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BIOHBV Kit

Kit for Hepatitis B Virus DNA detection (HBV) in human clinical samples

Instructions for Use

Ref. 90.053

PLEASE READ THE INSTRUCTIONS FOR USE THOROUGHLY BEFORE USING THE KIT, ESPECIALLY IF YOU ARE NOT FAMILIAR WITH THE PROTOCOL

BIOHBV Kit

For research use only (RUO)
Not for use in diagnostic procedures
Test results may be used for preliminary analysis only

Some of the applications which may be performed with this product may be covered by applicable patents in certain countries. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application. Biotools does not encourage the unlicensed use of patented applications.

PLEASE CHECK INTEGRITY OF KIT AND REAGENTS BEFORE USE. DETERIORATED KITS MAY CAUSE EQUIVOCAL RESULTS.

1. INTENDED USE

The BIOHBV Kit is a method for the qualitative determination of Hepatitis B Virus (HBV) in clinical samples by DNA amplification. The Kit uses a semi-nested amplification in one single reaction, achieving a high sensitivity and specificity. The kit has been tested with samples from European, Asian, African and Middle East origin.

The BIOHBV Kit is to be used with the following clinical samples:

- Blood samples stored in EDTA. Use of blood samples with heparine or citric acid has not been tested, and therefore, the kit may not render the expected results.
- Serum / plasma samples stored in heparine. Use of serum / plasma samples in other conditions has not been tested, and therefore, the kit may not render the expected results.
- PBMC samples stored at or bellow –20ºC. For prolonged storages of PBMC storage at –80ºC or in liquid nitrogen is recommended.
- Liver biopsies. For the use of the Kit with biopsies do not treat the tissue with acetic acid or iodide Paraffin-embedded tissues can be used provided the employed fixative reagents do not degrade or interact with DNA and deparaffination step is performed.

2. SUMMARY AND EXPLANATION OF THE TEST

Hepatitis B virus (HBV) is one of the main pathogens causing hepatitis in humans, being one of the most important health problems worldwide. It is estimated that 350 million individuals are chronically infected with HBV, and that 1 to 2 % will die each year from complications associated with the infection, the majority of these deaths occurring from cirrhosis of the liver and hepatocellular carcinoma[1].

Traditional diagnosis methods are based on serology, using immunological methods. Diagnosis based on antibodies does not generally differentiate between past and present infections. Viral antigen-based diagnosis is widely used as screening method. However, patients at early stages of the disease, or infected by mutant serotypes of the virus, can render false negative results, due to the lack of detectable antigen in the analysed sample[2].

DNA-based techniques allow early detection of the viral presence in the patient, as well as detection of most mutant serotypes. They can detect viral presence in the window period, or at minute levels of virus, so that they can be used for early diagnosis as well as treatment monitoring.

The BIOHBV Kit allows the detection of hepatitis B virus DNA in a wide variety of clinical samples (whole blood, serum, plasma, PBMCs, hepatic tissues, etc.).

3. PRINCIPLES OF THE PROCEDURE

The BIOHBV Kit is a test for qualitative analysis, using a DNA amplification technique for direct detection of DNA through agarose electrophoresis and ethidium bromide staining. Viral DNA present in positive samples is specifically amplified by using specific primers, which hybridise with homologous sequences in the viral genome.

[1] Allen *et al* (1999). J Clin Microbiol, 37 (10): 3338-3347.
[2] Van Deursen *et al.* (1998). J Clin Pathol, 51: 149-153.

The kit consists of a semi-nested amplification reaction, using three primers. One pair of primers (Pair 1 – S1/S2) hybridise with sequences common to HBV isolates known and tested so far. A third primer (S3) hybridises with the amplification product from S1 and S2, allowing an increase in the sensitivity and specificity of the detection reaction.

The first pair of primers renders a non-detectable product, except in samples with a high viraemia. The final product must render a 186 bp band. For band analysis, agarose electrophoresis must be performed, followed by ethidium bromide staining.

The detection process using BIOHBV Kit consists of three main steps: sample preparation, viral DNA amplification and detection by agarose electrophoresis and ethidium bromide staining.

A) Sample Preparation

The BIOHBV Kit is used with DNA purified from clinical samples. For viral DNA purification, **BIOTOOLS recommends the use of Speedtools DNA Extraction Kit** (Ref. 21.130/1/2) or **Speedtools Tissue DNA Extraction Kit** (Ref. 21.135/6/7) though other methods guaranteeing $A_{260/280}$=1.8 – 2.0, 50-100 ng/µl concentration and the absence of amplification inhibitors can also be employed.

Check the quality and suitability of the purified DNA for amplification reactions, for example, by performing control amplifications in parallel. For further information, please contact our Technical Dpt. (info@biotools.eu).

B) Amplification and Detection

Target selection

Selection of the target HBV sequences has been based on the study of highly conserved regions in the HBV genome (pre-S region). Selected regions have a high degree of conservation between the tested HBV variants.

Amplification

DNA amplification is performed with a thermostable DNA polymerase from *Thermus spp*. In the presence of magnesium, and with the suitable salt and ionic strenght conditions, the enzyme shows DNA polymerisation activity using as anchor a primer and a DNA molecule as template.

DNA purified from the sample to be analysed is added to the reaction mixture prepared with the reagents provided by the Kit, and containing all necessary reagents to perform the amplification reaction. Reaction mixture, already containing DNA, is incubated at different temperatures in order to allow the hybridisation of the primers to the DNA from the sample. Once the hybridisation has taken place, and in the presence of triphosphate deoxynucleotides, the DNA polymerase extends the primer and forms a DNA strand complementary to the template DNA. Cyclic repetition of this process results in exponential amplification of the sequences originally present in the sample included between the primer pairs.

Detection

Detection of amplified products is performed by agarose gel electrophoresis followed by ethidium bromide staining.

NOTE

Ethidium bromide is a highly mutagenic intercalating agent. We recommend use of gloves and the maximum caution in handling.

HBV presence is indicated by a band of approximately 186 bp. For samples with a high viraemia an additional band of 275 bp may appear, this band does not interfere with the final result.

4. REAGENTS

The Kit contains amplification reagents in liquid format for performance of 96 amplification reactions (Ref. 90.053). **Store all Kit vials at -15±8ºC**. Thaw and handle reagents on ice. Do not freeze/thaw Kit vials repeatedly. In case of frequent use, we recommend the aliquoting of the vial contents.

- **HBV Mixture:** Two vials: 2 x 1980 µl
  A Tris-HCl solution, containing <10 % glycerol, KCl, <0.001 % dATP, dCTP, dGTP, dTTP and primers. HBV mixture includes all amplification reagents for the detection of HBV DNA, except $MgCl_2$ and DNA polymerase, in the adequate ratios.

- **50 mM $MgCl_2$ Solution:** Vial: 1.8 ml
  Mix well before use.

- **Biotools DNA polymerase (1 U/µl):** Vial: 105 µl
  Add to reaction mixtures shortly before introduction of vials in thermal cycler.

- **Positive Control:** Vial: 300 µl
  Non-infective positive control of HBV. It consists of amplified DNA product, containing a generic sequence from HCB, flanked by the primers. To be analysed in a separate amplification reaction. It can also be used as intra-tube control.

5. INSTRUCTIONS FOR USE

NOTE

Thaw and keep reagents on ice while in use. For optimal results keep reaction mixtures and vials in refrigerated conditions until their introduction into the thermal cycler.

Proceed to Reagent Preparation Area in a laminar flow cabinet

1.- Final reaction volume is 50 µl. Prepare the **Reaction Mixture** following the table bellow (Table 1) in a 1.5 ml vial, according to the number of reactions to be performed (Reaction Mixture = HBV Mixture + $MgCl_2$ + DNA Polymerase). For each round of analysis include at least one positive control and one negative control. To ensure sufficient volume for all reactions, prepare the Reaction Mixture for n+1 reactions.

Table 1. Reaction Mixture

REAGENT	For 1 Reaction
HBV Mixture	37.5 µl
50 mM $MgCl_2$ Solution	1.5 µl
Biotools DNA Polymerase (1U/µl)	1 µl

2.- Aliquot **40 µl** of the **Reaction Mixture** in each amplification vial.

Remove vials from laminar flow cabinet and proceed to DNA Purification Area. Never introduce DNA from samples or positive controls in the laminar flow cabinet at the reagent preparation area. The amplification reaction must start in the next 10 minutes after adding purified DNA from samples and controls to the reaction mixture.

3.- Add **50-100 ng DNA** from the purified samples to each reaction vial. **Complete up to 50 µl final volume with sterile bidistilled water**.

NOTE

Clinical samples, depending on their nature and viraemia levels, may have different DNA concentrations, and therefore, amount of template to be added to the amplification reaction is expressed in ng rather than indicating sample volume. Quantity and purity of template must be calculated, e.g. by measuring $A_{260/280}$ values.

4.- **Positive Controls** should be prepared by adding **5 µl of Positive Control vial** (provided by the Kit) + 5 µl of sterile bidistilled water to an amplification vial with 40 µl of reaction mixture. **Negative Controls**

should be prepared adding **10 µl of sterile bidistilled water** to an amplification vial with 40 µl of reaction mixture.

5.- Close amplification vials. Place them in thermal cycler. Store remaining of all reagents at -15±8ºC.

6.- Perform the amplification according to the following program:

Amplification Program	
INITIAL DENATURING	94ºC / 5 min
CYCLIC AMPLIFICATION	94ºC / 30 sec 55ºC / 30 sec 72ºC / 30 sec
NUMBER OF CYCLES	50
FINAL ELONGATION	72ºC / 7 min
	4 ºC / ∞

NOTE

This protocol has been adapted for Eppendorf, MJ Research and Applied Biosystems GeneAmp™ thermal cyclers. For other thermal cyclers, optimisation of reaction parameters may be necessary. For any question, please contact our Technical Dpt. (info@biotools.eu).

6. INTERPRETATION OF RESULTS

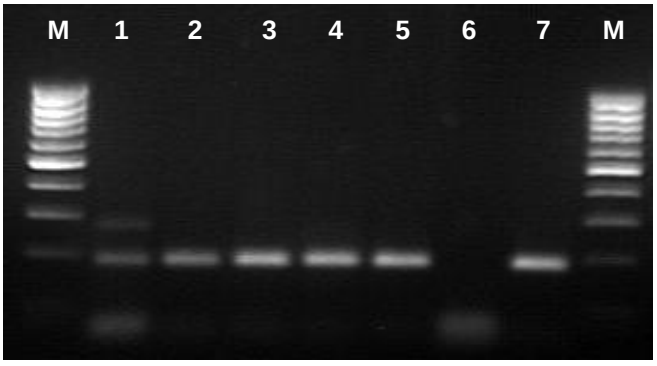
The analysis of amplification products is performed by horizontal electrophoresis in low EEO-agarose gels (e.g. MB Agarose, Ref. 20.011). Band visualisation is improved in 1.5-2 % gels using TAE 1X or TBE 0.5X as running buffers. It is recommended to add ethidium bromide in the agarose gel for a better resolution and visualisation.

NOTE

Ethidium bromide is a highly mutagenic intercalating agent. Use of gloves and maximum caution is recommended on handling this reagent.

Samples containing HBV sequences will render a band of 186 bp. For samples with a high viraemia an additional band of 277 bp may appear, This does not interfere with the results.

Figure 1: *Screening of serum samples from patients affected by hepatitis. Patient having a high HBV level (Lane 1); patients positives to HBV (Lanes 2 to 5); negative control (Lane 6); positive control (Lane 7); M: 100 bp Ladder (Ref. 31.006).*



7. MATERIALS REQUIRED BUT NOT PROVIDED

NOTE

For all equipments, regular maintenance and calibration is necessary. Follow manufacturer's instructions, and check working parameters regularly, specially for thermal cyclers and pipettes. Maintenance and calibration of instruments allows its correct functioning, and helps detecting problems that may render an incorrect analysis result.

Pre-amplification area (DNA purification and reagent preparation areas)

- Equipment, reagents and disposable material necessary for DNA purification (depending on the method, follow manufacturer's instructions)
- Timer
- Automatic pipettes[3] (10, 20 and 200 µl), filter or positive displacement tips, RNase-free[4]
- Disposable examination gloves, powder-free
- sterile bidistilled water
- Screw cap polypropylene tubes, 1.5 ml capacity, non siliconised, conical, sterile, RNase-free. It is recommended to use screw cap tubes, in order to avoid the potential contamination of samples and controls.
- Racks for 1.5 ml vials
- Containers for disposal of potentially-infectious material
- Disposable filter paper for working surface, cleaning paper for accidental spills
- Termi-DNA-Tor[5] or equivalent, in order to remove DNA from working surfaces

Amplification area

- Thermal cycler: Eppendorf MasterCycler™ Personal, MJ Research MiniCycler™ or Applied Biosystems GeneAmp™ 2700. Use of this kit in other equipments has not been tested. For further information, contact our Technical Dpt. (info@biotools.eu)
- Laminar flow cabinet
- Racks for reaction vials
- Reaction vials (0.2 ml, thin-walled)
- Sterile bidistilled water (Ref. 20.033 or equivalent)
- Automatic pipettes (10, 20 and 200 µl), filter or positive displacement tips, RNase-free
- Disposable examination gloves, powder-free
- Containers for disposal of potentially-infectious material
- Disposable filter paper for working surface, cleaning paper for accidental spills
- Termi-DNA-Tor or equivalent, in order to remove DNA from working surfaces

Post-amplification area

- Electrophoresis power supplies and tanks
- Gel Documentation system
- UV transilluminator
- Ethidium bromide
- Low EEO agarose (Ref. 20.011) or equivalent
- TAE or TBE
- DNA Ladder ranging between 150 to 700 bp (Ref. 31.006) or equivalent
- Electrophoresis loading buffer
- Automatic pipettes (10, 20 and 200 µl), filter or positive displacement tips, RNase-free
- Disposable examination gloves, powder-free
- Protective mask / googles for UV
- Microwave

8. WARNINGS AND PRECAUTIONS

Following is a list of warning and precautions. For more information, we recommend to read the Material Safety Data Sheet (MSDS), available in our webpage (www.biotools.eu), or by request to our Technical Dpt. (info@biotools.eu).

A. Research Use Only.
B. This test must be used with samples collected, handled and stored as indicated in the corresponding chapter. Efficiency of the test in other samples has not been tested.

[3] Precision of automatic pipettes must be in the range of 3 % of the indicated volume. If necessary, calibrate and check regularly, following manufacturer's instructions. It is recommended to use RNase-free filter tips and positive displacement tips, in order to avoid cross contamination between samples and amplicons.
[4] It is recommended to use different sets of pipettes for each reaction step (pre-amplification, amplification, post-amplification), in order to avoid contaminations that may render false positive results.
[5] Available in Biotools' catalogue (Ref. 22.001, 22.002).

C. The kit detects HBV DNA, and has been tested with different samples from different geographical origins (Europe, Middle East, Africa, Asia). Samples from other geographical areas have not been tested at the company laboratories. Some HBV isolates may present mutations at the primers' annealing sites, therefore affecting quality of the result. This applies for samples from geographical origins not tested in our laboratories, or for samples from the same geographical origin as those tested in our laboratories, but with mutations at the primers' annealing sites. The kit only detects presence/absence of HBV DNA, and is not intended for diagnosis or for quantification of viral loads.
D. Handle all samples and discarded material as infectious or potentially infectious.
E. Use powder-free examination gloves while handling reagents or samples, as well as lab coat. Wash hands thoroughly after performing the test.
F. Open and close reagent vials carefully. Observe temperature and light exposure instructions. After use, close vials and store at indicated temperatures.
G. All materials used with the kit, including reagents and samples, must be discarded as to inactivate all possible infectious agents
    1. **Solids**: autoclave
    2. **Liquids**: add sodium hypochloride[6] at a final concentration of 1 %, and incubate 30 minutes at room temperature before discarding any material.
H. Spills: wash spills with a 5 % solution of sodium hypochloride. Cover surface with absorbent material, saturated with a 5 % solution of sodium hypochloride. Let at least for 10 minutes. In order to avoid fume exposure, a plastic or glass cover can be used. All materials used for washing spills must be treated as infectious or potentially infectious material.
I. Do not use product after expiry or best before date.
J. Kit components have been tested as a whole. **Do not interchange components** with other kits, or components from different lots.
K. Nucleic acids are very sensitive to degradation by nucleases. Nucleases are present in human skin and surfaces that have been in contact with humans. Wash with Termi-DNA-Tor and cover working surfaces with suitable paper. Use powder-free examination gloves throughout the whole process.
L. Extreme care must be taken when aliquoting the different volumes in each reaction step. Mix well after addition of each reagent, unless otherwise noted. Read instructions for use of automatic pipettes.
M. Do not pipette by mouth.
N. Packaging material included with the kit is resistant to the indicated storage conditions. Storage at different conditions can cause breakage of the material, and possible contamination of kit contents.
O. Plastic material included with the kit is resistant in the normal conditions of use. Use of plastic material in extreme conditions may cause its breakage, and therefore, impossibility to use the kit.
P. Kit reagents, once used, must be discarded. Reagents cannot be reused once they have been used for the analysis of clinical samples, as this may cause false positive or false negative results.
Q. Laboratory workflow must be unidirectional, from pre-amplification area to post-amplification area. Specific equipment for each working area must be used, in order to avoid cross contaminations. Equipment used for amplification must remain in this area at all times.

9. STORAGE AND HANDLING INSTRUCTIONS

1. After reception, store the different reagents at the indicated temperatures at -15±8ºC. Use non frost-free freezers. Also, for frequent use (more than 1 time a week), aliquot the contents of the vials in different tubes, in order to avoid repeated freeze/thaw cycles.

2. Do not use the kit after expiry date. The closed kit is stable until the indicated date, if storage instructions are correctly followed. Do not mix reagents from other kits and/or other lots. If trace amounts of reagents remain, they must be discarded.

10. SAMPLE COLLECTION, TRANSPORT AND STORAGE

The different clinical samples recommended for use with the BIOHBV Kit are indicated at the beginning of this Manual. Samples taken with other methods or transported according to different specifications have not been tested for use with the kit. Biopsy samples can be used with the Kit but before applying acetic acid or iodide to the sample.

Samples stored in EDTA

Samples should be stored at 2-8 ºC and shipped at this temperature to the clinical analysis laboratory. For prolonged storages, we recommend freezing. Once in the laboratory, they can be stored at 2-8 ºC if analysis is to be performed in one week. If the analysis is to be performed later, store samples at -15±8ºC

[6] Commercial bleach usually contains sodium hypochloride at a concentration of 5 %. Bleach can be used, after performing the necessary calculations in order to achieve the indicated concentration.

or –70 ºC. Samples must be kept away from heat sources and preserved of environmental humidity. Bacterial growth must be avoided and DNA integrity must be kept.

Samples stored in heparine

Samples may be stored up to three days at 2-8 ºC, and shipped with refrigeration to the clinical analysis laboratory. For prolonged storages, we recommend temperatures of –20 ºC. Once in the laboratory, they should be stored at -15±8ºC or –70 ºC. Samples must be kept away from heat sources and preserved of environmental humidity. Bacterial growth must be avoided and DNA integrity must be kept.

Liver biopsies

Fresh biopsies of up to 5 mm must be used. Biopsy must be immediately placed in a preservative medium and stored at -15±8ºC. Biopsies can be shipped by express courier (24 hours maximum) at a maximum temperature of 25 ºC, and stored at -15±8ºC in the laboratory until performance of the test. Biopsies with a diametre under 2 mm must not be used.

Use of paraffin-embedded tissues is possible provided that tissue fixation method do not degrade DNA and purification of DNA is performed with methods specific for this kind of sample. For informative purposes, a protocol for purification of DNA from paraffin-embedded tissues is provided in our webpage (www.biotools.eu/eng/productos/paraffin.pdf).

PBMCs

PBMCs should be stored and shipped at -15±8ºC. For prolonged storages (more than one week), storage at –80 ºC or in liquid nitrogen is recommended.

In order to avoid accidents or accidental opening of the sample container, it is recommended to seal its closure with Parafilm® or equivalent before freezing.

Sample shipment must comply with local, national and international regulations for transport of etiological agents.

11. QUALITY CONTROL

It is recommended that at least one (1) Positive Control and one (1) Negative Control be run each time the test is performed. As with any new laboratory procedure, novel users should consider performing additional controls (both positive and negative) until a high degree of confidence is reached.

The Positive Control must render a 186 bp band. Vials containing negative control (sterile bidistilled water) must render no bands. Any analysis not fulfilling any of these results must be completely invalidated and discarded. It is necessary to repeat the process from its beginning, including DNA purification, processing other aliquot of the original sample. A failure of instruments during the test, indicated by error messages, also means that the test has not been valid. Repeat all the procedure for each sample from the amplification step.

For laboratories requiring an external control, this must contain a defined number of copies of the target sequence for HBV, and the viraemia levels in this control must be a multiply of the limit value of the testing procedure (e.g. WHO standards for HBV DNA).

12. PROCEDURAL PRECAUTIONS

1. Laboratory workflow must be unidirectional, from pre-amplification area to post-amplification area. Pre-amplification tasks must be initiated with the preparation of the reagents and sample purification. Equipments, materials and reagents must be dedicated and they must not be used for other activities or be transferred from one to another area. Gloves must be worn in each area, and must be discarded before proceeding to the next area. Equipments and materials used for setting-up of reactions must not be used for other activities, or for pipetting or processing amplified DNA or other DNA sources.

2. As with any analytical procedure, it is fundamental to use a good laboratory practice to obtain good results with this technique. Due to the high analytical sensitivity of the test, extreme care must be taken in order to keep the purity of all kit reagents and all reaction mixes. All reagents must be carefully checked in order to ascertain their purity. Discard all suspect reagents.

13. PROCEDURAL LIMITATIONS

1. Research Use Only.

2. Instructions must be followed in order to obtain correct results. Should the user have any questions, please contact our Technical Dpt. (info@biotools.eu).

3. This test has been validated for use with the reagents provided by the kit. The use of other amplification methods, or the use of equipment not fulfilling the specifications, may render equivocal results. User is responsible for validating the modifications for this test, in any of the indicated parameters.

4. This test has been validated with samples collected and shipped as per the "Sample Collection" chapter. Any modification has not been validated, and therefore, obtained results may not be correct.

5. Detection of HBV DNA depends on the number of viral particles present in the sample, and can be affected by sample collection methods, patient-related factors (e.g. age, symptoms), or for infection stage and sample size.

6. False negative results may be obtained due to polymerase inhibition. It is recommended to perform control reactions to distinguish between inhibition and true negatives.

7. Cross contamination between samples and exogenous DNA can only be avoided by following good laboratory practice. Instructions in this document must be strictly followed.

8. Use of this product is limited to qualified professional personnel, experienced in DNA purification and DNA amplification techniques.

9. It is important to pipette the indicated amounts, and mix well after each reagent addition. Check pipettes regularly.

14. WARRANTY

Products are guaranteed to conform to the quality and content indicated on each vial and external labels during their shelf life. BIOTOOLS obligation and purchaser's rights under this warranty are limited to the replacement by BIOTOOLS of any product that is shown defective in fabrication, and that must be returned to BIOTOOLS, freight prepaid, or at BIOTOOLS' option, replacement of the purchasing price.

Any complaint on damaged goods during transport must be directed to the handling or transport agent.

Product for Research Use Only. This product must be used by qualified professionals only. It is the responsibility of the user to ascertain that a given product is adequate for a given application. Any product not fulfilling the specifications included in the product sheet will be replaced. This warranty limits our responsibility to the replacement of the product. No other warranties, of any kind, express or implied, including, without limitation, implicit warranties of commercialisation ability or adequacy for a given purpose, are provided by BIOTOOLS. BIOTOOLS will not be held responsible for any direct, indirect, consequential or incidental damage resulting of the use, misuses, results of the use or inability to use any product.

Manufactured by:

BIOTOOLS, Biotechnological & Medical Laboratories, S.A. has been evaluated and certified to accomplish ISO 9001:2008 requirements for the following activities: Research and development of biotechnology products and manufacture of biotechnology and in vitro products.
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