

LPS SDS-Urea PAGE for Lewis Western blot

Materials:

Lysis buffer

Reagent	to 100mL
SDS	2g
2-Mercaptoethanol	4mL
Bromophenol Blue	0.003g
1M tris-Cl pH6.8	to 100mL

SDS-PAGE gel

10% separating gel	4% stacking gel
5.3mL 30% acrylamide/0.8% bisacrylamide	650µL 30% acrylamide/0.8% bisacrylamide
6.5mL water	3.05mL water
3.75mL 4x Tris-Cl/SDS pH 8.8	1.25mL 4x Tris-Cl/SDS pH 6.8
2.88g Urea	25µL 10%APS
50µL 10%APS	5µ TEMED
10µL TEMED	

Electroblotting buffer:

Reagent	In 4L	In 5L
Tris Base	9.664g	12.08g
Glycine	45.04g	56.3g
Methanol	800mL	1000mL
ddH ₂ O	To 4L	To 5L

Blocking Solution:

Reagent	for 50mL
Roche Blocking Reagent (10X)	5mL
PBS	45mL

Primary antibody Solution (on 0.5% blocking solution):

Reagent	for 10mL
Roche Blocking Reagent (10X)	500µL
Antibodies	
-anti-Lex (1:100)	100µL
-anti-Ley (1:100)	100µL
PBS	10mL

1XPBS-AT (PBS-Azide/Tween):

Reagent	In 1L	In 2L
10XPBS	100mL	200mL
NaN ₃	0.20g	0.40g
Tween20	0.5mL	1mL
ddH ₂ O	To 1L	To 2L

Store @4°C, no need to be sterile

Wash solution (0.5% blocking solution):

Reagent	
Roche Blocking Reagent (10X)	2.5mL
PBS	47.5mL

Secondary antibody solution (on 0.5% blocking solution)

Reagent	for 10mL
Roche Blocking Reagent (10X)	500μL
Antibody -Goat anti-mouse IgM HRP (1:2000)	5μL
PBS	10mL

Methods:**Preparing the samples:**

1. Resuspend cells on PBS.
2. Make a 1 mL 1:100 aliquot of the cells.
3. Determine OD at 600nm.
4. Calculate $(5/OD)1000 =$ amount of cells needed for 1mL 5OD resuspension.
5. Spin down at 8krpm for 5 min
6. Resuspend in 200μL Lysis buffer by pipetting up and down.
7. Boil for 10 mins.
8. Add 3 μL 20 μg/ μL Proteinase K; incubate at 60C for 1h.

Making the gel:

1. Install equipment.
2. Mix the reagents for the separating gel.
3. Pour separating gel, cover with a layer of ddH₂O; wait for 45 mins or until gel polymerizes. Take out water layer.
4. Mix reagents for stacking gel, and pour. Avoid bubbles.
5. Insert comb. Allow to polymerize for 45 mins or until gel polymerizes.
6. Install gel in the equipment. Add 1x SDS running buffer.
7. Load 6μL of Kaleidoskope Marker, and 10-5μL of each sample. Run at 35mAmp for 2h.

Transfer:

1. Cut the membrane and filters, while wearing gloves!!! Soak membrane, filters, and fiber pads in transfer buffers for about 15 minutes)

Prepare gel sandwich:

- a. place the cassette with the black side down, on a clean surface.
 - b. place one of the fiber pads on the black side.
 - c. place a sheet of filter paper on the fiber pad.
 - d. place the gel on the filter paper.
 - e. place the pre-wet membrane on the gel.
 - f. place the second sheet of filter paper on top of the membrane
 - g. add the last fiber pad.
 - h. remove bubbles
2. Place the sandwich on the electrode cell, and add transfer buffer until full.
3. Place cell on ice box, add ice.
4. Connect to power supply, and run for 1.5h at 1 amp.
5. Block membrane.

Detection of Lewis LPS:

1. Block the membrane overnight at 4°C without agitation (or for 1h at room temp. with agitation) using 1X blocking solution in PBS.
2. After blocking, incubate in primary antibody diluted as required in 0.5% blocking solution in PBS). Agitate for 1h at room temp.
3. Decant antibody (can reuse if stored at -20°C). Wash twice in 50mL 1XPBS-AT for 10 minutes each. Then wash twice with 50mL 0.5% blocking solution on PBS for 10 minutes each.
4. Incubate for an hour on the secondary antibody solution with agitation at room temperature.
5. Wash 4 times on 1XPBS-AT for 10 minutes each
- 5a. During this time prepare the cassettes with the film in the dark room. Turn on developer machine 10 minutes before.
- 6. Detection: Work quickly once membrane is exposed to detection reagents (1 membrane at a time).** Transfer membrane to a new tray, blotting off excess liquid. Mix 2mL of each of the two detection reagents in a 15mL falcon tube. Pipet the detection reagents over the membrane for 1-2 minutes, ensuring that the entire membrane is covered.
7. Blot the membrane to remove excess detection reagents. Encase in saran wrap and smooth out bubbles on the front (don't smear detection reagents on the surface of the saran wrap)
8. Change your gloves. Expose membrane to film in the dark room. Develop.