

Protocol for Multiple-locus Variable Number Tandem Repeat Analysis of *Streptococcus pneumoniae*

Version: MLVA-Spn-01-2011

Principle

The 8 VNTR loci (BOX elements) are amplified in 2 separate multiplex mixes each comprising 4 BOX loci. Each multiplex mixture contains 5 different primer sets, one for each BOX locus and 2 primer sets for BOX4 and BOX6. Each of the forward or reverse primers in a primer set carries a different 5'-fluorescent label. After the PCR, a size standard, which carries a 5th fluorescent label, is added to each of the 2 multiplex mixtures and an aliquot is separated on an automated DNA sequencer for accurate sizing. Subsequently the number of repeats in each BOX 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)
- Unlabeled oligonucleotide primers (e.g. Eurogentec, Seraing, Belgium, store at -20°C)
- Fluorescently labeled oligonucleotide primers (e.g. Applied Biosystems or Eurogentec, primers need to be HPLC purified, store at 4°C in the dark, do not freeze!)
- Qiagen multiplex PCR kit (Qiagen, Hilden, Germany; Art. No. 206145, store at -20°C)
- GeneScan 1200 LIZ Size Standard (Applied Biosystems, Foster City, USA; Art. No. 4379950, store at 4°C, 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. A loop full of colonies from cultures grown overnight on Columbia agar plates with 5% sheep blood at 37°C are suspended in 500 µl TE and heated for 10 min at 95°C. After the inactivation the lysate is used either directly or stored at -20°C until use in PCR.

PCR

PCRs of the 8 BOX loci are performed in 20-µl volumes in 2 multiplex PCRs. For each multiplex PCR 2 µl DNA (10 ng) or 2 µl *S. pneumoniae* lysate (diluted 1:10 in MilliQ water) is added to a mixture containing Qiagen multiplex master mix, and the primer as indicated in Table 2. The use of multiple primers for BOX4 and BOX6 was introduced to ensure amplification of these loci in strains in which the priming sites are altered. Both multiplex PCRs use the same PCR program.

Table 1. MLVA primer sequences

Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
BOX_01-Ff	CCAGAGACATTGATGAAGAGA	BOX_01-r	CGCTTTGATGAACTTGAGTT
BOX_02-Nf	TTGCTTGGTACAGAAAACAAA	BOX_02-r	CCCCATAAAACCCTCCTTATA
BOX_03-Vf	TCCAACACGACCTTTTATCC	BOX_03-r	TTCAGTAAACCCAGCTCGTA
BOX_04-f	TGGGTAAAAGTAGACAGGACT	BOX_04-Pr	CACTTCTACACTAGTTTGTAAAGCA
BOX_04-f2	AGGGGATTTACCCACTACAAA		
BOX_06-Nf	GAAAAAGGTCAGGAGTAGGTT	BOX_06-r	TCACTTGAGACAATCAGCC
BOX_06-f2	TTATGATTTTTGTCATTTTGC	BOX_06-Nr2	GAAATCTTTGAAAACTAGGATTT
BOX_11-Vf	TCCAATAATGACAGGTTTTCCCTC	BOX_11-r	TTCCAATCTACGCCTTTGAAG
BOX_12-Pf	TTGCCCTTTTCATCTTCGA	BOX_12-r	CAGCAACCATTGAAACGC
BOX_13-Ff	TCGCCTTTGCTAATCAAACA	BOX_13-r	CTGATTATATCGCTCACAAACCC

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)
BOX_01-Ff	0.6	BOX_06-Nf	0.8
BOX_01-r	0.6	BOX_06-r	0.8
BOX_02-Nf	0.8	BOX_06-f2	0.8
BOX_02-r	0.8	BOX_06-Nr2	0.8
BOX_03-Vf	0.8	BOX_11-Vf	0.8
BOX_03-r	0.8	BOX_11-r	0.8
BOX_04-f	0.8	BOX_12-Pf	0.8
BOX_04-f2	0.8	BOX_12-r	0.8
BOX_04-Pr	0.8	BOX_13-Ff	0.5
		BOX_13-r	0.5
HotStar mix	10.0	HotStar mix	10.0
MilliQ water	1.2	MilliQ water	0.6
Lysate or DNA	2.0	Lysate or DNA	2.0
Total	20.0	Total	20.0

All primers have a 10 µM concentration.

Table 3. PCR program

Time	Temperature	Cycles
15 min.	95°C	1
30 sec.	95°C	25
60 sec.	54°C	
60 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 1:200 in MilliQ water
- Mix 2 µl of the diluted samples with 10 µl of 1200 LIZ size standard (diluted 1:200 in MilliQ water)
- Heat denature for 5 min at 95°C 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:

The screenshot shows the 'Run Module Editor' window. The 'Name' field is 'GeneMapper50-POP7_3', 'Type' is 'REGULAR', and 'Template' is 'GeneMapper50_POP7'. The 'Description' field is empty. Below these fields is a table of 'Run Module Settings'.

Name	Value	Range
Oven_Temperature	63	18..70 DegC
Buffer_Temperature	35	30..35 DegC
PreRun_Voltage	15.0	0..15 kV
PreRun_Time	180	1..1800 sec
Injection_Voltage	1.6	0..15 kV
Injection_Time	25	1..90 sec
First_ReadOut_Time	200	100..16000 ms
Second_ReadOut_Time	200	100..16000 ms
Run_Voltage	8.0	0..15 kV
Voltage_Number_Of_Steps	10	0..100 Steps
Voltage_Step_Interval	20	0..180 secs
Voltage_Tolerance	0.6	0..6.0 kV
Current_Stability	30.0	0..2000 uA
Ramp_Delay	1	1..1800 sec
Data_Delay	500	1..1800 sec
Run_Time	6500	300..14000 sec

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 BOX 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 flanking sequences of some BOX loci may differ slightly in 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 (Spneumo MLVA allele size table vxxxx.xls) found on the 'Protocols and Tables' page. 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 Excel file also contains information on the nature of the aberrant alleles (e.g. allele 4_01) and the frequency in which they occur. The aberrant alleles have also been incorporated in the GeneMarker .xml files and designated as a different allele (e.g. allele 401). 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

BOX 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 BOX 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 BOX PCR.

Additional remarks

- For high throughput, PCRs can be performed in 96 well PCR trays (e.g. Greiner, Art. No. 652280).