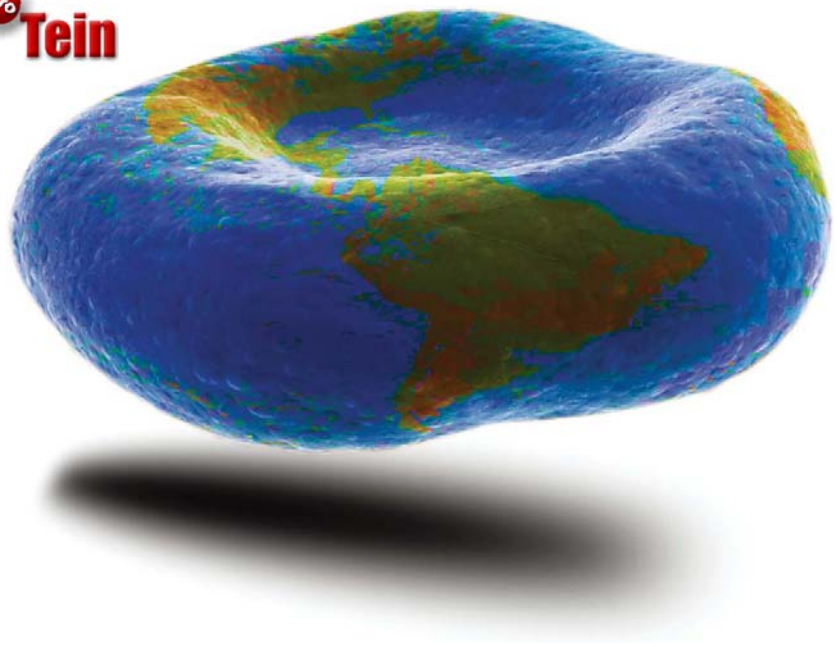

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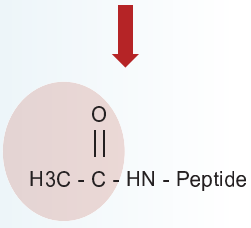
Free Modifications: N-Terminal Acetylation and C-Terminal Amidation

N-terminal Acetylation

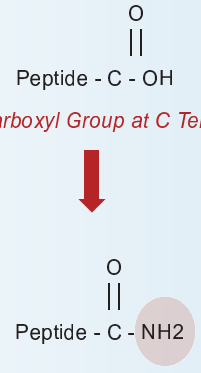
H2N - Peptide

(Free Amino Group at N Terminus)



(Acetylated N Terminus)

C-terminal Amidation



(Free Carboxyl Group at C Terminus)

(Amidated C Terminus)

Advantages:

- The altered peptide ends are uncharged, so modified peptides more closely mimic the native protein. This increases their ability to enter cells. We recommend modification for intracellular and in-vivo assays and for in-vitro functional studies.
- The modifications increase the metabolic stability of the peptides and their ability to resist enzymatic degradation by aminopetidases, exopeptidases, and synthetase. The modified peptides can then be used as substrates in enzyme assays.
- Amidation not only enhances the activity of peptide hormones; it also prolongs their shelf life. The changes reduce the influence of charged C- or N-termini during ELISA binding assays.

Tricine–SDS-PAGE

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Tricine–SDS-PAGE is commonly used to separate proteins in the mass range 1–100 kDa. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa. The concentrations of acrylamide used in the gels are lower than in other electrophoretic systems. These lower concentrations facilitate electroblotting, which is particularly crucial for hydrophobic proteins. Tricine–SDS-PAGE is also used preferentially for doubled SDS-PAGE (dSDS-PAGE), a proteomic tool used to isolate extremely hydrophobic proteins for mass spectrometric identification, and it offers advantages for resolution of the second dimension after blue-native PAGE (BN-PAGE) and clear-native PAGE (CN-PAGE). Here I describe a protocol for Tricine–SDS-PAGE, which includes efficient methods for Coomassie blue or silver staining and electroblotting, thereby increasing the versatility of the approach. This protocol can be completed in 1–2 d.

MATERIALS

REAGENTS

- Urea (Serva)
- Glycerol (Serva)
- Tetramethylethylenediamine (TEMED; Sigma)
- Mercaptoethanol (Sigma)
- Ammonium persulfate (Sigma)
- Reducing sample buffers:
  - Buffer A: 12% SDS (wt/vol), 6% mercaptoethanol (vol/vol), 30% glycerol (wt/vol), 0.05% Coomassie blue G-250 (Serva), 150 mM Tris/HCl (pH 7.0)
  - Buffer A/4: buffer A diluted with 3 volumes of water
  - Buffer C: buffer A without glycerol
- Nonreducing sample buffers:
  - Buffer B: 12% SDS (wt/vol), 30% glycerol (wt/vol), 0.05% Coomassie blue G-250 (Serva), 150 mM Tris/HCl (pH 7.0)
  - Buffer B/4: buffer B diluted with 3 volumes of water
  - Buffer D: buffer B without glycerol
- Electrode buffer (semidry transfer only): 300 mM Tris, 100 mM acetic acid (pH 8.6)

EQUIPMENT

- Vertical electrophoresis apparatus[27] without special cooling; most commercially available vertical protein gel electrophoresis systems are also suitable for Tricine–SDS-PAGE, for example, the SE 400 vertical unit (GE Healthcare) or the Protean II unit (Bio-Rad)
- Power supply (600 V, 500 mA) for SDS-PAGE and electroblotting ⊠CRITICAL For electroblotting, the power supply should have a minimal load resistance of ≤30 Ω
- Semidry blotter with glassy carbon electrodes (semidry transfer only)
- Polyvinylidene fluoride (PVDF) membranes (such as Immobilon P, Millipore; semidry transfer only) or nitrocellulose membranes
- Chromatography papers (17 CHR, Whatman; semidry transfer only)

REAGENT SETUP

Fixing solution (Coomassie or silver staining only) 50% methanol, 10% acetic acid, 100 mM ammonium acetate. Use essentially carbonyl-free methanol if mass spectrometric analyses are planned.⊠CRITICAL Ammonium ions are added several hours before using the fixing solution to deactivate minor amounts of aldehyde contaminants.

Electrode and gel buffers for Tricine–SDS-PAGE

	Anode buffer (10×)	Cathode buffer (10×)	Gel buffer (3×)
Tris (M)	1.0	1.0	3.0
Tricine (M)	—	1.0	—
HCl (M)	0.225	—	1.0
SDS (%)	—	1.0	0.3
pH	8.9	~8.25	8.45

Tricine obtained from Serva. Keep solutions at room temperature (20–25 °C). Do not correct the pH of the cathode buffer, which ideally should be close to 8.25.

AB-3 stock solution For the acrylamide-bisacrylamide (AB)-3 stock solution (49.5% T, 3% C mixture), which is normally used, dissolve 48 g of acrylamide and 1.5 g of bisacrylamide (each twice-crystallized; Serva) in 100 ml of water. For the AB-6 stock solution (49.5% T, 6% C mixture), which is needed only for optimal resolution of small proteins and peptides, dissolve 46.5 g of acrylamide and 3 g of bisacrylamide in 100 ml of water. ⊠CRITICAL Keep the solutions at 7–10°C, because crystallization occurs at 4°C.! CAUTIONAcrylamide and bisacrylamide are highly neurotoxic. When handling these chemicals, wear gloves and use a pipetting aid.

PROCEDURE

Casting the gel ⊠ TIMING ~2 h

1| Select and cast the appropriate base (separating) gel. The amounts of reagents required for two gels with dimensions 0.07 × 14 × 14 cm and containing 10% and 16% acrylamide are given in the table that follows. Do not degas the gel mixtures, because the gel buffer contains SDS. Overlay the poured gels with several drops of water. Leave the gels for about 30 min to polymerize. (Alternatively, precast gels can be purchased from vendors, and the protocol can be started from Step 3.)

⊠ CRITICAL STEP The freshly prepared ammonium persulfate (APS) solution and TEMED should be added last, immediately before pouring the gels, because these polymerize the gels.

		4% sample gel	10% gel	16% gel	16%/6 M urea
AB-3	(ml)	1	6	10	10
Gel buffer (3×)	(ml)	3	10	10	10
Glycerol	(g)	—	3	3	—
Urea	(g)	—	—	—	10.8
Add water to final volume	(ml)	12	30	30	30
Polymerize by adding:					
APS (10%)	(µl)	90	150	100	100
TEMED	(µl)	9	15	10	10

2| Overlay the polymerized separating gel (10% or 16%, or 16%/6 M urea) directly with a 4% sample (stacking) gel prepared as indicated in the table in Step 1, except if resolution of proteins <5 kDa is desired. If resolution of proteins <5 kDa is desired, then use AB-6 instead of AB-3 for the separating gel and overlay the separating gel with a 1-cm 10% gel, made up as described in the table. The 16% separating gel and the overlaid 10% 'spacer gel' can be polymerized together if no glycerol is added to the 10% acrylamide gel mixture (the common role of glycerol in SDS gels is to increase the density of solutions and to facilitate gel casting; it has no obvious effect on protein separation). Introducing a 10% 'spacer gel' between 4% stacking and 16% separating gels considerably sharpens the bands for proteins and peptides of 1–5 kDa.
? TROUBLESHOOTING

Sample preparation and protein loading ⏸ TIMING 30 min to 1 h
3| Adjust protein concentrations so that a suitable amount of protein can be loaded onto the gel. Concentrate samples, preferentially by techniques that do not increase the salt concentration (such as ultrafiltration). Roughly 0.2–1 µg of protein for each protein band (in 0.7 × 5 mm gel strips) is sufficient for Coomassie staining. Accordingly, the desired protein concentration in the sample is 0.1 mg ml^{-1} for each protein band. For silver staining, 100-fold less protein may be sufficient. Depending on the requirements of protein detection and analysis, concentration of the sample may be necessary.
⚠ CRITICAL STEP The maximal protein load can be limited by large amounts of neutral detergent in the sample and by high concentrations of lipid when solubilizing biological membranes. SDS must always be in large excess over neutral detergents and/or lipids. For isolated mitochondrial membranes (70% protein, 30% lipid), for example, the optimized maximal protein load of sample wells 0.7 × 5 mm is ⩽20 µg. Increasing the applied amount of protein to 40 µg, for example, may cause the individual protein bands to disappear in a diffuse background. Around 0.4% neutral detergent in the sample can be tolerated for direct mixing with SDS-containing sample incubation buffers and application to SDS gels. The incubation buffers and volumes described in Step 4 set a tenfold excess of SDS over the neutral detergent. Setting the SDS/neutral detergent ratio to<10 may result in a surprising result after staining: normally separated, large proteins may be detected in the upper gel areas, but the lower gel areas may be completely clear with no small proteins detectable.

4| Mix samples with SDS-containing sample buffers. The volume and buffer to be used depend on the origins of the samples. For low-density samples such as elution fractions from chromatographic columns, add 5µl of reducing or nonreducing sample incubation buffer A or B (see REAGENTS) to 15µl of sample. For high-density samples such as fractions from sucrose density gradients, add 5µl of sample buffer C or D to 15 µl of sample. For pellet samples, resuspend the pellet in 15–20 µl of buffer A/4 or B/4.

5| Incubate samples at 37 °C for 15 min or for up to 60 min for samples that were in pellet form.
⚠ CRITICAL STEP Avoid boiling samples, because membrane proteins can irreversibly aggregate in SDS at temperatures>50 °C.

6| Mount the gels in the vertical electrophoresis apparatus (see EQUIPMENT), and add anode buffer as the lower electrode buffer and cathode buffer as the upper

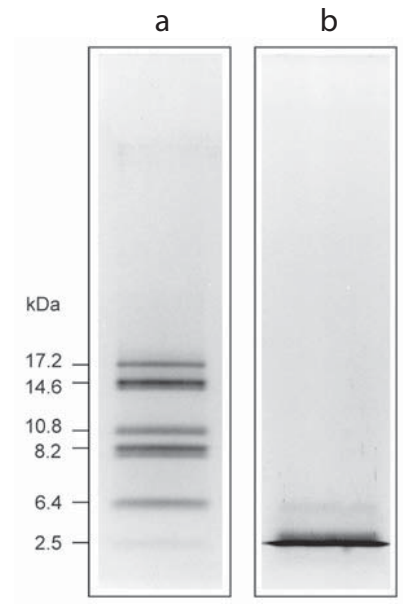

Figure 1 | Comparison of Tricine–SDS-PAGE and Laemmli–SDS-PAGE. Shown is the resolution of cyanogen bromide fragments of myoglobin by Tricine–SDS-PAGE (a) and Laemmli–SDS-PAGE (b) using 10% T, 3% C gels. Modified, with permission, from ref. 2.

electrode buffer (anodes and cathodes are commonly marked red and black, respectively, by the suppliers).

7| Load samples under the cathode buffer. Apply 10µl sample volumes to 0.7×5 mm sample wells.
▲ CRITICAL STEP For the optimal resolution of peptides of 1–5 kDa, reduce the amount of SDS in the incubation buffers, for example, by fourfold and reduce the volume applied to 5 µl. Reducing the amount of SDS facilitates electrophoretic separation of peptides from the bulk of SDS. To avoid streaking of peptide bands, peptides and bulk SDS must be separated before the faster-migrating bulk SDS and the peptides immediately following reach the separating gel.
▲ CRITICAL STEP The sample volume applied for Tricine–SDS-PAGE should not substantially exceed 10µl, because stacking of proteins in the range 20–100 kDa is less efficient than in Laemmli–SDS-PAGE.

Electrophoresis conditions ▲ TIMING 4–16 h
8| Set running conditions appropriate to your type of gel; guidance on appropriate running conditions are given in the table below. Start electrophoresis with an initial voltage of 30 V and maintain at this voltage until the sample has completely entered the stacking gel. The next appropriate voltage step can then be applied. The initial current may be as high as 80 mA for a 0.7-mm 10% gel. Gels may warm up, but the temperature should not exceed 35–40 °C. Approaching the end of the run, voltage can be gradually increased to shorten the total time of electrophoresis. Fast runs give better results than overnight runs, especially with 10% acrylamide gels. Alternatively, a constant power of▲10 W per gel might be set to ensure an even distribution of heat.
▲ CRITICAL STEP The specific settings for electrophoretic runs depend considerably on the apparatus used and its cooling capacity, the length and thickness of the gel, and the acrylamide concentration of the gel. The settings given above can be regarded only as general hints to approach reasonable experimental conditions. For initial runs, it seems advisable to test considerably lower voltage and wattage settings.

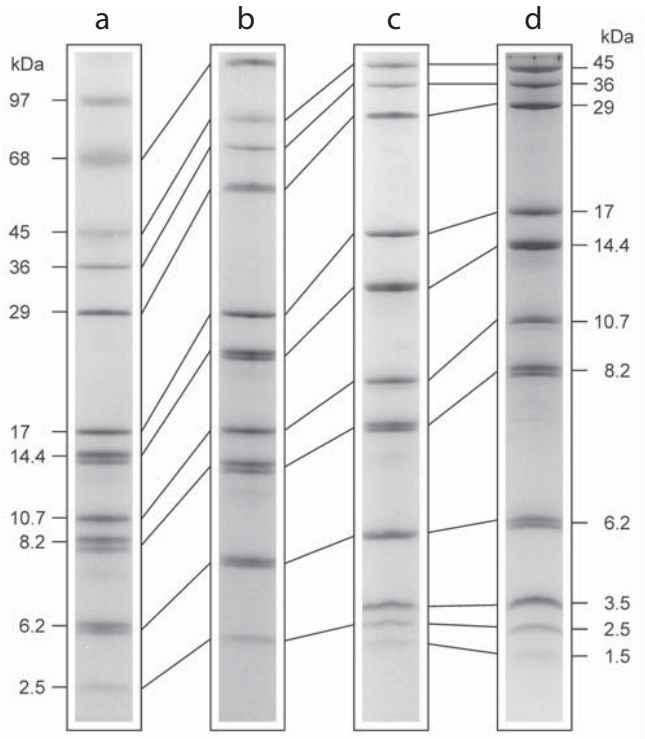

Figure 2 | Tricine–SDS-PAGE of marker proteins using various types of gel. Marker proteins with the indicated molecular masses were separated by gels comprising 10% T, 3% C (a); 16% T, 3% C (b); 16% T, 6% C (c); and 16% T, 6% C plus 6 M urea (d). Modified, with permission, from ref. 2.

	10% (0.7 mm)	16% (0.7 mm)	16% (1.6 mm)
Initial voltage	30 V	30 V	30 V
Next voltage step	190 V	200 V	90 V constant
Voltage at end of run	270 V	300 V	—
Time	3–4 h	5–6 h	Overnight

These data exemplify the voltage settings for thin (0.7-mm) and thick (1.6-mm) acrylamide slab gels (10% T, 3% C and 16% T, 3% C) with dimensions of 14×14 cm. The 4% sample gel is ▲2 cm, the separating gel is ▲12 cm. A simple apparatus[27] without special cooling is used.

Protein visualization
9| Protein can be visualized directly in the gel by Coomassie staining (A) or silver staining (B). Both Coomassie staining and silver staining can be done providing Coomassie staining is done first. Both procedures are compatible with subsequent mass spectrometric analysis. Alternatively, the proteins can be transferred to a PVDF membrane by electroblotting (C).
(A) Coomassie staining ▲ TIMING 1.5–5.5 h
(i) Incubate the gel in fixing solution. The length of incubation with fixing solution that is required depends on the gel type: 15 min for 0.7-mm 10% acrylamide gels; 30 min for 0.7-mm 16% acrylamide gels; or 60 min for 1.6-mm 16% acrylamide gels.
▲ PAUSE POINT Gels can be maintained in fixing solution for several days.

(ii) Stain the gel with 0.025% Coomassie dye in 10% acetic acid for twice the length of time used for fixing (Step 9Ai).
PAUSE POINT Gels can be maintained in staining solution for several days.
(iii) Destain the gel twice in 10% acetic acid. Each incubation should last 15–60 min.
(iv) Transfer the gel to water.
PAUSE POINT Gels can be maintained in water for several days.
(v) Coomassie-stained gels can be reused for silver staining after removing the protein-bound Coomassie dye by washing with 50% methanol, 50 mM ammonium hydrogen carbonate, followed by several washings with water.

(B) Silver staining TIMING 1.5–5.5 h
(i) Incubate the gel in fixing solution. The length of incubation with fixing solution required depends on the gel type: 15 min for 0.7-mm 10% acrylamide gels; 30 min for 0.7-mm 16% acrylamide gels; and 60 min for 1.6-mm 16% acrylamide gels.
PAUSE POINT Gels can be maintained in fixing solution for several days.
(ii) Wash the gel twice with water. Each incubation should last for the same amount of time as the gel was incubated in fixing solution.
PAUSE POINT Gels can be maintained in water for several days.
(iii) Sensitize the gel by incubating with 0.005% sodium thiosulfate ($Na_2S_2O_3$) for the same amount of time as the gel was incubated in fixing solution (15–60 min).
(iv) Incubate the gel with 0.1% silver nitrate for the same amount of time as the gel was incubated in the fixing solution (15–60 min).
(v) Wash the gel with water for seconds.
(vi) Add developer (0.036% formaldehyde, 2% sodium carbonate) to the gel for 1–2 min.
(vii) Stop development by incubating the gel in 50 mM EDTA for a total of 15–60 min.
CRITICAL STEP For mass spectrometric analyses, it might be advisable to replace the EDTA solution after 2–5 min to reduce the concentration of formaldehyde (residual from Step vi) as early as possible.
(viii) Wash twice with water.
PAUSE POINT Gels can be maintained in water for several days.

(C) Semidry electroblotting TIMING 17–25 h
(i) Soak a 6-mm stack of Whatman chromatography papers with electrode buffer and place half on the lower electrode of a semidry blotter (the cathode in this setup).
(ii) Wet a PVDF membrane with methanol and incubate it for 5–10 minutes with electrode buffer until the PVDF membrane is submerged in the buffer.
CRITICAL STEP If nitrocellulose membranes are used instead of PVDF membranes for western blots the membranes are immediately incubated with electrode buffer, since methanol dissolves nitrocellulose. After transfer to nitrocellulose membranes, the Coomassie-staining protocol described below should not be used, because nitrocellulose cannot be sufficiently destained. Staining by Ponceau S (0.1% in 1% acetic acid) and destaining with water may be used as a less-sensitive alternative.
(iii) Place the gel on top of the chromatography papers and cover the gel with the PVDF membrane.
(iv) Put the remaining 3-mm stack of chromatography papers soaked with electrode buffer on top.
(v) Place the anode on top.
(vi) Place a 5-kg load on top of the anode to avoid expansion of the gel during protein transfer.
(vii) Set the voltage to 15 V (actual voltage will be 7 V), limit the current to 0.4 mA per cm^2 of gel area (70 mA for a gel area of 12 × 14 cm) and electroblot for 16–24 h at room temperature.
(viii) Stain wet PVDF membranes for 5 min in 25% methanol, 10% acetic acid, 0.02% Coomassie blue G-250 dye.
(ix) Destain twice for 10 min with 25% methanol, 10% acetic acid; transfer to water; and then let the PVDF membrane dry. Protein bands on the dried membrane can be documented and are ready for N-terminal protein sequencing (Edman degradation).
CRITICAL STEP Do not use nitrocellulose instead of PVDF membranes if Edman degradation is desired, because nitrocellulose is dissolved by the organic solvents used.
(x) Destain the PVDF membrane with 100% methanol and transfer the membrane to aqueous western blotting buffers for immunodetection.

TIMING
Casting gel: 2 h
Sample preparation and loading: 30 min to 1 h
Electrophoresis: 4–16 h
Coomassie blue staining: 1.5–5 h (optional)

Silver staining: 1.5–5.5 h (optional)
Semidry electroblotting: 17–25 h (optional)

? TROUBLESHOOTING

Occasionally, proteins with similar masses migrate together. There are several options for separating these proteins. (i) Use gels containing 6 M urea, because urea (in addition to its general effects on the electrophoretic mobility of proteins in acrylamide gels) seems to alter SDS binding to proteins in a protein-dependent way. (ii) Vary the acrylamide concentration of the gels used. This affects the electrophoretic mobility of water-soluble and hydrophobic proteins differently; in other words, highly hydrophobic proteins show anomalous migration. The effects exerted by (i) and (ii) have been used to establish dSDS-PAGE as a technique to isolate hydrophobic proteins for mass spectrometric analysis[7]. (iii) Use reducing or nonreducing conditions; in other words, incubate the sample with SDS buffer containing or not containing thiol compounds such as mercaptoethanol. The electrophoretic mobility of proteins can vary with the reduction of internal disulfide bonds. This variation often appears as an apparent mass shift of 2–4 kDa in gels or, if disulfide bond breakage is incomplete, as a double band with differences of 2–4 kDa in apparent mass. (iv) Try Laemmli–SDS-PAGE instead of Tricine–SDS-PAGE and vice versa.

ANTICIPATED RESULTS

SDS is a strongly denaturing anionic detergent with unique characteristics. It unfolds and fully denatures all proteins, essentially disregarding specific secondary structures or hydrophobic domains, and generates SDS-protein complexes that are mostly characterized by a uniform charge-to-mass ratio. This makes SDS-PAGE in general a very simple and reliable technique for protein separation and (with exceptions) for mass determination.

The special utility of Tricine–SDS-PAGE for separating proteins in the low mass range as compared with Laemmli–SDS-PAGE is shown in Figure 1 . Using low-acrylamide gels for Tricine–SDS-PAGE (for example, 10% acrylamide gels) is advantageous for rapid separation, for covering a relatively wide mass range (2–100 kDa) and for efficient electroblotting and electroelution. The resolution power of Tricine–SDS-PAGE for small proteins can be further increased by using high-acrylamide gels and/or adding urea, as demonstrated by the gel variants shown inFigure 2 . For unknown reasons, urea reduces the electrophoretic mobility of proteins in general, but the migration of small proteins in particular. Therefore, the resolution of proteins in the low mass range is improved at the cost of a lower resolution for larger proteins[7,28].

Importantly, I would suggest that no concentrations of urea in excess of 6 M be used, because membrane proteins begin to oligomerize and to precipitate in spite of the presence of SDS.

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