LIPID MAPS THAWING AND PASSAGE PROCEDURE FOR RAW 264.7
Version 2, 08/11/04

The RAW 264.7 is a macrophage-like cell line derived from tumors induced in male BALB/c mice by the Abelson murine leukemia virus.

The RAW 264.7 cells to be used for the LIPID MAPS consortium were obtained from ATCC laboratories (cat# TIB-71; lot# 3002360), expanded to pass 3, and frozen down in aliquots. Each aliquot (vial) of cells shall be passed for no more than 20 passages. (1 vial per 2 months, 6 vials per year per lab).

To maximize uniformity across participating laboratories, RAW264.7 cells should be thawed from original LIPID MAPS frozen stocks on the first Monday of odd months (Jan, March, May etc.). Passing cells twice per week as described below will result in maximum passage numbers of 17 to 18 passages. This passage number limit is based on significant changes in transfection efficiency beyond 25 passages. Transcriptional activation of COX2 in response to KdO2 lipid A is not measurably different in passage 7 and passage 25 cells. Maximal passage numbers could be subject to change if additional studies document variation in lipid components as a function of passage number.

The RAW 264.7 cells adhere to tissue culture-grade plastic through cation–dependent integrin receptors and other cation-independent receptors. They are extremely sensitive to lipopolysaccharide (LPS) endotoxin from gram-negative bacteria, therefore, only sterile disposable tissue culture ware and solutions, buffers, and media with endotoxin tested distilled deionized water should be used.

**Thawing Procedure**

1. Warm the growth medium for 15 to 20 minutes in a 37° C water bath.
2. Place 15 mls of the warmed fresh medium into a new 75 $cm^2$ flask. (Barcode).
3. Enter the barcode of the vial containing the cells.
4. Thoroughly immerse the vial in 70% ethanol before thawing.
5. Thaw the cells by gently rubbing the vial between your hands (2-3min).
6. Carefully remove the cap and extract the cells with a pipette.
7. Dispense the cells in the newly prepared 75 $cm^2$ flask.
8. Maintain the cells at 37°, in a humidified atmosphere, with 5% $CO^2$.
9. After the cells have adhered, approximately 6 hrs, aspirate the old medium and add 15 mls of fresh medium.
10. When the cells are 80% confluent, pass them into a new vessel with fresh medium.

**Passage Procedure**

1. Warm the growth medium the same as in the thawing procedure.
2. Place a volume of the warmed fresh medium into new vessels. (See below for plate size, cell density, and medium volume recommendations)(Barcode vessels).
3. Aspirate the old medium from the cells.
4. Rinse the cells 1 X with DPBS.
5. Add fresh medium (10 ml to 150 $cm^2$ flask) to the cells.
6. Gently scrape the cells until all are dislodged.
7. Pipette the cell suspension, with a 10 ml pipette, up and down 5 X, without introducing bubbles.
8. Count the cells using a hemacytometer.
    a. Load 10 ul of the freshly suspended cells to the hemacytometer. Usually a 1:10 dilution in medium or DPBS of the suspended cells is needed before counting.
    b. The number of cells counted per square millimeter (middle 25 squares ruled on the hemacytometer) X dilution (if used) X 1 X $10^4$ = cell count per ml. This number X the total volume of the cell suspension = total cell count.
9. Dispense the cells into the new vessels. (e.g., 5 x $10^6$ cells per 150 $cm^2$ flask).
10. Grow the cells to no more than 80% confluence, 2-3 days, before passing or harvesting.

**LPS Preparation**

1. Resuspend KDO lipid A from Avanti in DPBS to a concentration of 1ug/ul.
2. Sonicate for 1 minute in an ultra sonic water bath.
3. Stimulate the RAW cells for 24 hours at a final concentration of 100ng/ml.

**Recommended cell seeding and medium volume for specific plate formats and tissue culture flasks to obtain approximately 80% confluence in 2-3 days:**

1. **150 mm plates: 5 x $10^6$/20 ml medium**
2. **100 mm plates: 2 x $10^6$/10 ml medium**
3. **60 mm plates: 5 x $10^5$/5 ml medium**
4. **6-well plates: 3 x $10^5$/well/3 ml medium**
5. **12-well plates: 7.5 x $10^4$/well/2 ml medium**
6. **24-well plates: 5 x $10^4$/well/1 ml medium**
7. **96-well plates: 2 x $10^4$/well/100 ul medium**
8. **150 $cm^2$ flask: 5 x $10^6$/20 ml-30 ml medium**
9. **75 $cm^2$ flask: 2.5 x $10^6$/10 ml-15 ml medium**
10. **25 $cm^2$ flask: 1 x $10^6$/5 ml-7 ml medium**