Standard Operating Procedure

Brucella MALDI-TOF

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 Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) of proteins to identify the *Brucella* genus in animal / human and environmental samples.

2 Normative and scientific references

Identification of Brucella by MALDI-TOF mass spectrometry. Fast and reliable identification from agar plates and blood cultures, Laura Ferreira , Silvia Vega Castaño, Fernando Sánchez-Juanes, Sandra González-Cabrero, Fabiola Menegotto, Antonio Orduña-Domingo, José Manuel González-Buitrago, Juan Luis Muñoz-Bellido, PLOS One, 2010 Dec 6;5(12):e14235. doi: 10.1371/journal.pone.0014235**.**

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.

4 Principle and reaction

4.1 Principle

Matrix-assisted laser desorption/ionization (MALDI) is an ionization technique that uses a laser energy-absorbing matrix to create ions from large molecules, such as proteins, with minimal damages or distortions. It has been applied to the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and carbohydrates) and various organic molecules since MALDI typically produces far fewer multi-charged ions than other similar techniques. MALDI methodology is a three-step process. First, the sample is mixed with a suitable matrix material and applied to a metal plate. Second, a pulsed laser irradiates the sample, triggering ablation and desorption of the sample and matrix material. Finally, the analyte molecules are ionized by being protonated or deprotonated in the hot plume of ablated gases,

and then they can be accelerated into whichever mass spectrometer is used to analyse them.

This method describes the preparation of bacterial strains and plate preparation to be read in MALDI-TOF machine. This analysis, for now, can be applied only to isolated colonies.

4.2 List of reagents, products

4.2.1 Bacterial culture

Enriched basal media (such as Blood agar based) as well as enriched selective media can be used to grow bacteria for MADLI-TOF analysis.

4.2.2 Reagents

The reagents used for MALDI-TOF are:

- Ethanol 70%,
- IVD BTS (Bacterial test standard),
- IVD HCCA,
- Ultrapure water (keep at +5°C ± 3°C),
- Solution OS : Organic solvent acetonitrile 50%, water 47.5% and tri-fluoroacetic acid 2.5%) bought either from Sigma-Aldrich (#19182) or VWR (#PRLS89449.230), recommended for solubilization of IVD HCCA.

Materials:

- PSM type II,
- Incubator at 37°C with CO2,
- Oese,
- 100 µl pipette tips and pipette,
- Plates: There are two types of MALDI-TOF plates: reusable and single use. The laboratory should choose which one to use according to its preferences and already existing protocols.
- If single use plates are used, the plate adapter is needed,
- Microtubes with screw-top covers,
- Plate transport box.

4.3 Equipments

MALDI-TOF machine.

4.4 Protocol

Growth of strains:

It is necessary to grow culture of potential Brucella strains on mentioned media for at least 48 H.

Preparation of reagents:

Precaution: Before any use of solvent solution (OS) it is highly recommended to mix vigorously the bottle minimum for one minute if not longer to homogenize the two phases (solvent, soluble). If the bottle pump is being used, it is recommended to pump five volumes and to throw it away. When the OS solution bottle is at 20% of its content, it is highly recommended to replace it in order to avoid misrepresentation of each chemical components in the bottle. After aliquoting, the bottle should be kept in a dark ventilated cabinet.

Reconstitution of BTS:

- Add 50 µl of standard solvent to lyophilized IVD BTS and reconstitute it by pipetting up and down minimum 20 times at room temperature (RT)? Avoid the bubbles during resuspension.
- Incubate the solution for five minutes and pipette it from above to below minimum 20 times.
- Centrifuge at room temperature at 13 000 RPM for 2 minutes.
- Aliquot 5 µl in microtubes to be used each time as a standard. Microtubes should be MALDI graded and closed with the screw-top cap. Aliquots should be stored at < -18°C.
  Precaution: Once reconstituted, the aliquots can be conserved next five months.

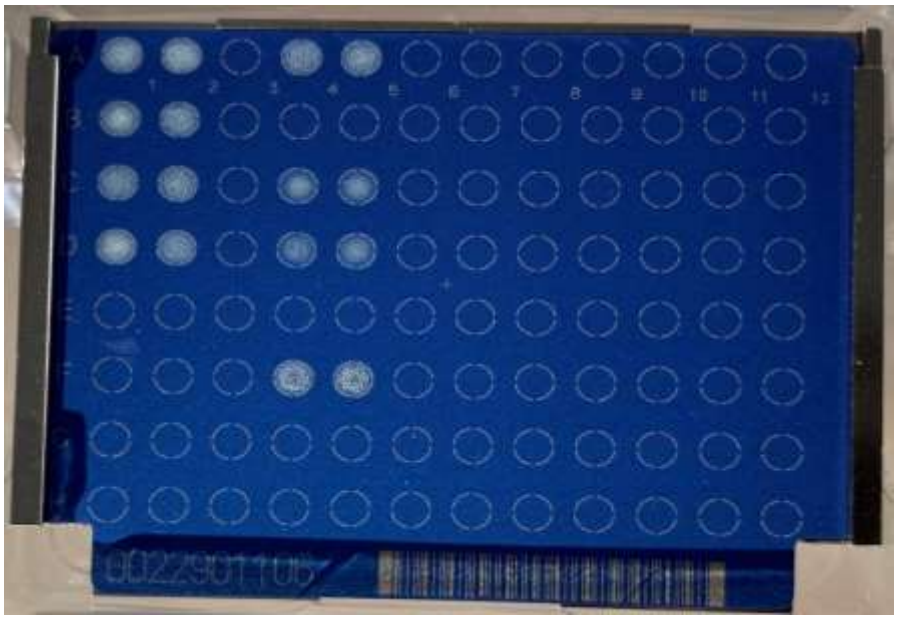
Reconstitution of matrix:

- Add 250 µl of OS to the IVD HCCA tube,
- Vortex the solution by avoiding the formation of bubbles,
- Incubate the solution at RT for 5 minutes,
- Make sure that all crystals are dissolved by visually inspecting the tube,
- Keep it at <-18°C and limit the number of freeze/thaw cycles.
  Precaution: Once reconstituted it can be used during one month.

Preparation of strains:

- Using ooze, transfer one bacterial colony to clean Eppendorf tube containing 300 µl of HPLC graded water. Turbulent suspension should be obtained.
- Add 900 µl of absolute Ethanol and mix the solution,
- Incubate overnight the colony in ethanol,
- Centrifuge 2 minutes at 13 000 – 15 000 RPM,
- Remove the supernatant using the pipette,
- Repeat the centrifugation step and remove again remaining ethanol,
- Leave it to air dry at RT for five minutes,
- Under the chemically graded biosafety cabinet, add 25 µl of 70% formic acid and mix by pipetting until the pellet dissolves,
- Add 25 µl of acetonitrile 100% and mix by pipetting two to three times,

- Centrifuge 2 minutes at 13 000 – 15 000 RPM,
- Transfer 1 µl of supernatant on a MALDI plate,
- Add 1 µl of IVD HCCA matrix and mix the samples by pipetting on the plate,
- Leave it to air dry at room temperature,
- Each sample should be mixed individually with matrix as soon as it is added onto the plate,
- Place the removable plate to the adapter in the transport box.,



Example of BRUKER MALDI-TOF plate

Plate should be read as soon as possible with maximum waiting time of 24h.

The use of BRUKER MALDI-TOF machine:

To start the machine and the PC for its control, please use the internal or dedicated platform protocol(s).

If there is a plate reader you can use it to read the plate bar code, and if not, it can be entered manually.

- Identify the wells on your plate,
- It is recommended to have at least one positive control with BTS for each plate,
- Start the reading of the plate.

Reading and interpretation:

The machine will read each identified well in order they were entered and using the spectral data base will identify the target. At the end of the analysis, it is possible to export the analysis report either in PDF or XLSX formats as well as spectra for each sample.

There are some limitations for interpretation and reading of the results:

- If BTS did not pass the quality control, the analysis of other samples on the same plate should not be performed.

- If the sample itself did not pass the quality control (orange/red cercle of the well position on the software) further interpretation of the result cannot be taken into account.
- The samples with the reading score of 1.99 and lower should be re-extracted and analysis should be repeated in order to confirm the results.

4.5 Critical points

General recommendations	▪ Prevent formation of aerosols and bubbles (each time tubes or plates have to be opened) ▪ Check the quality of pipettes calibration (volumetry) ▪ Use of tip with filters
Preparation of reagents	▪ Make sure that OS is well mixed before reconstitution of BTS and matrix stringently following instructions. ▪ Any further adaptations of this step require validation procedures to be performed
Bacterial inactivation	▪ Bacterial inactivation should be done in the BSL3 facility under the biosafety hood using freshly prepared ethanol ▪ Be careful to avoid contamination with sentinels
Distribution of samples, standards and matrix	▪ Pay attention to potential contaminations during mix preparation and distribution onto the plate ▪ It is not recommended to use multichannel pipettes
Interpretation	▪ See Reading and interpretation: