Protocol for Multiple-locus Variable Number Tandem Repeat Analysis of *Bordetella pertussis*

Version: MLVA-Bp-01-2010

Principle

The 5 VNTR loci are amplified in 5 separate PCRs. Each PCR mixture a fluorescently labeled forward primer and an unlabeled reverse primer. After the PCR a size standard, which carries a different fluorescent label, is added to each of the 5 PCR mixtures and an aliquot of each mixture 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

- Unlabeled oligonucleotide primers (e.g. Eurogentec, Seraing, Belgium, store at -20ºC)
- Fluorescently labeled oligonucleotide primers (e.g. Applied Biosystems or Eurogentec, primers need to be HPLC purified, store at 4ºC in the dark, do not freeze!)
- Qiagen HotStar Taq Master kit (Qiagen, Hilden, Germany; Art. No. 203445, store at -20ºC)
- Betain 5M solution (SIGMA, Art.No. B0300)
- 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)
- DNAse/RNAse free water (SIGMA Art.No. W4502)
- 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 or with bacterial lysates. The quality of DNA prepared using generally available rapid genomic DNA isolations kits will suffice. To prepare lysates, *Bordetella* culture grown to near confluence for 4 days on Bordet-Gengou agar, are suspended in 1 ml of DNAse/RNAse free water, and heated for 5 min at 95ºC. After the inactivation step, the lysate is centrifuged for 2 min at maximum speed in a microcentrifuge. The supernatant of the lysate is used either directly or is stored at -20°C until use in PCR.

PCR

PCRs of the 5 VNTR loci are performed in 20-µl volumes in 5 separate PCRs. For each PCR 2 µl of *B. pertussis* 1:400 diluted supernatant of lysate or 2 µl DNA (10 ng) is added to a mixture containing Qiagen master mix, a forward primer carrying a 5'-fluorescent label and an unlabeled reverse primer. The addition of betaine to PCRs improves the amplification of DNA by reducing the formation of secondary structure in GC-rich regions in GC-rich DNA such as *Bordetella* DNA. All 5 PCRs use the same PCR program.

Table 1. *B. pertussis*-MLVA primer sequences

Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
BP-VNTR1-Ff	CCTGGCGGCGGGAGACGTGGTGGTG	BP-VNTR1-r	AAAATTGCGGCATGTGGGCTGACTCTGA
BP-VNTR3-Ff	GCCTCGGCGAAATTGCTGAAC	BP-VNTR3-r	GCGGGCGAGGAAACGCCCGAGACC
BP-VNTR4-Ff	CGTGCCCTGCGCCTGGACCTG	BP-VNTR4-r	GCCGCTGCTCGACGCCAGGGACAA
BP-VNTR5-Ff	GAAGCCGGCCCACCCGAGCTCCAGGCTCTT	BP-VNTR5-r	TGCCGGGTTTCGGCATCTCGATGGGATACG
BP-VNTR6-Ff	CCAACGGCGGTCTGCTGGGTGGTC	BP-VNTR6-r	AGGGCGCTGGTCACGCCACCGAGGAT

The 5' labeling with fluorescent dye is indicated by uppercase character added to the primer name: F, FAM. The lowercase letters f and r denote forward and reverse character of the primer.

Table 2. The composition of the PCR mixture for VNTR1-VNTR5

Component	Amount (µl)
Forward primer	1
Reverse primer	1
Betaine 5 M	4
HotStar mix	10
DNAse/RNAse free water	2
Lysate or DNA	2
Total	20

All primers have a 10 pmol/ µl concentration.

Table 3. The composition of the PCR mixture for VNTR6

Component	Amount (µl)
Forward primer	1
Reverse primer	1
Betaine 5 M	6
HotStar mix	10
Lysate or DNA	2
Total	20

All primers have a 10 pmol/ µl concentration.

Table 4. PCR program

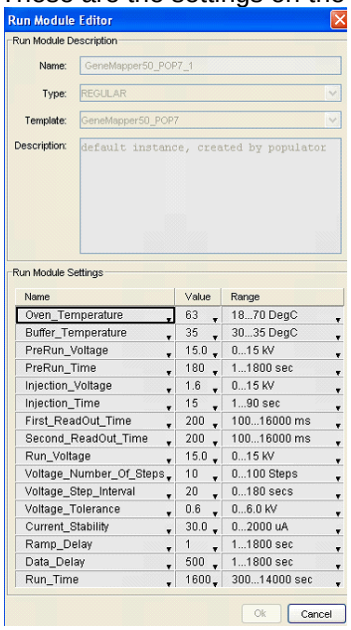
Time	Temperature	Cycles
15 min.	95°C	1
20 sec.	95°C	
30 sec.	67°C	25
60 sec.	72°C	
30 min.	68°C	1
Hold	20°C	1

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; Initially dilute 1:5 (20 µl PCR product + 80 µl DNAse/RNAse free water) and then 1:100 (5 µl 1:5 diluted PCR product + 95 µl DNAse/RNAse free water)
- Dilute the 500 LIZ size standard 1:2000 in Milli-Q grade water
- Mix 2 µl of the 1:200 diluted samples with 18 µl of 1:2000 diluted 500 LIZ size standard
- 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 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.

Although the PCR products are denatured when analyzed, refolding may occur leading to secondary structure of the PCR product. This may result in a apparent product size that differs from the theoretical size. Therefore, the use of a table containing the apparent sizes and the translation into the number of repeats is essential. 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)
- The MLVA profile of the Tohama strain is:

	VNTR1	VNTR3	VNTR4	VNTR5	VNTR6
Size of PCR product (bp)	383	104	208	113	274
Number of repeats	9	7	9	7	9

- This MLVA protocol has been succesfully used for *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*