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SHORT COMMUNICATION

Improvements to polar 2-D electrophoresis for proteomic applications

Rita Polati · Daniela Cecconi · Ryan Dosselli · Mattia Zaccarin · Renato Millioni

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Abstract Recently, we reported a new way of performing 2-DE, called P-dimensional electrophoresis (2-PE). In this approach, the second dimension is achieved in a radial gel which can accommodate up to six 7 cm long IPG strips simultaneously, improving reproducibility and throughput power in respect to 2-DE. Nevertheless, 2-PE was up to now limited to the use of only short strips because of technical difficulties. Here, we describe how to load longer strips (e.g., 18–24 cm) on 2-PE and report some representative images for a qualitative assessment.

Keywords P-dimensional electrophoresis · Spot resolution · Radial protein separation

P. Rita and C. Daniela have equally contributed to this work.

R. Polati · D. Cecconi
Department of Biotechnology, Proteomics and Mass Spectrometry Laboratory, University of Verona, Verona, Italy

R. Dosselli
Centre for Integrative Bee Research (CIBER), ARC CoE in Plant Energy Biology, The University of Western Australia, MCS Building M316, Crawley 6009, Australia

M. Zaccarin · R. Millioni (✉)
Proteomic Centre of Padova University, Padua, Italy
e-mail: millionirenato@gmail.com

M. Zaccarin
Department of Molecular Medicine, University of Padova, Padua, Italy

R. Millioni
Chair of Metabolism, Department of Medicine, University of Padova, Padua, Italy

2-DE is a popular method to resolve and analyze complex protein samples. In almost 40 years after his birth, this technique can boast a huge number of technical reports aimed at its improvement. However, some issues have not yet been completely eliminated, including the gel to gel experimental reproducibility and the reaching of an optimal resolution of the protein spots. A possible strategy to reduce 2-DE gel to gel variability is the so-called "multi-strip on one gel method" where two or three IPG strips with a length of, respectively, 12 and 7 cm are run simultaneously on the same 25 × 18 cm SDS-PAGE (Yuan et al. 2003). Recently, we reported a new application for 2-DE, called P-dimensional electrophoresis (2-PE) (Millioni et al. 2010a, b), which enables to take this approach a step further. In 2-PE, the second dimension run is not performed in a conventional rectangular-size (Cartesian) gel, but in a radial surface. This gel format (P-gel) can accommodate up to six 7 cm long strips simultaneously. Furthermore, we showed that the radial electrophoretic field applied during the second dimension of 2-PE allows to increase the resolution of closely adjacent spots compared to 2-DE controls (Millioni et al. 2012). In that study, radial and Cartesian maps were obtained using, respectively, 7 and 17 cm long strips and the Cartesian gels had an area that was approximately twice that of the radial ones. Despite this size difference, we observed that spots separation in the final maps showed equivalent quality in the two sets and concluded that shape of 2-D gels can be as influential as their size to increase spot detection. This represents the first attempt to introduce the second dimension gel shape as a new variable to be considered for 2-DE performance evaluation. Other factors have been studied that have a strong influence on spot resolution, among these the pros and cons of simply increasing the 2-D gel size (Millioni et al. 2010a, b; Campostrini et al. 2005;

Oguri et al. 2002; Poland et al. 2003; Lee et al. 2008; Lee and Pi 2009), and several indications on how to face the technical difficulties linked to these protocols have been suggested. In particular, Lee et al. (2008) and Lee and Pi (2009) performed systematic evaluations on the effect of gel size on spot detection varying strip lengths versus fixed SDS gel lengths and vice versa. They found that strip length had a far more important influence on spot resolution compared to SDS gel length as it should, considering the Svensson–Rilbe equation on resolution in IEF, by which said resolution is proportional to the square root of d(pH)/dx, (i.e., to how flat is the pH gradient over the separation axis, something that today users tend to ignore) (Righetti 1983). The number of spot detected was directly proportional to the length of strip, while only a little benefit was obtained by using second dimension separation distances greater than 12 cm. These data suggest that an optimized 2-D gel should ideally have a longer first dimension and a shorter second dimension compared to conventional ones. The shape of the P-gel may meet this requirement at best: indeed the area of the P-gel circular crown corresponds to that of a trapezium with the smaller base, the larger base and the height of, respectively, 40, 110 and 11 cm. Despite the fact that from a purely technical point of view a gel of such dimension would be very difficult to scan—even if the whole gel image can be acquired by step and recreated by using the "gel puzzling" tool which is available in many software packages used for the 2-DE analysis, such as Delta2D—the circular crown form of our P-gel (outer diameter = 35 cm) makes it suitable for single scanning step too. Therefore, to maximize the benefits of the 2-PE method we should be able to couple the use of very long IPG strip, (e.g., 40 cm), which appears to have the greatest influence on the overall results and the application of a radial electric field which acts increasing the resolution of strings of isobaric spots.

To achieve this challenging goal, we developed a new IPG strip loading method, which includes: (1) the reduction of IPG strip thickness, (2) the bending of such obtained strip, and (3) the embedding of the bended strip into agarose matrix.

However, we first had to overcome several technical difficulties that limited the applicability of 2-PE, as it was originally conceived, only to 7 cm long IPG strips. Indeed, short IPG strips can be easily loaded in the free circular space created by the insertion of a gasket between the glass plates during gel casting (Millioni et al. 2010a, b; Polati et al. 2012), but longer strips (including those 18- and 24-cm long) cannot be loaded this way. To address this limitation we tested different ways to load longer strips in

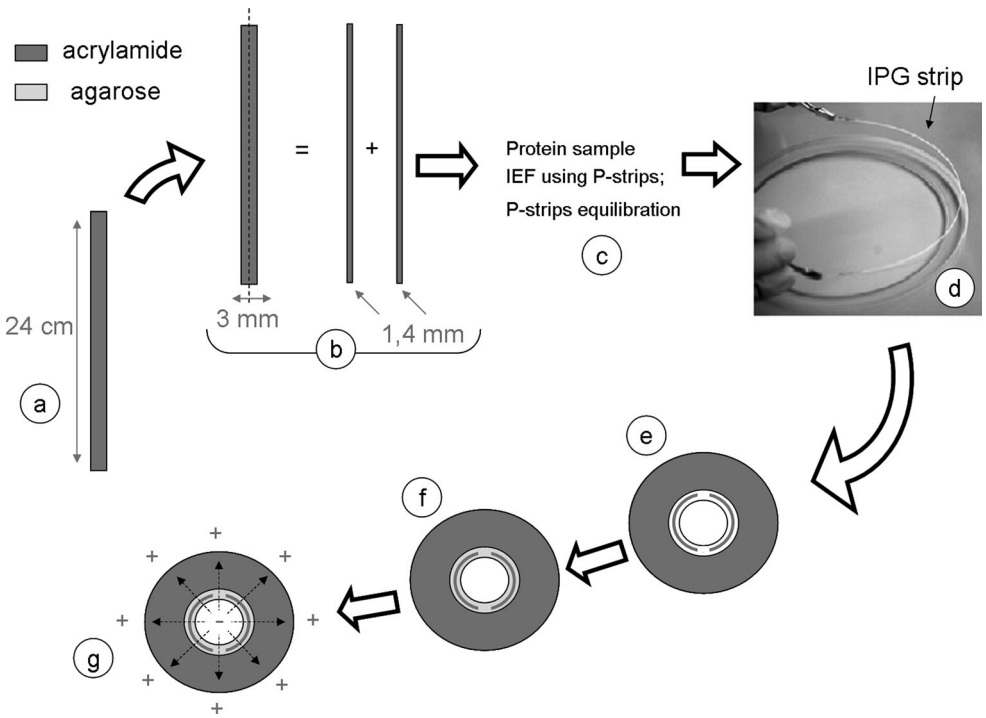

Fig. 1 Schematic protocol to load a 24 cm long IPG strip on a P-gel: *a, b* cut a commercial IPG strip in half obtaining two P-strips; *c* perform IEF on P-strips and equilibrate the P-strips following 2-DE standard protocols; *d, e* take the ends of P-strip with tweezers, bend the strip and insert it in the free circular space created by the insertion/removal of a gasket between the glass plates during the gel casting so that the strip acrylamide side will face the SDS gel. Each P-strip will occupy about one half of the inner circumference; *f* seal the P-strips in place adding hot agarose (0.5 %) containing bromophenol blue as tracking dye; *g* perform electrophoresis at 15 mA constant current with external cooling (20 °C) until the tracking dye migrated to the gel bottom

P-gels. At the beginning we used a thin fishing line to peel polyacrylamide away from the plastic backing. This system allowed to lay the matrix with focused proteins directly on the P-gel glass following its curvature, and to obtain a 2-PE map from a 24 cm long IPG strip (data not shown). Nevertheless, we choose to abandon this approach because the backing-free polyacrylamide gel strip, though very malleable, has the tendency to stretch and is difficult to handle. The best solution proved to be the bending of the strip sideways and its lodging along the P-gel inner circumference with the IPG gel side facing the second dimension gel. The backing of commercial strips is a flexible and thin polyester film (0.2 mm thick) which allows the strip to lean on the SDS gel inner edge, assuming the same curvature (Fig. 1). Thus, we reduced the width of traditional IPG strips from 3 to 1.4 mm to allow the strip to enter between the glass plates maintaining the thickness of the radial gel equal to that used for traditional Cartesian gels (i.e., about 1.5 mm). This modification is mandatory since the increase of the gel thickness is associated with a reduction of protein resolution. Therefore, failing to do so would result in the introduction of an untoward variable in the comparison of the two experimental sets. The strip cut was kindly performed by Elettrofor Company using a laser machine.

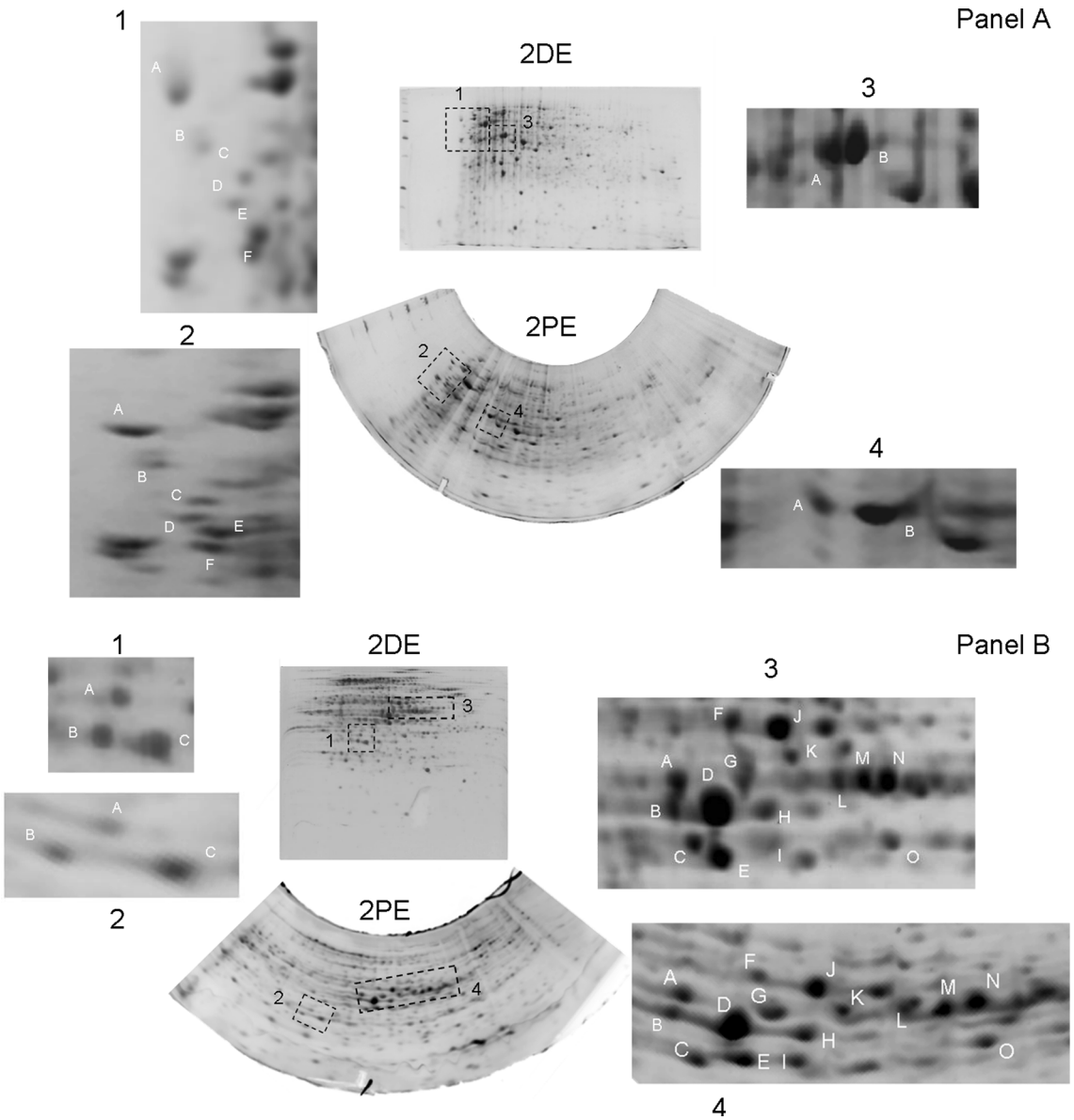

Fig. 2 *Panel a*: traditional and polar 2-D maps of cytoplasm proteins from *S. maltophilia* (400 µg). IPGs: 18-cm long non-linear 3–10 pH gradient. SDS-PAGE: 12 %T Tris–Glycine gels, Sypro Ruby staining. *Panel b*: traditional and polar 2-D maps of cytoplasm proteins from *E. coli* (400 µg). IPGs: 24-cm long non-linear 4–7 pH gradient. SDS-PAGE: 12 %T Tris–Glycine gels, Sypro Ruby staining. Boxes *1–4* represent enlarged sections of gel images to show the increased resolution obtained with 2-PE

We are aware that the thus modified strip (P-strip) has about half of the protein loading capacity of the parental one, but long strips have a loading capacity of up to 1 mg of proteins or even more if a narrow pH range is adopted (Lange et al. 2013). Therefore, the P-strip capacity is still more than sufficient to satisfy the majority of proteomic studies requirements. It should also be stressed that, to be comparable for sensitivity and reproducibility with other methods such as the advanced techniques of shotgun proteomics, the future of gel-based proteomics mainly lies on the use of fluorescent reagents for protein labelling (difference in-gel electrophoresis or DIGE) (Minden et al. 2009). Using these reagents, the amount of sample to be used is typically of few micrograms and therefore, if coupled with these reagents, the lower capacity of the P-strip will not be an issue.

We successfully tested this new way of interfacing the first and second dimension several times and we show here some results supporting the feasibility of this protocol. Indeed these data, obtained with 18 and 24 cm long strips and 400 μg of proteins per gel, extend the two main improvements on resolution achieved by performing 2-PE with 7 cm long strips. First, the effect of radial migration leads to an accentuated spot resolution directly proportional to the migration distance (see Fig. 2, panel a: box 3 vs. 4, spots A and B; panel b: box 1 vs. 2, spots B and C; box 3 vs. 4, spots L, M and N). Second, spots on the 2-PE map have a less rounded shape than the 2D map. The radial electric field geometry leads to the flattening of the spots which happens simultaneously to the distancing of spots during the electrophoretic migration, as can be appreciated for example in Fig. 2, panel a by comparing spots A, B, C, D, E, F of boxes 1 and 2 or in panel b by comparing spots A, B of boxes 3 and 4. This flattening, in turn, improves the resolution of spots corresponding to proteins with the same pI and very similar relative mass in the radial set in respect to the Cartesian one as shown in Fig. 2 by comparing panel 1 vs. 2, spots E and F or in Fig. 3 by comparing panel 3 vs. 4, spots A and B.

Gel images reported here have, at present, a purely qualitative value, and are intended to confirm the enhancement obtained by overcoming the limit on the length of 7 cm long IPG strip for 2-PE. Given the encouraging results of our previous publications (Millioni et al. 2012; Polati et al. 2012) and the confirmation of the improvement obtainable with longer strips brought by these preliminary data, we can suggest with confidence that the radial gel format should be considered as a valuable alternative to the universally adopted Cartesian one. The marketing of ready-to-use P-strips would also be of great help to the spread of this technology. Further tests including quantitative data are needed, and in progress, to verify this hypothesis.

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Conflict of interest The authors have declared no conflict of interest.

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