Preparation of Frozen Stocks of RAW 264.7 Cells  
AfCS Procedure Protocol PP00000180  
Version 1, 12/29/03

The RAW 264.7 cells used by the Alliance for Cellular Signaling (AfCS) were obtained from the American Type Culture Collection (ATCC; cat. no. TIB-71; lot no. 2263775). The vial of cells received from the ATCC was thawed using the Thaw Procedure for RAW 264.7 Cells (PP00000160). The thawed cells were expanded by maintenance in RAW 264.7 growth medium 1 (RAWGM1) and passage every two days (see Passage Procedure for RAW 264.7 Cells, PP00000159) until sufficient cells were obtained for preparation of frozen stocks. Numerous aliquots of frozen cells from similar passages provide a uniform source of RAW 264.7 cells for experimentation by AfCS laboratories.

Note: macrophages are extremely sensitive to lipopolysaccharide (LPS) endotoxin from Gram-negative bacteria. LPS has major effects on macrophage phenotype and function, including adhesion. All solutions, buffers, and media should be made with sterile, endotoxin-tested, distilled, deionized water.

Freezing Procedure
1. Prepare and bar code cryovials prior to removing cells from plates. (Note: in order to ensure that bar-code labels adhere to tube during freezing and storage, wrap a single layer of clear TZ tape around tube after bar-code label has been applied.)
2. Remove cells from plates (as indicated in Passage Procedure for RAW 264.7 Cells) to create a single cell suspension, and pool cells in a 250-ml conical tube. Each plate should yield 40 to 80 x 10⁶ cells, depending on the passage number. Newly thawed cells will yield lower quantities. (Note: cells thawed from ATCC were passaged on tissue culture plates for two passages and transferred to Valmark plates, where they were cultured in large volumes.)
3. Count cells as indicated in Passage Procedure for RAW 264.7 Cells.
4. Centrifuge tube of cells at 400 x g for 5 min at room temperature.
5. Remove all but approximately 500 µl of the supernatant.
6. Flick tube with an index finger to gently resuspend cell pellet.
7. Add RAW 264.7 freezing medium (RAWFM) to resuspend cells to a concentration of 5 x 10⁶ cells/ml.
8. Pipette up and down gently to resuspend cells in medium.
9. Transfer 1 ml of cells to each cryovial. (Note: this should be done as quickly as possible).
10. Cap cryovials containing cells.
11. Place cryovials into Nalgene cryo 1 °C freezing container (to achieve a 1 °C/minute rate of cooling).
12. Place freezing containers in freezer at −80 °C for at least 24 hr.
13. Transfer frozen cryovials containing cells to a storage vessel containing liquid nitrogen.

Reagents and Materials
Cryovial, exterior thread, 2 ml: Fisher Scientific; catalog no. 05-669-64
TZ tape, clear (24 mm, 1 inch): Brother Industries, Ltd.; catalog no. TZ-151

RAW 264.7 freezing medium (RAWFM): AfCS Solution Protocol ID PS00000568

Nalgene cryo 1 °C freezing container: Nalgene; catalog no. 5100-0001

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