Creative Diagnostics®

Version 08-11/24

User's Manual

Anti-Hepatitis A-Virus (HAV) IgG ELISA Kit

REF **DEIA-NB24-07G**

Σ **96T**

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

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

Anti-HAV IgG ELISA Kit 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, neutralising antibodies appear at the same time of Anti_x0002_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, where as in older population (≥50 years) HAV-Antibody was found in 40.3 % of the population. In countries with less favourable 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 paediatric. 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 IgG ELISA Kit 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 2 hours at 37 °C. Anti-HAV antibodies bind to the antigen. The conjugate (peroxidase labeled anti-HAV) is added and incubated again for 1 h at 37 °C. Free binding sites of the antigen are bound with conjugate. Excess conjugate is washed of the plate and the substrate is added and incubated for 30 min at room temperature. The bound conjugate changes the colour of the substrate to blue. The reaction is terminated by adding the stopping solution. The colour turns yellow. The absorbance of the coloured 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 serum 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 anti-Mouse Adiponectin antibody. Wells are separately breakable. (8x12) wells
2. **Conjugate Concentrate (KK):** 100-fold concentrated (peroxidase labeled Anti-HAV Ig). 1 x 100 µL
3. **Positive Control (PK):** ready for use, anti-HAV positive control serum >500 mIU/mL. 1 x 1 mL
4. **Negative Control (NK):** ready for use, anti-HAV negative control serum. 1 x 1 mL
5. **Dilution Buffer (VP):** ready for use. Please shake before use! 1 x 120 mL

6. **Washing Buffer (WP):** 20-fold concentrated solution. 1 x 50 mL
7. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate. 1 x 12 mL
8. **Stopping Solution (SL):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
9. **Sealing Tape:** for covering the microtiter plate. 2

Additional to Semi-Quantitative Test:

10. **Serum standard 1 (STD1):** ready for use, anti-HAV titer 50 mIU/mL. 1 x 1 mL
11. **Serum standard 2 (STD2):** ready for use, anti-HAV titer 30 mIU/mL. 1 x 1 mL
12. **Serum standard 3 (STD3):** ready for use, anti-HAV titer 10 mIU/mL. 1 x 1 mL

Materials Required But Not Supplied

1. Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer WP (A. dest.), 950 mL.
2. Precision pipettes and multichannel pipettes with disposable plastic tips
3. Polyethylene PE/Polypropylene PP tubes for dilution of samples
4. Anti-HAV antibody negative serum (for dilutions > 1:10 to the semi-quantiative measurement)
5. Incubator (suitable for incubations at 37°C)
6. Vortex-mixer
7. Microtiter plate washer (recommended)
8. Microplate reader ("ELISA-Reader") with filter for 450 and ≥590 nm

Storage

Store the kit at 2-8°C after receipt until its expiry date. Avoid repeated thawing and freezing.

The shelf life of the components after initial opening is warranted for 4 weeks, store the unused strips and microtiter wells airtight together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The 1:20 diluted Washing Buffer WP is 4 weeks stable at 2-8°C

Specimen Collection And Preparation

1. Sample type: Serum and Plasma

Serum and Heparin / EDTA-Plasma yield comparable values.

2. Specimen collection

Use standard venipuncture for the blood sampling. Haemolytic reactions are to be avoided.

3. Required sample volume: 25 µL

4. Sample stability

In firmly closable sample vials

• Storage at 4°C: max. 3 days

• Freeze-thaw cycles: max. 3

Freezing and thawing of samles should be minimized.

5. Interference

Anticoagulants like EDTA and heparin in the usual concentrations do not influence the test. Bilirubin concentrations do not interfere up to 200 µg/mL and triglyceride concentrationen up to 5 mg/mL. Strongly hemolyzed samples (hemoglobin > 10 mg/mL) can result in false negative or false low values. Strongly hemolyzed samples should not be used.

6. Sample dilution

• Qualitative Test: Dilution: 1:10 with Dilution Buffer VP

• Quantiatitve Test: Dilution 1:10 with Dilution Buffer VP, > 1:10 with VPN

Use a 10 % solution of anti-HAV antibody negative serum in Dilution Buffer VP (VPN) for all sample dilutions > 1:10

Example: Dilution 1:10, 25 µL Sample is added to 225 µL Dilutionbuffer VP.

Example Dilution 1:50, 1 mL anti-HAV Antibody negative Serum is mixed with 9 mL Dilutionbuffer VP (VPN). 10 µL Sample is added to 490 µL to this Dilution Buffer including negative Serum (VPN).

Reagent Preparation

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

1. The required volume of Conjugate Concentrate KK is prepared by 1:100 dilution with Dilution Buffer VP. (e.g. 10 µL KK + 990 µL VP) Prepare only the amount required. The diluted conjugate can be stored for at least one week at 4°C.
2. The required volume of Washing Buffer WP is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest. (e.g. 50 mL WP + 950 mL A.dest) Please dilute only according to daily requirements.
3. The required volume of negative Serum VPN is prepared by 1:10 dilution with Dilution Buffer VP. (e.g. 1 mL negative Serum + 9 mL VP)

Assay Procedure

Note

1. Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate Solution S, is photosensitive–store and incubation in the dark.
2. When performing the assay reagents and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, diluted Conjugate Concentrate KK, as well as the Substrate Solution S should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution SL should be added to the plate in the same order as Substrate Solution S. All determinations should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.
3. Proper washing is of basic importance for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the

examined samples. Effects like high background values or high variations may indicate washing problems. All washing must be performed with the provided Washing Buffer WP diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least.

Automatic washing: When using an Automatic microtiter plate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

Manual washing: Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

4. The danger of handling with potentially infectious material must be taken into account.

Assay Step

1. Set Standard 1-3, test samples (1:10 diluted), Positive Control, Negative Control wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate.
2. Prepare Standards: Aliquot 100ul of Standard 1-3, Positive Control, Negative Control, test samples into wells.
3. Incubate: Seal the plate with a cover and incubate at 37°C for 2 hours.
4. Add 50ul Conjugate Concentrate into above wells (standard, control and test samples). Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 37°C for 1 hour.
5. Wash: Aspirate the contents of the wells, and wash plate 3 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
6. Add 100ul of Substrate Solution S into each well, cover the plate and incubate at 20-25°C in dark within 30 minutes.
7. Stop: Add 100ul Stopping Solution SL into each well.
8. Measure the absorbance within 30 min at 450 nm, with ≥ 590 nm as reference wavelength.

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.

In order to evaluate the results it should be ensured that the absorbances of the blank (A1/A2) 0.1 do not exceed the Positive Control PK 0.15 OD450 units. The signal of the Negative Control NK must be greater than 1.0 OD450 units. The difference between the absorbance of the Negative Control NK and Positive Control PK must be at least 0.4 OD450 units. The absorbances of the samples should be within the standard curve (NK - STD3) in the semiquantitative method. If the absorbances are outside this range, for reliable determinations, they should be determined again at higher dilutions in a second test.

Calculation

First, the absorbance determined with the chosen reference wavelength (> 590 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.

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 enabeles the distinctions of positive and negative probands with a sensitivity of 98.26 % (n = 801).

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 = (Extinction Negative Control+ Extinction Positive Control) /2

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 below. The absorbance difference between negative and positive control is greater than 0.4 (1.390 to 0.024). The calculation of the cut-off value gives the following result.

cut-off = (NK+PK) /2 = (1.390 + 0.024) /2 = 0.707

The cut off value is therefore 0.707. 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 0.707 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 0.778 to 0.636 (= 0.707 + 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	Extinction	Mean
Negative Control	1.372	1.390
Negative Control	1.408	
Positive Control	0.024	
Serum Sample 1	1.461	1.387
Serum Sample 1	1.312	
Serum Sample 2	0.025	0.023
Serum Sample 2	0.021	
Serum Sample 3	0.735	0.739
Serum Sample 3	0.743	

Semi-Quantitative test analysis

1. Antibodytitre calculation

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 the Anti-HAV IgG ELISA Kit. Depending on the expected level of antibodies, the sample is diluted more or less. For these dilutions the Dilution Buffer including 10 % negative serum (VPN) is used. To determine the titer of the (cut-off) value the following formula is used

cut-off = (Extinction Negative Control+ Extinction Positive Control) /2

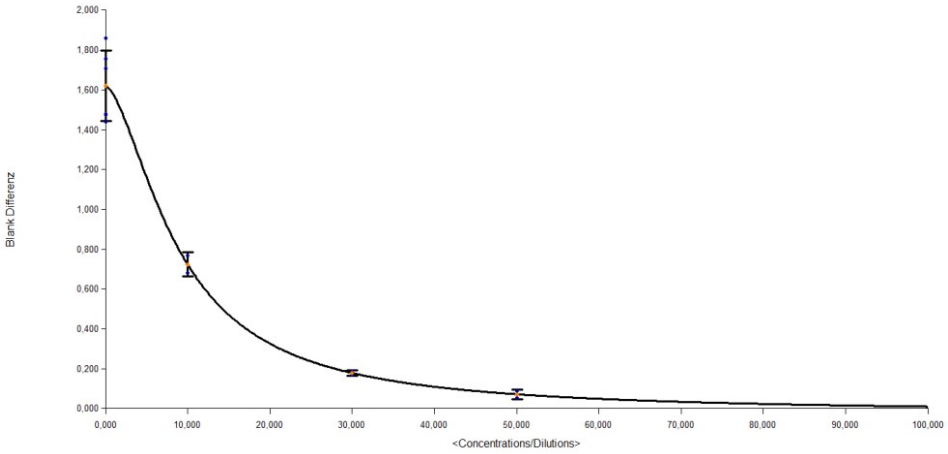
The serum dilution with an absorbance directly below the cut-off value represents the antibody titer.

2. Analysis with Creative Diagnostics standards

A more accurate determination of serum titer can be carried out in semi- quantitative test method. For this purpose, the test kit includes 3 standards, which contain a defined amount of anti-HAV antibodies. These are ready to use and can be tested directly in the assay.

These samples are used to estimate the anti-HAV titer based on a calibration curve. The absorbance values of each serum standard (STD1 - STD3) and the negative control (NK) as standard with the concentration 0 mIU/mL are given on the y-axis against the concentration (mIU/mL) of the antiHAV 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 > 0.9) 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 below.



In the table below, exemplary results of anti-HAV Creative Diagnostics antibody ELISA are shown.

Table 2: An example of an extinction with the semi-quantitative method when using the Creative Diagnostics standards including negative control as standard with 0 mIU/mL. The sample dilution >1:10 was carried out in dilution buffer with 10% negative serum (VPN). The calculation of the antibody content of the sample was performed using an evaluation program.

		Blank corrected OD450-620	Anti-HAV Titer in the diluted Probe (mIU/mL)	Anti-HAV Titer in the undiluted sample (mIU/mL)
Positive Control		0.01	-	-
Negative Control (Standard 4)		1.631	0	-
Standard 1		0.083	50	-
Standard 2		0.190	30	-
Standard 3		0.735	15	-
Sample	**Dilution**			
Serum 1	1:10	0.089	48.14	481
Serum 2	1:10	0.294	22.18	222
Serum 3	1:10	1.264	4.17	42
Serum 4	1:50	0.598	12.36	618
Serum 5	1:50	0.121	39.97	1998

3. 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 with 10% anti-HAV antibody negative serum (VPN). An examplary result is shown in Table 3. The cut off value for the evaluation of the results shown here is 0.724 OD450 units ((0.02 + 1.427)/2). The dilution of the sample, which absorbance is directly below the cut off value, is chosen for the calculation of the antibody content.

In serum 1 the absorbance of the 1:400 diluted sample is immediately below the cut-off. The anti- HAV titre in the diluted sample is 8.8 mIU/mL. The anti-HAV titer of the serum is therefore 8.8 x 400 = 3 520 mIU/mL.

In serum 2, the absorbance of 1:1600 diluted sample is immediately below the cut-off. The anti- HAV titre in the diluted sample is 8.5 mIU/mL. The anti-HAV titer in the undiluted serum is therefore 8.5 x 1600 = 13600 mIU/mL.

Table 3: Examplary 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 with 10 % negative serum (VPN) 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.

	Extinction-Meanvalue	Anti-HAV Titer in the diluted samples (mIU/mL)
Positive Control	0.020	-
Negative Control	1.427	-
Standard 1*	0.068	50
Standard 2*	0.157	30
Standard 3*	0.334	15
Standard 4*	0.542	10
Standard 5*	0.838	5
Standard 6*	0.953	3
Standard 7*	1.245	1
Serum 1:		
1:100 diluted	0.110	
1:200 diluted	0.262	
1:400 diluted	0.571	8.8 x 400= 3520
1:800 diluted	0.761	
1:1600 diluted	1.048	
Serum 2:		
1:100 diluted	0.013	
1:200 diluted	0.036	
1:400 diluted	0.088	
1:800 diluted	0.228	
1:1600 diluted	0.532	8.5 x 1600 = 13600

*Not included in the test kit

Precision

Serum samples were measured in at least 13 independent assays and the variation of the measurement result was calculated (Table 4).

Table 4: Inter-Assay Variation. SD= Standard deviation, CV% = Coefficient of Variation in %, Number = Number of the independent determinations.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
Mean [mIU/mL]	19303	3077	297	378	46	1210	45	27	9	5
SD	2557	378	36	54	13	156	5	3	2	1
CV%	**13**	**12**	**12**	**14**	**29**	**13**	**11**	**13**	**17**	**19**
Number	20	21	16	19	19	18	20	21	20	13

Sensitivity

The sensitivity of the test system was evaluated by the International standard NIBSC 97/646. The standard material was diluted and the dilution was applied as sample in the Anti-HAV IgG ELISA Kit. The measured signals (OD450) are shown in table 5.

Table 5: Analytical sensitivity. Shown are the measured signals (OD450).

NIBSC [mIU/mL]	0	2	5	10	20
Replicates-	1.577	1.327	1.136	0.925	0.564
	1.495	1.337	1.159	0.83	0.475
	1.587	1.297	1.108	0.898	0.557
	1.329	1.373	1.142	0,79	0,51
	1.562	1.071	1.111	0,819	0,505
	1.278	1.219	1.083	0,854	0,398
		1.225	1.093	0,812	0,464
		1.331	1.199	0,865	0,45
Mean	1.47	1.27	1.13	0,85	0,49
SD	0.13	0.10	0.04	0,05	0,06
CV [%]	9	8	3	5	11

The recalculated analytical sensitivity resulting from the 2fold standard deviation of the negative control (n=6) is 1.10 mIU/mL. In an additional experiment the theoretically calculated analytical sensitivity was proven by 1 mIU/mL diluted NIBSC 97/646. Here the negative control showed a signal of 2.5 (SD 0.18) and the diluted international standard had a signal of 2.12 (SD 0.2).

Furthermore, through the measurement of 801 human serum samples (of which 287 were positive and 514 negative for anti-HAV antibodies) can be shown that the Anti-HAV IgG ELISA Kit distinguishs very well between positive and negative samples, the sensitivity was 98.26 %.

Specificity

Assessment of the specificity of the ELISA was done with 25 samples negative for anti-HAV IgG antibody positive for IgG Antibodies against different viruses.

Linearity

The linearity of the assay was examplary by the dilution of two human sera with high anti-HAV antibody titer. The dilution of the samples was carried out until 1:10 in Dilution Buffer (VP) and for all dilutions > 1:10 in Dilution Buffer + 10 % anti -HAV antibody negative serum (VPN). In Figure below the dilutions of 1: 2.5 to 1:80 for serum 1 and 1:5 to 1:320 for serum 2 are shown. By means of linear regression can be shown, that the linearity of the test system is very high.

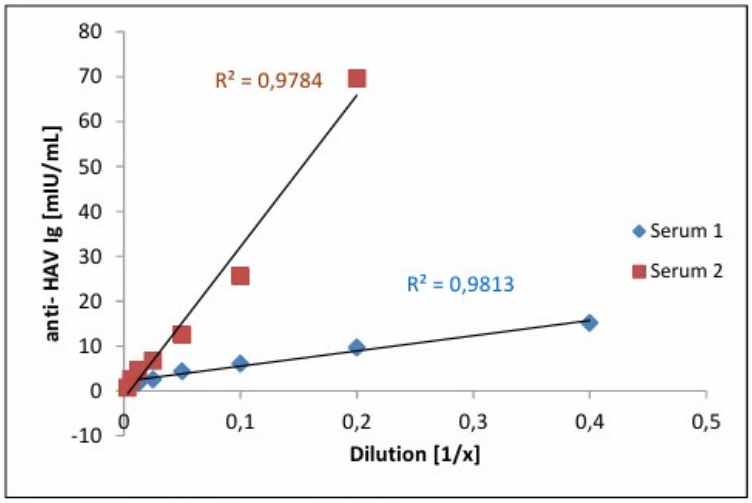


Figure 3 Linearity of the sample dilution.

Interferences

The influence of the heterophilic antibodies, rheuma factors and anti-species antibodies is reduced by the assay design, but can not be completely excluded. The interference of various physiological and pharmaceutical substances was tested for the concentrations indicated. Higher concentrations or other substances may interfere with the measurement.

Precautions

1. For research and professional use only. The Creative Diagnostics kit is suitable only for in vitro use and not for internal use in humans and animals.
2. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Creative Diagnostics will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided.
3. Do not use obviously damaged or microbial contaminated or spilled material.
4. This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.
5. Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.
6. Following components contain human serum: Positive Control, Negative Control, Conjugate Concentrate and Serum standard 1-3.

   The test plate MTP is coated with inactivated Antigen, which was negative in the rest infectivity test.

   Source human serum for the control sera provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.