NF-κB LUCIFERASE ASSAY

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BACKGROUND

- The **luciferase reporter assay** is a technique in molecular biology with a diverse range of applications, including the characterization of signaling pathways. In the Bowdish Lab, we use the luciferase assay to measure NF-κB levels in *in vitro* models of infection and inflammation.
- Luciferase is a family of enzymes produced naturally by a number of species, most notably those in the genus Lampyridae (firefly). These enzymes catalyze the oxidation of small, heterocyclic substrates called **luciferins**, resulting in the conversion of luciferin to an excited electronic state. When luciferin reverts back to ground state, luminescence results. This luminescence can then be detected by a luminometer.
- Molecular biologists take advantage of the luminescent property of luciferase by placing its gene under the control of promoters for a gene of interest. Subsequently, the transcriptional activation of this gene of interest can be monitored by luminescence.

NOTES

- This protocol is written for a procedure wherein HEK 293T cells are transiently transfected and then infected with *Streptococcus pneumoniae*. As such, it will be primarily of interest to Bowdish Lab members. **External users should modify this protocol where necessary.**
- **Plan for 5 consecutive days of lab work.** Day 1 to seed plates, Day 2 to transfect, Day 3 to infect and Day 5 to collect lysates and assay for luciferase activity.

EQUIPMENT

- Equipment:
    - Plate reader **with an injection system** capable of reading luminescence
    - **Opaque**, flat-bottom 96-well plates
    - Multi-channel pipette capable of operating in the 10-50µl range (optional, but would make your life much easier)
- Materials:
    - PEI transfection reagent
    - Luminescent β-galactosidase Detection Kit II (Clontech Laboratories, Cat# 631712)
    - Luciferase Assay Kit (Agilent Technologies, Cat# 219020)
    - DMEM (all of: complete, serum-free, 1% FBS)

PROTOCOL

Part I: Seeding Cells

- Preparatory Work:
    - Warm complete DMEM media and PBS to 37°C in water bath.
    - Have ready a source of growing HEK 293T cells from which to seed.
    - Prepare a BSL2 Biosafety Cabinet for work.

1. Remove media from growing HEK 293T cells.
2. Wash 1× with PBS.
3. Mechanically suspend cells in 20ml fresh media by washing bottom of flask repeatedly.
4. Assay cell count and viability. Typically, this is done through a Trypan blue stain.
5. Seed 6-well plates at a cell density of 3×10^5 cells per well in 3ml media.
    a. **Note: Do not plate at a density lower than 3×10^5 cells, or they will not grow!**
6. Incubate overnight at 37°C. Cells should be 50-80% confluent after 24h.

- Concluding Work:
    - Clean up BSL2 Biosafety Cabinet. Bleach and dispose of liquid waste.

Part II: Transfection

- Preparatory Work:
    - Warm **serum-free** DMEM media and PBS to 37°C in water bath.
    - Calculate the volumes of DNA needed for transfection. PEI transfection is optimal with a total of 2µg DNA. **Use extra to account for pipetting errors!**
    - Prepare a BSL2 Biosafety Cabinet for work.

1. Add DNA to incomplete DMEM, using 100µl media per well to transfect (**and extra to account for pipetting error**).
2. Add 12µl 1ng/ml PEI per 100µl media. Vortex 15s. Incubate 10min at room temperature.
3. Add a further 600µl media per 100µl media.
4. Add 700µl media with DNA and PEI **evenly** and **drop-wise** per well.
5. Incubate 3h at 37°C.
6. Add 2.7ml complete DMEM media.
7. Incubate 24h at 37°C.

- Concluding Work:
    - Clean up BSL2 Biosafety Cabinet. Bleach and dispose of liquid waste.

Part III: Infection

- Preparatory Work:
    - Ensure that you have a heat-killed, lysozyme digested *S. pneumoniae* strain 1121 stock ready. **The concentration that our stocks are made at is 5×10^8cells/ml**.
    - Warm **1% FBS** DMEM media to 37°C in water bath.
    - Calculate the volume of *S. pneumoniae* stock to use. **The optimal MOI is 25.**
    - Prepare a BSL2 Biosafety Cabinet for work.

1. Add appropriate volume of *S. pneumoniae* stock to 1% FBS DMEM (3ml media per well).
2. Remove media from transfected HEK 293T cells.
3. Wash 1× with PBS, 1ml per well.

4. Add 3ml of media with *S. pneumoniae*.
5. Incubate 48h at 37°C.

- Concluding Work:
    - Clean up BSL2 Biosafety Cabinet. Bleach and dispose of liquid waste.

Part IV: Collect Cell Lysates

- Preparatory Work:
    - Prepare 1× lysis buffer from MilliQ H_2O 5× lysis buffer supplied by the Luciferase Assay kit.
    - Prepare a bucket of ice. Bring with you to BSL2 Safety Cabinet.
    - Prepare BSL2 Biosafety Cabinet for work.

1. Remove media from HEK 293T cells.
2. Wash 2× with PBS, 1ml per well.
3. Cover cells with 300µl 1× lysis buffer.
4. Incubate 15min at room temperature. Swirl every ~3min.
5. Scrape cells into suspension in lysis buffer. Transfer to a 1.5ml Eppendorf tube on ice.
6. Vortex tubes 15s.
7. Centrifuge 2min at 12,000×g and 4°C.
8. Transfer supernatant to fresh 1.5ml Eppendorf tube on ice. Dispose of pellet.
9. Assay immediately (**Part V**) or store at -80°C.

- Concluding Work:
    - Clean BSL2 Biosafety Cabinet. Bleach and dispose of liquid waste.

Part V: Measuring Luminescence

- Preparatory Work:
    - Prepare luciferase substrate-assay by adding the entire assay buffer into the vial containing the lyophilized luciferase substrate (provided by Luciferase Assay kit). Mix well and divide into 1ml aliquots. Store at -80°C. **You should make at least 1.3ml more than necessary to account for dead volume in the plate reader injection system.**
    - Prepare β-galactosidase reaction substrate by warming buffer and substrate to room temperature and adding 1µl of substrate per 49µl buffer.
    - **Book the plate reader with injection system well beforehand!**

1. Using an opaque 96-well plate, transfer 20µl cell lysate per well in triplicate to test for luciferase activity.
2. Transfer 10µl cell lysate per well in triplicate to test for β-galactosidase activity. **This is separate from the lysates for luciferase activity.**
3. Add 50µl of β-galactosidase reaction mixture to each well used to test for β-galactosidase activity.
4. Incubate plate 30min at room temperature covered with aluminium foil.
5. Meanwhile, transfer enough luciferase reaction mixture (you will be using 50µl per well) to a 15ml Falcon tube (**adding enough to account for injector dead volume**). Cover with aluminium foil.
6. Measure luminescence of wells on a plate reader. For luciferase measurements, set injector to inject 50µl reaction mixture **immediately** prior to luminescence measurement.

- Concluding Work:
    - **Note: β-galactosidase is used as a transfection control and luciferase luminescence measurements are normalized to β-galactosidase luminescence measurements.**