Pre-infection experiments for CagA translocation

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Abstract

This is a variation of the CagA translocation assay performed in the lab.

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Protocol

Grow AGS cells

Step 1.

Grow cells in a 6-well plate until a 90-100% confluency in RPMI 1640 complemented with 10% FBS heat inactivated (Complete Media, CM). They should be preferable 48 h old. The day before synchronize cells overnight in G_0 arrest by incubating cells in RPMI 1640 media without FBS.

REAGENTS

RPMI 1640 medium 21875109 by Gibco - Thermo Fischer

Fetal Bovine Serum 10270106 by Gibco - Thermo Fischer

Prepare AGS cells for infection

Step 2.

30 min before infection, remove media without FCS and add 1 ml per well of new Complete Media (CM). If you need to add inhibitors, this is the time point to do it.

Prepare pre-infecting strain for infection

Step 3.

- Resuspend the pre-infecting bacteria (Bacteria A) for T_0 in PBS, NOT IN BROTH, and measure the OD_{550}. Try to resuspend bacteria shortly before infection, do not keep them in PBS too long.
- Calculate the amount necessary for the desired MOI. The standard MOI is 60 (OD_{550} 0,2/well). To calculate consider that an OD_{550} of 0,1/ml is equivalent to approx. $3x10^7$ cfu/ml.

NOTES

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Pre-infection

Step 4.

Add bacteria A to the cells and incubate at 37°C, 5% CO_2 for 1 hour.

Infection with CagA translocating strain

Step 5.

- Prepare the suspension of the translocating bacteria (Bacteria B) around 15 min before the 60 min of the pre-infection is due.
- After 60 min, add Bacteria B at an MOI of 60. Infect for 2 to 3 additional hours at 37°C, 5% CO_2.

Start the harvest

Step 6.

- Stop the infection by placing the plate on ice. For the rest of the procedure, maintain the samples cold to minimize protease activity.

Collect samples for cytokine measurements

Step 7.

Collect the media for cytokine measurements or remove the media with help of a vacuum pump. Add 1 ml PBS* (PBS (Ca-, Mg-) with 1 mM Sodium orthovanadate, 1mM PMSF, 1 µM Leupeptin, 1µM Pepstatin). Prevent the well's surface from drying out (crystal formation damages the cells).

REAGENTS

Sodium Orthovanadate S6508-10G by Sigma Aldrich

PMSF P7626 by Sigma Aldrich

Leupeptin View by Sigma Aldrich

Pepstatin A P5318 by Sigma Aldrich

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concentrations!!!

PBS, **1mM** PMSF, 1µM Leupeptin, 1µl Pepstatin, **1mM** Ortho-Vanadat

Collect cells

Step 8.

- With a cell scrapper detach the cells from the bottom of the plate. Prevent too much scrapping since this will damage the cells more than desired.
- Collect the cell suspension in a 1,5 ml tubes. Centrifuge the cells 500 g for 10 minutes at 4°C in a swing rotor.

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... damage the cells more **than** desired

Prepare samples for western blot analysis

Step 9.

- Discard ALL the supernatant. Resuspend the pellet in 20 µl of your favorite Lysis buffer containing protease inhibitors.
- Add immediately 25µl of 2X SDS loading buffer and boil the probes at 95°C for 10 min. To avoid condensation and stickiness of DNA, **place the tubes immediately in ice**. **Do not centrifuge!.**
- Store at -20°C until samples can be analyzed with western blot