



## HAV IgM IgM ANTIBODY TO HEPATITIS A VIRUS (HAV) ELISA KIT

### Two-step Incubation, Antibody Capture Principle

#### INTENDED USE

This kit is an enzyme-linked immunosorbent assay (ELISA) for qualitative determination of IgM-class antibodies to hepatitis A virus in human serum or plasma. It is intended for use in clinical laboratory for diagnosis of patients related to infection with hepatitis A.

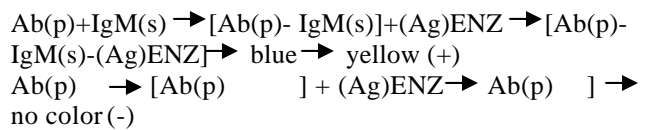
#### SUMMARY

Hepatitis A is a self-limited disease and chronic stage or other complications are rare. Infections occur early in life in areas where sanitation is poor and living conditions are crowded. With improved sanitation and hygiene, infections are delayed and consequently the number of persons susceptible to the disease increases. Because the disease is transmitted through the fecal-oral route in dense populated regions, an outbreak can arise from single contaminated source. The cause of hepatitis A is hepatitis A virus (HAV)-non enveloped positive strand RNA virus with a linear single strand genome, encoding for only one known serotype. HAV has four major, structural polypeptides and it localizes exclusively in the cytoplasm of human hepatocytes. The infection with HAV induces strong immunological response and elevated levels first of IgM and then IgG are detectable within a few days after the onset of the symptoms. The presence of anti-HAV IgM is an important serological marker for early detection and observation of the clinical manifestation of the disease. Increasing levels of anti-HAV IgM are detectable about three weeks after exposure with highest titer after four to six weeks later. Within six months after infection IgM concentration declines to non-detectable levels.

#### PRINCIPLE OF THE ASSAY

This HAV IgM ELISA kit is a solid phase, two-step incubation, antibody capture assay in which polystyrene microwell strips are pre-coated with antibodies directed to human IgM (anti- $\mu$ , chain). The patient's serum/plasma sample is added and during the first incubation, any IgM antibodies will be captured in the wells. After washing out all the other components of the sample and in particular IgG antibodies, the specific HAV IgM captured on the solid phase is detected by the addition of HAV antigens conjugated to horseradish peroxidase (HRP). During the second incubation, the conjugated antigens will specifically react only with the HAV IgM antibodies and after washing to remove unbound conjugates, Chromogen solutions are added to the wells. In presence of the (anti- $\mu$ )-(HAV-IgM)-(antigen-HRP) immunocomplex, the colorless Chromogens are hydrolyzed by the bound HRP conjugate to a blue colored product. The blue color turns yellow after stopping the reaction with

sulfuric acid. The amount of color can be measured and is proportional to the amount of antibody in the sample. Wells containing samples negative for HAV-IgM remain colorless. Assay Principle Scheme: Capture assay.



Incubation 1	Incubation 2	Immobilized Complex
Coloring	Results	
20 min.	→ 40 min.	→ 15 min.

Ab(p)<sup>-</sup> pre-coated anti-IgM antibodies (anti- $\mu$  chain); IgM(s)  
— HAV IgM antibodies in sample;  
(Ag)ENZ — HRP conjugated HAV antigens;

#### COMPONENTS

- **MICROWELL PLATE** 1 plate  
Blank microwell strips fixed on white strip holder. The plate is sealed in aluminum pouch with desiccant. Twelve 8-well strips per plate. Each well contains anti-IgM antibodies (anti- $\mu$  chain). The microwell strips can be broken to be used separately. Place unused wells in the plastic sealable storage bag together with the desiccant and return at 2~8 °C .
- **NEGATIVE CONTROL** 1 vial  
Yellowish liquid filled in vial with green screw cap  
0.5 ml per vial  
Protein-stabilized buffer tested non-reactive for HAV IgM. Preservatives: 0.1% ProClin 300. Ready to use as supplied. Once open, stable for one month at 2-8°C.
- **POSITIVE CONTROL** 1 vial  
Red color liquid filled in vial with red screw cap  
0.5 ml per vial  
Purified anti-HAV IgM antibodies diluted in protein-stabilized buffer containing preservatives: 0.1% ProClin 300. Ready to use as supplied. Once open, stable for one month at 2-8°C
- **HRP-CONJUGATE REAGENT** 2 vials  
Red liquid filled in a white vial with red screw cap.  
6 ml per vial  
Horseradish peroxidase-conjugated HAV antigens. Ready to use as supplied. Once open, stable for one month at 2-8°C.
- **STOCK WASH BUFFER** 1 bottle  
Colorless liquid. 50 ml per bottle  
PH 7.4 20<sup>x</sup> PBS(Containing Tween 20 as a detergent)
- **DILUTE BEFORE USE** -The concentrate must be diluted 1:20 with distilled/deionized water before use. Once diluted, stable for two weeks at room temperature or for one month at 2-8°C.
- **CHROMOGEN SOLUTION A** 1 vial  
Colorless liquid filled in white vial with green screw cap.  
7 ml per vial  
Urea peroxide solution. Ready to use as supplied. Once open, stable for one month at 2-8°C.
- **CHROMOGEN SOLUTION B** 1 vial  
Colorless liquid filled in the black vial with black screw cap.  
7 ml per vial

TMB solution. Tetramethylbenzidine dissolved in citric acid. Ready to use as supplied. Once open, stable for one month at 2-8°C.

**STOP SOLUTION** 1 vial  
Colorless liquid filled in white vial with white screw cap. 7ml per vial

Diluted sulfuric acid solution (2.0M H<sub>2</sub>SO<sub>4</sub>).  
**PLASTIC SEALABLE BAG** 1 Unit

For enclosing the strips not in use.

**CARDBOARD PLATE COVER** 2 Sheets

To cover the plates during incubation and prevent evaporation or contamination of the wells.

**PACKAGE INSERT** 1 Copy

#### **ADDITIONAL MATERIALS AND INSTRUMENTS REQUIRED BUT NOT PROVIDED**

1. Freshly distilled or deionized water.
2. Disposable gloves and timer.
3. Appropriate waste containers for potentially contaminated materials.
4. Disposable V-shaped troughs.
5. Dispensing system and/or pipette (single or multichannel). disposable pipette tips.
6. Absorbent tissue or clean towel.
7. Dry incubator or water bath, 37 ± 0.5°C .
8. Microshaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm.
10. Microwell aspiration/wash system.
11. Normal saline solution to dilute samples.

#### **SPECIMEN COLLECTION, TRANSPORTATION AND STORAGE**

1. Sample Collection: Either fresh serum or plasma samples can be used for this assay. Blood collected by venipuncture should be allowed to clot naturally and completely. Care should be taken to ensure that the serum samples are clear and not contaminated by microorganisms. Any visible particulate matters in the sample should be removed by centrifugation at 3000 RPM (round per minutes) for 20minutes at room temperature or by filtration on 0.22µ filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but highly lipaemic, icteric, or hemolized samples should not be used as they can give false results in the assay. Do not heat inactivate samples. This can cause sample deterioration.
2. Transportation and Storage: Store samples at 2-8°C. Samples not required for assay within 3 days should be stored frozen (-20°C or lower). Avoid multiple freeze-thaw cycles.
3. Sample preparation: Each sample must be diluted 1:1000 with normal saline.

#### **SPECIAL INSTRUCTIONS FOR WASHING**

1. A good washing procedure is essential to obtain correct and precise analytical data.
2. It is therefore recommended to use a good quality ELISA microplate washer, maintained at the best level of washing performances. In general, no less than 5 automatic washing cycles of 350-400µl/well are sufficient to avoid false positive reactions and high background.
3. To avoid contaminations of the plate with sample or HRP-conjugate, after incubation do not discard the content of

the wells but allow the plate washer to aspirate it automatically.

4. Anyway, we recommend calibrating the washing system on the kit itself in order to match the declared analytical performances. Assure that the microplate washer liquid dispensing channels are not blocked or contaminated and sufficient volume of Wash buffer is dispensed each time into the wells.

5. In case of manual washing, we suggest to carry out 5 cycles, dispensing 350-400µl/well and aspirating the liquid for 5 times. If poor results (high background) are observed, increase the washing cycles or soaking time per well.

6. In any case, the liquid aspirated out the strips must be treated with a sodium hypochlorite solution at a final concentration of 2.5% for 24 hours, before liquids are wasted in an appropriate way.

7. The concentrated Washing solution must be diluted 1:19 before use. For one plate, mix 50ml of the concentrate with 950ml of water for a final volume of 1000ml diluted Wash buffer. If less than a whole plate is used, prepare the proportional volume of solution.

#### **STORAGE AND STABILITY**

The components of the kit will remain stable through the expiration date indicated on the label and package when stored between 2-8 °C, do not freeze. To assure maximum performance of this HAV-IgM ELISA kit, protect the reagents from contamination with microorganism or chemicals during storage.

#### **PRECAUTIONS AND SAFETY**

This kit is intended FOR IN VITRO USE ONLY FOR PROFESSIONAL USE ONLY

The ELISA assay is time and temperature sensitive. To avoid incorrect result, strictly follow the test procedure steps and do not modify them.

1. Do not exchange reagents from different lots or use reagents from other commercially available kits. The components of the kit are precisely matched for optimal performance of the tests.
2. Make sure all the reagents are within the validity indicated on the kit box and of the same lot. Never use reagents beyond their expiry date stated on labels or boxes.
3. Allow the reagents and samples to reach room temperature (18-25°C) before use. Shake reagent gently before use.
4. Return at 2-8°C immediately after use.
5. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.
6. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.
7. Never allow the microplate wells to dry after the washing step. Immediately proceed to the next step. Avoid the formation of air bubbles when adding the reagents.
8. Avoid assay steps long time interruptions. Assure same working conditions for all the wells.
9. Calibrate the pipette frequently to assure the accuracy. Use different disposal pipette tips for each specimen and reagents in order to avoid cross-contaminations. Never pipette solutions by mouth.
10. The use of automatic pipettes and disposable tips is recommended.
11. Assure that the incubation temperature is 37 °C inside the incubator.
12. When adding samples, do not touch the well's bottom with the pipette tip.

13. When measuring with a plate reader, it is recommended to determine the absorbance at 450nm and with reference at 630nm.

14. All specimens from human origin should be considered as potentially infectious. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.

15. The pipette tips, vials, strips and sample containers should be collected and autoclaved for 1 hour at 121°C or treated with 10% sodium hypochlorite for 30 minutes to decontaminate before any further steps for disposal.

16. The Stop solution 2M H<sub>2</sub>SO<sub>4</sub> is a strong acid. CORROSIVE. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.

17. The enzymatic activity of the HRP-conjugate might be affected from dust, reactive chemical and substances like sodium hypochlorite, acids, alkalins etc. Do not perform the assay in the presence of these substances.

#### ASSAY PROCEDURE

**Step 1. Reagents Preparation:** Allow the reagents to reach room temperature. (18-25°C) Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the stock wash buffer 1:20 with distilled or deionized water. Use only clean vessels to dilute the Wash buffer. Mark three wells as Negative control (e.g. B1, C1, D1), two wells as Positive control (e.g. E1, F1) and one Blank. (A1-Neither samples or HRP-Conjugate should be added into the Blank well). Use only number of strips required for the test

**Step2 Sample Dilution:** Dilute the specimen 1:1000 with normal saline. Do not dilute the Controls, as they are ready for use as supplied

**Step3 Adding Sample:** Add 100µl of samples in each well and 100µl Positive and Negative controls and into their respective wells. **Note: Use a separate disposal pipette tip for each specimen, Negative Control, Positive Control to avoid cross-contamination**

**Step4 Sample Incubation:** Cover the plate with the plate cover and incubate for **20 minutes at 37°C**. It is recommended to use water tank to assure the temperature stability and humidity during incubation. If dry incubator is used, do not open the door frequently.

**Step5 Washing:** After the end of the incubation remove and discard the plate cover. Wash each well **5 times** with diluted Washing buffer. Each time allow the microwells to soak for **30-60 seconds**. After the final washing cycle, turn down the strips plate onto blotting paper or clean towel, and tap the plate to remove any remainders.

**Step6 Adding Conjugate:** Add 100µl of HRP-Conjugate Reagent into each well except the blank.

**Step7 HRP-Conjugate Incubation:** Cover the plate with the plate cover and incubate for **40 min at 37°C**.

**Step8 Washing:** Remove and discard the plate cover.

Aspirate the liquid and rinse each well **5 times** with Wash buffer (as step 5). After the final washing cycle, turn the strips plate and tap out any remainders.

**Step9 Coloring:** Add **50µl** of Chromogen A and **50µl** Chromogen B solution into each well including the **Blank** and mix gently. Incubate the plate at **37°C for 15 minutes avoiding light**. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in Positive control and HAV-IgM Positive sample wells.

**Step10 Stopping Reaction:** Using a multichannel pipette or manually add **50 µl** Stop solution into each well and mix gently. Intensive yellow color develops in Positive control and HAV IgM Positive sample wells.

**Step 11 Results Interpretation** Calibrate the plate reader with the Blank well and read the absorbance at **450nm**. If a dual filter instrument is used, set the reference wavelength at **630nm**. Calculate the Cut-off value and evaluate the results. (**Note:** read the absorbance within **5 minutes** after stopping the reaction)

#### INTERPRETATION OF RESULTS AND QUALITY CONTROL

Each microplate must be considered separately when calculating and interpreting results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample optical density (OD) value to the Cut-off value (C.O.) of the plate. If the Cut-off reading is based on Single filter plate reader, the results must be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on Dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls.

1. Calculation of Cut-off value (C.O.) = \*Nc x 2.1

\*Nc = the mean absorbance value for three negative controls Important: If the mean OD value of the negative control is lower than 0.05, take it as 0.05.

#### Example:

Calculation of Nc:

Well No	B 1	C 1	D
Negative controls OD value	0.02	0.012	0.016

Nc=0.016 (Nc is lower than 0.05, so take it as 0.05)

Calculation of Cut-off value: (C.O.)= 0.05 x 2.1= 0.105

If one of the Negative control values does not meet the Quality Control Range specifications, it should be discarded, and the mean value is calculated again using the remaining two values. If more than one control OD value does not meet the Quality Control Range specifications, the test is invalid and must be repeated.

#### **2. Quality control Range.**

1. The absorbance of the Blank well, which contains only Chromogens and Stop solution, is less than 0.080 at 450 nm.
2. The absorbance value OD of the Positive control must be equal to or greater than 0.800 after blanking.
3. The absorbance value OD of the Negative control must be less than 0.100 after blanking.

#### **3. Interpretations of results:**

( S = the individual absorbance (OD) of each specimen)

**Negative Results (S/C.O. <1):** samples giving absorbance less than the cut-off value are negative for this assay, which indicates that no IgM class antibodies to HAV have been detected with this HAV IgM ELISA kit. Therefore, there are no indications for recent infection and the patient is probably not infected with HAV.

**Positive Results (S/C.C.≥1):** samples giving an absorbance greater than or equal to the cut-off value are initially positive, which indicates that IgM class antibodies to HAV have probably been detected with this HAV IgM ELISA kit. Retesting in duplicates of any reactive sample is recommended. Repeatedly reactive samples can be considered positive for IgM antibodies to HAV and therefore there are indications for possible current infection with hepatitis A virus.

**Borderline: (S/CO = 0.9-1.1)** Samples with absorbance to Cut-off ratio between 0.9 and 1.1 are considered borderline. Retesting of these samples in duplicates is recommended positive for IgM antibodies to HAV.

Follow up and supplementary testing with other HAV tests is required to confirm the infection state.

### TEST PERFORMANCE AND EXPECTED RESULTS

This kit was standardized against Reference Standard from the Reference Laboratory for Immunology Product.

**Analytical Endpoint Sensitivity** (lower Detection limit) 0.1 NCU.

The clinical sensitivity of this kit was evaluated by testing samples obtained from 739 (288 children and 451 adults) individuals suspected for infection with HAV during outcome. Another group of samples from 1950 healthy blood donors was tested in order to determine the out in direct comparison with another commercially available HAV IgM ELISA kit used as a confirmation assay. The evaluation results are given below. Results obtained in individual laboratories may differ.

	CHILDREN				SENSITIVITY
	Tested	-	+	Confirmed	
Inapparent infection	148	3	145	145	100%
Anicteric /icteric	140	15	35	35	100%
TOTAL	288	18	180	180	100%

	ADULTS				SENSITIVITY
	Tested	-	+	Confirmed	
Inapparent infection	238	192	46	46	100%
Anicteric /icteric	213	120	190	190	100%
TOTAL	451	312	236	236	100%

### Clinical Specificity:

	CHILDREN			ADULTS		
	Tested	specificity	False positive	Tested	specificity	False positive
Healthy individuals	1220	>99%	5	730	>99%	4

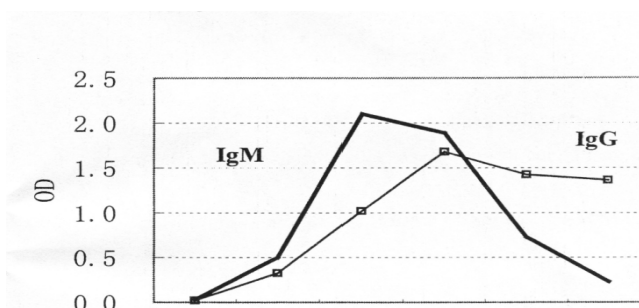
Sample dilution index	OD
1:1	2.543
1:500	2.234
1:5000	1.042
1:50 000	0.673
1:500 000	0.036

Days since infection	OD
0-20	0.031
21-40	0.521
41-50	2.143
51-60	1.890
61-80	0.736
81-100	0.231

**Linearity of sample dilution-undiluted sample:**

**Follow –up of individuals infected with HAV:**

**Follow –up of individuals infected with HAV:**



**Comparisons of this HAV IgM ELISA kit performance characteristic in follow up of individuals infected with HAV and reference anti-HAV IgG ELISA kit (squares).**

Reproducibility	Within run			Between run		
	Test	MeanOD	CV%	Test	MeanOD	CV%
Weak positive	10	0.428	8.1	10	0.395	8.5
Moderate positive	10	0.916	7.3	10	0.856	7.6
Strong positive	10	2.172	4.6	10	2.982	5.1

### Analytical Specificity:

1. No cross reactivity observed with samples from patients with HBV, HCV, HIV, CMV, and TP.
2. No interferences from elevated levels of rheumatoid factors up to 2000U/ml were observed during clinical testing.
3. The assay performance characteristics are unaffected from

elevated concentrations of bilirubin, hemoglobin, and triolein.

### LIMITATIONS

1. Non-repeatable reactive results may be obtained with any ELISA test due to the general characteristics of this type of assays. A negative result with an antibody detection test does not preclude the possibility of infection. Antibodies may be undetectable during the early stages of the disease and in some immunosuppressed individuals.
2. Any positive result must be interpreted in conjunction with the patient clinical information and other laboratory results
3. Common sources for mistakes: kits beyond the expiry date, bad washing procedures and wrong washing buffer concentration, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
4. The prevalence of the marker will affect the assay's predictive values.
5. False negative results can occur from inhibition of specific IgM in the presence of high titers of specific IgG. The removal of IgG can be helpful to prevent false negative results and methods for this are given elsewhere.

### REFERENCES:

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4. Berge JJ et al. The cost of hepatitis A infections in American adolescents and adults in 1997. *Hepatology*, 2000, 31(2): 469-473.
5. Burke DS, Graham RR, and Heisey GB. Hepatitis A virus in primates outside captivity. *Lancet*, 1981, 2:928.45(RR15):1-30.

### SUMMARY OF THE ASSAY PROCEDURE:

<b>Dilute the sample with normal saline</b>	<b>1:1000</b>
<b>Add sample</b>	<b>100 µl</b>
<b>Incubate</b>	<b>20 minutes</b>
<b>Wash</b>	<b>5times</b>
Add HRP-Conjugate	100µl
<b>Incubate</b>	<b>40min.</b>
<b>Wash</b>	<b>5 times</b>
<b>Coloring</b>	<b>500 A + 5091 B</b>
<b>Incubate</b>	<b>15 minutes</b>
<b>Stop the reaction</b>	<b>50µl stop solution</b>
<b>Read the absorbance</b>	<b>450nm or 450/630 nm</b>

### SUMMARY OF THE MAJOR COMPONENTS OF THE KIT:

Note: the components of individual kits are not interchangeable

<b>Microwell plate</b>	<b>Twelve 8-well strips</b>
<b>Negative control</b>	<b>One/ 0.5ml</b>
<b>Positive Control</b>	<b>One/ 0.5ml</b>
<b>HRP-Conjugate</b>	<b>Two/ 6ml</b>
<b>Wash Buffer</b>	<b>One/ 50ml</b>
<b>Chromogen A/B Stop solution</b>	<b>One each/7ml</b>

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