TLR4 BIOASSAY FOR THE DETECTION OF LPS IN BIOLOGICAL SAMPLES

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BACKGROUND

This assay is used for the detection of lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, which acts as an endotoxin and elicits strong immune responses in animals. This assay uses Human Embryonic Kidney 293 cells, which stably express receptors TLR4-MD2-CD14, which are involved in the NFkB-associated inflammatory response due to LPS binding. The SEAP-NFkB reporter plasmid allows for the detection of LPS levels via spectrophotometric analysis at 650nm.

Upon recognition of a PAMP, receptor signaling (i.e. TLR, Nod1/2 receptor) leads to the downstream activation and translocation of NFκB, a transcription factor that induces a pro-inflammatory response. In these cells, activation of NFκB leads to the expression of SEAP via the ELAM proximal promoter, which can be detected using HEK Blue Detection Media (Invivogen).

CULTURING:

☐This cell line grows at a slower rate than regular HEK293Ts. Split cells when approximately 80-90% in confluency

☐ Keep cells in 20mL of Complete DMEM (10% FBS, 5mL Pen-Strep, 5mL L-glutamine) with 100ug/mL of Normocin added. Cells do not grow well in the absence of Normocin

☐ Work in a biosafety hood in a tissue culture room. Ensure sterility by spraying everything with 70% ethanol

Splitting Procedure:

1. Discard medium by pipetting from flask into waste container

2. Add 10mL of Complete DMEM (37°C, containing antibiotics) and use pipette to lift cells off flask wall.

Note: HEK293s are loosely adherent, trypsin can be used if there is difficulty lifting

3. Aliquot 19mL of medium to a 600mL flask. Add 1mL of cell suspension to each flask. Mix gently.

4. Label flask(s) with cell line, passage number, date, and name. Place in 37°C incubator.

PERFORMING THE ASSAY: EQUIPMENT & MATERIALS:

- Level 2 Bio Hood, Tissue Culture Room

- 37°C, 5% CO2 Incubator, Tissue Culture Room

- Vortex, Tissue Culture Room

- Serum Free Media heated to 37°C in water bath (Incomplete Media or OptiMEM Media)

- 1% FBS Complete DMEM - HEK Blue Detection Media (Invivogen), heated 37°C in water bath - PEI working solution thawed on ice (1mg/1mL) - DNA of interest & empty vector, if transfecting other plasmids - 96-well plate(s) with seeded cells (see below)

DAY 1: SEEDING

Seed $4x10^3$ HEK293/TLR4-MD2-CD14 cells per well in a 96-plate in 200uL of media. The number of

plates will depend on the number of wells you need, given that all samples will be in duplicates.

DAY 2: TRANSFECTION

Necessary only if cells are not already stably transfected with the SEAP reporter plasmid

(for 3x96-well plates, adjust volumes if different number of plates)

1. Add the following in a 50mL conical tube: 10.5ug of empty pcDNA vector (optional, improves transfection efficiency) 6.5ug of SEAP-NFkB reporter plasmid 1800uL of Incomplete Media + 216ul PEI (last)

2. As soon as PEI is added, immediately pulse vortex transfection mixture for 15 seconds. 3. Incubate at room temperature for at least 10 minutes. 4. Add 18mL Incomplete Media to transfection mixture and gently mix by inverting tube. 5. Discard old media from previously seeded cells.

6. Using a multichannel pipette, add ~56uL of solution to the 96-well plates. There will be extra solution left over.

7. Incubate the plate(s) in the 37°C, CO2 Incubator for 2-3 hours. 8. After incubation, top off wells with 1% FBS Complete DMEM. DAY 3: Addition of Samples (Sera, Plasma, Proteins)

Note: For each plate, you may create a standard curve if you wish to know the concentration of LPS in your samples. If you just want to know whether your samples are contaminated with LPS, you may just use positive and negative controls, positive being 10ng/mL of LPS & negative, the HEK Blue Detection media alone.

Sample Preparation:

1. Dilute samples 1:5 in PBS, then 1:1 in sterile water (autoclaved MilliQ water works). The dilution will be1:10 overall.
2. Heat-inactivate samples at 75°C for 5 min.
3. Add 10uL of diluted sample & 190uL of HEK Blue Detection media into appropriate labeled tube, per well. *Each 10ul is 1ul of your sample. If you're doing duplicates, add 20uL sample + 380uL HBD media per tube.*
4. Once samples are prepared, discard ALL old media from 96-well plates, and then add 200ul of your different sample solutions. Incubate the plate(s) in the 37°C, CO2 Incubator for 24 hours.

DAY 3: Reading the Plates

After 24 hours, use a plate reader to read your plates at absorbance of 650nm. You may set your negative control as your blank to ease analyzing the data. Samples with readings close to and below 0 will be designated as not LPS contaminated.

References:

Circulating Muramyl Dipeptide Is Negatively Associated with Interleukin-10 in the Frail Elderly. Verschoor CP1, Naidoo A, Wallace JG, Johnstone J, Loeb M, Bramson JL, Bowdish DM. Inflammation. 2014 Oct 3. [Epub ahead of print]

http://www.bowdish.ca/lab/wp-content/uploads/2015/01/VerschoorMDP-IL10.pdf