Monobind Inc.
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AccuLite
CLIA Microwells

Alpha-Fetoprotein (AFP) Test System
Product Code: 1975-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Alpha-Fetoprotein (AFP) Concentration in Human Serum by a Microplate Enzyme Immunoassay, Chemiluminescence

2.0 SUMMARY AND EXPLANATION OF THE TEST

Alpha-Fetoprotein (AFP) is a glycoprotein with a molecular weight of 70 kDA. AFP shares considerable sequential homology with albumin and is normally produced during fetal development by the hepatocytes, yolk sac and to a lesser extent by the gastrointestinal tract. AFP appears as a major serum protein in the fetus, but its concentration declines rapidly towards birth. 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.

Ever since its first reported association with tumors, by Tatarinov in 1964 based on his work with liver carcinomas, AFP has been a subject of discussion with relation to many different tumors. Abnormal AFP levels have been associated with hepatocellular carcinoma, ovarian cancer, gastrointestinal cancer and pulmonary cancer and, most recently, with non-seminomatous testicular cancer. 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 light measurable with a luminometer.

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.

3.0 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:

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

$^{Btn}Ab_{(m)}$ = 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:
$^{ENZ}Ab_{..}\ Ag_{AFP} - {}^{Btn}Ab_{(m)} + Streptavidin_{C.W.} \Rightarrow$ 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, determined by reaction with a substrate that generates light, 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.

4.0 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. AFP Tracer Reagent – 13 ml/vial - Icon Ⓔ
One (1) vial containing enzyme labeled antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Light Reaction Wells - 96 wells – Icon ⇓
One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate – 20ml/vial - Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C (see Reagent Preparation Section).

E. Signal Reagent A – 7ml/vial - Icon C[A]
One (1) vial containing luminol in buffer. Store at 2-8°C (see Reagent Preparation Section).

F. Signal Reagent B – 7ml/vial - Icon C[B]
One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C (see Reagent Preparation Section).

G. Product Insert.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. **Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.**
Note 3: Above reagents are for a single 96-well microplate

4.1 Required but NOT provided:
1. Pipette capable of delivering 0.025ml (25µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Luminometer.
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.

5.0 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.

Safe Disposal of kit components must be according to local regulatory and statutory regulations.

6.0 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.

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 assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and high 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.

8.0 REAGENT PREPARATION

1. **Wash Buffer**
Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days.
2. **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 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

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

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).
*****Test procedure should be performed by a skilled individual or trained professional*****

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 AFP Tracer 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 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. Decant the wash and repeat four (4) additional times.**
8. Add 0.100 ml (100µl) of working signal reagent 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 SIGNAL ADDITION
9. Incubate for five (5) minutes in the dark.
10. Read the relative light units in each well for 0.2 – 1.0 seconds. **The results should be read within thirty (30) minutes of adding the substrate solution.**

10.0 CALCULATION OF RESULTS

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

1. Record the RLU's (*Relative Light Units*) obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLU's 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 RLU's for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLU's (23551) of the unknown intersects the calibration curve at (88ng/ml) AFP concentration (See Figure 1)*.

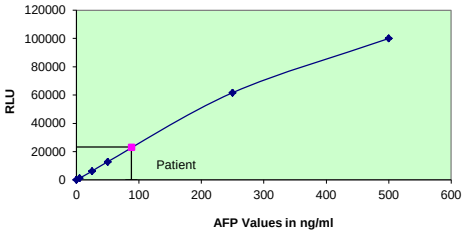
Note: Computer data reduction software designed for chemiluminescence assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

* The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay. In addition, the RLU's of the calibrators have been normalized to 100,000 RLU's for the F calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

EXAMPLE 1

Sample I.D.	Well Number	RLU (A)	Mean RLU(B)	Value (ng/ml)
Cal A	A1	69	79	0
	B1	89		
Cal B	C1	1281	1227	5
	D1	1172		
Cal C	E1	6219	6178	25
	F1	6136		
Cal D	G1	12717	12848	50
	H1	12979		
Cal E	A2	61923	61679	250
	B2	61434		
Cal F	C2	99061	100000	500
	D2	100939		
Cont 1	A3	5342	5229	22.3
	B3	5117		
Cont 2	C3	27884	28731	103.6
	D3	29577		
Patient	E3	23210	23551	88.0
	F3	23892		

Figure 1



11.0 Q.C. 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 MSDS and Risk Analysis Form for this product is 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 lipemic, 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 adhering 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. 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).
9. 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.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. 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.
12. Risk Analysis- as required by CE Mark IVD Directive 98/79/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 have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays (Boscato LM, Stuart MC. 'Heterophilic antibodies: a problem for all immunoassays' Clin. Chem. 1988:3427-33). For diagnostic purposes, the results from this assay should be in combination with clinical examination, patient history and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. 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.
7. 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.

13.0 EXPECTED RANGES OF VALUES

Approximately 97-98% of the normal healthy population has AFP levels less than 8.5ng/ml (8). 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 AccuLite® CLIA

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

Values for AFP for a normal, healthy population and pregnant women, during gestation cycle, are given in Table 2. The values depicted below represent limited in house studies in concordance with published literature.[9,10,11]

TABLE 2
Median Values during Gestation.

Gestation (Week)	AFP (ng/ml)
15	40.14
16	42.91
17	52.34
18	61.50
19	75.57
20	83.31
21	90.46

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.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precision of the AFP AccuLite® CLIA test system 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 3 and Table 4.

TABLE 3
Within Assay Precision (Values in ng/ml)

Sample	N	X	σ	C.V.
Level 1	20	12.4	0.9	7.0%
Level 2	20	92.3	3.6	4.0%
Level 3	20	203.9	9.5	5.0%

TABLE 4
Between Assay Precision* (Values in ng/ml)

Sample	N	X	σ	C.V.
Level 1	10	14.8	1.4	10.0%
Level 2	10	92.7	4.6	5.0%
Level 3	10	217.9	14.9	7.0%

*As measured in ten experiments in duplicate.

14.2 Sensitivity

The AFP AccuLite® CLIA Test System has a sensitivity of 0.003 ng. This is equivalent to a sample containing 0.134 ng/ml AFP concentration. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The AFP AccuLite® CLIA test system was compared with a reference method. Biological specimens from low, normal, and elevated concentrations were assayed. The total number of such specimens was 235. The least square regression equation and the correlation coefficient were computed for this test in comparison with the reference method. The data obtained is displayed in Table 5.

TABLE 5

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
Monobind (y)	96.6	y= 1.023(x) + -1.79	0.987
Reference (x)	96.15		

Only slight amounts of bias between the AFP AccuLite® CLIA test system 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.

14.4 Specificity

The cross-reactivity of the AFP AccuLite® CLIA test system to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of AFPA needed to produce the same light intensity.

Substance	Cross Reactivity	Conc.
Alfa-Fetoprotein (AFP)	1.0000	-
Follitropin (hFSH)	< 0.0001	1000ng/ml
Lutropin Hormone (hLH)	< 0.0001	1000ng/ml
Chorionic Gonadotropin (hCG)	< 0.0001	1000ng/ml
Carcinoembryonic Antigen (CEA)	< 0.0001	1000ng/ml
Prostatic Specific Antigen (PSA)	< 0.0001	1000 ng/ml
Prostatic Acid Phosphatase (PAP)	< 0.0001	1000 ng/ml
Cancer Antigen (CA-125)	< 0.0001	1000 U/ml
Cancer Antigen (CA 19-9)	< 0.0001	1000 ng/ml

15.0 REFERENCES

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Revision: 5 Date: 2021-Sep-23 **DCO: 1509**
MP1975 **Product Code: 1975-300**

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)

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IVD 2° 8° CE
EC REP CEpartner4U, Esdoornlaan 13 3951 DBMaarn, The Neatherlands www.cepartner4u.eu

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

Glossary of Symbols
(EN 980/ISO 15223)

IVD In Vitro - Diagnostic Medical Device	2° 8° Temperature Limitation Storage Condition (2-8° C)	Consult Instructions for Use
REF Catalogue Number	Σ Contains Sufficient Test for Σ	LOT Batch Code
Used By (Expiration Day)	Date of Manufacturer	Manufacturer
EC REP Authorized Rep in European Country	CE European Conformity	