Protocol for Multiple-locus Variable Number Tandem Repeat Analysis of *Haemophilus influenzae*

Version: MLVA-Hi-01-2010

Principle

The 6 VNTR loci are amplified in 2 separate multiplex mixes each comprising 3 VNTR loci. Each multiplex mixture contains 3 different primer sets, one for each VNTR locus and each of the 3 forward primers 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 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)
- 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 -20ºC)
- GeneScan 500 LIZ Size Standard (Applied Biosystems, Foster City, USA; Art. No. 4322682, 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. A loop full of colonies from bacteria grown overnight on Chocolate agar with 5% CO_2 at 37°C are suspended in 500 µl TE and heated for 10 min at 95ºC. After the inactivation step the lysate is used either directly or stored at -20°C until use in PCR. For use in the PCRs the lysates are dilute 1:10 in MilliQ water.

PCR

PCRs of the 6 VNTR loci are performed in 20-µl volumes in 2 multiplex PCRs. For each multiplex PCR *S. aureus* lysate is added to a mixture containing Qiagen multiplex master mix, 3 forward primers each carrying a different 5'-fluorescent label and 3 unlabeled reverse primers. Both multiplex PCRs use the same PCR program.

Table 1. Hi-MLVA primer sequences

Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
Hi-VNTR5-2Ff	AAAAATGCTTGTTGTAATGTGTG	Hi-VNTR5-2r	CCGATAAAAATCACTTTACAGAG
Hi-VNTR12-1Vf	TATCCGTCCGCTTTGTCT	Hi-VNTR12-1r	CCGTAATTGAGCAGACGA
Hi-VNTR12-2Pf	AAACCGAAATCGTAAAAATTTT	Hi-VNTR12-2r	CGATAGTATTCATACTGTTTCTG
Hi-VNTR6-1Nf	AATTTCTTGTTTTTCTGGTTCTG	Hi-VNTR6-1r	GGAAGAACCTGCTCCAGA
Hi-VNTR6-10Ff	CGCCATCACTAATATAACC	Hi-VNTR6-10r	CTTTTAATTGATAATTACCCTGT
Hi-VNTR8-1Ff	TCTAAAGACTTTTCTACATTACAA	Hi-VNTR8-1r	TAACGATTTTCAGCCAT

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)
Hi-VNTR5-2-Ff	0.3	Hi-VNTR-6-1Nf	0.8
Hi-VNTR5-2-r	0.3	Hi-VNTR-6-1r	0.8
Hi-VNTR12-1-Vf	0.2	Hi-VNTR6-10Ff	0.4
Hi-VNTR12-1-r	0.2	Hi-VNTR6-10r	0.4
Hi-VNTR12-2-Pf	0.8	Hi-VNTR8-1-Ff	0.8
Hi-VNTR12-2-r	0.8	Hi-VNTR8-1-r	0.8
Multiplex mix	10.0	Multiplex mix	10.0
MilliQ water	6.4	MilliQ water	5.0
Lysate (1:10) or 10 ng DNA	1.0	Lysate (1:10) or 10 ng DNA	1.0
Total	20.0	Total	20.0

All primers have a 10 pmol/ µl concentration.

Table 3. PCR program

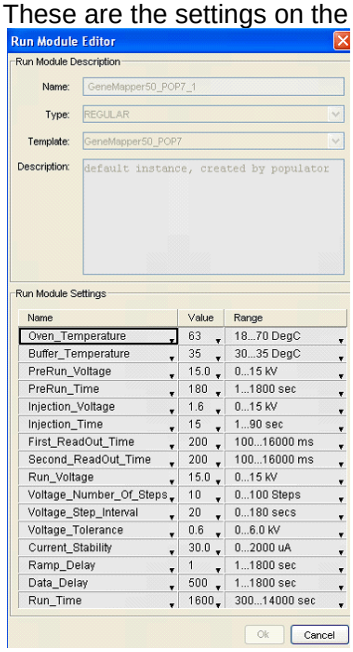
Time	Temperature	Cycles
15 min.	95°C	1
30 sec.	95°C	
60 sec.	54°C	22
60 sec.	72°C	
30 min.	68°C	1
Hold	4°C	1

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 (2 µl PCR product + 198 µl MilliQ water)
- Mix 2 µl of the diluted samples with 10 µl of 500 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 1 hour 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 500 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 Panel files and the 500 LIZ size standard file, 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 VNTR 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 (Panel files for Multiplex 1 and Multiplex2) 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 slightly if other types of 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. In exceptional cases the PCR has an aberrant size. Until now such aberrant sizes have been identified for the VNTR6-1 locus only. These aberrant alleles have been designated 50, 51, 52 etc.

Additional remarks

- For high throughput, PCRs can be performed in 96 well PCR trays (e.g. Greiner, Art. No. 652280).
- This MLVA works on capsulated and non-capsulated *H. influenzae*.
- Although 6 VNTR loci are amplified only 4 loci (VNTR5-2, VNTR12-1, VNTR12-2 and VNTR6-1) were used for the originally published method (*L.M. Schouls et al. 2005. J Clin Microbiol 43:2741-2749*) and these loci have been used to create the MLVA profiles and assign the MLVA-types. Two more loci are added in this protocol and one of these loci (VNTR6-10) is used to create a more discriminating profile. The website enables the use of both the 4-loci and 5-loci profiles for MLVA-assignment. The 6th VNTR locus (VNTR8-1) is not used for the assignment of MLVA types, but may serve to further discriminate isolates with identical $MLVA_4$ or $MLVA_5$ profiles.