

**Preparation of B-Lymphocyte RNA for Microarray Analysis**  
**AfCS Procedure Protocol PP0000000900**  
**Version 1, 01/08/02**

The following procedure permits the isolation of at least 5 µg of total RNA from a sample of purified mouse splenic B lymphocytes (see AfCS Protocol *Isolation of Resting B Lymphocytes from Sixteen Mouse Spleens*, PP0000001600). The quality of the RNA is assessed by separation of an aliquot through 1% agarose and staining with ethidium bromide as described in AfCS protocol *Visualization of RNA Preparations on 1% Agarose Gels*, PP0000002500. The isolated RNA is used for analysis of gene expression by microarray technology.

**Treatment of Samples and Termination of Reactions**

1. Suspend freshly isolated splenic B lymphocytes at  $16.7 \times 10^6$  cells/ml in Supplemented Iscove's Modified Dulbecco's Medium (SIMDM) and distribute 1.08 ml into individual wells of 12-well (growth area 3.8 cm<sup>2</sup>) tissue culture plates as needed.
2. Incubate at 37 °C in air with 5% CO<sub>2</sub> for 1 hr.
3. Transfer culture plates to an environmental chamber with an air atmosphere at 37 °C.
4. Add 0.12 ml of ligand (10X final concentration in SIMDM) or vehicle (in SIMDM) to appropriate wells to begin treatments in the 37 °C environmental chamber. (Note: vehicle controls constitute matching dilutions of solvents in which ligands are dissolved and stored.)
5. Place on shaker and rotate for 30 sec.
6. Resume incubation of cells at 37 °C in air with 5% CO<sub>2</sub> for desired times.
7. Transfer the cells from individual wells to 15-ml barcoded conical tubes at the end of the treatment time and centrifuge the samples at room temperature for 5 min at 400 x g in a tabletop centrifuge.
8. Remove the supernatants with 5-ml sterile plastic pipettes.
9. Add 1 ml of ice-cold TriPure to each of the wells and pipet up and down with 1-ml sterile plastic pipettes to lyse the cells.
10. Transfer the lysates with 1-ml sterile plastic pipettes to the corresponding conical tubes containing cell pellets.
11. Add 2 ml of TriPure to each conical tube to a final volume of 3 ml.
12. Pipette up and down with a 5-ml sterile plastic pipette and transfer the lysate to a 13-ml barcoded polypropylene tube.
13. Place the samples in a –80 °C freezer and store at –80 °C until ready for isolation of RNA. Otherwise, proceed immediately to step 14.

**Isolation of RNA**

14. Remove the samples from –80 °C freezer and thaw at room temperature.
15. Add 0.6 ml of chloroform (20% of TriPure volume) immediately after the samples are thawed.
16. Cap the tubes tightly and shake for 30 sec.
17. Incubate the mixtures at room temperature for 5 min.

18. Loosen the cap and centrifuge the samples at 4 °C for 15 min at 12,000 x g (e.g., 9,500 rpm in Beckman JA-17 rotor).
19. Transfer the colorless upper aqueous phase (about 1.8 ml) of each sample to a new 13-ml barcoded polypropylene tube.
20. Repeat the extraction by adding 2 ml of chloroform to the transferred, separated aqueous phase of each sample.
21. Cap the sample tubes tightly and shake for 30 sec.
22. Loosen the cap and centrifuge the samples at 4 °C for 15 min at 12,000 x g.
23. Transfer the colorless upper aqueous phase to a new 13-ml barcoded polypropylene tube.
24. Add 1.5 ml of isopropanol to each of the final, isolated aqueous phases (step 23), cap tightly, and mix by inverting.
25. Incubate at room temperature for 5 min.
26. Centrifuge the samples at 4 °C for 10 min at 12,000 x g.
27. Remove most of the supernatant gently, but leave about 0.5 ml containing the pellet of RNA.
28. Vortex briefly to disperse the pellet and transfer to a new barcoded Eppendorf tube.
29. Centrifuge for 5 min at 4 °C in a microfuge (Microfuge®18 Beckman Coulter) at 18,000 x g.
30. Remove the supernatant carefully without disturbing (losing) the pellet of RNA.
31. Add 1 ml of room temperature RNase-free 70% ethanol.
32. Vortex briefly to loosen the pellet and centrifuge at 18,000 x g in the microfuge for 5 min at 4 °C.
33. Remove the supernatant carefully and air-dry the sample for 30 min.
34. Add 10 µl of nuclease-free water to each sample.
35. Incubate the sample on ice for 20 min, vortexing at 5 min intervals.
36. Determine the concentration of total RNA by diluting 1 µl of the sample in 79 µl of Tris-EDTA buffer and reading the absorbance at 260 nm in a spectrophotometer.
37. Determine the integrity of total RNA by electrophoresis of 1 µg of total RNA on a 1.0% agarose gel and staining with ethidium bromide for 18S and 28S ribosomal RNA.
38. Place samples in a storage box and store at –80 °C prior to shipment to the Molecular Biology Laboratory for microarray analysis. Ship samples on dry ice.

### **Reagents and Materials**

Supplemented Iscove's Modified Dulbecco's Medium (SIMDM): AfCS Solution Protocol ID PS0000005600

Environmental chamber (Aluminum glove box with temperature control): Coy Laboratory Products; catalog no. 0850-003

Lab-Line Titer Plate Shaker: Lab-Line Instruments; catalog no. 4625

TriPure Isolation Reagent: Roche; catalog no. 1667165

Polypropylene tubes, 13 ml: Sarstedt; catalog no. 60.540

Chloroform: Sigma; catalog no. C5312

Isopropanol: Sigma; catalog no. I0398

Eppendorf tubes: Sarstedt; catalog no. 72.690

RNase-free 70% ethanol: dilute absolute ethanol to 70% (v/v) in nuclease-free water

Nuclease-free water: Ambion; catalog no. 9930

Tris-EDTA buffer, pH 8.0, 1X, 10 mM /1 mM : AfCS Solution Protocol ID PS0000006700

SYBR gold nucleic acid gel stain: Molecular Probes; catalog no. S11494

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