**In situ Preparation of Nuclear Matrix for Microscopy and Immunoblotting**

**Equipment and reagents**
- Tissue culture dishes (35 mm and 150 mm)
- Tissue culture plates (6-well)
- Glass coverslips (22X22 mm, No: 1.5) stored in 75% (v/v) ethanol
- Phosphate-buffered saline (PBS)
- 5X SDS sample buffer
- 2% paraformaldehyde in PBS, prepare fresh and keep on ice
- Cytoskeleton (CSK) buffer [100 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3mM MgCl₂, store in aliquots at –20°C. Before use add the following; 0.5% Triton X-100, 1 mM PMSF or AEBSF, Leupeptin 10 :M, Pepstatin A 10 :M, E-64 15 :M, Bestatin 50 :M, Recombinant RNasin (Promega) ribonuclease inhibitor 20 units/ml], after reconstitution keep on ice.
- Extraction buffer [250 mM ammonium sulfate, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100, 1 mM PMSF (or AEBSF), 10 :M Leupeptin, 10 :M Pepstatin A, 15 :M E-64, 50 :M Bestatin, Recombinant RNasin (Promega) ribonuclease inhibitor 20 units/ml], prepare fresh and keep on ice
- Digestion buffer [50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, store in aliquots at –20°C. Before use add the following; 0.5% Triton X-100, 1 mM PMSF (or AEBSF), 10 :M Leupeptin, 10 :M Pepstatin A, 15 :M E-64, 50 :M Bestatin, Recombinant RNasin (Promega) ribonuclease inhibitor 20 units/ml, RQ1 RNase-free RNase (Promega) 200-500 units/ml], prepare fresh and keep at room temperature
- DAPI (Molecular Probes)

**Method**
1. Remove coverslips from the 75% ethanol solution, flame to burn the ethanol off and place them into the 35-mm tissue culture dishes or into the wells of a 6-well tissue culture plate.\(^b\)
2. Plate the cells such that they reach 50-70% confluency at the day of experiment.

3. Wash the cells twice with PBS at 4°C.

4. Gently add 3 ml of CSK buffer to each dish and incubate for 10 min at 4°C.

5. Remove the CSK buffer completely by tilting the dishes and aspirating the buffer with a glass capillary pipette.

6. Transfer the coverslip #1 to a new well and fix it by adding 3 ml, 2% paraformaldehyde and incubate for 15 min at room temperature.

7. Gently add 3 ml of extraction buffer to each dish and incubate for 5 min at 4°C.

8. Remove the extraction buffer completely by tilting the dishes and aspirating the buffer with a glass capillary pipette.

9. Transfer the coverslip #2 to a new well and fix it by adding 3 ml, 2% paraformaldehyde and incubate for 15 min at room temperature.

10. Gently add 1 ml of digestion buffer and incubate for 30-60 min at room temperature.

11. Remove the digestion buffer completely by tilting the dishes and aspirating the buffer with a glass capillary pipette.

12. Terminate the digestion by gently adding 3 ml of extraction buffer and incubate for 5 min at 4°C.

13. Remove the extraction buffer and fix coverslip #3, 4, 5 by adding 3 ml, 2% paraformaldehyde, incubate for 15 min at room temperature.


It is essential that the DNase I is RNase-free.

Prepare at least 5 coverslips per sample. The first coverslip will be extracted only with Triton X-100. The second coverslip will be extracted with Triton X-100 followed by ammonium sulfate. The third and fourth coverslips will be extracted with Triton X-100, ammonium sulfate and digested with DNase I. The fourth coverslip is used as positive control (see below). The fifth coverslip is not extracted (control). It is generally convenient to use 6-well tissue culture plates when multiple samples need to be processed at the same time.

This also applies to transiently transfected cells.
If using suspension cells, approximately $10^6$ cells per extraction step will be adequate. Centrifuge the suspension cells at 650-1000 g for 5 min at 4°C between steps 3-13. Resuspend the cells at the each extraction step in the same volume of buffer as the adherent cells.

Incubate by simply placing the dishes on ice.

The duration of digestion and the necessary amount of DNase I will vary depending upon the cell type used and has to be optimized. A good starting point, particularly in mouse cell-lines (heterochromatin forms dense structural domains) is to use 500 units/ml DNase I and three different incubation times (15, 30 and 60 min) at room temperature. Monitor the release of chromatin by staining the cells with DAPI for fluorescence microscopy. DNase I treatment must lead to a complete loss of DAPI stain. If the digestion is insufficient, use 30 and 60 min digestions both at 32°C and 37°C.

For Western blot analysis, centrifuge the cells as above and measure the packed-cell volume. In order to extract proteins from the nuclear matrix, add 2-3 times the packed-cell volume of 5X SDS sample buffer to a final concentration of 1X SDS. Boil the samples for 5 min. Vortex or sonicate the samples (depending on the solubility of extract). Centrifuge the samples in a bench-top centrifuge at 16000 g for 10 min at room temperature.

It is very important to verify the integrity of nuclear matrix by processing coverslip #4 for indirect immunofluorescence microscopy. Use an antibody directed against one of the well-known matrix-associated proteins (e.g., NuMA). The hnRNPA1 and histone H1 proteins could be used as the negative controls for the detergent and high-salt extraction steps respectively.