

**Hemophilus Influenza B Real Time PCR Kit User Manual**

LT021210DR

For use with ABI Prism® 7000/7300/7500/7900/Step One Plus, iCycler iQ™4/iQ™5; Smart Cycler II, Bio-Rad CFX 96; Rotor Gene™ 6000; Mx3000P/3005P; MJ-Option2/Chromo4; LightCycler 480 Instrument

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**1. Intended Use**

By using real time PCR systems, Hemophilus Influenza B real time PCR kit is used for the detection of Hemophilus Influenza B in samples like nasal and pharyngeal secretions, sputum, pleural effusion, and etc.

**2. Principle of Real-Time PCR**

The principle of the real-time detection is based on the fluorogenic 5' nuclease assay. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially (Ct) is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

**3. Product Description**

Haemophilus influenzae type b, or Hib, formerly called Pfeiffer's bacillus or Bacillus influenzae, is a non-motile Gram-negative coccobacillus first described in 1892 by Richard Pfeiffer during an influenza pandemic. It is generally aerobic, but can grow as a facultative anaerobe. H. influenzae was mistakenly considered to be the cause of the common flu until 1933, when the viral etiology of the flu became apparent. Still, H. influenzae is responsible for a wide range of clinical diseases including some three million serious illnesses and an estimated 386,000 deaths per year, chiefly through meningitis and pneumonia. Almost all victims are children under the age of five, with those between four and 18 months of age especially vulnerable. Hib can be found in the upper respiratory system of humans, and cultured on Chocolate agar. The Hib genome consists of 1,830,140 base pairs of DNA in a single circular chromosome that contains 1740 protein-coding genes, 58 transfer RNA genes (tRNA, and 18 other RNA genes.

Hemophilus Influenza B real time PCR Kit contains a specific ready-to-use system for the detection of the Hemophilus Influenza B by polymerase chain reaction (PCR) in the real-time PCR system. The master contains reagents and enzymes for the specific amplification of the Hemophilus Influenza B DNA. Fluorescence is emitted and measured by the real time systems' optical unit during the PCR. The detection of amplified Hemophilus Influenza B DNA fragment is performed in fluorimeter channel FAM with the fluorescent quencher BHQ1. DNA extraction buffer is available in the kit. In addition, the kit contains a system to identify possible PCR inhibition by measuring the HEX/VIC/JOE fluorescence of the internal control (IC).

**4. Kit Contents**

Ref.	Type of Reagent	Presentation 25rxns
1	DNA Extraction Buffer	2 vials, 1.5ml
2	HIB Reaction Mix	1 vial, 950µl
3	PCR Enzyme Mix	1 vial, 12µl
4	Molecular Grade Water	1 vial, 400µl
5	Internal Control (IC)	1 vial, 30µl
6	HIB Positive Control	1 vial, 30µl

**Analysis sensitivity: 1 × 10<sup>4</sup> copies/ml**

Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors. If you use the DNA extraction buffer in the kit, the analysis sensitivity is the same as it declares. However, when the sample volume is dozens or even hundreds of times greater than elution volume by some concentrating method, it can be much higher.

**5. Storage**

- All reagents should be stored at -20°C. Storage at +4°C is not recommended.
- All reagents can be used until the expiration date indicated on the kit label.
- Repeated thawing and freezing (>3x) should be avoided, as this may reduce the sensitivity of the assay.
- Cool all reagents during the working steps.
- Reaction Mix should be stored in the dark.

**6. Additionally Required Materials and Devices**

- Biological cabinet
- Trypsin digestive Solution
- Real time PCR reaction tubes/plates
- Pipets (0.5 µl – 1000 µl)
- Sterile microtubes
- Biohazard waste container
- Tube racks
- Desktop microcentrifuge for "epENDORF" type tubes (RCF max. 16,000 x g)
- Real time PCR system
- Vortex mixer
- Cryo-container
- Sterile filter tips for micro pipets
- Disposable gloves, powderless
- Refrigerator and freezer

**7. Warnings and Precaution**

- Carefully read this instruction before starting the procedure.
- For in vitro diagnostic use only.
- This assay needs to be carried out by skilled personnel.
- Clinical samples should be regarded as potentially infectious materials and should be prepared in a laminar flow hood.
- This assay needs to be run according to Good Laboratory Practice.
- Do not use the kit after its expiration date.
- Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
- Once the reagents have been thawed, vortex and centrifuge briefly the tubes before use.
- Prepare quickly the Reaction mix on ice or in the cooling block.
- Set up two separate working areas: 1) Isolation of the RNA/ DNA and 2) Amplification/ detection of amplification products.
- Pipets, vials and other working materials should not circulate among working units.
- Use always sterile pipette tips with filters.
- Wear separate coats and gloves in each area.
- Avoid aerosols

**8. Sample Collection, Storage and transport**

- Collect samples in sterile tubes;
- Specimens can be extracted immediately or frozen at -20°C to -80°C.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents

**9. Procedure**

**9.1 DNA-Extraction**

DNA extraction buffer is supplied in the kit, please thaw the buffer thoroughly and spin down briefly in the centrifuge before use. It's better to use commercial kits for nucleic acid extraction.

**9.1.1 Sputum sample**

**1) Trypsin digestive Solution preparation**

Add 10g trypsin to 200ml sterile purified water and mix thoroughly. Adjust the PH value to 8.0 with 2%NaOH solution. Add 2ml 25mmol/L CaCl<sub>2</sub>, mix thoroughly and store at 4°C. **Please incubate at 37°C for 10 minutes before use.**

- 2) Estimate the volume of the sputum and add partes aequales of the trypsin digestive solution then vortex vigorously. Set at room temperature for 30 minutes. Transfer 0.5ml mixture to a new tube. Centrifuge the tube at 13000rpm for 5 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.
- 3) Add 1.0ml normal saline. Resuspend the pellet with vortex vigorously. Centrifuge at 13000rpm for 5 minutes. Carefully remove and discard supernatant from the tube without disturbing the pellet.
- 4) Repeat step 3)
- 5) Add 100µl DNA extraction buffer, closed the tube then resuspend the pellet with vortex vigorously. Spin down briefly in a table centrifuge.
- 6) Incubate the tube for 10 minutes at 100°C.
- 7) Centrifuge the tube at 13000rpm for 10 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

**9.1.2 Fluid samples (nasal and pharyngeal secretions and etc.)**

- 1) Take 1ml sample in a tube, centrifuge the tube at 13000rpm for 2min, and remove the supernatant and keep the pellet.
- 2) Add 100µl DNA extraction buffer to the pellet, close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
- 3) Incubate the tube for 10 minutes at 100°C.
- 4) Centrifuge the tube at 13000rpm for 10 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

**Attention:**

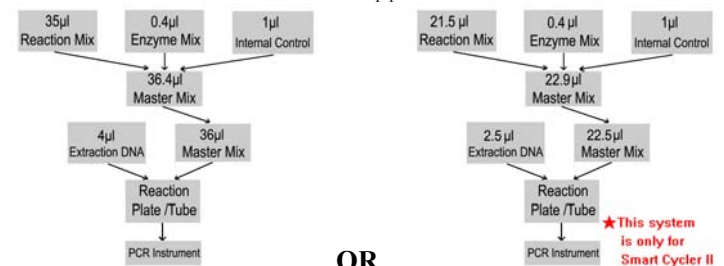
- A. During the incubation, make sure the tube is not open. Since the vapor will volatilize into the air and may cause contamination if the sample is positive.
- B. The extraction sample should be used in 3 hours or stored at -20°C for one month.
- C. DNA extraction kits are available from various manufacturers. You may use your own extraction systems or the commercial kit based on the yield. For the DNA extraction, please comply with the manufacturer's instructions.

**9.2 Internal Control**

It is necessary to add internal control (IC) in the reaction mix. Internal Control (IC) allows the user to determine and control the possibility of PCR inhibition. Add the internal control (IC) 1µl/rxn and the result will be shown in the HEX/VIC/JOE.

**9.3 PCR Protocol**

The Master Mix volume for each reaction should be pipetted as follows:



OR  
\*PCR system without HEX/VIC/JOE channel may be treated with 1µl Molecular Grade Water instead of 1µl IC.

- 1) The volumes of Reaction Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of controls, standards, and sample prepared. Molecular Grade Water is used as the negative control. For reasons of unprecise pipetting, always add an extra virtual sample. Mix completely then spin down briefly in a centrifuge.
- 2) Pipet 36µl (22.5µl for Smart Cycler II) Master Mix with micropipets of sterile filter tips to each Real time PCR reaction plate/tubes. Separately add 4µl (2.5µl for Smart Cycler II) DNA sample, positive and negative controls to different reaction plate/tubes. Immediately close the plate/tubes to avoid contamination.
- 3) Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.
- 4) Perform the following protocol in the instrument:

37°C for 2min	1cycle
94°C for 2min	1cycle
93°C for 15sec, 60°C for 1min	40cycles

( Fluorescence measured at 60°C)

Selection of fluorescence channels	
FAM	Target Nucleic Acid
HEX/VIC/JOE	IC

- 5) **⚠** If you use ABI Prism® system, please choose "none" as passive reference and quencher.

**10. Threshold setting:** just above the maximum level of molecular grade water.

**11. Quality control:** Negative control, positive control, internal control and QS curve must be performed correctly, otherwise the sample results is invalid.

Control	Channel	Ct value	
		FAM	HEX/VIC/JOE
Molecular Grade Water		UNDET	25~35
Positive Control(qualitative assay)		≤35	—

**12. Data Analysis and Interpretation**

The following sample results are possible:

	Ct value		Result Analysis
	FAM	HEX/VIC/JOE	
1#	UNDET	25~35	Below the detection limit or negative
2#	≤38	—	Positive
3#	38~40	25~35	Re-test; If it is still 38~40, report as 1#
4#	UNDET	UNDET	PCR Inhibition; No diagnosis can be concluded.