Winnie

Western blotting

✧ Materials

Protein marker @-30°C common fridge (upper door, the third box on the right side, red box): name->マーカー

TBST (0.05% Tween 20): 1790 mL ddH2O+200 mL 10X TBS+10 mL 10% Tween 20

✧ Procedures

Electrophoresis (SDS-PAGE)

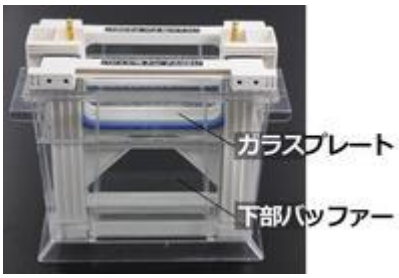
1. SDS-PAGE preparation: glassx2, siliconx1, clipsx2, combx1
2. Put all items on the brown tissue. Two glasses and comb are cleaned by 70% EtOH.

Comb: blue or white

3. Assemble silicon onto the thick glass (the bottom of ATTO is upward) and assemble the thin glass, clips one by one.
4. Put the thin glass facing to myself. Mark 3 cm on the thin glass from top to down.
5. Stand the package on the table.
6. Put appropriate amount of separating gel solution into the gap between the glass plates until reaching the mark. Add isopropanol for protecting the gel from air bubbles. Wait for at least 30 min.
7. Pour out isopropanol and wash the gel by DI water. Remove residual water by filter paper.
8. Put appropriate amount of stacking gel solution into the gap between the glass plates. Insert the well-forming comb to make wells without trapping air under the teeth. Wait for 20-30 min to let it gelate.
9. Remove the silicon. Gently remove the comb from gel.
10. Put the glass plate into the cast and then put the cast into the tank. The thin glass is facing to outside.

  blue cast

11. Add running buffer into the middle of cast.



12. Clean the well by the syringe. Remove all residual gel.
13. Gently put the samples and marker into the wells by loading tips (@tip cabinet, blue box) or ordinary p200 tips.
14. Cover the lid and set the program on power supply (BioRad).
    *Program: constant voltage, 80V for 15 min, 150V-200V for 60 min

Transferring (semi-dry)

15. Transfer buffer @4°C common fridge:
    高分子->10% methanol, 小分子->20% methanol
16. Cut the PVDF membrane and filter papers (above the PCR machines).
    10*8 for filter papers, 9*7 for PVDF membrane.
    *1 PVDF membrane and 6 filter papers for each gel
17. Prepare 2 cases for transfer buffer (beside water tank) and 1 case for methanol (RT drug cabinet, in front of professor's office).
18. Prepare the transferring machine. Put PVDF membrane into methanol case. Put six pieces of filter papers into the case containing transfer buffer.
19. After hydration, put PVDF membrane into the case containing transfer buffer.
20. Use the shovel to slice the gel gently. Put the gel into the case containing transfer buffer and over the PVDF membrane. Let the gel balance in transfer buffer for a while.
21. Put three wet filter papers on the semi-wet machine. Sliding the plastic stick to remove gas/bubbles.
22. Put the PVDF membrane and gel together onto the filter papers by tweezers.
23. Put three wet filter papers onto the gel. Sliding the plastic stick to remove gas/bubbles again. Cover the lid.
    *If you use old transfer machine, please put a thick book on the lid.
24. Set program: each membrane, constant A, 0.25A, maximum V=25V, for 50 min

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25. Wash the membrane with TBST twice. 5 min/each time
26. Blocking buffer: 5% skim milk (TBST), 2 or 5% BSA (TBST) or PVDF blocking buffer for can get signal (@4^{o}C common fridge)
27. Blocking for 30 min
28. Rinse the membrane with TBST once.
29. Prepare the primary antibody: 1uL Ab in 1 mL of appropriate buffer. (1:1000)
30. Take a small plastic bag (beside the sealing machine) and cut the bag by scissors. Cut the membrane.
31. Put the membrane into the bags. Seal the bag (left and right sides).
32. Add 1mL diluted primary antibody solution into the bag. Remove gas/bubbles.
33. Seal the bag (upper side, two lines).
34. Put the bag between glass plates (beside the shaker @RT) and then put them with heavy thing on the shaker.
35. Incubate in the cold room overnight.
36. Make a reservation of LAS-4000 in the common equipment center.
37. Wash the membrane with TBST for three times. 5 min/each time.
38. Prepare the secondary antibody: (1:10000)
    First, dilute 100X with can get signal buffer solution 2 into 1.5 ml tube.
    Subsequently, take 10uL of 100X diluted antibody into 1mL of appropriate buffer for membrane.
39. Take small plastic bag (beside the sealing machine) and cut the bag by scissors.
40. Put the membrane into the bags. Seal the bag (left and right sides).
41. Add 1mL diluted secondary antibody solution into the bag. Remove gas/bubbles.
42. Seal the bag (upper side, two lines).
43. Put the bag between glass plates (beside the shaker @RT) and then cover them with heavy thing on the shaker. Incubate at RT for one hour.
44. Rinse the membrane with TBST for three times and then wash the membrane with TBST for three times. 5 min/each time.
45. Prepare WB box for detection:
    Substrate: (choose one depending on the signal of primary antibody)
    I. Millipore (Forte) -> stronger ECL
    II. Promega (ECL Western Blotting Substrate) -> weak ECL
    Put solution A of ECL into the brown tube.
    3 tubes (solution A, solution B and mix tube)
    Tweezers.
    1 case.

46. Put the membrane on the plastic rap (each membrane/rap) or bring it in the box with TBST.
47. Press the start button of LAS-4000 machine and computer.
48. Open Software: not ready → ready (-25°C)
49. Put the membrane on the case.
50. Take 350 uL solution A + 350 uL solution B into the mix tube. Mix well and then add the substrate on the membrane.
51. Take out the membrane and put it into the plastic bag. Put the membrane into the machine. Change the tray to 1 position.
52. On LAS-4000 software:
    Focusing -> Tray position -> V Chemilu~ V Tray 1 -> Focusing
    -> For first time: Exposure type: Precisson
    time: Auto
    Sensitivity: Standard
    Image acquire: V on
    -> Also can use: Exposure type: Increment
    Interval time: 10 sec to 1 min
    Repeat time: 16 times
    Save file: folder-> Cell therapy-> own's folder (file:*set)
    Change the mode from Chemilu~ to Digital 1/30 sec for picturing marker and save the photo as Vimage~~ (*tif)
53. On Multiguage software:
    Open files (appropriate image and marker image)
    Process-> artihetric-> ok -> change contrast or scale type -> clip area -> clip image -> export as tiff file to own's folder