

Quantification of methicillin-resistant Staphylococcus aureus

mecA (penicillin binding protein 2)

And

S. aureus FEMB gene (chromosomal gene)

For general laboratory and research use only

Advanced kit





Contents

Introduction to <i>methicillin-resistant Staphylococcus aureus</i>	3
Reference	3
Specificity	4
Kit Contents	5
Reagents and equipment to be supplied by the user	5
Kit storage	5
Suitable sample material	6
Dynamic range of test	6
Principles of the test	7
Bench side Protocol	9
Amplification Protocol	12
Interpretation of Results	13
Notices and disclaimers	14
Trademarks	14



Introduction to *methicillin-resistant Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a specific strain of the *Staphylococcus aureus* bacterium that has developed antibiotic resistance to all penicillins, including methicillin and other narrow-spectrum β -lactamase-resistant penicillin antibiotics.^[1] The resistant strain, MRSA was first discovered in the UK in 1961 and is now widespread, particularly in the hospital setting where it is commonly termed a superbug.

MRSA may also be known as oxacillin-resistant *Staphylococcus aureus* (ORSA) and multiple-resistant *Staphylococcus aureus*, while non-methicillin resistant strains of *S. aureus* are sometimes called methicillin-susceptible *Staphylococcus aureus* (MSSA) if an explicit distinction must be made.

Although MRSA has traditionally been seen as a hospital-associated infection, community-acquired MRSA strains have appeared in recent years, notably in the US and Australia.^[2] The abbreviations CA-MRSA (community-associated MRSA) and HA-MRSA (hospital-associated MRSA) are now commonly seen in medical literature.

Methicillin resistance arises by acquisition of a staphylococcal cassette chromosome *SCCmec*, and is conferred by the *mecA* gene. Expression of this gene yields PBP2a, a penicillin binding protein with reduced affinity for β -lactam rings (the primary active-site of the β -lactam antibiotics).^[6]

Some strains of *S. aureus* over-express β -lactamase and appear to be resistant to oxacillin and, rarely, methicillin despite being *mecA*-negative. They have slightly raised minimum inhibitory concentrations (MICs) and may thus be described as "minimally resistant". Other strains express modified PBPs (not PBP2) and exhibit varying degrees of β -lactam antibiotic resistance.

Not only are MRSA strains resistant to the usual antibiotics, but a curious interbreeding with community staph has led to an additional worry. Many MRSA isolates found outside of medical facilities, and referred to as CAMRSA (community-acquired MRSA), have acquired the Panton-Valentine leukocidin factor,^[7] a gene that produces a series of chemicals that make these MRSA particularly invasive as well as resistant.

Reference

J Clin Microbiol. 2002 May;40(5):1821-3.



Specificity

The *PrimerDesign™ Quantification Kit for Methicillin-resistant Staphylococcus aureus*, Is designed to detect two markets. The *mecA* gene is a plasmid based gene responsible for antibiotic resistance. *FEMB* is a chromosomal gene specific to *S. aureus*. A positive result for both markers has been validated as a reliable test for MRSA. The primers and probe have 100% homology with all reference sequences in the NCBI data. base including those listed below.

MecA sequence specificity

AM292304, AB266532, AB266531, AB245471, AB245470, AB236888, AY894415, DQ106887, AY786579, AY271717, AB221124, AB221123, AB221122, AB221121, AB221120, AB221119, BA000017, AP006716, CP000046, AM048803, AM048802, CP000255, AJ810121, AJ810120, AB047089, Y14051.1, Y13095.1, Y13096.1, Y00688.1, X52593.1, BA000033, BA000018, AB121219, CP000029, X52592.1, AB063172, AB033763, D86934.2, AB096217, AB063173, AB037671,

FEMB sequence specificity

CP000703.1, BA000017.4, CP000046.1, CP000253.1, CP000255.1, X17688.1, X571857.1, BA000033.2, BA000018.3, BX571856.1



Kit Contents

- Pathogen specific primer/probe mix MecA (150 reactions **BROWN**)
- Pathogen specific primer/probe mix FemB (150 reactions **BROWN**)
- Pathogen positive control template MecA (for Standard curve **RED**)
- Pathogen positive control template FemB (for Standard curve **RED**)
- Internal extraction control DNA (2x 150 reactions **BLUE**)
- Internal extraction control primer/probe mix (2x 150 reactions **BROWN**)
- Endogenous ACTB primer/probe mix (2x 150 reactions **BROWN**)
- RNase/DNase free water

Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
- **Mastermix or Mastermix components**
This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x *Precision*[™] Mastermix.
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Thin walled 1.5 ml PCR reaction tubes**

Kit storage

This kit is stable at room temperature. Once the lyophilized components have been re-suspended the kit should be stored at -20°C. Unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.



Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions PrimerDesign pathogen detection kits have very high priming efficiencies of >95% and can detect between 1×10^8 and 1×10^2 copies of target template.



Principles of the test

Real-Time PCR

Two pathogen specific primer and probe mixes are provided (MecA and FemB) and the target sequences can be detected through the **FAM** channel. It is **NOT** therefore possible to perform a multiplex for the pathogen primers.

The primer and probe mix provided exploits the so-called TaqMan[®] principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5`-reporter dye and a downstream, 3`-quencher, During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real time PCR platforms.

Positive control

For copy number determination, and as a positive control for the PCR set up, the kit contains positive control template. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in your particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment.

Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources of contamination should first be explored and removed.



Internal DNA extraction control

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using real-time PCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the pathogen target DNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a CT value of 26+/-3.

Endogenous ACTB control

To confirm extraction of a valid biological template a primer and probe mix is included to detect the Actin Beta (ACTB) gene. Detection of ACTB is through the FAM channel and it is **NOT** therefore possible to perform a multiplex for ACTB and the pathogen primers. A poor ACTB signal may indicate that the sample did not contain sufficient human material.

Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PrimerDesign recommend the application of 0.2 U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).



Bench side Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Barrier tips are recommended for all pipetting steps.

1. Pulse-spin each tube in a centrifuge before opening.

This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. Reconstitute the kit components according to the table below

To ensure complete reconstitution, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
Pre-PCR box	
Primer/Probe mix (BROWN)	165 µl
Internal extraction control DNA (BLUE)	600 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Endogenous ACTB primer/probe mix (BROWN)	165 µl
Post-PCR bottle	
Positive Control Template (RED) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control DNA directly to the biological sample as this can lead to degradation and a loss in signal strength.

1. Add 4µl of Internal extraction control DNA (**BLUE**) to each sample in DNA lysis/extraction buffer
2. Complete DNA extraction according to the manufactures protocols



Real-time PCR detection

1. Prepare a reaction mix according to the table below

Include sufficient reactions for the standard curve wells (8 samples in duplicate) and also the negative control.

Pathogen detection mix

Component	l reaction
2X Precision™ Mastermix	10 µl
Pathogen primer/probe mix (BROWN)	1.0 µl
Internal extraction control primer/probe mix (BROWN)	1.0 µl
RNAse/DNAse Free water	3.0 µl
Final volume	15 µl

Endogenous ACTB detection mix (Optional)

Component	l reaction
2X Precision™ Mastermix	10 µl
Endogenous ACTB primer/probe mix (BROWN)	1.0 µl
RNAse/DNAse Free water	4.0 µl
Final volume	15 µl

2. Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.

3. Prepare sample DNA templates for each of your samples (suggested concentration 5ng/µl) in RNAse/DNAse free water.

If the concentration of DNA is not known, then dilute your DNA sample reactions 1:20 (10 µl of sample DNA and 190µl of water)

4. Pipette 5µl of diluted template into each well, according to your experimental plate set up.

For negative control wells use 5µl of RNAse/DNAse free water. The final volume in each well is 20µl



5. Preparation of standard curve dilution series

- 1) Pipette 900µl of RNase/DNase free water into 7 tubes and label 2-8
- 2) Pipette 100µl of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 100µl from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2×10^7 per µl
Tube 2	2×10^6 per µl
Tube 3	2×10^5 per µl
Tube 4	2×10^4 per µl
Tube 5	2×10^3 per µl
Tube 6	2×10^2 per µl
Tube 7	20 per µl
Tube 8	2 per µl

6. Pipette 5µl of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20µl.



Amplification Protocol

Amplification conditions using PrimerDesign 2X Precision™ MasterMix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37°C
	Enzyme Activation (if required)***	10 mins	95°C
50 cycles	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C

* Fluorogenic data for the control DNA should be collected during this step through the FAM and VIC channels

** Required if your mastermix includes UNG to prevent PCR carryover contamination

*** Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.



Interpretation of Results

Pathogen	Internal control	Negative control	Positive control	Interpretation
+ve	+ve	-ve	+ve	+ve
+ve	-ve	-ve	+ve	+ve
-ve	+ve	-ve	+ve	-ve
-ve	-ve	-ve	-ve	Exp Fail
+ve	+ve	+ve	+ve	Exp Fail

Internal PCR control

When used according to the above protocols, assuming a 100% extraction efficiency and that 1:20 of extracted DNA is used in the reaction, a CT value of 26 is expected. However this can vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. CT values of 26 ± 3 are within the normal range. When amplifying a pathogen sample with high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous ACTB control

The signal obtained from the ACTB primer and probe set will vary according to the amount of biological material present in a given sample.



Notices and disclaimers

During the warranty period PrimerDesign pathogen detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired.

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