

Preparation of B-Lymphocyte Lysates for Western Blot Analysis

AfCS Procedure Protocol PP0000001000

Version 1, 01/29/02

This procedure provides lysates of splenic B lymphocytes (B cells) ready for separation through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and for analysis of phosphoprotein content by immunoblotting. Sufficient sample is obtained for approximately four to five analyses as described in AfCS protocols *Western Blot Analysis—Phosphoprotein-Specific Antibody Mixture*, PP0000000700, and *Western Blot Analysis-Phosphotyrosine*, PP0000000600 .

Procedures

1. Suspend freshly isolated splenic B lymphocytes at 16.7×10^6 cells/ml in Supplemented Iscove's Modified Dulbecco's Medium (SIMDM) and distribute 1.08 ml in each well of 12-well (growth area 3.8 cm^2) tissue culture plates as needed.
2. Incubate at 37°C in air with 5% CO_2 for 1 hr.
3. Transfer culture plates to an environmental chamber containing air 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. 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% CO_2 .
7. Remove culture plates from the environmental chamber or tissue culture incubator 2.5 min prior to the planned end of the treatment (Note: this time is required for steps 8 through 13).
8. Transfer the cells into barcoded microfuge polyallomer tubes with a 1-ml micropipette. (Note: this is done in two 0.6-ml aliquots; repeat pipetting of the second aliquot is used to suspend cells at the bottom of the plate before transfer to the tube.
9. Pellet the cells by rapid centrifugation (about 10 sec; time to get up to full speed) in a microfuge at room temperature.
10. Remove the supernatants from the cell pellets by careful aspiration, leaving approximately 5 μl of liquid.
11. Loosen the cell pellets by quickly stroking the tubes 3 to 4 times along a rough surface, such as the top of an Eppendorf or other small test tube rack.
12. Add 90 μl of 1.5X sample buffer complete to each tube.
13. Flick the tube to disperse the pellets and lyse the cells.
14. Slip on cap locks to the top of the microfuge tube to keep the top from popping open.
15. Immediately heat the samples for 5 min at 95°C to 100°C in water in a heat block.
16. Cool the tubes on ice and subject to ultracentrifugation at 185,000 to 200,000 $\times g$ at 4°C for 1 hr (usually 55,000 rpm in Beckman TLA55 rotor). This step pellets the viscous DNA.

17. Using a transfer pipette, carefully collect the supernatant fraction into a bar-coded microfuge tube (any type, for storage) and measure the volume. The sample is ready for SDS-PAGE.
18. If the ultracentrifugation cannot be conducted immediately, centrifuge the tubes briefly in a microfuge to reduce any condensation (approximately 5 sec at 14,000 x g should be adequate).
19. Freeze and store the samples at -80°C , either before or after ultracentrifugation.

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

Sample buffer complete, 1.5X (1.5X SBC): AfCS Solution Protocol ID PP0000005000

Microfuge polyallomer tubes: Beckman; catalog no. 357448

Cap locks: PGC Scientifics; catalog no. 16-8126-12

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

Transfer pipette: Fisher Scientific; catalog no. 13-711-9A

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