HAV-Antigen ELISA

Enzyme Immunoassay for the Detection of Hepatitis A-Virus Antigen

Product-Code: E12

distributed in the US/Canada by:
EAGLE BIOSCIENCES, INC.
20A NW Blvd, Suite 112 Nashua, NH 03063
Phone: 617-419-2019 FAX: 617-419-1110
www.EagleBio.com • info@eaglebio.com

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Not for use in diagnostic procedures.

Gesellschaft für Forschung und Herstellung von Diagnostika GmbH
Aspenhastr. 25 • D-72770 Reutlingen / Germany
Phone: + 49 - (0) 7121 51484-0 • Fax: + 49 - (0) 7121 51484-10
E-Mail: contact@mediagnost.de • http://www.mediagnost.de
Read entire protocol before use!

INTENDED USE
INTRODUCTION
TEST PRINCIPLE
KIT CONTENTS
STORAGE
MATERIALS NOT PROVIDED
PRECAUTIONS
TECNICAL NOTES
TEST PREPARATION
SAMPLE PREPARATION
TEST PROTOCOL
RESULTS
POSITIVE SAMPLES
CALCULATION (example)
LIMITATIONS
Summary of the Assay
INTENDED USE
The mediagnost HAV-ANTIGEN EIA E12 is an enzyme immunoassay for research use and identifies Hepatitis A virus in stool and cell culture.

INTRODUCTION
A positive identification of Hepatitis A Virus (HAV) in human stool indicates a fresh and contagious infection with HAV. The passing begins about two weeks before the icteric phase of the disease and reached a peak after about one week before icterus. With the beginning of the icteric phase, the HAV passing drops steeply but HAV antigen could be found in the stool of some, not all, humans two weeks after onset of icterus.

The detection of HAV in specimen other than stool is also possible with the mediagnost HAV-ANTIGEN EIA E12, for example in lysates of HAV infected cells or in culture
supernatants. If necessary the specimen must be concentrated before testing (ultrafiltration i.e.). Specimen with high or low pH, high salt or detergents concentration should be dialysed against phosphate buffered saline (PBS).

TEST PRINCIPLE
The specimens are pipetted into wells of a microtiter plate previously coated with antibodies directed against HAV. The HAV antigen binds to the fixed antibody and after the incubation period of two hours at 37°C the plate is washed thoroughly. Bound HAV antigen is identified by conjugate addition (monoclonal anti-HAV, peroxidase conjugated) incubated for another two hours at 37°C. Excess conjugate is removed by washing and the substrate is added. After 30 minutes incubation at room temperature the reaction is terminated by adding stop solution. The blue colour of a positive reaction turns to yellow and is measured in a microplate reader at 450 nm. The intensity of the colour indicates the concentration of bound HAV antigen.
A positive reaction must be confirmed by neutralising with anti-HAV serum to discriminate false positive reactions which sometimes occur in stool.
KIT CONTENTS

1) **Microtiter Plate:**
   Microtiter plate with 96 wells, divided into 12 removable strips with 8 wells each, coated with monoclonal antibody against HAV antigen.

2) **Conjugate Concentrate (flask A):**
   1 vial (150 µl) conjugate, (mouse monoclonal anti HAV IgG, peroxidase conjugated) 100 x concentrated.

3) **Positive Control (flask B):**
   1 vial (500 µl) positive control. Hepatitis A-Virus antigen, inactivated, ready for use.

4) **Neutralising serum (flask C):**
   1 vial (500 µl) anti-HAV-positive serum, 10 x concentrated.

5) **Dilution buffer (flask D):**
   1 vial (120 ml) Dilution buffer for specimen und conjugate, red coloured, ready for use.

6) **Substrate (flask E):**
   1 vial (12 ml), ready for use.

7) **Stop solution (flask F):**
   1 vial (12 ml) stop solution, 0.2 M sulphuric acid, ready to use.
   Caution: Acid!

8) **Wash buffer (flask G):**
   1 vial (50 ml) wash buffer, 20 x concentrated.

9) **Sealing tape**
   for covering of the microtiter plate, 2 x, adhesive
STORAGE
All materials must be stored at 2-8 °C in the dark. Unused microtiterplate stripes have to be stored airtight together with the desiccant bag at 2-8°C. The shelf-life of the components after opening is not affected, if used appropriately.

MATERIALS NOT PROVIDED
- Distilled water for dilution of wash buffer
- Centrifuge for preparation of stool specimen.
- Incubator or water bath with an adaptor for microtiter plates.
- Precision pipettes with disposable tips.
- Microtiter Plate washer (recommended)
- Micro plate reader ("ELISA-Reader") with filter for 450 and ≥590 nm
- Polyethylen PE/Polypropylen PP tubes for dilution of samples

PRECAUTIONS
1) The mediagnost HAV-ANTIGEN EIA E12 is for in-vitro use only.
2) The antigen of the positive control has been inactivated with formaldehyde. Reagents of human origin have been tested for HBsAg and antibodies to HIV and HCV and been found to be negative. Nevertheless, such tests are unable to prove the complete absence of infectious agents. Therefore, all reagents should be handled with appropriate precautions.
3) Do not pipette by mouth. Wear disposable gloves throughout the test procedure. In case of spills, bench-tops and instruments must be disinfected.
4) Disposable materials should be treated as infectious waste.
5) The stop solution contains sulphuric acid and is therefore corrosive. On contact wash immediately with running water- if necessary contact a doctor.
6) Acidic waste should be neutralised before disposal.
7) The Reagents A, B, C, D contain as preservative (0.01%) 2-Methyl-4-isothiazolin-3-one Solution
R36/38 Irritating to eyes and skin
R43 Sensibilisation through skin contact possible
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

8) The Reagents A, B, C, D, G contain as preservative (0.01%) (w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-isothiazol-3-one
R36/38 Irritating to eyes and skin
R43 Sensibilisation through skin contact possible
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

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. The Stop Solution provided is an acid solution. Avoid direct contact. Wear eye, hand, face and clothing protection when using this material.

TECNICAL NOTES
Incubation at room temperature means: 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 for 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. Incubation at room temperature means: 20-25°C

TEST PREPARATION
1) Bring all reagents to room temperature (20-25°C) before use
2) **Wash Buffer (G):** Dilute the 20 x Wash Buffer 1:20 with distilled water. Attention: After dilution the Washing Buffer is only 4 weeks stable, please dilute only according to requirements.
3) Dilute the **neutralising serum (C)** for the confirmation of positive reactions 1:10 with dilution buffer (D). Dilute only the volume used in the test (50 µl per well). Diluted serum is stable for at least one week at 4°C.
4) Dilute the **100 x Conjugate Concentrate (A)** 1:100 with **Dilution Buffer (D)**. Dilute only the volume used in the test (100 µl per well). Diluted conjugate is stable for at least one week at 4°C.
SAMPLE PREPARATION
Prepare a 20% (w/v) suspension of stool in Dilution Buffer (D). Centrifuge the suspension with at least 2400 x g for 10 minutes at room temperature. The clear supernatant can be used in the test. If required repeat the centrifugation.

Supernatants of cell culture and cell lysates can be used directly. If required, they can be concentrated i.e. with ultracentrifugation.

TEST PROTOCOL
1) In every test, two negative and two positive controls should be performed, and also two positive controls under neutralising conditions.

   All wells needed are filled with either 50 µl Dilution Buffer (D) or 50 µl neutralising dilution buffer (1:10 diluted neutralising serum) each.

   Add Dilution Buffer (D) as negative control and the positive control (B), HAV antigen, 50 µl/well respectively to the preincubated wells. Stool and other specimen are also added 50 µl/well (double determination is recommended). Each well is filled with 100 µl liquid.

2) Seal the plate with adhesive tape and incubate it for 2 hours at 37°C.

3) At the end of the incubation period the wells are evacuated (attention: infective agent) and washed 3 times with 300 µl Wash Buffer (G) per well with 10 seconds incubation time respectively and empty the wells.

4) Add 100 µl diluted Conjugate Solution A per well, reseal the plate and incubate for another 2 hours at 37°C.

5) At the end of the incubation period the wells are evacuated and washed 3 times with 300 µl Wash Buffer
(G) per well with 10 seconds incubation time respectively and empty the wells.

6) Add **100 µl Substrate (E)** per well and incubate for 30 minutes in the dark.

7) After the incubation **100 µl Stop Solution (F)** is added into each well. The colour of positive reactions will turn from blue to yellow.

8) The **measurement** of the colour is performed at **450 nm**. The **reference wave length** in dual wave length mode should be ≥ **590 nm**.

**RESULTS**
Calculate the average of the multiple values. Subtract the negative control value (blank) from all measured values (could be done automatically by many readers as blank correction). The difference between the positive and negative control must be at least 0.5 otherwise the test is considered invalid.

The drop of the positive control value caused by neutralising serum must be more than 80 %.

The cut-off calculation is 10 % of the positive control value.

**POSITIVE SAMPLES**
Samples with extinction equal or **higher than the cut off value** are regarded as **positive**. The extinction of the positive samples should decline under neutralizing condition at least 25% otherwise the result is regarded not as positive. **Positive samples** with values higher than the **Positive Control (B)** which don’t decline more than 25% by neutralisation **must be diluted 1:10** in **Dilution Buffer (D)** and tested again. Sample values with a negative sign after subtraction of the blank could be found, nevertheless such test is valid.
CALCULATION (example)

positive control value 1 : 1.114
positive control value 2 : 1.162
Average : (1.114 + 1.162) : 2 = 1.138
negative control value 1 : 0.024
negative control value 2 : 0.030
Average : (0.024 + 0.030) : 2 = 0.027

Subtract the blank (negative control):

Positive control: 1.138 – 0.027 = 1.111
Cut-off : 1.111 / 10 = 0.111

Samples with extinction higher than 0.111 are regarded as positive if the value of the positive control on neutralizing conditions declines more than 80 % and the sample value itself declines more than 25 %.

LIMITATIONS

Test components are for in-vitro-use only.

Sensitivity:
ELISA-negative samples nevertheless can contain HAV particles.

Specificity:
91% of the elisa-positive samples were also HAV-PCR positive. Cross reactivity is not known.

Precision:
cut-off: 16% CV intra-assay
positive control: 3.5% CV intra-assay
### Summary of the Assay

<table>
<thead>
<tr>
<th>Reagent preparation:</th>
<th>Dilution:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate Concentrate A</td>
<td>1:100 with Dilution Buffer D</td>
</tr>
<tr>
<td></td>
<td>Dilute only the volume used in the test</td>
</tr>
<tr>
<td>Neutralising serum C</td>
<td>1:10 with Dilution Buffer D</td>
</tr>
<tr>
<td>Washing Buffer G</td>
<td>1:20 with Aqua dest.</td>
</tr>
<tr>
<td>Stool samples</td>
<td>Prepare a 20% (w/v) suspension of stool in Dilution Buffer D. Centrifuge the suspension with at least 2400 x g for 10 minutes at room temperature. The clear supernatant can be used in the test.</td>
</tr>
</tbody>
</table>

### Assay Procedure in Double Determination

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Reagents</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µl</td>
<td>Pipette Dilution Buffer D or Neutralising Dilution Buffer C (1:10 Dilution of the Neutralising Serum C)</td>
<td>In all wells required</td>
</tr>
<tr>
<td>50 µl</td>
<td>Add Dilution Buffer D (Negative-Control) in positions:</td>
<td>A1/A2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Add HAV-Antigene B (Positive-Control) in positions:</td>
<td>B1/B2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Add Samples in</td>
<td>following wells</td>
</tr>
</tbody>
</table>

Cover the wells with the sealing tape.

**Incubation: 2 h at 37°C**

3x 300 µl  Aspirate the contents of the wells and wash 3x with 300 µl Wash Buffer G.  each well

100 µl  Diluted Conjugate Solution A  each well

Cover the wells with the sealing tape.

**Incubation: 2 h at 37°C**

3x 300 µl  Aspirate the contents of the wells and wash 3x with 300 µl Wash Buffer G.  each well

100 µl  Substrate Solution E  each well

**Incubation: 30 min in the Dark at RT**

100 µl  Stopping solution F  each well

Measure the absorbance within 30 min at 450 nm (Reference wavelength ≥ 590)