LSOP Title	Western Blot
LSOP No.	LSOP38
Version	1.1
Location	UQ Node/Centre-wide
Policy/Procedure Link	*UQ- Equipment* *UQ -waste*
Risk Assessments	
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1.0 Scope

To outline the protocols for completing a western blot. This includes; Preparation, Running Gel, Transferring Gel, Primary Antibody, Secondary Antibody and Chemiluminescent Development. This protocol does not include how to isolate the protein for the experiment.

2.0 Definitions

TBS – Tris Buffered Saline (Odyssey Blocking Buffer)

PBS – Phosphate Buffered Solution

Tris-Gly – tris-glycerine

SDS – sodium dodecyl sulfate

3.0 Materials and Equipment

1. Running buffer
2. Western transfer buffer
3. Membranes
4. Filter paper

5. Loading mix
6. Eppendorf tubes
7. Centrifuge
8. Needle
9. Petri dish

10. Methanol
11. Spatula
12. Primary and Secondary Antibodies
13. TBS
14. TBS + 5% milk
15. Petri dishes
16. Tweezers
17. PBS
18. Pipette and Pipette Tips

19. SDS
20. Shaker
21. Gel System
22. Microwave

4.0 Prescribed Actions

Preparation:

1. Make running buffer: 100mL 5x Tris-Gly pH 8.3 buffer + 400mL of H_2O

2. Make Western transfer buffer (0.1% SDS, 20% Methanol) and place in fridge: 200mL 100% Methanol + 10mL 10% SDS + 790mL water
3. Check there are enough membranes and filter papers pre-cut

4. Calculate volume of protein needed for each sample (20ug of protein and the sample has 5ug/uL, then I will need 4uL of that sample for one well)

5. Calculate amount of loading mix needed for each sample. Loading mix (2x laemmli buffer + 8% β-mercaptoethanol)
6. Calculate volume of protein + loading mix to add to each well (max. 25uL + need same volume of loading mix as protein volume)
7. Label Eppendorf tubes for each sample + 1 tube for loading mix
8. Heat 500mL of water in microwave until boiling
9. Sample we are using: control, CK treatment, SL treatment, and decapitation treatment

Protocol:

10. Make loading mix as calculated prior: _ uL 2x Laemmli sample buffer + _ uL β-mercaptoethanol (i.e. 8%)

11. Combine same amount of sample and loading mix to make protein solutions as calculated prior.
12. Add protein solutions to boiling water for 5 minutes.
13. Remove green strip at the bottom of the gels before placing in the gel holder.
14. Add 2 gels to the gel holder (突起向外) & clamp in place.

15. Invert running buffer, fill inner chamber and bottom of gel holder up to the line.
16. Remove combs carefully from gels

17. Use a needle to clean out each well (squirt buffer into each well) & use needle to straighten sides of wells.
18. Remove protein solutions from boiling water, flick tubes and spin down quickly.
19. Add 6 ul of ladder to wells

20. Add the appropriate amounts of protein samples into each well as calculated prior.
21. Run at constant 25mA for ~1 hour
22. Check gel after 30 minutes to see how it is going.

<u>Prepare gels for Transfer</u>

23. Fill 1 Petri dish with methanol, 2 with Western transfer buffer, and 1 with water.
24. Use a spatula to pry apart the two plastic sides of the gel to release the gel inside. Remove the well sides to make the gel top smooth.
25. Label each membrane with a number using a PENCIL in corner of membrane. The side that is labelled is the side that the proteins will be transferred to.
26. Open gel holder cassette (sandwich).

27. Pre-wet pad (white fluffy thing) in transfer buffer and lay pad on the black side of the gel holder cassette.

28. Pre-wet filter paper in transfer buffer and lay on top of pad in gel holder cassette.

29. Place gel in Petri dish containing Western transfer buffer (0.1% SDS, 20% Methanol) for less than 5 minutes.
30. Wash gel in water and place on top of filter paper.

31. Soak membrane in methanol for a few minutes
32. Wash membrane in water and place on top of gel (position the # on the membrane above the notch on the gel, so that the # is facing down).

33. Pre-wet another square filter paper in transfer buffer and lay on top of membrane.

34. Pre-wet pad in transfer buffer and lay on top of membrane.
35. Shut white latch to close the gel holder cassette
36. Place the gel holder cassette in the gel holder with the black side of the cassette towards the black side of the gel holder.

37. Place a magnetic stirrer in the gel holder

38. Add COLD transfer buffer to the gel holder and turn the magnetic stirrer on.
39. Can also put ice next to the gel holder to keep the buffer cold.
40. Run at 100V for 1 hour
41. Watch that the gel doesn't get too hot

Primary antibody

42. Activate membrane by washing in methanol for 1 minute
43. Rinse membrane with ultra-pure water
44. Wet in 1X TBS and 5% milk + 1X TBS for 2 minutes
45. Block membrane by incubating in Odyssey Blocking Buffer (TBS) and 5% milk+1X TBS for 1 hour with gentle shaking
46. Pour off solution.
47. If necessary, can cut membranes in two if using different antibodies or blocking buffers
48. Add 1:5,000 dilution of primary antibody: 2uL of primary antibody + 10mL Odyssey Blocking Buffer (TBS) / 5% milk + 1X TBS
49. Rotate membrane so it is facing up and pour antibody solution onto each membrane.
50. Flip membrane face down and put on shaker on level 3 in 4°C room overnight. (or for 1-4 hours at room temperature)

Secondary antibody (red one)

51. Pour off liquid, turn membrane face up.
52. Rinse membrane with 5% milk + 1X TBST or 1X TBS-T
53. Cover membrane in ~20mL 5% milk + 1X TBST or 1X TBS-T and shake vigorously for 5 minutes (to rinse off antibodies that didn't bind).

54. Pour off liquid.
55. Repeat steps 51-52 three more times, the fourth time turn the membrane face down before putting on shaker.
56. Add 1:20,000 dilution of secondary antibody: 0.5ul of secondary antibody + 10mL Odyssey Blocking Buffer (TBS) or 5% milk + 1X TBS and 0.01% SDS
57. Incubate in dark for 1 hour at room temperature with gentle shaking
58. In dark: Pour off liquid, turn membrane face up.
59. In dark: Rinse membrane with 1X PBS-T or 1X TBS-T
60. In dark: Cover membrane in ~20mL 1x PBS-T or 1X TBS-T and shake vigorously for 5 minutes (to rinse off antibodies that didn't bind).
61. Pour off liquid.
62. Repeat steps 58-59 three more times, the fourth time turn the membrane face down before putting on shaker.

Chemiluminescent Development

63. Rinse with 1X TBS or 1X PBS with NO tween-20 (to remove Tween-20)
64. Can store membrane in 1X TBS or 1X PBS in dark at 4oC for up to 48 hours OR can air-dry membrane and store in dark at room temperature for longer
65. Image membrane using 169nm channel on Odyssey CL