The babies with neural tube defects. This option had been available, especially in developing countries, with conventional testing like ultrasound alone.

In this method, the combination calibrator (containing different levels of AFP, hCG and E3), patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies directed against distinct and different epitopes of AFP and hCG are added and the reactants mixed. Reaction between the various antibody specific antibodies and native analyte forms a sandwich complex that binds with the streptavidin coated to the well. In the case of E3 an analogous complex is formed with HRP (Followed by a biotinylated E3 antibody. A competition occurs between labeled E3 and the native E3 for a limited number of sites on the antibody.

After the completion of the required incubation period, the excess enzyme labeled antibody or antigen is washed off via a wash step. Allowing the enzyme labeled antibody or antigen to compete with enzyme labeled antibody or antigen in the wells for limited, specific binding sites. The intensity of the light is directly proportional to the concentration while in E3 it is inversely proportional to the concentration of the analyte.

The employment of several serum references of known levels of hCG, AFP and E3 permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen’s concentration can be interpolated.

### 3.0 PRINCIPLE

#### Immunoenzymometric assay (TYPE 3 for hCG - AFP):

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and antigens) and a serum containing the native antigen, enzyme labeled antibody, and competition. In excess, and native antigen, biotinylated (hCG/AFP) antibody.

After adding biotinylated antibody, and serum to the coated well, the enzyme labeled antibody and serum containing the native antigen, reaction results between the native antigen and competition. The competition, for the enzyme-labeled antibody, is as follows: to form a sandwich complex.

The interaction is illustrated by the following equation for AFP and hCG.

\[
\frac{dAb + Ag}{kB} \underset{AgAbBtn(\text{m})}{\rightarrow} \frac{AgAbBtn + Ab}{kB} \rightarrow \frac{AgAbBtn + Ab}{kB} \text{immobilized complex}
\]

### 4.0 REAGENTS

**Materials Provided: Reagents for 2x96 well Microplate**

A. Combi-Cal A: hCG/uE3 Concentration - 1mlvial (lyophilized) - lyophilized

B. Alpha-Fetoprotein

C. Beta-Human Chorionic Gonadotropin

D. Unconjugated Estriol (uE3) Concentration in Human Serum by a Microplate Enzyme Immunoassay, Chemiluminescence

**Triple Screen Panel Test System**

**Product Code:** 8575-300

#### 1.0 INTRODUCTION

**Intended Use:** The Quantitative Determination of Alpha-Fetoprotein (AFP), Beta-Human Chorionic Gonadotropin (hCG) and Unconjugated Estriol (uE3) Concentration in Human Serum by a Microplate Enzyme Immunoassay, Chemiluminescence

#### 2.0 SUMMARY AND EXPLANATION OF THE TEST

Monitoring of hCG, AFP and uE3 concentrations, at regular intervals, is considered to be very important in determining the fetal well-being. The collective information provided through these three assays (Triple Screen) provides the clinician with the comprehensive picture of the development of a healthy fetus and the health of the mother. These plates are usually run three times during the first trimester can be corrected unless it is caused by some genetic abnormality. Mononid provides the clinician with a single tool to monitor all three components, using 125 µl of patient’s sample (50µl for AFP, 50µl for uE3 and 25µl for hCG), in a single 75 minutes combination assay.

Alpha-Fetoprotein (AFP) is a glycoprotein with a molecular weight of 70 kDA. AFP is normally produced during fetal development by the hepatocytes, yolk sac and to a lesser extent by the gastrointestinal tract. Serum concentrations reach the highest level at twelve weeks of gestation. The peak level gradually decreases to less than 25 nM after one year of postpartum. Thereafter, the levels reduce further to less than 10 nM/g. The presence of abnormal levels in pregnant concentrations can become a risk marker for open neural tube defects (ONTD).

Elevated levels of AFP are found in patients with primary heptoma and yolk sac-derivated germ tumors. AFP is the most useful marker for the diagnosis and management of hepatocellular carcinoma.

### 5.0 SPECIMENS

#### 5.1 Preferred Specimens

A. Serum (50 µl) for AFP and hCG

B. Urine (50 µl) for uE3

#### 5.2 Acceptable Specimens

A. Serum (50 µl) for AFP and hCG

B. Urine (50 µl) for uE3

#### 5.3 Specimen Collection and Preparation

A. Serum specimens must be obtained fasting (at least 8 hours from last food or drink intake).

B. Whole blood or blood products should be separated (within 2 hours of collection) and kept refrigerated until the time of analysis.

C. Urine samples should be collected within 24 hours of specimen collection.

D. Urine samples must be refrigerated (4°C - 8°C) until analyzed.

### 5.4 LIMITATIONS

A. The test is not intended for the detection of non-malignant conditions such as previous pregnancy.

B. The test is not intended for the detection of non-cancerous conditions such as previous pregnancy.

C. Although the test is intended for the detection of cancerous conditions such as previous pregnancy, it may not be specific for all types of cancer.

### 5.5 INTERFERING FACTORS

#### 5.5.1 Natural Interfering Factors

A. Anti-AFP antibodies

B. Anti-hCG antibodies

C. Anti-uE3 antibodies

### 5.6 STABILITY

A. Serum specimens should be refrigerated (4°C - 8°C) until analyzed.

B. Urine samples should be refrigerated (4°C - 8°C) until analyzed.

### 5.7 SAFE DISPOSAL

A. All test materials and reagents should be disposed of in an appropriate manner.

### 6.0 SPECIMEN COLLECTION AND PREPARATION

**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 the Mumps, HIV, HBV & HCV Antibodies by FDA licensed reagents. Since no known test can be completely conclusive, infections that 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) 89-8350.

### 6.1 SPECIMEN COLLECTION

**A. Serum:**

- **Whole Blood:** Whole blood specimens should be collected in a red-top tube with EDTA or sodium or sodium citrate as an anticoagulant.
- **Serum:** Serum should be separated within 2 hours of collection and kept refrigerated until analyzed.

**B. Urine:** Urine specimens should be collected within 24 hours of specimen collection and kept refrigerated until analyzed.

### 6.2 SPECIMEN PREPARATION

**A. Serum:** Serum specimens should be separated within 2 hours of collection and kept refrigerated until analyzed.

**B. Urine:** Urine specimens should be collected within 24 hours of specimen collection and kept refrigerated until analyzed.

### 6.3 SPECIMEN TRANSPORT

**A. Serum:** Serum specimens should be transported at 4°C - 8°C until processed.

**B. Urine:** Urine specimens should be transported at 4°C - 8°C until processed.

### 6.4 SPECIMEN STORAGE

**A. Serum:** Serum specimens should be stored at 4°C - 8°C until processed.

**B. Urine:** Urine specimens should be stored at 4°C - 8°C until processed.

### 6.5 STABILITY

**A. Serum:** Serum specimens should be stored at 4°C - 8°C until processed.

**B. Urine:** Urine specimens should be stored at 4°C - 8°C until processed.
In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assessed within this time, the sample(s) may be stored at -20°C or cooler for up to 30 days, in smaller aliquots. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required for each of the three (3) parameters.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated 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 lot reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION:

1. Wash Buffer
   Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. The diluted wash buffer can be stored at 2-30°C for up to 60 days.

2. Patient Sample Preparation: For hCG patient samples* (first trimester), dilutions should be made as follows:
   Place 0.5 ml of Sample Diluent into a test tube and add 0.025ml (25µl) of patient sample. Vortex to mix (Dilution 1:2). Remove 0.025ml (25µl) of (1:2) dilution and dispense into another test tube containing 1.0ml (1000µl) of Sample Diluent (1/1) Final Dilution 1:861. Assay the 1:861 dilutions and multiply the results by the dilution factor 861.
   *if hCG from normal populations is to be run, no dilutions are required unless the patient’s hCG is suspected to be greater than 2500mIU/ml.

3. Working Signal Reagent Solution - Store at 2-8°C.
   Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 0.025ml of A and 1ml of B per two (2) eight well strips (a slight excess solution is made). Discard the unused portion if not used within 36 hours after mixing. Complete utilization of the reagents is anticipated, within the above time constraint, for the contents of Signal Reagent B into Signal Reagent A and label accordingly.

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

9.0 TEST PROCEDURE

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

*This procedure should be performed by a skilled individual or trained professional*

1. Format the microplates’ wells for each serum reference calibrator, control and patient specimen (as is and dilutions) to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.025ml (25µl) of the appropriate serum reference calibrator, control and specimens (diluted for hCG) into the assigned wells.

(For AFP and hCG):

3a. Add 0.100ml (100µl) of the AFP Tracer Reagent or hCG Tracer Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.

3b. Add 0.050ml (50µl) of the U-Estrilh Tracer Reagent to all wells. Swirl the plate gently for 20-30 seconds to mix the contents.

3c. Add 0.050ml (50µl) of the U-Estrilh Biotin reagent to all the wells.

4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Incubate 45 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

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. Discard the wash and repeat four (4) additional times.

8. Add 0.100ml (100µl) of working signal 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 PLATE AFTER SIGNAL ADDITION

9. Incubate at room temperature for five (5) minutes.

10. Read the RLUs (Relative Light Units) in each well in a microplate luminometer for at least 0.2 second/ well. The results can be read within 30 minutes of adding the signal solution.

10.0 CALCULATION OF RESULTS

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

1. Record the RLUs obtained from the printout of the luminometer as outlined in Example 1.

2. Plot the RLUs for each duplicate serum reference calibrator versus the corresponding calibrator concentration in corresponding units 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 analyte for an unknown, locate the average RLUs of the duplicates for each unknown on the axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the mean of duplicate RLUs of an unknown of 10,000RLUs/32777 intersects the AFP DRC at a concentration of 105.9 ng/ml (Figure 1 & Example 1).

Note: During regular monitoring of pregnancy, hCG levels rise exponentially and thus exceed the upper limits of the Dose Response Curve (DRC). It is essential to dilute these samples to obtain valid results. (Please see ‘Patient Sample Preparation’ under section ‘Reagent Preparation’). Also see the bottom of data table Example 2 for calculations of patient sample concentrations.

Note: Computer data reduction software designed for chemiluminescence 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 1-3 and Figure 1-3 is for illustration only and should not be used in lieu of a dose response curve prepared with each assay. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the calibrator with the greatest light output. This conversion minimizes differences cause by efficiency of the various instruments that can be used to measure light.

11.0 QC PARAMETERS

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

1. The dose response curve should be within established parameters.

2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSD5 and Risk Analysis Form for this product are available on 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 lipoemic, 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 signal reagent initiates a kinetic reaction; therefore, the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.

6. Failure to remove additional solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

7. Use components from the same lot. No intermixing of reagents from different batches.

8. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind’s IFU may yield inaccurate results.

9. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, may be strictly followed to ensure compliance and proper device usage.

10. 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.

11. Risk Analysis - as required by CE Marking Directive 93/42/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. The reagents for the test system procedure have been formulated to eliminate maximal interference; however, potential interaction between rare specimen reagents may cause erroneous results. Heterophile antibodies often cause such interactions and have been known to be problems for all kinds of immunoassays. (Boscato LM Stuart MC. 'Heterophile antibodies: a problem for all immunoassays' Clin Chem 1988;34-77).

14.1.2 Precision (hCG)

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>20</td>
<td>19.1</td>
<td>0.7</td>
<td>3.6%</td>
</tr>
<tr>
<td>Level 2</td>
<td>20</td>
<td>18.2</td>
<td>0.6</td>
<td>3.5%</td>
</tr>
<tr>
<td>Level 3</td>
<td>20</td>
<td>17.3</td>
<td>0.5</td>
<td>2.9%</td>
</tr>
</tbody>
</table>

TABLE 5. Within Assay Precision (hCG)

It is important to keep in mind that establishment of a range of values, which can be expected to be found by the 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 multiplicity 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.

14.1.4 Linearity & Hook Effect:

Massive amounts of related analytes were diluted in pooled human serum and tested, in linear dilutions to check the hook effect of the antibody system used in the Triple Screen Panel VAST® AccuLite® CLIA test system. The results are tabulated below in Table 14.

15.0 REFERENCES


Revised: 3 Date: 2019-Jul-16

MP8575 Product Code: 8575-300

<table>
<thead>
<tr>
<th>Size</th>
<th>Amount</th>
<th>(µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>3 (mg)</td>
<td>1 (mg)</td>
</tr>
<tr>
<td>B1</td>
<td>13(12)</td>
<td>13(13)</td>
</tr>
<tr>
<td>C1</td>
<td>13(12)</td>
<td>13(13)</td>
</tr>
<tr>
<td>D1</td>
<td>1(6)</td>
<td>1(6)</td>
</tr>
<tr>
<td>E1</td>
<td>1(6)</td>
<td>1(6)</td>
</tr>
<tr>
<td>F1</td>
<td>2(1)</td>
<td>2(1)</td>
</tr>
<tr>
<td>G1</td>
<td>2(1)</td>
<td>2(1)</td>
</tr>
<tr>
<td>H1</td>
<td>0(40)</td>
<td>0(75)</td>
</tr>
<tr>
<td>J1</td>
<td>2(7)</td>
<td>2(7)</td>
</tr>
<tr>
<td>K1</td>
<td>2(7)</td>
<td>2(7)</td>
</tr>
</tbody>
</table>

**TABLE 6. Between Assay Precision**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>20</td>
<td>22.1</td>
<td>1.18</td>
<td>5.3%</td>
</tr>
<tr>
<td>Level 2</td>
<td>20</td>
<td>154.3</td>
<td>8.87</td>
<td>5.6%</td>
</tr>
</tbody>
</table>

*As measured in ten experiments in duplicate

15.0 REFERENCES
