

Denaturing Reduced Protein Gels, Coomassie Staining, and Western Blot

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1. Denaturing Non-Reduced Proteins

Materials:

- Protein samples
- Deionized water
- NuPAGE Mini Cell
- Protein molecular weight standards - Kaleidoscope (Bio-Rad 161-0324), or Page Ruler (Fermentas SM0671).
- NuPAGE LDS Sample Buffer (4X)
- NuPage Reducing Agent (10X) (Life Technologies NP0004)
- PCR tubes
- PCR Machine
- NuPAGE MES SDS Running Buffer
- Plastic Container
- Coomassie Blue Stain
- Destain Solution
- 4-12% BisTris Gels 10 well x 1 mm

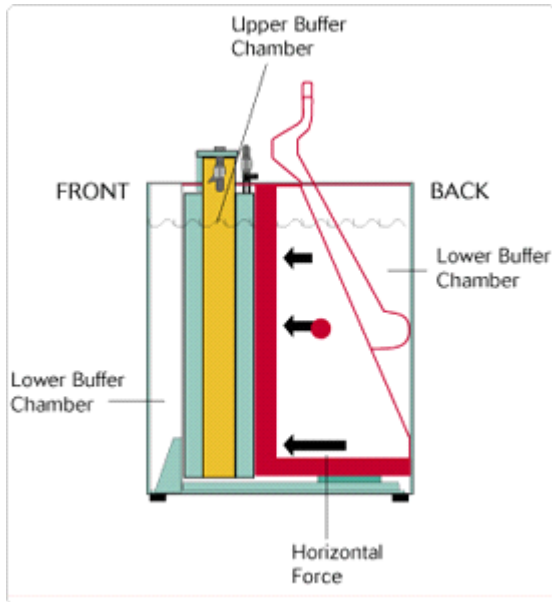
Denaturing Methods:

1. Prepare Samples on ice
Protein Samples (20 μ L/well)
+ 5 μ L LDS Sample Buffer (4X)
+ 12 μ L of 2 μ g/ μ L (~15 μ g/well)
Hint: Dilute protein down to 2 μ g/ μ L and use 12 μ L protein per well. Note that each well can hold a maximum of 25 μ L.
2. Prepare Protein Marker
Pick a protein marker and prepare as follows in a PCR tube:
Page Ruler SM0671 \rightarrow +5 μ L protein marker
Kaleidoscope 161-0324 \rightarrow +10 μ L protein marker
3. Heat the samples and the protein marker for denaturing electrophoresis at 70°C for 10 minutes for optimal results using a PCR machine. DO NOT BOIL.
4. After heating add 2 μ L NuPAGE Reducing Agent (10X)

Electrophoresis

5. Remove the gel from the pouch and rinse the gel cassette with deionized water. Peel off the tape from the bottom of the cassette.
6. Gently pull the comb out of the cassette and rinse the sample wells with 1X MES SDS running buffer. Repeat two more times.
7. Orient the two gels in the Mini-Cell such that the 'well' side of the cassette faces inwards toward the buffer core. Lock gel tension wedge into place.
Note: If you are running just one gel, use the plastic buffer dam in place of the second gel cassette to form the upper buffer chamber.
8. Fill the upper buffer chamber with a small amount of the running buffer to check for tightness of seal. If you detect a leak from upper to the lower buffer chamber, discard the buffer, reseal the chamber, and check again.

9. Once secure, fill the upper buffer chamber the buffer level exceeds the level of the wells. Do not use used buffer in the upper chamber.
10. Load 25 μ L of protein samples and protein marker into each well of the 10 well x 1mm gel.
11. Fill the lower buffer chamber with 600 mL of the MES 1X running buffer. You may use used buffer in the lower chamber.
12. Set the voltage to 200 V with expected current ranging start: 110–125 mA/gel and end: 70–80 mA/gel. Run time of 50 minutes.
13. Remove gel from the Mini-Cell and separate each of the three bonded sides using the gel knife. The well side of the cassette should face up.
14. Remove and discard the top plate. Cut off the entire foot of the gel and peel away the gel from the plate using the gel knife. In order to avoid breaking the gel, make sure the gel knife is wet before peeling the gel from the plate.



2. Coomassie Staining (gel)

NOTE: you can not run a western blot on a gel that has been stained

1. Place gel membrane in a clear plastic box and wash with deionized water three times for five minutes each (on orbital shaker 75 rpm)
2. Stain the membrane with Coomassie blue stain for 5 minutes (on orbital shaker 75 rpm)
3. Pour off excess Coomassie blue stain into the container labeled 'Used Coomassie Blue Stain' – it can be re-used.
4. Immerse membrane in destain solution for fifteen minutes twice (on orbital shaker at 75 rpm).
5. Pour off excess liquid and rinse with deionized water several times.
6. Air dry.

3. Western Blot:

Materials:

Rectangular Petri Dish (1/gel)
 GE Amersham Hybond PVDF Membrane (catalog #RPN303F)
 MeOH (Methanol)
 Distilled water (distilled DI water)

Whatman Blot Filter Paper (GB004 Item# 10427918)
Lasagna Dish
6 Blotting Pads
NuPAGE Mini Cell
Protein gel
Transfer Buffer (1X)
TBS-T
TBS-B
Primary Antibody (Sigma Cat # H1209)
Secondary Antibody (Invitrogen Cat #460693)
3 Falcon Tubes (50 mL)
Super Signal West Pico Chemiluminescent Substrate (catalog#34080)
Saran Wrap
Fujifilm LAS-3000 Intelligent Dark Box

Western Blot Methods:

1. Cut one 7.5cm x 8.5cm piece of PVDF membrane and wet with methanol for 10 seconds, until slight color change. Briefly rinse under distilled water and soak in distilled water for 5 minutes in rectangular petri dish. Then soak the membrane in transfer buffer for 10 minutes. Fold a corner of the membrane. The side that the corner points to will distinguish the side of the membrane that will hold the protein.
2. Soak 6 blotting pads in 1X transfer buffer (~600 mL) in a lasagna dish. Press out air bubbles. Soak two filter paper pieces in transfer buffer (per gel).
3. Place the piece of pre-soaked filter paper on top of the gel. Gently press out air bubbles.
4. Place two soaked blotting pads in the deeper (anode -) half of the blot module.
5. Place the PVDF membrane on top of the gel, folded corner facing up. Be careful to place the membrane on the first try, as the proteins will begin to transfer immediately after contact. Put the assembly membrane side up onto the two soaked blotting pads. Place the second piece of filter paper on top of the membrane.
6. Add four soaked blotting pads on top of the filter paper/membrane side, then add the second half (cathode+) half of the module.
*If two gels are being run at the same time, add one soaked blotting pad on top of the first filter paper/membrane assembly (instead of four) and then repeat the same process for the second gel. Add three blotting pads on top of the second filter paper/membrane assembly, and finally the cathode+ half of the module.
7. Hold the blot module together firmly and slide it into the guide rails of the lower buffer chamber of the Mini Cell. Put in the gel tension wedge and make sure they fit tightly.
8. Fill the blot module with transfer buffer just until the gels/membranes are covered. Make sure it does not leak.
9. Fill $\frac{3}{4}$ of the outer buffer chamber with 1X transfer buffer.
10. Put on the lid and run the blot at 30V for 1 hour.
11. Store the membrane in a covered petri dish with 1X transfer buffer.

Preparation for Immunodetection

All incubating and washing steps should be done in a rectangular petri dish on a rocking shaker at room temperature.

12. Wet the membrane in MeOH and rinse with distilled water.
13. Block the membrane by incubating in TBS-B for 1 hour.
14. Rinse in TBS-T for 5 minutes (3 times).
15. Incubate with the primary antibody 3 μ L/15mL TBS-B for 1 hour. Make 15mL for each membrane in a 50 mL falcon tube. Antibodies may be reused and stored at -20°C.

16. Wash with distilled water for 1 minute.
17. Wash with TBS-T for 5 minutes (2 times).
18. Incubate with the secondary antibody 0.75 μ L/15mL for 1 hour. Make 15mL for each membrane in a 50 mL falcon tube. Antibodies may be reused and stored at -20°C.
19. Wash with TBS-T for 10 minutes (3 times).
20. Mix a 1:1 ratio of Super Signal West Pico Stable Peroxide Solution and Super Signal West Pico Stable Luminol Enhancer Solution in a 50 mL falcon tube. Do not make too far in advance as this will not last more than a half day and cannot be reused. Make 16 μ L mixture/membrane and pour over membrane in the petri dish so that when on the rocker, liquid constantly moves across the top of the membrane. Incubate on rocker for 7 minutes.
21. Remove membrane from the petri dish and place protein side down on a flat piece of saran wrap. Fold the edges of saran wrap over the back side of the membrane. Gently press out any bubbles.
22. In the LAS-3000 dark box, place the membranes protein side up on top of the dark tray insert. Close the door and adhere to the following settings. Timing and exposure intensity will be variable depending on the protein. Note that the luminescent signal is good for one hour before beginning to wear off.
 - Methods/Tray Position: Tray 2, Chemiluminescence
 - Focusing: Brightness 3
 - Start with exposure time 10-20 seconds to get an initial idea of band intensity. One minute generally works well for the best picture, but it can take up to five in some cases.
23. Once desired image is found, save and print.

4. Coomassie Staining (membrane)

Materials:

Rectangular Petri Dish (1/gel)
 Coomassie Blue Stain
 Destain Solution
 Orbital Shaker

Method:

1. Stain the membrane with Coomassie blue stain for 7 minutes (on orbital shaker 75 rpm) in a rectangular petri dish.
2. Pour off excess Coomassie blue stain into the container labeled 'Used Coomassie Blue Stain' – it can be re-used.
3. Immerse membrane in destain solution for fifteen minutes (on orbital shaker at 75 rpm). Note that the membrane background will lighten when dry.
4. Pour off excess liquid and air dry. The membrane can be placed directly into a lab notebook when dry, and is reusable for staining or protein cutting.

Reagents:

1X Transfer Buffer

+ 50 mL 20X NuPAGE MES SDS buffer (Invitrogen)
 + 950 mL Distilled water

10X TBS

+ 200 mM Tris/HCl (pH to 7.6)
 + 1.37 M NaCl
 + Distilled water up to 1L

1X TBS

- + 100 mL 10X TBS
- + 900 mL Distilled water

TBS-T

- + 100 mL 10X TBS
- + 999 mL Distilled water
- + 1 mL Tween 20

TBS-B

- + 500 mL TBS-T
- + 15 g BSA (=3% w/v)

1X MES SDS Running Buffer

- + 50 mL MES Running Buffer (20X)
- + 950 mL Distilled water

Coomassie Blue Stain:

- + 0.06 g Brilliant Blue (Fisher - 825.99 g/mol)
- + 100 mL Methanol
- + 17.5 mL Acetic Acid
- + Distilled water up to 250 mL

Destain Solution

- + 125 mL Methanol
- + 17.5 mL Acetic Acid
- + Distilled Water up to 250 mL

Reference from Invitrogen's Website**NuPAGE® LDS Sample Buffer**

Use the NuPAGE® LDS Sample Buffer (4X) for preparing samples for denaturing gel electrophoresis with the NuPAGE® Gels. For native gel electrophoresis with NuPAGE® Tris-Acetate Gels, use the Novex® Tris-Glycine Native Sample Buffer (2X). For optimal sample preparation in all SDS-PAGE protocols, including the NuPAGE® system, denature and reduce the protein disulfide bonds under slightly alkaline pH conditions. Since the pH of the NuPAGE® LDS Sample Buffer is 8.4, sample reduction at this pH allows for maximal activity of the reducing agent.

The NuPAGE® LDS Sample Buffer is a 4X concentrated solution containing twice as much LDS as the 2X concentration of Novex® Tris-Glycine SDS or Tricine SDS Sample Buffer. This makes the NuPAGE® LDS Sample Buffer more viscous and difficult to pipet as compared to the Novex® Tris-Glycine or Tricine Buffers. The presence of more glycerol also increases the viscosity of the NuPAGE® LDS Sample Buffer. By bringing the NuPAGE® LDS Sample Buffer to room temperature (25°C), the buffer is more manageable

For reduced sample, add the reducing agent immediately prior to electrophoresis to obtain the best results.

Reagent	Reduced Sample	
Sample	x µl	

NuPAGE® LDS Sample Buffer (4X)	2.5 µl	
NuPAGE® Reducing Agent (10X)	1 µl	
Deionized Water	to 6.5 µl	
Total Volume	10 µl	

Reducing Agent

The NuPAGE® Reducing Agent contains 500 mM dithiothreitol (DTT) at a 10X concentration and is available in a ready-to-use, stabilized liquid form. Use the NuPAGE® Reducing Agent to prepare samples for reducing gel electrophoresis. *b*-mercaptoethanol is compatible with the NuPAGE® system and can be used with the NuPAGE® gels at a final concentration of 2.5%.

We recommend adding the reducing agent to the sample within an hour of loading the gel. Avoid storing reduced samples for long periods even if they are frozen. This will result in the reoxidation of samples during storage and produce inconsistent results.

NuPAGE® LDS Sample Buffer

The NuPAGE® LDS Sample Buffer (4X) is available from Invitrogen