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CagA phosphorylation assay and its semiquantitative analysis V.1

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Anna F. Zeitler[1], Luisa F Jimenez-Soto[2]

[1]Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Ludwig-Maximilians-Universität, Pettenkoferstraße 9a, D-80336 Munich, Germany;
[2]Walther Straub Institute for Pharmacology and Toxicology

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Luisa F Jimenez-Soto
Walther Straub Institute for Pharmacology and Toxicology

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We use this protocol and it is working.

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Disclaimer

These protocol is the result of transfer of information across several generation of PhD students under the guidance of Prof. Rainer Haas and PD. Dr. Wolfgang Fischer. The steps here are the authors' finished polished product of protocols already existent in the laboratory.

Abstract

CagA is the oncogenic toxic of *Helicobacter pylori*. The toxin is injected into host cells and tyrosine phosphorylated in the C-terminal EPIYA motifs by host kinases. Positive translocation of the CagA toxin into the cell is evaluated by detecting the phosphorylated form in the cell lysate using antibodies detecting phosphotyrosines. Previously detection was limited to a positive o negative results, but with new advances in semiquantitative western blots we are able to quantify changes on CagA translocation / Phosphorylation and get a better idea of the dynamics of injection.
The protocol described here has been used for evaluation of CagA phosphorylation and its semiquantitative analysis in the following publications:
DOI:**10.1111/cmi.12166**; DOI:**10.1038/srep23227**, and DOI:**10.1016/j.mimet.2016.04.016**

Guidelines

Make sure you follow the biosafety protocols and recommendations of your laboratory.

Materials

OXOID GC AGAR BASE (CM0367GC) Media was used. Its preparation follows the indications of the manufacturer. For the preparation of the vitamin mix stock solution (100X), contact directly Prof. Rainer Haas or PD. Dr. Wolfgang Fischer at the Max-von-Pettenkofer Institute in Munich, Germany.

Troubleshooting

Safety warnings

Components like Methanol and Acrylamide are know to cause damage to the nervous system. Work with them under the chemical hood to protect you from inhaling their vapors.

Ethics statement

These experiments do not need or include the use of animals or human material.

Before start

Make sure you know how to use all equipment in the lab, and read the safety warning of all chemicals used before you use them.

Prepare H. pylori bacteria for infection

1 *H. pylori* has a amazing capacity to change during growth. In order to keep the experiments reproducible and comparable, use relative fresh bacteria for all infection experiments.
On Friday afternoon, take from frozen stocks the bacteria by scratching the surface of the ice with the inoculation loop. A piece of "ice" will adhere to the loop. Do not distribute the material on the plate. Just place it on the agar and let *H. pylori* grow on the small drop of defrosted media, undisturbed. Place the agar plates to grow in an incubator with microaerophil conditions (10% CO_2, 5% O_2 and 75% N_2) at 37°C for the weekend.
On Monday, passage the bacteria to a new GC Cholesterol plate

Citation

Jiménez-Soto LF, Rohrer S, Jain U, Ertl C, Sewald X, Haas R (2012)
. Effects of cholesterol on Helicobacter pylori growth and virulence properties in vitro..

https://doi.org/10.1111/j.1523-5378.2011.00926.x LINK

, and pass them onto a new plate on Tuesday for Wednesday's work, where bacteria are fit enough to be used for infection.

Note

A "plate" corresponds to a Petri dish of 10 cm diameter with ca. 20 cm of agar growth media per plate.

Note

Optional: You can grow *H. pylori* in a 10% CO_2 incubator at 37°C. They will just take a bit longer to grow as they do in microaerophil enviroment.

Seed AGS cells 48 hours before infection will be performed.

2 The objective is to have a cellular confluency in the 6-well of 80-90% at the moment of infection. For you to achieve this, you have to know your cells and their multiplication speed. For AGS cells the usual splitting for 48 hours with synchronization (see below) was 1:8 (from a 90% - 100% confluent culture)

Synchronize the AGS cells 16h before infection

3 Remove serum-containing media (RPMI + 10% Inactivated FCS, Complete Media (CM)) and change it for media without serum (RPMI without serum) around 16 hours before infection, in order to synchronize cells in G_0.

Note

For each cell, a different method of synchronization might be needed. For example, I have gotten better results with HEK293 cells by using DMEM + Serum but without glutamine. Immune cells reacts very strong to lack of serum in their growth media. It can even cause changes in differentiation status.

Resuspend bacteria

4 For 1/4 of plate grown bacteria, collect all bacteria using a sterile cotton swab and introduce it in a tube containing at least 500 µl of PBS. Using circular movements, remove the bacteria culture attached to the cotton fibers using the PBS solution. The objective is to have as much bacteria in the solution, and reduce the amount of liquid attached to the cotton swab before discarding it.

Measure the optical density (OD) of the bacterial suspension at 550 nm

5 Make a 1:100 dilution of bacteria suspension in PBS (10µl bacterial suspension in 990µl PBS) and measure its optical density (OD) in a densitometer at 550 nm.

Calculate the amount of *H. pylori* colony forming units (CFU) per ml of suspension

6 Multiply the dilution factor to obtain the OD_{550} / ml of suspension.

Calculate the amount of bacteria in your suspension taking into consideration that and OD_{550} of 0,1 is equivalent to aprox. 3×10^7 CFU/ml.

Note

The value of CFU/ml in a OD550 of 0,1 tends to vary depending of the wild type strains or mutations. Some variations are negligible. Nonetheless, make sure your CFU/ml is similar to the value given here.

Infect your cells with an Multiplicity of Infection (MOI) of 60.

7 Multiplicity of Infection (MOI) of 60 means that you will add 60 bacteria for each cell in the well. For general calculations, each well of a 6-well cell culture plate has approx.

1×10^6 adherent cells at a confluency of 90% to 100%

Prepare the AGS cells 30 min before infection

8 Remove the serum-free media from the cells and add 1 ml of complete media (CM) per well around 30 min before infection

Infect the cells

9 Infect cells and incubate at 5% CO_2 37°C for 3 to 4 hours.

Note

The minimum time I have been able to infect synchronized cells and detect CagA phosphorylation in Western blot is 1 hour at MOI 60.

Stop infection for harvestinfection

10 Stop the infection by placing the plate on ice. For the rest of the procedure, maintain the samples cold.

Note

Since *H. pylori* is a Biosafetly Level 2 bacteria, make sure you can keep the surface of the ice free of contamination before disposing of the ice.

Collect supernatant for cytokine measurements

11 Collect the media for cytokine measurements. If no cytokine measurements are necessary, remove the media with help of a vacuum pump. Add 1 ml PBS* (PBS with protease and phosphatase inhibitors). Prevent the well's surface from drying out (crystal formation damages the cells).

Note

PBS*: PBS with Proteinase Inhibitors (1 mM PMSF, 1µM Leupeptin, 1µM Pepstatin, 1 mM Sodium Vanadat), fresh made

Detach cells

12 With a cell scrapper detach the cells from the bottom of the plate.

Note

Prevent too much scrapping since this will damage the cells more that desired. It helps to go methodically over the surface of the well. If you look carefully the reflection of the light will show you where cells have been already removed from the surface.

Collect cells

13 Collect the cells suspension in 1,5 ml tubes. Centrifuge the cells 500 g for 10 minutes at 4°C in a swing rotor centrifuge.

Prepare samples for Western blot

14
- Discard ALL the supernatant taking care of not loosing the pellet.
- 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 samples at 95°C for 10 min. To avoid condensation and stickiness of DNA, **place the tubes immediately in ice**. **Do not centrifuge!**

Note

If you have problems pipeting the samples on the SDS-Acrylamine gels (or similar) because of clumping of the samples, the placement of the tubes on ice after boiling was not fast enough.

Separation of proteins

15
- For separation of proteins, we use the protocol **Single Gel system based in Ahn T et al (2001)**

Transfer of proteins to PVDF membrane

16
- Activate gel for detection of proteins using the stainfree system for quantification of proteins present in the sample. These values allow us to do the normalization of the phosphorylation signals after immunodetection.
- We use the Bio-Rad **Gel Doc™ XR+ Gel Documentation System** for the stainfree detection.

- For transfer of proteins to the PVDF membrane, we use the protocol **Trans-Blot® Turbo™ Transfer with Home-made buffers**

Activate PVDF membranes

17 Place the membranes in contact with methanol to activate them.

Note

If you use nitrocellulose membranes, do not use methanol. It will destroy them.

Safety information

Methanol is toxic. Use your Personal Protective Equipment when handling methanol.

Western blot - Block the membranes

18 Block membrane with TBS-T (TBS + 0,075% Tween 20) with 3%BSA for 1 hour at room temperature.

Western blot - Primary antibody

19 Add the anti-phosphotyrosine antibody in a dilution 1:10000 dilution (Clone 4G10 antibody from Millipore), for 1 hour in PBS with 3% BSA at room temperature.

Western blot - Remove excess primary antibody

20 Wash 4 times, 15 minutes each, with 5 ml TBS-Tween (TBS + 0,075% Tween 20) at room temperature.

Western Blot- Secondary antibody

21 Add 1:10000 anti-mouse peroxidase conjugated antibody (Sigma) for 45 minutes in TBS 0,075% Tween 20 .

Western blot- Removal of excess secondary antibody

22 Wash 4 times 15 minutes with 5 ml TBS 0,075% Tween 20 at room temperature.

Western bot - Detection

23
- Remove ALL WASH BUFFER . Take care to not to let the membrane dry in this step!!!
- Add Millipore Immobilon Western (Cat Nr. WBKLS0500) developing solution (1 ml solution A, 1 ml solution B).
- Let the membrane roll with the developing solution for about 1 to 2 min
- Place membrane between to two transparencies.
- Place membrane in Gel documentation machine (Gel Doc™ XR+ Gel Documentation System) and detect the chemoluminiscence

Quantify signals relative to control infection

24 Using the software ImageLab from Bio-Rad, set lanes and bands. Quantify signals using relative values to the control infection.

Citation

Anna F. Zeitler, Luisa F. Jiménez Soto. Semi-quantitative analysis of western blot signals. protocols.io.

https://protocols.io/view/semi-quantitative-analysis-of-western-blot-signals-hjzb4p6
LINK

Normalize values

25 Using the same image and the image of the Stainfree gel, use the normalization option from the ImageLab software. Multiply the values obtained for the phosphorylation signal with the values obtained for the normalization.