The University of Iowa Dezii Translational Vision Research Group

TITLE: Western Blotting Protocol

SOP Number: D-DEZ-PRO-023 Revision Number: 0

Effective Date: 27 Apr 2015

Author: ______________________ Date: ______________

Reviewer: ______________________ Date: ______________

QA Approval: ______________________ Date: ______________

A. OBJECTIVE

This SOP is to provide information for Western blot analysis procedure used to detect specific proteins in a sample of tissue homogenate or extract in the DTVR GMP group.

B. APPLICABILITY

This procedure shall be performed by a trained analyst in association with the DTVR GMP group.

C. REFERENCES

D-DEZ-PRO-002 Plating Passaging and Freezing Fibroblast from Dermal Biopsy Punch
D-DEZ-PRO-022 Virus Production and Purification
D-DEZ-PRO-007 iPSC Culture and Differentiation

D. MATERIALS AND EQUIPMENT

XCell Sure Lock Mini-Cell Gel Electrophoresis system with associated equipment and plates (Life Technologies, Cat. #EI0001)
Mini Trans-Blot Cell and PowerPac Basic Power Supply (Bio-Rad, Cat. #170-3989)
QUBIT (Life Technologies)

For membrane stripping:
- Tris buffer (500mM, ph=7.4)
- Sodium Dodecyl Sulfate solution (20%)
- Beta-mercaptoethanol (14.7M, diluted to 100mM)

For SDS-PAGE
- Novex 4-20% Tris-Glycine Mini Protein Gels (Life Technologies, Cat. #EC6028BOX)
- Loading buffer (see details below)
- Glycine running buffers (see details below)

For Western Blot procedure:
- Chilled transfer buffer (1x, generated from 10x Tris-Glycine buffer plus 20% methanol)
- Tris Buffered Saline with Tween® 20 (TBST)
- PVDF Membrane (BioRad)

E. MEMBRANE STRIPPING PROTOCOL

(Note for LabGuru documentation: Double-click on step to mark as completed)

Recipe:
- 6.75 mL of 500mM Tris buffer (ph=7.4, final working concentration will be 67.5mM
- 5 mL 20% SDS (final working concentration will be 2%)
- 349 µL of Beta-mercaptoethanol (14.7M, final working concentration will be 100mM)

1. bring up to 50 mL with 1X PBS
2. shake at 55 degrees for 30 min
3. wash 3X with normal transfer buffer
4. re-block membrane for 30 min at room temperature
5. add primary antibody for actin or another antibody of interest O/N (1 hr to overnight) at 4 degrees

F. DAY ONE: SET UP, RUNNING, TRANSFER AND INITIAL PROBE

Set up of gel pouring apparatus, for Bio-Rad procedure, is as follows.

1. Open precast Mini-PROTEAN gel, remove tape from bottom of gel and remove comb, and rinse wells with running buffer.
2. Place gels in cassette and place cassette in apparatus (note: even if running only one gel both gels are required to make a seal and contain the running buffer).
3. Fill interior of apparatus (between gels) with 1X running buffer (Tris-glycine SDS running buffer stock, diluted in di water)
4. Fill exterior chamber with running buffer to the level of the bottom of the gel(s)

Protein preparation, loading and running the gel.

5. Protein samples should be prepared such that each condition has an equal amount of protein, generally 25-50 µg can be loaded into each lane.
6. Dilute each protein with 4x loading buffer (Laemmli buffer, BioRad) and boil prior to loading. *Optional step*: omit boiling for evaluation of hydrophobic proteins.

   Example: protein concentration as determined via BCA assay is 5µg/µL
   Add 10µL of protein and mix with 2µl of loading buffer.
7. Dilute stock 10X Tris Glycine SDS Running Buffer (BioRad) 1:1- with distilled water.
8. Add your pre-stained protein ladder to the first lane of the gel, if running two gels be sure to identify which is which (this can easily be done by varying ladder placement).
9. Add your protein samples one at a time to the desired lanes in the appropriate order, i.e. control, experimental condition 1, experimental condition 2, etc. (note each gel has to contain a control for quantitative/comparison purposes).
10. Place cap on the running apparatus, ensure that the wires are black to black and red to red, and run at 100V until the samples reach the bottom of the gel, as visualized by the presence of the blue dye in the loading buffer.
11. Align the cracker with the arrows on the precast gel and slowly crack the sides apart.
12. Remove the fringes with a razor blade. It can be helpful to leave a nub for lane one.
13. Remove gel from cassette and carefully handle with wet gloves
14. Move gel into a Tupperware of cold transfer buffer and equilibrate gel in TB for 15 minutes, on a rocker.

15. Pre-wet PVDF membrane with methanol, then store in transfer buffer while preparing cassette
16. Thoroughly wet sponges and filter paper. Assemble in order: black side of cassette; sponge; Whatman filter paper; gel; PVDF membrane; filter paper; sponge; and clear or red side of cassette, taking care to remove any bubbles.
17. Fasten clamps of sandwich. Add magnetic stir bar to apparatus, and assemble sandwich into transfer apparatus (black side of apparatus adjacent to black side of cassette). Fill apparatus with chilled transfer buffer and ice pack.
18. Place apparatus in ice bucket and place ice bucket on magnetic stir plate.
19. Connect wire to power source, red to red and black to black. Transfer for 1 hr at 100V while stirring.
20. Turn off power and remove leads. Remove membrane from gel. Dry membrane overnight between two fresh pieces of filter paper. (Optional: store blot in TBS in 4C refrigerator overnight).

G. DAY TWO: IMAGING AND ANALYSIS OF WESTERN BLOTS

1. Remove blots from the fridge, collect the primary antibody in a 15ml tube and freeze for further use (most antibodies can be used twice).
2. Rinse each blot with TBST, 2 times 10 Sec each and replace with fresh TBST.
3. Wash each blot with TBST 3 times 10 mins each by gentle rotation at room temperature.
4. Following final wash add Alexa-488 or Alexa-546-conjugated secondary antibody, appropriate species and concentration (generally 1:1000) and incubate for 1 hr at room temperature with gentle rotation.
5. Following secondary incubation, rinse and wash blots as previously.
6. Image the blots using BioRad VersaDoc.
7. Once imaged rinse blots strip if necessary, block and add primary antibody.
8. Repeat probes as many times as needed with different antibodies (generally a single western blot can undergo 4-5 rounds of stripping and reprobing).

H. HISTORY

Effective Date	Revision	Change
27 Apr 2015	0	Initial issue of SOP