MARCO TRANSFECTION AND LUCIFERASE ASSAY

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BACKGROUND

- The ***luciferase reporter assay*** is a molecular biological technique with many downstream applications including: characterization of putative regulatory sequences, transcription factors, and signalling pathway components. The ***protocol of this assay*** involves co-transfecting cells with a luciferase reporter plasmid downstream of the studied gene's (i.e. NF-κB) response elements, and plasmids encoding the desired immune receptors (i.e. MARCO/II, TLR2/4, MD2, and CD14). To account for variable transfection efficiency between samples, cells are also transfected with a constitutively expressed reporter plasmid such as β-galactosidase (β-gal). Similar to luciferase, β-gal emits light upon reaction with its substrate. Luciferase activity divided by β-gal activity gives a normalized value in relative light units. This value is representative of NF- κB activation following infection with heat-killed *S. pneumoniae.* The ***purpose of this assay*** is to facilitate the measurement of NF-κB activation since luciferase expression is easily measured and under the control of NF-κB.

NOTES

1. Plan for at least **5 consecutive days** of lab work. Day 1 to split cells/seed 6-well plates, Day 2 to transfect, Day 3 to infect, Day 4 to incubate, Day 5 to collect lysates/assay activity.
2. While the protocol calls for approximately 1 x 10^6 cells, transfected at 50-80% confluency, I find cells are overgrown by the time lysates are collected. I suggest seeding 0.5 x 10^6 cells, and transfecting at closer to 50% confluency.
3. It is best to do transfections in duplicate/triplicate as greater volumes are pipetted, thereby reducing pipetting error (ex. 100 ul→ 300 ul of MegaCell, 3 µl GeneJuice → 9 ul GeneJuice, scale up DNA volumes in a similar fashion).
4. Changing the serum-free media to complete media **5 hours** post-transfection is optimal.
5. For the first assay infect with a range of MOI (1, 5, 10, 50) to determine optimal MOI (you want a robust response without saturating the signal). I've found **25** to be best.
6. It is optimal to infect cells in 3 mL of media containing **1% FCS** complete media
7. For the control reporter gene (β-gal), apply the substrate and **wait 30 minutes** before measuring. However, activity decreases 60 minutes after application.
8. Make sure you calculate how much luciferase substrate you will need prior to measuring, **add a bit extra for priming the injector** (~ 0.5 mL).
9. When recording β-gal activity, make sure nothing is being injected with injector M (as residual luciferase substrate will contaminate the reading)

MATERIALS & EQUIPMENT

- Complete DMEM media
- Mega-Cell DMEM media (Sigma-Aldrich, Product# M3942)
- GeneJuice Transfection Reagent (EMD4Biosceinces/EMD Chemicals USA for Novagen, Cat# 70967)
- Luminescent B-galactosidase Detection Kit II (Clontech laboratories, Inc., Cat# 631712)
- Luciferase assay kit (Agilent technologies manufactured for Stratagene by Promega, Cat# 219020)
- Optiplate-96 (PerkinElmer Product# 6005290)
- Luminometer is needed to record signal of reporter genes

PROTOCOL

Part I: Transfection with Plasmids

1. Approximately 24 hours prior to transfection seed ~1 x 10^6 HEK293T cells per well of a 6-well tissue culture plate, with 3 mL of complete DMEM media (1% Penicillin/streptomycin (P/S), 1% L-glutamine (L-Glu), 10% Heat-inactivated Fetal Bovine Serum (HI-FBS)) (**see NOTE 1**).

2. The next day, observe confluency. If cells are ~50-80% confluent, then they are viable for transfection (**see NOTE 2**).

3. Place Mega-Cell serum-free media in 37°C water bath 30 minutes prior to transfection.

4. Transfection for 6-well plate using GeneJuice Transfection Reagent requires 1 µg of DNA. Calculate the volume of purified plasmid DNA needed according to its concentration, and the amount recommended by Dr. Cynthia Leifer: TLR2 or TLR4 (30 ng), CD14 (30 ng), NF-kB (100 ng), B-gal (100 ng), MARCO or MARCO II (300 ng). To total 1 µg, add empty vector (pcDNA3 or pBABE).

5. To 100 µl of Mega-Cell add 3 µl GeneJuice and vortex (**See NOTE 3**). Incubate mix for 5 minutes at room temperature.

6. Add calculated volumes of DNA to total 1 µg of the desired plasmids. Incubate for 15 minutes at room temperature.

7. While waiting, take 6-well plate out of the incubator and replace media with 3 ml of Mega-Cell.

8. Add 100 ul of DNA-GeneJuice mixture drop-wise to the desired well; rock the dish (don't swirl). Wait 4-8 hours (**see NOTE 4**) before replacing the media with complete DMEM media. Place cells back in the incubator and wait 24 hours before proceeding to part IIa.

Part IIA: Infection with Heat-Killed or *S. pneumoniae*

1. Heat-killed bacteria must be prepared in advance following the appropriate protocol (**SEE** Growing up *S. pneumoniae* protocol).

2. Calculate the volume of *S. pneumoniae* needed for the desired multiplicity of infection (MOI) (volume= cell # * MOI/ stock concentration) (**See NOTE 5**)

3. Change media of cells with fresh complete media (**see NOTE 6**) and add in required volume of *S. pneumoniae*. Leave cells for 48 Hours in the 37°C incubator.

Part IIB: Infection with LIVE *S. pneumoniae*

1. With live *S. pneumoniae* you must grow it the day of use (stop at an OD between 0.45 and 0.55).
2. Calculate the volume of *S. pneumoniae* needed for an MOI of 25 based on estimated stock concentration of 1-4 x 10^{-7} cfu/ul
3. Apply volume for 4-6 hours and then add gentamicin to prevent them from out-growing the HEK cells (~10ug/ul per well)
4. Change media of cells

Part III: Collecting Cell Lysates (Stratagene Kit protocol)

1. Remove the media from each well, wash well twice with 1X PBS (be careful not to dislodge your cells)
2. Remove PBS from wells.
3. Make 1X cell lysis buffer by adding 4 mL of dH2O per milliliter of 5X cell lysis buffer.
4. Cover cells by adding 200-500 ul of 1X cell lysis buffer.
5. Incubate the plate at room temperature for 15 minutes, swirl occasionally
6. Scrape the cells and buffer from each well into microcentrifuge tubes. Place tubes on ice.
7. Vortex the tubes for 10-15 seconds, spin tubes down in a microcentrifuge at 12, 000 x g for 15 seconds at room temperature
8. Transfer supernatant to microcentrifuge tube
9. Immediately assay or store at -20°C (for use within 1 month) or -80°C (for long-term storage). Note samples may lose activity with every freeze-thaw cycle.

Part IV: Making your Luciferase Substrate and β-gal Substrate

1. Prepare the **luciferase substrate-assay buffer** by adding the entire assay buffer (10 mL) to the vial containing the lyophilized luciferase substrate, and mix well.
2. Divide the luciferase substrate-assay buffer into aliquots (~ 1mL per tube, 10 tubes) to avoid multiple freeze-thaw cycles (store at -80°C, if using for longer than 1 month).
3. **Clontech β-gal reaction substrate:** warm reaction buffer and reaction substrate to room temperature. Prepare the master Reaction Buffer Mixture by adding 4 ul of reaction substrate to 196 ul of reaction buffer for each sample. For the purpose of my experiment I used 2.45 mL of the BIG bottle (reaction buffer); and 50 ul of small tube (reaction substrate). It depends on how many samples are being tested. Keep in mind 45-50 ul of substrate is added to each well of a 96-well plate.
4. Allow the substrate-assay buffer to reach room temperature

Part V: Loading Samples/Substrate and Assaying Activity

1. Using an **OPAQUE 96-well plate** (white plate) transfer 20 ul per well of supernatant to test Luciferase Activity, do this in triplicate. (Ex. For supernatant #1 you will have 3 wells on the 96-well plate containing 20 ul of its supernatant).

2. Transfer 10 ul per well of supernatant to test for β-galactosidase activity; do this in triplicate as explained above. (In total there should be 6 wells for each supernatant collected- 3 of 20 ul for testing luciferase, and 3 of 10 ul for testing β-gal)

3. Add 50 ul of the β-gal reaction mixture to EACH well containing 10 ul of supernatant.

4. Cover the plate with foil (to shield it from light) and **wait 30 minutes** prior to reading it (**see NOTE 7**)

5. In the meantime, transfer enough luciferase substrate to a 10-15 mL tube (**see NOTE 8**). You will be adding 50 ul to each well. This will be applied to the samples by an injector found on the luminometer.

6. Measure Luciferase Activity first, then set the injector to no injection (follow luminometer instructions) and measure B-gal (**see NOTE** 9).

ANALYSIS/SOFTWARE

- WinGlow software (or any other applicable software) used in conjunction with a Luminometer for reading plates
- Microsoft Excel to plot results