Enzyme Immunoassay for the quantitative determination of Alpha-Fetoprotein (AFP) in human serum

FOR IN VITRO DIAGNOSTIC USE ONLY

Store at 2 to 8°C.

INTENDED USE

For the quantitative determination of the Cancer Antigen AFP concentration in human serum.

INTRODUCTION

Alpha-fetoprotein (AFP) is a glycoprotein with a molecular weigh of approximately 70,000 daltons. AFP is normally produced during fetal and neonatal development by the liver, yolksac, and in small concentrations by the gastrointestinal tract. After birth, serum AFP concentrations decrease rapidly, and by the second year of life and thereafter only trace amounts are normally detected in serum.

Elevation of serum AFP to abnormally high values occurs in several malignant diseases, most notably nonseminomatous testicular cancer and primary hepatocellular carcinoma. In the case of nonseminomatous testicular cancer, a direct relationship has been observed between the incidence of elevated AFP levels and the stage of disease. Elevated AFP levels have also been observed in patients diagnosed with seminoma with nonseminomatous elements, but not in patients with pure seminoma.

In addition, elevated serum AFP concentrations have been measured in patients with other noncancerous diseases, including ataxia telangiectasia, hereditary tyrosinemia, neonatal hyperbilirubinemia, acute viral hepatitis, chronic active hepatitis, and cirrhosis. Elevated serum AFP concentrations are also observed in pregnant women. Therefore, AFP measurements are not recommended for use as a screening procedure to detect the presence of cancer in the general population.

PRINCIPLE OF THE TEST

The AFP ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a rabbit anti-AFP antibody directed against intact AFP for solid phase immobilization (on the microtiter wells). A monoclonal anti-AFP antibody conjugated to horseradish peroxidase (HRP) is in the antibody-enzyme conjugate solution. The test sample is allowed to react first with the immobilized rabbit antibody for 30 minutes. The
wells are washed to remove any unbound antigen. The monoclonal-HRP conjugate is then reacted with the immobilized antigen for 30

minutes at room temperature resulting in the AFP molecules being sandwiched between the solid phase and enzyme-linked antibodies.
The wells are washed with water to remove unbound labeled antibodies. A solution of TMB Reagent is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of Stop Solution changing the color to yellow. The concentration of AFP is directly proportional to the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

REAGENTS

Materials provided with the kit:

- AFP Antibody-Coated Wells (1 plate, 96 wells)
  Microtiter Wells coated with rabbit anti-AFP
- AFP Reference Standard Set (1.0 ml/vial)
  Contains 0, 5, 20, 50, 150, and 300 ng/ml of AFP (WHO, 72/225) in bovine serum with preservatives, lyophilized
- AFP Zero Buffer (13 ml)
  Contains tris buffer with preservatives
- AFP Enzyme Conjugate Reagent (18 ml)
  Contains AFP MoAb conjugated to horseradish peroxidase with preservatives
- TMB Reagent (11 ml)
  Contains 3, 3', 5, 5' tetramethylbenzidine (TMB) stabilized in buffer solution
- Stop Solution -1N HCl (11 ml)
  Diluted hydrochloric acid

Materials required but not provided:

- Precision pipettes: 20 µl, 100 µl, and 150 µl, and 1.0 ml
- Disposable pipette tips
- Distilled water
- Vortex mixer or equivalent
- Absorbent paper or paper towel
- Graph paper
- Microtiter plate reader

STORAGE CONDITIONS

1. Store the unopened kit at 2-8°C upon receipt and when it is not in use, until the expiration shown on the kit label. Refer to the package label for the expiration date.
2. Keep microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

INSTRUMENTATION

A microtiter well reader with a bandwidth of 10 nm or less and an optical density range of 0 to 3 OD or greater at 450 nm wavelength is acceptable for absorbance measurement.

WARNINGS AND PRECAUTIONS

1. CAUTION: This kit contains human material. The source material used for manufacture of this component tested negative for HBsAg, HIV 1/2 and HCV by FDA-approved methods. However, no method can completely assure absence of these agents. Therefore, all human blood products, including serum samples, should be considered potentially infectious. Handling should be as defined by an appropriate national biohazard safety guideline or regulation, where it exists.[25]

2. Avoid contact with 1N HCl. It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
3. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
4. Replace caps on reagents immediately. Do not switch caps.
5. Do not pipette reagents by mouth.
6. For in vitro diagnostic use.

SPECIMEN COLLECTION AND PREPARATION

1. The use of SERUM samples is required for this test.
   2. Specimens should be collected using standard venipuncture techniques. Remove serum from the coagulated or packed cells within 60 minutes after collection.
3. Specimens which cannot be assayed within 24 hours of collection should be frozen at –20°C or lower, and will be stable for up to six months.
4. Avoid grossly hemolytic (bright red), lipemic (milky), or turbid samples (after centrifugation).
5. Specimens should not be repeatedly frozen and thawed prior to testing. DO NOT store in "frost free" freezers, which may cause occasional thawing. Specimens which have been frozen, and those which are turbid and/or contain particulate matter, must be centrifuged prior to use.

PROCEDURAL NOTES

1. Pipetting recommendations (single and multi-channel): Pipetting of all standards, samples, and controls should be completed within 3 minutes.
2. All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same.
3. It is recommended that the wells be read within 15 minutes following addition of Stop Solution.

REAGENT PREPARATION

1. All reagents should be brought to room temperature (18-25°C) before use.
2. Reconstitute each lyophilized standard with 1.0 ml distilled water. Allow the reconstituted material to stand for at least 20 minutes and mix gently. Reconstituted standards will be stable for up to 30 days when stored sealed at 2-8°C.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 20 µl of standard, specimens, and controls into appropriate wells.
3. Dispense 100 µl of Zero Buffer into each well.
4. Thoroughly mix for 30 seconds. It is very important to have a complete mixing in this setup.
5. Incubate at room temperature (18-25°C) for 30 minutes.
6. Remove the incubation mixture by flicking plate content into a waste container.
7. Rinse and flick the microtiter wells 5 times with distilled or deionized water.
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense 150 µl of Enzyme Conjugate Reagent into each well. Gently mix for 10 seconds.
10. Incubate at room temperature for 30 minutes.
11. Remove the incubation mixture by flicking plate contents into a waste container.

12. Rinse and flick the microtiter wells 5 times with distilled or deionized water. (Please do not use tap water.)
13. Strike the wells sharply onto absorbent paper to remove residual water droplets.
14. Dispense 100 µl TMB Reagent into each well. Gentle mix for 10 seconds.
15. Incubate at room temperature for 20 minutes.
16. Stop the reaction by adding 100 µl of Stop Solution to each well.
17. Gently mix for 30 seconds. **It is important to make sure that all the blue color changes to yellow color completely.**
18. Read optical density at 450 nm with a microtiter reader **within 15 minutes.**

QUALITY CONTROL

Good laboratory practice requires that quality control specimens (controls) be run with each calibration curve to verify assay performance. To ensure proper performance, control material should be assayed repeatedly to establish mean values and acceptable ranges.

CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards, control, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of AFP in ng/ml from the standard curve.

EXAMPLE OF STANDARD CURVE

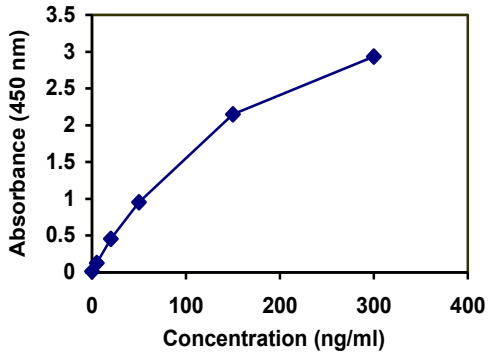
Results of a typical standard run with optical density readings at 450 nm shown in the Y axis against AFP concentrations shown in the X axis. This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own data and standard curve.

AFP (ng/ml)	Absorbance (450 nm)
0	0.012
5	0.127
20	0.455
50	0.952
150	2.150
300	2.932

EXPECTED VALUES

In high-risk patients, AFP values between 100 and 350 ng/ml suggest a diagnosis of hepatocellular carcinoma, and levels over 350 ng/ml usually indicate the disease. Approximately 97% of the healthy subjects have AFP levels less than 8.5 ng/ml. It is recommended that each laboratory establish its own normal range.

PERFORMANCE CHARACTERISTICS



1. **Accuracy**
   A statistical study using patient samples demonstrated good correlation of results with the commercially available kits as shown below:

   Comparisons between AFP ELISA and Abbott AxSym AFP ELISA kits provide the following data:

   N = 160
   Correlation coefficient = 0.9761
   Slope = 1.130
   Intercept = 1.4176
   AFP ELISA Test Mean = 14.3 ng/mL
   Abbott AxSym Mean = 17.6 ng/mL

2. **Sensitivity**
   The minimum detectable concentration of AFP by this assay is estimated to be 2.0 ng/ml.

3. **Precision**

   a. Intra-Assay Precision
   Within-run precision was determined by replicate determinations of four different control sera in one assay. Within-assay variability is shown below:

Serum Sample	**1**	**2**	**3**	**4**
Number of Replicates	24	24	24	24
Mean AFP (ng/mL)	189	81	20	4.8
Standard Deviation	7	3	1	0.4
Coefficient of Variation (%)	3.6	3.5	5.8	7.4

   b. Inter-Assay Precision
   Between-run precision was determined by replicate measurements of four different control sera in several different assays. Between-assay variability is shown below:

Serum Sample	1	2	3	4
Number of Replicates	20	20	20	20
Mean AFP (ng/mL)	218	87	21	4.7
Standard Deviation	14	4	1	0.3
Coefficient of Variation (%)	6.3	4.8	4.4	6.2

4. Recovery and Linearity Studies

a. Recovery

Various patient serum samples of known AFP levels were mixed and assayed in duplicate. The average recovery was 100.7%.

	Expected Concentration (ng/ml)	Observed Concentration (ng/ml)	% Recovery
1.	222.01	239.59	107.9
2.	187.83	209.33	111.4
3.	69.93	68.57	98.1
4.	61.64	59.63	96.7
5.	30.49	29.76	97.6
6.	16.58	16.82	101.4
7.	6.99	6.94	99.2
8.	4.15	3.88	93.5
	Average Recovery = 100.7%		

b. Linearity

Four patient samples were serially diluted with the zero standard in a linearity study. The average recovery was 102.6%.

#	Dilution	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1.	Undiluted	----	150.00	----
	1:2	75.00	78.53	104.7
	1:4	37.50	38.09	101.6
	1:8	18.75	18.88	100.7
	1:16	9.38	9.43	100.6
	1:32	4.69	4.45	94.9
	1:64	2.34	2.24	95.4

				Mean = 99.6 %
2.	Undiluted	----	267.26	----
	1:2	133.63	131.17	98.2
	1:4	66.82	64.35	96.3
	1:8	33.41	31.61	94.6
	1:16	16.70	16.13	96.6
	1:32	8.35	7.48	89.6
	1:64	4.18	3.57	85.4
				Mean = 93.4 %
3.	Undiluted	----	204.18	----
	1:2	102.09	113.01	110.7
	1:4	51.04	57.40	112.4
	1:8	25.52	29.94	117.3
	1:16	12.76	14.43	113.1
	1:32	6.38	6.76	106.0
	1:64	3.19	3.47	89.4
				Mean = 109.8%
4.	Undiluted	----	237.58	----
	1:2	118.79	127.17	107.1
	1:4	59.39	64.94	109.3
	1:8	29.70	33.80	113.8
	1:16	14.85	16.17	108.9
	1:32	7.42	7.59	102.2
				Mean = 107.6%

5. Specificity

The following substances were tested for cross-reactivity:

Analyte Tested	Concentration	Produced Intensity Equivalent to AFP (ng/mL)
PSA	250 ng/mL	0
	500 ng/mL	0
	1,000 ng/mL	0
	2,500 ng/mL	0
	5,000 ng/mL	0
CEA	2,500 ng/mL	0
	5,000 ng/mL	0
	10,000 ng/mL	0
CA 125	10,000 ng/mL	0
	50,000 ng/mL	0
CA 15-3	1,000 ng/mL	0
	2,000 ng/mL	0
	5,000 ng/mL	0

CA 19-9	5,000 ng/mL 10,000 ng/mL	0 0
PAP	6,000 ng/mL 12,000 ng/mL	0 0

LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.

2. This product is not intended to be used for the risk calculation of trisomy 21.
3. The results obtained from the use of this kit should be used only as an adjunct to other diagnostic procedures and information available to the physician.
4. Serum samples demonstrating gross lipemia, gross hemolysis, or turbidity should not be used with this test.
5. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

Reference

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