# Anti-HAV Antibody ELISA

**Enzyme Immunoassay for qualitative and quantitative detection of antibodies against**

## Hepatitis A-Virus

English

For Research Use Only.
Not for use in diagnostic procedures.

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## Symbols / Symbole

according to DIN EN 980 and EDMA recommendations Standard News 6 2001

| | | |
|---|---|---|
|  | | Expiry date / Verfallsdatum / Date de péremption / Data di scadenza /Fecha de caducidad / Data de validade / Uiterste gebruiksdatum / Udløbsdato / Utgångsdatum / Termin Ważności / Lejárati idő / Doba exspirácie / Doba exspirace |
|  | | Consider instructions for use / Bitte Gebrauchsanweisung beachten / Consultez la notice d'utilisation / Consultare le istruzioni per l'uso / Consulte las instrucciones de utilización / Consulte as instruções de utilização / Raadpleeg de gebruiksaanwijzing / Se brugsanvisningen / Läs anvisningarna före användning / Proszę przeczytać instrukcję wykonania / Vegyük figyelembe a használati utasításban foglaltakat / Postupujte podľa pokynov na použitie / Postupujte dle návodu k použití |
|  | | Lot-Batch Number / Charge-Chargennummer / Lot-Code du lot / Lotto-Numero di lotto / Lote-Código de lote / Lote-Código do lote / Lot-Partijnummer / Lot-Batchkode / Lot-Satskod / Numer serii / Lot-Batch szám / Číslo šarže / Číslo šarže |
|  | | Manufactured by / Hergestellt von / Fabriqué par / Prodotto da / Fabricado por / Fabricado por / Vervaardigd door / Fabrikation af / Tillverkad av / Wyprodukowane pr / Gyártotta / Vyrobené / Vyrobeno |
|  | | Catalogue Number / Bestellnummer / Numéro de référence / Numero di riferimento / Número de referencia / Número de referência / Referentienummer / Referencenummer / Katalognummer / Numer katalogowy / Katalñgusszám / Katalñgové číslo / Katalogové číslo |
|  | | Store at between / Lagerung bei zwischen / Conserver à entre / Conservare a tra / Conservar a temp. entre / Armazene a entre / Bewaar bij tussen / Opbevares mellem / Förvaras vid / Przechowywać w / Tároljuk ð ð között / Skladujte v rozsahu / Skladujte v rozmezí |
|  | | Contains sufficient for x tests / Inhalt ausreichend für x Tests / Contient suffisant pour x tests / Contenuto sufficiente per x test / Contiene suficiente para x pruebas / Contém suficiente para x testes / Bevat voldoende voor x bepalingen / Indeholder tilstrækkeligt til x prøver / Innehållet räcker till x analyser / Zawartość na x testñw / Tartalma X teszt elvégzésére elegendő / Obsahuje materiál pre x testov / Obsahuje materiál pro x testï |
|  | | Keep away from sunlight / Nicht dem Sonnenlicht aussetzen |
|  | | Incubation time / Inkubationszeit |
| °C | | Incubate at / Inkubation bei |
| MTP | | Miktrotiterplate/Mikrotiterplatte |
| SPE | | Sample / Probe |
| DILBUF | VP | Dilution Buffer / Verdünnungpuffer |
| CONJ | KK | Conjugate/ Konjugat |
| DILU X | | Dilute in Buffer X / Verdünnen in Puffer X |
| CAL X | Std 1- Std 2 - Std3 | Standard X / Standard X |
| Control | + / - | Control Serum positive / negative / Kontrollserum positiv/ negativ |
| WASHBUF 20x | WP | Washing Buffer Concentrate / Waschpufferkonzentrat |
| WASHBUF | | Washing Buffer / Waschpuffer |
| SUBST TMB | S | Substrate |
| $H_2SO_4$ | ST | Stop Solution / Stopp Lösung |
| TAPE | | Cover Plate with sealing tape / Platte abkleben |
| MEASURE | | Measure plate within 30 min at 450 nm (Referencefilter ≥590nm) / Ausmessung innerhalb von 30 min bei 450 nm (Referenzfilter ≥ 590 nm). |

# Read entire protocol before use!

## TECHNICAL FEATURES+APPLICATIONS

## INTENTED USE

Mediagnost anti-HAV EIA, E10 is an enzyme immunoassay for **research use only** it detects antibodies against Hepatitis A virus in Human Serum.

## INTRODUCTION

Positive detection of antibodies directed against the Hepatitis A virus (anti-HAV) is evidence of immunity to Hepatitis-A virus (1). After natural infection with the Hepatitis A-Virus, neutralising antibodies appear at the same time of Anti-HAV of IgG-Class formation (1).

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 (12).

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 (5,7,8).

## PRINCIPLE OF THE TEST:

Mediagnost anti-HAV EIA, E10 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 IgG) 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 subtrate 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 **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 PROVIDED

| | | |
|---|---|---|
| 1) | MTP | **Microtiter plate,** ready for use: Microtiter plate with 96 wells, divided up in 12 strips with 8 wells separately breakable, coated with inactivated HAV-antigen, packed in a laminate bag. |
| 2) | CONJ | **Conjugate Concentrate KK, 100 µl, 100fold concentrated** (peroxidase labeled Anti-HAV IgG) **Dilute 1:100 with VP.** The **diluted conjugate** can be stored for at least one week at 4°C. |
| 3) | BUF VP | **Dilution Buffer VP**, **120 ml,** ready for use, please use for the dilution of **Conjugate Concentrate KK**, and for sample dilution. |
| 4) | Control + | **Positive Control, PK 1 ml,** anti-HAV positive control serum >500 mIU/ml, ready for use. |
| 5) | Control - | **Negative Control, NK 1 ml,** anti-HAV negative control serum, ready for use |
| 6) | WASHBUF 20x | **Washing Buffer (WP), 50 ml,** 20 X concentrated solution. Dilute 1:20 with distilled water before use (e.g. add (50 ml) **WP** into a graduated flask and fill up with A.dest. to 1000 ml). Attention: After dilution the Washing Buffer is only 4 weeks stable, dilute only according to requirements. |
| 7) | SUBST | **Substrate (S), 12 ml,** ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised $H_2O_2$ Tetramethylbencidine. |
| 8) | $H_2SO_4$ | **Stopping Solution (SL), 12 ml,** ready for use, 0.2 M sulphuric acid, Caution acid! |
| 9) | | Sealing tape for covering of the microtiter plate, 2 x, adhesive. |

**Additionally for the Quantitative Test**

| | | |
|---|---|---|
| 10) | CAL STD1 | **Serum standard 1 (anti-HAV titer 50 mIU/ml) (STD1)**<br>1 vial (**1 ml) Serum Standard 1**, anti-HAV titer 50 mIU/ml, ready for use. |
| 11) | CAL STD2 | **Serum standard 2 (anti-HAV titer 30 mIU/ml) (STD 2)**<br>1 vial (**1 ml**) **Serum standard 2**, anti-HAV titer 30 mIU/ml, ready for use |
| 12) | CAL STD3 | **Serum standard 3 (anti-HAV titer 10 mIU/ml) (STD 3)**<br>1 vial (**1 ml**) **Serum Standard 3**, anti-HAV titer 10 mIU/ml, ready for use |

## MATERIALS REQUIRED BUT NOT PROVIDED

Distilled or deionized water for dilution of the Washing Buffer (WP)
Incubator or water bath with adaptor for microtiter plates
Vortex-mixer
Precision pipettes Micropipettes and multichannel pipettes with disposable plastic tips
Microtiter plate washer (recommended)
Micro plate reader ("ELISA-Reader") with filter for 450 and ≥590 nm
Polyethylene PE/Polypropylene PP tubes for dilution of samples

## STORAGE CONDITIONS

The microtiter plate wells and all undiluted reagents are stable until the expiry date if stored in the dark at 2-8°C.
Unused microtiterplate stripes have to be stored airtight together with the desiccant bag at 2-8°C.
The Substrate Solution (S), stabilised $H_2O_2$-Tetramethylbencidine, is photosensitive – store and incubate in the dark.
The shelf life of the components after opening is not affected, if used appropriately.

## TECHNICAL NOTES

**For research and professional use only.**
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.
Before use, all kit components should be mixed and brought **to room temperature at 20 - 25°C**. Precipitates in buffers should be dissolved before use by thorough mixing and warming. **Temperature WILL affect the absorbance** readings of the assay. However, Values for the samples will not be affected.
Do not mix reagents of different lots. Do not use expired reagents.
The microplate contains snap-off strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch and used in the frame provided.
**Incubation at room temperature means: Incubation at 20-25°C**
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 diluted to usage concentration. Washing volume per washing cycle and well must be 300 µl at least.

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

When using an automatic microtitre 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 is an adequate alternative option. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamically swinging out the microtitre 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.

## WARNINGS AND PRECAUTIONS

Caution: This kit contains material of human and/or animal origin. Source human serum for the Control Serum provided in this kit was tested by FDA recommended methods and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV) antibodies. No known test methods can offer total assurance of the absence of infectious agents; therefore all components and specimens should be treated as potentially infectious.
The antigen has been inactivated with formaldehyde.

**Stop solution contains 0.2 M Sulfuric Acid ($H_2SO_4$)**

R36/38 Irritating to eyes and skin
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1 After contact with skin, wash immediately with plenty of water
S36/37 Wear suitable protective clothing and gloves.

Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step. Use separate pipette tips for each sample, control and reagent to avoid cross contamination. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
Some reagents contain 0.01% **2-Methyl-4-Isothiazolin-3-one** or <0.01% **5-Chloro-2-Methyl-2H-Isothiazolin-3-one and 2-methyl-2H-Isothiazol-3-one,** R-Phrases: 34/43/36/38 S -Phrases: S26-28.1-36/37/45

**TMB-Substrate contains 3,3´,5,5´ Tetramethylbenzidine**.

| | |
|---|---|
| R20/21/R22 | Harmful by inhalation, in contact with skin and if swallowed |
| R36/37/38 | Irritating to eyes, respiratory system and skin |
| S26 | In case of contact with eyes, rinse immediately with plenty of water and seek medical advice |
| S28.1 | After contact with skin, wash immediately with plenty of water |
| S36/37 | Wear suitable protective clothing and gloves |

**General first aid procedures:**
Skin contact: Wash affected area thoroughly with water. Discard contaminated cloths and shoes.
Eye contact: In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes. In order to assure an effectual rinsing spread the eyelids.
Ingestion: If swallowed, wash out mouth thoroughly with water. Immediately see a physician.
Do not eat, drink or smoke in these areas.
Never pipette the materials with the mouth.
Spilled material must be wiped off immediately and should become disinfected. Clean contaminated areas and equipment with a suitable detergent.

**SPECIMEN COLLECTION, PREPARATION AND STORAGE**

***Sample Preparation***

The test can be performed either using human serum or plasma. Anticoagulants as EDTA or heparin in usual concentrations have no disturbing influence. Also bilirubin up to **200 µg/ml** and triglycerides up to 5 mg/ml don't disturb. Hemolytic samples (>10 mg/ml hemoglobin) can produce false negative or artificial low results. To store samples over 24 h freezing at **-20°C** is recommended. Avoid freezing/thawing.

Detection limit: 68 mIU/ml,
Measuring range: 3 - 50 mIU/ml

**The dilution of Serum samples:**

Qualitative Test: 1:10 with **Dilution Buffer (VP)**

Quantitative Test: **at least 1:10** with **Dilution Buffer (VP)**

Use **Dilution Buffer (VP)** with **10% negative anti-HAV serum** (e.g. the negative control) to dilute samples more than **1:10**.

# *ASSAY PROCEDURE*

NOTES: **Bring all reagents to room temperature (20 - 25°C) before use.** All determinations (Standards, Control Serum and Samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.
When performing the assay, the Standards, Control Serum and the samples should be pipette as fast as possible.
To avoid distortions due to differences in incubation times,
**Stop Solution ST** should be added to the plate in the same order as the Substrate Solution S.

# *QUALITATIVE PROTOCOL*

**Test Procedure**

1) Leave wells **A1/A2** (blank)
2) Pipette in positions **B1/2 100 µl of Positive Control (PK)**
   Pipette in positions **C1/2 100 µl of Negative Control (NK)**
3) Pipette sample dilution **100 µl** in the rest of the wells according the requirements. Double determination is recommended.
4) Seal strips with adhesive tape and incubate for **2 hours** at **37 °C**.
5) Add **50 µl diluted Conjugate (KK)** per well, except the first two wells (**A1/A2, blank**). **Don't empty or wash the plate.**
6) Seal the reaction chambers with tape and incubate again for **1 hour at 37°C**.
7) At the end of the incubation period empty the wells and wash **3 times** with **Washing Buffer (WP)** (**300 µl**/ **well**).
8) Add **100 µl** of **Substrate solution (S)** to each well, including (**A1/A2),** and incubate **30 minutes at room temperature (20 - 25°C) in the dark.**
9) Add **100 µl Stop Solution** into each well, including A1/A2. Attention: *acid!*
10) Blank the spectrophotometer at **450 nm** against the TMB **Substrate** blank (well **A1/A2**). Measure the absorbance of each sample at 450 nm. (For microplate photometers with dual wave length mode the reference wave length should be **≥590 nm**)

***RESULTS OF THE* QUALITATIVE *TESTS***

Calculate the mean values of absorbance of the negative controls and samples. The test is valid on the condition that:

The difference between the mean absorbance of **positive (PK)** and **negative control (NK)** is at least **0.4**

Otherwise the test must be repeated.

The cut- off is calculated as:

$$\frac{\text{(absorbance positive control + mean absorbance of negative controls)}}{2}$$

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

**Table 1:** Results of a qualitative anti-HAV determination as an example.

| **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 | |

Example

The test is valid because the following condition is complied with:
The Extinctions difference between Negative Control (**NK**) and Positive Control (**PK**) > 0.4

$$\text{Cut-off} = \frac{1.390 + 0.024}{2} = 0.707$$

The **Serum Sample 1** is **negative** because the extinction is higher than the cut-off value.
The **Serum Sample 2** is **positive**, because the extinction is lower than the cut-off value.

**The Extinction of Serum Sample 3 is with borderline values (0.778-0.636) (= 0.707 ± 10%).**
Samples ± 10% around the cut-off should be determined again.

**QUANTITATIVE PROTOCOL**

By means of the quantitative protocol the Serum titre can be estimated.

***Test Procedure***

Perform the test as described in the qualitative protocol.
Additionally to quantitative protocol:

Pipette 100 µl **Serum Standard (STD 1)** in the positions **D1/D2**

Pipette 100 µl **Serum Standard (STD 2)** in the positions **E1/E2**
Pipette 100 µl **Serum Standard (STD 3)** in the positions **F1/F2**
The test is valid, when following condition is fulfilled:
The difference between the mean value of extinction of the negative control and the positive control must be at least 0.4.

## PIPETTING SYSTEM, *QUANTITATIVE* PROTOCOL

- Dilute samples at least **1:10** with **Dilution Buffer VP,** use **Dilution Buffer (VP)** with **10% negative anti-HAV serum** (e.g. the negative control) to dilute samples more than **1:10**.
- **Positive Control (PK)**, **Negative Control (NK)** and **Serum Standards (STD)** are ready for use.
- Dilute Conjugate Concentrate **KK 1:100** with **Dilution Buffer**

| | |
|---|---|
| **Diluted Serum samples,<br>Positive Control,<br>Negative Control,<br>Serum Standard 1, 2, 3** | **100 µl** |
| Incubation: **2 hours at 37 ° C** | |
| **1:100 Diluted Conjugate** | **50 µl** |
| Incubation: **1** hour at **37°C,** Aspirate and Wash | |
| **Substrate** | **100 µl** |
| **Incubation:** 30 Minutes **at** RT **in the dark** | |
| **Stop Solution** | **100 µl** |
| Extinction (450 / ≥ 590 nm) | |

**Table 2**: An exemplary Extinction measurement with the quantitative method

| Sample-Nr. | Description | Extinction: | Average |
|---|---|---|---|
| 1 | Blank | 0 | |
| 2 | Negative Control | 1.312 | 1.292 |
| 3 | Negative Control | 1.272 | |
| 4 | Positive Control | 0.025 | |
| 5 | Serum Standard 1 | 0.128 | 0.139 |
| 6 | Serum Standard 1 | 0.150 | |
| 7 | Serum Standard 2 | 0.265 | 0.272 |
| 8 | Serum Standard 2 | 0.278 | |
| 9 | Serum Standard 3 | 0.717 | 0.711 |
| 10 | Serum Standard 3 | 0.705 | |
| 11 | Serum Sample 1 | 1.189 | 1.184 |
| 12 | Serum Sample 1 | 1.179 | |
| 13 | Serum Sample 2 | 1.340 | 1.346 |
| 14 | Serum Sample 2 | 1.352 | |
| 15 | Serum Sample 3 | 0.053 | 0.057 |
| 16 | Serum Sample 3 | 0.060 | |
| 17 | Serum Sample 4 | 0.032 | 0.030 |
| 18 | Serum Sample 4 | 0.027 | |
| 19 | Serum Sample 5 | 0.184 | 0.176 |

| | | | |
|---|---|---|---|
| 20 | Serum Sample 5 | 0.168 | |
| 21 | Serum Sample 6 | 0.624 | 0.628 |
| 22 | Serum Sample 6 | 0.632 | |
| 23 | Serum Sample 7 | 1.420 | 1.432 |

***Results of the Quantitative Assay***

Calculate the mean values of absorbance of the negative controls and samples. The difference between the mean absorbance of positive and negative control must be at least 0.4. Otherwise the test must be repeated.

Calculation of the serum titer can be performed in different ways:

**1) Fast semi quantitative evaluation**

The mean absorbance values of sample 1, 2 and 7 are higher than the mean absorbance of serum standard 3 (10 mIU/ml), so they are considered to be negative (anti-HAV titer in the 1:10 diluted samples are less than 10 mIU/ml). The undiluted samples have got titers less than 100 mIU/ml.
The mean absorbance value of the samples 3 and 4 are below the mean absorbance of serum standard 1 (50 mIU/ml), and are considered to be positive (the anti-HAV titer in the 1:10 diluted samples are more than 50 mIU/ml). The undiluted samples have got titers > 500 mIU/ml. Exacting determination can be made by titration (see Chapter Serum Titration).
The mean absorbance value of sample 5 is between the mean absorbencies of serum standard 1 (50 mIU/ml) and 2 (30 mIU/ml), which indicates the anti-HAV titer in the diluted sample to be higher than 30 and less than 50 mIU/ml. The undiluted serum has got the titer between 300 mIU/ml and 500 mIU/ml.
The mean absorbance value of sample 6 is between the mean absorbance of serum standard 2 (30 mIU/ml) and 3 (10 mIU/ml), which indicates the anti-HAV titer in the sample to be higher than 10 mIU/ml and less than 30 mIU/ml. The undiluted serum has got the titer between 100 mIU/ml and 300 mIU/ml.

**2) Anti-HAV titer by a standard curve:**

A titer calculation can be performed using a standard curve. The absorbance values of the standards are plotted on the y-axis, the logarithm of the anti-HAV titers on the x-axis and a straight line is drawn through the data points. Plot the serum sample extinctions parallel to the x-axis. The serum titer is determined by drawing a line down to the x-axis from where the standard curve and the parallel line cross. The titer of the diluted sample can be read from the x-axis and is to multiplicate 10-fold to get the titer of the undiluted sample (see Figure 1).

***SERUM TITRATION***

The *calibration of sera* for example for **preparation of an intern Standard** can be maid by titration the 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).

***Preparation of the titration***

To prepare the Standard curve prepare a Dilution of one Reference preparation* in Range of **0 to 100 mIU/ml**.

Dilution of Serum Samples:

The serum which is wished to be calibrated, should be diluted, that the excepted results are in the range of the Standard curve.

Titers of natural infections longer ago may reach about 1 -10 IU/ml. Titers of fresh infections (3 -6 month) reach titers of 100 to 300 IU/ml.

Choose dilutions like 1:100, 1:200, 1:400, etc.

To determine titers higher than 500 mIU/ml dilute the samples suitable steps.

**Dilution material:**

**Due to the matrix effect of serum proteins it is essential to use dilution buffer with 10% negative anti-HAV serum (e.g. the negative control) to dilute samples more than 1:10.**

**Performance of the titration of sera**

Perform test as described in “Quantitative Assay Procedure”. The Standard curve is prepared from a Reference reagent*.

**EVALUATION OF THE TITRATION**

To evaluate the results, you can use the method with linear regressive standard curve as described above or a evaluation with 4 parameter logistic.

These two evaluation methods were compared in testing two Anti-HAV-Positive Serum pools in five following days. The results are shown in the Table 3.

**Table 3:** Evaluation of titration by means of Linear Regression and 4 Parameter-Logistic.

| Evaluation method | Linear Regression | 4Parameter-Logistic |
|---|---|---|
| Anti-HAV Titre Pool 1 | 4.3 IU/ml | 3.9 IU/ml |
| Inter-Assay V.K. | 13.3 % | **10.9 %** |
| Anti-HAV Titre Pool 2 | 15.9 IU/ml | 14.4 IU/ml |
| Inter Assay V.K. | 15.4 % | **10.1 %** |

**a) Linear regression:**

Only the linear part of the standard curve can be used (5 to 50 mIU/ml).

The absorbance values of the standards are plotted on the y-axis, the logarithm of the anti-HAV titers on the x-axis and a straight line is drawn through the data points. Plot the serum sample extinctions parallel to the x-axis. The serum titer is determined by drawing a line down to the x-axis from where the standard curve and the parallel line cross. The titer of the diluted sample can be read from the x-axis.

The evaluation is performed in the middle range of the standard curve. Calculate the cut-off according the following form:

$$\text{Cut-off} = \frac{\text{Extinction positive Control} + \text{Extinction negative Control}}{2}$$

The sample dilution with the extinction just below the cut-off are best to evaluate. Find out the suitable titers to the measured extinctions of the samples and multiplicate with the dilution factor.

**Table 4:** Example of a Calibration of two sera (Serum 1 and Serum 2) with a reference reagent * (diluted to Standards 1*-7) (see Figure 2).

| | mean of Extinction | anti-HAV titer diluted sample (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

$$\text{cut-off} = \frac{0{,}020 + 1{,}427}{2} = 0{,}724$$

The Extinction of the 1:400 dilution of the serum 1 is just below the cut-off. The Anti-HAV Titre in the diluted samples is 8.8 mIU/ml. The Anti-HAV Titre of the serum is 8.8 x 400 = 3520 mIU/ml.
The Extinction of the 1:1600 dilution of the Serum 2 is just below the cut-off. The Anti-HAV Titre in the diluted sample is 8.5 mIU/ml. The Anti-HAV Titre in the undiluted serum is 8.5 x 1600= 13 600 mIU/ml.

**b) Computer aided:**
Using a suitable computer programme to calculate the interpolation of sigmoid curves (e.g. EasyFit, SLT, Crailsheim) it is possible to create a titration curve outwith the linear region of the curve, for example in the region between 0 and 100 mU/ml (this could be used to calibrate sera against reference preparations). Generating calibration curves and calculating HAV-titers is performed via a 4-parameter function.
Use the ascending part of the standard curve for evaluation. Calculate the cut-off-control.

$$\text{cut-off} = \frac{\text{Extinction positive Control} + \text{Extinction negative Control}}{2}$$

Take the sample values nearest to the cut off to evaluate the titre (see example above).
The Anti-HAV Titre in the diluted 1:400 samples of **serum 1** is 8.8 mIU/ml:
Titre of the undiluted sample: 18,8 x 400 = 3520 mIU/ml.
The Anti-HAV Titre in the diluted samples of **serum 2** is 8.6 mIU/ml:
Titre of the undiluted sample: 8,6 x 1600 = 13 760 mIU/ml

## Appendix / Anhang

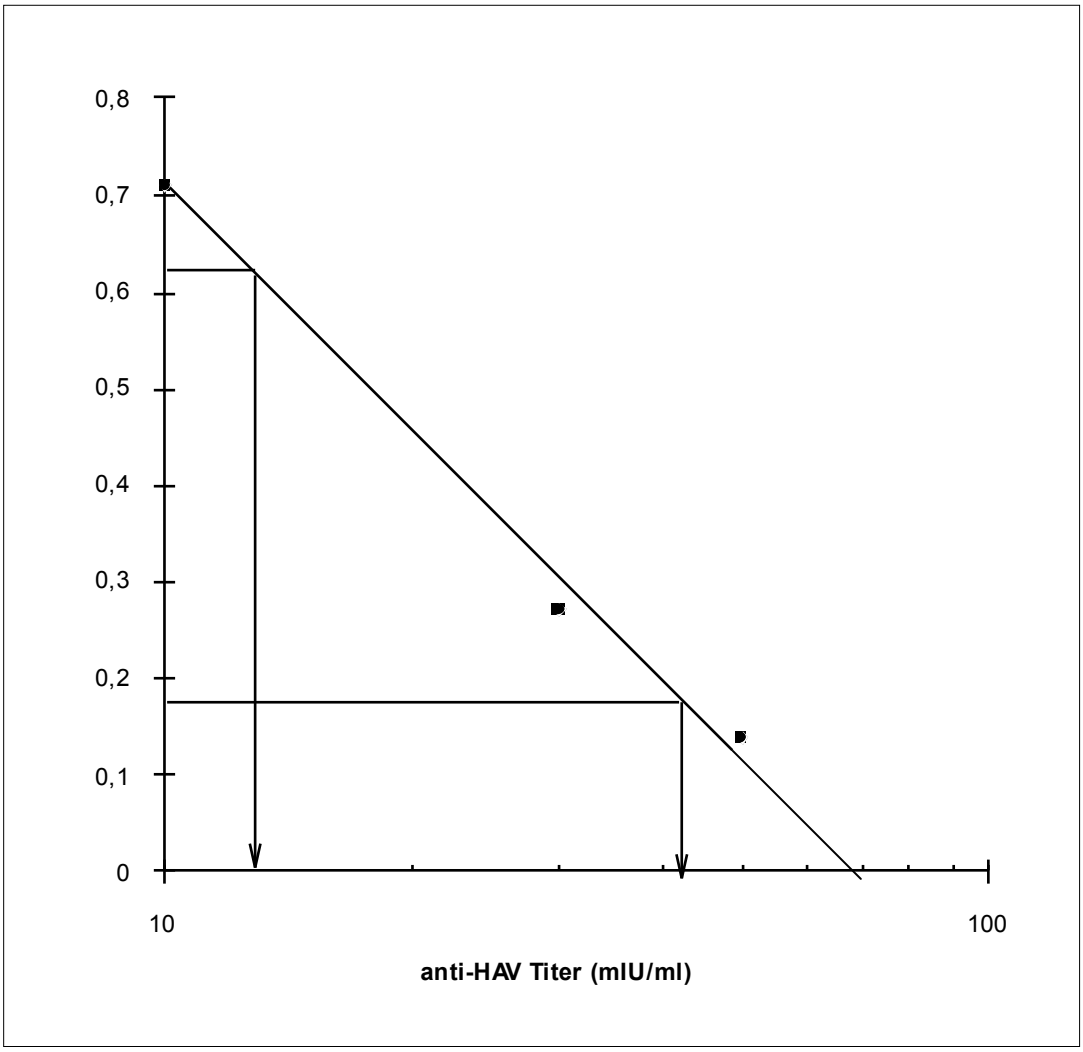


**Fig. 1:** The absorbance values of the standards are plotted on the y-axis, the logarithm of the anti-HAV titers on the x-axis and a straight line is drawn through the data points.

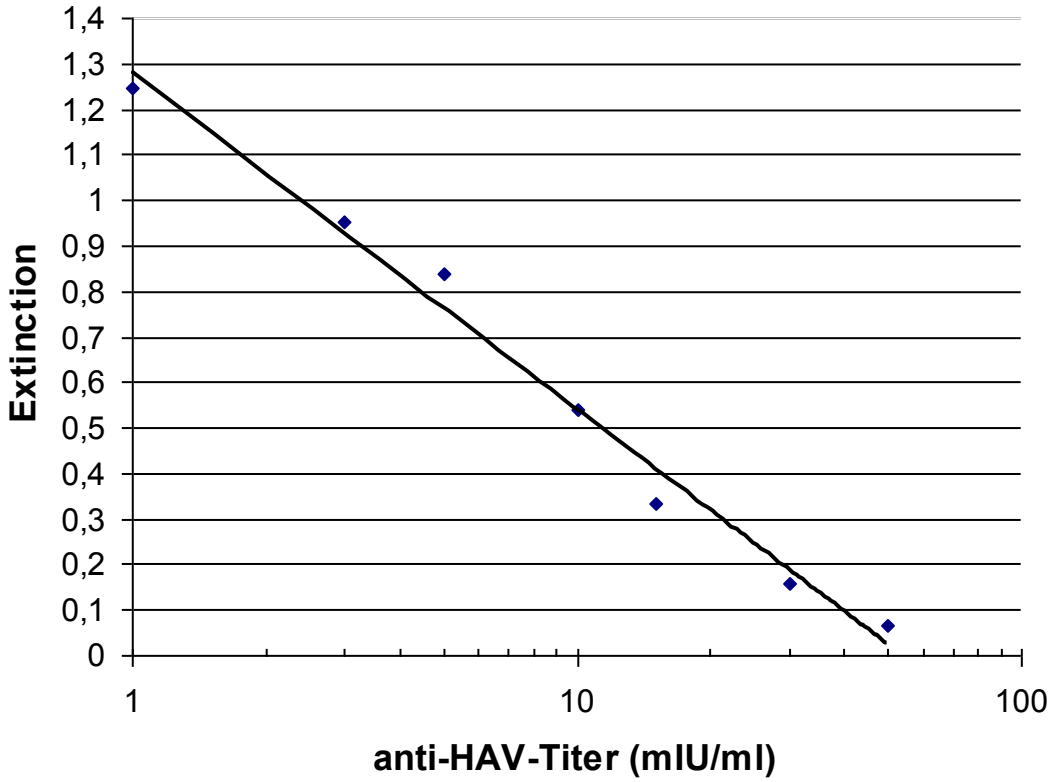


**Fig. 2:** Standard curve of a Reference reagent* with concentration of 50; 30, 15 10, 5, 3, 1 mIU/ml for Calibration of Sera (s.Tab. 4)

## LITERATUR / LITERATURE


1) Flehmig, B.: Hepatitis A. Baillière`s Clinical Gastroenterology, Vol.4 No. 3, p. 707, 1990.
2) Ambrosch, F. et al.: Comparison of HAV-Antibodies Induced by Vaccination, Passive Immunization, and Natural Infection. Viral Hepatitis and Liver Disease. S. 98 (1990). (Editors: Hollinger, Lemon, Margolis, published by Williams and Wilkins, Baltimore; ISBN 0-683-04120-7).
3) Delem, A. et al.: Characterization of the immune response of volunteers vaccinated with a killed vaccine against hepatitis A. Vaccine, Vol. 11, Issue 4, S. 479 (1993).
4) Heinricy, U. et al.: Schedule-dependent Immune Response to Hepatitis A Vaccination. Viral Hepatitis and Liver Disease. S. 108 (1990). (Editors: Hollinger, Lemon, Margolis, published by Williams and Wilkins, Baltimore; ISBN 0-683-04120-7).
5) Jilg, W.: Hepatitis-A-Impfung - Anmerkungen zur Diagnostik. Klin. Lab. 38, S.697 (1992).
6) Van Damme, P.; Cramm, M.: Hepatitis A vaccination for health care workers. British Medical Journal, Vol. 306, S1615, 1993).
7) Turner, P. C. et al: Screening Before Hepatitis-A Vaccination. Lancet 340/8828, S.1160 (1992).
8) Germanaud, J.; Causse, X.: Stratègie de la vaccination contre l`hepatite A. La Presse Médicale, 22, n° 21, S 1104, (1993).
9) Brede, H. D.: Hepatitis-A-Gefahr für Hong-Kong Touristen, BIOforum 6, S.222 (1993).
10) Euteneuer, B.: Hepatitis A und Immunprophylaxe. LABOR AKTUELL 5.93, Herausg. Bioscientia Ingelheim.
11) Karpinski, K. F. et al.: Statistical considerations in the quantitation of serum immunoglobulin levels using the enzyme-linked immunosorbent assay. Journal of Immunological Methods, 103, S.189 (1987).
12) Bölke, E.; Flehmig B.: New epidemiological patterns of Hepatitis A and B in Germany. Zbl.Hyg. 196, 511-514 (1995)

# SUMMARY - Mediagnost anti-HAV EIA E10

| **Dilution of Reagents and Samples** | | |
|---|---|---|
| **Conjugate concentrate KK** | **in Dilution Buffer VP** | **1:100** |
| **Washing Buffer WP** | **in Aqua dest**. (e.g. 50 ml **WP** : **A.dest** 950 ml ) | **1:20** |
| Dilute **samples 1:10** with **Dilution Buffer VP** (qualitative Test). *For quantitative Antibody determination** dilute at least **1:10** with **Dilution Buffer VP**, higher Dilutions s. S. 8. | | |

Proposal of Assay Procedure for double determination

| **Pipette** | **Reagents** | **Position** |
|---|---|---|
| | Blank | A1/A2 |
| 2 x 100 µl | Positive Control **PK** | B1/B2 |
| 2 x 100 µl | Negative Control **NK** | C1/C2 |
| ***Additional Protocol for the *quantitative determination* s. below** | | |
| 2 x 100 µl | Sample Dilution | in the rest of the wells according to requirements |
| Cover the wells with the sealing tape | | |
| **Incubate: 2 h at 37°C** | | |
| 50 µl | 1:100 diluted **Conjugate Concentrate KK** | Each well except A1/A2 |
| Cover the wells with the sealing tape | | |
| **Incubation: 1 h at 37°C** | | |
| 3 x 300 µl | Aspirate the contents of the wells and wash 3x with **300 µl** Wash Buffer **WP**. | Each well |
| 100 µl | Substrate Solution **S** | Each well |
| **Incubation: 30 min** in the dark at RT | | |
| 100 µl | Stopping Solution **SL** | Each well |
| Measure the absorbance within **30 min** at (450/≥590 nm) | | |

***The quantitative Protocol**

| **Pipette** | **Reagents** | **Position** |
|---|---|---|
| 2 x 100 µl | Standard 1 **STD 1** | D1/D2 |
| 2 x 100 µl | Standard 2 **STD 2** | E1/E2 |
| 2 x 100 µl | Standard 3 **STD 3** | F1/F2 |

**REF E10**  **International Test description for QUALITATIVE Assay**

| | | |
|---|---|---|
| **CONJ** | **KK** | **1:100 DILU VP** |
| **WASHBUF 20x** | **WP** | **1:20 DILU A. dest.** |

| | |
|---|---|
| **SPE** | **1:10 DILU VP** |
| **°C 20-25 °C** | |

| | | |
|---|---|---|
| | | A1/2 |
| 100 µl | **Control +** | B1/2 |
| 100 µl | **Control -** | C1/2 |
| 100 µl | **SPE** | |
| | **TAPE** | |

**2 h °C 37**

| | |
|---|---|
| 50 µl | **CONJ** |
| | **TAPE** |

**1 h °C 37**

| | |
|---|---|
| 3x 300 µl | **3x WASHBUF WP** |
| 100 µl | **SUBST TMB S** |

**30min °C 20-25**

| | |
|---|---|
| 100 µl | **$H_2SO_4$ SL** |
| | **MEASURE** |