Protocol for Multiple-locus Variable Number Tandem Repeat Analysis of *Staphylococcus aureus*

Version: MLVA-Sa-05-2011

Principle

The 8 VNTR loci are amplified in 2 separate multiplex mixes each comprising 4 VNTR loci. Each multiplex mixture contains 4 different primer sets, one for each VNTR locus and each of the 4 forward primers carries a different 5'-fluorescent label. After the PCR the 2 multiplex mixtures are mixed with a size standard which carries a 5^{th} fluorescent label and an aliquot is separated on an automated DNA sequencer for accurate sizing. Subsequently the number of repeats in each VNTR locus is calculated from the sizing data.

Reagents, equipment and software

- Tris-EDTA buffer (TE), 10 mM Tris.HCl, 1 mM EDTA pH 8.0 (store at room temperature)
- Lysostaphine (Sigma, Cat. No. L7368, store at -20°C)
- Unlabeled oligonucleotide primers (e.g. Eurogentec, Seraing, Belgium, store at -20°C)
- Fluorescently labeled oligonucleotide primers (e.g. Applied Biosystems or Eurogentec, store at 4°C in the dark, use HPLC purified primers only, do not freeze!)
- Qiagen multiplex PCR kit (Qiagen, Hilden, Germany; Art. No. 206145, store at -20ºC)
- GeneScan 1200 LIZ Size Standard (Applied Biosystems, Foster City, USA; Art. No. 4379950, store at 4ºC, Do not freeze!)
- MilliQ water (Water purified by the milliQ system, Millipore, Billerica, USA)
- PCR machine (e.g. Applied Biosystems GeneAmp PCR System 9700)
- Automated DNA sequencer that can separate 5 different fluorescent labels (e.g. Applied Biosystems 3730 DNA analyzer)

Optional

- GeneMarker software (v.1.51 or higher, Softgenetics, State College, USA)
- Bionumerics software (v.5.1 or higher, Applied Maths, Sint-Martens-Latem, Belgium)

Source of DNA

MLVA can be performed using 10 ng purified genomic DNA. However, the procedure has been optimized for use with bacterial lysates. Two colonies from cultures grown overnight on Columbia agar plates with 5% sheep blood at 37°C are suspended in 50 µl lysis mix in TE supplemented with 100 µg/ml lysostaphine, incubated for 35 min at 37ºC and heated for 10 min at 95ºC. After the inactivation step 450 µl TE is added and the lysate is used either directly or stored at -20°C until use in PCR.

PCR

PCRs of the 8 VNTR loci are performed in 25-µl volumes in 2 multiplex PCRs. For each multiplex PCR *S. aureus* lysate is added to a mixture containing Qiagen multiplex master mix, 4 forward primers each carrying a different 5'-fluorescent label and 4 unlabeled reverse primers. There is one exception in the first multiplex PCR where 5 pmol fluorescently labeled VNTR61_02Vf primer and also 5 pmol unlabeled primer VNTR61_02f are used to reduce the signal for this VNTR in the multiplex PCR. Both multiplex PCRs use the same PCR program.

Table 1. MRSA-MLVA primer sequences

Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
VNTR09_01Ff	ATAAGCATTGAAACCATTATGATG	VNTR09_01r	GCAACTTCTTAAAACAAAATATTG
VNTR61_01Nf	AATGCACATGAAACACTAATT	VNTR61_01r	GGTCAAGAATATTTAAAATCAATT
VNTR61_02Vf	CTGTGAAGTTAGATAGATGAGTTT	VNTR61_02r	GCAATTAACGATTTCTTCAC
VNTR61_02f	CTGTGAAGTTAGATAGATGAGTTT		
VNTR67_01Pf	CGTGAATCTCTTTTATAAGAGTGT	VNTR67_01r	CCCTCCTATTAATATATATACCGT
VNTR21_01Vf	GTCGATAAAGCATAAAGCTTT	VNTR21_01r	AGCAATGAATCAATAATTTTCA
VNTR24_01Pf	CAGCAGTAGTGCCGTT	VNTR24_01r	GTAACGGCTTCATCCA
VNTR63_01Ff	TGAAGATGTAGTAGGAATGTTAGT	VNTR63_01r	AGAAAAAGCTAAAGAAGTTGAA
VNTR81_01Nf	TTTGGATATGAAGCGAGA	VNTR81_01r	CATATGTCGCAGTACCATC
MLVA-MecA-Ff	AACGGTTTTAAGTGGAACG	MLVA-MecA-r	GCATATGAGATAGGCATCGT
MLVA-PVL-Ff	GGTGCATAATCTACAACGTTTAC	MLVA-PVL-r	AATACTCAAAGCTGCTGGAA

The 5' labeling with fluorescent dyes is indicated by uppercase characters added to the primer name: F, FAM; N, NED; V, VIC; P, PET. The lowercase letters f and r denote forward and reverse character of the primer.

Table 2. The composition of the PCR mixtures for the 2 multiplex PCRs

Multiplex 1		Multiplex 2	
Component	Amount (µl)	Component	Amount (µl)
VNTR09_01Ff	1.0	VNTR21_01Vf	1.0
VNTR09_01r	1.0	VNTR21_01r	1.0
VNTR61_01Nf	1.0	VNTR24_01Pf	1.0
VNTR61_01r	1.0	VNTR24_01r	1.0
VNTR61_02Vf	0.5	VNTR63_01Ff	1.0
VNTR61_02f	0.5	VNTR63_01r	1.0
VNTR61_02r	1.0	VNTR81_01Nf	1.0
VNTR67_01Pf	1.0	VNTR81_01f	1.0
VNTR67_01r	1.0	MLVA-PVL-Ff	1.0
MLVA-MecA-Ff	1.0	MLVA-PVL-r	1.0
MLVA-MecA-r	1.0		
Multiplex mix	12.5	Multiplex mix	12.5
MilliQ water	0.5	MilliQ water	0.5
Lysate	2.0	Lysate	2.0
Total	25.0	Total	25.0

All primers have a 10 pmol/ µl concentration.

Table 3. PCR program

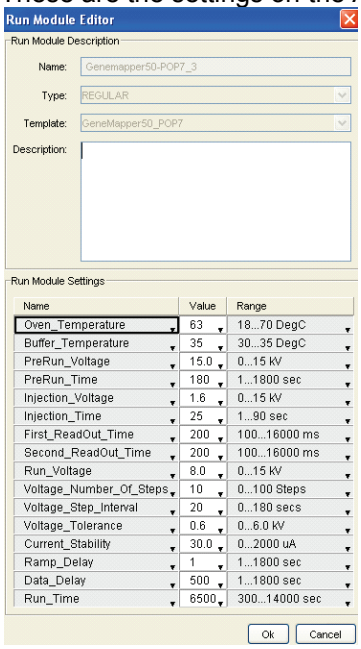
Time	Temperature	Cycles
15 min.	95°C	1
45 sec.	95°C	
45 sec.	54°C	20
90 sec.	72°C	
30 min.	68°C	1
Hold	4°C	1

The step of 30 min at 68°C is used to ensure complete terminal transferase activity of the Taq DNA polymerase. Omitting this step may result in double peaks.

Separation of PCR products for sizing

- Dilute PCR samples 1:100 (2 µl PCR product + 198 µl MilliQ water)
- Mix 1 µl of the diluted samples with 10 µl of 1200 LIZ size standard (diluted 1:100 in MilliQ water)
- Heat denature for 5 min at 95ºC in a PCR machine
- Separate the PCR products on an automated DNA sequencer. The protocol has been optimized using an approximately 2 hours run in 50 cm capillaries on an AB 3730 DNA analyzer.

These are the settings on the AB 3730 DNA analyzer for a run using the 1200 LIZ size standard:



Assessment of the number of repeats

Separation on the DNA sequencer results in .fsa files which can be imported and analyzed in the GeneMarker software to calculate the number of repeats of each VNTR locus. (GeneMarker Size Standard and Panel files, required for the correct sizing, are supplied for download). In order to store and analyze MLVA profiles a result table generated by GeneMarker can be imported into Bionumerics.

The sequences of the repeat units of virtually all VNTR loci used in this MLVA are slightly polymorphic. For some loci even the repeat unit sizes display small differences. Furthermore, the upstream flanking sequences of VNTR06_01 may also be polymorphic sequence and size. Therefore, the use of the table containing the apparent sizes and the translation into the number of repeats is recommended.. Please use the downloadable Genemarker specific .xml files or the Excel table (Bins for multiplex 1+2.xls) found on the webpage. The Excel table displays the allele (= number of repeat units), the apparent size for each allele and the binning sizes used to define the size limits for assigning the allele number. The bin sizes may differ if other DNA sequencers and/or other polymers are used. All alleles have been confirmed by DNA sequencing.

New and aberrant VNTR alleles

VNTR loci that do not yield a PCR product after repeated analysis are assigned allele number 99. Thus the assignment 99 may indicate the complete lack of the particular VNTR locus or mutations in the priming sites that prevent PCR. Each new allele is confirmed by DNA sequencing of the PCR product obtained in the VNTR PCR.

Additional remarks

- For high throughput, PCRs can be performed in 96 well PCR trays (e.g. Greiner, Art. No. 652280).
- VNTR24_01 represents the number of repeats in the *spa* gene. The number of repeats found in this locus always exceeds the number repeats found by *spa*-sequence typing by one repeat. Reason is the fact that the incomplete repeat present in the *spa* gene is ignored in *spa*-sequence typing, but counted in MLVA.
- This MLVA only works on *S. aureus* and not on coagulase-negative staphylococci.