SUMMARY AND EXPLANATION
Alpha-Fetoprotein (AFP) is a 68 kDa protein which is produced primarily during fetal life by the fetal liver yolk sac. 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. 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. 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.

Both AFP and hCG are measured in testicular cancer. Approximately 40% of patients with nonseminomatous germ cell tumors have elevation of only one marker. 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. 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. 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.

The usefulness of AFP measurements in the management of nonseminomatous testicular cancer patients undergoing cancer therapy has been well established. 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. Markers augment and complement information obtained from radiographic and other staging procedures.

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. AFP and/or hCG levels are elevated before orchectomy in about 60% of all Clinical Stage I patients but follow a normal decline after the testicle is removed.

For patients in clinical remission following treatment, AFP levels generally decrease. Post-operative AFP levels which fail to return to normal strongly suggest the presence of residual tumor. Following successful resection of primary or metastatic disease, AFP and hCG decline at a rate proportional to their respective half-lives. An elevated actual half-life of serum markers following orchectomy or retroperitoneal lymph node dissection may indicate the presence of occult, persistent disease.

As recently as the 1970s, nonseminomatosum germ cell tumors were often fatal. Due to advances in chemotherapy, most patients are cured, even those with disseminated disease. 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. 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. Tumor recurrence is often accompanied by a rise in serum AFP values prior to clinical evidence of progressive disease.

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. 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. 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.

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. However, a normalization of markers does not mean that all viable tumor has been eliminated.

Notably however, elevated serum AFP concentrations have also been reported in patients with noncancerous diseases such as ataxia telangiectasia, hereditary tyrosinemia, neonatal hyperbilirubinemia, acute viral hepatitis, chronic active hepatitis, cirrhosis, and other benign hepatic conditions. AFP is modestly elevated (up to 100 ng/mL) in 20% of patients with non-malignant liver disease. 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.
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, 2 mL, 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, 405 nm 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.

**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.

**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 tube that you use. Please reference the manufacturer’s instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tube 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.
contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.

5. 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

1. **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.
2. **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.
3. **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.

1. Reconstitute AFP Calibrator B-F and AFP Controls I & II each with 0.5 mL deionized water. Solubilize for **10 minutes**, Mix well.
2. Mark the microtitration strips to be used.
3. Pipet **25 μL** of the calibrators, controls and unknown samples to the appropriate wells.
4. Add **50 μL** of the AFP Assay Buffer to each well using a repeater pipette.
5. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **30 mins** at room temperature (**23 ± 2°C**).
6. Aspirate and wash each well **5 times** with the wash solution (**350 μL/well**) using an automatic microplate washer.
7. Add **100 μL** of the Antibody-enzyme conjugate ready to use solution to each well using a precision pipette.
8. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **30 mins** at room temperature (**23 ± 2°C**).
9. Aspirate and wash each well **5 times** with the wash solution (**350 μL/well**) using an automatic microplate washer.
10. Add **100 μL** of the TMB chromogen solution to each well using a repeater pipette. Avoid exposure to direct sunlight.
11. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-12 min** at room temperature (**23 ± 2°C**).
   **NOTE**: Visually monitor the color development to optimize the incubation time.
12. 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.

1. Optimum results can be obtained at incubation temperature of (**23 ± 2°C**).
2. Calculate the mean OD for each calibrator, control or diluted sample.
3. 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.
4. Determine the AFP concentrations of the controls and samples from the calibration curve by matching their mean OD readings with the corresponding AFP concentrations.
5. Any sample reading higher than the highest calibrator should be appropriately diluted using calibrator A/sample diluent and re-assayed.
6. 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.

QUALITY CONTROL

a. Each laboratory should establish mean values and acceptable ranges to assure proper performance.

b. AFP ELISA controls or other commercial controls should fall within established confidence limits.

c. The confidence limits for AFP ELISA controls are printed on the calibration card.

d. A full calibration curve, low and high level controls, should be included in each assay.

REPRESENTATIVE CALIBRATION CURVE DATA

<table>
<thead>
<tr>
<th>Well Number</th>
<th>Well Contents</th>
<th>Mean Absorbance</th>
<th>Conc (ng/mL)</th>
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<tbody>
<tr>
<td>A, A</td>
<td>Calibrators</td>
<td>0.006</td>
<td>0</td>
</tr>
<tr>
<td>B, B</td>
<td>Calibrators</td>
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<td>5</td>
</tr>
<tr>
<td>C, C</td>
<td>Controls</td>
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<td>Controls</td>
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<td>E, E</td>
<td>Controls</td>
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<tr>
<td>F, F</td>
<td>Controls</td>
<td>3.466</td>
<td>500</td>
</tr>
</tbody>
</table>

**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 42 replicates in 21 runs of calibrator A (0 ng/mL) and calibrator B (4 ng/mL) is 0.074 ng/mL.

**Imprecision**: Reproducibility of the AFP ELISA assay was determined in a study using two kit controls and two 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 EPS-A guidelines and are presented in the following table.

### 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 Inhibin A and the percent recovery was calculated.

### Interference:
When Potential interferents (hemoglobin, triglycerides and bilirubin) were added at a minimum of ten folds of their physiological concentration to control sample, AFP concentration were within ±10% of the control as represented in the following table. This study was based on NCCLS EP7-P.

### Analytical Specificity:
The antibody pair used in the AFP ELISA measures AFP and does not cross-react with following analytes.

### References:


