Project SOP D-JRP17-WP5.SOP1 – Protocol for Brucella in vitro infection assay

Workpackage 5

Responsible Partner: WBVR, ANSES

Contributing partners: APHA, BfR, FLI, IZSAM

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PROTOCOL FOR BRUCELLA IN VITRO INFECTION ASSAY

Infection conditions

- MOI: 100
- Tested for cell line: HeLa, THP-1, BoMac, DH82
- Medium: RPMI + 10% FCS + 1%X NEAA/ DMEM + 10% FCS

Day -4:

Inoculation of bacteria on plates (BSL3)

- Streak Brucella on non-selective agar plates (eg TSA, HIS agar) from -80°C stocks (table below)
- Incubate plates at 37°C, 5% CO_2 and wait until colonies are visible

Day -1

Cell culture preparation: early morning

- 24 h before starting the experiment, trypsinize and homogenize cells from pre-culture
- Count cells (determine amount of dead cells)
- Seed cells in the following densities in order to obtain 95-100% confluence in each well:

Cell line	24-wells plate (cells/well)	Cells per cm^2
HeLa	1×10^5	5.38×10^4
THP1	$2 – 5 \times 10^5$	13.45×10^4

Bacteria inoculation

Inoculate the *Brucella* strains in fresh liquid medium, so that $2*10^7$ bacteria per well are ready on the day of infection:

- Into a $25cm^2$ flask add 15 mL non-selective medium (e.g. HIS broth or TSB)
- Check the purity of the *Brucella* plate and if ok, dissolve 1 to 2 colonies into the media
- Incubate the flask at +37°C, 5% CO_2, shaking 170 rpm

Protocol is tested for the following reference strains:

NCTC number	Species	Biovar	Name	ATCC number
NCTC 10510	Brucella suis	biovar 2	Thomsen	
NCTC 10854	Brucella canis		RM-666	ATCC 23365
NCTC 10093	Brucella abortus	biovar 1	544	ATCC 23448
NCTC 10094	Brucella melitensis	biovar 1	16M	ATCC 23456
NCTC 10512	Brucella ovis		63/290	ATCC 25840
	Brucella microti		CCM 4915	
NCTC 10316	Brucella suis	biovar 1	1330	ATCC 23444;1330
NCTC 12890	Brucella	pinnipedialis		
NCTC 12891	Brucella	ceti		
	Brucella canis	Field strain		
BRU_2017_118	Ochrobactrum intermedium	X18004437-002	BSL3-HCU	Ringtestmonster Anses 2018
	Brucella abortus	Field strain		

Day 0

Infection of cell lines with Brucella (BSL3)

Preparation of bacterial inoculum

- In a 1.5 ml tube, put 1 ml of *Brucella* liquid culture (see day -1). If necessary, make more tubes for each strain
- Centrifuge at 4000 xg for 3 minutes
- Discard the supernatant and resuspend the pellet in 1 ml of PBS for washing
- Centrifuge at 4000 xg for 3 minutes
- Discard the supernatant and resuspend the pellet in 1 ml of PBS
- Measure OD600 and dilute inoculum with cell medium (RPMI + 10% FCS + 1X NEAA) to OD600 of 0.09 (OD/0.09 = dilution factor) in order to obtain a MOI of 100.
- Vortex inoculum
- Make serial dilutions (1:10) in RPMI + 10% FCS + 1%X NEAA) up to 10^{-7}, plate last 4 dilutions to verify MOI
- 500µl of inoculum (*Brucella* in RPMI + 10% FCS + 1%X NEAA) is used per well for the infection

Washing and infection of cells

- Pipet medium off cells in plates that were seeded yesterday
- Wash the wells 2x with PBS
- Carefully remove PBS after last wash
- Add bacterial inoculum (500 ul) to each well
- Spin the plates 800 xg, 15 min --> *timepoint 0 (start of infection*
- Incubate the 24 well plates for 1 hour at 37°C, 5% CO_2
- Carefully remove bacterial inoculum
- Add 1 mL medium + 50 ug/ml gentamicin per well
- Incubate plates at 37°C, 5% CO_2 until sampling

Timepoint of sampling

Harvesting of cells for RNA isolation

- After removal of the medium, immediately add 300 ul Trizol (see plate layout p 2)
- Incubate for 5 minutes at room temperature
- Scrape the cells loose with a pipet tip and put lysed cells in eppendorf tube
- Disinfect outside of tubes and take out of BSL3
- Isolate RNA according to protocol of kit supplier

RT-PCR

- Pipet off the medium of the wells
- Wash twice with PBS
- Pipet last PBS off very carefully
- Add 200 ul 0.1% - 0.2% TritonX-100 per well
- Incubate for 5 minutes at RT
- Homogenize cells with a P200 pipet tip
- Inactivate and lyse the cell suspension by pipetting 200 ul in 2 mL EasyMag lysis buffer
- Incubate overnight at RT
- Take out of BSL3 lab and proceed with DNA isolation followed by the PCR protocol provided

CFU plating

- Pipet medium out of well
- Wash the wells two times with PBS.
- Add 200 µL of Triton X-100 (0.1/0.2% in PBS) for lysing the cells
- Scratch the cell with a p200 hooked.
- Homogenizing cell lysing and incubation 5 min.
- Serial dilution (in 96-wells plate, PBS) with 20 µL in 180µL
- Plating 100µL of appropriate dilutions into TSA or BAB2 plates
- Incubation at 37°C +/- 5% CO_2 depending on Brucella species/strains

Dilutions to be plated:

Time post infection (hours)	***Brucella strain able to enters end replicates***	***Unknown Brucella strain***	***Brucella strain unable to enters end replicates***
1.5 – 2 (bacteria internalisation)	*From 10^{-2} to 10^{-4}*	*From 10^{0} to 10^{-4}*	*From 10^{0} to 10^{-2}*
5 – 6 (BCV maturation)	*From 10^{-2} to 10^{-4}*	*From 10^{0} to 10^{-4}*	*From 10^{0} to 10^{-2}*
20 – 24 (bacteria early replication)	*From 10^{-3} to 10^{-6}*	*From 10^{0} to 10^{-6}*	*From 10^{0} to 10^{-2}*
48 (end of bacteria replication)	*From 10^{-4} to 10^{-6}*	*From 10^{0} to 10^{-6}*	*From 10^{0} to 10^{-2}*