TopoGEN
Products and Services for **Cancer Research.**

Procedure for Making Extracts for Western Blotting

This procedure is designed optimally for HeLa cells; however, other cells lines will work without significant modification. Grow up HeLa cells to near confluence in any convenient culture dish. The following procedure is optimized for a 100 mm petri dish containing about 1-2 x 107 total cells.

• Decant the medium to waste and swirl/rinse the plate of cells with TD buffer (10-20 ml per wash/rinse).

• Scrape the cells into 2ml of TD buffer using a rubber policeman.

• Transfer cells into a 15 ml conical centrifuge tube and spin 1000 xg for 10 min at 4 C.

• Discard the supernate and resuspend cells in 2ml of buffer A (per plate)

• Incubate on ice for 10 min

• Spin at 1000xg for 10 min at 4 C to pellet nuclei.

• Discard supernatant, resuspend nuclei in 180 ul buffer A (per plate).

OPTIONAL: remove 5-10 ul of suspension place on microscope slideand evaluate % contamination of whole cells using phase microscope.

• Add 20ul of 10% SDS (sodium dodecyl sulfate, 10% w/v in H2O).

• Sonicate for 15 seconds.

• Add SDS-PAGE Loading buffer to give a final concentration of 25 mM Tris-Cl (pH 6.8), 1% SDS, 2.5% 2-mercaptoethanol,10% glycerol and 0.05% Bromophenol blue.

• Heat samples to 65 C for 5 min.

• Load various amounts of extract on the gel to determine optimal amount of protein that gives best signal:noise ratio on the Western blot. We recommend loading from 0.1 ul to 20 ul for example.

IMPORTANT: IF you overload the gels, a lot of extra smeary bands will appear. In this case you should load less protein.

Buffers:

• TD: 100mM NaCl, 20 mM KCl, 0.5mM Na2HPO4, 20 mM TRIS.

• BUFFER A: 100mM NaCl, 50mM KCL 0.1mM EDTA, 20 mM Tris-Cl, pH 7.5, 0.1mM

• PMSF, 10% Glycerol, 0.2% NP-40, 0.1% Triton-X 100

COMPLICATIONS AND IMPORTANT CONSIDERATIONS:

• Fresh extracts work best. Topoisomerase I is especially prone to degradation and you may get subbands that are undesirable as a result.

• Be careful not to overload the gels with excessive amounts of protein! Your gels will look very ugly and many non-specific bands may come up.

• Topo II is cell cycle regulated. If your cultures are very old or G1 or G0 arrested, your signal may be very weak.

• Topo I can often be present as proteoloytic fragments...protease inhibitors besides PMSF may help (and as noted, use fresh extracts).

Note that TopoGEN has several different antibodies (rabbit, mouse, human) so it is especially important that you use the appropriate secondary antibody. We find that 125 I- Protein A works best however, ECL and alkaline phosphatase based detections will also work if optimized.

The basic Western blotting method is standard. There are no other special tricks.