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Molecular Diagnosis of Viral Hepatitis B Infection

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We use this protocol and it's working

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Disclaimer

This is an optimized protocol for the quantitative detection of Hepatitis B using Zymo Quick-DNA Miniprep Kit (200 prep) and Bosphore HBV Quantitative Kit. The authors do not accept any liability for the collection and handling of both samples and reagents, results from the use of the protocol and its interpretation as well as any errors or omissions that may be made. The reader should make his/her own evaluation as to the appropriateness of the procedures described.

Abstract

With over 400 million HBV infections, viral hepatitis B remains a global public health concern. Diagnosis is primarily based on an immunological assay approach, which utilizes the Hepatitis B surface antigen in detection amongst other markers. This method, however, has several limitations which include the inability to detect mutation in the viral genome resulting in diagnostic escape and low antigen titers in study samples. Using an alternative approach, which is the molecular diagnostic technique such as real-time polymerase chain reaction (RT-PCR) would circumvent the limitations of immune detection. This protocol, thus, provides a step-by-step process of HBV diagnosis using RT-PCR which is a sensitive tool for diagnosis. The steps involved include sample collection and preparation, nuclei acid isolation, HBV detection and quantification using RT-PCR as well as the interpretation of results.

This protocol combines the high nuclei acid yield from isolation using Zymo Quick DNA Mini-prep Kit and Bosphore HBV Quantitative Kit for amplification of HBV DNA. The Bosphore HBV Quantitative Kit has a low detection limit of 1×10^{1} IU/ml and with a turn-around time of less than 4 hours when combined with Zymo Quick DNA Mini-prep Kit. The nucleic acid isolated in this protocol can be amplified using the Bosphore HBV Quantitative kit on several thermocyclers, which makes the protocol robust, cost-efficient, and cost-effective in resource-scarce areas.

Image Attribution

https://www.anatoliageneworks.com/en/diseases/hepatitis-b/

Guidelines

Reagent Preparation for Nucleic Acid Isolation
Nucleic Acid Isolation
Detection and Quantification of HBV Nucleic Acid
Results and Interpretation
Troubleshooting

Materials

Consumables

1. Microcentrifuge tube (1.5 ml)
2. 100 – 1000 µl filtered tips
3. 20 – 200 µl filtered tips
4. 5 – 20 µl filtered tips
5. 96-well PCR plate or 8-well PCR strip
6. Permanent marker

Reagents

1. ZYMO Quick DNA Miniprep Kit
2. Bosphore HBV Quantitative Kit (Includes internal control, standards (4), PCR master mix, positive control and nuclease-free water)
3. Absolute Ethanol (Molecular Grade)
4. Proteinase K

Equipment

1. Thermocycler (Real-Time PCR)
2. Incubator
3. Vortex
4. Microcentrifuge

Troubleshooting

Safety warnings

1. Handle all blood specimens and reagents as a potential biohazard.
2. Discard all waste materials in the appropriate receptacles.
3. β-mercaptoethanol has a pungent smell, it should be opened in a well-aerated space.
4. Ensure the use of the appropriate PPEs at all times

Before start

Sample Collection and Preparation

Aseptically collect venous blood from the antecubital vein of the forearm and dispense it into either a serum separator tube (SST), a plain tube (red top), or an EDTA tube (plasma).
Centrifuge the specimen at 3500 rpm for 10 minutes and transfer the serum or plasma into a sterile plain tube or a cryovial.

NB:

For samples collected into SST or plain tubes, allow the specimen to stand vertically undisturbed for at least 1 hour for the specimen to clot completely before centrifuging it to yield serum
Samples collected into EDTA tubes can be centrifuged immediately to yield plasma.
Care should be taken during sample collection and processing to avoid haemolysis of the specimen

Handling of PCR Reagents

Allow the PCR Master Mix and the other components of the Bosphore HBV Quantification kit to thaw completely at 4°C before use and avoid centrifuging to thaw.

Reagent Preparation for Nucleic Acid Isolation

1 Add 500 µL of β-mercaptoethanol to 100 mL of Genomic Lysis Buffer

Note

Cap the bottle tightly after each use

HBV Nucleic Acid Isolation

5h 2m

2 Transfer 200 µL of serum/plasma to a sterile 1.5 mL microcentrifuge tube (pre-labeled).

3 Add 5 µL of Internal Control (provided with the amplification kit) to each specimen and vortex for 00:00:30

30s

4 Add 400 µL of Genomic Lysis Buffer and 10 µL of Proteinase K to the sample.

5 Vortex and incubate the sample at 56 °C for 3 - 5 hours or Overnight .

5h

6 Vortex the sample at 3000 rpm for 00:00:30

30s

7 Transfer the entire content of the 1.5 ml microcentrifuge tube into a Zymo-Spin IIC Column in a collection tube.

8 Centrifuge at 10000 rpm for 00:01:00 . Discard the flow-through liquid.

1m

9 Transfer the Zymo-Spin IIC column into a new collection tube.

10 Add 200 µL of DNA Pre-Wash Buffer to the spin column and centrifuge 10000 rpm for 00:01:00. Discard the flow-through liquid. 1m

11 10. Add 500 µL of gDNA Wash Buffer to the spin column and centrifuge at 10000 rpm for 00:01:00. Discard the flow-through liquid. 1m

12 11. Transfer the Zymo-Spin IIC Column into a sterile 1.5 mL microcentrifuge tube

13 12. Add 100 µL (70 - 100) of DNA Elution Buffer to the spin column and incubate at Room temperature for 00:30:00 30m

14 13. Centrifuge at 13000 rpm for 00:00:30 to elute the DNA. 30s

15 14. Store the Viral Nucleic acid at -20 °C pending further analysis.

RT-PCR Detection and Quantification of HBV Nuclei Acid

16 The PCR is done in a 25 µL reaction as described below:

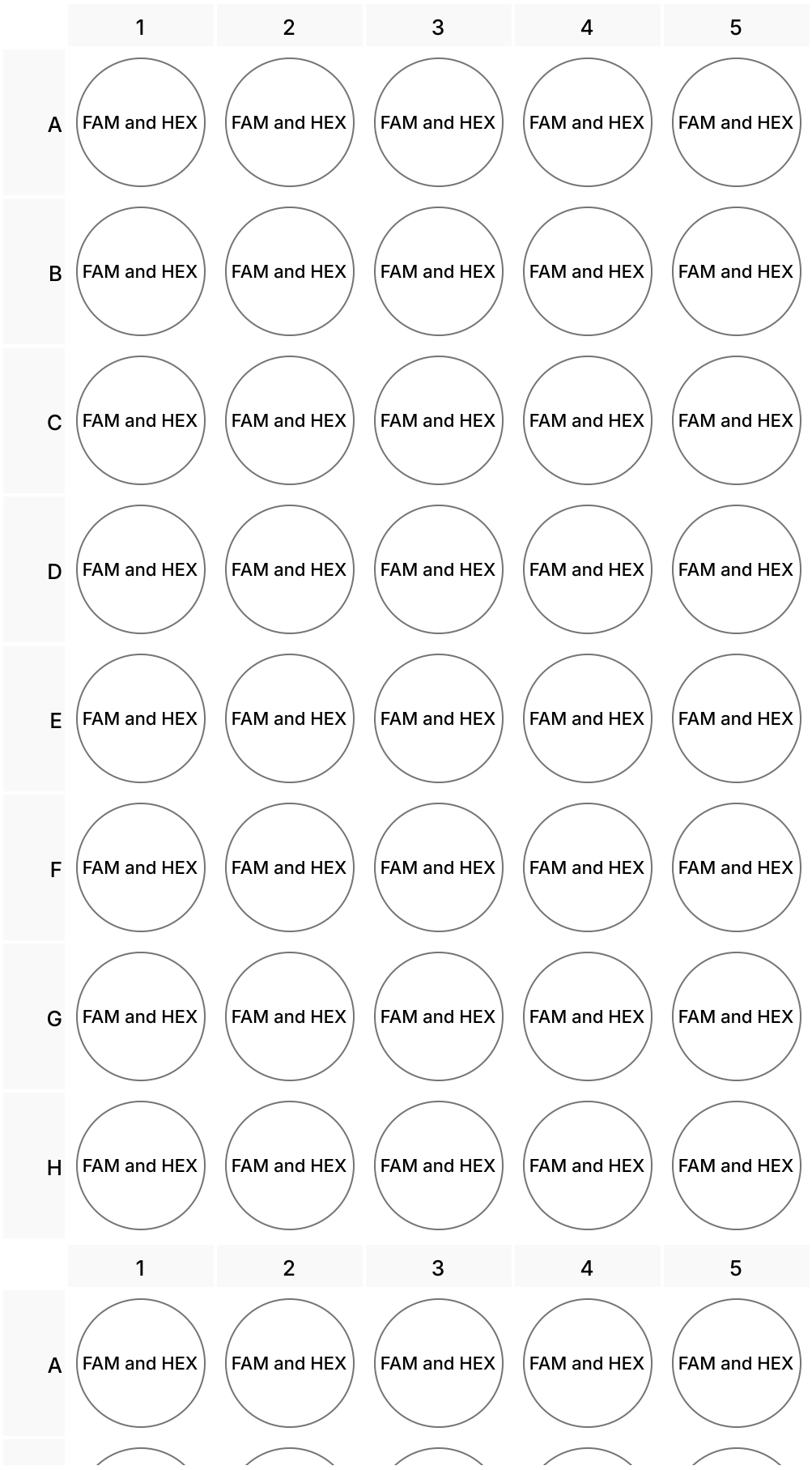
	A	B
	Reagent/Component	**X1 (µL)**
	PCR Master mix	15
	Test Nucleic acid/Standards/Negative Control/Positive Control	10
	Total Reaction Volume	**25 µL**

Allow the PCR Master Mix and the other components of the Bosphore HBV Quantification kit to thaw completely at 4°C before use and avoid centrifuging to thaw.

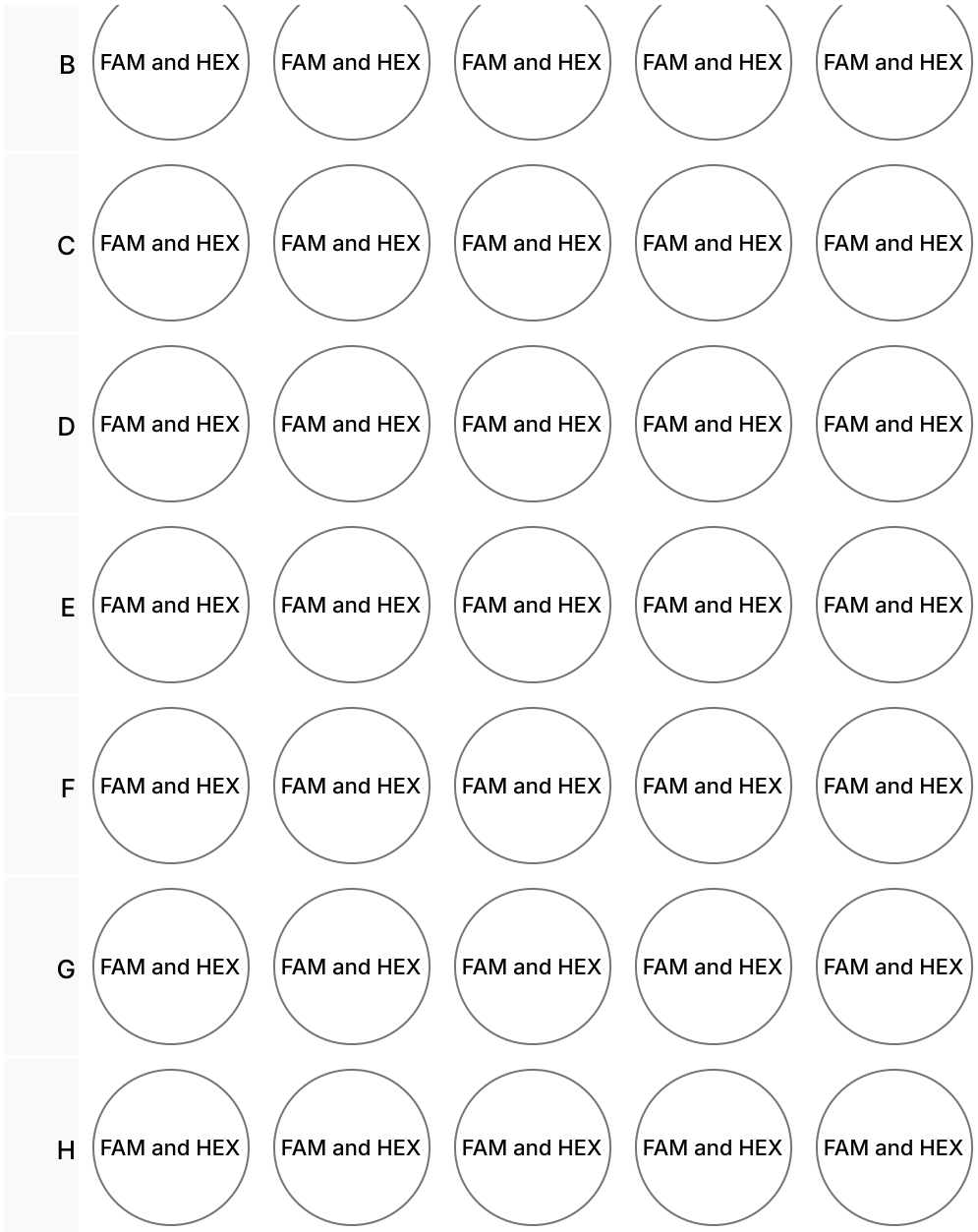
17 The reaction is done under the following cycling conditions:

	A	B	C	D
	Step	Temperature (°C)	Time (minutes)	Cycles
	Initial Denaturation	95	14:30	NA
	Denaturation	97	00:30	50 cycles
	Annealing and Synthesis (Data Collection)	54	01:30	
	Hold	32	01:00	NA

18 Select the appropriate fluorophore depending on the analyzer been used. For Bio-Rad CFX 1000 Series, select the **FAM dye for HBV** and **HEX for Internal Control** (IC).



1
2
3
4
5
A
FAM and HEX


B FAM and HEX FAM and HEX FAM and HEX FAM and HEX FAM and HEX
C FAM and HEX FAM and HEX FAM and HEX FAM and HEX FAM and HEX
D FAM and HEX FAM and HEX FAM and HEX FAM and HEX FAM and HEX
E FAM and HEX FAM and HEX FAM and HEX FAM and HEX FAM and HEX
F FAM and HEX FAM and HEX FAM and HEX FAM and HEX FAM and HEX
G FAM and HEX FAM and HEX FAM and HEX FAM and HEX FAM and HEX
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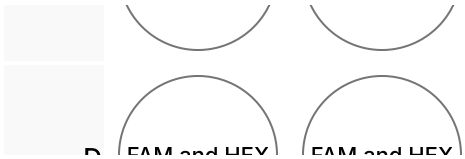
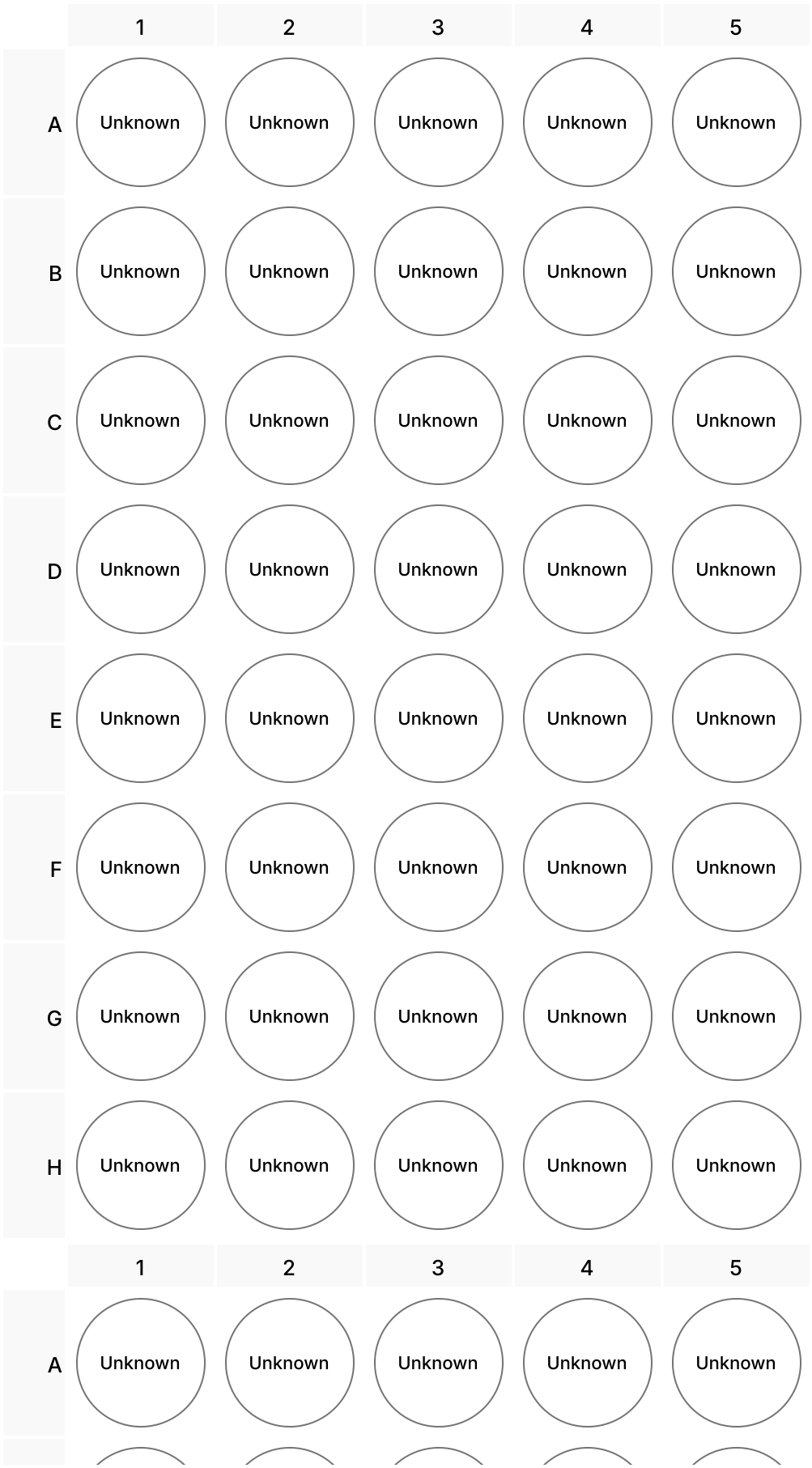
1 2
A FAM and HEX FAM and HEX
B FAM and HEX
C FAM and HEX FAM and HEX



19 Identify unknown samples (test samples), standards, positive control, and negative control on the plate as described below. Assign the right quantitative values to the standards


1
2
3
4
5
A
B
C
D
E
F
G
H
Unknown

B	Unknown	Unknown	Unknown	Unknown	Unknown
C	Unknown	Unknown	Unknown	Unknown	Unknown
D	Unknown	Unknown	Unknown	Unknown	Unknown
E	Unknown	Unknown	Unknown	Unknown	Unknown
F	Unknown	Unknown	Unknown	Unknown	Unknown
G	Unknown	Unknown	Unknown	Unknown	Unknown
H	Unknown	Unknown	Unknown	Unknown	Unknown

	1	2
A	Unknown	Negative Control
B	Unknown	
C	Unknown	Standard 1

20 Initiate the protocol

Results and Interpretation

21 Samples that cross the threshold in the FAM channel are displayed with their starting quantities and the corresponding Ct-values. Samples that do not cross the threshold are displayed as “No Ct” or “ –”

22 Possible outcomes from the PCR:

	A	B	C	D
	+	-	HBV Positive	The sample contains a high viral load of HBV and may suppress the amplification of the Internal Control. No need to check the HEX
	+	+	HBV Positive	The sample contains HBV Nucleic acid
	+	-	HBV Positive*	Caution: If the sample has a viral load <10 IU/ml and no Internal Control amplification, it indicates PCR inhibition which requires assay repetition (see troubleshooting)
	-	+	HBV Negative	No HBV Nucleic acid was detected. Internal control amplified indicating good DNA isolation and PCR procedures.
	-	-	Invalid**	Repeat the assay (see troubleshooting)

* and **: refer to the troubleshooting section

Troubleshooting

23 *If the sample has a viral load [M] <10 IU/ml and no Internal Control amplification, it requires that the assay is repeated once. Freeze-thaw[freeze the sample (temperature ≤ -10°C) for 20 minutes and defrost at room temperature for 10 minutes] the nucleic acid sample and dilute it with nuclease-free water in a ratio of 1:2. Use the diluted nucleic acid for repeating the assay

** Repeat the assay once taking into consideration possible pipetting errors in the first assay.

If both repeat as indicated above fail to yield the expected/better results, a new specimen should be collected from the patient and the process from nucleic acid isolation to amplification should be repeated with the new sample.

Protocol references

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