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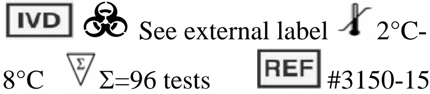


Catolog No. 3150-15

Neo-Natal T4 (Thyroxine)

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

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.

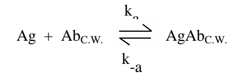
This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, calibrators, patient specimen, or controls, all made and dried in whole blood are first added to a microplate well. A buffer containing essential ingredients to isolate T4 from blood proteins is added. The blood from the filter paper dots is allowed to elute in the buffer overnight. 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. The next day excess blood is removed using a wash step. Enzyme-T4 conjugate is added. The enzyme labeled T4 binds to the sites on the antibody 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.

PRINCIPLE OF THE TEST

The essential reagents required for a solid phase sequential enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized antibody, and a whole blood sample containing the native antigen, a binding reaction results between the native antigen for a limited number of insolubulized binding sites. The interaction is illustrated by the followed equation:



$$Ag + Ab_{C.W.} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{C.W.}$$

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

Ag = Native Antigen (Variable Quantity)

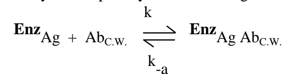
$AgAb_{C.W.}$ = Antigen-Antibody Complex

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Disassociation

$K = k_a / k_{-a}$ = Equilibrium Constant

After removing any unreacted native antigen by a wash step, the enzyme-conjugated antigen is introduced. The conjugate reacts with sites of the antibody unoccupied by the native antigen.



$$^{Enz}Ag + Ab_{C.W.} \underset{k_{-a}}{\overset{k}{\rightleftharpoons}} {}^{Enz}Ag\,Ab_{C.W.}$$

^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)

$^{Enz}Ag\,Ab_{C.W.}$ = Enzyme-antigen Conjugate -Antibody Complex

After a short second incubation, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different calibrators of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained

REAGENTS

Materials Provided:

A. Whole Blood Calibrators – (A-F)
Six (6) calibrators for thyroxine at varying concentrations (batch specific) made in whole blood spotted on S&S filter paper (Cat# 903) supplied in a zip-lock foil bag with a desiccant. Please see the bag label for actual concentrations for different calibrators. Store at 2-8°C. A preservative has been added.

B. Whole Blood Controls – (I, II & III)
Three (3) controls for thyroxine at varying concentrations (batch specific) made in whole blood spotted on S&S filter paper (Cat# 903) 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. Neo-T4 Elution Buffer:
One (1) vial containing 13 ml of buffer with binding protein inhibitors, surfactants and preservatives. Store at 2-8ºC.

D. Neo-T4 Enzyme Conjugate Diluent:
One (1) vial containing 13 ml of buffer, red dye, surfactants and preservatives. Store at 2-8°C.

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

F. T4 Antibody Coated Microplate – 96 wells- Icon
One 96-well microplate coated with purified Mouse anti-Thyroxine IgG and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

G. Wash Solution Concentrate -- 20ml – Icon-
One (1) vial containing surfactant, buffer and saline. Store at 2-30°C.

H. Substrate A –7.0ml/vial – Icon- S^A
Two (2) bottles containing tetramethylbenzidine (TMB) in a buffer. Store at 2-8°C.

I. Substrate B –7.0ml/vial - Icon S^B
One (1) bottle containing hydrogen peroxide (H_2O_2) in a buffer. Store at 2-8°C.

J. Stop Solution –8.0ml/vial – Icon- STOP
One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

K. Product Insert

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.
Note3: Do not use reagents that look cloudy or turbid. They may be contaminated.
Note4: Do not exchange reagents between different batches.

Required But Not Provided:

1. Pipette(s) capable of delivering 50µl & 100 µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate and substrate dilutions.
4. 1/8th inch 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 conjugate and working substrate.
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.

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.

SPECIMEN COLLECTION AND PREPARATION

The sampling from neonates is performed by lancing the heels of the infants and then spotting enough whole blood on S&S filter paper card (Cat# 903) to fill the marked circle. Allow the filter paper to dry at room temperature overnight away from heat and moisture. Enclose the dry blood specimen (DBS) in a moisture barrier plastic bag with desiccant and send to the laboratory.

The specimen should be collected 3-7 days post partum, Physical data including age and weight of the infant, whether a multiple birth, or a premature birth etc should accompany the sample. It is important for the clinician to know these facts in order to properly assess the thyroid status of the infant.

The dried blood samples are stable at 2-8°C for 2-3 weeks if stored in zip-lock, moisture resistant bags with desiccants.

REAGENT PREPARATION:

1. Working T4-Enzyme Conjugate Solution

Dilute the T4-enzyme conjugate 1:11 with Neo T4 Enzyme Conjugate Diluent in a suitable, clean 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 two-three hours for maximum performance of the assay.**

General Formula:

Amount of Buffer required = Number of wells * 0.1
Quantity of T4 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 T4 enzyme conjugate

2. Wash Buffer

Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at 2-30°C.

3. Working Substrate Solution:

Pour the contents of the vial labeled Solution 'A' into the vial labeled Solution 'B'. Mix and store at 2-8°C. Use within 60 days. Or for longer periods of usage 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).
Note: Do not use the working substrate if it looks blue.

TEST PROCEDURE

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

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 2-8°C.**
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 Elution Buffer 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). Seal with a plastic wrap.**
5. Incubate overnight (16-20 hrs) at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
7. 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. *NOTE: Make sure all the blood dots are removed at this point. There should* be no dot left in the microwells**
8. Add 0.1 ml (100µl) of working T4-Enzyme Conjugate solution to all the wells (***See Reagent Preparation Step #1).***
9. Swirl the microplate gently for 20-30 seconds to mix. Cover the plate with plate cover and incubate at room temperature for 60 minutes.
10. Repeat steps 6+7.

11. Add 0.100 ml (100µl) of working substrate solution to all wells (*see Reagent Preparation Step#3*).
12. Incubate at room temperature for fifteen (15) minutes.
13. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds.
    NOTE: Always add reagents in the same order to minimize reaction time differences between wells.
14. 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.**

QUALITY CONTROL

Each laboratory should assay outside controls at different levels to monitor batch to batch consistency. CDC (Centers for Disease Control) has an excellent neonatal controls program for monitoring neonatal thyroid assays.

Quality control trend data charts should be maintained to follow the performance of the supplied reagents.

RESULTS

A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.

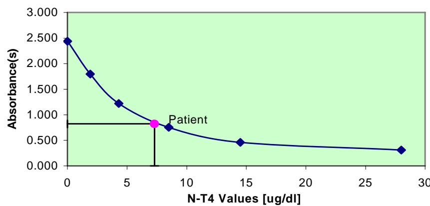
1. Record the absorbance obtained for each replicate from the printout of the microplate reader as outlined in Example 1.
2. Plot the mean absorbance for each duplicate whole blood calibrator versus the corresponding T4 concentration in µg/dl on a linear/ linear graph paper.
3. Connect the points to plot a best-fit to make a dose response curve (DRC).
4. Interpolate the T4 concentration in the unknown specimens from the DRC.

Q. C. PARAMETERS:

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

1. The absorbance (OD) of Calib '0' ng/ml should be $\geq$ 1.3.
2. Four out of six quality control pools should be within the established ranges.

Figure 1



*The data presented in Example 1 are 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 (A)	Mean Abs (B)	Value (µg/dl)
Cal A	A1	2.484	2.436	0
	B1	2.387		
Cal B	C1	1.790	1.798	1.9
	D1	1.806		
Cal C	E1	1.244	1.220	4.3
	F1	1.197		
Cal D	G1	0.781	0.752	8.5
	H1	0.724		
Cal E	A2	0.482	0.461	14.5
	B2	0.442		
Cal F	C2	0.302	0.310	28
	D2	0.318		
Cont - I	E2	1.225	1.262	***3.8***
	F2	1.298		
Cont – II	G2	0.937	0.941	***6.1***
	H2	0.946		
Cont - III	A3	0.541	0.526	***12.1***
	B3	0.512		
Patient	C3	0.765	0.767	***7.3***
	D3	0.769		

LIMITATIONS OF PROCEDURE

A. Assay Performance

1. 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.
2. It is very important that blood dots are completely removed from the wells during the first wash step.
3. 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 in the same sequence to eliminate any time deviation during reaction.
4. Plate readers measure vertically. Do not touch the bottom of the wells.
5. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and inaccurate results.
6. Microbiologically contaminated samples should not be used in the assay.

EXPECTED RANGES OF VALUES

Based on the limited number of samples at Diagnostic Automation 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.

PERFORMANCE CHARACTERISTICS:

Precision:

The within and between assay precision of the NT4 Microplate EIA Test System were determined by analyses on three different levels of dried blood controls. The number, mean values, standard deviation (σ) and coefficient of variation 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	S.D.	C.V.
Low	20	3.31	0.25	7.6%
Normal	20	8.11	0.73	9.1%
High	20	13.12	0.91	6.9%

TABLE 3

Between Assay Precision (Values in µg/dl)

Sample	N	X	S.D.	C.V.
Low	10	2.86	0.16	5.5%
Normal	10	7.72	0.82	10.6%
High	10	14.11	1.30	9.2%

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

B. Accuracy

The NT4 Microplate EIA 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 NT4 EIA in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

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

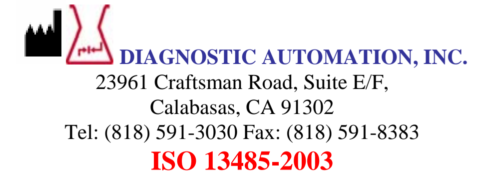
Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

C. Sensitivity

The NT4 EIA procedure has a sensitivity of 0.5 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.

REFERENCES

1. Harrison '*Internal Medicine'* Mosby Printing Philadelphia 12th Ed.
2. Dussault, J.H., Coloumbe, C., Laberge, C., Latarte, J., Guyde, H and Khoury, K., "iPreliminary report on Mass Screening Program for neonatal Hypothyroidism." J. Pediatr., **86**, 670-674. 1975.
3. Dussault, J.H.,& Laberge, C. "*Thyroxine (T4) determination in dried blood by radioimmunoassay: A screening Method for Neonatal Hypothyroidism."* Union Med. Con., **102**, 2062-2064. 1973.
4. Fisher, D.A., *Nenatal Thyroid Screening.* Pediatric Clinics of North America. **25**, 423-430. 1978.
5. Klein, A.., and T.P. Foley. (1974). Lancet 2:77.
6. Biosafety in Biomedical and Microbiological laboratories, 4th. Ed. U.S. Department of Health and Human Services, HHS Publication No. (CDC) 93-8395., Washington D.C. USA. 1999.
7. Young, D.S., Pestaner, L.C., and Gibberman, V. 'Clinical Chemistry' (1975) **21**:5.



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