

## Materials and Methods

### Equipment:

Bio-Rad Mini-Trans-Blot Cell  
Electrophoretic Blotting Apparatus  
Shaker (orbital type)

### Chemicals:

	Source	Cat. No.
ProBlott™	ABI	400994
Acrylamide	Bio-Rad	161-0100
Amido Black	Bio-Rad	161-0402
Ammonium persulfate	Bio-Rad	161-0700
Bromophenol blue	Bio-Rad	161-0404
Coomassie©Brilliant Blue R-250	Bio-Rad	161-0400
3-cyclohexylamino-1-propane- sulfonic acid (CAPS)	Aldrich	16,376-7
Glycerol	BRL	5514UA
Glycine	Bio-Rad	161-0717
2-mercaptoethanol	Sigma	M-6250
Methanol	ABI	400470
N,N'-methylene bisacrylamide	Bio-Rad	161-0200
N,N,N,N-Tetramethylene- diamine(TEMED)	Bio-Rad	161-0800
Ponceau	Sigma	P7767
Serva Blue G	Serva	35050
Sodium dodecyl sulfate (SDS)	Bio-Rad	161-0301
Sodium thioglycolate	Sigma	T-0632
Tricine	Aldrich	16,378-3
Tris (hydroxymethyl)aminomethane	Bio-Rad	161-0716
Triton X-100 (NP-40)	Bio-Rad	161-0407
Urea	Bio-Rad	161-0730

### SDS-PAGE Systems

A number of systems used with gel electrophoresis have been described<sup>4</sup>. These are the Tris-Glycine<sup>5</sup> and Tris-Tricine<sup>6</sup> buffer systems for mini-gels. Tris-glycine is used for separating proteins larger than 20kDa; the Tris-Tricine buffer system is used to resolve peptides and low-molecular weight proteins as well as larger proteins. Either buffer system can be used prior to electroblotting.

## Tris-Glycine stock solutions and buffers

*Lower tris (4 X)	Dissolve 36.34 g tris base and 0.8 g SDS in 150 mL D.I. H <sub>2</sub> O. Titrate with 6N HCl to pH 8.8. Add D.I. H <sub>2</sub> O to final volume of 200 mL.
*Upper tris (4 X)	Dissolve 12.11 g tris base and 0.8 g SDS in 150 mL D.I. H <sub>2</sub> O. Titrate with 6N HCl to pH 6.8. Add D.I. H <sub>2</sub> O to final volume of 200 mL.
*30% Acrylamide (30.8% T, 2.6% C)	Dissolve 30g acrylamide and 0.8 g bisacrylamide in D.I. H <sub>2</sub> O to final volume of 100 mL.
Electrophoresis Buffer	Dissolve 3.03 g tris base, 14.4 g glycine and 1 g SDS in 1 L D.I. H <sub>2</sub> O.
Ammonium persulfate (10%)	Dissolve 100 mg ammonium persulfate in 1 mL D.I. H <sub>2</sub> O. Store the solution at 5°C. Discard after one week.
Sample Preparation (2X) solution	Mix 1 mL glycerol, 0.5 mL 2-mercaptoethanol, 0.3 g SDS, 0.25 mL of 0.05% (w/v) bromophenol blue and 1.25 mL Upper Tris (4X) buffer in D.I. H <sub>2</sub> O to a final volume of 5 mL.

\*Filter solutions with 0.2 μ Nalgene© filter.

## Tris-Glycine Gel Solutions

The following proportions are for preparing 2 gels (0.5 x 60 x 90 mm) for mini-gel systems.

<b>Stock Solutions</b>	<b>10% Resolving Gel (8 mL)</b>	<b>15% Resolving Gel (8 mL)</b>	<b>4% Stacking Gel (4 mL)</b>
30% Acrylamide	2.66 mL	4.0 mL	0.52 mL
Upper tris (4X)	-	-	-
Lower tris (4X)	2.0 mL	2.0 mL	-
D.I. H <sub>2</sub> O	3.34 mL	2.0 mL	2.47 mL
De-gas for 5 minutes			
TEMED	4 µL	4 µL	4 µL
Ammonium persulfate (10%)	40 µL	40 µL	40 µL

## Electrophoresis Conditions for Tris-Glycine

1. Sample preparation: Mix one volume (1-10  $\mu\text{L}$ ) of sample solution with one volume of sample preparation solution (2X) and heat in boiling water bath for 2 minutes. The sample is now ready to load into the sample well for electrophoresis.
2. When using the mini-gel apparatus, the recommended electrophoresis condition for optimal resolution with minimal thermal band distortion is 7 mA constant current (50-90 volts) for approximately 1 hour. For maximum separation, allow the bromophenol blue dye to run to within 1 cm of the end of the gel.

## Tris-Tricine stock solutions and buffers

*Anode Buffer (10 X) (2.0 M Tris HCl)	Dissolve 121.9 g tris base in 400 mL D.I. $\text{H}_2\text{O}$ . Titrate to pH 8.9 with HCl. Add D.I. $\text{H}_2\text{O}$ to final volume 500 mL.
*Cathode Buffer (10 X) (1.0 M Tris, 1.0 M Tricine, SDS)	Dissolve 60.55 g, 89.58 g tricine and 5 g SDS in 400 mL D.I. $\text{H}_2\text{O}$ . Titrate to pH 8.25 with HCl. Add D.I. $\text{H}_2\text{O}$ to final 1.0% volume 500 mL.
*Acrylamide Solution (49.5% T, 3% C)	Dissolve 48 g acrylamide and 1.5 g bis-acrylamide in 100 mL D.I. $\text{H}_2\text{O}$ .
*Gel Buffer (3 X)	Dissolve 181.5 g tris base, 1.5 g SDS in 400 mL D.I. $\text{H}_2\text{O}$ . Titrate to pH 8.45 with HCl. Add D.I. $\text{H}_2\text{O}$ to final volume of 500 mL.
50% glycerol (v/v)	Dissolve 250 mL glycerol in 250 mL D.I. $\text{H}_2\text{O}$ .
Sample Preparation Solution (2X)	Mix 1.0 mL of 100% glycerol, 0.5 mL 2-mercaptoethanol, 2.5 mL of 20% (w/v) SDS, 0.62 mL Tris (pH 6.8, 1M), 0.01 mL of 10% Serva BlueG (w/v) in D.I. $\text{H}_2\text{O}$ to a final volume of 5 mL.

\*Filter solutions with 0.2  $\mu$  Nalgene© filter.

## Tris-Tricine Gel Solutions

The following proportions are preparing 2 gels (0.5 x 60 x 90 mm).

Stock Solutions	10% Resolving Gel (15 mL)	4% Stacking Gel (12.5 mL)
Acrylamide solution	3.0 mL	1.0 mL
50% glycerol	4.0 mL	-
Gel Buffer (3X)	5.0 mL	3.1 mL
H <sub>2</sub> O	3.0 mL	8.4 mL
De-gas for 5 minutes		
TEMED	7.5 µL	7.5 µL
Ammonium persulfate (10%)	75 µL	100 µL

## Electrophoresis Conditions for Tris-Tricine

1. Sample preparation: Mix 1 volume of sample with 1 volume of sample preparation solution (2 X) and heat in boiling water bath for 2 minutes.
2. Electrophoresis Conditions: 20 mA constant current for 2-3 hours. For maximum separation, allow the serva blue dye to run to within 1 cm of the end of the gel.

## Electroblotting

### CAPS Buffers<sup>1</sup>

Stock CAPS (10 X) Dissolve 22.13 g CAPS in 900 mL of D.I. water. Titrate with 2N NaOH (20 ml) to pH 11, and add D.I. water to a final volume of 1L. Store at 4°C.

Electroblotting buffer (10 mM CAPS in 10% MeOH) Prepare 2L by mixing 200 mL of the 10X CAPS buffer with 200 ml of methanol and 1600 mL of D.I. water.

### Tris-Glycine Buffer<sup>7,8</sup>

Electroblotting buffer mM Tris base, 192 mM glycine, 10% (v/v) methanol (pH 8.3)] Dissolve 3.0 g Tris and 14.4 g glycine in a solution of 900 mL H<sub>2</sub>O [25 and 100 mL methanol.

## Procedure:

1. Remove the gel from the electrophoresis cell and soak it in 100 mL of electroblotting buffer for 5 minutes.
2. Meanwhile, wet ProBlott™ with 100% methanol for a few seconds, then transfer it to the blotting buffer.

**Note: Be sure the ProBlott™ membrane is completely moistened with methanol before transferring it to the blotting buffer. If it dries out, wet it with methanol again.**

3. Dip the sponges and filter papers in a separate container of blotting buffer before starting to assemble the transblotting sandwich.
4. Assemble the transblot sandwich in the following order starting from anode side: sponge, filter paper, 2 sheets of ProBlott™, gel, filter paper, sponge.

**Make sure there are no bubbles between the gel and the membrane.**

6. Pour 1L of electroblotting buffer into the transblot cell and insert the transblot sandwich. Electroblotting conditions are dependent of the buffer, as follows:

CAPS:50V (170 mA-100mA) at room temperature for 30 minutes. Tris-Gly:40V (300 mA) at room temperature for 1-4 hours.

**Note: Electroblotting times vary and are dependent on protein sample. Longer electroblotting times may be necessary with proteins that are 70 kDa or larger.**

7. After transfer is complete, remove ProBlott™ from transblotting sandwich and rinse with D.I. water before staining.

## Protein Detection

Protein samples on ProBlott™ can be detected with conventional staining techniques, such as Coomassie© Brilliant Blue, Ponceau S or Amido Black.

### Coomassie© Brilliant Blue Staining Solution

0.1% Coomassie Blue R-250 in 1% acetic acid/40% MeOH

Dissolve 1.0 g Coomassie blue in 400mL of methanol. Stir for one hour. Add 10 mL acetic acid and 590 mL D.I. water. Stir for 30 min, then filter with a Nalgene© filter (0.45µ pore size).

## **Destaining solution**

### **50% MeOH in D.I. water**

Coomasie Blue Staining Procedure:

1. Remove the ProBlott™ membranes (both primary and secondary) from the transblot sandwich, and rinse them with D.I. water.
2. Saturate the ProBlott™ membranes with 100% MeOH for a few seconds.
3. Stain the ProBlott™ membranes (one at a time) with the staining solution using constant orbital shaking. Protein bands should appear within one minute.

**CAUTION: If the ProBlott™ membrane is over-stained, it may require a longer destaining time to prevent a high background.**

4. Destain the ProBlott™ membranes by soaking them in destaining solution. For quicker destaining and improved contrast, change the destaining solution several times, allowing the membranes to soak for 1-2 minutes between changes.
5. Rinse the membrane thoroughly with D.I. water, and excise the bands of interest with a clean razor blade.

## **Amido Black Stainig solution**

0.1% Amido Black in 1% acetic acid/40% MeOH

Dissolve 1.0 g Amido Black in 400 mL of methanol. Stir this solution for at least one hour. Add 10 mL acetic acid and 590 mL of D.I. water. Stir for another 30 minutes, then filter the solution with Nalgene filter (45µ pore size).

The staining procedure for Amido Black is the same as that described for Coomasie Blue, with the substitution of D.I. water as the destaining solution.

## **Ponceau S Staining solution**

0.2% Ponceau S in 1% acetic acid

Dissolve 0.4 g of Ponceau S in 198 mL of D.I. water and stir for 30 minutes. Add 2 mL of acetic acid to the mixture.

1. Remove the ProBlott™ membranes from the transblotting sandwich and rinse them with D.I. water.
2. Stain the ProBlott™ membranes in the Ponceau S staining solution with constant orbital shaking. Protein bands should appear within one minute.
3. Destain with a D.I. water rinse.
4. Excise the bands of interest with a clean razor blade.