MRSA Multiplex Real-time PCR Kit, IVD

REF MD04931, 96 reactions

For professional in vitro diagnostic use only

CE IVD

EN

Instructions for Use (IFU)

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1. Introduction

Worldwide, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most frequent human pathogens of nosocomial and community infections. *S. aureus* has a remarkable ability to acquire resistance to antibiotics, which has major implications for the therapeutic options of this pathogenic bacterium. In the 1960s, MRSA was first observed among clinical isolates from patients hospitalized in the United Kingdom but since the 1990s it has spread rapidly in the community. During the last decade, MRSA infections have increased considerably, making it a topic of high importance. MRSA infections are usually subcategorized into community-associated MRSA (CA-MRSA), healthcare-associated MRSA (HA-MRSA) and livestock-associated MRSA (LA-MRSA). *S. aureus* evolves into MRSA by uptake, via horizontal gene transfer, of the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element that encodes genes *mec*A or *mec*C which confer resistance to oxacillin and methicillin and, therefore, to most β-lactam antibiotics. Staphylococcal resistance to oxacillin/methicillin occurs when an isolate carries an altered penicillin-binding protein, PBP2a, which is encoded by the *mec*A (or *mec*C) gene. The new penicillin-binding protein binds β-lactams with lower avidity, which results in resistance to this class of antimicrobial agents. To date, 14 SCC*mec* cassette types have been described, of which types I to V occur most frequently. The SCC*mec* cassette type XI contains another *mec*A homolog, also termed *mec*C or *mecALGA*$_{251}$. Clinical samples often contain both coagulase-negative *Staphylococcus* (CoNS) and *S. aureus*, all of which can carry the *mecA* gene. Therefore, since the detection of *mecA*/*mecC* alone is insufficient to specifically identify MRSA, samples should be specifically tested for *S. aureus* in parallel with the detection of the *mecA*/*mecC gene*. Thus, to avoid the detection of false positives due to the presence of Methicillin-resistant-CoNS or Methicillin-sensitive *Staphylococcus aureus* (MSSA), and to accurately detect previously described MRSA in a clinical sample, a specific *S. aureus* region located between the conserved open reading frame *orfX* and SCC*mec* containing the *mecA*/*mecC* gene should be targeted. In addition, *S. aureus* produces an extracellular thermonuclease, encoded by the species-specific *nuc* gene, which is commonly used to distinguish *S. aureus* from the other *Staphylococcus spp.*

Culture-based detection of MRSA requires the isolation of pure colonies followed by antibiotic susceptibility testing, detection of the *mec*A gene or detection of the PBP2a protein. This process takes between 24 to 72 hours to implement. With the rapidity at which MRSA infections can spread, especially in healthcare settings where carriers are common, the capability of providing results of MRSA nasal carriage on the day of admission represents a definite advantage for infection prevention programs. Application of real-time PCR-based assays for MRSA screening from nasal swabs can decrease turnaround time to 1 to 2 hours. Fast and accurate MRSA screening allows infected patients to be specifically treated and appropriate hygiene methods to be introduced to prevent the transmission and spread of MRSA.

2. Intended Use

NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, is a molecular test based on real-time PCR technology, intended for the rapid detection and qualitative diagnosis of MRSA nucleic acids from nasal swabs collected in a transport kit containing Liquid Amies medium. This test is intended to be used as an aid in the diagnosis of MRSA infection in combination with the patient's clinical signs and symptoms and epidemiological risk factors. The test is not intended

to guide or monitor the treatment of MRSA infections. The real-time PCR technique allows the detection of specific MRSA DNA targets in the sample, if present. A negative result does not exclude nasal colonization of MRSA and should not be used as the sole instrument for the patient's treatment decision. There are no contraindications for using the MRSA Multiplex Real-time PCR Kit, IVD. Testing must be performed by specialized and qualified laboratory technicians, especially in real-time PCR technique and molecular *in vitro* Diagnostics. The kit should only be used as indicated in this user manual.

3. Principles of the Assay

NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, provides the set of reagents, enzymes, and oligonucleotides (primers and probes), for the qualitative detection of the Methicillin-resistant *Staphylococcus aureus* (MRSA), using the real-time PCR technique (see equipment specification requirements in **Section 6**). The kit detects target sequences of *mec*A, *mec*C and *nuc* genes, as well as the SCC*mec-orfX* junction. These genomic regions were previously described as specific genetic markers for MRSA identification. *S. aureus* resistance to methicillin/oxacillin and other β-lactam antibiotics is conferred by the *mec*A and *mec*C genes. The *mecA* gene is localized on the variable and unstable SCC*mec* gene cassette (staphylococcal cassette chromosome mec). The SCC*mec* cassette type XI (SCC*mec* XI) contains a *mecA* homolog, also termed *mecC*. The *mecC* gene, which has only 70% nucleotide homology to the conventional *mecA*, has been described in *S. aureus* isolates of humans and cattle. The SCC*mec-orfX* junction region was specifically selected to target the region between a conserved open reading frame *orfX* in *S. aureus* and the SCC*mec* containing the *mec*A gene. Finally, the *nuc* gene encodes a thermostable nuclease of *S. aureus* which allows distinguishing *S. aureus* from another *Staphylococcus spp*. The detection of these specific targets ensures that only MRSA is present in the sample.

NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, was designed to have a broad detection profile while remaining specific for MRSA detection. In addition, the set of oligonucleotides was particularly designed for the detection of this organism and does not show significant homology with other genomes, which reflects the high specificity and detection sensitivity of the test. As such, the kit was designed to be specific to MRSA and to avoid the detection of other organisms causing similar infections. The internal control, included in the kit, validates the efficacy of the nucleic acids extraction process as well as the absence of PCR inhibitors potentially present in the human biological samples. Periodically, NZYTech revisits MRSA target sequences and, if necessary, will release a new version of this kit. Additionally, the kit includes three external controls (two positive controls and a negative control) as described below. The positive controls consist of nucleic acid fragments that contain all target sequences detected by the kit.

In this kit, the qualitative detection of DNA is based on real-time PCR technology, which is a reference methodology in laboratory molecular diagnosis. It is a methodology of high sensitivity and specificity to accurately detect the presence of this organism. NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, is based on the principle of researching the presence of MRSA DNA, isolated and purified with an extraction system. The extracted DNA is subjected to a PCR amplification, in a single reaction, using five highly specific primer/probe sets exploiting the TaqMan® principle. In the presence of MRSA DNA, the TaqMan® probes bind to conserved

regions of *mecA*, *mecC* and *nuc* genes, as well as to a specific region of the *SCCmec-orfX* junction. Each of these four targets is flanked by two specific primers pairs. A fifth primers/probe set acts as an internal control, detecting the human *RNaseP* gene (RP). Detection of the internal control validates the efficacy of the extraction process, and it also allows confirming whether the PCR reaction was compromised by the presence/absence of inhibitors in clinical samples. To allow identifying the amplification of the five specific targets in a single reaction, MRSA (four primers/probe sets) and human RNase P, specific probes are differently labelled, namely with FAM™, HEX™, Texas Red® and Cy5™ reporter dyes, respectively. Thus, this kit consists of a pentaplex assay where the targets *mecA* & *mecC*, SCC*mec/orfX* junction and *nuc* are detected in the optical channels FAM, HEX/VIC/JOE and Texas Red/JUN, respectively, while the human RP gene is detected in the Cy5 optical channel. These oligonucleotides/probe sets are provided in optimized concentrations to ensure that human DNA, even when present at extremely high concentrations, does not limit the efficiency of the MRSA primers/probe sets.

4. Kit Composition

NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, provides a comprehensive set of reagents sufficient to perform 96 qPCR reactions in a single step.

Kit Component		Volume (per vial)	Number of tubes	Cap colour
MRSA MMix	NZYSupreme Multiplex qPCR Probe Master Mix (2x)	1050 µL	1	Neutral
MRSA PPMix	MRSA/RP Primer & Probe Mix (10x)	205 µL	1	Brown
MRSA POS 1	MRSA/RP Positive Control 1	105 µL	1	Red
MRSA POS 2	MRSA/RP Positive Control 2	105 µL	1	Red
NTC	No-Template Control	105 µL	1	Neutral

5. Storage, Stability and Handling Conditions

NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, is shipped refrigerated. Upon receipt of the kit, all components should be immediately stored at -85°C to -15°C. When in use, kit components should be promptly placed in the freezer after use to minimize the exposure time to room temperature. In addition:

- Minimise the number of freeze-thaw cycles by storing working aliquots. If appropriate, kit components may be aliquoted in smaller volumes after thawing. The kit is stable through a minimum of 6 freeze-thaw cycles.
- The MRSA PPMix component should be stored protected from light. Particularly, do not expose MRSA MMix to direct sunlight after combining with MRSA PPMix.
- If the package that protects the kit arrives damaged, please contact NZYTech.
- Beware of the expiry date indicated on the packaging. NZYTech does not recommend using the kit after the expiry date. On this date, the kit must be discarded following the disposal instructions in **Section 8.2**.

6. Materials and Instrumentation Required but Not Provided

- Real-time PCR equipment that detects FAM, HEX/VIC/JOE, Texas Red/JUN and Cy5 fluorescence channels (at emission wavelengths of 520, 556, 615 and 670 nm, respectively). See in **Section 11** the equipment models for which the kit was validated.
- Equipment and consumables for isolating DNA of biological/clinical samples.
- RNase/DNase-free qPCR plasticware: PCR tubes, strips, caps, 96-well plates, adhesive films.
- Pipettes and filter tips (RNase/DNase-free).
- Cooling block.
- Disposable gloves.
- Vortex and centrifuge.

7. Sample Collection and Preparation

MRSA Multiplex Real-time PCR Kit, IVD, is designed to detect DNA extracted from nasal swab samples. Nasal specimens should be collected from both nostrils, one at a time, using a unique swab (ESwab®, Copan). Place the swab in the transport tube containing Liquid Amies transport medium. Collection must be carried out in sterile tubes. Specimens should be tightly sealed in proper tubes or containers, correctly labelled, and then promptly transported to the laboratory. Collected samples should be tested as soon as possible. Inappropriate sample collection, handling and/or transport of specimens may result in a false result. Extracted nucleic acids constitute the starting material for the assay with NZYTech' MRSA Multiplex Real-time PCR Kit, IVD. NZYTech recommends the use of NZY Mag Viral RNA/DNA Isolation Kit, IVD (MD0488, NZYTech) for nucleic acids extraction, as this kit has been validated for the extraction of MRSA clinical samples. Please ensure that DNA samples are suitable in terms of purity, concentration, and nucleic acid integrity. Since ethanol is a strong inhibitor of real-time PCR, it is necessary to eliminate this component before the elution of nucleic acids during the extraction process. NZYTech' kit contains an internal control that targets human DNA co-purified with bacterial MRSA DNA. Human DNA is amplified with the set of oligonucleotides (primers and probe) from the human RP gene. The introduction of internal control is useful in assessing the efficiency of DNA extraction and isolation and/or in detecting the presence of potential inhibitors during sample processing.

8. Precautions and Warnings

Carefully follow the procedures and guidelines provided in this handbook to ensure that the test is performed correctly. Before using the test, check the product's integrity, namely the amount and type of kit components and their correct labelling. As in any analytical testing procedure, good laboratory practices are essential. Any deviation from good laboratory practices may result in assay failure or cause erroneous results. Due to high sensitivity of the kit, special care must be taken to keep reagents and PCR amplification mixes free from contamination.

8.1 Safety Information

Before using the kit, please consult the Safety Data Sheet (SDS) that is available on NZYTech website (www.nzytech.com). This kit detection should be performed only by staff trained in the

relevant technical and safety procedures in appropriately equipped laboratories. International and national guidelines on laboratory biosafety should be followed in all circumstances.

8.2 Handling and Procedural Requirements

- Only for professional *in vitro* diagnostic use.
- Do not use this kit after the expiration date.
- Do not use the test components if the kit sealing is damaged.
- Do not interchange reagents of different production lots.
- No reagents from other manufacturers should be used along with the reagents of this kit.
- DNase/RNase-free disposable plasticware and pipettes should be used in all procedures.
- Sample preparation, the reaction set up and amplification should be performed in different working areas. The order of tasks in the lab should be unidirectional. Always wear disposable gloves in each area and change them before entering a different area. If possible, change your coat.
- Select specific materials and equipment for each individual work area and do not transfer them from one area to another.
- Always use the NTC – No-template Control provided in the kit.
- Biological samples must be handled as if they were infectious and following appropriate biosafety precautions.
- Positive controls contain high copy number templates; they should be opened and processed away from test samples and kit components to avoid cross-contamination.
- Handle post-amplification plates with care and dispose them immediately after the end of the testing; plates should always be discarded into a proper biohazard container after use. Do not open post-amplification reaction tubes/plates to avoid amplicon contamination.
- At the end of each test, clean work surfaces and equipment with a DNA/RNA remover.
- Residues of chemicals and preparations are generally considered hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations.
- All results should be interpreted by a healthcare professional in the context of the patient's medical history and clinical symptoms.
- This test cannot exclude diseases caused by other pathogens.
- A negative result for any PCR test does not conclusively rule out the possibility of infection.
- Follow good laboratory practices, wear protective clothing, permanently wear disposable powder-free gloves, and use goggles and a mask. Do not eat, drink, or smoke in the working area.

9. Testing Procedure

Please read the instructions for use carefully before performing the assay. Beware that all pipetting steps and experimental plate set-up should be performed following good qPCR practices. After the plate is poured start immediately the qPCR protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artifacts that reduce the sensitivity of detection. Before the experiment, start by gently mixing the reaction tubes provided with the finger and centrifuge for five seconds to collect contents at the bottom of the tube. Place tubes on ice. **We strongly recommend pipetting MRSA POS 1 and MRSA POS 2 last to avoid cross-contaminations.**

9.1 Reaction set-up

1. Prepare a qPCR mix enough for the number of tests to be performed with a 5% additional volume for pipetting losses. Proceed according to the table below that specifies the volumes for 1 and *n* tests (where *n* corresponds to the total number of reactions):

Component	**1 test volume (µL)**	***n* tests * volume + 5% (µL)**
MRSA MMix **	10	*n* x 10.5
MRSA PPMix	2	*n* x 2.1
Volume final	**12**	***n* x 12.6**

** To calculate the total number of reactions needed for each assay, count the number of samples and add three more, to include the Negative and the two Positive controls.*
*** Please notice that a precipitate in the bottom of the master mix tube may be observed, in particular after multiple freeze/thaw cycles. To ensure optimal performance, please make sure all components are thawed and resuspended before use. In this case do not spin the master mix before pipetting.*

2. Pipette 12 µL of the qPCR mix into individual wells according to your real-time PCR experimental plate set-up.

3. For the negative control, add 8 µL of NTC instead of the DNA template into the negative control well. The final volume should be 20 µL.

4. For the biological samples, add 8 µL of each DNA sample into the sample wells, according to your experimental plate set-up. The final volume in each well should be 20 µL.

5. For the two positive controls, add 8 µL of MRSA POS 1 and add 8 µL of MRSA POS 2 instead of the DNA template into the positive control wells. The final volume should be 20 µL.

6. Cover and seal the plate with an appropriate optical adhesive film or caps before proceeding with the qPCR and detection steps.

7. Place the reaction plate in the real-time PCR equipment and run the qPCR protocol according to the section below.

9.2 Programming the real-time PCR equipment

The table below displays a standard protocol optimized on a few platforms. However, these conditions may be adapted and validated to suit different machine-specific protocols.

Cycles	**Temperature**	**Time**	**Step**
1	95 °C	5 min	*Polymerase activation*
45	95 °C	10 s	*Denaturation*
	60 °C	30 s	*Annealing/Extension**

** Depending on the qPCR equipment select the appropriate detection channels.Fluorogenic data should be collected during this step through channels FAM, HEX/VIC/JOE, Texas Red/JUN and Cy5.*

The fluorescent dyes used in this kit and their correspondent detection channels are the following:

Fluorescent Dyes & Detection Channels

Targets	Fluorescent dye	Detection channel
*mec*A/*mec*C	FAM™	FAM
SCC*mec*/*orfX junction*	HEX™	HEX/VIC/JOE
nuc	Texas Red®	Texas Red/JUN
RNaseP	Cy5™	Cy5
MRSA POS 1	FAM™ & HEX™ & Texas Red® & Cy5™	FAM & HEX/VIC/JOE & Texas Red/JUN Cy5
MRSA POS 2	FAM™ & HEX™ & Texas Red® & Cy5™	FAM & HEX/VIC/JOE & Texas Red/JUN & Cy5

MRSA Multiplex Real-time PCR Kit, IVD, was validated for the following Real Time PCR Systems: Applied Biosystems™ 7500 FAST, Applied Biosystems™ QuantStudio 5, Roche Lightcycler® 96 Instrument, Bio-Rad® CFX 96 and Bio-Rad® CFX Opus. If other equipment is used, the user should validate the kit using previously characterised samples (both positive and negative).

10. Data Analysis

10.1 Run Validation Criteria

Before analysing results, we recommend consulting the user manual of the respective qPCR device. Then verify that the real-time PCR test is valid. Thus, for each plate, please confirm if the results for Positive and Negative controls performed as expected, according to the following criteria:

- **Negative control (no template reaction):** no amplification is detected. Sample contamination may have occurred if the negative control has amplification curves (FAM, HEX/VIC/JOE, Texas Red/JUN and Cy5) with a sigmoidal shape. Repeat the test following good qPCR practices.

- **Positive control 1 (MRSA POS 1):** the amplification curves of FAM (*mecA*), HEX (SCC*mec*/*orfX* junction), Texas Red/JUN (*nuc*) and Cy5 (RP) are positive. Positive control is expected to amplify at Ct < 32, in the four channels. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

- **Positive control 2 (MRSA POS 2):** the amplification curves of FAM (*mecC*), HEX (SCC*mec*/*orfX* junction), Texas Red/JUN (*nuc*) and Cy5 (RP) are positive. Positive control is expected to amplify at Ct < 32, in the four channels. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

If the controls are according to the expected, the test is **valid**. Please proceed with the interpretation of the results for the tested samples.

If any of the controls do not exhibit the expected performance, the assay was compromised or executed improperly and should be considered **invalid**. **Please, repeat the test**. If the problem persists, contact the manufacturer.

10.2 Test Results Interpretation

NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, uses the following Ct cut-off values for results interpretation:

Ct value	Results interpretation
Ct ≤37	Detected (+) → **POSITIVE**
Ct >37	Not Detected (-) → **NEGATIVE**

MRSA is detected if the FAM (*mecA*/*mecC*), HEX/VIC/JOE (SCC*mec*/*orfX* junction) and Texas Red/JUN (*nuc*) amplification curves display a sigmoidal shape with a Ct≤37, regardless of what result is obtained for the RP (Cy5) assay (in this case the presence or absence of a signal in the Cy5 channel is not significant for the validity of the processed analysis).

MRSA is not detected if the FAM (*mecA*/*mecC*) and/or the HEX/VIC/JOE (SCC*mec*/*orfX* junction) and/or Texas Red/JUN (*nuc*) curves do not amplify or amplify at Ct>37, while the RP (Cy5) assay displays a positive sigmoidal curve (Ct≤45)

The test is invalid if the three MRSA amplification curves as well as the RP amplification curve are negative. The test should be repeated with nucleic acids re-purified from the sample.

The following table summarises the interpretation of principal results (evaluate the overall shape of the amplification curves; **only sigmoidal amplification curves are indicative of true amplification**).

mecA/mecC (FAM)	**SCC*mec/orfX*** (HEX/VIC/JOE)	***nuc*** (TexasRed/JUN)	**RP**[1] (Cy5)	**Negative control**	**Positive controls**	**Results interpretation**
+	+	+	+/-	-	+	**MRSA → POSITIVE** [2]
+	-	+	+/-	-	+	**MRSA → NEGATIVE** [3]
-	+	+	+/-	-	+	**MRSA → NEGATIVE**
-	-	+	+/-	-	+	**MRSA → NEGATIVE**
+	-	-	+/-	-	+	**MRSA → NEGATIVE** [4]
-	+	-	+/-	-	+	**MRSA → NEGATIVE**
+	+	-	+/-	-	+	**MRSA → NEGATIVE**
-	-	-	+	-	+	**MRSA → NEGATIVE**
-	-	-	-	-	+	**INVALID TEST** [5]

[1] *A high concentration/load of detectable MRSA DNA in the sample can lead to a reduced or absent internal control signal on the Cy5 channel (+: amplification curve detected; -: no amplification curve).*
[2] *MRSA: S. aureus resistant to methylicin.*
[3] *In the presence of a new or an unknown MRSA variant the HEX/VIC/JOE channel can be negative.*
[4] *MRCoNs: Coagulase-negative Staphylococcus resistant to methicillin/oxycillin.*
[5] *Repeat DNA extraction and run the qPCR test again.*

Note: Interpretation of results must account for the possibility of false negative and false positive results.

- False negative results may be caused by:
  - Unsuitable collection, handling and/or storage of samples.
  - Sample degradation.
  - Presence of qPCR inhibitors.
  - Mutations in the genome of the pathogenic agent; failure to detect new or unknown variants.
  - Failure to follow procedures in this handbook.
  - Use of unvalidated extraction kits or real-time PCR platforms.

- False positive results may be caused by:
  - Sample containing a mixture of multiple commensal pathogens.
  - Given the evidence of the resistance gene, a mixed infection of MSSA (methicillin-sensitive *S. aureus*) and CoNS (coagulates-negative staphylococci) may exist.
  - Cross-contamination with the positive controls due to its unsuitable handling.
  - Unsuitable handling of samples containing high concentrations of MRSA DNA. Due to the high susceptibility of the qPCR method for cross contaminations special care should be taken during DNA isolation.
  - Unsuitable handling of amplified product (post-amplification qPCR plate).

Negative results do not preclude MRSA infection and the test result should not be used as the sole basis for treatment or other patient management decisions. In addition, this test cannot rule out diseases caused by other pathogens.

11. Performance Evaluation

This kit performance was validated for the equipments specified in **Section 9.2** (see above). If other equipment is used, the user should validate the kit using previously characterised samples (both positive and negative).

11.1 Expected Results

Typical amplification plots, observed for clinical MRSA negative nasal swab specimen (Figure 1A) and of a nasal swab sample from a patient identified as a carrier of MRSA (Figure 1B), are presented below:

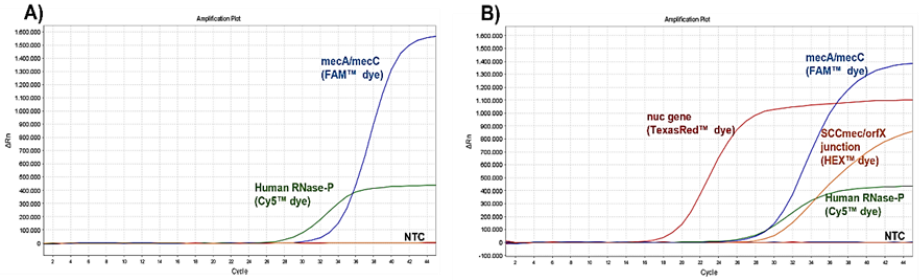


Figure 1. Detection of *mec*A/*mecC, nuc, SCCmec/orfX* junction fluorescence curves generated by the pentaplex MRSA Multiplex Real-time PCR Kit, IVD, in a clinical MRSA negative nasal swab specimens containing the *mecA* and/or *mecC* genes (Figure 1A) and a nasal swab sample from a patient identified as carrier of MRSA (Figure 1B). Blue curve: detection of DNA harbouring *mec*A/*mec*C targets through the FAM channel; Red curve: detection of DNA harbouring *nuc* target through the TexasRed/JUN channel; Orange curve: detection of a DNA harbouring the SCC*mec*/*orfX* junction target through the VIC/HEX/JOE channel; Green curve: detection a DNA harbouring the human RP target through the Cy5 channel.

11.2 Limit of Detection (LoD) - Analytical Sensitivity

The analytical sensitivity was defined as the lowest concentration of analyte that could be reliably detected with 95% confidence. This was assessed by testing MRSA nucleic acids at different copy numbers, spiked into DNA extracted from negative nasal swab samples, tested in 48 replicates per concentration, by two different operators, using 3 different kit batches following standard testing reaction conditions. Tests were repeated over 4 days. Together, the data revealed that NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, detects 0.15 copies/µL of *mec*A, *mec*C, *nuc* and SCC*mec*/*orfX* junction with confidence ≥95%. Thus, the tentative Limit of Detection (LoD) was determined to be 150 copies/mL for MRSA. All assays were performed using the Applied Biosystem® 7500 FAST Real Time PCR equipment and the analysis was performed using the equipment's software.

11.3 Analytical Specificity

11.3.1 Cross-Reactivity (Exclusion) and Specificity

Cross-reactivity and inclusivity were evaluated *in silico* by analysing all oligonucleotide probes and primers included in the kit against pathogens related to MRSA and normal pathogens that cause infections with similar symptoms, respectively. Assay primers and probes were screened against published genome sequences. Upon *in silico* analysis, the assay design was found to specifically detect MRSA and exhibited no reactivity with non-related species.

Cross-reactivity of the NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, was further evaluated *in vitro* by testing a panel consisting of well-characterized isolates of methicillin-sensitive *Staphylococcus aureus* (MSSA), coagulase-negative *Staphylococci* (CoNS), closely related genera and other pathogenic and commensal flora found in the nostrils. *In vitro* cross-reactivity of MRSA Multiplex Real-time PCR Kit, IVD, was assessed using the following pathogens: *Bacteroides ovatus, Bacteroides thetaiotaomicron, Burkholderia vietnamiensis, Dickeya dadantii, Enterobacter cloacae, Klebsiella pneumoniae, Mycobacterium intracellulare, Mycobacterium mageritense, Mycobacterium smegmatis, Nocardia nova, Pseudomonas mendocina, Streptococcus mutans, Streptococcus pneumoniae, Streptococcus pyogenes, Streptomyces avermitilis* and *Streptomyces albidoflavus.* Assays were performed using negative nasal specimens spiked with genomic DNA of the organisms referred to above. Furthermore, wet testing was performed using six inactive samples that are representative of true clinical human specimens, including *S. aureus* (MRSA Hospital Strain), *S. aureus* (MRSA Community Strain), *S. aureus* (*mecC*), *S. aureus* (MSSA Empty Cassette), *S. aureus* (MSSA) *and Staphylococcus epidermidis* (MSSE HER 1292) which is a CoNS (Zeptometrix). All tests were performed in triplicate using three kit batches. Samples were extracted in duplicate using Thermo Scientific KingFisher Flex Purification System with NZYTech' NZY Mag Viral RNA/DNA Isolation Kit, IVD (MD04881), and evaluated on the Applied Biosystems® 7500 FAST. None of the pathogens tested yielded a false-positive qPCR signal.

11.3.2 Interfering Substances

The impact of 26 potential interferent substances occasionally used in the nares or found in nasal swab specimens were evaluated for potential interference with the MRSA Multiplex Real-time PCR Kit, IVD. The assays were performed using negative nasal specimens spiked with MRSA positive specimens at ~2-3x LoD. Potential interfering substances were added to the contrived samples at concentrations representing the highest levels expected in human respiratory patient samples based on literature data. The experiment was performed over three days. All tests were performed in duplicate using one kit batch and compared to data obtained with a control test containing no interferents. At the concentrations tested, the results revealed that none of the molecules under test affected the sensitivity of the detection. The table below resumes the data collected under these experiments. Data revealed that none of the substances tested interfered with the sensitivity of detection of MRSA by the MRSA Multiplex Real-time PCR Kit, IVD.

Potential Interferent	Active Ingredients	Final concentration in the sample	Interference Yes (Y) or No (N)
Rhinomer® (Isotonic Sea Water)	Sea water	10% v/v	N
Strepfen® (Throat spray, oral anesthetic & analgesic)	Flurbiprofen	5% v/v	N
Vibrocil® (Nasal wash solution; Allergy spray)	Fluticasone propionate	5% v/v	N
Nasomet® (Nasal Corticosteroids spray)	Mometasone furoate	5% v/v	N
Pulmicort® (Nasal Corticosteroids spray)	Budesonide	5% v/v	N
Trobex® (Antimicrobial, systemic)	Trobamycin	10 µg/mL	N
Pyralvex® (Mouth analgesic, anti-inflammatory & antiseptic)	Rhubard extract, Salicylic acid	5% v/v	N
Eludril Gé (Mouthwash solution antiseptics)	Chlorhexidine gluconate, Chlorobutanol hemihydrate	5% v/v	N
Isophy® (Physiological Serum)	NaCl 0,9 %	10%	N
Saliva (human)	-	25% v/v	N
Mucolsovan® (Mucolytic)	Ambroxol hydrochloride	5% v/v	N
Whole Blood (human)	-	4% v/v	N
Bactroban® (Antibiotic, nasal ointment)	Mupirocin	5 mg/mL	N
Nasal mucus (mucin)	Mucus (human)	25% v/v	N
Allergodil® (Nasal spray Solution)	Azelastine hydrochloride	5% v/v	N
Aeromax nasal® (Nasal wash solution)	Budesonide	10% v/v	N
Avamys® (Nasal wash solution)	Fluticasone furoate	10% v/v	N
Bisolspray Nebulicina® (Nasal wash solution)	Oxymetazoline Hydrochloride	10% v/v	N
Mometasona Generis® (Nasal wash solution)	Mometasone	5% v/v	N
Nasorhinathiol® (Nasal wash solution	Oxymetazoline Hydrochloride	10% v/v	N
Rhinomer intense Eucalyptus® (Nasal wash solution)	hypertonic sea water with eucalyptus essential oil	10% v/v	N
Vibrocil Actilong® (Nasal wash solution)	Xylometazoline Hydrochloride	10% v/v	N
Tamiflu® (Antiviral drug)	Oseltamivir	5% v/v	N
Neo-Sinefrina® (Nasal wash solution)	Phenylephrine hydrochloride	10% v/v	N
Neo-Sinefrina Alergo® (Nasal wash solution)	Beclomethasone Dipropionate	10% v/v	N
Predniftalmina® (Ophthalmic Ointment)	Prednisolone and Chloramphenicol	10% v/v	N
Control without any interfering substance	H_2O	5% v/v	N

11.4 Precision

Assay precision for NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, was determined by the repeated testing of positive samples representing two bacterial load levels, 3x LoD and 30x LoD copies per reaction, spiked into DNA extracted from negative nasal swab specimens, using 3 different kit batches, and following typical testing reaction conditions. Precision was evaluated by measuring Cq average, Cq coefficient of variation and % of replicate detection, as described below for each case. The data is resumed in tables displayed below.

Precision of NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, while detecting *mec*A target gene

Variable tested		*mecA* (Copies/Reaction)	
		3x LoD	30x LoD
Repeatability	n	18	18
	Mean Cq	35,77	32,88
	Coefficient of Variation (%)	2,01	1,19
	% Replicate Detection	100	100
Daily Reproducibility	n	72	72
	Mean Cq	36,05	33,04
	Coefficient of Variation (%)	2,56	1,22
	% Replicate Detection	98,61	100
Lot-to-lot Reproducibility	n	126	126
	Mean Cq	36,07	33,06
	Coefficient of Variation (%)	2,14	1,10
	% Replicate Detection	99,21	100
Operator Reproducibility	n	36	36
	Mean Cq	36,35	33,18
	Coefficient of Variation (%)	1,51	1,14
	% Replicate Detection	100	100
Inter-instrument Reproducibility	n	90	90
	Mean Cq	35,93	32,92
	Coefficient of Variation (%)	2,19	1,23
	% Replicate Detection	100	100

Precision of NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, while detecting *mec*C target gene

Variable tested		*mecC* (Copies/Reaction)	
		3x LoD	30x LoD
Repeatability	n	18	18
	Mean Cq	35,56	33,02
	Coefficient of Variation (%)	2,57	0,95
	% Replicate Detection	100	100
Daily Reproducibility	n	72	72
	Mean Cq	35,68	33,07
	Coefficient of Variation (%)	2,54	1,40
	% Replicate Detection	97,22	100
Lot-to-lot Reproducibility	n	126	126
	Mean Cq	35,88	33,17
	Coefficient of Variation (%)	2,53	1,40
	% Replicate Detection	97,62	100
Operator Reproducibility	n	36	36
	Mean Cq	36,25	33,33
	Coefficient of Variation (%)	2,10	1,62
	% Replicate Detection	100	100
Inter-instrument Reproducibility	n	90	90
	Mean Cq	35,80	32,91
	Coefficient of Variation (%)	2,41	1,24
	% Replicate Detection	98,89	100

Precision of NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, while detecting *nuc S. aureus* specific target gene

Variable tested		*nuc* (Copies/Reaction)	
		3x LoD	30x LoD
Repeatability	n	18	18
	Mean Cq	35,63	32,76
	Coefficient of Variation (%)	2,03	1,09
	% Replicate Detection	100	100
Daily Reproducibility	n	72	72
	Mean Cq	35,68	32,88
	Coefficient of Variation (%)	2,29	1,41
	% Replicate Detection	100	100
Lot-to-lot Reproducibility	n	126	126
	Mean Cq	35,96	32,99
	Coefficient of Variation (%)	2,48	1,44
	% Replicate Detection	100	100
Operator Reproducibility	n	36	36
	Mean Cq	36,28	33,22
	Coefficient of Variation (%)	2,33	1,32
	% Replicate Detection	100	100
Inter-instrument Reproducibility	n	90	90
	Mean Cq	35,63	32,65
	Coefficient of Variation (%)	2,36	1,23
	% Replicate Detection	100	100

Precision of NZYTech' MRSA Multiplex Real-time PCR Kit, IVD, while detecting SCC*mec*/*orfX* junction target

Variable tested		SCC*mec*/*orfX* junction	
		3x LoD	30x LoD
Repeatability	n	18	18
	Mean Cq	36,09	33,27
	Coefficient of Variation (%)	1,44	0,77
	% Replicate Detection	100	100
Daily Reproducibility	n	72	72
	Mean Cq	36,25	33,33
	Coefficient of Variation (%)	2,04	1,16
	% Replicate Detection	100	100
Lot-to-lot Reproducibility	n	126	126
	Mean Cq	36,33	33,36
	Coefficient of Variation (%)	1,96	1,18
	% Replicate Detection	100	100
Operator Reproducibility	n	36	36
	Mean Cq	36,54	3,39
	Coefficient of Variation (%)	1,82	1,09
	% Replicate Detection	100	100
Inter-instrument Reproducibility	n	90	90
	Mean Cq	35,26	32,18
	Coefficient of Variation (%)	4,03	3,89
	% Replicate Detection	100	100

11.4.2 Repeatability

Repeatability was assessed by one operator by analysing 18 replicates of each sample (3x LoD and 30x LoD copies per reaction), accounting for a final number of 36 tests performed per target.

11.4.3 Daily Reproducibility

Daily reproducibility was assessed by one operator by analysing 72 replicates of each sample (3x LoD and 30x LoD copies per reaction), for 4 days, with 18 replicates of each concentration per day (a total of 144 assays per target were performed).

11.4.4 Lot-to-lot Reproducibility

Reproducibility between lots was assessed by one operator through the analysis of 126 replicates of each sample (3x LoD and 30x LoD copies per reaction) using 3 different kit batches with 84 replicates per batch.

11.4.5 Operator Reproducibility

Operator reproducibility was assessed by testing 36 replicates of each sample (3x LoD and 30x LoD copies per reaction), by three different operators with 12 replicates per operator, making a total of 24 replicates per operator, using 3 different kit batches.

11.4.6 Inter-instrument Reproducibility

Inter-instrument reproducibility was measured by one operator through the testing 90 replicates of each sample (3x LoD and 30x LoD copies per reaction), in five different qPCR equipments, in a total of 36 tests per equipment.

Real-time PCR equipment' manufacturer	Real-time PCR platform model
Applied Biosystem®	7500 Fast
	QuantStudio™ 5
Roche®	LightCycler™ 96 instrument
Bio-Rad®	CFX Opus 96 Real-Time PCR
	CFX96 Touch Real-Time PCR

11.5 Clinical evaluation

The clinical performance of NZYTech' MRSA Multiplex Real-time PCR Kit, IVD was evaluated using 157 nasal swab samples previously characterized using routine microbiological culture. Data revealed that 100% of clinical sensitivity (PPA) and 72,7% of clinical specificity (NPA) agreements were achieved for all positive and negative samples tested. Overall, results show high sensitivity and specificity to detect MRSA using NZYTech' MRSA Multiplex Real-time PCR Kit, IVD.

		Comparator assay		
		MRSA Positive	**MRSA Negative**	**Total**
MRSA Multiplex Real-time PCR Kit, IVD	**MRSA Positive**	58	27*	85
	MRSA Negative	0	72	72
	Total	58	99	157

PPA (Positive percent agreement): 100,0 % for MRSA
NPA (Negative percent agreement): 72,7 % for MRSA

* A possible explanation for those negative culture samples is loss of bacterial viability during specimen collection and/or transport, where the nonviable bacterial DNA remained available for amplification. In addition, this difference can be justified by the fact that qPCR technique is more sensitive than culture methods.

12. Quality Control

All components of NZYTech' MRSA Multiplex Real-time PCR Kit, IVD are tested following the protocols described above. The pentaplex real-time PCR system allows the detection of targets described for the identification of MRSA DNA and human RNase P DNA. Positive amplifications are observed for target genes, positive control and internal controls through Texas Red/JUN, FAM, HEX/VIC/JOE and Cy5 channels, according to respective primers/probe set reporter dyes.

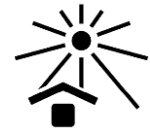
13. Technical Support

For Technical support, please contact our dedicated technical support team by Phone: +351 (0) 21 364 35 14 or Email: info@nzytech.com.

14. Trademarks and Disclaimers

All trademarks that appear in this manual are the property of their respective owners.

15. Explanation of Symbols

IVD	*in vitro* diagnostic medical device		Consult instructions for use
REF	Catalogue number		Manufacturer
LOT	Batch code		Use by
	Temperature limitation		Sufficient for
CONTROL +	Positive control		Keep away from the sun light (primer/probe mix)
CONTROL −	Negative control		

16. Conformity Declaration

Product Name: MRSA Multiplex Real-time PCR Kit, IVD

Catalogue Number: MD04931

Intended use: MRSA qualitative detection.

Manufacturer: NZYTech - Genes & Enzymes,

Estrada do Paço do Lumiar, Campus do Lumiar

Edifício E, R/C,

1649-038, Lisboa

Portugal

We, NZYTech, Lda – Genes & Enzymes, hereby declare that this product, to which this declaration of conformity relates, conforms with the following standards and other normative documents ISO 9001:2015, following the provisions of the 98/79/EC Directive on *in vitro* diagnostic medical devices as transposed into the national laws of the Member States of the European Union.

The product technical file is maintained at NZYTech, Estrada do Paço do Lumiar, Campus do Lumiar - Edifício E, R/C, 1649-038 Lisboa, Portugal.

Joana Brás, PhD

Technical Director

17. References

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