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VIM Producing Bacteria Real Time PCR Kit User Manual
For In Vitro Diagnostic Use Only

REF **QD-0276-01**

For use with LightCycler1.0/2.0 Instrument

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1. Intended Use

VIM producing bacteria real time PCR kit is used for the detection of VIM producing strains in sputum, S.C.F., lung biopsy and stool samples by 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

The widespread dissemination of metallo-beta-lactamase (MBL) resistance to carbapenem antibiotics among nonfermentative gram-negative pathogens has become a global concern. MBLs confer wide-spectrum resistance to all beta-lactams except for monobactams, and their catalytic activities are generally not inhibited by non-MBL inhibitors, such as clavulanic acid and tazobactam.Acquired MBLs have been reported mainly in clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter spp.*, sometimes from major clonal outbreaks, as well as in other non-fermenters; they have also been reported, less commonly, in members of the *Enterobacteriaceae*. The acquired MBLs so far described belong to five different families. The IMP and VIM types are the most widely reported. The genes for all these MBLs may be carried on mobile genetic elements, or may become chromosomally integrated. VIM producing bacteria real time PCR kit contains a specific ready-to-use system for the detection of VIM producing strain by polymerase chain reaction (PCR) in the real-time PCR system. The master contains reagents and enzymes for the specific amplification of VIM gene. Fluorescence is emitted and measured by the real time systems´ optical unit during PCR. The detection of amplified VIM gene DNA fragment is performed in fluorimeter **channel 530nm** 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 560nm fluorescence of the internal control (IC). An external positive control (1×10^7copies/ml) contained, allows the determination of the gene load. For further information, please refer to section 9.3 Quantitation.

4. Kit Contents

Ref.	Type of Reagent	Presentation 25rxns
1	DNA Extraction Buffer	2 vials, 1.5ml
2	VIM Reaction Mix	1 vial, 450µl
3	PCR Enzyme Mix	1 vial, 12µl
4	Molecular Grade Water	1 vial, 400µl
5	Internal Control (IC)	1 vial, 30µl
6	VIM Positive Control(1×10^7copies/ml)	1 vial, 30µl

Analysis Sensitivity：1×10^3 copies/ml； LOQ: 2×10^3～1×10^8copies/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
- Vortex mixer
- Cryo-container
- Sterile filter tips for micro pipets
- Disposable gloves, powderless
- Refrigerator and Freezer
- Real time PCR system
- Real time PCR reaction tubes/plates
- Pipets (0.5µl – 1000µl)
- Sterile microtubes
- Biohazard waste container
- Tube racks
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)

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 DNA-Extraction

DNA extraction buffer is supplied in the kit, please thaw the buffer thoroughly and spin down briefly in the centrifuge before use.

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 $CaCl_2$, mix thoroughly and store at 4℃. **Please incubate at 37℃ 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 50µl DNA extraction buffer, close 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 DNA extracted and can be used for PCR template.

9.1.2 Fluid samples (C.S.F. , and etc.)

1) Take 400µl (3ml for water sample) 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.

9.1.3 Lung biopsy or stool samples

1) Take about 50mg sample，wash the sample in 1ml normal saline and vortex vigorously. Centrifuge at 13000rpm for 2 minutes. Carefully remove and discard supernatant from the tube without disturbing the pellet.
2) Add 100µl DNA extraction buffer to the tube, closed the tube then vortex for 10 seconds.
3) Incubation the tube for 10 minutes at 100°C.
4) Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the extracted DNA and can be used for the template of the PCR.

Attention:

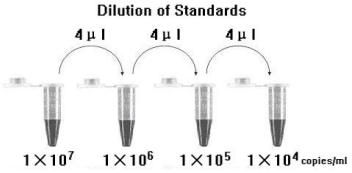
A. During the incubation, make sure the tube is not open，as the vapor will volatilize into the air and may cause contamination if the sample is positive.
B. The extraction sample should be used in 3 hours or store at -20°C for one month.
C. Different DNA extraction kits are available. You may use your 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 shown in the 560nm.

9.3 Quantitation

The kit can be used for **quantitative** or **qualitative** real-time PCR.
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 (1×10^7copies/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:



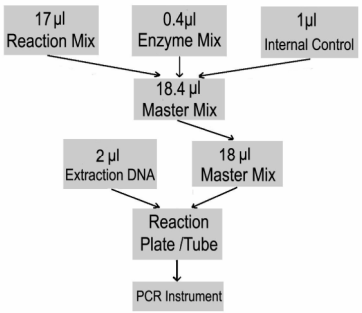
To generate a standard curve on the real-time system, all four dilution standards should be used and defined as standard with specification of the corresponding concentrations.

Attention:

A. Mix thoroughly before next transfer.
B. The positive control (1×10^7copies/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 follow:



※PCR system without 560nm 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 **18µl Master** Mix with micropipets of sterile filter tips to each real time PCR reaction plate/tubes. Separately add **2µl** 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 5sec, 60°C for 30sec (Fluorescence measured at 60°C)	40cycles

Selection of fluorescence channels	
530nm	Target Nucleic Acid
560nm	IC

10. Threshold setting: Choose **Arithmetic** as back ground and **none** as Noise Band method, then adjust the Noise band just above the maximum level of molecular grade water, and adjust the threshold just under the minimum of the positive control.

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.

Channel / Control	Crossing point value	
	530nm	560nm
Molecular Grade Water	Blank	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:

	Crossing point value		Result Analysis
	530nm	560nm	
1#	Blank	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#	Blank	Blank	PCR Inhibition; no diagnosis can be concluded.

For further questions or problems, please contact our technical support at **trade@liferiver.com.cn**