Stark Quantitative HBV Molecular Diagnostic Kit

IVD

CE

Contents

Description

The Stark Quantitative HBV Molecular Diagnostic Kit represents a state-of-the-art in vitro diagnostic assay meticulously crafted for the detection and precise quantification of Hepatitis B Virus (HBV) DNA. Employing cutting-edge TaqMan Real-Time PCR technology, this Kit demonstrates unparalleled sensitivity and specificity in amplifying HBV-specific target sequences within blood samples. Its incorporation of fluorescently labeled target-specific probes enables real-time monitoring of DNA amplification, facilitating the meticulous quantification of HBV DNA content. Equipped with an internal control system to counter potential PCR inhibitors, this Kit assures steadfast and dependable performance in identifying HBV infections. Trusted by healthcare professionals, the Stark Quantitative HBV Molecular Diagnostic Kit furnishes accurate results to assist in patient diagnosis and treatment strategies.

Intended Use

The Stark Quantitative HBV Molecular Diagnostic Kit is intended for in vitro diagnostic use by healthcare professionals. Purposefully crafted for the qualitative detection and quantitative determination of Hepatitis B Virus (HBV) DNA in blood samples, the results derived from this test are intended for clinical interpretation. They serve to aid healthcare professionals in diagnosing HBV infections, monitoring disease progression, and informing treatment decisions. Meticulous adherence to the provided instructions is imperative to ensure the attainment of accurate and dependable results.

Kit Content

Ingredients	25 Preps (REF: ST242005)	100 Preps (REF: ST242007)
Pro HBV Mix	220µl	875µl
QR-ROMAX, 4X	160µl	625µl
IC	125µl	500µl
HBV *QS1(1×105 IU/µl)	65µl	250µl
HBV *QS2(1×104 IU/µl)	65µl	250µl
HBV *QS3(1×103 IU/µl)	65µl	250µl
HBV *QS4(1×102 IU/µl)	65µl	250µl
HBV *QS5(1×101 IU/µl)	65µl	250µl
Water for Molecular Biology	125µl	500µl

* Quantitation standard

Storage

All components of the Stark Quantitative HBV Molecular Diagnostic Kit are pre-prepared and ready for immediate use upon arrival. Upon receipt, it is recommended to store all reagents at temperatures ranging from -15°C to -30°C. These conditions ensure stability and maintain the integrity of the components until the expiration date indicated on the label.

Guarantee and Warranty

CARBON Technologies LLC stands behind the efficacy of all manufactured Kits and reagents. If you need assistance in choosing the right Kits for your needs, our technical support team is available to provide guidance. Should the products not meet your expectations due to reasons other than misuse, please do not hesitate to contact our technical support team. In the rare event of issues arising from the manufacturing process, CARBON Technologies LLC will promptly replace the Kit.

Warning and Precautions

- This Kit is intended for in vitro diagnostic use only.
- Material Safety Data Sheets (MSDS) for all products and reagents can be found online at www.carbontechnologiesco.com.
- Adhere diligently to laboratory safety protocols.
- Familiarize yourself thoroughly with the guidelines before usage.
- Refrain from eating, drinking, smoking, chewing gum, applying cosmetics, or taking medicine in laboratories where hazardous materials and human samples are handled.
- Treat all patient samples and positive controls as potentially infectious.
- Use the Stark Genotyping HPV Molecular Diagnostic Kit under the supervision of a physician for emergency and in vitro diagnostic purposes.
- Ensure all procedure steps, including sampling, storage, shipping, and laboratory tests, comply with biosafety and molecular laboratory management standards.
- Equip clinical laboratories with instruments and operators in accordance with the regulations of the Ministry of Health.
- Any alteration or replacement of Kit contents may affect functionality and contravene product licensing.
- Utilize sterile and DNase-RNase-free pipette tips and microtubes to prevent contamination. Change filter pipette tips after each substance or sample addition.
- Dispose of waste following biosafety guidelines. Regularly sanitize desks and laboratory instruments with 70% Ethanol or 10% Sodium Hypochlorite.
- Shield Pro Mixes from sunlight exposure.
- Promptly clean and disinfect specimen spills using suitable disinfectants adhering to national and local regulations.
- Dispose of all specimens, reagents, and potentially contaminated materials following national and local regulations.
- The hazard and precautionary statements provided apply to the components of the Stark Genotyping HPV Molecular Diagnostic Kit.

Quality Control

The Stark Genotyping HPV Molecular Diagnostic Kit undergoes rigorous testing through predetermined experiments on a lot-to-lot basis to guarantee consistent product quality. Accessible results of these experiments can be obtained online by referencing the REF and Lot numbers at www.carbontechnologiesco.com.

Materials Required (but Not Provided)

- Always wear a suitable lab coat, disposable gloves, and protective goggles when working with chemicals. Refer to the appropriate safety data sheets (SDSs) from the product supplier for further information.
- Vortex mixer.
- Powder-free gloves (disposable).
- PCR tubes/strips in 0.2ml or PCR plates, 48/96 well, suitable for ABI (Applied Biosystems) instruments.
- DNA isolation Kit (DNJia Virus DNA Kit).
- Pipettes (adjustable).
- Sterile pipette tips with filters.
- Cooling block.
- Real-Time PCR machine.

Please note: The present Kit is compatible with all brands of ABI (Applied Biosystem) instruments.

Procedures

The Stark Quantitative HBV Molecular Diagnostic Kit is an in vitro diagnostic test designed for the determination and quantification of hepatitis virus DNA. It operates 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 quantity of amplified DNA is evaluated by monitoring fluorescence intensities in Real-Time PCR. Additionally, this method incorporates oligonucleotides with fluorophores attached at the 5' end with FAM as a reporter and at the 3' end with a quencher. Simultaneously, specific primers and probes are developed for the internal control (Exogenous internal control) with fluorophores VIC/HEX attached at the 5' end as a reporter. The internal control (IC) is added manually at the initial stages of the extraction or directly into the reaction. The specific probe for hepatitis virus DNA is labeled with FAM (green), while the internal control is labeled with VIC (yellow).

Applications

The Stark Quantitative HBV Molecular Diagnostic Kit offers a convenient and ready-to-use system for detecting Hepatitis B Virus DNA utilizing polymerase chain reaction (PCR) along with primers and specific fluorescent probes.

Storage and sample transportation

- Transport samples following specific precautionary procedures for pathogens, ensuring transportation does not exceed six hours.
- All samples must be transported at temperatures ranging from 2°C to 8°C, while plasma samples should be kept at -20°C.
- Whole blood should undergo separation into plasma and cellular components via centrifugation at 1200-1600 rpm for 20 minutes. Transfer the extracted plasma into sterile Eppendorf tubes.
- Avoid freezing blood samples, as routine freezing or prolonged storage can potentially reduce the assay's sensitivity.
- After extraction, isolated Hepatitis B Virus encapsulated DNA remains 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

Remove each component from the Kit and place them on the benchtop. Allow the reagents to equilibrate to room temperature. Subsequently, briefly vortex each tube in preparation for later use.

Molecular diagnostic tests based on nucleic acids utilizing polymerase chain reaction (PCR) represent highly sensitive and specific methods for detecting the Hepatitis B Virus in blood samples. Prior to PCR reaction, HBV antigen and present antibodies can be identified via ELISA. In cases of positivity or potential false-negative results, quantitative PCR testing becomes necessary, as the accuracy of diagnosis, assessment of drug resistance, and determination of illness severity rely on precise and effective estimation of blood virus load. Real-Time PCR emerges as the most convenient method for assessing isolated DNA and RNA concentration, utilizing an external standard control standard curve to quantify HBV-specific DNA in a sample. Consequently, a blank sample (consisting of a conserved sequence of HBV genome) is estimated against a standard curve of homologous DNA with varying concentrations (amplified HBV). The primary advantage of the provided Kit lies in its ability to cover a wide range of concentrations, facilitating the assessment of highly concentrated HBV without the need for elution. Thus, the HBV diagnostic Kit should possess the capability to specifically diagnose HBV virus and efficiently detect various strains of hepatitis virus.

The Stark Quantitative HBV Molecular Diagnostic Kit is based on Real-Time PCR technology, employing polymerase chain reaction to amplify HBV-specific target sequences and fluorescently labeled target-specific probes for DNA amplification. In addition to the HBV DNA-specific amplification and detection system, the present Kit includes oligonucleotides for amplification and detection of the internal control (IC), which helps mitigate potential inhibitions

during PCR reaction. This component can be introduced either at the beginning of the purification procedure to oversee the entire process (including isolation and PCR) or during the PCR reaction itself, effectively minimizing PCR inhibitors. Importantly, parallel control tests demonstrate no cross-reaction or impact on the HBV virus, human genome, or other common viruses present in human blood. Notably, the Stark Quantitative HBV Molecular Diagnostic Kit enables in vitro amplification of Hepatitis B Virus DNA followed by the quantitative determination of DNA content. This Kit is designed to operate across all brands of ABI (Applied Biosystems) instruments.

- Kit Contents Introduction

For mass screenings, swift and precise detection of HBV is paramount. Real-Time PCR HBV Kits, boasting high sensitivity, fulfill these requirements adeptly. Apart from the specific amplification of the HBV genome, this method incorporates oligonucleotides for the direct detection of the internal control (IC). The IC should be manually added at the onset of the nucleic acid purification procedure. Probes specific to Hepatitis B Virus DNA are labeled with the fluorophore FAM™ (green), while the IC is labeled with a fluorophore detectable in the VIC™ (yellow) channel.

QR-ROMAX and Pro HBV Mix solutions

These solutions encompass all essential components, including PCR buffer, DNA polymerase enzyme, magnesium salt, primers, and probes, facilitating the PCR-mediated amplification and target detection of HBV-specific DNA and the internal control (IC) in a single reaction setup.

Quantification Standards (QS)

The Quantification Standards (QS) comprise standard concentrations of HBV-specific DNA (refer to Table 1). These standards are calibrated against the International Standard for HBV DNA for Nucleic Acid Amplification Techniques and adhere to the Clinical Laboratory Standards Institute guidelines. The Quantification Standards serve the purpose of validating the functionality of the HBV DNA-specific amplification and detection system. Additionally, they are utilized to generate a standard curve, facilitating the quantification of HBV-specific DNA in a sample.

Table 1: Quantification Standards

Quantification Standards	IU/µl
HBV QS1	1×10^{5}
HBV QS2	1×10^{4}
HBV QS3	1×10^{3}
HBV QS4	1×10^{2}
HBV QS5	1×10^{1}

- NTC: No Template Control.
- NTC: Contains no HBV-specific DNA but includes the Internal Control template.
- The NTC serves as a negative control for the HBV DNA-specific Real-Time PCR and signifies potential contamination of QR-ROMAX and Pro HBV mix.

Features

Specific features of the Stark Quantitative HBV Molecular Diagnostic Kit.

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 a concentration of 200 IU/ml (code: 625607) in EDTA plasma was prepared, containing 10, 20, and 40 IU/ml. Each dilution was subjected to testing in 20 replicates, with the validated LOD aiming for 19 positive results out of every 20 replicates based on FAD. Data from all runs were consolidated, and probit analysis was conducted to ascertain the 95% LOD value. The limit of detection (LOD) of the Stark Quantitative HBV Molecular Diagnostic Kit is determined to be 40 IU/ml.
Diagnostic Specificity	CI95%: 99.06% –100%
Diagnostic sensitivity	(CI95%: 99.90% –100%) 97.87%
Linear range	109-102IU/ml
Dynamic range	109-40IU/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	ABI (Applied Biosystems) in all models
Fluorescent channels	Green-Yellow

Recommended Starting Material

- DNA Sample Requirement

Prior to commencing, add a 2-5cc blood sample into a tube containing EDTA. Following plasma isolation and DNA extraction, employ 10µl of the entire prepared sample in Real-Time PCR.

- Sample Storage and Preparation
  - The Stark Quantitative HBV Molecular Diagnostic Kit is intended for use with human EDTA plasma samples. Other sample materials have not been validated.
  - Blood should be collected using commercially available standard blood collection systems (e.g., Sarstedt, Becton Dickinson, Greiner, or equivalent).
  - Blood samples should be kept cooled at temperatures ranging from 2°C to 8°C.
  - To generate EDTA plasma, whole blood should be centrifuged according to the instructions provided by the manufacturer of the collection system within 24 hours after collection.
  - 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 before use.
  - Always handle samples as infectious and (bio-)hazardous, adhering to safe laboratory procedures. Promptly use an appropriate disinfectant for sample material spills and treat contaminated materials as biohazardous.
  - Frozen storage of samples does not compromise the performance of the Kit. Ensure that frozen samples

are completely thawed and properly mixed before use.

- Exercise caution regarding the health risks associated with positive samples, following necessary precautionary procedures at all stages, from collection and transportation to Kit application.
- Note that EDTA is the most suitable anticoagulative buffer for the Stark Quantitative HBV Molecular Diagnostic Kit.
- The use of other anticoagulants may not guarantee proper function and results.

Caution: Samples collected in tubes containing heparin as an anticoagulant should not be used.

Before start

- Prior to first use, ensure the intactness and completeness of the Kit contents and reagents.
- Avoid using samples other than human plasma to prevent incorrect in vitro diagnostic (IVD) examination results.
- Misuse of the reagents may result in contamination and invalid results.
- Utilize RNAse/DNAse free pipette tips with filters for sampling.

Buffer preparation

Refer to Table 2 and 3 for the required information to prepare the buffer.

Master Mix Preparation

Prepare the Master Mix according to the information provided in Table 4 and Table 5. Ensure to prepare the Master Mix for single-use only. Avoid adding QR-ROMAX to Pro HBV Mix if testing is not required. If using the internal control, refer to the relevant information in the handbook.

Table2: Reagents 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
QR-ROMAX, 4X	6.25µl
Purified DNA	10µl

Table 3: Required volumes for standard tubes

Standards	Volume per tube	Pro HBV Mix + QR-ROMAX, 4X per reaction
HBV QS1	10µl	15µl
HBV QS2	10µl	15µl
HBV QS3	10µl	15µl
HBV QS4	10µl	15µl
HBV QS5	10µl	15µl

Table 4: Required volumes for every single test tube

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

Table 5: Required volumes for negative control tubes

Volume per tube of water*	Pro HBV Mix + QR-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.

Pathogenesis

The Hepatitis B Virus (HBV) is responsible for the disease hepatitis B and possesses unique characteristics among human viral pathogens. It is a DNA virus that replicates via an RNA intermediate, placing it within the category of reverse transcribing DNA and RNA viruses. HBV belongs to the Hepadnaviridae family of viruses, which comprises genotypes A-H. The genomic structure of HBV is compact yet complex, capable of encoding seven distinct proteins within its 3.2 kb genome. These proteins include 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).

Transmission of HBV occurs primarily through blood or other body fluids and can survive outside the body for up to seven days. Common transmission routes include perinatal mother-to-infant transmission or horizontal transmission among children under 5 years old. Sources of infection may also include medical instruments used in surgery, tattooing needles, or razors contaminated with blood. The virus typically manifests 30 to 60 days after infection and can persist in the body, potentially leading to the development of chronic hepatitis B.

Symptoms of hepatitis B infection can vary from jaundice (yellowing of the skin and eyes) and dark urine to extreme fatigue, nausea, vomiting, and abdominal pain. Symptoms may persist for several weeks, although carriers of the virus can remain asymptomatic. The most severe outcomes include acute or chronic hepatitis, which may progress to liver cirrhosis or hepatocellular carcinoma (HCC). Currently, there is no cure for hepatitis B, but medications are available for symptom management and slowing the progression of cirrhosis.

Despite vaccination efforts, HBV infections remain prevalent worldwide, with approximately 240 million people suffering from chronic HBV infection and 887,000 HBV-related deaths annually (numbers increasing since 2015). The highest prevalence of Hepatitis B Virus is observed in the Western Pacific and Africa, where 6.2% and 6.1% of the adult population are infected, respectively. Infections are also prevalent in WHO-specified regions such as the Eastern Mediterranean, South-East Asia, Europe, and the Americas. Hence, there is a critical need for viral hepatitis B testing as an essential component of prevention and treatment efforts.

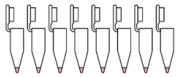
Workstation Preparation

All work areas, samplers, centrifuges, and related equipment must maintain sterility throughout the testing process. In case of nucleic acid contaminations, employ decontaminants such as Sodium hypochlorite 10%, ethanol 70%, and RNZO to ensure thorough decontamination.

Protocol

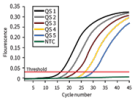
Step 1: Before extraction add 0.1µl internal control/µl of final elution to isolated nucleic acid

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



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

Step 1: Preparing Master Mix

Step 2: Add 15 µl Master Mix to new tube

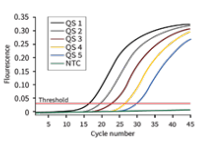
Step 3: : Add 1 µl internal control to step 2

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



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 QR-Romax,4×. For more information, refer Master mix preparation.

Process

- Switch on the ABI Step One/Step One Plus instrument.
- Turn on the computer and launch the ABI Step One/Step One Plus software.
- Begin the PCR setup according to the PCR Run Program outlined in Table 6.

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

- Ensure acquisition is obtained in both the green channel (HBV target detection channel) and the yellow channel (IC target detection channel). Refer to the ABI Step One/Step One Plus instruction manual for detailed guidance.
- Upon launching the ABI Step One/Step One Plus software, open a new experiment window by selecting "New Experiment," and fill in the required information and select the intended options as depicted in Figure 1.
- Depending on the instrument brand, choose the appropriate options:

    - For StepOnePlus instrument (96 wells) or StepOne instrument (48 wells)
    - Select "Quantitation standard curve" based on the Stark Quantitative HBV Molecular Diagnostic Kit.
    - Choose "TaqMan reagents."
    - Select "Standard" run mode, which takes approximately 2 hours to complete a run.

- In the next step, click on "plate setup" and define the first and second targets as FAM and VIC/HE, respectively, and identify their colors as green and yellow (refer to Figure 2).
- Click on "Assign target and sample" and enter both the sample and IC names based on the order in which the wells are filled. Set the Passive reference state to ROX.
- For identifying standards, in the "Assign target and sample" dialogue, define each well for each standard and its concentration in the "Define and set up standards" section (refer to Figure 4).
- In the "Run method" dialogue, optimize the PCR program based on the aforementioned thermal profile in the Kit's protocol. Click on "Add stage/step" to add more steps if needed. Click on "Run" and then "Save" to start the reaction (refer to Figures 1-5).

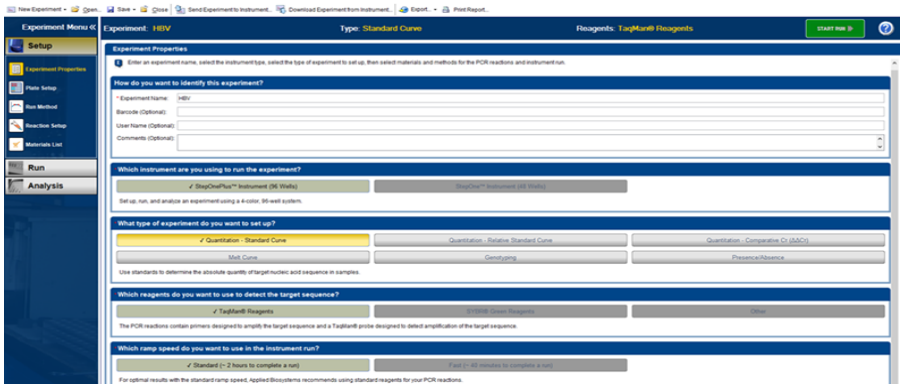


Figure1: Set up test is started from a New experiment.

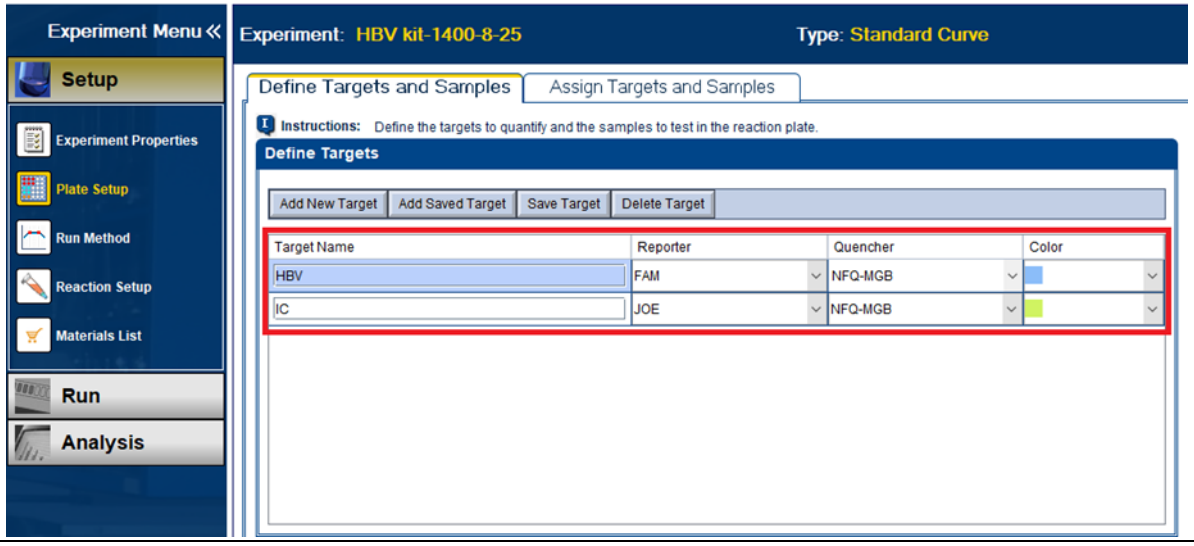


Figure 2: Define color the green and yellow for FAM and VIC/HEX.

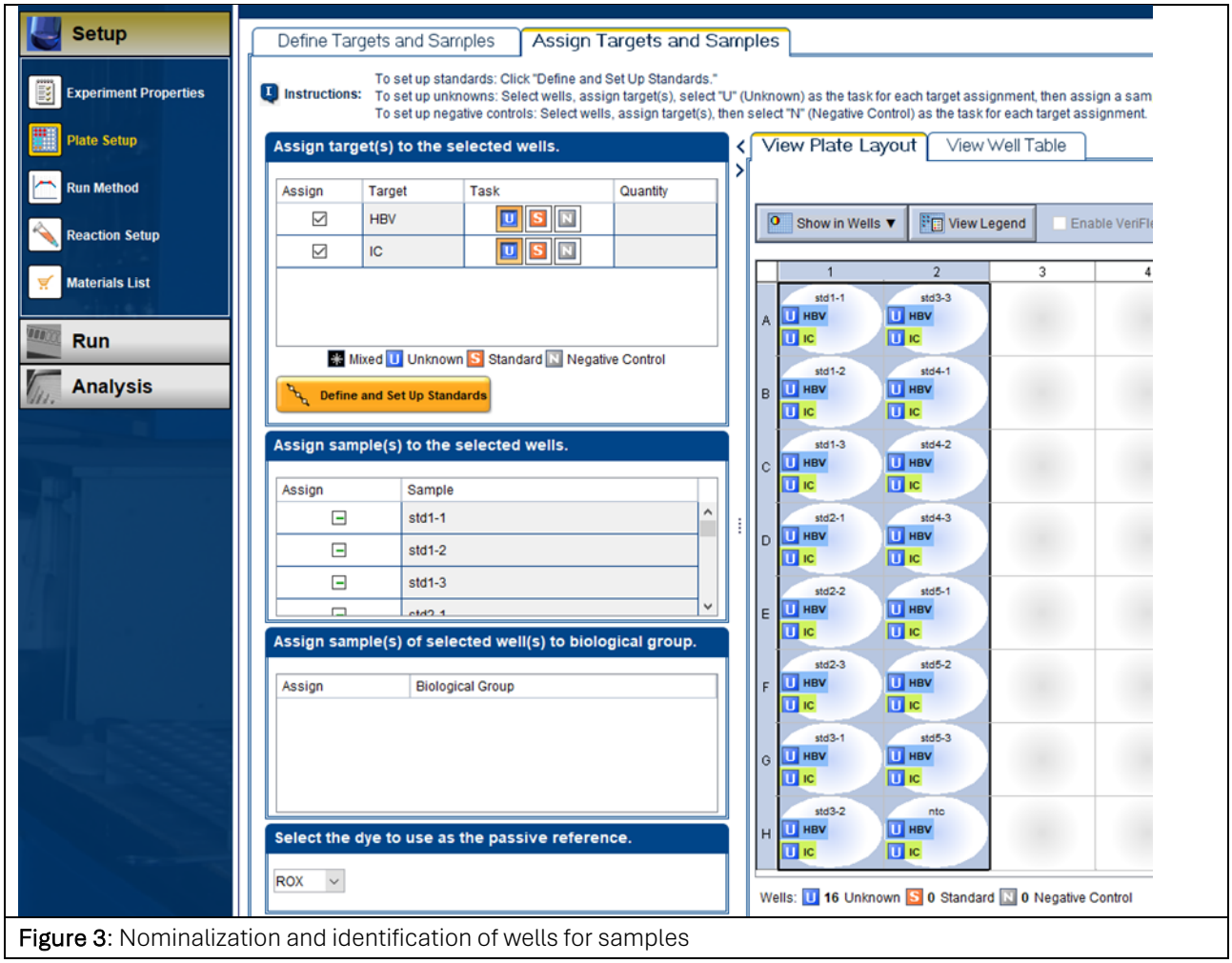


Figure 3: Nominalization and identification of wells for samples

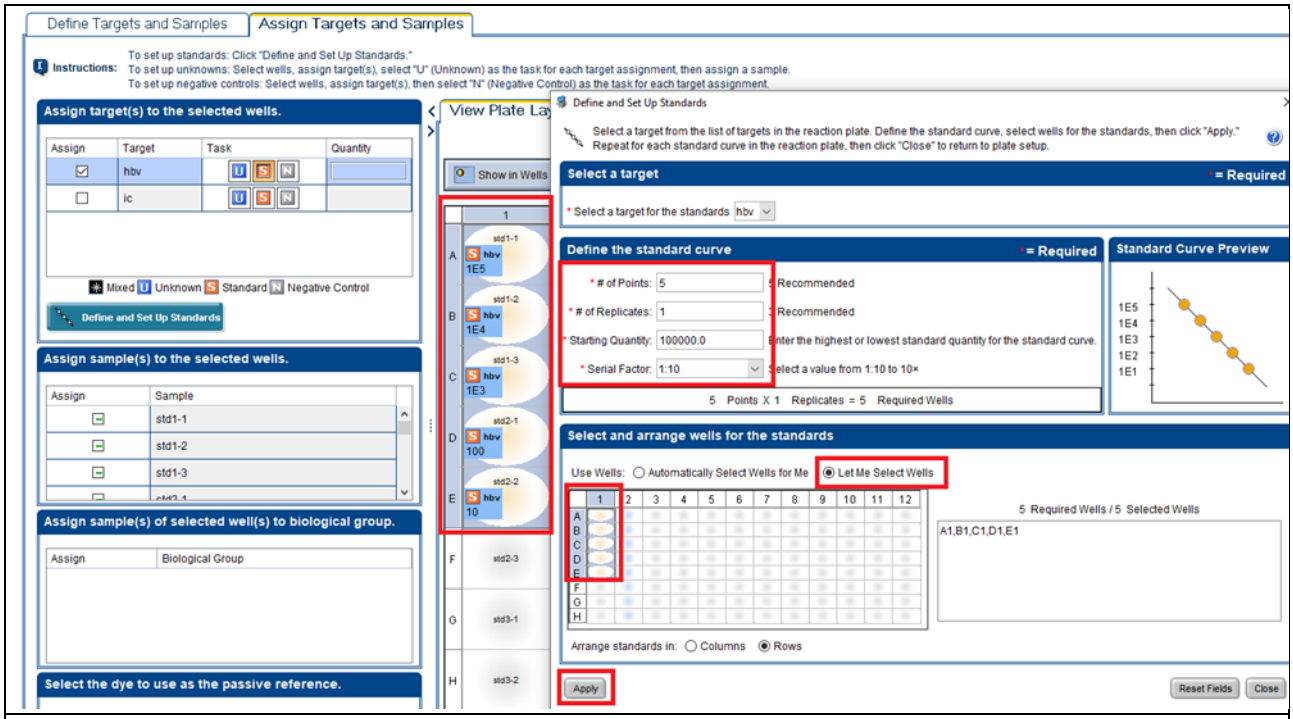


Figure 4: Definition of standards.

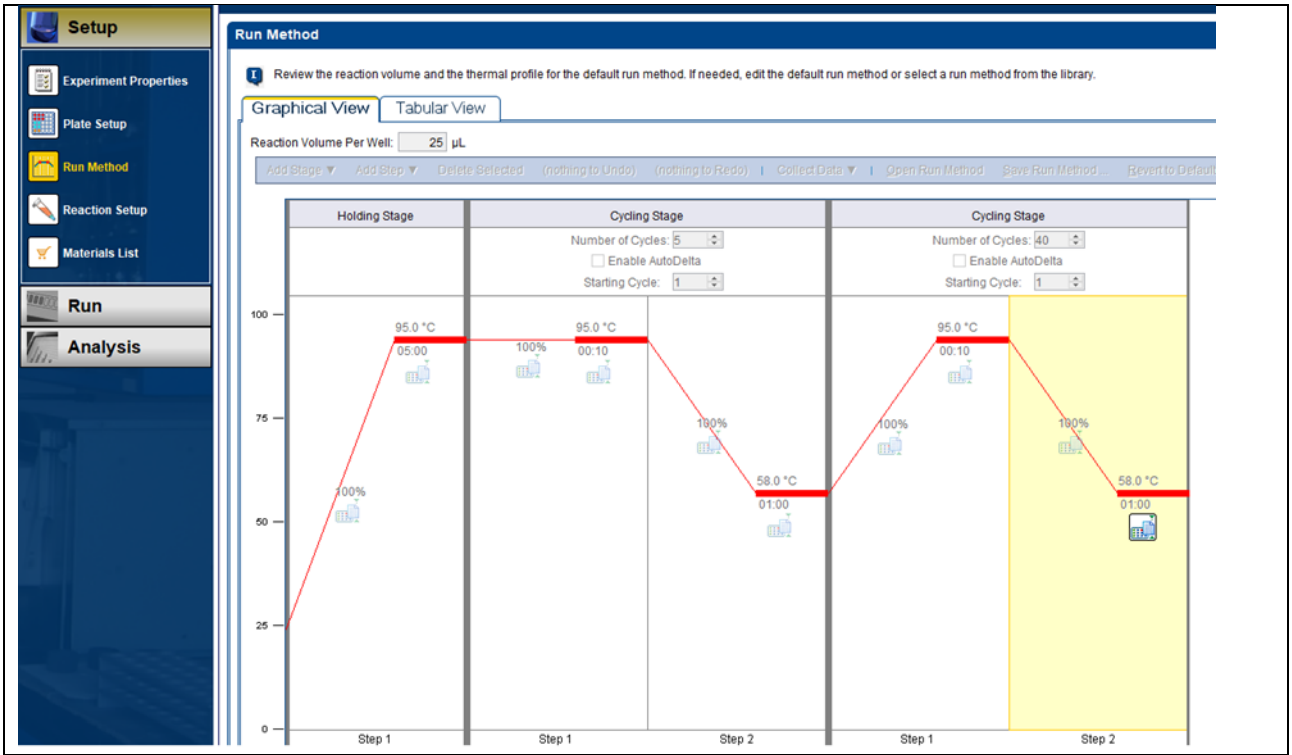


Figure 5: Optimization of thermal profile for PCR run.

Results Interpretation

The Quantification Standards provided in the Stark Quantitative HBV Molecular Diagnostic Kit utilize a standard panel comprising identified concentrations of HBV DNA. When preparing both sample and standard concentrations, add 10µl of isolated DNA to the 15µl Master Mix. The Quantification Standards serve to generate the standard curve, enabling the quantification of HBV-specific DNA concentration in the sample.

Enter the qualification standard in the ABI-specific software in IU/ml. Follow the provided formula to convert IU/µl, as supplied by the Kit contents.

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

If the volume of whole plasma were 200µl and elution 50µl, the first standard would be 2.5×10^{7} IU/ml entered in ABIe-specific software.

Validity of a Diagnostic PCR Run

A diagnostic PCR run is considered valid if it meets 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.

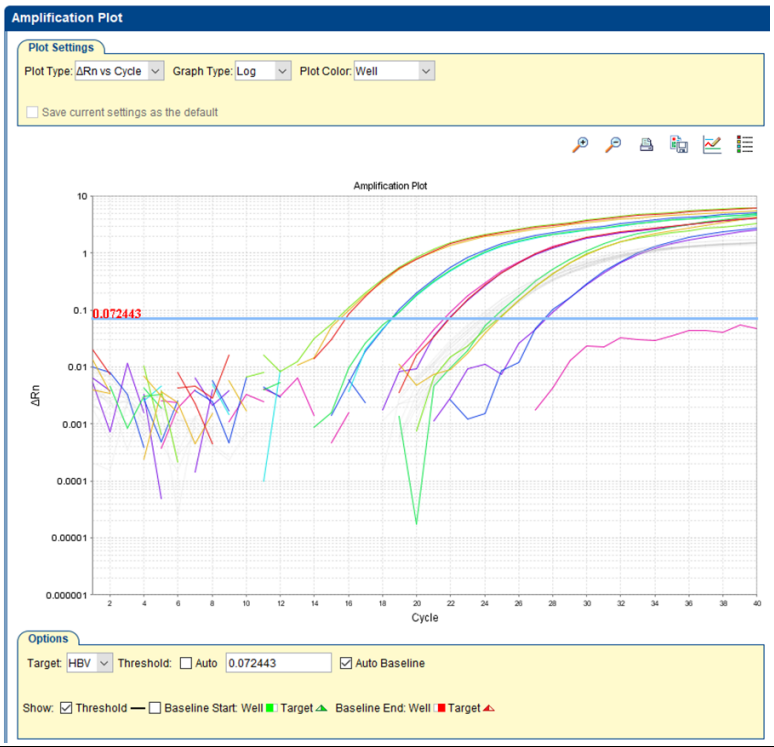


Figure 6: Standard curve for HBV in FAM channel provided by Step one/step one plus instrument.

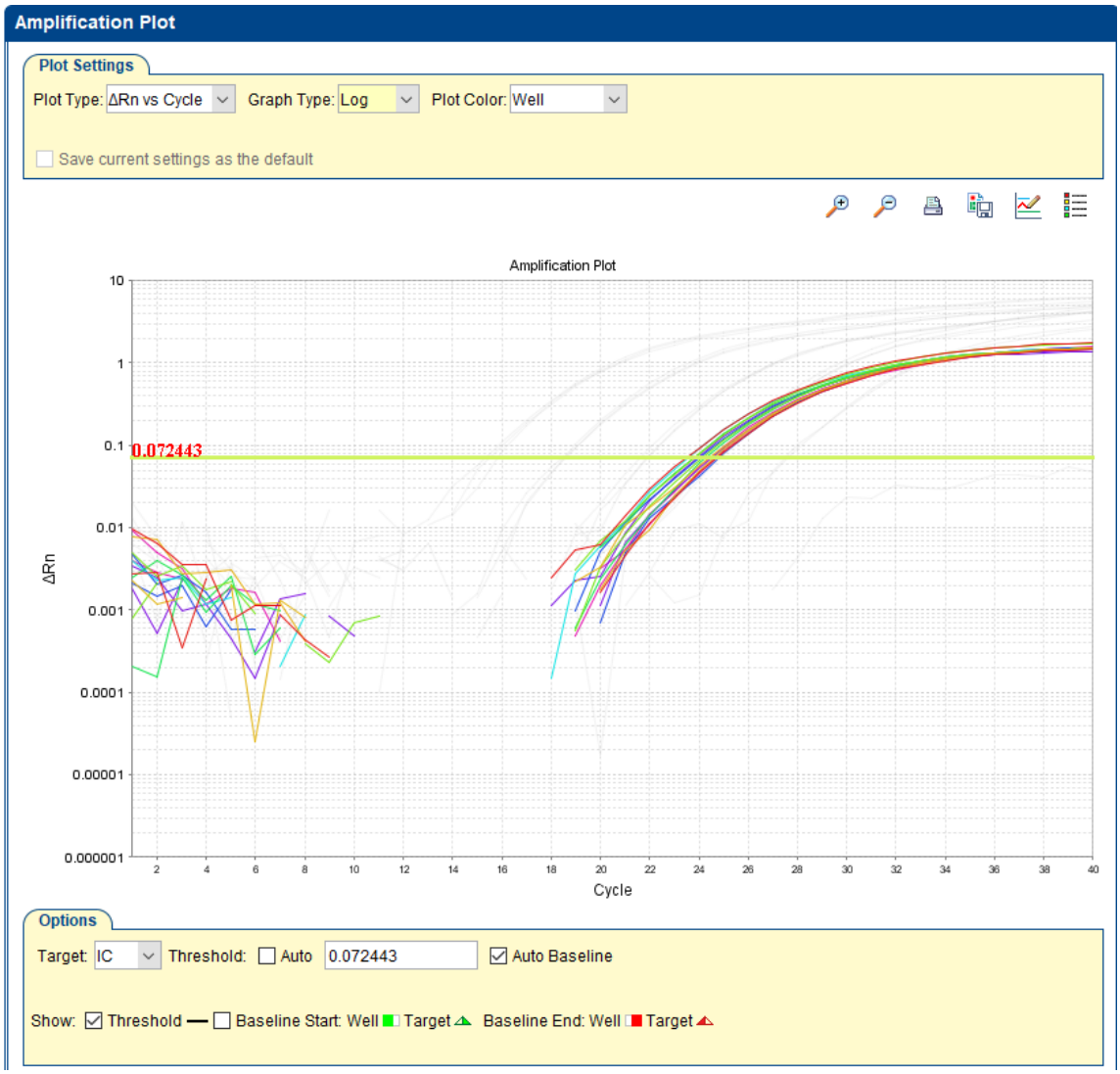


Figure 7: IC graph in VIC/HEX channel.

Test Limitation

- All reagents are exclusively intended for in-vitro diagnostics.
- The product is to be used by personnel specially instructed and trained in in-vitro diagnostics.
- Strict adherence to the user manual is necessary for optimal PCR results.
- Attention should be given to the 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 Stark Quantitative HBV Molecular Diagnostic Kit was tested using a dilution series of an international standard (Acrometrix: 625607) for diagnostic setups based on HBV DNA detection. The contents of the HBV Molecular Diagnostic Kit, which include plasmid, were determined based on Acrometrix and international standards.

- Analytical Specificity

The specificity of the Stark Quantitative HBV Molecular Diagnostic Kit is primarily ensured by the selection of primers and probes. In the initial step, HBV genome sequences submitted to NCBI were utilized to establish the virus genome Databank. Subsequently, primers and probes were designed using Beacon Designer 7 Software. The selected oligonucleotide pairs were then subjected to BLAST analysis to verify their specificity.

Standard Concentrations

The analytical sensitivity of the Stark Quantitative HBV Molecular Diagnostic Kit was assessed using a dilution series of an international standard (Acrometrix: 625607) for diagnostic setups based on HBV DNA detection. The contents of the HBV Molecular Diagnostic Kit, which include plasmid, were determined based on Acrometrix international standards.

Table 9: Analytical sensitivity results of Stark Quantitative HBV Molecular Diagnostic Kit.

	Viral load [IU/mL]	Average ct results for three repetitions
Diagnosis of Hepatitis B Virus DNA based on Stark Quantitative HBV Molecular Diagnostic Kit	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)

The LOQ is defined as the lowest level of analyte concentration that can be quantified in the assay with acceptable accuracy and precision. In this case, the LOQ was determined based on five standard dilution series ranging from 4,420 to 44,200,000 IU/ml, conducted with 14 replicates each. The quantification results indicate that the Stark Quantitative HBV Molecular Diagnostic Kit can accurately quantify HBV DNA concentrations up to 4,420 IU/ml. Results are provided in Table 10.

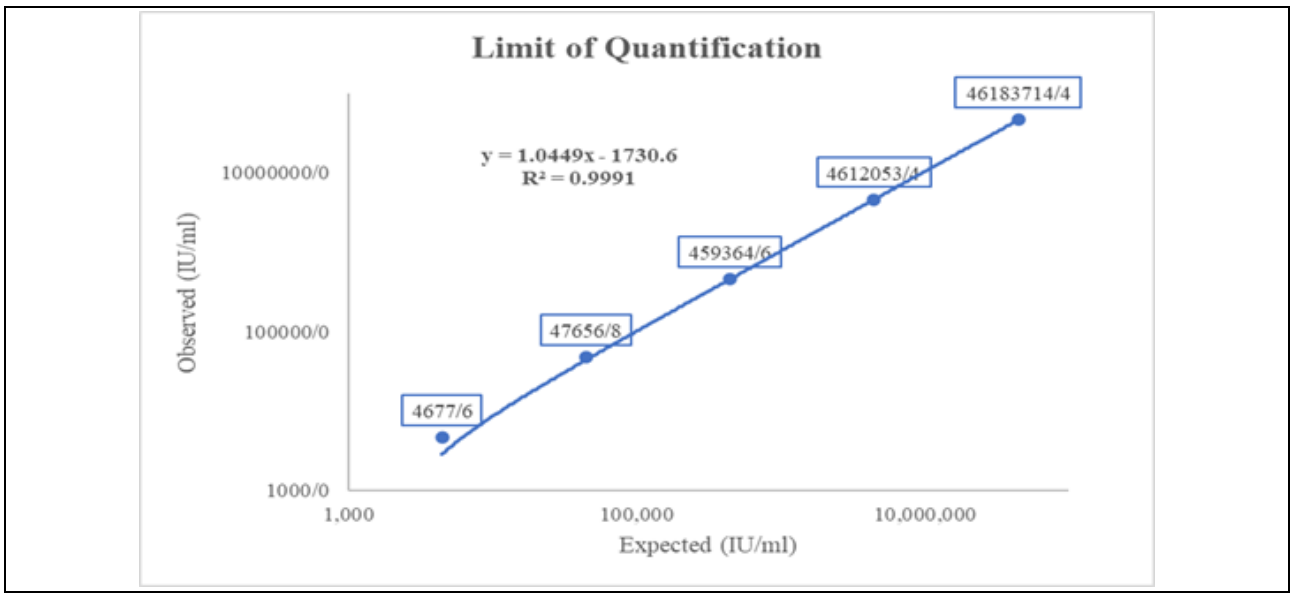


Table10: Limit of quantification results.

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

Figure 8: Limit of quantification for internal control

- Linearity

Linearity refers to the degree to which the response curve for a quantitative assay approximates a straight line, indicating that the assay output is directly proportional to the analyte concentration. The linear range of the Stark Quantitative HBV Molecular Diagnostic Kit ranges from 10^9 to 10^2 IU/ml, allowing for the evaluation of analyte concentrations within this limited range. This range represents the span of analyte concentrations for which the assay output demonstrates a linear relationship with the analyte concentration.

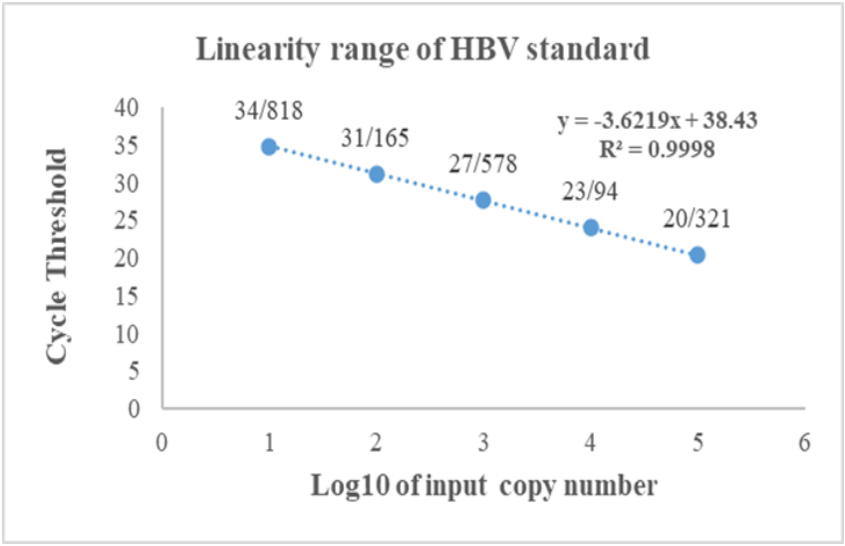


Figure 9: Liner variance for internal control

- Limit of detection (LOD)

The Limit of Detection (LoD) of the Stark Quantitative HBV Molecular Diagnostic Kit was determined through rigorous studies to establish the lowest detectable concentration of HBV DNA. These studies involved limiting dilution experiments using characterized samples.

The analytical sensitivity, considering the purification process utilizing the DNJia Virus DNA Kit, was assessed using a dilution series of the 5th ValiQuant HBV DNA Quantification Panel (Acrometrix) standards. These standards ranged

from 40 to nominal 10 HBV IU/ml spiked in clinical plasma specimens.

To confirm the LoD, 20 replicates were tested with dilution series (40, 20, 10 IU/ml) at the tentative limit of detection. The final LoD was determined as the lowest concentration in the dilution series resulting in positive detection in 19 out of 20 replicates.

The LoD of the Stark Quantitative HBV Molecular Diagnostic Kit, established using the DNJia Virus DNA Kit, was determined to be 40 IU/mL. This value indicates the lowest concentration of HBV DNA that can be reliably detected by the assay.

Table 11: LOD results gained from determining 10, 20, and 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 Stark Quantitative HBV Molecular Diagnostic Kitis 40 IU/ml.

- Inclusivity (analytical sensitivity)

The inclusivity of the primer/probe set utilized in the Stark Quantitative HBV Molecular Diagnostic Kit was assessed in silico using HBV sequences retrieved from the NCBI database. This analysis involved examining 8750 sequences accessed on September 6, 2021.

The primer/probe sets designed for the HBsAg gene sequence alignment analysis demonstrated complete inclusivity for HBV sequences identified from patient samples. Representative alignment results for the HBsAg gene are provided

in the table, confirming the robustness and effectiveness of the primer/probe set in detecting HBV sequences across a diverse range of samples.

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**

Wet testing of inclusivity using the DNJia Virus DNA Kit served as supplemental data, evaluating three HBV-positive specimens. These specimens were confirmed positive by the Stark Quantitative HBV Molecular Diagnostic Kit. Each specimen was diluted to concentrations of 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. (See table below).

Table 13: The results of diagnostic sensitivity of Stark Quantitative HBV Molecular Diagnostic Kitby CARBON Technologies LLC

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 Stark Quantitative HBV Molecular Diagnostic Kit was assessed through in silico analysis and wet testing of potentially cross-reactive whole pathogens or purified nucleic acid from clinical specimens. No cross-reactivity was detected.

In-silico mapping analysis of each primer/probe against various pathogens was conducted using the NCBI nr/nt database accessed on September 6, 2021, through the online BLASTN 2.10.0+ tool. Representative results are provided in the table below. Notably, 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 Stark Quantitative HBV Molecular Diagnostic Kitbased on a panel consisting of different concentrations of negative plasma samples. No potential cross-reactivities were observed with pathogens.

Table 15: Cross-reactivity of HBV resulted in Stark 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 the setup of the Stark Quantitative HBV Molecular Diagnostic Kit (as detailed in table 15), it was confirmed that RNA and DNA samples obtained from clinical specimens exhibit no cross-reactivity with other genomes.

- Accuracy

The precision data of the Stark Quantitative HBV Molecular Diagnostic Kit enables the determination of the total variance of the assay, encompassing both intra-assay and inter-assay variability.

Intra-assay variability

Intra-assay variability assesses the variability of multiple results of samples with the same concentration within a single experiment. This is determined by calculating the standard deviation and coefficient of variation (CV) for different cycle threshold (CT) values. Intra-assay variability was evaluated using the Quantitation Standards at five concentrations (QS 1- QS 5) with 14 replicates each.

The obtained range for the CV value was 0.42-0.74, falling within the valid range of ≥5%, indicating robust precision of the assay within a single experiment.

Table 16: Intra-assay variability test results

Standard	Mean of each run	SD of each run	% CV of each run

Std1	20.20214286	0.122422884	0.605989599
Std2	23.635	0.100747208	0.426262781
Std3	27.67071429	0.164759232	0.595428184
Std4	31.80142857	0.173243144	0.544765287
Std5	35.46357143	0.265115567	0.747571539

Inter-assay variability

Inter-assay variability in Real-Time PCR assesses the variability of multiple assay results generated on different instruments of the same type by different operators within one laboratory. It is utilized to determine the standard deviation and coefficient of variation (CV) for different cycle threshold (CT) values.

To evaluate inter-assay variability, the Quantitation Standards at five concentrations (QS 1- QS 5) were tested with 14 replicates each on three different days. The range obtained for the CV value was 0.35-1.76. It is noted that the valid range for the 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	20.32166667	0.119286427	0.586991358
Std2	23.94047619	0.265630107	1.109543958
Std3	27.57833333	0.096683086	0.350576246
Std4	31.165	0.551441062	1.769424232
Std5	34.81857143	0.559704277	1.607487768

- **Clinical evaluation**

The clinical performance of the Stark Quantitative HBV Molecular Diagnostic Kit was assessed using 194 plasma specimens obtained from patients suspected of HBV infection. The comparator method utilized was the AltoStar® HBV PCR Kit 1.5 (Altona), which is CE-IVD certified. The extraction method employed was the DNJia Virus DNA Kit. Both assays were conducted on ABI (Applied Biosystems) instruments.

The results, summarized in the analysis section, revealed a Positive Percent Agreement (PPA) of 97.87% and Negative Percent Agreement (NPA) of 100%. These findings underscore the robust clinical performance of the Stark Quantitative HBV Molecular Diagnostic Kit in accurately detecting HBV infection in clinical specimens.

Table 18: Clinical evaluation results

Test		AltoStar® HBV PCR Kit 1.5 (Altona)		Total
		Positive	Negative	
Stark 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

 Manufacturer

 Date of manufacture

 Use-by date

 Contains sufficient for <n> tests

 Consult instructions for use

 Biological risks

 Temperature limit

 CE Marking

 EU Representative

 In Vitro diagnostic medical device

 Catalogue Number

 Lot Number

Troubleshooting

For troubleshooting assistance with the Stark Genotyping HPV Molecular Diagnostic Kit, please consult the following guidelines. The CARBON Technologies LLC Technical Support Team is available to address any further questions or concerns you may have.

Symptoms	Problem	Suggestion
Signals with the negative controls in fluorescence channel is progressive (false positive curve)	Contamination occurred during preparation of the PCR	Repeat the PCR with new reagents in replicates based on instructions given in 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 isolated DNA Destruction in Real-Time PCR or other instruments.	- Repeat test with new extracted DNA - Repeat extraction with validate Kit - Elute extracted DNA at the ratio of 1µl per 10µl elution volume - Repeat test again. Contact with instruments' suppliers

Technical assistance

For technical assistance, CARBON Technologies LLC ensures your complete satisfaction. Our technical support team comprises highly trained and experienced scientists adept at troubleshooting most problems you may encounter. They can offer expert advice to help you select the most suitable product for your needs. You can contact our technical support team anytime through the following methods:

- Phone: +96897058350
- Directly submit your questions to the CARBON Technologies technical support team through our website: www.carbontechnologiesco.com
- Email your questions to: technicalsupport@carbontechnologiesco.com

Rest assured, our team is dedicated to providing prompt and effective assistance to address any inquiries or issues you may have. We are committed to ensuring your satisfaction and success in using our products.

Contact Information

EC REP Medunion S.L.
Carrer de Tapioles 33, 2-1, 08004, Barcelona, SPAIN.

Carbon Technologies LLC
Innovation Park Muscat (IPM), P.O. Box 92, Al Khoudh 123, Muscat, OMAN.

24-hour service hotline: +968-97058350

After-sale Service Center: Carbon Technologies LLC

CE
IVD

Release Date: … … … Date of Manufacture: … … …