METHOD USED TO EXTRACT TOTAL MUSCLE PROTEIN FOR WESTERN BLOT USING TRIS-EDTA BUFFER*

SOLUTIONS FOR SAMPLE EXTRACTION

1. Tris-EDTA Buffer, pH 8.3

	1 liter
50 mM Tris	6.06 g
10 mM EDTA	3.72 g

Adjust pH to 8.3; qs to 1 liter. Store at 4°C.

2. 0.5 M Tris, pH 6.8

	200 ml
Tris	12 g

Adjust pH to 6.8 with HCl; qs to 200 ml. Filter and store at 4°C.

3. 10% SDS

	500 ml
SDS	50 g

qs to 500 ml. Filter with Whatman filter paper. Store at room temperature

4. Filtered Distilled Water

5. 8 mg/ml Bromophenol Blue (0.8%)

400 mg/50 ml. Store at room temperature.
(This was changed from 4 mg/ml (0.4%) on 3/97 so the buffer would be darker).

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6. 2X Treatment Buffer minus MCE, pH 6.8

0.125 M Tris	2.5 ml solution (2)	50 ml solution (2)
4% SDS	4.0 ml solution (3)	80 ml solution (3)
20% glycerol	2.0 ml	40 ml
10% MCE	--------	-------
H_2O	0.5 ml	10 ml
	9.0 ml	180 ml

pH to 6.8. Store at room temperature.

7. 2X Treatment Buffer + MCE + Bromophenol Blue, pH 6.8

(changed 8/97 to avoid hazardous waste disposal)

9.0 ml 2X Treatment Buffer (Solution 6)
50 µl MCE
500 µl Bromophenol Blue (Solution 5)

Make fresh daily (or use aliquots that have been frozen). Any solution not used may be aliquoted and frozen for future use.

SOLUTIONS FOR GEL ELECTROPHORESIS

8. Stock Acrylamide (30%) (37.5:1)

	100 ml	500 ml
Acrylamide	29.2 g	146 g
Bisacrylamide	0.779 g	3.895 g

Mix (wrap beaker in foil to protect solution from light) and adjust to 100 ml. Filter and store in a dark bottle at 4°C. *We currently use pre-mixed Acrylamide solution from Bio-Rad.
Caution: Acrylamide is a neurotoxin. Wear gloves and face mask when working with it. Wash hands thoroughly after use. Polymerized gels can be disposed in the trash. Unpolymerized solutions are disposed as hazardous waste, contract disposal.

9. 1.5 M Tris base, pH 8.8

18.15 g/100 ml ddH_2O 90.75 g/500 ml ddH_2O
pH to 8.8 with HCl. Filter and store at 4°C.

10. 0.5 M Tris base, pH 6.8

6 g/100 ml ddH_2O 30 g/500 ml ddH_2O

pH to 6.8 with HCl. Filter and store at 4°C.

11. 10% SDS

10 g/100 ml ddH_2O

Filter (with filter paper) and store at room temperature up to 6 months. Some heat may be required to dissolve. Wear a face mask when preparing this solution.

12. 10% Ammonium Persulfate

0.1 g/1 ml ddH_2O

13. Running Buffer, pH 8.3

	10X
0.25 M Tris (F.W. 121.1)	30.0 g
1.92 M glycine	144.0 g
ddH_2O to 1 liter	

It is not necessary to check the pH of this solution. Store at room temperature.

To make 1X Running Buffer:	100 ml of 10X solution
	10 ml of 10% SDS
	890 ml ddH_2O

Make fresh for each gel run. Do not reuse the running buffer. Reusing the buffer can affect reproducibility since the ionic strength & pH of the buffer change during the run (per Bio-Rad).

14. Water Saturated Butanol

50 ml n-Butanol + 10 ml ddH_2O

Dispose excess as hazardous waste. Butanol that is used as gel overlay (approx. 1 ml), may be poured onto a paper towel and then allowed to evaporate in the hood.

SOLUTIONS FOR PROTEIN TRANSFER

15. Transfer Buffer

	2 liters	4 liters
Glycine	28.83 g	57.66 g
Tris	6.06 g	12.12 g
10% Methanol	200 ml(of 100%)	400 ml
ddH_2O	to 2 liters	to 4 liters

Should be pH 8.1 - 8.3 without pHing. This solution may be reused 1-2 times. Dispose in sewer system.

16. 0.1% Amido Black Staining Solution

	100 ml
Amido Black	100 mg
10% Methanol	10 ml
2% Acetic Acid	2 ml
ddH_2O	88 ml

Mix and filter. Store at room temperature. Dispose sewer system.

17. Destain

20% Methanol	2000 ml
7% Acetic Acid	700 ml
ddH_2O	7300 ml

Dispose in Methanol Hazardous Waste container.

SOLUTIONS FOR WESTERN BLOT

18. TBS, pH 7.4

	1 liter	or	10X	1 liter
20 mM Tris	2.4 g			24 g
137 mM NaCl	8.0 g			80 g
5 mM KCl	0.2 g			2 g
				(dilute to 1X to use)

pH with 1 N HCl. Filter and store at room temperature.

19. TTBS (0.05% Tween 20), pH 7.4

Add 250 µl Tween 20 to 500 ml TBS.

20. Blocking Solution (either solution may be used)

- Starting Block T20 Blocking Buffer*, Pierce #37543

(*A proprietary protein formulation in Tris buffered saline at pH 7.5 with 0.05% Tween)

- 5% Milk in TTBS, made from dried skimmed powdered milk

21. Bio-Rad Alkaline Phosphatase Substrate BCIP/NBT

200 µl “A” + 200 µl “B” + 20 ml Alkaline Phosphatase Buffer. Solutions A & B are light sensitive so add these to the buffer immediately before use. The buffer may be warmed to room temperature before use. Cold buffer slows the staining process. This is enough for 2 Blots. Hazardous waste disposal.

SAMPLE PREPARATION

1. Homogenize 1 gram of sample in 10 volumes (10 ml) 1X extraction buffer (Solution 1) for 20 sec with the Polytron at setting #4. Do this step in the cold room if using raw samples. For cooked core samples, this step may be done in the lab.

2. Immediately remove a 0.5 ml aliquot for solubilization and transfer to a 1.5 ml microcentrifuge tube.

3. Add 0.5 ml of 2X Treatment buffer (minus MCE). Mix well.

4. Heat samples in a 50°C waterbath for 20 minutes, repeat mixing using a pipetman , and reheat for 5 min. Nucleic acids may be stringy and viscous, but pipetting will help shear them.

5. Centrifuge for 20 min in a Eppendorf 5414 C centrifuge (maximum setting = 16,000 x g), to pellet insoluble material. Pellet should be small or undetectable.

6. Determine protein concentration of the supernatant (diluted 1:5 with 1x Treatment Buffer (10 µl sample + 40 µl buffer)) using the micro-BCA protein assay (use microtiter well plates). Do in triplicate.

   To each well add:

Sample:	10 µl diluted sample
or	
Standard:	10 µl (4, 2, 1, 0.5, 0 mg/ml BSA)

   Add 200 µl BCA reagent and incubate at 37°C for 30 min. Read plate on the microplate reader at 562 nm. If more than one plate is used, run a standard curve with each plate. Mix standards to contain the same concentration of potential interfering substances as the samples.

7. Dilute samples to 3 mg/ml using (or your desired protein concentration) 2X treatment buffer containing MCE and bromophenol blue (Solution 7). Mix samples well and heat in a 50°C waterbath for 10 min prior to loading on gel. Samples may be frozen at this point if gels cannot be run at this time.

GEL ELECTROPHORESIS

(If you are using the Bio-Rad Mini-Protean 3 Cell System, and the Bio-Rad Mini Transblot Electrophoretic System, use the Bio-Rad instructions for those systems)

1. The height of the separating gel is 5-1/2 cm. A 4% stacker is used.

		SEPARATING				
	4% Stacker	**15%**	**12.5%**	**10%**	**7.5%**	**5% Continuous**
1.5 M Tris, pH 8.8	----------	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml
0.5 M Tris, pH 6.8	1.88 ml	---------	---------	---------	---------	----------
30% Acrylamide	1.0 ml	9.98 ml	8.35 ml	6.65 ml	4.99 ml	100:1; 50% glycerol 3.3 ml
10% SDS	0.075 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Filtered ddH_2O	4.55 ml	4.72 ml	6.35 ml	8.05 ml	9.71 ml	11.2 ml
10% APS	50 µl	100 µl	100 µl	100 µl	100 µl	100 µl
TEMED	7.5 µl	10 µl	10 µl	10 µl	10 µl	10 µl

***This recipe is enough for 4 mini gels**

2. Mix separating gel and degas 15 minutes. Add APS and TEMED.

3. Pour gel (5-1/2 cm); overlay with water saturated Butanol and allow to polymerize 1 hour.

4. Make stacking gel and degas 15 minutes. Add APS and TEMED and mix immediately before stacker is to be poured (see step 5).

5. Pour off water saturated Butanol and rinse well with distilled water. Remove any residual water with a Kimwipe. Pour stacker making sure that no air bubbles are trapped under the wells. Place comb in between plates. Allow to polymerize 30 minutes.

6. Carefully remove comb and rinse wells with water. Remove residual water with a Kimwipe. Assemble gel rig using running buffer in the lower chamber and running buffer in the upper chamber.

7. Run gels at 200 volts for 45 minutes or until dye front just runs off the end of the gel.

8. While gel is running, prepare everything to transfer proteins from the gel to the membranes.

SAMPLE LOADING

1. If samples were frozen, heat thawed samples in a 50°C waterbath for 5 minutes.

2. A standard is run on every gel in triplicate. The standard preferably is a pooled sample of multiple animals collected at 0h postmortem. Based on the samples to be run, the standard must be species specific and muscle specific.

3. Always leave at least one outside lane on each side open to avoid sample smiling. The standard is run in the outside lanes and in the middle (see example).

Lane	
1	PDB
2	**0h Standard**
3	Sample
4	Sample
5	Sample
6	Sample
7	Sample
8	**0h Standard**
9	Sample
10	Sample
11	Sample
12	Sample
13	Sample
14	**0h Standard**
15	PDB

PROTEIN TRANSFER

1. Cut blotter paper (Whatman 3MMChr Chromatography paper) to 3” x 4”. Cut the PVDF Membranes to 2-1/2” x 3-1/2”. Notch the upper left hand corner of the membrane. This notch will correspond to lane 1 of the gel.

Using a pencil, mark your ID in this corner. Be careful to never touch the membranes with your hands - always wear gloves. Handle membranes with forceps.

2. Place transfer buffer in a tray. Assembling of the sandwiches will take place in this tray. Lay 1/2 of the plastic cassette in the tray. Place 1 buffer saturated sponge on top of this.

3. Remove one gel from rig and remove one glass plate. Remove all stacker. You may have to rub the glass plate with your finger to insure that all the stacker has been removed. Notch the gel at lane 1. Slide the gel into the tray containing transfer buffer.

4. Wet blotter paper (2 sets, 2 papers each) in transfer buffer.

5. Fill one tray (pipet tip box lids) with methanol and another with distilled water. Wet one membrane in methanol for approximately 5 seconds, making certain that the membrane is totally submerged. Transfer this membrane to the tray containing water for 30 seconds. Transfer to Transfer Buffer.

6. Transfer Stack Assembly. The sandwich is assembled so that the negative charge travels through the gel to the membrane. Assemble as follows: On top of the saturated sponge place 2 buffer saturated blotter papers. Remove air bubbles by rolling a 15x85 mm glass tube over the surface. Place the membrane on top of the blotter paper. Make sure there are no air bubbles under the membrane. Center the gel on top of the membrane. Gently remove air bubbles with your finger. Make certain your gloves are wet or the gel will tear. Place 2 buffer saturated blotter papers on the gel, from the center towards the edge. Roll the glass tube over the paper surface to remove air bubbles. During this process, keep all surfaces wet. Place 1 buffer saturated sponge on top of the blotter paper. Close the cassette. Place sandwich in tank with the gel on the negative electrode side. Fill tank with cold transfer buffer. Add the ice block to the tank. Place the tank on a stir plate with gentle stirring.

7. Transfer for 1 hour at 200 mA.

8. Rinse blot in water for ~ 10 seconds and place in blocking solution and store overnight at 4° or allow to air dry if membrane will be probed at a later time. If not, proceed with Western Immunoblot procedure after MW markers have been removed. While membrane is drying, lanes will be visible. Visualize molecular weight markers and cut off that lane. Stain MW markers with 0.1% Amido Black for 5-10 min. Destain for 5 min. Pour off into Methanol Hazardous Waste container. Rinse with water. Air dry. Air dried blots may be stored in a sealed bag at 4°C.

Western Immunoblot Procedure*

1. If blot was air dried re-wet blot in Methanol and then water as before.

All subsequent incubations performed in pipet tip box lids at room temperature with gentle rocking.

2. Place blot in 10 ml blocking solution for 30 minutes for Starting Block buffer or 60 minutes for 5% milk.

3. Pour off blocking solution and apply Primary Antibody made in blocking buffer. Incubate 1 h. The primary antibody can be saved and reused several times for future blotting procedures.

4. Wash blot 3X (5 min each) with TTBS.

5. Incubate with Secondary Antibody made in blocking buffer for 1 h.

6. Wash blot 3X (5 min each) with TTBS.

7. Apply Bio-Rad Alkaline Phosphatase Substrate BCIP/NBT until desired stain intensity.

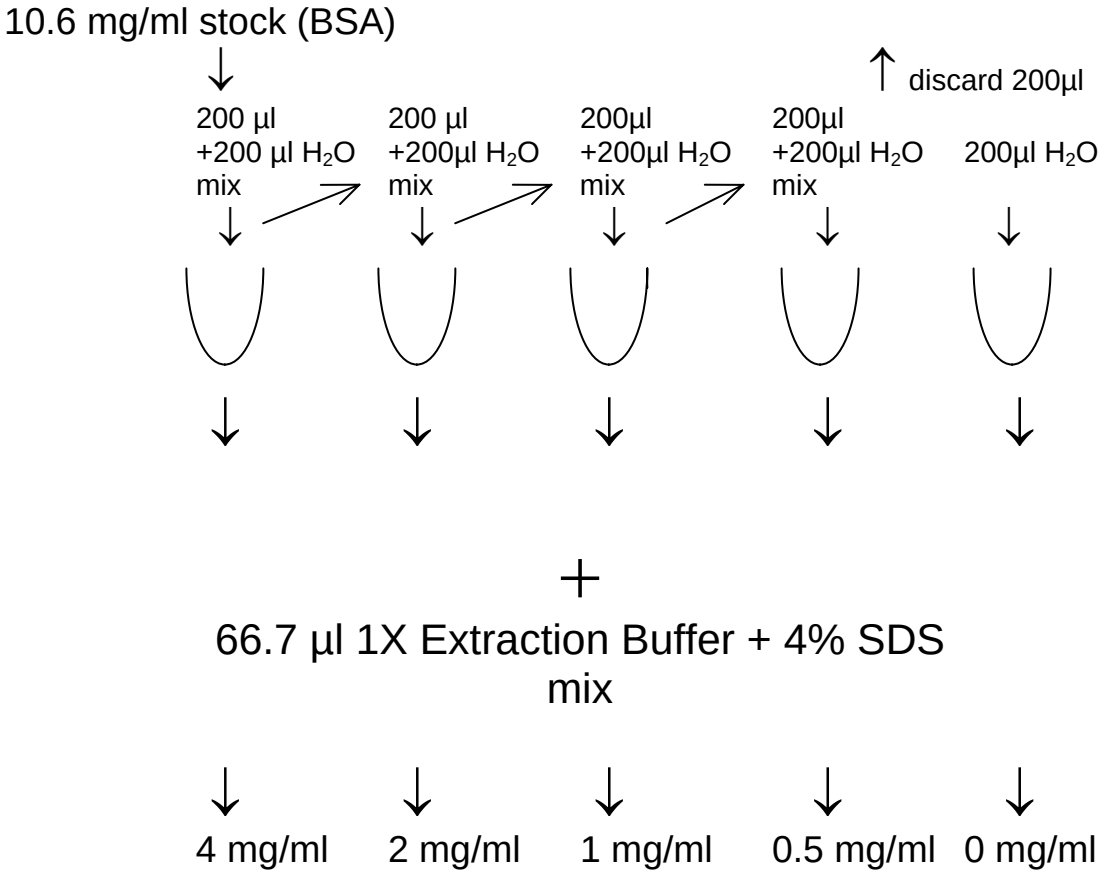
8. Wash blot extensively with water to remove substrate, air dry and store in the dark.

***Use this procedure if you are not using the chemiluminescence detection system for western blot.

STANDARD CURVE
FOR MICRO BCA PROTEIN ASSAY

A standard curve of 4, 2, 1, 0.5, and 0 mg/ml BSA is used for this procedure. Mix standards to contain same concentration of potential interfering substances as the sample. Standards should be made to contain 1% SDS and 2.5 mM EDTA (potential interfering substances). This can be done by serially diluting 10.6 mg/ml stock (200 µl/dilution) and adding 66.7 µl 50 mM Tris, 10 mM EDTA (1X Extraction Buffer, Solution 1) containing 4% SDS to each dilution. Freeze standards. Before using standards, heat thawed standards in 50°C water bath to solubilize SDS.

10.6 mg/ml →	5.3	2.65	1.33	.66	0
x .75 = 8 mg/ml	4	2	1	.5	0 w/ 12.5 mM Tris, 2.5 mM EDTA, 1% SDS (present in sample)
(200 µl/266.7 µl = .75)					



WESTERN BLOTTING PROTOCOL FOR PIERCE SUPERSIGNAL WEST DURA EXTENDED DURATION SUBSTRATE (DESMIN, clone D3)

*To achieve the desired antibody signal, all antibodies and protein amounts loaded onto a gel need to be titrated to ensure that the optimal signal is obtained.

1. Perform electrophoresis and transfer as listed in the protocol, Method Used to Extract Total Muscle Protein For Western Blot Using Tris-EDTA Buffer. If blot was air dried, re-wet blot in Methanol and then water.

2. 10 ml sufficiently covers 1 membrane. Non-specific binding sites are blocked by immersing the membrane in 5% non-fat dried milk (or Starting Block T20 buffer) at room temperature on an orbital shaker. Incubate for 30 minutes for the Starting Block Buffer or 60 minutes for the 5% milk.

3. During the blocking step dilute the primary antibody in TTBS.

4. Incubate the membrane in diluted primary antibody for 1 hour at room temperature. The diluted primary antibody may be saved and reused several times for future blotting.

5. Filtered TTBS (0.05% Tween) is used for the washes. Using a squirt bottle filled with TTBS, briefly rinse the membrane using 2 changes of TTBS. Wash once for 15 minutes and twice for 5 minutes with fresh changes of TTBS at room temperature.

6. During the washing step dilute the secondary antibody in TTBS. Use a secondary antibody that is peroxidase conjugated for use with the chemiluminescence detection system. Incubate the membrane in diluted secondary antibody for 1 hour at room temperature.

7. Using 20 mls/membrane, wash the membrane 1x15 minutes and 4x5 minutes in fresh changes of TTBS.

8. Detection with Pierce Supersignal West Dura Extended Duration Substrate. Incubate membrane for 5 min.

9. Using an Imaging system, capture the chemiluminescence image until the desired image is acquired.

STRIPPING OF ANTIBODIES FROM MEMBRANES

The Restore Western Blot Stripping Buffer (Pierce) provides a method of antibody removal that allows for several reprobings on the same membrane and is ideal for use with the SuperSignal West Chemiluminescent Substrates.

If the blot cannot be stripped immediately after ECL detection, the blot can be stored in TTBS at 4°C until the stripping procedure is to be performed.

1. Place the blot to be stripped in 10 ml Restore Western Blot Stripping Buffer and incubate for 15 min at room temperature. Higher affinity antibodies may require an incubation temperature of 37°C.

2. Remove the stripping buffer and dispose as hazardous waste. Wash in TTBS 1 x 15 min and 2 x 5 min. Your membrane should be ready for reprobing. To test for the removal of the antibodies and the immunodetection reagents, follow the instructions that come with the stripping buffer.

3. Re-blocking the membrane is not critical but may be required in some applications.

4. Begin immunoprobing with the primary antibody.

5. The blot can be stripped and probed several times but may require longer exposure times or a more sensitive chemiluminescence substrate.