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|  | SOP-BCR-7.4 | Western Blotting | Author: S. Clouthier  Alex Fox Sean McDermott Ali Quraishi Approved: M. Wicha  | Rev: 0 | Issued: 9/20/13 Revised: 4/27/15 |
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1.0 Purpose

SOP 7.4 outlines laboratory procedure and protocol for Western Blotting.

2.0 Scope

SOP 7.4 is intended to cover all resources, personnel and equipment in the BCR laboratory.

3.0 Procedure

3.1 Running 4-12% Bis-Tris gels in MES SDS Buffer:

1. Dilute 20x MES SDS running buffer to 1x.
 - Mix 950 mL ddH₂O with 50 mL 20x running buffer-mix well (stored at RT)
 - This will provide enough buffer for two gels to be run
2. Fill Bolt Mini Tank with running buffer, until the buffer level reaches the electrode (approx. 400 mL).
3. Remove cassette from packaging (Bis-Tris stored at RT, extra in 4°C walk-in; Tris-Gly stored at 4°C). Pull off white tape from bottom of cassette and carefully remove plastic comb from the top. Rinse the front and back of cassette with running buffer and rinse the wells 3x with running buffer.
4. Place cassette into brackets within gel tank (with wells towards the front), and move the taller part of the bracket forward, locking the cassette in place. (Make sure the electrodes sticking up from brackets are both to the center of the tank).
5. Repeat for number of gels that will be run.
6. Prepare samples for gel: (volumes below are for two 10-well gels, 30 uL volume per well).
 - 6 uL of 10x reducing agent (stored at 4°C)
 - 15 uL of 4x sample buffer (stored at 4°C)
 - 40 ug of protein (should be calculated by Bradford Assay)
 - dH₂O to bring total volume up to 60 uL
7. Load 10 uL of ladder (stored at 4°C) into well #1 in each gel.
8. Load 30 uL of protein sample into remaining wells in each gel.
 - 15-well gels will hold 20uL of sample/well
 - 17-well gels will hold 15uL of sample/well
9. Place top onto Bolt tanks, pressing down over each electrode to be sure the lid is firmly on so current can run through electrodes.
10. Plug red and black cords into power supply (adapters must be in first) and turn power supply on. Set voltage to a constant 165V, and timer to 35 minutes.
11. Press run on power supply. You will know everything is connected properly if bubbles can be seen moving up from electrode strip across the front of the tank.
12. After 35 minutes turn the power supply off and remove lid from tanks.
13. Brackets can be opened, and the gel cassette can be removed and placed into dH₂O in glass baking pan to be rinsed.

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14. To open cassette: insert metal gel knife into indents on the left and right sides of cassette and angle knife up and down to break the two sides of the cassette apart.
15. Place the beveled end of the gel knife into the line across the bottom of the cassette (where white tape was removed from earlier) and push the gel away from the plastic cassette. Gently remove the gel from the cassette and rinse in dH₂O.
 - Both halves of the cassette can be discarded once gel is removed
16. Using the beveled edge of the gel knife cut away the very bottom of the gel (the raised end) and top (where the wells were) and discard.
17. To clean the Bolt Mini Gel tank dump the running buffer in the sink and rinse the tanks out with dH₂O 3x and allow to air dry.

3.2 Transfer Process using iBlot 2:

1. Open either a PVDF or NC transfer stack (stored at RT) by removing the foil covering.
2. Remove the top copper layer with cathode gel, and place off to the side. Discard the white plastic separator beneath this.
3. The thin white paper that is visible now is the membrane. Remove gel from dH₂O and place onto the membrane, with the top of the gel towards the center of the transfer stack. (The ladder should be on the left side of the membrane if you are looking at the stack from the shorter end).
4. Two Bis-Tris or Tris-gly gels should be able to fit onto one PVDF or NCR transfer stack, with the top of each gel towards the middle of the stack.
5. Wet a piece of filter paper (in separate package with PVDF or NC stack box) with dH₂O and place over the gels. Use the mini roller to roll out any bubbles.
6. Place the copper and cathode gel back onto the filter paper, copper side facing up. Again use mini roller to roll out any bubbles.
7. Keeping the entire stack within the plastic tray it came in, place the stack into the iBlot 2 machine, with the small tab on the copper layer pointing to the left so it will line up with the electrical contact on the left side within the machine.
8. Place absorbent pad (also packaged separately within box) on top of copper layer, with the metal strip facing down so that it will touch the electrical contact to the front of the inside of the machine.
9. Close the lid and be sure it latches.
10. Check that the iBlot 2 is plugged in (adapter may be needed) and turn it on
11. Select the protocol that you want from the display screen and press start.
 - P0 is the standard protocol, with a duration of 7 minutes and three different voltages that the machine will adjust automatically
12. Once the transfer is complete, open the lid and discard the absorbent pad on top and remove the entire stack from the machine.
 - At this point, the iBlot 2 can be used immediately for another transfer; it does not need to be cleaned first nor does it need time to cool down

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13. Remove the top copper layer and peel the attached gel layer off; the gel can be discarded but the copper should be set aside for recycling.
14. The filter paper and two gels can be removed and discarded.
15. The membrane should be carefully removed and hung to dry or cut in half so one membrane can be run in the iBind.
16. The bottom copper layer should be peeled away from the cathode gel on it and set aside to recycle, all other layers can be discarded.
17. If membrane will not be stained with antibodies immediately it should be allowed to dry thoroughly, labeled, and then stored in a plastic bag or saran wrap at 4°C.
18. If membrane will be stained immediately, it should not be allowed to dry fully but should be placed in 5% BSA with sodium azide for blocking.

3.3 Using the iBind:

1. Blocking step- only before the **first** time blotting, not after membrane has been stripped.
 - Place membrane in small container and cover with about 20 mL of 5% BSA in 1xTBST with 0.04% sodium azide(store at 4°C), place onto rocker at RT for 30 minutes. Then rinse with dH2O and proceed to step 4
2. If membrane is dry and needs to be re-wet before staining:
 - For PVDF membrane- place membrane into 100% methanol for about 2 minutes, until the entire membrane is one consistent color, then rinse membrane well with dH2O
 - For NC membrane- membrane can be rewet with just dH2O. DO NOT place NC membrane into methanol to rewet
3. If membrane was stored in 1x PBS at 4°C:
 - For either PVDF or NC membrane- membrane should be rinsed well with dH2O
4. Make 1x iBind solution:
 - 6 mL 5x iBind solution (stored at 4°C)
 - 300 uL 100x iBind additive (stored at 4°C)
 - 23.7 mL dH2O

*Total of 30 mL 1x iBind solution
5. Cover wet membrane with 5 mL 1x iBind solution.
6. Place iBind card (stored at RT) into iBind device, and center card.
7. Using 5 mL 1x iBind solution wet iBind card, starting at the top of the card and moving down in a back and forth, zig-zag pattern.
8. Add another 1 mL 1x iBind solution to the center of the card where the membrane will be placed.
9. Place membrane onto iBind card, protein side facing down.
 - Try and center the membrane from either side and between the two lines on the side of the iBind device. DO NOT let membrane touch thick filter paper stack at the bottom of iBind card

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10. Use mini roller to roll out any air bubbles.
11. Close lid of iBind and make sure it is full latched, or iBind solution will leak.
12. Open small top lid for primary, secondary and washes.
13. Load 2 mL primary antibody mix into well #1.
 - Determine recommended concentration for primary
 - Calculate amount needed for 2 mL, and add to 2 mL 1x iBind solution
 - *Most Cell Signaling antibodies should work at a dilution of 1:1000
 - This dilution will work for phospho- and total AKT, MEK ½, MAPK (ERK), GSK3b, 4EBP1, s6 Ribosomal Protein, PTPN22
 - If a phosphorylated antibody does not work at 1:1000 dilution in 1x iBind solution, instead try diluting in 2mL 5% BSA in TBST and running this in the iBind device
 - *STEAP-1 (Santa Cruz) works at 1:200
 - Most primary Ab are in -20°C walk in
14. Load 2 mL 1x iBind solution into well #2 as a wash between primary and secondary.
15. Load 2 mL secondary antibody mix into well #3.
 - Determine recommended concentration for secondary, and multiply by 5
 - Calculate amount needed for 2 mL, and add to 2 mL of 1x iBind solution
 - *NA931 (α-mouse) and NA934 (α-rabbit) work well in 1:2000
 - Both stored in 4°C walk in
16. Load 6 mL 1x iBind solution into well #4 as a final wash.
17. Close top lid and let iBind device sit for 2.5 hours.
18. Lift small lid on top of iBind device, if iBind card within looks dry in all 4 wells then the run is complete. If there is any liquid in well #4 let iBind sit until this well is dry as well.
19. Once run is finished, open iBind device and remove membrane. iBind card can be discarded at this point.
20. Rinse membrane well in dH2O.
21. Blot off excess dH2O and lay membrane onto transparency.
22. For imaging using SuperSignal West **Pico** Chemiluminescent Substrate:
 - Mix 1.3 mL of each solution together in 15 mL tube (can be mixed up to an hour ahead of time) and fully cover membrane with 2.5 mL of this 1:1 mix
 - Let solution sit for 60 seconds, then blot off excess pico mix and replace membrane on transparency. Cover with second transparency, from an angle, so that no bubbles form between the two transparencies
 - Membrane can be taken to image
23. For imaging using SuperSignal West **Femto** Chemiluminescent Substrate: (for smaller amounts of protein)

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*Follow steps 22 first, activating with Pico substrate, then rinse membrane in TBST before activating with femto

- Mix 200 uL of each solution together in 500 uL tube (do not do too far ahead of time, femto should be used within 15 minutes of mixing)
 - Determine approximate location of protein on membrane based on molecular weight
 - Drip femto mix onto membrane where protein is expected- NOT on entire membrane
 - Let sit for 10-15 seconds then tip transparency and remove excess mix with paper towel
 - Cover membrane with second transparency
 - Membrane should be imaged immediately
24. If using imager on 3rd floor B20 (FluorcheM), chemiluminescence can be used to see bands of protein (photo will default white bands on black background) and chemi with markers will allow non-fluorescent ladder to be seen with bands.
25. After imaging membrane can be stripped or placed into 1x PBS in Ziploc bag and stored in 4°C walk-in.
- If stripping membrane- place on rocker at RT in 20 mL of Restore stripping buffer for 15 minutes. Rinse well with dH2O
 - Rinse on rocker for 30 minutes in TBST at RT
 - Can be place into 1x PBS for storage at 4°C or rinsed well with dH2O to be stained again

Imaging website for FluorcheM imager in building 20, 3rd floor, by Krebsbach lab:

http://10.21.250.46/run_viewer/browse_runs
 (can only be accessed from UM networks)

4.0 Applicable References

5.0 Change Description

| Revision | Date | Reference | Description of Change |
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