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MDR KPC/OXA Real-TM

Real Time PCR kit for detection and differentiation of Klebsiella pneumoniae carbapenemase (KPC) and OXA-carbapenemases in Enterobacteriaceae and Non Fermenting Gram Negative Bacteria (NFGNB)

Handbook

REF C2-100FRT

Σ **100**

NAME

MDR KPC/OXA Real-TM

INTRODUCTION

Nosocomial respiratory tract infections are major cause of excessive morbidity and mortality. Patients with serious underlying diseases have an especially high risk of acquiring these infections and that risk is magnified by exposure to respiratory therapy.

Beta-lactams remain a cornerstone for antimicrobial chemotherapy of a large number of bacterial infections, but their efficacy has been increasingly thwarted by dissemination of acquired resistance determinants among pathogenic bacteria. The exposure of bacterial strains to a multitude of β-lactams has induced a dynamic and continuous production and mutation of β-lactamase in many bacteria, expanding their activity even against later generation cephalosporins 5 and carbapenems by the production of extended-spectrum beta-lactamase (ESBL) and metallo-beta-lactamase (MBL) respectively. Since the genes that code for the production of ESBL are often linked to other resistance genes causing extended spectrum of drug resistance, this will result into fewer therapeutic alternatives.

ESBLs with hydrolytic activity against carbapenems are classified in three groups:

– Class A β-lactamases - *Klebsiella pneumoniae* carbapenemase (KPC)

– Class B - metallo-beta-lactamase (MBL) which includes New Delhi metallo-β-lactamase (NDM), Verona integron-encoded-metallo-β-lactamase (VIM) and imipenemase-metallo-β-lactamase (IMP)

– Class D - OXA-carbapenemases.

Main ESBL-producing pathogens are:

- Enterobacteriaceae
  - E. coli
  - K. Pneumoniae
  - K. Oxytoca
  - P. mirabilis
  - Enterobacter
  - Salmonella
- Non-fermentative Gram-negative
  - A. baumannii
  - P. aeruginosa

Methods of detection of ESBLs:

- Phenotypic methods (antibiotic susceptibility)
  - Used routinely in clinical laboratories
  - The accuracy of semiautomated microbiology systems is not optimal
- Genotypic methods (PCR-based amplification)
  - Used in reference laboratories
  - Discriminate between specific types of ESBLs
  - Need shorter time to detection (culture not required)
  - Have ability to detect low level resistance

INTENDED USE

MDR KPC/OXA Real-TM PCR kit is an *in vitro* nucleic acid amplification test for detection and differentiation of MDR genes using real-time hybridization-fluorescence detection of amplified products.

PRINCIPLE OF ASSAY

The detection of MDR genes includes DNA isolation from biological materials and real-time PCR amplification of DNA. MDR genes detection by the polymerase chain reaction (PCR) is based on the amplification of genome specific region using specific primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. The real-time monitoring of fluorescence intensities during the real-time PCR allows detection of the amplified product without re-opening the reaction tubes after the PCR run. **MDR KPC/OXA Real-TM** PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions.

- The carbapenemase group KPC is detected in the FAM/Green channel.
- The carbapenemase group OXA-48-similar (OXA-48 and OXA-162) is detected in the JOE/HEX/Yellow channel
- The *Internal Control (IC)* is detected in the Rox/Texas Red/Orange channel.

CONTENT

Reagent	*Volume (ml)*	*Amount*
PCR-mix-1-FRT KPC/OXA-48	1.2	1 tube
PCR-mix-2	0.3	2 tubes
Hot Start TaqF Polymerase	0.03	2 tubes
Pos2 KPC/OXA-48 (C+)	0.2	1 tube
DNA-buffer	0.2	1 tube
Negative Control (C–)*	1.2	2 tubes
Internal Control (IC)**	1.0	1 tube

**Must be used in the isolation procedure as Negative Control of Extraction: add 100 µl of C– (Negative Control) to labeled NCE tube;*
*** add 10 µl of Internal Control during the DNA purification procedure directly to the sample/lysis mixture*

MDR KPC/OXA Real-TM PCR kit is intended for 110 amplification reactions including controls.

MATERIALS REQUIRED BUT NOT PROVIDED

- Real Time Thermalcycler
- DNA isolation kit
- Desktop microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Disposable polypropylene PCR tubes or strips
- Tube racks

WARNINGS AND PRECAUTIONS

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Use routine laboratory precautions. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas. Do not pipette by mouth.
3. Do not use a kit after its expiration date.
4. Do not mix reagents from different kits.
5. Dispose all specimens and unused reagents in accordance with local regulations.
6. The use of heparinized specimens is not recommended.
7. Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
8. Once the reagents have been thawed, vortex and centrifuge briefly the tubes.
9. Prepare quickly the Reaction mix.
10. Specimens may be infectious. Use Universal Precautions when performing the assay.
11. Specimens and controls should be prepared in a laminar flow hood.
12. Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.
13. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant. Follow by wiping down the surface with 70% ethanol.
14. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
15. Material Safety Data Sheets (MSDS) are available on request.
16. Use of this product should be limited to personnel trained in the techniques of amplification.
17. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification Area. Do not return samples, equipment and reagents in the area where you performed previous step. Personnel should be using proper anti-contamination safeguards when moving between areas.

STABILITY AND STORAGE

MDR KPC/OXA Real-TM must be stored at 2-8°C. **TaqF Polymerase** and **PCR-mix-2** must be stored at -20°C. The kits can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

MDR KPC/OXA Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Note: Handle all specimens as if they are potentially infectious agents.

MDR KPC/OXA Real-TM PCR kit is intended for analysis of DNA extracted with DNA purification kits from the clinical/biological materials like haemoculture, bacterial culture, urine, swabs.

- *swabs*: swab area and place in “Eppendorf” tube with 0,5 ml of saline water or PBS sterile (Sacace Transport medium is recommended). Agitate vigorously. Repeat the swab and agitate in the same tube. Centrifuge at 1000g/min for 5 min. Discard the supernatant and leave about 100 µl of solution for DNA extraction.
- *Haemoculture, bacterial culture obtained by inoculation of clinical material in liquid enriched medium :* transfer 0,25 ml of culture in 1,5 ml tube and centrifuge at 10000 g for 10-15 min. Remove and discard the supernatant. Resuspend the pellet in 100 µl of Saline water.
- *urine* : collect 10-20 ml of first-catch urine in a sterile container. Centrifuge for 30 min at 3000 x g, carefully discard the supernatant and leave about 200 µl of solution. Resuspend the sediment. Use the suspension for the DNA extraction.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

The following kit is recommended:

⇨ **DNA-Sorb-A** (Sacace, REF K-1-1/A)

Please carry out DNA extraction according to the manufacture's instruction. Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture of all samples and Negative Extraction Control (NCE). If amplification is not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

PROTOCOL

PREPARING TUBES FOR PCR

1. Before starting work, thaw and vortex all reagents of the kit. Make sure that there are no drops on the caps of the tubes.
2. Take the required number of PCR tubes for amplification of clinical and control samples (negative control of extraction, negative and positive controls of amplification).
3. To prepare the reaction mixture, mix in a new sterile tube the reagents per one reaction:
   - **10 µl of PCR-mix-FRT KPC/OXA-48,**
   - **5 µl of RT-PCR-mix-2,**
   - **0.5 µl of Polymerase**

Thoroughly vortex the mixture, make sure that there are no drops on the caps of the tubes.

4. Add **15 µl** of the prepared reaction mixture to each PCR tube.
5. Add **10 µl** of **DNA samples** isolated from the clinical samples to each PCR tube.
6. Run the **control reactions**:

C– - Add **10 µl** of the **DNA sample** extracted from the Negative Control to the tube labeled NCE (Negative Control of Extraction)

C+ - Add **10 µl** of **Pos2 KPC/OXA-48 (C+)** to the tube labeled C+ (Positive Control of PCR).

NCA - Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

Make sure that there are no drops on the tube walls, otherwise vortex the tubes briefly.

Table. REACTION MIXTURE PREPARATION

		Reaction volume (with one extra sample)		
Reagent volume for one reaction, µl		**10.00**	**5.00**	**0.50**
N. samples	N. PCR reactions	PCR-mix-1	PCR-mix-2	Polymerase
4	7	80	40	4.0
6	9	100	50	5.0
8	11	120	60	6.0
10	13	140	70	7.0
12	15	160	80	8.0
14	17	180	90	9.0
16	19	200	100	10.0
18	21	220	110	11.0
20	23	240	120	12.0
22	25	260	130	13.0
34	37	380	190	19.0
46	49	500	250	25.0

AMPLIFICATION

Amplification program

	Rotor-type Instruments[1]			Plate- or modular type Instruments[2]		
Step	***Temperature °C***	***Time***	**Repeats**	***Temperature °C***	***Time***	**Repeats**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**95**	5 s	40	**95**	5 s	40
	60	20 s *fluorescent signal detection*		**60**	30 s *fluorescent signal detection*	
	72	15 s		**72**	15 s	

[1] *For example Rotor-Gene™ 6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)*

Fluorescent signal is detected in the channels for the FAM, JOE and ROX fluorophores.

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 6000, RotorGene Q)

Channel	**Calibrate / Gain Optimisation**	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	5FI - 10FI	0,1	10%	ON
JOE/Yellow	4FI – 8 FI	0,1	10%	ON
ROX/Orange	4FI – 8 FI	0,1	10%	ON

Plate- or modular type instruments (SaCycler-96™ (Sacace), *CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)).* For result analysis, set the threshold line at a level where curves of fluorescence are linear.

DATA ANALYSIS

Channel	**FAM**	**HEX**	**ROX**
MDR KPC/OXA Real-TM PCR kit	group **KPC**	group **OXA-48-similar**	**IC**

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve (in the middle of the linear section of the fluorescence curve for the positive control (C+) in logarithmic coordinates).

The result of amplification is considered **positive** if the fluorescence curve is characteristic of real-time PCR (S-shaped) and crosses the threshold line once in the significant fluorescence increase section and if the Ct value detected in the channel is below the threshold value specified in the below table.
The result of amplification is considered **negative** if the fluorescence curve is not S-shaped and if it does not cross the threshold line (the Ct value is absent).

RESULTS INTERPRETATION

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve.

Sample	Channels		
	FAM/ Green	JOE/ Yellow	ROX/ Orange
	KPC	OXA-48	IC
NCA	-	-	-
NCE	-	-	**<30**
Pos C+	**<30**	**<30**	**-**
Clinical samples	**<38**	**<38**	**<38**

Sample contains *genes group KPC* if the Ct value detected in the FAM channel is less than 38.

Sample contains *genes group OXA-48* if the Ct value detected in the JOE/HEX channel is less than 38.

The result is **invalid** if *Ct* value is not determined (absent) in the channel for FAM, JOE fluorophores, whereas the *Ct* value in the channel for the ROX fluorophore is not determined (absent) or greater than the specified boundary Ct value. In such cases, PCR analysis should be repeated starting from DNA extraction stage. If the same result is obtained in the second run, re-sampling of material is recommended

Results are accepted as significant only if both positive and negative controls of PCR and the negative control of DNA extraction passed correctly (see above the table for controls).

TROUBLESHOOTING

- The absence of positive signal in C+ in channels FAM/Green and JOE/HEX/Yellow may indicate incorrect amplification program or other errors made during PCR amplification. In this case, PCR should be carried out once again.
- Detection of any Ct value in NCA suggests contamination of reagents or samples. In this case, it is necessary to repeat the analysis of all tests starting from the isolation stage and to take measures for detecting and eliminating the source of contamination.

EXPLANATION OF SYMBOLS

	Manufacturer		Temperature limitation
	Use by	LOT	Batch code
REF	Catalogue number	VER	Version
	Consult instructions for use		Caution
	Contains sufficient for <n> tests	**C+**	Positive control of amplification
PCE	Positive Control of Extraction	**C–**	Negative control of extraction

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