ZERTIFIKAT ◆ CERTIFICATE ◆ 認證證書 ◆ СЕРТИФИКАТ ◆ CERTIFICADO ◆ CERTIFICAT

DAkkS Deutsche Akkreditierungsstelle D-ZM-11321-01-00

TÜV SÜD Product Service

Certificate

No. Q5 026709 0009 Rev. 01

Holder of Certificate: **DIALAB Produktion und Vertrieb von chemisch-technischen Produkten und Laborinstrumenten Gesellschaft m.b.H.**
IZ-NOE Sued
Hondastrasse, Objekt M55
2351 Wr. Neudorf
AUSTRIA

Certification Mark:

TÜV SÜD EN ISO 13485 tuvsud.com/ps-cert

Scope of Certificate: **Design, development, production and distribution of in-vitro diagnostic reagents and testkits in the areas of immunological detection of infectious diseases, immunochemistry/immunology/clinical chemistry biomarkers (analytes: enzymes, substrates, electrolytes reagents; controls/standards/calibrators), urinalysis, haematology, haemostasis and immunohaematology (blood grouping). Distribution of in-vitro diagnostic instruments including accessories for immunology, clinical chemistry, haematology, haemostasis and urinalysis.**

The Certification Body of TÜV SÜD Product Service GmbH certifies that the company mentioned above has established and is maintaining a quality management system, which meets the requirements of the listed standard(s). All applicable requirements of the testing and certification regulation of TÜV SÜD Group have to be complied with. For details and certificate validity see: www.tuvsud.com/ps-cert?q=cert:Q5 026709 0009 Rev. 01

Report No.: 713237224

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Date: 2023.05.04 16:31:52 EEST
Reason: MoldSign Signature
Location: Moldova

Valid from: 2022-03-29
Valid until: 2025-03-28

Date, 2022-03-17

Christoph Dicks
Head of Certification/Notified Body

TÜV SÜD Product Service GmbH • Certification Body • Ridlerstraße 65 • 80339 Munich • Germany

DAkkS Deutsche Akkreditierungsstelle D-ZM-11321-01-00

TÜV SÜD Product Service

Certificate

No. Q5 026709 0009 Rev. 01

Applied Standard(s):

EN ISO 13485:2016
Medical devices - Quality management systems - Requirements for regulatory purposes
(ISO 13485:2016)
DIN EN ISO 13485:2016

Facility(ies):

DIALAB Produktion und Vertrieb von chemisch-technischen Produkten und Laborinstrumenten Gesellschaft m.b.H.
IZ-NOE Sued, Hondastrasse, Objekt M55, 2351 Wr. Neudorf, AUSTRIA

See Scope of Certificate

Parameters: ./.

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TÜV®

SNAS
Reg. No. 575/P-051

Notified body 2854 | SKTC-180

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www.bqsgroup.eu

EC Certificate IVDD 22 003 0135

Full Quality Assurance System

Directive 98/79/EC on In Vitro Diagnostic Medical Devices
Annex IV excluding section 4 and section 6

Certificate holder: **DIALAB Produktion und Vertrieb von chemisch - technischen Produkten und Laborinstrumenten Gesellschaft m.b.H**

IZ NOE-Sued Hondastrasse Objekt M55, A-2351 Wiener Neudorf, Austria

Related audit report: AIVDD 2022NB003 I01

Other Facility(ies): -

The certificate was issued with respect to the following scope:

HIV 1&2 Ag/Ab

This certificate is effective from 24 May 2022 until 26 May 2025 and remains valid subject to execution of regular examinations and continuous compliance.
Initial version of the certificate was effective from 24 May 2022.

Certification has been authorized by

Digitally signed by Radovan Máčaj

Radovan Macaj
Head of Notified body

bqs.
Certified In Vitro diagnostic medical device

bqs issued the certificate on the basis of performed examination in accordance with Council Directive 98/79/EC, Slovak government decree No. 569/2001 Coll. of Laws and EN ISO/IEC 17065:2012. Notified Body has performed examination of quality assurance system in accordance with Annex IV excluding section 4 and section 6 of the directive and found that the quality assurance system meets the requirements laid down by Annex IV. For the placing on the market of List A devices an EC design-examination certificate according to Annex IV section 4 is required. Please see also notes overleaf if any.

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The Trust. We Make.

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Reg. No. 575/P-051

Notified body 2854 | SKTC-180

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Additional information on certification

Related to certificate number:

IVDD 22 003 0135

Description of product(s) within the certification scope:

HIV 1&2 Ag/Ab is an enzyme-linked immunosorbent assay (ELISA) intended for the qualitative detection of antigens and/or antibodies to Human Immunodeficiency Viruses (HIV) type 1 (group M -O) and/or type 2 in human serum or plasma samples. The method is also known as 4th generation ELISA for HIV detection. The kit is intended for screening of blood donors and as an aid in the diagnosis of clinical conditions related to infection with HIV-1 and/or HIV-2 –the etiological agents of the acquired immunodeficiency syndrome (AIDS).

Types/Categories/Models:	Z04380 (96 wells) Z13382 (480 wells)
Classification:	List A
Validity conditions:	-

This certificate is effective from 24 May 2022 until 26 May 2025 and remains valid subject to execution of regular examinations and continuous compliance.
Initial version of the certificate was effective from 24 May 2022.

bqs.
Certified In Vitro diagnostic medical device

bqs issued the certificate on the basis of performed examination in accordance with Council Directive 98/79/EC, Slovak government decree No. 569/2001 Coll. of Laws and EN ISO/IEC 17065:2012. Notified Body has performed examination of quality assurance system in accordance with Annex IV excluding section 4 and section 6 of the directive and found that the quality assurance system meets the requirements laid down by Annex IV. For the placing on the market of List A devices an EC design-examination certificate according to Annex IV section 4 is required. Please see also notes overleaf if any.

DIALAB Produktion und Vertrieb von chemisch-technischen Produkten und Laborinstrumenten Gesellschaft m.b.H.
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Phone: +43 (0) 2236 660910-0, Fax: +43 (0) 2236 660910-30, e-mail: office@dialab.at

HIV 1&2 Ag/Ab

(en) English

REF

Content

Z04380

-1 Microwell plate: 96 wells (12 x 8-well antigen and antibody coated strips, individual breakaway)
-1x 1 mL Antibody Positive Control I (HIV 1)
-1x 1 mL Antibody Positive Control II (HIV 2)
-1x 1 mL Antigen Positive Control
-1x 1 mL Negative Control
-1x 12 mL Enzyme Conjugate
-1x 3 mL Biotin Conjugate
-1x 6 mL Substrate Solution A
-1x 6 mL Substrate Solution B
-1x 6 mL Stop Solution
-1x 50 mL Wash Buffer
-3 Cardboard Plate Covers
-1 Plastic Bag
-1 Package Insert
-1 Certificate of Analysis

Σ **96**

Z13382

-5 Microwell plates: 96 wells (12 x 8-well antigen and antibody coated strips, individual breakaway)
-3x 1 mL Antibody Positive Control I (HIV 1)
-3x 1 mL Antibody Positive Control II (HIV 2)
-3x 1 mL Antigen Positive Control
-3x 1 mL Negative Control
-5x 12 mL Enzyme Conjugate
-5x 3 mL Biotin Conjugate
-1x 60 mL Substrate Solution A
-1x 60 mL Substrate Solution B
-1x 60 mL Stop Solution
-2x 125 mL Wash Buffer
-15 Cardboard Plate Covers
-5 Plastic Bags
-1 Package Insert
-1 Certificate of Analysis

Σ **480**

For professional in vitro diagnostic use only.

INTENDED USE

HIV 1&2 Ag/Ab is an enzyme-linked immunosorbent assay (ELISA) intended for the qualitative detection of antigens and/or antibodies to Human Immunodeficiency Viruses (HIV) type 1 (group M - O) and/or type 2 in human serum or plasma samples. The method is also known as 4th generation ELISA for HIV detection. The kit is intended for screening of blood donors and as an aid in the diagnosis of clinical conditions related to infection with HIV-1 and/or HIV-2 – the etiological agents of the acquired immunodeficiency syndrome (AIDS).

DIAGNOSTIC SIGNIFICANCE

The human immunodeficiency viruses type 1 and type 2 are the etiological agents of the acquired immunodeficiency syndrome (AIDS). HIV has been isolated from patients with AIDS, AIDS related complex (ARC) and from healthy individuals at high risk for AIDS. Infection with HIV is followed by an acute flu-like illness. This phase may remain unnoticed and the relationship to HIV infection may not be clear in many cases. The acute phase is typically followed by an asymptomatic carrier state, which progresses to clinical AIDS in about 50% of infected individuals within 10 years after seroconversion. Serological evidence of infection with HIV may be obtained by testing for presence of HIV antigens or antibodies in serum of individuals suspected for HIV infection. Antigens can generally be detected during both acute phase and the symptomatic phase of AIDS only. The Antibodies to HIV-1 and/or HIV-2 can be detected throughout virtually the whole infection period, starting at, or shortly after the acute phase and lasting till the end stage of AIDS. Apart from sexual transmission, the principal route of infection with HIV is blood transfusion. HIV can present both in cellular and cell-free fractions of human blood. Therefore, all donations of blood or plasma should be tested due to the risk of HIV transmission through contaminated blood.
The ELISA tests for detection of HIV infection are characterized with high sensitivity, specificity and simple operation procedure. There are most appropriate for testing of large numbers of specimens and currently, internationally available are hundreds of HIV tests used in routine blood screening or clinical diagnosis. Since the first HIV ELISA

tests were commercially introduced in 1985, four generations have been developed. The 1st generation tests were based on viral lysate antigens derived from viruses that are grown in human T-lymphocyte lines. The presence of traces of host cell components in which the virions have been propagated could lead to cross-contamination and thus to very high rates of false-positive results. With the cloning of the HIV genome, improved assays based on recombinant proteins and/or synthetic peptides (known as 2nd generation), became rapidly available. The utilization of biotechnology methods allow predominantly expression of the important immunoreactive regions of the proteins and also enabled the production of combined HIV-1/HIV-2 assays. The recombinant antigen could also be produced with considerably more purity and in large amounts, and they can be bond to solid-phase surface with much tighter control over protein ratios and concentrations. The first and second generations HIV kits were based on indirect ELISA method and could detect IgG antibodies only by enzyme-labeled anti-human IgG antibody. The 3rd generation ELISA utilized double antigen "sandwich" method: again with antigens coated on solid phase polystyrene plates, but with antibodies detection achieved with the help of another enzyme-labeled antigen. The 3rd generation assays could detect all antibodies in specimen (IgG, IgM, etc.) which significantly increases the assay's sensitivity comparing to the previous generations. In addition, the detection of IgM antibodies that are present only during the early stages of infection, much shortens the antibody detection "window" period (the period of time in which there is no detectable antibody production), and compare to the second generation, "sandwich" tests could detect antibodies 11 days earlier. To reduce even further the antibody detection "window" period, 4th generation HIV ELISAs that could simultaneously detect HIV antigens (p24) and antibodies have been developed and are commercially available since 1998. With detection of p24, the 4th generation tests shorten the "window" period to 16 days, or compare to the 3rd generation, HIV infection could be detected 8 days earlier.

TEST PRINCIPLE

DIALAB HIV 1&2 Ag/Ab ELSIA test is a two-step incubation, "sandwich" enzyme immunoassay kit, which uses polystyrene microwell strips pre-coated with recombinant HIV antigens (recombinant HIV-1 gp41, gp120, and recombinant HIV-2 gp36) and anti-HIV (p24) antibodies. As a first step, biotinylated anti-HIV (p24) antibodies together with the patient's serum or plasma specimen are added into the wells. During incubation, the specific HIV-1/2 antibodies if present in specimen, will be captured inside the wells. Simultaneously, if HIV p24 antigen is present in specimen, it will also be captured as a double antibody "sandwich" complex comprising of the coated antibodies-p24-biotinylated antibodies. The microwells are then washed to remove unbound serum proteins. The detection of the captured HIV p24 antigen-biotinylated antibody complex or HIV-1/2 antibodies is achieved during the second incubation step by adding of the enzyme Horseradish Peroxidase (HRP) which has been conjugated to second HIV 1+2 recombinant antigens and to avidin.

p24 detection: When p24 has been captured inside the wells, avidin will react with the biotin and attach HRP to the Ab-p24-Ab complex.

HIV 1&2 antibody detection: When HIV-1/2 antibodies have been captured inside the wells, the HRP-conjugated antigens will bind to the captured antibodies forming Ag-Ab-Ag (HRP) "sandwich" immunocomplex. The microwells are washed to remove unbound conjugate, and Chromogen solutions are added to the wells. In wells containing the Ag-Ab-Ag (HRP) and/or Ab-p24-Ab (HRP) "sandwich" immunocomplexes, the colorless Chromogens are hydrolyzed by the bound HRP to a blue colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color intensity can be measured and is proportional to the amount of antibodies or p24 captured in the wells, and to the specimen respectively. Wells containing specimens negative for anti-HIV-1/2 or p24 remain colorless.

REAGENT COMPOSITION

Component	Description
Microwell plate	Blank microwell strips fixed on white strip holder. The plate is sealed in aluminum pouch with desiccant. Each well contains recombinant HIV 1/2 antigens and anti-p24 antibodies. The microwell strips can be broken to be used separately. Place unused strips in the provided plastic storage bag together with the desiccant and return to 2-8°C. Once opened, the plate strips are stable for 4 weeks when stored at 2-8°C together with the desiccant. The microwell strips are for SINGLE USE only. Do not use if the vacuum sealing has been damaged when first time taken of out the box.
Antibody Positive Control I (HIV 1)	Red-colored liquid filled in a vial with red screw cap. Preservative: 0.1 % ProClin™ 300. Protein-stabilized buffer solution tested positive for antibodies to HIV-1. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Antibody Positive Control II (HIV 2)	Red-colored liquid filled in a vial with yellow screw cap. Preservative: 0.1 % ProClin™ 300. Protein-stabilized buffer solution tested positive for antibodies to HIV-2. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Antigen Positive Control	Red-colored liquid filled in a vial with blue screw cap. Preservative: 0.1 % ProClin™ 300. Protein-stabilized buffer solution tested positive for HIV p24 recombinant antigen. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.

Negative Control	Yellow-colored liquid filled in a vial with green screw cap. Preservative: 0.1 % ProClin™ 300.Protein-stabilized buffer tested non-reactive for HBsAg and antibodies to HIV 1/2, HCV, TP. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Enzyme Conjugate	Red-colored liquid in a white vial with red screw cap. Preservative: 0.1 % ProClin™ 300. Horseradish peroxidase-conjugated recombinant HIV 1+2 antigens. Horseradish peroxidase conjugated avidin. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Biotin Conjugate	Blue-colored liquid in a white vial with blue screw cap. Preservative: 0.1 % ProClin™ 300. Biotinylated anti-HIV p24 antibodies diluted in protein-stabilized buffer. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Substrate Solution A	Colorless liquid filled in a white vial with green screw cap. Urea peroxide solution. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Substrate Solution B	Colorless liquid filled in a black vial with black screw cap. TMB (Tetramethyl benzidine), N,N-dimethylformamide. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Stop Solution	Colorless liquid in a white vial with yellow screw cap. Diluted sulfuric acid solution (0.5M H_2SO_4). Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Wash Buffer	Colorless liquid filled in a clear bottle with white screw cap. Detergent: Tween-20. Buffer solution containing surfactant. The concentrate must be diluted 1 to 20 with distilled/ deionized water before use. Once diluted, stable for 1 week at room temperature, or for 2 weeks when stored at 2-8°C.

MATERIAL REQUIRED BUT NOT PROVIDED

- Freshly distilled or deionized water
- Disposable gloves and timer
- Appropriate waste containers for potentially contaminated materials
- Dispensing system and/or pipette (single or multichannel), disposable pipette tips
- Absorbent tissue or clean towel
- Dry incubator or water bath, 37±1°C
- Plate reader, single wavelength 450 nm or dual wavelength 450 nm and 600-650 nm
- Microwell aspiration/wash system

--- Microplate reader and microplate washers are available from Dialab. ---

REAGENT PREPARATION

Allow the reagents and samples to reach room temperature **(18-30°C)**. Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed, 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 buffer. All other reagents are **ready to use as supplied**.

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 HIV 1&2 Ag/Ab ELISA during storage, protect the reagents from contamination with microorganisms or chemicals.

WARNINGS AND PRECAUTIONS

Warning

ProClin™ 300

H317: May cause an allergic skin reaction.
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P333+P313: If skin irritation or rash occurs: Get medical advice/attention.
P363: Wash contaminated clothing before reuse.

Danger

N,N-dimethylformamide:

H360D:	May damage the unborn child.
P201:	Obtain special instructions before use.
P280:	Wear protective gloves/protective clothing/eye protection/face protection.
P308+P313:	IF exposed or concerned: Get medical advice/attention.

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.

- 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.
- Make sure all the reagents are within the validity indicated on the kit box and of the same lot. Never use reagents beyond their expiry date stated on labels or boxes.
- **CAUTION - CRITICAL STEP:** Allow the reagents and samples to stabilize at room temperature (18-30°C) before use. Shake reagent gently before, and return to 2-8°C immediately after use.
- Use only sufficient volume of sample as indicated in the procedure steps. Failure to do so may cause in low sensitivity of the assay.
- 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.
- 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.
- Avoid assay steps long time interruptions. Assure same working conditions for all wells.
- Calibrate the pipette frequently to assure the accuracy of samples/reagents dispensing. Use different disposal pipette tips for each specimen and reagents as to avoid cross-contaminations.
- Assure that the incubation temperature is 37°C inside the incubator.
- When adding samples, avoid touching the well's bottom with the pipette tip.
- When measuring with a plate reader, it is required to determine the absorbance at 450 nm or at 450/600-650 nm.
- 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.
- 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.
- All specimens from human origin should be considered as potentially infectious. Strict adherence to GLP (Good Laboratory Practices) can ensure the personal safety.
- **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 derived sera have been used for stabilizing of the positive and negative controls. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE-free geographical areas.
- Never eat, drink, smoke or apply cosmetics in the assay laboratory. Never pipette solutions by mouth.
- Chemicals should be handled and disposed of only in accordance with the current GLP (Good Laboratory Practices) and the local or national regulations.
- The pipette tips, vials, strips and sample containers should be collected and autoclaved for not less than two hours at 121°C or treated with 10% sodium hypochlorite for 30 minutes to decontaminate before any further steps for disposal. Solutions containing sodium hypochlorite should NEVER be autoclaved. Material Safety Data Sheet (MSDS) available upon request.
- 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 Solution, Substrate Solutions and the Wash Buffer.
- The Stop solution (0.5M H_2SO_4) is an acid. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes.
- ProClin™ 300 0.1% used as a preservative can cause sensation of the skin. Wipe up spills immediately or wash with water if come into contact with the skin or eyes.

Indication of instability and deterioration of the reagent:
Before use, please check the vials for presence of turbidity and/or particles. If present, this is indication of possible microbial contamination and the reagents should not be used. 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 specimens must be retested. In case of constant erroneous results and proven deterioration or instability of the reagents, immediately substitute the reagents with new one or contact Dialab's technical support for further assistance.

SPECIMEN COLLECTION AND STORAGE

- **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 as to avoid haemolysis of the RBC. Care should be taken to ensure that the serum specimens are clear and not contaminated by microorganisms. Any visible particulate matters in the specimen should be removed by centrifugation at 3000 RPM (round per minutes) for 20 minutes at room temperature or by filtration.
- Plasma specimens collected into EDTA, sodium citrate or 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. Specimens with visible microbial contamination should never be used.
- Dialab HIV 1&2 Ag/Ab ELISA test is intended **ONLY** for testing of individual serum or plasma specimens. Do not use the assay for testing of cadaver specimens, saliva, urine or other body fluids or pooled (mixed) blood.
- **Transportation and Storage:** Store samples at 2-8°C. Samples not required for assaying within 1 week should be stored frozen (-20°C or lower). Multiple freeze-thaw cycles should be avoided. For shipment, samples should be packaged and labeled in accordance with the existing local and international regulations for transport of clinical samples and ethological agents.

TEST PROCEDURE

Step 1 **Preparation:** Mark three wells as Negative control (e.g. B1, C1, D1), three wells as Positive control (e.g. E1 for HIV-1, F1 for HIV-2 and G1 for HIV-Ag) and one Blank (e.g. A1, neither specimens nor 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 Biotin Conjugate:** Add **20 µL** of Biotin Conjugate into each well except in the Blank.

Step 3 **Adding Samples:** Add **100 µL** of Positive Controls, Negative Control and Samples into their respective wells, mix gently. **Note: Use a separate disposal pipette tip for each specimen, Negative Control, Positive Control to avoid cross-contamination.**

Step 4 **Incubating:** Cover the plate with the plate cover and incubate for **60 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 Wash buffer. Each time, allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn the plate down onto blotting paper or clean towel, and tap it as to remove any remaining liquids.

Step 6 **Adding Enzyme Conjugate:** Add **100 µL** Enzyme Conjugate into each well except in the Blank.

Step 7 **Incubating:** 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 Wash buffer. Each time, allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn the plate down onto blotting paper or clean towel, and tap it as to remove any remaining liquids.

Step 9 **Coloring:** Add **50 µL** of Substrate Solution A and then **50 µL** of Substrate Solution B into each well including the Blank, mix gently. Incubate the plate at **37°C for 30 minutes avoiding light**. The enzymatic reaction between the Substrate Solutions and the Enzyme Conjugate produces blue color in Positive Controls and HIV 1/2 positive for antigens/antibodies sample wells.

Step 10 **Stopping Reaction:** Using a multichannel pipette or manually, add **50 µL** of Stop Solution into each well and mix gently. Intensive yellow color develops in Positive control and HIV 1/2 positive for antigens / antibodies specimen wells.

Step 11 **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at **450 nm**. If a dual filter instrument is used, set the reference wavelength at **600-650 nm**. Calculate the Cut-off value and evaluate the results.

Note: read the absorbance within 10 minutes after stopping the reaction.

Instructions for Washing:

- A good washing procedure is essential in order to obtain correct and precise analytical data.

- 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.
- To avoid cross-contaminations of the plate with specimen or HRP-conjugate, after incubation do not discard the content of the wells but allow the plate washer to aspirate it automatically.
- 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.
- In case of manual washing, we suggest to carry out **5 washing 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.
- In any case, the liquid aspirated out the strips should be treated with a sodium hypochlorite solution (final concentration of 2.5%) for 24 hours, before liquids are disposed in an appropriate way.
- The concentrated Wash buffer should be diluted **1:20** before use. If less than a whole plate is used, prepare the proportional volume of solution.

INTERPRETATION OF RESULTS

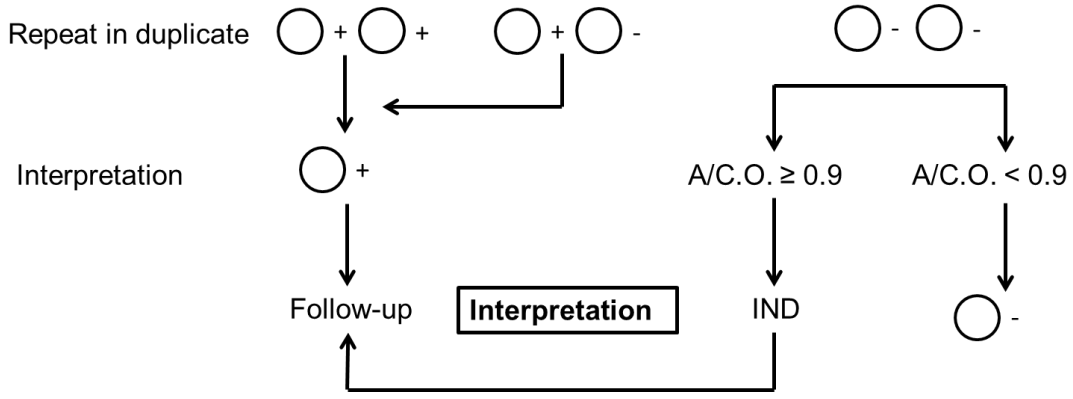
Negative Results (OD/C.O.<1): Samples giving absorbance less than the Cut-off value are negative for this assay, which indicates that no HIV 1&2 antibodies or p24 antigen have been detected with the Dialab HIV 1&2 Ag/Ab ELISA kit, therefore the patient is probably not infected or the blood unit do not contain antibodies to HIV 1&2 or p24 antigen and could be transfused in case that other infectious diseases markers are also absent.

Positive Results (OD/C.O.≥1): Samples giving an absorbance equal to or greater than the Cut-off value are considered initially reactive, which indicates that HIV 1&2 antibodies and/or p24 antigen have probably been detected using Dialab HIV 1&2 Ag/Ab ELISA kit. All initially reactive specimens should be retested in duplicate using Dialab HIV 1&2 Ag/Ab ELISA kit before the final assay results interpretation. Repeatedly reactive specimens can be considered positive for antibodies to HIV 1/2 and/or p24 antigen with Dialab HIV 1&2 Ag/Ab ELISA kit.

Borderline (OD/C.O.=0.9-1.1): Samples with absorbance to Cut-off ratio between 0.9 and 1.1 are considered borderline and retesting of these samples in duplicates is recommended to confirm the results.

Follow-up, confirmation and supplementary testing of any positive specimen with other analytical system (e.g. WB, 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 result interpretation and follow up:



IND = non interpretable

- If, after retesting of the initially reactive samples, both wells are negative results (OD/CO<0.9), these samples should be considered as non-repeatable positive (or, false positive) and recorded as negative. As with many very sensitive ELISA assays, false positive results can occur due to the several reasons, most of which are connected with, but not limited to, inadequate washing step.
- 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 can be considered positive for antibodies to HIV 1&2 and/or p24 antigen and therefore the patient is probably infected with HIV 1&2 and the blood unit must be discarded.
- 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.

QUALITY CONTROL AND CALIBRATION

Each microplate must 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

must 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 value from the print report values of specimens and controls

Calculation of Cut-off value: (C.O.) = *Nc + 0.12

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

Quality control (assay validation): The test results are valid if the Quality Control criteria are verified. 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.

Quality control criteria:

1. The OD value of the Blank well, which contains only Substrate and Stop Solution, must be < 0.080 at 450 nm.

2. The OD value of the Positive Controls must be ≥ 0.800 at 450/600-650 nm or at 450 nm after blanking.

3. The OD value of the Negative Control must be ≤ 0.100 at 450/600-650 nm or at 450nm after blanking.

If one of the Negative Control OD values does not meet the Quality Control criteria, it should be discarded and the mean value calculated again using the remaining two values. If more than one Negative Control OD values do not meet the Quality Control Range specifications, the test is invalid and must be repeated.

Example:

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

Well No.:	**B1**	**C1**	**D1**
Negative Control OD values after blanking:	0.020	0.012	0.016
Well No.:	**E1**	**F1**	**G1**
Positive Control OD values after blanking:	2.421	2.369	2.893

All control values are within the stated quality control range
2. Calculation of Nc: = (0.020 + 0.012 + 0.016)/3 = 0.016
3. Calculation of the Cut-off: (C.O.) = 0.016 + 0.12 = 0.136

PERFORMANCE CHARACTERISTICS

The analytical and clinical performance characteristics of Dialab HIV 1&2 Ag/Ab ELISA kit were evaluated by two external evaluation centres.

Diagnostic sensitivity:

100% (500/500 positive samples) tested on 310 anti-HIV-1, 100 anti-HIV-2 and 40 anti-HIV-1 non B subtypes (A, C, D, F, G, H, J, K, O, CRF01_AE and other circulating recombinant forms) serum/plasma samples and 50 anti-HIV-Ab / HIV-1 Ag positive samples.

The kit represents state of the art on 20 seroconversion panels:

- On the 38 (of a total of 68) early seroconversion samples that were positive on one or more HIV Ag/Ab tests, the Dialab HIV 1&2 Ag/Ab ELISA kit detected 34 samples
- All seroconversion HIV samples were positive on the Dialab HIV 1&2 Ag/Ab ELISA kit

Same day samples: no complement interference was observed on 25 same day fresh samples spiked with a small amount of an HIV Ag/Ab positive sample.

Diagnostic specificity: 99.96% tested on 5004 negative plasma samples of blood donors – based on the results after repeat testing of initially reactive samples.

Analytical specificity: 200/200 hospitalized patients were negative on the Dialab HIV 1&2 Ag/Ab ELISA kit. 95/101 samples containing potentially cross reactive substances, including samples from pregnant women, were negative on the Dialab HIV 1&2 Ag/Ab ELISA kit. Serum to plasma equivalence is demonstrated on 25 positive and 25 negative serum / EDTA plasma / heparin plasma / sodium citrate plasma couples.

p24 antigen analytical sensitivity: 1.25 U/mL

TRACEABILITY

HIV p24 antigen international reference standard (WHO 90/636) was used as a reference for the Dialab HIV 1&2 Ag/Ab ELISA.

EXPECTED VALUES

Dialab HIV 1&2 Ag/Ab is a qualitative assay and cannot be used to measure the antigen concentration, therefore the concept of expected values is not applicable. Example values for absorbance can be found in the chapter QUALITY CONTROL AND CALIBRATION.

LIMITATIONS

- Positive results must be confirmed with another available method and interpreted in conjunction with the patient clinical information.
- Antibodies or p24 antigen may be undetectable during the early stage of the disease and in some immunosuppressed individuals. Therefore, negative results obtained Dialab HIV 1&2 Ag/Ab ELISA kit are only indication that the specimen does not contain detectable level of HIV1/2 antibodies or p24 antigen and any negative result should not be considered as conclusive evidence that the individual is not infected with HIV 1/2 or the blood unit is not infected with HIV 1/2.
- If, after retesting of the initially reactive specimens, the assay results are negative, these specimens 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 the several reasons, most of which are related but not limited to inadequate washing step.
- 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.
- The prevalence of the marker will affect the assay's predictive values.
- This assay cannot be utilized to test pooled (mixed) serum or plasma. Dialab HIV 1&2 Ag/Ab ELISA kit has been evaluated only with individual serum or plasma specimens.
- Dialab HIV 1&2 Ag/Ab ELISA kit is a qualitative assay and the results cannot be used to measure antibody or antigen concentration. This assay cannot distinguish infections with HIV-1 and HIV-2. This assay cannot distinguish antibody and p24 antigen positive results.

WASTE MANAGEMENT

Reagents must be disposed of in accordance with local regulations.

LITERATURE

1. Barbe, F. et al., (1994) Early detection of anti bodies to HIV-1 by a third generation enzyme immunoassay. Ann. Biol. Clin. (Paris), 52: 341-345.
2. Barre-Sinoussi, F. et al., (1984) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immunodeficiency syndrome (AIDS), Science, 220: 868-871.
3. Clave, F. et al. (1991) Solution conformation preferences of immunogenic peptides derived from the principal neutralization determination of the HIV-1 envelope glycoprotein gp120. Biochemistry. 30: 9187-9194.
4. Constantine, N., T. et al., (1993) Serologic test for the retroviruses: approaching a decade of evolution. AIDS, 7: 1-13Gnann JW et al. (1987) Science; 237: 1346-1349.
5. Barbe, F. et al.,(1994) Early detection of antibodies to HIV-1 by a third generation enzyme immunoassay. Ann.Biol.Clin.(Paris),52, 341-345.
6. Barr P.J. et al.,(1987) Antigenicity of domains of the HIV envelope polypeptide expressed in the yeast Saccharomyces cerevisiae.Vaccine,5:90-101.

USED SYMBOLS

Symbol	Description
Cont.	Content

CE 2854 IVD 2°C 8°C

www.dialab.at

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

Microwell Method

HAV IgM

REF Z08372

For in vitro Diagnostic Use

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

Enzyme Linked Immunosorbent Assay for qualitative determination of IgM-class antibodies to human hepatitis A virus (HAV IgM) in serum or plasma.

Microwell Method - 96 wells

(12 x 8-well Antigen coated Strips)

Individual breakaway

GENERAL INFORMATION

- ❑ Wavelength Measurement Filter: 450nm
  Optional Reference Filter: 630nm
- ❑ Enzyme Conjugate
  HRP (Horseradish Peroxidase)
- ❑ Substrate
  TMB (3,3´,5,5´-Tetramethyl-benzidine)
- ❑ Sample
  Serum or Plasma
- ❑ Incubation Time
  75 minutes at 37°C (20/40/15)
- ❑ Shelf life and Stability of Kit Components

Kit:	see expiration date on the label
Kit Components:	see expiration date on the label

INTENDED USE

This kit is an enzyme-linked immunosorbent assay (ELISA) for qualitative determination of IgM-class antibodies to hepatitis A virus in human serum or plasma. It is intended for use in clinical laboratories for diagnosis of acute hepatitis A and managements of patients related to infection with hepatitis A virus.

KIT COMPONENTS

MICROWELL PLATE	1	**12×8-well** strips per plate. Blank microwell strips fixed on white strip holder. The plate is sealed in aluminium 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 plastic sealable storage bag together with the desiccant and return to 2-8°C.
POSITIVE CONTROL	1	0.5 mL per vial. Red-colored color liquid filled in a vial with red screw cap. Purified anti-HAV IgM antibodies diluted in protein-stabilized buffer. Preservatives: 0.1% ProClin300. Ready to use as supplied. Once open, stable for one month at 2-8°C.
NEGATIVE CONTROL	1	0.5 mL per vial. Yellowish liquid filled in a vial with green screw cap. Protein-stabilized buffer tested non-reactive for HAV IgM. Preservatives: 0.1% ProClin300. Ready to use as supplied. Once open, stable for one month at 2-8°C.
ENZYME CONJUGATE	1	12 mL per vial. Red-colored liquid filled in a white vial with red screw cap. Horseradish peroxidase-conjugated HAV antigens. 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 white vial with green 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 solution (Tetramethyl benzidine dissolved in citric acid). Ready to use as supplied. Once open, stable for one month at 2-8°C.
STOP SOLUTION	1	7 mL per vial. Colorless liquid filled in a white vial with white screw cap. Diluted sulfuric acid solution (0.5 M H_2SO_4).
WASH BUFFER	1	50 mL per bottle. Colorless liquid filled in a clear bottle with white screw cap, pH 7.4, 20x PBS (contains Tween-20 as a detergent) **DILUTE BEFORE USE:** The concentrate must be diluted **1:20** with distilled/deionized water before use. Once diluted, stable for one week at room temperature or for two weeks at 2-8°C.
CARDBOARD PLATE COVER SHEETS	2	
PLASTIC SEALABLE BAG	1	
PACKAGE INSERT	1	

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. Microshaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450 nm or dual wavelength 450 nm and 630 nm.
10. Microwell aspiration/wash system.
11. Normal saline solution to dilute samples.

SUMMARY AND EXPLANATION

Hepatitis A is a self-limited disease and chronic stage or other complications are rare. Infections occur early in life in areas where sanitation is poor and living conditions are crowded. With improved sanitation and hygiene, infections are delayed and consequently the number of persons susceptible to the disease increases. Because the disease is transmitted through the fecal-oral route in dense populated regions, an outbreak can arise from single contaminated source. The cause of hepatitis A is hepatitis A virus (HAV)-non enveloped positive strand RNA virus with a linear single strand genome, encoding for only one known serotype. HAV has four major, structural polypeptides and it localizes exclusively in the cytoplasm of human hepatocytes. The infection with HAV induces strong immunological response and elevated levels first of IgM and then IgG are detectable within a few days after the onset of the symptoms. The presence of anti-HAV IgM is an important serological marker for early detection and observation of the clinical manifestation of the disease. Increasing levels of anti-HAV IgM are detectable about three weeks after exposure with highest titter after four to six weeks later. Within six months after infection IgM concentration declines to non-detectable levels.

TEST PRINCIPLE

This kit is a solid phase, two-step incubation, antibody capture ELISA assay in which, poly-styrene microwell strips are pre-coated with antibodies directed to human immunoglobulin M proteins (anti-μ chain). The patient's serum/plasma sample is added and during the first incubation, any IgM antibodies will be captured in the wells. After washing out all the other components of the sample and in particular IgG antibodies, the specific HAV IgM captured on the solid phase is detected by the addition of HAV antigens conjugated to horseradish peroxidase (HRP-Conjugate). During the second incubation, the HRP-conjugated antigens will specifically react only with the HAV IgM antibodies and after washing to remove unbound HRP-conjugate, Chromogen solutions are added to the wells.
In presence of the (anti-μ)-(HAV IgM)-(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 intensity can be measured and is proportional to the amount of antibody captured in the wells and to the sample, respectively. Wells containing samples negative for HAV IgM remain colorless.

Ab(p)+IgM(s) → [Ab(p)–IgM(s)]+(Ag)ENZ→ [Ab(p)–IgM(s)–(Ag)ENZ] →blue→yellow				(+)
Ab(p) → [Ab(p)]+ Ag)ENZ→ [Ab(p)] → no color				(-)
Incubation 1	Incubation 2	Immobilized Complex	Coloring	Results
20 min.	40 min.	15 min.		

Ab(p)–pre-coated anti-IgM antibodies (anti-μ chain);
IgM(s) – HAV IgM antibodies in sample;
(Ag)ENZ– HRP conjugated HAV antigens;

SPECIMEN COLLECTION, TRANSPORTATION AND STORAGE

1. **Sample Collection:** Either fresh serum or plasma samples can be used for 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 as to avoid hemolysis of the RBC. 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 for at least 20 minutes at room temperature, or by filtration on 0.22μm filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but highly lipaemic, icteric, or hemolized samples should not be used as they could give erroneous results in the assay. Do not heat inactivate samples. This can cause sample deterioration.
2. **Transportation and Storage:** Store samples at 2-8°C. Samples not required for assaying within 3 days should be stored frozen (-20°C or lower). Multiple freeze-thaw cycles should be avoided. For shipment, samples should be packaged and labeled in accordance with the existing local and international regulations for transport of clinical samples and ethological agents.
3. **Sample preparation:** Each sample must be diluted 1:1000 with normal saline.

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 with dispensing of 350-400μl/well, are sufficient to avoid false positive reactions and high background (all wells turn yellow).
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's 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 perform at least 5cycles, dispensing 350-400μl/well and aspirating the liquid for 5times. 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 (final concentration of 2.5%) for 24 hours, before liquids are disposed in an appropriate way.
7. The concentrated Washing solution should be diluted **1 to 20** before use. For one plate, mix 50 mL of the concentrate with 950 mL of water for a final volume of 1000 mL 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 HAV IgM ELISA kit, during storage protect the reagents from contamination with microorganism or chemicals.

PRECAUTIONS AND SAFETY

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

FOR PROFESSIONAL USE ONLY

The ELISA assay is a time and temperature sensitive method. 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 as to achieve optimal performance during testing.
2. Make sure that all reagents are within the validity indicated on the kit box and are of the same lot. Never use reagents beyond the expiry date stated on reagents labels or on the kit box.
3. **CAUTION - CRITICAL STEP:** Allow the reagents and samples to stabilize at room temperature (18-30°C) before use. Shake reagent gently before, 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 in low sensitivity of the assay.
5. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.
6. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.
7. 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.
8. Avoid assay steps long time interruptions. Assure same working conditions for all wells.
9. Calibrate the pipette frequently to assure the accuracy of samples/reagents dispensing. Always use different disposal pipette tips for each specimen and reagents as to avoid cross-contaminations. Never pipette solutions by mouth.
10. The use of automatic pipettes is recommended.
11. Assure that the incubation temperature is 37°C inside the incubator.
12. When adding samples, avoid touching the well's bottom with the pipette tip.
13. When reading the results with a plate reader, it is recommended to determine the absorbance at 450 nm or at 450 nm with reference at 630 nm.
14. All specimens from human origin should be considered as potentially infectious.
15. Materials from human origin may have been used in the kit. These materials have been tested with tests 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. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.
16. Bovine derived sera may have been used in this kit. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE free-geographical areas.
17. The pipette tips, vials, strips and sample containers should be collected and autoclaved

for 1hour at 121°C or treated with 10% sodium hypochlorite for 30minutes to decontaminate before any further steps for disposal.

18. The Stop solution (0.5 M H_2SO_4) is a strong acid. Corrosive. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.
19. The enzymatic activity of the HRP-conjugate might be affected from dust, reactive chemical, and substances like sodium hypochlorite, acids, alkalins etc. Do not perform the assay in the presence of such substances.
20. Materials Safety Data Sheet (MSDS) available upon request.
21. If using fully automated microplate processing system, 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.

PROCEDURE

Step 1: Reagents Preparation: Allow all reagents and samples to reach room temperature. (18-30°C) for at least 15-30 minutes. 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 stock wash buffer **1 to 20** with distilled or deionized water. Use only clean vessels to dilute the Wash buffer. Mark three wells as Negative control **(e.g. B1, C1, D1),** two wells as Positive control **(e.g. E1, F1)** and one Blank well. (**e.g. A1**, neither samples or Enzyme 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: Diluting Sample: Dilute the specimen 1:1000 with normal saline. Do not dilute the Controls, as they are ready for use as supplied

Step 3: Adding Sample: Add **100 µL** of samples into each well and **100 µL** Positive and Negative controls into their respective wells. **Note: Use a separate disposal pipette tip for each specimen, Negative and Positive Controls as to avoid cross-contamination**

Step 4: Incubating (1) Sample: Cover the plate with the plate cover and incubate for **20 minutes at 37°C**. It is recommended to use thermostat-controlled water tank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step 5: Washing (1): 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 plate onto blotting paper or clean towel, and tap it as to remove any remaining liquids.

Step 6: Adding Conjugate: Add **100 µL** of Enzyme Conjugate Reagent into each well except the blank.

Step 7: Enzyme Conjugate Incubation (2): Cover the plate with the plate cover and incubate for **40 min at 37°C**.

Step 8: Washing (2): Remove and discard the plate cover. Aspirate the liquid and rinse each well **5times** with Wash buffer (as step 5). After the final washing cycle, turn the plate and tap out any remainders.

Step 9: Coloring: Add **50 µL** of Substrate Solution A and **50 µL** Substrate Solution B solution into each well including the **Blank** and mix by tapping the plate gently. Incubate the plate at **37°C for 15 minutes avoiding light**. The enzymatic reaction

between the Substrate Solutions and the Enzyme Conjugate produces blue color in Positive control and HAV 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 HAV IgM positive sample wells.

Step 11: Measuring the Absorbance: Calibrate the plate reader with the Blank well and read the absorbance at **450 nm**.If a dual filter instrument is used, set the reference wavelength at **630 nm**. Calculate the Cut-off value and evaluate the results.
(**Note:** read the absorbance within **5**minutes after stopping the reaction)

INTERPRETATION OF RESULTS AND QUALITY CONTROL

Each microplate must 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's 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 must 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

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

***Nc** = the mean absorbance value of the three negative controls

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

Example:
Calculation of Nc:

Well No	B1	C1	D1
Negative controls OD value	0.02	0.012	0.016

Nc=0.016 (Nc is lower than 0.05, so take it as 0.05)
Calculation of Cut-off value: (C.O.)= 0.05 x 2.1= 0.105

If one of the Negative control values does not meet the Quality control range specifications, it should be discarded, and the mean value is calculated again using the remaining two values. If more than one negative control OD value does not meet the Quality control range specifications, the test is invalid and must be repeated.

2. Quality control range:

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

3. Interpretations of the results:

(S = the individual absorbance (OD) of each specimen)

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 HAV have been detected with this HAV IgM ELISA kit. Therefore, there are no serological indications for recent infection, and the patient is probably not infected with hepatitis A virus .

Positive Results (**S/C.O.≥1):** samples giving an absorbance greater than, or equal to the Cut-off value are initially positive, which indicates that IgM class antibodies to HAV have probably been detected with this HAV IgM ELISA kit. Retesting in duplicates of any reactive sample is recommended. Repeatedly reactive samples can be considered positive for IgM antibodies to HAV and therefore there are serological indications for current infection with hepatitis A 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. Retesting of these samples in duplicates is recommended. Repeatedly reactive samples can be considered positive for IgM antibodies to HAV.
Follow-up and supplementary testing of any positive samples with other HAV tests is required to confirm the infection state.

TEST PERFORMANCE AND EXPECTED RESULTS

The **clinical sensitivity** of this kit was evaluated by testing samples obtained from 739 (288 children and 451 adults) individuals suspected for infection with HAV during outcome. Another group of samples from 1950 healthy blood donors was tested in order to determine the clinical specificity of the test. These evaluation studies were carried out in direct comparison with another commercially available HAV IgM ELISA kit used as a confirmation assay. The evaluation results are given below.

Clinical Specificity:

	CHILDREN		**ADULTS**	
	Tested specificity	False positive	Tested specificity	False positive
Donors	1220 >99%	5	730 >99%	4

Clinical Sensitivity:

	CHILDREN				
	Tested	-	+	confirmed	SENSITIVITY
Inapparent infection	148	3	145	145	100%
Anicteric /icteric	140	15	35	35	100%
TOTAL	288	18	180	180	100%

	ADULTS				
	Tested	-	+	confirmed	SENSITIVITY
Inapparent infection	238	192	46	46	100%
Anicteric /icteric	213	120	190	190	100%
TOTAL	451	312	236	236	100%

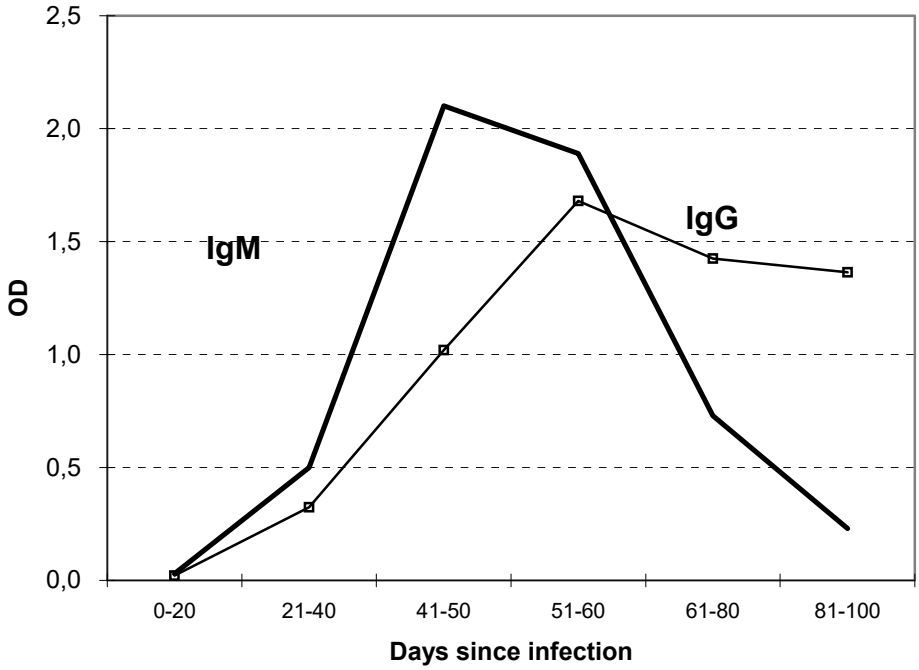
Linearity of sample dilution- undiluted sample:

Sample dilution index	OD
1:1	2.543
1:500	2.234
1:5000	1:042
1:50 000	0.673
1:500 000	0.036

Follow-up of individuals infected with HAV:

Days since infection	OD
0-20	0.031
21-40	0.521
41-50	2.143
51-60	1.890
61-80	0.736
81-100	0.231

Follow-up of individuals infected with HAV:



Comparisons of this HAV IgM ELISA kit performance characteristic in follow-up of individuals infected with HAV, and reference anti-HAV IgG ELISA kit (squares).

Analytical Specificity:

1. No cross reactivity observed with samples from patients confirmed to be infected with HBV, HCV, HIV, CMV, and TP.
2. No interferences from elevated levels of rheumatoid factors up to 2000U/ml were observed during clinical testing.
3. The assay performance characteristics are unaffected from elevated concentrations of bilirubin, hemoglobin, and triolein.

Reproducibility	**Within run**			**Between run**		
Specimen	Test	MeanOD	CV%	Test	MeanOD	CV%
Weak positive	10	0.428	8.1	10	0.395	8.5
Moderate positive	10	0.916	7.3	10	0.856	7.6
Strong positive	10	2.172	4.6	10	2.982	5.1

LIMITATIONS

1. Non- repeatable reactive results may be obtained with any ELISA test due to the general characteristics of this diagnostic method. A negative result with an antibody detection test does not preclude the possibility of infection. Antibodies may be undetectable during the early stages of the disease and in some immunosuppressed individuals.
2. 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 the several reasons, most of which are related but not limited to inadequate washing step.
3. Any positive results must be interpreted in conjunction with the patient clinical information and other laboratory results
4. Common sources for mistakes: kits beyond the expiry date, bad washing procedures and wrong washing buffer concentration, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
5. The prevalence of the marker will affect the assay's predictive values.
6. False negative results can occur from inhibition of specific IgM in the presence of high titers of specific IgG. The removal of IgG can be helpful to prevent false negative results and methods for this are given elsewhere.
7. 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.

INDICATIONS OF INSTABILITY OR DETERIORATION OF THE REAGENTS

1. Values of the Positive or Negative controls, which are out of the indicated Quality control range, are indicator 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 classified as due to deterioration or instability of the reagents, immediately substitute the reagents with new ones.
2. If after mixing of the Chromogen A and B solutions into the wells, the, the color of the mixture turns blue within few minutes, do not continue carrying out the testing and replace the reagents with fresh ones.

VALIDITY

Please do not use this kit beyond the expiration indicated on the kit box and reagent labels.

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

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

Microwell Method

HBc IgM capture

REF: Z01365

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 HBc IgM (IgM class antibodies to hepatitis B virus core antigen) 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:	see expiration date on the label
Kit Components:	see expiration date on the label

INTENDED USE

This anti-HBc IgM kit is an enzyme-linked immunosorbent assay (ELISA) for qualitative determination of IgM class antibodies to hepatitis B virus core antigen in human serum or plasma. It is intended for use in clinical laboratories for diagnosis and management of patients related to infection with hepatitis B.

KIT COMPONENTS

MICROWELL PLATE	1	The plate is sealed in aluminium pouch with desiccant. **12×8-well strips per plate. Each well contains anti-IgM antibodies (anti-µ chain).** The microwell strips can be broken to be used separately. Place unused wells in the plastic sealable storage bag together with the desiccant and return to 2-8°C.
POSITIVE CONTROL	1	0.5 mL per vial. anti-HBc 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. Protein-stabilized buffer tested non reactive for anti-HBc 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. Horseradish peroxidase-conjugated purified HBcAg, labeled with monoclonal anti-HBc. Ready to use as supplied. Once open, stable for one month at 2-8°C.

SUBSTRATE SOLUTION A	1	7 mL per vial. 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. TMB solution. Tetramethylbenzidine dissolved in citric acid. Ready to use as supplied. Once open, stable for one month at 2-8°C.
STOP SOLUTION	1	7 mL per vial. Diluted sulfuric acid solution (0.5 M H_2SO_4).
WASH BUFFER	1	50 mL per bottle. pH 7.4, 20x PBS (Containing Tween-20 as a detergent).
CARDBOARD 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. Microshaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm.
10. Microwell aspiration/wash system.
11. Normal saline solution for dilution of the samples.

SUMMARY AND EXPLANATION

Hepatitis B virus (HBV) is an enveloped, double-stranded DNA virus belonging to the Hepadnaviridae family and is recognized as the major cause of blood transmitted hepatitis together with hepatitis C virus (HCV). Infection with HBV induces a spectrum of clinical manifestations ranging from mild, inapparent disease to fulminant hepatitis, severe chronic liver diseases, which in some cases can lead to cirrhosis and carcinoma of the liver. Classification of a hepatitis B infection requires the identification of a number of serological markers expressed during three phases (incubation, acute and convalescent) of the infection. Now several diagnostic test are used for screening, clinical diagnosis and management of the disease. Hepatitis B “core” antigen (HBcAg) is a major component of the viral structure. HBcAg is composed of a single polypeptide of about 17 kD that is released upon disaggregation of the core particles; the antigen contains at least one immunological determinant. Antibodies to HBcAg (anti-HBc total antibody and IgM) appear shortly after the appearance of HBsAg and persist for life both in persons who have recovered from a hepatitis B infection and in those who develop HBsAg-carrier status but in rare cases, an HBV infection can also run its course without the appearance of immunologically detectable anti-HBc (usually in immunosuppressed patients).
In chronic hepatitis, however, spikes of anti-HBc IgM synthesis are present, confirming reactivation of HBV in hepatocytes and giving origin to permanent IgM low titers. Presence of IgM and total anti-HBc indicates an ongoing or recent HBV infection. When used in conjunction with tests for other HBV serological markers, a laboratory diagnosis or a rule out of HBV infection can be achieved.

TEST PRINCIPLE

This anti-HBc IgM ELISA kit is a two-step incubation, solid phase antibody capture assay in which polystyrene microwell strips are pre-coated with antibodies directed to human IgM (anti-μ chain). The patient's serum/plasma sample is added and during the first incubation step, any IgM-class antibodies will be captured inside the wells. After washing out all the other components of the sample and in particular IgG-class antibodies, the specific anti-HBc IgM captured on the solid phase is detected by the addition of purified HBcAg, labeled with a anti-HBc monoclonal antibody conjugated to horseradish peroxidase (HRP). During the second incubation, the HRP-conjugated antigens will specifically react only with anti-HBc IgM antibodies and after washing to remove the unbound HRP-conjugate, Chromogen solutions are added to the wells. In presence of the (anti**-**μ chain**)**-(anti-HBc IgM)-(HBcAg-Ab (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 anti-HBc IgM remain colorless.

Assay principle scheme: Antibody Capture ELISA

Ab(p)+IgM(s)	**→[Ab(p)–IgM(s)]+ENZ**	**→[Ab(p)–IgM(s)–ENZ]**	**→blue→yellow**	**(+)**
Ab(p)	**→[Ab(p)]+ENZ**	**→[Ab(p)]**	**→ no color**	**(-)**
Incubation1	**Incubation2**	**Immobilized Complex**	**Coloring**	**Results**
30min.	**30 min.**		**15 min.**	

Ab(p)–pre-coated anti-IgM antibodies (anti-μ chain)
IgM(s)–anti-HBc IgM antibodies in sample
ENZ– HRP conjugated HBcAg labeled with antibody

SPECIMEN COLLECTION, TRANSPORTATION AND STORAGE

1. **Sample Collection:** Either fresh serum or plasma samples can be used for this assay. Blood collected by venipuncture should be allowed to clot naturally and completely. 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 on 0.22 μm filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but highly lipaemic, icteric, or hemolized samples should not be used as they can give false results in the assay. Do not heat inactivate samples. This can cause sample deterioration.
2. **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 Enzyme 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 5times. 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 1000 mL 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 HBc 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 DIAGNOSTIC 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. 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. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.
5. 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. Never pipette solutions by mouth.
9. The use of automatic pipettes and disposable tips is recommended.

10. Assure that the incubation temperature is 37°C inside the incubator.
11. When adding samples avoid touching the well's bottom with the pipette tip.
12. When reading the absorbance with a plate reader, it is recommended to determine the absorbance at 450nm or at 450nm with reference at 630nm.
13. All specimens from human origin should be considered as potentially infectious. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.
14. The pipette tips, vials, strips and sample containers should be collected and autoclaved for 1 hour at 121°C or treated with 10% sodium hypochlorite for 30 minutes to decontaminate before any further steps for disposal.
15. The Stop Solution contains 0.5M H_2SO_4. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.
16. The enzymatic activity of the HRP-Conjugate might be affected from dust, reactive chemical, and substances like sodium hypochlorite, acids, alkalins etc. Do not perform the assay in the presence of such substances.

PROCEDURE

Step1 **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. 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 or Enzyme Conjugate should be added into the Blank well). Use only number of strips required for the test.

Step2 **Diluting Sample:** Dilute each sample **1:1000** with normal saline (Do not dilute the controls, they are ready to use as supplied).

Step3 **Adding Sample:** Add **100 µL** of samples and **100 µL** Positive and Negative controls and into their respective wells. **Note: Use a separate disposal pipette tip for each specimen, Negative Control and Positive Control as to avoid cross-contamination.**

Step4 **Sample Incubation:** Cover the plate with the plate cover and incubate for **30 minutes at 37°C**. It is recommended to use water tank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step5 **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.

Step6 **Adding Enzyme Conjugate:** Add **100 µL** of Enzyme Conjugate Reagent into each well except for the Blank.

Step7 **Incubating Enzyme Conjugate:** Cover the plate with the plate cover and incubate for **30 minutes at 37°C**.

Step8 **Washing:** Remove and discard the plate cover. Aspirate the liquid and rinse each well **5times** with Wash buffer (as step 5). After the final washing cycle, turn the strip plate and tap out any remainders.

Step9 **Coloring:** Add **50 µL of Substrate Solution A and after that 50 µL 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 Enzyme Conjugate produces blue color in Positive control and anti-HBc IgM Positive sample wells.

Step10 **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 anti-HBc IgM Positive sample wells.

Step11 **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at **450 nm**. If a dual filter instrument is used, set the reference wavelength at **630 nm**. Calculate the Cut-off value and evaluate the results.
Note: read the absorbance within **5** minutes after stopping the reaction.

INTERPRETATION OF RESULTS AND QUALITY CONTROL

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.

Example:
1. Calculation of Nc:

Well No	B1	C1	D
Negative controls OD value	0.02	0.012	0.016

Nc=0.016 (Nc is lower than 0.05 so take it as 0.05)
2. Calculation of Cut-off value (C.O.)= 0.05 x 2.1= 0.105

If one of the Negative control values does not meet the Quality Control Range specifications, it should be discarded, and the mean value is calculated again using the remaining two values. If more than one control OD value does not meet the Quality control range specifications, the test is invalid and must be repeated.

Quality control range

The test results are valid if the Quality Control criteria are verified. 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 Chromogens 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 450/630 nm or at 450nm after blanking.

3. The absorbance value OD of the Negative control must be less than 0.100 at 450/630 nm or at 450nm after blanking.

Interpretations of the results
(S = the individual absorbance (OD) of each specimen)

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 hepatitis B core antigen have been detected with this anti-HBc IgM ELISA kit. Therefore, there are no evidences for resent infections with HBV and the patients is probably not infected with HBV

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 hepatitis B core antigen have probably been detected with this anti-HBc IgM ELISA kit. Any reactive samples must be retested in duplicates. Repeatedly reactive samples can be considered positive for anti-HBc IgM. Positive results with anti-HBc IgM detection indicate possible recent infection with HBV.

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 recommended. Repeatedly positive samples can be considered positive for anti-HBc IgM.
The result from this assay should not be used alone to establish the infection state.

TEST PERFORMANCE AND EXPECTED RESULTS

Analytical Endpoint Sensitivity: 26PEI U/ml

The **clinical specificity** of this assay has been determined by a panel of samples obtained from 2500 healthy blood donors and 230 undiagnosed hospitalized patients. The repeatedly reactive samples and samples confirmed positive with the reference test were not included in the calculation of specificity.

The **clinical sensitivity** of this anti-HBc IgM ELISA kit has been calculated by a panel of samples obtained from 548 hepatitis B patients with well-characterized clinical history based upon reference assays for detection of HBsAg, HBeAg, anti-HBs, anti-HBe, and anti-HBc. This panel included samples from acute, chronic and recovered hepatitis B patients. Licensed anti-HBc IgM ELISA test was used as a confirmatory assay. The evaluation results are given below. Results obtained in individual laboratories may differ.

Specificity	Samples	-	+	Confirmed positive	Specificity	False positive
Blood donors	2500	2492	8	5	99.87%	3
Hospitalized patients	230	210	20	20	100%	0
TOTAL	**2730**	**2702**	**28**	**25**	**99.93%**	**3**
Sensitivity	Samples	-	+	Confirmed positive	**Sensitivity**	False negative
Acute	318	3	314	315	99.68%	1
Chronic	128	110	18	18	100%	0
TOTAL	**446**	**113**	**332**	**333**	**99.84%**	**1**
Recovery	102	101	1	1	100%	0

Marker prevalence in follow up of patients infected with HBV:

Days since infection	Number of samples	+	-	Detected prevalence of anti-HBc IgM
0	10	2	8	20%
1-10	12	3	8	25%
11-20	13	4	7	30%
21-30	9	8	1	88%
31-50	9	9	0	100%
51-70	14	14	14	100%
71-100	11	11	11	100%
101-120	8	8	8	100%
121-150	3	3	3	100%
151-170	2	1	1	50%
171-200	1	0	1	0%
Total:	92	63	29	73.66%

Analytical Specificity:

No cross reactivity observed with samples from patients infected with HAV, HCV, HIV, CMV, TP, and HTLV.

No interferences from rheumatoid factors up to 2000 U/mL were observed during clinical testing. The assay performance characteristics are unaffected from elevated concentrations of bilirubin, hemoglobin, and triolein.

Reproducibility		Within run		Between run	
Specimen Type	N0.	Mean OD	CV%	Mean OD	CV%
Weak positive	10	0.352	8.1%	0.302	8.5%
Moderate positive	10	0.884	7.3%	0.805	7.6%
Strong positive	10	1.821	4.6%	1.783	5.1%
Positive control	10	2.0	4.3%	1.958	4.4%

LIMITATIONS

1. Non-repeatable reactive results may be obtained with any ELISA test due to the general characteristics of this type of assays. A negative result with an antibody detection test does not preclude the possibility of infection. Antibodies may be undetectable during the early stages of the disease and in some immunosuppresed individuals.
2. Any positive results must be interpreted in conjunction with the patient clinical information and other laboratory results.
3. Common sources for mistakes: kits beyond the expiry date, bad washing procedures and wrong washing buffer concentration, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
4. The prevalence of the marker will affect the assay's predictive values.
5. False negative results can occur from inhibition of specific IgM in the presence of high titers of specific IgG. The removal of IgG can be helpful to prevent false negative results and methods for this are given elsewhere.

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ELISA ENZYME **L**INKED **I**MMUNO**S**ORBENT **A**SSAY

Microwell Method

HBcAb

REF Z00364

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 antibodies to Hepatitis B core antigen (HBcAg) in human serum or plasma.

Microwell Method - 96 wells

(12 x 8-well antigen coated strips

Individual breakaway)

INTRODUCTION

Hepatitis B core Antigen (or HBcAg) is the major component of the core particles of Hepatitis B virus (or HBV). Particles have a size of 27 nm and contain a circular double-stranded DNA molecule, a specific DNA-polymerase and HBeAg; HBcAg is composed of a single polypeptide of about 17 kD that is released upon dis-aggregation of the core particles; the antigen contains at least one immunological determinant. Upon primary infection, anti HBcAg antibodies are one of the first markers of HBV hepatitis appearing in the serum of the patient, together or slightly later than HBsAg, the viral surface antigen. Anti HBcAg antibodies are produced usually at high titers and their presence is detectable even years after infection. Isolated HBcAb, in absence of other HBV markers, have been observed in blood units, suggesting the use of this test for screening HBV, in addition of HBsAg. The determination of HBcAb has become important for the classification of the viral agent, together with the detection of the other markers of HBV infection, in sera and plasma.

PRINCIPLE OF THE ASSAY

This anti-HBc ELISA kit is based on solid phase, one step incubation competitive principle ELISA. Anti-HBc if present in the sample competes with monoclonal anti-HBc conjugated to horseradish peroxidase (HRP) for a fixed amount of purified HBcAg pre-coated in the wells. When no anti-HBc is present in the sample, the HRP labeled anti-HBc will be bound with the antigens inside the wells and any unbound HRP-Conjugate is removed during washing. Chromogen B and A solutions are added into the wells and during incubation 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. No or low color developing suggests the presence of antibodies to HBcAg in the sample.

Assay principle scheme: Competition ELISA

Ag(p)	**+**	**Ab(s)+(Ab)ENZ**	**→**	**[Ag(p)–Ab(s)]**	**→**	**No color**	**(+)**
Ag(p	**+**	**(Ab)ENZ**	**→**	**[Ag(p) – (Ab)ENZ]**	**→ Blue →**	**Yellow Color**	**(-)**
Incubation				**Immobilized Complex**		**Coloring**	**Results**
60 min.					**15 min.**		

Ag(p)–pre-coated HBcAg;
Ab(s)– anti-HBc in sample;
(Ab)ENZ– HRP conjugated anti-HBc ;

TEST CONDITIONS AND NOTICES

1. All the reagents contained in the kit are for “in vitro” diagnostic use only.
2. Do not use the kit or reagents after the expiry date stated on labels. Do not mix reagents of different lots.
3. Procedures should be performed carefully in order to obtain reliable results and clinical interpretations.
4. Bring all the reagents to room temperature for at least 60 min, before the test is started.
5. Avoid any contamination of reagents when taking them out of vials. We recommend use of automatic pipettes and disposable tips. When dispensing reagents, do not touch the wall of microplate wells with tips, in order to avoid any cross-contamination.
6. In the washing procedure, use only the Wash Buffer provided with the kit and follow carefully the indications reported in the “WASHING INSTRUCTIONS” section of this insert.

7. Ensure that the Substrate A/B mixture does not come in contact with oxidizing agents or metallic surfaces; avoid any intense light exposure during the incubation step or the reagent preparation.
8. Samples and materials potentially infective have to be handled with care as they could transmit infection.

   All objects come in direct contact with samples and all residuals of the assay should be treated or wasted as potentially infective. Best procedures for inactivation are treatments with autoclave at 121°C for 30 min or with sodium hypochlorite at a final concentration of 2.5% for 24 hrs. This last method can be used for the treatment of the liquid waste after that it has been neutralized with NaOH.
9. Avoid any contact of liquids with skin and mucosas.
   Use always protective talk-free gloves, glasses and laboratory coats, according to the safety regulations.

CONTENT OF THE KIT

Microwell Plate	Blank microwell strips fixed on a white strip holder. The plate is sealed in aluminum pouch with desiccant. 12×8-well strips per plate. Each well contains purified HBcAg. The microwell strips can be broken to be used separately. Place unused wells in the plastic sealable storage bag together with the desiccant and return to 2-8°C.
Enzyme Conjugate	6.5 mL per vial. Horseradish peroxidase-conjugated anti-HBc. Ready to use as supplied. Once open, stable for one month at 2-8°C.
Wash Buffer	30 mL per bottle, pH 7.4, 20x PBS (containing Tween-20 as a detergent). The concentrate must be diluted 1:20 with distilled or deionized water before use. Once diluted, stable for one week at room temperature or for two weeks at 2-8°C. **DILUTE BEFORE USE!**
Substrate Solution A	7 mL per vial. Urea peroxide solution. Ready to use as supplied. Once open, stable for one month at 2-8°C.
Substrate Solution B	7 mL per vial. TMB solution- Tetramethylbenzidine dissolved in citric acid. Ready to use as supplied. Once open, stable for one month at 2-8°C.
Stop Solution	7 mL per bottle. Diluted sulfuric acid solution (0.5 M H_2SO_4). Ready to use as supplied.
Negative Control	1 mL per vial. Protein-stabilized buffer tested non-reactive for anti-HBc. Preservatives: 0.1% ProClin 300. Ready to use as supplied. Once open, stable for one month at 2-8°C.
Positive Control	1 mL per vial. Purified anti-HBc 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.
Cardboard Sealer	1 piece. To cover the plates during incubation and to prevent the well from evaporation or contamination.

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 anti-HBc ELISA kit, during storage protect the reagents from contamination with microorganism or chemicals.

MATERIALS 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. Microshaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450 nm or dual wavelength 450 nm and 630 nm.
10. Microwell aspiration/wash system.

SPECIMEN COLLECTION, TRANSPORTATION AND STORAGE

1. Sample Collection: Either fresh serum or plasma samples can be used for this assay. Blood collected by venipuncture should be allowed to clot naturally and completely. 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 (rounds per minute) for 20 minutes at room temperature or by filtration on 0.22 µm filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but highly lipaemic, icteric, or hemolysed samples should not be used as they can give false results in the assay. Do not heat inactivate samples. This can cause sample deterioration.
2. Transportation and Storage: Store samples at 2-8°C. Samples not required for assaying 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 Enzyme 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 5times. 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 30 mL of the concentrate with 570 mL of water for a final volume of 600 mL diluted Wash Buffer. If less than a whole plate is used, prepare the proportional volume of solution.

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. 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. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.
5. 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. Never pipette solutions by mouth.
9. The use of automatic pipettes and disposable tips is recommended.
10. Assure that the incubation temperature is 37°C inside the incubator.
11. When adding samples avoid touching the well's bottom with the pipette tip.
12. When reading the absorbance with a plate reader, it is recommended to determine the absorbance at 450 nm or at 450 nm with reference at 630 nm.
13. All specimens from human origin should be considered as potentially infectious. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke or apply cosmetics in the assay laboratory.
14. The pipette tips, vials, strips and sample containers should be collected and autoclaved for 1 hour at 121°C or treated with 10% sodium hypochlorite for 30 minutes to decontaminate before any further steps for disposal.

15. The Stop Solution contains 0.5 M H_2SO_4. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.
16. The enzymatic activity of the Enzyme Conjugate might be affected from dust, reactive chemical and substances like sodium hypochlorite, acids, alkalins etc. Do not perform the assay in the presence of such substances.

ASSAY 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 stock Wash Buffer 1:20 with distilled or deionized water. Use only clean vessels to dilute the buffer.

Step 2 Numbering Wells: Set the strips needed in strip-holder and number sufficient number of wells including three Negative Controls (e.g. B1, C1, D1) two Positive Controls (e.g. E1, F1) and one Blank (e.g. A1 neither samples nor Enzyme Conjugate should be added into the Blank well). Use only number of strips required for the test.

Step 3 Adding Sample and Enzyme Conjugate: Add 50 µL of Positive Control, Negative Control, and Specimen into their respective wells. Note: Use a separate disposal pipette tip for each specimen, Negative Control and Positive Control to avoid cross-contamination. Add 50 µL of Enzyme Conjugate to each well except the Blank and mix by tapping the plate gently.

Step 4 Incubating: Cover the plate with the plate cover and incubate for 60 minutes at 37°C. It is recommended to use water tank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step 5 Washing: At the end of the incubation, remove and discard the plate cover. Wash each well 5 times with diluted Wash 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 Coloring: Dispense 50 µL of Substrate Solution A and after that 50 µL Substrate Solution B into each well including the Blank. Incubate the plate at 37°C for 15minutes, avoiding light. The enzymatic reaction between the Substrate Solutions and the Enzyme Conjugate will produce blue color in Negative Control and anti-HBc negative sample wells.

Step 7 Stopping Reaction: Using a multichannel pipette or manually add 50 µL Stop Solution into each well and mix gently. Intensive yellow color develops in Negative control and anti-HBc negative sample wells.

Step 8 Measuring the Absorbance: Calibrate the plate reader with the Blank well and read the absorbance at 450 nm. If a dual filter instrument is used, set the reference wavelength at 630 nm. Calculate the Cut-off value and evaluate the results. (Note: read the absorbance within 5 minutes after stopping the reaction).

INTERPRETATION OF RESULTS AND QUALITY CONTROL

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 × 0.5
*Nc = the mean absorbance value for three negative controls.

If one of the Negative control values does not meet the Quality Control Range specifications, it should be discarded and the mean value is calculated again using the remaining two values. If more than one control OD value does not meet the Quality control range specifications, the test is invalid and must be repeated.

Example: of Cut-off calculation:
1. Calculation of Nc

Well No:	B1	C1	D1
Negative controls OD value	1.720	1.715	1.717

Nc=1.717
2. Calculation of Cut-off (C.O.)= 1.729 × 0.5 =0.858

QUALITY CONTROL RANGE

The test results are valid if the Quality Control criteria are verified. 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 Chromogens and Stop solution, is less than 0.080 at 450 nm.

2. The absorbance value OD of the Negative control must be equal to or greater than 0.800 at 450/630 nm or at 450 nm after blanking.

3. The absorbance value OD of the Positive control must be less than 0.100 at 450/630 nm or at 450 nm after blanking .

INTERPRETATIONS OF THE RESULTS

(S = the individual absorbance (OD) of each specimen)

Negative Results (S/C.O.>1):

Samples giving an absorbance greater than the Cut-off value are considered negative, which indicates that no antibodies to HBV core antigen have been detected using this anti-HBc ELISA kit. This result should not be used alone to establish the infection state.

Positive Results (S/C.O.≤1):

Samples giving absorbance less than or equal to the Cut-off value are initially reactive for this assay, which indicates that antibodies to HBV core antigen have probably been detected with this anti-HBc ELISA kit. Any initially reactive samples must be retested in duplicates. Repeatedly reactive samples can be considered positive for anti-HBc. A positive result with anti-HBc detection is an indication of acute HBV infection. Monitoring of anti-HBc concentrations can be used in follow up of chronic HBV patients. However, any positive result should not be used alone to establish the infection state.

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 is recommended. Repeatedly reactive samples can be considered positive for anti-HBc.

TEST PERFORMANCE AND EXPECTED RESULTS

Analytical Endpoint Sensitivity: 0.8PEI U/mL

The **clinical specificity** of this assay has been determined by a panel of samples obtained from 1683 healthy blood donors and 145 undiagnosed hospitalized patients. The Repeatedly reactive samples and samples confirmed positive with the reference test were not included in the calculation of the specificity.

The **clinical sensitivity** of this anti-HBc ELISA kit have been calculated by a panel of samples obtained from 975 hepatitis B patients with well-characterized clinical history based upon reference assays for detection of HBsAg, HBeAg, anti-HBs, anti-HBe, and anti-HBc. This panel included samples from acute, chronic and recovered hepatitis B patients. Licensed anti-HBc ELISA test was used as a confirmatory assay. The evaluation results are given below. Results obtained in individual laboratories may differ.

Specificity	Samples	-	+	Confirmed positive	**Specificity**	False Positive
Blood donors	1683	566	1117	1115	99.64%	2
Hospitalized patients	145	80	65	65	100%	0
TOTAL	**1828**	**646**	**1182**	**1180**	**99.82**	**2**

Sensitivity	Samples	-	+	Confirmed positive	**Sensitivity**	False Negative
Acute	429	11	417	418	99.76%	1
Chronic	105	0	105	105	100%	0
Recovery	441	5	436	436	100%	0
TOTAL	**975**	**16**	**958**	**959**	**99.92**	**1**

Analytical Specificity:

1. No cross reactivity observed with samples from patients infected with HAV, HCV HIV, CMV, and TP.
2. No interference from rheumatoid factors up to 2000U/mL observed during clinical testing.
3. The assay performance characteristics are unaffected from elevated concentrations of bilirubin, hemoglobin, and triolein.
4. Frozen specimens have been tested to check for interferences due to collection and storage.

Reproducibility	No runs	**Within run**		**Between run**	
		Mean OD	CV%	Mean OD	CV%
Weak positive	10	0.639	5.8%	0.645	6.4%
Moderate positive	10	0.394	7.4%	0.404	8.0%
Strong positive	10	0.012	21%	0.017	22%
Negative control	10	1.768	4.5%	1.702	4.6%

LIMITATIONS

1. Non-repeatable positive result may occur due to the general biological and biochemical characteristics of ELISA assays. The test is designed to achieve very high performance characteristics of sensitivity and specificity. However, in very rare cases some HBV mutants or subtypes can remain undetectable. Antibodies may be undetectable during the early stages of the disease and in some immunosuppressed individuals.
2. Any positive results must be interpreted in conjunction with patient clinical information and other laboratory testing results.
3. Common sources for mistakes: kits beyond the expiry date, inappropriate washing procedures, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
4. The prevalence of the marker will affect the assay's predictive values.

VALIDITY

Please do not use this kit beyond the expiry date indicated on the kit box and reagent labels!

REFERENCES

1. Hansson, B.G. (1977). Persistence of Serum Antibody to Hepatitis B Core Antigen. J. Clin. Microbiol. 6, 209.
2. Hoofnagle, J.H., Gerety, R.J. and Barker, L.F. (1973). Antibody to Hepatitis B Virus Core in man. Lancet, 869.
3. Hoofnagle, J.H., Gerety, R.J., Ni, L.Y. and Barker, L.F. (1974).
4. Antibody to Hepatitis B Core Antigen. N. Engl. J. Med., 290, 1336
5. Mushahwar, I.K.,Dienstag,J .L.,Polesky,H.F et al (1981) Interpretation of Various Serological Profiles of Hepatitis B Virus Infection.Am J. Clin Pathol,76,773

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ELISA ENZYME **L**INKED **I**MMUNO**S**ORBENT **A**SSAY

Microwell Method

HBsAb

REF: Z00361

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 Antibodies to Hepatitis B surface antigen (HBsAb) in human serum or plasma. It is intended for use in medical laboratories for diagnosis and management of patients related to infection with hepatitis B virus.

Microwell Method - 96 wells

(12 x 8-well Antigen coated strips

Individual breakaway)

INTRODUCTION

Hepatitis B virus (HBV) is an enveloped, double-stranded DNA virus belonging to the Hepadnaviridae family and is recognized as the major cause of blood transmitted hepatitis together with hepatitis C virus (HCV). Infection with HBV induces a spectrum of clinical manifestations ranging from mild, unapparent disease to fulminant hepatitis, severe chronic liver diseases, which in some cases can lead to cirrhosis and carcinoma of the liver. Classification of a hepatitis B infection requires the identification of several serological markers expressed during three phases (incubation, acute and convalescent) of the infection. Now several diagnostic tests are used for screening, clinical diagnosis and management of the disease.
Hepatitis B surface antigen (HBsAg), which appears shortly after infection, is an important protein of the envelope structure of the virus. HBsAg is a key serological marker for detection and diagnosis of HBV and is detectable in blood during the acute phase of the disease. Clearance after treatment shows recovery while presence for more than half year after infection indicates possible progression to long chronic carrier stage. During the acute phase of the infection, strong immunological response develops and increasing titers of HBsAg neutralizing antibodies (anti-HBs) are marker for recovery. The serological detection of anti-HBs has become important method for the follow up of patients infected by HBV, prospective prevalence studies, and the monitoring of recipients upon vaccination with synthetic and natural HBsAg based vaccines.

PRINCIPLE OF THE ASSAY

For detection of anti-HBs, this kit uses antigen “sandwich” ELISA method where polystyrene microwell strips are pre-coated with recombinant HBsAg. Patient’s serum or plasma sample is added to the microwells together with a second HBsAg conjugated to Horseradish Peroxidase (Enzyme Conjugate). In case of presence of anti-HBs in the sample, the pre-coated and conjugated antigens will be bound to the two variable domains of the antibody and during incubation, the specific immunocomplex formed is captured on the solid phase. After washing to remove sample serum proteins and unbound Enzyme Conjugates, Substrate Solutions containing Tetramethylbenzidine (TMB) and urea peroxide are added to the wells. In presence of the antigen-antibody-antigen (HRP) “sandwich” complex, the colorless Substrate Solutions are hydrolyzed by the bound Enzyme Conjugate to a blue-colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color intensity can be measured and is proportional to the amount of antibody captured in the wells, and to the sample respectively. Wells containing samples negative for anti-HBs remain colorless.

CONTENT OF THE KIT

Microwell Plate	Blank microwell strips fixed on white strip holder. The plate is sealed in aluminum pouch with desiccant. Each well contains recombinant HBsAg. 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 opened, stable for 4 weeks at 2-8°C.
Negative Control	1x 1 mL, yellowish liquid filled in a vial with green screw cap. Protein-stabilized buffer tested non-reactive for anti-HBs. Preservative: 0.1 % ProClin™ 300. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Positive Control	1x 1 mL, Red-colored liquid filled in a vial with red screw cap. anti-HBs diluted in protein-stabilized buffer. Preservative: 0.1 % ProClin™ 300. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Enzyme Conjugate	1x 6.5 mL, red-colored liquid in a white vial with red screw cap. Horseradish peroxidase-conjugated HBsAg. Preservative: 0.1 % ProClin™ 300. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Wash Buffer	1x 30 mL, Colorless liquid filled in a white bottle with white screw

	cap. Buffer solution containing surfactant. Detergent: Tween-20. The concentrate must be diluted **1 to 20** with distilled/deionized water before use. Once diluted, stable for 1 week at room temperature, or for 2 weeks when stored at 2-8°C.
Substrate Solution A	1x 7 mL, colorless liquid filled in a white vial with green screw cap. Urea peroxide solution. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Substrate Solution B	1x 7 mL, colorless liquid filled in a black vial with black screw cap. TMB (Tetramethyl benzidine), N,N- dimethylformamide. Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.
Stop Solution	1x 7 mL, colorless liquid in a white vial with white screw cap. Diluted sulfuric acid solution (0.5 M H_2SO_4). Ready to use as supplied. Once opened, stable for 4 weeks at 2-8°C.

MATERIALS REQUIRED BUT NOT PROVIDED

- Freshly distilled or deionized water
- Disposable gloves and timer
- Appropriate waste containers for potentially contaminated materials
- Dispensing system and/or pipette
- Disposable pipette tips
- Absorbent tissue or clean towel
- Dry incubator or water bath, 37±1°C
- Plate reader, single wavelength 450 nm or dual wavelength 450/600-650 nm
- Microwell aspiration/wash system

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 ELISA, protect the reagents from contamination with microorganism or chemicals during storage.

SAMPLE COLLECTION

1. Specimen 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 as to avoid haemolysis of the RBC. Care should be taken to ensure that the serum specimens are clear and not contaminated by microorganisms. Any visible particulate matters in the specimen 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 or 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. This ELISA is intended ONLY for testing of individual serum or plasma samples. Do not use the assay for testing of cadaver samples, saliva, urine or other body fluids, or pooled (mixed) blood.
4. Transportation and Storage: Store specimens at 2-8°C. Specimens not required for assaying within 1 week should be stored frozen (-20°C or lower). Multiple freeze-thaw cycles should be avoided. For shipment, samples should be packaged and labeled in accordance with the existing local and international regulations for transportation of clinical samples and ethological agents.

PRECAUTIONS AND SAFETY

TO BE USED ONLY BY QUALIFIED PROFESSIONALS.
The ELISA assays are 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 their expiry date stated on labels or boxes.
3. CAUTION - CRITICAL STEP: Allow the reagents and specimens to reach room temperature (18-30°C) before use. Shake reagent gently before use. Return at 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 in low sensitivity of the assay.
5. Do not touch the exterior bottom of the wells; fingerprints or scratches may interfere with the 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 long time interruptions of assay steps. Assure same working conditions for all wells.
8. Calibrate the pipette frequently to assure the accuracy of samples/reagents dispensing. 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 specimens, do not touch the well's bottom with the pipette tip.
11. When measuring with a plate reader, determine the absorbance at 450 nm or at 450/600-650 nm.
12. The enzymatic activity of the Enzyme 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 tests kits with accepted performance and found negative for HBsAg and antibodies to HIV 1/2, HCV, TP. 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 derived sera have been used for stabilizing of the positive and negative controls. 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. Materials Safety Data Sheet (MSDS) 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 solution, the Chromogens, and the 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 come into contact with the skin or eyes.
21. ProClin^{TM}300 0.1% used as preservative, can cause sensation of the skin. Wipe up spills immediately or wash with water if come into contact with the 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 with new one or contact Wantai technical support for further assistance.

WASHING INSTRUCTIONS

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 Enzyme 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 30 mL of the concentrate with 570 mL of water for a final volume of 600 mL diluted Wash Buffer. If less than a whole plate is used, prepare the proportional volume of solution.

ASSAY 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 stock Wash Buffer **1:20** with distilled or deionized water as indicated in the instructions for washing. Use only clean vessels to dilute the buffer.

Step 2 **Numbering Wells:** Set the strips needed in strip-holder and number sufficient number of wells including three Negative controls **(e.g. B1, C1, D1)**, two Positive controls **(e.g. E1, F1)** and one Blank (**e.g. A1**, neither samples nor Enzyme Conjugate should be added into the Blank well). Use only the number of strips required for the test.

Step 3 **Adding Sample and Enzyme Conjugate:** Add **50 µL** of Positive control, Negative control, and Specimen into their respective wells. **Note: Use a separate disposal pipette tip for each specimen, Negative Control and Positive Control as to avoid cross-contamination.** Add **50 µL Enzyme Conjugate** to each well except the Blank and mix by tapping the plate gently.

Step 4 **Incubating:** Cover the plate with the plate cover and incubate for **30 minutes at 37°C**. It is recommended to use water tank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step 5 **Washing:** At the end of the incubation, remove and discard the plate cover. Wash each well **5** times with diluted Wash 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 **Coloring:** Dispense **50 µL of Substrate Solution A and after that 50 µL of Substrate Solution B** into each well including the **Blank**, and mix by tapping the plate gently. Incubate the plate at **37°C for 15 minutes avoiding light**. The enzymatic reaction

between the Substrate Solutions and the Enzyme Conjugate produces blue color in Positive control and in anti-HBs Positive sample wells.

Step 7 **Stopping Reaction:** Using a multichannel pipette or manually add **50 µL** Stop Solution into each well. Intensive yellow color develops in Positive control and anti-HBs Positive sample wells.

Step 8 **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at **450 nm**. If a dual filter instrument is used, set the reference wavelength at **630 nm**. Calculate the Cut-off value and evaluate the results. (**Note:** read the absorbance within **5 minutes** after stopping the reaction)

QUALITY CONTROL AND CALCULATION OF 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:
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.

- The A value of the Blank well, which contains only Chromogen and Stop solution, is < 0.080 at 450 nm.
- The A values of the Positive Control must be ≥ 0.800 at 450/600~650nm or at 450nm after blanking.
- The A values of the Negative Control must be ≤ 0.100 at 450/600~650nm or at 450nm after blanking.

If one of the Negative Control A values does not meet the Quality Control criteria, it should be discarded and the mean value calculated again using the remaining two values. If more than one Negative control A values do not meet the Quality Control Range specifications, the test is invalid and must be repeated.

INTERPRETATIONS OF THE RESULTS

(S = the individual absorbance (OD) of each specimen)

Negative Results (**S/C.O.<1):** samples giving absorbance less than the Cut-off value are negative for this assay, which indicates that no antibodies to hepatitis B virus surface antigen have been detected with this anti-HBs ELISA kit. Therefore, there are no indications for past infection and the individual is not immune to infection with HBV.

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 antibodies to HBV surfaces antigen have been detected using this anti-HBs ELISA kit. Retesting in duplicates of any reactive samples is recommended. Repeatedly reactive samples can be considered positive for anti-HBs. Elevated concentrations of anti-HBs are indication for recovery and immunity to HBV.

Borderline (S/C.O.=0.9-1.1): samples with absorbance to Cut-off ratio between 0.9 and 1.1 are considered borderline and retesting of these samples in duplicates is recommended to confirm the results. Repeatedly positive samples can be considered positive for antibodies to HBsAg.

Follow-up, confirmation and supplementary testing of any positive specimen with other analytical system is required. Clinical diagnosis should not be established based on a single test result. It should integrate clinical and other laboratory data and findings.

TEST PERFORMANCE AND EXPECTED VALUES

Analytical Endpoint Sensitivity (lower detection limits)**:** This assay shows sensitivity near the Cut-off of 5 mIU/mL.

Clinical Specificity: The clinical specificity of the assay has been determined by a panel of samples obtained from 1500 healthy blood donors and 250 undiagnosed hospitalized patients.

Specificity	Samples			True positive	Specificity	False positive
	No.	-	+			
Blood Donors	1500	869	631	630	99.89%	1
Hospitalized Patients	250	140	110	109	99.29%	1
TOTAL	1750	1009	741	739	99.80%	2

Clinical Sensitivity: The clinical sensitivity of the assay has been calculated by a panel of samples obtained from 580 hepatitis B patients with well-characterized clinical history based upon reference assays for detection of HBsAg, HBeAg, anti-HBs, anti-HBe, and anti-HBc. Licensed anti-HBs ELISA test was applied as a confirmatory assay. For establishing the test performance characteristics during monitoring of post-vaccination antibody response, additional group of samples from 200 individuals receiving HBV vaccine was tested for anti-HBs.

Sensitivity	Samples			True positive	Sensitivity	False negatives
	No.	-	+			
Acute	350	345	5	5	100%	0
Chronic	130	130	0	0	100%	0
Recovery	100	5	95	95	100%	0
Vaccine recipients	200	7	193	193	100%	0
TOTAL	780	486	293	293	100%	0

Analytical Specificity:

1. No cross reactivity observed with samples from patients infected with HAV, HCV, HIV, CMV, TP.
2. No interference was observed from rheumatoid factors up to 2000 U/mL.
3. The assay performance characteristics are unaffected from elevated concentrations of bilirubin, hemoglobin, and triolein.
4. No high dose hook effect up to 150,000 mIU/mL.
5. Frozen specimens have been tested too 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. In very rare cases some HBV mutants or subtypes can remain undetectable. A negative result with an antibody detection test does not preclude the possibility of infection.
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 the several reasons, most of which are related but not limited to inadequate washing step.

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 concentration.

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