Standard Operating Procedure

Brucella MLVA Typing

SAFETY PRECAUTIONS

The laboratory shall take all precautions in order to guarantee the necessary safety, for both the operator and the environment, against the biological and chemical hazards due to the activities conducted according to this document.

1 Scope

The present document describes a standard technique aiming at MLVA (Multiple Locus VNTR Analysis) typing for diagnostic and epidemiological use in animal / human brucellosis, through 16 markers: eight minisatellite (panel 1) and eight microsatellite (panel 2) markers. This method has been adjusted from Al Dahouk et al. (2007).

2 Normative and scientific references

- Brucellosis (*Brucella abortus*, *B. melitensis* and *B. suis*) (infection with *B. abortus*, *B. melitensis* and *B. suis*), *In*: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017, Chapter 2.1.4, OIE, Paris, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.04_BRUCELLOSIS.pdf

- Quality Management in Veterinary testing Laboratories. *In*: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017, Chapter 1.1.5., OIE, Paris http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.05_QUALITY_MANAGEMENT.pdf

- Al Dahouk S, Le Flèche P, Nöckler K, Jacques I, Grayon M, Scholz HC, Tomaso H, Vergnaud G, Neubauer H., 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. J Microbiol Methods. 2007 Apr;69(1):137-145. Epub 2007 Jan 3.

- Le Flèche P, Jacques I, Grayon M, Al Dahouk S, Bouchon P, Denoeud F, Nöckler K, Neubauer H, Guilloteau LA, Vergnaud G., 2006. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiology 2006, 6:9 doi:10.1186/1471-2180-6-9

- MLVA-NET: http://mlva.u-psud.fr/brucella/

- Microbes Genotyping: http://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/907

3 Definitions

- **Variable number tandem repeat (VNTR):**

A variable number tandem repeat (or VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and often show variations in length (number of repeats) among individuals. Each variant acts as an inherited allele, allowing them to be used for personal or parental identification. Their analysis is useful in genetics and biology research, forensics, and DNA fingerprinting.

- **Multiple loci VNTR analysis (MLVA):**

Multiple loci VNTR analysis (MLVA) is a method employed for the genetic analysis of particular microorganisms, such as pathogenic bacteria, that takes advantage of the polymorphism of tandemly repeated DNA sequences.

- **DeoxyriboNucleotide TriPhosphate (dNTP):**

dNTP stands for deoxyribonucleotide triphosphate. Each dNTP is made up of a phosphate group, a deoxyribose sugar and a nitrogenous base. There are four different dNTPs that can be split into two groups: the purines (adenine and guanine) and the pyrimidines (cytosine and thymine).

- **Taq polymerase:**

A heat stable DNA polymerase that is normally used in the Polymerase Chain Reaction (PCR). It was isolated from *Thermus aquaticus*.

- **PCR Master Mix:**

PCR Master Mix is a solution containing *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer at optimal concentrations for efficient PCR amplification of DNA templates.

- **GelRed:**

GelRed is an intercalating nucleic acid stain used in molecular biology for agarose gel electrophoresis. GelRed is structurally closely related to ethidium bromide and consists of two ethidium subunits that are bridged by a linear spacer. Its fluorophore, and therefore its optical propertiesare essentially identical to those of ethidium bromide. When exposed to ultraviolet light, it will fluoresce with an orange color that strongly intensifies after binding to DNA.

- **Capillary electrophoresis:**

MLVA can also be analysed on capillary electrophoresis. Using this approach, allele calling is automated and small repeats can differentiated. Multiplexing is also easier to do. The cost of this analysis is higher than a classical agarosis gel, it is more appropriate to studies with a significant number of strains to type.

4 Principle and reaction

4.1 Principle

Sixteen well-selected and characterised (in terms of mutation rate and diversity) loci are amplified by PCR, so that the size of each locus can be measured, usually by electrophoresis of the amplification products together with reference DNA fragments (a so-called DNA size marker). From this size estimate, the number of repeat units at each locus can be deduced. The resulting information is a code which can be easily compared to reference databases once the assay has been harmonised and standardised.

4.2 List of reagents, products

4.2.1 PCR amplification step

- Ultrapure water (keep at +5°C ± 3°C),
- dNTP mixture [25mM] (final concentration 200 µM ; keep frozen at tp < -16°C),
- GoTaq® Polymerase [5U/µL) (final concentration 0.04 U/µL ; keep frozen at tp < -16°C),
- 5X GoTaq® Reaction Buffer [Green buffer], which contains $MgCl_2$ at a concentration of 7.5mM for a final concentration of 1.5mM in the 1X reaction. The 5X Green GoTaq® Reaction Buffer has two dyes (a blue dye and a yellow dye) that separate during electrophoresis to show migration progress (final concentration 1X ; keep frozen at tp < -16°C),
- Primers [100 µM] (final concentration 0.3 µM; keep frozen at tp < -16°C).

Primers list

Primer Name		Sequence	Number of bases	Panel
Bruce 06	Forward	ATGGGATGTGGTAGGGTAATCG	22	Panel1
	Reverse	GCGTGACAATCGACTTTTTGTC	22	
Bruce 08	Forward	ATTATTCGCAGGCTCGTGATTC	22	
	Reverse	ACAGAAGGTTTTCCAGCTCGTC	22	
Bruce 11	Forward	CTGTTGATCTGACCTTGCAACC	22	
	Reverse	CCAGACAACAACCTACGTCCTG	22	
Bruce 12	Forward	CGGTAAATCAATTGTCCCATGA	22	
	Reverse	GCCCAAGTTCAACAGGAGTTTC	22	
Bruce 42	Forward	CATCGCCTCAACTATACCGTCA	22	
	Reverse	ACCGCAAAATTTACGCATCG	20	
Bruce 43	Forward	TCTCAAGCCCGATATGGAGAAT	22	
	Reverse	TATTTTCCGCCTGCCCATAAAC	22	
Bruce 45	Forward	ATCCTTGCCTCTCCCTACCAG	21	
	Reverse	CGGGTAAATATCAATGGCTTGG	22	
Bruce 55	Forward	TCAGGCTGTTTCGTCATGTCTT	22	
	Reverse	AATCTGGCGTTCGAGTTGTTCT	22	
Bruce 18	Forward	TATGTTAGGGCAATAGGGCAGT	22	Panel 2A
	Reverse	GATGGTTGAGAGCATTGTGAAG	22	
Bruce 19	Forward	GACGACCCGGACCATGTCT	19	
	Reverse	ACTTCACCGTAACGTCGTGGA	22	
Bruce 21	Forward	CTCATGCGCAACCAAAACA	19	
	Reverse	GATCTCGTGGTCGATAATCTCATT	24	
Bruce 04	Forward	CTGACGAAGGGAAGGCAATAAG	22	Panel 2B
	Reverse	CGATCTGGAGATTATCGGGAAG	22	
Bruce 07	Forward	GCTGACGGGGAAGAACATCTAT	22	
	Reverse	ACCCTTTTTCAGTCAAGGCAAA	22	
Bruce 09	Forward	GCGGATTCGTTCTTCAGTTATC	22	
	Reverse	GGGAGTATGTTTTGGTTGTACATAG	25	
Bruce 16	Forward	ACGGGAGTTTTTGTTGCTCAAT	22	
	Reverse	GGCCATGTTTCCGTTGATTTAT	22	
Bruce 30	Forward	TGACCGCAAAACCATATCCTTC	22	
	Reverse	TATGTGCAGAGCTTCATGTTCG	22	

4.2.2 Gel preparation

- TAE buffer (Tris, Acétate, EDTA) [0.5X] ; (keep at +5°C ± 3°C)
- SeaKem®METAPHOR agarose (keep at room temperature) (final concentration: 1.5%)
- SeaKem®GTG agarose (keep at room temperature) (final concentration: 1.5%)
- GelRed (keep at +5°C ± 3°C),
- DNA Ladder Plus 100-bp (keep at +5°C ± 3°C).

4.3 Equipments

- Thermocyclers : Biorad, Eppendorf, Applied…
- Electrophoresis systems (gels or capillary)

4.4 Protocol

4.4.1 Mix preparation

- Dilute DNA extracts with water to obtain approximately 5 ng of DNA.

- Thaw mix reagents (except Taq to be thawed at the last moment because it is not a hot start Taq polymerase).

- Pulse reagents (don't vortex) and then draw up / empty the pipette 3 – 5 times to homogenize them.

- Dilute primers at 100 µM, to obtain a final concentration of 3 µM (10 µL of primers + 33 µL of water).

- First, prepare a MASTER Mix as described in the table (**without primers**).

	Reagents for one sample (µl)	**Reagents for 8 samples (µl)**	Batch
Ultrapure water	*12.6*	**100.8**	-
Green buffer [5X] (1X)	*5*	**40**	
dNTPs [25mM] (200µM)	*0.2*	**1.6**	
Go Taq	*0.2*	**1.6**	
Forward primer [3 µM] (0.3 µM)	*2.5*	**20**	
Reverse primer [3 µM] (0.3 µM)	*2.5*	**20**	
DNA **(5 ng)**	*2*	**16**	-
Total	*25*	*200*	

Mix volume per well **23**

-Then, distribute the Master Mix in tubes corresponding to the different loci and add the corresponding primers (forward / reverse).

-Then, distribute 2 µL of DNA in each well and seal the plate, using a PCR plastic film.

4.4.2 Amplification

- Put the plate into the thermocycler and run the following program

Number of cycles	Temperature (°C)	Minutes	Seconds
1	95	2	0
30	96	0	30
	60	0	30
	70	1	0
1	70	5	0
1	12	∞	

- Following amplification, keep the plate with protection against the light at +5°C ± 3°C if you use the amplification products during the day or freeze it to use it later.

4.4.3 Gel preparation and electrophoresis

- **Gel preparation (final concentration of 3 %)**

- First, weight SeaKem®METAPHOR (1.5 %) and add 0.5X TAE buffer;

- Add SeaKem®GTG agarose (1.5 %) and then shake for 10 min with a magnetic plate stirrer

(As an example, weight 6 g of SeaKem®METAPHOR agarose, add 400 mL of TAE buffer, add 6 g of SeaKem®GTG agarose and then shake for 10 min.)

-Thoroughly melt in the microwave oven.

- Put the agarose solution in a water bath at 60°C to drive the temperature down.

- Add Gelred and cast the gel when the gel is at moderate temperature (not to hot)

- **Electrophoresis**

- Distribute 5µL of mix (DNA amplified + loading buffer) and 5µL of size markers into gel wells.

- A short initial phase of high voltage (3 min, 130 V) is performed for the DNA to enter into the gel, and then, electrophoresis is carried out overnight (at low voltage, 50-60 V).

- Cover the electrophoresis gel with an aluminum sheet to avoid troubles with the light.

- **Gel images**

- Adjust carefully brightness and / or contrast, sharpness and focus to obtain a clear and easy-to-read picture of the gel.

4.4.4 Reading and interpretation

- For each band (marker), estimate the size of amplified DNA, compare it to the included reference strain (16 M / Thomsen…) and the ladder, using a ruler or a software

- Infer the number of repeat units for each marker using the allele assignment table (Annex 2) and the MLVA profile. As an example, the correspondence table for the Panel 1 is presented for the 16M reference strain.
(source: MLVA-net ; http://mlva.u-psud.fr/brucella/IMG/pdf/PROTOCOL_FOR_MLVA_TYPING_version3.pdf)

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

MLVA profile should be compared to public databases using MLVA-net and/or microbes genotyping.

4.5 Critical points

General recommendations	▪ Prevent formation of aerosols (each time tubes or plates have to be opened) ▪ Check the quality of pipettes calibration (volumetry) ▪ Use of tip with filters
DNA extraction	▪ No thermic extraction (DNA extraction is preferable using phenol/chloroform or a classic extraction kit) ▪ DNA concentration has to be standardized (according to extraction protocol)
Mix preparation	▪ Taq has to be thawed at the last moment, just before being added to the mix. ▪ Pay attention to potential contaminations during mix preparation (different reagents / primers / buffer…)
Distribution of DNA and mix into plates	▪ Pay attention to contaminations during distribution ▪ In case of multichannel pipettes, verify volumes to be distributed ▪ Prefer to organize the plate by loci (instead of sample)
Gel preparation	▪ Use at least 1.5 % METAPHOR for a better resolution ▪ Use a magnetic plate stirrer for at least 10 min before dissolving the agarose solution ▪ Perfect agarose dissolution ▪ Avoid bubbles formation during gel casting
Electrophoresis	▪ Following a short time of sustained voltage (3 minutes), a slow migration (overnight) is recommended ▪ Cover the gel during the migration with an aluminum sheet
Picture	▪ Ensure a high picture quality of an image, including the brightness and / or contrast, sharpness and focus.
Interpretation	▪ Take your time and a ruler

Annex 1: MLVA plate

Example drafted for 16 markers, 3 strains to be typed, 2 reference strains, 1 Mix control

Panel 1: Bruce 6, 8, 11, 12, 42, 43, 45, 55

Panel 2A: Bruce 18, 19, 21

Panel 2B: Bruce 04, 07, 09, 16, 30

	1	2	3	4	5	6	7	8	9	10	11	12
A	**1** Bruce 06	**2** Bruce 06	**3** Bruce 06	**4** Bruce 06	**5** Bruce 06	**6** Bruce 06	**1** Bruce 18	**2** Bruce 18	**3** Bruce 18	**4** Bruce 18	**5** Bruce 18	**6** Bruce 18
B	**1** Bruce 08	**2** Bruce 08	**3** Bruce 08	**4** Bruce 08	**5** Bruce 08	**6** Bruce 08	**1** Bruce 19	**2** Bruce 19	**3** Bruce 19	**4** Bruce 19	**5** Bruce 19	**6** Bruce 19
C	**1** Bruce 11	**2** Bruce 11	**3** Bruce 11	**4** Bruce 11	**5** Bruce 11	**6** Bruce 11	**1** Bruce 21	**2** Bruce 21	**3** Bruce 21	**4** Bruce 21	**5** Bruce 21	**6** Bruce 21
D	**1** Bruce 12	**2** Bruce 12	**3** Bruce 12	**4** Bruce 12	**5** Bruce 12	**6** Bruce 12	**1** Bruce 04	**2** Bruce 04	**3** Bruce 04	**4** Bruce 04	**5** Bruce 04	**6** Bruce 04
E	**1** Bruce 42	**2** Bruce 42	**3** Bruce 42	**4** Bruce 42	**5** Bruce 42	**6** Bruce 42	**1** Bruce 07	**2** Bruce 07	**3** Bruce 07	**4** Bruce 07	**5** Bruce 07	**6** Bruce 07
F	**1** Bruce 43	**2** Bruce 43	**3** Bruce 43	**4** Bruce 43	**5** Bruce 43	**6** Bruce 43	**1** Bruce 09	**2** Bruce 09	**3** Bruce 09	**4** Bruce 09	**5** Bruce 09	**6** Bruce 09
G	**1** Bruce 45	**2** Bruce 45	**3** Bruce 45	**4** Bruce 45	**5** Bruce 45	**6** Bruce 45	**1** Bruce 16	**2** Bruce 16	**3** Bruce 16	**4** Bruce 16	**5** Bruce 16	**6** Bruce 16
H	**1** Bruce 55	**2** Bruce 55	**3** Bruce 55	**4** Bruce 55	**5** Bruce 55	**6** Bruce 55	**1** Bruce 30	**2** Bruce 30	**3** Bruce 30	**4** Bruce 30	**5** Bruce 30	**6** Bruce 30

1	*B.melitensis* 16M		3	C267	5	D223
2	*B.suis* Thomsen		4	C365	6	T- Mix

Annex 2: *Brucella* MLVA table for alleles assignement

(source: MLVA-net ; http://mlva.u-psud.fr/brucella/IMG/pdf/bru_table_allele_assignement_version3.6.pdf)

Brucella **table for alleles assignement [allele calling convention to convert alleles size (bp) into number of repeats (u)]**

Panel 1 (agarose2%)															
bruce06-BRU1322_134bp_408bp_3u	140 (1u)	274 (2u)	**408 (3u)**	542 (4u)											
bruce08-BRU1134_18bp_348bp_4u	312 (2u)	330 (3u)	**348 (4u)**	366 (5u)	384 (6u)										
bruce11-BRU211_63bp_257bp_2u	**257 (2u)**	320 (3u)	383 (4u)	509 (6u)	635 (8u)	698 (9u)	887 (12u)	1013 (14u)	1076 (15u)						
bruce12-BRU73_15bp_392bp_13u	[b]302 (7u)	[b]317 (8)	327-332 (9u)	342-347 (10u)	362 (11u)	377 (12u)	**392-397 (13u)**	407-411 (14u)	422 (14u)	437 (14u)	452 (14u)				
bruce42-BRU424_125bp_539bp_4u	164 (1u)	289 (2u)	414 (3u)	**539 (4u)**	664 (5u)	789 (6u)	914 (7u)								
bruce43-BRU379_12bp_182bp_2u	170 (1u)	**182 (2u)**	194 (3u)												
bruce45-BRU233_18bp_151bp_3u	133 (2u)	**151 (3u)**	169 (4u)	187 (5u)											
bruce55-BRU2066_40bp_273bp_3u	193 (1u)	233 (2u)	**273 (3u)**	313 (4u)	353 (5u)	393 (6u)	433 (7u)		[a]553 (10u)						
Panel 2A (agarose3%)															
bruce18-BRU339_8bp_146bp_5u	130 (3u)	138 (4u)	**146 (5u)**	154 (6u)	162 (7u)	170 (8u)	178 (9u)	[a]186 (10u)							
bruce21-BRU329_8bp_148bp_6u	140 (5u)	**148 (6u)**	156 (7u)	164 (8u)	172 - 175 (9u)										
Panel 2B (agarose3%)															
bruce04-BRU1543_8bp_152bp_2u	144 (1u)	**152 (2u)**	160 (3u)	168 (4u)	176 (5u)	184 (6u)	192 (7u)	200 (8u)	208 (9u)	216 (10u)	224 (11u)	232 (12u)	240 (13u)	248 (14u)	
	256 (15u)	264 (16u)	272 (17u)	280 (18u)	288 (19u)	296 (20u)	304 (21u)	312 (22u)	320 (23u)		360 (28u)				
bruce07-BRU1250_8bp_158bp_5u	134 (2u)	142 (3u)	150 (4u)	**158 (5u)**	166 (6u)	174 (7u)	182 (8u)	190 (9u)	198 (10u)	206 (11u)	214 (12u)	222 (13u)	230 (14u)		
	246 (16u)														
bruce09-BRU588_8bp_156bp_7u	124 (3u)	132 (4u)	140 (5u)	148 (6u)	**156 (7u)**	164* (8u)	172 (9u)	180 (10u)	188 (11u)	196 (12u)	204 (13u)	212 (14u)	220 (15u)	228 (16u)	
	236 (17u)	244 (18u)	252 (19u)	260 (20u)	268 (21u)	276 (22u)	284 (23u)	292 (24u)							
bruce16-BRU548_8bp_152bp_3u	144 (2u)	**152 (3u)**	160 (4u)	168 (5u)	176 (6u)	184 (7u)	192 (8u)	200 (9u)	208 (10u)	216 (11u)	224 (12u)	[a]232 (13u)	240 (14u)	248 (15u)	
	[b]254 (16u)		[b]270 (18u)												
bruce30-BRU1505_8bp_151bp_6u	119 (2u)	127 (3u)	135 (4u)	143 (5u)	**151 (6u)**	159 (7u)	167 (8u)	175 (9u)	183 (10u)	191 (11u)	199 (12u)				
Previous Panel 2A member															
bruce19-Bru324_3bp_163bp_36u	76 (7u)		82 (9u)		[b]88 (11u)		**163 (36u)**		169 (38u)	[c]172 (39u)	[c]175 (40u)	178 (41u)		184 (43u)	
	187 (44u)	190 (45u)	193 (46u)		202 (49u)										

darker gray is the *in silico* inferred size in 16M genome sequence (NC_003317 and NC_003318) * observed size in Nouzilly-Orsay 16M reference strain

The indicated PCR product sizes are as obtained when using the primers published in Le Flèche et al. 2006 BMC microbiology. They need to be ajusted if alternative primers are used.

DNA sizing equipment must be calibrated by using reference strains as raw size estimates may need to be adjusted.

[a] *Alleles observed in B. microti isolates* [b] *Alleles observed in B. ceti isolates* [c] *Allele observed in B. inopinata*

Le Flèche et al. 2006 version 3.6 (last modified April 22 2013)

Version 3.6 minor format errors; Version 3.5 and 3.4 modifications co

for locus bruce12-BRU73_15bp_392bp_13u: (327-332)bp encoded 9u

locus Bruce19-BRU324_6bp_163bp_18u is renamed bruce19-BRU324