Monobind Inc.
Lake Forest, CA 92630, USA

AccuBind
ELISA Microwells

Alpha-Fetoprotein (AFP) ELISA

Product Code: 1925-300

Intended Use: The Quantitative Determination of Alpha-Fetoprotein (AFP) Concentration in Human Serum by a Microplate Immunoenzymometric assay
(For Research Use Only)

SUMMARY AND EXPLANATION OF THE TEST

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 a peak level of up to 10 mg/ml at twelve weeks of gestation (1). This peak level gradually decreases to less than 25 ng/ml after one year of postpartum. Thereafter, the levels reduce further to less than 10 ng/ml.

Elevated levels of AFP are found in patients with primary heptatoma and yolk sac-derived germ tumors. AFP is the most useful marker for the diagnosis and management of hepatocellular carcinoma (2).

AFP is also elevated in pregnant women. Presence of abnormally high AFP concentrations in pregnant women provides a risk marker for Down syndrome (3),

In this method, AFP calibrator, 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) are added and the reactants mixed. Reaction between the various AFP antibodies and native AFP forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the enzyme-AFP antibody bound conjugate is separated from the unbound enzyme-AFP conjugate by aspiration or decantation. 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 serum references of known alpha-fetoprotein (AFP) levels permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with AFP concentration.

PRINCIPLE

Immunoenzymometric assay (TYPE 3):

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, **in excess,** and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-AFP antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, 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:

$$Enz_{Ab} + Ag_{AFP} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} Enz_{Ab}\text{-}Ag_{AFP}\text{-}{}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

Ag_{AFP} = Native Antigen (Variable Quantity)

^{Enz}Ab = Enzyme labeled Antibody (Excess Quantity)

Enz_{Ab} - Ag_{AFP}-$^{Btn}Ab_{(m)}$ = Antigen-Antibodies Sandwich Complex

k_a = Rate Constant of Association

k_{-a} = 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:

Enz_{Ab} .. Ag_{AFP}-$^{Btn}Ab_{(m)}$ + $Streptavidin_{C.W.}$ ⇒ Immobilized complex

$Streptavidin_{C.W.}$ = Streptavidin immobilized on well

Immobilized complex = 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 - Icons A-F**
Six (6) vials of references AFP antigen at levels of 0(**A**), 5(**B**), 25(**C**), 50(**D**), 250(**E**) and 500(**F**)ng/ml. Store at 2-8°C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the WHO 1st IRP # 72/225.

B. **Anti-AFP Enzyme Reagent —13ml/vial - Icon**
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**
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. **Wash Solution Concentrate – 20 ml - Icon**
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

E. **Substrate A --7ml/vial - Icon S[A]**
One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

F. **Substrate B -- 7ml/vial - Icon S[B]**
One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

G. **Stop Solution -- 8ml/vial - Icon** STOP
One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

H. **Product Instructions.**

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.
Note 3: Above reagents are for a single 96-well microplate

Required But Not Provided:

1. Pipette(s) capable of delivering 25µl, 50µl 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. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Quality control materials

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) 88-8395.

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

Samples may be refrigerated at 2-8^{0}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^{0}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 references 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. Pipette 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 wash. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100µl) of working substrate 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 THE PLATE AFTER SUBSTRATE ADDITION
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. **Always add reagents in the same order to minimize reaction time differences between wells.**
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 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 kit reagents. Fresh reagents should be used to determine the reason for the variations.

RESULTS

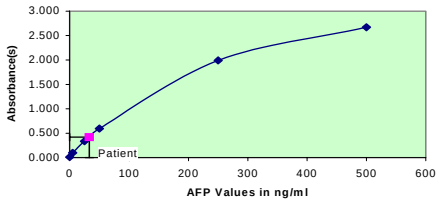
A dose response curve is used to ascertain the concentration of AFP in unknown specimens.

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 unknown 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).

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (ng/ml)
Cal A	A1	0.012	0.011	0
	B1	0.011		
Cal B	C1	0.100	0.098	5
	D1	0.097		
Cal C	E1	0.336	0.335	25
	F1	0.333		
Cal D	G1	0.612	0.594	50
	H1	0.577		
Cal E	A2	2.005	1.990	250
	B2	1.975		
Cal F	C2	2.664	2.672	500
	D2	2.680		
Patient	E2	0.427	0.420	**33.2**
	F2	0.413		

Figure 1



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

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 lipemeic 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 cance**r 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 8.5ng/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.

TABLE I
Expected Values for the AFP AccuBind™ Elisa Test System

Male and Female	<8.5ng/ml (97-98%)

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

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precision of the AFP AccuBind™ ELISA Microplate Procedure were determined by analyses on three different levels of control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in ng/ml)

Sample	N	X	σ	C.V.
Level 1	20	33.1	1.85	5.6%
Level 2	20	140.5	7.45	5.3%
Level 3	20	230.5	10.45	4.5%

TABLE 3
Between Assay Precision* (Values in ng/ml)

Sample	N	X	σ	C.V.
Level 1	10	31.5	1.75	5.6%
Level 2	10	135.8	8.54	6.3%
Level 3	10	244.5	9.58	3.9%

*As measured in ten experiments in duplicate.

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.

TABLE 4

Method	Mean	Least Square Regression Analysis	Correlation Coefficient
This Method (Y)	6.60	y = -0.7514+0.9639*(X)	0.978
Reference (X)	6.43		

Only slight amounts of bias between the Monobind AFP AccuBind™ Microplate Elisa Procedure 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.

D. Linearity & Hook Effect:

Three different lot preparations of the Monobind AFP AccuBind™ Microplate Elisa reagents were used to assess the linearity and hook effect. Massive concentrations of AFP (> 100,000 ng/ml) were used for linear dilutions in pooled human patient sera.

The test showed no hook effect upto concentrations of 10,000 ng/ml and a within dose recovery of 97.0 to 109.4%.

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 IU/ml
hLH	10 IU/ml
hTSH	100 mIU/ml
hPRL	100 µg/ml

REFERENCES

1. Wild D, *The Immunoassay Handbook*, Stockton Press, 445 (1994).
2. Henry JB, "*Clinical Diagnosis and Management by Laboratory Methods",* WB Saunders Company, 1075 (1996).
3. Wild D, *The Immunoassay Handbook,* Stockton Press **p400-02.** (1994)
4. Li D, Mallory T, Satomura S, "AFP; a new generation of tumor marker for hepatocellulor carcinoma", *Clin Chem Acta,* **313,** 15-9 (2001).
5. Mizejewski GJ, 'Alfa-fetoprotein structure and function; relevant to isoforms, epitopes and conformational variants' *Exp Biol Med*, 226**,** 337-408 (2001).
6. Johnson OJ, Williams R, 'Cirrhosis and etiology of hepatocellular carcinoma', *J Hepatology,* **4,** 140-147 (1987).
7. Javadpour N, 'The role of biologic tumor markers in testicular cancer', *Cancer*, 45**,** 1755-61 (1980).

Revision: B **Date: 102506**

Cat #: 1925-300

Size		96(A)	192(B)
Reagent (fill)	A)	1ml set	1ml set
	B)	1 (13ml)	2 (13ml)
	C)	1 plate	2 plates
	D)	1 (20ml)	1 (20ml)
	E)	1 (7ml)	2 (7ml)
	F)	1 (7ml)	2 (7ml)
	G)	1 (8ml)	2 (8ml)

For Orders and Inquiries, please contact

Monobind Inc.
100 North Pointe Drive
Lake Forest, CA 92630 USA

Tel: 949-951-2665
Fax: 949-951-3539
Email: info@monobind.com
On the Web: www.monobind.com

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

IVD CE 2° 8°

EC REP **CEpartner4U**, 3951 DB; 13.NL
Tel: +31 (0) 6-516.536.26