Version 13-07/24

User's Manual

Human Anti-Hepatitis A Virus ELISA kit

REF **DEIASL276Q**

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RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

Anti-HAV ELISA is an enzyme immunoassay for the detection of antibodies against Hepatitis A virus in Human Serum for research use.

General Description

After infection with the Hepatitis A-Virus, neutralizing antibodies appear at the same time of Anti- HAV of IgG-Class formation. The Anti-HAV titers 3 to 6 months after naturally occurring infections are very high, within the range of 100 to more than 300 International Units per ml (IU/ml). Even after more than 10 years the titers usually remain at more than 1 to 10 IU/ml.

A value of 10 - 30 (mIU/ml) milli International Units can be considered the minimal protective level. Since the incidence of HAV infection in children has diminished in Northern Europe in recent years, children and juveniles are predominantly Anti-HAV negative. In an adult population could be observed, that the percentage of anti-HAV positive individuals raises with increasing age: only 3.9 % of the young people (18-24 years) show antibody against HAV, in older population (≥50 years) HAV-Antibody was found in 40.3 % of the population.

In countries with less favorable sanitary conditions around the Mediterranean area, Africa or Asia, the incidence is very high. Higher risk is also given for clinical staff, especially in the pediatric.

Since a vaccine against Hepatitis A virus infections is available, vaccinations are recommended for people travelling to countries where a high risk of HAV infections exists and for health care employees.

Principles of Testing

Anti-HAV ELISA is a pseudo-competitive enzyme immunoassay. Serum or plasma samples are added to the wells of a microtiter plate, which have been previously coated with inactivated HAV antigen, and incubated for 1 hours at 37 °C. Anti-HAV antibodies in samples bind to the antigen. The mouse anti-HAV antibody is added and incubated for 1h at 37 °C. Free binding sites of the antigen are bound with mouse anti-HAV antibody. The conjugate (enzyme linked polyclonal antibody specific for mouse antibody) is pipetted and incubated again for 1 h at 37 °C. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution (TMB) is added to microwells and incubated for 15 min at 37 °C. The bound conjugate changes the color of the substrate to blue. The reaction is terminated by adding the stopping solution. The color turns yellow. The absorbance of the colored reaction product is measured on a microtiter plate reader. The extinction is reciprocal to the anti-HAV titer.

For semi-quantitative determination use the included standards.

The preparation of titration curve e.g. for calibration of sera by means of standard reagents is also possible.

Reagents And Materials Provided

1.Microtiter plate, ready for use, coated with inactivated HAV- antigen. Wells are separately breakable, (8×12) wells.

2.Mouse anti-HAV antibody (100×), 100-fold concentrated solution, 1×120 µL.

3.Conjugate, ready for use, polyclonal antibody specific for mouse antibody, 1×12 mL.

4.Positive Control (PC), anti-HAV positive control, lyophilized powder.

5.Negative Control (NC), anti-HAV negative control, lyophilized powder.

6.Dilution Buffer, ready for use, 1×50 mL

7.Wash Buffer, 20-fold concentrated solution, 1×50 mL

8.Substrate, ready for use, 2×6 mL

9.Stop Solution, ready for use, 1×7 mL

10.Serum Standard (100 mIU), anti-HAV standard 100 mIU, lyophilized powder. For semi-quantitative test.

Materials Required But Not Supplied

1. Freshly distilled or deionized water
2. Disposable gloves and timer
3. Appropriate waste containers for potentially contaminated materials
4. Dispensing system and/or pipette
5. Disposable pipette tips
6. Absorbent tissue or clean towel
7. Dry incubator or water bath, 37±0.5°C
8. Plate reader, single wavelength 450nm or dual wavelength 450/620nm
9. Microwell aspiration/wash system

Storage

After you receive the kit, all the components should be stored in the refrigerator (4-8°C) also up to 1 year. Long term storage, improper storage conditions and large temperature fluctuation cycles may cause precipitates in the TMB solution. These precipitates should not affect the assay noticeably. Nevertheless, if you observe such precipitates, we recommend to avoid them by allowing them to sink to the bottom.

Specimen Collection And Preparation

1. Specimen Collection: No special patient's preparation required. Collect the specimen in accordance with the normal laboratory practice. Either fresh serum or plasma specimens can be used with this assay. Blood collected by venipuncture should be allowed to clot naturally and completely - the serum/plasma must be separated from the clot as early as possible as to avoid hemolysis of the RBC. Care should be taken to ensure that the serum specimens are clear and not contaminated by microorganisms. Any visible particulate matters in the specimen should be removed by centrifugation at 3000 RPM (round per minutes) for 20 minutes at room temperature or by filtration.
2. Plasma specimens collected into EDTA, sodium citrate or heparin can be tested, but highly lipemic, icteric, or hemolytic specimens should not be used as they can give false results in the assay. Do not heat inactivate specimens. This can cause deterioration of the target analyte. Samples with visible microbial

contamination should never be used.

3. CD Anti-HAV ELISA Kit is intended ONLY for testing of individual serum or plasma samples. Do not use the assay for testing of cadaver samples, saliva, urine or other body fluids, or pooled (mixed) blood.
4. Transportation and Storage: Store specimens at 2-8°C. Specimens not required for assaying within 7 days should be stored frozen (-20°C or lower). Multiple freeze-thaw cycles should be avoided. For shipment, samples should be packaged and labeled in accordance with the existing local and international regulations for transportation of clinical samples and ethological agents.

Reagent Preparation

Note: Bring all reagents to room temperature (15-30°C) prior to use.

1. To run the assay more than once, ensure that reagents are stored at conditions stated on the label. Prepare only the appropriate amount necessary for each run. The kit can be used up to 4 times within the expiry date stated on the label.
2. Preparation of the Wash Buffer (1X): Dilute Wash Buffer to 1X with ultrapure Water before use (50 mL Wash Buffer (20X) + 950 mL ultrapure water). Mix well. The Wash Buffer is stable at 2-8°C until the expiry date stated on the label. Wash buffer (1X) can be stored in a closed flask at 2-8°C for 1 month.
3. Preparation of PC and NC: The PC and NC is provided as a lyophilized powder. Reconstitute PC and NC with 1 mL ultrapure water respectively and vortex gently. The reconstituted reagent should be aliquoted and stored below -20°C.
4. Preparation of Mouse anti-HAV antibody (1X): Dilute Mouse anti-HAV antibody to 1X with Dilution Buffer before use (50µL Mouse anti-HAV antibody (100X) + 4.95 mL Dilution Buffer). Mix well.
5. Preparation of Serum Standard(100 mIU/ml): The Serum Standard (100 mIU) is provided as a lyophilized powder. Reconstitute it with 1 mL ultrapure water and vortex gently. The reconstituted reagent should be aliquoted and stored below -20°C.

For semi-quantitative test:

Preparation of anti-HAV standards: Diluent Serum Standard(100 mIU/ml)with Dilution Buffer as described below.

Suggested Preparation of anti-HAV Standards			
	mIU/ml	Range: 0 to 50 mIU/ml	
Stock	100		
S1	50	Add 150µl Stock	+150µl Dilution Buffer
S2	20	Add 50µl Stock	+200µl Dilution Buffer
S3	5	Add 15µl Stock	+285µl Dilution Buffer

Assay Procedure

Assay Procedure in Double Determination			
Pipette	**Reagents**		**Position**
100 µL		-	A1/A2
100 µL	Positive Control	PC	B1/B2
100 µL	Negative Control	NC	C1/C2
100 µL	Standard 1 50 mIU/ml （only in semi-quantitative measurement)	S1	D1/D2
100 µL	Standard 2 20 mIU/ml （only in semi-quantitative measurement)	S2	E1/E2
100 µL	Standard 3 5 mIU/ml （only in semi-quantitative measurement)	S3	F1/F2
100 µL	Samples e.g. 1:20 diluted	according to demand	
Cover the wells with the sealing tape.			
Incubation: 1 h at 37 ℃			
5x 300 µL	Aspirate the contents of the wells and wash 5x with 300 µL Washing Buffer	in each well	
100 µL	Diluted Mouse anti-HAV antibody (1X)	from B1 in each well	
Cover the wells with the sealing tape.			
Incubation: 1 h at 37 ℃			
5x 300 µL	Aspirate the contents of the wells and wash 5x with 300 µL Washing Buffer	in each well	
100 µL	Conjugate	from B1 in each well	
Cover the wells with the sealing tape.			
Incubation: 1 h at 37 ℃			
5x 300 µL	Aspirate the contents of the wells and wash 5x with 300 µL Washing Buffer	in each well	
100 µL	Substrate	in each well	
Cover the wells with the sealing tape.			
Incubation: 15 minutes in the dark at 37 ℃			
50 µL	Stop Solution	in each well	
Measure the absorbance within 30 min at 450 nm (reference filter 620 nm)			

Instead of the standards contained in the test, an internal standard can be used. This may be the dilution of a reference preparation, or prepared by titration of serum against a reference preparation.

Reference preparations are available from the Paul-Ehrlich-Institute, (Postfach 17 40, 63207 Langen, Tel. 06103/770, Telefax: 06103/77123, anti-HAV Reference sample, "PEI-Standard") or from the Central laboratory of the Netherlands Red Cross (Plesmanlaan 125, 1006 CX Amsterdam, Telefax 31 20 5123474, WHO International Reference Preparations, 97/646 anti-hepatitis A immunoglobulin, WHO-Standard).

Quality Control

GLP requires that controls be run with each calibration curve. A statistically significant number of controls should be frequently assayed to establish mean values and acceptable ranges to assure proper performance.

Quality criteria

In order to evaluate the result, it should be ensured that the **Positive Control PC** must be lower than **0.5** OD450 units. The signal of the **Negative Control NC** must be greater than **0.8** OD450 units.

The absorbances of the samples should be within the standard curve (S0 - S3) in the semi-quantitative method. If the absorbances are outside this range, for reliable determinations, they should be determined again at higher dilutions in a second test.

Evaluation

First, the absorbance determined with the chosen reference wavelength (620 nm) is subtracted from each absorbance value at 450 nm, regardless of the chosen evaluation method. Following this, the absorbance of

the blanks (A1/A2) is also subtracted from all values. The measured values thus obtained are the basis for all further analyzes.

1. Qualitative test analysis

For the qualitative determination of anti-HAV antibodies in human serum samples a method was created in the context of product development, which enables the distinctions of positive and negative probands with a sensitivity of 95.9 % (n = 47).

For this purpose a cut-off value is calculated from the signal of the positive control and negative control according to the following formula.

$$cut-off = \frac{Extinction\ Negative\ Control + Extinction\ Positive\ Control}{1.3}$$

Samples with mean absorbance values higher than the cut-off value are considered to be negative. Samples with absorbance values less than the cut-off are considered to be positive. Samples ± 10% around the cut-off should be determined again.

An exemplary test result is shown in Table 1. The calculation of the cut-off value gives the following result.

$$cut-off = \frac{NC + PC}{1.3} = \frac{1.343 + 0.095}{1.3} = 1.106$$

The cut off value is therefore 1.106. Thus, all samples whose signal is higher than this are classified as negative (containing no anti-HAV antibody) and all samples in which the signal is lower than 1.106 are assessed as positive (containing anti-HAV antibody).

The absorbance of serum sample 1 is greater than the cut-off value, it is thus negative. The absorbance of serum sample 2 is less than the cut-off value, it is positive. In serum sample 3, the absorbance value is in the range of 0.9955 to 1.2167 (= 1.106 + 10 %), the determination must be repeated in the above example.

Table 1: Examples of absorbances of anti-HAV determinations using the qualitative test methods are shown as the difference between the absorbances at 450 nm signal and the signal at 620 nm as a reference.

Sample	OD450nm	Mean
NC	1.387	1.343
NC	1.298	
PC	0.102	0.095
PC	0.087	
Serum Sample 1	1.402	1.464
Serum Sample 1	1.526	
Serum Sample 2	0.775	0.769
Serum Sample 2	0.762	
Serum Sample 3	1.102	1.092
Serum Sample 3	1.082	

2. Semi-Quantitative test analysis

In addition to the calculation of the antibody content in mIU/ml by using a defined standard, a semi-

quantitative analysis can be carried out by the determination of the antibody titer. For this the unknown sample is used in various dilutions in this ELISA. Depending on the expected level of antibodies, the sample is diluted more or less. For these dilutions the Dilution Buffer is used. To determine the titer of the (cut-off) value the following formula is used

$$cut - off = \frac{Extinction\ Negative\ Control + Extinction\ Positive\ Control}{1.3}$$

The serum dilution with an absorbance directly below the cut-off value represents the antibody titer.

3. Analysis with CD standards

A more accurate determination of serum titer can be carried out in semi- quantitative test method.

These samples are used to estimate the anti-HAV titer based on a calibration curve. The absorbance values of each serum standard (S1 - S3) and the negative control (NC) as standard with the concentration 0 mIU/mL are given on the y-axis against the concentration (mIU/mL) of the anti- HAV antibody on the x-axis and the regression line is placed through the points.

For computational evaluation various regression methods are useful. The method with the best curve fit (at least R2 > 0.99) should be selected. Ideally, the calculation of the antibody content of the sample is performed by means of an evaluation program. An exemplary standard curve determined by 4- parameter logistics is shown in Figure 1.

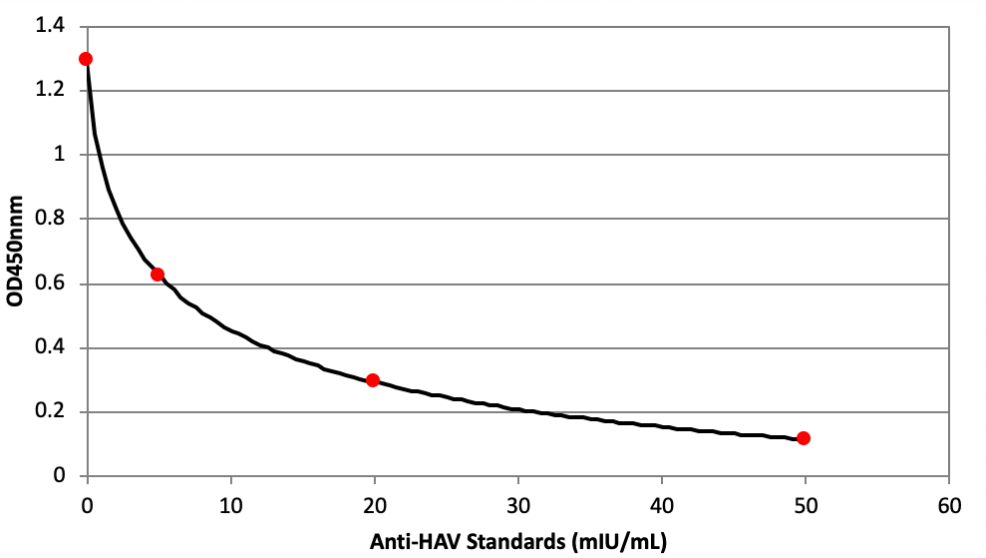

Figure 1: Exemplary standard curve with negative control as standard with the concentration of 0 mIU/mL.

In the table 2, exemplary results of anti-HAV antibody ELISA are shown.

Table 2: An example of an extinction with the semi-quantitative method when using the CD standards including negative control as standard with 0 mIU/mL. The sample dilution >1:20 was carried out in dilution buffer. The calculation of the antibody content of the sample was performed using an evaluation program.

	OD450nm	Anti-HAV titer in the diluted Probe(mIU/mL)	Anti-HAV titer in the diluted Probe(mIU/mL)
PC	0.112	-	
NC	1.403	-	
S1	0.116	50	
S2	0.302	20	
S3	0.616	5	
Sample			
Serum 1, 1:20	0.103	75.89	1517.70
Serum 2, 1:20	1.384	0.02	0.48
Serum 3, 1:20	0.578	5.82	116.48
Serum 4, 1:20	0.465	8.75	175.06
Serum 5, 1:20	0.127	58.01	1160.14
Serum 6, 1:20	0.772	3.02	60.38

4. Analysis with reference preparation

In addition, it is also possible to prepare standards in the desired concentrations from reference material to quantify the anti-HAV antibody.

The reference material should be diluted with the Dilution Buffer. An exemplary result is shown in Table 3.

The cut off value for the evaluation of the results shown here is 1.105 OD450 units ((0.112 + 1.324)/1.3). The dilution of the sample, which absorbance is directly below the cut off value, is chosen for the calculation of the antibody content.

In the sample the absorbance of the 1:1600 diluted sample is immediately below the cut-off. The anti-HAV titer in the diluted sample is 1.694 mIU/mL. The anti-HAV titer of the serum is therefore 1.694 x 1600 = 2710 mIU/mL.

Table 3: Exemplary extinction measurement by the semi-quantitative method using the reference preparation (NIBSC 97/646): The reference preparation and the unknown samples were diluted in Dilution Buffer to the concentration as given in the table and used in the assay. The calculation of the antibody content of the sample was performed using an evaluation program.

	OD450nm	Anti-HAV titer (mIU/mL)
PC	0.112	-
NC	1.324	-
Cut-off	1.105	
S1	1.185	0.625
S2	1.071	1.25
S3	0.935	2.5
S4	0.623	5
S5	0.390	10
S6	0.293	20
S7	0.115	50
Sample		
1:100 diluted	0.204	
1:200 diluted	0.348	
1:400 diluted	0.507	
1:800 diluted	0.735	
1:1600 diluted	0.987	1.694×1600=2710
1:3200 diluted	1.141	

Precision

Intra-CV < 15%

Inter-CV < 15%

Sensitivity

The Detection Threshold for the assay is 0.65 mIU/mL.

Precautions

1. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
2. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
3. All reagents should be handled as if potentially infectious. Operators should wear gloves and protective clothing when handling any patient sera or serum-based products.
4. Some reagents within the kit may contain antimicrobial agents, and acid. Avoid contact with the skin and eyes. Rinse immediately with plenty of water if any contact occurs.
5. Any liquid brought into contact with potentially infectious material needs to be discarded in a container with a disinfectant. Disposal must be performed in accordance with local regulations.
6. Only trained laboratory personnel should execute this test.