

Proteomics in Practice

By Reiner Westermeier and Tom Naven
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Addendum

Status: February 2003

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Topic: Basic pH gradients

Stabilisation of protein thiol groups as disulfides

The main reason for the frequently observed horizontal spot streaking in basic gradients and the basic regions of wide gradients is the weak acid nature of DTT and DTE. The reductants become negatively charged, start to migrate towards the anode and leave the thiol groups of the cysteins unprotected. This leads to

- oxidation of some thiols, resulting in additional artifactual spots.
- backfolding of tertiary structures, resulting in pI changes and streaking.
- inter-subunit aggregates, resulting in pI changes and smearing.

As already mentioned on page 17 of *Proteomics in Practice*, the use of alternative reductants like 2-mercaptoethanol, tributylphosphine, triscarboxyethylphosphine etc. cannot be recommended because of other problems, which are not discussed here.

The phenomenon of streaking and appearance of artifactual spots in basic regions is abolished, when the first dimension IEF is run with an excess of hydroxyethyl-disulphide (HED, “DeStreak”) according to Olsson *et al.* (2002). The protein cysteinyl groups are then oxidized to mixed disulfides. In this way all thiol groups are converted into one defined form. The resulting pattern shows round spots, the number of spots is reduced compared to separations in presence of reductant, and there is a light shift of the basic pIs towards basic pH because of the missing negative charges of the thiols.

Note: proteins are extracted in the conventional way with a reductant like DTT or DTE; also alternative reductants like 2-mercaptoethanol, TBP (Tributylphosphine) or TCEP (Tris(2-hydroxyethyl)phosphine) can be used. The lysis buffer does not contain HED. The sample is diluted with HED-containing rehydration solution and applied with cup-loading at the anodal end of the pre-rehydrated IPG strip, containing 100 mmol/L HED. The running conditions are not changed. The reductant content is very critical when rehydration loading is used. However, when the sample has been diluted with rehydration buffer down to as low as 1 mmol/L DTT content, good results can also be obtained with rehydration loading (Bengt Bjellqvist, unpublished result).

For the second dimension SDS electrophoresis the IPG strips are equilibrated in the conventional way with SDS, Tris-Cl, urea, glycerol and DTT, followed by alkylation with iodoacetamide.

Reference

Olsson I, Larsson K, Palmgren R, Bjellqvist B. Organic disulfides as a means to generate streak-free two-dimensional maps with narrow range basic immobilized pH gradient strips as first dimension. *Proteomics* 2 (2002) 1630-1632.

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Topic: Three-dimensional electrophoresis

The analysis of smaller subsets of protein mixtures frequently provides improved pattern and allows to detect proteins which otherwise do not enter the gel. A possible procedure is the three-dimensional electrophoresis procedure introduced by Werhan and Braun (2002). The first dimension is the separation of protein complexes by blue native polyacrylamide electrophoresis according to Schagger and von Jagow (1991). This technique had been developed for the isolation of enzymatically active membrane proteins at pH 7.5. Coomassie Blue G-250 is added to the cathodal buffer of a native electrophoresis. It competes with the nonionic detergents used for solubilization of membrane proteins and complexes for binding sites on the surface. Proteins and complexes bind the dye and become negatively charged and stay soluble in detergent-free solution. The visible bands are eluted electrophoretically from the gel, destained, and further analysed by standard denaturing 2-D electrophoresis: IEF / SDS-PAGE. Interestingly, this method reveals also proteins with high hydrophobicity; most probably due to the reduction of complexity of the protein mixture.

References

- Werhahn W, Braun H-P. Biochemical dissection of the mitochondrial proteome from *Arabidopsis thaliana* by three-dimensional gel electrophoresis. *Electrophoresis* 23 (2002) 640-646.
- Schagger H, von Jagow G. Coomassie-SDS-PAGE for direct visualisation of polypeptides during electrophoresis. *Anal Biochem.* 199 (1991) 223-231.

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Topic: Prefractionation of the sample by electrophoresis and isoelectric focusing Denaturing IEF in a granulated gel bed

Görg *et al.* (2002) have developed a very quick, reproducible and efficient prefractionation method: preseparation of complex protein mixtures by carrier ampholyte IEF in a granulated gel bed. The procedure is performed on a standard flatbed electrophoresis equipment at an exactly adjusted temperature of 20°C. The technique is simple: the sample is mixed with Sephadex G-200 superfine, which has been reswollen with rehydration solution containing urea, CHAPS, DTT and carrier ampholytes. This slurry is pipetted into a trough and covered with a thin layer of silicone oil. After 4 hours isoelectric focusing the pH fractions are removed with a spatula and directly applied onto rehydrated IPG strips. In the electric field the proteins migrate out from the granulated gel into the polyacrylamide gel of the IPG strip. The fractions can also be stored in a freezer at -70°C.

The technique is also desalting the sample – no microdialysis is required. The other benefits of this method are obvious:

- Standard laboratory equipment is used.
- The technique is easy to carry out.
- The separation is quick.
- Denaturing sample prefractionation according to pI in presence of urea, detergents etc. No protein losses.
- It can be done with high and low protein amounts
- It allows maximum protein load on narrow range IPG strips.

Subsequent separations of the pH fractions in the respective narrow pH interval IPG strips show a considerable increase in spot number. Many more of the lower expressed proteins can be detected in this way.

Reference

Görg A, Boguth G, Köpf A, Reil G, Parlar H, Weiss W. Sample prefractionation with Sephadex isoelectric focusing prior to narrow pH range two-dimensional gels. *Proteomics* 2 (2002) 1652-1657.

Insert into Page 62**Topic: Buffer systems**

Tris-acetate / Tris-tricine

Reference

Olsson I, Stålberg R. A new semi-dry method for horizontal electrophoresis in ultrathin polyacrylamide gels. In Electrophoresis '86. Proceedings of the fifth meeting of the international Electrophoresis Society. Dunn MJ, Ed. VCH Verlagsgesellschaft, Weinheim. (1986) 230-232.

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Topic: 2D-DIGE

Image acquisition

The gel is scanned three times with a laser scanner at different excitation wave length. To avoid cross-talk of the emission signals, different narrow-band pass filters are inserted for each dye. The most accurate and sensitive results are obtained by using a scanner as described on pages 88 to 90 in *Proteomics in Practice*.

Co-detection of spots

For DIGE the image analysis software must have a special algorithm to co-detect the spots. In this case the boundaries of the related spots are identical. The measurement of the spot volumes and the relative quantification of the different protein spots become thus very accurate.

Internal standard

The strongest feature of the DIGE technique is the possibility to analyse the protein mixtures with an internal standard for each protein. Aliquots of each sample of a biological experiment are pooled and labeled with one of the dyes. When the internal standard is co-run and co-detected with the samples, the spot volumes of the samples inside each gel and between different gels can be normalized to the spot volumes of the internal standard. In this way gel-to-gel variations are equalized to prevent wrong conclusions on biological variations. The figure 41 in *Proteomics in Practice* can be replaced with the following cartoon:

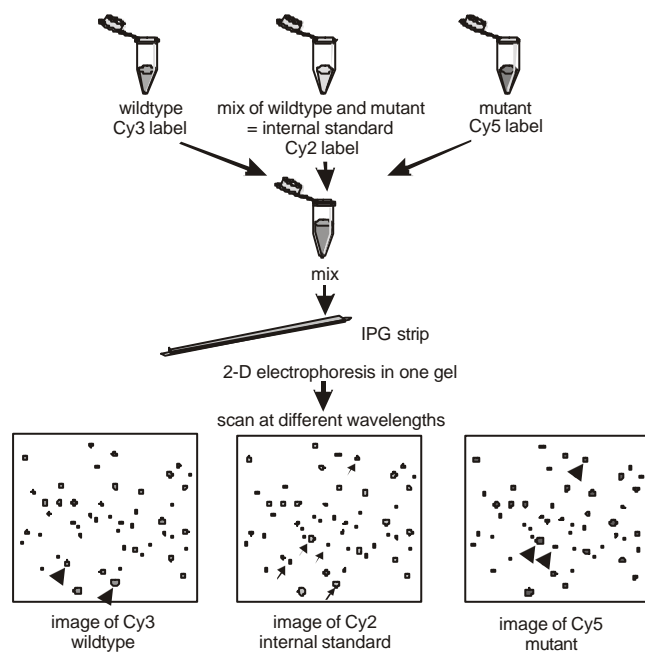


Fig. 41: Difference gel 2-D electrophoresis. The sample proteins and the pooled internal standard are labeled with different fluorescent tags. The images are acquired with a fluorescence scanner at different wavelengths.

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Topic: Spot cutting

Spot picking from unbacked gels

The advantages of the concept described above apply also on unbacked gels. In order to prevent shrinking or swelling of the gel it has to be stabilized during scanning and spotpicking. The following procedure, developed by Burghardt Scheibe, is easy to carry out and has proved itself in practice:

A cellophan sheet is soaked in water and clamped into gel drying frames as described on page 238. The screws are removed from the frame for this procedure. The upper cellophane side is dried with lint-free tissue paper and the reference markers are stuck to the cellophane at the appropriate positions. The frame-cellophane assembly is then turned around to form a tray. The gel slab is soaked in water to swell as much as possible and placed onto the cellophane. The gel is allowed to dry on the cellophane for about 15 minutes. Now the gel can be scanned in a visible light or fluorescence scanner. A picking list is created with the image analysis software.

The picking tray is removed from the spot picker and a gel casting cassette is placed into this position. In order to achieve a good contrast for the reference markers a black plastic sheet is placed onto the gel casting cassette. The camera needs to be recalibrated, because the picking level is lower than with the original tray. The frame with the gel is placed on the black plastic sheet and the spots are picked like from a film- or glass-supported gel. In order to prevent curling up of the gel edges, every half hour a few millilitres of water needs to be pipetted on the gel.

Insert into Pages 178 and 179

Topic: Precipitation

Practice shows, that for some samples too much of the precipitant is left in the pellet, resulting in salt effects like streaking of the 2-D pattern close to the lateral sides of the gel. As a remedy, an additional step can be inserted into the precipitation procedure after step no. 6:

Procedure A: for sample volumes of 1 – 100 μ L

Insert between steps 6 and 7:

6a. Without disturbing the pellet, layer 40 μ L of co-precipitant on top of the pellet. Let the tube sit on ice for 5 minutes.

6b. Carefully reposition the tube in the centrifuge. Centrifuge the tube again for 5 minutes, use a pipette tip, remove and discard the wash.

Continue with step 7.

Procedure B: for dilute samples of more than 100 μ L

Insert between steps 6 and 7:

6a. Add co-precipitant 3–4 times the volume of the pellet. Let the tube sit on ice for 5 minutes.

6b. Carefully reposition the tube in the centrifuge. Centrifuge the tube again for 5 minutes, use a pipette tip, remove and discard the wash.

Continue with step 7.

Improved Keratin tables:

Replace original tables with the following ones with values to four decimal places:

Tab. 1: K2C1_HUMAN: KERATIN, TYPE II CYTOSKELETAL 1 (K1 skin)

Nominal mass (M_r): 65847; Calculated pI value: 8.16

Position in sequence	Experimental mass [M+H] ⁺	Sequence
185 – 196	1383.6903	SLNNQFASFIDK
211 – 222	1475.7489	WELLQQVDTSTR
199 – 210	1475.7852	FLEQQNQVLQTK
417 - 431	1716.8511	QISNLQQSISDAEQR
223 - 238	1993.9766	THNLEPYFESFINNLR
518 - 548	2383.9518	GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR
549 – 587	3312.31081	GSYSGSGSSYGSGGGSYGSGGGGGHGSYSGSSSSGGYR

Tab. 2: K22E keratin, 67K type II epidermal - human

Nominal mass (M_r): 65825; Calculated pI value: 8.07

Position in sequence	Experimental mass [M+H] ⁺	Sequence
245 – 253	1037.5262	YLDGLTAER
381 – 390	1193.6160	YEELQVTVGR
46 - 61	1320.5828	HGGGGGGFSGGGGFGSR
71 - 92	1838.9143	GGGFSGGSGFSGGSGFSGGSGFSGGGFSGGGFSGGGR
93 – 128	2831.1940	SISISVAGGGGGFGAAGGFGR

Tab. 3: K.9. Cytokeratin 9 (Skin), type I, cytoskeletal - human

Nominal mass (M_r): 61950; Calculated pI value: 5.14

Position in sequence	Experimental mass [M+H] ⁺	Sequence
233 – 239	897.4135	MTLDDFR
224 – 232	1060.5633	TLLDIDNTR
242 – 249	1066.4986	FEMEQLNR
449 – 471	2510.1317	EIETYHNLLEGGQEDFESSGAGK
63 - 94	2705.1610	GGSGFGYSYGGGSGGGFSASSLGGGFSGGSR

Tab. 4: K10 (K1CJ-human) keratin 10, type I, cytoskeletal – human

Nominal mass: 59492; Calculated pI value: 5.17

Position in sequence	Experimental mass [M+H] ⁺	Sequence
442 – 450	1165.5848	LENEIQTYR
323 – 333	1365.6393	SQYEQLAEQNR
166 – 177	1381.6481	ALEESNYELEGK
41 - 59	1707.7721	GSLGGGFSSGGFSGGFSR
423 – 439	2025.9433	AETECQNTTEYQQLLDIK
208 – 228	2367.2626	NQILNLTDDNANILLQIDNAR