimen's activity can be extrapolated from the DRC.

3.0 PRINCIPLE

Sequential Competitive ELISA Method, Type 6:
The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing the biotinylated antibody, in extraction buffer, with a dried blood spot (DBS) containing the antigen, a reaction results between the antigen and the antibody. The interaction is illustrated by the following equation:

$$Ag + Ab_{(Btn)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{(Btn)}$$

$Ab_{(Btn)}$ = Specific Biotinylated Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
Ag $Ab_{(Btn)}$ = Antigen-Antibody complex (Variable Quantity)
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation
$K = k_a / k_{-a}$ = Equilibrium Constant

Simultaneously, the immune complex is immobilized through the interaction with streptavidin coated to the well. Unbound reactants are removed at the end of the incubation time by a wash step:

$AgAb_{(Btn)} + Ab_{(Btn)} + S_{CW} \Rightarrow$ Immobilized Complex(IC) + Ab_{CW}

S_{CW} = Streptavidin immobilized on well
Immobilized complex (IC) = $AgAb_{(Btn)}$ bound to the well
Ab_{CW} = Excess antibody bound to the well

Enzyme-conjugated antigen is then introduced. The conjugate reacts with sites of the antibody unoccupied by the native antigen.

$$^{Enz}Ag + Ab_{CW} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}AgAb_{CW}$$

^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$^{Enz}AgAb_{CW}$ = Enzyme-antigen Conjugate -Antibody Complex

After a wash step, substrate is added resulting in reaction with the enzyme bound on the wall of the microwells. The enzymatic reaction is terminated with an acid. The end product is measured at 450 nm. The T4 of the unknown DBS is determined using a calibration curve generated with known concentration of N-T4.

$$^{Enz}AgAb_{(CW)} + \text{Substrate} \rightarrow \text{Color (450nm)}$$

4.0 REAGENTS

Materials Provided:

A. N-T4 Calibrators – Dried Blood Spots (Two rows by six dots levels - 2 x 6)
Six (6) levels of T4 Antigen in dried blood spots at approximate concentrations of 0(**A**), 1.5 (**B**), 3.5(**C**), 7(**D**), 14(**E**) and 25(**F**) µg/dl placed on S&S type 903 filter paper. Store at 2-8°C. A preservative has been added.
Note 1: The exact values are printed on the outside of the aluminum pouch.
Note 2: The ***Lot Specific*** calibrators, whole human blood based, were calibrated using analytically pure T4 (greater than 99% by weight). This material exceeds the specifications set by USP.

B. N-T4 Controls – (I, II & III)
Three (3) controls for thyroxine at varying concentrations (batch specific) made in whole blood spotted on S&S type 903 filter paper supplied in a zip-lock foil bag with a desiccant. Please see the bag label for ranges for different controls. Store at 2-8°C. A preservative has been added.

C. N-T4 Elution Reagent – 12 ml/vial
One (1) vial contains buffer with binding protein inhibitors, surfactants and preservatives. Store at 2-8ºC.

D. N-T4 Conjugate Buffer – 13 ml/ml – Icon Ⓑ
One (1) vial contains buffer, dye, surfactants and preservatives. Store at 2-8°C.

E. N-T4 Enzyme Reagent – 1.5 ml/vial – Icon Ⓔ
One (1) vial contains thyroxine-horseradish peroxidase (HRP) conjugate in a protein-stabilizing matrix. A preservative has been added. Store at 2-8°C.

F. N-T4 Biotin Reagent – 1.2 ml/vial – Icon ∇
One (1) vial contains biotinylated anti-thyroxine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

G. 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.

H. Wash Solution Concentrate – 20ml/vial – Icon
One (1) vial contains surfactant, buffer and saline. Store at 2-8°C.

I. Substrate Solution – 12ml/vial – Icon S^N
One (1) vial contains tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

J. Stop Solution – 8ml/vial – Icon STOP
One (1) vial contains a strong acid (0.5M H_2SO_4). Store at 2-8°C

K. 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 2-8ºC. Kit and component stability are identified on label.**
Note3: Above reagents are for a single 96-well microplate.
Note 4: Do not use reagents that look cloudy or turbid. They may be contaminated.
Note5: Do not exchange reagents between different batches.

4.1 Required But Not Provided:
1. Laboratory Shaker capable of 150rpm rotation.
2. Dispenser(s) for repetitive deliveries of, 0.050ml (50µl), 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for dilutions.
4. 1/8thinch hole punch.
5. Microplate washer or a squeeze bottle (optional).
6. Microplate Reader capable of absorbance readings at 450nm and 620nm.
7. Test tubes for making working enzyme and biotin solutions.
8. Absorbent Paper for blotting the microplate wells.
9. Plastic wrap and microplate cover for incubation steps.
10. Vacuum aspirator (optional) for wash steps.
11. Timer.
12. External 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 blood 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.

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

6.0 SPECIMEN COLLECTION AND PREPARATION

Follow the guidelines in the NCCLS publication LA4T[7] for collecting blood samples in the neonatal screening program, which can be obtained from: NCCLS, 771 E. Lancaster Ave, Villanova, PA 19085. Use WHATMAN type 903 filter paper. For samples screening for CAH, collect samples 3 to 5 days after birth. Use disposable lancets with tips less than 2.5 mm to prick the medial or lateral sides of the bottom of the heel. Allow a drop of blood to form with sufficient volume to fill a 5/8 inch diameter spot on filter paper. Gently touch the drop of blood with the filter paper. DO NOT PRESS AGAINST THE SKIN. DO NOT TOUCH SPOTTED AREA. Suspend spotted papers horizontally and allow drying at room temperature for a minimum of 3 hours. Avoid spots touching other surfaces and keep away from direct light. The samples should be transported to the laboratory within 24 hours after collection in appropriate storage container. The laboratory should store the specimens at 2-8 °C protected from moisture and direct light.

The dried blood spots are stable for at least 3 weeks at 2-8 °C protected from light and moisture. Reject samples with the following conditions:
1. Specimens not collected on WHATMAN type 903 filter paper.
2. Blood spots not completely saturated on both sides.
3. Blood spots with appearance of caking or clotting.
4. Blood spots with appearance of moisture.

7.0 QUALITY CONTROL

Each laboratory should assay external 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. The individual laboratory should set acceptable assay performance limits. 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. Working N-T4-Biotin Reagent Solution
Dilute the N-T4 Biotin Reagent 1:11 with N-T4 Elution Reagent in a suitable, clean container. For example, dilute 160µl of Biotin Reagent with 1.6ml of Elution Reagent for 16 wells (A slight excess of solution is made.) This reagent should be used within two to three (2-3) hours for maximum performance of the assay.
General Formula:
Amount of Elution required = Number of wells * 0.1
Quantity of N-T4 Biotin necessary = # of wells ∗ 0.01
i.e. = 16 x 0.1 = 1.6ml for N-T4 Elution Reagent
16 x 0.01 = 0.16ml (160µl) for N-T4 Biotin Reagent

2. Wash Buffer
Dilute contents of wash concentrate (20ml) to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

3. Working N-T4-Enzyme Reagent Solution
Dilute the N-T4 Enzyme reagent 1:11 with N-T4 Enzyme Conjugate Buffer in a suitable, clean container. For example, dilute 160µl of enzyme reagent with 1.6ml of conjugate buffer for 16 wells. (A slight excess of solution is made.) **This reagent should be used within twenty four (24) hours for maximum performance of the assay.**
General Formula:
Amount of Buffer required = Number of wells * 0.1
Quantity of N-T4 Enzyme necessary = # of wells ∗ 0.01
i.e. = 16 x 0.1 = 1.6ml for N-T4 Conjugate Buffer
16 x 0.01 = 0.16ml (160µl) for N-T4 Enzyme Reagent

Note: Do not use reagents that are contaminated or have bacteria growth

9.0 TEST PROCEDURE

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

1. Assemble the required number of microwells for each calibrator, control and patient sample to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8ºC.**
2. Punch out 1/8" blood dot out of each calibrator, control and specimens into the assigned wells. ***(NOTE:*** **Do not punch blood dots from areas that are printed or that are near the edge of the blood spot).**
3. Add 0.100 ml (100µl) of **Working N-T4 Biotin Reagent** Solution to all the wells.
4. Shake the microplate gently for 20-30 seconds to mix. **(NOTE: Make sure that all blood dots are fully submerged in the liquid and not stuck to the walls of the microwells).**
5. Cover with a microplate cover and rotate for 90 minutes at ambient temperature using a laboratory rotator set @ 150rpm.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper. **NOTE: Make sure all the blood dots are removed at this point. There should be no dots left in the microwells.**
7. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) 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 four (4) additional times.**

8. Add 100 µl of **Working N-T4 Enzyme Reagent** to each well.
9. Cover the microplate and rotate for 45 minutes at ambient temperature using a laboratory rotator set @ 150rpm.
10. Repeat wash step #7.
11. Add 0.100 ml (100µl) of substrate solution to each well.
12. Cover the microplate and incubate for 15 minutes at ambient temperature. **No rotation is required for this step**.
13. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds.
14. **NOTE: Always add reagents in the same order to minimize reaction time differences between wells.**
15. 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 fifteen (15) minutes of adding the stop solution.**

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of T4 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 T4 concentration in µg/dl 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 T4 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 µg/dl) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.719) intersects the dose response curve at (10.8µg/dl) T4 concentration (See Figure 1).

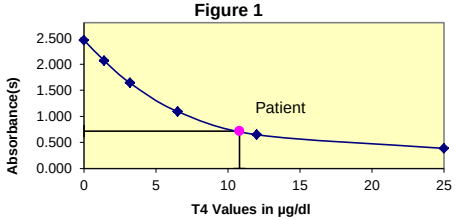
Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

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

EXAMPLE 1

Sample I.D.	Well Number	Abs.	Mean	Value (µg/dl)
Cal A	A1	2.528	2.462	0
	B1	2.398		
Cal B	C1	2.082	2.070	1.4
	D1	2.059		
Cal C	E1	1.667	1.641	3.2
	F1	1.616		
Cal D	G1	1.131	1.094	6.5
	H1	1.058		
Cal E	A2	0.648	0.649	13
	B2	0.651		
Cal F	C2	0.386	0.387	25
	D2	0.388		
Cont - I	E2	1.874	1.855	2.3
	F2	1.836		
Cont – II	G2	1.447	1.436	4.3
	H2	1.425		
Cont - III	A3	0.830	0.785	9.8
	B3	0.740		
Patient	C3	0.698	0.719	10.8
	D3	0.739		

Figure 1



11.0 Q. C. PARAMETERS

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

1. The absorbance (OD) of Calibrator '0' µg/dl should be ≥ 1.3.
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The SDS and Risk Analysis Form for this product are available upon 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, 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. 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.
11. 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.
12. 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. ***Measurements 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. 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.

13.0 EXPECTED RANGES OF VALUES

Based on the limited number of samples at Monobind Inc., and as suggested in the printed literature, the normal range for healthy neonates is assigned at 8 - 23 µg/dl.

It is important to keep in mind that any normal range establishment is dependent upon a multiplicity of factors like the specificity of the method, the locale, the population tested and the precision of the method in the hands of technicians. 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 technicians 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 N-T4 Accubind® ELISA test system were determined by analyses on three different levels of dried blood controls. The number (N), mean values (X), standard deviation (σ) and coefficient of variation (C.V.) for each of these controls are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in µg/dl)

Sample	N	X	σ	C.V.
Low	20	2.76	0.30	10.9%
Normal	20	5.15	0.45	8.8%
High	20	11.30	0.88	7.8%

TABLE 3
Between Assay Precision (Values in µg/dl)

Sample	N	X	σ	C.V.
Low	10	2.86	0.24	8.4%
Normal	10	5.24	0.35	6.7%
High	10	11.10	0.88	7.9%

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

14.2 Sensitivity

The N-T4 Accubind® ELISA test system has a sensitivity of 1.1 µg/dl. The sensitivity was ascertained by determining the variability of the 0 µg/dl calibrator and using the 2 σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The N-T4 Accubind® ELISA test system was compared with an automated fluorescent methodology. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.5 µg/dl – 46 µg/dl). The total number of such specimens was 370. The least square regression equation and the correlation coefficient were computed for this N-T4 Accubind® ELISA method in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean	Least Square Regression Analysis	Correlation Coefficient
Monobind (y)	15.63	y = 0.604+0.941(x)	0.955
Reference (x)	15.96		

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.

14.4 Specificity

The cross reactivity of the thyroxine antibody used for N-T4 AccuBind® ELISA to selected substances was evaluated by adding massive amounts of the interfering substance to a serum matrix. The cross-reactivity was calculated by deriving a ratio between doses of interfering substance to dose of thyroxine needed to displace the same amount of the conjugate.

Substance	Cross Reactivity	Concentration
l–Thyroxine	1.0000	----
d-Thyroxine	0.9800	10µg/dl
d-Triiodothyronine	0.0150	100µg/dl
l–Triiodothyronine	0.0300	100µg/dl
Iodothyrosine	0.0001	100µg/ml
Diiodotyrosine	0.0001	100µg/ml

15.0 REFERENCES

1. Harrison, *Internal Medicine,* Mosby Printing, Philadelphia, 12th Ed.
2. Dussault JH, Coloumbe C, Laberge C, Latarte J, Guyde H and Khoury K, "Preliminary report on Mass Screening Program for neonatal Hypothyroidism", *J. Pediatr,* 86, 670-674 (1975).
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7. *Biosafety in Biomedical and Microbiological laboratories,* 4th Ed, U.S. Department of Health and Human Services, HHS Publication No 93-8395 (CDC), Washington DC, USA (1999).
8. Young DS, Pestaner LC, & Gibberman V, *Clinical Chemistry*, 21, 5 (1975).

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MP2625 **Product Code: 2625-300**

Size / Item	1 Plate 96 Test	2 Plate 192 Test	5 Plate 480 Test	10 Plate 960 Test	20 Plate 1920
A)	1 Set	1 Set	2 Sets	4 Sets	8 Sets
B)	1 Set	1 Set	2 Sets	4 Sets	8 Sets
C)	1 x 13 ml	2 x 13 ml	1x 52 ml	2 x 52 ml	4 x 60 ml
D)	1 x 13 ml	2 x 13 ml	1x 52 ml	2 x 52 ml	4 x 60 ml
E)	1 x 1.5 ml	2 x1.5 ml	1 x 8 ml	2 x 8 ml	4 x 8ml
F)	1 x 1.5 ml	2 x1.5 ml	1 x 8 ml	2 x 8 ml	4 x 8ml
G)	1	2	5	10	20
H)	1 x 20 ml	2 x 20 ml	1 x 60 ml	2 x 60 ml	4 x 60 ml
I)	1 x 12 ml	2 x 12 ml	1 x 52 ml	2 x 52 ml	4 x 52 ml
J)	1 x 8 ml	2 x 8 ml	1 x 30 ml	2 x 30 ml	4 x 30 ml

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Please visit our website to learn more about our products and services.

Glossary of Symbols
(EN 980/ISO 15223)

IVD — In Vitro - Diagnostic Medical Device
2°–8° — Temperature Limitation Storage Condition (2-8° C)
Consult Instructions for Use
REF — Catalogue Number
Σ — Contains Sufficient Test for Σ
LOT — Batch Code
Used By (Expiration Day)
Date of Manufacturer
Manufacturer
EC REP — Authorized Rep in European Country
CE — European Conformity