



# Protocol #13

## SDS-PAGE and Western blot

### Introduction

Proteins are extracted from cells and separated by size using polyacrylamide gel electrophoresis (PAGE). This method uses precast gels which are quicker, safer and give better resolution and reproducibility than self-prepared gels. Recipes for self-prepared are provided in the index. Following transfer of the proteins, the nitrocellulose membrane will be blocked, and probed with a primary antibody. Bound primary will then be detected with HRP-conjugated secondary antibody and this detected using ECL reagent.

### Aims

You will be provided with HEK/COS cells that have been transfected with four constructs in differing amounts as shown below. Note, in all cases, the total amount of DNA used for the transfection is 2.0 µg. Working in groups of four, take two samples each and share one gel between the group. Note – each gel kit holds two gels.

Sample	pP2X2	pP2X2-EE	antisense	pcDNA3.1	ratio
A	–	–	–	2.0 µg	N/A
B	2.0 µg	–	–	–	N/A
C	–	0.2 µg	–	1.8 µg	N/A
D	–	1.0 µg	–	1.0 µg	N/A
E	–	1.8 µg	–	0.2 µg	N/A
F	–	0.2 µg	1.8 µg	–	1:9
G	–	1.0 µg	1.0 µg	–	1:1
H	–	1.8 µg	0.2 µg	–	9:1

A and B are negative controls

C, D and E are positive controls

F, G and H are experimental clones and should be compared to C, D and E respectively

### Timetable

*Day 1* Prepare samples, assemble apparatus, run gel, transfer, block, 1° Ab o/n

*Day 2* Add 2° Ab and visualise with ECL

## **Gel assembly and sample preparation**

### *Materials required*

Western blot apparatus  
3MM Whatmann paper  
ECL membrane

### **Caution**

Non-polymerised acrylamide is a neurotoxin – avoid inhalation and wear gloves at all times. Avoid powdered solutions – if possible, purchase pre-mixed reagent.

## **Experimental procedures**

1. Remove medium from cells, and wash once with PBS.
2. Add about 1 ml PBS and scrape cells off dish – transfer into an eppendorf tube
3. Spin cells for 1 min – discard supernatant and resuspend in 50  $\mu$ l RIPA buffer
4. Incubate at 4 °C for 30 mins on quake.
5. Spin for 10 mins to remove cell debris and nuclei
6. Remove 40  $\mu$ l supernatant to a fresh tube
7. Add 25  $\mu$ l H<sub>2</sub>O and 25  $\mu$ l 4 x NuPAGE LDS sample buffer
8. Heat to 70°C for 10 mins.
9. Add 10  $\mu$ l NuPAGE 10 x reducing agent

## **Gel**

1. Assemble gel apparatus.
2. Place gel in tank, fill tank with running buffer and load samples into wells.
3. Electrophorese the gel for approximately 1 h at 200 V .
4. Stop gel and transfer to nitrocellulose membrane 37 V for 1 hour.

**Western Blot***Materials required*

Autoradiograph	Tweezers or forceps	ECL reagent
Primary Ab	Secondary Ab	Cling film

Solutions

1 x TBS

1 x TBS/0.1% Tween 20

1 x TBS/0.1% Tween 20/4 % Marvel (nonfat milk powder)

20 x TBS (1 litre)

48.4g Tris, 160 g NaCl, pH 7.6 (HCl)

Acid Strip

4.35 g NaCl, 3.75 g Glycine pH 2.6 (30 mins at RT)

**ECL reagent – Caution**

ECL reagent may be a potential carcinogen – wear gloves.

**Disassemble transfer apparatus/Block/Primary Ab**

1. Carefully disassemble the transfer apparatus. Using tweezers or forceps, place the nitrocellulose membrane in container and wash for 10 min in 1 x TBS
2. Block the membrane with 1 x TBS/0.1% Tween 20/4 % Marvel for 1-2 hours.
3. Add primary antibody to blot at the desired concentration (1:1000), and incubate overnight with gentle rocking in cold room.

**Wash/secondary Ab**

1. Wash three times for 5 mins with 1 x TBS/0.1% Tween 20.
2. Dilute the secondary antibody to the desired concentration (1:1000) with 1 x TBS/0.1% Tween 20/4 % Marvel and add to blot - incubate for 1 hour with gentle rocking at RT
3. Wash three times in H<sub>2</sub>O
4. Wash five times for 5 mins with 1 x TBS/0.1% Tween 20.
5. Wash fivetimes in H<sub>2</sub>O

**Visualize with ECL**

1. Mix the detection reagent (ECL): 1.5 ml Reagent 1 and 1.5 ml Reagent 2
2. Add the mixed ECL reagent to the surface of the membrane for 1 min.
3. Drain off the ECL reagent and wrap the blot in Saran wrap.
4. Expose the membrane to XS-1 autoradiograph film for the desired amount of time (e.g. 30 s to 3 min).

**Western Blot - Appendix**

*Alternatives to pre-cast gels*

Hybaid and Biorad sell gel apparatus to run non-precast gels.

*Gel recipes*

As an alternative to pre-cast gels, you may wish to prepare your own gels. These recipes are for 8, 10 or 12% gels. Check recipes before use. The ratio of Acrylamide:bis-acrylamide varies depending on the gel type. For most protein gels, a ratio of 38:1 is used.

Bis-Acrylamide (30%)	Casting gel apparatus
APS	1.5M Tris-HCl (pH 8.8)
SDS (25% (w/v))	0.5M Tris-HCl (pH6.8)
TEMED	Butan-2-ol

Resolving gel

	<u>8%</u>	<u>10%</u>	<u>12%</u>	
Acrylamide/Bis-acrylamide (30%; 38:1)	2.67	3.33	4.00	ml
1.5M Tris-HCl (pH 8.8)	2.50	2.50	2.50	ml
10% (w/v) SDS (25%)	100	100	100	µl
0.06% (w/v) APS	100	100	100	µl
0.4% (v/v) TEMED	5	5	5	µl
<u>dH<sub>2</sub>O</u>	<u>5.68</u>	<u>4.00</u>	<u>3.35</u>	<u>ml</u>
<u>Total volume</u>	<u>10</u>	<u>10</u>	<u>10</u>	<u>ml</u>

Pipette 1 ml butan-2-ol on top of the resolving gel while the gel is solidifying to occlude air and enable polymerisation of the gel. Prior to adding the stacking gel, remove the butan-2-ol and the rinse the surface of the running gel with dH<sub>2</sub>O.

4% Stacking gel recipe

	<u>Volume</u>	
Acrylamide/Bis-acrylamide (5%)	670	µl
0.5M Tris-HCl (pH6.8)	1.25	ml
10% SDS (w/v) (25%)	50	µl
0.06% (w/v) APS	25	µl
0.04% (v/v) TEMED	5	µl
<u>dH<sub>2</sub>O</u>	<u>3.05</u>	<u>ml</u>
<u>Total volume</u>	<u>5</u>	<u>ml</u>

Pour this on top of the resolving gel and allow 30 min to solidify. Do not forget to place the comb into the stacking gel to make wells!