

## MATERIALS AND METHODS

## Materials and methods

The following list of chemicals, reagents and equipment were used in this study.

Aprotinin, **DAPI**, DNase I, Digitonin, 3, 5-**diiodosalicylate** (lithium salt), DTT, PMSF, RNase A, single strand DNA cellulose, spermidine, spermine, thiodiglycol, triton-X 100, tween-20, trypsin, trasylol, molecular weight markers such as bovine albumin, egg albumin, carbonic anhydrase, chymotrypsinogen, **glyceraldehyde-3-phosphate** dehydrogenase,  $\alpha$ -**lactalbumin**, soyabean inhibitor, trypsinogen were purchased from Sigma chemical company, USA.

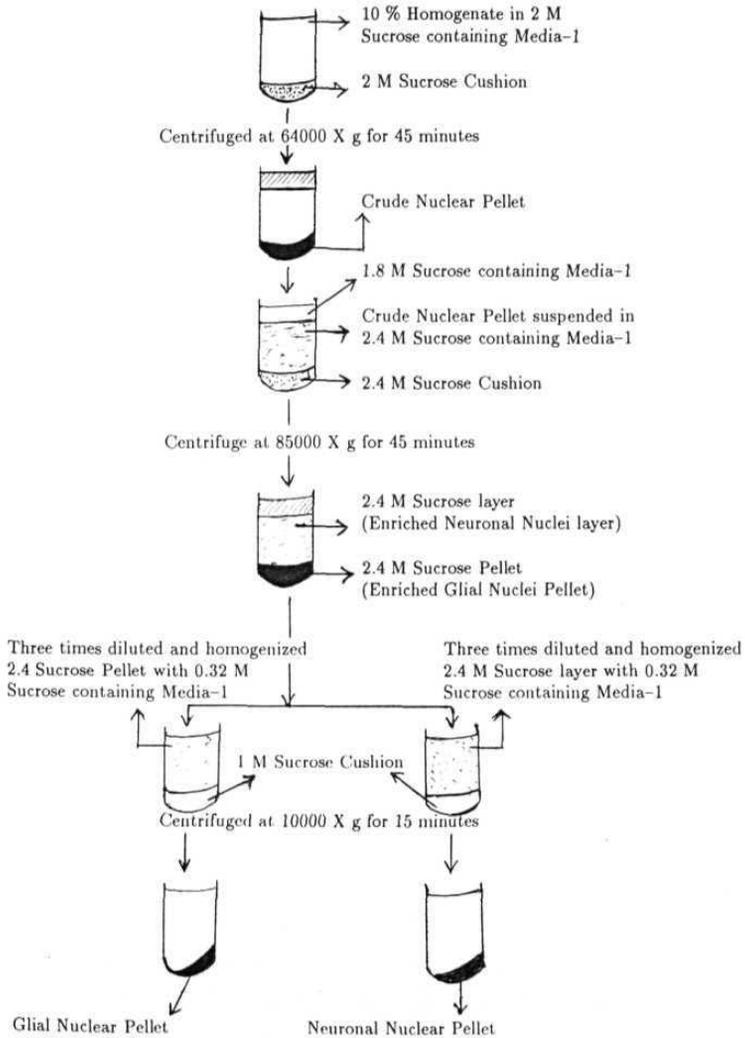
Acrylamide, agarose, coomassie brilliant blue,  $\beta$ -**mercaptoethanol**, N, N-**methylene-bis-acrylamide**, SDS, sucrose, Tris were obtained from Sisico Research Laboratories, India.

Tissue culture media and chemicals such as Nutrient mixture **F-10** HAM, Dulbecco's modified medium, MEM vitamins, pencillin, streptomycin, **gentamycin**, **L-glutamine**, sodium pyruvate, phenol red, horse serum, fetal bovine serum procured from Himedia Laboratories pvt. ltd, Bombay, India.

Nitrocellulose sheets, DEAE Cellulose were obtained from Whatman company. Ampholine, Surface pH electrode, High Voltage power supply, Fraction collector, Novablot unit were purchased from LKB, **Bromma**, USA. The **4-chloro-1-naphthol** was obtained from Pierce.

All the other chemicals and reagents were used in this study were of analytical grade, locally available.

**ISOLATION OF NEURONAL AND GLIAL NUCLEI FROM THE BRAIN**



Wistar strain albino rats were killed by decapitation, brain regions were rapidly removed and placed in chilled 0.32 M sucrose containing 1 mM **MgCl<sub>2</sub>**, and 0.1 mM **PMSF (Media-1)**. Nuclei were isolated by the method of Thompson (1973) with minor modifications. All the operations were performed at 4°C unless specified and all sucrose densities were prepared in media-1. Cerebral cortices were separated, homogenized to 20 % (w/v) in 2.0 M sucrose using Dounce tissue homogenizer with pestle 'B'. The homogenate was further diluted to 10 % with 2.0 M sucrose and filtered through one layer of muslin cloth. The filtrate was layered over a 2.0 M sucrose cushion and centrifuged at 64000 x g for 45 minutes using SW-28 rotor in **Beckman** ultracentrifuge. The pellet was suspended in 2.5 ml of 2.4 M sucrose and was layered on 1 ml cushion of 2.4 M sucrose (with this cushion, purity of glial nuclei will be increased). This gradient was further overlaid with 1.3 ml of 1.8 M sucrose in 4.8 ml tubes of SW-60 rotor and centrifuged at 85000 x g for 45 minutes, which resulted in three fractions

- a) 1.8 M sucrose layer (contains lipids, broken nuclei and membranes) was removed with pasture pipette and discarded.
- b) 2.4 M sucrose cushion overlay (enriched neuronal nuclei)
- c) 2.4 M sucrose pellet (enriched glial nuclei)

Both the neuronal and glial enriched fractions were diluted separately with two volumes of 0.32 M sucrose, layered over 1.0 M sucrose cushion and centrifuged at 10000 x g for 15 minutes to obtain pure neuronal and glial nuclear pellets. Purity and integrity of nuclei was checked by 4, 6-diamidino-phenyl indole (**DAPI**) fluorescence and phase

contrast microscopy. The nuclei were stored at  $-20^{\circ}\text{C}$  till further use in 0.32 M sucrose prepared in isolation buffer (IB) (3.75 mM Tris-HCl pH 7.4, 0.05 mM spermine, 0.0125 mM spermidine, 1 % thiodiglycol, 20 mM KCl and 0.1 mM PMSF).

## Cancer cell lines-cell culture

All the cell lines were obtained from **NFATCC**, Pune. The following cancer cell lines were grown in 25 cm<sup>2</sup> T-flasks with their respective medium and conditions described separately. All the cell lines were maintained in logarithmic growth in CO<sub>2</sub> incubator.

### C6 glioma cells

Rat C6 glioma cells (39 th passage ; C6 glioma was cloned from a rat glial brain tumor induced by N-nitroso-methylurea) were maintained under standard tissue culture conditions ( $37^{\circ}\text{C}$ , 5 % CO<sub>2</sub> and 95 % humid atmosphere) in 82.5 % nutrient mixture F-10 (HAM) medium supplemented with 15 % horse serum, 2.5 % fetal bovine serum and 0.015 mg / ml phenol red, 10 mM sodium pyruvate, 2 mM L-glutamine, 1 % (v/v) MEM Vitamins, 1 % (v/v) non-essential amino acids, 100 U / ml penicillin, 0.1 mg / ml streptomycin and 0.03 mg/ml gentamycin.

### Neuro-2A

Neuronal cell types and small round stem cells containing neuroblastoma were established from a spontaneous tumor of strain A albino mouse. The 172 passage of this neuroblastoma was cultured in minimum essential medium with non-essential amino acids and BSS (90 %), fetal bovine serum (10 %) and 0.015 mg / ml phenol red, 10 mM sodium pyruvate,

2 mM L-glutamine, Vitamins, 100 U / ml pencillin, 0.1 mg / ml streptomycin and 0.03 mg / ml **gentamycin** under standard tissue culture conditions (37°C, 5 % CO<sub>2</sub> and 95 % humid atmosphere).

### **IMR-32**

It is a mixture of two morphologically distinct cell types (a small neuroblast-like cell and a large hyaline fibroblast) and established from an abdominal mass occurring in a 13 month old male Caucasian. The 52 passage of this neuroblastoma was cultured in minimum essential medium with non-essential amino acids and BSS (90 %), fetal bovine serum (inactivated) 10 %, 0.015 mg / ml phenol red, 10 mM sodium pyruvate, 2 mM L-glutamine, 1 % (v/v) MEM vitamins, 100 U / ml pencillin, 0.1 mg / ml streptomycin and 0.03 mg / ml gentamycin under standard tissue culture conditions (37°C, 5 % CO<sub>2</sub> and 95 % humid atmosphere).

### **SK-N-SH**

Epithelial like human neuroblasoma, metastasis to bone marrow cell line, has neurogenic origin, exhibits a large doubling time and high levels of **dopamine- $\beta$ -hydroxylase**. The cell line was maintained in Dulbecco's modified medium with non-essential amino acids and Earle's BSS (90 %), fetal bovine serum (10 %), 0.015 mg / ml phenol red, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 % (v/v) MEM Vitamins, 100 U / ml pencillin, 0.1 mg / ml streptomycin and 0.03 mg / ml gentamycin under standard tissue culture conditions (37°C, 5 % CO<sub>2</sub> and 95 % humid atmosphere).

### **U-373MG**

Epithelial like human glioblastoma, astrocytoma grade III cell line derived from a malignant **glioma** brain. The cell line was maintained in

Dulbecco's modified medium with non-essential amino acids and earle's BSS (90 %), fetal bovine serum (10 %), 0.015 mg / ml phenol red, 10 mM sodium pyruvate, 2 mM L-glutamine, 1 % (v/v) MEM vitamins, 100 U / ml penicillin, 0.1 mg / ml streptomycin and 0.03 mg/ml gentamycin under standard tissue culture conditions (37°C, 5 % CO<sub>2</sub> and 95 % humid atmosphere).

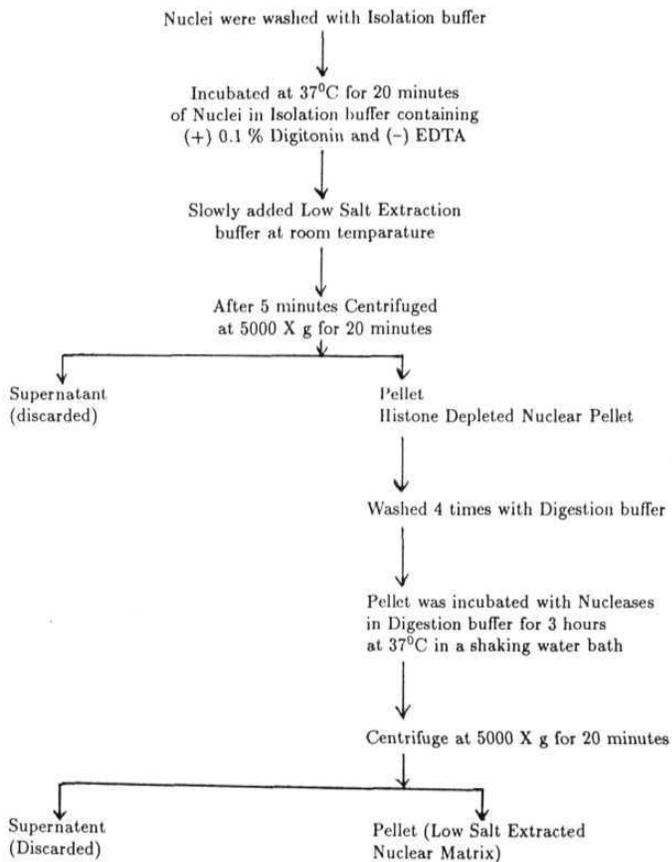
The cell lines, as soon as they reached **confluency** were passaged. The cells were given fresh medium for every three days. The monolayer cells were detached by trypsinization (0.1 % trypsin for 5 minutes). Following detachment, the trypsin was inactivated by adding 10 ml of medium containing serum. Cells were harvested by centrifugation at 1000 g for 10 minutes, followed by washing with medium. The cell pellet was suspended in less volume of medium and cell number was counted using a haemocytometer.

### Isolation of nuclei from cancer cell lines

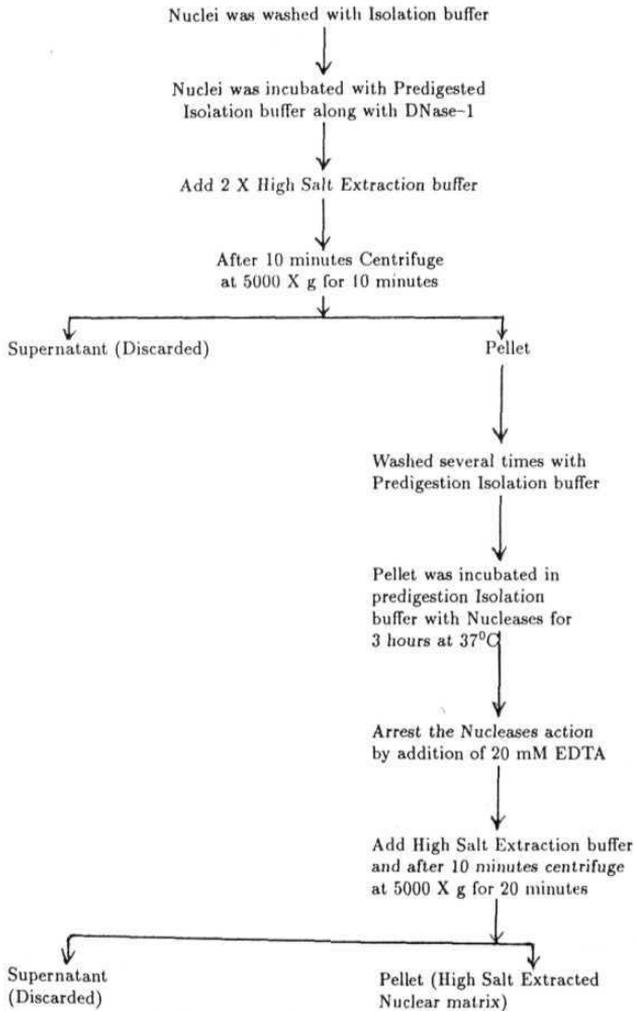
Cells were collected by centrifugation at 4000 x g for 10 minutes. The cell pellet was washed three times with IB and suspended in IB containing 0.1 % digitonin. The cells were homogenized manually in Dounce tissue **homogenizer** using pestle 'B' with 20 up and down strokes to get nuclei from the cells. The crude nuclei were collected by centrifugation at 3000 g for 10 minutes as a pellet. The crude nuclear pellet was resuspended in 2.0 M sucrose and the suspension was overlaid on 2.0 M sucrose cushion and centrifuged at 64,000 x g for 45 minutes in SW-60 rotor in Beckman Ultracentrifuge to get pure nuclei.

The neuronal, **glial** and cancer cell nuclear pellets were washed twice with IB separately and were suspended finally in IB. Optical density of the nuclei was measured at 260 **nm**.

## NUCLEAR MATRIX EXTRACTED BY LOW SALT METHOD



## NUCLEAR MATRIX PREPARATION BY HIGH SALT METHOD



## Preparation of nuclear matrix

Nuclear matrices were prepared by two ways (1) Low salt method (LSM) (2) High salt method (**HSM**) following the method of Mirkovitch *et al.*, (1984) with minor modifications.

### (1) Low salt method (LSM)

Ten O.D 260 units of nuclei were incubated at 37°C for 20 minutes in 100  $\mu$ l of IB containing 0.1 % digitonin. To this 7 ml of low salt extraction buffer (LSEB) [(0.5 mM Hepes / NaOH pH 7.4, 0.25 mM spermidine, 2 mM EDTA / KOH pH 7.4, 2 mM KCl, 0.1 % Triton X-100, 25 mM 3-5 lithium diiodosalicylic acid] was slowly added. After 5 minutes, histone depleted nuclear pellets were recovered by centrifugation at 3000 x g for 20 minutes in a microfuge. The nuclear pellet was washed four times with digestion buffer (DB) (20 mM Tris-HCl pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 0.02 M KCl, 70 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.1 % Triton X-100, 100 KIU / ml trasylol and 0.1 mM PMSF). The final pellet was incubated at 37°C for 3 hours in shaking water bath with DB containing both DNase 1 and RNase A (each 150  $\mu$ g / ml concentration). The nuclease activity was terminated by adding EDTA to a final concentration of 20 mM and nuclear matrices were pelleted by centrifugation at 3000 x g for 10 minutes. The pellet was designated as nuclear matrix of low salt method (NM-LSM).

### (2) High salt method (HSM)

Five O.D 260 units of nuclei were suspended in 300  $\mu$ l of 4X predigested isolation buffer (**PIB**) (predigestion was done by adding of 10 mM MgCl<sub>2</sub>, 50 mM NaCl and 250  $\mu$ g / ml DNase 1 to the IB and incubated at 37°C for 4 hours) containing 0.1 % Triton X-100 and 0.1 % digitonin. After

10 minutes equal volume of 2X high salt extraction buffer (HSEB) (2.0 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM PMSF, 0.2 mM MgCl<sub>2</sub>, 0.02 mM DTT and 0.4 % Triton X-100) was added and centrifuged at 3,000 x g for 10 minutes. The pellet was washed three times with PIB and was finally incubated for 3 hours at 37°C in 2 ml PIB containing both DNase I and RNase A (each 150  $\mu$ g / ml). Nuclease action was terminated by adding EDTA to a final concentration of 20 mM in a equal volume of 1X HSEB. After 5 minutes, nuclear matrices were recovered by centrifuging at 3,000 g for 10 minutes. The pellet was designated as nuclear matrix of high salt method (NM-HSM).

### Extraction of proteins from the nuclei and nuclear matrix

Ten O.D of 260 units of nuclei were incubated in 100  $\mu$ l of the TMN buffer [10 mM Tris-HCl pH 7.4, 10 mM MgCl<sub>2</sub>, DNase I (50  $\mu$ g / ml), RNase A (50  $\mu$ g / ml) and aprotinin (5  $\mu$ g / ml)] for 2 hours at 37°C before extracting the proteins.

Total proteins were extracted from the nuclei (after incubation with TMN buffer) and nuclear matrix in 5 ml of TUMP buffer (50 mM Tris-HCl pH 7.5, 5 M urea, 5 mM MgCl<sub>2</sub> and 1 mM PMSF) by homogenizing with pestle 'A' in Dounce homogenizer. The supernatant containing proteins was recovered by spinning at 10,000 x g for 10 minutes and was dialysed against the TMP buffer (10 mM Tris-HCl pH 7.5, 7 mM  $\beta$ -mercaptoethanol and 1 mM PMSF). The dialysate was further centrifuged at 10000 x g for 10 minutes and the clear supernatant containing proteins was used for further analysis.

## Scanning electron microscopic studies

Nuclei and nuclear matrix pellets were fixed in 4 % glutaraldehyde for 90 minutes. They were washed with phosphate buffer saline (10 mM sodium phosphate buffer pH 7.4 and 150 mM NaCl) twice (at 1000 x g for 5 minutes) and final pellets were suspended in 1 % ammonium molybdate solution. This suspension was used for scanning in scanning electron microscope Joel **JSM-35** after gold vapor coating. Photographs were taken at 20 KV with a 60 $\mu$  objective aperture in a Joel JSM-35 scanning electron microscope equipped with a roll film camera.

## Protein estimation

The concentration of protein in different samples was estimated by Lowry *et al.*, (1951) method using bovine serum albumin as standard.

## DEAE-cellulose chromatography

The protein samples were dialysed extensively against equilibration buffer (20 mM Hepes pH 7.4, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 5 % glycerol and 0.1 mM PMSF) containing 50 mM NaCl. Preswollen DEAE was suspended in the same buffer and a 10 ml column was packed, washed and equilibrated with the same buffer. The dialysed protein sample was loaded onto the column. The flow through (unbound proteins) was collected and the column was washed with minimum 6-8 bed volumes of equilibration buffer (or till the absorbance of the wash was zero). The bound proteins were eluted with a stepwise gradient of NaCl (0.2, 0.4, 0.6, **0.8**, 1.0, 1.5, 2.0 M) in equilibration buffer and 2 ml fractions were collected using a LKB-fraction collector. The optical density of the fractions was measured at 280 nm and peak fractions were analyzed by electrophoresis (SDS-PAGE).

## HPLC analysis of the proteins

HPLC analysis was performed in Shimpac PA-DEAE column of Shimadzu SCL-6AV. The protein sample was dialysed extensively against  $\text{Na}_2\text{HPO}_4$  - Citric acid buffer (9 : 1 mix). The dialysed samples were centrifuged (at 10,000 x g for 15 minutes) and the clear supernatant was used for HPLC analysis. Before loading sample (0.2 ml) to the column, it was equilibrated with  $\text{Na}_2\text{HPO}_4$  - Citric acid buffer. The bound proteins were eluted by binary linear gradient of  $\text{Na}_2\text{HPO}_4$  - Citric acid buffer. The peak fractions were collected and analysed by electrophoresis.

## single strand DNA-cellulose chromatography

The protein sample was dialysed against TEDP buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT and 0.1 mM PMSF) containing 50 mM NaCl. The ssDNA-cellulose was suspended in TEDP buffer and left for 2 hours, further equilibrated with TEDP buffer containing 50 mM NaCl. A 5 ml column was packed and equilibrated with the same buffer. The dialysed protein sample was loaded on to the column, flow through (unbound proteins) was collected and washed with minimum 6-8 bed volumes of TEDP buffer containing 50 mM NaCl. The DNA binding proteins were eluted by a stepwise gradient of NaCl (0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 M) in TEDP buffer and 1 ml fractions were collected using LKB-fraction collector. The optical density of the fractions was measured at 280 nm and were analysed by electrophoresis.

## Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

### Sample preparation for slab electrophoresis

Nuclei and / or nuclear matrix proteins in TMP buffer were precipi-

tated by adding 5 volumes chilled acetone, kept overnight at  $-70^{\circ}$  and centrifuged at  $10,000 \times g$  for 5 minutes in a microfuge. The protein pellets were dissolved in SDS sample buffer (8 % sucrose, 1 mM DTT, 0.015 % bromophenol blue and 1 % SDS) and heated at  $100^{\circ}\text{C}$  for 5 minutes, and allowed to cool for 5 minutes on ice. The samples were cleared by centrifugation at  $10,000 \times g$  for 5 minutes and the clear supernatant with known quantity of protein was loaded on to the gel.

Proteins were separated by electrophoresis in 18 % polyacrylamide gels as described by Thomas and **Khornberg** (1975) with minor modifications as suggested by Reddy and Suryanarayana (1989). In brief, the modifications include :

1. the **bis-acrylamide** concentration in the resolving gel was decreased from 0.4 M to 0.2 M.
2. Tris concentration in the resolving buffer was increased from 375 mM to 750 mM and the pH was also increased from 8.8 to 9.1.
3. glycine concentration in the running buffer was reduced to the half of the original concentration (from 380 mM to 190 mM).

Electrophoretic runs were carried out in the slab gel of 0.1 cm thick and 16 cm long at 170 Volts for approximately 4 hours or till the dye front reaches to the bottom.

### Staining with coomassie blue

After electrophoresis the gels were removed carefully and fixed in 7.5 % glacial acetic acid for 20 minutes on a shaker. The proteins in the gel were stained with staining solution (7.5 % glacial acetic acid, 50 % **methanol**

and 0.01 % coomassie brilliant blue R-250) for 1 hour and destained with the destaining solution (7.5 % glacial acetic acid and 5 % methanol) with several changes on the shaker.

## Two-dimensional electrophoresis (2DE) [IEF-SDS-PAGE

### Sample preparation for isoelectrofocusing (IEF)

Nuclei and / or nuclear matrix proteins in TMP buffer were concentrated by acetone precipitation. The protein in the sample was solubilized in 40  $\mu$ l of sample lysis buffer [9.5 M urea, 2 % ampholines (1.6 % of 5-7, 0.4 % of 3-10.5), 2% NP-40 and 5 %  $\beta$ -mercaptoethanol and 2 % SDS] by swirling at room temperature, followed by one freezing and thawing cycle. The IEF protein mix was centrifuged at 10,000 x g for 5 minutes and the clear supernatant was loaded on to the IEF gel.

Two-dimensional electrophoresis was done according to O'Farrel (1975) with minor modifications. In the first dimension, IEF tube gel was pre-focused by applying the voltage ; 200 V for 15 minutes, 300 V for 30 minutes and 400 V for 30 minutes. After prefocussing the samples were loaded and the gels were run for 9000 volt-hours [600 V for 12 hours, 700 V for one and half hour and 750 V for last one hour]. The IEF gels were removed and equilibrated in a equilibration buffer (1 % glycerol, 5 raM DTT, 2.3 % SDS and 0.625 M Tris-HCl pH 6.8) for half an hour to remove ampholines. The tube gels were transferred on to second dimension run (SDS-PAGE) with the stacking gel (4.75 % acrylamide and bis-acrylamide) over the resolving gel (11.27 % acrylamide and bis-acrylamide). The contact between IEF tube gel and second dimension slab gel was established by embedding with a hot solution of 1 % (w/v) agarose containing 0.0025 % (w/v) bromo-phenol blue (BPB). The second dimension run was performed at

15 mA till the tracking BPB dye enters into the separating gel, then at 20 mA till the dye reaches to the bottom. After completion of second dimension, gels were silver stained to identify the proteins.

### **Silver staining**

Proteins were visualized by a sensitive silver staining procedure (Blum *et al.*, 1987) with minor modifications. After completion of the electrophoresis the stacking gel was removed and the separating gel was fixed in the fixing solution (50 % methanol, 12 % glacial acetic acid) for 1 hour on a shaker with gentle shaking and transferred into 50 % ethanol for 1 hour. The gels were treated with sodium thiosulphate (0.2 g / liter) for 1 minute and washed with distilled water for 1 minute, including three changes for every 20 seconds. Further, the gels were impregnated with silver nitrate (1 g / liter) containing 0.75 ml of 37 % formaldehyde for 30 minutes. After washing the gels with distilled water for 1 minute, the protein spots were developed with sodium carbonate (60 g / liter) containing 0.5 ml of 37 % formaldehyde per liter. After the protein spots developed, the gels were washed with distilled water and placed in fixing solution for 1 hour. The gels were immediately photographed and stored in 50 % ethanol.

### **Identification of proteins**

In 2DE the proteins were identified basing on their molecular weight (Mr) and isoelectric point (p.I).

#### **1. Determination of Molecular weight of the proteins**

Molecular weight of the proteins was determined based on their relative co-migration along with known protein standard markers (bovine albumin-66000 ; egg albumin-45000 ; glyceraldehyde-3- Phosphate

dehydrogenase-36000 ; carbonic anhydrase-29000 ; chymotrypsinogen-25000 ; trypsinogen-24000 ; soyabean trypsin inhibitor-20100 ;  $\alpha$ -lactalbumin-14200).

## 2. Determination of the Isoelectric point of Protein

Isoelectric point of a specific protein was determined by comparison with pH gradient observed in the IEF tube gel with surface pH electrode (LKB-Producter) as well as gel extrusion into deionized water. The IEF gel was cut into the equal size (0.5 cm length) pieces and each gel piece was placed in 1 ml distilled water and allowed to stay overnight with gentle shaking. The pH extruded from the gel into the distilled water was measured with a pH meter. A standard graph was drawn by plotting gel length on X-axis and pH on the Y-axis.

## Production of polyclonal antibodies

Polyclonal antibodies to Nuclear matrix proteins were raised according to Stoffler and Wittmann (1971) in albino rabbits. Nuclear matrix protein (1 mg / ml) present in TMP buffer was emulsified with equal volume of the Freund's complete adjuvant and injected subcutaneously into rabbit at multiple sites. After 4 weeks, booster injections each of 0.5 mg / ml of protein in Freund's incomplete adjuvant were given subcutaneously at 4 th, 5 th and 6 th weeks. Rabbits were bled after 3 rd booster injection through the pinna vein. Serum was collected after centrifugation of the clotted blood.

## Immunodiffusion

Immunodiffusion (Ouchterlony) was performed as described by Stoffler and Wittmann (1971). Agarose (1.5 %) (w/v) was dissolved in 0.9 % NaCl and 19 mM sodium barbital buffer pH 8.4 by heating in boiling water bath

for 45 minutes. This was poured into immunodiffusion plates to a height of 0.3 cm and allowed to cool at room temperature. After cutting the wells, the test samples and antisera were placed in respective wells. The diffusion was allowed to take place for 12 hours at room temperature in moisture containing box. The immunodiffusion plates were photographed against dark background with scattering light. The immunodiffusion gels were extensively washed with 0.09 % NaCl and finally with water. The gels were dried and stained with coomassie brilliant blue as in SDS-PAGE gels.

### Western blotting

Western blotting was performed according to Towbin *et al.*, (1979) with minor modifications. The nuclear matrix proteins were electrophoresed in the gel and transferred on to a nitrocellulose sheet (NC) (0.45  $\mu$  pore size) using TGM buffer (25 mM Tris-HCl pH 8.3, 0.192 M glycine and 20 % methanol) as a electrode buffer at 0.8 mA / cm<sup>2</sup> transunit (total gel length x breadth) in LKB-Multiphore II Nova blot for 2 hours. The electroblots were washed with TNT buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1 % Tween-20) for 10 minutes and blocked with blocking buffer (5 % skimmed dry milk in TNT buffer) for 1 hour. The NC sheet was then incubated with 2 % primary antibody in blocking buffer for 2 hours with gentle shaking and washed six times each for 10 minutes with TNT buffer. The blots were incubated with secondary antibody 1 : 2000 dilution of peroxidase conjugated anti-rabbit IgG in blocking buffer for 2 hours followed by washing six times each for 10 minutes with TNT buffer. For the colour reaction, the blots were soaked in TNHC buffer (10 mM Tris-HCl, 150 mM NaCl, 0.03 % hydrogen peroxide and 0.275 mg of 4-chloro-1-naphthol (4C1N). The reaction was terminated by washing the blot with water.