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**Enterobacter sakazakii Real Time PCR Kit User Manual**

# LT024310DD50

***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***

**Life Technologies (India)Pvt. Ltd.**
**306, Aggarwal City Mall, Opposite M2K Pitampura Ne Delhi-110034 (INDIA). Ph: +91-11-42208000, 42208111,42208222, Mobile: +91-9810521400, Fax: +91-11-42208444**
**Email: customerservice@lifetechindia.com Website: www.lifetechindia.com**

Σ 50 | −20 °C | CE

## 1. Intended Use

Enterobacter sakazakii real time PCR kit is used for the detection of Enterobacter sakazakii in enrichment broth of milk powder by using real time PCR systems.

## 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

Enterobacter sakazakii is a Gram-negative rod-shaped pathogenic bacterium of the genus Enterobacter. It is a rare cause of invasive infection with historically high case fatality rates (40–80%) in infants. It can cause bacteraemia, meningitis and necrotising enterocolitis. E. sakazakii infection has been associated with the use of powdered infant formula even after extended period of storage for more than 2 years. E. sakazakii was defined as a new species in 1980 by Farmer et al. 1980. DNA-DNA hybridization showed that E. sakazakii was 53–54% related to species in two different genera, Enterobacter and Citrobacter. However diverse biogroups were described and Farmer et al. suggested that these may represent different species.

Enterobacter sakazakii real time PCR kit contains a specific ready-to-use system for the detection of the Enterobacter sakazakii through polymerase chain reaction in the real-time PCR system. The master contains reagents and enzymes for the specific amplification of the Enterobacter sakazakii DNA. Fluorescence is emitted and measured by the real time systems´optical unit during the PCR. The detection of amplified Enterobacter sakazakii DNA fragment is performed in fluorimeter **channel FAM** with the fluorescent quencher BHQ1. In addition, the kit contains a system to identify possible PCR inhibition by measuring the HEX/VIC/JOE fluorescence of the internal control (IC). An external positive control defined as $1\times10^7$ copies/ml is supplied which allow the determination of the gene load. For further information, please refer to section 9.3Quantitation.

## 4. Kit Contents

| Ref. | Type of Reagent | Presentation 50 rxns |
|---|---|---|
| 1 | DNA Extraction Buffer | 2 vials, 1.5ml x2 |
| 2 | E. sakazakii Reaction Mix | 1 vial, 950µl x2 |
| 3 | PCR Enzyme Mix | 1 vial, 12µl x2 |
| 4 | Molecular Grade Water | 1 vial, 400µl x2 |
| 5 | Internal Control | 1 vial, 30µl x2 |
| 6 | E. sakazakii Positive Control($1\times10^7$ copies/ml) | 1 vial, 30µl x2 |

**Analysis sensitivity: $5\times10^3$ copies/ml; LOQ: $1\times10^4$～$1\times10^8$ 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
- Real time PCR system
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)
- Vortex mixer
- Real time PCR reaction tubes/plates
- Cryo-container
- Pipets (0.5µl – 1000µl)
- Sterile filter tips for micro pipets
- Sterile microtubes
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator and Freezer
- Tube racks

## 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.
- Quickly prepare 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.

## 8. Sample Collection, Storage and transportation

- 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 Sampling and DNA extraction

#### 9.1.1 Sampling and increasing bacteria

1) Sterilize the sample package and sampling tools before sampling.
2) Take 100g, 10g and 1g milk powder into three different culture bottles of 2L, 250ml and 125ml respectively.
3) Add 9 times volume sterilized water into these three culture bottles(M/V=1:9), and incubate them under 36±1℃ for 18~22h..
4) Take 10ml culture medium from the culture bottles into 90ml EE broth respectively, and incubate them for 18~22h.

#### 9.1.2 DNA-Extraction

DNA extraction buffer is contained 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.

1) Take about 1ml culture medium form each bottle of EE broth to a 1.5ml tube. Centrifuge the tubes at 8000r/min for 5 minutes, carefully remove and discard supernatant from the tubes without disturbing the pellet.
2) Add 100µl DNA extraction buffer into each tube, close the tubes, then resuspend the pellet with vortex vigorously. Spin down briefly in a table centrifuge.
3) Incubate the tubes for 5 minutes at 100 °C.
4) Centrifuge the tubes at 12000rpm for 5 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， as 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 store at -20 ℃ for one month.
**C.** Different DNA extraction kits are available. 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 got in the HEX/VIC/JOE channel.

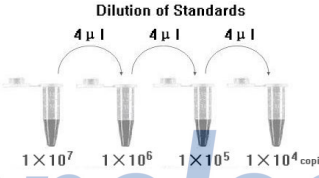
### 9.3 Quantitation

The kit can be used for **quantitative** or **qualitative** real-time PCR. A positive control defined as $1\times10^7$ copies/ml is supplied in the kit.

**For performance of quantitative real-time PCR, Standard dilutions must prepare first as follows. Molecular Grade Water is used for dilution.**

**The step of dilution is not needed for performance of qualitative real-time PCR.**

Take positive control ($1\times10^7$ copies/ml) as the starting high standard in the first tube. Respectively pipette **36ul** of Molecular Grade Water into next three tubes. Do three dilutions as the following figures:



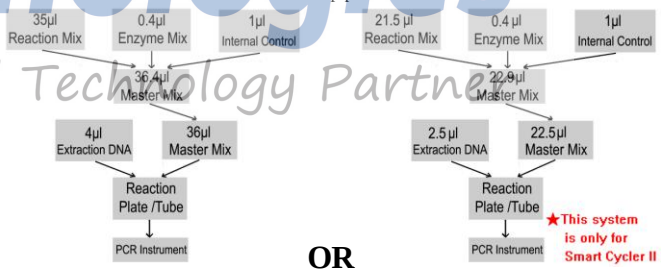

To generate a standard curve on the real-time system, all four dilution standards should be used and defined as standard with specification of the corresponding concentrations.

**Attention:**

**A.** Mix thoroughly before next transfer.
**B.** The positive control ($1\times10^7$ copies/ml) contains high concentration of the target DNA. Therefore, be careful during the dilution in order to avoid contamination.

### 9.4 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 1 µl Molecular Grade Water instead of 1 µl IC.

1) The volumes of Reaction Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of the 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 the master mix completely then spin down briefly in a centrifuge.
2) Pipet **36µl (22.5µl for SmartCycer II)** Master Mix with micropipets of sterile filter tips to each *Real time* PCR reaction plate/tube. Then separately add **4µl (2.5µl for SmartCycer 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 (Fluorescence measured at 60 °C) | 40cycles |

| 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.Calibration for quantitative detection:** Input each concentration of standard controls at the end of run, and a standard curve will be automatically formed.

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

| | Ct value | |
|---|---|---|
| | FAM | HEX/VIC/JOE |
| Molecular Grade Water | UNDET | 25~35 |
| Positive Control(qualitative assay) | ≤35 | —— |
| QS（quantitative detection） | Correlation coefficient of QS curve≤—0.98 | |

## 13. Data Analysis and Interpretation

The following results are possible:

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