Standard Operating Procedure

Brucella real time PCR

IDBRU

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 ID Gene® *Brucella* spp Triplex (IDBRU) kit is a Reel-time Polymerase Chain Reaction (Rt-PCR) kit for specific *Brucella* spp. sequence amplification based on TaqMan® technology. This is a qualitative Triplex system that enables simultaneous amplification of the target DNA and internal endogenous and exogenous controls.

The present document describes a standard technique aiming at real-time Polymerase Chain Reaction (PCR) to detect the presence / absence of *Brucella* DNA in animal / human samples. Prior to PCR, the DNA extraction protocols have to be validated for each type of sample handled in the laboratory in order to assure standardized performances.

2 Normative and scientific references

HOLZAPFEL M., 2018. De l'épidémiologie moléculaire aux analyses fonctionnelles de Brucella chez les ruminants, une approche intégrée pour l'identification et l'étude de la diversité phénotypique d'un genre génétiquement homogène. Thèse de doctorat en Microbiologie, soutenue le 26-11-2018 à Paris Est, dans le cadre de l'École doctorale ABIES, 200 pages. Online document: http://www.theses.fr/2018PESC1141/document

3 Definitions

- **Analyte:** Genomic DNA of *Brucella*
- **Matrix:** tissues from various organs, tissue swabs, secretions, organ shredding, bacteria suspension.
- **Background noise** qualifies the non-characteristic part of the curves observed during the first 10 to 15 cycles.
- **Amplification** refers to the presence of a curve with an increasing linear part followed by a plateau phase. Any curve that does not have this appearance will be considered a non-characteristic curve.
- **The threshold** should be placed above the background noise, preferably in the middle of the linear part (in "logarithmic scale" display) common to all amplification curves. Ct (Cycle threshold)

4 Principle and reaction

4.1 Principle

In a first step, the total DNA present in a given sample is extracted and purified using a kit after the addition of exogenous DNA called NTPCBru (Enxogenous Positive control non-target) to the matrix.

The extracted DNA is then amplified in the PCR step using the target-specific forward and reverse primers present in the kit's reaction mix. For *Brucella* detection, the PCR system implemented is based on the use of a pair of specific oligonucleotide primers and a labelled probe for all species in this genus detected with FAM reporter.

Detection of the exogenous gene added to the extraction step is performed using the IDBRU PCR kit, with the reaction mix also containing the specific primers and probe. This internal control system verifies the DNA extraction process, as well as the presence of any PCR inhibitors. It provides a check of the extraction steps.

5 Material and method

5.1 Material

- Instruments:

  Thermocycler Reel Time PCR with channels for reading the following fluorophores: FAM™, HEX™ or VIC® and Cyanine 5 (Cy5).
- Equipment and consumables:

  The equipment suitable for molecular biology.
  - **PEC-BRU:** Positive Extraction Control: Inactivated *Brucella ovis* strain,
  - **PAC-BRU:** Positive Amplification Control: synthetic nucleic acid
  - **T-Lysis**: Negative Lysis Control
  - **T- Ext**: Negative Extraction Control
  - **T- PCR**: Negative Amplification Control
  - **NTPC-BRU:** Exogenous non-target positive control: Non-pathogenic bacteria acting as a target mimic.
  - **NTPCen:** Endogenous non-target positive control.
  - **ARM-BRU:** Ready-to-use reaction mixture containing Taq polymerase, primers, hydrolysis probes and oligonucleotides for the amplification and detection of the *Brucella* spp genome, endogenous and exogenous non-target positive controls.

5.2 Method

- Prepare Real-time PCR analysis worksheet with all sample and controls
- Defrost the IDBRU kit a room temperature

Prepare in two distinct places

DNA Free:

- Homogenize the ARM-BRU tube (vortex) and centrifuge briefly.
- Dispense 8 µl of ARM-BRU per well. Use PCR plates or tubes suitable for the thermal cycler.

DNA deposit:

- Deposit 5 µl of DNA according to the plate plan.
- Seal the plate with a plastic PCR film.
- Centrifuge the plate to ensure that the liquid will reach the bottom of each well.

- Put the plate into a thermocycler and run the following program:
- Program the thermal cycler to read the following detectors for each well to be analyzed:
  - *Brucella* spp: FAMTM (non-fluorescent quencher*)
  - NTPCen: VIC® / HEXTM (non-fluorescent quencher*)
  - NTPC-BRU: Cy5 (Non-fluorescent quencher*)

Step	**Polymerase activation**	**DNA denaturation**	**Primer's fixation Elongation**
Temperature	95°C	95°C	60°C
Time	2min	10 sec	20sec
Number of cycles		x45	

- Select a final volume of 13 µl per PCR. If different volumes are combined in the same analysis, indicate the largest plate volume to the thermal cycler.

- Place the plate in the thermal cycler, if not already done, and start the run.

6 Results validation and interpretation

- **PAC-BRU**: detect in FAM™, refer to the Cq value given on the quality control sheet of the corresponding batch
- **Negative Extraction Control:** detect in VIC™ and Cy5, The Cq value obtaine for NTPC-BRU in Cy5 should be considered as the NTPC-BRU reference value
- **Negative Amplification Control**: No characteristic curve at all
- **PAC-BRU**
- **PEC-BRU**

If all controls are ok, then tested samples can be analyzed.

***Brucella* spp. signal (FAM)**	**NTPC-BRU signal (Cy5)**	**NTPCen signal (VIC)**	**INTERPRETATION**
Detected	Detected or undetected	Detected or undetected	**Result interpreted as positive for *Brucella* spp**
Undetected	Detected	Detected	**Result interpreted as negative for *Brucella* spp**
Undetected	Detected	Undetected	**Problem with DNA deposit or with extraction step or the PCR is inhibited**
Undetected	Undetected	Detected	**PCR reaction is inhibited**
Undetected	Undetected	Undetected	**Problem with DNA deposit or with extraction step or the PCR is inhibited**

The results are exported from the thermal cycler software for interpretation in the Real-time PCR analysis worksheet.

The result is returned as "**target DNA not detected**" when the Ct obtained with the PCR is indeterminate.

The result is returned as "**doubtful**" when the Ct obtained is between 38 and 40.

The result is reported as "**target DNA detected**" when the sample has a target Ct < 38 with a characteristic amplification curve (sigmoidal appearance in logarithmic display).

6.1 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 the positive control
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