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Acetic Acid–Urea Polyacrylamide Gel Electrophoresis of Basic Proteins

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1. Introduction

Panyim and Chalkley described in 1969 a continuous acetic acid–urea (AU) gel system that could separate very similar basic proteins based on differences in size and effective charge *(1)*. For instance, unmodified histone H4 can be separated from its monoacetylated or monophosphorylated forms *(2)*. At the acidic pH 3 of this gel system, basic proteins with a high isoelectric point will clearly have a net positive charge that will be the major determinant of electrophoretic mobility. If a single of these positive charges is removed, for example, by in vivo acetylation of one of the positively charged ε-amino lysine side chain residues in the small histone H4 protein (102 residues), a significant decrease in effective gel mobility is observed. Similarly, addition of a phosphate moiety decreases the net positive charge of the protein during gel electrophoresis by one. Separation between similarly sized and charged proteins, for example, the partially acetylated H2A, H2B, and H3 histones of most organisms, can typically be achieved only by inclusion of a nonionic detergent such as Triton X-100 (*see* Chapter 17).

In 1980, Bonner and co-workers introduced a discontinuous acetic acid–urea–Triton (AUT) variation that avoids the necessity for exhaustive preelectrophoresis *(3)*, prevents deformation of sample wells *(4)* and generally produces much sharper, straighter bands. Omission of Triton from this method creates the high capacity and high resolution AU gel electrophoresis protocol described in **Subheading 3. Figure 1** shows an example of the possibilities and limitations of the AU gel system. Yeast histones were extracted from four parallel cultures using a novel method that preserves all postsynthetic modifications *(5)*. The separation of single-charge differences, proven to be caused by acetylation of lysines, is clearly demonstrated for each histone species (**Fig. 1**). It is clear that, without fractionation, the patterns of H2B, H2A, and H3 histones would overlap, preventing quantitation of each protein and its acetylation. Protein band shapes are generally sharp and straight. Compression of band shapes, visible for some high-abundance nonhistone proteins with lower gel mobilities shown in **Fig. 1**, can be minimized by decreasing the protein concentration during gel stacking by increasing gel thickness.

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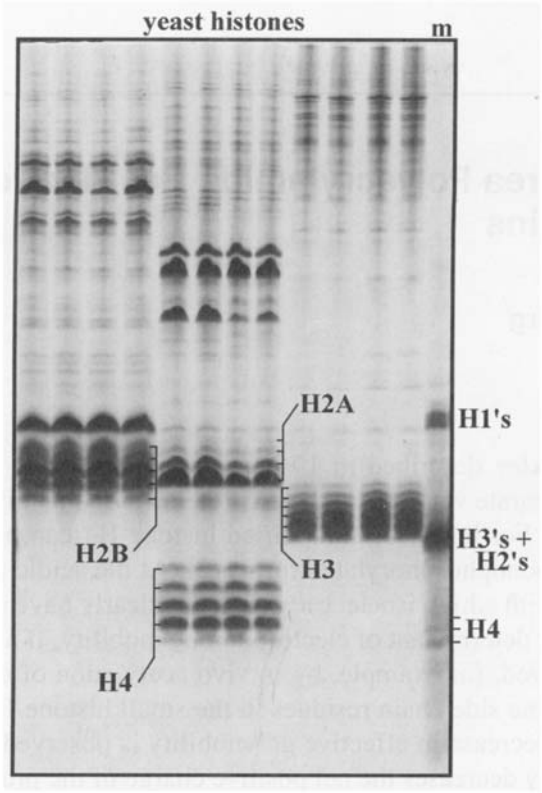

Fig. 1. Histones of the yeast *Saccharomyces cerevisiae* were extracted from crude nuclei, prepared in such a way that protein integrity and postsynthetic modifications were maintained ***(5)***. Histones from four parallel cultures (approx 2×10^{10} cells) were extracted, fractionated by reversed-phase HPLC into pools containing histone H2B (*left four lanes*), coeluting histone H2A and H4 (*center four lanes*) and histone H3 (*right four lanes*). In each histone species, the fastest mobility band represents nonacetylated histone. In each slower moving species one more lysine has been acetylated, neutralizing the positive charge of the lysine side chain, reducing the protein gel mobility by one charge. Virtually all low-mobility nonhistone proteins in this gel can be removed from yeast histone preparations by 100,000 mol wt ultrafiltration ***(5)***. The "m" indicates a marker lane with total calf thymus histones. Calf histone species are marked along the side. Note the closely overlapping pattern of H2A, H2B, and H3 histones. The long AU separating gel was 1 mm thick, 19 cm wide, and 27 cm long. The top of the gel is visible at the top of the figure. Electrophoresis was at 250 V constant voltage for 20 h with electrical current decreasing from 22 mA to 6 mA. The electrophoretic front, visualized using methylene blue, was below the lower edge of the figure, close to the lower margin of the gel. The gel was stained overnight in Coomassie, destained in 4 h with two aliquots of polyurethane foam, and digitized under standard conditions on a UMAX Powerlook II flatbed scanner with transilluminator.

AU gels are currently used for very dissimilar proteins with isoelectric points lower than those of histones but sufficiently above pH 3 to be positively charged during gel electrophoresis. Examples include neutrophil defensins ***(6)***, antimicrobiol nasal secretions ***(7)***, enzymes like tyrosinase (***8***), serum isoenzymes ***(9)***, chemokines ***(10)*** and basic protamines ***(11)***. Typically curved, somewhat diffuse protein bands, characteristic for native, non-stacking electrophoretic gel separations are obtained. The superior discontinuous gel system described here has only been used for histones, producing clearer bands in a shorter period of time than achievable in continuous AU gel modes ***(1–2)***.

2. Materials

1. Vertical gel apparatus for short (15 cm) or long (30 cm) slab gels. A gel electrophoresis apparatus that allows gel polymerization between the glass plates with spacers, without necessarily being assembled in the apparatus, is preferable. This facilitates the even and complete photopolymerization of the acrylamide gel. In this type of apparatus the glass–gel sandwich is typically clamped to the lower buffer reservoir, which acts as a stand, after which the upper buffer reservoir is clamped to the top of the gel assembly.

   Details of the procedure are described for a fairly standard and flexible gel apparatus that uses two rectangular glass plates (4 mm thick standard plate glass with sanded edges), 21 cm wide and 32.5 and 35.5 cm long, respectively. The Plexiglas bottom buffer reservoir with platinum electrode is 22.5 cm wide with three sides 5 cm high and one of the long sides 12.5 cm high. The glass plates are clamped to this side. The upper buffer reservoir with platinum electrode and a similar buffer capacity is 18 cm wide with one long side enlarged to measure 21 cm wide by 10 cm high. It contains a cutout of 18 cm wide and 3.5 cm high that allows access of the upper reservoir buffer to the top of the gel. The 21-cm-wide Plexiglas plate is masked with 5-mm-thick closed-cell neoprene tape (weather strip) and provides a clamping ridge for attachment to the top of the glass-gel sandwich.
2. Spacers and combs are cut from 1-mm Teflon sheeting. High-efficiency fluorography may benefit from 0.5-mm spacers. Teflon up to 3 mm thick is less easy to cut but yields very high capacity gels (*see* **Note 1**).

   Two side spacers (1.5 × 35 cm) and one bottom spacer (0.5 × 24 cm) are required. Added to the top of the side spacers is 3 cm adhesive, closed-cell neoprene tape (weather strip, 14 mm wide and 5 mm thick). This is not required if a more expensive glass plate with "rabbit ears" is used instead of the rectangular shorter plate.

   Combs have teeth 5–10 mm wide and 25–50 mm long, separated by gaps of at least 2.5 mm. For the detailed protocol described, a 15-cm-wide comb with 20 teeth of 5 × 30 mm is used.
3. Vaseline pure petroleum jelly.
4. Acrylamide stock solution: 60% (w/v) acrylamide, highest quality available, in water (*see* **Note 2**). The acrylamide is dissolved by stirring. Application of heat should be avoided, if possible, to prevent generating acrylic acid. The solution can be kept at least for 3 mo on the laboratory shelf at room temperature. Storage at 4°C can exceed 2 yr without detectable effects.
5. *N,N'*-methylene bis-acrylamide stock solution: 2.5% (w/v) in water (*see* **Note 2**).
6. Glacial acetic acid (HAc): 17.5 *M*.
7. Concentrated ammonium hydroxide: NH_4OH, 28–30%, approx 15 *M*.
8. *N,N,N',N'*-Tetramethylenediamine (TEMED), stored at 4°C.

9. Riboflavin-5'-phosphate (R5P) solution: 0.006% (w/v) in water. This solution is stable for more than 6 mo if kept dark and stored at 4°C.
10. Urea, ultrapure quality.
11. Side-arm suction flasks with stoppers, magnetic stirrer, stirrer bar, and water-aspirator vacuum; measuring cylinders with silicon-rubber stoppers; pipets, and pipetting bulbs or mechanical pipetting aids; 1- and 5-mL plastic syringes, with 20-gauge needles.
12. Fluorescent light box with diffuser for even light output and with the possibility to stand vertically. Light intensity should equal or exceed 5 klx at a distance of 5–10 cm. A high-quality X-ray viewing light box with three 40-W bulbs typically will meet this specification.
13. Aluminum foil.
14. Electrophoresis power supply with constant voltage mode at 300–500 V with up to 50 mA current, preferably with a constant power mode option.
15. Urea stock solution: 8 *M* urea in water. An aliquot of 40 mL with 1 g of mixed-bed resin (Bio-Rad [Hercules, CA] AG 501-X8) can be used repeatedly over a period of months if refrozen and stored between use at –20°C (*see* **Note 3**).
16. Phenolphthalein indicator solution: 1% (w/v) in 95% ethanol, stored indefinitely at room temperature in a closed tube.
17. Dithiothreitol (DTT, Cleland's reagent) is stored at 4°C and is weighed freshly for each use.
18. Methylene blue running front indicator dye solution: 2% (w/v) in sample buffer (*see* **Subheading 3., step 22**).
19. Reference histones: Total calf thymus histones (Worthington, Freehold, NJ), stored dry at 4°C indefinitely or in solution at –80°C in 50-µL aliquots of 5 mg/mL in water for more than 1 yr (*see* **Subheading 3., step 23**).
20. Glass Hamilton microsyringe (100 µL) with Teflon-tipped plunger.
21. Electrophoresis buffer: 1 *M* acetic acid, 0.1 *M* glycine (*see* **Note 4**). This solution can be made in bulk and stored indefinitely at room temperature.
22. Destaining solution: 20% (v/v) methanol, 7% (v/v) acetic acid in water.
23. Staining solution: Dissolve a fresh 0.5 g of Coomassie Brilliant Blue R250 in 500 mL of destaining solution for overnight gel staining (*see* **Note 5**). For rapid staining within the hour the dye concentration should be increased to 1% (w/v). If the dye dissolves incompletely, the solution should be filtered through Whatman no. 1 paper to prevent staining artifacts.
24. Glass tray for gel staining and destaining.
25. Rotary or alternating table top shaker.
26. Destaining aids: Polyurethane foam for Coomassie-stained gels or Bio-Rad ion-exchange resin AG1-X8 (20–50-mesh) for Amido Black-stained gels.

3. Method

1. Assemble a sandwich of two clean glass plates with two side spacers and a bottom spacer, lightly greased with Vaseline to obtain a good seal, clamped along all sides with 2-in binder clamps. The triangular shape of these clamps facilitates the vertical, freestanding position of the gel assembly a few centimeters in front of the vertical light box.
2. Separating gel solution: Pipet into a 100-mL measuring cylinder 17.5 mL of acrylamide stock solution, 2.8 mL of *bis*-acrylamide stock solution, 4.2 mL of glacial acetic acid, and 0.23 mL of concentrated ammonium hydroxide (*see* **Notes 2** and **6**).
3. Add 33.6 g of urea and add distilled water to a total volume of 65 mL.
4. Stopper the measuring cylinder, and place on a rotary mixer until all urea has dissolved. Add water to 65 mL, if necessary.
5. Transfer this solution to a 200-mL sidearm flask with magnetic stir bar on a magnetic stirrer. While stirring vigorously, stopper the flask and apply water-aspirator vacuum.

Initially, a cloud of small bubbles of dissolved gas arises, which clears after just a few seconds. Terminate vacuum immediately to prevent excessive loss of ammonia.

6. Add 0.35 mL of TEMED and 4.67 mL of R5P (*see* **Note 7**), mix, and pipet immediately between the glass plates to a marking line 5 cm below the top of the shorter plate (*see* **Notes 2** and **8**).
7. Carefully apply 1 mL of distilled water from a 1-mL syringe with needle along one of the glass plates to the top of the separating gel solution to obtain a flat separation surface.
8. Switch the light box on, and place a reflective layer of aluminum foil behind the gel to increase light intensity and homogeneity (*see* **Note 1**). Gel polymerization becomes detectable within 2 min and is complete in 15–30 min.
9. Switch the light box off, completely drain the water from between the plates, and insert the comb 2.5 cm between the glass plates. The tops of the teeth should always remain above the top of the short glass plate.
10. Stacking gel solution, made in parallel to **steps 2–4**: Into a 25-mL measuring cylinder, pipet 1.34 mL of acrylamide stock solution, 1.28 mL of *bis*-acrylamide stock solution, 1.14 mL of glacial acetic acid, and 0.07 mL of concentrated ammonium hydroxide (*see* **Notes 2** and **6**).
11. Add 9.6 g of urea, and add distilled water to a total volume of 18.6 mL.
12. Stopper the measuring cylinder, and place on a rotary mixer until all urea has dissolved. Add water to 18.6 mL, if necessary.
13. Once the separating gel has polymerized, transfer the stacking gel solution to a 50-mL sidearm flask with stir bar and degas as described under **step 5**.
14. Add 0.1 mL of TEMED and 1.3 mL of R5P, mix, and pipet between the plates between the comb teeth. Displace air bubbles.
15. Switch the light box on, and allow complete gel polymerization in 30–60 min.
16. Prepare sample buffer freshly when the separation gel is polymerizing (**step 8**). The preferred protein sample is a salt-free lyophilisate (*see* **Note 9**). Determine the approximate volume of sample buffer required, depending on the number of samples.
17. Weigh DTT into a sample buffer preparation tube for a final concentration of 1 *M*, that is, 7.7 mg/mL.
18. Per 7.7 mg of DTT add 0.9 mL of 8 *M* urea stock solution, 0.05 mL of phenolphtalein, and 0.05 mL of NH_4OH to the tube to obtain the intensely pink sample buffer.
19. Add 0.05 mL of sample buffer/sample tube with lyophilized protein to be analyzed in one gel lane (*see* **Note 10**). To ensure full reduction of all proteins by DTT, the pH must be above 8.0. If the pink phenolphtalein color disappears owing to residual acid in the sample, a few microliters of concentrated ammonium hydroxide should be added to reach an alkaline pH.
20. Limit the time for sample solubilization and reduction to 5 min at room temperature to minimize the possibility of protein modification at alkaline pH by reactive urea side reactions, for example, by modification of cysteine residues by cyanate.
21. Acidify the sample by adding 1/20 volume of glacial acetic acid.
22. To each sample add 2 µL of methylene blue running front dye (*see* **Note 11**).
23. Prepare appropriate reference protein samples: To 2 and 6 µL of reference histone solution with 10 and 30 µg total calf thymus histones, add 40 µL of sample buffer (**step 18**), 2.5 µL of glacial acetic acid, and 2 µL of methylene blue.
24. When stacking gel polymerization is complete, remove the comb. Drain the wells completely, using a paper tissue as wick, to remove residual unpolymerized gel solution. At comb and spacer surfaces, gel polymerization is typically incomplete. The high urea concentration of unpolymerized gel solution interferes with the tight application of samples.

25. Remove the bottom spacer from the bottom of the gel assembly and use it to remove any residual Vaseline from the lower surface of the gel.
26. Clamp the gel assembly into the electrophoresis apparatus and fill the lower buffer reservoir with electrophoresis buffer.
27. Use a 5-mL syringe with a bent syringe needle to displace any air bubbles from the bottom of the gel.
28. Samples are applied deep into individual sample wells using a Hamilton microsyringe (rinsed with water between samples) (*see* **Note 12**). For the combination of comb and gel dimensions listed, 50-µL sample will reach a height of 1 cm (*see* **Note 8**). Samples can also be applied to sample wells by any micropipetter with plastic disposable tip. Pipet each sample solution against the long glass plate and let it run to the bottom of the well.
29. Apply reference samples in the outer lanes, which frequently show a slight loss of resolution due to edge effects. The threefold difference in reference protein amounts facilitates correct orientation of the gel following staining and destaining and obviates the need for additional markings. Optionally, apply 50 µL of acidified sample buffer to unused lanes.
30. Gently overlayer the samples with electrophoresis buffer, dispensed from a 5-mL syringe fitted with a 21-gauge needle until all wells are full.
31. Fill the upper buffer reservoir with electrophoresis buffer.
32. Attach the electrical leads between power supply and electrophoresis system: the + lead to the upper and the – lead to the lower reservoir. Note that this is opposite to the SDS gel electrophoresis configuration. Remember, basic proteins such as histones are positively charged and will move toward the cathode (negative electrode).
33. Long (30-cm) gels require 15–20 h of electrophoresis at 300 V in constant voltage mode. They are most easily run overnight. For maximum resolution and stacking capacity, the initial current through a 1-mm thick and 18-cm-wide gel should not exceed 25 mA. Gel electrophoresis is completed in the shortest amount of time in constant power mode with limits of 300 V, 25 mA, and 5 W. The current will drop towards completion of electrophoresis to 6 mA at 300 V.

    Short (15-cm) gels are run at 250 V in constant voltage mode with a similar maximal current, or in constant power mode starting at 25 mA. In the latter example, electrophoresis starts at 25 mA and 135 V, and is complete in 5.5 h at 13 mA and 290 V (*see* **Note 13**).
34. Electrophoresis is complete just before the methylene blue dye exits the gel. Obviously, electrophoresis may be terminated if lesser band resolution is acceptable, or may be prolonged to enhance separation of basic proteins with low gel mobilities, for example, histone H1 variants or phosphorylated forms of histone H1.
35. Open the glass–gel sandwich, and place the separating gel into staining solution, which is gently agitated continuously overnight on a shaker (*see* **Note 14**).
36. Decant the staining solution. The gel can be given a very short rinse in water to remove all residual staining solution.
37. Place the gel in ample destaining solution (*see* **Note 15**). Diffusion of unbound Coomassie dye from the gel is facilitated by the addition of polyurethane foam as an absorbent for free Coomassie dye. To avoid overdestaining and potential loss of protein from the gel (*see* **Note 14**), destaining aids in limited amounts are added to only the first and second destaining solutions. Final destaining is done in the absence of any destaining aids.
38. Record the protein pattern of the gel on film or on a flatbed digital scanner (*see* **Note 16**), possibly with quantitative densitometry ***(12)***. Subsequently the gel may be discarded, dried, eluted, blotted ***(6)***, or prepared for autoradiography or fluorography as required.

4. Notes

1. Owing to absorbance of the light that initiates gel polymerization, gels thicker than 1.5 mm tend to polymerize better near the light source and produce protein bands that are not perpendicular to the gel surface. For very thick gels, two high-intensity light boxes, placed at either side of the gel assembly, may be required for optimal gel polymerization and resolution.
2. All acrylamide and *bis*-acrylamide solutions are potent neurotoxins and should be dispensed by mechanical pipetting devices.
3. Storage of urea solutions at –20°C minimizes creation of ionic contaminants such as cyanate. The mixed-bed resin ensures that any ions formed are removed. Care should be taken to exclude resin beads from solution taken, for example, by filtration through Whatman no. 1 paper.
4. The stacking ions between which the positively charged proteins and peptides are compressed within the stacking gel during the initial phase of gel electrophoresis are NH_4^+ within the gel compartment and glycine$^+$ in the electrophoresis buffer. Chloride ions interfere with the discontinuous stacking system (*see* **Note 7**). This requires that protein samples should (preferably) be free of chloride salts, and that glycine base rather than glycine salt should be used in the electrophoresis buffer.
5. Amido Black is an alternate staining dye which stains less intensely and destains much slower than Coomassie, but is the better stain for peptides shorter than 30–50 residues.
6. The separating and stacking gels contain 15 vs 4% acrylamide and 0.1 vs 0.16% *N,N'*-methylene (*bis*-acrylamide), respectively, in 1 *M* acetic acid, 0.5% TEMED, 50 m*M* NH_4OH, 8 *M* urea, and 0.0004 % riboflavin-5'-phosphate.

   We have observed that 8M urea produces the highest resolution of histones in these gels when Triton X-100 is present (*see* **Chapter 17**). Equal or superior resolution of basic proteins has been reported for AU gels when the urea concentration is reduced to 5 *M*.
7. Acrylamide is photopolymerized with riboflavin or riboflavin 5'-phosphate as initiator, because the ions generated by ammonium persulfate initiated gel polymerization, as used for SDS polyacrylamide gels, interfere with stacking (*see* **Note 4**).
8. The height of stacking gel below the comb determines the volume of samples that can be applied and fully stacked before destacking at the surface of the separating gel occurs. In our experience, 2.5-cm stacking gel height suffices for samples that almost completely fill equally long sample wells. In general, the single blue line of completely stacked proteins and methylene blue dye should be established 1 cm above the separating gel surface. Thus, a 1–1.5 cm stacking gel height will suffice for small-volume samples.
9. Salt-free samples are routinely prepared by exhaustive dialysis against 2.5% (v/v) acetic acid in 3500 molecular weight cutoff dialysis membranes, followed by freezing at –70°C and lyophilization in conical polypropylene tubes (1.5-, 15-, or 50-mL, filled up to half of nominal capacity) with caps punctured by 21-gauge needle stabs. This method gives essentially quantitative recovery of histones, even if very dilute.

   Alternatively, basic proteins can be precipitated with trichloroacetic acid or acetone, acidified by hydrochloric acid to 0.02 *N*, provided that excess salt and acidity is removed by multiple washes with acetone.

   Solutions of basic proteins can be used directly, provided that the solution is free of salts that interfere with gel electrophoresis (*see* **Note 11**) and that the concentration of protein is high enough to compensate for the 1.8-fold dilution that occurs during sample preparation. Add 480 mg of urea, 0.05 mL of phenolphthalein, 0.05 mL of concentrated ammonium hydroxide, and 0.05 mL of 1 *M* DTT (freshly prepared) per milliliter sample. If not pink, add more ammonium hydroxide. Leave for 5 min at room temperature. Add 0.05 mL of glacial acetic acid. Measure an aliquot for one gel lane and continue at **step 22**.

10. The amount of protein that can be analyzed in one gel lane depends highly on the complexity of the protein composition. As a guideline, 5–50 μg of total calf thymus histones with five major proteins (modified to varying extent) represents the range between very lightly to heavily Coomassie-stained individual protein bands in 1-mm-thick, 30-cm-long gels using 5-mm-wide comb teeth.
11. Methylene blue is a single blue dye that remains in the gel discontinuity stack of 15% acrylamide separating gels (*see* **Note 4**). Methyl green is an alternate dye marker that contains methylene blue together with yellow and green dye components that remain together in discontinuous mode but that in continuous gel electrophoresis show progressively slower gel mobilities (*see* **Note 17**).
12. As an alternative to **step 28**, electrophoresis buffer is added to the upper buffer reservoir, and all sample wells are filled. Samples are layered under the buffer when dispensed by a Hamilton microsyringe near the bottom of each well. The standard procedure tends to prevent mixing of sample with buffer and thus retains all potential stacking capability.
13. Long gels used in overnight electrophoresis are made on the day that electrophoresis is started. The time for preparation of short gels may prevent electrophoresis on the same day. The nature of the stacking system of the gel (*see* **Note 4**) allows one to prepare a gel on day 1, to store it at room temperature overnight and to initiate electrophoresis on the morning of day 2. To prevent precipitation of urea, gels should not be stored in a refrigerator. The gels should not be stored under electrophoresis buffer as glycine would start to diffuse into the gel and destroy the stacking capability of the system. We routinely store short gels overnight once polymerization is complete (*see* **step 15**) and before bottom spacer and comb are removed. Saran Wrap® is used to prevent exposed gel surfaces from drying.
14. Be warned that small and strongly basic proteins such as histones are not fixed effectively inside 15% acrylamide gels in methanol–acetic acid without Coomassie. Comparison of identical gels, one fixed and stained as described and the other placed first in destain solution alone for several hours, followed by regular staining by Coomassie, reveals that 90% or more of core histones are lost from the gel. We speculate that the Coomassie dye helps to retain histones within the gel matrix. This is consistent with the observation that gradual loss of Coomassie intensity of histone bands is observed upon exhaustive removal of soluble dye.
15. Note that the gel increases significantly in size when transferred from the gel plates into the staining solution. The compositions of staining and detaining solvent mixtures are identical and changes in gel size are not observed upon destaining.
16. A standard method to record the protein staining pattern in Coomassie-stained polyacrylamide gels has been Polaroid photography, using an orange filter to increase contrast, with the gel placed on the fluorescent light box, covered by a glass plate to prevent Coomassie staining of the typical plastic surface. Polaroid negatives can be scanned but suffer from a nonlinear response of density, even within the range in which careful Coomassie staining leads to near-linear intensity of protein band staining.

    With the advent of 24+ bit color flatbed scanners with transmitted light capabilities and linear density capabilities in excess of three optical densities, it has become easy to record, and quantitate, intensity of protein staining patterns. Care should be taken to develop a standard scanning setup, using the full dynamic range (typically all three colors used at their full range, 0–255 for a 24-bit scanner, excluding automatic adjustments for density and contrast). A standard gamma correction value should be determined, using an optical density wedge (Kodak), to ensure that the density response is linear. Placing a detained polyacrylamide gel on the gel scanner, one should cover the top of the gel with an acetate

film to prevent surface reflection abnormalities and prevent touching of the transilluminating light source surface to the film, avoiding moiré interference patterns. Recording gel patterns at 300 dpi in full color and saving loss-less tiff files facilitates faithful replication of experimental results (**Fig. 1**). Quantitative densitometry programs can use the image files.

17. Gel preelectrophoresis until the methylene blue dye, and thus all ammonium ions (*see* **Note 4**), have exited the separating gel converts this gel system into a continuous one. This option can be used to separate small proteins and peptides that do not destack at the boundary with the separating gel. Although this option is available, one should consider alternatives, such as increasing the acrylamide concentration of the separating gel. West and co-workers have developed a system with 40–50% polyacrylamide gels that is similar to the one described here and that has been optimized for the separation of small basic peptides ***(13)***.

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