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

SOLUTIONS FOR SAMPLE EXTRACTION

1. Tris-EDTA Extraction 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.

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.

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

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.
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.

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

1g/10 ml ddH_2O.
Store in a dark bottle at 4°C.

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

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 4-5 times.

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.

17. Destain

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

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

2.5% Sheep Serum	12.5 ml of crude prep sheep serum
TTBS	500 ml

Filter and store at 4°C.

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.

SAMPLE PREPARATION

1. Homogenize 1 gram of sample in 10 volumes (10 ml) extraction buffer (Solution 1) for 20 sec with the Polytron at setting #4. Do this step in the cold room.

2. Immediately remove a 0.5 ml aliquot for solubilization and transfer to a 1.5 ml microcentrifuge tube. Do this step in the cold room.

3. Add 0.5 ml of 2X Treatment buffer (minus MCE). Mix well by repeatedly pipetting. Nucleic acids may be stringy and viscous, but pipetting will help shear them.

4. Heat samples in a 50°C waterbath for 20 minutes, repeat mixing, and reheat for 5 min.

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)

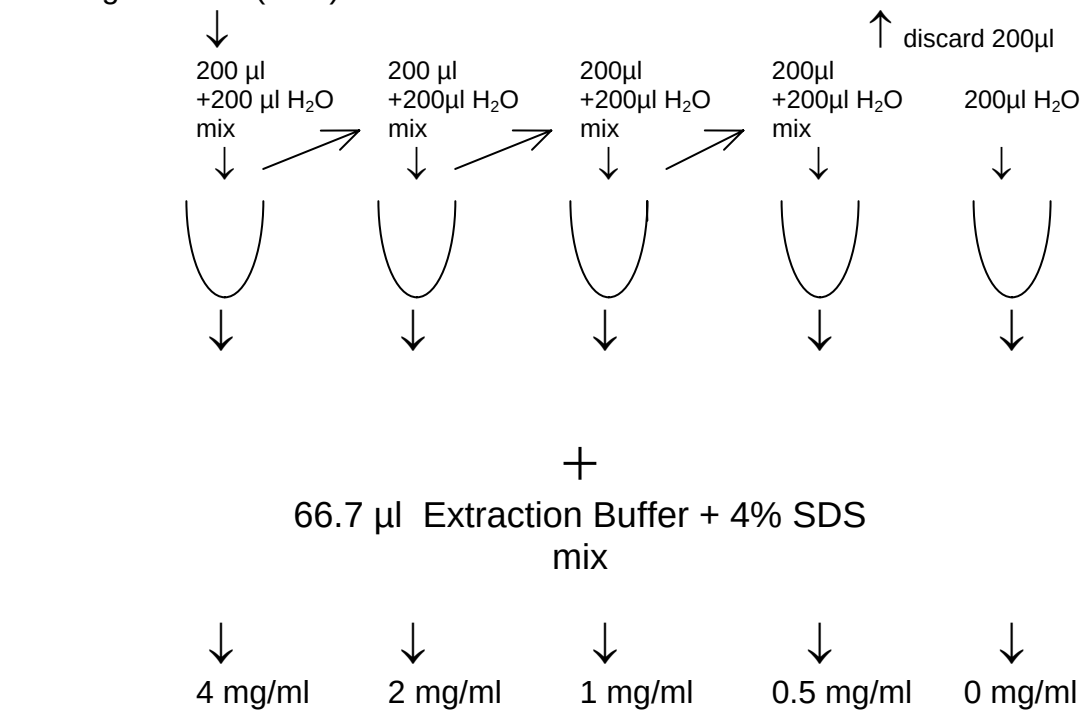
Add 200 µl BCA reagent and incubate at 37°C for 30 min. Read plate on the microplate reader at 562 nm. If more than one plate is used, run a standard curve with each plate. Mix standards to contain the same concentration of potential interfering substances as the samples.

7. Dilute samples to 2 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.

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 (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)						



*Above calculations are based on a BSA stock concentration of 10.6 mg/ml. If the stock concentration is different, refer to the next page for example calculations for diluting BSA standards to correct the concentrations of interfering substances.

The concentration of interfering substances in the extraction buffer must be adjusted for each different BSA stock concentration. For 10.05 mg/ml, instead of adding
66.7 µl of extraction buffer to 200 µl of stock BSA (to get 8 mg/ml from 10.6 mg/ml) you need 51.3 µl (to get 8 mg/ml from 10.05 mg/ml).

$\frac{200}{251.3} = 0.80$ $\quad 0.80 \times 10.05 = 8.0$ mg/ml

$\frac{51.3}{66.7} = 0.769$; inverse = 1.30

1.30 x 50 mM Tris = 65 mM Tris
1.30 x 10 mM EDTA = 13 mM EDTA
1.30 x 4% SDS = 5.2% SDS

Thus, the adjusted concentration of interfering substances in the extraction buffer for 10.05 mg/ml are:

65 mM Tris
13 mM EDTA
5.2% SDS

Perform serial dilutions as described on the previous page.

GEL ELECTROPHORESIS

It is suggested that users check each equipment's operating manuals (electrophoresis units, transfer units, ECL kits, imaging systems) for the suggested operating conditions. Users may need to adapt the following protocol to suit their specific needs.

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. Place comb in between plates. Pour stacker making sure that no air bubbles are trapped under the wells. Allow to polymerize 30 minutes.

6. Carefully remove comb and rinse wells with water. Remove residual water with a Kimwipe. Assemble gel rig and add running buffer as instructed in operating manual.

7. If samples were frozen, heat thawed samples in a 50°C waterbath for 5 minutes. See attachment to determine how much protein to load.

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

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

SAMPLE LOADING

1. 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 and muscle specific.

2. Always leave one or two outside lanes open (to avoid sample smiling). The standard is run in the outside lanes and the middle lane (see example).

Lane	
1	PDB
2	PDB
3	**0h standard**
4	sample
5	sample
6	sample
7	sample
8	**0h standard**
9	sample
10	sample
11	sample
12	sample
13	**0h standard**
14	PDB
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 15 ml conical over the surface. Place the membrane on top of the blotter paper, curved side up. 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 a 15 ml conical 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. Place a plastic cassette on top of this. Hold the sandwich together with two rubber bands. Place sandwich in tank with the gel on the negative electrode side (black = negative; red = positive). Fill tank with cold transfer buffer. Transfer is done at 4°C. Place the tank on a stir plate with gentle stirring.

7. Transfer. 12.5% gels transferred at 200 mA for 1 h @ 4°C (TnT)
10% gels transferred at 200 mA for 1 h @ 4°C (vinculin, desmin) (Towbin transfer buffer with 10% MeOH)
5% gels transferred at 170 mA overnight at 4°C
(Towbin transfer buffer, no MeOH, + .005% SDS)

8. Rinse blot in water 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. While the membrane is drying, lanes will be visible. Visualize molecular weight markers (if used) and cut off that lane from the membrane. Stain MW markers with 0.1% Amido Black for 5-10 min. Destain for 5 min. 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 $\geq$ 60 min to block membrane.

3. Pour off blocking solution and apply Primary Antibody made in blocking buffer. Incubate 1 h.

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 BioRad 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.

WESTERN BLOTTING PROTOCOL FOR PIERCE SUPERSIGNAL WEST DURA EXTENDED DURATION SUBSTRATE

10 ml sufficiently covers 1 membrane.

1. Perform electrophoresis and transfer as listed in the protocol, Method Used to Extract Total Muscle Protein For Western Blot Using Tris-EDTA Buffer.

2. If blot was air dried, re-wet blot in Methanol and then water.

3. Non-specific binding sites are blocked by immersing the membrane in TTBS + 2.5% Sheep Serum for one hour at room temperature on an orbital shaker. Membranes may be left in the blocking solution overnight in a refrigerator.

4. During the blocking step dilute the primary antibody in TTBS. See attachment for the desired antibody dilution.

5. Incubate the membrane in diluted primary antibody for 1 hour at room temperature.

6. 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.

7. During the washing step dilute the secondary antibody in TTBS. Use the Pierce ECL antibody anti-mouse or anti-rabbit. See attachment for the desired antibody dilution.

8. Incubate the membrane in diluted secondary antibody for 1 hour at room temperature.

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

10. Detection.

DETECTION

Using An Imaging System Equipped With A Cooled CCD Camera:

1. Using the Pierce SuperSignal West Dura Extended Duration Substrate Kit, mix an equal volume of West Dura Luminol/Enhancer solution with West Dura Stable Peroxide Solution to give sufficient coverage of the membrane. 3.0 ml will cover one membrane. This substrate can be reused consecutively two to four times without significant loss of signal.

2. Place membrane in a tray, protein side up.

3. Add the detection solution and incubate for 5 minute at room temperature without shaking.

4. Drain off excess detection reagent and transfer membrane to a sheet protector. Gently smooth out air pockets.

5. Place the membrane, protein side up, in the light box. Adjust the camera for image enlargement, focus and light.

6. Expose the membranes for the desired time.

7. Save the images after exposure is complete.

ALTERNATIVE METHOD OF DETECTION:

Exposing the ECL incubated membrane to autoradiograph film is suitable for protein detection. The primary and secondary antibody dilutions would need to be titrated for optimal results with this method. The X-ray film can then be scanned using a reflective densitometer or by using an imaging system.

References:

Wheeler, T. L. and M. Koohmaraie. 1999. The extent of proteolysis is independent of sarcomere length in lamb longissimus and psoas major. J. Anim. Sci. 77:2444-2451.

Wheeler, T. L., S. D. Shackelford and M. Koohmaraie. 2002. Technical note: Sampling methodology for relating sarcomere length, collagen concentration, and the extent of postmortem proteolysis to beef and pork longissimus tenderness. J. Anim. Sci. 80:982-987.

Rhee, M. S., T. L. Wheeler, S. D. Shackelford and M. Koohmaraie. 2004. Variation in palatability and biochemical traits within and among eleven beef muscles. J. Anim. Sci. 82:534-550.

OPTIMAL CONDITIONS FOR ECL ANTIBODY DETECTION
PIERCE SUPER SIGNAL WEST DURA EXTENDED DURATION SUBSTRATE
(For Use With a Cooled CCD Camera Imaging System)

These parameters may have to be modified to work under different conditions.

TROPONIN-T (SIGMA T6277, Clone JLT-12, mouse IgG)

12 1/2% gel (30% acrylamide, 37.5:1)
Transfer 1 hr @ 4°C @ 200 mA
Blocking Agent: TTBS + 2.5% Sheep Serum
Protein Concentration: 10 µg (15 well gel)
Primary Dilution: 1:10,000 (Lot 68H4877)
Secondary Dilution: 1:250,000
ECL Detection:
Incubation: Pierce West Dura Substrate; 5 min incubation
Exposure: 2 min exposure, 1 min post substrate

DESMIN (Developmental Studies Hybridoma Bank, Clone D3)

10% gel (30% acrylamide, 37.5:1)
Transfer 1 hr @ 4°C @ 200 mA
Blocking Agent: TTBS + 2.5% Sheep Serum
Protein Concentration: 10 µg (15 well gel)
Primary Dilution: 1:50 (Batch 2/2/00)
Secondary Dilution: 1:25,000 Pierce antibody, anti-mouse
ECL Detection:
Incubation: Pierce West Dura Substrate; 5 minute incubation.
Exposure: 5 minute exposure, 1 min post substrate

VINCULIN (Accurate Chemical Clone V284)

10% gel (30% acrylamide, 37.5:1)
Transfer 1 hr @ 4°C @ 200 mA
Blocking Agent: TTBS + 2.5% Sheep Serum
Protein Concentration: 10 µg (15 well gel)
Primary Dilution: 1:200 (Lot P00D644)
Secondary Dilution: 1:25,000 Pierce antibody, anti-mouse
ECL Detection:
Incubation: Pierce West Dura Substrate; 5 minute incubation.
Exposure: 1 minute exposure, 1 min post substrate