



PROTOCOL

ATTACH AND INVASION / VIABILITY ASSAY

DAY 1

- Growth the bacteria from stock plate (take two or 3 colonies) in a 3 mL tube with the respective media (i.e. TSB for *Aeromonas salmonicida*).
- Place the tube in the roller or shaker at the right temperature (i.e. 15°C for *Aeromonas salmonicida* or *Renibacterium salmoninarum*), left there for about 24 hours.

***All the procedure must be conducted using a Bunsen burner to avoid contamination.**

DAY 2

- In a 250 mL flask add 30 mL of the respective media (i.e. TSB for *Aeromonas salmonicida*) and add 300 μ L of the bacteria inoculum previously prepared.
- Label the flask and place it in a shaker (180 rpm) at the right temperature (i.e. 15°C for *Aeromonas salmonicida* or *Renibacterium salmoninarum*).
- In the case of *Aeromonas salmonicida*, the flask must be prepared in the morning to obtain 0.7 OD (600 nm) early the next day, but it will be different for each bacteria.

***All the procedure must be conducted using a Bunsen burner to avoid contamination.**

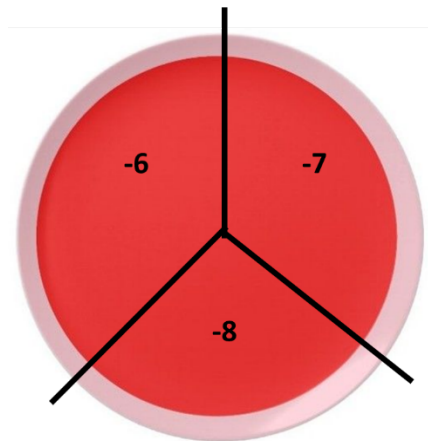
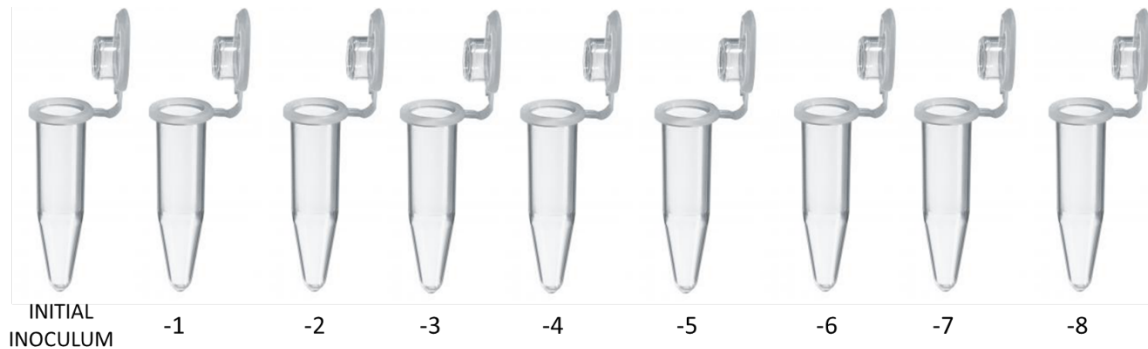
DAY 3

- 1) Put inside the biosafety cabinet the next material:
 - a) Culture media without antibiotics (non penn/strep)
 - b) DBPS
 - c) Gentamicin
 - d) Triton 100X
 - e) Trypsin
 - f) Plastic container for waste and liquid waste

***All the media must be added at room temperature.**



- 2) Bacteria procedure (example about *Aeromonas salmonicida*):
- Check the OD in the spectrophotometer using plastic columns.
 - Wash the columns before add the bacteria, using bleach and after mili-Q water.
 - One control column must contain 500 uL of the respective media (i.e. TSB for *Aeromonas salmonicida*), and the other one must contain 500 uL of bacteria inoculum.
 - Aeromonas salmonicida* must have an OD close to 0.7 ($\pm 0,02$), if it is more than 0.7, make a dilution with the same media until get 0.7 OD, if it is less than 0.7, wait until get 0.7 OD.
 - Put 30 mL of inoculum in a falcon tube and centrifuge for 10 min, 6000 rpm, at 4°C.
 - Discard the supernatant and re-suspend the pellet in 1 mL of DPBS.
 - Centrifuge for 10 min, 6000 rpm, at 4°C.
 - Discard the supernatant and re-suspend the pellet in 1 mL of DPBS, transfer to a 1.7 mL Eppendorf tube.
 - Centrifuge Eppendorf tube for 5 min, 5000 rpm, at room temperature.
 - Discard the supernatant and re-suspend the pellet in 300 uL of DPBS (initial inoculum).
 - Make serial dilutions until -8 and plate 100 uL in TSA-CR (Congo red) plates.



***All the procedure must be conducted using a Bunsen burner to avoid contamination.**

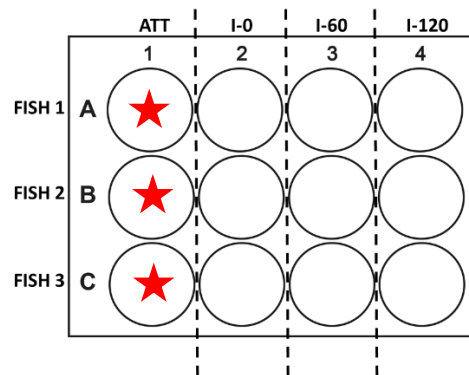


- 3) Wells procedure (macrophages and ASK cell line):
 - a) Take wells previously prepared and put inside the biosafety cabinet.
 - b) Remove media from cells carefully and add 1 mL of DPBS (room temperature) gently.
 - c) Remove DPBS and add 1 mL of 5% FBS culture media without antibiotics (macrophages culture media) or 1 mL of 20% FBS culture media without antibiotics (cell line culture media).
 - d) Storage the wells in the incubator until infection.

***All the procedure must be conducted inside the biosafety cabinet.**

ATTACH – INVASSION ASSAY

- 1) Wells infection
 - a) Use the -2 dilution (1:10 infection) or -3 dilution (1:1 infection) to start the assay. Usually will be necessary prepare 2 Eppendorf tubes for each plate (total volume: 1.8 mL).
 - b) Add in each well 100 uL of -2 or -3 bacteria inoculum.
 - c) Left inside the incubator for 1 hour at 15°C.
- 2) ATTACHMENT

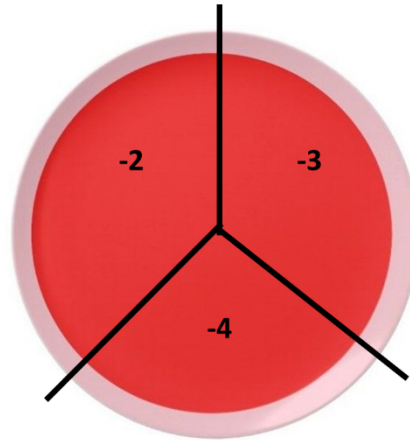


- a) Put the plate inside the biosafety cabinet.
- b) Remove media and wash gently with 1 mL of DPBS.
- c) Remove DPBS and add 400 uL of Triton 100X.
- d) Add 600 uL of DPBS and pipette up/down to remove cell from the bottom of the plate.
- e) Transfer to a label 1.7 Eppendorf tube (initial ATT inoculum).
- f) Make serial dilution from de initial ATT inoculum until -4 or until the necessary dilution (different number of dilutions for each bacteria or for each amount of bacteria).
- g) Wait until the drops are well absorbed in the plate, then, place the plates inside the incubator at 15°C.



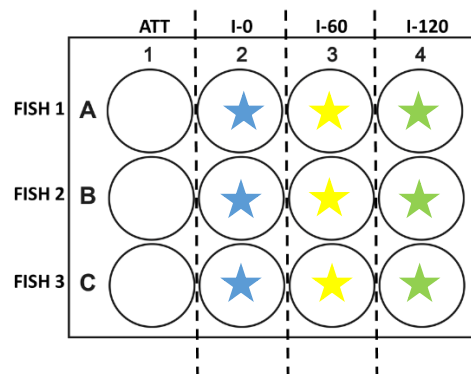
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- h) Wait until colony formation. Count colonies (between 20 and 300 per dilution it is recommended, discard data from dilutions with less than 20 colonies and more than 300 colonies) and then process the data.



***The procedure must be conducted inside the biosafety cabinet until the letter (e), then, from the letter (f) until letter (g) the procedure must be conducted using a Bunsen burner outside the biosafety cabinet to avoid contamination.**

3) INVASION

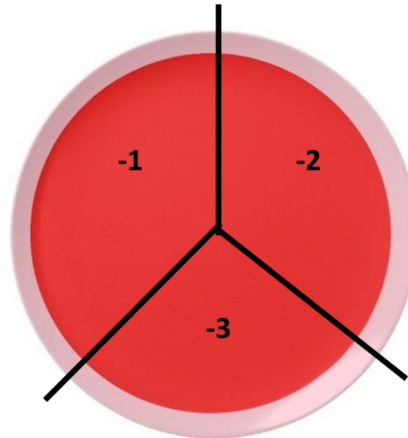


- Remove media of I-0 (blue), I-60 (yellow) and I-120 (green). Wash gently with 1 mL of DPBS.
- Add gently 1 mL of culture media without antibiotic.
- Add 2 uL of gentamicin in each plate and put inside the incubator for 1 hour at 15°C.
***At this point start the infection, be sure that you added correctly the gentamicin.**
***For invasion I-0**
- After 1 hour, remove media of I-0 (blue) only. Wash gently with 1 mL of DPBS.
- Remove DPBS and add 400 uL of Triton 100X.
- Add 600 uL of DPBS and pipette up/down to remove cell from the bottom of the plate.
- Transfer to a label 1.7 Eppendorf tube (initial I-0 inoculum).



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- h) Make serial dilution from de initial I-0 inoculum until -3 or until the necessary dilution (different number of dilutions for each bacteria or for each amount of bacteria).
- i) Wait until the drops are well absorbed in the plate, then, place the plates inside the incubator at 15°C.



***For invasion I-60**

- j) After 1 hour, remove media of I-60 (yellow) only. Wash gently with 1 mL of DPBS.
- k) Remove DPBS and add 400 uL of Triton 100X.
- l) Add 600 uL of DPBS and pipette up/down to remove cell from the bottom of the plate.
- m) Transfer to a label 1.7 Eppendorf tube (initial I-60 inoculum).
- n) Make serial dilution from de initial I-60 inoculum until -3 or until the necessary dilution (different number of dilutions for each bacteria or for each amount of bacteria).
- o) Wait until the drops are well absorbed in the plate, then, place the plates inside the incubator at 15°C.

***For invasion I-120**

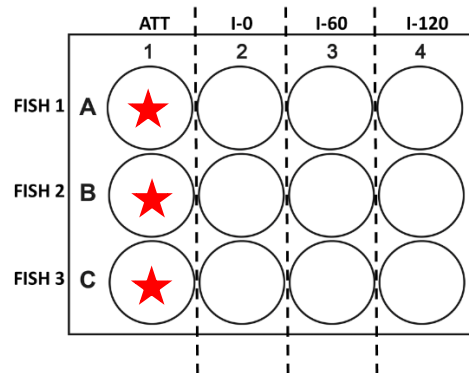
- p) After 1 hour, remove media of I-120 (green) only. Wash gently with 1 mL of DPBS.
- q) Remove DPBS and add 400 uL of Triton 100X.
- r) Add 600 uL of DPBS and pipette up/down to remove cell from the bottom of the plate.
- s) Transfer to a label 1.7 Eppendorf tube (initial I-120 inoculum).
- t) Make serial dilution from de initial I-120 inoculum until -3 or until the necessary dilution (different number of dilutions for each bacteria or for each amount of bacteria).
- u) Wait until the drops are well absorbed in the plate, then, place the plates inside the incubator at 15°C.

The procedure must be conducted inside the biosafety cabinet and then using a Bunsen burner outside the biosafety cabinet to avoid contamination (similar to the instruction described for attachment).



VIABILITY ASSAY

1) VIABILITY for attachment.



- Put the plate inside the biosafety cabinet.
- Remove media and wash gently with 1 mL of DPBS.
- Remove DPBS and add 500 μ L of Trypsin for 10 min.
- After 10 min., add 500 μ L of culture media without antibiotics and pipette carefully up/down to remove all the cell from the bottom of the plate.
- Transfer to a label 1.7 Eppendorf tube.
- Transfer 10 μ L to a new 1.7 Eppendorf tube and add 90 μ L of culture media without antibiotics.
- Take 10 μ L of the new dilution and mix in a new 1.7 Eppendorf tube with 10 μ L of trypan blue.
- Put 10 μ L in count cell plates and check cell viability.

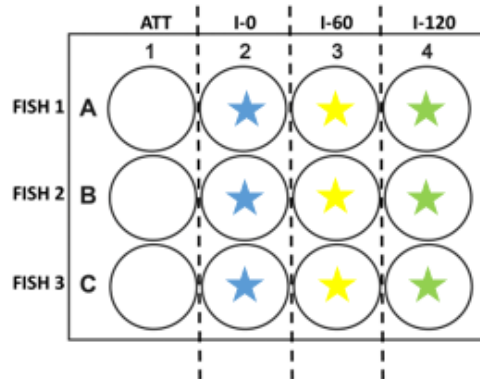
***If you have too much cells, make an extra dilution and count cells again. If you have a low number of cells, restart the process for that sample (from letter f).**

***All the procedure must be conducted inside the biosafety cabinet, except count cells.**





2) VIABILITY for invasion



- a) Remove media of I-0 (blue), I-60 (yellow) and I-120 (green). Wash gently with 1 mL of DPBS.
- b) Add gently 1 mL of culture media without antibiotic.
- c) Add 2 uL of gentamicin in each plate and put inside the incubator for 1 hour at 15°C.
***At this point start the infection, be sure that you added correctly the gentamicin.**
***For VIABILITY invasion I-0**
- d) Remove media and wash gently with 1 mL of DPBS.
- e) Remove DPBS and add 500 uL of Trypsin for 10 min.
- f) After 10 min., add 500 uL of culture media without antibiotics and pipette carefully up/down to remove all the cell from the bottom of the plate.
- g) Transfer to a label 1.7 Eppendorf tube.
- h) Transfer 10 uL to a new 1.7 Eppendorf tube and add 90 uL of culture media without antibiotics.
- i) Take 10 uL of the new dilution and mix in a new 1.7 Eppendorf tube with 10 uL of trypan blue.
- j) Put 10 uL in count cell plates and check cell viability.
***For VIABILITY invasion I-60**
- k) Remove media and wash gently with 1 mL of DPBS.
- l) Remove DPBS and add 500 uL of Trypsin for 10 min.
- m) After 10 min., add 500 uL of culture media without antibiotics and pipette carefully up/down to remove all the cell from the bottom of the plate.
- n) Transfer to a label 1.7 Eppendorf tube.
- o) Transfer 10 uL to a new 1.7 Eppendorf tube and add 90 uL of culture media without antibiotics.
- p) Take 10 uL of the new dilution and mix in a new 1.7 Eppendorf tube with 10 uL of trypan blue.
- q) Put 10 uL in count cell plates and check cell viability.
***For VIABILITY invasion I-120**
- r) Remove media and wash gently with 1 mL of DPBS.

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- s) Remove DPBS and add 500 uL of Trypsin for 10 min.
- t) After 10 min., add 500 uL of culture media without antibiotics and pipette carefully up/down to remove all the cell from the bottom of the plate.
- u) Transfer to a label 1.7 Eppendorf tube.
- v) Transfer 10 uL to a new 1.7 Eppendorf tube and add 90 uL of culture media without antibiotics.
- w) Take 10 uL of the new dilution and mix in a new 1.7 Eppendorf tube with 10 uL of trypan blue.
- x) Put 10 uL in count cell plates and check cell viability.

***If you have too much cells, make an extra dilution and count cells again. If you have a low number of cells, restart the process for that sample (from letter f).**

***All the procedure must be conducted inside the biosafety cabinet, except count cells.**