Laboratory Procedure Manual

Analyte:	**IgG Hepatitis E Antibody**
Matrix:	**Serum**
Method:	DSI DS-EIA-ANTI-HEV-G
Method No.:	
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as performed by:	*Assay Development and Diagnostic Reference Laboratory* *Division of Viral Hepatitis* *National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention,*
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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_F	LBDHEG	Hepatitis E IgG (anti-HEV)

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

CONTENTS OF THE DS-EIA-ANTI-HEV-G KIT

Table 1.

LABEL	NATURE OF THE REAGENTS	PRESENTATION
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.	1 plate
Sample diluent	Transparent or slightly opalescent liquid, violet-blue colored, sediment may form which completely dissolves at shaking. Preserving agent: 0.01 % thimerosal. Store at 2-8°C until expiration date in a tightly sealed vial.	1 vial 12.5 ml
Conjugate (concentrated 21-fold)	Monoclonal mouse antibodies against human IgG, 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.	1 vial 0.75 ml
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 tight1y sealed vial.	1 vial 13.5 ml
Positive Control Inactivated	Heat inactivated human serum positive for anti-HEV-IgG, 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.	1 vial 1.5 ml
Negative Control Inactivated	Heat inactivated human serum negative for anti-HEV-IgG, 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.	1 vial 2.5 ml
Washing Solution (concentrated 25-fold)	Transparent or slightly opalescent liquid, colorless, or pale yellow, sediment may form that dissolves at 35-39°C and shaking. Store at 2-8°C until expiration date in a tightly sealed vial.	1 vial 50.0 ml

Substrate Buffer	Citric acid and sodium acetate solution, pH 4.1-4.3, containing H_2O_2. Transparent colorless liquid. Preserving agent: 0.05% ProClin 300. Store at 2-8°C until expiration date in a tightly sealed vial.	1 vial 15.0 ml
TMB (concentrated 21-fold)	Solution containing Tetramethylbenzidine (TMB). Transparent colorless liquid. Store at 2-8°C until expiration date in a tightly sealed vial.	1 vial 1.5 ml
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.	1 vial 25.0 ml

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.

PRECAUTIONS

The reliability of the results depends on correct implementation of the following Good Laboratory Practices:

- The temperature in the lab should be 18-24°C.
- Do not use expired reagents.
- Do not mix reagents from different lots within a given test run.
- Before use, it is necessary to wait 30 minutes for the reagents to stabilize to room temperature (18-24°C).
- Carefully reconstitute the reagents avoiding any contamination.
- Do not carry out the test in the presence of reactive vapors (acid, alkaline, aldehyde vapors) or dust that could alter the enzyme activity of the conjugates.
- Use glassware thoroughly washed and rinsed with deionized water or preferably, disposable material.
- Do not allow the microplate to dry between the end of the washing operation and the reagent distribution.
- The enzyme reaction is very sensitive to metal ions. Consequently, do not allow any metal element to come into contact with the various conjugate or substrate solutions.
- Use a new distribution tip for each sample.
- Well washing is a critical step in this procedure: respect the recommended number of washing cycles and make sure that all wells are completely filled and then completely emptied. Incorrect washing may lead to inaccurate results.
- Never use the same container to distribute conjugate and development solution.
- Check the pipettes and other equipment for accuracy and correct operation.
- Do not change the assay procedure.
- Use high quality water.
- Avoid exposure of the reagents to excessive heat or sunlight during storage and incubation.

HEALTH AND SAFETY INSTRUCTIONS

- 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 infections agents are absent, handle reagents and patients samples as if capable of transmitting infections 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 into the container. Samples and reagent of human origin, as well as, contaminated material and products must be discarded after decontamination: either 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. Also solid wastes should be disinfected by autoclaving for 1 hour at temperature 124-128°C and pressure 1.5 kHz/sm^2 (0.15 MPa). Also liquid wastes can be disinfected by boiling treatment for 30 min or by autoclaving for 1 hour at temperature 124-128°C and pressure 1.5 kHz/sm^2 (0.15 MPa). Tools and equipment should be wiped 2 times by 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.

MATERIALS AND EQUPMENT REQUIRED BUT NOT PROVIDED WITH THE KIT:

- 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 ± 1.0)^{o}C.
- Automatic microplate washer.
- Microplate reader equipped with 450 nm or with 450 nm and 620-680 nm filters.
- Disposable gloves.

COLLECTION AND HANDLING OF SPECIMENS

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 not more than for 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, hyperlipidemia must not be analyzed.

PREPARATION OF THE 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 content of the vial with concentrated Washing Solution (x25). Dilute the required volume of concentrated Washing Solution with corresponding volume of purified water prior to use (See Table 2). Mix the solution thoroughly. The prepared Working Washing Solution is stable at least for 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 2). 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 2). 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-foJd), 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 within 6 months after opening when stored at 2-8°C.

ASSAY PROCEDURE

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 table 2.

Table 2. Table of consumable components of the kit

	Working Wash Solution		Working Solution of Conjugate		Substrate Mixture	
Number of Used strips	**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

EIA procedure

1. Wash the coated strips with Working Washing Solution two times before the assay. Add into each well 380-400 µl of Working Washing Solution. Allow a soak time 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 by plate lid or a protective film and incubate during 30 minutes in microplate incubator at (37.0±1.0)°C.

3. Remove the content of the wells and wash the plate 4 times with the Working Washing Solution as described in procedure 1.

4. Pipette 100 µl of Working Solution of Conjugate into each of the wells. Cover the plate by plate lid or a protective film and incubate during 30 minutes in microplate incubator at (37.0±1.0)°C.

5. Remove the content of the wells and wash the plate 4 times with the Working Washing Solution as described in procedure 1.

6. Pipette 100 µl of Substrate Mixture into each well. Keep the plate in a dark place during 20 min at 18-24°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. Scheme of the assay is represented in Annex.

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 not less than 0.6, average OD value of Negative Control is not greater than 0.2.

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.

Interpretation of Results:
Sample is Negative: if the OD value is < Cut-off
Sample is Positive: if the OD value is ≥ Cut-off

PERFORMANCE CHARACTERISTICS OF DS-EIA-ANTI-HEV-G

Sensitivity and specificity
The following groups of sera were used in the trials:

1. Healthy donors serum samples (n=600).
2. Serum samples, containing different infection markers:
    - Anti-HAV-IgM positive (n=27)
    - HBsAg positive (n=109)
    - Anti-HCV IgG positive (n=323)
    - CMV PCR positive (n=28)
3. Serum samples of hepatitis E patients, collected in Central Asia (n=13)
4. Serum samples of persons who had contacts with hepatitis E patients (Afghanistan war veterans from hyperendemic regions of the country) n=317, and samples from those who had no contacts (veterans from non-endemic regions) n = 208.
5. Serum samples of volunteers infected with hepatitis E. (sera were kindly' provided by M.S. Balayan and had been kept frozen at -70°C).

6. HEab Panel: chimpanzee serum samples containing (sera № 3-8) and not containing (sera № 1-2) HEV antibodies (n=8).
7. Serum samples, containing and not containing HEV antibodies form working Manufacturer's panel (RPP No 18.12.02.04), n=16.

Sensitivity and specificity of the DS-EIA-ANTI-HEV-G was tested on serum samples containing and not containing HEV antibodies from HEab and RPP No 18.12.02.04. RPP No 18.12.02.04 is designed by DSI S.r.l. for technological control of the manufacturing process. The panel consists of 16 serum samples, containing and not containing HEV IgG antibodies.

Positive sera were collected from patients with clinically confirmed diagnosis of HEV infection at infectious diseases hospitals and blood transfusion centers. Presence or absence of antibodies against HEV was detected by Abbott HEV EIA (Abbott diagnostics, USA) kits.

All serum samples have been characterized in the passport of the panel
HEab panel (CDC, USA) consists of chimpanzee serum samples, not containing (sera № 1 and № 2) anti-HEV IgG, containing anti-HEV IgM and IgG (serum № 3), and containing anti-HEV IgG in various concentration (sera № 4-8). Results of the trials are shown in Table 3.

Table 3. Sensitivity and specificity evaluation of 3 laboratory batches of the DS-EIA-ANTI-HEV-G kit

Serum Number	RPP 18.12.02.04 (DSI S.r.l.)		HEab	
	OD (mean±δ of 3 laboratory batches)	OD/Cut-off	OD (mean±δ of 3 laboratory batches)	OD/Cut-off
1	0.849±0.039	3.61	0.032±0.005	0.152
2	0.961±0.045	4.09	0.045±0.005	0.210
3	2.112±0.076	8.99	2.456±0.056	11.695
4	1.661±0.056	7.06	2.560±0.065	12.19
5	1.218±0.087	5.18	2.559±0.046	12.185
6	1.902±0.072	8.09	2.612±0.038	12.438
7	1.648±0.048	7.01	2.733±0.047	13.014
8	1.009±0.048	4.29	2.650±0.056	12.619
9	0.057±0.0032	0.24		
10	0.042±0.0034	0.18		
11	0.043±0.0041	0.18		
12	0.043±0.0013	0.18		
13	0.041±0.0054	0.18		
14	0.051±0.0035	0.22		
15	0.055±0.0056	0.23		
16	0.060±0.0029	0.26		

As it is shown in the table 3, sensitivity of the DS-EIA-ANTI-HEV-*G* is 100%. Positive results are obtained with all positive sera from panels HEab and RPP. The OD in cases of negative sera was lower than cut-off that shows on 100% specificity of the assay.

Comparative evaluation of diagnostic performance of DS-EIA-ANTI-HEV-G and Abbott HEV EIA has been carried out using RPP 18.12.02.04. All serum samples were tested with both kits. Results are shown in table 4.

Table 4. Comparative evaluation of diagnostic performance of DS-EIA-ANTI-HEV-G and Abbott HEV EIA

Serum Number	DS-EIA-ANTI-HEV-G RPP 18.12.02.04 OD/Cut-off	Abbott HEV EIA RPP 18.12.02.04 OD/Cut-off
1	11.496	5.52
2	2.524	1.84
3	9.424	4.75
4	9.600	4.5
5	1.130	1.01
6	8.800	5.00
7	4.485	4.2
8	4.485	3.82
9	0.045	0.064
10	0.125	0.240
11	0.111	0.190
12	0.098	0.120
13	0.088	0.094
14	0.103	
15	0.123	
16	0.067	

Shown data suggest that diagnostic performance of both compared kits is equal. However, the positivity coefficient was higher with DS-EIA-ANTI-HEV-G,

Different groups of the population with various infectious diseases or blood donors were tested to reveal prevalence of HEV markers. DS-EIA-ANTI-HEV-G was used to conduct the research. Obtained data are shown in table 5.

Table 5. Anti-HEV reactivity of the sera collected from different groups of the population

Patient groups	DS-EIA-ANTI-HEV-G		
	Total studied sera	HEV Ab positive sera number	
		Amount	percentage
Blood donors	600	22	3.6
Hep. A patients	27	1	3.7
Hep. B patients	109	9	8.2
Hep. C patients	323	23	7.1
CMV patients	28	0	0

As it can be seen from the Table 5, DS-EIA-ANTI-HEV-G has revealed 3.6% of positive sera among blood donors, 6.3% among other acute hepatitis patients. Observed situation corresponds to other studies descried in literature (Balayan MS, Mikhailov MI, Viral Hepatitis, 1999, Amipress).

Table 6 shows results of the comparative study of waterborne outbreak in Uzbekistan. 13 serum samples were obtained during this outbreak from acute hepatitis E patients.

Table 6. Comparative evaluation of anti-HEV IgG detection with DS-EIA-ANTI-HEV-G and Abbott HEV EIA in serum samples from patients during reconvalescence period (Uzbekistan, Kyrgyzia)

Serum Number	DS-EIA-ANTI-HEV-G		Abbott HEV EIA	
	OD	Result	OD	Result
2163-3	0.054	-	0.054	-
2181-2	0.910	+	0.203	-
2166-1	1.164	+	2.000	+
2168-1	1.054	+	1.096	+
2170	0.883	+	2.000	+
2172-2	1.153	+	0.656	+
2178-2	1.008	-	2.000	-
7410	0.057	+	0.150	+
7412	1.201	+	2.000	+
7413	1.018	+	2.000	+
7414	0.073	-	0.261	+
7415	1.242	+	0.972	+
7419	0.661	+	0.398	+
Cutoff	0.245		0.219	

Data show that both kits revealed positive results in 10 samples and 2 samples were negative, Sample № 2181-2 was defined positive with DS-EIA-ANTI-HEV-G (OD=O.910) and negative with Abbott HEV EIA (OD=0.203). Sample № 7414 was defined negative with DS-EIA-ANTI-HEV-G (OD=0.073) and positive with Abbott HEV EIA (OD=0.261). It should be noted that OD was only slightly higher than the cut-off value in case of sample № 7414 and Abbott HEV EIA. Shown results indicate that both systems have similar sensitivity and specificity parameters.

CONDITIONS OF STORAGE AND TRANSPORTATION

Shelf life is 15 months. Keep in dark dry place at 2-8°C.

Transportation may be done by all kinds of covered transport at temperature 9-20°C not more than during ten (10) days. Freezing is prohibited.

SUMMARY STATISTICS AND QC GRAPHS

Since hepatitis E is qualitative data there are no summary statistics or qc graphs.

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ANNEX

Schedule of the assay

1	Wash the plate	Working Washing Solution, not less than 380 µl, 2 times
2	Add	100 µl of Positive Control, Negative Control
3	Add	90 µl of Sample diluent
4	Add	10 µl of tested samples
5	Incubate	30 min, (37.0 ± 1.0)°C, microplate incubator
6	Wash the plate	Working Washing Solution, not less than 380 µl, 4 times
7	Add	100 µl of Working Solution of Conjugate
8	Incubate	30 min, (37.0 ± 1.0)°C, microplate incubator
9	Wash the plate	Working Washing Solution, not less than 380 µl, 4 times
10	Add	100 µl of Substrate Mixture
11	Incubate	20 min, 18-24°C in the dark place
12	Add	50 µl of Stopping Reagent
13	Read the optical density	450 nm/620-680 nm or 450 nm