Standard Operating Procedure

Bruce Ladder

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 Bruce Ladder typing for identification of several *Brucella* species, including *B. abortus*, *B. melitensis, B. ovis, B. suis, B. canis, B. neotomae* and some vaccine strains (S19, RB51 and Rev1). This method has been published by Garcia-Yoldi et al. (2006).

2 Normative and scientific references

Multiplex PCR Assay for the Identification and Differentiation of all *Brucella* Species and the Vaccine Strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1 (2006) David Garcıa-Yoldi, Clara M. Marın, Marıa J. de Miguel, Pilar M. Munoz, Jose L. Vizmanos and Ignacio Lopez-Goni (https://academic.oup.com/clinchem/article/52/4/779/5626963)

Evaluation of a Multiplex PCR Assay (Bruce-ladder) for Molecular Typing of All *Brucella* Species, Including the Vaccine Strains (2008), I. Lopez-Goni, D. García-Yoldi, C. M. Marín, M. J. de Miguel, P. M. Munoz, J. M. Blasco, I. Jacques, M. Grayon, A. Cloeckaert, A. C. Ferreira, R. Cardoso, M. I. Correa de Sa, K. Walravens, D. Albert, and B. Garin-Bastuji. (https://jcm.asm.org/content/jcm/46/10/3484.full.pdf)

New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis* (2011), Ignacio Lopez-Goni, David Garcıa-Yoldi, Clara M. Marın, Marıa J. de Miguel, Elıas Barquero-Calvo, Caterina Guzman-Verri, David Albert and Bruno Garin-Bastuji, Veterinary Microbiology 154, p 152-155. (https://www.sciencedirect.com/science/article/pii/S0378113511003634?via%3Dihub)

3 Definitions

- **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).

- **Immolase:**

IMMOLASE™ is a heat-activated, thermostable DNA polymerase. IMMOLASE provides high yield and improved specificity when compared to standard polymerases and can eliminate the presence of non-specific binding, such as primer-dimers and mis-primed products.

- **PCR Master Mix:**

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

4 Principle and reaction

4.1 Principle

Bruce-ladder is a multiplex PCR designed to identify several *Brucella* species and vaccine strains.

4.2 List of reagents, products

4.2.1 PCR amplification step

- Ultrapure water (keep at +5°C ± 3°C),
- Buffer [10X] (final concentration 1X ; keep frozen at tp < -16°C),
- MgCl2 [25mM] (final concentration 2mM ; keep frozen at tp < -16°C),
- dNTP mixture [25mM] (final concentration 200 µM ; keep frozen at tp < -16°C),
- Immolase [5U/µL] (final concentration 1 U ; keep frozen at tp < -16°C),
- Primers [2.5 µM] (final concentration 12.5 pM; keep frozen at tp < -16°C).

Primers for Bruce-ladder 2.0:

Primer Name	Primer Alias	Primer sequence	Primer Size
BMEI0998f	BME A1	atc cta ttg ccc cga taa gg	20
BMEI0997r	BME A2	gct tcg cat ttt cac tgt agc	21
BMEI0535f	BME B1	gcg cat tct tcg gtt atg aa	20
BMEI0536r	BME B2	cgc agg cga aaa cag cta taa	21
BMEII0843f	BME C1	ttt aca cag gca atc cag ca	20
BMEII0844r	BME C2	gcg tcc agt tgt tgt tga tg	20
BMEI1426f		tcg tcg gtg gac tgg atg ac	20
BMEI1427r		atg gtc cgc aag gtg ctt tt	20
BMEII0428f	BME E1	gcc gct att atg tgg act gg	20
BMEII0428r	BME E2	aat gac ttc acg gtc gtt cg	20
BR0953f	BR F1	gga aca cta cgc cac ctt gt	20
BR0953r	BR F2	gat gga gca aac gct gaa g	19
BMEI0752f	BME G1	cag gca aac cct cag aag c	19
BMEI0752r	BME G2	gat gtg gta acg cac acc aa	20
BMEII0987f	BME H1	cgc aga cag tga cca tca aa	20
BMEII0987r	BME H2	gta ttc agc ccc cgt tac ct	20

4.2.2 Gel preparation

- TAE buffer (Tris, Acétate, EDTA) [0.5X] ; (keep at +5°C ± 3°C)
- SeaKem®LE agarose (powder) (keep at room temperature)
- SeaKem®GTG agarose (keep at room temperature)
- Gel-Red
- Loading buffer [10X] (final concentration 1X ; keep at +5°C ± 3°C)
- DNA Ladder Plus 100-bp [0.5 µg/µl] (keep at +5°C ± 3°C).

4.3 Equipments

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

4.4 Protocol

DNA is previously extracted from isolated strains.

4.4.1 Mix preparation

Rq: this first part is realized in a "no-amplicon" room of the molecular biology laboratory.

- Thaw mix reagents

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

- First, prepare a MASTER Mix as described in the table below

	For 1 sample (µl)
Ultrapure water	15,1
Buffer [10X] (1X)	2,5
MgCl2 [50 mM] (2mM)	1
Primers mix [2,5µM] (12,5 pM)	5
dNTPs mix [25 mM] (200µM)	0,2
Immolase [5U/µl] (1U)	0,2
DNA (1µg/ml)	1
Final volume	25

-Then, distribute 24µl of the PCR Mix in each well according to the plate loading plan.

Ex:

Plan de plaque PCR-Bruce Ladder suis

	1	2	3	4	5	6	7	8	9	10	11	12
A	**1** Multiplex suis	**2** Multiplex suis	**3** Multiplex suis	**4** Multiplex suis	**5** Multiplex suis	**6** Multiplex suis	**7** Multiplex suis	**8** Multiplex suis	**9** Multiplex suis	**10** Multiplex suis	**11** Multiplex suis	**12** Multiplex suis
B	**13** Multiplex suis	**14** Multiplex suis	**15** Multiplex suis	**16** Multiplex suis	**17** Multiplex suis	**18** Multiplex suis	**19** Multiplex suis	**20** Multiplex suis	**21** Multiplex suis	**22** Multiplex suis	**23** Multiplex suis	**24** Multiplex suis
C	**25** Multiplex suis	**26** Multiplex suis	**27** Multiplex suis	**28** Multiplex suis	**29** Multiplex suis	**30** Multiplex suis	**31** Multiplex suis	**32** Multiplex suis	**33** Multiplex suis	**34** Multiplex suis	**35** Multiplex suis	**36** Multiplex suis
D	**37** Multiplex suis	**38** Multiplex suis	**39** Multiplex suis	**40** Multiplex suis	**41** Multiplex suis	**42** Multiplex suis	**43** Multiplex suis	**44** Multiplex suis	**45** Multiplex suis	**46** Multiplex suis	**47** Multiplex suis	**48** Multiplex suis
E	**49** Multiplex suis	**50** Multiplex suis	**51** Multiplex suis	**52** Multiplex suis	**53** Multiplex suis	**54** Multiplex suis	**55** Multiplex suis	**56** Multiplex suis	**57** Multiplex suis	**58** Multiplex suis	**59** Multiplex suis	**60** Multiplex suis
F	**61** Multiplex suis	**62** Multiplex suis	**63** Multiplex suis	**64** Multiplex suis	**65** Multiplex suis	**66** Multiplex suis	**67** Multiplex suis	**68** Multiplex suis	**69** Multiplex suis	**70** Multiplex suis	**71** Multiplex suis	**72** Multiplex suis
G	**73** Multiplex suis	**74** Multiplex suis	**75** Multiplex suis	**76** Multiplex suis	**77** Multiplex suis	**78** Multiplex suis	**79** Multiplex suis	**80** Multiplex suis	**81** Multiplex suis	**82** Multiplex suis	**83** Multiplex suis	**84** Multiplex suis
H	**85** Multiplex suis	**86** Multiplex suis	**87** Multiplex suis	**88** Multiplex suis	**89** Multiplex suis	**90** Multiplex suis	**91** Multiplex suis	**92** Multiplex suis	**93** Multiplex suis	**94** Multiplex suis	**95** Multiplex suis	**96** Multiplex suis

1	Brucella suis 1	17		33		49		65		81	
2	Brucella suis 2	18		34		50		66		82	
3	Brucella suis 3	19		35		51		67		83	
4	Brucella suis 4	20		36		52		68		84	
5	Brucella suis 5	21		37		53		69		85	
6		22		38		54		70		86	
7		23		39		55		71		87	
8		24		40		56		72		88	
9		25		41		57		73		89	
10		26		42		58		74		90	
11		27		43		59		75		91	
12		28		44		60		76		92	
13		29		45		61		77		93	
14		30		46		62		78		94	
15		31		47		63		79		95	
16		32		48		64		80		96	T- Mix

-Then, distribute 1 µL of DNA in each well and seal the plate, using a PCR plastic film.

4.4.2 Amplification

Rq: this second part is realized in a “amplicon” room of the molecular biology laboratory.

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

Nb of cycle	Temperature (°C)	Time	
		Minutes	Seconds
1	95	7	0
25	95	0	35
	64	0	45
	72	3	0
1	72	6	0
1	4	∞	

Total time of the program: 3 hours

- Following amplification, keep the plate at +5°C ± 3°.

4.4.3 Gel preparation and electrophoresis

- **<u>Gel preparation (final concentration of 2.4 %)</u>**

- First, weight Agarose powder (50% Agarose LE and 50% Agarose GTG) and add 0.5X cold TAE buffer for a final concentration of 1.2% (ex: 1.2 g of Agarose LE + 1.2 g of Agarose GTG in 200 ml of TAE 0.5X);

Melt at the microwave until complete dissolution with regularly agitation (use a glove to avoid burning).

<u>Attention:</u> risk of boiling! Melt with different steps.

Put the agarose solution in a water bath at 50-60°C to drive the temperature down until the solution can be take bare hands without burning.

Agitate and cast the gel at moderate temperature. Add 3 drops of Gel-Red.

<u>Attention:</u> The cuve should be clean to cast the gel. If not, wash the cuve with distilled water.

- In a 96 well plate or in a parafilm sheet, deposit 1 µl of loading buffer and 8µl of PCR product or DNA molecular weight ladder.

- **<u>Electrophoresis</u>**

- Place the gel into the cuve and deposit 5-7 µl of PCR product or DNA molecular weight ladder.

- A short initial phase of high voltage (5 min, 120 V) is performed for the DNA to enter into the gel, and then, electrophoresis is carried out during 2h30 (100 V).

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

- **<u>Gel images</u>**

- 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), compare it to the included reference strain (*B. melitensis*, *B. abortus*…) and the ladder.

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	▪ 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
Gel preparation	▪ 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	▪ 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	▪ Compare with reference strains