

Western Blotting: Mini-gels

Materials

⌘ Protein Extraction Buffer (for callus or kernel), 100 ml

Solution	Stock	Final	Volume
Tris-HCl pH 8.0	1 M	200 mM	20 ml
NaCl	4 M	100 mM	2.5 ml
Sucrose	2 M	400 mM	20 ml
EDTA	0.5 M	10 mM	2 ml
β -mercaptoethanol	14 M	14 mM	100 μ l
Tween-20	20 %	0.05 %	250 μ l
Water			55.15 ml

⌘ Sample Loading Buffer (SDS reducing buffer), 9.5 ml

0.5M Tris-HCl pH 6.8	1.25 ml
10 % SDS	2.0 ml
0.5 % Bromophenol Blue	0.2 ml
Glycerol	2.5 ml
Water	3.55 ml

Add 50 μ l β -mercaptoethanol to 950 μ l of sample loading buffer prior to use. Dilute the sample at least 1:2 with the sample loading buffer

⌘ 10X Electrode Running Buffer, 1L

Tris base	30.3 g
Glycine	144 g
SDS	10 g

⌘ 1X Electrode Running Buffer

10X electrode running buffer	100 ml
Water	900 ml

⌘ Transfer Buffer, 4L

Tris base	12.12 g
Glycine	57.6 g
Methanol	800 ml
Volume to 4 L	

Chill to 4°C before use.

⌘ **10 % APS**

Ammonium persulfate 50 mg
 Sterile water 500 µl
 Vortex well.

Note: APS must be made fresh each time.

⌘ **Resolving Gel Buffer**

1.5M Tris-HCl pH 8.8

⌘ **Stacking Buffer**

0.5M Tris-HCl pH 6.8

⌘ **Prestained SDS-PAGE Standards, broad range (Bio-Rad: 161-0318, Control 98148)**

Protein	Calibrated MW (daltons) on Tris-HCL gel	Calibrated MW (daltons) on Bis-Tris- HCl gel	Calibrated MW (daltons) on Tris- Acetate gel
Myosin	198,089	195,755	197,455
B-galactosidase	113,586	107,181	110,535
Bovine Serum Albumin	96,368	59,299	69,966
Ovalbumin	52,989	41,220	46,139
Carbonic Anhydrase	35,960	27,578	37,570
Soybean Trypsin Inhibitor	28,491	20,514	
Lysozyme	18,533	15,189	
Aprotinin	5,736	6,458	

⌘ **Staining Solution**

0.25 % Coomassie Brilliant Blue R
 50 % Methanol
 7 % Acetic Acid

⌘ **30 % Methanol**

⌘ **PBS, 10X**

Na₂HPO₄ 15 g
 KH₂PO₄ 4 g
 NaCl 61.2 g

⌘ **PBS, 1X**

10X PBS 100 ml
 Water 900 ml

⌘ **PBST, 1X**

10X PBS 100 ml
 Tween-20, 100 % 500 µl
 Water 900 ml

⌘ **Blocking Solution**

5 g non-fat dry milk in 100 ml PBS

⌘ **TEMED (source)**

⌘ **PVDF Membrane (source)**

⌘ **1 % Milk**

1 g non-fat dry milk in 100 ml PBS

⌘ **Primary Antibody**

⌘ **Secondary Antibody**

⌘ **AP (Alkaline Phosphatase) Substrate Buffer, 1L**

NaCl	5.84 g
Tris base	12.11 g
MgCl ₂ *6H ₂ O	1.02 g
pH to 9.5	

⌘ **AP Substrate Solution A (BioRad: #)**

⌘ **AP Substrate Solution B (BioRad: #)**

Gels (1.5 mm)

⌘ **Stacking Gel (5 %), 5 ml**

Water	2.85 ml
30 % Acrylamide/Bis	0.85 ml
Gel Buffer	1.25 ml
10 % SDS	50 µl
10 % APS	50 µl

⌘ **Resolving Gel (12 %), 15 ml**

Water	5.1 ml
30 % Acrylamide/Bis	6 ml
Gel Buffer	3.75 ml
10 % SDS	150 µl
10 % APS	150 µl

Methods

⌘ **Preparation of gels**

1. Mix the components for the stacking gel and resolving gel in separate tubes. Vortex well.

2. Add 7.5 μ l of TEMED to the **Resolving Gel**, vortex well. TEMED is a neurotoxin, handle with care.
3. Pipet the resolving gel into 2 mini-gels in the gel cassette quickly taking care not to create bubbles.
4. Immediately add 1 ml of water to the gel cassette to completely cover the surface of the resolving gel. This creates a smooth surface to the resolving gel
5. Let the resolving gel solidify in the gel cassette for 20 minutes.
6. After the resolving gel has solidified, use filter paper to wick the water off the surface of the gel.
7. Add 5 μ l of TEMED to the **Stacking Gel**, vortex well.
8. Pipet the stacking gel on top of the resolving gel in the 2 mini-gels in the gel cassette.
9. Immediately insert a 1.5 mm comb into each mini-gel
10. Let the stacking gel solidify in the gel cassette for 20 minutes.
11. Remove the mini-gels from the gel cassette, wrap in wet paper towels, seal in a plastic bag, and store at 4°C until use (not more than 1-2 days).

⌘ Preparation of samples

1. Samples are usually run in duplicate, boiled and unboiled. Dilute the samples at least 1:2 with the sample loading buffer, for example 10 μ l sample + 10 μ l of buffer. Boil the boiled samples for 5 minutes at 95°C to denature and load the gel.

⌘ Running of gels

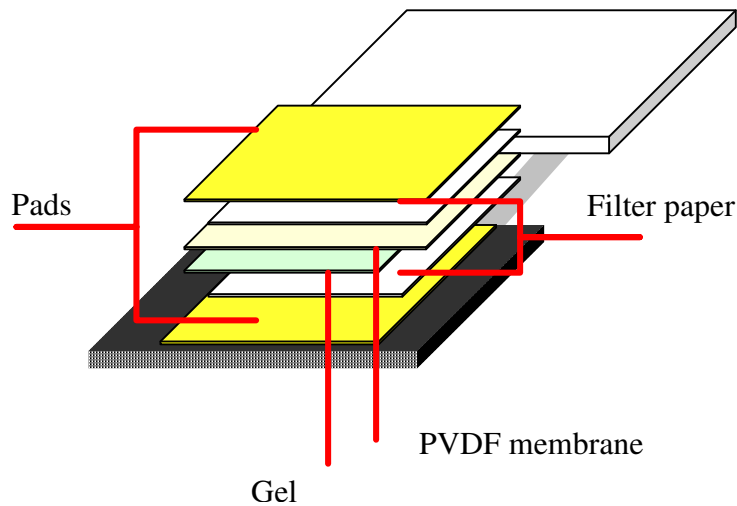
1. Assemble gel cassettes in gel tank and fill with 1X electrode running buffer
2. Load between 5 and 50 μ l of sample slowly into the wells. Load sample loading buffer into empty wells to ensure even voltage along the gel
3. Assemble electrophoresis apparatus and run gel at 125V for about 1.5 hours or until the bromophenol blue is 0.5 cm from the edge of the gel.

⌘ Staining of gels

1. Incubate the gel in staining solution for 1 hour at room temperature.
2. De-stain the gel in 30 % methanol, changing the solution several times over a 24 hour period.

⌘ Western blot

1. Soak 2 pads, filter paper, and membrane for each gel in cold transfer buffer.
2. Cut the lower right corner of the gel to ensure proper orientation in the transfer apparatus.
3. Assemble transfer apparatus.



4. Place the cassettes in the tank with a stir bar and an ice pack in the outer reservoir. Fill the tank with pre-chilled transfer buffer.
5. Place the tank on a magnetic stir plate in the cold room and run at 150V for at least 2 hours.
6. After transfer, remove the membranes from the cassette and wash 1 minute in PBST on a platform shaker at room temperature.
7. Incubate with blocking solution for 1 hour on a platform shaker at room temperature. Alternatively, membranes can be incubated at 4°C overnight.
8. Wash membranes 5 minutes in PBST on a platform shaker at room temperature.
9. Incubate with primary antibody for 1 hour on a platform shaker at room temperature.
10. Wash membranes 3 x 5 minutes in PBST on a platform shaker at room temperature.
11. Incubate with secondary antibody for 1 hour on a platform shaker at room temperature.
12. Wash membranes 3 x 5 minutes in PBST on a platform shaker at room temperature.
13. Wash membranes 1 minute in AP substrate buffer on a platform shaker at room temperature to remove any residual detergent.
14. Incubate each membrane in 15 ml AP substrate buffer containing 150 µl each of substrate a and b solutions for 1 minute on a platform shaker at room temperature.
15. Color should appear in 30 minutes or less.
16. Stop the reaction by adding deionized water.
17. Air dry the membranes, wrap in plastic, and place in the dark at room temperature.

References

Weber and Osborn, 1969