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 $MgCl_2$, 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 $MgCl_2$, 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 $MgCl_2$, 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[a] DNase (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.[c]
3. Wash the cells twice with PBS at 4°C.[d]
4. Gently add 3 ml of CSK buffer to each dish and incubate for 10 min at 4°C.[e]
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.[f]
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.[g]
13. Remove the extraction buffer and fix coverslip #3, 4, 5 by adding 3 ml, 2% paraformaldehyde, incubate for 15 min at room temperature.
14. Process all samples for immunofluorescence microscopy.[h]

[a] It is essential that the DNase I is RNase-free.

[b] 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.

[c] This also applies to transiently transfected cells.

[d] 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.

[e] Incubate by simply placing the dishes on ice.

[f] 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.

[g] 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.

[h] 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.