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Received November 16, 2012  
Revised January 18, 2013  
Accepted February 14, 2013

## Research Article

# The new horizon in 2D electrophoresis: New technology to increase resolution and sensitivity

A principally new type of an electrophoresis setup for the second dimension of 2DE named HPE (high performance electrophoresis) has recently become available that provides excellent reproducibility much superior to traditional 2DE. It takes up ideas from early beginnings of 2DE which could not be satisfactory realized at that time. The new HPE system is in contrast to all other established systems a horizontal electrophoresis that employs a new type of precast polyacrylamide gels on film-backing and runs on a multilevel flatbed electrophoresis apparatus. In a systematic approach we compared its features to traditional 2DE for the cytosolic proteome of *Bacillus subtilis*. Not only the reproducibility is enhanced, but also nearly all qualitative parameters as resolution, sensitivity, the number of protein spots (25% more), and the number of different proteins (also additional 25%) are markedly increased. More than 200 proteins were exclusively found in HPE. This new electrophoresis system does not use buffer tanks. No glass plates are needed. Therefore handling of gels is greatly facilitated and very simple to use even for personnel with low technical skills. The new HPE system is technically at the beginnings and further development with increased performance can be expected.

### Keywords:

*Bacillus subtilis* / High performance electrophoresis / Horizontal SDS-PAGE / Protein quantification / Two-dimensional gel electrophoresis

DOI 10.1002/elps.201200618



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## 1 Introduction

Proteome analysis implies many challenges to researchers mainly caused by the complexity of samples, including large differences of proteins in size, *pI* and hydrophobicity, a highly dynamic range of their abundance in cells and a considerable number of post-translationally modified isoforms that are frequently difficult to detect and to characterize. To master these challenges a number of different methodologies have been developed. Of these, a 2DE based workflow was in the past the most widely adopted and had the most general applicability. However, 2DE based methods have been increasingly criticized as being difficult, unreliable, and poorly reproducible.

The first approach of proteome analysis [1] was solely based on high-resolution 2DE that had been developed by O'Farrell [2] almost 40 years ago. Major features were the introduction of completely denaturing conditions for proteins and a combined separation of proteins in an IEF in thin gel rods in presence of 9 M urea as first dimension and in discontinuous polyacrylamide gels in vertical glass cassettes in presence of SDS as second dimension. Since that time, several attempts have been made to increase the number of detected proteins and to improve its reproducibility, but very few of them could fulfill the expectations in practice. For example, increased resolution by enlarging gel size [3] demands very high technical skills, thus—unfortunately—only very few laboratories reached the ability to achieve reproducible results. The largest step forward was the implementation of IPG for the first dimension by development of precast film-backed gels containing a pH gradient polymerized onto the gel matrix. These gels were washed and dried and cut into narrow, 3 mm wide, strips [4]. Therefore, usage of poorly reproducible

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**Abbreviation:** HPE, High performance electrophoresis

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carrier ampholytes could be strongly reduced leading to a much better reproducible and easy-to-use first dimension. It offered stable pH gradients of different shape and a choice of different ways for sample application [5]. Especially last item became important to reduce the unpredictable precipitation of proteins during their entry into gels. Handling of IPG strips is much easier than that of thin gel rods and they maintain their pH gradient profile independently from sample composition. Because of all these advantages use of IPG strips became the standard method for IEF in 2DE.

In contrast, the technology of the second dimension remained unchanged in principle over decades. Running SDS polyacrylamide gels in vertical glass cassettes in large buffer tanks is still standard. However, in several laboratories including ours daily practice gave rise to doubts about reproducibility and standardization. Indices for serious problems of this technique could be increasingly observed. General difficulties of 2DE, especially in reproducibility, seemed to be caused by problems of SDS-PAGE and not of IEF as believed for a long while. Some problems of traditional SDS-PAGE were already recognized in the early period of 2DE. Almost 20 years ago Görg et al. showed that thin horizontal, flatbed SDS gels offer higher resolution and sharper protein spots than the conventional 1 or 1.5 mm thick vertical gels [6]. The disadvantage of this method was a nonquantitative migration of proteins out of the IPG strip into the SDS gel. Incomplete and unpredictable transfer of proteins was probably the major reason why this approach was not generally accepted.

In a systematic approach we compared features of the new horizontal high performance electrophoresis (HPE) to traditional 2DE. We evaluated resolution, sensitivity, reproducibility, and compatibility with subsequent MS analysis using a complex mixture of cytosolic proteins from the model organism *Bacillus subtilis*.

## 2 Material and methods

### 2.1 Growth conditions and preparation of protein extracts

*B. subtilis* wildtype strain 168 was grown at 37°C under agitation (180 rpm) in minimal medium according to Stülke et al. [7] that was modified containing 0.2% glucose and 1 mM potassium dihydrogen phosphate. Growth was monitored by measurement of OD at 500 nm and bacteria were harvested at  $OD_{500nm} = 0.5 (\pm 0.1)$ . Protein extracts of cytosolic proteins were prepared as described in Eymann et al. [8]. Aliquots of the protein extracts have been stored at –70°C. Protein concentrations were determined by RotiNanoquant (Roth, Karlsruhe, Germany).

### 2.2 2DE and visualization of proteins

IEF was done in the pH range 4–7 with IPG strips of 18 cm (SERVA, Heidelberg, Germany; GE Healthcare Europe, Freiburg, Germany), loaded with 200 µg protein in

rehydration solution as described in Eymann et al. [8]. Focusing occurred in the Multiphor II system (GE Healthcare) in a three stepped voltage gradient (gradient to 500 V until 500 Vh, 500 V until 2500 Vh, gradient to 3500 V until 10 kVh, and 3500 V until 35 kVh had been reached). IPG strips have been subsequently stored at –70°C until further application. SDS-PAGE was alternatively done in the traditional, vertical Investigator electrophoresis system (Genomic solutions, Chelmsford, MA, USA) using self-cast Laemmli gels of 12.5% T and 2.6% C in the resolving gel as described in Büttner et al. [9] or in the horizontal system HPE™ Tower (SERVA) using 2D HPE™ Large Gel NF 12.5% gels (SERVA) of 12.5% T and 2% C in the resolving gel according to the instructions of the manufacturer. Thickness of these two gel types is different with 1 mm for the traditional, vertical gels, and 650 µm for the horizontal HPE gels. Both gel types were stained with LavaPurple™ (SERVA) according to the instructions of the manufacturer. Stained proteins were detected with a Typhoon 9400 multi-mode imager (GE Healthcare) by excitation with the green laser (532 nm) and detection with a 560 nm long pass filter. Resolution was set to 100 microns per pixel and intensity was adapted by the photomultiplier to obtain an image displaying the most intense protein spot slightly below saturation. Because the dynamic range of proteins in bacterial cells is  $10^6$ , much higher than dynamic range of the resolution of the human eye, for visualization of low abundant proteins it was necessary to enhance the contrast of the images to be able to detect faint spots and to cut them out for identification. The same is true for presentation of results. Image analysis and in silico enhancement was accomplished with software Delta2D 4.3 (Decodon, Greifswald, Germany).

### 2.3 Spot processing for MS analysis

All spots visualized by the fluorescent stain were selected for identification with MALDI-TOF-MS. The huge number of spots requested automatic image analysis and spot processing. Image analysis was done with Delta2D 4.3 software (Decodon). Sample gel images were aligned using the warping technology and a pick list was generated containing the coordinates of the proteins to be identified, sorted from the lowest to the highest abundant protein spots. Protein spots were excised from stained gels using Ettan spot picker (Amersham™ Biosciences, Uppsala, Sweden) with a picker head of 2 mm diameter and spots of similar staining intensity were sorted into 96 well microplates greiner bio one (Greiner, Frickenhausen, Germany). The tryptic digest with subsequent spotting onto a MALDI target was carried out automatically with the Ettan Spot Handling Workstation (Amersham Biosciences). Gel pieces were washed twice with 100 µL buffer of 50% v/v CH<sub>3</sub>OH and 50% v/v of 50 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 min and once with 100 µL of 75% v/v CH<sub>3</sub>CN for 10 min. After drying at 37°C for 17 min 10 µL trypsin solution (Promega, Madison, WI, USA) were added and incubated at 37°C for 120 min. Concentration of trypsin was adapted to the intensity of protein spots which therefore were classified into three groups. Most intense spots were digested using highly purified

water from AriumProUV (Sartorius, Göttingen, Germany) containing 4  $\mu\text{g/mL}$  trypsin, weaker spots that were visible without image enhancement were digested with 2  $\mu\text{g/mL}$ , and low abundant proteins, whose spots became visible only after virtual contrast enhancement, were digested with a trypsin concentration of 1  $\mu\text{g/mL}$ . For extraction the gel pieces were covered with 60  $\mu\text{L}$  of 0.1% v/v TFA in 50% v/v  $\text{CH}_3\text{CN}$  and incubated for 30 min at room temperature. Peptide containing supernatant was transferred into a new microplate and extraction was repeated with 40  $\mu\text{L}$  of the same solution. Supernatants were pooled and dried completely at 40°C for 220 min. Dried peptides were resuspended in 0.9  $\mu\text{L}$  solution of 50%/49.5%/0.5% v/v/v  $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{TFA}$  containing 3.3 mg/mL matrix (CHCA), and 0.7  $\mu\text{L}$  of dissolved peptides were spotted onto MALDI target. Samples were allowed to dry on target at room temperature for 10 to 15 min before measurement.

## 2.4 MS and MS/MS analysis

MALDI-TOF measurement was carried out on the AB SCIEX TOF/TOF™ 5800 Analyzer (AB Sciex, Framingham, MA, USA). This instrument is designed for high throughput analysis, able to measure samples, calibrate spectra and analyze data automatically using the TOF/TOF™ Series Explorer™ Software V4.1.0. Spectra were recorded in a mass range from 900 to 3700 Da with a focus mass of 1700 Da. For one main spectrum 25 subspectra with 100 shots per subspectrum were accumulated using a random search pattern. If the autoproteolytic fragment of trypsin with the mono-isotopic  $(\text{M}+\text{H})^+$   $m/z$  at 2211.104 reached a S/N of at least 40, an internal calibration was automatically performed as one-point-calibration using this peak. The standard mass deviation was below 0.15 Da. Only if the automatic mode failed (in less than 1%) calibration was carried out manually. The five most intense peaks from these TOF-spectra were selected for MS/MS analysis. For one main spectrum 20 sub-spectra with 125 shots per subspectrum were accumulated using a random search pattern. The internal calibration was automatically performed as one-point-calibration with the mono-isotopic arginine  $(\text{M}+\text{H})^+$   $m/z$  at 175.119 or lysine  $(\text{M}+\text{H})^+$   $m/z$  at 147.107 if these peaks reached a S/N of at least 5.

## 2.5 Data processing for identification

Peak lists were created using GPS Explorer™ Software Version 3.6 (build 332) with the MS settings 900–3700 Da mass range, 20 peaks per 200 Da peak density, minimal S/N of 15, and maximal 65 peaks per spot. MS/MS settings were a mass range from 60 Da to reduced mass of precursor (–20 Da), a peak density of 50 peaks per 200 Da and maximal 65 peaks per precursor. Peak lists were created for a minimal S/N of 10. All peak lists were analyzed using Mascot search engine version 2.4.0 (Matrix Science Ltd, London, UK) with a specific database for *B. subtilis*. Identifications were considered as correct if a protein was identified at least twice in two

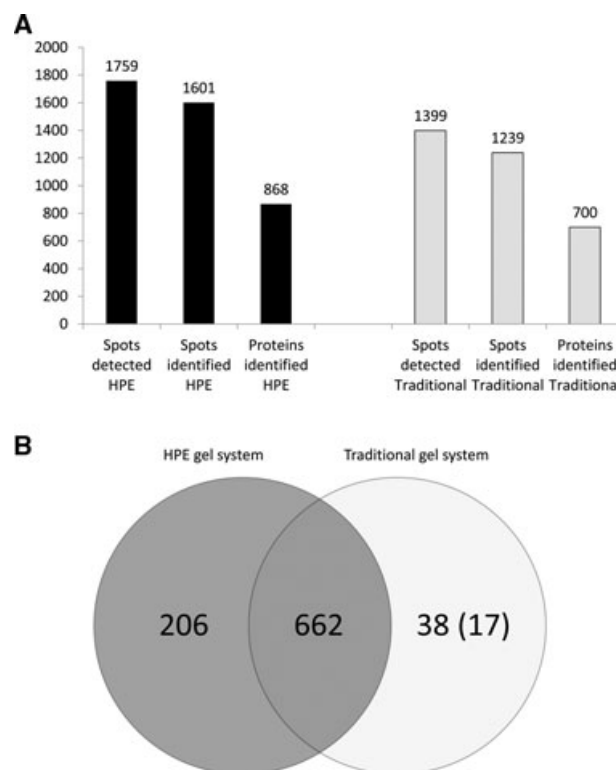


**Figure 1.** Graphic representation of results obtained from the 2D separation of cytosolic proteins from *B. subtilis* 168 using IPG strips in the pI range of 4–7 and in the following alternatively either horizontal HPE system or traditional, vertical gel system and subsequent identification of protein spots by MALDI-TOF-MS/MS. (A) Number of detected spots, number of identified spots, and number of different proteins within these spots (without multiple spots) are shown for these different electrophoresis techniques. (B) Overlap and specificity of different proteins found by these different electrophoresis techniques. Omitting rules of identification acceptance (at least two identifications in independent experiments), we found 21 of the 38 proteins specific for conventional gels once in HPE gels indicating their principal detectability in HPE and a probable reduction of proteins exclusively found in vertical gels to 17 (number in parenthesis) in future.

independent analyses. Protein spots were assigned using the SubtiWiki gene names from update 01. 06. 2011.

## 3 Results

Protein extracts of *B. subtilis* 168 containing mainly cytosolic proteins were prepared from exponentially growing cells. These extracts were used for IEF in the pH gradient of 4–7 and subsequent SDS-PAGE with either the HPE system or the conventional, vertical system followed by staining with LavaPurple™ fluorescent stain. We detected 1759 protein spots in HPE and 1399 protein spots in traditional gels, revealing a clearly better resolution in HPE gels. Approximately 90% of these protein spots were identified by MALDI-TOF-MS in both gel types, 1601 spots in HPE (91%) and 1239 spots in traditional gels (88.5%; Fig. 1A). Neglecting

isoforms of proteins occurring in multiple spots, within all spots in horizontal HPE 868 different proteins could be identified (Supporting Information Table 1), while 700 different proteins were identified in conventional, vertical gels (Supporting Information Table 2). In total 906 different proteins could be found, 662 of them in both gel types, 206 proteins were exclusively identified in HPE and only 38 proteins could be solely found in traditional gels (Fig. 1B). Because most of these exclusively found proteins are low abundant, the ratio of 206/38 demonstrates a considerable better sensitivity of HPE gels. Moreover, omitting strict rules for identification acceptance (at least two identifications in independent experiments), we found 21 of the 38 proteins specific for conventional gels once in HPE. Contrarily at no time a protein specific for HPE could be identified in traditional gels. These one-hit-spots are regularly located in overcrowded regions of the gels. All of them belong to mixed spots in HPE, while most of them could be identified in single spots in traditional gels. This indicates that enhanced resolution of proteins in one gel region can lead to formation of new mixed protein spots in another region.

To evaluate reproducibility during a period of more than two years at least six different batches of HPE gels were used for the separation of aliquots of the very same protein extract. An excellent reproducibility was ascertained as to be seen in Fig. 2A: In dual-channel images different gels are present in the colors red and green and placed on top of the other [10]. Spots in mixed color yellow indicate a perfect match, red or green spots show displaced locations in different gels. Using technical replicates the dual-channel image should appear in yellow completely. In contrast to horizontal HPE gels, pronounced differences occurred in traditional, vertical gels. Whereas HPE gels can be almost perfectly overlaid with a minimum of computational work because of their reproducibility, for vertical gels a tremendously higher effort is needed to reach a comparable result. The example for traditional gels shown in Fig. 2B is representative. Figure 2B displays an intermediate situation during image analysis after an investment of time comparable to the image analysis of HPE gels. The quality of single gels themselves is recognizably high, but mutual comparison offers problems in reproducibility. It is possible to cope with these problems, but it demands extensive manual work of image analysis by setting a lot of match vectors to correct gel distortions. In case of HPE gels the time for alignment was markedly reduced, since much less match vectors were needed. After running a high number of gels of both types, the difference in reproducibility can now be expressed in numbers. A relatively independent quality standard for reproducibility is the suitability of gels for quantitative analysis of proteins according to Maass et al. [11]. It is based on a spot shape where smear should only be detectable at extremely low level that the real spot boundaries in gel and the theoretical spot boundaries calculated by software package Delta2D should completely cover each other. Criteria for assignment of gels to the different quality classes were set as follows: gels suitable for absolute protein quantification comprise at least 90% spots

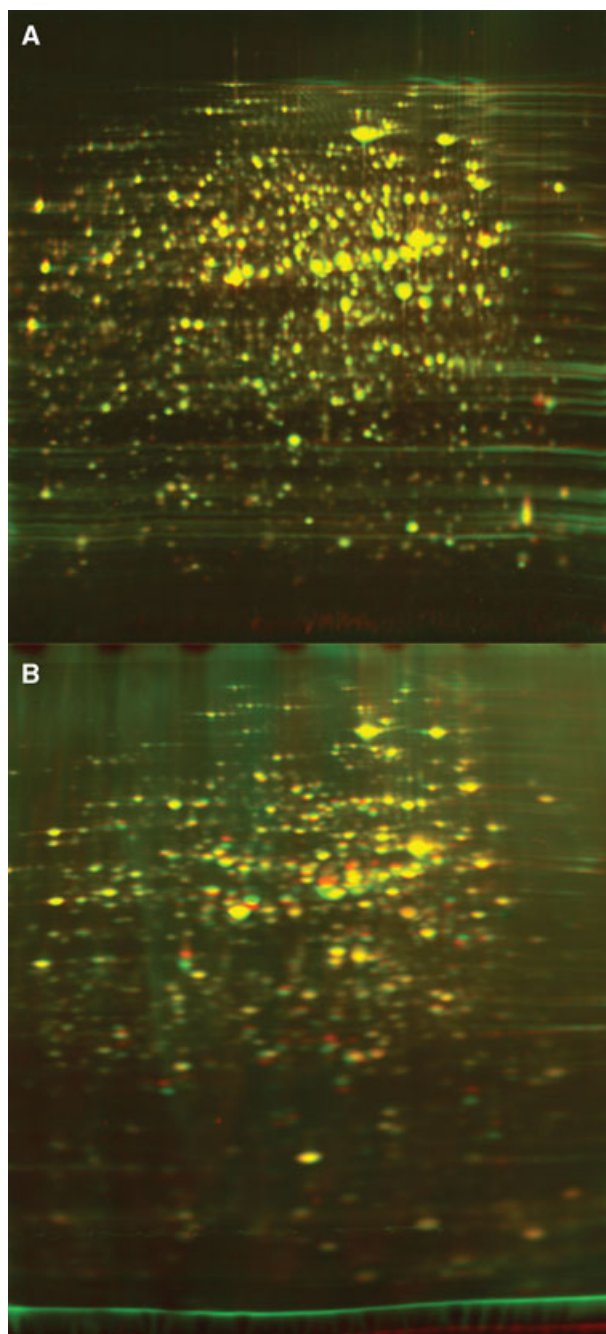


**Figure 2.** Dual channel images of representative replicates of gels using the very same protein extract from *B. subtilis* 168 separated with IPG strips in the *pI* range 4–7 and either the horizontal HPE system (A) or alternatively the traditional, vertical gel system (B). Six different batches of HPE gels have been used that had been produced in a span of nearly 2 years. The two batches with the longest interval in between are shown here. Traditional gels have been prepared within 1 week, in different labs using identical protocols. Problems of reproducibility become obvious with regard to nonlinear running differences that occurred in the vertical gel system. In silico contrast enhancement of the gel images was applied to visualize low abundant protein spots as described in Section 2.2.

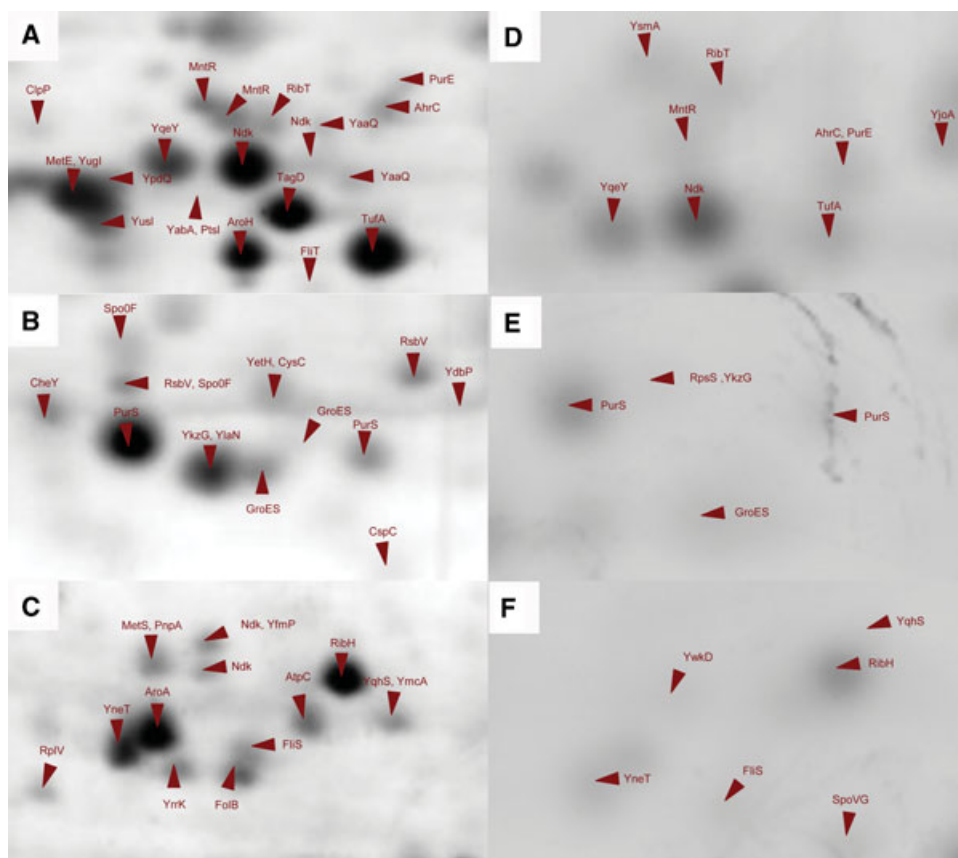


**Figure 3.** Corresponding gel image regions of 2D gels revealing spots of low molecular weight proteins are shown from horizontal HPE system (A–C) and the traditional, vertical gel system (D–F). A and D represent a region of around 15 kDa, B and E of about 10 kDa, C and F of approximately 13 kDa. Enhanced focusing of low molecular weight proteins in HPE gels becomes obvious. This is the combined result of decreased thickness of HPE gels, use of higher electrical field strength, and improved dissipation of heat. In silico contrast enhancement of the gel images was applied to visualize low abundant protein spots as described in Section 2.2.

of that type, gels for relative protein quantification comprise at least 75% and gels suitable for identification only comprise at least 50% such spots, respectively. This classification method was verified for its relevance by a gel-free orthogonal MS approach using AQUA peptides and could be approved. Only 130 (60.7%) out of 214 conventional, vertical gels reached highest quality and could be used for absolute protein quantification, additional 29 (13.6%) were suitable for relative quantification, further 20 (9.3%) could be only used for protein identifications. However, for HPE gels a nonquantifiable quality originating from problems with SDS-PAGE is a rare exception. From approximately 700 HPE gels run in our institute in different projects only about 3.5% showed too low reproducibility leading to their exclusion (Wachlin, personal communication). It should be noted here that the numbers investigated for the described vertical system are generally comparable to others. With many years of experience we are driving several conventional electrophoresis systems from different suppliers and their performance is not much different.

Not all proteins benefit from advantages in HPE gel performance equally. Three different groups are particularly affected, proteins of low molecular weight, low abundant proteins and proteins of very similar molecular weight. An overview for each group is separately presented in the Figs. 3, 4, and 5. Figure 3 shows exemplarily low molecular weight regions revealing faint spots on vertical gel images, while fo-

cusing and intensity of these spots were greatly enhanced in images obtained from HPE gels. Also low abundant proteins appeared in much more focused and intense spots in images of HPE gels. Frequently this feature enabled us to detect and identify low abundant proteins, which were undetectable in traditional gels (Fig. 4). In traditional gels the absolute protein amount of faint spots could be determined via AQUA peptides [11]. The lowest amount was found for protein PolX with 10 ng which is equivalent to a copy number of approximately 100 molecules per cell. The copy number seems to be the more suitable scale in general because the absolute amount of protein does not consider the molecular size. In HPE gels these investigations have not been conducted until now because the considerable larger number of low abundant proteins found therein needs to be investigated with a corresponding effort and the experimental adaptations necessary due to the lower limit of protein amount of these spots in HPE gels are not finished yet. In addition, in HPE gels we observed an enhanced resolution of proteins with very similar molecular weight, and which formerly could not be separated, thus suggesting positive effects of HPE on migration of SDS charged molecules (Fig. 5). Furthermore, we observed an increase of mixed spots containing more than one protein in HPE gels to 29% of all spots found, whereas this portion in traditional gels is only 19%. This phenomenon can be easily explained by the smaller area of spots caused by improved focusing in HPE. In the larger spots of conventional gels

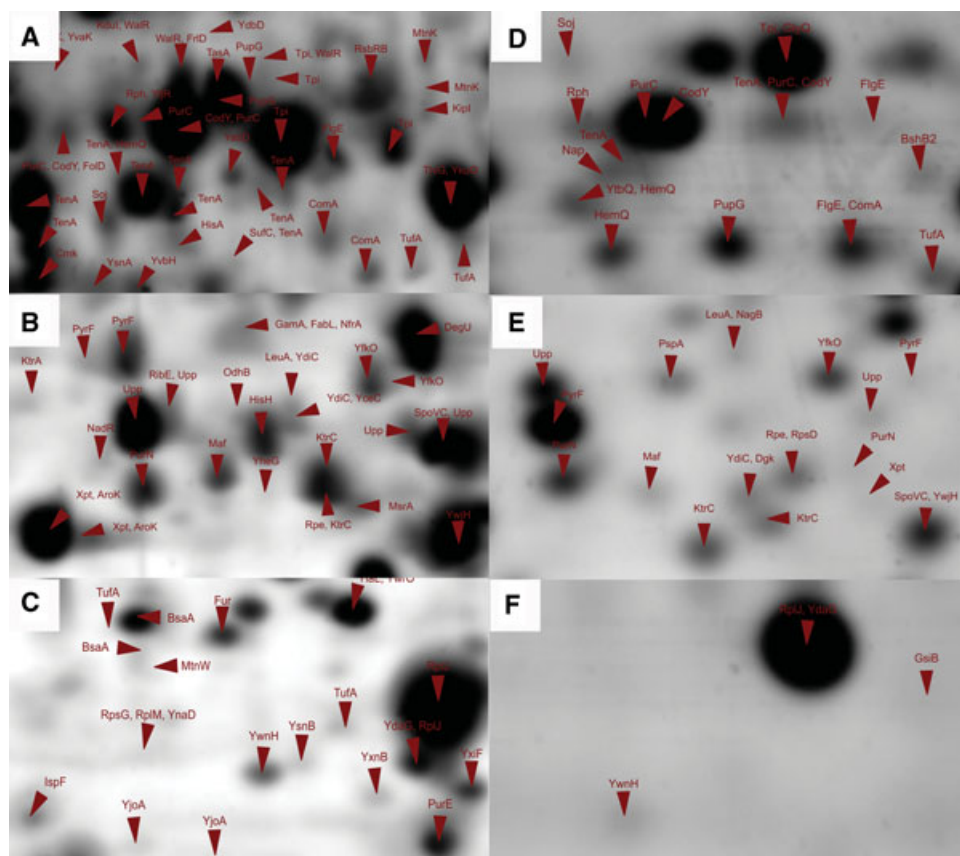


**Figure 4.** Corresponding gel image regions containing low abundant protein spots are shown from HPE system (A–C) and the traditional, vertical gel system (D–F). A and D represent a region of around 25 kDa, B and E of about 24 kDa, C and F of approximately 17 kDa. Various proteins could only be detected in HPE but not in vertical gels, for example YabD (A, D), YheG (B, E), YxnB (C, F). Much better resolution and sensitivity is a combined result of decreased thickness of HPE gels, use of higher electrical field strength and improved dissipation of heat. In silico contrast enhancement of the gel images was applied to visualize the low abundant protein spots as described in Section 2.2.

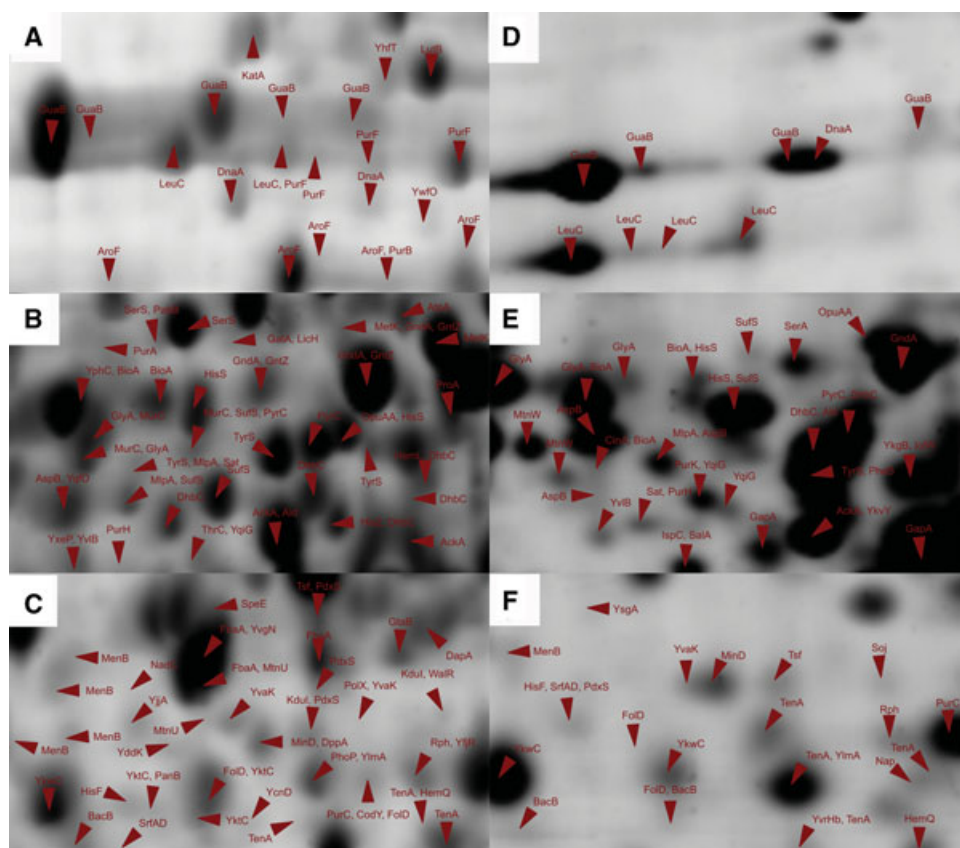
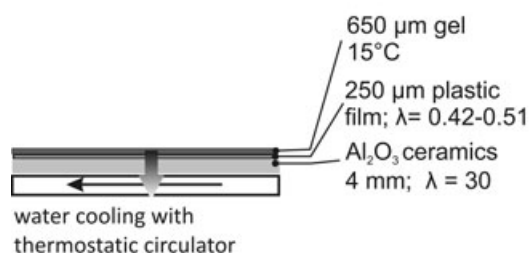


**Figure 5.** Corresponding gel image regions from HPE system (A–C) and the traditional, vertical gel system (D–F) revealing enhanced resolution of the HPE system even for those spots of higher abundance which could be found in traditional gels as mixed spots only. A and D represent a region of around 50 kDa, B and E of about 40 kDa, C and F of approximately 30 kDa. In these sections centrally located abundant spots GuaB and DnaA (A, D), HisS and SufS (B, E) or MinD and YvaK (C, F) were separated by a shift in Molecular Weight demonstrating better resolution in HPE gels in second dimension. In silico contrast enhancement of the gel images was applied to visualize low abundant protein spots as described in Section 2.2.

## HPE horizontal system



## Conventional vertical system

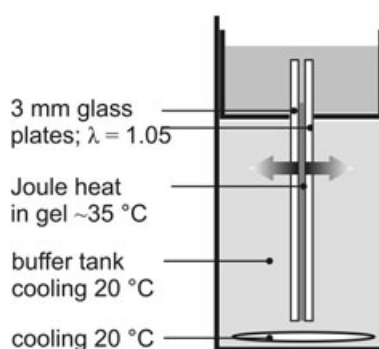


**Figure 6.** Schematic representation of temperature control in a conventional vertical buffer tank and on horizontal cooling plates. The temperature conductivity coefficient  $\lambda$  is defined as power  $P$  that is conducted through a material over the distance  $L$  between two surfaces that differ in temperature  $T$ ; the higher coefficient  $\lambda$  and the thinner the layer, the more efficient is heat transfer.

additional components of these spots might be less frequently cut because of the larger spot area, and their comparatively low local protein concentration might be insufficient for detection as well.

Finally, we want to state our opinion, that superior reproducibility is the main advantage of the horizontal HPE system. Altogether we detected 25% additional spots and identified approximately 25% more different proteins in HPE gels. More than 200 proteins were exclusively found in HPE; whereas 38 proteins could only be identified in traditional gels. However, 21 of these 38 proteins were identified at least once in HPE gels, and most of them are within gel regions showing a very high spot density, thus indicating that they are incorporated into newly formed mixed spots. A detailed picture of all these differences and obvious advantages can be found in the Supporting Information showing the master gels for HPE (Supporting Information Fig. 1) and traditional gel system (Supporting Information Fig. 2). In addition, we should mention the very simple use of HPE gels even by unskilled personnel, which leads, together with considerably decreased runtime of electrophoresis (to a third) and significantly reduced computational work because of superior reproducibility, to a considerable reduction of work to be done, especially in larger projects.

## 4 Discussion

### 4.1 Technical principles

It is general knowledge, that during electrophoresis the temperature in gels rises due to development of Joule heat. If gels are not cooled, the separation front will become U-shaped (also known as “smiling effect”), and the zones will become blurred because of an increase of diffusion.

The conventional way of running SDS polyacrylamide gels employs buffer tanks, which use high volumes of buffers as a heat sink to dissipate the Joule heat. For large gels the separation takes several hours to overnight, therefore the buffer itself needs to be cooled with a heat exchanger. As shown in Fig. 6, heat dissipation from electrophoresis gels occurs across several steps: the heat exchanger cools the buffer and

the buffer removes heat from gels through the glass plates. Usually glass plates are at least 3 mm thick and have a poor temperature coefficient ( $\lambda = 1.05$ ). Furthermore, vertical gels need to be at least 1 mm thick in order to accommodate the equilibrated IPG strip; thickness of IPGs film-backing is 200  $\mu\text{m}$  and that of the gel is about 700  $\mu\text{m}$ .

In the alternative horizontal setup the situation is totally different: Gel layers can be considerably thinner, because gels are running without a glass cassette.

The thermal conductivity coefficient  $\lambda$  is defined as power  $P$  [W] that is conducted through a material over the distance  $L$  [m] between two surfaces that differ in temperature  $dT$  [K] (see equation 1, in [http://www.engineeringtoolbox.com/thermal-conductivity-d\\_429.html](http://www.engineeringtoolbox.com/thermal-conductivity-d_429.html)).

$$\lambda = \frac{P}{LdT} \quad (1)$$

The higher coefficient  $\lambda$  and the thinner the layer is, the more efficient is the heat transfer. Plastic film-backing consists of polyethylene. It is much thinner than glass plates (250  $\mu\text{m}$ ) and has, although its temperature coefficient is worse ( $\lambda = 0.42\text{--}0.51$ ) versus  $\lambda = 1.05$  of glass ([http://www.engineeringtoolbox.com/thermal-conductivity-d\\_429.html](http://www.engineeringtoolbox.com/thermal-conductivity-d_429.html)), a higher temperature transfer efficiency. The cooling plates are made from aluminum oxide ceramics, which have a much better temperature coefficient ( $\lambda = 30$ ), and are directly cooled with water delivered from a thermostatic circulator. Electrophoretic separations in thinner gels produce sharper zones: this had been shown a few decades ago by Sanger and Coulson for DNA fragments [12] and Görg et al. [13] for proteins. Also it could already been observed a considerable advantage of thinner gel layers in spot visualization even for the classical stains of that time with their comparatively low sensitivity [13]. The detection of low abundant protein spots and superior focusing of low molecular weight proteins within the HPE gels (Figs. 3 and 4) clearly demonstrate the benefits of thinner gels.

With sharper zones a higher protein concentration per gel volume is obtained, which results in higher sensitivity of detection, and probably higher yield of tryptic digestion for subsequent MS analysis. Application of all these effects

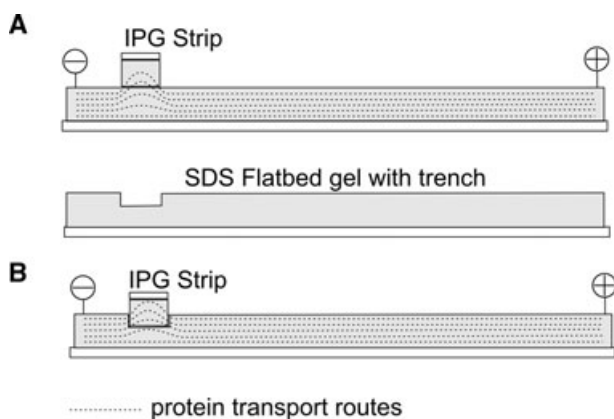


**Figure 7.** Schematic representation of protein transport from IPG strip to horizontal SDS flatbed gel. Situation A: Gel with even surface. Situation B: Gel with trench to accommodate IPG strip.

together improves the resolution and zone sharpness of the electrophoretic separation.

However, in order to exploit the advantages of thin horizontal flatbed gel technology, the method needed to be modified to secure the complete transfer of all proteins from IPG strip into SDS gel. The best solution is to introduce a narrow gel indentation into the stacking gel area with the width of 3 mm, exactly matching the standard width of all commercially available IPG strips. Figure 7 shows the comparison of protein transport routes into a SDS gel with an even surface, and into a SDS gel containing a trench. It is important, that there is still a gel layer at the bottom of the trench ensuring as much contact surface between the two gels as possible.

Staining of IPG strips after protein transfer showed a complete removal of proteins from the IPG strip, no proteins were left in the strip. There is a second advantage of this gel geometry: in contrast to gels with an even surface there is a defined location in the SDS gel to apply the strip: this improves the reproducibility of the protein pattern.

However, introducing such a restriction of the gel thickness results in another challenge: during application of the electric field gel will dry out at position of the IPG strip and start to burn already after a couple of minutes due to electroendosmosis. In this context it should be mentioned, that there has always been a technical challenge when combining IPG and SDS gels [14]: during equilibration of IPG strips in basic SDS buffer the carboxylic groups of the IPG become deprotonated, which results in fixed negative charges in the gel. In an electric field these negative charges are attracted by the anode, and this leads to the electroendosmosis effect: transport of positively charged water molecules into direction of the cathode. Generally, during the initial phase of the second dimension run, when proteins are transferred from IPG strip into SDS polyacrylamide gel, the electric field must be kept low, because otherwise this electroosmotic flow will carry a part of the proteins towards the opposite direction, the cathodal side. Additional measures, adding 6 M urea to equilibration solution, including a second equilibration step adding

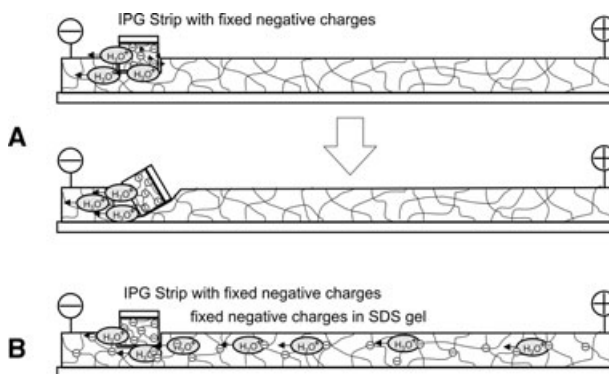


**Figure 8.** Schematic representation of electroosmotic water flow in situation A: SDS gel with standard polyacrylamide composition, and situation B: SDS polyacrylamide gel contains also fixed negative charges. (A) The electroosmotic effect, caused by fixed negative charges in the IPG strip, leads to drying out of the gel layer. (B) However, when also the SDS gel contains fixed negative charges, the trench will not dry out, because there is a small electroosmotic water flow generated throughout the SDS gel.

iodoacetamide, and prolongation of equilibration steps to 15 min each, reduced the electroosmotic flow between these two gels considerably [15]. When proteins have completely migrated into SDS gel, separation can be run with high electric field strength. No further measures need to be taken in vertical gel systems, because IPG strip and SDS gel edge are covered with cathodal buffer. However, when horizontal SDS gels were employed for second dimension, the IPG strip had to be removed from the gel as soon as possible after the proteins transfer phase, and the cathodal electrode wick had to be moved further onto contact area between IPG strip and SDS gel [6]. As another remedy incorporation of fixed charged groups into the second dimension polyacrylamide gel considerably reduced the electroendosmosis effects [14]. In Fig. 8 the explanation for this phenomenon is demonstrated: (A) the second dimension gel is polymerized with standard monomer solutions, and (B) for the second dimension gel acidic acrylamide derivatives are co-polymerized with acrylamide monomers. When the SDS gel contains fixed negative charges, the contact zone between IPG strip and SDS gel will not dry out, because there is a light electroosmotic water flow throughout the entire gel layer.

New horizontal HPE gels for 2DE contain therefore both modifications: a trench for accommodation of the IPG strip for a complete transfer of proteins into second dimension, and fixed acidic groups in the matrix to prevent drying of the contact area in gel.

The HPE gels used in this study had a thickness of 650  $\mu\text{m}$ , were 260 mm wide and 200 mm long. The trench is located in the 25 mm long stacking gel area containing 6% T and 3% C; the resolving gel contained 12.5% T and 2% C. The traditional Laemmli gels we used have a similar geometry beside thickness of 1 mm and were 230 mm wide and 220 mm long. Also the gel composition concerning acrylamide is similar, the resolving gel contained 12.5% T and 2.6% C. The traditional gels are slightly longer, the portion of

crosslinker is slightly higher but their performance is lower. Therefore an influence of acrylamide composition and principal geometry beside thickness is improbable.

In order to maintain long-term stability for commercial purposes and to prevent alkaline hydrolysis of the matrix, HPE gels are polymerized with a buffer having a pH value below pH 7. Consequently, it is necessary to replace the glycine in the running buffer of traditional Laemmli gels by tricine in case of HPE gels, in order to achieve a good separation quality. This has an additional benefit: with tricine in the running buffer the resolution in a HPE gel is extended down to 6 kDa in contrast to a minimum of 10 kDa in a conventional glycine buffer system. It might be expected, that such a modified buffer composition causes some modifications in the separation pattern in SDS electrophoresis due to an increased polymerization efficiency and interferences of the SDS-protein micelles with the different trailing ions. Also the addition of fixed negative charges to the matrix might have an influence on the separation parameters. However, so far we have not found any displacements of the respective protein spots, when conventional and HPE gels have been compared.

## 4.2 Outlook on technology development

A further reduction of gel thickness would improve the resolution further, however during our optimization experiments we had found out, that this would result in a loss of loading capacity: highly abundant protein spots showed a tendency of vertical streaking (not published). In addition: when a gel layer is thinner than 0.5 mm, spot picking robots show a tendency to lose gel plugs during their transfer from gel to microplate.

Currently experiments are performed on hardware side, using material with markedly higher temperature conductivity coefficients and thinner coatings with high electric resistance for even more efficient heat dissipation. We expect, that this will enable us to apply higher electric field strength on gel, in order to reduce running time and to increase resolution and sensitivity further. This may inspire the introduction of gel-based high-throughput screening projects, as such screenings on the protein level with 2DE have not technically been possible until now, but have the advantage of displaying possible post-translational modifications and protein isoforms. Combined with better access to low abundant proteins, which frequently act as regulators, and which are often post-translationally modified to fulfill their role, such a screening might open new perspectives for investigation of interesting biological questions.

*We thank Angelika Görg for inspiring and supporting alternative ideas of fundamental developments in technology of clas-*

*sical 2DE over the last decades. We also like to thank Gerhild Wachlin for her careful statistics about HPE gel runs in daily laboratory routine. This investigation received financial support from the Bundesministerium für Bildung und Forschung within the projects 0315592B and 03Z1C51.*

*The authors have declared the following potential conflict of interest: Dr. Reiner Westermeier is employed by SERVA Electrophoresis GmbH Heidelberg, Germany, a supplier of products for life sciences research covering electrophoresis reagents and equipment, fine biochemical quality reagents, and microbiology and cell culture specialties. All other authors are without commercial interests.*

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