Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and biotinylated monoclonal antibody, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

\[ \text{EnzAb} + \text{Ag} \xrightarrow{\text{ka}} \text{EnzAb-\text{Ag}} \xrightarrow{\text{k-a}} \text{EnzAb} \]

\[ \text{IgG} \text{AFP} = \text{Biotinylated Monoclonal Antibody (Excess Quantity)} \]

\[ \text{EnzAb} = \text{Enzyme Antibody (Excess Quantity)} \]

\[ \text{EnzAb} \cdot \text{Ag} \cdot \text{AFP} \cdot \text{IgG} = \text{Antigen-Antibodies Sandwich Complex} \]

\[ k_a = \text{Rate Constant of Association} \]

\[ k_a = \text{Rate Constant of Dissociation} \]

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[ \text{EnzAb} \cdot \text{Ag} \cdot \text{AFP} \cdot \text{IgG} \rightarrow \text{Streptavidin} \rightarrow \text{Immobilized complex} \]

\[ \text{Streptavidin} \cdot \text{IgG} = \text{Streptavidin immobilized on well} \]

\[ \text{Immobilized complex} \rightarrow \text{sandwich complex bound to the well} \]

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

### REAGENTS

**Materials Provided:**

A. **Alpha-Fetoprotein (AFP) — 1 ml/vial - Icon A**

   - Six (6) vials of reference standards of AFP antigen at levels of 0(A), 25(B), 50(C), 100(D), 150(E) and 200(F)ng/ml. Store at 2-8°C. A preservative has been added. Store at 2-8°C.

B. **Anti-AFP Enzyme Reagent — 13ml/vial - Icon A**

   - One (1) vial containing enzyme labeled antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. **Streptavidin Coated Microplate — 96 wells - Icon A**

   - One (1) vial containing streptavidin coated well. Immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated to the well and exogenously added biotinylated monoclonal anti-AFP antibody.

D. **Wash Solution Concentrate — 20 ml - Icon A**

   - One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

E. **Substrate A — 7ml/vial - Icon A**

   - One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

F. **Substrate B — 7ml/vial - Icon A**

   - One (1) bottle containing hydrogen peroxide (H2O2) in buffer. Store at 2-8°C.

G. **Stop Solution — 8ml/vial - Icon A**

   - One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

H. **Product Instructions.**

### 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 licensed reagents. 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) 88-3895.

### SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum 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 specimen should be obtained. The blood should be collected in a plain red-top venipuncture tube without additives or anti-coagulants. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

Specimens may be refrigerated at 2-4°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, 0.050ml of the specimen is required.

**REAGENT PREPARATION:**

1. **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.

2. **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 and controls to room temperature (20 - 27°C).

1. 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.

2. Pipet 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.

3. Add 0.100 ml (100µl) of the anti-AFP Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.

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

5. Incubate 60 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 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 reagents in the same order to minimize reaction time differences between wells.

8. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Add the various AFP antibodies and native AFP forms a sandwich complex that binds with the streptavidin coated to the well.

9. Incubate at room temperature for fifteen (15) minutes.

10. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds.

11. 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 controls at levels in the low, normal and high range of the assay. The control material should be monitored for monitored 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 uncontrolled change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variation.
RESULTS

A dose response curve is used to ascertain the concentration of AFP 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 AFP concentration in ng/ml 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 AFP 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 ng/ml) from the horizontal axis of the graph (the duplicates of the unknowns may be averaged as indicated).

In the following example, the average absorbance (0.420) intersects the dose response curve at (33.2 ng/ml) AFP concentration (See Figure 1).

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 F should be ≥ 1.3.
2. The absorbance (OD) of calibrator A should be ≤ 0.05.
3. Four out of six quality control pools should be within the established ranges.

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. 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.
3. Plate readers measure vertically. Do not touch the bottom of the wells.
4. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
5. Sample(s), which are contaminated microbiologically, should not be used in the assay. Highly lipemic or hemolysed specimen(s) should similarly not be used.
6. Patient specimens with AFP concentrations above 500 ng/ml may be diluted (for example 1/10 or higher) with normal male serum (AFP < 10 ng/ml) and re-assayed. The sample’s concentration is obtained by multiplying the result by the dilution factor (X10).
7. Each component in one assay should be of the same lot number and stored under identical conditions.

B. Interpretation

1. 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.
2. AFP has a low clinical sensitivity and specificity as a tumor marker. Clinically an elevated AFP value alone is not of diagnostic value as a test for cancer and should only be used in conjunction with other clinical manifestations (observations) and diagnostic parameters. AFP levels are known to be elevated in a number of benign diseases and conditions including pregnancy and non-malignant liver diseases such as hepatitis and cirrhosis.

EXPECTED RANGES OF VALUES

Approximately 97-98% of the normal healthy population has AFP levels less than 8.5 ng/ml (4). In high-risk patients, AFP values between 100-350 ng/ml suggest hepatocellular carcinoma. Concentrations over 350 ng/ml usually are indication of the disease.

E. Specificity

No interference was detected with the performance of Monobind AccuBind™ AFP Elisa upon addition of massive amounts of the following substances to a human serum pool.

- Acetylsalicylic Acid  100 µg/ml
- Amethopterin   100 µg/ml
- Ascorbic Acid  100 µg/ml
- Atropine   100 µg/ml
- Caffeine   100 µg/ml
- CEA   10 µg/ml
- PSA   1.0 µg/ml
- CA-125 10,000 U/ml
- hCG 1000 U/ml
- hLH 10 U/ml
- hTSH 10 U/ml
- hPRL 100 µg/ml

TABLE 2

<table>
<thead>
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<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
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<tbody>
<tr>
<td>Level 1</td>
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<td>1.75</td>
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<tr>
<td>Level 2</td>
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<td>135.8</td>
<td>8.54</td>
<td>6.3%</td>
</tr>
<tr>
<td>Level 3</td>
<td>10</td>
<td>244.5</td>
<td>9.58</td>
<td>3.9%</td>
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</table>

*As measured in ten experiments in duplicate.

TABLE 4

<table>
<thead>
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<th>Method</th>
<th>Mean</th>
<th>SD</th>
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<td>Reference (X)</td>
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<tr>
<td>Monobind AFP AccuBind™ Microplate Elisa Procedure</td>
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B. Sensitivity

The AFP procedure has a sensitivity of 0.025 ng. This is equivalent to a sample containing 1 ng/ml AFP concentration.

C. Accuracy

The Monobind AFP AccuBind™ Microplate Elisa Procedure was compared with a reference Elisa method. Biological specimens ranging from 2.5 to 601 ng/ml concentrations were assayed. The total number of such specimens was 301. The least square regression equation and the correlation coefficient were computed for the AFP AccuBind™ in comparison with the reference method. The data obtained is displayed in Table 4.

E. Specificity

No interference was detected with the performance of Monobind AccuBind™ AFP Elisa upon addition of massive amounts of the following substances to a human serum pool.

Acetylsalicylic Acid  100 µg/ml
Amethopterin   100 µg/ml
Ascorbic Acid  100 µg/ml
Atropine   100 µg/ml
Caffeine   100 µg/ml
CEA   10 µg/ml
PSA   1.0 µg/ml
CA-125 10,000 U/ml
hCG 1000 U/ml
hLH 10 U/ml
hTSH 10 U/ml
hPRL 100 µg/ml

REFERENCES