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Protocol status: Working
We use this protocol and it's working

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TNF ELISA Protocol

caitlynhenry[1]

[1]Department of Biology, Misericordia University

caitlynhenry

ABSTRACT

The purpose of this experiment is to investigate the effects of forskolin-mediated cAMP activation on TNF-α secretion by LPS-treated Schwann cells. The immortalized rat RT4-D6P2T cell line (ATCC #CRL-2768) was cultured and received one of the following treatments: 0.1, 1, or 10 µg/mL of LPS, in N2 media (control) or N2 media supplemented with 2 µM of forskolin, for 3 hours. Cell media samples were collected, and the Rat TNF-alpha ELISA kit (RayBiotech, Cat #ELR-TNFa-1, Norcross, GA) was performed to quantify changes in TNF-α secretion in response to the different treatment combinations.

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To prepare RT4-D6P2T cell media samples (for three 6-well plates):

1 Aseptically culture immortalized rat RT4-D6P2T Schwann cells (ATCC, Cat #CRL-2768, Manassas, VA) in Dulbecco's Modified Eagle Medium (DMEM) (ATCC, Cat #30-2002, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher, Cat #16000044, Waltham, VA) and 1% penicillin/streptomycin (Pen-strep) (GIBCO, Cat #15140-015, Gaithersburg, MD)/amphotericin B (R&D Systems, Cat #B23192, Minneapolis, MN) at 37°C and 5% CO_2 in poly-L-lysine (PLL)-coated dishes.

2 At 80% confluency, split and seed cells into DMEM (2 mL DMEM/well) in three PLL-coated 6-well plates at a density of ~300,000 cells/well.

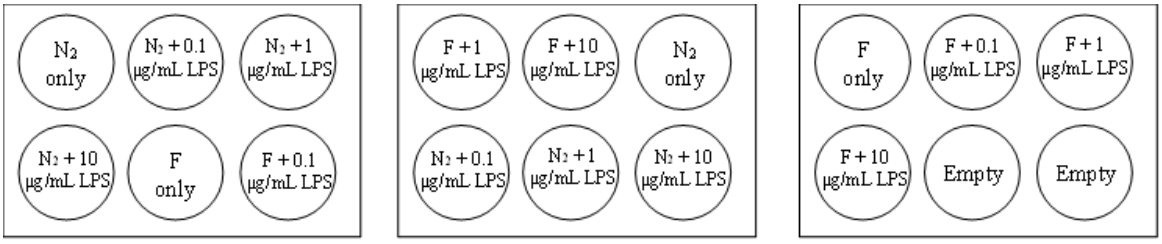
3 Incubate cells in DMEM for 24 hours.

4 After 24 hours, aspirate the DMEM and wash each well 2-3x with 2 mL HBSS. After the last wash, add 2 mL N_2 media (DMEM/F12, no phenol red [Thermo Fisher, Cat #21041025, Waltham, MA] supplemented with 5 µg/mL insulin [Sigma, Cat #91077C, St. Louis, MO] and 100 µg/mL apo-transferrin [Sigma, Cat #T1147, St. Louis, MO]) to each well.

5 Incubate cells in N_2 media for 24 hours.

6 After 24 hours, prepare the forskolin-supplemented media by adding 10 µL of a 2 mM forskolin stock to 20 mL of N_2 media.

7 Add 2 mL of the appropriate medium to each well following the plate layout (see example below).



8 After adding the media, add the appropriate LPS dose to each well following the plate layout.

0.1 µg/mL LPS: 2 µL of 100 µg/mL LPS stock OR 20 µL of 10 µg/mL LPS stock

1 µg/mL LPS: 2 µL of 1 mg/mL LPS stock OR 20 µL of 100 µg/mL LPS stock

10 µg/mL LPS: 20 µL of 1 mg/mL LPS stock

9 Allow cells to incubate in the different treatment combinations for the required incubation time (3 hours).

10 After the required incubation time, remove the 6-well plates from the incubator and collect desired volume of media from each well.

11 Store media samples at -80°C for future use.

To perform TNF ELISA (using RayBiotech Rat TNF-alpha ELISA kit [Cat #E…

12 Bring all reagents/samples to room temperature.

13 Make the appropriate volume of 1X Diluent B by adding DI H_2O to 5X Diluent B (Item E).

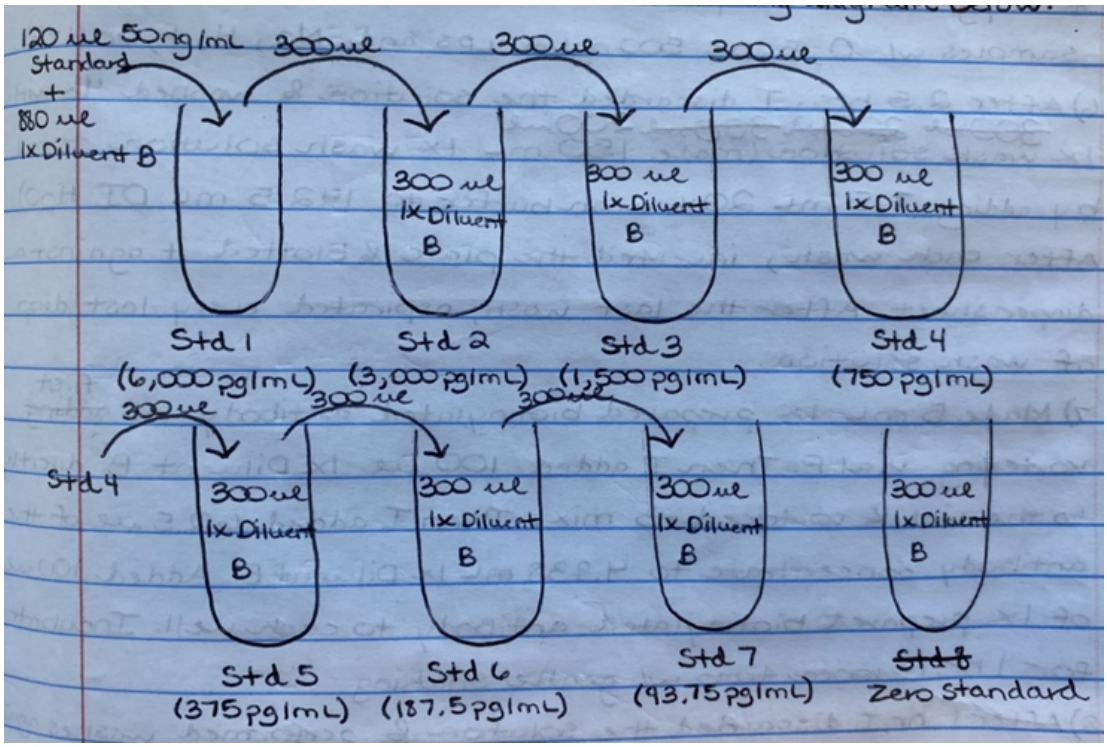
14 Standard Preparation:

14.1 Gently vortex a vial of Standard Protein (Item C).

14.2 Add 400 µL 1X Diluent B directly to Item C and gently vortex to mix.

14.3 Add 120 µL diluted standard to 880 µL 1X Diluent B.

14.4 Perform a serial dilution to achieve the required standard concentrations (see example below).



15 Sample Preparation:

15.1 Dilute samples in 1X Diluent B.

15.2 For RT4-D6P2T media samples, use a 1:2 or 1:1 dilution.

1:2 dilution: 83.3 µL sample + 166.7 µL 1X Diluent B
1:1 dilution: 125 µL sample + 125 µL 1X Diluent B

16 Add 100 µL of each standard and sample into the appropriate wells of the 96-well plate following the plate layout (see example plate layout below).

	STD1	STD1	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9
	STD2	STD2	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10
	STD3	STD3	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11
	STD4	STD4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12
	STD5	STD5	Sample 5	Sample 5	Sample 5			
	STD6	STD6	Sample 6	Sample 6	Sample 6			
	STD7	STD7	Sample 7	Sample 7	Sample 7			
	ZERO STD	ZERO STD	Sample 8	Sample 8	Sample 8			

17 Cover the plate and incubate for 2.5 hours on a rocker at room temperature.

18 While waiting, prepare the desired volume of 1X wash solution by adding DI H_2O to 20X wash buffer.

19 Antibody Preparation:

19.1 Gently vortex the detection antibody (Item F).

19.2 Add 100 µL 1X Diluent B directly to the vial and gently pipette up and down to mix.

19.3 Dilute the detection antibody concentrate 80-fold using 1X Diluent B.

20 After 2.5 hours, discard the solution from each well.

21 Wash each well 4x with 1X wash solution. After each wash, invert the plate and blot it against a diaper sheet until the diaper sheet becomes dry. After the last wash, aspirate every last drop of wash solution.

22 Add 100 µL of the prepared detection antibody to each well, cover the plate, and incubate for 1 hour on a rocker at room temperature.

23 HRP-Streptavidin Preparation:

23.1 Gently vortex HRP-Streptavidin Concentrate (Item G) and pipette up and down to mix.

23.2 Dilute the HRP-Streptavidin Concentrate 200-fold using 1X Diluent B.

24 After 1 hour, discard the solution from each well and repeat washes following Step 10.

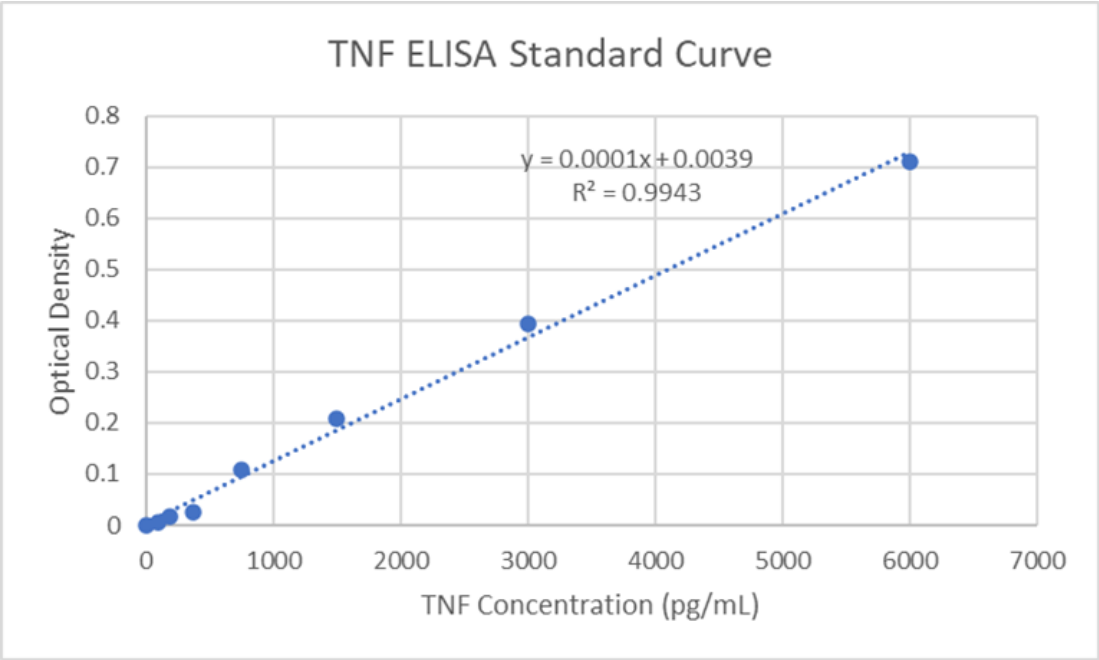
25 Add 100 µL of the prepared HRP-Streptavidin solution to each well, cover the plate, and incubate for 45 minutes on a rocker at room temperature.

26 After 45 minutes, discard the solution from each well and repeat washes following Step 10.

27 Add 100 µL TMB One-Step Substrate Reagent (Item H) to each well, cover the plate completely in aluminum foil, and incubate for 30 minutes on a rocker at room temperature in the dark.

28 After 30 minutes, add 50 µL Stop Solution (Item I) to each well. Immediately read the plate on the plate reader at 450 nm.

29 Plot the standard curve (see example below). Use the standard curve to approximate the concentration of TNF-alpha present in each sample. NOTE: Do not forget to correct for the dilution factor (for example, if you did a 1:1 dilution, multiply the TNF-alpha concentrations by 2).


TNF ELISA Standard Curve
y = 0.0001x + 0.0039
R² = 0.9943
Optical Density
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1000
2000
3000
4000
5000
6000
7000
TNF Concentration (pg/mL)