

Revised 26 Jan. 2006

1 INTENDED USE

Enzyme Immunoassay (ELISA) for the determination of IgM antibodies to Hepatitis E Virus in human plasma and sera. The kit may be used for the follow-up of HEV-infected patients.

For “in vitro” diagnostic use only.

2 INTRODUCTION

Hepatitis E Virus or HEV is a recently discovered agent of enterically transmitted viral hepatitis. HEV is an unenveloped single-strand RNA virus structurally similar to Calicivirus and is found in the stool of infected patients. HEV is a serious problem in many developing countries and its first outbreak was reported in 1955 in New Delhi, India. Hepatitis E has never been associated with chronic infection; however a high case-fatality rate has been found among pregnant women. The cloning and sequencing of HEV genome have lead to the development of serological tests for the detection of anti HEV antibodies. These tests are based on synthetic immunodominant antigens derived from conservative regions of the virus.

Tests for IgM are used to determine the nature of the infective agent in patients showing symptoms of hepatitis, in order to rule out the possibility of other most severe viral infections (HBV, HDV, HCV).

3 PRINCIPLE OF THE TEST

Microplates are coated with HEV-specific synthetic antigens encoding for conservative and immunodominant determinants derived from Mexican and Burma virus strains. The solid phase is first treated with the diluted sample and anti HEV IgM are captured, if present, by the antigens adsorbed on wells. After washing out all the other components of the sample, in the 2nd incubation bound anti HEV IgM antibodies are detected by the addition of polyclonal specific anti hIgM antibodies, labeled with peroxidase (HRP). The enzyme captured on the solid phase, acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the amount of anti HEV antibodies present in the sample.

A cut-off value let optical densities be interpreted into HEV antibody negative and positive results.

Neutralization of IgG anti-HEV and Rheumatoid Factor, carried out directly in the well, is performed in the assay in order to block such kind of interferences.

4 COMPONENTS

The kit contains reagents for 96 tests.

1. **Microplate** MICROPLATE 1 microplate, 12 strips of 8 microwells coated with HEV specific synthetic antigens derived from ORF2 and ORF3 regions. Plates are sealed into a bag with desiccant. Allow the microplate to reach room temperature before opening; reseal unused strips in the bag with desiccant and store at 4°C.
2. **Negative Control** CONTROL - 1 x 2.0ml/vial; Ready to use control. It contains 1% goat serum proteins, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives. The negative control is yellow colour coded.

Revised 26 Jan. 2006

3. **Positive Control** CONTROL + 1 x 2.0ml/vial; Ready to use control.
It contains 1% goat serum proteins, human anti HEV IgM, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives.
The Positive Control is dark green colour coded.
4. **Wash buffer concentrate** WASHBUF 20X ; 1 x 60ml/bottle. 20x concentrated solution.
Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0+/-0.2, 0.05% Tween 20 and 0.1% Kathon GC.
5. **Enzyme Conjugate** CONJ ; 1 x 16ml/vial. Ready to use and red colour coded reagent.
It contains Horseradish Peroxidase conjugated goat polyclonal antibodies to human IgM, 5% BSA, 10 mM Tris buffer pH 6.8+/-0.1, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives.
6. **Chromogen/Substrate** SUBS TMB ; 1 x 16ml/vial. Ready-to-use component.
It contains 50 mM citrate-phosphate buffer pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide or H₂O₂.
Note: To be stored protected from light as sensitive to strong illumination.
7. **Sulphuric Acid** H₂SO₄ 0.3 M ; 1 x 15ml/vial.
It contains 0.3 M H₂SO₄ solution.
Attention: Irritant (Xi R36/38; S2/26/30)
8. **Specimen Diluent:** DILSPE ; 2 x 60ml/vial.
It contains 2% casein, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.1% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives.
To be used to dilute the sample.
9. **Neutralizing Reagent:** SOLN NTR ; 1x8ml/vial. Ready-to-use Reagent.
It contains goat anti hIgG, 2% casein, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.1% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives.
10. **Plate sealing foils** 2 x
11. **Package insert** 1 x

5 MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (200ul and 10ul) and disposable plastic tips.
2. EIA grade water (bidistilled or deionized, 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 capable to provide a temperature of +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and possibly with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

6 WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.

Revised 26 Jan. 2006

2. All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
3. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
4. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-borne microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen/Substrate from strong light and avoid vibration of the bench surface where the test is undertaken.
5. Upon receipt, store the kit at 2-8°C into a temperature controlled refrigerator or cold room.
6. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
7. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
8. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.
9. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
10. Do not use the kit after the expiration date stated on the external container and internal (vials) labels.
11. Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
12. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
13. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before waste. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 minutes.
14. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
15. The Sulphuric Acid is an irritant. In case of spills, wash the surface with plenty of water
16. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

7 SPECIMEN: PREPARATION AND RECOMMENDATIONS

1. Blood is drawn aseptically by venipuncture 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 electronic reading is strongly recommended.

Revised 26 Jan. 2006

3. Hemolyzed (red) and visibly hyperlipemic (“milky”) samples have to be discarded as they could generate false results. 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 frozen/thawed more than once as this 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 0.2-0.8µ filters to clean up the sample for testing.

8 PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-use of the device and up to 6 months.

Microplates:

Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant is not turned to dark green, indicating a defect of manufacturing. In this case call DRG[®]'s customer service. Unused strips have to be placed back into the aluminium pouch, in presence of desiccant supplied, firmly zipped and stored at +2-8°C. When opened the first time, residual strips are stable till the indicator of humidity inside the desiccant bag turns from yellow to green.

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

Positive Control:

Ready to use. Mix well on vortex before use. Handle this component as potentially infective, even if HCV, eventually present in the control, has been chemically inactivated.

Wash buffer concentrate:

The whole content of the 20x concentrated solution has to be diluted with bidistilled water up to 1200 ml and mixed gently end-over-end before use.

Once diluted, the wash solution is stable for 1 week at +2-8°C. In the preparation avoid foaming as the presence of bubbles could give origin to a bad washing efficiency.

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

Enzyme conjugate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.

If this component has to be transferred use only plastic, possibly sterile disposable containers.

Chromogen/Substrate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes. Do not expose to strong illumination, oxidizing agents and metallic surfaces. If this component has to be transferred use only plastic, possible sterile disposable container.

Revised 26 Jan. 2006

Neutralizing Reagent: Ready to use component. Mix carefully on vortex before use.

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

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.

9 INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume 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 in order to show a precision of 1% and a trueness of +/-2%. Decontamination of spills or residues of kit components should also be carried out regularly.
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. Usually 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 sections "Validation of Test" and "Assay Performances". 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 microplate reader has to be equipped with a reading filter of 450nm and ideally with a second filter (620-630nm) for blanking purposes. Its standard performances should be
 - (a) bandwidth < 10 nm;
 - (b) absorbance range from 0 to > 2.0;
 - (c) linearity to > 2.0; repeatability > 1%.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 that the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.

Revised 26 Jan. 2006

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 sections "Validation of Test" and "Assay Performances". 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.

10 PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label (primary container). Do not use if expired.
2. Check that the liquid components are not contaminated by visible particles or aggregates.
3. Check that the Chromogen (TMB) is colourless or pale blue by aspirating a small volume of it with a sterile plastic pipette.
4. 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.
5. Dissolve the content of the Control Serum as reported.
6. Dilute all the content of the 20x concentrated Wash Solution as described above.
7. Allow all the other components to reach room temperature (about 1 hr) and then mix gently on vortex all liquid reagents.
8. Set the ELISA incubator at +37°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.
9. Check that the ELISA reader is turned on or ensure it will be turned on at least 20 minutes before reading.
10. If using an automated work station, turn on, check settings and be sure to use the right assay protocol.
11. Check that the micropipettes are set to the required volume.
12. Check that all the other equipment is available and ready to use.
13. In case of problems, do not proceed further with the test and advise the supervisor.

11 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** into a properly defined dilution tube (example: 1000 µl Sample Diluent + 10 µl sample). Do not dilute the Calibration Set as calibrators are ready to use.
Mix carefully all the liquid components on vortex and then proceed as described below.
2. Place the required number of Microwells in the microwell holder.
Leave A1 well empty for the operation of blanking.
3. Dispense 50 µl of the Neutralizing Reagent (SOLN NTR) in all the wells of the samples.
Do not add it in the wells used for Controls !
4. Dispense 100 µl of Negative Control in duplicate and 100 µl of Positive control in single.
Then dispense 100 µl of diluted samples in each properly identified well.

Revised 26 Jan. 2006

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. Wash the microplate with an automatic washer by delivering and aspirating 300 µl/well of diluted washing solution as reported previously (section 9.3).
7. Pipette 100 µl Enzyme Conjugate into each well, except the A1 well, and cover with the sealer.
Check that this red coloured component has been dispensed in all the wells, 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.

8. Incubate the microplate for 60 min at +37°C.
9. Wash microwells as in step 6.
10. 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.

11. Pipette 100 µl Sulphuric Acid into all the wells using the same pipetting sequence as in step 10.
Addition of acid will turn the positive calibrators, the control serum and the positive samples from yellow to blue.
12. 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.

General 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.

Revised 26 Jan. 2006

12 ASSAY SCHEME

Method	Operations
Neutralizing Reagent; (in sample wells only !)	50 µl
Negative and Positive Controls	100 µl
Samples diluted 1:101	100 µl
1st incubation	60 min
Temperature	+37°C
Wash step	4-5 cycles
Enzyme conjugate	100 µl
2nd incubation	60 min
Temperature	+37°C
Wash step	4-5 cycles
TMB/H ₂ O ₂	100 µl
3rd incubation	20 min
Temperature	r.t.
Sulphuric Acid	100 µl
Reading OD	450nm

An example of dispensation scheme is reported below:

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S5										
B	NC	S6										
C	NC	S7										
D	PC	S8										
E	S1	S9										
F	S2	S10										
G	S3	S11										
H	S4	S12										

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

13 INTERNAL QUALITY CONTROL

A check is carried out on the controls and the calibrator any time the kit is used in order to verify whether their OD450nm values are as expected and reported in the table below.

Revised 26 Jan. 2006

Check	Requirements
Blank well	< 0.100 OD450nm value
Negative Control (NC)	< 0.100 mean OD450nm value after blanking
Positive Control	> 0.500 OD450nm value

- If the results of the test match the requirements stated above, proceed to the next section.
- If they do not, do not proceed any further and operate as follows:

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Substrate solution has not got contaminated during the assay
Negative Control (NC) > 0.100 OD450nm after blanking	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (dispensation of positive control instead of negative control); 4. that no contamination of the negative control or of their wells has occurred due to positive samples, to spills or to the enzyme conjugate; 5. that micropipettes haven't got contaminated with positive samples or with the enzyme conjugate 6. that the washer needles are not blocked or partially obstructed.
Positive Control < 0.500 OD450nm	1. that the procedure has been correctly executed; 2. that no mistake has been done in the distribution of controls (dispensation of negative control instead of positive control. In this case, the negative control will have an OD450nm value > 0.150, too. 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the positive control has occurred.

Should these problems happen, after checking, report any residual problem to the supervisor for further actions.

14 CALCULATION OF THE CUT-OFF

The tests results are calculated by means of a cut-off value determined with the following formula:

$$\text{Cut-Off} = \text{NC mean OD450nm} + 0.250$$

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



DRG[®] HEV IgM ELISA (EIA-4417)



Revised 26 Jan. 2006

Important note:

When the calculation of results is done by the operative system of an ELISA automated work station be sure that the proper formulation is used to calculate the cut-off value and generate the right interpretations of results.

15 INTERPRETATION OF RESULTS

Test results are interpreted as ratio of the sample OD450nm and the Cut-Off value (or S/Co) according to the following table:

<u>S/Co</u>	<u>Interpretation</u>
< 1.0	Negative
1.0 – 1.2	Equivocal
> 1.2	Positive

A negative result indicates that the patient has no detectable anti HEV IgM reactivity.

Any patient showing an equivocal result should be tested again on a second sample taken 1-2 weeks later from the patient and examined.

A positive result is indicative of HEV infection and therefore the patient should be treated accordingly.

Important notes:

1. Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.
2. Any positive result should be confirmed by an alternative method before a diagnosis of viral hepatitis is formulated.
3. When test results are transmitted from the laboratory to an informatics center, attention has to be done to avoid erroneous data transfer.
4. Diagnosis of viral hepatitis E infection has to be done and released to the patient only by a qualified medical doctor.



DRG[®] HEV IgM ELISA (EIA-4417)



Revised 26 Jan. 2006

An example of calculation is reported below:

The following data must not be used instead of real figures obtained by the user.

Negative Control: 0.060 – 0.080 OD450nm

Mean Value: 0.070 OD450nm

→ Lower than 0.100 – Accepted

Positive Control: 1.589 OD450nm

→ Higher than 0.500 – Accepted

Cut-Off = $0.070 + 0.250 = 0.320$

Sample 1: 0.070 OD450nm → Sample 1 S/Co < 1.0 = negative

Sample 2: 1.690 OD450nm → Sample 2 S/Co > 1.2 = positive

16 PERFORMANCES

Evaluation of Performances has been conducted on negative and positive samples in an external clinical center with reference to a FDA approved kit.

16.1 LIMIT OF DETECTION

The limit of detection of the product has been checked on the international reference reagent for HEV antibody supplied by NIBSC/WHO with code n° 95/584. This material was assessed to be positive also for anti HEV IgM, low titer. This material when used as provided by the supplier in the assay conditions was detected positive with a S/Co value > 1.0.

16.2 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

These parameters were checked on about 700 sample derived from different patients and healthy individuals.

16.2.1 Diagnostic specificity:

It is defined as the probability of the assay of scoring negative in the absence of specific analyte.

A total of more 500 unselected donors and HEV negative hospitalized patients, including 1st time donors, were examined. The diagnostic specificity was assessed against a kit US FDA approved. A diagnostic specificity of **≥ 98%** was observed. Moreover, diagnostic specificity was assessed by testing more than 100 potentially interfering specimens (other infectious diseases, patients affected by non viral hepatic diseases, dialysis patients, pregnant women, haemolized, lipemic, etc.). A value of specificity of 100% was assessed.

No false reactivity due to the method of specimen preparation has been observed. Both plasma, derived with different standard techniques of preparation (citrate, EDTA and heparin), and sera have been used to determine the value of specificity. Frozen specimens have been tested, as well, to check for interferences due to collection and storage.

No interference was observed.

High reactive RF positive samples were observed to give origin to some false positive results.



DRG[®] HEV IgM ELISA (EIA-4417)



Revised 26 Jan. 2006

16.2.2 Diagnostic Sensitivity

It defined as the probability of the assay of scoring positive in the presence of specific analyte.

The diagnostic sensitivity has been assessed externally and internally on a total number of 100 positive specimens coming from Germany, Mexico and from Burma.

A diagnostic sensitivity of $\geq 98\%$ was found.

16.3 PRECISION:

It has been calculated on two samples, one negative and one positive, examined in 16 replicates in three separate runs.

CV values ranging between 5-15%, depending on OD450nm values, were found.

The variability seen did not result in sample misclassification.

17 LIMITATIONS

Repeatable false positive results were assessed for high titer RF positive samples, escaping the effect of the Neutralizing Reagent.

Frozen samples containing fibrin particles or aggregates after thawing have been observed to generate some false results.

18 REFERENCES

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