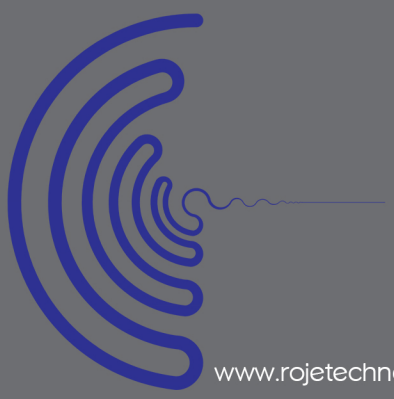

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ROJETECHNOLOGIES

Viga Quantitative HBV Molecular Diagnostic Kit

Quantitative RT-PCR Assay

Molecular HBV Diagnostic kit based on Quantitative Real Time-PCR

For In Vitro Diagnostic Use

By ROJE
Edition, 01/2022

ROJETechnologies has been founded since 2014, and manufactures a wide range of molecular biology kits. We research, develop and create our products in order to make easier and more comfortable approaches to do research in molecular biology. Our target is offering high-quality affordable Molecular and diagnostic Kits and reagents, comparable of the world leaders, to research centers, laboratories, clinics, hospitals and diagnostic centers all over the world.

Contents

Kit Content

Viga Quantitative HBV Molecular Diagnostic Kit

Components	25 Preps	100 Preps
Pro HBV Mix	220µl	875µl
QD-ROMAX, 4X	160µl	625µl
IC	125µl	500µl
HBV *QS1(1×10^4 IU/µl)	65µl	250µl
HBV *QS2(1×10^3 IU/µl)	65µl	250µl
HBV *QS3(1×10^2 IU/µl)	65µl	250µl
HBV *QS4(1×10^1 IU/µl)	65µl	250µl
Water for Molecular Biology	125µl	500µl

* Quantitation standard

Storage

The components of Viga Quantitative HBV Molecular Diagnostic Kit are ready to use. After arrival, all reagents should be stored at –15°C to –30°C and are stable until the expiration date stated on the label.

Intended Use

The Roje Viga Quantitative HBV Molecular Diagnostic Kit is an in vitro diagnostic test, based on Real Time-PCR technology, for detecting and quantifying human hepatitis B virus (HBV) specific DNA (genotypes A to H) in human EDTA plasma.

Guarantee & Warranty

ROJETechnologies guarantee the efficiency of all manufactured kits and reagents. For more information on choosing proper kits based on your needs, please contact our technical support team. If any product does not satisfy you due to reasons other than misuse, please contact our

technical support team. If the problem is due to the manufacturing process, the ROJE team will replace the Kit for you.

Notice to Purchaser

This product is only for experiments and not for commercial use of any kind. No right to resell it or any components. For information about out-licensing or distributors, contact ROJE business team.

Warning and Precautions

- Before first use, check the product and its components for completeness concerning number, type, and filling. Do not use a defective or incomplete product; performance could be compromised.
- Do not use other sample types! The use of different sample types may compromise the product performance.
- PCR inhibitors (e.g., heparin) may cause false-negative or invalid results.
- Improper storage conditions may lead to compromised product performance.
- A lack of centrifugation of the product components after thawing could contaminate of the components with reagent residues in the lids and, consequently, a compromised product performance.
- Repeated thawing and freezing (>2x) should be avoided, as this may reduce assay performance.
- Do not use product components beyond the expiration date printed on the component label.
- Improper handling of product components and samples may lead to contamination causing incorrect IVD examination results.
    - Do not interchange vial or bottle caps, as cross-contamination may occur.
    - To minimize the risk of carryover contamination, store positive and potentially positive material separated from the kit components.
    - Use separated working areas for sample preparation/reaction setup and amplification/detection activities.
    - Always wear disposable gloves.

    - Do not open the PCR plates post amplification to avoid contamination with amplicons.
- Do not exceed the PCR Mix storage time. This could lead to compromised product performance.
- Always treat samples as infectious and (bio-)hazardous by safe laboratory procedures. For sample material spills, promptly use an appropriate disinfectant. Handle contaminated materials as biohazardous.
- Dispose of hazardous and biological waste only in compliance with local and national regulations to avoid environmental contamination.
- As with any diagnostic test, results should be interpreted in consideration of all clinical and laboratory findings.
- Potential mutations within the target regions of the HBV genome covered by the primers and probes used in the kit may fail to detect the presence of the pathogen.

General precautions

- Use sterile and RNA/DNA-free pipet tips with filters.
- All steps in storage and purification of samples containing HBV (samples and PCR products) should be done in a separate place from that of Master Mix.
- Thaw all components thoroughly at room temperature (15°C to 25°C) before starting an assay.
- When thawed, mix the components (by pipetting repeatedly or by pulse vertexing) and centrifuge briefly. Ensure that no foam or bubbles are present in the reagent tubes.
- Work quickly and keep PCR reagents on ice or in the cooling block before loading.

Quality Control

All ROJETechnologies products are tested against predetermined experiments on a lot-to-lot basis according to ROJETechnologies ISO-certified quality management system to ensure consistent product quality. For more information about the result, enter the labeled REF and LOT number in the "certificate of analysis" on www.rojetechnoloes.com.

Procedure

Viga Quantitative HBV Molecular Diagnostic Kit is an in vitro diagnostic test for the determination and quantification of hepatitis virus DNA. It is based on TaqMan Real Time-PCR technology, utilizing polymerase chain reaction (PCR) to amplify HBV-specific target sequences and fluorescently labeled target-specific probes to the amplified DNA. The final amount of amplified DNA is assessable by monitoring the fluorescence intensities in Real Time-PCR. In addition, this method contains oligonucleotides attached to fluorophores at the 5' end with FAM as a reporter and the 3’ end with a quencher. In the meantime, specific primers and probes were developed as the internal control (Exogenous internal control) with fluorophores VIC/HEX attached at the 5’ end as a reporter. IC (internal control) is added manually at the first stages of the extraction or added into the reaction. Specific prob for hepatitis virus DNA is labeled by FAM (green) and internal control by VIC (yellow).

Equipment & Reagents to Be Supplied by User

- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. Consult the appropriate safety data sheets (SDSs) available from the product supplier for more information.
- Vortex mixer
- Powder-free gloves (disposable)
- DNA isolation kit (DNJia Virus DNA Kit)
- Pipets (adjustable)
- Sterile pipet tips with filters
- Cooling block
- Real Time-PCR

Kit is operative in the following instruments

- Rotor-Gene Q, 2plex
- Corbett Rotor-Gene 3000&6000
- Mic qPCR Cycler

Sample collection

Storage and sample transportation

- Transport samples under specific precaution procedure for pathogens and make sure transportation should not exceed six hours.
- All samples must be transported at 2 - 8 °C and plasma samples at -20°C.
- Whole blood should be separated into plasma and cellular components by centrifugation at 1200-1600 rpm for 20 min. Transfer extracted plasma into sterile Eppendorf.
- Avoid freezing blood samples as the assay's sensitivity can be reduced if you freeze the samples as a matter of routine or store them for a longer period of time.
- After extraction, the isolated Hepatitis B virus encapsulated DNA is stable for up to 14 days if stored at +4°C, for 12 weeks if stored at -20°C, and up to one year when stored at -70°C.

Before use

Take out each component from the kit and place them on bench top. Allow the reagents to equilibrate to room temperature, then briefly vortex each tube for later use.

Applications

The Viga Quantitative HBV Molecular Diagnostic Kit constitutes a ready-to-use system for detecting hepatitis B virus DNA using polymerase chain reaction (PCR) and primers and specific fluorescent probs.

Description

Molecular diagnostic tests based on nucleic acids utilizing polymerase chain reaction are high-sensitive and high-specific methods for detecting the Hepatitis B virus in blood samples. Before PCR reaction, HBV antigen and present antibody can be identified by ELISA. Either in positive cases or possible false-negative results, then a quantitative PCR test will be necessary, as all diagnostic accuracy, drug resistance, and illness severity depend on the accurate and effective estimation of blood virus load. Real Time-PCR is the most convenient method for following estimation of isolated DNA and RNA concentration based on an external standard control standard curve, which allows the quantification of HBV-specific DNA in a sample. Accordingly, a blank sample (here conserved sequence of HBV genome) is estimated based on a standard curve of

homologous DNA with distinct concentrations (here amplified HBV). The main advantage of the provided kit is coverage of a wide range of concentrations, as high concentrated HBV is assessable without necessary elution. Thus, HBV diagnostic kit should be able to diagnose the HBV virus specifically and detect various strains of hepatitis virus efficiently. Viga Quantitative HBV Molecular Diagnostic Kit is based on Real Time-PCR technology, utilizing polymerase chain reaction (PCR) to amplify of HBV specific target sequences and fluorescently labeled target-specific probes to amplify DNA. In addition to the HBV DNA specific amplification and detection system, inter/intra assay, linear range, and standard control, the present kit contains oligonucleotides for the amplification and detection of the IC, which restrict probable inhibitions during PCR reaction. This item can be added either at the begging of the purification procedure to control the whole process (involving isolation and PCR) or just during the duration of the PCR reaction, which can knock down PCR inhibitors. Noticeably, the parallel control test has neither any cross-reaction nor effect on the HBV virus or human genome or other types of common viruses existing in human blood. Importantly, Viga Quantitative HBV Molecular Diagnostic Kit can be operated in vitro hepatitis B virus DNA amplification and then the quantitative determination of DNA content. This kit is designed to be operated in Rotor-Gene 3000, Rotor-Gene 6000, and Rotor-Gene Q.

Kit content introduction

For mass screenings, rapid and accurate detection of HBV is of pivotal importance. Real Time-PCR HBV kits with high sensitivity meet these objectives. In addition to specific amplification of the HBV genome, this method contains oligonucleotides for direct detection of IC (internal control). IC should be manually added at the beginning of the nucleic acid purification procedure. Probes specific for hepatitis B virus DNA are labeled with the fluorophore FAM™(green), and IC is labeled with a fluorophore detectable in the VIC™ (yellow) channel.

QD-ROMAX and Pro HBV Mix solutions

Contain all components like PCR buffer, DNA polymerase enzyme, magnesium salt, primers, and probes that allow the PCR mediated amplification and target detection of HBV-specific DNA and of the IC in one reaction setup.

- **Quantification Standards (QS)**

The Quantification Standards (QS) contain standard concentrations of HBV-specific DNA (see Table 1). They were calibrated against the International Standard for HBV DNA for Nucleic Acid Amplification Techniques and Clinical a Laboratory Standards Institute. The Quantification Standards are used to verify the functionality of the HBV DNA specific amplification and detection system as well as to generate a standard curve, which allows the quantification of HBV specific DNA in a sample.

Table 1: Quantification Standards

Quantification Standards	IU/µl
HBV QS1	1×10^4
HBV QS2	1×10^3
HBV QS3	1×10^2
HBV QS4	1×10^1

- NTC: no template control
- NTC: contains no HBV-specific DNA but does contain the Internal Control template.
- The NTC is used as a negative control for the HBV DNA specific Real Time-PCR and indicates possible Q-ROMAX and Pro HBV mix contamination.

Features

Specific features of Viga Quantitative HBV Molecular Diagnostic Kit are listed here in Table 2.

Table 2. Viga Quantitative HBV Molecular Diagnostic Kit features and specifications

Technology	Real Time-PCR
Analysis type	Quantitative
Target Gene	HBsAg gene
Analytical Feature	Enable to determine A to H genotypes of hepatitis B virus DNA and negative HBV with 100% specificity.
Analytical sensitivity	To determine the limit of detection (LOD), a dilution series of the 5th Acrometrix International Standard for HBV DNA for Nucleic Acid Amplification Techniques with 200 IU/ml (code: 625607) in EDTA plasma containing 10, 20, 40 IU/ml was generated. Each dilution was tested in

	20 replicates (based on FAD, the validated LOD should be 19 positive results in every 20 replicates). Data from all runs were combined,and a probit analysis was performed to determine the 95% LOD value. The limit of detection (LOD) of Viga Quantitative the HBV Molecular Diagnostic Kit is 40 IU/ml.
Diagnostic Specificity	CI95%: 99.06% –100%
Diagnostic sensitivity	(CI95%: 99.90% –100%) 97.87%
Linear range	10^9-10^2IU/ml
Dynamic range	10^9-40IU/ml
Report unit	IU/ml
International standard	Acrometrix code: 625607
PCR contamination and DNA extraction efficiency control	PCR inhibition and DNA extraction efficiency control
Sample	Plasma/serum
Storage	-15 to -30 °C
Recommended extraction method	DNJia Virus DNA Kit (DN983056)
Recommended equipment	Rotor-Gene Q, 2plex, Corbett Rotor-Gene 3000&6000, Mic qPCR Cycler
Fluorescent channels	Green-Yellow

Recommended Starting Material

DNA Sample Requirement

Before starting, add a 2-5 cc blood sample into a tube containing EDTA. After plasma isolation and DNA extraction, utilize 10µl of the whole prepared sample in Real Time-PCR.

Sample storage and Preparation

- Viga Quantitative HBV Molecular Diagnostic Kit has been performed using human EDTA plasma samples. Other sample materials are not validated.
- Blood has to be collected with commercially available standard blood collection systems (e.g., Sarstedt, Becton Dickinson, Greiner, or equivalent)
- The blood samples should be shipped cooled (2°C to 8°C).
- For a generation of EDTA plasma, whole blood should be centrifuged according to the instructions provided by the manufacturer of the collection system within 24 hours after collection.

- Before use, EDTA plasma should not be stored for more than two days at room temperature (20°C to 25°C), five days at 2°C to 8°C, or two months at -25°C to -15°C.

- Always treat samples as infectious and (bio-)hazardous in accordance with safe laboratory procedures. For sample material spills, promptly use an appropriate disinfectant. Handle contaminated materials as biohazardous.
- Frozen storage of samples does not compromise kit performance. When working with frozen samples, make sure samples are completely thawed and properly mixed before use.
- Be careful about the health risk of positive samples and follow necessary precaution procedures in all stages, from collection and transportation to kit application.
- Notice that EDTA is the most suitable anticoagulative buffer for Viga Quantitative HBV Molecular Diagnostic Kit; otherwise, there is no guarantee for accurate function and result.
- **Notice:** Samples collected in tubes containing heparin as an anticoagulant should not be used.

Before start

- Before first use, make sure about the intactness and completeness of kit contents and regents.
- Avoid utilizing samples other than human plasma to prevent incorrect IVD examination results.
- Misusing the regents may lead to contamination and invalid results.
- Use RNAase/DNAase free tip sampler with filter.

Buffer preparation

Master mix preparation

The total volume of the prepared sample used in this test is 10µl. Refer table2. Required information for preparing tubes is available in table 3,4,5. If you use internal control, refer to the following information in the handbook.

Notice: prepare Master mix just for single-use and avoid adding QD-ROMAX to Pro HBV mix if you do not need to test.

Table2: Regents preparation per one single reaction (DNA isolation efficiency and PCR inhibition are controlled by adding internal control in the purification stage).

Required component	volume
Pro HBV Mix	8.75µl
QD-ROMAX, 4X	6.25µl
Purified DNA	10µl

Table 3: Required volumes for standard tubes

Standards	Volume per tube	Pro HBV Mix + QD-ROMAX, 4X per reaction
HBV QS1	10µl	15µl
HBV QS2	10µl	15µl
HBV QS3	10µl	15µl
HBV QS4	10µl	15µl

Table 4: Required volumes for every single test tube

Volume per tube of an unknown sample	Pro HBV Mix + QD-ROMAX, 4X per reaction
10µl	15µl

Table 5: Required volumes for negative control tubes

Volume per tube of water*	Pro HBV Mix + QD-ROMAX, 4X per reaction
10µl	15µl

Notice: pay attention to using the NTC tube in each run.

*Sample is changed with water in NTC tube, controlling contamination in reaction.

pathogenicity

The hepatitis B virus (HBV) causes the disease hepatitis B. The hepatitis B virus is unique among human viral pathogens. It is a DNA virus that replicates via an RNA intermediate and thus belongs to the reverse transcribing DNA and RNA viruses. HBV is part of the Hepadnaviridae family of viruses, which consists of genotype A-H.

The genomic structure of HBV is both compact and complex, with the ability to encode seven distinct proteins with only 3.2 kb. These proteins are the polymerase protein (Pol gene); core antigen (HBsAg) and e antigen (HBeAg); large, medium, and small surface-antigen proteins (S gene); and the X protein (X gene).

HBV is transmitted via blood or other body fluids and can survive outside the body for seven days. The most common transmission types are perinatal mother-to-infant transmission or horizontal transmission between children up to 5 years. Sources of infection are also medical surgery instruments, tattooing needles, or razors that are contaminated with blood.

The virus may be evidenced 30 to 60 days after infection and can persist in the body. The infected person can develop chronic hepatitis B. Symptoms can reach from yellowing of skin and eyes (jaundice) and dark urine to extreme fatigue, nausea, vomiting, and abdominal pain, which can last for several weeks, but carriers can be asymptomatic as well. The worst-case scenario is the development of acute or chronic hepatitis, with progression to liver cirrhosis or hepatocellular carcinoma (HCC).

Hepatitis B cannot be cured so far. However, medication for symptom treatment is available, and the slowdown of cirrhosis progression is possible. Despite vaccinations, hepatitis B virus infections are still prevalent worldwide. Hepatitis B remains a global health problem, with about 240 million people suffering from chronic HBV infection and 887,000 HBV-related deaths per year (numbers increasing again since 2015).

Hepatitis B virus prevalence is highest in the Western Pacific and Africa, where 6.2% and 6.1% of the adult population is infected. Infections also occur in the WHO-specified regions, such as the Eastern Mediterranean, South-East Asia, Europe, and the Americas [3]. Therefore, there is a

high demand for viral hepatitis B testing as an important part of all prevention and treatment efforts.

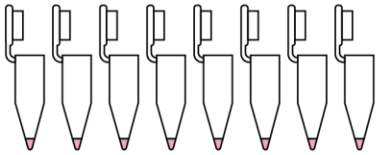
workstation preparation

All work areas, samplers, centrifuges, and all related equipment must be sterile. Use decontaminants like Sodium hypochlorite 10%, ethanol 70%, and RNZO in nucleic acid contaminations.

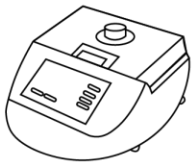
Protocol

Step 1: Before extraction add 0.1µl internal control/µl of final elution to isolated nucleic acid

Step 2: Mix 15 µl Master Mix and 10 µl prepared DNA



Step 3: Run the Real-Time PCR program



Step 4: Interpret the result

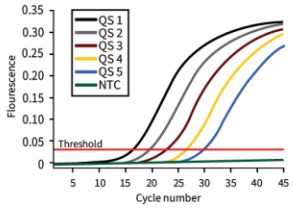


Figure 1: Addition of internal control during DNA amplification in PCR. In this case, Master mix involves Pro HBV Mix and QD-Romax,4×. For more information, refer Master mix preparation.

Step 1: Preparing Master Mix

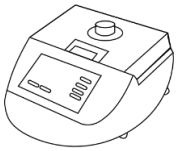
Step 2: Add 15 µl Master Mix to new tube

Step 3: : Add 1 µl internal control to step 2

Step 4: Transfer 15 µl of prepared mixture to a new tube

Step 5: Add 10 µl isolated nucleic acid to step 4

Step 6: Run the Real-Time PCR program



Step 7: Interpret the result

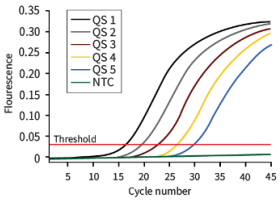


Figure2: Addition of internal control to Master mix. Notice that there is not any addition of internal control during the purification stage. In this case, Master mix involves Pro HBV Mix and QD-Romax,4×. For more information, refer Master mix preparation.

Table 6: PCR program for HBV

stage	Temperature	Incubation Time	Cycle Number
Pre-Denaturation	95°C	5 min	1
Denaturation **Annealing and Extension**	95°C 58°C	10 sec 60 sec	5
Denaturation **Annealing and Extension and acquisition on channels Green and Yellow**	95°C 58°C	10 sec 60 sec	40

Process

- Switch on Rotor-Gene.
- Switch on the computer and then Rotor-Gene software.
- Start PCR setup based on PCR Run Program (table 6).
  - In the **New Run Wizard** dialog, click **Edit Profile** in the **Temperature Profile** box to enter the PCR program.
    - Set **Hold Temperature** to 95°C and **Hold Time** to 5 min required for hot-start enzyme activation and Pre-Denaturation.
    - Optimize temperature and time for each cycle: 95°C for 10 sec required for denaturation and 58°C for 60 sec for annealing and extension
- The acquisition should be gained in the green channel (HBV target detection channel) and the yellow channel (IC target detection channel).

- In the **Edit Profile** dialog, select both **green** and **yellow** in the Channel Setup box.
- Click **Gain Optimization** and then **Optimize Acquiring** to set the acquisition.
- Set the temperature to 58°C and click **Start**

- After starting Rotor-Gene software, click **Next** in the **New Run Wizard** window and enter the PCR program in the **Edit Profile** dialog.

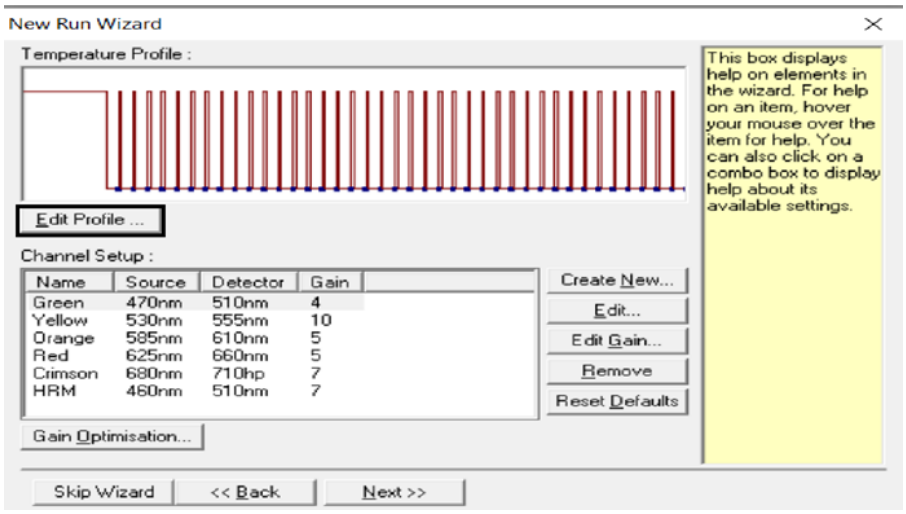


Figure1: Set PCR program in the **Edit Profile.**

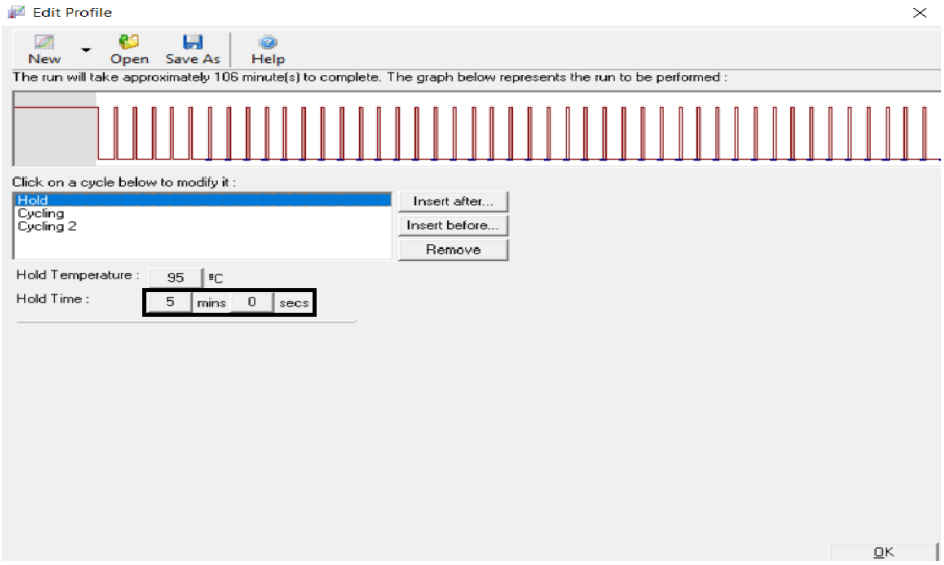


Figure 2: Optimize temperature and time for the hot-start enzyme.

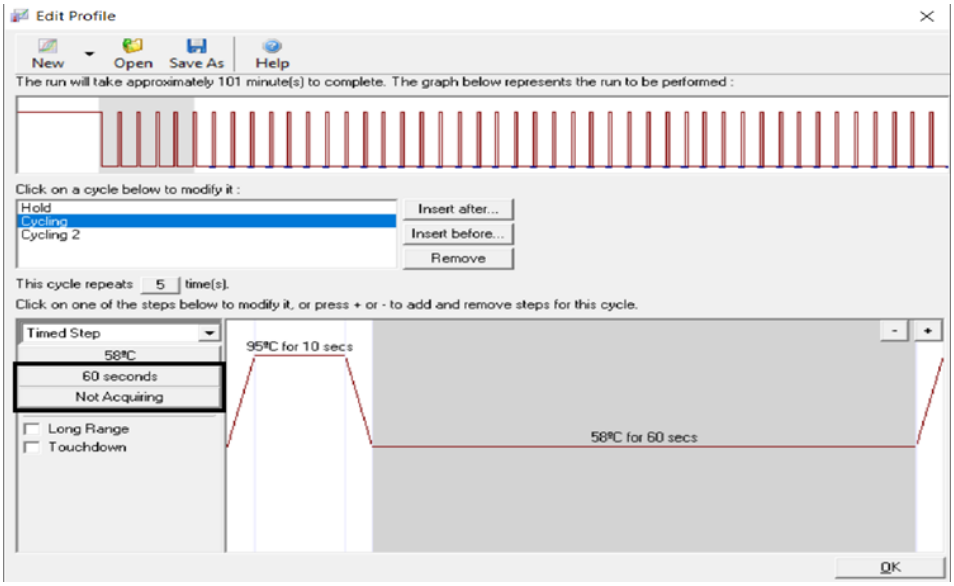


Figure 3: Set DNA amplification program

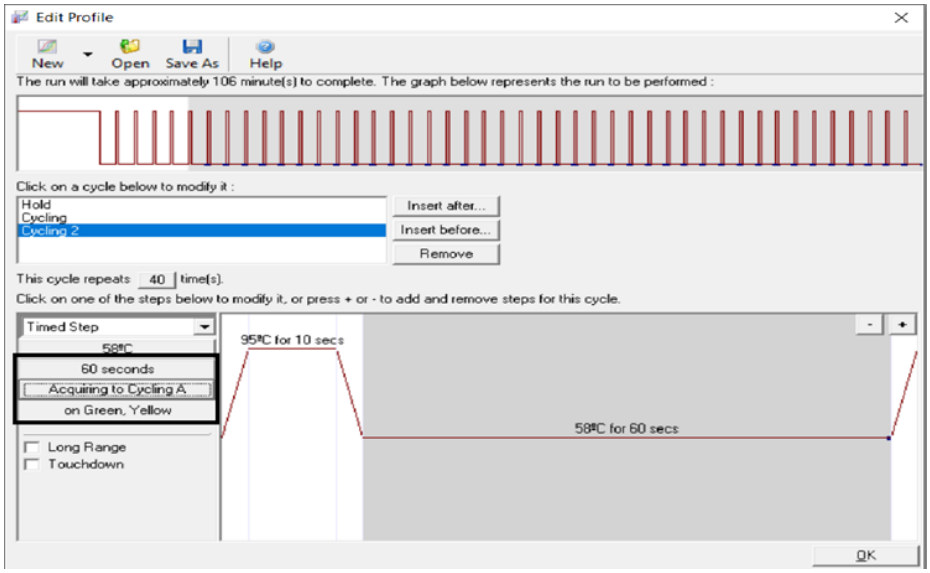


Figure4: 40 cycles DNA amplification setup

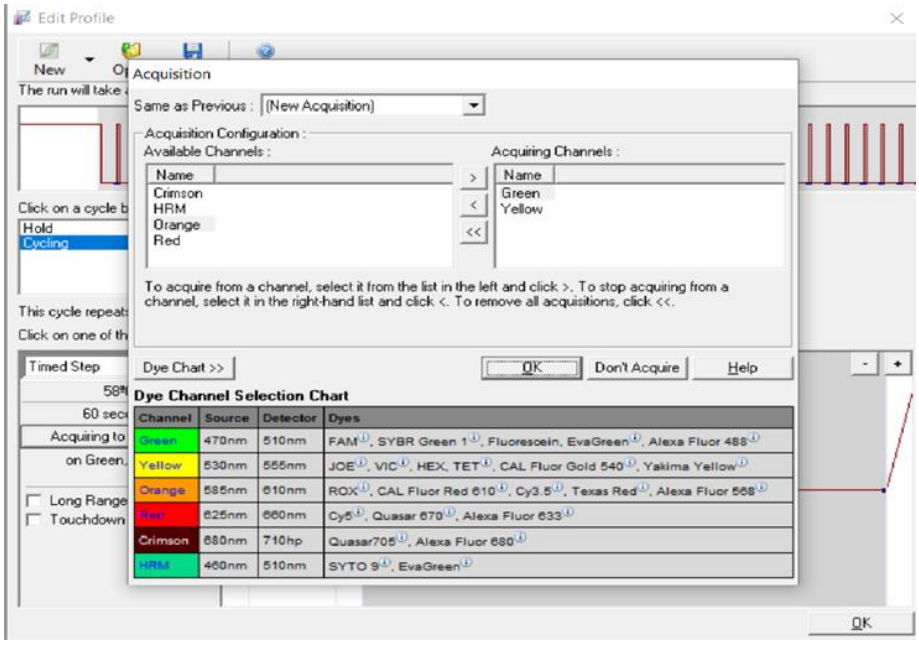


Figure 5: Acquisition setup based on green and yellow channels

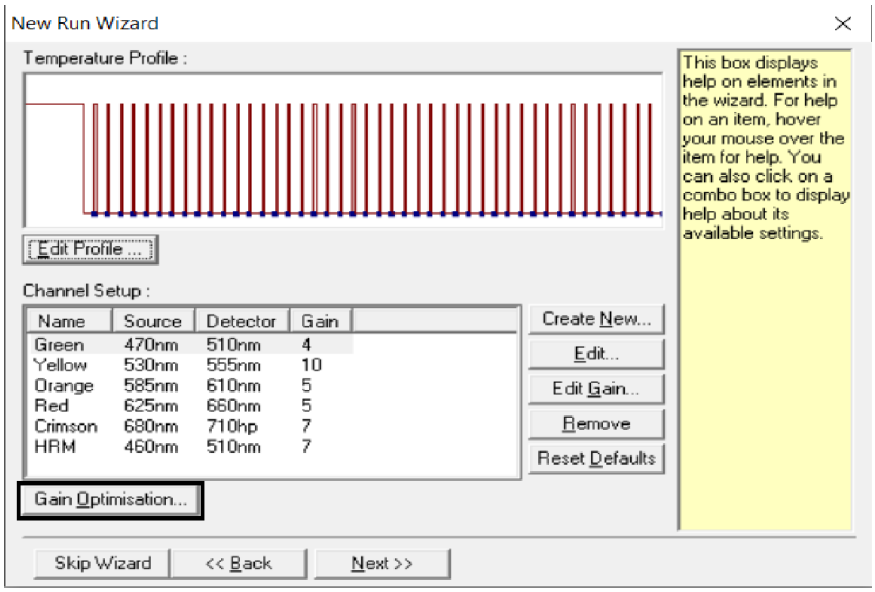


Figure6: optimize PCR signals for specific fluorescence (green and yellow) detection. Click **Gain Optimization** in the **New Run Wizard** window. Then, in the**Auto-Gain Optimization Setup** dialog, set the calibration temperature to 58°C, which is compatible with annulling temperature

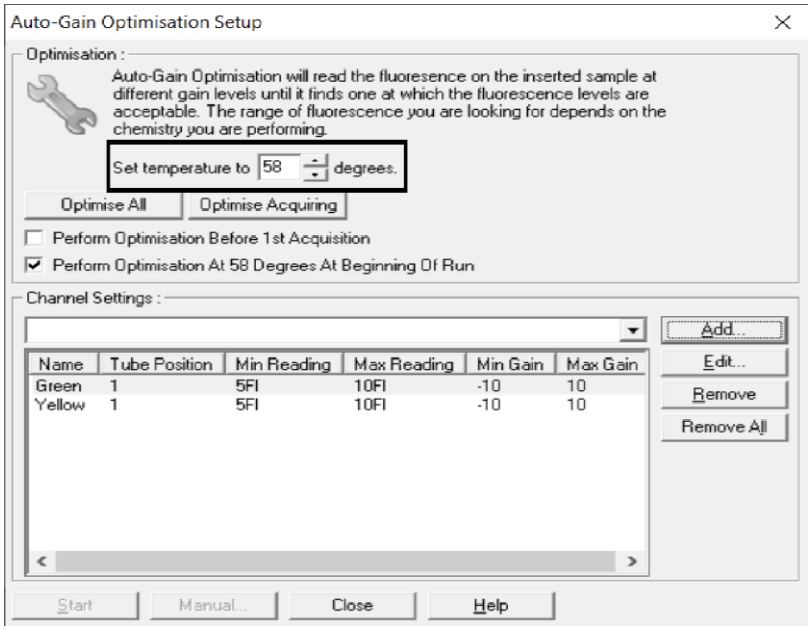


Figure7: setting channels for probs. Notice select FAM/Syber and JOE if work with Rotor-Gene 3000)

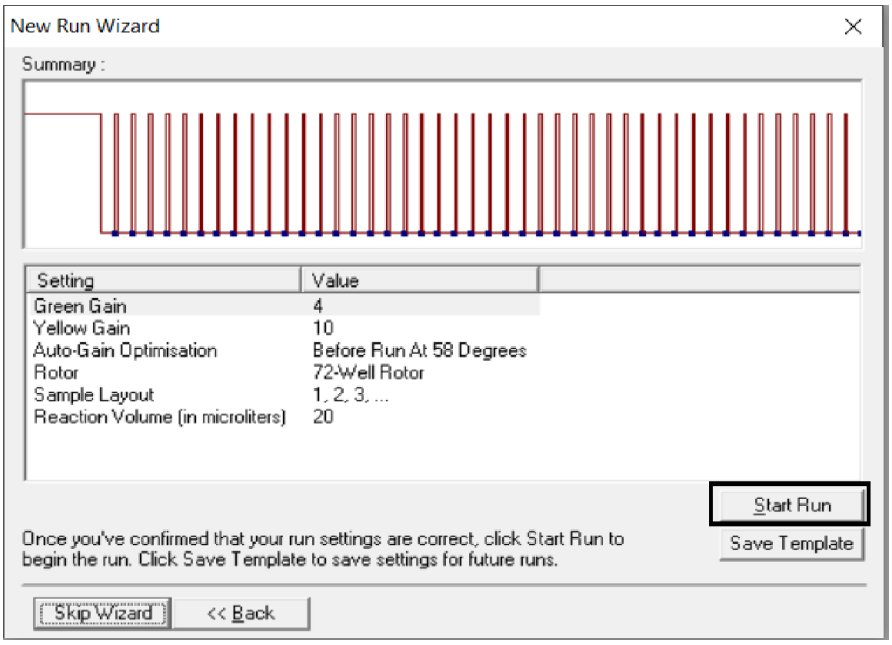


Figure 8: Click **Start Run** in **New Run Wizard.** Notice select FAM/Syber and JOE if work with Rotor-Gene 3000)

Results Interpretation

Quantification Standards in Viga Quantitative HBV Molecular Diagnostic Kit use a standard panel consisting of identified concentrations of HBV DNA. In the preparation of both sample and standard concentrations, 10µl of isolated DNA should be added to the 15µl Master Mix. Quantification Standards are used to generate the standard curve, which allows the quantification of HBV-specific DNA concentration in the sample. Enter qualification standard in Rotor Gene-specific software just in IU/ml. Follow provided formula to convert IU/µl by which kit contents are supplied.

$$\text{Result (IU/ml)} = \frac{Result\left(\frac{IU}{\mu l}\right) x\ Elution\ Volume\ (\mu l)}{\text{Sample Volume (ml)}}$$

If the volume of whole plasma were 200µl and elution 50µl, the first standard would be 2.5×10^6 IU/ml entered in Rotor Gene-specific software.

Validity of a Diagnostic PCR Run

A diagnostic PCR Run is valid if met the following control condition:

Table7: Control conditions for a valid PCR Run

Control	Detection Channel	
	FAM™ (HBV target)	VIC™ (Internal Control)
Quantification Standard (Std / Pos)	+	not applicable
NTC negative control	-	+

The generated standard curve reaches the following control parameter value:

Table 8: Standard curve control parameter

Control Parameter	Valid Value
R square (R2)	≥ 0.98

The standard curve's control parameter is displayed below the standard curve graph in the Data Analysis window.

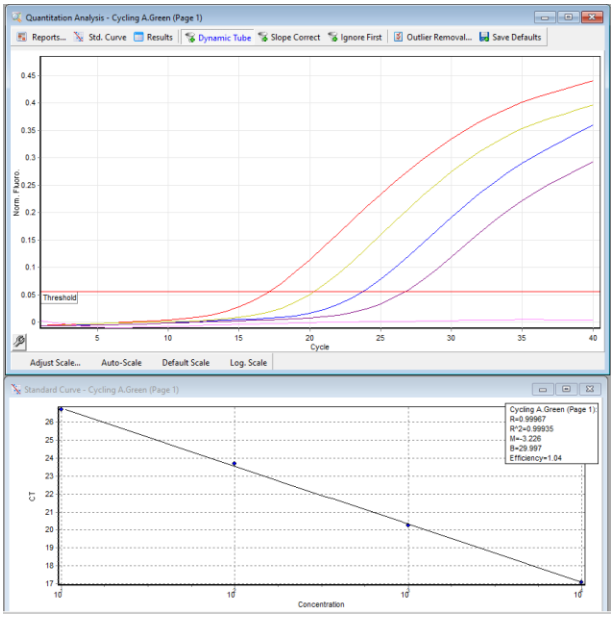


Figure 9: Standard curve is provided for each qualification standard. PCR Efficiency, Correlation Coefficient, and Intercept are revealed in provided graph.

Test Limitation

- Notice that all reagents may exclusively be used in in-vitro diagnostics.
- The product is to be used by personnel specially instructed and trained in the in-vitro diagnostics only.
- Strict compliance with the user manual is required for optimal PCR results.
- Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

Performance Characteristics

Analytical sensitivity

The analytical sensitivity of the Viga Quantitative HBV Molecular Diagnostic Kit was tasted against dilution series in an international standard (Acrometrix: 625607) for diagnostic setups based on HBV DNA detection. HBV Molecular Diagnostic kit contents (contains plasmid) were determined based on Acrometrix and international standards.

Analytical Specificity

The specificity of the Viga Quantitative HBV Molecular Diagnostic Kit is first and foremost ensured by selecting the primers and probs. In the first step, the HBV genome sequences submitted in NCBI were used to establish the virus genome Databank. The design of primers and probes was carried out using Beacon Designer 7 Software. The oligonucleotide pairs selected were checked by BLAST analysis.

Standard Concentrations

The analytical sensitivity of the Viga Quantitative HBV Molecular Diagnostic Kit was tasted against dilution series an international standard (Acrometrix: 625607) for diagnostic setups based on HBV DNA detection. HBV Molecular Diagnostic kit contents (contains plasmid) were determined based on Acrometrix international standards.

Table 9: Analytical sensitivity results of Viga Quantitative HBV Molecular Diagnostic kit

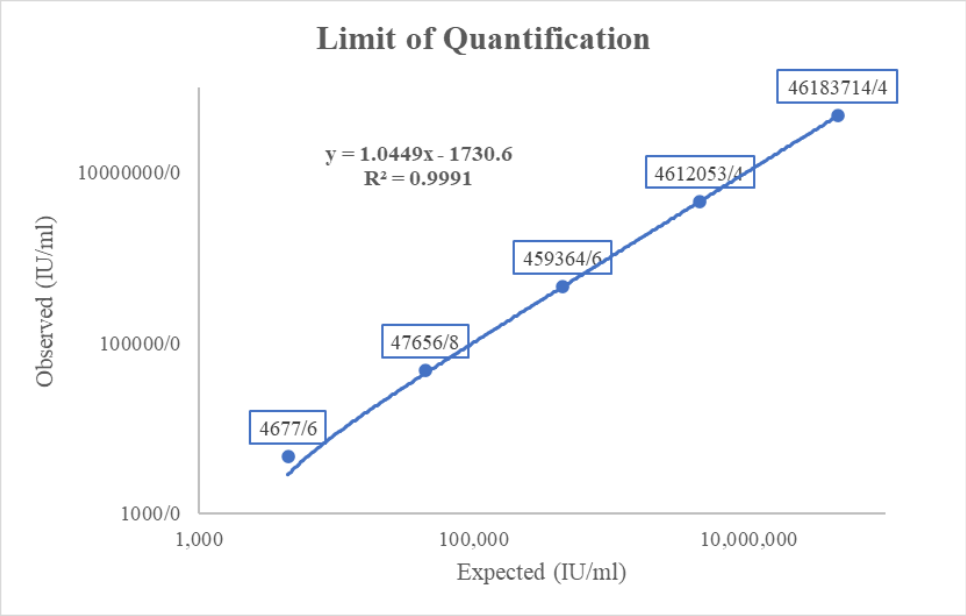
Diagnosis of hepatitis B virus DNA based on Viga Quantitative HBV molecular diagnostic kit	Viral load [IU/mL]	Average ct results for three repetition
	2,000,000	23.94
	200,000	27.57
	20,000	31.16
	2,000	34.81
	2,00	37.18

Limit of Quantification (LOQ)

Determine the lowest level of analyte concentration that can be quantified in the assay, with acceptable accuracy and precision. LOQ is determined based on five standard dilution series from 4,420 to 44,200,000 IU/ml carried out on 14 replicates. The quantification results show that the Viga quantitative HBV molecular diagnostic kit can be quantified up to a concentration of 4420 IU/ml with acceptable accuracy. Results are provided in table 10.

Table10: Limit of quantification results

HBV DNA Calibrator	Expected Results (IU/ml)	Mean of three runs for each concentration	CV of three runs for each concentration
Neg	0	0	N/A
E1	4,420	4677.6	4.0
E2	44,200	47656.8	6.1
E3	442,000	459364.6	1.7
E4	4,420,000	4612053.4	2.4
E5	44,200,000	46183714.4	3.6



Linearity

Determine the degree to which the response curve for a quantitative assay approximates a straight line by evaluating the span of analyte concentrations for which the assay output is directly proportional to the analyte concentration. The Linear range of Viga Quantitative HBV Molecular Diagnostic Kit ranging 10^9-10^2IU/ml allows evaluation of limited concentration range.

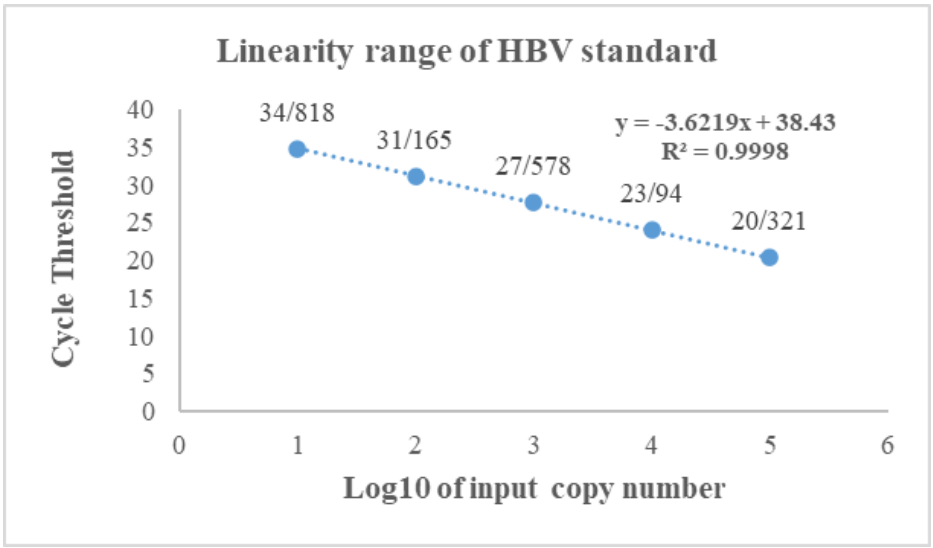


Figure 11: Liner variance for internal control

Limit of detection (LOD)

LoD studies were used to determine the lowest detectable concentration of HBV DNA, at which approximately 95% of all (true positive) replicates test positive. The LoD was determined by limiting dilution studies using characterized samples.

The analytical sensitivity in consideration of the purification (DNJia Virus DNA Kit) of the Viga quantitative HBV molecular diagnostic kit was determined using a dilution series of the 5st ValiQuant HBV DNA Quantification Panel (Acrometrix) standards from 40 to nominal 10 HBV IU/ml spiked in clinical plasma specimens.

The LoD of each test was then confirmed by testing 20 replicates with a dilution series (40, 20, 10 IU / ml) at the tentative limit of detection. The final LoD of each test was determined to be the lowest dilution series resulting in positive detection of 19 out of 20 replicates.

The LoD of the Viga quantitative HBV molecular diagnostic kit was established using DNJia Virus DNA Kit. The results demonstrated that the LoD of assay is 40 IU/mL.

Table 11: LOD results gained from determining 10, 20, 40 IU/ml dilutions after 20 replicates

Test Number	**concentration (IU/mL)**		
	HBV		
	40	20	10
1	41.95	44.53	Undetermined
2	43.9	44.92	Undetermined
3	39.11	40.59	Undetermined
4	41.26	Undetermined	Undetermined
5	41.29	Undetermined	44.9

6	43.59	Undetermined	43.36
7	42.59	Undetermined	Undetermined
8	43.08	Undetermined	Undetermined
9	42.73	Undetermined	Undetermined
10	43.21	42.51	43.48
11	40.71	41.38	43.01
12	41.0	Undetermined	Undetermined
13	43.23	Undetermined	Undetermined
14	40.59	Undetermined	Undetermined
15	40.54	42.54	Undetermined
16	42.51	44.0	44.23
17	40.55	Undetermined	43.5
18	43.25	Undetermined	Undetermined
19	43.44	Undetermined	Undetermined
20	40.21	Undetermined	Undetermined
Positive percentage in each concentration	100%	35%	30%

The limit of detection (LoD) of the Viga Quantitative HBV Molecular Diagnostic Kit is 40 IU/ml.

Inclusivity (analytical sensitivity)

Inclusivity of the primer/probe set used in the Viga quantitative HBV molecular diagnostic kit was analyzed in silico based on HBV sequences from NCBI (8750 sequences) database accessed on September 6, 2021. The primer/probe sets for HBsAg gene sequence alignment analysis demonstrate 100% inclusivity for HBV sequences identified from patient samples. The representative alignment results for HBsAg gene are shown in the table.

Table 12: the results of in silico experiments

Strain	Target	Accession	% Homology Test Forward primer%	% Homology Test Reverse primer%	% Homology Test Probe%
Hepatitis B virus isolate EG7207	HBs-Ag	MW784518.1	100	100	100
Hepatitis B virus OM-AHB	HBs-Ag	LC592170.1	100	100	100
Hepatitis B virus isolate SEN-WS300633	HBs-Ag	MW567980.1	100	100	100
Hepatitis B virus isolate GMB-EG9060	HBs-Ag	MW567973.1	100	100	100
Hepatitis B virus isolate NG/HBV/BD-021	HBs-Ag	MN819062.1	100	100	100
Hepatitis B virus isolate NG/HBV/PH-069	HBs-Ag	MN819056.1	100	100	100
Hepatitis B virus isolate NG/HBV/SD-106	HBs-Ag	MN819055.1	100	100	100
Hepatitis B virus isolate 8990	HBs-Ag	MN845924.1	100	100	100
Hepatitis B virus isolate 3448	HBs-Ag	MN845908.1	100	100	100
Hepatitis B virus isolate 2920	HBs-Ag	MN845902.1	100	100	100

Hepatitis B virus isolate 9-HBV2	HBs-Ag	MW234358.1	100	100	100
Hepatitis B virus isolate 2-HBVA	HBs-Ag	MW234356.1	100	100	100
Hepatitis B virus isolate 2-hbvD	HBs-Ag	MW234355.1	100	100	100
Hepatitis B virus isolate HD-18-098	HBs-Ag	MN996914.1	100	100	100
Hepatitis B virus isolate HD-18-094	HBs-Ag	MN996913.1	100	100	100
Hepatitis B virus isolate P3_D0	HBs-Ag	MW082640.1	100	100	100
Hepatitis B virus isolate coThai1	HBs-Ag	MT111596.1	100	100	100
Hepatitis B virus isolate I9	HBs-Ag	MN562231.2	100	100	100
Hepatitis B virus isolate I4	HBs-Ag	MN562226.2	100	100	100

Based on gained alignment results, oligonucleotides (primers and probes) sequence comparison analysis against publicly available sequences shows 100% overlapping.

Clinical Sensitivity

The wet testing of inclusivity using the DNJia Virus DNA Kit was evaluated as supplemental data by testing three HBV positive specimens. These specimens were confirmed positive by Viga quantitative HBV molecular diagnostic kit. Each specimen was diluted to 400, 4000, and 40000 IU/ml (3log10> LOD, 2log10> LOD, 1log10> LOD) in a negative specimen matrix (Plasma specimen) and tested in the tenth replicate. (Below table).

Table 13: the results of diagnostic sensitivity of Viga Quantitative HBV Molecular Diagnostic Kit ROJETechnology

HBV Negative Samples	Sample	Concentration [IU/ml]	Ct
3log10> LOD	plasma	40000 IU/ml	31.07
			30.83
			30.96
			30.68
			30.79
			30.99

			30.75
			30.96
			30.88
			30.92
2log10 > LOD	plasma	4000 IU/ml	34.19
			34.34
			34.47
			34.3
			34.52
			34.21
			34.46
			34.7
			34.56
			34.56
1log10 > LOD	plasma	400 IU/ml	38.44
			38.21
			38.57
			38.69
			38.38
			38.52
			38.50
			38.49
			38.25
			38.54

Based on table 13, diluted specimens (3log10> LOD, 2log10> LOD, 1log10> LOD) were positive.

Cross-Reactivity (analytical)

Cross-reactivity of the Viga quantitative HBV molecular diagnostic kit was evaluated both in silico analysis and by wet testing potentially cross-reactive whole pathogens or purified nucleic acid from clinical specimens. No cross-reactivity was detected. The in-silico mapping analysis of each primer/probe against several pathogens is based on the NCBI nr/nt database accessed September 6, 2021, using the online BLASTN 2.10.0+, and the representative results are shown below the table. No cross-reactivity was observed for other listed blood-borne pathogens based on both in silico and wet-testing.

Table 14: The In-Silico Specificity Analysis of Primer and Probe Set for Other blood-borne pathogens.

Pathogen (Taxonomy ID)	Strain	Target	GenBank Acc#	% Homology Test FP	% Homology Test RP	% Homology Test Probe
Human immunodeficiency virus	R1041_QCL27_Enr_CE	HBs-Ag	MK512792.1	63%	58%	52%
Hepatitis C virus subtype 1a	HCV-1a/US/BID-V324/2001	HBs-Ag	EU256097.1	45%	58%	44%
HSV-1	DOCK8	HBs-Ag	MN401207.1	54%	64%	56%
Human papillomavirus	HPV-mSK_091	HBs-Ag	MH777234.1	63%	52%	44%
HSV-2	SYD-SCT1	HBs-Ag	MT044485.1	59%	64%	64%
Mycoplasma genitalium	M2288	HBs-Ag	CP003773.1	45%	41%	36%
Chlamydia trachomatis	tet9	HBs-Ag	CP035484.1	90%	58%	52%
Streptococcus agalactiae	Sag27	HBs-Ag	CP031556.1	90%	58%	64%
Human T-cell leukemia virus type I	IR (26)	HBs-Ag	MN453013.1	36%	47%	68%
Human gammaherpesvirus	NPCT115	HBs-Ag	MK540470.1	54%	52%	60%
Human alphaherpesvirus	KPZ13-372	HBs-Ag	MH709376.1	68%	88%	72%
Human T-lymphotropic virus 2	BRSP56501-15	HBs-Ag	KY928507.1	31%	47%	36%
Human parvovirus B19	C39 NS1	HBs-Ag	DQ293995.2	40%	52%	36%
JC polyomavirus	JCV255-01	HBs-Ag	JF425441.1	41%	40%	32%
Neisseria gonorrhoeae	TUM15748	HBs-Ag	AP023071.1	81%	58%	64%
Trichomonas vaginalis	TVAG_228310	HBs-Ag	XM_001580504.1	54%	64%	48%

Cross Reactivity (clinical)

Determination of clinical cross-reactivity was carried out by Viga Quantitative HBV Molecular Diagnostic Kit based on a panel consisting of different concentrations of negative plasma samples. No potential cross-reactivities observed with pathogens.

Table 15: Cross-reactivity of HBV resulted Viga Quantitative HBV Molecular Diagnostic Kit setup

Virus/Bacteria/Parasite	Source/ Sample type	Ct Value (ORF1ab gene/N gene)
Human immunodeficiency virus-1	Clinical sample	**-/-**
Hepatitis C virus	Clinical sample	**-/-**
Cytomegalovirus	Clinical sample	**-/-**
Herpes simplex virus type 1	Clinical sample	**-/-**
Herpes simplex virus type 2	Clinical sample	**-/-**
Human papillomavirus	Clinical sample	**-/-**
Epstein-Barr virus	Clinical sample	**-/-**
Adenovirus	Clinical sample	**-/-**
Influenza A	Clinical sample	**-/-**
Influenza B	Clinical sample	**-/-**

Legionella pneumophila	Clinical sample	**-/-**
Cryptococcus neoformans	Clinical sample	**-/-**
Chlamydia pneumonia	Clinical sample	**-/-**
Streptococcus pneumoniae	Clinical sample	**-/-**
Respiratory Syncytial Virus	Clinical sample	**-/-**
Mycoplasma pneumoniae	Clinical sample	**-/-**
Streptococcus pyogenes	Clinical sample	**-/-**
Mycobacterium tuberculosis	Clinical sample	**-/-**
10 Pooled human genomes	Clinical sample	**-/-**

Based on Viga Quantitative HBV Molecular Diagnostic Kit setup (table 15), RNA and DNA samples supplied from clinical samples ensured no cross-reactivity with other genomes.

Precision

The precision data of the Viga Quantitative HBV Molecular Diagnostic Kit allows the determination of the total variance of the assay. The total variance consists of the intra-assay variability (variability of multiple results of samples of the same concentration within one experiment), the inter-assay variability (variability of multiple results of the assay generated on different instruments of the same type by different operators within one laboratory). Intra-assay variability. Intra-assay variability in Real Time-PCR determines the variability of multiple results of samples of the same concentration within one experiment and use to determine the standard deviation and CV value for different CT values. The intra-assay variability was collected using the

Quantitation Standard of the four concentrations (QS 1- QS 4). Testing was performed with 14 replicates. The obtained range for CV value was 0.42-0.74. The valid range for CV value is ≥5%.

Table 16: Intra-assay variability test results

Standard	Mean of each run	SD of each run	% CV of each run
Std1	23.635	0.100747208	0.426262781
Std2	27.67071429	0.164759232	0.595428184
Std3	31.80142857	0.173243144	0.544765287
Std4	35.46357143	0.265115567	0.747571539

Inter-assay variability

The inter-assay variability in Real Time-PCR determines the variability of multiple assay results generated on different instruments of the same type by different operators within one laboratory. It is used to determine the standard deviation and CV value for different CT values. The inter-assay variability was collected using the Quantitation Standard of the four concentrations (QS 1-QS 4). Testing was performed with 14 replicates on 3 different days. The obtained range for CV value was 0.35-1.76. The valid range for CV value is ≥10%.

Table 17: Inter-assay variability test results

Standard	Mean of three runs	SD of three runs	CV of three runs
Std1	23.94047619	0.265630107	1.109543958
Std2	27.57833333	0.096683086	0.350576246
Std3	31.165	0.551441062	1.769424232
Std4	34.81857143	0.559704277	1.607487768

Clinical evaluation

The clinical performance of the Viga quantitative HBV molecular diagnostic kit was established using 194 plasma specimens collected from patients who were suspected of HBV. The comparator method was the AltoStar® HBV PCR Kit 1.5 (Altona), which received CE-IVD. The extraction

method was the DNJia Virus DNA Kit. Both assays were run on Rotor Q (Qiagen). The results are summarized in the analysis part and demonstrated a PPA of 97.87% and NPA of 100%.

Table 19: Clinical evaluation results

Test		**AltoStar® HBV PCR Kit 1.5 (Altona)**		**Total**
		Positive	Negative	
Viga quantitative HBV molecular diagnostic kit	**Positive**	92	0	92
	Negative	2	100	102
Total		94	100	194

- Positive Agreement Rate: 92/94 ×100%=97.87%
- Negative Agreement Rate: 100/100×100%= 100%
- Overall rates of agreement: (92+ 100) / (92+ 0+ 100+2) ×100%=98.96%

Symbols

symbols	meaning	symbols	meaning
	Date of manufacture		manufacturer
	Expiration Date		Temperature limitation
IVD	In Vitro Diagnostics	LOT	Lot number
		REF	Reference number

Troubleshooting

Here we try to cover as many problems as you may see in using this product; however, scientists in ROJE Technical Support Team are eager to answer all your questions. Do not hesitate to contact us for more information.

Symptoms	Problem	Suggestion
Signals with the negative controls in thefluorescence channel are progressive (false positive curve)	Contamination occurred during the preparation of the PCR	• Repeat the PCR with new reagents in replicates based on instructions given in the handbook
Weak or no signal of the internal control of a negative plasma sample	DNA was not identified	• The PCR conditions do not comply with the protocol • Incorrect application of the instruments • The PCR was inhibited or DNA was lost during extraction • Absence of human cells for amplification
Fluorescent signal is intense or typical S shape is not appeared	Poor quality of extracted DNA Destruction in Real-Time PCR or other instruments.	• repeat test with new DNA • Repeat extraction with validate Kit • Elute extracted DNA at the ratio of 1 l per 10µl elution volume • Repeat test again. Contact with instruments' suppliers

Ordering Information

Category	Product Name	Cat NO.	Size
Molecular Diagnostics	Viga Quantitative HBV Molecular Diagnostic Kit	MD003059	100 Preps
	Viga Quantitative HBV Molecular Diagnostic Kit	MD003058	25 Preps
Related Product	DNall VirAll Kit	DN983053	100 Preps

Technical Assistance

ROJETechnologies guarantee your complete satisfaction. ROJE technical support team is composed of highly trained, experienced scientists; who can troubleshoot most problems you face. Our technical support team can offer expert advice which may help you select a suitable product.

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- You can submit your question directly to ROJE Technical Support Team from our website (www.ROJETechnologies. com)
- Or send your questions to this email address, technicalsupport@rojetechnologies.com.

Factory address:

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