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Western blotting using NuPAGE system

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We use this protocol and it's working

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Disclaimer

Note that any protocol involving animals should be reviewed and approved by your Institutional Animal Care and Use Committee (IACUC) before use.

Abstract

This protocol describes the procedure for western blotting with the NuPAGE system used by the Seifert Lab.

It supplements the manufacturer's guide to the NuPAGE electrophoresis system:
https://tools.thermofisher.com/content/sfs/manuals/nupage_tech_man.pdf

Materials

For sample preparation and electrophoresis:

- Quantified protein samples
- NuPAGE™ LDS Sample Buffer (4X) **Invitrogen - Thermo Fisher Catalog #**NP0008 (stored at 4 °C)
- NuPAGE Sample Reducing Agent (10X) **Thermo Fisher Scientific Catalog #**NP0009 (stored at 4 °C)
- NuPAGE Antioxidant **Thermo Fisher Scientific Catalog #**NP0005 (stored at 4 °C)
- NuPAGE gel for electrophoresis (stored at 4 °C)
- NuPAGE™ MES SDS Running Buffer (20X) **Thermo Fisher Catalog #**NP0002 (stored at Room temperature)
- NuPAGE™ MOPS SDS Running Buffer (20X) **Thermo Fisher Catalog #**NP0001 (stored at Room temperature)
- Novex Sharp Pre-stained Protein Standard **Thermo Fisher Scientific Catalog #**LC5800 (stored at -20 °C)

For transfer:

- iBlot™ Transfer Stack, PVDF, mini **Thermo Fisher Catalog #**IB401002
- Tris-Glycine transfer buffer (10X)
- ~ 1.5 L 1X Tris-Glycine transfer buffer with 20% methanol

Protocol materials

NuPAGE Antioxidant **Thermo Fisher Scientific Catalog #**NP0005

Novex Sharp Pre-stained Protein Standard **Thermo Fisher Scientific Catalog #**LC5800

iBlot™ Transfer Stack, PVDF, mini **Thermo Fisher Catalog #**IB401002

NuPAGE Sample Reducing Agent (10X) **Thermo Fisher Scientific Catalog #**NP0009

NuPAGE™ LDS Sample Buffer (4X) **Invitrogen - Thermo Fisher Catalog #**NP0008

NuPAGE Antioxidant **Thermo Fisher Scientific Catalog #**NP0005

NuPAGE™ MOPS SDS Running Buffer (20X) **Thermo Fisher Catalog #**NP0001

Novex Sharp Pre-stained Protein Standard **Thermo Fisher Scientific Catalog #**LC5800

NuPAGE™ MES SDS Running Buffer (20X) **Thermo Fisher Catalog #**NP0002

iBlot™ Transfer Stack, PVDF, mini **Thermo Fisher Catalog #**IB401002

Troubleshooting

Sample Preparation and Electrophoresis

1 Prepare samples On ice in individual 0.5 mL tubes according to the following chart:

	A	B	C	D	E
	Reagent	8 wells	10 wells	12 wells	15 wells
	Protein Extract (maximum)	40 µg	40 µg	30 µg	30 µg
	ddH2O	up to 19.50 µL	up to 16.25 µL	up to 13.00 µL	up to 9.75 µL
	4X LDS Sample Buffer	7.50 µL	6.25 µL	5.00 µL	3.75 µL
	10X Reducing Agent	3.00 µL	2.50 µL	2.00 µL	1.50 µL
	Total Volume (maximum)	30.0 µL	25.0 µL	20.0 µL	15.0 µL

Note

- Maximum amount of protein and volume depends on size of wells
- Remember to keep a well open for a standard
- Any wells without samples should be filled with loading buffer to maintain an equal charge across the gel
- Be sure to keep the samples on ice to minimize degradation!

2 Incubate prepared samples for 00:10:00 at 70 °C to denature proteins and bind SDS

3 While samples are incubating, prepare the gel and the electrophoresis apparatus

3.1 Make 800 mL of running buffer by diluting 40 mL stock buffer with 760 mL ddH_2O

Note

The use of MES or MOPS depends on antigen resolution desired. Refer to the NuPAGE migration guide: https://www.thermofisher.com/us/en/home/technical-resources/research-tools/image-gallery/image-gallery-detail.3436.html

3.2 Choose desired gel and open package

Note

- Wear gloves, as the buffer in the package contains sodium azide
- The gel percentage and matrix depend on the antigen resolution desired. Refer to the NuPAGE migration guide: https://www.thermofisher.com/us/en/home/technical-resources/research-tools/image-gallery/image-gallery-detail.3436.html

3.3 Rinse the gel cassette with ddH_2O

3.4 Remove the comb from the top and the strip of tape from the bottom

3.5 Place the gel cassette into the apparatus and clamp shut

3.6 Add 1X running buffer to the middle chamber, ensuring that buffer covers the wells, and then to the outer chamber

3.7 Add 500 µL of NuPAGE Antioxidant **Thermo Fisher Scientific Catalog #**NP0005 to the inner chamber

4 Load 15 µL of sample into individual wells using gel loading pipet tips

5 Add 10 µL
Novex Sharp Pre-stained Protein Standard **Thermo Fisher Scientific Catalog #**LC5800
to one well

6 Attach the top of the electrophoresis apparatus, plug the leads into the power supply and turn the power supply on

7 Run at a constant voltage of 180 V until the blue dye front has reached the bottom of the gel

Note

- the yellow front is ~5 kDa
- run time is typically about 00:45:00 for MES and about 01:00:00 for MOPS

Transfer

8 Remove the gel cassette from the apparatus and place onto paper towels. (The cassette is two pieces of plastic that enclose the gel.)

9 Using the provided metal tool, break the cassette at all edges by leveraging the tool between the plastic pieces

10 Making sure not to tear the gel, carefully separate the plastic pieces so that the gel remains on one

11 Using two hands, carefully pick up the gel, separating it from the remaining plastic piece, and place into ddH_2O

12 Use the metal tool to cut off the wells and the bottom of the gel

13 *Optional: The gel can be stained with Coomassie blue to visualize all protein*

14 Activate the PVDF membrane in 100% methanol

15 Assemble the iBlot™ Transfer Stack, PVDF, mini **Thermo Fisher Catalog #**IB401002 in 1X Tris-Glycine transfer buffer containing 20% methanol in the following order: two filter papers, gel, PVDF membrane, two filter papers, sponge.

Note

Be sure to orient the gel onto the membrane in a logical manner. (i.e. keep the largest protein of the ladder on upper left). Use a plastic roller to roll out any bubbles after each stage of the transfer stack has been added.

16 Close and insert the cassette into the transfer box and close the lid, connecting the red lead to the red side and the black to black

17 Run at 75 V for 02:00:00 or at 55 V for 04:00:00 for higher molecular weight proteins

18 When finished, open the lid, remove the cassette, and carefully remove the sponge, filter papers and gel from the membrane, checking that the ladder has transferred from the gel to the membrane. Using forceps, quickly remove the membrane and place in a tray with 1X TBST

19 *Optional: The membrane can be stained with Ponceau S to visualize all protein*