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2-DE with IPGs

In order to overcome the limitations of carrier ampholyte generated pH gradients, IPGs were developed in the late 1970s. However, the 2-DE pattern we included in the first publication on IEF with IPGs [Bjellqvist *et al.*, *J. Biochem. Biophys. Methods* 1982, 6, 317–339] was far from being competitive to O'Farrell's high-resolution 2-DE with carrier ampholytes. Our 2-DE pattern in this article was, more or less, only a proof of principle. It was, however, the beginning of a long journey of stepwise improved 2-DE protocols we developed in our laboratory and summarized in the reviews published in *Electrophoresis* 1988, 9, 531–546 and in *Electrophoresis* 2000, 21, 1037–1053. Milestones were the design of the IPG strip, and the "reduction-alkylation equilibration protocol" of IPG strips after IEF for the efficient transfer of proteins from first to second dimension. The protocol of 2-DE with IPGs has been constantly refined, *e.g.* by the generation of tailor-made IPGs with different pH intervals from the acidic to the basic extremes (pH 2.5–12), and extended separation distances for improved resolution. In the present review, a historical outline from the technical difficulties encountered during the development of 2-DE with IPGs, to the establishment of the actual "standard protocol" will be given, as well as the modified procedures for the separation of very acidic, very alkaline, low-abundance and hydrophobic proteins, followed by a brief discussion of the advantages and technical challenges of gel-based proteomic technologies.

Keywords:

IPG / IPG-Dalt / 2-DE

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1 History

The rapid development of proteomics is technology-driven, and 2-DE for the separation of complex protein mixtures was the first proteomic approach. Meanwhile, 2-DE, MS and bioinformatic tools are the key components of an approach that has been termed "the classical proteomic methodology". However, in view of proteome complexity and numerous new technologies that have emerged in the last years, the question may arise: "Is 2-DE outdated or still indispensable?" The limitations of 2-DE are repeatedly listed and described, with respect to (i) resolution, (ii) reproducibility, separation of (iii) very acidic and (iv) very basic proteins, detection of (v) low-abundance proteins and (vi) integral membrane proteins and/or constraints to (vii)

detect all the proteins present in the sample with a wide range in expression levels and differences in solubility.

1.1 From 2-D maps to high-resolution 2-DE

The *resolution* of 2-DE was dramatically improved by O'Farrell in 1975 [1] introducing chaotropes and detergents for protein solubilization and separation in both dimensions replacing the former 2-D maps (native IEF × native PAGE) with limited resolution and spot numbers (*e.g.* Macko *et al.* [2], Dale and Latner [3], Kenrick and Margolis [4], Wrigley [5], Domschke *et al.* [6] and Klose [7]). In contrast to former results using native 2-DE, with, *e.g.* 275 protein spots in the 2-DE map of fetal mouse liver, and about 230 spots in whole mouse embryos as reported by Klose [7], O'Farrell succeeded for the first time to reveal more than 1100 protein spots of *Escherichia coli* lysates [1]. However, despite his breathtaking success in 2-DE, O'Farrell already critically described in his pioneering publication that the *reproducibility* of 2-D patterns is limited due to batch-to-batch variability of carrier ampholyte synthesis and pH gradient instability over focusing time. Moreover, due to the cathodic drift [8] and the so-called "plateau phenomenon" [9] of carrier amphi-

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Abbreviation: DIGE, difference gel electrophoresis

lyte-generated pH gradients, in practice, the final pH gradient did not extend far beyond pH 7.5, with resultant loss of alkaline proteins [1]. Hence, O'Farrell *et al.* in 1977 introduced an alternative procedure, known as non-equilibrium pH gradient electrophoresis [10] with short focusing times for the separation of alkaline proteins. Under these non-equilibrium IEF conditions, alkaline proteins are still present in the 2-DE gel, however, at the expense of reproducibility, since this procedure is difficult to standardize.

1.2 From carrier ampholytes to IPGs

In order to overcome the limitations of carrier-ampholyte-generated pH gradients, immobilized pH gradients were developed by Gasparic, Bjellqvist and Rosengren [11]. IPGs are based on the bifunctional Immobililine[®] reagents, which are chemically well-defined acrylamide derivatives with the general structure $\text{CH}_2 = \text{CH-CO-NH-R}$, where R contains either an amino or a carboxyl group, and form a series of acrylamido buffers with different p*K* values between p*K* 1.0 and 13. Since the reactive end is co-polymerized with the acrylamide matrix, extremely stable pH gradients are generated, holding the promise of true steady-state IEF with increased reproducibility (see E. Gianazza and P.G. Righetti, this issue).

The Immobililine concept was published for the first time in 1982 [12] as the result of a collaboration of three groups that started in 1981, after an Electrophoresis Society Meeting in Charleston (USA): Bengt Bjellqvist and Kristina Ek from LKB (Bromma, Sweden), Elisabetta Gianazza and Pier Giorgio Righetti from the University of Milano (Italy) and Angelika Görg, Reiner Westermeier and Wilhelm Postel from the Technische Universität München (Germany). LKB provided the "patent" and theory of Immobililines, Pier Giorgio Righetti was to that time the unchallenged "pope of IEF", whereas Angelika Görg was nick-named "the ultrathin lady", as she had challenged the conventional 2 mm thick polyacrylamide gels for 2-DE by introducing thin (≤ 0.5 mm) polyacrylamide gels on plastic backing for IEF and SDS PAGE [13, 14]. The casting of thin gradient gels on plastic backing was one of the prerequisite conditions to realize the "Immobililine Project", which was at that time only nick-named within LKB as the "Smear project" due to the disappointing results.

1.3 From proof of principle to standard procedure

Despite the rapid progress we made in the next six months after the Charleston meeting, our first 2-DE pattern, included in this publication (Fig. 11 in [12]), was far from being competitive to O'Farrell's high-resolution carrier ampholyte 2-DE. The 2-DE pattern in this article was, more or less, only a proof of principle. It was, however, the beginning of a long journey of stepwise improved 2-DE

protocols we developed in our lab and summarized in the reviews published in 1988 [15] and 2000 [16]. During the next ten years after the publication of our basic protocol in 1988 [15], 2-DE technology with IPGs became commonly accepted and has now largely superseded carrier ampholyte-based 2-DE. Both review papers have been cited almost 600 times and more than 800 times, respectively. However, there were many more original articles in which we demonstrated, *e.g.* the increased reproducibility of 2-DE with IPGs in several inter-laboratory comparisons [17, 18], the higher resolution by the ability to generate (ultra-) narrow pH gradients (*e.g.* IPG 4.9–5.2 over 24 cm; $\Delta pI = 0.001$) for complex samples such as mouse liver [19–21], the detection of low-abundance proteins after sample prefractionation in Sephadex-IEF gels [22, 23], and the highly reproducible separation of alkaline proteins [24–28] using IPGs up to pH 12 and steady-state IEF running conditions replacing O'Farrell's non-equilibrium pH gradient electrophoresis [10] technology. Consequently, IEF with IPGs is nowadays predominantly used for the first dimension of 2-DE in proteome analysis. The IPG-Dalt protocol summarized below has become of common use and has been established as the standard procedure that is nowadays described in the manufacturers' manuals or applied in scientific publications without being cited any longer.

2 Milestones

The protocol of 2-DE with IPGs has been constantly refined. Milestones were the design of the IPG strip (Fig. 1) [15], the "reduction-alkylation equilibration protocol" [29] for IPG strips for the efficient transfer of proteins from first to second dimension and for improved pattern quality and, finally, the generation of tailor-made pH gradients with different pH intervals from pH 2.5 to 12 and/or extended separation distances for improved resolution and for the separation of very acidic and/or very alkaline proteins [24–28].

2.1 Development and design of IPG strips

Of prime importance was the development and design of IPG strips (Fig. 1) [15, 30], which finally went into industrial production in 1991. Attempts by Righetti's [31] and Hochstrasser's [32] groups to run IPG IEF in capillaries or gel rods – complementary to carrier ampholyte 2-DE in gel rods – were not successful. In contrast to those attempts, our approach was based on flat-bed IPG gels. Our first attempt in 1982 [12] was to use thin IPG gel plates cast on plastic backing for IEF of a number of samples run in parallel. After IEF, the proteins separated in the gel plates were fixed and stained with Coomassie Blue. The IEF lanes containing the visualized samples were cut out using sharp scissors and placed on horizontal SDS-PAGE gels (Fig. 1A). However, depending on the different salt content of the

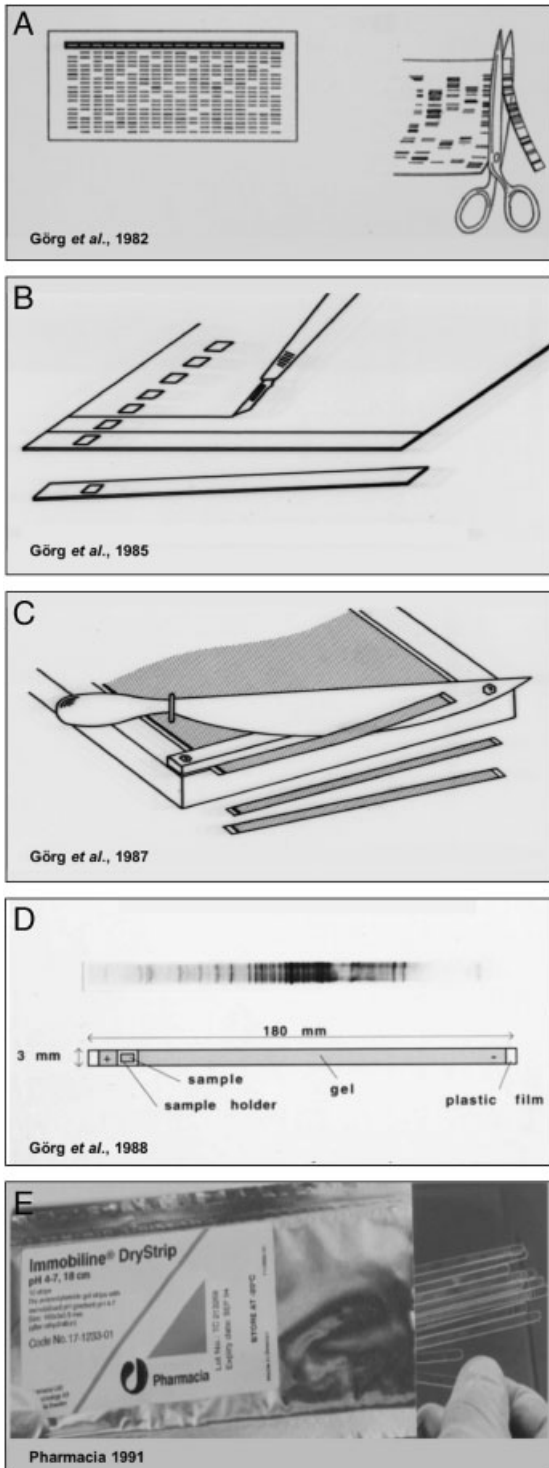


Figure 1. Development and design of IPG strips according to Görg *et al.*: IEF in IPG plates on plastic backing. (A) Cutting IEF sample tracks after Coomassie staining (Görg and co-workers, 1982 [12]). IEF in individual IPG strips (B) by slicing a rehydrated IPG plate into single IPG strips *before* IEF (Görg *et al.*, 1985 [34]); (C) by cutting the IPG dryplate into strips *prior to rehydration and IEF* (Görg *et al.*, 1987 [35]). (D) Design of IPG gel strips cast on plastic backing (Görg *et al.*, 1988 [15]). (E) Industrial production of the first ready-made IPG DryStrips[®] (Pharmacia, 1991).

samples applied in parallel, the IEF bands were extending horizontally (lateral band-spreading) and running in “curved” tracks. Therefore, gel troughs on the plate were produced by removing small gel lanes between the sample application sites using pipette tips connected to a water-jet vacuum pump, as suggested by Righetti [33]. Alternatively, we cut the rehydrated IPG plate into individual 10 mm wide strips prior to IEF using a scalpel (Fig. 1B) [34], but it was a tedious procedure to get through the rigid plastic film and the soft rehydrated gel without gel distortions. Finally, as a by-product of a fruitful collaboration with Sam Hanash’s 2-DE lab in Ann Arbor (USA), we decided to cut the washed and dried IPG plates into individual strips using a paper cutter (Fig. 1C) [15, 35]. Also, we realized that narrower (*i.e.* 3–5 mm wide) IPG strips were optimal in order to reduce detergent smear and electroendosmotic effects on horizontal SDS gels [30, 35]. The design of the strip is shown in Fig. 1D and is still valid until today. The industrial production of the first IPG DryStrips (Pharmacia), adopting this design, started in 1991 (Fig. 1E).

2.2 The equilibration step

The “equilibration protocol” of IPG strips we introduced in 1987 [29] has been widely accepted and is nowadays part of the standard protocol of IPG-Dalt used for the proper transfer of proteins from first to second dimension and for improved pattern quality. Regrettably, this paper has been cited only rarely (157 times), probably due to the fact that papers published in *Electrophoresis* before 1987 are not listed in MedLine.

It is essential that the IPG strips be equilibrated after IEF to allow the separated proteins to fully interact with SDS. Due to the observation that the focused proteins bind more strongly to the fixed charged groups of the IPG gel matrix than to carrier ampholyte gels, we prolonged the equilibration time recommended by O’Farrell from approximately 2 min to 10–15 min, and added urea and glycerol to the equilibration solution to reduce electroendosmotic effects inherent to the fixed charges of IPG strips in contact with SDS gels and to improve protein transfer from the first to the second dimension. The most important step of our equilibration protocol [29] is to incubate the IPG strips for 10–15 min in a Tris-HCl buffer containing 2% SDS, 1% DTT, 6 M urea and 30% glycerol. This is followed by a further 10–15 min equilibration in the same solution containing 4% w/v iodoacetamide instead of DTT. The latter step is used to alkylate any free DTT, as otherwise it migrates through the second-dimension SDS-PAGE gel – binding to any dust particles – resulting in an artifact known as point-streaking that can be observed as very sharp, thin vertical lines in silver-stained gels. More importantly, iodoacetamide alkylates the sulfhydryl groups of the proteins to prevent their potential reoxidation during the SDS PAGE run with subsequent vertical spot streaking. This step is also highly recommended for spot identification by MS. After equilibration, the IPG strips are applied onto

the surface of the vertical or, respectively, horizontal SDS-PAGE gels [36].

Loss of proteins during the equilibration step and subsequent transfer from the first to the second dimension has been reported and is primarily due to proteins that remain in the IPG strip because of adsorption to the IPG gel matrix and/or insufficient equilibration times, and because of wash-off effects. For better control of potential protein losses due to protein adsorption to the IPG strip, strips may be stained with Coomassie Brilliant Blue after 2-DE. Investigations using radio-labelled proteins revealed that up to 20% of the proteins get lost during equilibration [37]. The majority of these proteins (most probably, those located near the surface of the IPG strip) are lost during the very first minutes of equilibration and are quite reproducible for any given sample, whereas protein losses in the second equilibration step are insignificant.

3 The IPG-Dalt standard protocol

The original protocol of 2-DE with immobilized pH gradients (IPG-Dalt) as described in 1988 [15], updated in 1995 [36] and 2000 [16]) summarizes our own experiences over the years, and the step-wise improvements, the critical parameters we experienced inherent to IEF with IPGs and a number of different experimental conditions and samples. Moreover, the protocols were tested by more than 500 participants in approximately one-week hands-on courses, performed in our lab between 1990 and 2008, with the participants' samples with unknown properties, not to mention the prominent black specks engraved in the cooling plates indicating samples that "burned" during the first experiments. We did not count all the tutorials and hands-on courses we ran worldwide, but in summary it was an excellent opportunity to transfer knowledge and to receive precious feed-back and input from the participants. As a result of all those activities, a standard protocol has been established that can be summarized as follows: The first dimension of IPG-Dalt, IPG-IEF, is performed in individual, 3 mm wide and up to 24 cm long IPG gel strips cast on GelBond PAGfilm (laboratory-made or commercial Immobiline DryStrips). After IEF, the IPG strips are equilibrated with SDS buffer in the presence of urea, glycerol, DTT and iodoacetamide [15, 29], and applied onto horizontal or vertical SDS gels in the second dimension [36].

3.1 Preparation of IPG strips

Linear or non-linear wide (*e.g.* IPG 3–12), medium (*e.g.* IPG 4–7), narrow (*e.g.* IPG 4.5–5.5), and/or ultra-narrow (*e.g.* IPG 4.9–5.3) IPGs can be cast in different pH ranges between 2.5 and 12, as well as in different lengths, usually from 7 to 24 cm (however, IPG strips up to 54 cm long have been generated [38]). Narrow or ultra-narrow (≤ 1 pH unit) linear IPG can be calculated with relative ease with the help of the Henderson–Hasselbalch equation, or by using nomograms (for

details see [33]), whereas computer-assisted programs as developed by Righetti's [39] and Altland's [40] group are obligatory for the calculation of wider (> 1 pH unit) and/or complex (*e.g.* non-linear) IPGs. IPG gels are formed by mixing two immobiline starter solutions in a gradient mixer according to the gradient casting technique for ultrathin gels on plastic backing initially developed by Görg *et al.* [13, 14]. Besides Immobiline[®] chemicals, the gel-casting solutions contain an acrylamide/bisacrylamide mixture. For narrow-range alkaline IPG gels (*e.g.* IPG 9–12), acrylamide may be substituted by *N,N'*-dimethyl-acrylamide for improved stability of the gel matrix [24]. After polymerization at 50°C for 60 min, the IPG gel is washed with deionized water, impregnated in aqueous glycerol and dried at room temperature. The surface of the dry IPG gel is protected with a sheet of plastic film before it is stored in a sealed plastic bag at -20°C . Prior to use, the dried gel is cut into 3 mm wide IPG dry strips with a paper cutter (Fig. 1C) [15, 30]. Besides these laboratory-made IPG gels, a multitude of commercial IPG dry strips can now be purchased from different suppliers. These ready-made IPG dry strips are increasingly popular due to simplified handling, and standardized pH gradient slopes by using computer-driven burette systems of Altland [41, 42] with, consequently, improved inter-laboratory reproducibility and comparability of data [17, 18]. Those ready-made IPG Dry Strips have significantly contributed to the widespread application of 2-DE in proteomics.

3.2 Sample application

Prior to IEF, the IPG dry strips are rehydrated to their original thickness with a buffer containing urea (or, alternatively, urea/thiourea [43]), nonionic- or zwitterionic detergent(s), a reductant and small amounts of carrier ampholytes [44]. Although the IPG strips are typically rehydrated for instantaneous use, they may also be stored at -70°C up to several weeks for later usage without loss of pattern quality.

Samples can be applied onto IPG strips in two different ways: either onto the rehydrated IPG strips by "cup loading" or, alternatively, IPG dry strips are rehydrated with rehydration buffer already containing sample ("sample in-gel rehydration") [45]. Although sample application by in-gel rehydration is more convenient than cup-loading, it is discouraged for samples containing (i) very high molecular weight, (ii) very alkaline and/or (iii) hydrophobic proteins, since these are taken up into the gel only with difficulty, *e.g.* due to hydrophobic interactions between proteins and the wall of the tray, or because of size-exclusion effects of the gel matrix. Improved entry of higher molecular weight proteins ($M_r > 100$ kDa) into the IPG gel matrix is facilitated by "active" rehydration, *i.e.* by applying low voltages (30–50 V) during reswelling [16, 26], but for quantitative analysis, sample in-gel rehydration is still somewhat less reliable than cup-loading [46].

For *cup-loading*, IPG dry strips are reswollen in rehydration buffer. After rehydration, samples ($> 50 \mu\text{L}$) are

applied into disposable plastic cups placed onto the surface of the IPG strip. Best results are typically obtained when the samples are applied at the pH extremes, *i.e.* either near anode or cathode. Sample application near the anode proved to be superior to cathodic application in most cases. When using basic pH gradients such as IPGs 6–10, 6–12 or 9–12, anodic application was found to be mandatory for all kinds of samples investigated [24–28].

3.3 IEF running conditions

Depending on the protein amount, sample volume and conductivity of the sample solution (*e.g.* salt, carrier ampholytes, *etc.*), voltage and current should be limited to 150 V and 50 μ A *per* IPG strip during the initial stage (1–2 h) of IEF to ensure proper sample entry and/or to avoid Joule heating due to salt ions in the sample. As the run proceeds, the salt ions migrate towards the electrodes, resulting in decreased conductivity and allowing high voltages (up to 12 000 V) to be applied. Sample entry time with restricted voltages (50–100 V) should be prolonged up to 5 h for samples with high salt contents or for micropreparative separations with high sample loads. Several changes of the electrode filter paper strips in which the ions collect are recommended [16, 36].

Temperature during IEF has an important effect on the reproducibility of the 2-DE patterns, and was investigated by us in detail [47]. It turned out that focusing temperature does not only affect pattern quality, but also has an influence on the spot positions of the resulting 2-D polypeptide patterns since spot positions vary along the pH axis with different applied temperatures. Shifts in spot positions were primarily found at the extremes of the pH gradient, whereas in the pH region between 6 and 7, position shifts were less marked [47]. It is thus extremely important to run the separations at an actively controlled temperature, where 20°C proved to provide the optimal conditions.

The number of *volthours* required for steady-state IEF depend on the pH gradient (wide or narrow), the separation distance and the amount of protein loaded onto the IPG strip. Hence, the optimum focusing time must be established empirically for each combination of protein sample, protein loading and the particular pH range and length of IPG gel strip used. Detailed protocols including optimum focusing parameters for a number of different wide and narrow pH range IPGs have been published by us [15, 16, 48] and are also available on our web-site (<http://www.wzw.tum.de/proteomik>).

3.4 Tailor-made IPG gels

The *choice of pH gradient* primarily depends on the sample's protein complexity. Broad range IPGs, such as IPG 3–12, are typically used to analyze simple proteomes (*i.e.* small genome, organelles or subfraction) or to obtain an overview

of a more complex proteome. Although the resolution of 2-DE is impressive, it is, however, still far from being comprehensive in view of the enormous diversity of proteins from higher eukaryotic proteomes, where extensive post-translational modifications of proteins and differential gene splicing lead to expression of more proteins than the total number of genes in their genomes. With samples such as total lysates of eukaryotic cells or tissues, 2-DE on a single wide-range pH gradient reveals only a small percentage of the whole proteome. The best approach, preferentially in combination with prefractionation procedures, is to use *multiple narrow overlapping* IPGs (“zoom-in” gels, *e.g.* IPG 4–5, IPG 4.5–5.5 and/or extended separation distances (up to 24 cm, or even longer) to achieve an optimal resolution to avoid multiple proteins species in apparent single spots. The potential of narrow IPGs and extended separation distances has been exemplified by the separation of pea proteins as early as 1988 [15]. Later, we have systematically investigated the improved separation and visualization capabilities of narrow and or ultra-narrow IPGs (maximum resolution: $\Delta pI = 0.0015$) by analyzing the *Saccharomyces cerevisiae* [21] and mouse liver proteomes [19]. In addition, Westbrook *et al.* [49] demonstrated not only the improved resolution and higher spot numbers by using narrow-range IPGs, but also the identification by MS of additional protein species and isoforms of proteins from apparent single spots on broader-range IPGs. However, it should be kept in mind that despite the relatively high sample loads (mg quantities) that may be applied onto narrow pH range IPGs, proteins from genes with codon bias values of <0.1 (*i.e.* lower-abundance proteins) remain usually undetected unless samples are prefractionated prior to IPG IEF (*cf.* [22, 50]).

3.5 Giant and mini gels

Very long (≥ 30 cm) separation distances for maximum resolution of complex protein patterns have been applied occasionally [51]. Although size stability and handling of the fragile tube gels used in carrier-ampholyte 2-DE is often a problem, IPG gel strips are cast on plastic backings so that they can neither stretch nor shrink, and thus contribute significantly to improved reproducibility. Twenty-four centimeter long IPG gel strips [26] are meanwhile routine, but also 54 cm long IPG strips were run successfully [38]. On the other hand, short separation distances (≤ 40 mm) in both dimensions have been applied for 2-DE analysis of samples containing a limited number of proteins only, *e.g.* for purity control of recombinant proteins or for high throughput analysis, using the PHASTTM electrophoresis system [52].

3.6 Second dimension: SDS-PAGE

Surprisingly enough, it is often neglected that SDS-PAGE is, in contrast to IPG-IEF, not a steady-state separation, and

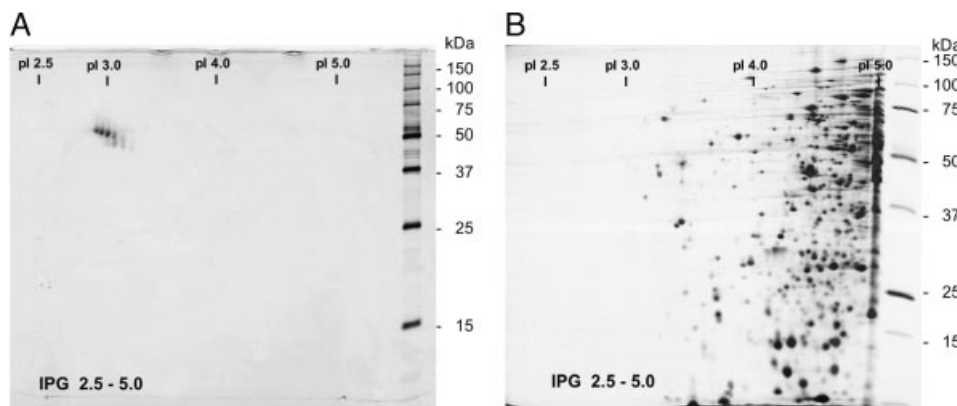


Figure 2. IPG-Dalt of very acidic proteins. IPG 2.5–5.0. Anodic sample application. Sample entry: step-wise increased voltage to the steady-state according to our standard protocol [16]. Samples: (A) Hyperglycosylated recombinant erythropoietin anlogon (Aranesp[®]). (B) *C. glutamicum* proteins.

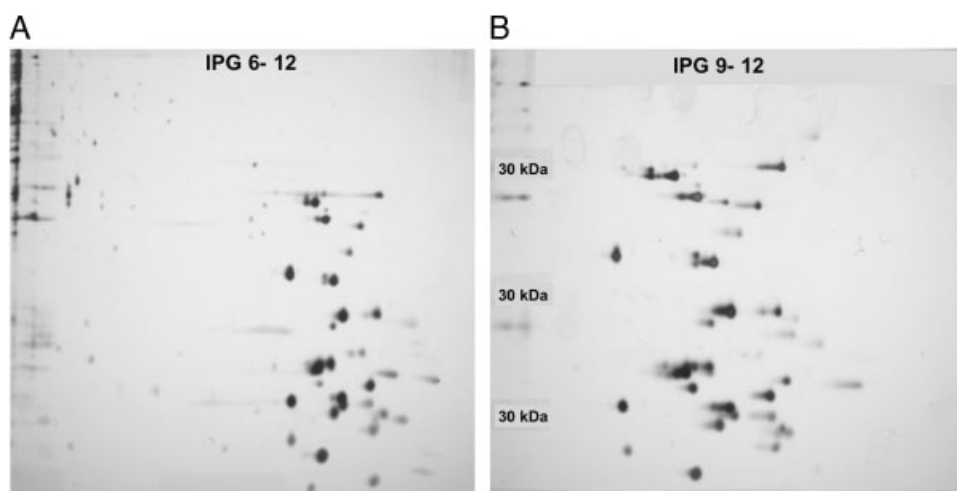


Figure 3. IPG-Dalt of very alkaline *L. lactis* proteins. IPG 6–12 and 9–12. Sample application at the anode. Sample entry: limited to 2 h with 150–500 V, continued to the steady state with 8000 V according to our modified protocol [27, 28]. Reference maps and proteome databases of very alkaline proteins see Drews and Görg [76] (also available on our webpage: <http://www.wzw.tum.de/proteomik/lactis/index.htm>).

thus adds a number of variabilities in 2-D patterns. Therefore, highly standardized running conditions and electrophoresis systems should be used. SDS PAGE can be performed on horizontal or vertical systems [36]. Horizontal setups [53] are ideally suited for ready-made gels (*e.g.* ExcelGel; GE Healthcare), whereas vertical systems are preferred for multiple (up to 20) runs in parallel, in particular for large-scale proteome analysis, which usually requires simultaneous electrophoresis of batches of second-dimension SDS-PAGE gels for higher throughput and maximal reproducibility [54]. In contrast to horizontal SDS-PAGE systems, it is not necessary to use stacking gels with vertical setups, as the protein zones within the IPG strips are already concentrated and the nonrestrictive, low polyacrylamide concentration IEF gel acts as a stacking gel.

4 Challenges in proteome analysis

Limitations and challenges of 2-DE are repeatedly reported in different reviews with respect to the analysis of very acidic and very alkaline, low-abundant and/or very hydrophobic proteins.

4.1 Very acidic proteins

By using acrylamido buffer pK 1.0, very acidic IPGs can be generated. IPGs from 2.5 to 5 are easily established and run under standard conditions without electroendosmotic flow *etc.* However, according to our experience not too many very acidic proteins with pIs below 3.0 are present in the majority of samples, and, hence, detectable (A. Görg *et al.*, unpublished results; *see* Fig. 2).

4.2 Very alkaline proteins

Theoretical 2-D maps calculated from sequenced genomes indicate that approximately 30% of all proteins possess alkaline pIs up to pH 12, but it is frequently reported that very alkaline proteins are not accessible in gel-based proteomics. However, by generating IPGs up to pH 12 [24–26], *e.g.* IPGs 6–12 and 9–12 (Fig. 3), and modifying the standard protocol of IPG-Dalt [24, 27, 28], highly reproducible 2-D patterns of, *e.g.* ribosomal proteins with pIs between 10.5 and 11.8 were obtained and reference maps of *S. cerevisiae*, *Lactococcus lactis* and *Corynebacterium glutamicum* cells could be established [27, 28].

The protocol for very alkaline proteins, which was established after a series of experiments, is rather simple, taking into account that it took us almost 1 year of trial and error experiments and encouraging coffee breaks to get rid of the most reproducible horizontal streaks in the 2-DE patterns of J. J. Madjar's ribosomal proteins of HeLa cells with *pI*s from 10.5 to 11.8 (see Fig. 3 in [24]). Using IPGs 9–12, there was a strong water transport from the cathode to the anode (see Fig. 4 in [24]), indicated by desiccated paper wicks at the cathode, glued to the gel and waterlogged paper strips at anode. Suggestions such as the substitution of dimethyl-acrylamide for acrylamide [24], the use of an additional paper strip soaked with DTT applied near the cathode providing a continuous influx of DTT during IEF to compensate for the loss of DTT (which is a weak acid and migrates out of the basic part of the gel) [36, 55] and the addition of isopropanol to the IPG rehydration solution in order to suppress the reverse electroosmotic flow, which causes highly streaky 2-D patterns in narrow pH range IPGs of 9–12 and 10–12 [24], were, although helpful to a certain extent, not really satisfying.

Finally, it turned out that for the separation of very alkaline proteins, cup-loading at the anode is mandatory, and the use of

high voltages (final settings up to 8000 V) after a short sample entry time with limited voltages, is the most efficient and simple remedy [26–28]. Moreover, the sulfhydryl groups of cysteines in proteins should be stabilized as mixed disulfides by using hydroxyethyl-disulfide (DeStreak™) reagent in the IPG strip rehydration solution instead of a reductant [56].

In addition, wide pH gradients up to pH 12, such as IPG 3–12 [26] and IPG 4–12 [25] are ideally suited to provide an overview of the proteome of a cell or tissue. In particular, the IPG 4–12, which is flattened between pH 9 and 12, proved to be an extremely useful pH gradient for the separation of very alkaline proteins. For eukaryotic organisms, procedures such as TCA/acetone precipitation of proteins are recommended for enrichment of basic proteins exceeding *pI* 10, since these are usually underrepresented in lysis buffer extracts [26].

4.3 Low-abundant proteins

Since there is no amplification step for proteins analogous to the polymerase chain reaction method for amplifying

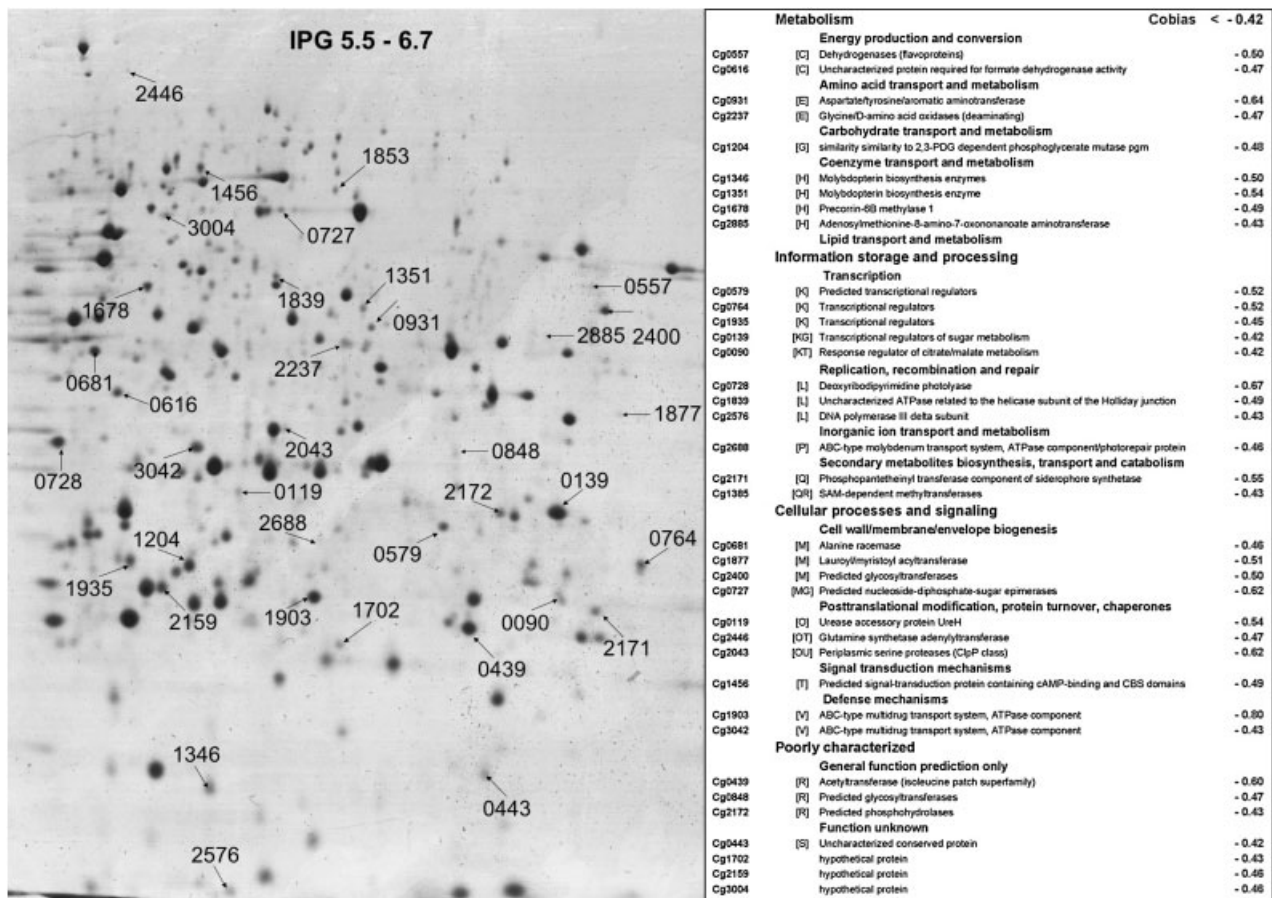


Figure 4. IPG-Dalt of putative low-abundance *C. glutamicum* proteins after sample pre-fractionation with flat-bed Sephadex IEF according to Görg *et al.* [22, 23]. IPG 5.5–6.7. A protein fraction separated in a Sephadex IEF gel is applied on top of a rehydrated IPG strip and covered with silicone oil. IEF running conditions according to standard protocol [16]. Carsten Lück, PhD-Thesis, Technische Universität München, 2008 (<http://mediatum2.ub.tum.de/node?id=650128>).

nucleic acids, and due to the high dynamic range and diversity of expressed proteins, particularly in eukaryotic tissues, the analysis of low-abundance proteins is a major problem encountered in almost all proteome analysis technologies. Strategies for increasing the comprehensiveness of analysis of complex proteomes are to subdivide the proteome either by targeting a specific sub-proteome or by applying sample prefractionation methods. Popular approaches are based on electrophoretic prefractionation such as preparative IEF, IEF in a rotating, multi-chamber device, free-flow electrophoresis or the use of a multifunctional electrokinetic membrane apparatus in which samples are separated by charge and/or size (reviewed by Simpson [57]). A multicompartment electrolyzer with isoelectric membranes has been developed by Righetti and co-workers and was later adapted to the requirements of proteome analysis [58, 59]. A simple, cheap and fast prefractionation procedure based on flat-bed IEF in granulated gels has been devised by Görg *et al.* [22, 23]. The protein fractions are simply loaded onto the surface of the corresponding narrow IPG strips. It has been demonstrated that this type of prefractionation allows higher protein load (6- to 30-fold) on narrow IPG gels without detrimental protein precipitation at the electrodes and horizontal streaking [50]. The improved resolution of very narrow IPGs in combination with milligram-amounts of prefractionation

enables the detection of low-abundance proteins (see Fig. 4).

4.4 Membrane proteins

Membrane proteins, which constitute a significant proportion (approximately 30%) of the cell's proteins, are of particular interest since they are supposed to play key functions in various important cellular processes and are, therefore, important targets for drug development. Despite their importance, recent proteomic studies have demonstrated that very hydrophobic proteins and, in particular, membrane proteins are extremely underrepresented on 2-DE gels, and that the analysis of very hydrophobic proteins, such as integral membrane proteins, remains a challenge for 2-DE (but also LC-MS) based proteomic approaches. This under-representation may be attributed to several factors: Besides their low solubility and their tendency to aggregate and precipitate in aqueous media, many membrane proteins possess basic pIs and/or are expressed in low copy numbers [60, 61]. Certain "loss" of membrane proteins on 2-D gels may also be attributed to the fact that these proteins, once solubilized, may in fact enter the IPG strips and also focus properly, but do not elute during the transfer step from first to second dimension [62,

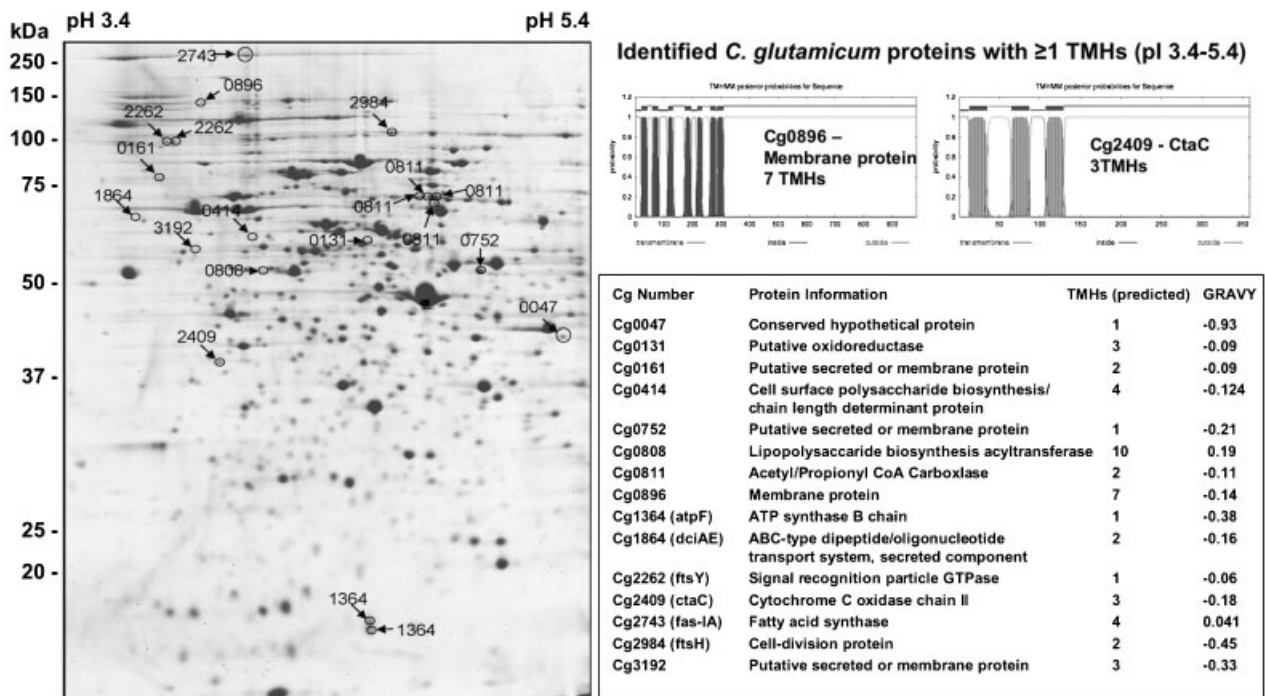


Figure 5. IPG-Dalt of *C. glutamicum* membrane proteins. IPG 3.4–5.4. Sample preparation: Membrane pellet was extracted with a buffer containing 7 M urea, 2 M thiourea, 1% octyl- β -D-galactopyranoside, 1% NP-40, 1% Triton X-100, 0.5% DTT and 0.5% Pharmalyte in 50% acetonitrile. IPG strip rehydration: same buffer, except 6 M urea instead of 7 M. Sample application: at the anode. Sample entry: 3 h, followed by IEF to the steady state according to our standard protocol [16]. Transmembrane helices were predicted with the TmPred (http://www.ch.embnet.org/software/TMPRED_form.html) and the Jvrigel (www.jvrigel.de) software programs. (F. Weiland, C. Lück, A. Klaus, A. Görg, *Protome analysis of C. glutamicum: Strategies for the detection/identification of alkaline and hydrophobic proteins*. Presented at the 7th Siena meeting "From Genome to Proteome", 2006) (<http://www.expasy.ch/ch2d/siena/poster06.pdf>).

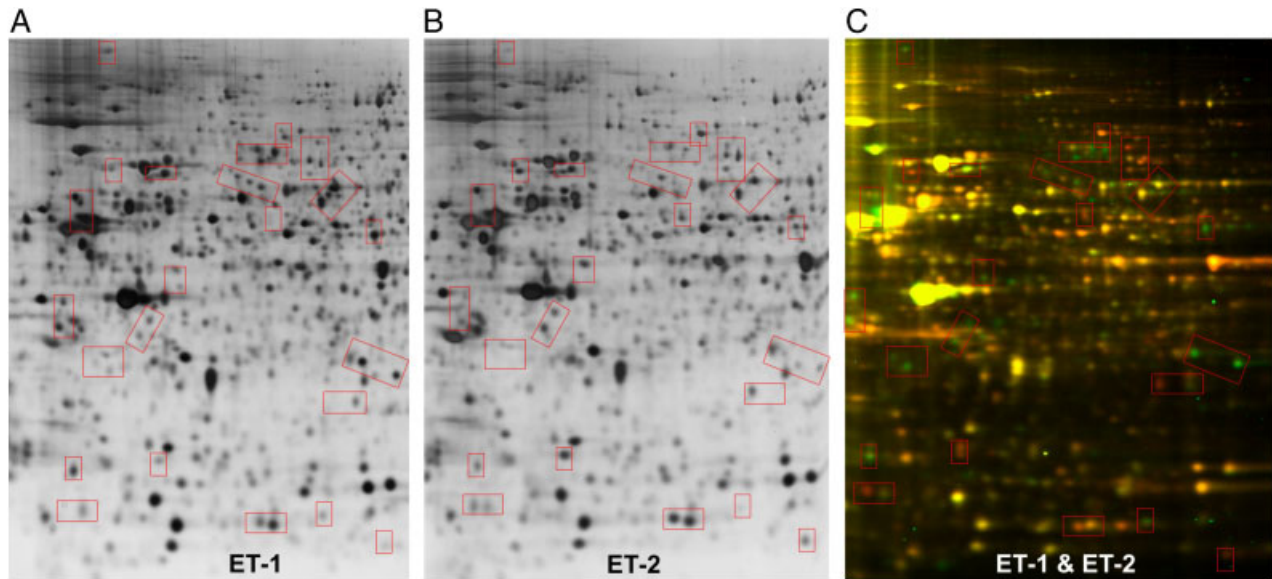


Figure 6. Comparison of Ewing tumor cell lines ET-1 and ET-2. IPG 4–8. (A and B) Silver stain. (C) Image overlay using DIGE technology for the unambiguous detection and quantitation of differentially expressed proteins. Squares indicate differentially expressed proteins (F. Weiland, G. Richter, S. Burdach, A. Görg, in preparation).

63]. Thiourea has been recommended in the IPG equilibration protocol for more efficient transfer of hydrophobic proteins [63], but may also cause vertical streaks in the 2-D pattern. Progress has been made towards improving solubilization and separation of hydrophobic proteins, *e.g.* by the introduction of thiourea and novel zwitterionic detergents [64–66], and the inclusion of acetonitrile into the IPG DryStrip rehydration solution (see Fig. 5), which enable the display of at least several membrane proteins on 2-DE gels, however predominantly those with low GRAVY scores, *i.e.* proteins with only one or two transmembrane helices, or those with a rather hydrophilic (*e.g.* glycosylated) “tail”, so that their overall hydrophobicity is not too high (*cf.* Fig. 5). These studies demonstrate that a unique procedure to solubilize and separate all membrane proteins does not exist yet, and that most integral membrane proteins still cannot be adequately solubilized with nonionic or zwitterionic detergents or organic solvents. More or less, the best strategy of gel-based proteomics is the combination of sample prefractionation procedures and SDS-PAGE analysis of membrane fractions in combination with LC-MS/MS (*cf.* [67]).

4.5 Quantitative proteomics: difference gel electrophoresis

Quantitation of differentially expressed proteins in a series of samples is – despite novel technologies – not an easy effort, independent from using gel-based or non-gel-based proteomic technologies. With the introduction of fluorescent difference gel electrophoresis (DIGE) technology for 2-DE by Minden and co-workers ([68]), and by using an internal standard [69], quantitation of differently expressed

proteins in a series of samples became reality. In DIGE, two – or more – samples are labeled *in vitro* using different fluorescent cyanine minimal dyes differing in their excitation and emission wave-lengths, then mixed before IEF and separated on a single 2-D gel. After consecutive excitation with the appropriate wavelengths, the images are overlaid and “subtracted” (normalized), whereby only differences (*e.g.* up/downregulated, and/or post-translationally modified proteins) between the two samples are visualized (Fig. 6). Due to the comigration of both samples, methodological variations in spot positions and protein abundance are excluded, and, consequently, image analysis is considerably facilitated. Since three cyanine dyes are available, it is possible to include an internal standard, which is run on all gels within a series of experiments. This internal standard, typically a pooled mixture of all the samples in the experiment labelled with this dye, is used for normalization of data between gels thereby minimizing experimental variation and increasing the confidence in matching and quantitation of different gels in complex experimental designs [69].

5 Outlook

5.1 2-DE for proteome analysis: Outdated or still indispensable?

There is a long history of the application of 2-DE, *e.g.* medical, clinical, biological, genetic or toxicological research, which is reflected in thousands of publications during the past 30 years. After its steep rise in the late 1970s and the early 1980s after O’Farrell’s outstanding publication, there was a slow decline in the late 1980s due to the inability to identify the gel-separated

proteins of interest on a large scale. However, after the introduction of large-scale genome sequencing, the development of MS methods for the analysis of proteins and peptides, progress in bioinformatic tools and the rise of Proteomics in general, 2-DE – in particular with IPGs – has experienced a revival and is today by far the most commonly applied protein separation technology in proteome research. However, despite its inherent advantages, 2-DE is far from being perfect for analyzing the total proteome of complex eukaryotic organisms. Almost ten years ago, S. J. Fey and P. Mose Larsen wrote: “2D gel electrophoresis is the technology that everyone loves to hate. Although almost everyone would like to replace it, the resolution and sensitivity it offers are exquisite and unsurpassed if one wants a global view of cellular activity” [70]. Despite numerous attempts to replace 2-DE, which led to the development of several highly-sophisticated non-gel-based proteomic technologies, we consider this statement still valid at large. Each proteome analysis technology currently available resembles characteristic technical advantages, but also limitations [71]. For example, when LC/LC-MS-based proteomic analysis is repeated on the same protein extract, the average of overlap of identified protein species between experiments is typically only 60% [72]. In contrast to the 2-DE approaches, information about protein abundances is initially unavailable in the non-gel-based technologies, unless stable isotope labelling is applied. Moreover, 2-DE is highly parallel and unsurpassed for its ability to run as far as 20 2-D gels at a time with thousands of proteins *per* gel. Post-translationally modified proteins can be readily located in 2-DE gels because they appear as distinctive spot clusters, which can be subsequently identified by MS analysis. For a global view, PTMs (e.g. glycosylation or phosphorylation) can be visualized with specific fluorescent dyes (e.g. [73, 74]). However, there are challenges, in particular, with respect to detection of low-abundance proteins and, particularly, of integral membrane proteins, whereas non-gel-based methods are unsurpassed by their potential to cover the whole proteome [75]).

In conclusion, due to the wide diversity of protein abundance and properties in complex proteomes, it is anticipated that no single proteome analysis technology will be able to effectively address all proteome analysis requirements. 2-DE gels will probably remain the “gold standard” within the foreseeable future to which any competing method should be compared, and to which it should display clear advantages.

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6 References

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Professor Görg has been the key researcher in developing the technology and use of IPG based 2-DE that have ensured that 2-DE based proteomics remains a key tool in most proteomics projects until today. Angelika Görg received scientific awards from the British and Japanese Electrophoresis Societies, the Heinz-Maier-Leibnitz Medal, and the HUPO Distinguished Achievement Award in Proteomics "in recognition of her indispensable contributions to the field of proteomic sciences", not to mention her bi-annual international "Proteomic Forum" conferences in Munich.