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Acid–Urea–Triton Polyacrylamide Gel Electrophoresis of Histones

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1. Introduction

Acid–urea polyacrylamide gels are capable of separating basic histone proteins provided they differ sufficiently in size and/or effective charge (*see* Chapter 16). Separation between similarly sized and charged molecules, such as the histones H2A, H2B, and the H3 forms of most organisms, can typically not be achieved. Zweidler discovered that core histones but not linker histones (*see* **Note 1**) bind the nonionic detergent Triton *(1)*. Generally, Triton is added to an acetic acid–urea (AU) gel system to separate core histone sequence variants and histone species with overlapping AU gel patterns. This type of gel is known as an AUT or a TAU gel. To date, a single example is known where addition of Triton X-100 has allowed separation of a nonhistone primary sequence variation, the hydrophobic replacement variant of phenylalanine by leucine in fetal hemoglobin *(2)*.

The binding of Triton to a core histone increases the effective mass of the protein within the gel without affecting its charge, and thus reduces its mobility during electrophoresis. Separation between most or all core histone proteins of diverse species can virtually always be obtained by adjusting concentrations of Triton and of urea, which appears to act as a counteracting, dissociating agent *(3)*. Experimentally, an optimal balance can be determined by gradient gel electrophoresis with a gradient of urea *(4)* or Triton *(5)*. The Triton gradient protocol in the discontinuous gel system, developed by Bonner and co-workers *(6)*, is described in **Subheading 3.** It has a distinct advantage over the urea gradient protocol. Generally, it can identify a core histone protein band as belonging to histone H4, H2B, H3, or H2A. In this order the apparent affinities for Triton X-100 increase sharply *(5,7,8)*. An example of such a separation of a crude mixture of histones with nonhistone proteins from a tobacco callus culture is shown in **Fig. 1A**. In addition, a detailed working protocol for a long AUT gel at 9 m*M* Triton and 8 *M* urea is provided. It describes the protocol used extensively in my laboratory for the analysis of core histones, especially of histone H3, in dicot *(7)* and monocot plants *(8)*, in the green alga *Chlamydomonas (9)* and in budding yeast *Saccharomyces*

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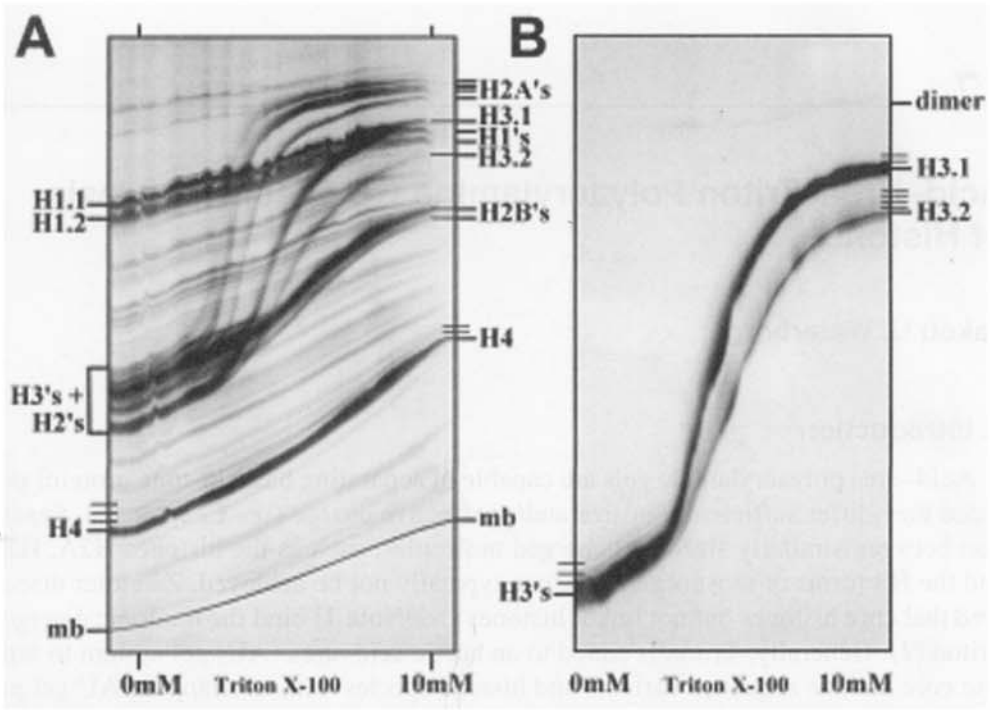

Fig. 1. Acid–urea gradient gel electrophoresis of tobacco histones. **(A)** A crude preparation of basic proteins, extracted from callus cultures of tobacco *(7)*, was electrophoresed on the gradient AUT gel and stained with Coomassie, as described. Marks at top and bottom indicate the joints between the gel compartments, from left to right, 0 m*M* Triton, 0–10 m*M* Triton gradient, and 10 m*M* Triton. The buffer front is marked by the methylene blue dye (mb). The identified histone bands are marked: two variants for histone H1, histone H4 with detectable mono- through triacetylation, two histone H2B variants, two histone H3 variants (H3.1 and the more highly acetylated histone H3.2) and, at least four histone H2A variant forms. Note that the Triton affinity of the core histones increases in the typical order of H4, H2B, H3, and H_2A. **(B)** Tobacco histone H3, purified by reversed-phase HPLC as a mixture of low acetylated histone H3.1 and highly acetylated histone H3.2, on a gradient AUT gel. The 0 and 10 m*M* Triton concentrations coincide with the edge of the figure. The presence of a small amount of histone H3 dimer is indicated.

cerevisiae (10). **Figure 1B** shows an example of the differentially acetylated histone H3 variant proteins of tobacco, purified by reversed-phase high performance liquid chromatography (HPLC) *(7)*. The protocol description parallels directly the acid–urea gel protocol described in Chapter 16, which also provides details for the use of different gel dimensions.

2. Materials

1. Vertical gel apparatus for long (30-cm) slab gels. A gel electrophoresis apparatus that allows gel polymerization between the glass plates with spacers, without being assembled in the apparatus, is required. This allows the even and complete photopolymerization of the acrylamide gel compartments in both orientations used. 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 wide with three sides 5 cm high and one of the long sides 12.5 cm high. To this side the glass plates are clamped. The upper buffer reservoir with platinum electrode and with 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. Required are two side spacers (1.5 × 35 cm), one bottom spacer (0.5 × 24 cm) and, for the gradient gel, one temporary spacer (1.5 × 21 cm). 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). A 15-cm-wide comb with 20 teeth of 5 × 30 mm, cut from a rectangle of 15 × 5 cm Teflon, is used with the teeth pointing down for the regular gel and with the teeth pointing up as a block comb for the gradient gel.
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. Triton X-100 stock solution: 25% (w/v) in water (0.4 *M*) is used, as it is much easier to dispense accurately than 100%.
9. *N,N,N',N'*-Tetramethylenediamine (TEMED), stored at 4°C.
10. Riboflavin 5'-phosphate solutions: 0.006% (R5P) and 0.06% (R5P-hi) (w/v) in water. The lower concentration solution is used for the regular gel and for all stacking gels. It is stable for more than 6 mo if kept dark and stored at 4°C. Riboflavin 5'-phosphate readily dissolves at the higher concentration, which is used for the gradient gel formulation, but it cannot be stored for more than 1 d at room temperature in the dark, as precipitates form readily upon storage at 4°C.
11. Glycerol.
12. Urea, ultrapure quality.
13. 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. Plastic syringes, 1 and 5 mL, with 20-gauge needles.
14. 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.
15. Aluminum foil.
16. Gradient mixer to prepare a linear concentration gradient with a volume of 30 mL. We use with success the 50-mL Jule gradient maker (Research Products International, Mt. Prospect, IL) with two 25-mL reservoirs.
17. Electrophoresis power supply with constant voltage mode at 300–500 V with up to 50 mA current, preferably with a constant power mode option.

18. 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 uses at –20°C (*see* **Note 3**).
19. Phenolphthalein indicator solution: 1% (w/v) in 95% ethanol, stored indefinitely at room temperature in a closed tube.
20. Dithiothreitol (DTT, Cleland's reagent) is stored at 4°C and is weighed freshly for each use.
21. Methylene blue running front indicator dye solution: 2% (w/v) in sample buffer (*see* **Subheading 3., step 16**).
22. 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 17**).
23. Glass Hamilton microsyringe (100 µL) with Teflon-tipped plunger.
24. 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.
25. Destaining solution: 20% (v/v) methanol, 7% (v/v) acetic acid in water.
26. Staining solution: Dissolve a fresh 0.5 g of Coomassie Brilliant Blue R250 in 500 mL of destaining solution. If the dye dissolves incompletely, the solution should be filtered through Whatman no. 1 paper to prevent staining artifacts.
27. Glass tray for gel staining and destaining.
28. Rotary or alternating table top shaker.
29. Destaining aid: Polyurethane foam.

3. Method

1. For the AUT regular gel, follow **steps a–i** and continue at **step 3**.
   a. 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 (**Fig. 2A**).
   b. 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 solutions (*see* **Note 5**).
   c. Add 33.6 g of urea and add distilled water to a total volume of 63.5 mL.
   d. Stopper the measuring cylinder and place on a rotary mixer until all urea has dissolved. Add water to 63.5 mL, if necessary.
   e. 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.
   f. Add 1.575 mL of Triton, 0.35 mL of TEMED and 4.67 mL of R5P (*see* **Note 6**), mix, and pipet immediately between the glass plates to a marking line 5 cm below the top of the shorter plate (*see* **Note 7**).
   g. 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.
   h. Switch the light box on and place a reflective layer of aluminum foil behind the gel to increase light intensity and homogeneity. Gel polymerization becomes detectable within 2 min and is complete in 15–30 min.

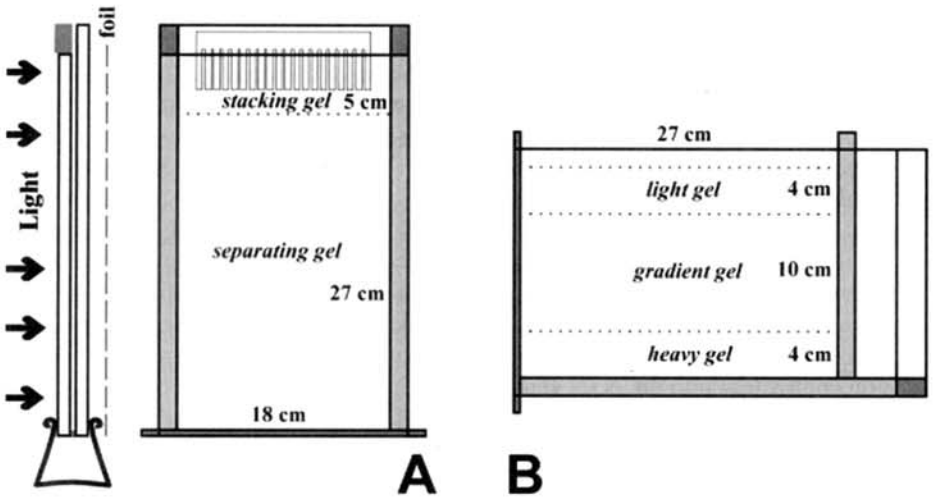

Fig. 2. **(A)** Side and front view of a long AUT gel assembly for 20 samples. The Teflon side spacers are shown in light gray, the bottom Teflon spacer and the neoprene blocks in dark gray. The fluorescent light source and reflecting aluminum foil are shown. One clamp is shown in the side view to demonstrate the independent vertical stand of this assembly and to represent the clamps all around the assembly at all spacer locations. **(B)** Front view of the AUT gradient assembly as used when the separation gel partitions are formed and polymerized.

i. 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.

2. For the Triton gradient gel, follow **steps a–q** and continue at **step 3**.
   a. Assemble a sandwich of two clean glass plates with one side spacer, a bottom spacer, and a temporary spacer, clamped with large binder clamps (**Fig. 2B**). The spacers are lightly greased with Vaseline to obtain a good seal but the amount of grease on the temporary spacer should be as low as possible. No grease should be present on the side of this spacer facing the buffer compartment to assure a flawless destacking surface. Place magic marker guidance lines at 4, 14, and 18 cm above the long side spacer (**Fig. 2B**). Place the assembly horizontally a few centimeters in front of the vertical light box and add a reflective layer of aluminum behind the gel.
   b. Heavy gel separating gel solution: Pipet into a 50-mL graduated, capped polypropylene tube, 4.0 mL of glycerol, 10 mL of acrylamide stock solution, 1.6 mL of *bis*-acrylamide stock solution, 2.4 mL of glacial acetic acid, and 0.13 mL of concentrated ammonium hydroxide. Add 19.2 g of urea. Add distilled water to a total volume of 38.5 mL (*see* **Note 5**).
   c. Light gel separating gel solution: Pipet into a 50-mL graduated, capped polypropylene tube, 10 mL of acrylamide stock solution, 1.6 mL of *bis*-acrylamide stock solution, 2.4 mL of glacial acetic acid, and 0.13 mL of concentrated ammonium hydroxide. Add 19.2 g of urea. Add distilled water to a total volume of 39.5 mL.
   d. Place the closed tubes on a rotary mixer until all urea has dissolved. Add water to the required final volume, if necessary.
   e. Transfer each solution to a 100-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.

f. Return the gel solutions after degassing to the capped tubes.
g. Add to the heavy gel solution 1.0 mL of Triton stock solution.
h. Wrap each tube into aluminum foil to protect the gel solution from light (*see* **Note 6**).
i. Prepare freshly concentrated (0.06%) riboflavin 5'-phosphate (R5P-hi) solution (*see* **Subheading 2., step 10**).
j. Add to each gel solution 0.20 mL of TEMED and 0.27 mL of R5P-hi, and mix.
k. Under conditions of darkness (or very reduced light levels), pipet approx 12 mL of heavy gel solution between the plates, up to the 4-cm heavy gel surface mark (**Fig. 2B**).
l. Turn the light box on for 2 min only. This allows the heavy gel to initiate polymerization and gelling, but retains sufficient unpolymerized acrylamide to fuse this gel partition completely into the gel layered on top.
m. In darkness, set up the gradient maker with 15 mL of heavy gel and 15 mL of light gel solutions and create slowly, over a period of at least several minutes, a linear gradient that flows slowly and carefully between the glass plates. Empirically, we have observed that a 10% increase over the calculated volume of the $27 \times 10 \times 0.1$ cm gradient partition will create a gradient that is complete near the marker line between gradient and light partition (**Fig. 2B**).
n. Turn the light box on for 2 min only.
o. In darkness, slowly add light gel solution on top of the partially polymerized gradient gel until the upper marker line.
p. Switch the light box on for complete gel polymerization in 15–30 min.
q. Remove the temporary spacer from the assembly. Insert a slightly greased spacer along the polymerized light gel. Reclamp the assembly. Insert the 20-well comb in the middle and upside down as a block comb for 2.5 cm below the edge of the shorter glass plate. Reposition the assembly vertically between light box and aluminum foil (**Fig. 2A**).

3. Stacking gel solution, made in parallel to **steps 1b–d** or **steps 2b–d**: Pipet into a 25-mL measuring cylinder 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* **Note 5**).
4. Add 9.6 g of urea and add distilled water to a total volume of 18.6 mL.
5. Stopper the measuring cylinder and place on a rotary mixer until all urea has dissolved. Add water to 18.6 mL, if necessary.
6. Once the separating gel has polymerized, transfer the stacking gel solution to a 50-mL sidearm flask with stir bar and degas as in **step 1e**.
7. Add 0.1 mL of TEMED and 1.3 mL of R5P, mix and pipet between the plates between the comb teeth.
8. Displace air bubbles, especially in the gradient assembly where residual Vaseline may interfere with gel solution flow. In this case, all (mini) bubbles should be carefully removed from the separation gel surface that will act as the destacking boundary.
9. Switch the light box on and allow complete gel polymerization in 30–60 min.
10. Sample buffer is freshly prepared when the separation gel is polymerizing. The preferred protein sample is a salt-free lyophilisate (*see* **Note 8**). For regular gels, determine the approximate volume of sample buffer required, depending on the number of samples. For a gradient gel 0.5–1.5 mL sample buffer appears optimal.
11. Weigh DTT into a sample buffer preparation tube for a final concentration of 1 *M*, that is, 7.7 mg/mL.
12. Per 7.7 mg of DTT, add 0.9 mL of 8 *M* urea stock solution, 0.05 mL of phenolphthalein, and 0.05 mL of concentrated ammonium hydroxide to the tube to obtain the intensely pink sample buffer.

13. Add 0.05 mL of sample buffer per sample tube with lyophilized protein to be analyzed in one gel lane (*see* **Note 9**). Add 1 mL of sample buffer to sample for one gradient gel. To assure full reduction of all proteins by DTT, the pH must be above 8.0. If the pink phenolphthalein color disappears because of residual acid in the sample, a few microliters of concentrated ammonium hydroxide should be added to reach an alkaline pH.
14. 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.
15. Acidify the sample by addition of 1/20 volume of glacial acetic acid.
16. Add methylene blue running front dye: 2 µL per gel lane or 50 µL for a gradient gel.
17. For a regular AUT gel, prepare reference histone samples: To 2 and 6 µL reference histone solution with 10 and 30 µg of total calf thymus histones, one adds 40 µL sample buffer (**step 12**), 2.5 µL of glacial acetic acid, and 2 µL of methylene blue.
18. 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.
19. Remove the bottom spacer from the gel assembly gel assembly and use it to remove any residual Vaseline from the lower surface of the gel.
20. Clamp the gel assembly into the electrophoresis apparatus and fill the lower buffer reservoir with electrophoresis buffer.
21. Use a 5-mL syringe with a bent syringe needle to displace any air bubbles from the bottom of the gel.
22. For regular gel, follow **steps a–c** and continue at **step 24**.
    a. Samples are applied deep into individual sample wells by Hamilton microsyringe (rinsed with water between samples) (*see* **Note 10**). For the combination of comb and gel dimensions listed, a 50-µL sample will reach a height of 1 cm (*see* **Note 7**). 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.
    b. 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.
    c. Gently overlayer the samples with electrophoresis buffer, dispensed from a 5-mL syringe fitted with a 21-gauge needle until all wells are full.
23. For gradient gel, follow **steps a–c** and continue at **step 24**.
    a. Fill the block well with electrophoresis buffer.
    b. Use a level to confirm that the bottom of the preparative well is exactly horizontal.
    c. Distribute the total sample evenly across the width of the well using a 250-µL Hamilton microsyringe. Limited mixing of sample and electrophoresis buffer will facilitate even loading and is easily dealt with by the strong stacking capability of the gel system (*see* **Note 11**).
24. Fill the upper buffer reservoir with electrophoresis buffer.
25. 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).

26. 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 toward completion of electrophoresis to 6 mA at 300 V.
    Gradient gel electrophoresis typically takes a few more hours. At 300 V constant voltage the electrophoretic current is reduced, in particular at the heavy side of the gel due to the reduced water concentration caused by the inclusion of glycerol. The ion front with methylene blue dye reflects this in a distinct curvature (**Fig. 1A**).
27. Electrophoresis is complete just before the methylene blue dye exits the gel. Obviously, electrophoresis may be terminated earlier if lesser band resolution is acceptable, or may be prolonged to enhance separation of histones with low gel mobilities, for example, histone H3 variants (**Fig. 1B**).
28. Open the glass–gel sandwich and place the separating gel into staining solution, which is gently agitated continuously overnight on a shaker (*see* **Note 12**).
29. Decant the staining solution. The gel can be given a very short rinse in water to remove all residual staining solution.
30. Place the gel in ample destaining solution. 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, polyurethane foam in limited amounts is added to only the first and second destaining solutions. Final destaining is done in the absence of any foam.
31. Record the protein pattern of the gel on film (**Fig. 1**) or on a flatbed digital scanner (*see* **Note 13**). Subsequently the gel may be discarded, dried, or prepared for autoradiography or fluorography as required. Blotting of AUT gels requires removal of Triton to allow successful histone transfer (*see* **Note 14**).

4. Notes

1. Core histones share a 65-basepair helix-turn-helix-turn-helix histone-fold motif which is the basis of the hydrophobic pairwise interaction between histones H2A and H2B and between histones H3 and H4. Our studies of histone H3 variant proteins from HeLa, *Physarum* ***(11)***, *Chlamydomonas* ***(9)*** and several plant species ***(5,7,8)*** support the notion that the characteristic differences in Triton X-100 affinity—Triton affinity: H2A > H3 > H2B > H4 (**Fig. 1**) —depend on sequence differences at residues whose side chains are mapped to the inside of the histone fold ***(12,13)***. To date, many proteins involved in DNA organization and transcription have been shown to share the histone fold, creating homo- and hetero-dimer complexes ***(14)***. However, none have yet been analyzed by AUT gradient gel electrophoresis to confirm the predicted interaction with Triton X-100.
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 assures 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. 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. 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. The concentration of Triton X-100 in the separating gel of the regular system is 9 m*M*, optimal for the separation desired in our research for histone H3 variant forms of plants and algae.
   In the gradient system, glycerol and Triton concentrations change in parallel: 10% (v/v) glycerol and 10 m*M* Triton in the heavy gel (**Fig. 2B**) and a gradient between 10 and 0% (v/v) glycerol and between 10 and 0 m*M* Triton in the gradient from heavy to light (**Fig. 2B**).
6. 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**).
7. 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, a 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, 1–1.5 cm stacking gel height will suffice for small volume samples.
8. Salt-free samples are routinely prepared by exhaustive dialysis against 2.5% (v/v) acetic acid in 3500 mol wt 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. (For alternative methods, *see* Chapter 16).
9. 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.
   The optimal amount of protein for a gradient gel also depends on the number of protein species that must be analyzed. In general, a gradient using the block comb used, equivalent to the width of 30 gel lanes, should be loaded with 30 times the sample for one lane.
10. As an alternative to **step 22**, 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.
11. The preparative well of a gradient gel should not be loaded with sample prior to the addition of electrophoresis buffer. Uneven distribution of sample cannot be avoided when buffer is added.
12. For unknown reasons, the polyacrylamide gel below the buffer front tends to stick tightly to the glass in an almost crystalline fashion. This may cause tearing of a gradient gel at the heavy gel side below the buffer front. Since attachment to one of the gel plates is typically much stronger, one can release the gel without problems by immersing the glass plate, with gel attached, in the staining solution.
13. 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 where 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 assure that the density response is linear. Placing a destained 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.

14. Triton X-100 interferes with native-mode electrotransfer of histones to nitrocellulose ***(15)***. Exchange of Triton by SDS under acidic conditions allows Western blotting ***(16)*** (*see* Chapter 42).

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