2-D Gel Electrophoresis: First-Dimension Separation

Protocol

Bulletin 6240

First-Dimension Separation Methods

Master 2-D techniques before proceeding to separate your own samples with the ReadyPrep™ 2-D starter kit. Premixed reagents, a standardized sample, and a detailed optimized protocol allow you to get familiar with 2-D techniques and to validate your 2-D system.

Protein Load for 2-D Gels

Table 1 shows generally recommended protein loads for 2-D gels. Because of sample-to-sample variation, the amounts are a guide only. For narrower pH range IPG strips, more protein can be loaded, because proteins outside the range of pI resolution will not remain on the strip to enter the 2-D gel. For single-pH-unit IPG strips, the amount that can be loaded can be as much as 4–5 times more, which allows better detection of low-abundance proteins. For further discussion of factors related to protein load.

Table 1. Approximate protein loads for IPG strips.

IPG Strip Length	Analytical Load (Silver or SYPRO Ruby staining)	Preparative Load (Coomassie staining)
7 cm	10–100 µg protein	200–500 µg protein
11 cm	50–200 µg protein	250–1,000 µg protein
17 cm	100–300 µg protein	1–3 mg protein

IPG Strip Rehydration

Solutions used to rehydrate IPG strips prior to loading a sample are the same as those used to solubilize or dilute samples for in-gel rehydration. Methods for rehydration of strips in buffers (with or without sample) are described in the following sections.

Passive Rehydration With Sample

Passive sample application during rehydration is performed by placing the IPG strip gel side down in the channel of a focusing or rehydration tray that contains the sample in an appropriate rehydration solution. Use the sample volumes given in Table 2. This procedure will result in rehydration of the strips to their original thickness of 0.5 mm. Larger or smaller volumes can be used and the strips will swell to accommodate more liquid up to a point (Görg et al. 2000). A minimum of 11 hr total rehydration time is recommended. It is important that the strips be left in the well for the entire time, even if it appears that all of the liquid has been absorbed. High MW proteins cannot enter the gel until the pores are large enough to accept them, which only occurs when the pores have swelled to their maximum size.

Table 2. Approximate volumes to hydrate ReadyStrip™ IPG strips.

ReadyStrip Strip Length IPG	Volume
7 cm	125 µl
11 cm	200 µl
17 cm	300 µl

If too much solution remains outside the gel in the focusing tray during electrophoresis, a parallel current path along the surface of the strip can form in which the proteins will not be focused. This can result in protein loss and streaking. To minimize the possibility of a parallel current path, rehydrate the strips in a disposable rehydration tray, then transfer them to the focusing tray. During transfer, carefully blot excess liquid from the strip with moist filter paper prior to beginning the run.

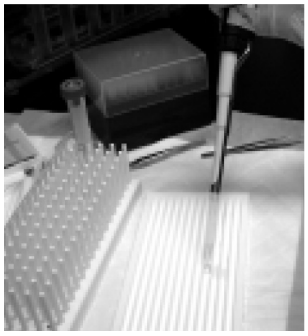
Remove the IPG strip from the protective cover using gloved hands and forceps. Carefully place the IPG strip in the rehydration buffer, gel side down, making sure the entire strip is wetted. There is no “best way” to place dry IPG strips in contact with solution in the trays. Any of the methods illustrated in Figure 1 are suitable.

A

B

Fig. 1A and 1B. Strip rehydration method 1. Prop up one of the long edges of the tray at an angle to the lab bench. Pipet the rehydration solution along the entire length of the lower corner of each channel (A); place the strip, edge first, into the liquid (B). Then place the tray flat on the benchtop.

C



D

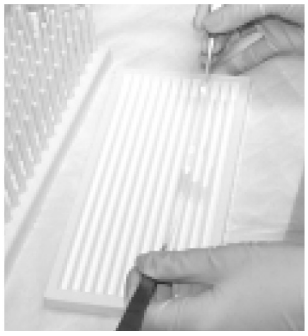


Fig. 1C and 1D. Strip rehydration method 2. Pipet the rehydration solution into the middle of each tray channel (C); bend the strip into a “U” shape and lower it into the liquid from the center out to the edges (D).

E

Fig. 1E. Strip rehydration method 3. Pipet the rehydration solution into one end of each tray channel. Butt the strip up to the same end of the channel and lower it into the liquid toward the opposite end (E).

It is helpful to add a trace of Bromophenol Blue to the sample solution to observe the hydration process. Allow the liquid to distribute for about 1 hr before covering the strips with mineral oil. The IPG strips must be covered to prevent evaporation, which will cause the urea to precipitate as it becomes more concentrated. As a precaution against evaporation, mineral oil should be gently layered on top of each channel until it completely covers each strip.

Active Rehydration

For active rehydration of IPG strips with sample in a focusing tray, run the IEF cell under low voltage (50 V). Ensure that the liquid extends past the electrode wires at each end so that the entire strip rehydrates and no dry area creates a discontinuity in the current path. It might be necessary to lift the ends of the IPG strip slightly to get the liquid to flow to the ends of the strip. After the sample has been in contact with the strips for 1 hr, add mineral oil to cover each strip. The PROTEAN® i12™ IEF cell can be programmed for active rehydration and to transition automatically into a focusing run. Alternatively, a pause may be incorporated to allow the operator to insert a wick under each end of the strip (see the section below on performing IEF). If this method of sample application causes a disproportionate ratio of large proteins to small proteins, try passive rehydration.

Performing IEF

The PROTEAN i12 IEF cell with integrated power supply and Peltier cooling is recommended for IEF protocols in this manual. It can simultaneously run up to twelve 11 or 17 cm IPG strips or up to twenty-four 7 cm strips. Running conditions can be better controlled by running the same type of sample, buffer, and IPG strip pH range together.

Positioning Strips and Use of Wicks

After the strips have rehydrated, move them to the i12 focusing tray if they were rehydrated in other trays. Carefully blot excess liquid from the strip with moist filter paper. Wicks are highly recommended because they collect salts and other contaminants in the sample. Without wicks, salts collect at the anode and cathode, producing high conductivity that can alter the gradient, cause discontinuities in the gel, and cause “hot spots” or burns. Place a dry wick on each electrode that is used (Figure 2). Position the wicks within the indentations of the channels. Pipet 5–8 µl of water on each wick before positioning the IPG strips.

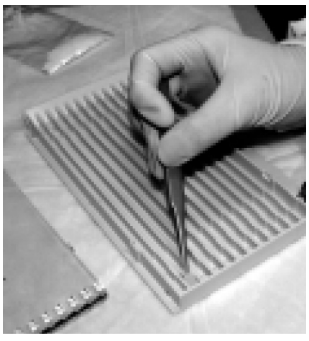


Fig. 2. Placement of wicks on the electrodes in each channel that will be used. IPG strips will be placed on top of the wicks.

Alternatively, if strips are rehydrated in the focusing trays (either actively or passively), the ends of each strip can be lifted with forceps and wet wicks inserted between the strip and the electrodes (Figure 3). Wicks should be wetted but not soaked. Blot wetted wicks before placing them in the tray.

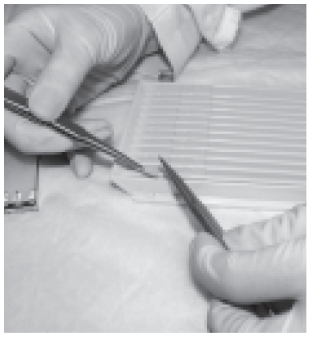


Fig. 3. Insertion of wicks under both ends of an IPG strip that has been rehydrated in a focusing tray.

Cover the strip with mineral oil before starting the focusing run to prevent evaporation and carbon dioxide absorption during focusing. Channels should be filled nearly to the top but should not be overflowing. The i12 focusing tray has rounded corners at both ends of the individual channels that prevent mineral oil movement into the adjacent channels. The rounded corners also reduce salt buildup due to inadequate cleaning between IEF runs. It is important to clean the focusing trays properly between runs. Channel-to-channel leakage is common when salts accumulate in the channels.

Focusing Conditions for IPG Strips on the PROTEAN i12 IEF Cell

Table 3 gives suggested total volthours for IPG strip runs. These conditions are intended as a guide; individual samples may require more or less time.

Table 3. Broad and narrow ranges.

	Start Voltage	Find Voltage	Volt-Hours	Ramp	Temperature
ReadyStrip pH 3–10, 3–10 NL, 4–7, 5–8*					
7 cm	0 V	4,000 V	8–15,000 V-hr	Rapid	20°C
11 cm	0 V	8,000 V	20–35,000 V-hr	Rapid	20°C
17 cm and 18 cm	0 V	10,000 V	40–60,000 V-hr	Rapid	20°C
24 cm	0 V	10,000 V	60–80,000 V-hr	Rapid	20°C
ReadyStrip pH 3–6 Focusing Conditions, ***					
7 cm	0 V	4,000 V	8–10,000 V-hr	Rapid	20°C
11 cm	0 V	8,000 V	15–20,000 V-hr	Rapid	20°C
17 cm and 18 cm	0 V	10,000 V	30–40,000 V-hr	Rapid	20°C
24 cm	0 V	10,000 V	40–55,000 V-hr	Rapid	20°C
ReadyStrip pH 7–10, †**					
7 cm	0 V	4,000 V	8–16,000 V-hr	Rapid	20°C
11 cm	0 V	8,000 V	20–30,000 V-hr	Rapid	20°C
17 cm and 18 cm	0 V	10,000 V	40–50,000 V-hr	Rapid	20°C
24 cm	0 V	10,000 V	60–70,000 V-hr	Rapid	20°C

Voltage Ramping Modes

Voltage ramping can replace traditional stepwise voltage programming with continuous voltage changes. The PROTEAN i12 IEF cell (Figure 4) includes three voltage ramping modes: rapid, linear, and slow. Each ramping mode is appropriate for the resistance of particular samples. The combined resistance of the IPG strips, the rehydration buffer, and the sample determines which ramping mode should be used. During the focusing process, charged contaminants move to the electrodes and proteins move to the pH equal to their pI. While the proteins are being focused, the resistance of the IPG strip gradually increases until it reaches a maximum.

Fig. 4. The PROTEAN i12 IEF cell and accessories.

Each voltage ramping mode controls the rate of voltage change as follows:

Rapid ramping mode — In rapid ramping mode, salts and other ionic contaminants are driven from the IPG strips as rapidly as possible. The limiting factor in reaching the maximum set voltage is the current limit per strip. The maximum voltage can be reached in ≤2 hr for high-resistance (low-ionic-strength) samples, or in >6 hr for low-resistance samples. In both cases the power supply will run at the set current limit until a steady state is reached. This is the mode of choice for many samples, and is particularly useful to minimize low-resistance sample run time.

Linear ramping mode — In linear ramping mode, the voltage increases linearly within the programmed time frame, starting with the final voltage of the previous step and ending with the maximum voltage programmed. The resistance of the sample/rehydration buffer system will determine whether the maximum set voltage can be reached in the programmed time. This mode is used for samples of intermediate resistance.

Slow ramping mode — In this mode, the voltage is increased quadratically:

$$V = B + (N^2 \times (E - B)/T^2)$$

where B = starting voltage, E = ending voltage, N = elapsed time, and T = total time. The run will continue below or at the current limit. This mode is used for high-resistance sample/rehydration buffer systems to minimize high power input initially while achieving high voltage as quickly as possible.

Note: The default current limit in the PROTEAN i12 IEF cell is 50 µA per strip. A higher current limit, up to 99 µA per strip, can be programmed into a method. All preset methods have a fixed current limit of 50 µA per strip. In the rapid ramping mode, the system runs at the set current limit and adjusts the voltage until the maximum voltage is reached. In the linear or slow ramping modes, the system follows a specific algorithm and does not always run at the current limit. The factor that determines the time needed to reach maximum voltage is the composition of the sample solution. Systems with high salt concentration and high sample loads require a long time to reach steady state. It is not always possible to reach the maximum set voltage within the programmed time. High ampholyte concentrations and high protein load also limit the final attainable voltage.

Storage of IPG Strips After IEF

Because the pH gradient is fixed in the IPG strip gel, focused proteins are more stable at their pI than in conventional IEF gels. Focused IPG strips can be stored at –20°C indefinitely without affecting the final 2-D pattern. IPG strips are bound to a plastic sheet, so gel cracking, which results from expansion and contraction during freezing and thawing, is avoided and the IPG strips retain their original dimensions after thawing. It is convenient to store IPG strips in rehydration trays or screwcap plastic tubes, which can then be used to equilibrate the strips for the second dimension.

* The final voltage for each pH range may not be reached, but the total volt-hours given above are sufficient to properly focus samples with final voltages as low as 3,000 V (7 cm), 5,000 V (11 cm), and 7,000 V (17 cm, 18 cm, and 24 cm). A lower final voltage will increase total run time.

** The final voltage for this pH range may not be reached, but the total volt-hours given above are sufficient to properly focus samples with final voltages as low as 2,000 V (7 cm), 3,000 V (11 cm), and 6,000 V (17 cm, 18 cm, and 24 cm). A lower final voltage will increase total run time.

*** Enhanced resolution and separation of proteins may be achieved using cup loading with sample application at the cathode (–) end of the IPG strip.

† To ensure success with basic range IPG strips, performing two additional steps is strongly recommended. The first step is to treat the sample using the ReadyPrep reduction-alkylation kit (catalog #163-2090). This reduces streaking caused by disulfide bond formation, which is more problematic with basic range proteins. The second step is to use cup loading when loading samples for isoelectric focusing. For more information, refer to the ReadyPrep reduction-alkylation kit instruction manual (bulletin 4110063).

This is an excerpt from Bio-Rad's comprehensive manual, 2-D Electrophoresis for Proteomics (Bulletin 2651).

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