

Laboratory Procedure Manual

Analyte: **IgM Hepatitis E Antibody**

Matrix: **Serum**

Method: **DSI DS-EIA-ANTI-HEV-M (Ref E-152)**

Method No.:

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As performed by: **Assay Development and Diagnostic Reference Laboratory
Laboratory Branch
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Important Information for Users

The National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Data File Name	Variable Name	SAS Label
HEPE_J	LBDHEM	Hepatitis E IgM antibody (IgM anti-HEV)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

DS-EIA-ANTI-HEV-M is an enzyme immunoassay kit intended for the detection of IgM antibodies to hepatitis E virus in human serum or plasma.

Hepatitis E virus (HEV) infection is a major public health problem in many developing countries and an emerging health threat in developed countries. Notable large HEV outbreaks resulting in significant morbidity have occurred in India and China resulting in 79,000 cases and 119,000 cases, respectively. In countries with suboptimal sanitary conditions, HEV is the single most important cause of sporadic and epidemic hepatitis. In susceptible populations, high attack rates have been observed. Case-fatality rates in outbreaks range from 0.2% to 4%, but pregnant women, especially during the third trimester, may have a case-fatality rate of 10%–25%.

The true prevalence, incidence, and risk of HEV infection in developed countries are not clearly understood. There have been less than half a dozen cases of acute hepatitis E thought to be acquired domestically and reported in the United States from 1997–2010. These hepatitis E cases were all caused by genotype 3, all occurred in persons aged ≥ 40 years, and no clear source of infection was found despite intensive investigation. In contrast, asymptomatic but apparently widespread infection with HEV is observed. A previous analysis of >18,000 serum samples from the Third National Health and Nutrition Examination Survey (NHANES III), 1988–1994, showed that 21% of US residents were seropositive for anti-HEV IgG. Similarly high prevalence of anti-HEV was documented among blood donors, veterinary workers, injections drugs users from different parts of the US in and other developed countries. Although usually self limiting, HEV infection can cause chronic hepatitis in recipients of solid organ transplants and other immune-compromised individuals such as those who are HIV infected.

2. SAFETY PRECAUTIONS

All reagents included in the kit are intended for "in vitro diagnostic use".

Human origin material used in the preparation of the Negative Control and the Positive Control, has been tested and found non-reactive for hepatitis B surface antigen (HBsAg), antibodies to HCV and antibodies to human immunodeficiency virus (HIV-1 and HIV-2).

Because no known test method can offer complete assurance that infectious agents are absent, handle reagents and patients samples as if capable of transmitting infectious disease.

Do not eat, drink, smoke, or apply cosmetics where immunodiagnostic materials are being handled.

Do not pipette by mouth.

Any equipment directly in contact with specimens and reagents as well as washing solutions should be considered as contaminated products and treated as such.

Wear lab coats and disposable gloves when handling reagents and samples and thoroughly wash your hands after handling them.

Avoid spilling samples or solutions containing samples.

Avoid any contact of the Substrate Buffer, the TMB and the Stopping Reagent with the skin and mucosa.

Provide adequate ventilation.

Do not forget to neutralize and/or autoclave the washing wastes or any fluids containing biological samples before discarding them. Samples and reagent of human origin, as well as, contaminated material and products must be discarded after decontamination by immersion in bleach at a final concentration of 5% of sodium hypochlorite (1 volume of bleach for 10 volumes of contaminated fluid or water) for 30 minutes. Solid wastes should be disinfected by autoclaving for 1 hour at temperature 124-128°C and pressure 1.5 kHz/sm² (0.15 MPa). Liquid wastes can also be disinfected by boiling treatment for 30 min or by autoclaving for 1 hour at temperature 124-128°C and pressure 1.5 kHz/sm² (0.15 MPa). Tools and equipment should be wiped 2 times with 70 % ethanol before and after work.

Some reagents contain ProClin 300 (0.05 %). *Irritant. May cause sensitization by skin contact. After contact with skin, wash immediately with plenty of soap and water.*

3. COMPUTERIZATION; DATA MANAGEMENT

Raw data are transcribed manually from instrument readout sheets into a computerized database. This database was custom-designed for the management of CDC Assay Development and Diagnostic Reference Laboratory (ADDRL) test results, and functions within SQL Server software (Microsoft, Redmond, WA) with a .NET (Microsoft, Redmond, WA) user interface. Test values are compared with a cutoff value calculated from controls. Results are expressed as "positive" or "negative" for anti-HEV. Other information in the database may typically include the ADDRL identification number, the specimen number, the date collected, the date tested, and results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer.

Finished data are reviewed by the laboratory supervisor and transmitted to the NCHS along with other NHANES data.

Files stored on the CDC Local Area Network (LAN) are automatically backed up nightly to tape by CDC Data Center staff.

Documentation for data system maintenance is maintained with printed copies of data records for 2 years.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Note: Handle all specimens as if they are capable of transmitting infectious agents.

No special patient preparation before collection is necessary.

Collection of blood samples should be implemented according to the current practices. Serum or plasma may be used. Separate serum or plasma from blood cells as soon as possible to avoid any hemolysis. Extensive hemolysis may affect test performance. Specimens with observable particulate matter should be clarified by centrifugation prior to testing. Suspended fibrin particles or aggregates may yield falsely positive results. Do not heat the samples.

Samples can be stored at 2-8°C for not more than 48 hours; they may be deep-frozen at -20°C. Plasma must be quickly thawed by warming for a few minutes at 40°C (to avoid fibrin precipitation). Avoid repeated freeze/thaw cycles. Samples that have been frozen and defrosted more than 1 time cannot be used. Samples with expressed hemolysis or hyperlipidemia must not be analyzed.

Specimens and controls should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories* and in the CLSI Document M29-A. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

a. Instrumentation and Software

Microplate reader

b. Reagents

1. Ready for use reagents:
 - Negative Control

- Positive Control
- Conjugate diluent
- Sample diluent
- Stopping Reagent

2. Reagents to prepare:

- HEV-Ag Coated Strips. Each plate containing 12 strips is wrapped in a sealed foil-lined bag. Open the bag and remove the plate. Select the number of coated strips required for the assay. Unused strips should be placed back in the bag. After the bag has been opened, the strips are stable for 6 months at 2-8°C, provided that the foil bag is resealed with the clip or the foil bag is resealed in self-sealing plastic bag. The silica gel bag should not be removed from the foil packaging.
- Working Washing Solution. Thoroughly mix the vial of concentrated Washing Solution (x25). Dilute the required volume of concentrated Washing Solution with corresponding volume of purified water prior to use (See Table). Mix the solution thoroughly. The prepared Working Washing Solution is stable for at least 14 days at 18-24°C or for 28 days at 2-8°C when used in GLP condition.
- Working Solution of Conjugate. Dilute the necessary volume of thoroughly mixed concentrate of Conjugate with the corresponding volume of Conjugate diluent (See Table). Mix thoroughly until diluted avoiding foaming. Do not apply intensive mixing. Prepare before use. The working solution of Conjugate can be stored not more than 12 hours in the dark at 18-24°C.
- Substrate Mixture. Dilute the required volume of TMB (concentrated 21-fold) with the corresponding volume of Substrate Buffer (1:20 ratio) (See Table). Mix thoroughly until diluted. The Substrate Mixture should be prepared before use. Mixture is stable not more than 10 hours when stored in a dark place at 18-24°C in clean vials. **Substrate Mixture should be colorless!**

3. Storage of unused reagents

After opening the vials with unused reagents: Negative Control, Positive Control, Substrate Buffer, Sample diluent, Conjugate diluent, Washing Solution (concentrated 25-fold), Stopping Reagent, TMB (concentrated 21-fold), Conjugate (concentrated 21-fold) can be stored in tightly sealed vials until the kit expiration date at 2-8°C. HEV-Ag Coated Strips are stable for 6 months after opening when stored at 2-8°C.

4. Kit contents

HEV-Ag Coated Strips: Polystyrene stripped plate with colorless transparent wells coated with mix of recombinant antigens of HEV. Store at 2-8°C until expiration date.

Sample diluent: Transparent or slightly opalescent liquid, violet-blue colored; sediment may form which completely dissolves with shaking. Preserving agent: 0.01 % thimerosal. Store at 2-8°C until expiration date in a tightly sealed vial.

Conjugate (concentrated 21-fold): Monoclonal mouse antibodies against human IgM, labeled horse-radish peroxidase. Transparent or slightly opalescent liquid, light yellow colored. Preserving agent: 0.04% ProClin 300, 0.04% gentamycin sulfate. Store at 2-8°C until expiration date in a tightly sealed vial.

Conjugate diluent: Transparent yellow liquid at temperature of 2-8°C; opalescent yellow color liquid at temperature of 18-24°C. Preserving agent: 0.01% thimerosal. Store at 2-8°C until expiration date in a tightly sealed vial.

Positive Control Inactivated: Heat inactivated human serum positive for anti-HEV-IgM, negative for anti-HIV-1,2, HBsAg and anti-HCV. Transparent or slightly opalescent liquid, red colored. Preserving agent: 0.04% ProClin 300, 0.1% sodium azide. Store at 2-8°C until expiration date in a tightly sealed vial.

Negative Control Inactivated: Heat inactivated human serum negative for anti-HEV-IgM, anti-HIV-1,2, HBsAg and anti-HCV. Transparent or slightly opalescent liquid, green colored. Preserving agent: 0.01% thimerosal, 0.1% sodium azide. Store at 2-8°C until expiration date in a tightly sealed vial

Washing Solution (concentrated 25-fold): Transparent or slightly opalescent liquid, colorless or pale yellow; sediment may form that dissolves at 35-39°C with shaking. Store at 2-8°C until expiration date in a tightly sealed vial.

Substrate Buffer: Citric acid and sodium acetate solution, pH 4.1-4.3, containing H₂O₂. Transparent colorless liquid. Preserving agent: 0.05% ProClin 300. Store at 2-8°C until expiration date in a tightly sealed vial.

TMB (concentrated 21-fold): Solution containing Tetramethylbenzidine (TMB). Transparent colorless liquid. Store at 2-8°C until expiration date in a tightly sealed vial.

Stopping Reagent: 0.75 M/L sulphuric acid solution. Transparent colorless liquid. Store at 2-8°C until expiration date in a tightly sealed vial.

Additionally the following may be included in the delivery set:

- a lid for polystyrene 96-well plates or a protective film for EIA plates;
- disposable tips;

- a plastic dish for liquid reagents;
- a plastic clip or self-sealing plastic bag.

c. Other materials required but not provided

Purified water.

Automatic or semiautomatic, adjustable or preset pipettes or multipipettes to measure and dispense 10 µl, 50 µl, 90 µl, and 100 µl.

Disposable pipette tips.

Microplate incubator at $(37.0 \pm 1.0)^{\circ}\text{C}$.

Automatic microplate washer.

Microplate reader equipped with 450 nm or with 450 nm and 620-680 nm filters.

Disposable gloves.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

No calibrations are performed. The result is compared to a cutoff calculated from the OD value of the negative control.

b. **Verification**

Not Applicable

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

The samples are brought to room temperature.

Note: Before use, allow reagents to reach room temperature (18-24°C) for 30 min.

The required volumes of reagents depending on the number of used strips are presented in the table below.

Table of consumable components of the kit

Number of Used strips	Working Wash Solution		Working Solution of Conjugate		Substrate Mixture	
	Washing Solution (concentrated 25-fold) (ml)	Purified water (ml)	Conjugate (concentrated 21-fold) (ml)	Conjugate diluent (ml)	Substrate Buffer (ml)	TMB (concentrated 21-fold) (ml)
1	4.0	96.0	0.05	1.0	1.0	0.05

2	8.0	192.0	0.10	2.0	2.0	0.10
3	12.0	288.0	0.15	3.0	3.0	0.15
4	16.0	384.0	0.20	4.0	4.0	0.20
5	20.0	480.0	0.25	5.0	5.0	0.25
6	24.0	576.0	0.30	6.0	6.0	0.30
7	28.0	672.0	0.35	7.0	7.0	0.35
8	32.0	768.0	0.40	8.0	8.0	0.40
9	36.0	864.0	0.45	9.0	9.0	0.45
10	40.0	960.0	0.50	10.0	10.0	0.50
11	44.0	1056.0	0.55	11.0	11.0	0.55
12	50.0	1200.0	0.65	13.0	13.0	0.65

b. Operation of the Assay Procedure

1. Wash the coated strips with Working Washing Solution two times before the assay. Add to each well 380-400 µl of Working Washing Solution. Allow a soak time of at least 40 seconds and aspirate. Do not leave any fluid in the wells. Use of an automatic microplate washer is strongly recommended. Incomplete washing will adversely affect assay precision.
2. Pipette 100 µl of Positive Control and Negative Control in duplicates. Pipette 90 µl of Sample diluent and 10 µl of tested specimens into the rest of the wells. Violet-blue color of Sample diluent should change to blue-green when specimens were added. Cover the plate with a plate lid or protective film and incubate 30 minutes in microplate incubator at $(37.0 \pm 1.0)^{\circ}\text{C}$.
3. Remove the content of the wells and wash the plate 4 times with the Working Washing Solution as described in step 1.
4. Pipette 100 µl of Working Solution of Conjugate into each of the wells. Cover the plate with a plate lid or protective film and incubate 30 minutes in microplate incubator at $(37.0 \pm 1.0)^{\circ}\text{C}$.
5. Remove the content of the wells and wash the plate 4 times with the Working Washing Solution as described in step 1.
6. Pipette 100 µl of Substrate Mixture into each well. Keep the plate in a dark place for 20 min at $18-24^{\circ}\text{C}$.
7. Stop the reaction by adding 50 µl Stopping Reagent to each well and read the optical density at 450/620-680 nm using a microplate reader. Reading the absorbance at 450 nm only is possible.

c. Reporting results

The presence or absence of antibodies to hepatitis E virus is determined by the ratio of the OD of each sample to the calculated cut-off value.

For the assay to be valid, OD value of Positive Control must be not less than 0.6 and average OD value of Negative Control must not be greater than 0.2.

d. Interpretation of Results

Sample is Negative: if the OD value is $<$ Cut-off.

Sample is Positive: if the OD value is \geq Cut-off.

e. Recording of Data

Positive and negative control reagents are supplied with each test kit. The assay cutoff value is calculated from values obtained from these controls and entered in the DMS by the analyst.

Raw optical density values for each specimen are manually entered by the analyst into the Data Management System (DMS), where the data are processed and interpreted according to the cutoff value calculated automatically by the DMS based on the formula provided in the Instructions for Use.

If **Negative Control or Positive Control** is invalid then the entire run is invalid; repeat the entire run including control and sample preparation.

f. Calculations

Calculate Cut-Off value as:

Cut-Off = average OD value of Negative Control + 0.200

0.200 is a coefficient defined by manufacturer during statistical processing for each lot.

9. REPORTABLE RANGE OF RESULTS

Final results are expressed qualitatively as positive or negative for the presence of IgM anti-HEV antibody in the sample. No quantitative results are determined.

10. QUALITY CONTROL (QC) PROCEDURES

For the assay to be valid OD value of Positive Control not less than 0.6, average OD value of Negative Control is not greater than 0.2.

The precision of these procedures is as claimed for licensure and is maintained by the manufacturer.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

The entire run is considered to be invalid if one or both controls are not within specified limits.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The sample is restricted to human serum or plasma.

No interfering substances have been identified.

13. REFERENCE RANGES (NORMAL VALUES)

All normal, noninfected humans should have negative values for Hepatitis E antibodies.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may remain at 20-25°C during preparation and testing only.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Other tests for Hepatitis E may be substituted but must be accompanied by validation data to show substantial equivalence with this assay. Substitution of test methods may not be done without approval from the NCHS.

Alternate storage is not recommended.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Test results are documented through the lab management database to track specimens. Specimens in long-term storage are arranged by study group. The storage location of each sample is listed with the test data. For NHANES, residual specimens are stored frozen and returned to the NCHS specimen bank after testing for each cycle has been completed.

19. SUMMARY STATISTICS AND QC GRAPHS

Qualitative assays are assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

CLSI. Statistical Quality Control for Quantitative Measurements: Principles and Definitions; Approved Guideline - Third Edition. CLSI document C24-A3 [ISBN 1-56238-613-1]. CLSI, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA, 2006.

NCCLS. *Interference Testing in Clinical Chemistry; Proposed Guideline*. NCCLS document EP7-P (ISBN 1-56238-020-6). CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087, 1986.

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NCCLS. *Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline - Second Edition*. NCCLS document EP5-A2 [ISBN 1-56238-542-9]. CLSI, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA, 2004.