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 insolubilized binding sites. The interaction is illustrated by the following equation:

\[ \text{Ag} + \text{Ab}_{cw} \underset{k_{d}}{\overset{k_{a}}{\rightleftharpoons}} \text{AgAb}_{cw} \]

\( k_{a} \) = Rate Constant of Association

\( k_{d} \) = Rate Constant of Disassociation

\( k_{a}/k_{d} \) = 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.

\[ \text{EnzAg} + \text{Ab}_{cw} \underset{k_{d}}{\overset{k_{a}}{\rightleftharpoons}} \text{EnzAgAb}_{cw} \]

\( k_{a} \) = Rate Constant of Association

\( k_{d} \) = Rate Constant of Disassociation

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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 insolubilized binding sites. The interaction is illustrated by the following equation:

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\( k_{a} \) = Rate Constant of Association

\( k_{d} \) = Rate Constant of Disassociation

\( k_{a}/k_{d} \) = Equilibrium Constant

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 FOR 192-well MICROLATE 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 Euton Buffer:
One (1) vial containing 50 µL of buffer with binding protein inhibitors, surfactants and preservatives. Store at 2-8°C.
D. Neo-T4 Enzyme Conjugate Diluent:
One (1) vial containing 24 mL of buffer, red dye, surfactants and preservatives. Store at 2-8°C.
E. T-4HPP Enzyme Conjugate – 2.5 mL/vial
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. T-4 Antibody Coated Microplate – 96 wells
Two 96-well microplate coated with purified Mouse anti-Thyroxine IgG and packaged in an aluminum bag with a desiccant. Store at 2-8°C.
G. Wash Solution Concentrate – 20 mL
One (1) vial containing surfactant, buffer and saline. Store at 2-30°C.
H. Substrate A – 7.0 mL/vial - S1
Two (2) bottles containing tetramethylbenzidine (TMB) in a buffer. Store at 2-8°C.
I. Substrate B – 7.0 mL/vial - S2
Two (2) bottles containing hydrogen peroxide (H2O2) in a buffer. Store at 2-8°C.
J. Stop Solution – 8.0 mL/vial
Two (2) bottles containing a strong acid (1N HCl). Store at 2-30°C.
K. Product Insert (1)
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.

Required But Not Provided:
1. Pipette(s) capable of delivering 50 µL & 100 µL volumes with a precision of better than 1.5%.
2. Dispenser(s) for repeat deliveries of 0.100 mL and 0.300 mL volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200 µL) and (200-1000 µL) dispenser(s) for conjugate and substrate dilutions.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Reader capable of absorbance readings at 450nm and 620nm.
6. Microplate Reader capable of absorbance readings at 450nm and 620nm.
7. Test tubes for making 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.

PRECAUTIONS
Not for Internal or External Use in Humans or Animals
1. 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) 88-3835.
2. Do not exchange reagents between different batches.
3. Do not use reagents beyond the kit expiration date.
4. Do not use reagents that look cloudy or turbid. They may be contaminated.
5. Do not use the T4 enzyme conjugate solution after 2-3 hours of preparation. Discard the unused portion.

SPECIMEN COLLECTION AND PREPARATION
The specimen from neonates is prepared by lancing the heel of the infant and then spotting enough whole blood on S&S filter paper (Cat# 903) to fill the marked circle. Allow the filter paper to dry at room temperature overnight before being folded to the laboratory 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 160 µL of conjugate with 1.6 mL 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. Store at 2-8°C.

General Formula
Amount of Buffer required = Number of wells * 0.1

2. Wash Buffer
Dilute contents of Wash Concentrate to 1000 mL 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 1 mL of A and 1 mL 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 – 25°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 specimen 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 (100 µL) 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 (22-25°C).
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
7. Add 300 µL 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 0.1 mL (100 µL) of working substrate solution to all the wells. See the Reagent Preparation Section #3.
9. Incubate at room temperature for fifteen (15) minutes.
10. Wash three times just as in Step #7.
11. Add 0.100 mL (100 µL) of working substrate solution to all the wells (see Reagent Preparation Section #3).
12. Incubate at room temperature for fifteen (15) minutes.
13. Add 0.050 mL (50 µL) of stop solution to each well and gently mix for 15-20 seconds.
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. Extrapolate the T4 concentration in the unknown specimens from the DRC.

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 removing 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 MP Biomicals, Inc., and as suggested in the printed literature the normal range for healthy neonates is assigned at 6 – 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 (s) and coefficient of variation for each of these controls are presented in Table 2 and Table 3.

TABLE 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Well</th>
<th>Mean</th>
<th>S.D.</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A</td>
<td>2.484</td>
<td>2.436</td>
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<tr>
<td></td>
<td>B</td>
<td>2.387</td>
<td></td>
<td></td>
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<tr>
<td>Cal B</td>
<td>C</td>
<td>1.790</td>
<td>1.798</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>1.806</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal C</td>
<td>E</td>
<td>1.244</td>
<td>1.220</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal D</td>
<td>G</td>
<td>0.781</td>
<td>0.752</td>
<td>8.5</td>
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<tr>
<td></td>
<td>H</td>
<td>0.724</td>
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<td></td>
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<tr>
<td>Cal E</td>
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<td>0.461</td>
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<td></td>
<td>B</td>
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<td></td>
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<tr>
<td>Cal F</td>
<td>C</td>
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<td>0.310</td>
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<tr>
<td></td>
<td>D</td>
<td>0.318</td>
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<td></td>
</tr>
<tr>
<td>Cont – I</td>
<td>E</td>
<td>1.225</td>
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<tr>
<td></td>
<td>F</td>
<td>1.298</td>
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<td></td>
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<tr>
<td>Cont – II</td>
<td>G</td>
<td>0.937</td>
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<td>H</td>
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<td></td>
</tr>
<tr>
<td>Cont – III</td>
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<tr>
<td>Patient</td>
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<td>0.767</td>
<td>7.3</td>
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<tr>
<td></td>
<td>D</td>
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</tbody>
</table>

*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.

TABLE 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>X</th>
<th>S.D.</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>2.86</td>
<td>0.16</td>
<td>5.5</td>
</tr>
<tr>
<td>Normal</td>
<td>7.72</td>
<td>0.82</td>
<td>10.6</td>
</tr>
<tr>
<td>High</td>
<td>14.11</td>
<td>1.30</td>
<td>9.2</td>
</tr>
</tbody>
</table>

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

REFERENCES


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