Brucella MLVA workshop, Orsay March 27th 2006

revised April 14th by GV (minor corrections linked to renumbering Bruce08 and Bruce55)
last edited : September 2007

PROTOCOL FOR MLVA TYPING, application to *Brucella* MLVA typing

Summary : this overview describes the MLVA assay from the PCR reaction set-up to the visual analysis of gel images (8 panel 1 markers).

1- DNA

It is recommended to use good PCR-quality DNA.

A reference strain (such as *Brucella melitensis* 16M or one of the widely available vaccine strains) for which the expected size is known for each VNTR locus is needed.

Typical set-up to genotype 30 strains : it may be convenient to make a DNA master plate using a 96 wells pcr plate (or V-type wells) :

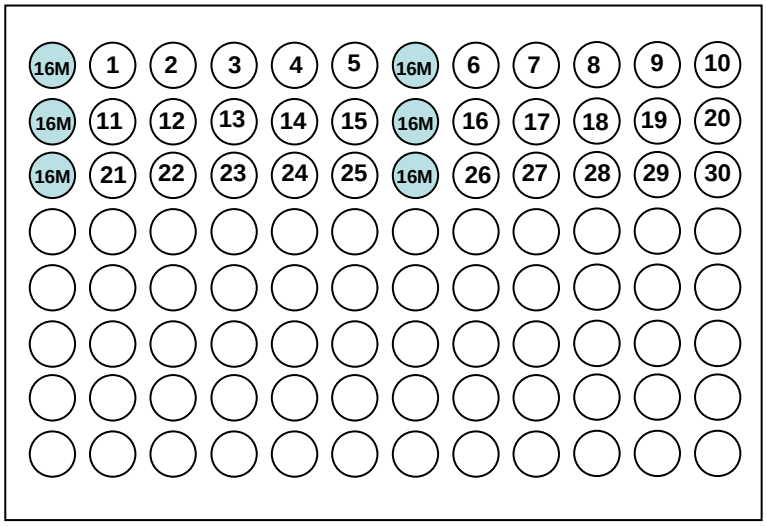

Figure 1 : DNA master plate (1 to 5 ng/µl) including the reference DNA (16M in the present case)

2- VNTR PCR amplification and genotyping

2.1 PCR conditions :

Reactions are made in a15µl final volume including :

-3 to 15ng of DNA

-10x PCR Reaction Buffer

-5x Betaine (5 M solution)

-1U of *Taq* DNA polymerase

-200µM of each deoxynucleotide triphosphate (2 mM each dNTP master solution)

-0.5 µM of each flanking primer (primer sequences, Le Flèche 2006).

	1 PCR reaction	30 PCR reactions	+10%
Primers mix	1.5µl	45µl	50 µl
10x PCR buffer	1.5µl	45µl	50 µl
5x Betaine	3 µl	90 µl	100 µl
dNTP (2 mM each)	1.5µl	45µl	50 µl
Taq (5U/µl)	0.2 µl	6 µl	7 µl
H_2O	4.5 µl	135µl	146µl
Distribute 12 µl of above mix per well			
DNA	Distribute 3µl per well		
Final volume	15µl per well		

Table 1 : PCR mix for 30 reactions

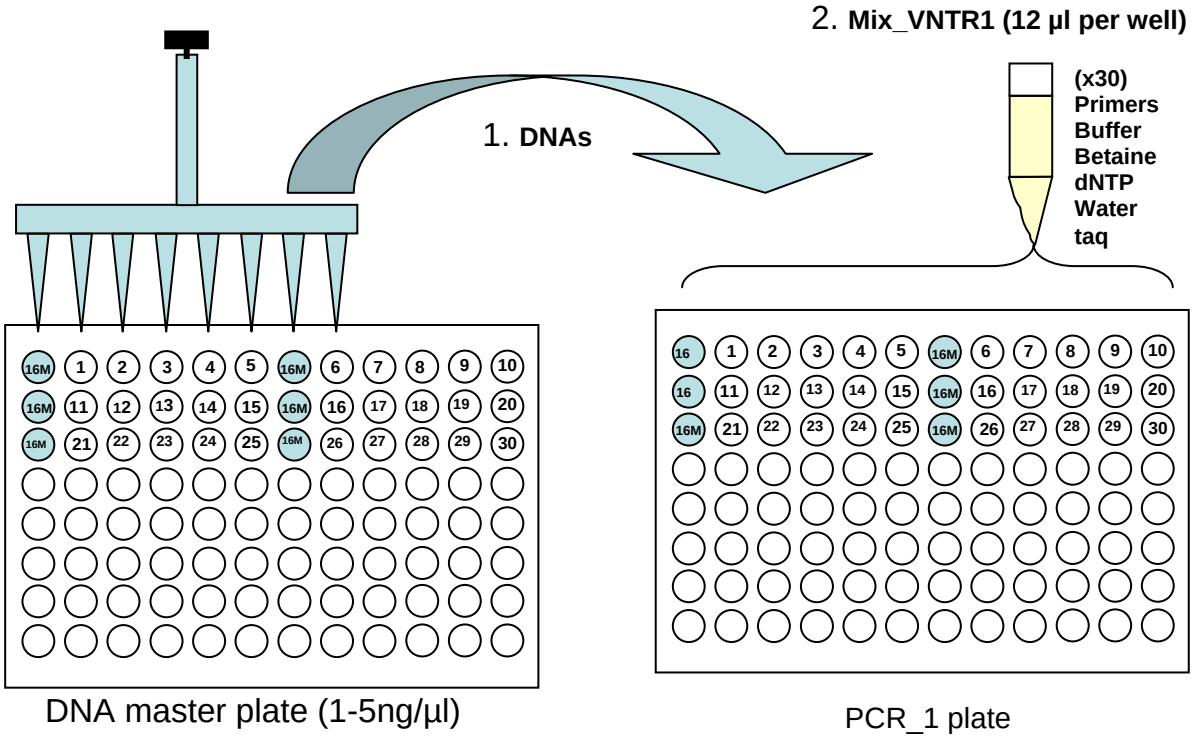

Figure 2 : distributing reaction mix and DNAs into PCR plate for VNTR locus 1

2.2 Amplification :

-Initial denaturation step at 96°C for 5 minutes

-followed by 30 cycles of

denaturation at 96°C for 30s,

primer annealing at 60°C for 30 s,

extension at 70°C for 1 min.

-Final extension step : 70°C for 5 min.

2.3 Electrophoresis

The PCR reaction products (3-5µl) are run on standard agarose in 0.5x TBE buffer until the bromophenol blue has run for 20 cm (2 combs on a 40 cm long gel).

For Panel 1 VNTRs, use a 2% standard agarose gel.

The size markers used are a 100-bp ladder (Bio-Rad, EZ Load 100pb PCR Molecular Ruler) or 20-bp ladder (Bio-Rad, EZ Load 20pb Molecular Ruler) according to the VNTR unit length.

Three to five microliters of amplification product load on gel **(3 by 3)** with a multi-channel pipette.

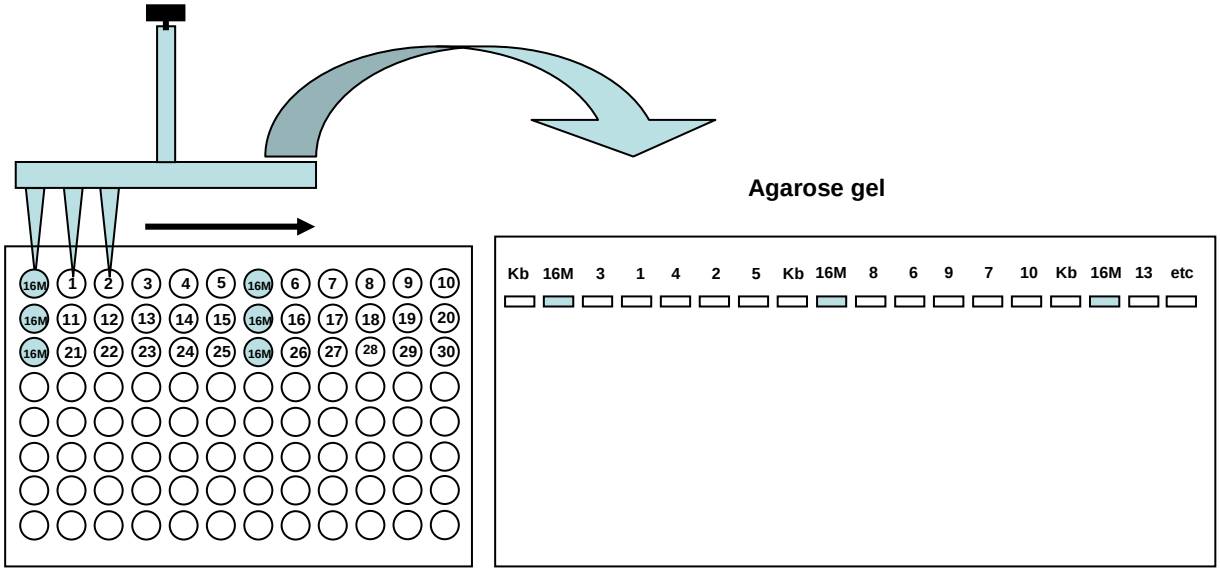

Figure 3 : Loading of PCR products on the agarose gel

2.4 Ethidium bromide staining and photograph

Gels are stained with ethidium bromide ([0.25 to 0.5 µg/ml] ; 10µl to 20µl of 10 mg/ml stock solution in 400ml 0.5x TBE) for 30-60 minutes and photographed under U.V. light.

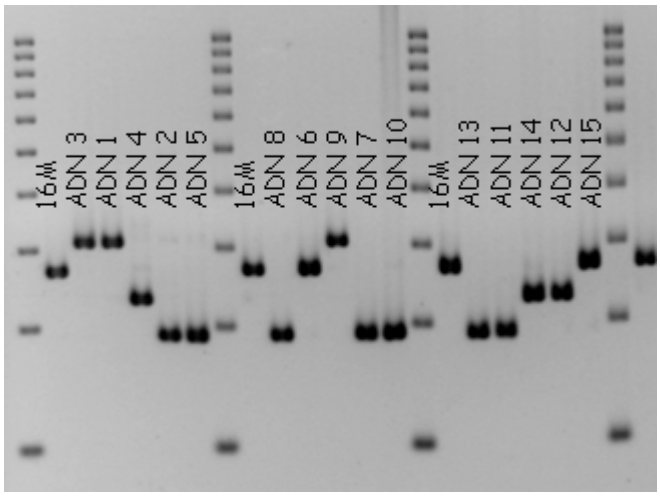

Figure 4 : gel image, locus bruce55-BRU2066_40bp_273bp_3u (bruce55). Migration on 2% agarose gel

3- Data analysis

The number of repeat units can be deduced from the allele size using reference strain for comparison.

VNTR name[a]	Size on agarose gel	Number of units
bruce06-BRU1322_**134bp**_408bp_3u	408bp	3U
bruce08-BRU1134_**18bp**_348bp_4u	348bp	4U
bruce11-BRU211_**63bp**_257bp_2u	257bp	2U
bruce12-BRU73_**15bp**_392bp_13u	392bp	13U
bruce42-BRU424_**125bp**_539bp_4u	539bp	4U
bruce43-BRU379_**12bp**_182bp_2u	182bp	2U
bruce45-BRU233_**18bp**_151bp_3u	151bp	3U
bruce55-BRU2066_**40bp**_273bp_3u	273bp	3U

[a] *nomenclature* : for example *bruce55-BRU2066_40bp_273bp*_3u is a VNTR at position 2066 Kb in the *B.melitensis* 16M genome with a 40 bp motif , a total PCR product length of 273 bp (in 16M) with the primers used . The allele size corresponds to 3 units

Table 2 :Panel 1 correspondence table for the 16M reference strain

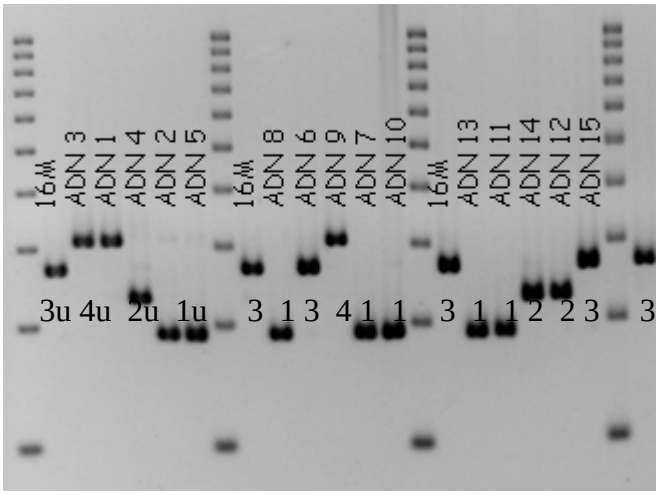

Figure 5 : conversion of the Figure 4 gel image into repeat copy number for each band. For bruce55, 1U = 40 bp.

16M	3u	273bp
adn1	4u	313bp
adn2	1u	193bp
adn3	4u	313bp
adn4	2u	233bp
adn5	1u	193bp
etc...		

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