Western Blot Protocol

1. Cast Gel:

    Assemble minigel apparatus(Be sure no leaking)

    Make resolution gel (recipe) and mix
    Make stacking gel (recipe) → diwater—acrylamide/bis

    Add 7.5ml of res gel to plates; Add diwater (to make sure it's flat.)

    (While waiting for resolution gel to harden (30min)
    Clean up
    Prepare running if needed (50ml 10X tris-sds→500ml dwater)

    Resolution gel should be hard now, pour off water, blot with filter paper

    Finish stacking gel (temed, aps),shake gently. Add stacking gel until at top, not overflowing. ***Hurry and insert comb** **no air bubbles*****
    Wait ~ 30min for gel to harden.

    While waiting for stacking gel to harden:

2. Prepare samples:

    1) Get ice, turn heat block on

    2) Take samples out of freezer→into waterbath to thaw if needed→ then into ice bucket.

    3) Label eppendorf tubes

    4) Add ________ul of <u>sample</u> to tubes (λ= ug/ul)

    5) Add _____________ to <u>marker</u> tube *make sure marker is at bottom of tube*

    6) Add _____________ of <u>2X western blot</u> dilution/sample buffer(Blue) to all

    poke holes→ boil all tubes for 1-3 min.

3. Run gel:

    Remove combs
    Put both gels on plastic thing

    Add running buffer (pour on side to prevent bubbles)

    eye level
    To load samples, press 1st stop, place tip in bottom of tube, lift sample slowly, no bubbles!!!

Load ______ul marker to lane 2; Load ______ul samples to wells

Assemble box Run ~1 hr at ~132v

4. Transfer electrophoresis:
   Cut filter squares
   Cut membrane film →soak in plastic dish with methanol.
   Soak foam pads in transfer buffer
   No bubbles in anything

   In box with transfer buffer, assemble sandwich

   Gel is on big plate, so you know marker is on left side
   Sandwich: 2 foam pads→sheet of filter paper place on gel →1st gel (no bubbles)→membrane(no bubbles)→filter paper→2foam pads.

   Place in box apperatus--clamp--fill with transfer buffer--no leaking—pour into side → Run ~2hr at ~21v

   Replace running buffer
   Prepare plastic square boxes with 1X PBS

   Disassemble box and place the membranes in PBS wash / remove PBS
   Add 20ml milk buffer and rocker 20min.
   Pour out

   Add 10ml milk buffer with *1st AB __________________________ul in plates and rock 1hr.
   (Could stop here and put in 4° over night.)

   SAVE AB

   Add 10ml milk buffer→rock 10 min. / pour out
   Repeat

   Add 10ml milk buffer→ add 2nd AB ______________________________ul
   → Rock 30 min. / pour out milk

   Add 10ml milk→ rock 10 min. / pour out
   Repeat

   Wash with 15ml plain buffer 20 min.

5. Develop film:

    Take saran wrap and cut a piece for each membrane

    In plastic sq boxes add detection reagent 3 ml. #1, and 3 ml. #2 ***mix***

    Blot membrane on sides

    Place membrane in detection reagent → shake 1 min.

    Blot membrane lightly on sides, place on saran wrap(in cassette) and fold over

    Tape them down on the cassette / close
    In darkroom (red light) → place film on membrane and close box → for _1_min
    Place film in autodev. Machine → check film.

    If good results, label film using marker, date, #, Ab used, protein used.

Western Blot Recipes

1. **resolution Gel:**	50ml	20ml	10ml
dH_2O	24.2ml	9.48ml	4.78ml
1.5M Tris-HCL(pH8.8)	12.5ml	5.0ml	2.5ml
10% SDS	500ul	200ul	100ul
40% Acrylamide/Bis	12.5ml	5.1ml	2.5ml
TEMED	50ul	20ul	10ul
10% APS(Fresh)	250ul	200ul	100ul

2. **Stacking Gel:**	20ml	10ml	5ml
dH_2O	12.66ml	6.28ml	3.1ml
0.5M Tris-HCL(pH6.8)	5ml	2.5ml	1.25ml
10% SDS	200ul	100ul	50ul
40% Acrylamide/Bis	1.95ml	1000ul	600ul
TEMED	40ul	20ul	10ul
10% APS	150ul	100ul	50ul

3. **transfer Buffer:**	1500ml
Tris Base(no HCL)	4.54g
Glycine	21.7g
dH_2O	500ml and mix
Methanol	300ml mix with 200ml dH2O
Bring to 1500ml with dH_2O	

4. **milk buffer:**	1000ml	500ml	250ml	100ml
Mix milk in beaker with 50ml dH_2O 1st				
Dry milk	50g	25g	12.5g	5g
Mix with rest in cylinder:				
1M Tris-HCL(pH 7.5)	20ml	10ml	5ml	2ml
5M NaCl	27ml	13.5ml	6.7ml	2.68ml
Tween-20 (100%)	1ml	0.5ml	0.25ml	100ul
Bring up with dH_2O to	1000ml	500ml	250ml	100ml

5. **Plain buffer:**

	100ml	50ml	25ml
1M Tris-HCL(pH7.5)	2ml	1ml	500ul(.5ml)
5M NaCl	2.75ml	1.37ml	680ul(.68ml)
Tween-20(100%)	100ul	50ul	25ul
Bring up with dH_2O to	100ml	50ml	25ml

6. **2X Dilution/Sample buffer** 4ml

0.5M Tris-HCL(pH6.8)	1ml
Glycerol (100%)	0.8ml
10% SDS(Laryl Sulfate)	1.6ml
2-ME (β-mercaptoethanol)	0.4ml
0.05% Bromophenol Blue(dye)	0.2ml

7. **Stripping Buffer:** see protocol

8. **0.5M Tris-HCL:** 400ml

6.05g Tris base in 40ml dH_2O
Adjust pH 6.8 with 1 N HCL
Add dH_2O to 400ml total volume

9. **1.5M Tris-HCL:** 500ml

91g Tris base in 300ml dH_2O
Adjust pH 8.8 with 1N HCL
Add dH_2O to 500ml total volume

10. **10X SDS Running Buffer/electrophoresis buffer:**

Dissolve 10g SDS in beaker of dH_2O (may have to heat)
Dissolve 30.2g Tris base
Dissolve 144g Glycine
Place all in cylinder add dH_2O to 1000ml

To use, make 1X dilution: 50ml 10X→ add dH_2O up to 500ml
100ml → add dH_2O up to 1000ml

11. **Coomassie blue G-250 staining solution:**

Acetic acid	20ml
dH_2O	180ml
Coomassie blue G-250	0.05g

Mix 1 hr. store RT

12. **Destaining buffer:**

50 parts	acetic acid
165 parts	methanol
785 parts	dH_2O