Pay attention to changes!
ENZYME IMMUNOASSAY FOR
QUALITATIVE DETERMINATION OF
TOTAL ANTIBODIES TO HEPATITIS DELTA ANTIGEN (anti-HD)
IN SERUM SAMPLES
For in vitro use only

1. INTRODUCTION
The Delta antigen/antibody system (HDAg/anti-HD) is related to HBV infection but immunologically distinct from its known reactivities; it is the expression of the Delta virus (HDV, Hepatitis D Virus), cause of severe liver disease in HBsAg carriers.
HDV is a 35-37nm particle containing low molecular weight RNA and HDAg, with an outer coat of HBsAg obtained from HBV. HDV is a defective virus and its replication requires helper functions provided by HBV.
HDAg has been detected in liver and in serum and induces a specific antibody response (anti-HD antibodies) both of IgG and IgM class.
Detection of total anti-HD antibodies represents the method of choice for identification of HDV infection. Anti-HD antibodies can be detected in high titres in HBsAg chronic carriers (superinfection) and, at lower titres, in patients with acute type B hepatitis (coinfection).
Based on detection of circulating total anti-HD, worldwide epidemiological studies demonstrate that HDV infection is ubiquitous, though its prevalence varies in different Countries.
The typical hepatic damage to HBsAg chronic carriers with HDV infection is chronic active hepatitis, though chronic persistent and lobular hepatitides are also observed. In patients with HDV infection the picture of lobular or persistent chronic damage, though histologically benign, often evolves towards cirrhosis. In HBsAg chronic carriers the presence of HDAg in the liver and of anti-HD in the serum suggests progressive liver disease and is therefore an unfavourable prognostic sign.
In addition, primary HDV/HBV infection often results in severe hepatitis which is not infrequently fulminant.

2. PRINCIPLE OF THE ASSAY
The method for qualitative anti-HD determination is a simultaneous competitive assay, illustrated in Fig. 1, based on the ELISA technique (Enzyme-Linked Immunosorbent Assay).
Anti-HD present in the sample and labelled anti-HD antibodies compete for a fixed quantity of HDAg bound to the solid phase. The quantity of enzyme tracer bound to the solid phase and consequently the enzyme activity are inversely proportional to the anti-HD concentration present in samples or controls.

Enzyme activity is measured by adding a colourless chromogen/substrate solution. The enzyme action on chromogen/substrate produces a colour which is measured with a photometer.

### 3. REAGENTS AND ACCESSORIES

#### 3.1. Reagents provided in the kit

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coated strips</td>
<td>12 8-well strips</td>
</tr>
<tr>
<td>Enzyme tracer</td>
<td>1 vial, 0.5 mL</td>
</tr>
<tr>
<td>Negative control</td>
<td>1 vial, 2 mL</td>
</tr>
<tr>
<td>Positive control</td>
<td>1 vial, 2 mL</td>
</tr>
<tr>
<td>Tracer diluent</td>
<td>1 vial, 17.15 mL</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>1 vial, 40 mL</td>
</tr>
<tr>
<td>Chromogen</td>
<td>1 vial, 9 mL</td>
</tr>
<tr>
<td>Substrate</td>
<td>1 vial, 9 mL</td>
</tr>
<tr>
<td>Blocking reagent</td>
<td>1 vial, 30 mL</td>
</tr>
<tr>
<td>Number of tests</td>
<td>96</td>
</tr>
</tbody>
</table>

**STORAGE:** Upon receipt, store all reagents at 2-8°C, away from intense light. Do not freeze. Once opened, the reagents of this kit are stable for eight weeks when properly stored, unless otherwise stated. The kit is stable for one work week when used throughout the day for eight hours at room temperature and stored overnight at 2-8°C. Reagents should not be used past the expiry date. The expiry date of the kit is reported on the external label. The expiry date of each component is reported on the respective vial label.
Specific reagents (see §4.a) from different batches must not be mixed. Common reagents (see §4.b) are interchangeable between batches.

3.2. Materials provided with the kit
- 2 precut cardboard sealers suitable for 1 to 12 strips
- 2 cardboard sealers suitable for 12 strips (one plate)
- pouch sealer.

3.3. Equipment and materials required, but not supplied
- Vertical reading photometer (such as ETI-SYSTEM reader or equivalent) with the following instrument specifications:
  - wavelength: dual wavelength, 450 nm and 620-630 nm
  - bandwidth: ≤ 10 nm
  - absorbance range: 0 absorbance units to ≥ 2.5 absorbance units
  - repeatability: better than or equal to 0.005 absorbance units, or 1%, whichever is greater
  - linearity or trueness: better than or equal to 0.010 absorbance units, or 2%, whichever is greater
  - drift: less than 0.005 absorbance units per hour.
- Thermostatically-controlled humid chamber with the following specifications:
  - temperature: 37°C ± 1°C.
- Manual or automatic equipment for rinsing wells (such as ETI-SYSTEM washer or equivalent) with the following instrument specifications:
  - volume dispensed: 300-370 μL
  - number of wash cycles: 5
  - soak time: 30 seconds
  - aspirate the last aliquot of dispensed liquid: yes.
- Micropipettes with disposable tips (50, 100 μL) (50 μL: trueness ± 3%, precision 2%; 100 μL: trueness ± 2%, precision 1%).
- Glassware.
- Distilled water.
ETI-LAB or ETI-MAX 3000 equipment may be used for automated test processing.

4. COMPOSITION AND PREPARATION OF REAGENTS
All serum and plasma units used to produce the components provided in this kit have been tested for the presence of HBsAg, anti-HCV and anti-HIV-1/2 and found to be non-reactive, except for the material positive for anti-HD, which is reactive for HBsAg and anti-HCV. The presence of HBsAg and HCV in the final reagents is however excluded, because HBsAg is removed by purification and HCV-RNA is found negative. However, as no test method can offer absolute assurance that pathogens are absent, all specimens of human origin should be considered potentially infectious and handled with care.

a) SPECIFIC KIT REAGENTS

4.1. Coated strips
Wells are coated with biotinylated anti-HD IgG (human) and recombinant HDAg. Ready to use, the strips should be kept at 2-8°C.

*Bring the coated strips to room temperature before opening the pouch to avoid development of condensed water in the wells. Place unused strips in the pouch, securely reseal and store at 2-8°C.*
4.2. Enzyme tracer (conjugate)
The vial contains 0.5 mL human anti-HD Fab fragments conjugated to horseradish peroxidase (HRP), phosphate buffer, BSA, stabilizers and preservatives. The solution should be diluted 1:50 with tracer diluent (4.5) (e.g. 100 μL tracer + 4.9 mL diluent). Prepare only the amount of working enzyme tracer needed for the run and keep the concentrated enzyme tracer at 2-8°C.
The working enzyme tracer can be stored for one week at 2-8°C after preparation.

4.3. Negative control
The vial contains 2 mL human serum/plasma non-reactive for anti-HD and preservatives. Ready to use, the reagent should be stored at 2-8°C up to the expiry date.

4.4. Positive control
The vial contains 2 mL human anti-HD antibodies, PBS buffer and preservatives. Ready to use, the reagent should be stored at 2-8°C up to the expiry date.

4.5. Tracer diluent
The vial contains 17.15 mL human serum/plasma, PBS buffer, newborn calf serum and preservatives. Ready to use, the reagent should be stored at 2-8°C up to the expiry date. The solution is used to dilute the enzyme tracer (4.2).

4.6. Wash buffer (25x)
The vial contains 40 mL PBS buffer, Tween® 20 and preservatives. Dilute the vial contents to one litre with distilled water and store for one week at 2-8°C. The reagent is used to rinse wells.
If crystallization occurs at 2-8°C, warm the wash buffer to 37°C and mix well before diluting.

4.7. Chromogen
The vial contains 9 mL tetramethylbenzidine derivative in buffer solution. Mix the solution 1:1 with substrate (4.8) (e.g. 1 mL chromogen + 1 mL substrate). After dilution, chromogen/substrate can be stored for 8 hours at room temperature, away from the light.

4.8. Substrate
The vial contains 9 mL buffer solution containing H₂O₂. The solution should be mixed 1:1 with chromogen (4.7).

4.9. Blocking reagent
The vial contains 30 mL 1N sulphuric acid (R 36/38, S 26). Ready to use, the reagent should be stored at 2-8°C up to the expiry date.

b) REAGENTS COMMON TO OTHER ETI KITS

5. SPECIMEN COLLECTION AND PREPARATION
Only human serum may be used. Blood should be collected aseptically by venipuncture, allowed to clot, and the serum separated from the clot as soon as possible. Samples having particulate matter, turbidity, lipaemia, or erythrocyte debris may require clarification by filtration or centrifugation before testing. Grossly haemolyzed or lipaemic samples as well as samples containing particulate matter or exhibiting obvious microbial contamination should not be tested. If the assay is performed within 48 hours of sample collection, the samples should be kept at 2-8°C; otherwise they should be aliquoted and stored deep-frozen (~20°C or below). If samples are stored frozen, mix thawed samples
well before testing. Avoid repeated freeze-thaw cycles. Samples containing sodium azide should not be assayed.

6. ASSAY PROCEDURE

Bring reagents to room temperature (20-25°C) before assaying. Perform all assay steps without stopping.

Number sufficient strips to run 3 negative controls, 2 positive controls and the unknowns in singlicate. Samples and controls should be subjected to the same process and incubation time.

Prepare one blank well containing chromogen/substrate only.

A disposable tip should be used for dispensing each sample and control.

Dispense the samples and controls as illustrated in the scheme herebelow:

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<tbody>
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<td>S2</td>
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</tr>
</tbody>
</table>
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Key: O = Well NC = Negative control BLK = Blank PC = Positive control S = Samples.

Adjust the thermostatically-controlled humid chamber to 37° ± 1°C.

1. **Dispense 50 µL negative control, positive control and samples** into their respective wells.
2. **Dispense 100 µL diluted enzyme tracer** (4.2) into all wells, except for the blank well.
3. Apply a cardboard sealer in order to prevent evaporation.
4. **Incubate for three hours ± 15 min at 37° ± 1°C.**
5. Prepare chromogen/substrate just before the end of the incubation.

6. When incubation has been completed, discard the cardboard sealer and rinse the strips. Use a suitable automatic or semiautomatic washer for washing strips. Aspirate the liquid and rinse each well five times with a volume of wash buffer ranging from 0.30 to 0.37 mL. Avoid overflows from the reaction wells. When either an automatic or semiautomatic washer is used, the soak time between each rinse should be 30 seconds.

After rinsing, turn the strips mouth down onto blotting paper and gently tap to remove any liquid residue. Leave the strips overturned on blotting paper until they have been
rinsed.

7. **Dispense 100 μL chromogen/substrate** solution into all wells.
8. **Incubate for 30 ± 2 min at room temperature**, away from intense light.
9. **Dispense 100 μL blocking reagent** into all wells in the same order and at the same rate as for chromogen/substrate.
10. **Measure the absorbance** of specimens with a photometer at 450/630 nm within one hour of adding the blocking reagent.

When dedicated equipment is used, absorbance values are provided automatically after selecting the suitable protocol. When another vertical reading photometer is used, blank the instrument with the blank, record the absorbance at 450/630 nm for each specimen and subtract the 630 nm absorbance value from the 450 nm absorbance value.

**ETI-LAB or ETI-MAX 3000 equipment may be used for automated test processing.**

### 7. CALCULATION OF RESULTS

#### 7.1. Calculation of cut-off value

The cut-off value is determined by adding the mean absorbance for the negative control values (NCx) multiplied by 0.5 to the mean absorbance for the positive control values (PCx) multiplied by 0.5.

Cut-off value = 0.5 NCx + 0.5 PCx.

#### 7.2. Run validation criteria

The following criteria should be used to validate quality control when evaluating results. Always calculate and evaluate mean control values for each plate, even if plates are combined to form a single batch.

- The absorbance value for the blank well must range between 0.000 and 0.150.
  
- The mean negative control absorbance value must be greater than or equal to 0.600.
  
- The mean positive control absorbance value must be less than or equal to 0.080.

The difference between the mean negative control absorbance value and the mean positive control absorbance value (N – P) must be greater than or equal to 0.500.

NCx – PCx ≥ 0.500.

If not, the run is invalid and must be repeated.

#### 7.3. Interpretation of results

The presence or absence of anti-HD is determined by comparing the absorbance of the unknown samples to that of the cut-off value.

- **Samples with absorbance values within ± 10% of the cut-off value must be retested in order to confirm the initial result.**  **Samples which are repeatedly reactive should be considered positive.**  **Samples which are non-reactive at the second test should be considered negative.**
7.4. Calculation example

The following data must only be considered an example and should not be employed instead of the data obtained by the user.

Absorbance for the negative control

<table>
<thead>
<tr>
<th>Negative control</th>
<th>Net absorbance at 450/630 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.273</td>
</tr>
<tr>
<td>2</td>
<td>1.253</td>
</tr>
<tr>
<td>3</td>
<td>1.289</td>
</tr>
</tbody>
</table>

Mean absorbance, \( \text{NC} \bar{x} = 1.272 \)

Absorbance for the positive control

<table>
<thead>
<tr>
<th>Positive control</th>
<th>Net absorbance at 450/630 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.016</td>
</tr>
<tr>
<td>2</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Mean absorbance, \( \text{PC} \bar{x} = 0.017 \)

Cut-off value \( (0.5 \text{NC} \bar{x} + 0.5 \text{PC} \bar{x}) = 0.5 \times 1.272 + 0.5 \times 0.017 = 0.645 \).

Negative – Positive control difference \( \text{N – P} = 1.272 – 0.017 = 1.255 \).

The \( \text{N – P} \) difference is greater than 0.500; thus the technique is acceptable and data should be considered valid.

Screening of unknown samples

Sample 1 = absorbance 0.182
Sample 2 = absorbance 1.083

Sample no. 1 should be considered reactive for anti-HD and sample no. 2 non-reactive, as the cut-off value is 0.645.

8. SPECIFIC PERFORMANCE CHARACTERISTICS

8.1. Analytical specificity

Analytical specificity may be defined as the ability of the assay to accurately detect specific analyte in the presence of potentially interfering factors in the sample matrix (e.g., haemolysis, effects of sample treatment), or cross-reactive antibodies.

Controlled studies of potentially interfering substances or conditions showed that the assay performance was not affected by haemolysis (up to 100 mg/dL haemoglobin), lipaemia (up to 3000 mg/dL triglycerides), bilirubinaemia (up to 20 mg/dL bilirubin), or by antibodies to other infectious agents.
8.2. Precision
Different sample pools (from A to C), containing different titres of specific analyte, were assayed to determine repeatability and reproducibility of the assay (i.e., within- and between-assay variability). The variability shown in the tables below did not result in sample misclassification.

<table>
<thead>
<tr>
<th>Repeatability</th>
<th>Pool A</th>
<th>Pool B</th>
<th>Pool C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of determinations</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean absorbance</td>
<td>0.116</td>
<td>0.462</td>
<td>1.423</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.007</td>
<td>0.026</td>
<td>0.092</td>
</tr>
<tr>
<td>Coefficient of variation, %</td>
<td>5.8</td>
<td>5.7</td>
<td>6.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reproducibility</th>
<th>Pool A</th>
<th>Pool B</th>
<th>Pool C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of determinations</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Mean absorbance</td>
<td>0.112</td>
<td>0.453</td>
<td>1.424</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.006</td>
<td>0.024</td>
<td>0.075</td>
</tr>
<tr>
<td>Coefficient of variation, %</td>
<td>5.3</td>
<td>5.3</td>
<td>5.3</td>
</tr>
</tbody>
</table>

8.3. Diagnostic specificity
Diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of specific analyte. Diagnostic specificity was assessed by testing 593 specimens selected from an expected anti-HD-negative population (blood donors, patients affected by infectious diseases with similar symptomatology and HBsAg asymptomatic carriers). Six positive results were observed in the population studied - diagnostic specificity: 98.99% (95% confidence interval: 97.81-99.63%).

8.4. Diagnostic sensitivity
Diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of specific analyte. Diagnostic sensitivity was assessed by testing 172 specimens selected from an expected anti-HD-positive population (HBsAg asymptomatic carriers and anti-HD-positive subjects). One negative result was observed in the population studied - diagnostic sensitivity: 99.42% (95% confidence interval: 96.81-99.99%).

8.5. High-dose saturation effect
Whenever samples containing extremely high antibody concentrations are tested in a competitive test, misestimated results may be excluded, because the analytical signals remain consistently low (saturation curve). Analysis of saturation effect was evaluated by testing four high-titred samples positive for anti-HD. These samples resulted in an absorbance value around zero that would be expected with high-titred sera, indicating no sample misclassification.

9. LIMITATIONS OF THE PROCEDURE
Bacterial contamination, repeated freeze-thaw cycles or heat inactivation of the specimens may affect the absorbance values of the samples with consequent alteration of anti-HD levels. Diagnosis of an infectious disease should not be established on the basis of a single test result. A precise diagnosis, in fact, should take into consideration the patient's clinical history, symptomatology, as well as serological data.
10. WARNINGS AND PRECAUTIONS
A skilful technique and strict adherence to the instructions are necessary to obtain reliable results. In particular, precise pipetting, aspiration and washing are essential. Non-repeatedly reactive samples might derive from various methodological factors, such as:
- cross-exchange of vial caps
- use of the same tip when withdrawing from different vials or dispensing different samples
- exposure of reagents or samples to intense heat or heavy sources of bacterial contamination
- inadequate rinsing of wells
- contamination of well rims by tracer or samples
- use of reagents from different master lots.
In addition the following precautions are required:
- to avoid contamination, use a clean dedicated dispenser for the enzyme tracer solution
- wash buffer should be stored in clean containers to prevent contamination with enzyme-inactivating substances
- it is essential that clean labware be used for chromogen/substrate preparation.

11. SAFETY PRECAUTIONS
- Handle with care chromogen, substrate and blocking reagent. Avoid chromogen, substrate and blocking reagent coming into contact with oxidizing agents or metallic surfaces.
- Do not eat, drink, smoke or apply cosmetics in the assay laboratory.
- Do not pipette solutions by mouth.
- Avoid direct contact with all potentially infectious materials by using protective articles such as lab coats, protective glasses and disposable gloves. Wash hands thoroughly at the end of each assay.
- Avoid splashing or forming an aerosol. Any reagent spills should be washed with a 5% sodium hypochlorite solution and disposed of as though potentially infectious.
- All samples, biological reagents and materials used in the assay must be considered potentially able to transmit infectious agents. They should therefore be disposed of in accordance with the prevailing regulations and guidelines of the agencies holding jurisdiction over the laboratory, and the regulations of each Country. Disposable materials must be incinerated; liquid waste must be decontaminated with sodium hypochlorite at a final concentration of 5% for at least half an hour. Liquid waste containing acid must be neutralized before treatment. Any materials to be reused must be autoclaved using an overkill approach (USP 24, 2000, p. 2143). A minimum of one hour at 121°C is usually considered adequate, though the users must check the effectiveness of their decontamination cycle by initially validating it and routinely using biological indicators.
  R 36/38  – Irritating to eyes and skin.
  S 26  – In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
ADJUST THE THERMOSTATICALLY-CONTROLLED HUMID CHAMBER TO 37° ± 1°C.

1 - DISPENSE REAGENTS INTO THE STRIP WELLS ACCORDING TO THE FOLLOWING SCHEME, LEAVING AN EMPTY WELL FOR THE BLANK:

<table>
<thead>
<tr>
<th>WELLS</th>
<th>NEGATIVE CONTROL (3x)</th>
<th>POSITIVE CONTROL (2x)</th>
<th>SAMPLES (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REAGENTS</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NEGATIVE CONTROL</td>
<td>50 μL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>POSITIVE CONTROL</td>
<td>-</td>
<td>50 μL</td>
<td>-</td>
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<tr>
<td>SAMPLES</td>
<td>100 μL</td>
<td>100 μL</td>
<td>50 μL</td>
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<tr>
<td>ENZYME TRACER</td>
<td></td>
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<td>100 μL</td>
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</tbody>
</table>

2 - INCUBATE FOR 3 HOURS ± 15 MIN AT 37° ± 1°C.
3 - ASPIRATE THE LIQUID. REPEATEDLY WASH WITH WASH BUFFER.
4 - DISPENSE 100 μL CHROMOGEN/SUBSTRATE INTO ALL WELLS.
5 - INCUBATE FOR 30 ± 2 MIN AT ROOM TEMPERATURE IN THE DARK.
6 - DISPENSE 100 μL BLOCKING REAGENT INTO ALL WELLS.
7 - READ ABSORBANCES WITH A PHOTOMETER AT 450/630 nm.