Systems *Bioengineering* Laboratory

Western Blotting

Prepared by: Evren Azeloglu, Anthony Mendoza
Version: 2.0
Date: 27-January-2022

Materials

Sample Preparation and Lysis

- 1.5mL Eppendorf tubes
- Cell scrapers for each sample (Fisher, Cat: 08-773-2)
- Phosphate Buffered Saline 10X Molecular Biology Grade (Corning, Cat. 46-013-CM)
- RIPA Buffer (Thermo Fisher Scientific, Cat. 89901)
- Halt™ Protease/Phosphatase Inhibitor Single-Use Cocktail (100X) (Thermo Fisher Scientific, Cat. 78442)
- Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific, Cat. 23200)
- BrandTech™ BRAND™ Plastic Cuvettes (Fisher Scientific, Cat. 14-377-013)
- 4X Bolt™ LDS Sample Buffer (Thermo Fisher Scientific, Cat. B0007)
- 10X Bolt™ Sample Reducing Agent (Thermo Fisher Scientific, Cat. B0009)
- ddH_2O

Gel electrophoresis and semi-dry protein transfer

- PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Fisher Scientific, Cat. 26619)
- Bolt™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 15-well (Thermo Fisher Scientific, Cat. NW04125BOX)
- Mini Gel Tank (Thermo Fisher Scientific, Cat. 25977)
- Bolt™ Antioxidant (Thermo Fisher Scientific, Cat. BT0005)
- 20X Bolt™ MES SDS Running Buffer (Thermo Scientific, Cat. B0002)
- Trans-Blot® SD Semi-Dry Transfer Cell (Bio-rad, Cat. 1703940)
- Western Blotting Filter Paper, Extra Thick, 8.5 cm x 9 cm (Thermo Fisher Scientific, Cat. 88610)
- Bolt™ Transfer Buffer (20X) (Thermo Fisher Scientific, Cat. BT00061)
- Supported Nitrocellulose Membrane, 0.45 μm (Bio-rad, Cat. 1620094)
- Methanol
- ddH_2O

Protein probing and detection

- TRIS-buffered saline (TBS, 10X) pH 7.4 (Thermo Fisher Scientific, Cat. J60764)
- Tween™ 20, Fisher BioReagents™™™ (Fisher Scientific, Cat. BP337-100)
- Andwin Scientific NON-FAT DRY MILK, 25.6 ounces in a pack of 5 (Fisher Scientific, Cat. 190915ASC)
- IRDye® 680LT Donkey anti-Mouse IgG Secondary Antibody (Li-cor, Cat. 926-68022)
- IRDye® 800CW Donkey anti-Rabbit IgG Secondary Antibody (Li-cor, Cat. 926-32213)
- IRDye® 800CW Donkey anti-Goat IgG Secondary Antibody (Li-cor, Cat. 926-32214)
- Black Western Blot Incubation Boxes (Li-cor, Cat. 929-97205)
- LI-COR Odyssey® XF Imaging System

Methods

Sample Lysis and Normalization of Protein Concentration

1. Load wide bucket with ice
2. Label sample tubes and place on ice
3. Label 50 mL tubes for cell scrape collection and place them on ice
4. Prepare RIPA 2X protease/phosphatase lysis buffer (Recipe for 1 mL RIPA 2X: 1 mL of RIPA + 20 μL of Halt™ Protease/Phosphatase Inhibitor Single-Use Cocktail (100X))
5. Prepare PBS 1X by diluting PBS 10X in ddH_2O.
6. Allow for PBS 1X to reach 4°C on ice.
7. Place sample plates or dishes on ice
8. Evenly wash the samples with cold PBS 1X twice
9. Evenly coat the samples with PBS 1X and scrape adherent cells into the pre-cooled 50 mL tubes.
10. Spin scraped cells in cold PBS 1X at 1,250 RPM for 3 minutes.
11. Aspirate the PBS 1X from the cell pellet
12. Resuspend the cell pellet in 40 μL of RIPA 2X (determine the volume of lysis buffer for your sample type empirically)
13. Vortex lysates every ten minutes for 5 seconds while allowing them to incubate over ice for 30 minutes
14. Separate soluble and insoluble proteins by spinning down for 30min @ 13000 RPM at 4°C
15. While the samples are spinning, label fresh 1.5 mL tubes and allow to cool over ice
16. After completed centrifugation, collect the supernatant and discard the insoluble residue
17. Place the samples on ice
18. Pipette 1 mL of Coomassie (Bradford) protein assay reagent into absorbance cuvettes for standards (working range of 1-25 μg/mL)
19. Add 1μL of each sample lysate to 1 mL of Coomassie protein assay reagent
20. Allow the samples to incubate at room temperature for 10 minutes to increase the consistency of results
21. Turn on the spectrophotometer and select “Absorbance” from “Basic Modes”. Accept the default settings (595nm absorbance wavelength)
22. Vortex each standard cuvette until well mixed (homogeneous in color), and place into the carousel aligning the clear (arrowed) side of the cuvettes with the light emitter (notched side) of the carousel slots. The blank sample goes into the blue (initial) slot
23. Measure the absorbance of each standard by pressing the green button while recording the values into the lab book
24. Place the standards aside (keep them in case a new run is necessary)
25. Use zero-intercept linear regression to compute protein concentration of each sample and normalize all by diluting to a lowest common concentration with the RIPA solution
26. Samples can be frozen at -80ºC at this point

Sample preparation for gel electrophoresis

Total volume and protein content for loading sample should be determined empirically. We will use a total loading sample volume of 40

27. Label a fresh set of Eppendorf 1.5 mL tubes
28. Standardize the total volume to be loaded for each sample
29. Determine the total volume of protein to be loaded per sample while keeping the total protein content loaded per sample constant
30. Add 10 μL of 4X Bolt™ LDS Sample Buffer to each sample tube for a 1X Bolt™ LDS sample buffer concentration per sample
31. Add dH_2O to reach 40 uL of each sample to be loaded
32. Add 4 uL of 10X Bolt™ Sample Reducing Agent to each sample tube for a 1X Bolt™ Sample Reducing Agent concentration per sample
33. Boil the samples for 2 minutes at 90 °C or for 10 minutes at 70 °C

Gel electrophoresis and semi-dry protein transfer

34. Prepare the Bolt™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 15-well by removing the bottom seal and locking it into the Mini Gel Tank chamber
35. Prepare 1X Bolt™ MES SDS Running Buffer by diluting the 20X Bolt™ MES SDS Running Buffer in dH_2O.
36. Fill the tank with 1X Bolt™ MES SDS Running Buffer.
37. Load 7 μL of PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa
38. Load the samples
39. Add 500 μL of Bolt™ Antioxidant to the tank
40. Run the gel at 200V for 23 minutes

Approximately 5 – 10 minutes before completion of the electrophoretic run

41. Prepare the Trans-Blot® SD Semi-Dry Transfer Cell by incubating a western blotting filter paper in 1X Bolt™ Transfer Buffer (Recipe for 1L: 50 mL of Bolt™ Transfer Buffer (20X) + 100 mL of methanol (10 – 20% methanol, determine empirically) + 850 mL of ddH_2O) on the anode plate)
42. Trace a cut out from the supported nitrocellulose membrane using a dry western blotting filter paper
43. Using membrane forceps, place the supported nitrocellulose membrane on the wet filter paper so that the interior portion of the supported nitrocellulose membrane contacts the gel
44. Once electrophoresis of the sample is complete, remove the gel from the Mini Gel Tank
45. While holding the gel in hand, separate the gel cassettes using a gel knife, careful to not tare the gel
46. The gel should adhere to one side of the cassette, discard the other side
47. Using the gel knife, remove the edges of the gel
48. Place your index finger between the gel and cassette
49. With the index finger under the gel, flip the gel onto your middle finger, ring finger, and little finger
50. Place the gel on the wet nitrocellulose membrane so that the membrane will read left to right
51. Wet the membrane with transfer buffer
52. Using a soft roller, remove visible air bubbles between the gel and membrane (roll in one direction)
53. Wet another western blotting filter paper and place it on top of the membrane
54. Roll the top of the gel sandwich
55. Latch the cathode plate on to the base
56. Place the safety lid on
57. Perform the semi-dry transfer at 25V for 30 minutes
58. Once the semi-dry transfer is complete, place the membrane in a LI-COR western blot incubation box in blocking buffer (5% dry milk w/v in TBS 1X)

Protein probing and detection

59. Block the membrane in 5% dry milk w/v TBS 1X with gentle agitation for an hour at RT
60. Wash thrice for 5 minutes each with TBST (TBS 1X with 0.1% Tween™ 20)
61. Dilute primary antibody as recommended by its product data sheet in 5% BSA in TBST with 0.01% sodium azide
62. Incubate the membrane with primary antibody solution overnight at 4 °C with gentle agitation
63. Recollect the primary antibody and store at 4 °C for reuse up to 4x
64. Wash thrice for 5 minutes with TBST
65. Dilute LI-COR secondary antibodies as recommended by the product data sheet in 5% dry milk in TBST
66. Incubate the membrane with the secondary antibody solution prepared for 1 hour at RT with gentle agitation
67. Wash thrice for 5 minutes with TBST
68. Detect your protein using the LI-COR Odyssey® XF Imaging System