Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

Aerobic complex Real-TM Quant

Handbook

Real Time PCR Kit for quantitative detection and differentiation of Enterobacteriaceae (E.coli, Klebsiella spp., Proteus spp. etc.), Staphylococcus spp. and Streptococcus spp.

REF B88-100FRT

Σ **100**

NAME

Aerobic complex Real-TM Quant

INTENDED USE

Aerobic complex Real-TM Quant kit is a Real-Time test for the qualitative and quantitative detection of *Enterobacteriaceae* (*E.coli, Klebsiella* spp.*, Proteus* spp. etc.), *Staphylococcus* spp. and *Streptococcus* spp. in biological materials by using real-time hybridization-fluorescence detection.

PRINCIPLE OF ASSAY

Detection and quantitation of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection contains two steps: DNA extraction from the biological material and amplification of DNA fragment of microorganism with real-time hybridization-fluorescence detection. The DNA extraction from the clinical material is carried out with the presence of the Internal Control (Internal Control-FL), which allows controlling the procedure of examination of each sample. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run. The results of amplification of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA are detected separately for each type by three different channels.

Channel for fluorophore	FAM/Green	JOE/Hex/Yellow	ROX/Orange	Cy5/Red
DNA-target	*Enterobacteriaceae*	*Staphylococcus* spp.	*Streptococcus* spp.	IC

The DNA quantitation by real-time PCR is based on the existence of linear dependence between the logarithm of initial DNA-target concentration in the sample and start of the exponential growth of the fluorescent signal (threshold cycle, *Ct*). For the quantitative analysis simultaneous carried out real-time amplification with real-time detection for the DNA samples obtained from the test samples and DNA-standards (the samples with the certain concentration of DNA-target). According to the results of amplification DNA- standards built a calibration line on which the determination of the concentration of DNA-target in the test samples.

MATERIALS PROVIDED

- **PCR-mix-1 Aerobes**, 1,2 ml;
- **PCR-buffer FRT**, 0,6 ml;
- **Hot Start DNA Polymerase**, 0,06 ml;
- **TE-buffer**, 0,2 ml;
- **Negative Control C-***, 1,2 ml;
- **Internal Control IC ****, 1,0 ml;
- **Standards**:
  - **QSG1**, 0,2 ml;
  - **QSG2**, 0,2 ml.

Contains reagents for 100 tests

* must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg;
** add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit
- Real Time Thermalcycler
- Tubes or PCR plate
- Workstation
- Pipettes with sterile, RNase-free filters tips
- Tube racks
- Disposable powder-free gloves and laboratory coat
- Refrigerator for 2–8 °C.
- Deep-freezer at minus 24 to minus 16 °C.
- Reservoir for used tips.

STORAGE INSTRUCTIONS

Aerobic complex Real-TM Quant must be stored at -20°C. The **Aerobic complex Real-TM Quant** kit can be shipped at 2-8°C but should be immediately stored at -20°C on receipt.

STABILITY

Aerobic complex Real-TM Quant Test 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

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes 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 DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

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. 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

Aerobic complex Real-TM Quant can analyze DNA extracted from:

- *swabs;*
- *plasma;*
- *CSF;*

Specimens can be stored at +2-8°C for no longer than 12 hours, or freeze at -20°C to -80°C. 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 kit:

- ⇨ **DNA/RNA-Prep** (Sacace, REF K-2-9);
- ⇨ **DNA-Sorb-A** (Sacace, REF K-1-1/A) for swabs;
- ⇨ **SaMag Bacterial DNA Extraction kit** (Sacace, REF SM006).

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.

PROTOCOL

1. Prepare required quantity of tubes or PCR plate.
2. Prepare for each sample in the new sterile tube **10*N µl** of **PCR-mix-1 Aerobes**, **5*N µl** of **PCR-buffer FRT** and **0,5*N** of **Hot Start DNA Polymerase.**
3. Add **15 µl** of **Reaction Mix** into each tube.
4. Add **10 µl** of **extracted DNA** sample to appropriate tube with Reaction Mix.
5. Prepare for qualitative run 1 positive control and 1 negative control:
   - add **10 µl** of **QSG2** to the tube labeled C*pos*;
   - add **10 µl** of **TE-buffer** to the tube labeled C*neg*;
6. For quantitative analysis prepare 4 tubes and perform QSG1 and QSG2* standards twice.
   **QSG1 and QSG2 values are specific for each lot and are reported in the Quant Data Card provided in the kit.*

Close tubes and transfer them into the instrument in this order: samples, negative controls, positive control, Standards.

Create a temperature profile on your Real-time instrument as follows:

	Rotor type instruments[1]				Plate type or modular instruments[2]			
Stage	**Temp, °C**	**Time**	**Fluorescence detection**	**Cycle repeats**	**Temp,°C**	**Time**	**Fluorescence detection**	**Cycle repeats**
Hold	95	15 min	–	1	95	15 min	–	1
Cycling	95	5 s	–	5	95	5 s	–	5
	60	20 s	–		60	20 s	–	
	72	15 s	–		72	15 s	–	
Cycling 2	95	5 s	–	40	95	5 s	–	40
	60	20 s	FAM(Green), JOE(Yellow), Rox (Orange), Red		60	30 s	FAM, JOE/HEX/Cy3, Rox/TexasRed, Cy5/Red	
	72	15 s	–		72	15 s	–	

[1] For example Rotor-Gene™ 6000/Q (Corbett Research, Qiagen)
[2] For example, *SaCycler-96™ (Sacace),CFX96™*/ iQ5™/iQ iCycler™ (BioRad); Mx3000P/Mx3005P™ (Stratagene), Applied Biosystems® 7500 Real Time PCR (Applera), SmartCycler® (Cepheid)

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 6000, RotorGene Q)

Channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	0.1	10-20 %	on
JOE/Yellow	0.1	10-20 %	on
Rox/Orange	0.1	5-15 %	on
Cy5/Red	0.1	10-30 %	on

Plate- or modular type instruments

For result analysis, set the threshold line at a level corresponding to 10–20% of the maximum fluorescence signal obtained for QSG2 sample during the last amplification cycle.

RESULTS INTERPRETATION

Analysis curve of fluorescence signal accumulation in four channels:

- The signal of the *Enterobacteriaceae* DNA amplification product is detected in the channel for the FAM/Green fluorophore.
- The signal of the *Staphylococcus* spp. DNA amplification product is detected in the channel for the JOE/Hex/Yellow fluorophore.
- The signal of the *Streptococcus* spp. DNA amplification product is detected in the channel for the ROX/Orange fluorophore.
- The signal of the IC DNA amplification product is detected in the channel for the Cy5/Red fluorophore.

Results are interpreted by the crossing (or not crossing) of the fluorescence curve with the threshold line that set at the level of exponential growth of fluorescence. That determines presence (or absence) of *Ct* (cycle threshold) value of a sample in the appropriate cell of the result grid. Obtained *Ct* values are used for plotting a calibration line and determination of concentration of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA in samples.

The values of DNA standards concentrations are specified in the *Data Card* for the PCR kit.

According to the obtained values of *Ct* and the calibration line, the device program automatically calculated values of the number of copies of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA in the reaction tube, and issued in the corresponding column in the result grid. The obtained values are used to calculate the number of genomic equivalents of the microorganisms DNA contained in 1 ml of the sample of biological material according to the formula:

[Number of copies] DNA of microorganisms x K = [Number of genomic equivalents] per 1 ml (GE/ml)

The coefficient K for calculation the result in GE/ml is specified in the *Data Card* for the PCR kit.

The DNA concentration values of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. reflect the general content of the certain microorganisms in the biological material inserted into transport medium.

If the obtained value is less than $1x10^4$ GE/ml then the output result is “less than $1x10^4$ GE/ml”, if the obtained value is more than $1x10^8$ GE/ml then the output result is “more than $1x10^8$ GE/ml” (according to the linear range of the kit).

The clinical interpretation of the test results should be carried out by the doctor only on the basis of complex examination of the patient according to the anamnesis data, clinical and epidemiological status, keeping into account the existed clinical and methodological recommendations. The result of the PCR is considered reliable only if the results obtained for Negative Control of amplification as well as for the Negative Control of extraction of DNA and DNA standards are according to the Table "Results for Controls" and the amplification efficiency index E stays in the limits which are specified in the *Data Card* enclosed in the PCR kit.

Results for controls

Control	Stage for control	FAM (Green)	JOE(Yellow)/HEX/Cy3	Rox (Orange)/TexasRed	Cy5/Red	Interpretation
NCE	DNA isolation	Concentration value is absent or < 2000 GE/ml	Concentration value is absent or < 2000 GE/ml	Concentration value is absent or < 2000 GE/ml	Pos (Ct <boundary value)	OK
NCA	PCR	Concentration value is absent or < 1000 GE/ml	Concentration value is absent or < 1000 GE/ml	Concentration value is absent or < 1000 GE/ml	–	OK
QSG2	PCR	Pos (Ct <boundary value)	Pos (Ct <boundary value)	Pos (Ct <boundary value)	Pos (Ct <boundary value)	OK

Boundary value of the cycle threshold, Ct

Sample type	Channel for fluorophore	Ct boundary value	
		Rotor-type instruments	Plate-type instruments
QSG2	FAM/Green, JOE/Yellow/Hex/Cy3, ROX/Orange	33	36
Clinical samples, NCE, QSG2	Cy5/Red	35	38

- The sample is considered to be positive for *Enterobacteriaceae* DNA if in the channel Fam/Green the value of **Ct** is different from zero (Ct<boundary value)
- The sample is considered to be positive for *Staphylococcus* spp. DNA if in the channel JOE(Yellow)/HEX the value of **Ct** is different from zero (Ct< boundary value);
- The sample is considered to be positive for *Streptococcus* spp. DNA if in the channel Rox/Orange the value of **Ct** is different from zero (Ct< boundary value);
- The sample is considered to be uncertain if its Ct value is more than boundary value. Additional double study of this sample should be conducted;

- Specimens with Ct < boundary value in the Cy5/Red channel and absent fluorescence signal in other channels are interpreted as negative.
- Specimens with absent signal (or with Ct > boundary value) in the Cy5/Red channel are interpreted as invalid.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific primers and probes. The specificity of the kit Aerobic complex Real-TM Quant was 100%. The potential cross-reactivity of the kit Aerobic complex Real-TM Quant was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit Aerobic complex Real – TM Quant allows to detect *aerobes* DNA in 100% of the tests with a sensitivity of not less than **2000 GE/ml.** Linear range of the kit is **$1x10^4$ – $1x10^8$ GE/ml**.

TROUBLESHOOTING

1. Weak or no signal of the IC (Cy5/Red channel).
    - The PCR was inhibited.
        ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
    - The reagents storage conditions didn't comply with the instructions.
        ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. JOE(Yellow)/HEX, Fam(Green) or Rox(Orange) signal with Negative Control of extraction with concentration more than than boundary values.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        ⇒ Repeat the DNA extraction with the new set of reagents.
4. Signal with Negative Control of Amplification (TE-buffer) in Cy5 (Red) channel.
    - Contamination during PCR preparation procedure. All samples results are invalid.
        ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        ⇒ Repeat the PCR preparation with the new set of reagents.

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
* CFX96™, iCycler™ and iQ5™ are trademarks of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Corbett Research
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