



STIMULATION & CULTURING OF HEK293Ts STABLY EXPRESSING mNOD2 & SEAP-NFκB REPORTER

Created by: Aveen Naidoo

Date: June 2013

Updated by: Grace Teskey

Date: October 2017

Bowdish Lab, McMaster University
Hamilton, ON, Canada
www.bowdish.ca

BACKGROUND:

This cell line is originally derived from human embryonic kidney (HEK293) cells grown in tissue culture. The HEK293T variants contain the SV40 Large T-antigen, which allows for episomal replication of transfected plasmids containing the SV40 origin of replication. In comparison to HEK293s, HEK293Ts are more easily transfectable. This cell line has been stably transfected to express mNod2, an intracellular PRR that detects bacterial peptidoglycan-derived muramyl dipeptide (MDP), and the SEAP-NFκB Reporter. SEAP is the secreted form of human embryonic alkaline phosphatase. Upon recognition of a PAMP, Nod signaling leads to the downstream activation of NFκB, a transcription factor that induces a pro-inflammatory response. In these cells, activation of NFκB leads to the expression of SEAP, 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) and under constant antibiotic selection 50uL of 10mg/mL Zeocin & 100uL of 1mg/mL Blasticidin

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: HEK293Ts 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.

Detection of MDP in Mouse Serum

EQUIPMENT & MATERIALS:

- Level 2 Bio Hood, Tissue Culture Room
- 37°C, 5% CO₂ Incubator, Tissue Culture Room
- Vortex, Tissue Culture Room
- Heating block
- Spectramax Plate Reader

Note: any plate reader will do that can read absorbance at 650nm

- Complete DMEM (10% FBS, 1% L-glut & Pen-Strep) - HEK293T line stably expressing mNod2 & SEAP-NFκB (P<10) - HEK Blue Detection Media (Invivogen), heated 37°C in water bath - Mouse serum (*minimize freeze-thaw cycles*) - Phosphate-Buffered Saline - Endotoxin-free H₂O (autoclaved MilliQ water works as well)

PROTOCOL:

DAY 1: CELL PREPARATION

1. Seed 4000 cells/well in a 96-well format in a volume of 200uL in Complete DMEM media

Seeding needs to be done ~ 24 hours before beginning the assay to give cells enough time to adhere.

DAY 2: SAMPLE PREPARATION & STIMULATION

1. Dilute serum samples 1:5 in PBS, then 1:1 in sterile water (autoclaved MilliQ water

works). *The dilution will be 1:10 overall. For this assay, I aim for 1uL of serum per well and perform the assay in triplicate.*

2. Heat-inactivate samples at 75°C for 5 min.

The purpose of the previous dilution was to allow for proper heat inactivation without impairing the integrity of your samples. I have tried MANY different heat inactivation temperatures, dilutions, and proteinase-K/ β -mercaptoethanol treatments. This method was found to work best. If you do not dilute your samples, the serum can adopt a gel-like consistency. If you do not HI your samples, clumping will occur in wells.

3. Add 10uL of diluted sample & 190uL of HEK Blue Detection media (per well) into appropriately labeled tube. *Each 10ul is 1ul of your sample. If you're doing duplicates, add 20uL sample + 380uL HBD media per tube. You can try different volumes.*

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.

To save time, I used a multichannel pipette to discard old media. Try your best to minimize the amount of time that wells are free of media i.e. remove media from one section of the 96-well plate at a time.

DAY 3: ABSORBANCE SPECTROSCOPY FOR DETECTION OF MDP

1. 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 – there usually is background SEAP expression. Samples with readings close to and below 0 will be designated as below detection limit.

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Notes:

ϕ For each plate, you may create a standard curve if you wish to know the concentration of MDP in your samples (0 to 0.5ug/mL). If you just want to know relative differences in your samples, you may just use positive and negative controls, **positive** being 0.5ug/mL of MDP & **negative**, the HEK Blue Detection **media alone** and/or **gnotobiotic serum**.

φ If you wish to transfect in serum, you will get a more robust response. However, the assay is responsive enough to add serum directly into media without transfecting. Furthermore, opting not to transfect would better emulate the biological response *in vivo*.

φ Literature suggests that acidification of media aids in cellular uptake of MDP

Dose Response to MDP in 293T/mNOD2 Cells

