Preparation of WEHI-231 Positive Control B for Western Blot Analysis
AfCS Procedure Protocol ID PS00000469
Version 1, 07/09/03

WEHI-231 cells are stimulated by IL-10 and anti-CD40 and extracted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer to be used as a positive control for Western blots processed with phosphospecific antibodies. (AfCS Procedure Protocol Western Blot Analysis—Phosphoprotein-Specific Antibody Mixture, PP00000007).

Stimulation of Cells
1. Transfer a known quantity of WEHI-231 cells into a centrifuge tube (15-ml or 50-ml conical tube).
2. Centrifuge at 400 x g for 5 min at 4 °C in a J6-HC swing bucket centrifuge or equivalent centrifuge.
3. Aspirate the supernatant, leaving approximately 15 µl of liquid (this volume remains on top of the pellet).
4. Cap the tube and tap the bottom of the tube to resuspend the cell pellet.
5. Add WEHI-231 assay medium (WHAM) to bring the concentration of WEHI cells to 5.5 x 10^6 cells/ml.
6. Aliquot 900 µl of cell suspension into ultracentrifuge microfuge tubes.
7. Incubate tubes in a 37 °C water bath for 45 min.
8. Add 100 µl of the 10X ligand solution in WHAM (3.38 nM IL-10 and 1.33 µM anti-CD40) to each tube. If the total sample number is greater than 5, stimulate samples in sets (5 samples or less per set), staggering each set by at least 2.5 min.
9. Invert quickly and shake the tube to mix.
10. Incubate tubes in a 37 °C water bath for 12.5 min.
11. Centrifuge at 30,000 x g in a microcentrifuge for 15 sec (this includes acceleration and deceleration time).
12. Aspirate the supernatant.
13. Cap tube and loosen the cell pellet by quickly stroking the tube 3 to 4 times across a rough surface, such as the top of a microfuge tube rack.
14. Add 300 µl of sample buffer complete, 1.5X (1.5X SBC).
15. Flick the tube to mix.
16. Lock the top of the microfuge tube.
17. Immediately heat the samples for 5 min at 95 °C to 100 °C in water in a heat block.
18. Cool the tubes on ice and subject to ultracentrifugation at 185,000 to 200,000 x g at 4 °C for 1 hr (usually 55,000 rpm in Beckman TLA55 rotor). This step pellets the viscous DNA.
19. Using a transfer pipette, carefully collect the supernatant fractions into a single, appropriately sized tube with tight fitting cap and mix well by inversion.
20. Pipette mixture as one 25-µl aliquot (for protein determination) and the remainder as 100-µl aliquots (into any type of microfuge tube).
21. Freeze and store the samples at –80 °C.
23. Thaw one 100 µl-aliquot of lysate mixture on ice and dilute it to 1.5 mg/ml with 1.5X SBC. Divide the dilution into 25-µl aliquots and store at –80 °C.

Note: for Western blotting, thaw one tube of 1.5 mg/ml positive control, and load 10 µl per lane (usually two lanes per gel). Any remaining lysate from this thaw must be used the same day or discarded.

**Reagents and Materials**
- Conical tubes, 15 ml: Greiner; catalog no. 188261
- Conical tubes, 50 ml: Greiner; catalog no. 4943
- J6 centrifuge with a JS 4.2 swinging bucket rotor: Beckman Coulter; catalog no. 360271
- WEHI-231 assay medium (WHAM): AfCS Solution Protocol ID PS00000436
- Microfuge polyallomer tubes: Beckman Coulter; catalog no. 357448
- Sample buffer complete, 1.5X (1.5X SBC): AfCS Solution Protocol ID PS00000050
- 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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