



# Hepa AFP-IC

ELISA Kit for the detection of Alpha-Fetoprotein (AFP)

Immune Complexes in Hepatocellular Carcinoma (HCC)

**PRODUCT PROFILE**

**FOR RESEARCH USE ONLY**

**40-941-330002**



# Hepa AFP-IC

## PRODUCT PROFILE

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- Hepatocellular Carcinoma (HCC): from diagnosis to treatment
- Serological tumor markers for HCC diagnosis: the role of Alpha-Fetoprotein (AFP)
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## HEPATOCELLULAR CARCINOMA (HCC): from diagnosis to treatment

Hepatocellular Carcinoma (HCC) is one of the most common fatal cancers worldwide, the fourth one for incidence rate, in particular. It is the most frequent form of primary liver tumors. Mortality index for this kind of neoplasm is very high: most patients with HCC die within few years after diagnosis, and less than 5% of affected individuals survives to five years (1, 2).

Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) infections, exposure to Aflatoxin B and excessive intake of alcohol have been identified as the major risk factors for HCC development. HBV and, above all, HCV infections are the main causes of chronic liver disease, condition that strongly increases probability of hepatocytes neoplastic transformation. Every year, about 0.5% of chronic liver disease affected individuals develops HCC. This population is defined at high risk of HCC incidence. 300 millions or 170 millions people are HBV or HCV infected all over the world, respectively: this means that about 2,5 millions of HBV or HCV infected persons should be monitored for HCC growth.

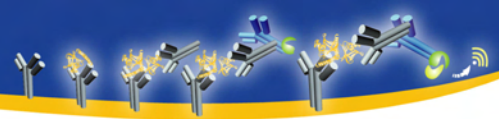
Cirrhosis is among the leading causes of death (the third one, precisely) and is also an important risk factor for HCC, irrespective of the etiology of the pathology. The annual risk of developing HCC among persons with cirrhosis is between 1 and 6% and detection of HCC at an early stage is mandatory to improve the poor prognosis of this disease. Risk patients including chronic carriers of hepatitis B and individuals with cirrhosis should be involved in screening programs (3,4).

The clinical outcome of patients with HCC is very poor, since diagnosis is usually established late, treatment is generally unsatisfactory and death often occurs within few years. Except for the presence of  $\alpha$ -fetoprotein (AFP) in serum, biochemical tests for the detection of HCC biomarkers are of little help in HCC diagnosis, as at the moment there is no tumor marker specific and sensitive enough to detect HCC in an early phase of development. Sensitivity and specificity of AFP serum level are limited. Only 50-70% of patients with HCC have elevated levels of AFP, whereas only approximately one-third of patients with small HCCs (< 3 cm) have a serum AFP above 200 ng/mL. At a cutoff point of 100 ng/mL, the sensitivity is only of 60%. Prothrombin Induced by Vitamin K Absence II (PIVKA-II) or Des- $\gamma$ -Carboxy Prothrombin (DCP) serum concentrations also increase in HCC patients and are sometimes used for HCC diagnosis. However, specificity and sensitivity is still low to give clinical significance to these assays (TAB.1) (5).

METHODS	PROS	CONS
<b>AFP DETERMINATION</b>	Non invasive (blood samples)	May lead to false positive (> 50 %) and false negative (>30 %) results
<b>DCP DETERMINATION</b>	Non invasive (blood samples)	Accurate only for late stage HCC
<b>COMPUTERIZED TOMOGRAPHY (CT) SCAN</b>	Non invasive	Accurate when neoplastic nodules are clearly evident (> 3 cm diameter)
<b>ULTRASOUNDS (US)</b>	Non invasive	Accurate when neoplastic nodules are clearly evident (> 3 cm diameter)
<b>MAGNETIC RESONANCE IMAGING (MRI)</b>	Non invasive	Accurate when neoplastic nodules are clearly evident (> 3 cm diameter)
<b>HISTOLOGY (LIVER BIOPSY)</b>	May confirm the diagnosis for lesions $\leq$ 2 cm	Invasive (liver biopsy) Expert pathologist needed

**TAB.1:** Comparison among different current methods of HCC diagnosis.

Detection of HCC at an early stage may significantly reduce mortality. This particular cancer develops in more than 90% of cases in patients affected by cirrhosis, a well defined high risk population, and mass screening could be justified since 1) the at-risk population can be easily identified, 2) tumor resection at an early stage can be curative, 3) HCC tends to grow slowly and stay confined to the liver. However massive screening should be justified only when sensitive and specific diagnostic procedures are available. Controlled trials for HCC screening/surveillance in high risk patients have been published and are in progress towards completion.



Ultrasonography and  $\alpha$ -Fetoprotein (AFP) monitoring are at present the only reasonable screening strategies to detect HCC nodules of small dimensions, but not totally satisfactory to justify massive screening programs (6, 7).

*It is therefore important to identify highly specific and sensitive markers in liver tissue, that can predict tumor and tumor staging in an identified at-risk population and in an early stage of development, in order to carry out a timely intervention.*

As far as prognosis is concerned, there are many molecular factors (TAB.2), which lately have been considered useful in HCC for therapy response, tumor recurrence and patient survival monitoring. Proliferation markers, cell cycle/apoptosis regulators, adhesion molecules, angiogenesis promoters are often considered as significant indicators of HCC prognosis, not always with clear results. However, neither one of them nor more put together, at present, provide all the features needed to become a clinical relevant prognostic marker (8).

<b>MARKERS</b>	<b>PROS</b>	<b>CONS</b>
<b>Proliferation markers (PCNA, Ki-67, Mcm2, Mib-1)</b>	Malignant grade evaluation Recurrence time prediction Long-term survival prediction	Low sensitivity Low specificity
<b>Nuclear morphology markers (AgNOR)</b>	Tumour stage evaluation Recurrence prediction Progression prediction	Low sensitivity Low specificity
<b>p53 and MDM2</b>	Long-term survival prediction	Low sensitivity Low specificity
<b>Cell Cycle regulators (CyclinE, Cdc2, p27)</b>	Recurrence time prediction Long-term survival prediction	Low sensitivity Low specificity
<b>Tumor promoters (ras, c-myc, c-erbB-2, EGF-R)</b>	Recurrence time prediction Progression prediction	Low sensitivity Low specificity
<b>Apoptosis regulators (Fas, Fas L)</b>	Recurrence time prediction	Low sensitivity Low specificity
<b>Adhesion molecules (E-cadherin, ICAM-1, CD44 isoforms)</b>	Tumour stage evaluation	Low sensitivity Low specificity
<b>Cancer invasion markers (MMP, uPA)</b>	Recurrence time prediction Long-term survival prediction	Low specificity
<b>Angiogenesis promoters (VEGF, bFGF)</b>	Long-term survival prediction	Low sensitivity Low specificity

**TAB.2:** Comparison among molecular biomarkers studied for prognostic relevance in HCC.

*It becomes of great importance for HCC control to discover high sensitive and specific prognostic biomarkers.*

Therapy of HCC is often just palliative care, based above all on controlling disease and helping patients live longer and better. Localized resectable liver cancer is cancer that can be removed during surgery. Surgical resection provides the best hope but is suitable only in few cases. Patients with small-localized tumors may have prolonged survival after resection, but the diagnosis is usually established late and liver tumor has frequently spread through



the liver. Patients with unresectable cancer may receive other treatments to extend life (TAB.3). HCC is not radiosensitive, and chemotherapy is usually unsuccessful. Moderately good long-term survival rates have been reported after liver transplantation (9).

TREATMENT	METHOD
<b>Radiofrequency ablation</b>	Cancer cells necrosis with heat
<b>Laser thermal ablation</b>	Cancer cells necrosis with laser-induced heat
<b>Percutaneous ethanol injection</b>	Cancer cells necrosis with ultrasound-guided alcohol infusion
<b>Hepatic arterial infusion</b>	Cancer cells death with anti-tumoral drug infusion into hepatic artery
<b>Chemoembolization</b>	Cancer cells death with anti-tumoral drug infusion into hepatic artery, followed by blocking the flow of blood through the artery

**TAB.3.** *Treatment choices other than surgery in HCC therapy. At this time, liver cancer can be cured only when it is found at an early stage and only if the patient is healthy enough to have an operation.*



## SEROLOGICAL TUMOR MARKERS FOR HCC DIAGNOSIS: THE ROLE OF ALPHA-FETOPROTEIN (AFP)

The most widely used serologic marker to detect HCC is Alpha-Fetoprotein (AFP), which is elevated (>20 ng/mL) in a wide number of HCC patients (30-60%). USA surveillance programs for at risk patients consist in fact in the periodical screening of the liver by ultrasound imaging and determination of serum alpha-fetoprotein (AFP) levels (3, 10, 11, 12). A considerable number of patients with chronic liver disease may have AFP levels in the range 20-200 ng/mL (13-15). In addition, AFP serum levels in cirrhotic and HCC patients often overlap and higher AFP cut off values (>100 ng/mL) have been used to increase specificity but reducing sensitivity to extremely low values (5-15%) (16). Although these limitations, nowadays the combination of ultrasound imaging and serum alpha-fetoprotein detection is considered the most effective method for liver chronic diseases monitoring and early detection of liver malignant lesions. Of course every technique which could improve diagnostic sensitivity and/or specificity in this field is of strategic importance patient management.

### Hepa AFP-IC

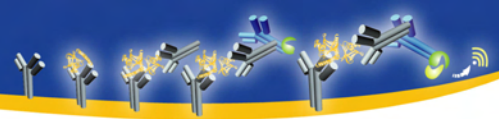
#### *ELISA Kit for the detection of Alpha-Fetoprotein (AFP) Immune Complexes in Hepatocellular Carcinoma (HCC)*

Early detection of HCC is still difficult due to the lack of adequate biomarkers to clearly differentiate HCC from benign liver lesion with high sensitivity and high specificity. Measurement of serum level of AFP, the only biomarker currently used in liver diseases diagnostic routine, provides an indication for the diagnosis and management of HCC (17). At a cut off level of 20 ng/mL, AFP shows 60-80% sensitivity but this value drastically diminishes in small HCC lowering to 40% (18). In addition in cases of benign hepatic parenchymal regeneration AFP may be elevated and overlaps the AFP levels in HCC. In fact, 15-58% of patients with chronic hepatitis and 11-47% with cirrhosis have AFP values in the range between 20 and 200 ng/mL (16, 18), so that the specificity of AFP test for HCC is very low. This represents a serious clinical problem, which confounds the interpretation of the test for diagnostic confirmation in HCC. Adoption of higher AFP cut off value to discriminate HCC from non malignant liver disease declines dramatically assay sensitivity to 5-15% (16). GenWay has developed an ELISA assay to assess the presence of AFP as circulating IgM immune complexes (IC) and the diagnostic accuracy for AFP-IgM IC, Free AFP (FAFP) and their combination have been evaluated in patients affected by HCC, cirrhosis, chronic hepatitis (CH) and in healthy subjects. Results provide the first evidence of AFP-IgM immune complexes occurrence in patients affected by liver diseases, with serum levels increasing according to the severity of liver injury. The gain achieved in the diagnosis of hepatocellular carcinoma by using the combination of both FAFP and its immune complexed form suggests that this approach might be useful in clinical practice for early diagnosis of primary liver cancer (19).

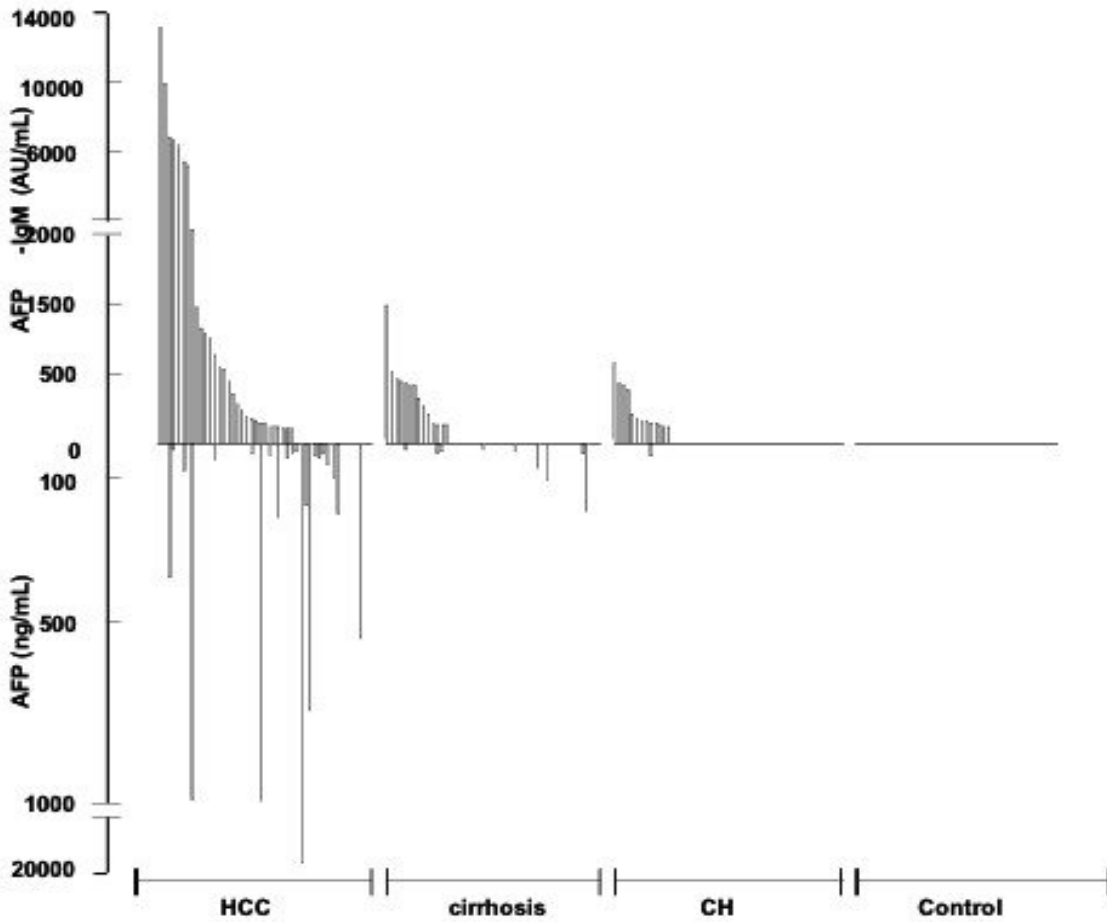
## DETAILED SEROLOGICAL AND MOLECULAR STUDIES

A total of 200 sera were analyzed in parallel by AFP-IgM IC and FAFP assays. FAFP cut off value was set at 20 ng/mL and for AFP-IgM IC at 120 AU/mL, in order to clearly discriminate normal healthy subjects from patients with liver diseases. The mean serum concentration of circulating AFP complexed with IgM in HCC (mean  $\pm$  SD: 1378.3  $\pm$  2935.7 AU/mL) was significantly higher than in the cirrhosis (129.8  $\pm$  261.4 AU/mL) and chronic hepatitis (80.9  $\pm$  168.9 AU/mL) groups ( $p < 0.01$ , Student's t test) (**FIG. 1**). The distribution of serum levels of AFP-IgM IC in HCC was significantly higher compared to cirrhosis and chronic hepatitis ( $p < 0.01$ , Mann-Whitney U test), and in terms of sensitivity AFP-IgM IC was detected above the cut off level in 30 out of 50 HCC patients (60%). FAFP was elevated only in 44% (23/50) of HCC patients (fig. 1). FAFP levels were above the cut off in 18% (9/50) and 2% (1/50) of cirrhosis and chronic hepatitis, respectively, compared to 28% (14/50) and 26% (13/50) of positive patients for AFP-IgM IC in the same groups (**Tab. 4**). The diagnostic accuracy measured as the area under the receiver operating characteristic (ROC) curve (AUROC) for FAFP and AFP-IgM IC was not significantly different for discriminating HCC from cirrhosis (0.648 vs. 0.676,  $p = 0.709$ ) (**FIG. 2A**) and from chronic hepatitis (0.712 vs. 0.699,  $p = 0.844$ ) (**FIG. 2B**). Since no correlation was found among the serum levels of the two biomarkers, co-determination of both markedly improved the indexes of diagnostic accuracy for liver cancer diagnosis. As reported in Table I, 82% (41/50) of HCC patients were positive for at least one marker. Furthermore the detection of both markers in parallel significantly increased the efficiency for discriminating HCC from cirrhosis (combination 71% vs. 66, 63% AFP-IgM IC and FAFP, respectively) and HCC from chronic hepatitis (combination 78% vs. 67, 71% AFP-IgM IC and FAFP, respectively).

This study demonstrates that AFP is detectable in serum of HCC patients coupled to IgM's to form circulating immune complexes. AFP-IgM IC levels measured by Hepa AFP-IC ELISA Kit are significantly elevated (> 120 AU/mL) in a much more elevated number of patients (30/50, 60%) compared to FAFP levels (22/50, 44%).



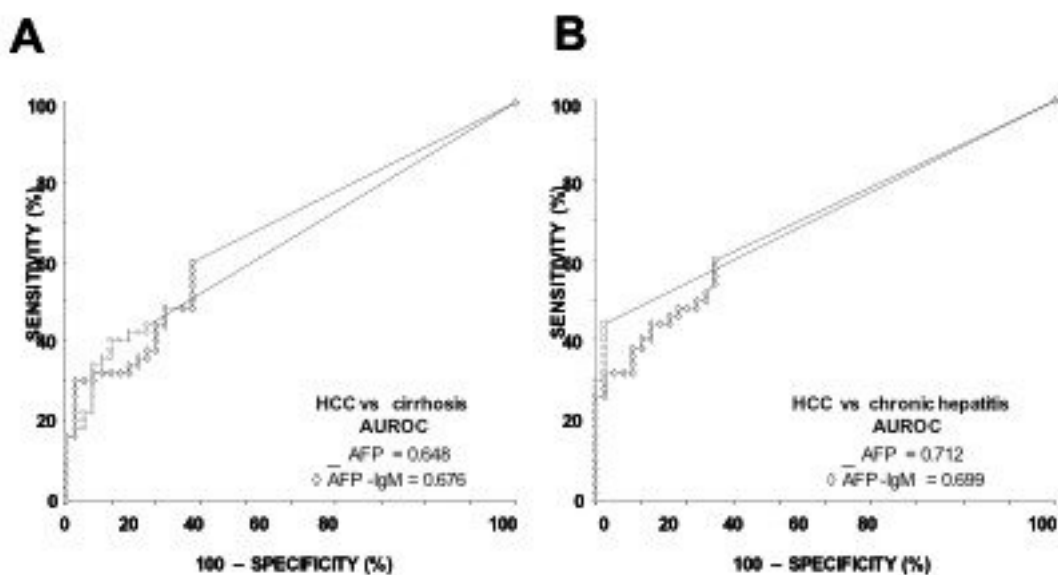
Furthermore, the co-determination of the two forms of circulating AFP significantly increases the efficiency for discriminating HCC from non neoplastic liver diseases, thus improving diagnosis of liver cancer (19).



**FIG.1:** Significantly elevated serum levels of AFP-IgM IC and FAFP in HCC (n= 50), cirrhosis (n= 50), chronic hepatitis (CH, n=50) and in healthy subjects (control, n=50). The significant levels (cut off) for AFP-IgM IC and FAFP were 120 AU/mL and 20 ng/mL, respectively.

BIOMARKER	Sensitivity	Specificity	PPV	NPV	Efficiency
<b>AFP-IgM cff=120 AU/mL</b>					
HCC vs control		<b>100</b>	<b>100</b>	<b>71</b>	<b>80</b>
HCC vs cirrhosis	<b>60</b>	<b>72</b>	<b>68</b>	<b>64</b>	<b>66</b>
HCC vs CH		<b>74</b>	<b>70</b>	<b>65</b>	<b>67</b>
<b>Free AFP cff=20 ng/mL</b>					
HCC vs control		<b>100</b>	<b>100</b>	<b>64</b>	<b>72</b>
HCC vs cirrhosis	<b>44</b>	<b>82</b>	<b>71</b>	<b>59</b>	<b>63</b>
HCC vs CH		<b>98</b>	<b>96</b>	<b>64</b>	<b>71</b>
<b>AFP-IgM cff=120 AU/mL &amp; Free AFP cff=20 ng/mL</b>					
HCC vs control		<b>100</b>	<b>100</b>	<b>85</b>	<b>91</b>
HCC vs cirrhosis	<b>82</b>	<b>64</b>	<b>73</b>	<b>77</b>	<b>71</b>
HCC vs CH		<b>80</b>	<b>83</b>	<b>80</b>	<b>78</b>

**Tab 4:** Comparison of the indexes of diagnostic accuracy for AFP-IgM IC, FAFP, both in combination, in differentiation of patients with HCC from those with cirrhosis, chronic hepatitis and healthy subjects (control). PPV (Positive predictive value) = True Positive (TP) / TP + False Positive (FP); NPV (Negative predictive value) = True Negative (TN) / False Negative (FN) + TN; Efficiency = TP + TN / TP+ FP + TN + FN.



**FIG.2:** ROC curves comparing the distribution of serum levels above the cutoff for AFP-IgM IC (cut off=120 AU/mL) and FAFP (cut off=20ng/mL) in HCC patients versus those with cirrhosis (**A**) and chronic hepatitis (**B**).

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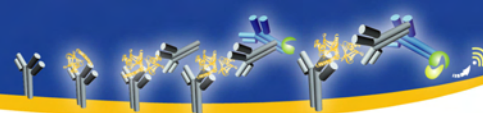




# Hepa AFP-IC

*ELISA Kit for the detection of Alpha-Fetoprotein (AFP)  
Immune Complexes in Hepatocellular Carcinoma (HCC)*

## INSTRUCTIONS FOR USE



## Hepa AFP-IC: Instructions for use

### PRODUCT CODE

**Hepa AFP-IC XG005**

### INTENDED USE

Hepa AFP-IC is an enzyme linked immunosorbent assay (ELISA) for the quantitative measurement of alpha-fetoprotein (AFP) immune complexes (AFP-IgM) in serum samples.

### EXPLANATION

AFP-IgM belongs to a novel generation of in vitro diagnostic methods based on the detection of AFP as circulating Immune Complexes (IC). AFP-IgM is a highly specific and sensitive ELISA assay for HCC detection designed to measure AFP-IgM in patient sera. The amount of AFP-IgM is expressed in Arbitrary Units (AU), using a AFP-IgM purified standard of human origin as reference. Studies have demonstrated that the measurement of AFP-IgM levels in HCC patients detects liver cancer with higher sensitivity with respect to AFP, the well-established serum HCC biomarker, without compromising specificity (1-3). Furthermore, the occurrence of the free and IgM-complexed form of the circulating AFP does not overlap, indicating that AFP-IgM is complementary to AFP and the use of both markers can increase sensitivity to over 80% (1-4).

### PRINCIPLE OF THE TEST

Standard Calibrators and specimens are simultaneously incubated with anti-AFP antibodies coated to the wells of a microtiter plate. The immune complexes AFP-IgM are detected by the addition of an enzyme conjugated secondary antibody and an enzyme substrate (ABTS). The developed color is proportional to the amount of the analyte in the sample.

### REAGENTS AND MATERIALS PROVIDED

- **XG005-PL:** 96 wells multi-strip Assay-Plate, pre-coated with affinity purified rabbit anti-AFP.
- **XG005-IC:** Human purified AFP Immune Complexes (AFP-IgM) Standard Calibrators Solution. (Red cap)
- **XG-EA:** Enzyme-conjugated secondary antibody solution. (Green cap)
- **XG-CH4:** Chromogen solution ABTS (2,2'-AZINO-bis(3-ETHYLBENZOTHAZOLINE-6-SULFONIC ACID)).
- **XG-SB:** Enzyme substrate solution. (Blue cap)
- **XG-DB:** Dilution Buffer.
- **XG-WB:** Washing Buffer.

### EQUIPMENT REQUIRED

- Microplate washer



- Microplate readers

## STORAGE

### ***Avoid repeated freeze and thaw cycles***

#### **Storage at 4°C:**

- **XG005-PL:** 96 wells multi-strip Assay-Plate, pre-coated with affinity purified rabbit anti-SCCA
- **XG-CH4:** Chromogen Solution\*†
- **XG-SB:** Enzyme substrate
- **XG-DB:** Dilution Buffer\*
- **XG-WB:** Washing Buffer\*

#### **Storage at -20°C:**

- **XG005-IC:** Standard solution
- **XG-EA:** Enzyme-conjugated secondary antibody solution

(\*) Must be used within one month of reconstitution

(†) Must be stored in a dark location

## EXPIRATION DATE

**SEE LABEL ON VIAL.**

## WARNINGS

### **POTENTIAL BIOHAZARDOUS MATERIALS:**

The standard calibrator **XG005-IC** is of **human origin**. The reference material was tested using an approved method of evaluation for the presence of the antibodies to HIV, antibodies to the hepatitis C virus and hepatitis B surface antigens, and found to be negative. **Since no test method can offer complete assurance that HIV, hepatitis B virus, hepatitis C virus, or other infectious agents are absent, all human sourced materials should be considered potentially infectious.** It is recommended that these reagents and human specimens be handled in accordance with the OSHA Standard on Bloodborne Pathogens (4). Biosafety Level 2 (5) or other appropriate biosafety practices (6, 7) should be used for materials that contain or are suspected of containing infectious agents.

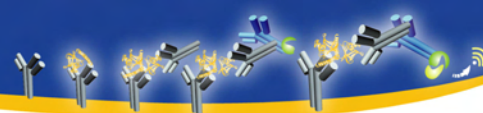
## SPECIMEN COLLECTION AND PREPARATION

The use of serum samples are recommended for the Hepa AFP-IC assay. Serum specimens should be collected aseptically, avoiding hemolysis if possible. Specimens should be stored at 2-8°C if the assay will be performed within 24 hours after collection. Specimens should be stored frozen if testing will occur after 24 hours.

If frozen, specimens should be mixed thoroughly after thawing to ensure consistency in the results. Avoid repeated freezing and thawing. Specimens showing particulate matter, erythrocytes, or turbidity must be clarified by centrifugation before testing.

## PROCEDURAL NOTES

1. Allow samples and reagents to reach room temperature prior to testing. Do not use water baths to thaw samples or reagents.
2. Mix samples and all reagents thoroughly before use.
3. Avoid excessive foaming of reagents. Also avoid exposure of reagents to



excessive heat or light during storage and incubation.

4. Avoid handling the tops of the wells both before and after filling.
5. Standards and samples should be assayed in duplicate.
6. Run a separate standard curve for each assay.
7. Use only coated wells from the same reagent batch for each assay. Also do not mix reagents from different kit lots.
8. Perform incubations in a sealed box containing a wet paper towel, in order to prevent evaporation.

## **INSTRUCTIONS FOR USE**

### **Reagents preparation**

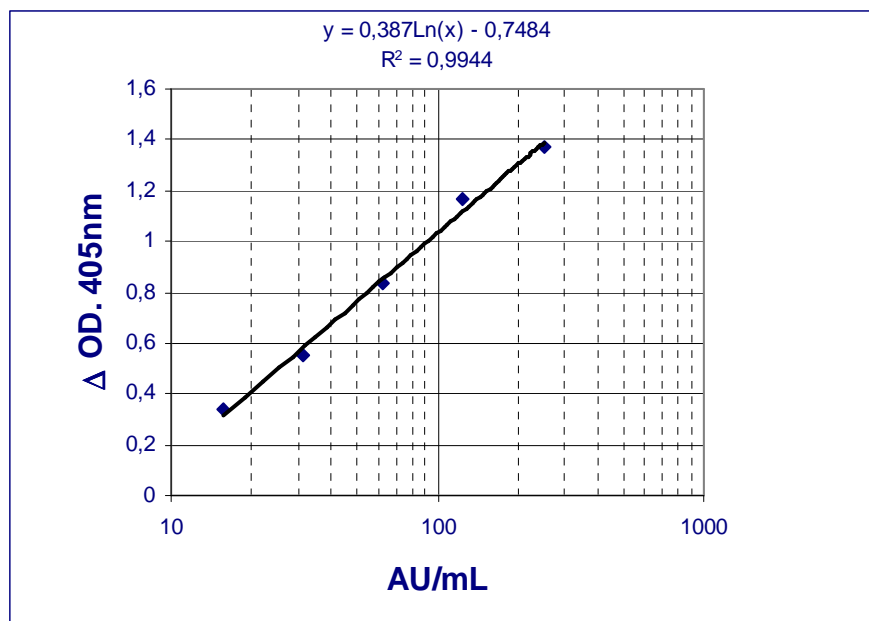
- Reconstitute **XG-CH4** chromogen solution with 20 mL of distilled water.
- Reconstitute **XG-DB** dilution buffer with 25 mL of distilled water.
- Reconstitute **XG-WB** washing buffer with 1 L of distilled water.
- Prepare the required amount of **XG-EA** enzyme-conjugated secondary antibody solution diluting 10-fold in reconstituted **XG-DB** dilution buffer.

### **Assay protocol**

1. Prepare assay reagents as described above.
2. Set up the microtiter plate with sufficient wells to enable the running of all required standards and samples.
3. Remove excess microtiter plate strips from the frame and store in the re-sealable foil bag with the desiccant provided.
4. Wash the microtiter plate strips three times with **XG-WB** washing buffer (300  $\mu$ L/well).
5. Dispense 100  $\mu$ L/well of standard calibrators (in duplicate) starting from 250 AU/mL and performing in-plate 2-fold serial dilutions to a final concentration of 15,6 AU/mL. Use **XG-DB** dilution buffer as diluent. Also dispense 100  $\mu$ L/well of **XG-DB** dilution buffer as blank, in duplicate.
6. Dispense 100  $\mu$ L/well of eight fold (1:8) dilution of samples (in duplicate). Use **XG-DB** dilution buffer as diluent.
7. Incubate 1hour at room temperature.
8. Wash six times with **XG-WB** washing buffer (300  $\mu$ L/well).
9. Add 100  $\mu$ L/well of diluted **XG-EA** enzyme-conjugated secondary antibody solution.
10. Incubate 1h at room temperature.
11. Wash six times with **XG-WB** washing buffer (300  $\mu$ L/well).
12. Prepare the required amount of chromogen-enzyme substrate solution adding 1  $\mu$ L of **XG-SB** enzyme substrate solution per 3 mL of **XG-CH4** chromogen solution. The chromogen-enzyme substrate solution must be used within 24 hours.
13. Apply 150  $\mu$ L/well of freshly prepared chromogen-enzyme substrate solution. Allow color to develop for 20 min. at 37°C in the dark and measure OD values of each well using an ELISA plate reader set to 405 nm. Plot the standard curve  $\Delta$ OD values as described in the next section: Processing of the results.



**FIG. 1:** Range of linearity of a typical standard curve for Hepa AFP-IC after 20 minutes of substrate incubation.



#### PROCESSING OF THE RESULTS

Average the duplicate readings for each standard calibrator and sample, and subtract the zero standard optical density. The standard calibrators may be used to construct a standard curve with values reported in AU/mL (see Fig. 1). This data deduction may be performed through computer methods using curve fitting routines or may also be manually deduced by plotting the absorbance values of the standard on the y-axis versus concentration on the logarithmic x-axis and drawing the standard curve. The immune complexes (AFP-IgM) concentration in the biological sample can be calculated directly from the standard curve by interpolation. The value obtained must be multiplied by the dilution factor.

#### QUALITY CONTROL

The intra- and inter-assay coefficients of variation were determined on 4 typical standard curves and the results were less than 10 %. For optimal performance, the absorbance of the zero standard should be  $< 0.2 OD_{405}$ . It is recommended that each laboratory assays appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

#### INTERPRETATION

The AFP-IgM cut-off value was 120 AU/mL for differentiating HCC from non-malignant chronic liver diseases (2,3).

#### SPECIFIC PERFORMANCE CHARACTERISTICS

##### AFP-IgM analytical range of the assay

The range of calibration is 15-250 AU/mL.

##### Hook effect

The Hook effect could occur for concentrations  $> 250 AU/mL$ . The sample with values above 250 AU/mL should be further diluted and re-measured.



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