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ELISA **E**NZYME **L**INKED **I**MMUNO**S**ORBENT **A**SSAY

Microwell Method

HDV IgM

Ref. Z14301

For in vitro Diagnostic Use

P r o d u c t I n s e r t

Enzyme Linked Immunosorbent Assay for the **cut - off** determination of HDV IgM (IgM class antibodies to hepatitis D virus) in human serum or plasma.

Microwell Method - 96 wells

(12 x 8-well Antigen coated Strips)

Individual breakaway

GENERAL INFORMATION

- **Wavelength**
  Measurement Filter: 450 nm
  Optional Reference Filter: 630 nm

- **Enzyme Conjugate**
  HRP (Horseradish Peroxidase)

- **Substrate**
  TMB (3,3´,5,5´-Tetramethyl-benzidine)

- **Sample**
  Serum or Plasma

- **Incubation Time**
  75 minutes at 37°C (30/30/15)

- **Shelf life and Stability of Kit Components**

Kit:	12 months from production date
Kit Components:	see expiration date on the label

KIT COMPONENTS

MICROWELL PLATE	1	Blank microwell strips fixed on white strip holder. The plate is sealed in aluminum pouch with desiccant. Each well contains anti-IgM-antibodies (anti-µ-chain). The microwell strips can be broken to be used separately. Place unused wells or strips in the provided plastic sealable storage bag together with the desiccant and return to 2-8°C. Once open, stable for one month at 2-8°C.
POSITIVE CONTROL	1	0.5 ml per vial. Red-colored liquid filled in a vial with red screw cap. Purified HDV IgM antibodies diluted in protein-stabilized buffer. Preservatives: 0.1% ProClin 300. Ready to use as supplied. Once open, stable for one month at 2-8°C.
NEGATIVE CONTROL	1	0.5 ml per vial. Blue-colored liquid filled in a vial with green screw cap. Protein-stabilized buffer tested non-reactive for HDV IgM. Preservatives: 0.1% ProClin 300. Ready to use as supplied. Once open, stable for one month at 2-8°C.
ENZYME CONJUGATE	1	12 ml per vial. clear bottle with blue cap. Horseradish peroxidase-conjugated recombinant HDV antigens. Ready to use as supplied. Once open, stable for one month at 2-8°C.
SAMPLE DILUENT	1	12 ml per vial. Blue-colored liquid in a clear bottle with black screw cap. Serum base, casein and sucrose solution. Preservative 0.1% ProClin™ 300. Ready to use as supplied. Once open, stable for one month at 2-8°C.
SUBSTRATE SOLUTION A	1	7 ml per vial. Colorless liquid filled in a clear bottle with white screw cap. Urea peroxide solution. Ready to use as supplied. Once open, stable for one month at 2-8°C.
SUBSTRATE SOLUTION B	1	7 ml per vial. Colorless liquid filled in a black vial with black screw cap. TMB (Tetramethylbenzidine) solution. Ready to use as supplied. Once open, stable for one month at 2-8°C.
STOP SOLUTION	1	7ml per vial. Colorless liquid in a clear vial with red screw cap. Diluted sulfuric acid solution (0.5M H_2SO_4).
WASH BUFFER	1	50ml per bottle. Colorless liquid filled in a white bottle with white screw cap. pH 7.4, 20 × PBS. The concentrate must be diluted 1 to 20 with distilled water before use. Once diluted, stable for one week at room temperature, or for two weeks when stored at 2-8°C.
PLATE COVER SHEETS	2	

MATERIALS REQUIRED BUT NOT PROVIDED

1. Freshly distilled or deionized water.
2. Disposable gloves and timer.
3. Appropriate waste containers for potentially contaminated materials.
4. Disposable V-shaped troughs.
5. Dispensing system and/or pipette (single or multichannel), disposable pipette tips.

6. Absorbent tissue or clean towel.
7. Dry incubator or water bath, 37±0.5°C.
8. Microwell plate reader, single wavelength 450 nm or dual wavelength 450 nm and 630 nm.
9. Microwell aspiration/wash system.

SUMMARY AND EXPLANATION

Hepatitis D is caused by Hepatitis D virus (Delta agent) – a defective (36 – 43nm) enveloped RNA virus, which requires co-infection with Hepatitis B virus (HBV) for its replication. Transmitted percutaneously or sexually through contact with infected blood or blood products, HDV is associated with the most severe forms of chronic and acute hepatitis in many HBsAg positive patients. Since the infection with HDC requires infection with HBV, the development of the disease depends on whether the two viruses infect simultaneously (co-infection) or whether the newly infected HDV patient is also a chronic HBV carrier (super-infection). The co-infection with HDV can lead to severe acute hepatitis disease with low risk of chronic stage development. Chronic HBV carrier patients super-infected with HDV are at risk to develop chronic HDV disease, which can lead to cirrhosis in 70-80% of the patient. The serological diagnosis of Hepatitis D is based on detection of specific HDV antibodies (anti-HDV) or antigens. IgM anti-HDV is the first antibody to appear in acute infection. IgM anti-HDV is transient and rapidly replaced by IgG anti-HDV. However, persistence of IgM anti-HDV will indicate chronic HDV infection, which continues to replicate. During HBV-HDV co-infection, detectable concentrations of HDV antibodies appear after the tenth week of exposure and clearance during convalescence indicates recovery. The serological detection of IgM class antibodies is an important marker for diagnosis and monitoring of patients during early infection period and post treatment recovery. Decreasing of low titers of IgM suggest early recovery during HDV co- and acute super-infection while constantly elevated level of IgM indicates possible progression to chronic carrier stage.

TEST PRINCIPLE

Dialab HDV IgM ELISA is a solid phase, two-step incubation, antibody capture ELISA method in which polystyrene microwell strips are pre-coated with antibodies directed to human immunoglobulin M proteins (anti-M chain). The patient's serum/plasma sample is diluted and added into the wells, and during the first incubation step, any IgM antibodies will be captured. After washing out all the other components of the sample and in particular IgG antibodies, the specific HDV IgM captured on the solid phase is detected by the addition of purified HDC antigens conjugated to horseradish peroxidase (HRP-conjugate). During the second incubation step, the conjugated antigens will react only with the specific HDV IgM antibodies and after washing to remove unbound HRP-conjugate, Chromogen solutions are added into the wells. In presence of the (anti-µ)-(HDV-IgM)-(HDV antigen-HRP) immunocomplex, the colorless Chromogens are hydrolyzed by the bound HRP conjugate to a blue-colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color can be measured and is proportional to the amount of antibody in the sample. Wells containing samples negative for HDV IgM remain colorless.

SPECIMEN COLLECTION, TRANSPORTATION AND STORAGE

1. **Sample Collection:** No special patient's preparation required. Collect the specimen in accordance with the normal laboratory practice. Either fresh serum or plasma specimens can be used with this assay. Blood collected by venipuncture should be allowed to clot

naturally and completely- the serum/plasma must be separated from the clot as early as possible to avoid hemolysis of the red blood cells. Care should be taken to ensure that the serum samples are clear and not contaminated by microorganisms. Any visible particulate matters in the sample should be removed by centrifugation at 3000 RPM (round per minutes) for 20 minutes at room temperature or by filtration.

2. Plasma specimens collected into EDTA, sodium citrate of heparin can be tested, but ***highly lipaemic, icteric or hemolytic*** specimens should not be used as they can give false results in the assay. ***Do not heat inactivate specimens.*** This can cause deterioration of the target analyte. Samples with visible microbial contamination should never be used.
3. Dialab HDV IgM ELISA is intended ONLY for testing of individual serum or plasma samples.
4. **Transportation and Storage:** Store samples at 2-8°C. Samples not required for assay within 3 days should be stored frozen (-20°C or lower). Avoid multiple freeze-thaw cycles.

SPECIAL INSTRUCTIONS FOR WASHING

1. A good washing procedure is essential to obtain correct and precise analytical data.
2. It is therefore recommended to use a good quality ELISA microplate washer, maintained at the best level of washing performances. In general, no less than **5** automatic washing cycles of 350-400 µl/well are sufficient to avoid false positive reactions and high background.
3. To avoid cross-contaminations of the plate with sample or HRP-conjugate, after incubation do not discard the content of the wells but allow the plate washer to aspirate it automatically.
4. Anyway, we recommend calibrating the washing system on the kit itself in order to match the declared analytical performances. Assure that the microplate washer liquid dispensing channels are not blocked or contaminated and sufficient volume of Wash buffer is dispensed each time into the wells.
5. In case of manual washing, we suggest to carry out at least 5 cycles, dispensing 350-400 µl/well and aspirating the liquid for **5 times**. If poor results (high background) are observed, increase the washing cycles or soaking time per well.
6. In any case, the liquid aspirated out the strips should be treated with a sodium hypochlorite solution at a final concentration of 2.5% for 24 hours, before liquids are wasted in an appropriate way.
7. The concentrated Washing solution should be diluted **1:20** before use. For one plate, mix 50 ml of the concentrate with 950 ml of water for a final volume of 1000ml diluted Wash Buffer. If less than a whole plate is used, prepare the proportional volume of solution.

STORAGE AND STABILITY

The components of the kit will remain stable through the expiration date indicated on the label and package when stored between 2-8°C, **do not freeze.** To assure maximum performance of this HDV IgM ELISA kit, protect the reagents from contamination with microorganisms or chemicals during storage.

PRECAUTIONS AND SAFETY

This kit is intended **FOR PROFESSIONAL IN VITRO USE ONLY**

The ELISA assay is time and temperature sensitive. To avoid incorrect result, strictly follow the test procedure steps and do not modify them.

1. Do not exchange reagents from different lots or use reagents from other commercially available kits. The components of the kit are precisely matched for optimal performance of the tests.
2. Make sure that all reagents are within the validity indicated on the kit box and of the same lot. Never use reagents beyond the expiry date stated on labels or boxes.
3. **CAUTION – CRITICAL STEP:** Allow the reagents and samples to reach room temperature (18-30°C) before use. Shake reagent gently before use and return to 2-8°C immediately after use.
4. Use only sufficient volume of sample as indicated in the procedure steps. Failure to do so, may cause low sensitivity of the assay.
5. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.
6. Never allow the microplate wells to dry after the washing step. Immediately proceed to the next step. Avoid the formation of air bubbles when adding the reagents.
7. Avoid assay steps long time interruptions. Assure same working conditions for all the wells.
8. Calibrate the pipette frequently to assure the accuracy. Use different disposal pipette tips for each specimen and reagents in order to avoid cross-contaminations.
9. Assure that the incubation temperature is 37°C inside the incubator.
10. When adding samples avoid touching the well's bottom with the pipette tip.
11. When reading the absorbance with a plate reader, it is recommended to determine the absorbance at 450nm or at 450nm with reference at 630nm.
12. The enzymatic activity of the HRP-conjugate might be affected from dust and reactive chemical and substances like sodium hypochlorite, acids, alkalis, etc. Do not perform the assay in the presence of these substances.
13. If using fully automated equipment, during incubation, do not cover the plates with the plate cover. The tapping out of the remainders inside the plate after washing can also be omitted.
14. All specimens from human origin should be considered as potentially infectious. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety.
15. **WARNING:** Materials from human origin may have been used in the preparation of the Negative Control of the kit. These materials have been tested with test kits with accepted performance and found negative for antibodies to HIV 1/2, HCV, TP and HBsAg. However, there is no analytical method that can assure that infectious agents in the specimens or reagents are completely absent. Therefore, handle reagents and specimens with extreme caution as if capable of transmitting infectious diseases. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE-free geographical areas.
16. Never eat, drink, smoke or apply cosmetics in the assay laboratory. Never pipette solutions by mouth.
17. Chemical should be handled and disposed of only in accordance with the current GLP (Good Laboratory Practices) and the local or national regulations.
18. The pipette tips, vials, strips and specimen containers should be collected and autoclaved for not less than 2 hours at 121°C or treated with 10% sodium hypochlorite for 30 minutes to decontaminate before any further steps of disposal. Solutions containing

sodium hypochlorite should NEVER be autoclaved. Material Safety Data Sheet (MSDS) are available upon request.

19. Some reagents may cause toxicity, irritation, burns or have carcinogenic effect as raw materials. Contact with the skin and the mucosa should be avoided but not limited to the following reagents: Stop Solutions, Chromogen Solutions, Wash Buffer.
20. The Stop Solution 0.5 M H_2SO_4 is an acid. Use it with appropriate care. Wipe up spills immediately and wash with water if there is contact with skin or eyes.
21. ProClin^{TM} 300 0.1% is used as a preservative and can cause sensation of the skin. Wipe up spills immediately or wash with water if it comes in contact with skin or eyes.

INDICATIONS OF INSTABILITY/DETERIORATION OF THE REAGENT: Values of the Positive or Negative Controls, which are out of the indicated quality control range, are indicators of possible deterioration of the reagents and/or operator or equipment errors. In such case, the results should be considered as invalid and the samples must be retested. In case of constant erroneous results and proven deterioration or instability of the reagents, immediately substitute the reagents wit new ones or contact Dialab technical support for further assistance.

PROCEDURE

Step 1 **Reagents Preparation:** Allow the reagents to reach room temperature (18-30°C). Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the Wash Buffer **1:20** with distilled or deionized water. Use only clean vessels to dilute the Wash buffer. All other reagents are ready to use as supplied.
Preparation: Mark three wells as Negative control **(e.g. B1, C1, D1),** two wells as Positive control **(e.g. E1, F1)** and one Blank. (**e.g. A1**, neither samples nor HRP-Conjugate should be added into the Blank well). If the results will be determined by using dual wavelength plate reader, the requirement for use of Blank well could be omitted. Use only number of strips required for the test.

Step 2 **Adding Diluent:** Add **100 µl** of Specimen Diluent into each well except the Blank.

Step 3 **Adding Sample:** Add **10 µl** Positive Control, Negative Control and Specimen into their respective wells except the Blank. **Note: Use a separate disposal pipette tip for each specimen, Negative Control and Positive Control as to avoid cross-contamination. Mix by tapping the plate gently.**

Step 4 **Incubation:** Cover the plate with the plate cover and incubate for **30 minutes at 37°C**.

Step 5 **Washing:** At the end of the incubation remove and discard the plate cover. Wash each well **5 times** with diluted Washing buffer. Each time allow the microwells to soak for **30-60 seconds**. After the final washing cycle, turn down the strip plate onto blotting paper or clean towel and tap the plate to remove any remainders.

Step 6 **Adding HRP-Conjugate:** Add **100 µl** of HRP-Conjugate into each well except for the Blank.

Step 7 **Incubation:** Cover the plate with the plate cover and incubate for **30 minutes at 37°C**.

Step 8 **Washing:** At the end of the incubation remove and discard the plate cover. Wash each well **5 times** with diluted Washing buffer. Each time allow the microwells to soak for **30-60 seconds**. After the final washing cycle, turn down the strip plate onto blotting paper or clean towel and tap the plate to remove any remainders.

Step 9 **Coloring:** Add **50 µl** of Substrate Solution A and **50 µl** of Substrate Solution B into each well including the **Blank**. Incubate the plate at **37°C for 15 minutes avoiding light**. The enzymatic reaction between the Substrate Solutions and the HRP-Conjugate produces blue color in Positive control and HDV IgM-positive sample wells.

Step 10 **Stopping Reaction:** Using a multichannel pipette or manually add **50 µl** Stop solution into each well and mix gently. Intensive yellow color develops in Positive control and HDV IgM Positive sample wells.

Step 11 **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at **450nm**. If a dual filter instrument is used, set the reference wavelength at **630nm**. Calculate the Cut-off value and evaluate the results.
Note: read the absorbance within **10** minutes after stopping the reaction.

QUALITY CONTROL AND CALCULATION OF THE RESULTS

Each microplate should be considered separately when calculating and interpreting results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample optical density (OD) value to the Cut-off value (C.O.) of the plate. If the Cut-off reading is based on single filter plate reader, the results should be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on Dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls.

Calculation of Cut-off value (C.O.) = *Nc × 2.1

*Nc = the mean absorbance value for three negative controls.

Important: If the mean OD value of the negative control is lower than 0.05, take it as 0.05.

Quality control (assay validation)

The test results are valid if the Quality Control criteria are fulfilled. It is recommended that each laboratory must establish appropriate quality control system with quality control material similar to or identical with the patient sample being analyzed.

1. The absorbance of the Blank well, which contains only Substrate Solutions and Stop Solution, is less than 0.080 at 450 nm.
2. The absorbance value OD of the Positive control must be equal to or greater than 0.800 at 450/630nm or at 450nm after blanking.
3. The absorbance value OD of the Negative control must be less than 0.100 at 450/630nm or at 450nm after blanking.

Example:

1. Quality Control
   Blank well A value: A1 = 0.025 at 450 nm (Note: Blanking is only required when reading with single filter at 450 nm)

Well No.:	B1	C1	D1
Negative Control after blanking	0.012	0.010	0.011
Well No.:	E1	F1	
Positive Control after blanking	2.363	2.436	

   All control values are within the stated quality control range.

2. Calculation of Nc:

$$Nc = \frac{0.012 + 0.010 + 0.011}{3} = 0.011$$

(Nc is lower than 0.05, so take it as 0.05)

3. Calculation of the Cut-off (C.O.) = 0.05 x 2.1 = 0.105

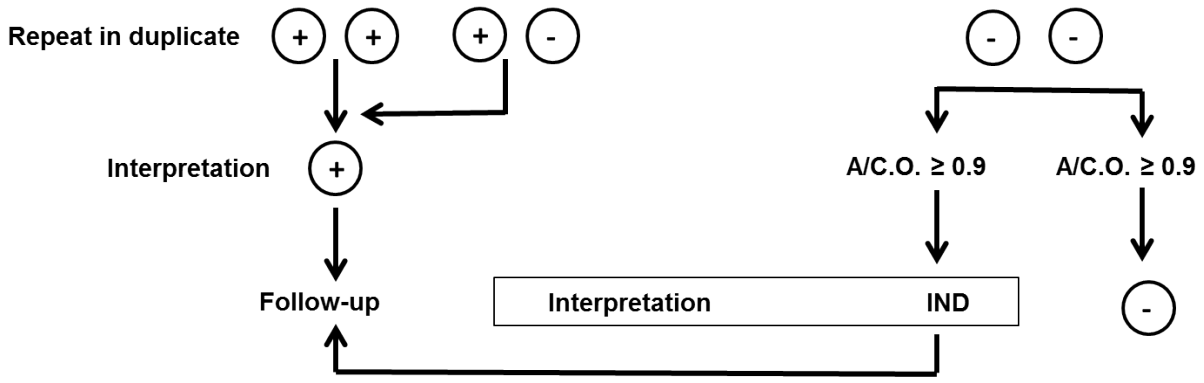
Interpretations of the results

Negative Results (S/C.O.<1): samples giving absorbance less than the Cut-off value are negative for this assay, which indicates that no IgM-class antibodies to HDV core antigen have been detected with this HDV IgM ELISA kit. Therefore, there are no evidences for resent infections with HDV and the patients is probably not infected with HDV.

Positive Results (S/C.O.≥1): samples giving an absorbance greater than or equal to the Cut-off value are initially reactive, which indicates that IgM-class antibodies to HDV have probably been detected with this HDV IgM ELISA kit. Any initially reactive samples must be retested in duplicates. Repeatedly reactive samples can be considered positive for IgM-antibodies to HDV. Therefore, there are indications for possible current infection with hepatitis D virus.

Borderline (S/C.O.=0.9-1.1): Samples with absorbance to Cut-off ratio between 0.9 and 1.1 are considered borderline samples and retesting of these samples in duplicates is required to confirm the initial results.
Follow-up, confirmation and supplementary testing of any positive specimen with other analytical system (e.g. PCR) is required. Clinical diagnosis should not be established based on a single test result. It should integrate clinical and other laboratory data and findings.

INITIAL RESULTS INTERPRETATION AND FOLLOW-UP
ALL INITIALLY REACTIVE OR BORDERLINE SAMPLES.



IND = non-interpretable

- If, after retesting of the initially reactive samples, both wells are negative results (A/C.O.<0.9), these samples should be considered as non-repeatable positive (or false positive) and recorded as negative. As with many very sensitive ELISA assay, false positive results can occur due to the several reasons, most of which are connected with, but not limited to, inadequate washing steps.
- If after retesting in duplicates, one or both wells are positive results, the final result from this ELISA test should be recorded as repeatedly reactive. Repeatedly reactive specimens could be considered positive for IgM antibodies to HDV and therefore, the

patient is probably infected with HDV.
- After retesting in duplicates, samples with values close to the Cut-off value should be interpreted with caution and considered as “borderline” zone sample, or uninterpretable for the time of testing.

PERFORMANCE CHARACTERISTICS

1. **Sensitivity:** The clinical sensitivity of this assay has been calculated by a panel of samples obtained from 2500 hepatitis B acute and chronic patients in which, 2400 samples were found HBsAg positive. After testing with HDV RT-PCR, 150 individuals were diagnosed infected with HDV. During testing with this HSV IgM ELISA kit, 107 of the HDV RT-PCR confirmed positive samples were found positive for HDV IgM and 107 samples were confirmed HDV IgM positive when tested with another commercially available HDV IgM ELISA kit. Sensitivity 100%.
2. **Specificity:** The clinical specificity of this assay has been evaluated by a panel of samples obtained from 500 healthy individuals. No false positive results observed which indicates 100% specificity of the test.
3. **Analytical Specificity:** No interferences have been observed when testing patients with other HDV-unrelated clinical conditions like HIV, HCV, HAV and TP.
   No interference was observed from rheumatoid factors up to 2000 U/mL.
   The assay performance characteristics are unaffected from elevated concentrations of bilirubin, hemoglobin and triolein.
   Frozen specimens have been tested as well to check for interferences due to collection and storage.

LIMITATIONS

1. Positive results must be confirmed with another available method and interpreted in conjunction with the patient clinical information.
2. Antibodies may be undetectable during the early stage of the disease and in some immunosuppressed individuals. Therefore, negative results obtained with this HDV IgM ELISA are only indication that the sample does not contain detectable level of IgM antibodies to HDC and any negative result should not be considered as conclusive evidence that the individual is not infected with HDV.
3. If, after retesting of the initially reactive samples, the assay results are negative, these samples should be considered as non-repeatable (false positive) and interpreted as negative. As with many very sensitive ELISA assays, false positive results can occur due to several reasons, most of which are related but not limited to inadequate washing steps.
4. The most common assay mistakes are:
   - Using kits beyond the expiry date
   - Bad washing procedures
   - Contaminated reagents
   - Incorrect assay procedure steps
   - Insufficient aspiration during washing
   - Failure to add specimens or reagents
   - Improper operation with the laboratory equipment
   - Timing errors
   - The use of highly hemolyzed specimens or specimens containing fibrin
   - Incompletely clotted serum specimens
5. The prevalence of the marker will affect the assay’s predictive values.

6. This kit is intended ONLY for testing of individual serum or plasma samples. Do not use it for testing of cadaver samples, saliva, urine or other body fluids or pooled (mixed) blood.
7. This kit is a qualitative assay and the results cannot be used to measure antibody concentrations.

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ELISA **E**nzyme **L**inked **I**mmuno**s**orbent **A**ssay

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