Western Blotting



**Reagents Needed:**

**10X Transfer Buffer:**

480 mM Tris

390 mM glycine

Bring to 1L with dH2O

**1X Transfer Buffer with 20% Methanol:**

100 mL 10X Transfer Buffer

200 mL 100% Methanol

Bring to 1L with dH2O

**100% Methanol**

**1X Tris Buffered Saline, 0.05% Tween-20 (TBST):**

20 mM Tris

150 mM NaCl

500 μL Tween-20

pH 7.5

Bring to 1L with dH2O

**10,000X Fold 1°Ab (mouse-α-polyhistidine) Solution:**

5 μL 1° Ab (mouse-α-polyhistidine) [stored in -20°C in Hurlbert Lab]

2.5 g Nonfat Dry Milk

49.995 mL TBST

**2,500X Fold 2°Ab (goat-α-mouse IgG Alkaline Phosphatase):**

20 μL 2°Ab (goat-α-mouse IgG Alkaline Phosphatase) [stored in 4°C in Hurlbert Lab]

5μL 5,000x StrepTactin-AP [stored in -20°C in Hurlbert Lab]

49,975 mL TBST (NO MILK!!)

**BCIP/NBT Solution** [Stored in 4°C in Hurlbert Lab]

**Procedure:**

1. Follow SDS-PAGE gel protocol online using target protein with hexahistidine tag and appropriate controls. Use the molecular weight ladder located in the -20°C (Hurlbert Lab) in the Western Blotting bag
2. After the gel is removed from the glass panes, soak the gel in 1X Transfer Buffer with 20% methanol
3. Cut the polyvinyldifluoride (PVDF) membrane [Millipore, lot# K6JN0371B] into squares the size of the SDS-PAGE gel and soak the PVDF membrane in 100% methanol to remove all bubbles
4. Soak the PVDF in 1x Transfer Buffer with 20% methanol
5. Soak saturation pad [Gelman Instrument Company, Cat# 51334] in 1x Transfer Buffer with 20% methanol and place on the Trans-blot SD (Biorad)
6. Soak filter paper (150 mm circles, Cat# 1450 150) in 1x Transfer Buffer with 20% methanol and place on the saturation pad
7. Remove the soaking PVDF membrane and place it on top of the filter paper
8. Remove the soaking SDS-PAGE gel and place it on top of the PVDF membrane
9. Place another 150 mm filter paper and saturation pad soaked in 1x Transfer Buffer with 20% methanol **(MAKE SURE THERE ARE NO BUBBLES! BREAK A 10 ML PIPETTE AND USE IT TO ROLL OUT BUBBLES)**
10. You should now have what I like to call the gel sandwich. From bottom to top: saturation pad, filter paper, PVDF, SDS-PAGE, filter paper, saturation pad.
11. Attach the Trans-blot SD to the voltmeter and set it to 15 volts for 15 minutes. **(After the transfer, check the PVDF membrane. If you cannot see the colored ladder on the PVDF membrane then run the voltage longer)**
12. Place the blotted PVDF in the 1°Ab (mouse-α-polyhistidine) solution
13. Incubate the blotted PVDF with the 1°Ab while shaking in the cold room over night
14. Wash the blotted membrane in TBST (no milk) four times at 15 minute intervals while shaking in the cold room
15. Add the secondary antibody to the PVDF membrane and let it shake in the cold room for at least 1 hour
16. Wash the blotted PVDF membrane with TBST (no milk) while shaking in the cold room over night or longer
17. Wash the blotted PVDF with fresh TBST (no milk) for 15 minutes while shaking in the cold room
18. Bring BCIP/NBT solution to room temperature
19. Wash the blotted PVDF membrane with deionized water to remove all TBST
20. Add a thin layer of BCIP/NBT solution to the blotted PVDF membrane and keep it covered with paper towels (light sensitive). Gently shake the solution and periodically check to see color change. Bands on the gel containing a hexahistidine tag will become dark purple. The ladder will become dark purple due to the StrepTactin AP conjugate in the 2° Ab solution. If the membrane background begins to become purple, immediately dump the BCIP/NBT solution and add water to stop the reaction.
21. Store the membrane in paper towels and allow to dry