Core D Procedure Protocol
ATP, Kdo2 Lipid A Treatment
5-12-2009

Overview

1. One large experiment will be completed to provide samples to all cores.
2. Untreated cells will be harvested at 0 hours. Treated cells will be harvested at 0.15, 0.30, 1, 2, 4, 8, 20 hours. See Figure 1 for an overview of the treatment time course.
3. Table 1 for an overview of the treatment.
4. Only one replicate will be made for each time and treatment. 10 aliquots will be generated from each replicate. Cores E, G, and J will receive one aliquot, cores H, I and K will receive two aliquots and core G will receive 20 mL of medium from each treatment.
5. Data will be normalized to DNA content (pmol lipid per µg DNA). DNA and TNFa data will be generated by core D. DNA and TNFa assay data will be emailed to each core.

Setup

1. Seed 2 - 100 mm TC plates with 1 x 10^7 cells in 10 mL Bone Marrow-derived Macrophage Growth Medium 2 (PS0000003200) each.
2. Incubate 16 hours at 37°C.

Reagent Preparation

1. ATP – (PS0000004000)
2. Kdo2 Lipid A working solution – prepare from Kdo2 Lipid A stock solution (PS0000001401)

Treatment

1. Treat each plate with the appropriate reagents:

Pretreatment (priming) 4h Kdo2 or DPBS followed by	10 µL 100 ug/mL Kdo2 (FC-100 ng/mL) 10 µL DPBS
ATP or 10 mM Hepes/10 mM DTT time course: .15, .30, 1h, 2h, 4h, 8h and 20h	250 µL 200 mM/mL ATP (FC-5 mM/mL) 250 µL 10 mM Hepes/10 mM DTT

2. Note the time and return the plates to the incubator.
3. For 0 hours, do not treat with anything. Proceed with harvest.

Harvest

1. At the appropriate time, remove the plates from the incubator.
2. Immediately remove medium from each plate and place in labeled 50 mL polypropylene tubes for Core G.

3. Remove .5 mL of medium from each treatment and place in labeled Eppendorf tubes for the TNFα assay.
4. Place the plates on ice and wash twice with 5 mL of cold DPBS.
5. Add 5 mL of cold DPBS to the first plate and 5.5 mL to the second plate of each treatment and scrape the cells with a scraper.
6. Transfer the cells to a labeled 50 mL conical tube on ice.
7. Gently suspend cells by swirling or lightly vortexing for 10 secs.
8. Remove 200 µL of the cell suspension to an Eppendorf on ice for the DNA assay.
9. Divide the 10 mL cells into aliquots of **1 mL/2 x 10^6 cells each**, placing into 13x100mm glass tubes with Teflon-lined screw caps.
10. Freeze and store cells at -80°C.

Medium Processing for TNFα Assay

1. Centrifuge the TNFα aliquots at top speed in refrigerated microfuge for 2 min, collect an aliquot of supernatant (~0.4 mL) and place in a new labeled Eppendorf tube. Freeze the aliquots at -20°C.
2. TNFα is to be assayed by Jay Westcott at ELISA Tech (Aurora, CO).

Cell Processing for DNA Assay

1. Store at -20°C.
2. DNA is to be assayed by core D according to the LIPID MAPS DNA Assay (PP0000002700).

All samples will be shipped to their cores on dry ice

Figure 1

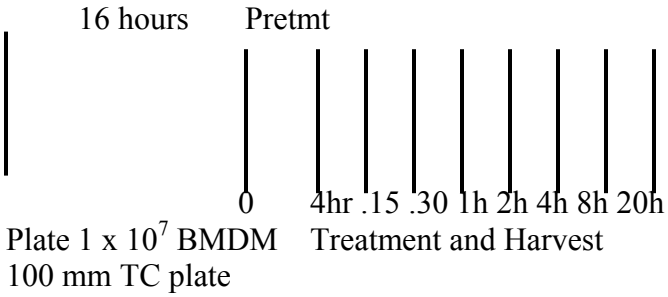

Table 1: Number labeling of samples, time and treatment

Sample #	Pretmt Hour	Tmt Hour	Rx
1	0	0	-K-A
2	4	.15	-K-A
3	4	.15	+K-A
4	4	.15	-K+A
5	4	.15	+K+A
6	4	.30	-K-A
7	4	.30	+K-A
8	4	.30	-K+A
9	4	.30	+K+A
10	4	1	-K-A
11	4	1	+K-A
12	4	1	-K+A
13	4	1	+K+A
14	4	2	-K-A
15	4	2	+K-A
16	4	2	-K+A
17	4	2	+K+A
18	4	4	-K-A
19	4	4	+K-A
20	4	4	-K+A
21	4	4	+K+A
22	4	8	-K-A
23	4	8	+K-A
24	4	8	-K+A
25	4	8	+K+A
26	4	20	-K-A
27	4	20	+K-A
28	4	20	-K+A
29	4	20	+K+A

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