IVD For *in Vitro* Diagnostic Use

CE

Bac Multi-Resist Real-TM

Handbook

Real Time PCR kit for detection of of bacteria resistant to glycopeptide and betalactam antibiotics (imp, TBM4, oxa-51-like, ctx-M-1, tem, van A/B, mec A, oxa-48-like, oxa-40-like, vim, kpc, oxa-23-like, ndm, shv, ges)

REF R1-P026-24-S

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NAME

Bac Multi-Resist Real-TM

INTRODUCTION

Most infections are caused by bacteria, many of which are ever-evolving and resistant to nearly all available antibiotics. β-Lactams and glycopeptides are used to combat these infections by inhibiting bacterial cell-wall synthesis. This mechanism remains an interesting target in the search for new antibiotics in light of failed genomic approaches and the limited input of major pharmaceutical companies

INTENDED USE

Bac Multi-Resist Real-TM PCR kit is intended to detect DNA of bacteria resistant to glycopeptide and betalactam antibiotics (imp, TBM4, oxa-51-like, ctx-M-1, tem, van A/B, mec A, oxa-48-like, oxa-40-like, vim, kpc, oxa-23-like, ndm, shv, ges). The kit is intended to be used as an aid for management of opportunistic infections which include resistance to antibiotics. The results of this test should not be used as the sole basis for diagnosis, treatment or patient management decisions.

PRINCIPLE OF ASSAY

The detection of isolated DNA is performed by real-time PCR amplification of DNA 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.

The reagent kit includes mixtures for amplification, specific for the detection of DNA of bacteria and also contains an internal control (IC), which an indicator of the quality of the reaction in each individual tube (except tube No. 3, see table 1).

To control the location of the strips in the thermalblock of the PCR device, an oligonucleotide with a fluorescent label Rox was added to the mixture for amplification of tube No. 8. It is used by the device as a marker for determining the position of strips in the thermalblock.

MATERIALS PROVIDED

Reagent	*Amount*	*Volume*
PCR-Reaction mix-1 (paraffin sealed)	24 strips x 8 tubes each	20 µl each tube
Taq Polymerase	4 tubes	500 µl each tube
Positive control (C+)	1 tube	320 µl
Cap strips	24 strips x 8 caps each	/
Negative control (C-)*	1 tube	1000 µl

Contains reagents for 24 tests

* *must be used in the isolation procedure as Negative Control of Extraction (NCE).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- 65°C ± 2°C dry heat block
- Vortex mixer
- Pipettes with sterile, RNase-free filters tips
- 1,5 ml polypropylene sterile tubes
- Disposable gloves, powderless
- Tube racks

Zone 2: Real Time amplification:

- Real Time Thermalcycler with 4 fluorescence channels
- Workstation
- Pipettes with sterile, RNase-free filters tips
- Tube racks

STORAGE INSTRUCTIONS

Bac Multi-Resist Real-TM must be stored from +2 to +8 °C. The kit can be shipped at room temperature for 3-4 days and should be stored at +2 to +8 °C immediately on receipt.

STABILITY

Bac Multi-Resist 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.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

- Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
- Use routine laboratory precautions. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas. Do not pipette by mouth.
- Do not use a kit after its expiration date.
- Do not mix reagents from different kits.
- Dispose all specimens and unused reagents in accordance with local regulations.
- The use of heparinized specimens is not recommended.
- 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.
- Prepare quickly the Reaction mix.
- Specimens may be infectious. Use Universal Precautions when performing the assay.
- Specimens and controls should be prepared in a laminar flow hood.
- Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.
- 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.
- 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.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of amplification.
- 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.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

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

Bac Multi-Resist Real-TM kit is designed for DNA analysis of bacteria resistant to glycopeptide (G) and beta-lactam (L) antibiotics (A) in DNA material obtained from phlegm, urine, smears/scrapes from respiratory tract, gastro-intestinal and urogenital tracts, faeces, aspirates, exudates and bacterial cultures:

- *sputum:* take the material in an amount of at least 1.0 ml in a disposable graduated sterile vial with a wide neck and screw caps.
- *urine:* for analysis take the first portion of morning urine in an amount of not less than 20-30 ml. Urine collection is carried out in a special sterile container equipped with a screwed lid.
- swabs;
- *scrapings from the respiratory tract, gastrointestinal and urogenital tract:* sampling is carried out using special sterile disposable instruments like probes, cytobrushes or swabs, depending on the source of the clinical material and according to the established procedure. After taking the clinical material, transfer it into a tube with a suitable transport medium. Rinse the probe or swab with clinical material into the transport medium thoroughly for 10–15 sec, avoiding liquid spatter. Then remove the probe or swab from the solution, pressing it against the wall of the tube, squeeze out the excess liquid, and discard. Close the tube cap tightly and label.
- *Feces:* take a sample of feces with a mass (volume) of approximately 1-3 g (1-3 ml). A sample in the amount of 1 g (approximately) with a separate tip with a filter or disposable blades is transferred to a special sterile dry bottle. After collection of feces, close the tube cap tightly and label.
- *Aspirates:* the sampling is carried out in a disposable sterile tube. After taking the material, close the tube cap tightly and label.
- *Exudates:* sampling is carried out using special sterile disposable instruments like probes, cytobrushes or swabs, depending on the source of the clinical material and according to the established procedure. After taking the clinical material, transfer it into a tube with a suitable transport medium. Rinse the probe or swab with clinical material into the transport medium thoroughly for 10–15 sec, avoiding liquid spatter. Then remove the probe or swab from the solution, pressing it against the wall of the tube, squeeze out the excess liquid, and discard. Close the tube cap tightly and label.
- *bacterial cultures:* material is taken from liquid and solid media using a disposable microbiological loop or spatula. Place a single colony of cells or 100 µl of growth medium in a 1.5-2.0 ml vial with 500 µl containing a physiological saline solution. After taking the material, close the tube cap tightly and label.

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

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇨ **DNA/RNA Prep NA** (Sacace, REF K-2-9/2);
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006).

Please carry out DNA extraction according to the manufacture's instruction.

A negative control sample should go through all stages of DNA extraction. Sterile physiological saline solution can be used as a negative control sample with the volume according to DNA extraction kit used.

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 (Reaction volume 35 µl):

PREPARING TUBES FOR PCR

1. Take out from the refrigerator all the reagents.
2. For each clinical sample and controls are required 1 strip of **PCR-Reaction mix-1**. Prepare the required number of strips, including N strips for N clinical samples, one for Positive Control Amplification (PCA) and one for Negative Control of Extraction (NCE).
3. Vortex shortly and spin for 3-5 sec the **Taq polymerase** tube. Mix by pipetting and **add 10 µl** to each PCR tube without damaging the paraffin layer.
4. Add **5 µl** of **DNA samples** isolated from the clinical samples to each PCR tube without damaging the paraffin layer.
5. Run the **control reactions**:
   - **C–** - Add **5 µl** of the **Negative (C-)** extracted from the Negative Control sample to the tube labeled NCE (Negative Control of Extraction) without damaging the paraffin layer.
   - **C+** - Add **5 µl** of **Positive control (C+)** to the tube labeled C+ (Positive Control of Amplification) without damaging the paraffin layer.
6. Cap and spin down briefly (2-3 seconds) all the strips.
7. Transfer the tubes into the thermalcycler. First tube of strip contains blue buffer.

AMPLIFICATION

1. Create a temperature profile on your instrument as follows:

Step	Temperature °C	Min.	Sec.	Fluorescence signal detection**	Repeats
1	80	0	30		1
	94	1	30		
2	94	0	30		5
	64	0	15		
3	94	0	10		45
	64	0	15*	**Fluorescence detection**	

*For example, SaCycler-96™ (Sacace), CFX-96™*** Deep Well / iQ5™ (BioRad); Mx3005P™/Mx3000P™ (Agilent), ABI® 7500 Real Time PCR (Applied Biosystems);*
** On ABI® 7500 Real Time PCR instrument, please set the fluorescence acquisition time to 30 seconds.*
*** Fluorescence detection on channels FAM/Green, Joe/HEX/Yellow, ROX/Orange, Cy5/Red*
***NOTE:* FOR CFX-96** *and other plate type instruments: it is recommended to use at least two additional empty strips placing them in the last left and right columns of the thermal block to better uniform the thermolid pressure in case of not filling the complete plate.*

Targets are detected by a fluorescent signal in the channels FAM, HEX, ROX and Cy5 fluorophores according to the table below:

№ TUBE	FAM	HEX	ROX	CY5	COLOR LABELING OF BUFFER
1	imp	IC	-	-	Blue
2	TBM[1]	IC	-	oxa-51-	Colorless
3	ctx-M-1	-	-	tem	
4	van A\B	IC	-	mec A	
5	oxa-48-	IC	-	oxa-40-	
6	vim	IC	-	kpc	
7	oxa-23-	IC	-	ndm	
8	shv	IC	Marker	ges	

[1] TBM – total bacterial mass.

INSTRUMENT SETTINGS

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples, approximately 10% of the fluorescence value in the last amplification cycle for the positive control of amplification.

DATA ANALYSIS

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.

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).

The analysis of the PCR results is automatically performed by the *SaCycler-96™ (Sacace)* device software Real Time PCR according to the table below:

Detection channel				Result	Interpretation
Fam	**Hex**	**Rox**	**Cy5**		
Ig and Cp values for samples					
Ig is specified[2] (for one or more tubes N°1,3-8)	Not considered	-	Ig is specified[2] (for one or more tubes N°2-8)	+	Genes associated with antibiotic resistance are detected
Ig is not specified (for one or more tubes N°1,3-8)	Cp is specified (for the same tubes as on Fam\Cy5 channels), tube N°3 – does not contain IC	-	Ig is not specified (for one or more tubes N°2-8)	-	Genes associated with antibiotic resistance are not detected
Cp is not specified (for one or more tubes N°1-8)	Cp is not specified (for the same tubes as on Fam\Cy5), tube N°3 – does not contain IC	-	Cp is not specified (for one or more tubes N°2-8)	invalid	Invalid result[3]
Ig and Cp values for positive control sample					
Ig is specified (for all tubes)	Not considered	-	Ig is specified (for tubes N°2-8)	+	Positive result
Ig and Cp values for negative control sample					
Ig is not specified (for all tubes, for TBM: Ig≤3,5 is acceptable)	Cp is specified (for all tubes except tube N°3 – which does not contain IC).	-	Ig is not specified (for all tubes)	-	Negative result

[2] Ig values of genes associated with antibiotic resistance are calculated automatically. By comparing the Ig values it is possible to perform semi-quantitative analysis. It can be performed by estimating a proportion of genes compared to each other and to TBM. Ig value for genes associated with antibiotic resistance should be lower than Ig TBM+0,5. If it is not, the semi-quantitative analysis cannot be performed, and results should be reported as qualitative result. In this case the result is reported as positive, and in “Data analysis” window result is “+”, and in the specific report - “detected”. It can be caused by PCR technique violation, in this case for semi-quantitative analysis see note 6. Ig value less than 3,0 are not shown, but Cp values for this samples are shown. It is interpreted as negative result and in “Data analysis” window result is “-”, and in the specific report - “not detected”.

[3] Repeat PCR amplification or repeat DNA extraction is performed sequentially.

PERFORMANCE

Sensitivity

The detection limit for bacterial DNA is 2.0 x 10^3 copies / ml. The detection limit is established by analyzing serial dilutions of laboratory control samples.

The detection limit depends on the type of biomaterial, the kit / set of reagents used for DNA isolation and the final volume of elution (dilution) of the extracted DNA.

Specificity

The analytical specificity of **Bac Multi-Resist Real-TM** PCR kit was assessed by bioinformatics analysis using available on-line databases with up-to-date comprehensive genetic information. The specific oligonucleotides used in the test were checked against GenBank database sequences. None of the sequences showed sufficient similarity for unspecific detection.

The clinical specificity of **Bac Multi-Resist Real-TM** PCR kit was confirmed in laboratory clinical trials.

Diagnostic characteristics:

Number of samples (n) - 105;

Diagnostic sensitivity (95% CI) - 100% (98.7-100%);

Diagnostic specificity (95% CI) – 100% (99.8-100%).

Repeatability and reproducibility

Sample	Genes of resistance in the sample	The number of conformed results	
		Repeatability (conducting PCR in the same day by the same operator, device and kit batch)	Reproducibility (Conducting PCR in different days by different operators, devices and kit batches)
N°1	van A\B	3 repeats of 3	4 repeats of 4
N°2	ndm	3 repeats of 3	4 repeats of 4
N°3	mec A	3 repeats of 3	4 repeats of 4
N°4	ctx-M-1, tem, vim	3 repeats of 3	4 repeats of 4
N°5	oxa-51-like, tem oxa-40-like	3 repeats of 3	4 repeats of 4

TROUBLESHOOTING

- The absence of positive signal in C+ in channels FAM, HEX, ROX and Cy5 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 NCE (except in HEX channel) 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.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* MX3005P® is a registered trademark of Agilent Technologies
* ABI® is a registered trademark of Applied Biosystems

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