Standard Operating Procedure

Brucella HRM-PCR

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, the High Resolution Melting (HRM) real-time Polymerase Chain Reaction (PCR) to identify the species and five biovars of *Brucella* in animal / human samples.

2 Normative and scientific references

High-Resolution Melting PCR as Rapid Genotyping Tool for *Brucella* Species, Guillaume GIRAULT, Ludivine PERROT, Virginie MICK, Claire PONSART, *Microorganisms 10(336)*

3 Definitions

- **Single Nucleotide Polymorphism (SNP)**

A SNP is a single nucleotide mutation in the genome of an organism.

- **Master Mix:**

Kit 1: The LightCycler® 480 High Resolution Melting Master is a ready-to-use 2x-concentrated hot start reaction mix, containing hot start PCR enzyme and ResoLight, a saturating DNA dye, for amplification and detection of heteroduplex regions in PCR amplicons. Additional MgCl2 is required (included in the commercial kit).

Kit 2: The Applied Biosystems™ MeltDoctor™ HRM Reagent Kit is ready-to-use hot start reaction mix, containing hot start AppliTaq Gold™ DNA polymerase and MeltDoctor™ HRM Dye, a stabilized for of the fluorescent SYTO™ 9 double stranded nucleic acid stain.

- **Primers:**

Specific primers have been designed to identify each species and biovars from the *Brucella* genus specifically.

- **Expected allele:**

The expected allele refers to the SNP allele of the species/biovar which is targeted.

- **Other allele:**

The other allele refers to the SNP allele of all other species/biovar than the one targeted.

4 Principle and reaction

4.1 Principle

High-resolution melting (HRM) is a post-PCR technique that determines with high precision the

melt profile of PCR products. A new generation dye is incorporated into a double-stranded DNA. Using a slow constant increase in temperature, fluorescence acquisition allows the distinction between two different populations of amplicons. The method can be used to interrogate small number of SNPs.

Complete genomes of the *Brucella* genus have been aligned to identify SNPs. The filtered SNP matrix, composed of relevant variants, is used to identify and select species or biovars specific SNPs, i.e. having the expected allele. Once the fixed variants are identified, the 100 bp SNP flanking region is extracted and primers are designed. A maximum amplicon size of 100 bp is expected, but most of the time, smaller amplicons are targeted.

4.2 List of reagents, products

4.2.1 PCR amplification step LightCycler® 480 High Resolution Melting Master

- Ultrapure water (keep at +5°C ± 3°C),
- Kit 1. LightCycler® 480 High Resolution Melting Master [2X] (final concentration 1X ; keep frozen at temperature < -16°C).
- MgCl2 [25 mM] (final concentration 2,5 µM ; keep frozen at temperature < -16°C),
- Primers [20 µM] (final concentration 0,2 µM ; keep frozen at tp < -16°C),

4.2.2 PCR amplification step Applied Biosystems™ MeltDoctor™ HRM Reagent Kit

- Ultrapure water (keep at +5°C ± 3°C),
- MeltDoctor™ HRM Reagent kit (keep frozen at temperature < -16°C).
- Primers [20 µM] (final concentration 0,2 µM ; keep frozen at tp < -16°C),

Primers list

Species targeted	Forward primer (5'-3')	Reverse primer (5'-3')	Expected Allele (related to column 1)	Other Allele (related to all other *Brucella)*
B. abortus	acgaagaagcgatctcgatg	aggaaaggccgatgatgtaa	T	C
B. melitensis	cggtccgggccacctttacg	ggcccggcaattgctcctga	C	T
B. suis-B. canis	ctggcggaaaaggatttgat	aatcacgacaaaccacagca	T	C
B. suis biovar 1	tgacatggaccctgttttcc	cagcgtgacactgaacatgg	G	A
B. suis biovar 2	agaccttgcgcttgaacg	gccacactgctgagttcg	T	C
B. suis biovar 3	gtatggcggaatgcagga	cacaaacgccagtgaacg	A	G
B. suis biovar 4	aagatcgccgtcgtctcg	ggccacaacagcctgaac	A	G
B. suis biovar 5	cttccgttgaagggcaatc	gcctcgaaaacgaaatcatc	C	T
B. canis	gagaactgacccgatggaaa	caagggaaccgaatatctgc	C	T
B. microti	aactgccggatgtgaaaaag	aaggatcgaggcgtcataaa	C	T
Marine *Brucella*	gcgatttcattgcccttg	ttgaaatgggcttcatcca	A	G
B. ceti 1	aatgccgcaatcttcatctt	cctctgcgcgacagtttaag	A	C
B. ceti 2	ctcgctcccaaacactaccc	cgttcgccccttatatttga	C	T
B. pinnipedialis	tgcgggatttcaaggataag	aagatcgccagatcgtgct	T	C
B. ovis	atgggctttggcggtatt	cgcccaggtagagctttg	T	C
B. neotomae	atggcgaattcgatgaaaag	tgtcttcacagacgggaatg	T	G

B. meliensis Rev 1	cttcacgccatgcttctttt	atgctcaccaccttcaacg	T	C

4.3 Equipments

Thermocyclers : ViiA7 (Life technologies), QS7 (Life technologies), any real time thermocycler with a HRM module.

4.4 Protocol

Amplification step:

The DNA is extracted from a pure colony prior to the amplification step. The reagents must be thawed before use and used at room temperature. All reagents are homogenised and centrifuged.

Kit 1: The PCR mix is prepared according to the following calculations for LightCycler® 480 HRM master mix [2x]:

HRM Mix	
	Reagents for 1 sample (µl)
Ultrapure water	2.8 µl
LightCycler® 480 HRM master mix [2x] (1x)	5
MgCl2 [25 mM] (2,5 µM)	1
Primer Fd [20 µM] (0,2 µM)	0.1
Primer Rv [20 µM] (0,2 µM)	0.1
DNA (1 µg/ml)	1
Total volume	10

In a 96 real time PCR plate, deposit 9 µl of PCR mix in each well according to the plate plan.

Kit 2: The PCR mix is prepared according to the following calculations for MeltDoctor™ HRM Reagent kit:

HRM Mix	
	Reagents for 1 sample (µl)
Ultrapure water	8.4 µl
MeltDoctor™ HRM Reagent kit (1x)	10
Primer Fd [20 µM] (0,2 µM)	0.3
Primer Rv [20 µM] (0,2 µM)	0.3
DNA (1 µg/ml)	1
Total volume	20

In a 96 real time PCR plate, deposit 19 µl of PCR mix in each well according to the plate plan.

Proceed in the same manner when using Kit 1 or Kit 2.

Deposit 1 µl of DNA according to the plate plan and seal the plate with a plastic PCR film. For each HRM assay, a positive and negative expected allele sample is always added, to compare the tested DNAs.

Centrifuge the plate to ensure that the liquid will reach the bottom of each well. Put the plate into a thermocycler and run the PCR HRM program

Kit 1:

Number of cycles	Temperature (°C)	Time	
		Minutes	Seconds
1	95	10	0
40	95	0	10
	58	0	10
	72	0	20
1	95	0	30
1	65	1	0
From 65°C to 94°C (rate of 1°C/s with 25 acquisitions/°C)	HRM step		
1	60	0	15

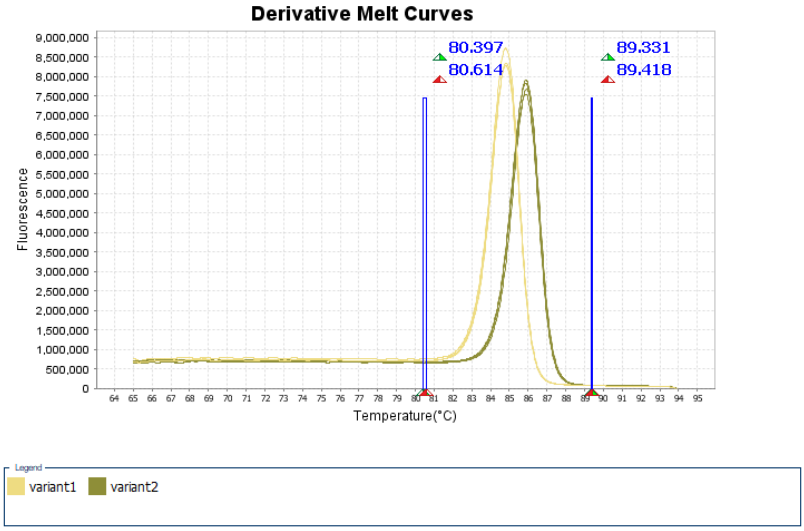
Kit 2:

Number of cycles	Temperature (°C)	Time	
		Minutes	Seconds
1	95	10	0
40	95	0	15
	60	1	0
1	95	0	10
1	65	1	0
From 65°C to 94°C (rate of 1°C/s with 25 acquisitions/°C)	HRM step		
1	60	0	15

4.4.4 Reading and interpretation

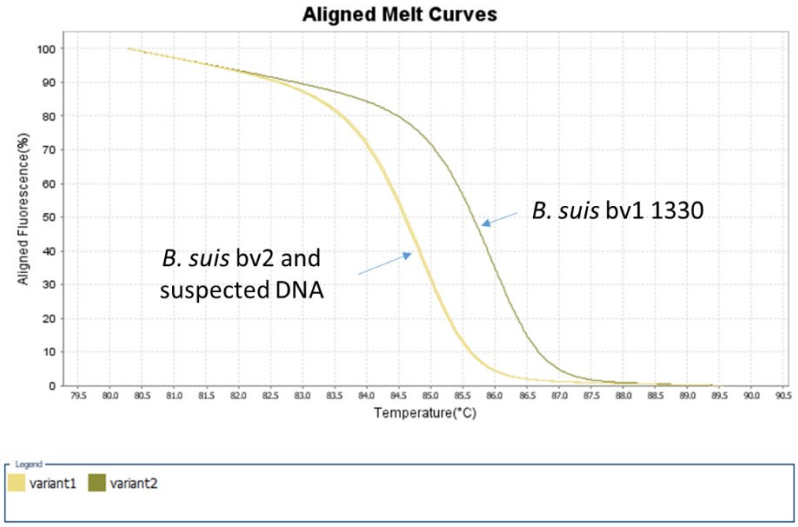
The melting temperatures and melting curve profiles are analysed by the QuantStudio™ Real-Time PCR Software (version 1.2).

The positive and negative allele's sentinels are analysed, to determine the allelic profile. First, amplification curves are checked (PCR step). Then, bordering bars are correctly adjusted to the melting curves, (just before the start of curve increase, see figure below). This step is important to correctly analyse the curves.



Then, each targeted DNA is analysed in comparison to the sentinels. The melting temperature is exported and analysed, so each DNA is then attributed to the species, according to the melting temperature and the allelic profile of the sentinels. An example described the whole process below.

Example: suspected DNA belonging to *Brucella* genus were subjected to HRM PCR to identify the species involved. In this case, *B. suis* primers related to biovars are analysed (biovar 1 versus biovar 2). DNA from strain 1330 (biovar 1) and Thomsen (biovar 2) were used as sentinels. The DNA from the isolates have the same HRM profile than *B. suis* biovar 2 DNA, so they belong to *B. suis* bivoar 2.



The melting temperature are then extracted and compared

Well Position	Sample Name	Target Name	Tm1	Delta Tm	Species	biovar
A5	Thomsen	Bsuis bv2	84,827	-1,101	B. suis	biovar 2
B5	1330	Bsuis bv2	85,928	1,101	B. suis	biovar 1
F5	H763	Bsuis bv2	84,804	-1,124	B. suis	biovar 2
G5	H763	Bsuis bv2	84,805	-1,123	B. suis	biovar 2

The difference in melting temperature allow the attribution of a sample to a species / biovar. In this example, sample H763 has been tested in duplicate, and Tm is 84,804 and 84,805. The difference with the Tm of *B. suis* biovar 1 strain 1330 is more than 1°C, and in this case, very similar to the Tm of *B. suis* biovar 2 strain Thomsen (0,022 and 0,023°C). In this case, sample H763 is confirmed to belong to *B. suis* biovar 2.

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	▪ DNA extraction is preferable using a classic extraction kit ▪ Be careful to avoid contamination with sentinels
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
Interpretation	▪ See 4.4.4