Preparation of RAW 264.7 Cell Lysates for FXM Western Blot Analysis
AfCS Procedure Protocol PP00000168
Version 1, 8/13/04

This procedure provides lysates of cultured RAW 264.7 cells that are ready for separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysis by Western immunoblotting. Sufficient sample is obtained for approximately four to five analyses as described in AfCS protocols such as Western Blot Analysis—Phosphoprotein-Specific Antibody Mixture, PP00000007, and Western Blot Analysis—Phosphotyrosine, PP00000006.

Procedure
1. Suspend RAW 264.7 cells at 3.3 x 10^5 cells/ml in RAW 264.7 growth medium for FXM (RGMFXM), and distribute 3 ml of the cell suspension into each well of a tissue culture (TC)-treated 6-well plate or individual 35-mm TC-treated dishes (growth area 9.5 cm^2).
2. Incubate at 37 °C in air with 5% CO₂ for 18 to 24 hr.
3. Transfer containers from incubator to tissue culture hood.
4. Aspirate the growth medium.
5. Add 1.35 ml of RAW 264.7 assay medium (RAWAM).
6. Incubate at 37 °C in air with 5% CO₂ for 1 hr.
7. Transfer dishes to an environmental chamber containing air at 37 ºC.
8. Add 0.15 ml of ligand (10X final concentration in RAWAM) or vehicle (in RAWAM) to appropriate wells or dishes. (Note: vehicle controls constitute matching dilutions of solvents in which ligands are dissolved and stored.)
9. Place on shaker and rotate for 10 sec (this only works for 6-well plates).
10. Incubate at 37 °C in the environmental chamber for the desired times. Exception: if the time of exposure to ligand is greater than 20 min, plates are incubated at 37 °C in air with 5% CO₂.
11. Remove culture plates from environmental chamber or tissue culture incubator 10 to 20 sec prior to planned end of treatment. (Note: this is the time range required to process three samples simultaneously for steps 12 through 15.)
12. At end of treatment time, aspirate medium carefully with a glass pipette connected to a vacuum. With plate held at a 45-degree angle, remove all of medium.
13. Add 150 µl sample buffer complete, 1.5X/EDTA, 7.5 mM (1.5X SBCe), which includes dithiothreitol, 2-mercaptoethanol, protease inhibitors, and phosphatase inhibitors.
14. Harvest cell lysate immediately using a cell lifter. (Note: scraping takes about 30 sec.)
15. Transfer lysis mixture to a bar-coded microfuge polyallomer tube using a 200-µl micropipette set at 100 µl. Repeat to collect remaining solution (solution is viscous).
16. Slip cap locks onto tops of microfuge tubes to keep tops sealed.
17. Immediately heat samples for 5 min at 95 to 100 °C in a heat block (with water in wells of heat block to efficiently transfer heat to tubes).
18. Cool tubes on ice.
19. Centrifuge samples at 185,000 to 200,000 x g for 1 hr at 4 °C (usually 55,000 rpm in Beckman TLA55 rotor). This step pellets the viscous DNA. If centrifuge is not available, freeze and store samples at –80 °C until samples can be centrifuged.

20. Measure and transfer supernatant to a new tube with label.

21. Freeze and store sample at –80 °C if sample will not be used immediately.

22. After thawing, centrifuge briefly to collect condensate; keep tubes in a rack at room temperature.

23. Vortex to mix before loading samples on gel.

**Use of Environmental Chamber**

An environmental chamber with temperature control is used to maintain uniform temperature during experimental treatments. High humidity is maintained with an open tray of water in the chamber.

**Reagents and Materials**

RAW 264.7 growth medium for FXM (RGMFXM): AfCS Solution Protocol PS00000636

Falcon cell culture plate, 6 well: BD Biosciences; catalog no. 353046

Falcon culture dish, 35 x 10 mm: BD Biosciences; catalog no. 353001

Incubator: Forma Scientific; model 3110 series II

RAW 264.7 assay medium (RAWAM): AfCS Solution Protocol ID PS00000556

Environmental chamber (aluminum/plexiglass glove box with temperature control): Coy Laboratory Products Inc.; catalog no. 8307-060

Sample buffer complete, 1.5X/EDTA, 7.5 mM (1.5X SBCe): AfCS Solution Protocol PS00000533

Cell lifter: Costar; catalog no. 3008

Dry block heater: VWR Scientific Products; catalog no. 13259-032

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**Date:** 08/26/04

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