USER GUIDE

novex® by life technologies™

NativePAGE™ Novex® Bis-Tris Gel System

A system for native gel electrophoresis

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Kit Contents and Storage

Types of Products

This manual is shipped with the following products: For ordering information, go to **www.lifetechnologies.com/support** or contact Technical Support (page 40).

Product	Quantity
NativePAGE™ Novex® 3–12% Bis-Tris Gels	Box of 10 gels
NativePAGE™ Novex® 4–16% Bis-Tris Gels	Box of 10 gels

Shipping and Storage

The NativePAGE™ Novex® Bis-Tris Gels are shipped on blue ice. Upon receipt, store the gels at 2ºC to 8ºC.

Do not freeze NativePAGE™ Gels.

The **expiration date** is printed on the gel. To obtain the best results, avoid using expired gels or improperly stored gels.

Product use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

Introduction

Overview

Introduction

The NativePAGE™ Novex® Bis-Tris Gel system is a near neutral pH, pre-cast polyacrylamide mini gel system to perform native (non-denaturing) electrophoresis. The near neutral pH 7.5 environment during electrophoresis results in maximum stability of both proteins and gel matrix, providing better band resolution than other gel systems including the traditional Tris-glycine native electrophoresis (Laemmle) system. The NativePAGE™ Novex® Bis-Tris Gel system provides a sensitive and high-resolution method for analysis of native membrane protein complexes, native soluble proteins, molecular mass estimations, and assessing the purity of native proteins.

NativePAGE™ Gel System

The NativePAGE™ Gel system is based on the Blue Native Polyacrylamide Gel Electrophoresis (BN PAGE) technique developed by Schägger and von Jagow (Schägger & von Jagow, 1991) that uses Coomassie G-250 as a charge-shift molecule. For details on the NativePAGE™ Gel system, see page 4.

In standard SDS-PAGE, the charge-shift molecule is SDS. The SDS denatures proteins and binds to proteins conferring a net negative charge allowing the proteins to migrate in one direction towards the anode. The SDS is present in the sample buffer and running buffer.

In BN PAGE, the Coomassie G-250 binds to proteins and confers a net negative charge while maintaining the proteins in their native state without any protein denaturation. The G-250 is present in the cathode buffer to provide a continuous flow of G-250 into the gel, and is added to samples containing non-ionic detergent prior to loading the samples onto the gel. The gels do not contain any G-250.

The binding of G-250 to proteins offers the following advantages resulting in high-resolution native electrophoresis (Schägger, 2001):

- Proteins with basic isoelectric points (pI) normally have a net positive charge that are converted to proteins with a net negative charge, allowing the proteins to migrate in one direction towards the anode.
- Membrane proteins and proteins with significant surface-exposed hydrophobic area are less prone to aggregation as G-250 binds non-specifically to hydrophobic sites converting them to negatively charged sites.

Applications

The NativePAGE™ Novex® Bis-Tris Gel system is suited for:

- Analyzing native membrane protein complexes or soluble protein complexes
- Determining the purity of native proteins, and estimating molecular masses of native proteins and complexes
- Performing Two-Dimensional Native/SDS-PAGE to resolve complex samples
- Analyzing protein complexes purified using NativePure™ Native Complex Purification System from Life Technologies (page 38)
- Performing in-gel or solution activity assays

Continued on next page

Types of Gels

The NativePAGE™ Novex® Bis-Tris Gels are available in different acrylamide concentrations and well formats (see the following table). Gels are available in 1.0-mm thickness only.

Feature	Bis-Tris Gels
Gel Acrylamide Concentration	3–12% and 4–16%
Well Format	10 and 15 wells

Compatibility

The size of a NativePAGE™ Novex® Bis-Tris Gel is 10 × 10 cm (the gel size is 8 × 8 cm). We recommend using the XCell™ *SureLock*™ Mini-Cell (page 37) for the electrophoresis of NativePAGE™ Novex® Bis-Tris Gels to obtain optimal and consistent performance.

Purpose of the Manual

This manual provides the following information:

- An overview of the NativePAGE™ Electrophoresis System
- Instructions for preparing samples and running buffer
- Instructions for performing native gel electrophoresis using the XCell™ *SureLock*™ Mini-Cell
- Two-Dimensional native/SDS-PAGE protocol
- Protocols for staining using Coomassie and silver staining
- Western blotting protocol using the XCell II™ Blot Module
- Examples of expected results
- Troubleshooting

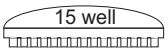
NativePAGE™ Novex® Bis-Tris Gel Specifications

Specifications

Gel Matrix:	Acrylamide/Bisacrylamide
Gel Thickness:	1.0 mm
Gel Size:	8 cm × 8 cm
Cassette Size:	10 cm × 10 cm
Cassette Material:	Styrene Copolymer (recycle code 7)
Sample Well Configuration:	10- and 15-well

Loading Volumes

The recommended loading volumes and protein load per band by the detection method are provided in the following table.

Well Types	Recommended Maximum Load Volume	Maximum Protein Load Per Band by Detection Method		
		Coomassie Staining	Silver Staining	Immuno-blotting
10 Well 	25 µL	1.0 µg/band	Scale your sample load for the sensitivity of your silver staining kit. For use with the SilverQuest™ or SilverXpress ® Silver Staining Kits, we recommend a protein load of ~50 ng/band.	Scale your sample load according to the sensitivity of your detection method.
15 Well 	15 µL	0.5 µg/band		

NativePAGE™ Gel System

Introduction

The ability to maintain native protein conformation and provide high-resolution native electrophoresis makes the NativePAGE™ Bis-Tris Gel System a powerful system for analyzing native protein complexes as compared to traditional native electrophoresis systems such as the Tris-Glycine system (Schägger *et al.*, 1994).

The traditional Tris-Glycine (Laemmle) gel system is the most widely used native electrophoresis system but offers the following limitations:

- The high operative pH of the Tris-Glycine system adversely affects some proteins that are sensitive to high pH conditions
- It is incompatible with native samples that require a non-ionic detergent for protein solubilization

System components and general information on the NativePAGE™ Gel system are included in this section. For system overview, see page 1.

System Components

The NativePAGE™ Novex® Bis-Tris Gel System consists of:

- NativePAGE™ Novex® Bis-Tris [Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl] Mini Gels for separating proteins and protein complexes
- NativePAGE™ Sample Buffer (4X) and NativePAGE™ 5% G-250 Sample Additive for sample preparation
- NativePAGE™ Running Buffer (20X) and NativePAGE™ Cathode Buffer Additive (20X) for native electrophoresis
- NuPAGE® Transfer Buffer for blotting of NativePAGE™ Novex® Bis-Tris Gels

NativePAGE™ Novex® Bis-Tris Gels

The NativePAGE™ Novex® Bis-Tris Gel is a 1.0 mm thick, 8 x 8 cm mini gel used for **native** (non-denaturing) gel electrophoresis of protein samples.

The NativePAGE™ Novex® Bis-Tris Gels are used with NativePAGE™ Running Buffers (see page 5) to produce a non-denaturing electrophoresis system operating at near neutral pH. The near neutral pH environment during electrophoresis results in maximum stability of both proteins and gel matrix, providing better band resolution than other gel systems.

Continued on next page

NativePAGE™ Gel System, Continued

Estimating Size

The use of G-250 charge-shift in NativePAGE™ gels results in protein resolution based upon protein size allowing accurate size estimation of native proteins and protein complexes (Schägger *et al.*, 1994).

However, since the proteins maintain their native conformation, the size estimation may have an expected size estimation error of ~15%. For example, if you estimated the molecular mass of a protein to be 450 kDa using NativePAGE™ gels, the actual mass may vary between 380-520 kDa. Due to the large diversity of protein structure and characteristics, we recommend verifying the molecular mass of native proteins using other techniques such as gel filtration or mass spectrometry.

Differences in size estimations using NativePAGE™ gels are produced by slow migration and overestimation of mass which can arise due to:

- Non-ideal binding of G-250 produces an incomplete or absent charge-shift
- Protein structures that significantly deviate from globularity or that have open interior space have a size, or diameter, that is unusually large for their mass
- Proteins with acidic pI's and compact structures may migrate faster or glycosylated proteins may migrate slower and resolve into diffuse bands due to heterogeneity in glycosylation
- Proteins that bind lipids may migrate at different rates when prepared with different concentrations of detergent due to variation in the amount lipid remaining on the protein at different detergent concentrations.

NativePAGE™ Bis-Tris Buffer System

The NativePAGE™ Bis-Tris non-denaturing buffer system involves three ions:

- Chloride ($^-$) is supplied by the gel buffer and serves as a leading ion due to its ion mobility as compared to other anions in the system. The gel buffer ions are BisTris ($^+$) and Cl^- (pH 6.8).
- Tricine ($^-$) serves as the trailing ion. The running buffer ions are BisTris and Tricine (pH 6.8
- BisTris ($^+$) is the common ion present in the gel buffer and running buffer. During electrophoresis, the operative pH is 7.5.

Advantages

The operating near neutral pH of NativePAGE™ Novex® Bis-Tris Gels and buffers provide the following advantages over the Tris-Glycine (Laemmle) Gel system:

- Longer shelf life of up to 6 months due to improved gel stability
- Allows the protein to retain the native structure and activity as demonstrated by in-gel and in solution activity of proteins after NativePAGE™ electrophoresis (Schägger & von Jagow, 1991; Zerbetto *et al.*, 1997)
- Improved protein stability during electrophoresis at near neutral pH resulting in sharper band resolution and accurate results

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NativePAGE™ Gel System, Continued

Separation Range

The NativePAGE™ Novex® Bis-Tris Gels have a wide range of separation throughout the low and high molecular weight ranges.

The NativePAGE™ Novex® 3–12% Bis-Tris Gels resolve proteins in the molecular weight range of 30-10,000 kDa.

The NativePAGE™ Novex® 4–16% Bis-Tris Gels resolve proteins in the molecular weight range of 15-1,000 kDa.

To choose the correct NativePAGE™ Novex® Bis-Tris Gel for your application, refer to **www.lifetechnologies.com/support**.

Downstream Applications

The NativePAGE™ Novex® Bis-Tris Gels are compatible with most staining protocols including silver, and Coomassie stains.

The SilverQuest™ Silver Staining Kit or SilverXpress® Silver Staining Kit (page 25) is suitable for silver staining of NativePAGE™ Gels. For best results and better background, we recommend using the SilverQuest™ Silver Staining Kit.

The NativePAGE™ Novex® Bis-Tris Gels are compatible with any of the standard Coomassie staining procedures. The Novex® Colloidal Blue Staining Kit (page 22) is recommended for staining NativePAGE™ Gels.

For Western blotting applications, we recommend using a semi-wet transfer apparatus such as the XCell II™ Blot Module (page 29) to blot NativePAGE™ Gels.

Methods

Prepare Samples

Important

Due to the large diversity of proteins present in different cells and tissues, it is not possible to offer a sample preparation protocol that is suitable for all proteins. Based on the starting material and goal of the experiment, the sample preparation protocol needs to be determined empirically.

Brief procedures for sample preparation are described on the following pages. You may use this procedure as a starting point for your lysate and then optimize the procedure based on the initial results.

Objectives of Sample Preparation

The major objectives of sample preparation are to:

- Completely solubilize the proteins
- Maintain proteins in solution in their native state during electrophoresis
- Prevent protein modifications and proteolysis

NativePAGE™ Sample Prep Kit

The NativePAGE™ Sample Prep Kit (page 38) includes sample preparation reagents for native gel electrophoresis. The kit includes ready-to-use detergent solutions (10% DDM and 5% Digitonin) that improve the solubility of hydrophobic and membrane proteins during sample preparation.

The samples prepared with 10% DDM (n-dodecyl-β-D-maltoside), 5% Digitonin, or the NativePAGE™ Sample Prep Kit are compatible with NativePAGE™ Novex® Bis-Tris Gels for native gel electrophoresis showing increased resolution and reduced streaking.

NativePAGE™ 5% G-250 Sample Additive

The NativePAGE™ 5% G-250 Sample Additive is a concentrated stock solution of Coomassie G-250 designed for use with detergent (non-ionic) containing samples prepared for NativePAGE™ gel electrophoresis.

The G-250 dye displaces detergent or loosely bound lipid molecules from membrane proteins and protein complexes prepared in native buffers containing non-ionic detergents, converting hydrophobic sites to negatively charged sites required for NativePAGE™ electrophoresis (see page 1 for details). This prevents membrane proteins from aggregating during separation on a NativePAGE™ gel which does not contain any solubilizing detergent. The G-250 dye also binds to detergent molecules in the sample and carries them in the dye-front, ahead of resolving proteins to minimize vertical streaking.

The NativePAGE™ 5% G-250 Sample Additive is added to detergent containing samples just prior to loading samples onto a NativePAGE™ gel such that the final G-250 concentration in the sample is $1/4^{th}$ to $1/10^{th}$ of the detergent concentration (Schägger, 2001).

Continued on next page

Prepare Samples, Continued

NativePAGE™ Sample Buffer (4X)

Use the NativePAGE™ Sample Buffer (4X) to prepare samples for native (non-denaturing) gel electrophoresis with the NativePAGE™ Novex® Bis-Tris Gels.

The NativePAGE™ Sample Buffer (4X) is formulated for native gel electrophoresis and contains BisTris buffer, pH 7.2, NaCl, glycerol, and Ponceau S.

General Guidelines

- Solubilize the proteins or protein complexes using the minimum amount of detergent necessary for maximal solubilization.
- Maintain the samples on ice during sample preparation and **do not heat** samples prior to electrophoresis.
- You may need to prepare your protein samples with 10% DDM (page 38), 5% Digitonin (page 37), or other detergents to determine the best solubilizer for your protein (Eubel *et al.*, 2005; Schägger, 2001).
- Maintain the salt concentration of the sample at < 50 mM.
- For detergent containing samples, always add NativePAGE™ 5% G-250 Sample Additive prior to loading samples onto the gel.
- For detergent-free samples, addition of NativePAGE™ 5% G-250 Sample Additive is optional.
- Prepare samples in 1X NativePAGE™ Sample Buffer, if possible.
- If your sample is in a SDS-PAGE sample buffer, prepare a fresh lysate **without** SDS using the detergents included in the sample prep kit. **Do not** use SDS-PAGE samples for native gel electrophoresis.
- You may add protease inhibitors in your sample preparation. Various protease inhibitor cocktails are commercially available.
- Avoid using a complex sample preparation strategy as it may result in protein loss.

Note

- If a precipitate forms in the 5% Digitonin solution, heat the solution at 95°C for 5 minutes and vortex slowly to dissolve the precipitate. Cool to room temperature prior to use. The 5% Digitonin will stay in solution at room temperature for up to a week. You may reheat the solution multiple times without any loss in activity.
- Always wear gloves, protective eyewear, and a laboratory coat while handling the detergents. Digitonin is toxic and handle with care, avoid any exposure of Digitonin to skin.

NativeMark™ Unstained Protein Standard

NativeMark™ Unstained Protein Standard (page 38) is specifically designed for use with NativePAGE™ Novex® Bis-Tris Gels and consists of 8 protein bands that allow accurate molecular weight estimation in the range of ~20–1200 kDa. The standard is supplied in a ready-to-use format and is easily visualized with Coomassie or silver staining, and also with membrane stains such as Ponceau S, or Coomassie after western transfer.

Continued on next page

Prepare Samples, Continued

Materials Needed

You will need the following items. See page 38 for ordering information.

- Protein sample
- NativeMark™ Unstained Protein Standard
- NativePAGE™ Sample Buffer (4X)
- Deionized water
- Homogenization unit for tissue samples
- *Optional:* Protease inhibitor cocktail and Benzonase nuclease (Sigma, cat. no. E-1014)

For samples that need detergent solubilization:

- NativePAGE™ Sample Prep Kit, 10% DDM (n-dodecyl-β-D-maltoside), or 5% Digitonin
- NativePAGE™ 5% G-250 Sample Additive

Prepare Cell/Tissue Lysates

Use this procedure as a starting point; optimize detergent concentration as described in **Optimizing Detergent Concentration** (next page), especially if you are solubilizing the sample for the first time.

1. To 10–50 mg (wet weight) minced animal/plant tissue, *E. coli* cells, or mammalian cells, add the following to the sample with a final volume of 1 mL:

Reagent	Final Conc.
NativePAGE™ Sample Buffer (4X)	1X
10% DDM or 5% Digitonin	1%

2. Homogenize the samples on ice as follows:
   - **For *E. coli***, sonicate the sample **on ice** for 3 rounds of 15 seconds each at ~50% power with cooling the sample on ice between sonications.
   - **For mammalian cells**, pipet the solution up and down several times.
   - **For tissue samples**, use an appropriate homogenization unit.
3. Centrifuge the lysate at 20,000 × g for 30 minutes at 4°C. You may need to use ultracentrifugation at 100,000 × g for 15 minutes for some samples to clarify the lysate.
4. ***Optional Benzonase treatment:*** For samples with high DNA content such as tissue or cell samples, we recommend a benzonase (endonuclease) treatment to reduce protein streaking as follows:

   To the sample from Step 3, add $MgCl_2$ to a final concentration of 2 mM and 1–2 units benzonase per µL of sample. Mix well and incubate at room temperature for 30–60 minutes. Perform centrifugation as described on Step 3.
5. Aliquot the supernatant into sterile microcentrifuge tubes and store at −80ºC until use. Discard the pellet.
6. Determine the lysate protein concentration using the Qubit® Protein Assay Kit (page 38) or BCA protein assay.

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Prepare Organelle Extracts

Protocol for preparing extracts from isolated organelles such as chloroplasts or mitochondria (Graham & Rickwood, 1997) is described below.

Use this procedure as a starting point; optimize detergent concentration as described in **Optimizing Detergent Concentration**, below, especially if you are solubilizing the sample for the first time.

1. Thaw an aliquot of the isolated, pelleted organelle sample on ice before extraction.
2. Solubilize the organelle proteins in cold 1X NativePAGE™ Sample Buffer containing 0.5–2% DDM or Digitonin. Mix by pipetting up and down and by inversion.
3. Incubate the sample on ice for 15 minutes.
4. Centrifuge the lysates at 20,000 × g for 30 minutes at 4ºC.
5. Aliquot the supernatant into sterile microcentrifuge tubes and store at −80ºC until use. Discard the pellet.
6. Determine the lysate protein concentration using Qubit® Protein Assay Kit (page 38) or BCA protein assay.

Optimize Detergent Concentration

To obtain optimal solubilization of membrane proteins from your samples, you may need to optimize the detergent concentrations based on your initial results.

As a starting point, we recommend using DDM or Digitonin at a final concentration of 1%. For optimal results, you may vary the final DDM concentration from 0.5–5% and final Digitonin concentrations from 0.5–2.5% for your samples. If you need to use higher detergent concentration, you may need to purchase the detergent powder. Contact Technical Support (page 40) for details. Increasing the incubation time of the sample with detergents may also increase protein solubilization.

Continued on next page

Prepare Samples, Continued

Prepare Samples for NativePAGE™ Gels

See page 3 for the recommended protein load.

For samples prepared in 1X NativePAGE™ Sample Buffer and detergents (pages 9–10), add ONLY the NativePAGE™ 5% G-250 Sample Additive immediately prior to electrophoresis as described below. There is no need to add NativePAGE™ Sample Buffer.

For samples prepared in buffers other than 1X NativePAGE™ Sample Buffer, prepare your samples in a total volume of 10 µL as described below. If you need to prepare samples in a volume of 20–40 µL, adjust the volume accordingly.

Reagent	Sample with Detergent	Detergent-free Sample
Sample	*x* µL	*x* µL
NativePAGE™ Sample Buffer (4X)	2.5 µL	2.5 µL
NativePAGE™ 5% G-250 Sample Additive	0.25–1 µL*	optional
De onized Water	to 10 µL	to 10 µL

Mix well. **Do not heat samples for native gel electrophoresis.**

*Ensure the G-250 concentration is 1/4th the detergent concentration.

Prepare Running Buffer

Running Buffers

Two types of NativePAGE™ Running Buffers are used for native gel electrophoresis of NativePAGE™ Novex® Bis-Tris Gels. See page 38 for purchasing pre-made buffers or page 40 for buffer recipes.

- NativePAGE™ 20X Running Buffer
- NativePAGE™ 20X Cathode Buffer Additive, contains 0.4% Coomassie G-250 (added to NativePAGE™ Running Buffer to generate the Cathode Buffer)

Choose the Appropriate Cathode Buffers

Two types of Cathode Buffer are used in the NativePAGE™ Gel Electrophoresis system:

- Dark Blue Cathode Buffer (contains 0.02% G-250)
- Light Blue Cathode Buffer (contains 0.002% G-250)

The choice of Cathode Buffer depends on the sample type and downstream applications (see table below) as Dark Blue Cathode Buffer interferes with some applications.

If you are using….	and performing….	then Choose the
Detergent Samples	Coomassie Staining	Dark Blue Cathode Buffer.
	Silver Staining	Light Blue Cathode Buffer.
	Western Blotting or Two-dimensional (2D) electrophoresis	Dark Blue Cathode Buffer until the dye front migration is 1/3rd of the gel, pause the run, remove the Dark Blue Cathode Buffer with a pipet, and replace buffer with the Light Blue Cathode Buffer before resuming the run.
Non-detergent Samples	Coomassie Staining	Dark or Light Blue Cathode Buffer
	Silver Staining	Light Blue Cathode Buffer
	Western Blotting or 2D electrophoresis	Light Blue Cathode Buffer

Continued on next page

Prepare Running Buffer, Continued

Materials Needed

You will need the following items. See page 38 for ordering information.

- NativePAGE™ Running Buffer (20X)
- NativePAGE™ Cathode Buffer Additive (20X)
- Deionized water

Prepare Anode Buffer

Instructions to prepare 1000 mL 1X NativePAGE™ Anode Buffer are described below. Scale-up the volume of reagents accordingly if more buffer is needed.

1. Prepare 1000 mL 1X NativePAGE™ Anode Buffer using NativePAGE™ Running Buffer (20X) as follows:

NativePAGE™ Running Buffer (20X)	50 mL
Deionized Water	950 mL
Total Volume	1000 mL

2. Mix thoroughly and use ~600 mL of the 1X NativePAGE™ Anode Buffer in the Lower (Outer) Buffer Chamber.

Prepare Cathode Buffer

Instructions to prepare 200 mL 1X NativePAGE™ Cathode Buffer (Dark or Light Blue) are described below. Scale-up the volume of reagents accordingly if more buffer is needed.

1. Prepare 200 mL 1X NativePAGE™ Cathode Buffer using NativePAGE™ Running Buffer (20X) and NativePAGE™ 20X Cathode Additive as follows:

Reagents	**Dark Blue**	**Light Blue**
NativePAGE™ Running Buffer (20X)	10 mL	10 mL
NativePAGE™ Cathode Additive (20X)	10 mL	1 mL
Deionized Water	180 mL	189 mL
Total Volume	200 mL	200 mL

2. Mix thoroughly and use ~200 mL of the appropriate 1X NativePAGE™ Cathode Buffer in the Upper (Inner) Buffer Chamber.

Perform Electrophoresis

Introduction

Instructions are provided below for electrophoresis of the NativePAGE™ Novex® Bis-Tris Gels using the XCell™ *SureLock*™ Mini-Cell from Life Technologies (page 38). For more information on the XCell™ *SureLock*™ Mini-Cell, refer to the manual supplied with the unit or from **www.lifetechnologies.com/manuals**.

If you are using the NativePAGE™ Gels with any other electrophoresis units, follow the manufacturer's recommendations.

Gels are individually packaged in clear pouches with Packaging Buffer. The Packaging Buffer contains low levels of residual acrylamide monomer and 0.02% sodium azide. Wear gloves at all time when handling gels.

Warning: This product contains a chemical (acrylamide) known to the state of California to cause cancer. To obtain a Safety Data Sheet (SDS), see page 40.

Important

To ensure success with the NativePAGE™ electrophoresis system, remember the important points listed below:

- Under **no** circumstances, use NuPAGE® MES or MOPS SDS Running Buffers, or Tris-Glycine SDS buffers with NativePAGE™ Gels
- Use **only** NativePAGE™ Running buffers (see previous page)
- **Do not heat or boil** samples
- Inner and Outer Buffer Chambers MUST be filled with the recommended amount of running buffer (page 16) to obtain optimal results

Materials Needed

You will need the following items:

- Protein sample (see page 7 for sample preparation)
- NativeMark™ Unstained Protein Standard (page 38)
- 1X NativePAGE™ Anode and Cathode Buffers (page 12)
- Gel loading tips
- XCell™ *SureLock*™ Mini-Cell and power supply (page 38)
- Appropriate NativePAGE™ Novex® Bis-Tris Gels

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Perform Electrophoresis, Continued

Follow these recommendations to obtain the best results:

- Load samples onto the gel prior to filling the Upper Buffer chamber to provide easy visualization of the sample wells containing the blue Cathode Buffer.
- Perform electrophoresis in the cold room with pre-chilled buffers or at room temperature with pre-chilled buffers depending on your protein sample.
- To promote a uniform running of the stacking front, load sample buffer in all empty wells.
- Run the gels immediately after loading the samples.
- Use the appropriate Dark or Light Blue Cathode Buffer for electrophoresis based on your sample type and downstream applications (page 12).
- To remove NativePAGE™ Novex® 3–12% Bis-Tris Gels from the cassettes, allow the gel to fall off of the plastic cassette by inverting the cassette over the staining container and pushing the foot of the gel through the slot with a gel knife (page 17). Since NativePAGE™ Novex® 3–12% Bis-Tris Gels contain low acrylamide percentage making the gels more fragile, handle these gels carefully by only handling the bottom, higher percentage acrylamide part of the gel.

Prepare Gel Cassettes

1. Cut open the gel cassette pouch and drain away the gel packaging buffer.
2. Remove the gel from the pouch.
3. Rinse the gel cassette with deionized water. Peel off the tape from the bottom of the cassette.
4. In one smooth motion, gently pull the comb out of the cassette.
5. Rinse the sample wells with 1X NativePAGE™ Cathode Buffer. Invert the gel and shake to remove the buffer. Repeat two more times. Fill the wells with 1X NativePAGE™ Cathode Buffer. Be sure to displace all air bubbles from the wells, as they will affect sample running.

Note

- Always handle the cassette by its edges only.
- Upon removal of the comb, a thin layer of polyacrylamide may be observed in some sample wells of NativePAGE™ Gels. The sample loading or gel performance is not affected by the thin polyacrylamide layer.

Continued on next page

Perform Electrophoresis, Continued

Procedure Using XCell™ *SureLock*™ Mini-Cell

Instructions for performing electrophoresis using the XCell™ *SureLock*™ Mini-Cell are described below.

XCell™ *SureLock*™ Mini-Cell requires ~200 mL buffer for the Upper Buffer Chamber and ~600 mL buffer for the Lower Buffer Chamber.

1. Orient the two gels in the Mini-Cell such that the notched "well" side of the cassette faces inwards toward the Buffer Core. Seat the gels on the bottom of the Mini-Cell and lock into place with the Gel Tension Wedge. Refer to the XCell™ *SureLock*™ Mini-Cell manual for detailed instructions.

   Note: If you are using only one gel, replace the second gel cassette with the plastic Buffer Dam.

2. Load an appropriate volume of the protein sample at the desired protein concentration onto the gel (see page 3 for recommended loading volumes).

   Note: Samples are loaded before filling the Upper Buffer chamber to provide easy visualization of the sample wells containing the blue Cathode Buffer. If you try to load the samples after filling the buffer chamber, sample wells are not clearly visible causing erroneous sample loading.

3. Load 5 µL (10-well gel) or 3 µL (15-well gel) of NativeMark™ Unstained Protein Standard.

4. 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 refill.

5. Once the seal is tight, fill the Upper Buffer Chamber (inner) with ~200 mL of the appropriate (Dark or Light) 1X Cathode Buffer (see page 12 to choose the appropriate Cathode buffer). The buffer level must exceed the level of the wells.

6. Fill the Lower (outer) Buffer Chamber with ~600 mL of the 1X Anode Buffer (page 13).

7. Place the lid on the assembled Mini-Cell. The lid firmly seats if the (-) and (+) electrodes are properly aligned.

8. With the power **off**, connect the electrode cords to power supply. Turn on the power. See next page for running conditions.

Continued on next page

Perform Electrophoresis, Continued

Run Conditions

Perform electrophoresis as described in the table below. Current readings are per gel.

Note: If you are using samples with detergents and performing Western Blotting or 2D electrophoresis, be sure to replace the Dark Blue Cathode Buffer with Light Blue Cathode Buffer once the dye front migration is 1/3rd of the gel (see page 12).

NativePAGE™ Bis-Tris Gel	Voltage	Run Time	Expected Current
Standard, Room Temperature Run	150 V Constant	90–115 minutes (3–12% gel) 105–120 minutes (4–16% gel)	Start: 12–16 mA End: 2–4 mA
Low Temperature (4ºC) Run	150 V Constant for 60 minutes, then increase voltage to 250 V Constant for the remainder of the run (30–90 minutes)		Start: 8–10 mA End: 2–4 mA

Remove Gel after Electrophoresis

1. After electrophoresis is complete, shut off the power, disconnect electrodes, and remove gel(s) from the XCell™ *SureLock*™ Mini-Cell.
2. If you are performing second dimension SDS-PAGE (page 19), mark the lanes on the cassette that will be excised prior to opening the cassette.
3. Separate each of the three bonded sides of the cassette by inserting the Gel Knife into the gap between the cassette's two plates. The notched ("well") side of the cassette faces up.
4. Push down gently on the knife handle to separate the plates. Repeat on each side of the cassette until the plates are completely separated.

   Caution: Use caution while inserting the gel knife between the two plates to avoid excessive pressure towards the gel.
5. Carefully remove and discard the top plate, allowing the gel to remain on the bottom (slotted) plate.
6. Hold the plate such that the gel is facing downwards over a container (containing the appropriate staining reagent for staining or transfer buffer for Western blotting) and push the gel foot through the slot with a Gel Knife to allow the gel to peel from the plate and into the container.
7. Excise the gel foot and sample wells with a Gel Knife once the gel is in the container.

Continued on next page

Perform Electrophoresis, Continued

Note

The NativePAGE™ Gels appear deep blue in color after electrophoresis and some highly abundant protein bands may be visible due to staining with the Coomassie G-250 in the sample additive and Cathode Buffer.

To obtain sensitive staining, you need to denature the proteins to expose more hydrophobic sites for dye-binding and perform fixing, staining, and destaining steps. For detailed staining protocols, see pages 22–25.

Activity Assays

Since the NativePAGE™ gels employ native (non-denaturing) conditions; the proteins may still remain native and active after electrophoresis. The activity of the proteins can be verified using in-gel or solution assays (Manchenko, 1994). See page 33 for an example of results.

Note: Protein activity after NativePAGE™ electrophoresis is usually dependent on the sensitivity of the proteins to the buffers used in electrophoresis.

Two-Dimensional Native/SDS-PAGE

Introduction

Two-Dimensional (2D) native/SDS-PAGE combines native gel electrophoresis using NativePAGE™ Gels in the first dimension followed by analyzing proteins (usually from one lane of the gel) using second dimension SDS-PAGE.

Instructions to perform second dimension SDS-PAGE are described below.

Second Dimension SDS-PAGE

The 2D electrophoresis procedure involves reducing and alkylating the proteins separated on the NativePAGE™ Gel in buffers, loading the gel (lane) strip on a second dimension SDS gel, and performing SDS-PAGE.

SDS Gel

We recommend using the following for 2D SDS-PAGE:

- NuPAGE® or Tris-Glycine SDS Gel with a 2D-well. The length of the 2D-well of a Novex® SDS gel is 6.5 cm.
- NuPAGE® or Tris-Glycine ZOOM® Gel with an IPG-well. The length of the IPG-well of a ZOOM® Gel is 7.1 cm.

Note

For 2D analysis, we recommend that you run the sample in duplicates on the NativePAGE™ gel. Excise each lane and stain one lane with a protein stain to visualize the protein bands and process the second lane for second dimension SDS-PAGE. This will allow you to orient the protein spots obtained after 2D analysis.

Materials Needed

You will need the following items. See page 38 for ordering information.

- NuPAGE® LDS Sample Buffer (4X)
- NuPAGE® Sample Reducing Agent
- NuPAGE® Novex® Bis-Tris Gel or Novex® Tris-Glycine Gel with 2D-well or IPG-well
- Appropriate running buffer depending on the type of gel you are using
- Sterile 15 mL conical tubes
- Ethanol
- N,N-Dimethylacrylamide (DMA); Aldrich, Cat. no. 27413-5

Continued on next page

Two-Dimensional Native/SDS-PAGE, Continued

Note

Incubating the NativePAGE™ gel strip in NuPAGE® LDS Sample Buffer equilibrates the proteins on the strip in SDS buffer and prepares the proteins for 2D SDS-PAGE. We recommend using the NuPAGE® LDS Sample Buffer with NuPAGE® Novex® Bis-Tris Gels or Novex® Tris-Glycine ZOOM® Gels. See the following sections for the procedure to equilibrate the gel strip.

Prepare Buffers

Prepare the following buffers:

Reducing Solution

1. Dilute NuPAGE® LDS Sample Buffer (4X) to 1X with deionized water. You will need ~20 mL for each strip.
2. Add 0.5 mL NuPAGE® Sample Reducing Agent (10X) to 4.5 mL 1X NuPAGE® LDS Sample Buffer from Step 1 in a 15 mL conical tube to obtain 1X NuPAGE® LDS Sample Buffer with 50 mM DTT (dithiothreitol)

Alkylating Solution

Add 28 µL of DMA to 5 mL 1X NuPAGE® LDS Sample Buffer from Step 1 to obtain 1X NuPAGE® LDS Sample Buffer with 50 mM DMA. Mix well.

Quenching Solution

Add 0.05 mL NuPAGE® Sample Reducing Agent (10X) and 1 mL ethanol to 4 mL 1X NuPAGE® LDS Sample Buffer from Step 1 in a 15-mL conical tube to obtain 1X NuPAGE® LDS Sample Buffer with 5 mM DTT and 20% ethanol.

Equilibrate the Gel Strip

1. After electrophoresis, mark the lane on the cassette that will be excised prior to opening the cassette.
2. Remove the gel from the cassette as described on page 17.
3. With the gel adhering to one plate, excise the desired gel strip (lane) along the markings on the cassette made in Step 1 using a Gel Knife.
4. Carefully transfer each gel strip to a sterile 15-mL conical tube. Be sure to handle the gel strip around the high percentage acrylamide area only.
5. Add 5-mL Reducing Solution (see recipe above) to each tube.
6. Incubate for 15–30 minutes at room temperature. Decant the Reducing Solution.
7. Add 5 mL Alkylating Solution (see recipe above) to each tube.
8. Incubate for 15–30 minutes at room temperature. Decant the Alkylating Solution.
9. Add 5 mL Quenching Solutions (see recipe above) to each tube.
10. Incubate for 15 minutes at room temperature. Decant the Quenching Solution.

Use the equilibrated gel strip immediately for SDS-PAGE.

Continued on next page

Two-Dimensional Native/SDS-PAGE, Continued

Apply the Gel Strip

Apply the equilibrated gel strip to the second dimension SDS gel as described below:

1. Remove the appropriate gel cassette from the pouch and peel off the tape covering the slot on the back of the gel cassette.
2. In one smooth motion, gently pull the comb out of the cassette.
3. If the molecular weight marker well in the gel is bent, straighten the well using a gel loading tip.
4. Fill the 2D-well or the IPG-well with 1X appropriate running buffer and lay the cassette flat on a benchtop.
5. Transfer the equilibrated gel strip onto the plastic plate above the sample well of the gel. Align the gel strip exactly above the sample well.
6. Trim the gel strip with a scalpel to ensure that the gel strip fits into the sample well.
7. Hold the gel cassette to a vertical position and gently slide the gel strip into the sample well using a thin plastic tool such as a ruler. Make sure the gel strip is in contact with the second dimension SDS gel and there are no bubbles between the gel strip and second dimension SDS gel.
8. Overlay the gel strip with a total of 60 µL 1X NuPAGE® LDS Sample Buffer by adding 20 µL buffer to the left side of the lane, 20 µL buffer to the middle of the lane, and 20 µL to the right side of the lane such that the buffer forms a thin layer on top of the gel strip.
9. Insert gel into the mini-cell, fill the buffer chambers with 1X SDS Running Buffer, and perform SDS-PAGE using the appropriate run parameters.

See page 34 for an example of 2D electrophoresis results.

Coomassie Staining of NativePAGE™ Gels

Introduction

Coomassie staining instructions for NativePAGE™ Novex® Bis-Tris Gels are described in this section.

General Staining Guidelines

Follow the general guidelines listed below to obtain the best results:

- The volume of fixing, staining, and destaining solutions will depend on the volume of your staining container. To obtain good results, the volume of the solution must be sufficient to cover the gel completely and to allow the gel to move freely during all of the steps.
- When using a microwave oven for staining, be sure the gel is completely covered in the solution and use a microwaveable staining container. Use caution while using staining reagents in a microwave oven. Do not overheat the staining solutions.

Materials Needed

You will need the following items:

- Appropriate staining containers
- Shaker
- Deionized water
- Fix Solution (40% methanol, 10% acetic acid)
- Destain Solution (8% acetic acid)
- Microwave oven (~1100 Watts)
- Colloidal Blue Staining Kit (for sensitive staining protocol)
- 0.02% Coomassie R-250 in 30% methanol and 10% acetic acid (for Coomassie R-250 protocol)

Note

Use the Fast Coomassie G-250 staining protocol to quickly stain the NativePAGE™ Gels (sensitivity: ~60 ng BSA) as described on the next page.

If you need sensitive Coomassie G-250 staining, use the High Sensitivity staining protocol (sensitivity: ~30 ng BSA) as described on page 24.

Continued on next page

Coomassie Staining of NativePAGE™ Gels, Continued

Coomassie R-250 Staining

A Coomassie R-250 staining protocol for NativePAGE™ Gels is described below.

The total staining time is ~4–5 hours and sensitivity is **~50 ng** BSA. For a more sensitive staining protocol, see next page.

1. Place gel in 100 mL Fix solution (40% methanol, 10% acetic acid) and microwave on high (950–1100 watts) for 45 seconds.
2. Shake the gel on an orbital shaker for 15–30 minutes at room temperature. Decant Fix Solution.
3. Repeat Steps 1 and 2 once **for NativePAGE™ Novex® 4–16% Bis-Tris Gels only**.
4. Add 100 mL Coomassie R-250 Stain (0.02% Coomassie R-250 in 30% methanol and 10% acetic acid) and microwave on high for 45 seconds.
5. Shake the gel on an orbital shaker for 15–30 minutes. Decant the stain.
6. Add 100 mL Destain Solution (8% acetic acid) and microwave on high (950–1100 watts) 45 seconds.
7. Shake the gel on an orbital shaker at room temperature until the desired background is obtained.

Fast Coomassie G-250 Staining

A fast staining protocol for NativePAGE™ Gels using the Coomassie G-250 from the cathode buffer additive is described below.

Be sure to use the **Dark Blue Cathode Buffer** for the electrophoresis run, if you plan to stain the gel using the Fast Coomassie G-250 staining protocol.

The total staining time is ~2–3 hours and sensitivity is **~60 ng** BSA. For a more sensitive staining protocol, see next page.

1. Place gel in 100 mL Fix solution (40% methanol, 10% acetic acid) and microwave on high (950–1100 watts) for 45 seconds.
2. Shake the gel on an orbital shaker for 15 minutes at room temperature. Decant Fix Solution.
3. Add 100 mL Destain Solution (8% acetic acid) and microwave on high (950–1100 watts) 45 seconds.
4. Shake the gel on an orbital shaker at room temperature until the desired background is obtained.

See page 32 for an example of results.

Continued on next page

Coomassie Staining of NativePAGE™ Gels, Continued

High Sensitivity Coomassie G-250 Staining

A sensitive staining protocol for NativePAGE™ Gels using the Colloidal Blue Staining Kit is described below.

The total staining time is ~16 hours and sensitivity is **~30 ng** BSA.

1. Place gel in 100 mL Fix solution (40% methanol, 10% acetic acid) and microwave on high (950–1100 watts) for 45 seconds.
2. Shake the gel on an orbital shaker for 15–30 minutes at room temperature. Decant Fix Solution.
3. During the gel incubation, prepare 100 mL Stain Solution using the reagents included with the Colloidal Blue Staining Kit (page 37) as follows. Be sure to shake Stainer B before using.

Stainer A	20 mL
Stainer B	5 mL
Methanol	20 mL
Deionized water	55 mL

4. Repeat Steps 1 and 2 once **for NativePAGE™ Novex® 4–16% Bis-Tris Gels only**.
5. Add 100 mL Stain Solution from Step 3 and shake the gel on an orbital shaker overnight at room temperature. Decant the stain solution.
6. Add 100 mL Destain Solution (8% acetic acid) and shake the gel on an orbital shaker at room temperature for 5 minutes. Decant the Destain Solution.
7. Add 100 mL deionized water and shake on an oribital shaker until the desired background is obtained.

Drying NativePAGE™ Gels

The stained NativePAGE™ Gels can be dried for storage or analysis by vacuum-drying or air-drying. We recommend using the DryEase® Mini Gel Drying Kit (page 38) to air-dry the gel.

Silver Staining of NativePAGE™ Gels

Introduction

Silver staining instructions for NativePAGE™ Novex® Bis-Tris Gels are described in this section. For details, refer to the SilverQuest™ Silver Staining Kit or SilverXpress® Silver Staining Kit manual supplied with the staining kits or available from **www.lifetechnologies.com/manuals**.

Note

For silver staining of NativePAGE™ Gels, the Fixative (40% ethanol, 10% acetic acid in ultrapure water) is used to fix the proteins and also as a wash solution to remove any remaining G-250 after electrophoresis. Be sure to perform the fixing and washing as indicated to obtain a low background.

Follow these guidelines to obtain the best results:

- The volume of fixing, staining, and destaining solutions will depend on the volume of your staining container. To obtain good results, the volume of the solution must be sufficient to cover the gel completely and to allow the gel to move freely during all of the steps.
- Always use ultrapure water of >18 megohm/cm resistance, clean glass containers, and Teflon-coated stir bars for preparing and handling all solutions.
- Use freshly made solutions.
- Perform all incubations on a rotary shaker rotating at a speed of 1 revolution/second at room temperature.
- Avoid touching the gel with bare hands or metal objects.
- Be sure to use the Light Blue Cathode Buffer during electrophoresis (page 12).
- Maintain the volumes and incubation times of steps as described in the protocol.

Materials Required But Not Supplied

You will need the following items:

- Incubation Trays (page 38) or appropriate staining containers
- Shaker
- Ultra pure water
- Ethanol
- SilverQuest™ Silver Staining Kit or SilverXpress® Silver Staining Kit (page 38)
- Fixative (40% ethanol, 10% acetic acid in ultrapure water)

Continued on next page

Silver Staining of NativePAGE™ Gels, Continued

Preparing Solutions for SilverQuest™ Staining Protocol

Use the reagents provided in the kit to prepare the following solutions for **SilverQuest**™ Silver staining protocol:

- Sensitizing solution

Ethanol	30 mL
Sensitizer	10 mL
Ultrapure water	to 100 mL

- Staining solution

Stainer	1 mL
Ultrapure water	to 100 mL

- Developing solution

Developer	10 mL
Developer enhancer	1 drop
Ultrapure water	to 100 mL

Preparing Solutions for SilverXpress® Staining Protocol

Use the reagents provided in the kit to prepare the following solutions for **SilverXpress**® Silver staining protocol:

- Sensitizing solution

Methanol	100 mL
Sensitizer	5 mL
Ultrapure water	105 mL

- Staining solution

Stainer A	5 mL
Stainer B	5 mL
Ultrapure water	90 mL

- Developing solution

Developer	5 mL
Ultrapure water	95 mL

- Stopping Solution

Stopper	5 mL

Continued on next page

Silver Staining of NativePAGE™ Gels, Continued

SilverQuest™ Silver Staining Protocol

Instructions for staining gels with SilverQuest™ Silver Staining Kit are included in this section.

1. After electrophoresis, remove the gel and place the gel in a clean staining tray containing 100 mL Fixative.
2. Fix the gel in 100 mL Fixative for 1–2 hours with shaking.
3. Decant Fixative. Add 100 mL fresh Fixative and continue the fixing step for 8–16 hours. Decant Fixative.
4. Add 100 mL fresh Fixative and incubate for 2 hours.
5. Decant Fixative and wash the gel in 30% ethanol for 10 minutes.
6. Decant the ethanol and add 100 mL Sensitizing solution. Incubate for 10 minutes.
7. Decant the Sensitizing solution and wash the gel in 100 mL 30% ethanol for 10 minutes.
8. Wash the gel in 100 mL ultrapure water for 10 minutes.
9. Incubate the gel in 100 mL Staining solution for 15 minutes.
10. After staining is complete, decant the Staining solution and wash the gel with 100 mL ultrapure water for 20–60 seconds.

    Note: Washing the gel for more than a minute removes silver ions from the gel resulting in decreased sensitivity.
11. Incubate the gel in 100 mL Developing solution for 4–8 minutes until the desired band intensity is reached.
12. When the desired staining intensity is achieved, immediately add 10 mL Stopper directly to the gel still immersed in Developing solution. Gently agitate the gel for 10 minutes. The color changes from pink to colorless indicating the end of development.
13. Decant the Stopper solution and wash the gel with 100 mL ultrapure water for 10 minutes.

See page 32 for an example of silver staining results.

Continued on next page

Silver Staining of NativePAGE™ Gels, Continued

SilverXpress® Silver Staining Protocol

Instructions for staining gels with SilverXpress® Silver Staining Kit are described below.

Step	Solution	Vol/Gel	Time
1A	Incubate the gel in three changes of Fixative	100 mL	1–2 hours
1B		100 mL	8–16 hours
1C		100 mL	2 hours
2A	Decant the Fixing Solution and incubate the gel in two changes of Sensitizing Solution.	100 mL	30 minutes
2B		100 mL	30 minutes
3A	Decant the Sensitizing Solution and rinse the gel twice with ultra pure water.	200 mL	5 minutes
3B		200 mL	5 minutes
4	Incubate the gel in Staining Solution.	100 mL	15 minutes
5A	Decant the Staining Solution and rinse the gel twice with ultra pure water.	200 mL	5 minutes
5B		200 mL	5 minutes
6	Incubate the gel in Developing Solution.	100 mL	3–15 minutes
7	Add the Stopping Solution directly to the gel when the desired staining intensity is reached.	5 mL	10 minutes
8A	Decant the Stopping Solution and wash the gel three times in ultra pure water.	200 mL	10 minutes
8B		200 mL	10 minutes
8C		200 mL	10 minutes

Drying NativePAGE™ Gels

The stained NativePAGE™ Gels can be dried for storage or analysis by vacuum-drying or air-drying. We recommend using the DryEase® Mini Gel Drying Kit (page 38) to air-dry the gel.

Western Blotting

Introduction

Instructions for Western blotting of NativePAGE™ Gels using the XCell II™ Blot Module are described in this section.

If you are using any other blotting apparatus, follow the manufacturer's recommendations.

For details, refer to the XCell II™ Blot Module manual supplied with the unit or available from **www.lifetechnologies.com/manuals**.

NuPAGE® Transfer Buffer

The NuPAGE® Transfer Buffer is recommended for western transfer of proteins from NativePAGE™ Novex® Bis-Tris Gels. The transfer buffer maintains the neutral pH environment established during gel electrophoresis, protects against modification of the amino acid side chains, and is compatible with N-terminal protein sequencing using Edman degradation. Pre-made NuPAGE® Transfer Buffer (20X) is available separately (page 38) or refer to the NuPAGE® Technical Guide, which is available from **www.lifetechnologies.com/manuals**, for buffer recipes.

Blotting Membrane

PVDF is the recommended blotting membrane for western blotting with NativePAGE™ Gels. Nitrocellulose is **not** compatible for blotting NativePAGE™ Gels since the nitrocellulose membrane binds the Coomassie G-250 dye very tightly and is not compatible with alcohol-containing solutions used to destain the membrane and fix the proteins (page 31).

- Be sure to replace the Dark Blue Cathode Buffer with Light Blue Cathode Buffer once the dye front migration is 1/3 of the gel (page 12), if you are using detergent containing samples.
- Wear gloves at all times during the entire blotting procedure to prevent contamination of gels and membranes, and to avoid exposing your skin to irritants commonly used in blotting procedures.
- Do not touch the membrane or gel with bare hands. This may contaminate the gel or membrane and interfere with further analysis.

Materials Required But Not Supplied

You will need the following items. See page 38 for ordering information.

- XCell II™ Blot Module
- Methanol
- NuPAGE® Transfer Buffer (20X)
- Blotting membranes: Invitrolon™/Filter Paper Sandwich or PVDF
- Blotting Roller
- Incubation Tray

Continued on next page

Western Blotting, Continued

Preparing 1X Transfer Buffer

Prepare 1000 mL of 1X NuPAGE® Transfer Buffer with using the NuPAGE® Transfer Buffer (20X) as follows:

NuPAGE® Transfer Buffer (20X)	50 mL
Deionized Water	to 1000 mL

Preparing Blotting Pads, Membrane, and Filter Paper

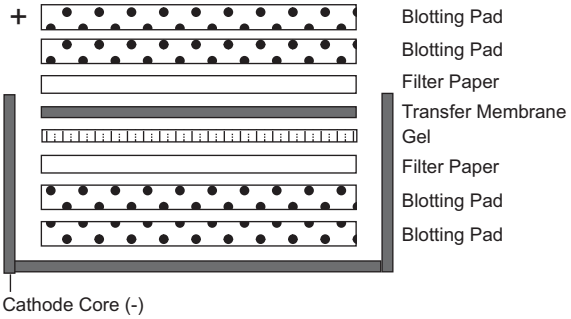
- **Blotting Pads:** Use ~ 700 mL of 1X transfer buffer to soak the blotting pads until saturated. Remove air bubbles by squeezing the blotting pads while they are submerged in buffer. Removing air bubbles is essential as they can block the transfer of biomolecules.
- **PVDF membrane**: Pre-wet the PVDF membrane for 30 seconds in methanol, ethanol, or isopropanol. Briefly rinse in deionized water and then place the membrane in an Incubation Tray containing 1X transfer buffer for several minutes.
- **Filter paper**: Soak briefly in 1X transfer buffer immediately prior to use.

Continued on next page

Transferring One Gel

For transferring 1 gel using the XCell II™ Blot Module:

1. Place 2 soaked blotting pads onto the cathode (-) core of the blot module. The cathode core is deeper of the 2 cores.
2. Replace the blue transfer buffer from the tray containing the gel (Step 6, page 17) with fresh 1X Transfer Buffer. Avoid incubating the gel in 1X Transfer Buffer for more than 10 minutes.
3. Position a piece of pre-soaked filter paper under the gel and remove the gel from the tray using the filter paper. Place the filter paper with the gel on top of the blotting pads so the gel is facing up.
4. Place the pre-soaked PVDF membrane on the gel. Remove any air bubbles using the Blotting Roller or a glass pipette.
5. Place the other pre-soaked filter paper on top of the transfer membrane. Remove any trapped air bubbles.
6. Add enough pre-soaked blotting pads to rise 0.5-cm over the rim of the cathode core. Place the anode (+) core on top of the pads. The final assembly contains the gel closest to the cathode plate (see figure below).



7. Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber.
8. Insert the Gel Tension Wedge into the Lower Buffer Chamber and lock the Wedge into position.
9. Fill the Inner Buffer Chamber with 1X NuPAGE® Transfer Buffer until the gel/membrane assembly is covered.
10. Fill Outer Buffer Chamber with 650 mL deionized water.
11. Attach lid, connect the leads to the power supply, and perform transfer at 25 V constant for 1 hour. *The expected start current is 130 mA and end current is 80 mA.*
12. After transfer, incubate the membrane in 20 mL of 8% acetic acid for 15 minutes to fix the proteins. Rinse with deionized water and air-dry the membrane.

    Note: If you do not wish to air-dry the membrane, you can proceed directly with immunodetection. However, there is some residual Coomassie G-250 dye that is bound to the membrane which is eventually washed away during the blocking, washing, and antibody incubation steps.
13. If you have air-dried the membrane, rewet the membrane with methanol (also removes any background dye bound to the membrane), then rinse with deionized water prior to immunodetection.

Expected Results

Introduction

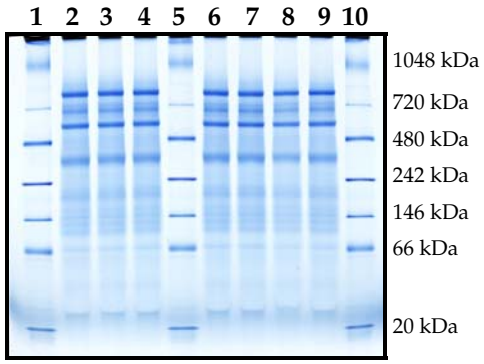
Examples of results obtained after performing various downstream applications with NativePAGE™ Novex® Bis-Tris Gels are shown in this section.

Coomassie Staining Results

An example of a Coomassie stained NativePAGE™ Novex® 4-16% Bis-Tris Gel using the fast staining protocol with Coomassie G-250 from the cathode buffer additive as described in this manual is shown below.

Lanes 1, 5, 10: NativeMark™ Unstained Protein Standard (5 µL)

Lanes 2, 3, 4, 6, 7, 8, 9: Bovine mitochondrial extract (18 µg)



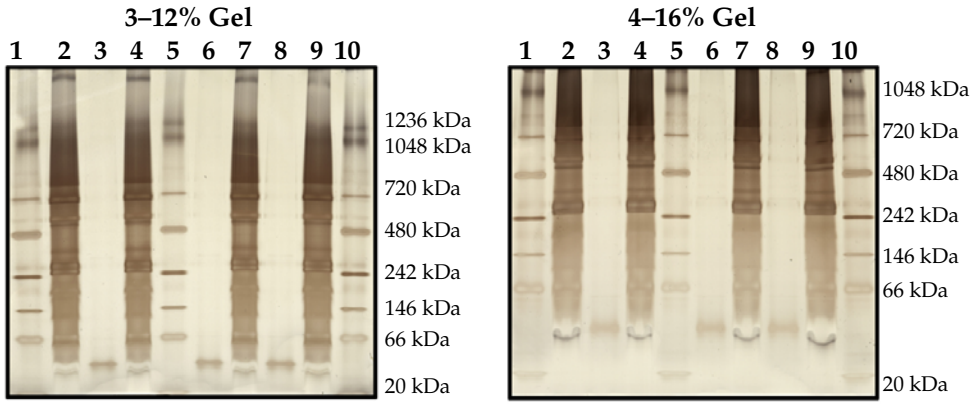
Silver Staining Results

An example of a NativePAGE™ Novex® Bis-Tris Gel silver stained using the SilverQuest™ Silver Staining Kit as described in this manual is shown below.

Lanes 1, 5, 10: 1:20 diluted NativeMark™ Unstained Protein Standard (5 µL)

Lanes 2, 4, 7, 9: Bovine mitochondrial extract (675 ng)

Lanes : 3, 6, 8: Purified green fluorescent protein (37.5 ng)



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In-gel Activity Assay Results

Examples of activity assays are shown below.

β-galactosidase Activity

An in-gel colorimetric assay of β-galactosidase activity (Manchenko, 1994) was performed to assess the activity of the enzyme. After NativePAGE™ electrophoresis, gels were incubated for 5 minutes in PBS (phosphate buffered saline), pH 7.0. The PBS was decanted and the gel was incubated in substrate solution (0.01% nitroblue tetrazolium and 0.05% X-gal in PBS, pH 7.0) for 20 minutes at room temperature. After developing the gel for activity, the gel was stained with Coomassie staining as described in this manual for visualizing the protein standard bands to assign molecular weights.

As shown in the gel below, dark purple bands indicate β-galactosidase activity at 465 kDa with some faint purple bands indicating activity for higher oligomers of β-galactosidase. β-galactosidase is a tetrameric enzyme with subunits of 116.3 kDa.

Lane 1: NativeMark™ Unstained Protein Standard (5 µL).

Lanes 2–9: Purified β-galactosidase (0.0125 µg, 0.025 µg, 0.05 µg, 0.1 µg, 0.125 µg, 0.25 µg, 0.5 µg, and 1 µg, respectively)

Green Fluorescent Protein (GFP) Activity

The fluorescence of GFP was detected after NativePAGE™ electrophoresis to assess the activity of the protein.

After NativePAGE™ electrophoresis, the sample fluorescence in the gel was examined using the FujiFilm LAS-1000's CCD camera (477 nm excitation with a 520–640 nm band pass emission filter) with an exposure time of 40 seconds.

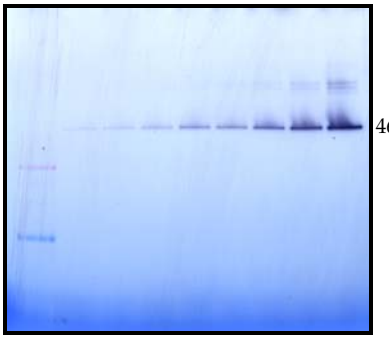
As shown in the gel below, fluorescent bands for GFP (33 kDa) are visible indicating proper folding of the protein. **Note:** The fluorescent band in the NativeMark™ Unstained Protein Standard is due to the fluorescence of phycoerythrin.

Lanes 1, 5, 10: NativeMark™ Unstained Protein Standard (5 µL).

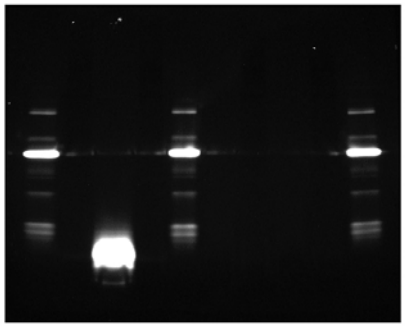
Lane 3: 20 µg purified GFP

Lanes 2, 4, 6, 7, 8, 9: 18 µg Bovine mitochondrial extract

β-galactosidase activity (4-16% Gel)



GFP (4-16% Gel)



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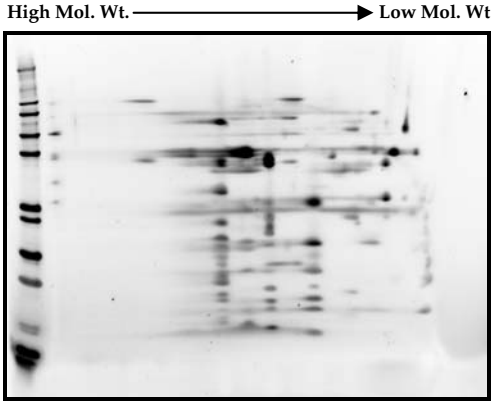
2D Results

An example of results obtained with bovine mitochondrial extract (18 µg) sample subjected to two-dimensional native/SDS-PAGE.

Two-Dimensional Native/SDS-PAGE was performed with bovine mitochondrial extract solubilized with 1% DDM. Samples were analyzed on a NativePAGE™ Novex® 3–12% Bis-Tris Gel in the first dimension. A gel strip from the first dimension gel was equilibrated, reduced, and alkylated as described in this manual and loaded onto a second dimension NuPAGE® Novex® 4–12% Bis-Tris Gel. SDS-PAGE was performed using NuPAGE® MES SDS Running Buffer with XCell™ *SureLock*™ Mini-Cell using standard conditions and proteins were stained with SYPRO® Ruby Protein Stain. Mark12™ Unstained Protein Standard (2 µL) was used as the protein standard.



Troubleshooting

Introduction

Review the information below to troubleshoot your experiments with NativePAGE™ Gels. To troubleshoot your staining or Western blotting experiments, refer to the manuals supplied with staining or blotting kits.

Observation	Cause	Solution
Low or no current during the run	Incomplete circuit	• Remove the tape from the bottom of the cassette prior to electrophoresis. • Make sure the buffer covers the sample wells. • Check the wire connections on the buffer core to make sure the connections are intact.
Run taking longer time	Running buffer too dilute	Make fresh running buffer as described on page 12 and do not adjust the pH of the 1X running buffer. Use the recommended buffers (page 12).
	Upper buffer chamber is leaking	Make sure the buffer core is firmly seated, the gaskets are in place, and the gel tension lever is locked.
	Voltage is set too low	Set the correct voltage (page 17).
Run is faster than normal with poor resolution	Buffers are too concentrated or incorrect.	Prepare the 1X Running Buffers as described on page 12. If you are preparing your own buffer, check the buffer recipe and remake if necessary.
	Voltage, current, or wattage is set at a higher limit	Decrease power conditions to the recommended running conditions (page 17).
No bands	Low protein load	Increase the protein load. Use an accurate and sensitive protein estimation method.
	Improper sample preparation	• Increase the amount of detergents used for solubilization. Try different detergents to obtain optimal solubilization of your protein of interest. • Check to make sure there is no precipitate in the Digitonin solution (page 8).
	Insensitive detection method	Use sensitive detection methods such as silver staining or immunoblotting.
No distinct spots after 2D electrophoresis	Air bubbles between the gel strip and 2D gel	Smooth out any air bubbles.
	Gel strip not correctly loaded	Align the strip properly as described on page 21. Be sure the gel is in contact with the SDS gel.

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Troubleshooting, Continued

Observation	Cause	Solution
Streaking of proteins	• Sample overload • High salt in the sample • Sample precipitates • DNA complexes in the sample • Particulate material in your sample	• Load the appropriate amount of protein (page 3). • Decrease the salt concentration of your sample using dialysis or gel filtration. • Increase the concentration of detergents in your sample and be sure to add the NativePAGE™ 5% G-250 Sample Additive to detergent containing samples. • Perform benzonase treatment during sample preparation (page 9). • Clarify the lysate using centrifugation or ultracentrifugation.
Poor resolution, bands are not very sharp (fuzzy, streaking)	Incorrect sample or running buffer used	Use the recommended sample buffer and 1X running buffer based on the gel type. Do not use the NuPAGE® MOP/MES or Tris-Glycine Running Buffer with SDS for native gel electrophoresis.
Protein smearing	Protein degradation	Add protease inhibitors during sample preparation (page 8).
Faint shadow or "ghost" band below the expected protein band	Used expired gels or improperly stored gels	Store gels at 4ºC. Do not freeze the gels. Avoid using expired gels. Use fresh gels.
Cannot see the sample wells to load sample	Cathode Buffer loaded into the cathode chamber	To allow better visualization of sample wells, we recommend loading the samples into the wells containing cathode buffer prior to filling the cathode chamber. If you are an experienced user, you may load the samples into the wells prior to inserting the gel cassette into the mini-cell.
Gels break during handling	Gels contain low acrylamide percentage making the gels more fragile	Handle NativePAGE™ Novex® 3–12% Bis-Tris gels carefully by only handling the bottom, higher percentage acrylamide part of the gel.
No activity observed after electrophoresis	Protein denatured or degraded	• **Do not heat** samples for native electrophoresis. • Perform electrophoresis at 4ºC using chilled buffers. • Use protease inhibitors during sample preparation. • The protein activity is usually dependent on the sensitivity of the proteins to the buffers used in NativePAGE™ electrophoresis.

Appendix

Buffer Recipes

NativePAGE™ Running Buffer

The NativePAGE™ Running Buffer (20X) is available separately (page 38).

The final concentration of the 1X buffer is given below:
50 mM BisTris
50 mM Tricine
pH 6.8

1. To prepare 1000 mL of NativePAGE™ Running Buffer (20X), dissolve the following reagents in 700 mL ultrapure water:

BisTris	209.2 g
Tricine	179.2 g

2. Mix well and adjust the volume to 1000 mL with ultrapure water.
3. Store at room temperature. The buffer is stable for 6 months when stored at room temperature.
4. For electrophoresis, dilute this buffer to 1X with water (page 13).

NativePAGE™ Sample Buffer

The NativePAGE™ Sample Buffer (4X) is available separately (page 38).

The final concentration of the buffer at 1X is given below.
50 mM BisTris
6 N HCl
50 mM NaCl
10% w/v Glycerol
0.001% Ponceau S
pH 7.2

1. To prepare 10 mL of NativePAGE™ Sample Buffer (4X), dissolve the following reagents in 5 mL ultrapure water:

BisTris	0.418 g
6 N HCl	0.107 mL
Glycerol	4 g
NaCl	0.117 g
Ponceau S	0.4 mg

2. Mix well and adjust the volume to 10 mL with ultrapure water.
3. Store at 4ºC. The buffer is stable for 6 months when stored at 4ºC.

NativePAGE™ Cathode Buffer (20X)

The NativePAGE™ Cathode Buffer Additive (20X) is available separately (page 38).

0.4% Coomassie G-250

1. To prepare 250 mL of NativePAGE™ Cathode Buffer Additive (20X), dissolve 1 g Coomassie G-250 dye in 250 mL ultrapure water:
2. Mix well and store the buffer at room temperature. The buffer is stable for 6 months when stored at room temperature.
3. For electrophoresis, prepare the 1X Dark Blue or Light Blue Cathode Buffer as described on page 13.

Accessory Products

Additional Products

Ordering information for electrophoresis products available separately is provided below. For detailed information, visit **www.lifetechnologies.com/support** or call Technical Support (page 40).

Product	Quantity	Cat. no.
XCell™ *SureLock*™ Mini-Cell	1 unit	EI0001
XCell II™ Blot Module	1 unit	EI9051
NativePAGE™ Running Buffer (20X)	1 L	BN2001
NativePAGE™ Cathode Buffer Additive (20X)	250 mL	BN2002
NativePAGE™ Sample Buffer (4X)	10 mL	BN2003
NativePAGE™ 5% G-250 Sample Additive	0.5 mL	BN2004
NativePAGE™ Running Buffer Kit	1 kit	BN2007
NativePAGE™ Sample Prep Kit	1 kit	BN2008
NuPAGE® Transfer Buffer (20X)	1 L	NP0006-1
10% DDM (n-dodecyl-β-D-maltoside)	1 mL	BN2005
5% Digitonin	1 mL	BN2006
Qubit® Protein Assay Kit	1 kit	Q33211
Stains and Standards		
Colloidal Blue Staining Kit	1 kit	LC6025
SilverQuest™ Silver Staining Kit	1 kit	LC6070
SilverXpress® Silver Staining Kit	1 kit	LC6100
DryEase® Mini-Gel Drying Kit	1 kit	NI2387
NativeMark™ Unstained Protein Standard	5 × 50 µL	LC0725

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Accessory Products, Continued

Product	Quantity	Cat. no.
Blotting Products		
Invitrolon™ PVDF (0.45-µm)/Filter Sandwiches	20 each	LC2005
Nitrocellulose (0.45-µm)/Filter Sandwiches	20 each	LC2001
Blotting Roller	1 roller	LC2100
Incubation Tray	8 each	LC2102
WesternBreeze® Chromogenic Kit, Anti-Mouse	1 kit	WB7103
WesternBreeze® Chromogenic Kit Anti-Rabbit	1 kit	WB7105
WesternBreeze® Chemiluminescent Kit, Anti-Mouse	1 kit	WB7104
WesternBreeze® Chemiluminescent Kit, Anti-Rabbit	1 kit	WB7106
Power Supply		
ZOOM® Dual Power Supply (100–120 VAC, 50/60 Hz)	1 each	ZP10001
PowerEase® 500 Power Supply (100–120 VAC, 50/60 Hz)	1 each	EI8600
PowerEase® 500 Power Supply (220/240 VAC, 50/60 Hz)	1 each	EI8700

NativePure™ Native Complex Purification System

The NativePure™ Native Complex Purification System is available for analysis of native protein complexes. The system includes NativePure™ Gateway® vectors that allow fusion of your protein of interest to a BioEase™ tag, which facilitates *in vivo* biotinylation of your recombinant fusion protein. The biotin-tagged protein is then used as a bait to identify proteins that interact with your protein of interest in mammalian cells. The protein complexes are purified using NativePure™ Affinity Purification Kit with Streptavidin Agarose under native conditions. The purified protein complexes are then analyzed by NativePAGE™ gels, SDS-PAGE, Western analysis, or mass spectrometry.

Product	Quantity	Cat. no.
NativePure™ pcDNA™ Gateway® Vector Kit	1 kit	BN3002
NativePure™ Mammalian Affinity Purification Kit	1 kit	BN3006
NativePure™ Affinity Purification Kit	1 kit	BN3003

Technical Support

Obtaining support

For the latest services and support information for all locations, go to **www.lifetechnologies.com/support**.

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- Obtain information about customer training
- Download software updates and patches

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