DRG® HAV IgM ELISA (EIA-4432)

REVISED 16 SEPT. 2010 RM (VERS. 1.1) **USA: RUO**

Please use only the valid version of the package insert provided with the kit.

1 INTENDED USE

Enzyme ImmunoAssay (ELISA) for the determination of IgM class antibodies to Hepatitis A Virus in human plasma and sera with the "capture" system.

For Research Use Only.

2 PRINCIPLE OF THE TEST

The assay is based on the principle of "IgM capture" where IgM class antibodies in the sample are first captured by the solid phase coated with anti hIgM antibody.
After washing out all the other components of the sample and in particular IgG antibodies, the specific IgM captured on the solid phase are detected by the addition of a purified preparation of inactivated HAV, labelled with an antibody conjugated with peroxidase (HRP).
After incubation, microwells are washed to remove unbound conjugate and then the chromogen/substrate is added.
In the presence of peroxidase the colorless substrate is hydrolyzed to a colored end-product, whose optical density may be detected and is proportional to the amount of antibodies to HAV present in the sample.

3 COMPONENTS

The kit contains reagents for 96 tests.

1. **Microplate:** MICROPLATE:
   12 strips of 8 breakable wells coated with anti human IgM antibody, affinity purified, and sealed into a bag with desiccant. Bring the microplate to room temperature before opening the bag. Unused strips have to be returned into the bag and the bag has to be sealed and stored back to 2-8°C, in presence of the desiccant.
2. **Negative Control:** CONTROL –
   1 x 2.0 ml/vial. *Ready to use* control. It contains goat serum proteins, 10 mM tris buffer pH 6.0+/-0.1, 0.1% Tween 20, 0.09% sodium azide and 0.1% Kathon GC as preservatives.
   The negative control is colourless.
3. **Positive Control:** CONTROL +
   1 x2.0 ml/vial. *Ready to use* control. It contains anti HAV IgM, goat serum proteins, 10 mM tris buffer pH 6.0+/-0.1, 0.1% Tween 20, 0.09% sodium azide and 0.1% Kathon GC as preservatives.
   The positive control is green colour coded.

4. **Calibrator:** CAL…
   N° 1 *lyophilized* vial. To be dissolved with EIA grade water as reported in the label.
   It contains anti HAV IgM, 2% BSA, 10 mM tris buffer pH 6.0+/-0.1, 0.09% sodium azide and 0.1% Kathon GC as preservatives.
   Note: The volume necessary to dissolve the content of the vial may vary from lot to lot. Please use the right volume reported on the label.
5. **Wash buffer concentrate:** WASHBUF 20X
   1 x 60 ml/bottle. 20x concentrated solution.
   Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0+/-0.2, 0.05% Tween 20 and 0.05% Kathon GC.
6. **Enzyme conjugate 20X:** CONJ
   1 x 0.8 ml/vial. 20X concentrated solution.
   It contains Horseradish peroxidase conjugated antibody specific to HAV in presence of 10 mM Tris buffer pH 6.8+/-0.1, 2% BSA, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives.
7. **HAV Antigen:** Ag HAV
   1 x16 ml/vial. *Ready-to-use* solution.
   It contains inactivated and stabilised HAV in presence of 10 mM Tris buffer pH 6.8+/-0.1, 2% BSA, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives. The reagent is red colour coded.
8. **Specimen Diluent:** DILSPE
   2 x 60.0 ml/vial. Proteic buffered solution for the dilution of samples.
   It contains goat serum proteins, 10 mM tris buffer pH 6.0+/-0.1, 0.1% Tween 20, 0.09% sodium azide and 0.1% Kathon GC as preservatives. The reagent is blue colour coded.
9. **Chromogen/Substrate:** SUBS TMB
   1 x 16 ml/vial. It contains a 50 mM citrate-phosphate buffered solution at pH 3.5-3.8, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide of H_2O_2.
   Note: To be stored protected from light as sensitive to strong illumination.
10. **Sulphuric Acid:** H_2SO_4 0.3 M
    1 x 15 ml/vial. It contains 0.3 M H_2SO_4 solution. **Attention:** Irritant (Xi R36/38; S2/26/30)
11. **Plate sealing foils**
12. **Package insert**

4 MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes of 10µl, 100µl and 1000µl and disposable plastic tips.
2. EIA grade water (double distilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator (dry or wet) set at +37°C (+/-0.1°C tolerance).
6. Calibrated ELISA microwell reader with 450nm (reading) and if possible with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.

8. Vortex or similar mixing tools.

5 SPECIMEN: PREPARATION AND RECOMMENDATIONS

1. Blood is drawn aseptically by venepuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.
2. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and reading is strongly recommended.
3. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
4. Sera and plasma can be stored at +2-8°C for up to five days after collection. For longer storage periods, samples can be stored frozen at –20°C for several months. Any frozen samples should not be freeze/thawed more than once as IgM antibodies may get damaged and as this procedure may generate particles that could affect the test result.
5. If particles are present, centrifuge at 2.000 rpm for 20 min or filter using 0.2-0.8 µ filters to clean up the sample for testing.

6 PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 3 months.

1. Antibody coated microwells:
Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant has not turned dark green, indicating a defect in conservation. In this case, call customer service. Unused strips have to be placed back into the aluminium pouch, with the desiccant supplied, firmly zipped and stored at +2–8°C. When opened the first time, unused strips are stable until the humidity indicator inside the desiccant bag turns from yellow to green.

2. Negative Control:
Ready to use. Mix well on vortex before use.

3. Positive Control:
Ready to use. Mix well on vortex before use. Handle this component as potentially infectious, even if HAV, eventually present in the control, has been chemically inactivated.

4. Calibrator:
Add the volume of ELISA grade water, reported on the label, to the lyophilised powder; let fully dissolve and then gently mix on vortex. The solution is not stable. Store the Calibrator frozen in aliquots at –20°C.
Note: When dissolved the Calibrator is not stable. Store in aliquots at –20°C.

5. Wash buffer concentrate:
The whole content of the concentrated solution has to be diluted 20x with bidistilled water and mixed gently end-over-end before use.
Once diluted, the wash solution is stable for 1 week at 2–8°C. During preparation avoid foaming as the presence of bubbles could impact on the efficiency of the washing cycles.

Note: Once diluted, the wash solution is stable for 1 week at +2–8° C.

6. Enzyme conjugate:
20X preparation. Mix well on vortex.
Avoid contamination of the liquid with oxidizing chemicals, dust or microbes when the reagent is aspirated to be used.

7. HAV Antigen:
Ready to use. Mix well on vortex before use.
Handle this component as potentially infectious, even if HAV has been chemically inactivated.

6+7. HAV Antigen/Antibody complex:
About 5-10 min before its use, dilute the 20X concentrated Enzyme Conjugate in the proper volume of HAV Antigen, necessary for the assay. Then mix on vortex carefully.

Example: To run 2 strips, dilute 100 µl Enzyme Conjugate 20X into 2 ml of HAV Antigen.
Note: *This immunocomplex is not stable; discard the exceeding volume.*

8. Sample Diluent:
Ready to use. Mix well on vortex before use.

9. Chromogen/Substrate:
Ready to use. Mix well on vortex before use.
Avoid contamination of the liquid with oxidizing chemicals, air-driven dust or microbes. Do not expose to strong light, oxidizing agents and metallic surfaces. If this component has to be transferred use only plastic, and if possible, sterile disposable container.

10. Sulphuric Acid:
Ready to use. Mix well on vortex before use. Attention: Irritant (Xi R36/38; S2/26/30)
Legenda: R 36/38 = Irritating to eyes and skin.
S 2/26/30 = In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

7 INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume (tolerance +/-5%) required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained. Decontamination of spills or residues of kit components should also be carried out regularly. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-2%.
2. The ELISA incubator has to be set at +37°C (tolerance of +/-0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.

3. The ELISA washer is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimized using the kit controls and reference panels, before using the kit for routine laboratory tests. 4-5 washing cycles (aspiration + dispensation of 300µl/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls and well characterized negative and positive reference samples, and check to match the values reported below in the section O'. Regular calibration of the volumes delivered by, and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.
4. Incubation times have a tolerance of +/-5%.
5. The ELISA reader has to be equipped with a reading filter of 450nm and ideally with a second filter (620-630nm) for blanking purposes Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure the correct optical density is measured. It should be regularly maintained according to the manufacturer 's instructions.
6. When using an ELISA automated work station, all critical steps (dispensation, incubation, washing, reading, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the section O "Internal Quality Control'. The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader.
   In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells. The use of ELISA automated work stations is recommended when the number of samples to be tested exceed 20-30 units per run.
7. DRG´s customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

8 PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label (primary container). Do not use the device if expired.
2. Check that the liquid components are not contaminated by visible particles or aggregates. Check that the Chromogen/Substrate is colourless or pale blue by aspirating a small volume of it with a sterile plastic pipette. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box (primary container). Check that the aluminium pouch, containing the microplate, is not punctured or damaged.
3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dissolve the Calibrator as described above and gently mix.
5. Allow all the other components to reach room temperature (about 1 hr) and then mix gently on vortex all liquid reagents.
6. Set the ELISA incubator at +37°C +/-0.1°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
7. Check that the ELISA reader is turned on or ensure it will be turned on at least 20 minutes before reading.
8. If using an automated work station, turn on, check settings and be sure to use the right assay protocol.
9. Check that the micropipettes are set to the required volume.
10. Check that all the other equipment is available and ready to use.
11. In case of problems, do not proceed further with the test and advise the supervisor.

9 ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

1. Dilute samples 1:101 by dispensing first 10 µl sample and then 1 ml Sample Diluent into a dilution tube; mix gently on vortex.
2. Place the required number of Microwells in the microwell holder. Leave the 1st well empty for the operation of blanking.
3. Dispense 100 µl Negative Control in triplicate, 100 µl Positive Control in single and 100 µl Calibrator in duplicate in proper wells.
   Do not dilute controls and the calibrator as they are ready to use!
4. Dispense 100 µl diluted samples in the proper sample wells and then check that all the samples wells are blue coloured and that controls and calibrator have been dispensed.
5. Incubate the microplate for **60 min at +37°C**.
   Important note: *Strips have to be sealed with the adhesive sealing foil, supplied, only when the test is carried out manually. Do not cover strips when using ELISA automatic instruments.*
6. About 5-10 minutes before use, prepare the HAV Antigen/Antibody immunocomplex as described previously.
7. Wash the microplate with an automatic washer by delivering and aspirating 300 µl/well of diluted washing solution as reported previously (section 9.3).
8. Pipette 100 µl HAV Antigen/Antibody complex into each well, except the 1st blanking well, and cover with the sealer. Check that all wells are red coloured, except A1.
   Important note: *Be careful not to touch the plastic inner surface of the well with the tip filled with the Enzyme Conjugate. Contamination might occur.*
9. Incubate the microplate for **60 min at +37°C**.
10. Wash microwells as in step 7.
11. Pipette 100 µl Chromogen/Substrate mixture into each well, the blank well included. Then incubate the microplate at **room temperature (18-24°C) for 20 minutes**.
    Important note: *Do not expose to strong direct illumination. High background might be generated.*
12. Pipette 100 µl Sulphuric Acid into all the wells to stop the enzymatic reaction using the same pipetting sequence as in step 10. Addition of acid will turn the positive control and positive samples from yellow to blue.
13. Measure the colour intensity of the solution in each well, as described in section 9.5, at 450nm filter (reading) and possibly at 620-630nm (background subtraction), blanking the instrument on A1.

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Important notes:

1. If the second filter is not available ensure that no finger prints are present on the bottom of the microwell before reading at 450nm. Finger prints could generate false positive results on reading.
2. Reading has to be carried out just after the addition of the Stop Solution and anyway not any longer than 20 minutes after its addition. Some self oxidation of the chromogen can occur leading to high background.

10 ASSAY SCHEME

Controls & samples diluted 1:101	**100 µl**
1st incubation	**60 min**
Temperature	+37°C
Washing	4-5 cycles
HAV & Tracer	100 µl
2nd incubation	**60 min**
Temperature	+37°C
Washing	4-5 cycles
TMB/H_2O_2 mix	100 µl
3rd incubation	**20 min**
Temperature	r.t.
Sulphuric Acid	100 µl
Reading OD	450nm

An example of dispensation scheme is reported in the table below:

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S2										
B	NC	S3										
C	NC	S4										
D	NC	S5										
E	CAL	S6										
F	CAL	S7										
G	PC	S8										
H	S1	S9										

Legenda: BLK = Blank, NC = Negative Control, CAL = Calibrator, PC = Positive Control, S = Sample

11 CALCULATION OF THE CUT-OFF

The test results are calculated by means of the mean OD450nm value of the Negative Control (NC) and a mathematical calculation, in order to define the following cut-off formulation:

Cut-Off = NC + 0.250

The value found for the test is used for the interpretation of results as described in the next paragraph.

Important note: *When the calculation of results is performed by the operating system of an ELISA automated work station, ensure that the proper formulation is used to calculate the cut-off value and generate the correct interpretation of results.*

12 REFERENCES / LITERATURE

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