

AFP ELISA

RUO

AL-104

INTENDED USE

The AFP Enzyme-Linked Immunosorbent Assay (ELISA) Kit provides materials for the quantitative measurement of AFP in serum. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

The concentration of AFP in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity. The results reported by the laboratory to the physician must include the identity of the AFP assay used. Values obtained with different AFP assays cannot be used interchangeably. Before changing assays, the laboratory must confirm baseline values for patients being serially monitored.

SUMMARY AND EXPLANATION

Alpha-Fetoprotein (AFP) is a 68 kDa protein which is produced primarily during fetal life by the fetal liver yolk sac.¹ Elevated AFP levels are seen in patients with nonseminomatous testicular cancer. More than 95% of testicular cancers belong to a heterogeneous group called germ-cell tumors because it is widely believed that they arise in primordial germ cells.² Germ cell tumors (GCTs) are classified either as seminomatous or as nonseminomatous. The latter can be further classified as embryonal carcinoma, teratoma, or choriocarcinoma. The seminoma histologic subtype can be found in 40% of all germ cell tumors while the nonseminoma histologic subtype can be found in 60% of germ cell tumors.³ The different histologic types of germ cell tumors may occur singly or in various combinations. Elevated AFP levels have been observed in patients diagnosed as having seminomatous testicular cancer with nonseminomatous elements, but not in patients with pure seminoma.⁴⁻⁹ Both AFP and hCG are measured in testicular cancer. Approximately 40% of patients with nonseminomatous germ cell tumors have elevation of only one marker.¹⁰ During the clinical course of the disease, the levels of the two markers do not always parallel each other.

A direct relationship has been observed between the incidence of elevated AFP levels in nonseminomatous testicular cancer and the stage of the disease.^{4,6} Elevation of AFP (>10 IU/L or 12.1 ng/mL) occurs in 80% of metastatic and in 57% of stage 1 nonseminomatous germ cell tumors.¹⁰ In Clinical Stage 2B or higher, AFP and/or hCG are elevated in 65-80% of the cases with increasing frequency according to the bulk of the disease.¹²

The usefulness of AFP measurements in the management of nonseminomatous testicular cancer patients undergoing cancer therapy has been well established.^{4,6,13} Current management of testicular germ cell tumors relies upon the use of serum tumor markers, which can indicate the presence of small foci of active tumor that cannot be detected by currently available imaging techniques.¹⁰ Markers augment and complement information obtained from radiographic and other staging procedures.¹⁴ Also, the short half-lives of tumor markers facilitate their use in assessing tumor burden during therapy. AFP has a serum half-life of 3.5 - 6 days.¹⁵ AFP and/or hCG levels are elevated before orchiectomy in about 60% of all Clinical Stage I patients but follow a normal decline after the testicle is removed.¹²

For patients in clinical remission following treatment, AFP levels generally decrease.⁵ Post-operative AFP levels which fail to return to normal strongly suggest the presence of residual tumor.^{4,6,16} Following successful resection of

primary or metastatic disease, AFP and hCG decline at a rate proportional to their respective half-lives.¹⁵ An elevated actual half-life of serum markers following orchiectomy or retroperitoneal lymph node dissection may indicate the presence of occult, persistent disease.¹⁴

As recently as the 1970s, nonseminomatous germ cell tumors were often fatal. Due to advances in chemotherapy, most patients are cured, even those with disseminated disease.² The clinical use of AFP and hCG measurements has been essential to this success. Many patients have a marker surge during the first week of chemotherapy, presumably secondary to tumor lysis. AFP may increase from 20% to 200% over pretreatment levels.¹⁴ Chemotherapeutic responses are accompanied by a decline in marker levels. Persistent marker elevation is usually the result of residual malignancy. Rising marker values may occur before or after clinical recurrence and one marker may rise in discordance with the other.¹⁴ Tumor recurrence is often accompanied by a rise in serum AFP values prior to clinical evidence of progressive disease.⁴⁻⁵

Elevated serum levels of AFP are also associated with some non-testicular cancers. Increased serum concentrations of AFP were first observed in human subjects with primary hepatocellular carcinoma.¹¹ Subsequently, elevated serum AFP values have been associated with other malignant diseases such as teratocarcinoma (with yolk sac components) of the ovary, endodermal sinus tumors, certain gastrointestinal tumors (with and without liver metastasis), and tumors of other tissues.^{12-13, 16-20} A study performed at the National Institutes of Health and the Mayo Clinic demonstrated elevated AFP values in patients with pancreatic, gastric, colon, and lung cancer.¹⁴

In additional studies, AFP was elevated in 60-80% of patients with hepatocellular cancer, in 23% of patients with gastrointestinal cancer and in 10% of patients with liver metastasis from various tumor types.¹² However, a normalization of markers does not mean that all viable tumor has been eliminated.¹⁴

Notably however, elevated serum AFP concentrations have also been reported in patients with noncancerous diseases such as ataxia telangiectasia, heredity tyrosinemia, neonatal hyperbilirubinemia, acute viral hepatitis, chronic active hepatitis, cirrhosis, and other benign hepatic conditions.^{14,16,21-26} AFP is modestly elevated (up to 100 ng/mL) in 20% of patients with non-malignant liver disease.¹² Due to its lack of specificity for malignant conditions, AFP testing is not recommended as a screening procedure to detect cancer in the general population.

PRINCIPLE OF THE TEST

The AFP ELISA is a quantitative two-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown diluted samples are added to anti-AFP antibody coated microtiter wells and incubated. After first incubation and washing step, the wells are incubated with horseradish peroxidase labelled antibody conjugate. After a second incubation and washing step, the wells are incubated with substrate solution (TMB). After TMB incubation, an acidic stopping solution is added. In principle, the antibody-HRP conjugate binds to the solid phase antibody-antigen complex. Finally, the antibody-antigen and conjugate complex bound to the well is detected by addition of enzyme-substrate reaction. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as reference filter. The absorbance measured is

directly proportional to the concentration of AFP in the samples and calibrators.

MATERIALS SUPPLIED

CAL-104A AFP Calibrator A/Sample Diluent

One vial, 2mL, labeled AFP Cal A/Sample Diluent, containing 0 ng/mL AFP in a protein-based buffer with a non-mercury preservative. Store unopened at 2-8°C or frozen until the expiration date.

CAL-104B - CAL-104F AFP Calibrators B-F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 5-500 ng/mL AFP in protein based buffer with non-mercury preservative. Refer to **calibration card** for exact concentrations. Store unopened vial at 2 to 8°C until the expiration date. Reconstitute calibrators B-F with 0.5 mL deionized water. Solubilize, Mix well and use after reconstitution. Aliquot and freeze at 20°C or colder for up to one year. Avoid repeated freeze thaws.

CTR-104-I & CTR-104-II AFP Controls (Lyophilized)

Two vials, labeled Levels I and II containing low and high AFP in protein based buffer with a non-mercury preservative. Refer to **calibration card** for exact control ranges. Store unopened at 2 to 8°C until the expiration date. Reconstitute control Levels I and II with 0.5 mL deionized water. Solubilize, mix well and use after reconstitution. Aliquot and freeze at 20°C or colder for up to one year. Avoid repeated freeze thaws.

PLT-104 Anti-AFP Antibody Coated Microtitration Strips

One strip holder, containing 96 polystyrene microtitration wells with anti-AFP antibody immobilized to the inside wall of each well. Store at 2 to 8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

ASB-104 AFP Assay Buffer

One bottle, 8 mL, containing a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.

ECR-104 AFP Antibody Enzyme Conjugate Ready-To-Use

One bottle, 12 mL, containing anti-AFP antibody conjugated to the enzyme horseradish peroxidase in a protein-based (BSA) buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.

TMB-100 TMB Chromogen Solution

One bottle, 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2 to 8°C until expiration date.

WSH-100 Wash Concentrate A

One bottle, 60 mL, containing buffered saline with a nonionic detergent. Store at 2 to 30°C until expiration date. Dilute 25-fold with deionized water prior to use.

STP-100 Stopping Solution

One bottle, 12 mL, containing 0.2 M sulfuric acid. Store at 2 to 30°C until expiration date.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microtitration plate reader capable of absorbance measurement at 450 nm, 405nm and 630 nm.
2. Microtitration orbital plate shaker.
3. Microtitration plate washer.
4. Semi-automated/manual precision pipette to deliver 2–250 µL.
5. Semi-automated/manual precision pipette to deliver 1–10 µL.

6. Repeater Pipette.
7. Vortex mixer.
8. Deionized water.

WARNINGS AND PRECAUTIONS

For *in vitro* diagnostic use.

The following precautions should be observed:

- a) Follow good laboratory practice.
- b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.
- c) Handle and dispose of all reagents and material in compliance with applicable regulations

WARNING: Potential Biohazardous Material

This reagent may contain some human source material (e.g. serum) or materials used in conjunction with human source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, 2007.²⁸

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 as a preservative. Pro-Clean 400 and peroxide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

SAMPLE COLLECTION AND PREPARATION

- a. Serum is the recommended sample type.
- b. Sample handling, processing, and storage requirements depend on the brand of blood collection tube²⁹ that you use. Please reference the manufacturer's instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.
- c. Samples may be stored at 4°C if assayed within 24 hours; otherwise samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.
- d. Avoid assaying lipemic, hemolyzed or icteric samples.
- e. Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.
- f. For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens.

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the AFP ELISA assay. It is the responsibility of the customer to validate the assay for their use. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.
2. A calibration curve must be included with each assay.
3. Bring all kit reagents to room temperature before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix different lots of any kit component and do not use any component beyond the expiration date.
4. Use a clean disposable pipette tip for each reagent, calibrator, control or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the HRP conjugates. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial

contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.

- Incomplete washing will adversely affect the outcome and assay precision. Care should be taken to add TMB into the wells accurately and efficiently to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight. TMB chromogen solution should be colorless. Development of a blue color may indicate reagent contamination or instability.

PREPARATION OF REAGENTS

- AFP Calibrators B-F and AFP Controls I & II:** Tap and reconstitute AFP Calibrator B-F and AFP Controls I & II each with 0.5 mL deionized water. Solubilize, mix well and use after reconstitution.
- Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature when stored in a tightly sealed bottle.
- Microtitration Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

ASSAY PROCEDURE

Allow all samples and reagents to reach room temperature. Mix reagents thoroughly by gentle inversion before use. After reconstitution of reagents, mix thoroughly, avoiding foam. Calibrators, controls and samples should be assayed in duplicate.

- Reconstitute AFP Calibrator B-F and AFP Controls I & II each with **0.5 mL** deionized water. Solubilize for **10 minutes**, Mix well.
- Mark the microtitration strips to be used.
- Pipet **25 µL** of the **calibrators, controls and unknown samples** to the appropriate wells.
- Add **50 µL** of the **AFP Assay Buffer** to each well using a repeater pipette.
- Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **30 mins** at room temperature ($23 \pm 2^\circ\text{C}$).
- Aspirate and wash each well **5 times** with the wash solution (**350 µL/well**) using an automatic microplate washer.
- Add **100 µL** of the **Antibody-enzyme conjugate ready to use solution** to each well using a precision pipette.
- Incubate the wells, shaking at 600–800 rpm on an orbital microplate shaker, for **30 mins** at room temperature ($23 \pm 2^\circ\text{C}$).
- Aspirate and wash each well **5 times** with the wash solution (**350 µL/well**) using an automatic microplate washer.
- Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
- Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-12 min** at room temperature ($23 \pm 2^\circ\text{C}$).
NOTE: Visually monitor the color development to optimize the incubation time.
- Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm**.
NOTE: Zero calibrator should be programmed as “Blank” while reading the optical density. If instrument has a wavelength correction, set the instrument to dual wavelength measurement at **450 nm** with background wavelength correction at **630 nm**.

RESULTS

NOTE: The results in this package insert were calculated by plotting the **log optical density (OD) data on the y-axis and log AFP concentration on X-axis** using a cubic regression curve-fit. Other data reduction methods may give slightly different results.

- Optimum results can be obtained at incubation temperature of ($23 \pm 2^\circ\text{C}$).
- Calculate the mean OD for each calibrator, control or diluted sample.
- Plot the log of the mean OD readings for each of the Calibrators along the y-axis versus log of the AFP concentrations in ng/mL on the x-axis, using a cubic regression curve fit.
- Determine the AFP concentrations of the controls and samples from the calibration curve by matching their mean OD readings with the corresponding AFP concentrations.
- Any sample reading higher than the highest calibrator should be appropriately diluted using calibrator A/sample diluent and re-assayed.
- Any sample reading lower than the analytical sensitivity should be reported as such.

LIMITATIONS

The reagents supplied in this kit are optimized to measure AFP levels in human serum. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples.³⁰⁻³³

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- AFP ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for AFP ELISA controls are printed on the calibration card.
- A full calibration curve, low and high level controls, should be included in each assay.

REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Well Contents	Mean Absorbance	Conc (ng/mL)
	Calibrators	0.006	
A1, A2	A	(Blank)	0
B1, B2	B	0.065	5
C1, C2	C	0.247	20
D1, D2	D	0.900	80
E1, E2	E	2.085	200
F1, F2	F	3.467	500

CAUTION: The above data must not be employed in lieu of data obtained by the user in the laboratory.

ANALYTICAL CHARACTERISTICS

All analytical characteristics are stated in ng/mL.

Analytical Sensitivity:

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviation of 24 replicates of calibrator A (0 ng/mL) and calibrator B (5 ng/mL) is 0.44 ng/mL.

Imprecision:

Reproducibility of the AFP ELISA assay was determined in a study using two kit controls and three serum samples. The study included a total of 19 assays, four replicates of each per assay (n=76). Representative data were calculated based on NCCLS EP5-A guidelines and are presented in the following table.

Sample	Mean Conc.	Within Run	Between Run	Total

	(ng/mL)	SD	%CV	SD	%CV	SD	%CV
CI	23.4	1.0	4.2	1.0	4.2	1.4	5.9
CII	201.1	6.2	3.1	10.5	5.2	12.2	6.1
S1	174.0	4.1	2.4	7.4	4.2	8.4	4.9
S2	30.3	1.7	5.7	1.9	6.2	2.5	8.4
S3	32.3	1.7	5.1	2.7	9.8	3.2	9.8

Recovery:

Known amounts of AFP were added to four serum samples containing different levels of endogenous AFP. The concentration of AFP was determined before and after the addition of exogenous AFP and the percent recovery was calculated.

Sample ID	Endogenous Value in ng/ml	Expected in ng/mL	Observed in ng/mL	% Recovery	Average %Recovery
S1	26.1	49.8	49.0	98%	93%
		73.5	64.8	88%	
		97.2	89.4	92%	
S2	33.4	56.7	58.1	102%	94%
		80.0	72.8	91%	
		103.4	92.1	89%	
S3	22.0	45.9	47.2	103%	95%
		69.8	61.4	88%	
		93.7	87.4	93%	
S4	30.7	54.1	52.0	96%	90%
		77.6	67.4	87%	
		101.1	88.6	88%	

Linearity:

Based on NCCLS EP-6-P multiple dilutions of Calibrator F and the four serum samples containing various AFP levels were diluted with extraction buffer. The % recovery on individual samples is represented in the following table.

Sample ID	Dilution factor	Expected Value	Observed Value	% Recovery	Average % Recovery
F Dilution	NEAT VALUE	500.0	NA	NA	101%
	2	250.0	257.2	103%	
	4	125.0	122.3	98%	
	8	62.5	61.7	99%	
	16	31.3	31.7	101%	
	32	15.6	16.5	105%	
S1	NEAT VALUE	63.5	NA	NA	97%
	2	31.7	30.9	97%	
	4	15.9	16.0	101%	
	8	7.9	7.5	95%	
S2	NEAT VALUE	56.6	NA	NA	97%
	2	28.3	29.8	105%	
	4	14.1	14.5	102%	
	8	7.1	5.9	83%	
S3	NEAT VALUE	72.8	NA	NA	101%
	2	36.4	35.8	98%	
	4	18.2	18.4	101%	
	8	9.1	9.5	104%	
S4	NEAT VALUE	69.1	NA	NA	95%
	2	34.5	32.3	94%	
	4	17.3	16.7	97%	
	8	8.6	8.3	96%	

Analytical Specificity:

The antibody pair used in the AFP ELISA measures AFP and does not cross-react with following analytes at specified concentrations:

Sr No	Cross-reactant	Conc. (ng/mL)	% Recovery
1	Alpha-2-macroglobulin	65	ND
2	IhCG	1000	ND
3	Estriol	50	ND
4	βhCG	1000	ND
5	Inhibin A	100	ND
6	AMH	50	ND
7	rPAPP-A	1000	ND
8	htPAPP-A	1000	ND
9	Activin A	50	ND

Interference:

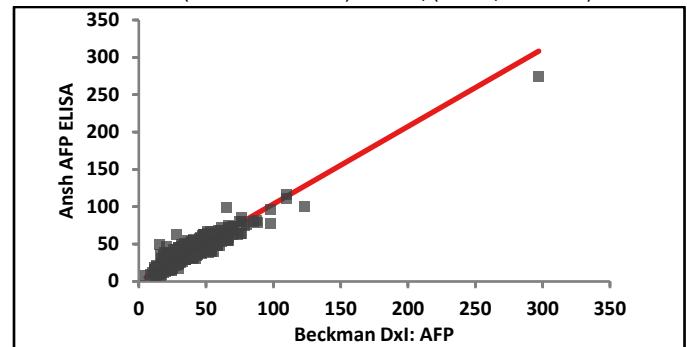
When Potential interferents (hemoglobin, triglycerides and bilirubin) were added at a minimum of ten folds of their physiological concentration to control sample, AFP concentrations were within $\pm 20\%$ of the control as represented in the following table. This study was based on NCCLS EP7-P.

Interferents	Analyte Conc. (mg/mL)	Unspiked Sample Value (ng/mL)	Spiked Sample Value (ng/mL)	% Difference
Hemoglobin	1.35	26.0	26.0	0.0
		50.6	55.9	10.4
		21.1	21.9	3.4
		29.5	31.7	7.5
		26.0	25.5	-2.0
Triglycerides	5	50.6	48.9	-3.4
		21.1	20.7	-2.0
		29.5	28.2	-4.5
		26.3	29.0	10.2
		49.7	58.5	17.7
Bilirubin	0.6	21.1	23.1	9.5
		26.2	31.0	18.2
		26.2	31.0	18.2

Method Comparison:

The AFP ELISA has been compared to Commercial kit using 849 second trimester serum samples. Passing Bablok analysis of the results yielded the following Regression:

$$\text{Ansh ELISA} = 1.038 (\text{Beckman Dxl: AFP}) - 0.1174, (r=0.93; P<0.0000)$$



Expected Value:

Serum samples were analyzed using Ansh AFP ELISA. The expected ranges for AFP were calculated using 95% non-parametric estimation using Analyse-It® for Microsoft Excel.

Population (Gestation Age)	No of specimens (n)	Median conc. (ng/mL)	95% CI (ng/mL)
22 Weeks	9	63.4	52.6 – 80.3
21 Weeks	12	42.8	39.5 – 67.9
20 Weeks	26	53.4	47.3 – 62.8
19 Weeks	48	47.8	41.6 – 53.5

18 Weeks	108	39.3	35.0 – 42.9
17 Weeks	158	34.9	32.6 – 38.2
16 Weeks	259	30.8	28.4 – 31.9
15 Weeks	228	26.8	25.2 – 28.5


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