Omunis

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Instruction Manual

PUMA HBV Kit

PCR. Universal. Molecular. Access.

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Notice d'utilisation : user manual

TEST PRINCIPLE

1. Purpose of use

The PUMA HBV kit is a real-time PCR test that allows the detection and quantification of the hepatitis B virus (viral load) in human plasma/serum. Combined with the clinical presentation and other biological markers of the status of the disease, the measurement of the HBV viral load is to be used for the surveillance of the progression of the disease and for the follow-up of patients infected with HBV that are or are not receiving an anti-viral treatment. The test is based on the principle of real-time amplification of the viral DNA (S gene) present in the samples, which allows for the precise quantification of PCR products during the exponential phase of the amplification process.

PUMA HBV is intended for Research use only (RUO). It must not be used for diagnostic purposes.

2. Advantages

With real-time detection of the fluorescent signal during and/or after each PCR cycle, the PCR quantitative data can be obtained in a very brief period of time. Thus, no post-PCR treatment is required, which not only leads to a considerable reduction in the risk of contamination of the PCR product but also permits the avoidance ulterior contamination of the samples by amplicons produced by previous reactions.

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3. Molecular mechanisms

The PUMA HBV test exploits the principle of PCR by hydrolysis of a nucleotide probe marked with both a 5' fluorescent reporter grouping (ex : FAM^{TM}) and a 3' non-fluorescent quencher grouping (ex : TAMRA). During PCR, the forward and reverse primers hybridize to a specific sequence at the amplicon level. The probe contained in the same reaction mixture hybridizes to a target sequence of the amplicon. When the probe is intact, the spatial proximity between the reporter and the quencher inhibits the fluorescence of the reporter, mainly by a transfer of Förster type energy. During PCR, the probe sets itself up specifically between the two sites where the forward and reverse primers are hybridized, and inhibits all activity of the Taq polymerase. Simultaneously, its 5'-3' exonuclease function, that cleaves the probe between the reporter and the quencher is activated. The reporter, freed from the quencher, emits a fluorescent signal, which is registered in real-time by the sensors. Thus rid from probe fragments, the target sequence can be read and amplified by the Taq polymerase.

The augmentation of the fluorescent signal is only detected if the target sequence is complementary to the probe and if it is amplified during PCR. Thus, a non-specific amplification cannot be detected. With this reaction principle, the fluorescent signal is directly proportional to the amplification of the target during PCR.

4. Reaction phases

The evolution of the amplification is represented by a sigmoid progression curve which can be divided in to two phases:

a) An exponential amplification phase, over the course of which the amount of PCR product obtained at every moment is a direct function of the number of initial copies. At the beginning of the

exponential amplification phase, the moment where the signal exits peak noise, corresponds to a number of cycles called Ct (cycle threshold).

b) The exponential amplification phase is followed by a plateau phase, which corresponds to a slow in amplification, due to the depletion of the reagents.

5. Operational steps

The PUMA HBV kit has two main operational steps:

a) Preparation of the sample

The viral DNA is extracted, purified and concentrated by means of a chosen extraction technique (reagents not provided in this kit).
E.g. : QIAmp DNA minikit (Qiagen) ; sample volume: 250 µL ; elution volume : 50 µL.

b) Amplification / Analysis: Quantification and measurement of HBV DNA

The viral load of the tested sample is obtained by extrapolating the calibration curve (range of standards provided in the kit), to the Ct value of the sample.

The PUMA HBV kit allows for the testing of up to 92 patient samples (100 tests in total, including the range of standards and the controls).

Réactifs	Type	Volume or quantity / tube	Nomber of tubes	State	Storage
HBV Enzyme Mix	DNA polymérase and buffer	450 µl	1	Liquid	-20°C
HBV DNA Panel	Plasmid at 10^7 UI/ml et tampon	50 µl	1	Liquid	-20°C
HBV + IC Oligos Set	Primers and probes for HBV and internal control	297 µl	1	Liquid	-20°C
Water	Water for molecular biology	1,5 ml	1	Liquid	-20°C
DNA Internal Control	Extraction and amplification Internal Control	550 µl	1	Liquid	-20°C
HBV Positive Control *	HBV Plasmid at 10^4 UI/ml and buffer	50 µl	1	Liquid	-20°C
HBV Negative Control **	Buffer	50 µl	1	Liquid	-20°C

COMPOSITION OF THE KIT

* *The point in the range is given at 10^7 UI/ml, diluted in a TE + 10% glycerol buffer, balanced at pH=7,5. This Standard is necessary for the making of the range (dilutions) for the performance of the test.*
** *The given positive control is a point at 10^4 UI/ml diluted in TE + 10% glycerol buffer balanced at pH=7,5. The negative control corresponds to a TE buffer balanced at pH=7,5.*

REAGENT STORAGE

- The PUMA HBV kit is transported at -20°C.
- Upon receipt, the box must be stored at -20°C. Stored under these conditions, the reagents will remain stable until the expiry date indicated on the label.

- Once prepared from the “HBV DNA” tube, the 5 points of the range can be frozen at -20°C. It is up to the user to ascertain the impact of the freezing/unfreezing cycles before any handling.

In all cases, it is advised to prepare non pyrogenic and “nuclease free” aliquots in sterile microvials, to close them with the appropriate lids and to label them carefully, indicating the name of the reagent, the batch and the expiry date.

MATERIAL REQUIRED BUT NOT PROVIDED

- 1-10 µL, 20-200 µL and 100-1000 µL pipettes.
- Pipette tips with filters.
- Benchtop centrifuges.
- Benchtop vortex.
- Open real-time PCR thermocycler,
- Single use gloves.
- Sterile microvials.
- Microplates with adhesive film.
- Manual or automated nucleic acid (DNA) extraction kit, suitable for collection.
- Refrigerator.
- Tube racks.

The real-time PCR instrument used for the test must be an « open » system with at least the following main characteristics:

- quantitative real-time PCR trials
- a programmable thermocycling block
- an excitation source : LEDs, light or laser;
- Filtration sets (length of excitation/emission wave) suitable for the detection of reporter fluorophores of FAM and Cy5 probes.
- Connection to a computer using specific analysis software allowing for the collection of fluorescence data, the conducting of trials of absolute quantification and the interpretation of results.

USE PRECAUTIONS

The PUMA HBV kit is a test that is to be exclusively used by qualified laboratory personnel subject to biology laboratory good practice, conscious of the biological risks and trained in molecular biology analysis. The interpretation of the results obtained from this test is the responsibility of the laboratory’s chief biologist or of a duly authorised laboratory technician.

Differences in the treatment of samples and in the technical procedure can lead to non-interpretable results.

- For all handling, gloves and a lab coat must be worn. The wearing of protective goggles is strongly recommended for the handling of blood/plasma samples and other components of the kit presenting a potential risk of infection.
- Do not eat, drink or smoke in the different work zones of the laboratory.
- Never pipette the reagents by mouth. Avoid contact with skin and mucous membranes.

- In case of contact of skin or eyes with samples or reagents, wash thoroughly with a large quantity of water and contact a doctor if irritation develops.
- Use new cones and filters to avoid cross-contamination between the samples and the reagents.
- Use nonpyrogenic and nuclease free consumables (pipettes, tubes, cones etc).
- The samples must be handled and eliminated as if they present an infective risk. They require use precautions such as those described in "*Biosafety in Microbiological and Biomedical Laboratories* (BMBL)" (*www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf*).
- Carefully clean and disinfect all work surfaces with an extemporaneously prepared solution of 0.5% sodium hypochlorite in demineralised or distilled water.
- After each trial, the consumables (and waste) must be considered contaminated and treated with a 0.5% sodium hypochlorite solution or with any other inactivating agent.
- Carefully wash hands after all handling.
- Dispose of unused reagents, waste and tested samples in accordance with the rules in place in your country at the local, regional and national levels.

The reagents were specially formulated to be used in this real-time PCR quantitative test. For optimal performance, no substitutions or modifications should be made.

The components of this kit are tested as a single group: do not mix the reagents from different batches.

The quality of the amplification also depends on the quality of the DNA extraction and on its conservation. Preference should be given to the recommended extraction techniques. Omunis is not responsible for the quality of the results obtained with non-recommended elements, material and protocol.

Do not use extraction reagents after the indicated expiry date has passed.

Do not use the kit after its expiry date has passed.

TERMS OF USE

The workflow in the molecular biology laboratory must be unidirectional; starting in the pre-amplification zone and ending in the amplification zone.

The reaction mixture must be prepared in a separate room or within a biological safety cabinet.

To assure the separation of the phases of preparation of the sample, mixture and amplification, each work zone and/or room must have its own equipment (cf. required materials that are not provided) including pipettes, cones, reagents, etc… This equipment must not leave the zone it has been assigned to.

The use of the method for detection and quantification of the viral load of HBV DNA must be limited to personnel that are well trained in molecular biology techniques and more specifically, in the process in its entirety (preparation of the sample, extraction of nucleic acids and real-time PCR trial).

BIOLOGICAL SAMPLE COLLECTION AND PREPARATION

1. Sample collection

Serum or plasma samples can be tested indifferently with the PUMA HBV kit. In the case of an analysis of plasma, the blood must be collected in sterile tubes with citrate or an EDTA type

anticoagulant. Heparin inhibits the real-time PCR reaction and must not be used with the PUMA HBV kit. The addition of anticoagulants is not necessary in the case of serum (dry tube) analysis.

2. Sample preparation

Once collected, the samples can be conserved either at room temperature for a maximum of 4h, or at -20°C for a longer period of time. A serum/plasma sample can be frozen and unfrozen twice without any significant negative impact on the extracted DNA yielded, and thus on the signal obtained after amplification.

If cryoprecipitate is visible, it can be removed by brief centrifugation and the samples must be treated immediately. This step will have no effect on the test results.

The extraction and purification of HBV DNA from a serum/plasma sample will be done with a non-provided extraction kit. The PUMA HBV kit is currently validated with the use of:

- the QIAmp DNA Mini Kit (Qiagen) manual extraction kit,
- the Viral NA (DiaSorin) automated extraction kit, on the Arrow (NorDiag) automate, according to the manufacturers instructions

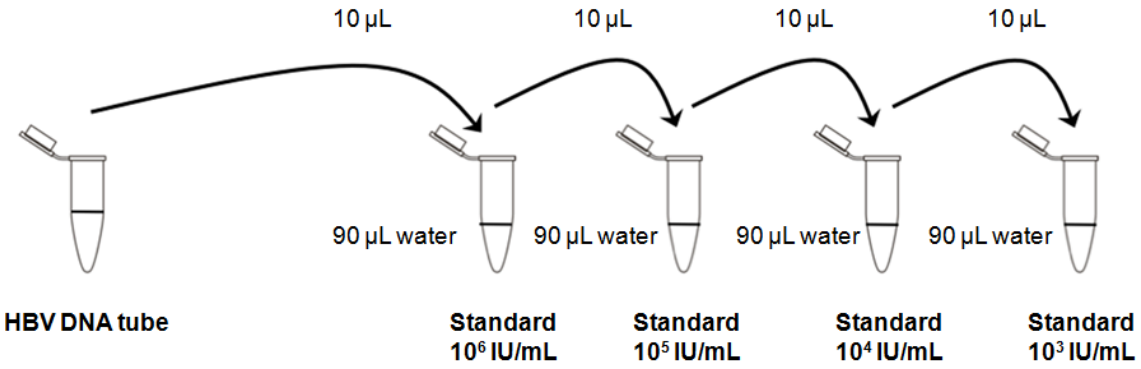
ADDING AND EXRACTION OF THE INTERNAL CONTROL

The PUMA HBV kit includes a DNA extraction and amplification internal control. This must be added directly to the sample before extraction, at 5µL per sample. The DNA extraction can then be done in accordance with the manufacturer's instructions.

PREPARATION OF THE RANGE OF STANDARDS AND OF THE CONTROLS

The PUMA HBV kit includes a standard with a viral load of 10^7 IU/mL ("HBV DNA tube"). In order to prepare the range of standards that will be used in the real-time PCR reaction for the quantification of the HBV viral load of the samples, a serial dilution from this tube is necessary. After dilution the range will include 5 points: 10^7, 10^6, 10^5, 10^4 and 10^3 IU/mL

- Prepare 4 new 0,5 mL sterile nuclease free tubes (not provided), for each point of range to be prepared (10^6 to 10^3 IU/mL).
- Add 90 µL of water to each tube.
- In the first tube, add 10 µL of previously homogenised standard HBV to 10^7 IU/mL (provided "HBV DNA" tube). Vortex for 10 seconds. Briefly centrifuge the tube before opening to make the drops in the lid fall. This tube is the 10^6 IU/mL point of the range.
- In the second tube, add 10 µL of the standard HBV at 10^6 IU/mL prepared above. Vortex for 10 seconds. Briefly centrifuge the tube before opening to make the drops in the lid fall. This tube is the 10^5 IU/mL point of the range.
- In the third tube, add 10 µL of the standard HBV at 10^5 IU/mL prepared above. Vortex for 10 seconds. Briefly centrifuge the tube before opening to make the drops in the lid fall. This tube is the 10^4 IU/mL point on the scale
- In the fourth and last tube, add 10 µL of the standard HBV at 10^4 IU/mL prepared above. Vortex for 10 seconds. Briefly centrifuge the tube before opening to make the drops in the lid fall. This tube is the 10^3 IU/mL point on the scale



⚠ *After each dilution, homogenise the mixture by briefly vortexing the tubes.*

PREPARATION OF THE PCR REACTION

1. Preparation of the reaction mixture

Calculate the number of samples to test, including the 5 HBV standards with decreasing viral charges (10^7, 10^6, 10^5, 10^4, et 10^3 IU/mL), the positive control, the negative control and an additional negative control (e.g. water for molecular biology).

In a sterile, nuclease free, 1.5 or 2 ml microvial, prepare the following reactive mixture for each sample. For N samples, multiply each of the indicated volumes by N+10%:

Reagents	Volume (µl)
HBV + IC Oligos Set	2,7 µl
HBV Enzyme Mix	4 µl
Water	9,3 µl
Total volume of reaction	16 µl

2. Preparation of the PCR plate

- Homogenise the solution yielded by vortexing. Briefly centrifuge the tube to collect any droplets present on the edges or in the lid of the tube.
- Allocate 16 µL of the previous reaction volume to a PCR microplate suitable for the amplification system.

⚠ *Be sure not to make any bubbles when pipetting in the microplate*.

- Homogenise each tube containing the points in the range (10^7, 10^6, 10^5, 10^4 et 10^3 IU/mL), the Controls (positive and negative) and the samples (patient or water) by vortexing for a few

seconds, then briefly centrifuge the tubes before opening to make the drops in the lid fall. Put 4 µL of each point in the range, control and sample in the wells of the plate containing 16 µl of reaction mixture, to be tested. Each well to be tested therefore contains a total volume of 20 µl.

Note : for detailed instructions concerning a quantification trial, refer to the user manual specific to the real-time PCR instrument and software.

a) After having carefully closed the PCR plate with the suitable adhesive film, place the plate inside the real-time PCR instrument.

Note : From this step and up to the elimination of the analysed plate containing the amplified targets, do not move or remove the adhesive film covering the plate.

b) Conduct the real-time PCR trial based on the programming of the following parameters:

Steps	Repetition	Temperature	Duration
Incubating UNG	1	50°C	10 minutes
Enzyme activation	1	95°C	5 minutes
Denaturation	50	95°C	15 seconds
Amplification	50	60°C	1 minute

The PUMA HBV kit can be used with different open amplification/analysis kits. To date, the tests have been optimised with the following PCR thermocyclers:

- **LightCycler 480 II** Real-Time PCR (Roche).
- **FluoroCycler 96** (Hain Lifescience)
- **CFX96** Touch Real-Time PCR Detection System (Bio-Rad).

⚠ *It is necessary to verify that the systems being used have been installed, calibrated and verified per the instructions and recommendations of the manufacturer.*

DATA ANALYSIS AND INTERPRETATION OF RESULTS

1. Software Configuration

When specific software for the real-time PCR system is used, consult the instruction manual describing data collection and the operating method for the quantification of the viral charge.

The software must at least allow one to:

- Choose the type of fluorophore ;
- Adjust the threshold or establish the threshold automatically for each well test ;
- Carry forward the spread of range points, controls and samples to test in the plate plan
- Map the calibration curve used for the quantitative dose

- Establish a results sheet describing the data of each well test, including : the position of the well, the fluorophore measured, the identification of the sample, the Ct value and the initial target quantity in the sample (e.g. value in UI/ml or $\log_{10}$ of the quantity).
- Report all of the analytical data

After the real-time PCR trial and data collection:

1. Verify and determine the threshold setting;
2. Note de Ct values of each point in the HBV range (intersection between the threshold and the amplification curve);
3. Verify the validation requirements of the trial before interpreting the results of each sample.

2. Trial validation requirements

✓ **Range of Standards**:

- The value of the slope is between -3,6 and 2,9 (included).
- The correlation coefficient R^2 of the curve, is between 0,95 and 1,00 (included), ideally $R^2 \geq 0,98$.

✓ **Negative Control**:

Ideally, the fluorescence emitted by a negative control must not intersect the threshold. It is a non-specific amplification indicator. If the fluorescence passes the threshold, check for an atypical curve (see below). In the case of an amplification curve, a contamination or distribution error in the microplate is to be assumed.

✓ **Positive Control**:

The value of the positive control must preferably be measured before 32 cycles. The absence of amplification of a positive control is indicative of the presence of a PCR inhibitor or an error in the distribution of the reagents. In this case, it is necessary to repeat the analysis series.

✓ **Atypical Curve**:

Some samples present atypical curves that are not characteristic of amplification curves. In this case, the result is considered to not be interpretable and the analysis of the sample must be repeated.

✓ **Extraction and amplification internal control** (« HBV Internal Control » tube)

For a use of the internal control as mentioned in the protocol, assuming the extraction was 100% effective and using 1:10 of the DNA extracted in the reaction, a Ct value of 32 is to be expected. However, this value can vary significantly depending on the effectiveness of the extraction and the parameters of the analysis instrument used. Ct values in a range of 32±3, are to be considered normal.

When the amplification of the target gene yields a high copy number, the internal control provided may not produce the amplification curve (especially for viral loads over 100 000 IU/mL). This result is normal and should not invalidate the test. It must be interpreted as a positive result, despite the absence of the internal control signal. This phenomenon is the result of a competition between the internal control and the viral target during amplification of the target, in the case of a high copy number

3. Interpretation of the results

- If the properties of the range of standards are not reached, or if the validation requirements of the positive and negative controls are not fulfilled, the entire series of analyses must be repeated.

- A result is considered to be positive when the HBV viral load measured is above the threshold of 90 IU/mL (detection limit of the test)
- Based on the analytical sensibility of the test, the quantification limit is estimated at 900 IU/ml. An HBV viral load result detected that is inferior to this 900 IU/ml limit is positive (detectable), but the value of the viral load is not precisely quantifiable. It is advised that for these samples, “detectable under the qualification threshold” be indicated.
- A result is negative or “not detected” when no value is obtained or if the value is below the detection threshold (<90 IU/ml).

PERFORMANCE

The performance of the PUMA HBV test was compared with that of the COBAS Taqman HBV Test v2.0 (Roche Diagnostics) test.

1. Material used

The extraction of DNA from the HBV was conducted through the automatic Arrow Viral NA 3.0 technique, from a collection of 250 µL of serum and with an elution of 50 µL. The amplification was carried out with the LightCycler 480 II Real-Time PCR (Roche).

2. Biological material used

A total of 59 samples of serum from patients who tested positive for HBV (median concentration of viral load = 5,66 $\log_{10}$ IU/mL) and 33 samples of serum from patients who tested negative for HBV were provided by the specialised services of an external hospital laboratory (samples tested using COBAS Taqman HBV Test v2.0 by Roche).

3. Performance

Sensitivity
98% (59/59) of the samples were found to be positive by the PUMA HBV test (median concentration of the viral load = 5,38 $\log_{10}$ IU/mL).

Specificity
100% (33/33) of the samples were found to be negative by the PUMA HBV test.

Linearity of the range of Standards
The linearity of the range of Standards was measured by undertaking 12 independent analyses for each dilution point of the range: R^2 = 0,999.

Detection limit
The detection limit of the test is 90 IU/ml. It was calculated using 6 concentrations (50; 80; 90; 100; 110; 120 IU/ml) of a reference sample ("*3rd WHO International Standard for HBV DNA Nucleic Acid Amplification Technique*" *(NIBSC)*) in 33 different tests.

Genotypes
100% of the genotypes tested (A-G) could be detected and quantified by the PUMA HBV test.

Correlation

The correlation of the values of the viral loads between the two techniques is R^2 = 0.9576.

The Bland-Altman analysis shows that 91% of the samples are spread in the limits of the average of differences (-0.19 ± 2SD IU/mL). The two techniques show equivalent HBV viral load values (difference < 0.5 $\log_{10}$ IU/mL) for 70 % of samples.

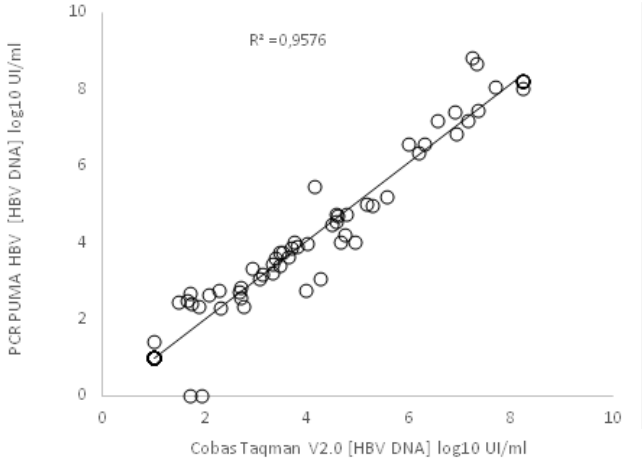


Fig. 1. Correlation between PUMA HBV Kit and Cobas.

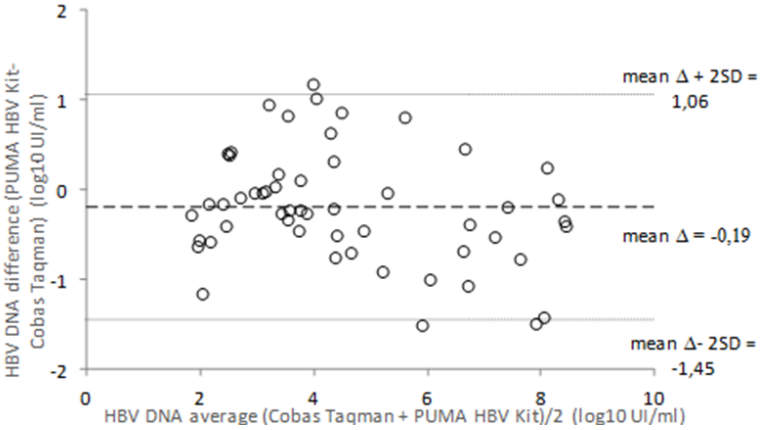


Fig. 2. Bland-Altman between Cobas and PUMA HBV Kit.

*Comparison of the **Roche technique** and the **PUMA HBV** test for the quantification of HBV DNA in the serum of 92 patients.*

NOTE

The manufacturer and the competent authority in the member state where the user is established should be notified in the case of any serious incident linked to the device.

SIGNIFICATION DES SYMBOLES UTILISES

REF	Reference		Expiration date
Σ	Number of reactions	LOT	Batch number
	Storage temperature		Attention
	Manufacturer		