

Western protocol

Note: keep protein samples on wet ice throughout.

Day1

Cell Lysis and Quantitation

Lyse cells in H₂O or detergent depending on the protein's solubility.

Sonicate sample to break up genomic DNA.

Spin sample at 10,000 rpm for 5 minutes.

Transfer supernatant to a new tube.

Quantitate protein concentration by Pierce BCA protein assay kit.

Gel Preparation

Use GeneMate pre-cast gels (4-20% Tris-Hcl) with Bio-Rad vertical mini-gel apparatus (lane openings face in).

Fill inner chamber with 1x Tank Buffer to top of the gel.

Fill outer chamber with 1x Tank Buffer to about 3cm below the top of the gel.

Sample Preparation and Gel Loading

Set up the following mix (20ul total volume):

Sample Volume for		
<u>5-20ug protein</u>	<u>H₂O to 10ul</u>	<u>2X SDS sample buffer</u>
x	y	10ul

Boil samples in heat block for 2 minutes.

Load samples in to gel with gel loading tips.

Fill any empty lanes with H₂O + SDS sample buffer (20ul total) to ensure straight migration.

Run gel at 90V for 2.5 hours.

Gel Transfer

Wet your gloves with Transfer Buffer.

Disassemble gel, cut off lane separators with razor blade and place in small clean container containing about 75ml Transfer Buffer.

Let gel soak in transfer buffer as transfer sandwich is made.

Cut out two blotting pads and one piece of nitrocellulose to exact measurements of gel to be transferred.

Soak all components in transfer buffer.

Assemble sandwich in semi-dry transfer cell as follows:

Top

Blotting pad

Gel

Nitrocellulose

Blotting pad

Bottom

Transfer at 15V for 40 minutes

Blocking

Place nitrocellulose (called blot from now on) in 300ml TBST/5% milk (only use Carnation nonfat dehydrated milk).

Let soak at 4^oC overnight.

Day2

Wash blot in 100ml TBST for 30 seconds with mild shaking

Primary Antibody Stain

Place blot in clean small western blot container.

In TBST/0.5% milk add primary antibody

Cover blot with stain (5ml is sufficient for our western blot boxes).

Incubate at room temperature for a minimum of 2 hours with mild shaking.

For best staining, incubate overnight at 4⁰C with mild shaking.

Day2 cont. or Day3

Wash blot in 100mlTBST for 30 seconds with mild shaking.

Wash blot 3 times with 200ml TBST/0.5% milk for 10 minutes each time with mild shaking.

Secondary Antibody Stain

Place blot in clean small western blot container.

In TBST/0.5% milk add secondary antibody (We use 1:2500 HRP-conjugated secondary)

Incubate at room temperature for minimum of 1 hour with mild shaking.

Wash blot 3 times with 200ml TBST/0.5% milk for 15 minutes each time with mild shaking.

Wash blot 2 times with 200ml TBST for 15 minutes each time with mild shaking.

Chemiluminescence

Place blot in clean small western blot container.

Mix Lumi-light reagents 1:1 and add 10ml to cover the blot

Incubate at room temperature for 5 minutes with minimal light exposure.

Take the blot out and place two kim-wipes over the top to soak up excess liquid.

Place the blot between two transparent plastic sheets and remove all air bubbles.

Place in film cassette and visualize by autoradiography.

Take a 1 minute exposure and gauge whether it's too light or too dark.

Take multiple additional exposures.

NOTE: Lumi-light is most active from 10-30 minutes after addition to blot.