

## Western Blot Protocol (optimized for Xrcc2 detection)

### I. POLYACRYLAMIDE GEL PROCEDURE

#### Resolving Gel:

1. Assemble your gel cassette, glass plates with spacers into block and fit tightly on gasket.
2. Depending on the proteins you intend to resolve, you will want to choose the appropriate concentration of acrylamide. I find that 10% works for most proteins. The recipes below make enough for 4 gels, scale down appropriately.

	<b>7%</b>	<b>10%</b>	<b>12%</b>	<b>15%</b>	<b>20%</b>
<b>H<sub>2</sub>O</b>	15.3 ml	<b>12.3 ml</b>	10.2 ml	7.2 ml	0.55 mL
<b>1.5 M Tris-HCl, pH 8.8</b>	7.5 ml	<b>7.5 ml</b>	7.5 ml	7.5 ml	1.875 mL
<b>20% (w/v) SDS</b>	0.15 ml	<b>0.15 ml</b>	0.15 ml	0.15 ml	37.5 uL
<b>10% (w/v) ammonium persulfate (APS)</b>	0.15 ml	<b>0.15 ml</b>	0.15 ml	0.15 ml	
<b>Add when ready to pour gel:</b>					
<b>TEMED</b>	0.02 ml	<b>0.02 ml</b>	0.02 ml	0.02 ml	
<b>Acrylamide/Bis-acrylamide (30%/0.8% w/v)</b>	6.9 ml	<b>9.9 ml</b>	12.0 ml	15.0 ml	5 uL

3. Mix the reagents in a 50ml conical tube, leaving the APS and TEMED to be added at the very end, immediately before you pour your gel. Using a plastic pasture pipette, add the gel mixture to the cassette.
4. Fill about 2/3 the way up (you want to leave enough room for ~ 1-2 cm of stacking gel below the combs. See image on next page)
5. Top the gel with ultra 100% methanol using a plastic pasture pipette. This will help remove bubbles and insure the gel polymerizes consistently on the edges.
6. Allow gel to polymerize for 10-20min, pour off water, or until the remaining solution in the tube becomes solid.
7. Make staking gel (see next page), add APS and TEMED immediately before adding the staking gel to resolving gel.

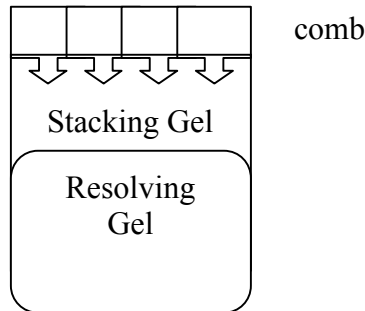
#### Stacking Gel Solution (4% Acrylamide):

<b>H<sub>2</sub>O</b>	3.075 ml
<b>0.5 M Tris-HCl, pH 6.8</b>	1.25 ml
<b>20% (w/v) SDS</b>	0.025 ml
<b>Acrylamide/Bis-acrylamide (30%/0.8% w/v)</b>	0.67 ml

**Add when ready to pour gel:**

**10% (w/v) ammonium persulfate (APS)** 0.025 ml  
**TEMED** 0.005 ml

8. Pour off methanol.
9. Add Stacking gel to resolving gel with a plastic pasture pipette all the way to the top of the cassette. Insert combs making sure there are no bubbles between wells (it's ok if some of the gel spills out).
10. Add 100% methanol if there are air bubbles.
11. Allow gel to polymerize for ~20min, or until combs can be removed easily without disturbing the sides of the wells.



## II. WESTERN PREPARATION & PROCEDURE

### A. SOLUTIONS:

#### **5X Electrode (Running) Buffer (1L)**

**\*\*\*Use all Running & Washing Buffers @ 1X concentration**

Reagent	Amount	1x Final Concentration
Tris Base	15.14 g	25mM
Glycine	75.07 g	200mM
10% SDS	50 mL	0.1%
di-H <sub>2</sub> O	Bring up to 1L	-----

#### **10X TBST (Washing) Buffer (1L)**

Reagent	Amount	1x Final Concentration
1M Tris-HCl; pH 7.5	200 mL	20mM
NaCl	75.07g	200mM
Tween-20	50mL	0.1%
di-H <sub>2</sub> O	Bring up to 1L	-----

#### **2X SDS Sample Buffer (25mL)**

Reagent	Amount	Final Concentration
1M Tris-HCl; pH 6.8	4 mL	160mM
Glycerol	2.5 g	10%
10% SDS	5 mL	2%
β-Mercaptoethanol	625 uL	2.5%

Coomassie Blue	Pinch	-----
di-H <sub>2</sub> O	Bring up to 1L	-----

**1X Towbin (Transfer) Buffer (1L)**

Reagent	Amount	Final Concentration
Tris Base	3.03 g	25mM
Glycine	14.4 g	192mM
<b>100% Methanol</b>	<b>200mL</b>	<b>20%</b>
di-H <sub>2</sub> O	Bring up to 1L	-----

Add MeOH  
right before use

**Blocking Solution (50mL)**

-5% Dried Milk in 1X TBST : 2.5 g Dried Milk; bring up to 50mL with 1X TBST

**B. PROCEDURE:**

1. Assemble electrophoresis apparatus with gel, remove comb, and fill front and back reservoirs with Electrode buffer.
2. Using a pipette tip, flush wells with electrode buffer.
3. Add equal amounts of 2X sample buffer to volume of sample you want to run, denature samples on hot plate at 96°C for 5 min. Load Marker, and load (up to 30ul total/ well) denatured samples to gel.
4. Connect to energy source, run gel for 3 hours at 125V. (or until noted marker band reaches the bottom of gel, time could go up to 5 hours) Make sure to keep an eye on gel, if amps get too low, you could be leaking buffer or running the gel too hot.
5. Turn off electricity source. Discard buffer and remove gel from apparatus (move on immediately to semidry transfer).
6. Make sure to wash all parts of the gel tank well with DI water to remove salts.

**Semidry Transfer:**

1. Cut PVDF membrane to fit gel size, and 6 pieces of filter paper, the same size or slightly smaller than membrane.
2. Activate membrane in methanol for 10 min (on orbital shaker), wash with DI water 2x for 2 min.
3. Mark membrane for the side that proteins will be transferred to and equilibrate membrane in towbin transfer buffer for 10 min.
4. Trim edges off of gel and equilibrate in towbin transfer buffer for 10 min.
5. Pour just enough towbin transfer buffer into transfer dock so you can see it above transfer mesh.
6. Place three pieces of dampened filter paper down on transfer plate rolling out bubbles with a glass rod. Add saran wrap around filter paper so that the wrap is under the very edge of the filter paper. Place activated/equilibrated membrane on top of the filter paper. Place gel on top of membrane, rolling out bubbles with glass rod. Finally place three more dampened filter paper pieces on top of the gel, again rolling out bubbles.
7. Pour a little more towbin transfer buffer onto stack. Attach cover and turn on machine.

8. Run for 65 min at 45V (dependent upon size of gel; see semidry transfer manual).
9. Turn off machine, remove cover and discard filter paper and gel.
10. Wash membrane in 1xTBST for 1 min.
11. Rinse Semidry apparatus with DI water and wipe with paper towels and allow to dry
12. Proceed to western steps below.

Western (all incubations and washes are done with slight agitation on orbital shaker):

1. Block membrane with 5% milk solution in TBST for 1hr at room temp.
  2. Dilute primary antibody to optimized concentration for primary incubation in 5% milk in 1X TBST plus 2uL/10mL of 10% sodium azide and incubate overnight at 4°C.
- Xrcc2 1° antibody (1:200 dilution): Xrcc2 (N-20) [Santa Cruz; Cat. # sc-5895]
  - RAD51d 1° antibody (1:200 dilution): RAD51d (C-16) [Santa Cruz; Cat. # sc-33090]
  - $\beta$ -actin 1° antibody (1:5000 dilution): Rb pAb to  $\beta$ -actin [Abcam; Cat. # ab8227-50]
3. Equilibrate buffers and membrane to RT.
  4. Wash membrane in 1x TBST once for 15 min. and then 5min, 3X.  
(if using an HRP secondary antibody proceed to step 5, if primary antibody is conjugated with HRP move on to development.
  5. Dilute secondary antibody in 5% milk/TBST, and incubate for 1hr at room temp.
  6. 2° to Xrcc2 and RAD51d (1:5,000 dilution): donkey anti-goat IgG-HRP [Santa Cruz; Cat. # sc-2020] 2° to  $\beta$ -actin (1:10,000 dilution): ECL donkey anti-rabbit IgG-HRP [GE Healthcare; Cat. # NA934V]
  7. Wash membrane in 1x TBST once for 15 min. and then 5min, 3X.

Exposing and Developing:

1. Using Amersham ECL western blotting detection reagents [GE Healthcare Life Sci.; Cat. # RPN2132]: remove from 4°C to equilibrate to RT.
2. Perform under low light: for approximately 8x7cm membrane Mix 5ml solution A with 125ml solution B in 15 ml conical tube. Mix reagents by inversion.
3. Remove membrane from last wash with tweezers and allow for excess buffer to drip off.
4. Lay protein side up on clean plastic wrap. Pour detection reagents on to membrane, making sure to cover the entire surface. Incubate at RT for 5 min in dark.
5. Lift membrane from detection reagents with tweezers allowing for excess to drip off and place protein side down on fresh plastic wrap.
6. Fold edges of plastic wrap around membrane keep from drying out. Place in protective film cassette.
7. Proceed to dark room for exposure and development.
8. Place the membrane protein side up and expose on film for the following times: 1s, 10s and 30 sec. If bands are detected decide on length of exposure (3, 5, 10, or 15 min. adjust as needed) If no band is detectable, expose for 20min or longer.
9. Run film through developer machine.

