Transfecting and Plating RAW 264.7 Cells with Lipofectamine 2000
AfCS Procedure Protocol PP00000182
Version 1, 10/31/03

The following procedure is for simultaneous transfection and plating of RAW 264.7 cells. This protocol results in approximately 50% to 70% cell viability, and of those viable cells, 20% to 40% are transfected when using pEYFP-N1 from Clontech.

Day 1—Procedure for Splitting Cells before Transfection
Cells are growing in RAW 264.7 growth medium 1 (RAWGM1).
1. On the day prior to transfection, plate RAW 264.7 cells at 1 x 10^5 cells/ml in one or more petri dishes in RAWGM1.
2. Grow cells overnight. The cell count should be between 3 and 8 x 10^5 cells/ml, 16 to 24 hr later.

Day 2—Procedure for Preparing Lipofectamine 2000 and DNA
1. Warm RAWGM1 (~10 ml) to 37 °C and equilibrate in 5% CO₂ incubator. Use a tissue culture flask with a vent cap. The RAWGM1 will be used in steps 8 and 13.
2. For each transfection, add 1 µl Lipofectamine 2000 to 25 µl Opti-MEM in a 1.8-ml Eppendorf tube. Mix and allow to sit for 5 min at room temperature.
3. Determine the volume of DNA needed for 0.5 µg. Add DNA to another tube of 25 µl Opti-MEM for each transfection.
4. After the 5 min incubation is complete, add the 25 µl DNA mixture to the 25 µl Lipofectamine 2000 mixture. Mix by tapping or pipetting.
5. Allow this mixture to incubate for 20 min at room temperature. Do not allow mix to sit for more than 20 min.
6. Prepare cells as described below (see Preparation of RAW 264.7 Cells for Transfection).
7. Determine how much RAWGM1 to add to each well of an 8-well coverglass chamber by subtracting the volume of cells needed and the 50 µl of Lipofectamine/DNA mix from 400 µl (e.g., 400 µl - (50 µl mix + 75 µl cells) = 275 µl medium).
8. Add the required amount of RAWGM1 to each well.
9. Add the Lipofectamine/DNA mix to the medium in each well (after 20 min incubation is completed).
10. Add 5 x 10^4 cells to each well (see Preparation of RAW 264.7 Cells for Transfection, below).
11. Incubate in 37 °C 5% CO₂ incubator for 3 hr.
12. After 3 hr, carefully remove most of medium, leaving enough so that cells will not dry out.
13. Add 400 µl of prewarmed CO₂-equilibrated fresh RAWGM1.
14. Place coverglass chamber back into incubator.
15. Incubate overnight.
16. Cells are ready for imaging.
Preparation of RAW 264.7 Cells for Transfection
The following is a procedure for harvesting RAW 264.7 cells, growing in RAWGM1, prior to transfection.
1. Pool dishes of cells that have been prepared for transfection.
2. Rinse bottom of each dish with medium at least 10 times by pipetting with a 5-ml disposable pipette. Use a 5-ml disposable pipette to transfer cells from dishes to a labeled 15-ml conical tube.
3. Add cold PBS-EDTA, pH 7.35, to each dish, and let sit in tissue culture hood for 5 min. Do not allow solution to sit for more than 5 min.
4. Rinse bottom of each dish with PBS-EDTA at least 8 times by pipetting solution with a 5-ml disposable pipette. Use a 5-ml disposable pipette to transfer cells from dishes to labeled 50-ml conical tube.
5. Centrifuge for 6 to 8 min at 1000 rpm in Beckman centrifuge.
6. Carefully remove supernatant.
7. Resuspend pelleted cells in 1 ml PBS-EDTA. Triturate at least 8 times to break up clumps of cells.
9. Remove 100 µl and add to Coulter cuvette containing 9.9 ml of sheath fluid. Invert 3 times to mix.
10. Count cells using a Coulter Counter.
11. Determine volume of cells required for 5 x 10^4 cells.

Reagents and Materials
pEYFP-N1: BD Biosciences Clontech; catalog no. 6006-1
RAW 264.7 growth medium 1 (RAWGM1); AfCS Solution Protocol ID PS00000510
Petri dishes, 100 x 25 mm: Kord-Valmark/Midwest Scientific; catalog no. 906
Tissue culture flask with vent cap, 75 cm^2: Corning Inc.; catalog no. 430641
Lipofectamine 2000: Invitrogen; catalog no. 11668027 or 11668019
Opti-MEM; Invitrogen; catalog no. 31985070
Chambered coverglass, 8-well; Nunc Lab-Tek; catalog no. 155411
Phosphate buffered saline, pH 7.35, 1X, with EDTA, 2 mM (low endotoxin) (PBS-EDTA, pH 7.35); AfCS Solution Protocol ID PS00000575
Allegra 6R centrifuge with GH-3.8A rotor: Beckman Coulter; catalog no. BK366816 (centrifuge), BK366650 (rotor)
Z1 Coulter Counter: Beckman Coulter; catalog no. 6605698.