

Western Blot

DAY I

Streak strains for colonies

DAY II - Days II – IV can be condensed into one day's work

Grow cells to mid log

Take OD. ** This is especially important for quantitative work

Pellet 1mL

Pull of all sup with pipetman, not aspirator

If you do not have time to finish the next phase, pellets may be stored at -20C

DAY III

SDS gel: (1.5 – 2 hr)

0.75mm 12% SDS-PAGE

Resuspend pellet in 100 μ L GTE + 2 mg/mL lysozyme, and 1 μ L AEBSF

Make a stock solution, and do not shake or vortex the lysozyme solution!

Incubate @ 37C for 15 minutes

Incubate on ice for 15 minutes

Check to see if cells have lysed

Add 100 μ L 2X sample buffer

Boil for 5 minutes

Load gel, normalizing to OD (use MV=MV, standardizing to 10 μ L of the lowest OD)

Use 10 μ L BioRAD prestained ladder

If probing different proteins, run a ladder for each set so you can cut the blot

Run gel

Western Transfer: (20 – 30 min)

Set up while the gel is running (putting all liquids in appropriate trays):

Cut PVDF membrane using template in box

Dip membrane in 100% methanol to activate; put only a thin layer of methanol (<5mL) in tray

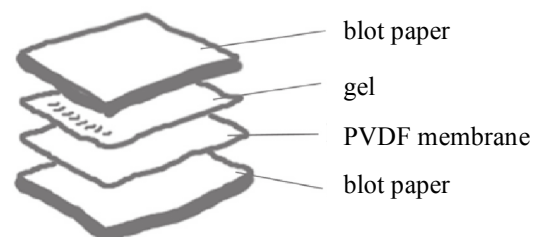
Soak for 5 min in H₂O

Let equilibrate in transfer buffer 10+ minutes

Cut two fat pads of blot paper to same size as membrane; this

reduces waste and increases the efficiency of transfer

Soak blot paper in transfer buffer



When gel has finished running, cut off the combs and ease the gel into transfer buffer using the green paint-knife tool.

Soak the gel in transfer buffer for 10-20 min; do not soak >20 min!

Set up gel sandwich! Mmm.

Press out bubbles by rolling a test tube over each layer.

Run for 20 min at 20V

Use righthand power box (PowerPac 200)

Lift up corner of gel to check transfer

Cleanup:

Do not leave any transfer buffer on electrodes.

Wash promptly w/ ddH₂O and Kimwipe, let dry.

Store with blot paper between metal electrodes!

Don't let the metal surfaces touch!

Block: (30 min – O/N)

Use 5% milk in 1X PBS (2.5g milk in 50mL 1X PBS)

Incubate 30 min – O/N

** If incubating O/N, cover gel in saran wrap and place on shifter in cold room

DAY IV

Probe: (3.5 hours – O/N) All of this can be done in ambient light, @ RT.

Primary antibody stain for 2-5 hrs (can run O/N if nonspecific binding isn't a problem)

Make 1% milk (dilute the 5% 1:5, or just add some flecks to 1X PBS; not precise)

10mL 1% milk/probe/blot (so 20mL/blot for primary and secondary)

Label trays; have a tray for each probe

Don't let the blot dry out

Put blot in 10mL 1% milk

Add probe at appropriate dilution (1:1000 for rabbit Spo0J, 1:5000 for rabbit FtsZ)

Make sure you use the primary antibody that complements your secondary (rabbit, etc)

Put on shaker for 2+ hours; chicken antibody usually needs more than rabbit

Wash 3 times in 1X PBS

Pour on, pour off a few times

For thorough final wash, you may place on shaker in 1X PBS for ~10 min

Secondary antibody stain for 1 hr – not longer than 1 hr!!

Rabbit secondary is 1:5000

Store at -20C, and do not let warm up; it is conjugated to enzyme HRP (horseradish peroxidase)

Wash 3 times in 1X PBS

Develop: (1 hr)

Pour off all remaining PBS

Developing HRP-conjugated secondary using Detection Reagents 1 and 2 (in 4C fridge)

DON'T LET THE REAGENTS MIX IN THEIR BOTTLES! They'll react and go bad.

Add 1mL of each reagent (2 mL total), mix in tray

Pipet mixture all over blot repeatedly

Pick up blot with tweezers, shake off, dab edge on Kimwipe

Place between sheets of transparency film or Saran Wrap

Take Pictures:

Sign up for Fuji Imager in Kranz Lab (sign up for an hour in case some steps run long)

Set shelf # based on gel size

Make sure settings are for "Chemiluminescent Samples"

Make sure tray is pushed all the way in

Program Settings – don't adjust temperature:

FUJIFILM LAS 1000

Operation Mode: Precision Dark Frame Subtraction

Image Type: Image Spotting

Exposure: 30 seconds Flat-Frame Correction

Binning pixels Distortion Correction

Visible Frame Grab Invert Pixels

Image Data Offset: 256

Save As: file type "Fuji Film (Quantable)"