2-D Gel Electrophoresis Protocol

I. Sample preparation (analytical gels)

Sample preparation and solubilization are crucial factors for the overall performance of the 2-D PAGE technique. Protein complexes and aggregates should be completely disrupted in order to avoid appearance of new spots due to a partial protein solubilization. The SWISS-2DPAGE samples were prepared as followed:

Human samples

CEC (Colon epithelial cell): Within a maximum of 30 min, a large right or left colon tissue sample (5 x 7 cm) was prepared on ice in the operating room, rinsed with cooled phosphate buffer saline, pH 7.2 to further remove cell debris and blood and then immersed into an iced PBS solution containing 3 mM EDTA, 50 µg/ml leupeptine, 0.2 mM PMSF and 0.8 mM benzamidine. Crypts were scraped away from the basal membrane with a scalpel. The tissue was then gently pressed through a mesh with a pore size of 300 µm to separate epithelial cells from stroma and filtered through a nylon mesh with 200 µm pores. After centrifugation at 350 g at 4o C for 10 min, the membranes were permeabilized in 70 % chilled ethanol and the cells were shaken overnight at 4°C. After washing with PBS, the cells were incubated with fluorescein-conjugated anticytokeratin antibodies (CAM 5.2) for 45 min and then sorted by FACS. The pellet was mixed with 100 µl per 10^6 cells of a solution containing urea (8 M), CHAPS (4 % w/v), DTE (65 mM), Tris (40 mM) and a trace of bromophenol blue. 100 µl of the final diluted colon epithelial cell sample was loaded onto the IPG gel strip.

CSF (cerebrospinal fluid): An aliquot of 250 µl of human CSF was mixed with 500 µl of ice cold acetone and centrifuged at 10000 g at 4°C for 10 minutes. The pellet was mixed with 10 µl of a solution containing SDS (10 % w/v) and DTE (2.3 % w/v). The sample was heated to
95°C for 5 minutes and then diluted to 60 µl with a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted CSF sample (45 µg) was loaded on the first dimensional separation.

ELC (erythroleukemia cell line): A monolayer culture of human ELC was grown, rinsed trypsinized and washed as explained in the HEPG2 sample preparation. A pellet of 0.8 x 10_6 cells were mixed and solubilized with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted ELC sample was loaded on the first dimensional separation.

HEPG2 (hepatoblastoma carcinoma derived cell line): A monolayer culture of human hepatoblastoma carcinoma derived cell line was grown in Dulbecco's modified Eagle medium (DMEM) containing 10 % fetal calf serum (FCS). Cells were rinsed once with DMEM without FCS and removed from the flask by incubating them with a solution containing trypsin (0.5 g/l) and EDTA (0.2 g/l). After 3 minutes, DMEM containing FCS was added into the flask to stop the action of the trypsin. The cells were detached from the surface of the flask by squirting the solution onto the cells. The suspension was transferred into a tube and the cells were centrifuged at 1000 g during 5 minutes. Supernatant was discarded and the cells were washed with DMEM without FCS. After centrifugation and removal of DMEM, 0.8 x 10^6 cells were mixed and solubilized with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted HEPG2 sample was loaded on the first dimensional separation.

HEPG2SP (hepatoblastoma carcinoma derived cell line secreted proteins): 5 ml of supernatant HEPG2 culture media were concentrated down to 30 µl in a MicrosepTM Concentrators. The concentrated sample was mixed with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted HEPG2SP sample was loaded on the first dimensional separation.

HL60 (promyelocytic leukemia cells): A monolayer culture of a human promyelocytic leukemia cell line was grown in Dulbecco's modified Eagle medium (DMEM) containing 10 % fetal calf serum (FCS). Cells were rinsed once with DMEM without FCS and removed from the flask by incubating them with a solution containing trypsin (0.5 g/l) and EDTA (0.2 g/l). After 3 minutes, DMEM containing FCS was added into the flask to stop the action of the trypsin. The cells were detached from the surface of the flask by squirting the solution onto the cells. The suspension was transferred into a tube and the cells were centrifuged at 1000 g during 5 minutes. Supernatant was discarded and the cells were washed with DMEM without FCS. After centrifugation and removal of DMEM, 0.8 x 10^6 cells were mixed and solubilized with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted HL60

sample was loaded on the first dimensional separation.

Kidney: Tissue resections were washed several times in cold rinse buffer (glutamine-free RPMI 1640 medium containing 5 % fetal calf serum, 0.2 mM phenylmethylsulfonyl fluoridem, 1mM EDTA, and antibiotics: oxacillin 25 µg/ml, gentamycin 50 µg/ml, penicillin 100 U/ml, streptomycin 100 µg/ml, amphotericin B 0.25 µg/ml, nistatin 50 U/ml) to further remove cell debris and blood, and were frozen by immersion in liquid nitrogen. They were then submitted to mechanical dissociation by scraping and gentle squeezing. The cell suspension was washed several times in cold phosphate buffer saline, pH 7.2. The cellular pellet was denatured with 100 µl per 10^6 cells of a solution containing urea (8 M), CHAPS (4 % w/v), DTE (65 mM), Tris (40 mM) and a trace of bromophenol blue. 100 µl of the final diluted kidney cell sample was loaded onto the IPG gel strip.

Liver protocole 1: Five frozen slices (20 µm x 5 mm x 10 mm) of a human liver biopsy were mixed with 300 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. 60 µl (45 µg) of the final diluted liver sample was loaded on the first dimensional separation.

Liver protocole 2: 10 mg of a human liver frozen biopsy was lyophillized for 48 h. It was then crushed in a mortar containing liquid nitrogen and mixed with 1.5 ml of a solution containing urea (8 M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. 60 µl (100 µg) of the final diluted liver sample was loaded on the first dimensional separation.

Lynphoma: Five frozen slices (20 µm x 5 mm x 10 mm) of a human lymphoma biopsy were mixed with 300 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. 60 µl (45 µg) of the final diluted lymphoma sample was loaded on the first dimensional separation.

Plasma: An aliquot of 6.25 µl of human plasma was mixed with 10 µl of a solution containing SDS (10 % w/v) and DTE (2.3 % w/v). The sample was heated to 95°C for 5 minutes and then diluted to 500 µl with a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. 60 µl (45 µg) of the final diluted plasma sample was loaded on the first dimensional separation.

Platelet: Fresh blood has been centrifuged at 1000 g for 5 minutes. The supernatant (platelet-rich plasma: RPR) was then centrifuged at 5000 g for 10 minutes. 106 washed platelets were suspended

in 500 µl of lysis buffer containing Tris-HCl pH 8.0 (10 mM), $MgCl_2$ (1.5 mM), KCl (10 mM), DTE (0.5 mM) and PMSF (0.5 mM) and incubated on ice for 10 minutes. Mechanical lysis was achieved with a potter homogenizer and the resulting lysate was centrifuged at 3000 g for 10 minutes. Then 1/10 volume of a solution containing Tris-HCl pH 8.0 (0.3 M), KCl (1.4 M) and $MgCl_2$ (30 mM) was added to the supernatant and ultracentrifuged at 54000 g for 2 hours. The supernatant was diluted with 3 volumes of distilled water to decrease the salt concentration and then concentrated down to 30 µl in a Microsep™ Concentrator. The concentrated protein sample was mixed and solubilized with 70 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted platelet sample was loaded on the first dimensional separation.

RBC (red blood cells): Fresh blood was centrifuged at 2500 g at 4°C for 10 minutes. Plasma and buffy coat were removed and RBC were washed three times with the same volume of PBS pH 7.4. An aliquot of 7 µl of RBC was mixed with 483 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. 40 µl (45 µg) of the final diluted RBC sample was loaded on the first dimensional separation.

U937 (macrophage like cell line): A monolayer culture of human U937 was grown at a concentration of 0.5 million/ml in RPMI 1640 containing 1 % FCS. The cells were then rinsed, trypsinized and washed as explained in the HEPG2 sample preparation. After centrifugation and removal of RPMI, 0.8 x 10^6 cells were mixed and solubilized with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted U937 sample was loaded on the first dimensional separation.

Mouse samples

BAT (Brown adipose tissue): 400 µg of dried brown adipose tissue was mixed with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM), SDS (0.05 % w/v) and a trace of bromophenol blue. The whole final diluted sample (150 µg) was loaded in a cup at the cathodic end of the IPG gels.

Islet (Pancreatic islet cells): 200 µg of of small and large pancreatic islets were mixed with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM), SDS (0.05 % w/v) and a trace of bromophenol blue. The whole final diluted sample (100 µg) was loaded in a cup at the cathodic end of the IPG gels.

Liver: 200 µg of dried liver was mixed with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM), SDS (0.05 % w/v) and a trace of bromophenol blue. The whole final diluted sample (100 µg) was loaded in a cup at the cathodic end of the IPG gels.

Liver Nuclei (Soluble nuclear proteins and matrix from liver tissue): Nuclear proteins were solubilized in a buffer containing 5M urea, 2M thiourea, 2 % CHAPS (w/v), 2 % sulfobetains (w/v), 65mM DTE, 40mM Tris (pH 6.8), 0.8 % Resolyte 3.5-10 and a trace of bromophenol blue.

Muscle (Gastrocnemius muscle): 200 µg of dried gastrocnemius muscle was mixed with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM), SDS (0.05 % w/v) and a trace of bromophenol blue. The whole final diluted sample (100 µg) was loaded in a cup at the cathodic end of the IPG gels.

WAT (White adipose tissue): 16n mg of dried white adipose tissue was mixed with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM), SDS (0.05 % w/v) and a trace of bromophenol blue. The whole final diluted sample (150 µg) was loaded in a cup at the cathodic end of the IPG gels.

Other samples

Dicty (Dictyostelium discoideum): A WS380B wild type strain was used here. Slugs (0.9 mg dry weight) were resuspended in 40 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. 9 µl of this sample was diluted with 60 µl of the same solution. This whole final Dicty diluted sample was loaded onto the IPG gel strip.

E.coli. (Escherichia coli): Cells were grown aerobically in glucose minimal morpholinopropane sulfonate (MOPS), plus thiamine at 37°C. Growth was stopped in the late exponential phase at an OD of 1 at 600 nm. 500 ml of culture medium was centrifuged for 30 min at 3000 rpm at 4°C and the pellet was washed 4 times for 10 min at 4000 rpm in 10 ml low salt washing sample buffer: KCl 3.0 mM, KH_2PO_4 1.5 mM, NaCl 68 mM, NaH2PO4 9.0 mM. The pellet was then resuspended in 600 µl of a buffer containing 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTE, 0.5 mM Pefabloc SC (protease inhibitor), 0.1 % SDS, and stored at -20°C. 1 µl of the latter was mixed with 60 µl of a solution containing Urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. After centrifugation at 10000 g for 5 minutes, the whole final diluted E. coli sample was loaded onto the IPG gel strip.

Yeast (Saccharomyces cerevisiae): Cells were washed twice with PGSK (NH_2PO_4-H2O 0.52 g/l, Na_2HPO_4-2H2O 8.8 g/l, NaCl 2.83 g/l, KCl 0.372 g/l and glucose 11 g/l), centrifuged at 3000 rpm for 5 min (4°C) and the supernatant removed. The pellet was resuspended in 1 volume of PGSK and 1 volume of glass beads (425-600 µm diameter) and shaked for 10 min at 4°C in a bead beater. The extracts were centrifuged at 3000 rpm for 10 min at 4°C, the supernatant retained and the pellet subjected these procedures a second time. The supernatants were pooled and 100 µg (measured by modified Lowry) of yeast proteins was mixed with 60 µl of a solution containing urea (8 M), CHAPS (4 % w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole yeast diluted sample was loaded onto the IPG gel strip.

II. Immobilized pH gradient (IPG) as first dimension

A non-linear immobilized pH gradient (3.5-10.0 NL IPG 18 cm) was used as the first dimension. It offered high resolution, great reproducibility and allowed high protein loads. Based on our specifications, the non-linear pH gradient strips were prepared by Pharmacia-Hoeffer Biotechnology AB and are commercially available. The strips were 3 mm wide and 180 mm long.

IPG gel strips rehydration

Hydration was performed overnight in the Pharmacia re-swelling cassette with 25 ml of a solution containing urea (8 M), CHAPS (2 % w/v), DTE (10 mM), Resolyte pH 3.5-10 (2 % v/v) and a trace of Bromophenol Blue.

Sample application

When the rehydration cassette had been thoroughly emptied and opened, the strips were transferred to the Pharmacia strip tray. After placing IPG strips, humid electrode wicks, electrodes and sample cups in position, the strips and cups were covered with low viscosity paraffin oil. Samples were applied at the cathodic end of the IPG strips in a slow and continuous manner, without touching the gels.

Running conditions

The voltage was linearly increased from 300 to 3500 V during 3 hours, followed by 3 additional hours at 3500 V, whereupon the voltage was increased to 5000 V. A total volt hour product of 100 kvh was used in an overnight run.

IPG gel strips equilibration

After the first dimension run the strips were equilibrated in order to resolubilize the proteins and to reduce -S-S- bonds. The strips were equilibrated within the strip tray with 100 ml of a solution containing Tris-HCl (50 mM) pH 8.4, urea (6 M), glycerol (30 % v/v), SDS (2 % w/v) and DTE (2 % w/v) for 12 min. -SH groups were subsequently blocked with 100 ml of a solution containing Tris-HCl (50 mM) pH 6.8, urea (6 M), glycerol (30 % v/v), SDS (2 % w/v), iodoacetamide (2.5 % w/v) and a trace of Bromophenol Blue for 5 min.

III. SDS-PAGE as second dimension

In second dimension, a vertical gradient slab gel with the Laemmli-SDS-discontinuous system was used with some small modifications.

1. Gels are not polymerized in the presence of SDS. This seems to prevent the formation of micelles which contain acrylamide monomer, thus increasing the homogeneity of pore size and reducing the concentration of unpolymerized monomer in the polyacrylamide. The SDS used in the gel running buffer is sufficient to maintain the necessary negative charge on proteins.

2. Piperazine diacrylyl (PDA) is used as crosslinker. We believe this reduces N-teminal protein blockage, gives better protein resolution, and reduces diamine silver staining background.

3. Sodium thiosulfate is used as an additive to reduce background in the silver staining of gels.

4. The combination of the IPG strip and agarose avoids the need for a stacking gel. In addition, the gels were cast with the Angelique system from LargeScaleBiology, which it is an efficient and easy to use PC control equipment that allowed to cast simultaneously 10 to 60 gels.

Gel composition and dimension

•Dimension: 160 x 200 x 1.5 mm
•Resolving gel: Acrylamide/PDA (9-16 % T / 2.6 % C)
•Stacking gel: No stacking
•Leading buffer: Tris-HCl (0.375 M) pH 8.8
•Trailing buffer: Tris-glycine-SDS (25 mM-198 mM-0.1 % w/v) pH 8.3
•Additives: Sodium thiosulfate (5 mM)
•Polymerization agents: TEMED (0.05 %) , APS (0.1 %)

The gels were poured until 0.7 cm. from the top of the plates and overlayered with sec-butanol for about two hours. After the removal of the overlay and its replacement with water the gels were left overnight.

IPG gel strips transfer

After the equilibration, the IPG gel strips were cut to size. Six mm were removed from the anodic end and 14 mm from the cathodic end. The second dimension gels were overlayered with a solution containing agarose (0.5 % w/v) and Tris-glycine-SDS (25 mM-198 mM-0.1 % w/v) pH 8.3 heated at about 70°C and the IPG gel strips were immediately loaded through it.

Running conditions

Current: 40 mA/gel (constant)

Voltage: The voltage is non-limiting, but usually requires 100 to 400 V.

Temperature: 8-12°C

Time: 5 hours

IV. Protein detection

The application of the 2-D PAGE technology to separate, analyse and characterize proteins contained in biological samples would not have been possible without the development of complementary detection methods. Perhaps today one of the most popular non-radioactive protein detection is the silver staining which is 100-fold more sensitive than Coomassie Brilliant Blue staining. The different masters shown in SWISS-2DPAGE were stained with the ammoniacal silver staining as followed:

Silver staining protocol

All steps were performed on an orbital shaker at 36 rpm.

1. At the end of the second dimension run, the gels were removed from the glass plates and washed in deionized water for 5 min.

2. Soaked in ethanol: acetic acid: water (40: 10: 50) for 1 hour.

3. Soaked in ethanol: acetic acid: water (5: 5: 90) for 2 hours or overnight.

4. Washed in deionized water for 5 min.

5. Soaked in a solution containing glutaraldehyde (1 %) and sodium acetate (0.5 M) for 30 min.

6. Washed 3 times in deionized water for 10 min.

7. In order to obtain homogeneous dark brown staining of proteins, gels were soaked twice in a 2,7 naphtalene-disulfonic acid solution (0.05 % w/v) for 30 min.

8. Rinsed 4 times in deionized water for 15 min.

9. Gels were stained in a freshly made ammoniacal silver nitrate solution for 30 minutes. To prepare 750 ml of this solution, 6 g of silver nitrate were dissolved in 30 ml of deionized water, which was slowly mixed into a solution containing 160 ml of water, 10 ml of concentrated ammonia (25 %) and 1.5 ml of sodium hydroxide (10 N). A transient brown precipitate might form. After it cleared, water was added to give the final volume.

10. After staining, the gels were washed 4 times in deionized water for 4 min.

11. The images were developed in a solution containing citric acid (0.01 % w/v) and formaldehyde (0.1 % v/v) for 5 to 10 min.

12. When a slight background stain appeared, development was stopped with a solution containing Tris (5 % w/v) and acetic acid (2 % v/v).

Scanning

The Laser Densitometer (4000 x 5000 pixels; 12 bits/pixel) from Molecular Dynamics and the GS-700 from Bio-Rad have been used as scanning devices. These scanners were linked to Sparc workstations and Macintosh computers.