Instruction for Use

diarellaMRSA 3.0 TM

real time PCR Kit

For qualitive *in vitro* detection of Methicillin Resistant Staphylococcus aureus (MRSA) DNA in clinical specimens.

REF	G01100-32	G01100-96
Σ	32	96

- 18°C

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1 Intended Use

The diarellaMRSA 3.0 TM real time PCR is an assay for the detection of the DNA of *MRSA* in clinical specimens.

2 Pathogen Information

Staphylococcus aureus are gram-positive coccal bacteria which are ubiquitously found in the environment. About 25-30 % of the human population are long-term carriers of *S. aureus* because the bacteria are frequently part of the skin flora found in the nose and on skin. *S. aureus* can cause a range of illnesses such as minor skin infections, like furuncles and abscesses, pyomyositis, but also life-threatening diseases such as pneumonia, endocarditis, toxic shock syndrome (TSS), and sepsis.

Of increasing importance worldwide are Methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Especially in hospitals MRSA present a danger, because they are resistant to all ß-lactam antibiotics (e.g. penicillin) and often possess further resistances to other anitbiotics.

3 Principle of the Test

The diarellaMRSA 3.0 TM real time PCR contains specific primers and dual-labeled probes for the amplification and detection of *MRSA* DNA in clinical specimens. The PCR targets the orfX/SSCmec junction and allows for the detection of MRSA in clinical samples, even those containing Coagulase-Negative Staphylococci. Furthermore, diarellaMRSA 3.0 real time PCR Kit allows the detection of the methicillin resistance gene mecA/mecC, to eliminate false positive results through dropout mutants.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The fluorescence of the pathogen-specific probes is measured in the FAM channel. The fluorescence of the mecA/mecC gene specific probes is measured in the Cy5 channel. For a positive MRSA result, both channels need to show an amplification.

Furthermore, diarellaMRSA 3.0 TM real time PCR contains a Control DNA, which is added during DNA extraction and detected in the same reaction by a differently labeled probe.

The Control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimen. The fluorescence of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel.

4 Package Contents

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of the diarellaMRSA 3.0 TM real time PCR Kit

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 512 µl	2 x 768 µl
Positive Control	red	1 x 50 µl	1 x 100 µl
Negative Control	green	1 x 50 µl	1 x 100 µl
Control DNA	colourless	1 x 160 µl	2 x 240 µl

5 Equipment and Reagents to be Supplied by User

- DNA isolation kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation
- Optional: BLP-DNA (Bacterium-Like Particles, please look at chapter ‚Control DNA' for details.)

6 Transport, Storage and Stability

The diarellaMRSA 3.0 TM real time PCR Kit is shipped on dry ice or cool packs. All components must be stored at -18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package.
Up to 20 freeze and thaw cycles are possible.
For convenience, opened reagents can be stored at +2-8°C for up to 6 months.
Protect kit components from direct sunlight during the complete test run.

7 Important Notes

- The diarellaMRSA 3.0 TM real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 General Precautions

- Stick to the protocol described in the Instruction for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine diarellaMRSA 3.0 TM real time PCR components of different lot numbers.

9 Sample Material

Starting material for the diarellaMRSA 3.0 TM real time PCR is DNA isolated or released from clinical specimens (e.g. throat swabs, nasal swabs, bronchial lavage, wound swabs).

10 Sample Preparation

The diarellaMRSA 3.0 TM real time PCR is suitable for the detection of *MRSA* DNA isolated from clinical specimens with appropriate isolation methods.
Commercial kits for DNA isolation such as the following are recommended:

- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

Important:
In addition to the samples always run a ‚water control' in your extraction. Treat this water control analogous to a sample.
Comparing the amplification of the Control DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

Please note the chapter ‚Control DNA'.

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the manufacturer.

11 Control DNA

A Control DNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.
The Bacterium-Like Particles (BLP-DNA) is not supplied.

DNA isolation from throat swabs, nasal swabs, bronchial lavage samples

a) Control DNA or BLP-DNA used as Extraction Control:

diarellaMRSA 3.0 TM real time PCR Control DNA or BLP-DNA is added to the DNA extraction.
Add 5 µl Control DNA or BLP-DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions. Please follow protocol A.

The Control DNA must be added to the Lysis Buffer of the extraction kit.

b) Control DNA used as Internal Control of the real time PCR:

If only inhibition will be checked please follow protocol B.

12 Real time PCR

12.1 Important Points Before Starting:

- Please pay attention to the chapter 7 ‚Important Notes'.
- Before setting up the real time PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run one Positive Control and one Negative Control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed, and centrifuged very briefly.

12.2 Procedure

If the Control DNA or BLP-DNA is used to control both, the real time PCR and the DNA isolation procedure, please follow protocol A. If the Control DNA is solely used to detect possible inhibition of the real time PCR, please follow protocol B.

Protocol A

The Control DNA or BLP-DNA was added during DNA extraction (chapter 11 ‚Control DNA'). In this case, prepare the Master Mix according to Table 2.

The Master Mix contains all of the components needed for PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2:Preparation of the Master Mix (Control DNA was added during DNA extraction)

Volume per Reaction	Volume Master Mix
16.0 µl Reaction Mix	16.0 µl x (N+1)

Protocol B

The Control DNA is used for the control of the real time PCR only (see chapter 11 ‚Control DNA'). In this case, prepare the Master Mix according to Table 3.

The Master Mix contains all of the components needed for real PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the Master Mix (Control DNA is added directly to the Master Mix)

Volume per Reaction	Volume Master Mix
16.0 µl Reaction Mix	16.0 µl x (N+1)
0.5 µl Control DNA*	0.5 µl x (N+1)*

*The increase in volume caused by adding the Control DNA is not taken into account when preparing the PCR assay.

Protocol A and B: real time PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube.
- Add **4 µl** of the eluates from the DNA isolation (including the eluate of the water control), the Positive Control and the Negative Control to the corresponding optical PCR reaction tube (Table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

12.3 Instrument Settings

For the real time PCR use the thermal profile shown in Table 5.

Table 5: real time PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of DNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec Aquisition at the end of this step	60°C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to Table 6.

Table 6: Overview of the instrument settings required for the diarellaMRSA 3.0 TM real time PCR.

Real time PCR Instrument	Parameter	Detection Channel	Notes		
LightCycler 480II			Colour Compensation Kit Multiplex 1 (G070MP1-cc) not equired		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	MRSA SCCmec	FAM (465-510)	1	10	1
	Control DNA	HEX (533-580)	1	10	2
	mecA/mecC Mutation/Deletion	CY5 (618-660)	1	10	3
Stratagene Mx3000P / Mx3005P	MRSA SCCmec	FAM	Gain 8		
	Control DNA	HEX	Gain 1	Reference Dye: None	
	mecA/mecC Mutation/Deletion	Cy5	Gain 4		
ABI 7500	MRSA SCCmec	FAM			
	Control DNA	JOE	Option Reference Dye ROX: NO		
	mecA/mecC Mutation/Deletion	Cy5			
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	MRSA SCCmec	Green	Gain 5		
	Control DNA	Yellow	Gain 5		
	mecA/mecC Mutation/Deletion	Red	Gain 5		

13 Data Analysis

The *MRSA* specific amplification is measured in the FAM channel. The fluorescence of the mecA/mecC-Gene specific probes is measured in the Cy5 channel.
The amplification of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel.

Following results can occur:

Ct Values			Interpretation
FAM Channel SCCmec	**Cy5 Channel** resistance gene mecA/mecC	**HEX Channel**	
pos	pos	pos or neg	**Positive result, the sample contains *MRSA* DNA.** The result for the Control DNA is irrelevant.
pos	neg	pos or neg	**Negative result, the sample contains MS-*MRSA* DNA.** The result for the Control DNA is irrelevant.
neg	pos	pos or neg	**Negative result, the sample contains MR-CoNS DNA.** The result for the Control DNA is irrelevant.
neg	neg	27-35*	**Negative result, the sample contains no *MRSA/ MS-MRSA and MR-CoNS* DNA.**
neg	neg	> 35*/ neg	**No diagnostic statement can be made.** The real time PCR is either inhibited or errors occurred while DNA extraction.

*Depending on the PCR instrument and/or the chosen extraction method, the Ct values might be shifted. The water control can be used as reference. If the HEX Ct value of a sample differs a lot from the water control, partial inhibition has occurred, leading to false negative results in case of weak positive samples.

Figure 1 and **Figure 2** show examples for positive and negative real time PCR results.

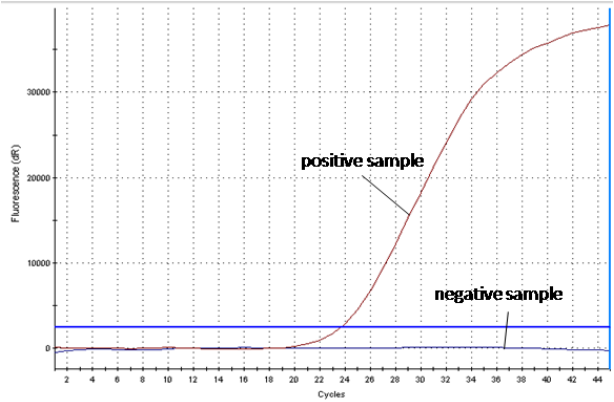

Figure 1: The positive sample shows amplification signal in the bacteria specific channel (FAM/Cy5), whereas no fluorescence signal is detected in the negative sample.

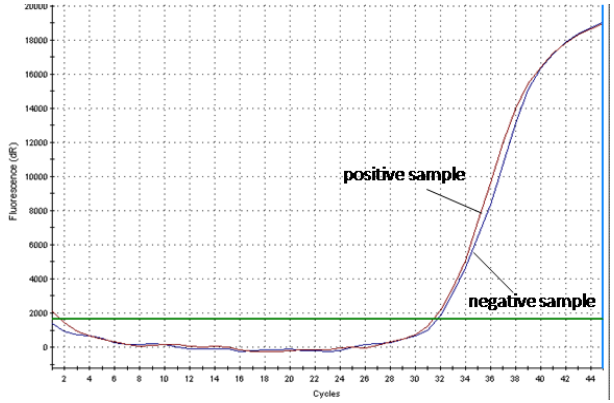

Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the bacteria specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

14 Assay Validation

Set a threshold as follows:

Negative Controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive Controls

All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal Controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a C_T of 33.

15 Limitations of the Method

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.
A negative test result does not exclude an *MRSA* infection.

16 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the FAM channel or Cy5-channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the *MRSA* specific amplification, the Cy5 channel for the mecA/mecC specific amplification and the VIC®/HEX/JOE™/TET channel for the amplification of the Control DNA.
Incorrect configuration of the real time PCR	Check your work steps and compare with ‚Procedure' on page 7.
The programming of the	Compare the thermal profile with the protocol (Table 5, page

thermal profile is incorrect	9).
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport, Storage and Stability', page 4.
Weak or no signal of the Control DNA and simultaneous absence of a signal in the bacteria specific FAM channel or Cy5 channel.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 7).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see chapter ‚Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.
DNA loss during isolation process	In case the Control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in ‚Transport, Storage and Stability'.
Detection of a fluorescence signal in the FAM channel or Cy5 channel of the Negative Control	
Contamination during preparation of the PCR	Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

17 Kit Performance

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the diarellaMRSA 3.0 TM real time PCR 14 positive and 13 negative samples were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 %

The positive predictive value was found to be 100 %, the negative predictive value showed to be 100 %.

Table 7: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	negative samples
diarellaMRSA 3.0 positive	14	0
diarellaMRSA 3.0 negative	0	13
Sensitivity	100%	
Specificity	100%	

17.2 Analytical Sensitivity

The limit of detection (LoD) of diarellaMRSA 3.0 TM real time PCR was determined using serial dilutions of synthetic target DNA-sequences in a Stratagene Mx3000 real time PCR instrument. The LoD of diarellaMRSA 3.0 TM real time PCR for *MRSA* is at least 1 cfu per reaction each.

17.3 Analytical Specificity

The specificity of the diarellaMRSA 3.0 TM real time PCR was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:

The diarellaMRSA 3.0 TM real time PCR showed a positive result for the sample containing *MRSA,* whereas samples containing other pathogens were reliably tested negative. The results are shown in Table 8.

Table 8: Bacterial and viral pathogens tested for the determination of the analytical sensitivity of the diarellaMRSA 3.0 TM real time PCR Kit.

Strain	Expected Result	Result
Streptococcus agalactiae	negative	negative
Coxsackievirus Strain P.B.	negative	negative
Coxsackievirus Strain B.S.	negative	negative
Herpes simplex virus	negative	negative
Borrelia burgdorferi	negative	negative
Tick borne encephalitis	negative	negative
Influenca A	negative	negative
Influenca B	negative	negative
Respiratiory syncytial virus A	negative	negative
Respiratiory syncytial virus B	negative	negative
Legionella pneumophila Serogroup 1	negative	negative
Cytomegalovirus	negative	negative
MRSA	positive	positive

18 Abbreviations and Symbols

MRSA	Methicillin-resistant *Staphylococcus aureus*
MS-MRSA	Methicillin-suceptible MRSA, mecA dropout mutant
OrfX/SCCmec	Junction for *S. aureus* DNA and SCCmec cassette
MSSA	Methicillin-suceptible *Staphylococcus aureus*
MR-ConS	Methicillin-resistant coagulase negative *Staphylococcus*
mecA / mecC	Two varaints of the methicillin resistance gene
DNA	Deoxyribonucleid Acid
PCR	Polymerase Chain Reaction
REACTION MIX	Reaction Mix
CONTROL +	Positive Control
CONTROL −	Negative Control
CONTROL DNA IC	Control DNA

REF	Catalog number
Σ	Contains sufficient for <n> test
- 18°C	Upper limit of temperature
	Manufacturer
	Use by YYYY-MM
LOT	Batch code
CONT	Content
	Consult instructions for use
IVD	*In vitro* diagnostic medical device
CE	European Conformity

19 Literature

[1] Bundesgesundheitsbl 2014, 57, 696–732: Empfehlungen zur Prävention und Kontrolle von Methicillin-resistenten Staphylococcus aureus-Stämmen (MRSA) in medizinischen und pflegerischen Einrichtungen.

[2] Centers for Disease Control and Prevention: Methicillin-resistant Staphylococcus aureus. www.cdc.gov/mrsa. May 16, 2016.