AFP

ELISA Kit (RUO)

Kit ID # AFP-001

96 assays

Manual Version: 01

Investigational Use Only (IUO)

www.imcarebiotech.com

Intended use

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

This IUO product is intended for product testing and determining performance characteristics in determining the levels of AFP in human serum. This product should not be used in clinical diagnosis and patient management.

Product description

The AFP ELISA Kit is an enzyme-linked immunosorbent assay for quantitative detection of human AFP in serum samples.

Background

Liver cancer, primarily in the form of hepatocellular carcinoma (HCC), is the second most lethal cancer worldwide. HCC accounts for 90% of liver cancer cases and is closely linked to risk factors such as chronic viral infections (HBV/HCV), fatty liver, cirrhosis, alcohol abuse, and metabolic diseases like diabetes. In 2020, HCC led to 906,000 new cases and 830,000 deaths globally, highlighting the urgent need for effective surveillance and early detection to improve patient survival. [1-4]

Serum biomarkers are essential tools for the surveillance and diagnosis of HCC. Alpha-fetoprotein (AFP), a glycoprotein normally produced during fetal development, is the most widely used and best-established biomarker for HCC [5]. After birth, AFP levels decrease to very low concentrations. However, levels can become significantly elevated in the presence of HCC, as the tumor cells re-express the protein. For decades, AFP measurement, often combined with liver ultrasonography, has been the cornerstone of HCC surveillance programs for high-risk populations [6].

Despite its widespread use, the diagnostic performance of AFP has notable limitations. Its sensitivity for detecting early-stage HCC is suboptimal, ranging from 40% to 65%, meaning a significant number of early, potentially curable tumors can be missed [7-8]. Furthermore, AFP lacks specificity for HCC given that moderately elevated AFP levels can also be found in patients with other conditions, including chronic hepatitis, cirrhosis, and other malignancies such as germ cell tumors and gastric cancer, leading to potential false-positive results [9]. Due to these limitations, clinical guidelines often recommend against using AFP as a standalone test for diagnosis, emphasizing its role in surveillance and risk stratification alongside imaging [6-7]. Multiplexing AFP with other HCC related biomarkers can improve the accuracy of early HCC detection.

Principle of the Assay

This is a quantitative ELISA that measures the concentration of AFP in human serum to aid in the assessment of HCC in at-risk patients.

The AFP ELISA kit uses a 96-well strip well microplate pre-coated with a capture antibody that specifically binds to AFP, the target analyte (1). When experimental samples, such as patient serum or standards, are added to the wells, the AFP in the sample binds to the immobilized capture antibody selectively (2). A horseradish peroxidase (HRP)-conjugated detection antibody that binds to a different epitope on the AFP facilitates detection when added to the assay wells. Tetramethylbenzidine (TMB) substrate solution is then added to the wells, where it reacts with the HRP enzyme, producing a blue color to the assay wells. The intensity of the blue color is proportional to the amount of AFP in the sample (3). The reaction is stopped by the addition of an acidic stop solution, turning the color from blue to yellow. The absorbance of the resulting yellow color is measured at 450 nm, and this absorbance is directly proportional to the concentration of AFP in the sample. Figure 1 demonstrates the assay principle of the sandwich ELISA in an assay well of a 96-well plate. The concentration of AFP in each sample is determined by comparing the absorbance values to a 4-parameter logistic (4PL) standard curve constructed from known calibrator concentration.

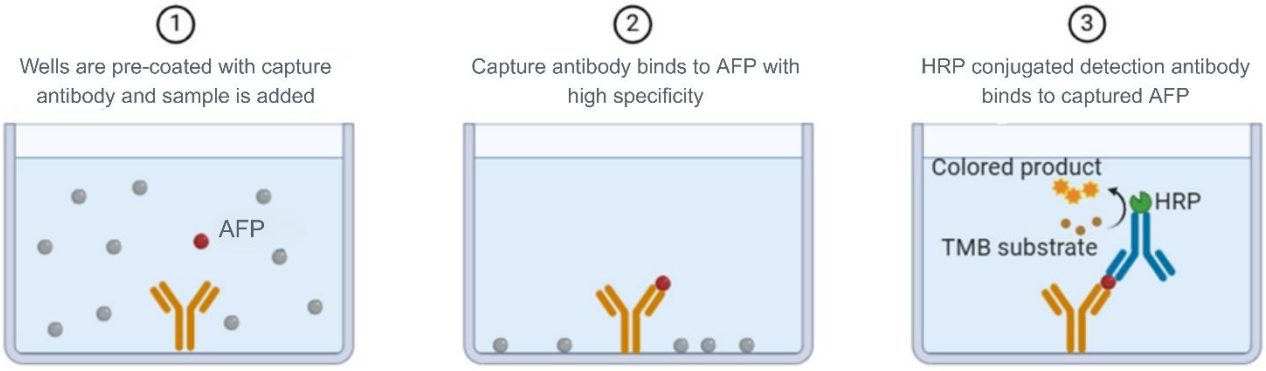


Figure 1: Schematic representation of ELISA assay principle demonstrating capture antibody coated at the bottom of the ELISA well. AFP present in the samples were captured by capture antibody while all the non-specific proteins are washed away. The captured LC-SPIK is detected by addition of HRP conjugated capture antibody where TMB substrate results in color read-out measured by absorbance reader at 450 nm.

Contents & Storage

Unopened test kits should be stored at 2-8°C upon receipt. The unopened kit may be stored at 2-8°C for up to 12 months. The shelf-life of the kit is shortened after opening the kit. The microtiter plate should be kept in a sealed bag with desiccants.

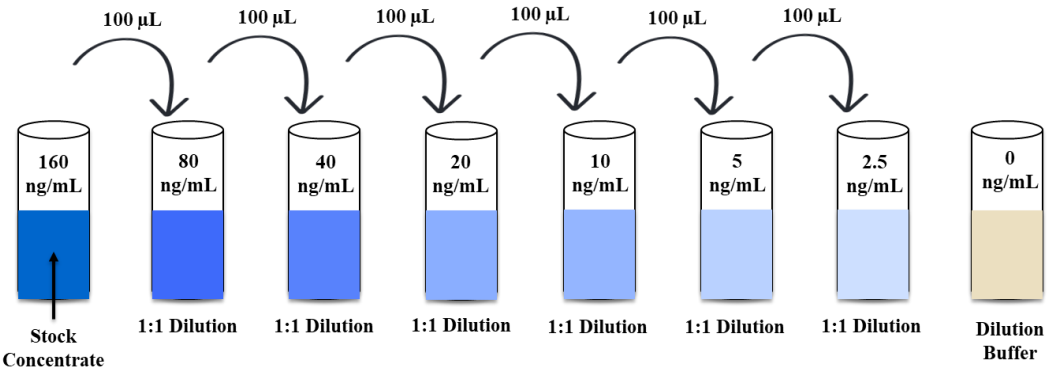
Component	Amount
96-strip well microplate – 12 modules of 8 wells each *Supplied in a foil pouch. Ready to use.*	96 (8x12) wells
Human AFP Standard (160 ng/mL) *Recombinant AFP in stabilized buffer at 100X concentration. Ready to use.*	2 vials, 200 µL each
Dilution Buffer contains PBS, BSA *Ready to use.*	25 mL
Detection antibody - Enzyme conjugate, HRP labeled *Supplied at 100X concentration. Ready to use.*	150 µL
TMB Solution *Ready to use.*	11 mL
Stop solution, contains acid *Ready to use.*	11 mL
Wash Buffer - contains detergent *Supplied at 20x concentration. Ready to use.*	2 bottles, 50 mL

Materials required but not supplied

- Microplate reader capable of endpoint measurements at 450 nm
- 10 µL to 1000 µL adjustable single/multi-channel micropipettes with disposable tips
- Plate washer – optional
- Distilled or deionized water for dilutions
- Mixer for plate shaking
- Data reduction software for 4PL curve fit (available at imcarebiotech.com)

Reagent Preparation

- All reagents should be allowed to reach room temperature (18-25 °C) before use.
- All reagents are provided with 2 mL tubes, we recommend spinning the tubes prior to use.
- **Standard and Samples:** The Human AFP Standard is provided as a 100X concentrate of 160 ng/mL (200 µL), corresponding to the top calibrator level of 16 ng/mL after dilution – referred to as level 7. To begin, dilute the 10X stock solution by mixing 100 µL of the 160 ng/mL AFP stock with 100 µL of the provided dilution buffer to 80 ng/mL (1:1) – referred to as level 6.
- Using 80 ng/mL as the new working solution, perform serial dilutions in dilution buffer to generate the standard curve. Mix thoroughly between each step to ensure homogeneity. Use the dilution buffer alone as the Zero calibrator (0 ng/mL). Visual representation to produce dilution series (below).
- The standards prepared at 10X along with samples will be further diluted 10x in the plate by first adding 90 µL dilution buffer to the plate followed by 10 µL standard/controls/QCs/sample.



- **Wash Buffer:** If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute the concentrated wash buffer 20-fold with distilled water (add 50 mL concentrated wash buffer into 950 mL distilled water). Prime plate washer with wash buffer.
- **HRP Labeled Detection Antibody Solution:** Prepare no more than 1 hour before experiment. Calculate the total volume of working solution required. (100 µL x number of wells x 1.1). Dilute 100x detection antibody 100-fold with dilution buffer and mix thoroughly (do not vortex).
- **TMB (Substrate) Solution:** Protect from light, use 100 µL per well.

Assay Procedure

Equilibrate all kit components and samples to room temperature prior to use. The approximate assay time is 2 hour 15 minutes.

1. Secure the desired number of coated wells in the holder.

2. **Prepare Plate & Sample addition:** Set standard and test sample wells on the pre-coated plate and record their positions. Add 90 µL dilution buffer solution to the bottom of all wells without scraping the side walls.
    a. Add 10 µL of the diluted standards into the standard wells.
    b. Add 10 µL 0 ng/mL calibrator and dilution buffer to blank wells.
    c. Add 10 µL of human serum samples into test sample wells.
    d. Place plate on plate mixer and mix for 30 seconds at 1000 RPM. It is very important to have complete mixing in this step.

3. **Sample Incubation:** Cover the plate and incubate at 37°C for 60 minutes.

4. **Wash:** Remove cover and wash plate 5 times with wash buffer and dry by flipping the plate and tapping on a dry paper towel.

5. **Detection Antibody:** Add 100 µL of 1X HRP labeled detection antibody solution to all wells without touching the sides of the wells.

6. **Detection Antibody Incubation:** Cover plate and incubate at 37°C for 45 minutes.

7. **Wash:** Remove cover and wash plate 5 times with wash buffer and dry by flipping the plate and tapping on a dry paper towel.

8. **Substrate:** Add 100 µL of TMB substrate to all the wells.

9. **Substrate Incubation:** Cover the plate and incubate at room temperature in dark for 20 minutes.

10. **Stop Solution:** Add 100 µL stop solution to all the wells.

11. **Measurement:** Read OD absorbance at 450 nm in microplate reader immediately after adding stop solution. **Calculation of Results**

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 percent of the mean value.
- Construct a standard curve by plotting the mean absorbance obtained for each standard calibrator against its concentration in ng/ml with absorbance on y-axis and concentration on x-axis.
- Drawing a best fit curve through the points of the graph, we recommend a 4PL Curve as best curve fit to calculate sample concentrations using mean absorbance value for each sample.
- If instructions in this protocol have been followed, samples have been diluted 1:10 (10 µL sample + 90 µL dilution buffer). Thus, concentrations read from the standard curve must be multiplied by the dilution factor of x10.
- Calculation of samples with a concentration exceeding 100 ng/mL may result in incorrect AFP levels. Such samples require further dilution according to expected human AFP values with dilution buffer in order to have accurate concentration evaluation.

Example of Standard Curve

Results of a typical standard run with OD readings at 450 nm shown on the Y-axis against AFP standard concentrations shown on 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 their own data and standard curve.

AFP (ng/mL)	OD at 450 nm
16.0	3.92
8.00	2.34
4.00	1.24
2.00	0.60
1.00	0.32
0.50	0.17
0.25	0.10
0.00	0.01

4PL Fitting Properties	Value
Top	9.77
Bottom	0.00
IC50	23.03
logIC50	1.36
Hill Slop	1.11
Span	9.77
RSQ	1.00
LOD	0.01

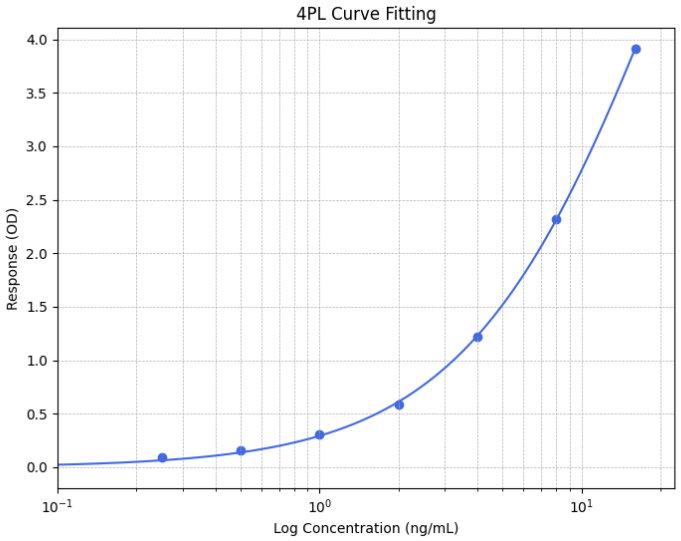


Figure 2: Representative standard curve for AFP ELISA kit.

Best Practices

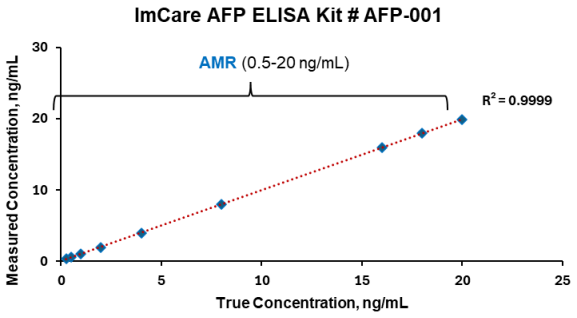
- Wear appropriate Personal Protective Equipment (PPE) when handing the samples and other kit components.
- A new standard curve must be created for each assay run, as conditions can vary from one assay to another for accurate sample analysis.
- Proper washing at each step is crucial. Inadequate or improper washing can result in false positive or false negative results. Ensure the assay wells are empty prior to the addition of follow-up reagents.
- Ensure wells are not left uncovered or allowed to dry for long periods.
- Bacterial or fungal contamination in either the screen samples or reagents, or cross-contamination between reagents, can lead to inaccurate results.

- Use disposable pipette tips, flasks, and glassware when possible. If reusable glassware is used, ensure it is thoroughly cleaned and rinsed to remove all detergent residues.

Performance Characteristics

Sensitivity: The limit of detection (LoD) of AFP is defined as the lowest concentration of analyte in a sample that can be distinguished from background noise with high confidence (Mean of blank + 3x standard deviation). LoD of the AFP ELISA kit was determined to be ~ 0.043 ng/mL. The limit of quantitation (LoQ) defined as Mean of blank + 10x standard deviation was determined to be ~ 0.055 ng/mL.

Analytical Measurement Range (AMR): AMR of the AFP ELISA kit defines the range over which the assay provides precise measurements of the analyte. The AMR was established by evaluating the linearity and recovery of known analyte concentrations across multiple assays runs. Measured concentrations were compared to expected values, and %recovery was assessed against an acceptance criterion of 80%–120%.AMR of the AFP ELISA kit was determined as 0.50 to 20.00 ng/mL, demonstrating high correlation (R^2 = 0.999) with true values ensuring reliable quantitation of the AFP.



True Value (ng/mL)	Measured Value (ng/mL)	% recovery
20.00	19.83	99.1
18.00	18.01	100.0
16.00	15.98	99.9
8.00	8.05	100.6
4.00	3.98	99.5
2.00	1.91	95.8
1.00	1.04	103.6
0.50	0.57	114.5
0.25	0.34	134.2

Figure 3: Representative linearity assessment curve for AFP ELISA kit.

Repeatability:

Five individual assays were performed to assess variance and %CV values, establishing the repeatability of the test. In each run, three independent measurements were taken for each test sample, which included two test samples and negative control (n=15 measurements). Test sample 1 consisted of human serum with approximately 20 ng/mL of AFP, and test sample 2 contained human serum with approximately 70 ng/mL of AFP, while the negative control contained human serum with approximately 1.0 ng/mL of AFP. 2 standard curves were run on each plate. Internal quality control (QC) levels - HQC,

MQC, and LQC - were included in each run to monitor assay performance, demonstrating inter-assay CV% values < 10%.

Variance Limits (±20% Tolerance Range)

Level	Observed Variance (S^2)	Acceptable Range for S^2
Level 1	2.452	1.962 - 2.943
Level 2	23.792	19.033 – 28.550

Coefficient of Variation (CV%) Limits (±20% Tolerance Range)

Level	Observed CV %	Acceptable Range for CV
Level 1	7.754	6.203 % - 9.305 %
Level 2	7.308	5.846 % – 8.769 %

Interference

The interference study demonstrated that the assay is generally robust against common interferents found in human serum; however, notable effects were observed with hemolysis and lipemia. Hemoglobin levels above 5 mg/dL and triglyceride concentrations exceeding 15 mg/mL caused mild to strong interference, particularly at higher analyte levels, and should be avoided. Free bilirubin showed mild interference at lower analyte levels, while conjugated bilirubin had no effect. Vitamins such as B6 and C caused mild interference, likely due to interaction with assay components. Other tested substances, including common medications and serum proteins, had no significant impact on assay performance. Overall, the assay met total allowable error (TEa) criteria under most conditions, supporting its reliability when used with properly handled samples. For optimal results, it is recommended to avoid using grossly hemolyzed or lipemic samples

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