



Formerly Cell Biosciences

# NanoPro Assay Development Guide

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NanoPro Assay Development Guide

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# Table of Contents

<b>Preface</b> .....	iii	<i>3.4: Run the Basic Screening Protocol</i> .....	21
<b>Chapter 1:</b>		<b>Chapter 3:</b>	
<b>General Information</b> .....	1	<b>Assay Optimization</b> .....	23
<i>Introduction to NanoPro Technology</i> .....	2	<i>Assay Optimization</i> .....	24
<i>Safety</i> .....	4	<i>Optimization of the Separation Conditions</i> .....	24
<i>User Attention Notifications</i> .....	4	<i>Choice of Gradient</i> .....	24
<i>Chemical Hazards</i> .....	4	<i>IEF Separation (Focusing) Time</i> .....	27
<i>Material Safety Data Sheets</i> .....	5	<i>Separation Mode</i> .....	29
<i>Customer Service and Technical Support</i> .....	5	<i>Immobilization Conditions</i> .....	31
<b>Chapter 2:</b>		<i>Optimization of Antibody Conditions</i> .....	33
<b>Getting Started</b> .....	7	<i>Antibody Affinity Considerations</i> .....	33
<i>NanoPro Assay Development Overview</i> .....	8	<i>Optimization of Antibody Incubation Times and Concentrations</i> .....	35
<i>Step 1: Create Lysates Suitable for Assay Development</i> .....	8	<i>Determination of Dynamic Range Using Optimized Conditions</i> .....	36
<i>General Guidelines</i> .....	8	<i>Signal Burn-Out</i> .....	37
<i>Procedure for Lysis of Adherent Cells</i> .....	10	<i>Enriched Protein Samples and Limit of Detection (LOD)</i> .....	39
<i>Procedure for Lysis of Suspension Cells</i> .....	12	<b>Chapter 4:</b>	
<i>Step 2: Determine Lysate Protein Concentration</i> .....	14	<b>Sample Preparation</b> .....	43
<i>Guidelines for Choosing an Optimal Protein Concentration</i> .....	14	<i>Reagent Considerations</i> .....	44
<i>Step 3: Screen Antibodies</i> .....	14	<i>Buffer Components</i> .....	44
3.1 <i>Choosing an Antibody</i> .....	14	<i>Sample Preparation Buffers</i> .....	47
3.2 <i>Assay Component Considerations</i> .....	15	<i>Cell Lines - Guidelines and Preparation</i> .....	50
3.3 <i>Assay Plate Preparation</i> .....	16		

<i>Fine Needle Aspirate (FNA) - Guidelines and Preparation</i> .....	50	<i>Dilution of Denatured Lysates</i> .....	73
<i>Practical Considerations</i> .....	50	<i>IEF Gradient</i> .....	74
<i>Sample Procurement and Processing</i> .....	50	<i>Choosing an Antibody</i> .....	74
<i>Preclinical FNA Procedure</i> .....	50	<i>Antibody Incubation Times and Concentrations</i> ..	74
<i>Clinical FNA Procedure</i> .....	51	<i>Lysate Titration</i> .....	76
<i>Sample Stability</i> .....	51	<i>UV Immobilization Times</i> .....	76
<i>Cell Yields</i> .....	51	<i>Recommended Protocol for Optimization of the</i>	
<i>Hemoglobin Contamination</i> .....	52	<i>NanoPro Denaturing Assay</i> .....	78
<i>Effect of Residual Cell Media on NanoPro Assay</i>		<i>Items Needed</i> .....	78
<i>Performance</i> .....	54	<i>Procedure</i> .....	78
<i>Snap Freezing</i> .....	54	<i>References</i> .....	81
<i>Tissue Origin</i> .....	54		
<i>FNA Preparation Procedures</i> .....	56		
<i>References</i> .....	58		
<i>Peripheral Blood Mononuclear Cells (PBMC) - Guidelines and Preparation</i> .....	59		
<i>Introduction</i> .....	59		
<i>PBMC Preparation Procedures</i> .....	59		
<i>Example NanoPro Analysis of p-STAT</i> .....	63		
<b>Chapter 5:</b>			
<b>Denaturing Assays</b> .....	67		
<i>Introduction</i> .....	68		
<i>General Denaturing Considerations for NanoPro</i>			
<i>Assays</i> .....	69		
<i>Comparison of Native and Urea/DTT-Treated Protein</i>			
<i>Profiles</i> .....	69		
<i>Optimization of Denaturing Procedure for NanoPro</i>			
<i>Assays</i> .....	73		
<i>Sample Preparation</i> .....	73		
<i>Urea/DTT Concentration</i> .....	73		
<i>Denaturing Temperature and Incubation Time</i> ..	73		

# Preface

The goal of the NanoPro Assay Development Guide is to summarize ProteinSimple's experience in developing nanoimmunoassays in a comprehensive way to allow NanoPro system users to quickly start their own assay development. This guide also provides an in-depth discussion of assay development principles for the NanoPro platform. Examples for specific sample sources, assay development tools and applications are also provided.

ProteinSimple updates the NanoPro Assay Development Guide on a regular basis in order to provide our users with the most current information on assay development. New chapters and topics will be added in the near future. We invite our users to visit [www.cellbiociences.com/literature](http://www.cellbiociences.com/literature) often to download the latest version of this guide.

For ordering and general information on the ProteinSimple assay reagents used in this guide, please call (888) 607-9692 or visit [www.proteinsimple.com](http://www.proteinsimple.com)



## **Chapter 1:**

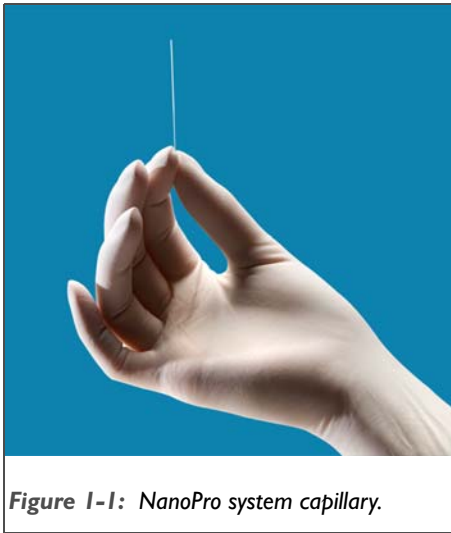
# General Information

### **Chapter Overview**

- Introduction to NanoPro Technology
- Safety
- Customer Service and Technical Support

## Introduction to NanoPro Technology

The ProteinSimple NanoPro™ systems enable detailed characterization of cell signaling processes in extremely small biological samples. Whereas traditional protein analysis techniques require thousands to millions of cells, NanoPro systems require as few as 25 cells per assay. Biological samples typically too small for traditional protein characterization techniques such as primary cells, fine needle tumor aspirates, FACS-sorted cells, microdissected tissue sections and isolated stem cell populations can be analyzed using straightforward protocols.



NanoPro systems are automated, capillary-based immunoassay platforms. As in western blot analysis, proteins from complex biological samples are separated, immobilized and probed with specific antibodies. However, NanoPro systems use capillary isoelectric focusing (cIEF) separation to resolve the various modification states of signaling proteins (Figure 1-1).

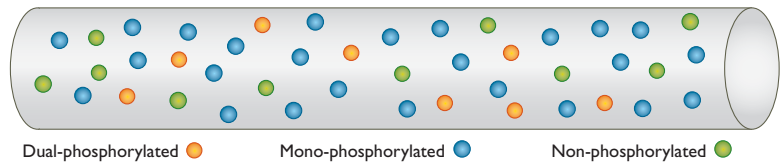
The capillary format provides rapid, high-resolution separations, with an average separation time of 40 minutes. After separation, proteins are chemically linked to the capillary wall. This linkage step is achieved through UV-light activation of a proprietary photoactive capture chemistry coated on the inner surface of each capillary. Proteins immobilized on the capillary wall are then washed and probed with primary and secondary antibodies. Secondary antibodies are conjugated to horseradish peroxidase (HRP), which enables ultrasensitive chemiluminescence detection. After addition of Luminol and Peroxide detection

reagents, chemiluminescence signal is collected through the capillary wall (Figure 1-2). Analysis is performed in Compass software and the resulting data are presented in electropherogram format along with a digital image of the separated proteins in the capillary (Figure 1-3).

## The Process

**Load**

The capillary is filled with a 400-nL mixture of sample, fluorescently labeled pI standards, and ampholytes.

**Separate**

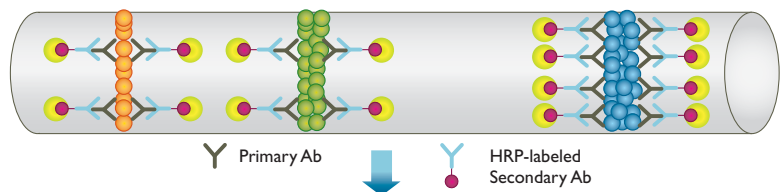
Voltage is applied across the capillary to drive the IEF separation. Individual proteins and pI standards concentrate at their isoelectric points, and the position of each standard in the capillary is recorded.

**Immobilize**

The capillary is exposed to UV light, activating the proprietary linking chemistry and locking the separated protein isoforms to the capillary wall.

**Immunoprobe**

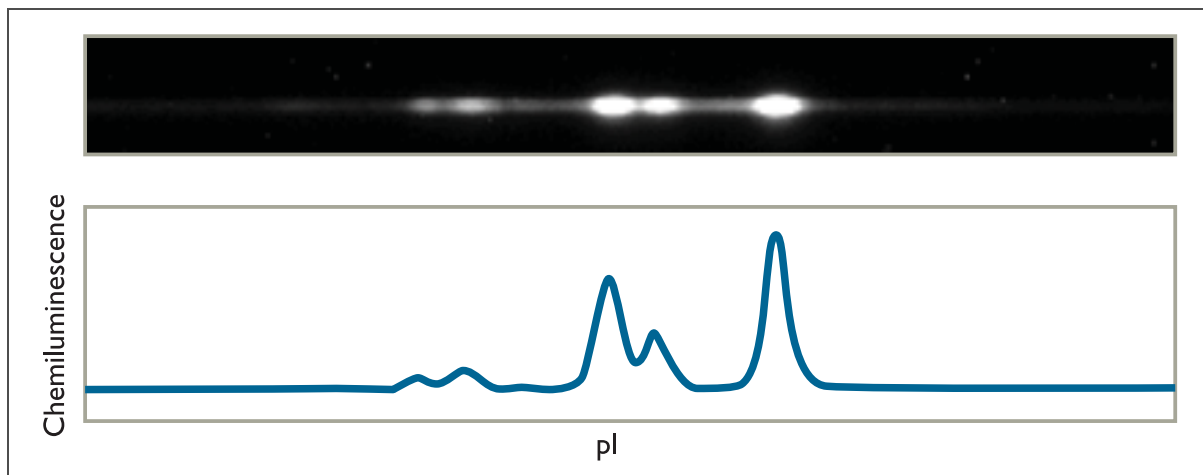
The capillary is rinsed and immunoprobed for specific proteins. Luminol and Peroxide are added to catalyze the generation of chemiluminescent light, which is captured by a CCD camera.

**Quantitate**

The digital image is analyzed and quantitative results are presented in the software.



**Figure 1-2:** NanoPro system immunoassay process. All steps are carried out in a single capillary.



**Figure 1-3:** NanoPro system sample data. Chemiluminescent image of separated proteins in the capillary and corresponding electropherogram.

## Safety

### User Attention Notifications

Several user attention phrases are used throughout this manual. Each phrase should draw the following level of attention from the user:

**NOTE** Points out useful information.

**IMPORTANT** Indicates information necessary for proper instrument operation.

**CAUTION** Cautions users regarding potentially hazardous situations in regard to user injury or damage to the instrument if the information is not heeded.

**!WARNING!** Warns users that serious physical injury can result if warning precautions are not heeded.

### Chemical Hazards

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#### **!WARNING! CHEMICAL HAZARD**

Some chemicals used can be potentially hazardous, and can cause injury or illness.

---

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (e.g., safety glasses, gloves, or clothing). For additional safety guidelines consult the MSDS.
- Do not leave chemical containers open.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

## Material Safety Data Sheets

Some chemicals used with the NanoPro system may be listed as hazardous. Warnings are displayed on the labels of all chemicals when hazards exist.

MSDSs provide users with safety information needed to store, handle, transport and dispose of the chemicals safely. ProteinSimple recommends updating laboratory MSDS records periodically.

Material Safety Data Sheets for ProteinSimple reagents are available online at [www.proteinsimple.com/literature](http://www.proteinsimple.com/literature) or by calling (888) 607-9692. Otherwise call the chemical manufacturer directly or visit their web site.

## Customer Service and Technical Support

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## **Chapter 2:**

# Getting Started

### **Chapter Overview**

- NanoPro Assay Development Overview
- Step 1: Create Lysates Suitable for Assay Development
- Step 2: Determine Lysate Protein Concentration
- Step 3: Screen Antibodies

## NanoPro Assay Development Overview

The assay development process for NanoPro systems is very similar to that for other immunoassay formats such as ELISA and Western Blot. ProteinSimple recommends following the process outlined in this chapter when developing NanoPro assays for the first time, or when analyzing unknown samples.

At the onset of assay development, the following three steps are applied and will be described in this chapter:

- **Step 1:** Create lysates suitable for assay development
- **Step 2:** Determine lysate protein concentration
- **Step 3:** Screen antibodies

Subsequent chapters will describe the full assay development process in detail. Information will also be provided on custom assay development for specific sample types and reagents, as well as particular considerations for NanoPro assays.

## Step 1: Create Lysates Suitable for Assay Development

Lysate quality is one of the main determinants of assay success. ProteinSimple lysis protocols are designed with two primary sample objectives:

- Preserving the status of signaling proteins
- Maintaining samples at low ionic strength

### General Guidelines

- **Choose an easily accessible model system.** Whenever possible, conduct assay development using cultured cell lines rather than primary cells or tissues. Although analysis of precious biological samples may be the final objective, ProteinSimple recommends performing assay development using readily available material.
- **Choose a relevant model system.** The choice of the right biological system depends upon the protein target. The cell model system chosen should be well established for the protein of interest and yield a strong biological response to treatment. During assay development, it is useful to screen antibodies against treated and untreated biological samples. This approach allows identification not only of antibodies that produce the most significant peaks, but also those that are the most sensitive to measuring biological changes. For a range of examples, please refer to our Application Briefs which can be downloaded at [www.proteinsimple.com/literature](http://www.proteinsimple.com/literature).

## Guidelines for Preserving the Status of Signaling Proteins

- Work quickly and efficiently when washing and lysing cells.
- Keep samples on ice at all times. Use ice-cold wash and lysis buffers.
- Always use protease and phosphatase inhibitor cocktails. ProteinSimple provides the following inhibitor mixes, listed below at their final assay concentrations:

### Aqueous Inhibitor Mix:

- 40 mM NaF
- 12 mM Glycerophosphate
- 1 mM Sodium Orthovanadate
- 0.5 mM EDTA
- 2.5 mM EGTA

### DMSO Inhibitor Mix:

- 1 mM AEBSF
- 5 µg/mL Aprotinin
- 50 µM Bestatin
- 5 µg/mL E-64 Protease Inhibitor
- 10 µg/mL Leupeptin
- 7 µg/mL Pepstatin A
- Phosphatase inhibitor cocktail (with effective concentrations of Cantharidin, Bromotetramisole, and Microcystin LR)

## Guidelines for Maintaining Low Sample Ionic Strength

- Use the recommended ProteinSimple lysis buffers:
  - **Preferred:** ProteinSimple Bicine/Chaps Lysis Buffer - 20 mM Bicine (pH 7.6), 0.6% CHAPS
  - **Alternate:** ProteinSimple RIPA Lysis Buffer - 20 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP40 alternative, 0.25% Sodium Deoxycholate, 10% Glycerol

---

*NOTE: Lysates made in RIPA will need to be diluted at least 1:20 in a low salt sample diluent (such as the ProteinSimple Sample Diluent) before analysis.*

---

- Use a low ionic strength cell wash buffer:
  - **Preferred:** ProteinSimple Cell Wash Buffer - 20 mM Bicine, 250 mM Sucrose, 0.1% Kathon CG (pH 7.5)

---

*NOTE: The total ion concentration of the sample after all dilution steps should not exceed 50 mM.*

---

Please refer to Chapter 4, "Sample Preparation" for a discussion of various custom lysis buffer components and their effect on NanoPro assay performance.

## **Procedure for Lysis of Adherent Cells**

---

### *NOTES:*

*The following procedure was designed for a single, 10 cm dish. Volumes should be scaled appropriately to the number of cells being analyzed.*

*Except where noted otherwise, all steps in the procedure should be performed on ice or at 4 °C. All reagents used should be kept on ice at all times.*

*Microfuge tubes used in the procedure should be pre-chilled before use.*

---

**IMPORTANT:** Combine the Lysis Buffer and inhibitors as detailed in Step 2 immediately before use. Do not prepare in advance.

---

## **Items Needed**

### **ProteinSimple Reagents**

- Cell Wash Buffer
- Lysis Buffer
- Aqueous and DMSO Inhibitor Mixes

### **Materials and Equipment**

- Ice and an ice bucket
- Liquid nitrogen or dry ice
- Cell scraper
- Microfuge tubes

- Microfuge centrifuge
- Vortexer
- Pipettors and tips

### **Procedure**

1. Cool Cell Wash Buffer and Lysis Buffer on ice.
2. Add inhibitors to Lysis Buffer:
  - Dilute Aqueous Inhibitor Mix 1:25 in Lysis Buffer
  - Dilute DMSO Inhibitor Mix 1:50 in Lysis BufferExample: To prepare 2 mL of Lysis Buffer with inhibitors, add 80  $\mu$ L of Aqueous Inhibitor Mix and 40  $\mu$ L of DMSO Inhibitor Mix to 1.88 mL ice-cold Lysis Buffer.
3. Wash cells 2 times with 5-10 mL of ice-cold Cell Wash Buffer.
4. After the second wash, tilt dish to aspirate Cell Wash Buffer completely. Ensure the dish remains on ice during aspiration.
5. Add 400  $\mu$ L of ice-cold Lysis Buffer containing inhibitors to dish. Swirl around to ensure good coverage and incubate for 5 minutes on ice.
6. Scrape cells from the dish with cell scraper and transfer to a pre-chilled microfuge tube.
7. Pipette up-and-down 5 times to resuspend cells and aid lysis.
8. Incubate for 30 minutes on ice. During incubation, vortex briefly every 5 minutes.
9. Clarify lysate by centrifugation for 15 minutes at 14,000  $\times$  g at 4  $^{\circ}$ C.
10. Immediately transfer supernatant to a clean microfuge tube.
11. Transfer 5-30  $\mu$ L aliquots of supernatant into individual microfuge tubes.
12. Snap-freeze aliquots on dry ice or in liquid nitrogen.
13. Store aliquots at -80  $^{\circ}$ C.

## Procedure for Lysis of Suspension Cells

---

### NOTES:

Except where noted otherwise, all steps should be performed on ice or at 4 °C. All reagents used should be kept on ice at all times.

Microfuge tubes used in the procedure should be pre-chilled before use.

---

**IMPORTANT:** Combine the Lysis Buffer and inhibitors as detailed in Step 3 immediately before use. Do not prepare in advance.

---

### Items Needed

#### ProteinSimple Reagents

- Cell Wash Buffer
- Lysis Buffer
- Aqueous and DMSO Inhibitor Mixes

#### Materials and Equipment

- Ice and an ice bucket
- Liquid nitrogen or dry ice
- Microfuge tubes
- Microfuge centrifuge
- Vortexer
- Pipettors and tips

### Procedure

1. Cool Cell Wash Buffer and Lysis Buffer on ice.
2. Determine the amount of Lysis Buffer to prepare (see Table 2-1).
3. Add inhibitors to Lysis Buffer:
  - Dilute Aqueous Inhibitor Mix 1:25 in Lysis Buffer.
  - Dilute DMSO Inhibitor Mix 1:50 in Lysis Buffer.

Example: To prepare 2 mL of Lysis Buffer with inhibitors, add 80  $\mu$ L of Aqueous Inhibitor Mix and 40  $\mu$ L of DMSO Inhibitor Mix to 1.88 mL ice-cold Lysis Buffer.

4. Collect cells by centrifugation for 5 minutes at 500  $\times$  g at room temperature.
5. Wash cells by suspension once with 10-15 mL of ice-cold Cell Wash Buffer. Follow with centrifugation for 5 minutes at 500  $\times$  g at 4  $^{\circ}$ C.
6. Aspirate the supernatant completely from the cell pellet.
7. Add ice-cold Lysis Buffer containing inhibitors to the cell pellet as indicated in Table 2-1 to resuspend cells.
8. Incubate for 5 minutes on ice.
9. Pipette up-and-down 5 times to break up the cell pellet.
10. Incubate for 30 minutes on ice. During incubation, vortex briefly every 5 minutes.
11. Clarify lysate by centrifugation for 15 minutes at 14,000  $\times$  g at 4  $^{\circ}$ C.
12. Immediately transfer supernatant to a clean microfuge tube.
13. Transfer 5-30  $\mu$ L aliquots of supernatant into individual microfuge tubes.
14. Snap-freeze aliquots on dry ice or in liquid nitrogen.
15. Store aliquots at -80  $^{\circ}$ C.

Number of Cells	Volume of Lysis Buffer
>100,000	10 $\mu$ L - 20 $\mu$ L
100,000 - 1,000,000	20 $\mu$ L - 50 $\mu$ L
1,000,000	50 $\mu$ L - 100 $\mu$ L

**Table 2-1:** Lysis Buffer volumes for lysis of suspension cells.

## Step 2: Determine Lysate Protein Concentration

ProteinSimple recommends accurate measurement of total protein concentration in lysates. Advanced knowledge of concentration provides insight into protein signal changes and when comparing signal data between different cycles and runs.

---

*NOTE: ProteinSimple recommends using the BCA Protein Assay Kit (Pierce, 23225) for measurement of total protein concentration.*

---

### Guidelines for Choosing an Optimal Protein Concentration

- **Screen against two accurately determined sample protein concentrations.** Protein signals should change with concentration and as a response to treatment. Therefore, it is important to accurately determine the total protein concentration for each sample. In addition, running two different lysate concentrations for each antibody will provide a broader window of evaluation. Protein concentrations should span a 3 to 5-fold range. ProteinSimple recommends using total protein concentrations of 0.02 mg/mL and 0.1 mg/mL, and testing of both treated and untreated samples at each concentration for an initial assay.

## Step 3: Screen Antibodies

### 3.1 Choosing an Antibody

There are no general rules for predicting antibody activity on the NanoPro platform. Antibodies that are reported to work in several different applications are preferred. Antibodies raised against whole proteins as opposed to peptides are also favored. However, these parameters alone do not guarantee success - each antibody must be functionally tested.

#### **Antibody-Related Considerations**

- **Antibody Dilution.** At the beginning of the assay development process, increased background signals can be tolerated in order to detect all promising antibodies. Therefore, ProteinSimple recommends starting with relatively high antibody concentrations. For the Basic Screening Protocol, the standard dilution is 1:50.
- **Antibody Diluent.** The ProteinSimple Antibody Diluent is strongly recommended as it has been proven superior to any other buffer systems tested.

- **Antibody Incubation Time.** Antibody incubation times should be sufficient for antibody binding to approach equilibrium. The Basic Screening Protocol uses a two-hour incubation for primary antibodies followed by a one-hour secondary antibody incubation, which are the recommended initial incubation times. These times can then be adjusted as necessary during assay development.

### 3.2: Assay Component Considerations

Assay component parameters and recommendations for the Basic Screening Protocol are summarized in Table 2-2.

- **IEF gradient.** The pIs observed with the NanoPro platform are typically quite close to the theoretical pIs for the protein of interest. However, because assays are run under semi-native conditions, occasional complex formation or partial exposure of charged residues on the protein surface can cause significant shifts of observed pIs. Therefore, the Basic Screening Protocol uses a wide gradient (pH 3-10) to capture all sample protein peaks.

Assay Component	Parameter	Recommendation
<b>Lysate</b>	Biological system	Strong, robust response of protein of interest
	Protein concentrations	0.02 mg/mL and 0.1 mg/mL
	Sample diluent	ProteinSimple Bicine/CHAPS Lysis Buffer with DMSO Inhibitor Mix
<b>Premix</b>	Separation gradient	pH 3-10
<b>pI Standards</b>	Individual Standards or Standard Ladder	pI Standard Ladder I
<b>Antibody</b>	<i>Primary</i>	Active in multiple applications, raised against complete protein
	Initial dilution	1:50
	Initial diluent	ProteinSimple Antibody Diluent
	<i>Secondary</i>	HRP-conjugate specific to species of primary antibody
Initial dilution	1:100	
Initial diluent	ProteinSimple Antibody Diluent	

**Table 2-2:** Basic Screening Protocol assay component parameters.

### 3.3: Assay Plate Preparation

ProteinSimple recommends using the assay plate layout shown in Figure 2-1. The following procedure is for this plate layout, which only uses quadrant I of the 384-well plate.

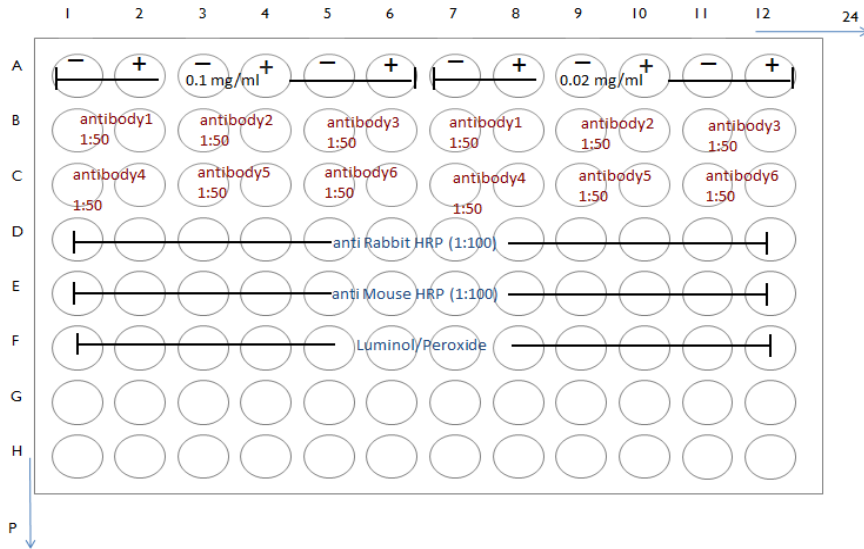


Figure 2-1: Recommended assay plate layout for the Basic Screening Protocol.

#### Procedure for Preparation of Recommended Assay Plate Layout

NOTE: The total number of antibodies screened depends on the number of available antibodies. The plate layout should be adjusted accordingly. The NanoPro 1000 system is capable of running 8 cycles unattended, allowing a maximum of 24 primary antibodies to be screened in one run using the recommended plate layout.

#### Items Needed

##### ProteinSimple Reagents

- Premix G2
- pI Standard Ladder I
- DMSO Inhibitor Mix

- Sample Diluent
- Antibody Diluent
- Luminol
- Peroxide
- Secondary antibodies

### **Customer Reagents**

- Primary antibodies
- Lysate samples

### **Material and Equipment**

- Microfuge tubes
- 384-well plate
- Centrifuge
- Microtiter plate adapter for centrifuge
- Vortexer
- Pipettors and tips

### **Procedure**

#### **Sample Mix Preparation**

For a flow diagram of sample mix preparation, please see Figure 2-2.

1. Combine 3.3  $\mu$ L of pl Standard Ladder 1 with 146.7  $\mu$ L of Premix G2 pH 3-10. Mix by vortexing.

---

#### *NOTES:*

*All pl Standard Ladders are provided at 60X of the required final concentration for NanoPro assays.*

*ProteinSimple recommends using the Bicine/CHAPS Lysis Buffer as the sample diluent.*

---

2. Combine 4  $\mu$ L of DMSO Inhibitor Mix with 46  $\mu$ L of Sample Diluent and mix.

---

*NOTE: DMSO Inhibitor Mix is provided at 50X of the required final concentration for NanoPro assays.*

---

3. Dilute lysate to 4X the final protein concentration in the Sample Diluent containing inhibitors (prepared in step 2). Dilution for the recommended lysate concentrations is as follows:

---

*NOTE: Lysate dilution volumes assume the total lysate protein concentration is 1 mg/mL.*

---

- **0.1 mg/mL Lysates:** For each treated and untreated lysate, combine 6  $\mu\text{L}$  Sample Diluent containing inhibitors with 4  $\mu\text{L}$  of lysate and mix.
  - **0.02 mg/mL Lysates:** For each treated and untreated lysate, combine 9.2  $\mu\text{L}$  Sample Diluent containing inhibitors with 0.8  $\mu\text{L}$  of lysate and mix.
4. Combine 10  $\mu\text{L}$  of diluted lysate prepared in step 3 with 30  $\mu\text{L}$  of Premix G2 containing pI Standards prepared in step 1 (a 1:3 ratio). Vortex to mix.
- 

### **IMPORTANT**

Ampholyte premix solutions are very viscous. It is imperative for assay performance to mix the Premix, pI Standards and lysates thoroughly by vortexing for at least 15 seconds.

---

### ***Antibody Preparation***

1. Combine 2  $\mu\text{L}$  of each stock primary antibody with 98  $\mu\text{L}$  Antibody Diluent for a 1:50 dilution and mix well.
2. Combine 3  $\mu\text{L}$  of each stock secondary antibody (HRP conjugate) with 293  $\mu\text{L}$  Antibody Diluent for a 1:100 dilution and mix well.

### ***Luminol-Peroxide Solution Preparation***

1. Combine 150  $\mu\text{L}$  Luminol and 150  $\mu\text{L}$  Peroxide (1:1 ratio) and mix.

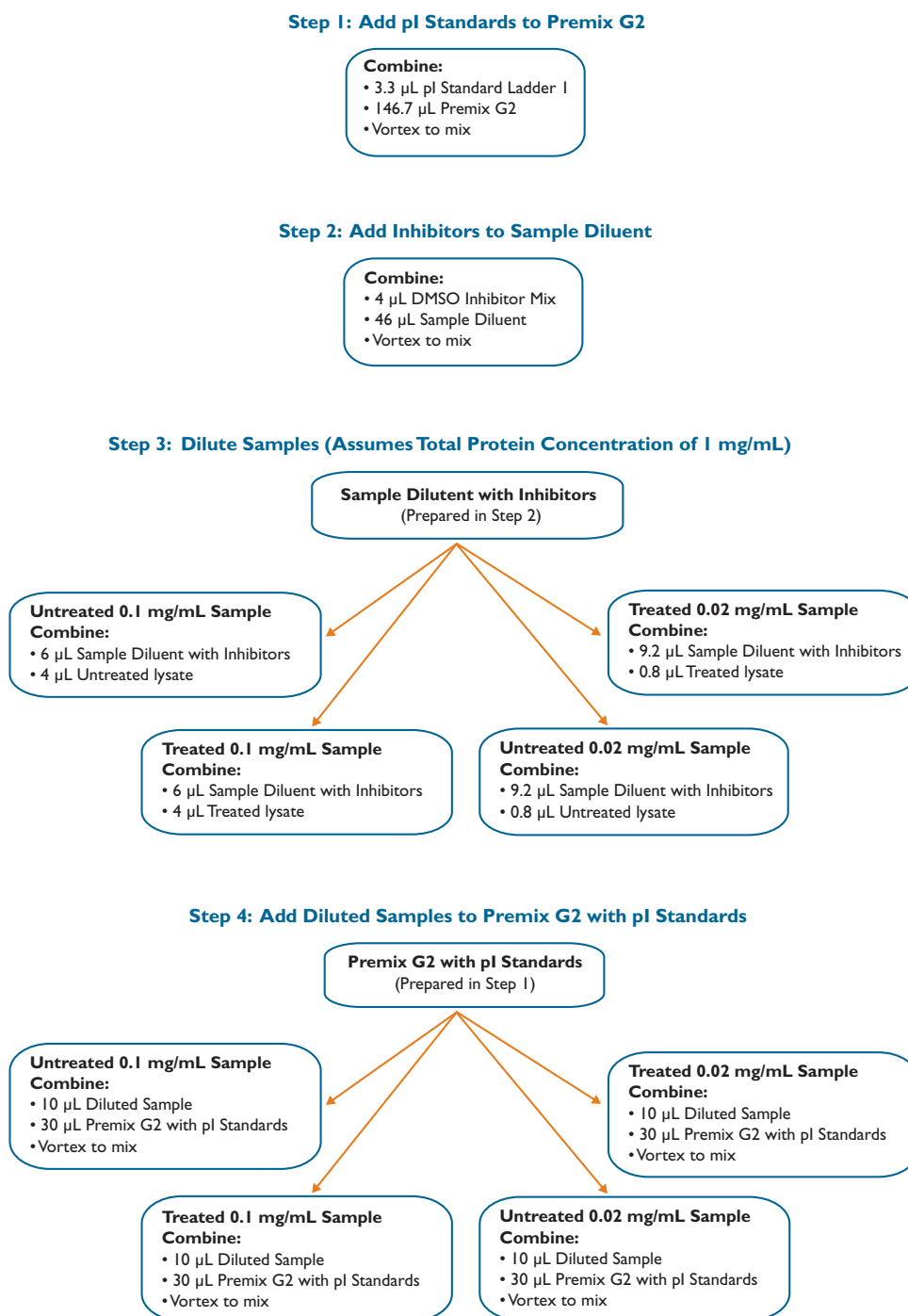


Figure 2-2: Sample mix preparation.

## Adding Reagents to the Assay Plate

---

*NOTE: The recommended plate well volume for samples is 10  $\mu$ L. For all other assay reagents, the recommended plate well volume is 20  $\mu$ L.*

---

1. Pipette 10  $\mu$ L of diluted sample into the wells of row A as follows:
    - Untreated 0.1 mg/mL sample: A1, A3 and A5
    - Treated 0.1 mg/mL sample: A2, A4 and A6
    - Untreated 0.02 mg/mL sample: A7, A9 and A11
    - Treated 0.02 mg/mL sample: A8, A10 and A12
  2. Pipette 20  $\mu$ L of primary antibodies into the wells of rows B and C as follows:
- 

*NOTE: In the recommended plate layout, six primary antibodies are screened. Use more plate rows if more than six primary antibodies are available.*

---

- Primary antibody 1: B1-B2 and B7-B8
  - Primary antibody 2: B3-B4 and B9-B10
  - Primary antibody 3: B5-B6 and B11-B12
  - Primary antibody 4: C1-C2 and C7-C8
  - Primary antibody 5: C3-C4 and C9-C10
  - Primary antibody 6: C5-C6 and C11-C12
3. Pipette 20  $\mu$ L of diluted secondary antibody into each well of row D and E, or the next available rows if more than two rows of primary antibodies will be used.
  4. Pipette 20  $\mu$ L of Luminol-Peroxide solution into each well of row F, or the next available row if the plate layout was adjusted to use additional primary and secondary antibodies.
  5. Centrifuge the plate at 2500  $\times$  g for 5 minutes to spin down the liquid and eliminate bubbles.
- 

*NOTE: The presence of bubbles in plate wells can degrade assay separation efficiency.*

---

### 3.4: Run the Basic Screening Protocol

The NanoPro system settings used in the Basic Screening Protocol are summarized in Table 2-3.

#### **Basic Screening Protocol System Considerations**

- **UV immobilization time.** The optimal UV immobilization time can be protein dependent. An optimal protein signal can typically be obtained between 80 and 140 seconds of UV exposure. The Basic Screening Protocol uses an immobilization time of 100 seconds, which produces a good signal for most proteins.
- **Chemiluminescence exposure time.** A wide range of exposure times should be used in order to obtain accurate data. If the exposure time is too long, the signal may burn-out due to depletion of chemiluminescent HRP substrate. Conversely, insufficient exposure time may result in minimized signals. To gather all chemiluminescence signals, the recommended times are: 30, 90, 270, 480 seconds.

<b>Instrument Setting</b>	<b>Recommendation</b>
<b>Separation Conditions</b>	15000 $\mu$ W, 40 minutes
<b>UV Immobilization Time</b>	100 seconds
<b>Wash 1</b>	2 washes, 150 seconds each (default)
<b>Primary Antibody Incubation</b>	120 minutes (default)
<b>Wash 2</b>	2 washes, 150 seconds each (default)
<b>Secondary Antibody Incubation</b>	60 minutes (default)
<b>Wash 3</b>	2 washes, 150 seconds each (default)
<b>Chemiluminescence Exposure Time</b>	30, 90, 270 and 480 seconds

*Table 2-3: Basic Screening Protocol system settings.*

#### **Data Interpretation**

The goal of the Basic Screening Protocol is to identify potential antibodies for further optimization. Promising antibodies should result in reproducible data - protein peaks will be observed at the same pIs between cycles, and also between different protein concentrations. Signal changes between treated and un-treated samples should be in accordance with the expected biology. In addition, antibodies with similar specificities from different vendors should recognize the same proteins.

At the initial screening step, the response for some proteins may be very small and background signals may be high. However, protein response can be improved over the course of assay development. Antibodies that produce small peaks can be used to validate results observed with other antibodies that produce large peaks, as long as the small peaks are reproducible and are at the same position as the large peaks. Signals should also increase proportionately with protein concentration.

One can expect the phospho isoforms for any given protein to be more acidic, as negative charges are added through this modification. However, different trends might be observed if this phosphorylation leads to the formation of stable complexes with other proteins.

Once the initial antibodies are identified, the following conditions are varied to optimize the assay:

- Primary and secondary antibody concentrations and incubation times
- Premix gradients
- Separation conditions
- UV immobilization time
- Lysate dilution to establish linear range

Details on these variables and other assay development considerations are found in Chapter 3, “*Assay Optimization*”.

## **Chapter 3:**

# Assay Optimization

### **Chapter Overview**

- Assay Optimization
- Optimization of the Separation Conditions
- Immobilization Conditions
- Optimization of Antibody Conditions
- Determination of Dynamic Range Using Optimized Conditions

## Assay Optimization

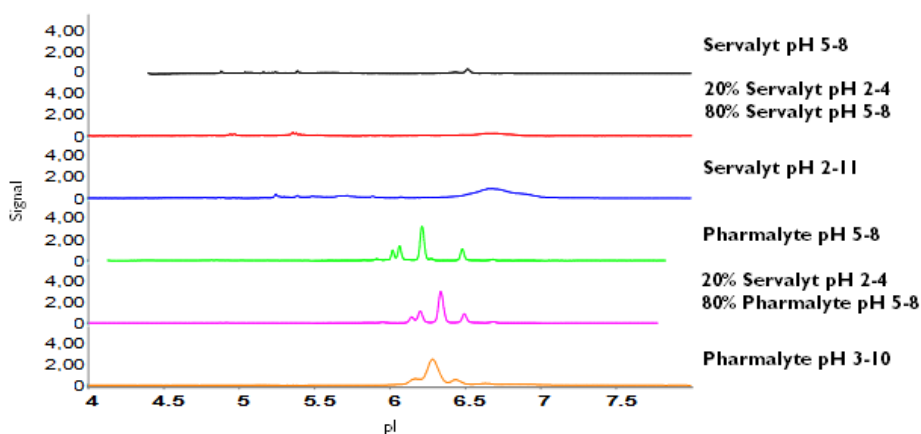
At this point, assay protein peaks should be reproducible between runs and peak signals should change with treatment. Ideally, different antibodies should recognize the same peaks. For guidelines on initial assay development, please refer to Chapter 2, “Getting Started”.

## Optimization of the Separation Conditions

The range of protein peak pIs determined using a broad ampholyte gradient provide insight into whether or not subsequent gradient optimization will improve peak resolution.

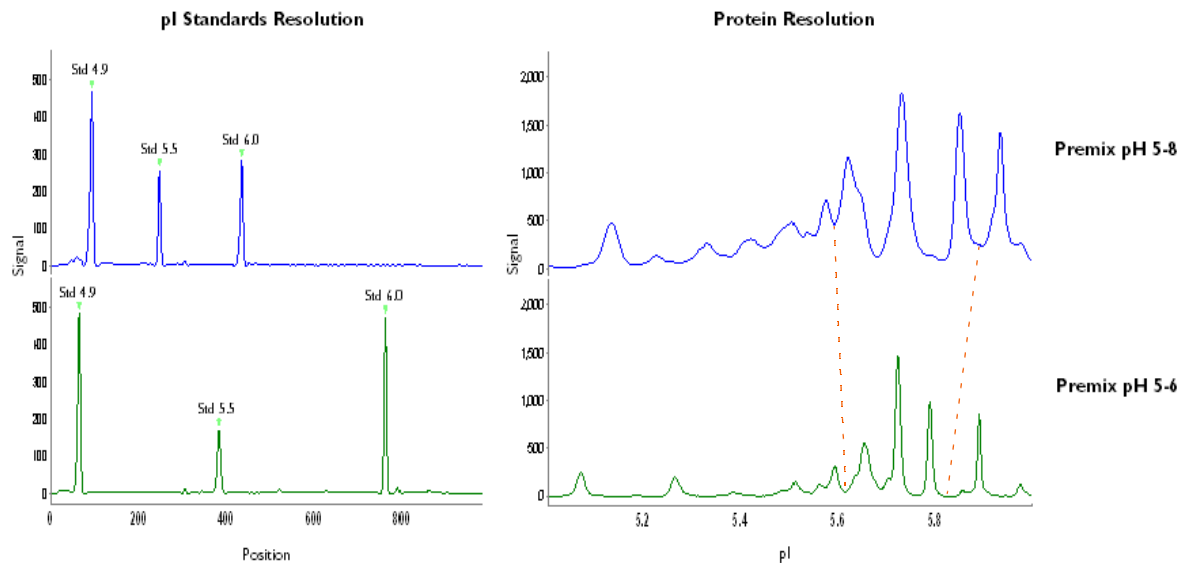
### Choice of Gradient

By definition, ampholyte gradients are random mixtures of molecules with different pIs. Hence, it is not possible to predict with absolute confidence the suitability of one vendor’s ampholyte gradient over another. During separation, proteins migrate to specific positions in the capillary, and their pIs are calculated from these positions. We have determined that the same ampholyte gradient from various vendors can result in slightly different protein positions in the capillary during isoelectric focusing (IEF), and consequently the calculated protein pIs can also be different. Our experience indicates that ampholyte premixes based on Pharmalyte® gradients are very reliable. However, it has also been discovered that in some cases resolution can be improved with Servalyt® gradients (Figure 3-1). ProteinSimple offers ampholyte premixes based on several different gradients as well as an ampholyte-free premix to maximize optimization.



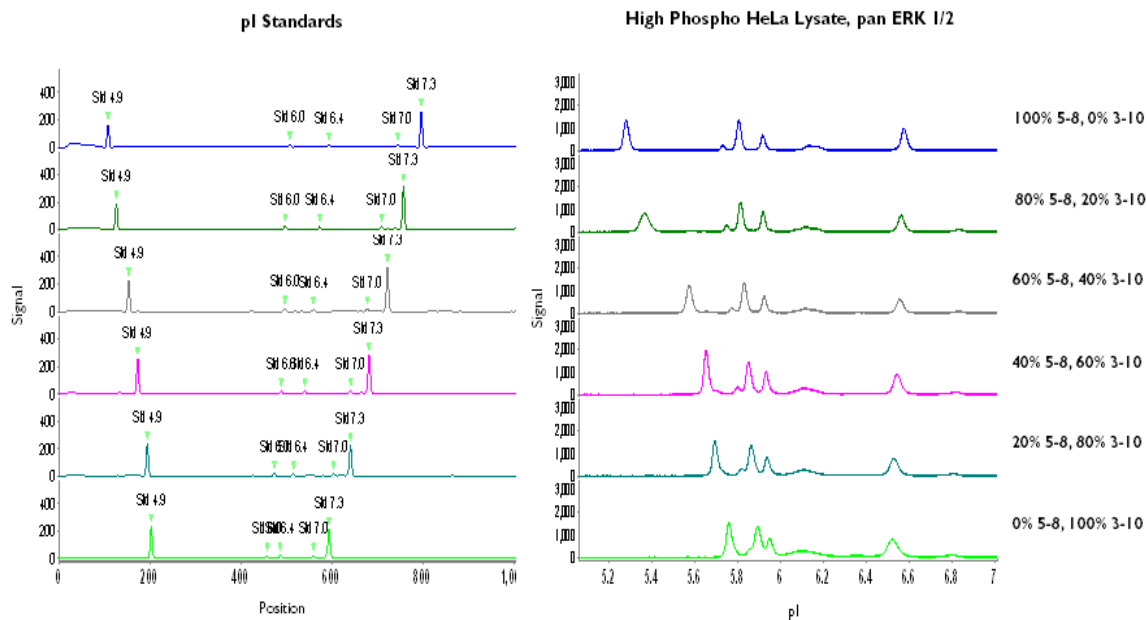
**Figure 3-1:** Effect of various ampholyte gradients on protein resolution. MCF7 cells (serum starved), 0.05 mg/mL. Antibody - Anti-p70S6 (Santa Cruz Biotechnology, SC-8418).

Resolution can sometimes be improved by using premixes with narrower gradients. The premixes provided by ProteinSimple are a great starting point. However, the ampholyte-free premix is a good choice for special ampholyte compositions during final assay optimization. Figure 3-2 illustrates how a narrow gradient can improve resolution.



**Figure 3-2:** Effect of a narrow pH 5-6 gradient on resolution. Sample - HeLa cells. Antibody - Anti PAK1 (Santa Cruz Biotechnology, SC-881).

When using premixes with narrower gradients, the gradient formed in the capillary may be more sensitive to flow phenomena. One option for addressing this is to mix gradients. Mixing a premix with a narrow gradient into a small amount of a premix with a wide gradient is referred to as nesting. Nesting can retain the resolution of the narrow gradient while stabilizing the separation in the capillary at the same time (Figure 3-3). Insufficient use of a premix with a narrow gradient will lead to decreased improvement in resolution, while insufficient use of a premix with a wide gradient will eliminate its stabilizing effect.



**Figure 3-3:** Effect of nested gradients.

Sample composition can also affect final positioning of the gradient in the capillary. If the gradient position shifts toward the end of the capillary, a one-sided plug can be used to push the gradient inward. Plugs can consist of a narrow, extreme gradient such as an acidic pH 2-4 ampholyte, or a small molecule with an extreme pI such as TEMED (pI 12.5). Plugs and the desired ampholyte are added to the ampholyte-free pre-mix. Figure 3-4 shows how low concentrations of TEMED can shift the gradient to be more acidic, but it can lead to gradient compression if used at high concentrations. Gradient compression will not be uniform throughout all regions, so it is important to titrate the plug for each assay system.

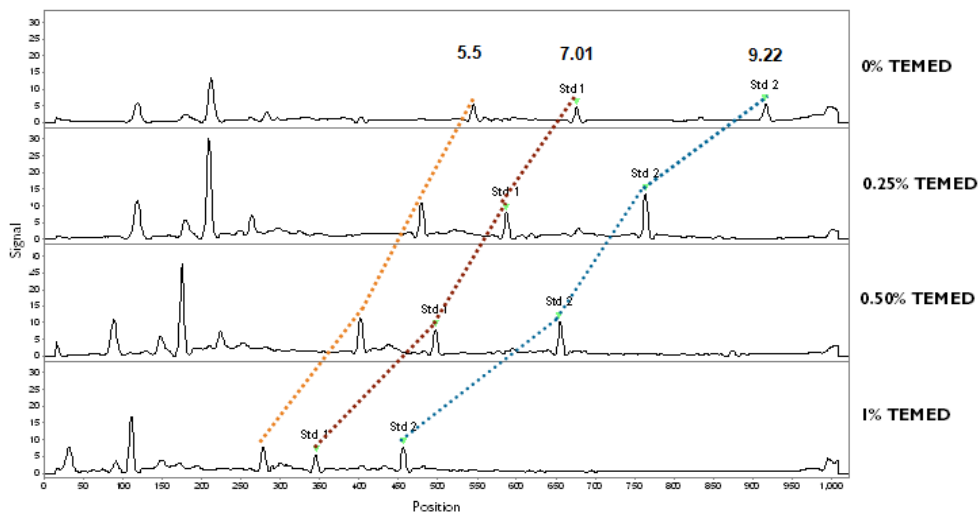


Figure 3-4: TEMED concentration effect on protein resolution.

## IEF Separation (Focusing) Time

The optimal separation time (also known as focusing time) is approximately 40 minutes for most protein systems. However, separation time is influenced by the pH range of the premix as well as sample buffer composition. In general, separation should continue until all proteins are fully focused. Proteins will separate more slowly than fluorescent pI standards. Hence, it is not sufficient to run the separation only until the standards are fully focused. If the ideal focusing time is significantly extended, the resistance in the capillary will become high which can lead to electroosmotic flow. In addition, even with low hydrodynamic flow, extended separation times will amplify this effect. Hydrodynamic flow due to any source will significantly impair data quality, even if it does not cause samples or standards to flow out of the capillary. Again, the goal of assay development is to find the optimum time.

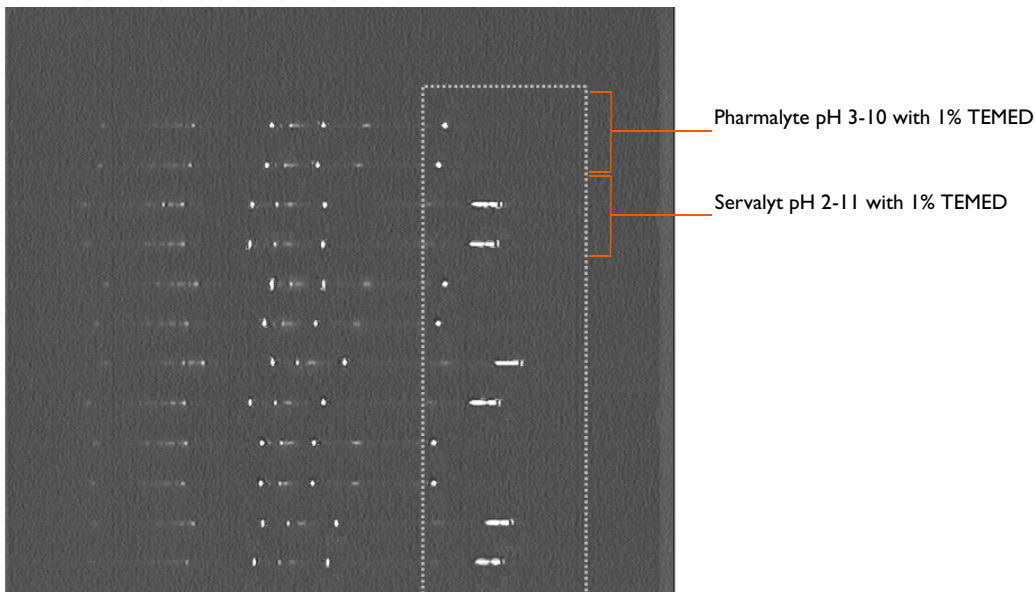
### Electroosmotic flow (EOF)

The overall fluid movement caused by positively-charged ions migrating towards a negative electrode and carrying molecules in the same direction or vice versa. In capillary IEF, EOF is induced by charged capillary walls. If, for example, the wall is negatively charged, the corresponding positive counter ions will move in the electric field toward the basic reservoir and pull the surrounding premix with them. This effect results in a net flow of sample to the basic (right) side of the capillary. Positively charged capillary walls cause the opposite effect, resulting in net flow to the acidic (left) side of the capillary.

### Hydrodynamic flow (HDF)

Hydrodynamic flow is a net movement of analytes in a capillary that causes a pressure difference at the ends of the capillary. This can be caused by differences in fluid levels at either end of the capillary or if the capillary is not completely level.

The focusing time for proteins is influenced by the pH gradient of the premix used as well as the pI of the protein. Figure 3-5 demonstrates that the basic (right) side of the gradient separates more slowly than the acidic (left) side on the NanoPro platform. Comparing Servalyt and Pharmalyte gradients, the Pharmalyte gradient focuses faster in the basic range and is therefore preferred for this pI range. As seen in Figure 3-5, GSK3 reaches its pI of 9 in a Pharmalyte pH 3-10 gradient in 50 minutes, whereas with the Servalyt pH 2-11 gradient complete resolution is not obtained.



**Figure 3-5:** Comparison of Pharmalyte and Servalyt gradient premixes. Separation conditions - 1200  $\mu$ W, 50 minutes.

Parameters to consider in choosing separation conditions are: reproducibility of pI, protein peak signal strength, and peak resolution.

**The resolution between two peaks (a & b) is given by:**

$$R_s = 2 (pI_a - pI_b) / (W_a + W_b)$$

**Where:**

**pI** = pI of peak

**W** = peak width of peak

**R<sub>s</sub>** = resolution ( $R_s > 1.5$  for baseline resolution)

## Separation Mode

Isoelectric focusing is used to separate proteins based on small differences in electric charge. Higher electric field strengths produce more highly resolved peaks. However, care must be taken to avoid excessive current which can lead to Joule heating and protein precipitation.

As focusing proceeds, the molecules in the capillary approach their isoelectric point and become less charged, lowering the conductivity in the capillary. As the conductivity drops, the voltage can be increased without risking excessive current. The NanoPro 100 system provides two options for this step-wise increase in voltage.

### Constant Power Mode

In **Constant Power** mode, the user selects one power setting for the 12 capillaries (generally 15000  $\mu$ W). The NanoPro 100 instrument will monitor the current, and gradually increase the voltage as the separation proceeds. This mode has the advantage of maximizing the rate at which the voltage is increased, while correcting for variability in the salt content of the samples (i.e. the voltage increase will be slower for samples with higher conductivity, eliminating the risk of heating).

If the conductivity of the samples is different between capillaries due to different cell preparations or pH gradients, **Constant Power** mode will not give reproducible results and should not be used. While the **Constant Power** mode ensures the total power applied to the 12 capillaries stays constant, if sample conductivity differs, the power in each capillary can differ greatly. In this scenario, there is a risk of overheating the samples with higher conductivity and sacrificing resolution for the samples with lower conductivity.

### Constant Voltage Mode

In **Constant Voltage** mode, the user defines a series of voltage settings to ramp the electric field strength over time. In this mode, the electric field strength will be completely reproducible from cycle to cycle, but the voltage settings may need to be tailored for specific sample matrices to avoid heating.

If the conductivity of the samples to be run in the same cycle are considerably different due to cell preparations or pH gradients, **Constant Voltage** must be used to obtain reproducible results. The voltage settings and focusing time will need to be determined empirically according to Ohm's Law:

$$V = IR$$

To check voltage settings for heating, the current must be monitored at the time the voltage is increased. Ohm's Law states that for a sample of a given resistance the voltage will be proportional to the current. For example, increasing the voltage from 100 V to 200 V will produce about a 2-fold increase in current. If the capillaries suffer from Joule heating, this current increase will be greater than 2-fold as the increased temperature will reduce the resistance in the capillary.

If this type of non-linearity is occurring with a given set of conditions, the time allotted for the voltage steps should be increased and/or the voltage applied should be slightly increased.

### **Recommendation for Initial Screening**

ProteinSimple recommends constant power mode separations (15,000  $\mu$ W) for initial screening. In constant power mode, the voltage starts low as many ions can contribute to the current. It then rises over time and stabilizes when most of the ions have left the capillary or have settled at their pl.

$$P = R \cdot I^2$$

**Where:**

**P** = Power

**I** = Current

**R** = Resistance

If **P** remains constant, the initial ion concentration will be relatively high. These ions can carry high current, therefore the resistance is low. As the separation proceeds, more and more ions either settle at their pl or leave the capillary. As such, fewer ions are able to carry current, and the resistance in the capillary goes up.

In this mode, extremely high currents are avoided. High currents can contribute to temperature increases in the capillary which can lead to protein precipitation.

The disadvantage of constant power mode is that the power is regulated over all capillaries in the cycle, and voltage is adjusted for all capillaries equally. Hence, all capillaries are connected and influence each other. For most experiments, differences in ionic strength and composition between samples should be minimal and therefore all capillaries will be electrically similar. If great differences in gradient and sample composition exist among capillaries in a cycle, it might be advantageous to work in constant voltage mode with a step gradient for the separation (see Table 3-1). This strategy will force similar conditions for all capillaries.

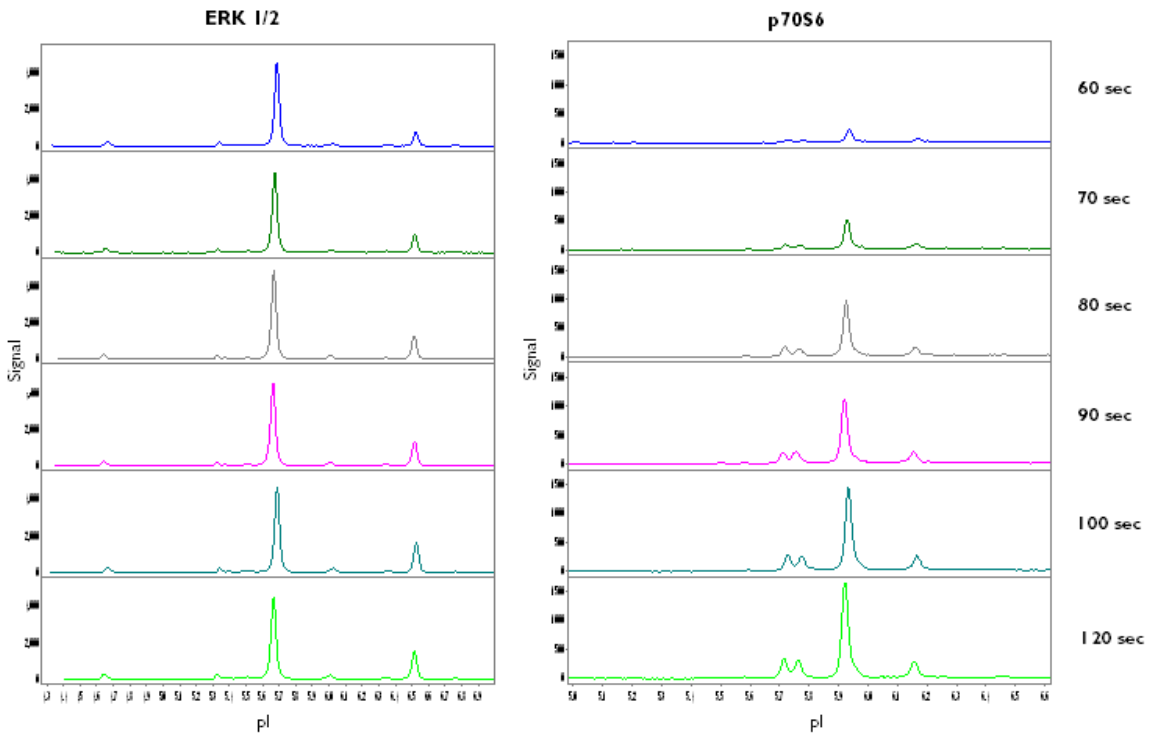
Voltage (V)	Time (min)
100	2
200	2
300	2
500	5
800	5
1000	5
1500	20

**Table 3-1:** Recommended step gradient voltage and time settings.

## Immobilization Conditions

Exposure to UV light immobilizes sample proteins to the capillary wall through a reaction with the proprietary coating on the inside of the capillary. Most proteins show a gaussian immobilization response curve to UV exposure times. While a broad maximum is typically seen at around 100 seconds for most proteins, some respond differently. Hence, it is important to perform a protein response to UV exposure experiment for each new assay developed.

Figure 3-6 shows examples of determining the optimal UV exposure time for two proteins. The data indicates that ERK was very robust over the UV exposure range tested, while p70S6 displayed an optimal response at approximately 100 seconds.



**Figure 3-6:** Response to different UV exposure times for ERK 1/2 and p70S6.

The NanoPro 1000 system allows up to eight cycles (12 capillaries each) of experimental data to be run automatically. When setting up a full, eight-cycle UV exposure time response experiment, it is important to control for potential drops in cycle response. To account for this, the same UV exposure time is repeated in multiple cycles. For example, an 80 or 100 second UV exposure time will be included in the first, middle and last cycle in the experiment. Additional UV exposure times between 30 and 180 seconds are randomly distributed across the remaining cycles. Table 3-2 shows a suggested set up for testing protein response to UV exposure time. Through experimentation, we have determined that the optimal UV exposure time is a function of the protein and not the antibody, and can therefore be applied across antibodies.

Cycle	Time (sec)
1	80
2	100
3	120
4	60
5	80
6	140
7	40
8	80

**Table 3-2:** Recommended cycle distribution to determine optimal UV exposure time.

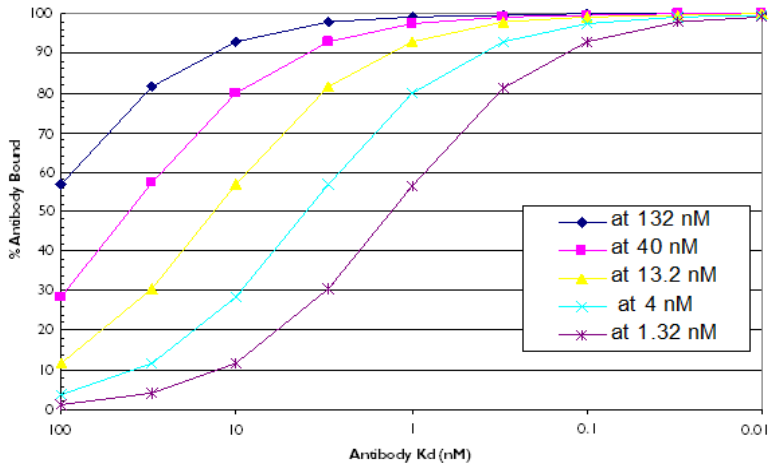
## Optimization of Antibody Conditions

The approach to optimization of antibody conditions is very similar to that used for ELISA development. In both cases, the primary and secondary antibodies should be at saturating concentrations to ensure that signal change is due only to the protein of interest and not to small changes in antibody dilution.

### Antibody Affinity Considerations

The sensitivity of NanoPro technology provides detection of analyte concentrations below most antibody  $K_d$  values.

Figure 3-7 shows the theoretical dependency of analyte binding on antibody concentration and affinity. A 132 nM antibody is equivalent to a 1:50 dilution of an antibody at 1 mg/mL. At a theoretical  $K_d$  of 1 nM and a 1:500 dilution, this antibody binds more than 90% of the analyte present, while at a theoretical  $K_d$  of 10 nM, the same antibody would only bind 60% of the analyte present. It can be concluded that the primary defining factor for assay sensitivity is the quality (affinity and specificity) of the primary antibody used. Interestingly, the analyte concentration does not influence the equilibrium significantly.



**Figure 3-7:** Theoretical analyte saturation curve at different antibody concentrations (0.04 nM analyte).

Unfortunately, not many antibodies have such a high affinity. It is important to be aware of the affinity of the antibody used in order to properly interpret data, especially if quantitative conclusions are to be drawn. Figure 3-8 shows an example of an antibody dilution curve for an Hsp70 antibody (Novus Biologicals, NB600-571). Based on these data, this antibody has an excellent affinity of approximately 0.5 nM. Hence, a dilution of 1:300 is in the saturating range.

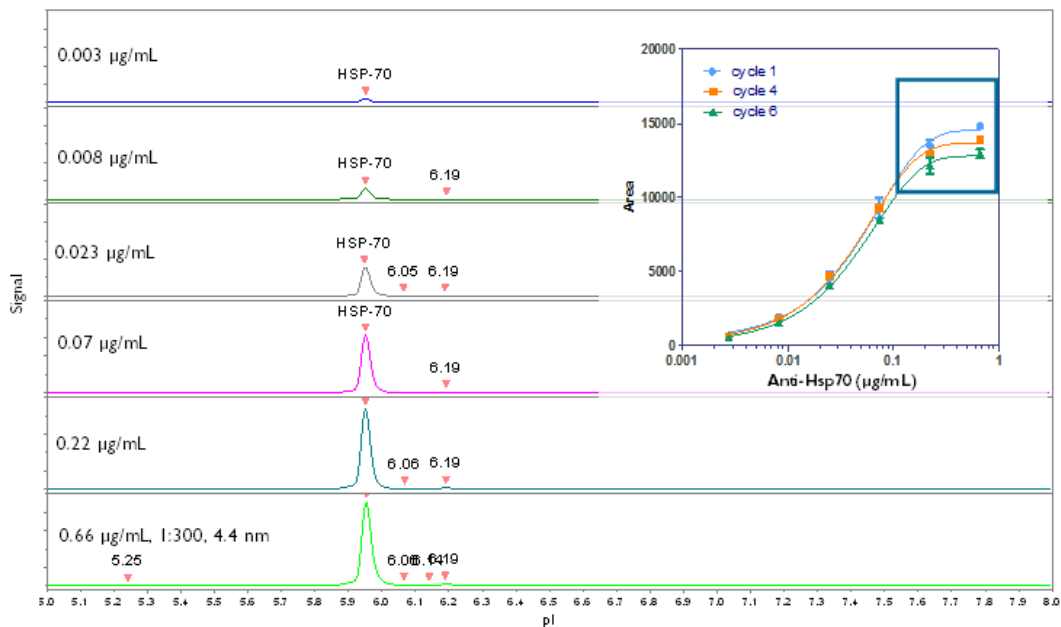
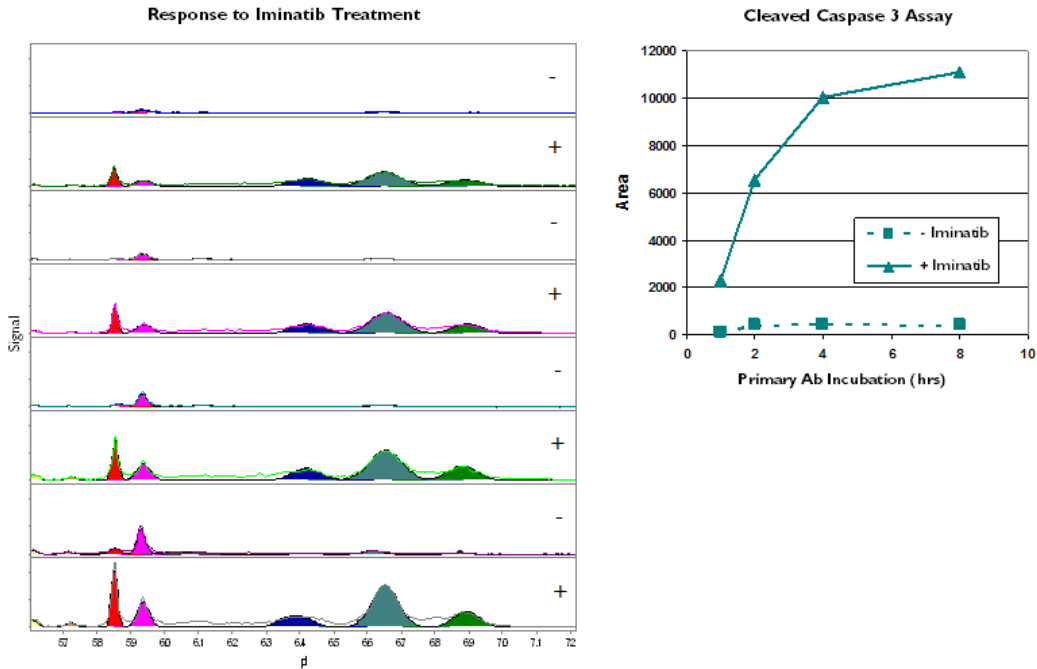


Figure 3-8: Dilution curve for an Hsp70 antibody. High phospho HeLa lysate (0.01 mg/mL).

## Optimization of Antibody Incubation Times and Concentrations

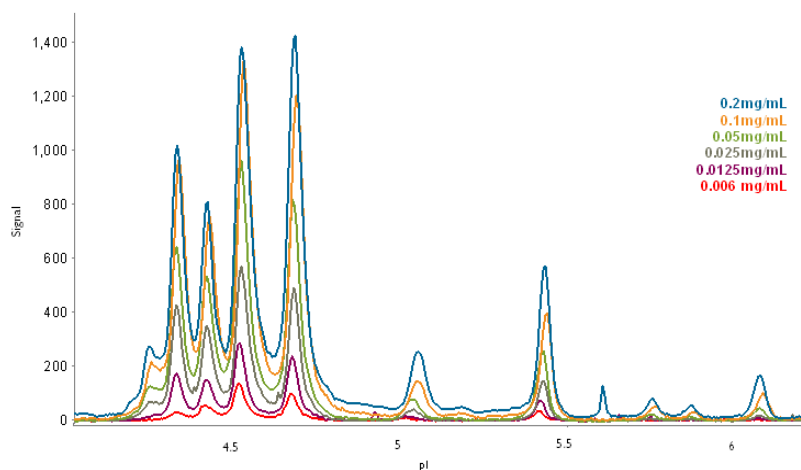
It is important that equilibrium, as defined by  $K_d$ , has actually been reached in an assay. Thus, determination of both incubation time and antibody concentration is an important step in assay development. The incubation time must be long enough for antibody binding to reach equilibrium. However, some antibodies have limited stability over time. Therefore, determination of the optimum incubation time through experimentation is necessary. Figure 3-9 shows protein response to incubation time for an anti-cleaved Caspase3 antibody. The signal is close to saturation after six hours of incubation, with no significant increase of background.



**Figure 3-9:** Protein response to incubation time. K562 cells at 0.15 mg/mL, untreated and treated for 24 hours with 5 μM Iminatib (Gleevec®). Antibody - cleaved Caspase 3 (Cell Signaling Technology, 9661), 1:50 dilution.

## Determination of Dynamic Range Using Optimized Conditions

The primary goal of all assay development steps is to reach conditions in which a change in signal is a response only to a concentration change in the measured protein, and not due to changes in other parameters. This can be assessed by a lysate dilution curve once the previous optimization experiments have been performed. Ideally, the lysate concentration used is directly proportional to the signal measured. This dilution experiment will give the scientist an idea of the dynamic range of the assay. In the example shown in Figure 3-10, 0.1 mg/mL is the top end of the recommended range, as 0.2 mg/mL does not show the expected increase.

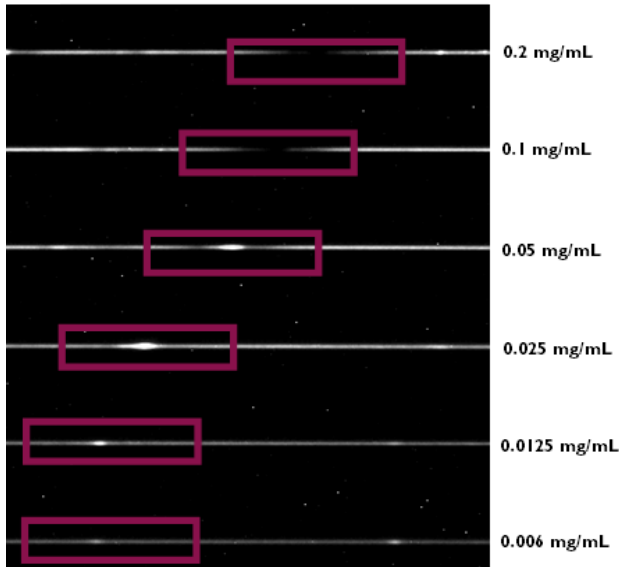


**Figure 3-10:** Serial dilution of AKT. MCF7 cells treated with IGF-1. Antibody - pan-AKT (Santa Cruz Biotechnology, SC-8312).

## Signal Burn-Out

Lack of signal, or burn-out, caused by peroxidase substrate depletion is a phenomenon seen at high protein or lysate concentrations. Modest beginnings of burn-out will present as reduced linearity of the dilution curve. This phenomenon can be identified through evaluation of different chemiluminescence exposure times. The signal/time coefficient should be constant for all exposure times used. If the peroxidase substrate starts to be limiting, the signal/time coefficient will be reduced for subsequent exposure times.

Significant burn-out manifests as no signal at high lysate or protein concentrations, together with increasing signals at lower concentrations. Figure 3-11 shows an example of Hsp70 detection at different concentrations of HeLa lysate. The presence of burn-out is indicated by the lack of detected protein signal at the two highest concentrations.

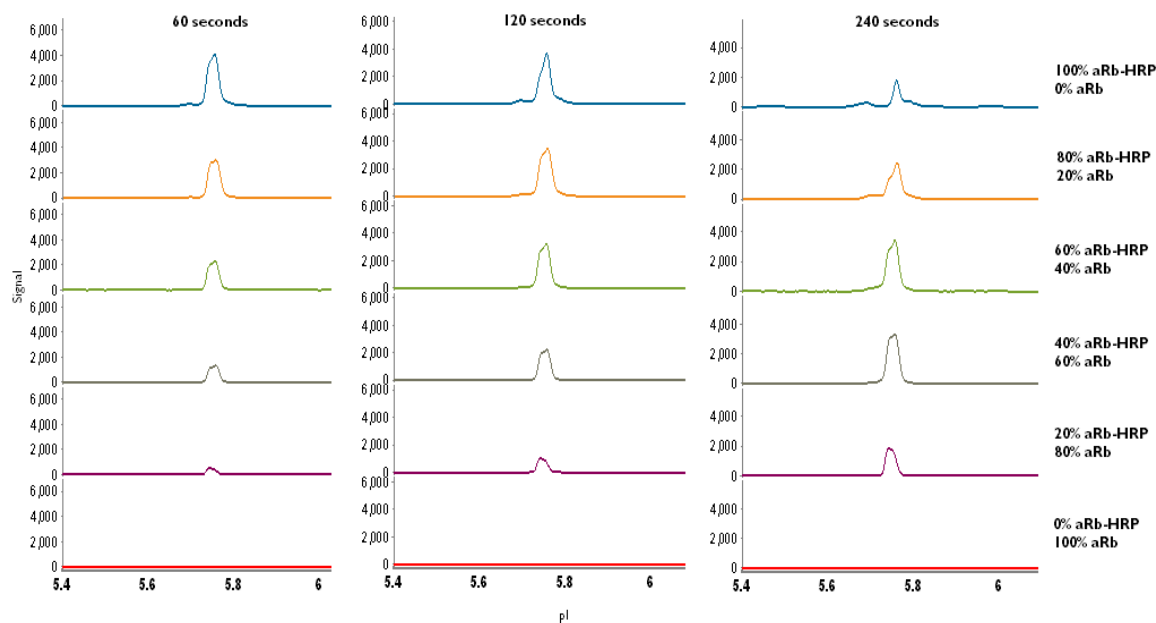


**Figure 3-11:** Detection of Hsp70 in different HeLa lysate concentrations. Burn-out occurs at 0.1 mg/mL.

Burn-out can be addressed by lowering the lysate or protein concentration in the assay. It should never be addressed by lowering the primary antibody concentration, as this could impair the linearity of the assay and may also reduce sensitivity and reproducibility.

In certain circumstances, it may be desirable to probe for two or more proteins in a single assay, as in the case of precious samples or when a loading control must be detected in the same sample as a protein of interest. The ideal concentration for one protein might lead to burn-out for another protein. To keep the signal for multiple proteins in the same sample within optimal range while avoiding burn-out or non-saturating antibody conditions, the HRP-secondary antibody can be mixed with the same unlabeled secondary antibody. This step reduces both the specific activity of the detection antibody and the signal size. It also maintains a constant concentration of the total secondary antibody as well as optimum saturation and equilibrium conditions.

In Figure 3-12, burn-out of the Hsp70 signal is clearly observed. Shorter exposure times under these conditions also displayed non-linear peak behavior. However, the specific activity of the secondary antibody HRP conjugate was reduced by mixing it with identical non-labeled antibody. This both reduced the peak height and lead to the expected signal increase between exposure times, which is a good indicator for linear behavior of the assay in general.

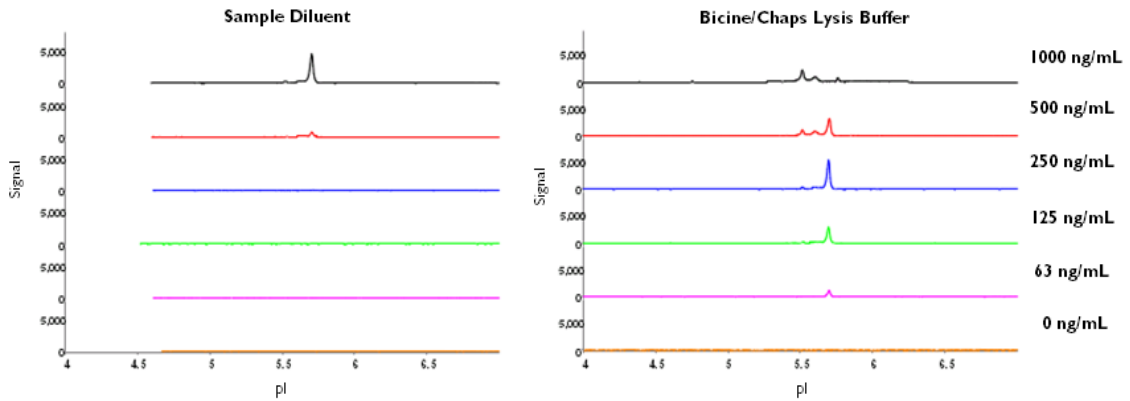


**Figure 3-12:** Demonstration of non-linear peak behavior (burn-out) at high lysate concentrations. Linearity achieved by adding non-labeled secondary antibody. K562 cell lysate, 0.15 mg/mL. Antibody - Anti-Hsp70 (Novus Biologicals, NB600-571), 1:500 dilution. Detection - 60, 120 and 240 second chemiluminescence exposures.

## Enriched Protein Samples and Limit of Detection (LOD)

When assessing recombinant or other purified proteins, potential interactions between proteins at low concentrations and plastic surfaces (i.e. pipette tips and tubes) must be taken in account. This interaction can manifest as an abrupt drop in a dilution curve at lower protein concentrations when well above the expected detection limit, or as overall decreased sensitivity of the assay. As in any other assay, the use of a carrier protein in the sample diluent can eliminate this problem. This approach would apply for recombinant protein analysis, immuno-precipitation eluates, or very high lysate dilutions.

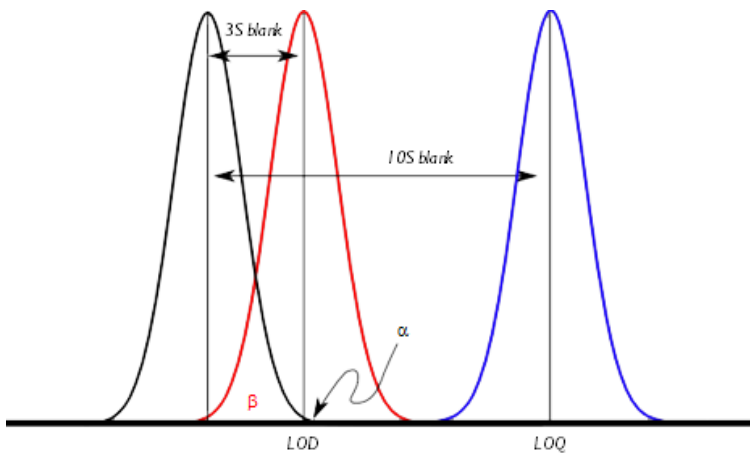
The most common carrier in other methods is Bovine Serum Albumin (BSA). For an IEF method like the NanoPro assay, BSA is not an ideal carrier protein as it will focus at its specific pI and potentially interfere with sample protein pI detection. There is also potential for BSA to precipitate and clog the capillary. The ProteinSimple Bicine/CHAPS Lysis Buffer works well as a sample diluent as it is both compatible with NanoPro assays and the CHAPS detergent itself can act as a carrier. A comparison of recombinant Hsp70 in ProteinSimple Sample Diluent (no carrier) and Bicine/CHAPS Lysis Buffer (carrier) is shown in Figure 3-13.



**Figure 3-13:** Effect of carrier protein in sample diluent on dynamic range and limit of detection. Varying concentrations of recombinant Hsp70 in ProteinSimple Sample Diluent and Bicine/Chaps Lysis Buffer used as the sample diluent.

In select cases, it is also possible to use a complex lysate carrier that does not contain the protein of interest, if such a lysate can be identified. This approach is not always plausible, and there is always potential for cross-reactivity with endogenous proteins.

Having a well defined and optimized carrier system can expand the dynamic range and lower the limit of detection significantly as shown in Figure 3-13.



**Figure 3-14:** Relationship between sample blank, limit of detection (LOD) and limit of quantitation (LOQ).

The figure illustrates the relationship between the blank, the limit of detection (LOD), and the limit of quantification (LOQ). It shows the probability density function for normally distributed measurements at the blank, the LOD (defined as 3x standard deviation of the blank), and the LOQ (defined as 10x standard deviation of

the blank). For a signal at the LOD, the alpha error (probability of false positive) is small at 1%. However, the beta error (probability of a false negative) is 50% for a sample that has a concentration at the LOD (red line). This means a sample could contain an impurity at the LOD, but there is a 50% chance that a measurement would give a result less than the LOD. At the LOQ (blue line), there is minimal chance of a false negative.



## **Chapter 4:**

# Sample Preparation

### **Chapter Overview**

- Reagent Considerations
- Cell Lines - Guidelines and Preparation
- Fine Needle Aspirate (FNA) - Guidelines and Preparation
- Peripheral Blood Mononuclear Cells (PBMC) - Guidelines and Preparation

## Reagent Considerations

The success of assay development is highly dependent on sample preparation. In addition, IEF-based technologies, including the NanoPro platform, are sensitive to sample matrix composition. Therefore, every step in the sample preparation process that may impact the final sample composition in the capillary must be carefully considered. This section describes general recommendations when choosing lysis system components. Guidelines for specific sample types and their preparation will be discussed later in the chapter.

### Buffer Components

Most buffers used for protein extraction contain some or all of the following components:

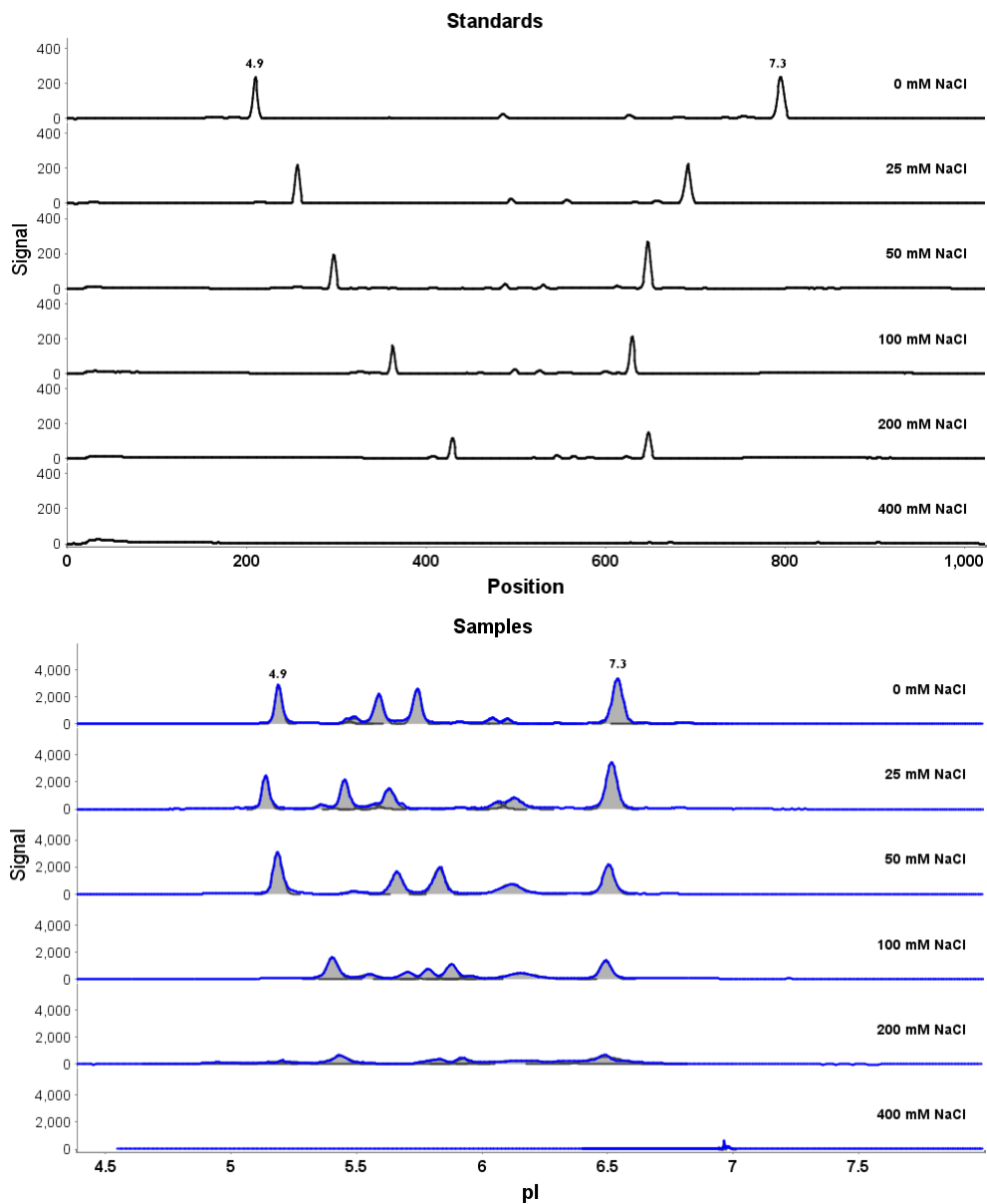
- NaCl
- Buffering agent
- Detergents
- Protease and Phosphatase inhibitors

### Salts

Since salts carry charge, they will increase the electrical current during IEF separation. Increased current can result in elevated temperatures in the capillary and may lead to precipitation. High salt concentrations can also compress the gradient and therefore affect peak resolution. The total ionic strength of the sample is defined by all components carrying a charge. NaCl is generally the main contributor. However, buffering agents, detergents, and ionic inhibitors can also contribute significantly and need to be taken in account.

In Figure 4-1, high salt concentrations not only decrease resolution but also reduce peak heights – no peaks are detected at 400 mM NaCl. Data at 0 mM NaCl represent typical results when using Bicine/CHAPS as the sample diluent, and data at 25 mM NaCl imitate typical results when using the ProteinSimple Sample Diluent. Both diluents can be efficiently used in NanoPro assays.

- **Maximum Concentration.** The final total salt concentration must not exceed 50 mM in the capillary.



**Figure 4-1:** Sample - high phospho HeLa cells at 0.03 mg/mL. Antibody - pan ERK. Bicine/CHAPS Lysis Buffer used as sample diluent and spiked with corresponding concentrations of NaCl.

## ***Buffering Agent***

In our experience, the NanoPro system is most compatible with zwitter-ionic compounds that do not introduce multiple charges. Bicine has less influence than HEPES, and HEPES has much less influence on the separation than Tris.

- **Maximum Concentration.** The buffering agent concentration must not exceed 10 mM in the capillary.

## ***Detergents***

As with buffering agents, zwitter-ionic detergents are preferred as they do not contribute to the ionic strength of the sample. It is important to avoid the use of SDS and other charged detergents. The binding of SDS to the protein of interest can also change the pI of the protein. Although protein-SDS binding is utilized for SDS-PAGE, it is counterproductive in IEF separations.

## ***Recommended Detergents***

- CHAPS:  $\leq 0.3\%$  in capillary
- NP-40:  $\leq 0.1\%$  in capillary
- Triton X-100:  $\leq 0.05\%$  in capillary

## ***Protease and Phosphatase Inhibitors***

Inhibitors are often overlooked as contributors to the overall ionic strength of a sample preparation buffer. For large sample volumes derived from tissue culture cells or tissues, this contribution will generally be diluted out in the sample diluent and will not affect the assay. For small sample preparations, however, this contribution must be taken into account. The inhibitors in the ProteinSimple Aqueous Inhibitor Mix (Table 4-1) can also contribute up to 35 mM salt to the final total salt concentration in the capillary.

Aqueous Inhibitor Mix Component	Concentration in Lysis Buffer
Sodium fluoride	40 mM
Beta glycerophosphate	17 mM
Sodium orthovanadate	1 mM
EDTA	2 mM
EGTA	10 mM
<b>Total</b>	<b>70 mM</b>

*Table 4-1: Final concentration of Aqueous Inhibitor Mix components in Lysis Buffer.*

## Sample Preparation Buffers

The buffers needed for sample preparation can be divided into three categories:

- Cell washing buffers
- Lysis buffers
- Sample dilution buffers

The ideal lysis system would result in minimal matrix changes and a high sample matrix uniformity in the final mix of assay components in the capillary. The compatibility of each buffer used with the NanoPro system should be optimized.

### Cell Wash Buffer

Phosphate-buffered saline (PBS) and HEPES-buffered saline (HBS) are the most commonly used buffers for washing cells. Both contain 150 mM NaCl. Both are well tolerated by cells, and induce minimal changes in cell physiology.

As discussed prior, a maximum of 50 mM total salt is acceptable in IEF. Thus, these cell wash buffers are acceptable only if their carry-over into the lysed sample is minimized. To achieve optimal reproducibility, it is preferred to replace the NaCl with another component that can support the required physiological osmolarity without adding to the total salt concentration.

- **NaCl Alternative.** Sucrose is highly water soluble, does not carry charge and is widely accepted by conventional IEF users as a replacement for NaCl. We have shown that 250 mM sucrose in combination with HEPES or Bicine buffers is a very suitable cell wash buffer.

## **Lysis Buffer**

The lysis buffer must efficiently extract and stabilize the protein of interest from the cell matrix. It also must be compatible with IEF separations. We routinely use two lysis buffers: RIPA and Bicine/CHAPS.

- **Preferred Lysis Buffer:** Bicine/CHAPS is highly compatible with the NanoPro system and can be used in direct dilution with the premix. Therefore, Bicine/CHAPS is the preferred choice for precious samples with low protein concentrations.
- **Alternate Lysis Buffer:** RIPA is well known for its ability to extract a vast variety of proteins. However, samples prepared with RIPA must be diluted at least 20-fold before analysis to reduce the sample's ionic strength.

The lysis buffer also needs to stabilize the protein modification of interest. For assessing various levels of phosphorylation, we use a mix of protease and phosphatase inhibitors. However, to study other post-translational modifications, different inhibitor mixes may be needed.

## **Sample Dilution Buffer**

Ideally, the lysis buffer would also be used as the sample diluent. The Bicine/CHAPS system has this characteristic (see Table 2). Just before sample dilution, the DMSO-based protease and phosphatase Inhibitor Mix is added. The Aqueous Inhibitor Mix is not added to the Sample Diluent due to its relatively high salt concentration.

RIPA cannot be used as a sample diluent. We therefore developed a Sample Diluent with a low NaCl concentration and identical buffering agents (no detergents) for use with RIPA (see Table 4-2). Both the ProteinSimple Sample Diluent and the Bicine/CHAPS Lysis Buffer used as a sample diluent can be combined with either lysis buffer:

Component	RIPA System	Bicine/CHAPS System
Cell Wash Buffer	20 mM HEPES (pH 7.5) 250 mM Sucrose	20 mM Bicine (pH 7.5) 250 mM Sucrose
Lysis Buffer	<b>RIPA</b> <ul style="list-style-type: none"> <li>• 20 mM HEPES (pH 7.5)</li> <li>• 150 mM NaCl</li> <li>• 1% NP-40</li> <li>• 0.25% Sodium Deoxycholate</li> <li>• 10% Glycerol</li> <li>• 1X DMSO and Aqueous Inhibitor Mixes</li> </ul>	<b>Bicine/CHAPS</b> <ul style="list-style-type: none"> <li>• 20 mM Bicine (pH 7.5)</li> <li>• 0.6% CHAPS</li> <li>• 1X DMSO and Aqueous Inhibitor Mixes</li> </ul>
Sample Diluent	<b>Sample Diluent</b> <ul style="list-style-type: none"> <li>• 20 mM HEPES (pH 7.5)</li> <li>• 25 mM NaCl</li> <li>• 10% Glycerol</li> <li>• 1X DMSO Inhibitor Mix</li> </ul>	<b>Bicine/CHAPS Lysis Buffer</b> <ul style="list-style-type: none"> <li>• 20 mM Bicine (pH 7.5)</li> <li>• 0.6% CHAPS</li> <li>• 1X DMSO Inhibitor Mix</li> </ul>

**Table 4-2:** Sample dilution buffer components.

## Cell Lines - Guidelines and Preparation

Both adherent and suspension cell lines are commonly used for assay development. Basic considerations and lysis procedures can be found in Chapter 2, “Getting Started”.

## Fine Needle Aspirate (FNA) - Guidelines and Preparation

The considerations, procedures and data provided in this section are courtesy of Dr. Alice Fan, Stanford University School of Medicine, Palo Alto, CA.

### Practical Considerations

Fine needle aspiration (FNA), is a procedure used to sample cells from superficial (just under the skin) lumps or masses. In this technique, a thin, hollow needle is inserted into the mass to extract cells. The advantage of FNAs is that they are minimally invasive. However, they produce a comparatively low cell number, often not sufficient for multiple protein measurements by traditional analytical technologies like Western Blot. NanoPro technology allows for the interrogation of signaling proteins in these small FNA samples.

In this section, we present sample preparation considerations and NanoPro assay protocols for samples obtained by FNA. Please refer to “FNA Preparation Procedures” on page 56 for details on FNA sample procurement, freezing and lysate preparation prior to NanoPro analysis.

### Sample Procurement and Processing

General considerations are provided below for procurement of FNAs for subsequent NanoPro system analysis. However, actual animal protocols and human subjects protocols for these procedures will be investigator-specific and must be approved by the appropriate institutional review committee before they can be performed for research purposes. All FNAs should be performed by qualified personnel trained in the procedure.

### Preclinical FNA Procedure

Preclinical refers to, for example, obtaining cells from subcutaneous tumors in xenografts. Temporary anesthesia or restraint of the animal may be considered in order to facilitate the sample collection. The procedure can be performed with a sterile 16-25 gauge needle attached to a 3 mL syringe. To prevent possible clotting from blood in the specimen, the syringe may be pre-filled with 1 mL PBS. Collection of 5-10 passes through the tumor generally yields an adequate number of cells for NIA analysis. Immediately after acquiring the aspirate material, empty contents of the syringe into 4 mL of cold PBS.

## Clinical FNA Procedure

Sample procurement from patients should be performed by an experienced cytopathologist. The cytopathologist may want to immediately check the number and quality of cells to assess if additional passes are needed. Collect the sample into RPMI 1640 media.

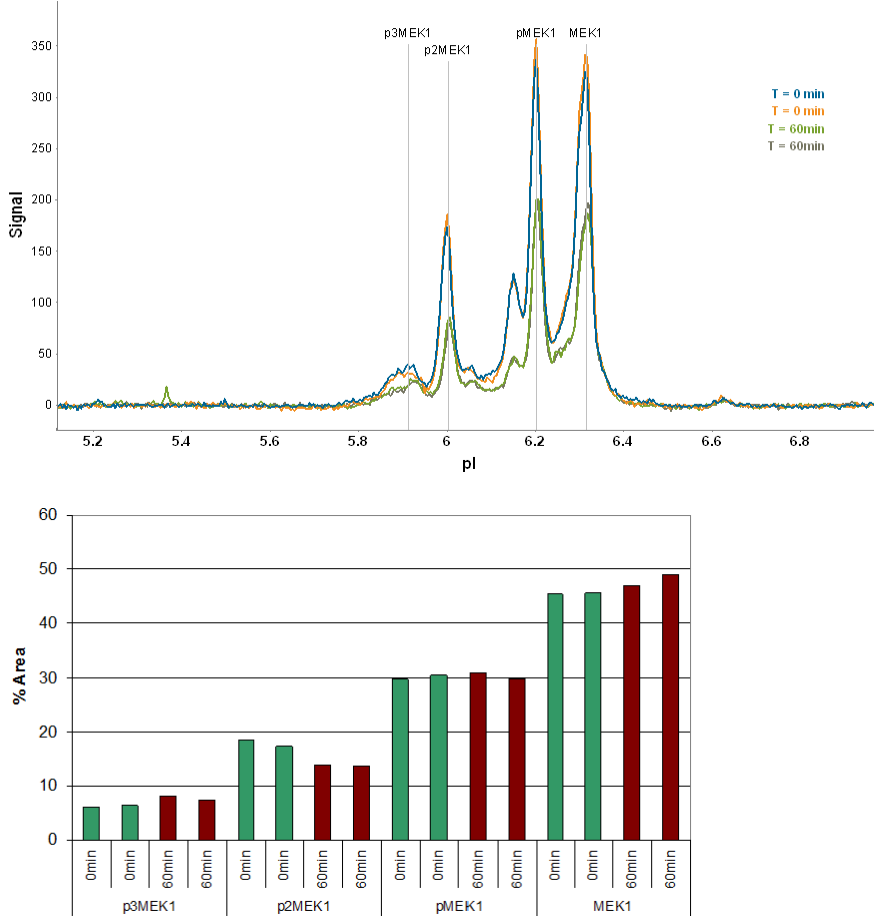
## Sample Stability

In a clinical setting, sample stability can be a challenge due to logistical issues (such as proximity of the clinic to the laboratory) that increase the amount time between clinical sample acquisition and subsequent processing of the sample for research. During this time, it is important to keep the cell suspension strictly on ice. For ERK (data not shown) and MEK (Figure 4-2) it is possible to keep FNA samples from human tumors up to one hour on ice without a change in the relative phosphorylation profile. However, addition of phosphatase or protease inhibitors to the RPMI may further improve sample stability. Investigators analyzing proteins from tissues with higher phosphatase activity or proteins with more labile phospho groups may wish to confirm an appropriate time frame specific for their own specimens.

## Cell Yields

FNA collection can vary greatly based on tumor type and operator, although yields are typically between 10,000 and 10 million cells. As an approximation, obtaining a visible pellet indicates sufficient cells for a subsequently successful lysis.

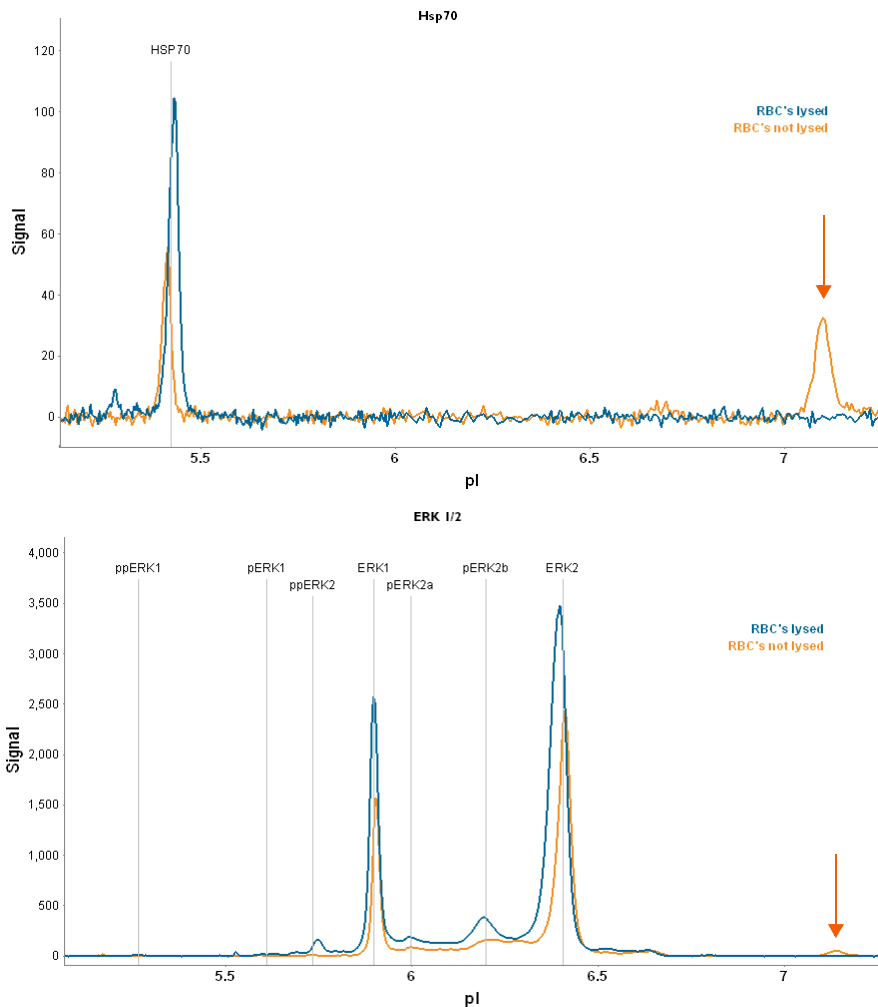
The lysis conditions described in Chapter 2, "Getting Started" were optimized to achieve sample protein concentrations of 2-5 mg/mL, approximately equivalent to lysing 1 million cells in 20  $\mu$ L of lysis buffer. Bicine/CHAPS buffer was chosen because it is tolerated in the NanoPro assay at 50% dilution and thus minimizes buffer based dilution artifacts.



**Figure 4-2:** Effect of time specimen spent on ice on MEK signal. Representative measurements for FNA of solid tumor kept on ice for 0 and 60 minutes. Although the signal drops (top), the relative percent phosphorylation of all MEK isoforms remains unchanged in samples kept on ice for 60 minutes compared to 0 minutes (bottom). No significant difference between groups corrected for multiple testing observed.

## Hemoglobin Contamination

The iron in hemoglobin acts as a catalyst for the chemiluminescence reaction that causes luminol to glow. Hence, blood contamination of samples can result in an antibody-independent, luminol-dependent hemoglobin peak detected near a pI of 7. (Figure 4-3).



**Figure 4-3:** Effect of PharmLyse treatment on Hsp70 (top) and ERK 1/2 (bottom) response. Blue - RBC cells treated with PharmLyse. Orange - RBC cells not treated with PharmLyse. PharmLyse removes the hemoglobin peak at pI 7.0, however greater signal variability is observed in treated samples.

There are several commercially available methods to lyse red blood cells and remove the hemoglobin from a sample of interest. Each method involves further manipulation of the sample, which can theoretically increase the variability between samples. One of the most common methods used for clinical specimens is treatment of samples with PharmLyse™ (BD Biosciences, 555899). Example data is shown in Figure 4-3. However, while use of PharmLyse is compatible with the NanoPro platform and does remove the hemoglobin peak, Dr. Fan indicates that variability between samples can significantly increase. Therefore, when the peaks of interest do

not overlap with the hemoglobin peak, it is not suggested to remove the red blood cells from the samples. For analysis of proteins of interest that might resolve in the same pI region as hemoglobin, we recommend weighing the advantages with potential pitfalls when using this method.

A protocol for the use of PharmLyse with specimens to be analyzed on the NanoPro system is presented on page 57.

## Effect of Residual Cell Media on NanoPro Assay Performance

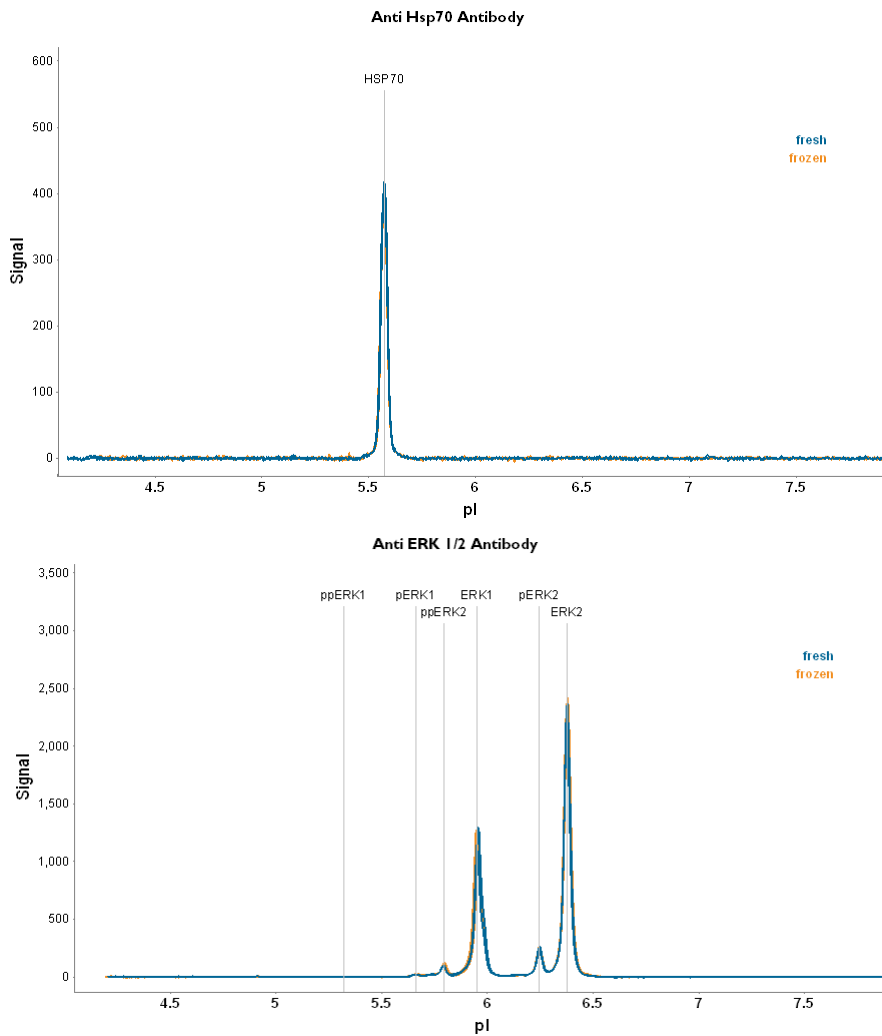
After sample collection, cells are spun down and the RPMI (Cellgro RPMI 1640, 10-041-CV) is removed. As RPMI contains physiological concentrations of NaCl plus other salts, this removal must be as complete as possible to avoid high or variable salt content in the sample, as described earlier in this chapter. Washing the cells at this point with a sucrose containing wash buffer, as described in “Salts” on page 44 can reduce the salt contamination. However, every additional manipulation to the sample will contribute to the variability of the final analyzed sample. We recommend taking great care in complete removal of the RPMI - for example, by using a gel loading tip to remove the last microliters of RPMI.

## Snap Freezing

Freezing the cell pellets in liquid nitrogen after removal of RPMI has significant logistical advantages, especially when working in a clinical setting with variable sample collection opportunities. In addition, this step eliminates the variability associated with the lysis step itself since samples can be lysed simultaneously and batch analyzed regardless of their collection date. Snap freezing of pellets does not affect quantification of ERK1/2 phosphorylation in an FNA sample from a patient. (Figure 4-4).

## Tissue Origin

The tissue the specimen is collected from is another factor to consider for successful sample preparation. For example, it was observed that FNA samples drawn from lung tissue often have a very viscous supernatant after spinning the cells down. This is likely due to a high mucus content which also correlates to low and variable signals. High fat content also hinders sample preparation, and care must be taken when removing the fat layer after spinning the lysate.



**Figure 4-4:** Effect of snap freezing FNA pellets versus fresh FNA samples on signal. HSP70 (top) and ERK 1/2 (bottom) shown.

## FNA Preparation Procedures

### Sample Procurement and Freezing

Always use BSL-2 safety precautions when handling samples. For example, work in hood, dispose of tips and supernatants in bleach, etc.

1. Investigator (for preclinical specimens) or cytopathologist (for clinical specimens) performs at least 10 passes through tissue and collects into 4 mL RPMI-1640.
2. Keep the FNA suspension on ice for transport or store at 4 °C for as brief a time possible.

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*NOTE: Minimize time between collection and snap freezing of the FNA pellets, preferably less than one hour.*

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3. Divide FNA suspension equally into three 1.5 mL tubes. Perform optional PharmLyse step here if required using protocol that follows.
4. Spin 1.5 mL tubes at 5000 × g for 5 minutes at 4 °C.
5. Remove RPMI very carefully and avoid disturbing the pellet as much as possible. Minimize residual supernatant through use of a gel-loading tip. Expect 1-2 µL max residual supernatant volume.
6. Snap freeze pellets in liquid nitrogen. Store pellets at -80 °C until ready for lysis.

### Lysis

1. Prepare Bicine/CHAPS Lysis Buffer containing inhibitors:
  - Dilute Aqueous Inhibitor Mix 1:25 in Lysis Buffer
  - Dilute DMSO Inhibitor Mix 1:50 in Lysis BufferExample: To prepare 2 mL of Lysis Buffer with inhibitors, add 80 µL of Aqueous Inhibitor Mix and 40 µL of DMSO Inhibitor Mix to 1.88 mL ice-cold Lysis Buffer.
2. Thaw cell pellet on ice for 1-2 minutes.
3. Add 20 µL of lysis buffer containing inhibitors to a pellet approximately equivalent in size to 1 million cells.
4. Completely resuspend cells in lysis buffer by pipetting up and down or via quick, low speed vortex.
5. Leave on ice for 30 minutes. Briefly vortex after 15 minutes.
6. Centrifuge at 14,000 × g for 10 minutes at 4 °C.
7. Collect supernatant with P200 filter tip. This is the lysate.

8. Aliquot 5-10  $\mu\text{L}$  into individual microfuge tubes and snap freeze in liquid nitrogen. Store at  $-80\text{ }^{\circ}\text{C}$ .
9. Determine protein concentration of lysate using the Pierce BCA kit in one of the aliquots.

### **PharmLyse (Optional)**

1. Prepare a 1X solution of PharmLyse at room temperature.
2. Spin FNA sample in RPMI in 1.5 mL tubes at  $1500 \times g$  for 5 minutes at  $4\text{ }^{\circ}\text{C}$ .
3. Discard supernatant as described in step 5 of "*Sample Procurement and Freezing*".
4. Resuspend cells in 500  $\mu\text{L}$  of 1X PharmLyse.
5. Incubate for 10 minutes at room temperature.
6. Add 750  $\mu\text{L}$  sterile PBS.
7. Spin at  $5000 \times g$  for 5 minutes at  $4\text{ }^{\circ}\text{C}$ .
8. Remove supernatant and follow protocol from step 6 of "*Sample Procurement and Freezing*".

### **NanoPro Assay**

Protein concentrations between 0.03 and 0.10 mg/mL in the capillary were generally run for proteins tested. Optimal concentration will depend on sample origin and the protein of interest, and may require some assay optimization. All other conditions are equivalent to general recommendations.

## References

### **Human FNA**

1. Buley, I.D., Fine needle aspiration of lymph nodes, *J Clin Pathol.*, 1998 Dec, 51(12): 881-5.
2. Lever, J.V., Trott, P.A., Webb, A.J., Fine needle aspiration cytology, *J Clin Pathol.*, 1985 Jan, 38(1): 1-11.
3. Linsik, J., Franzen, S., Clinical aspiration cytology, Philadelphia: Lippincott Williams and Wilkins Publishers, 1989.

### **Mouse FNA**

4. Hidalgo, M., Amador, M.L., Jimeno, A., Mezzadra, H., Patel, P., Chan, A., Nielsen, M.E., Maitra, A., Altio, S., Assessment of gefitinib- and CI-1040-mediated changes in epidermal growth factor receptor signaling in HuCCT-1 human cholangiocarcinoma by serial fine needle aspiration, *Mol Cancer Ther.*, 2006 Jul, 5(7): 1895-903.
5. Jimeno, A., Chan, A., Cusatis, G., Zhang, X., Wheelhouse, J., Solomon, A., Chan, F., Zhao, M., Cosenza, S.C., Ramana Reddy, M.V., Rudek, M.A., Kulesza, P., Donehower, R.C., Reddy, E.P., Hidalgo, M., Evaluation of the novel mitotic modulator ON 01910.Na in pancreatic cancer and preclinical development of an ex vivo predictive assay, *Oncogene*, 2009 Jan 29, 28(4): 610-8.

## Peripheral Blood Mononuclear Cells (PBMC) - Guidelines and Preparation

The considerations, procedures and data provided on PBMC are courtesy of Fernando Shahjarian and Dr. Holden Maecker, Stanford University, Human Immune Monitoring Center, Palo Alto, CA.

### Introduction

Peripheral blood mononuclear cells (PBMC) are widely used in research and clinical uses every day. Many scientists conducting research in the fields of immunology (including auto-immune disorders), infectious diseases, hematological malignancies, vaccine development, transplant immunology, and high-throughput screening are frequent users of PBMC. In many cases, PBMC are obtained from blood banks. Whenever human PBMC are used, the specific handling precautions dictated by each state or institution must be followed.

### PBMC Preparation Procedures

This section provides isolation and preparation procedures for PBMC from human blood for use on the NanoPro platform.

#### *Isolation of PBMC*

1. Place heparin tubes (BD Vacutainer sodium heparin blood collection tubes, 10 mL, green top) filled with whole blood on a rocker for at least 5 minutes (average volume of blood is 8-10 mL).
2. Transfer the whole blood from the heparin tubes into a 50 mL conical tube.
3. Dilute the whole blood by adding PBS (GIBCO, 14190 containing no calcium or magnesium) into the conical tube to approximately the 30 mL mark for a final dilution of at least 2-3X.
4. Ficoll (GE Healthcare, 17-1440-02) will be added next. Invert the ficoll bottle a few times to mix prior to opening.
5. Aspirate 13-15 mL of ficoll into a serological pipette. To underlay the ficoll, place the pipette tip at the very bottom of the conical tube containing the diluted blood. Next, remove the bulb or Pipet-Aid from the pipette, allowing the ficoll to slowly release into the tube. This prevents unnecessary mixing of ficoll and diluted blood, which provides much better separation during centrifugation.
6. As the ficoll fills the bottom of the tube, it will push the diluted blood layer up. When most of the ficoll has drained, plug the top of the pipette and slowly remove it from the tube. Removing the pipette in this manner helps prevent the ficoll from mixing with the blood.
7. Gently transfer the conical tube from the bio-safety cabinet to a centrifuge, being careful to not jostle the ficoll and diluted blood layers. Centrifuge for 30-40 minutes at  $400 \times g$  at room temperature.

8. After centrifugation, 4 layers will be present in the tube: a yellowish layer on top (plasma), a thin, white, buffy coat under the plasma (PBMC), a clear fluid under the buffy coat (PBS), and a red blood cell (RBC) and platelet layer at the very bottom. Gently transfer the tube back to the bio-safety cabinet, again being careful not to jostle the layers.
9. Using a 10 mL pipette, gently aspirate the buffy coat layer. The pipette should be placed directly above the buffy coat layer during aspiration. Be careful to not disturb the bottom red layer as this may draw RBCs into the pipette. Once mixed, RBCs cannot easily be removed from PBMC.

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*NOTE: Any plasma or PBS aspirated with the buffy coat will be washed out in a later step. Multiple aspirations may be needed in order to obtain as much of the buffy layer as possible without aspirating RBCs in the process.*

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10. Transfer the buffy coat (PBMC) layer into a fresh 50 mL conical tube.
11. Wash the PBMC by adding PBS into the conical tube to approximately the 50 mL mark.
12. Centrifuge at  $350 \times g$  for 10 minutes at room temperature.
13. After centrifugation, aspirate the supernatant making sure to not disturb the pellet at the bottom of the tube.
14. Perform a second wash by adding 30-50 mL of PBS to the pellet in the tube and repeating steps 12 and 13.
15. If needed, a cell count can now be performed. Resuspend the pellet in 1 mL of Complete-RPMI (C-RPMI) media (RPMI 1640, 10% FBS, 1% L-Glut, 1% Pen/Strep), and aspirate a small aliquot for counting.
16. If needed, the pellet can be frozen in a DMSO, FBS, C-RPMI solution. Prepare a 20% DMSO/80% FBS solution and dilute 1:2 with 1 mL of C-RPMI media. This will make a final concentration of 10% DMSO/40% FBS/60% C-RPMI media. Aliquot 1 mL (approximately 5-10 million cells) into cryovials for freezing.

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*NOTE: It is recommended to store vials at  $-80\text{ }^{\circ}\text{C}$  for 24 hours prior to transferring them to a liquid nitrogen freezer for long-term storage.*

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## Thawing PBMC Vials

1. Warm media (C-RPMI, Pen/Strep, L-Glut is recommended) to 37 °C in a water bath. Each sample will require 20 mL of media with benzonase (Sigma-Aldrich, E8263-25KU), and no more than 10 samples should be thawed at a time. Calculate the volume of media needed for the number of samples that will be thawed, then prepare the appropriate amount of warm media at a 1:10000 media/benzonase (25 U/mL) ratio.
2. Remove samples from liquid nitrogen and transport to lab on dry ice.
3. Place 10 mL of warm benzonase media into 15 mL conical tubes. Prepare a separate tube for each sample to be thawed, making sure to label each tube with the sample it will receive.
4. Thaw the frozen vials in the 37 °C water bath. When the cells are almost completely thawed, move the samples to the bio-safety cabinet.
5. Slowly add 1 mL of warm benzonase media from the appropriate media tube prepared in step 3 to the cell vial. Next, transfer the 1 mL of cells in benzonase media back into the conical tube containing the remaining benzonase media. Continue rinsing the cell vial with additional media until all cells are retrieved. Repeat this step for each of the remaining samples, working as quickly as possible.
6. Centrifuge the conical tubes containing the cells for 10 minutes at 190 × g.
7. Aspirate the supernatant from the cells and resuspend in 1 mL of warm benzonase media. Pump the pipette up and down to mix. Add an additional 9 mL of media to bring the final volume to 10 mL.
8. Centrifuge the conical tubes containing the cells for 10 minutes at 190 × g.
9. Aspirate the supernatant from the cells and resuspend in 1 mL PBS.
10. If needed, count cells using a Vi-CELL<sup>®</sup> or hemocytometer. To prepare an aliquot for counting with a Vi-CELL, add 20 µL of cells to 480 µL of PBS in the Vi-CELL counting chamber. Load in Vi-CELL and select PBMC for cell type and a 1:25 dilution factor.

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### NOTES:

*After thawing and before treatment, resting the thawed PBMC for 1 hour in C-RPMI 1640, 10% FBS, 1% L-Glut, 1% Pen/Strep is recommended.*

*For an example of treatment for induction of STAT3 and STAT5 phosphorylation, please refer to “Example NanoPro Analysis of p-STAT” on page 63.*

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## Lysis

The procedure that follows is for PBMC treated with IL-2.

1. Prepare lysis buffer by adding the appropriate phosphatase and protease inhibitors to the Bicine/CHAPS Lysis Buffer:
  - Dilute Aqueous Inhibitor Mix 1:25 in Lysis Buffer
  - Dilute DMSO Inhibitor Mix 1:50 in Lysis BufferExample: To prepare 2 mL of Lysis Buffer with inhibitors, add 80  $\mu\text{L}$  of Aqueous Inhibitor Mix and 40  $\mu\text{L}$  of DMSO Inhibitor Mix to 1.88 mL of ice cold Lysis Buffer.
2. Resuspend cells in 1 mL of PBS.
3. Centrifuge cells in PBS for approximately one minute at 500  $\times$  g.
4. Aspirate PBS supernatant and add 30-50  $\mu\text{L}$  of Bicine/CHAPS Lysis Buffer containing inhibitors to each sample.

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*NOTE: PBMC generally yield less protein than other cell types. Minimizing lysing volumes is recommended regardless of cell count. An acceptable cell count range is  $3 \times 10^5 - 10 \times 10^6$  cells.*

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5. Resuspend the cells in Bicine/CHAPS Lysis Buffer containing inhibitors and place on ice for 30 minutes. Flick or gently shake the tube every 10 minutes to ensure proper mixing and aid lysis.
6. Centrifuge cells at 14000  $\times$  g for 10 minutes at 4  $^{\circ}\text{C}$ .
7. Transfer supernatant to fresh tubes for flash freezing in liquid nitrogen or dry ice. Aliquots of 5-10  $\mu\text{L}$  are recommended in order to prevent protein degradation caused by freeze/thaw cycles.
8. Use one aliquot to measure total protein concentration using a BCA protein assay.

## Example NanoPro Analysis of p-STAT

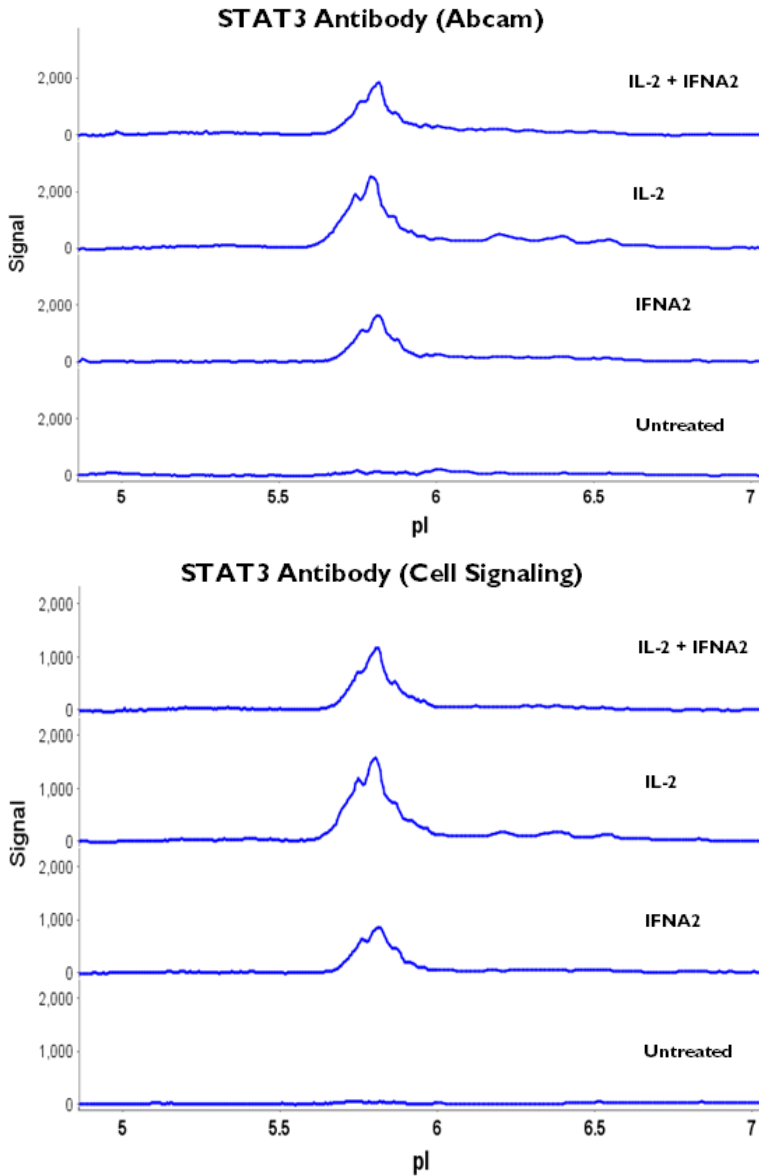
This section provides a general overview of p-STAT3 and p-STAT5 analysis in PBMC. Treatment protocols for PBMC are expected to be dependent on each application. The data that follow are for PBMC treated with IL-2 followed with Human Interferon alpha2.

### Cell Preparation

After thawing, PBMC cells (donor from Stanford Blood Center) were rested for 1 hour in Complete-RPMI (C-RPMI) media at 37° C, 5% CO<sub>2</sub>. Cells were then treated with 500 ng/mL IL-2 (Interleukin-2, BD, 554603) in C-RPMI medium for 24 hours at 37° C, 5% CO<sub>2</sub>. After 24 hours with IL-2 stimulation, the cells were treated with 5000U IFNA2 (Human Interferon alpha2, Invitrogen, 11105-1) for 5 minutes.

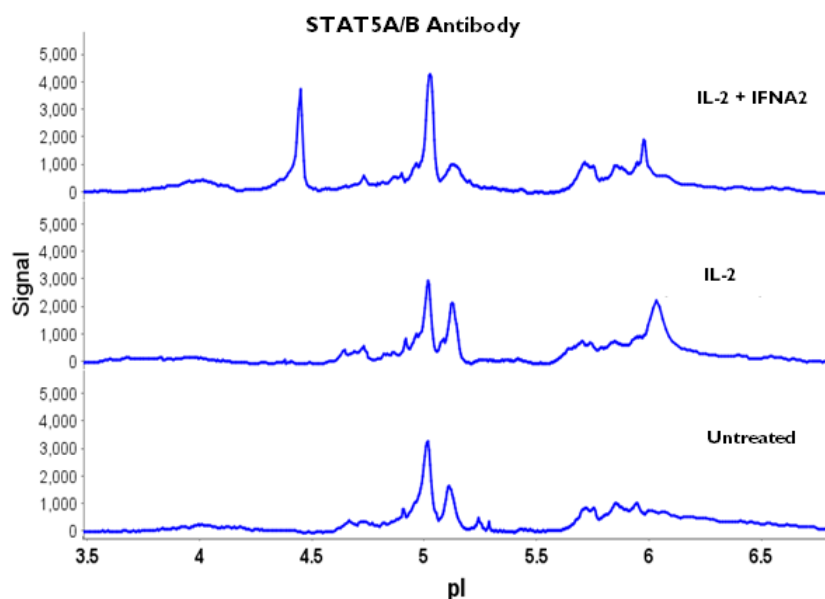
### Treatment Results

Treating cells with IL-2 and IFNA2 increases STAT3 phosphorylation in PBMC. Samples were analyzed on the NanoPro system. Donor PBMC were treated with either IL-2 + IFNA2, only IL-2 or only IFNA2. All expressed a prominent peak which was recognized by the anti-phospho STAT3 (Y705) antibody (Abcam, 30646) which was not present in the untreated PBMC sample (Figure 4-5, top). In a different analysis, the same prominent peak was also recognized by the anti-phospho STAT3 (Tyr705) antibody (Cell Signaling, 9131) which is shown in Figure 4-5 (bottom).



**Figure 4-5:** STAT3 phosphorylation in PBMC treated with IL-2 and IFNA-2 (first trace), IL-2 treatment only (second trace), IFNA2 treatment only, (third trace) and untreated (fourth trace). Primary Antibody (top) - Anti-phospho-STAT3 (Y705) (Abcam, 30646). Primary Antibody (bottom) - Anti-phospho-STAT3 (Tyr705) (Cell Signaling, 9131). Secondary Antibody - Anti-Rabbit HRP (ProteinSimple, 040-656). Final protein concentration in capillary - 0.1 mg/mL as determined by a BCA assay. Separation gradient - Premix 4-9.

Treating cells with IL-2 and IFNA2 also increases STAT5 phosphorylation in PBMC. Samples were again analyzed on the NanoPro system. The anti-phospho STAT5A/B antibody (Upstate, 06-867) recognizes several peaks in PBMC (Figure 4-6, top). Data for cells treated only with IL-2 for 24 hours (Figure 4-6, middle) and for untreated cells (Figure 4-6, bottom) are also shown.



**Figure 4-6:** STAT5 phosphorylation in PBMC treated with IL-2 and IFNA2 (top), IL-2 treatment only (middle), and untreated (bottom). Primary Antibody - Anti-phospho-STAT5A/B (Upstate 06-867). Secondary Antibody - Anti-Rabbit HRP (ProteinSimple, 040-656). Final protein concentration in capillary - 0.1 mg/mL as determined by a BCA assay. Separation gradient - Premix 4-9.

## NanoPro Assay

The final concentrations of proteins tested were 0.1 mg/mL in the capillary. Optimal concentration will depend on sample origin and the protein of interest, and may require some assay optimization. All other conditions are equivalent to general recommendations.



## **Chapter 5:**

# Denaturing Assays

### **Chapter Overview**

- Introduction
- General Denaturing Considerations for NanoPro Assays
- Comparison of Native and Urea/DTT-Treated Protein Profiles
- Optimization of Denaturing Procedure for NanoPro Assays
- Recommended Protocol for Optimization of the NanoPro Denaturing Assay

## Introduction

The NanoPro assay separates, immobilizes and detects cell proteins in their native state. Since lysis buffer typically contains only a limited amount of zwitterionic detergent (0.6% CHAPS), it is expected that at least some of the protein complexes and quaternary structures found in cell signaling pathways are also retained.

These results are extremely valuable, and can be better understood and interpreted with the development of complementary assay conditions in which protein lysates are denatured. This allows dissociation of complexes and partial or complete protein unfolding. Running an assay under denaturing conditions in conjunction with the native state IEF separation allows identification of protein complexes and their components. This is done via comparison and analysis of changes in protein profiles upon dissociation, similar to 2D gel analysis. In addition, greater resolution and peak efficiency can be achieved by reducing the number of non-specific interactions.

It is important to remember, however; the essential difference between 2D gel analysis and NanoPro system analysis. Instead of IEF followed by size-based protein separation, NanoPro technology employs parallel IEF-based separations performed on differentially treated samples.

Also, the mechanism and end results of protein denaturing are significantly different when mediated by SDS/heat methods compared to chaotropic agents like urea. It is assumed that SDS causes tertiary structure unfolding in the submicellar and chain expansion in the micellar range of concentrations. The chain expansion, driven by coulombic repulsion between the protein-bound micelles, promotes the formation of cylindrical or rod-like structures with uniform charge density (1, 2, 3).

On the other hand, urea denatures proteins by altering water structure within the hydration shell, facilitating the exposure of nonpolar parts of the solvent, and possibly also by direct interaction with the protein backbone. It has been speculated that this first mechanism is prevalent at lower urea concentrations, and results primarily in dissociation of protein units of quaternary structure. Whereas at high concentrations, urea also interacts with the protein directly and facilitates its unfolding into a "random coil" (4).

In some NanoPro assays, the dissociation of protein complexes that occurs at a 3-5 M urea concentration may be the most interesting part of denaturing process. However, other proteins appear to have high signal intensity and stable profiles after treatment with high urea concentrations (up to 7.25 M).

Interestingly, the latest research shows that residual secondary structure is often preserved in proteins denatured by urea, to various degrees. It has been shown that integral membrane proteins and other proteins with long hydrophobic sequences will retain most of their secondary structure (5).

Initial results indicate that membrane proteins can tolerate higher urea concentrations with no detrimental effects on assay performance. The other group that performs very well with denatured assays is small proteins, which can partially renature their secondary structure once the denatured lysate is diluted. We speculate that the residual secondary structure may promote better immobilization of the proteins, but the mechanism of this process is unclear.

## General Denaturing Considerations for NanoPro Assays

Since IEF separation requires low ionic strength, it is not compatible with SDS/heat mediated protein denaturation unless highly diluted. In our experience SDS, even diluted to a residual <0.002% in the capillary, invariably resulted in absence of signal. Since the fluorescent peptide pI Standards appeared to be separated and immobilized normally, we speculate that loss of signal is related to the initial SDS treatment, and not to residual SDS in the capillary.

Historically, proteins separated by IEF were denatured by urea or a combination of urea/thiourea supplemented with nonionic detergents and reducing agents which further denatured the proteins by reducing disulfide linkages. In particular, the urea/thiourea mixture in combination with certain detergents is often recommended for denaturation of membrane proteins, since it's shown to be beneficial in resolving solubility problems common to this species.

We have tested urea and thiourea as denaturing agents in lysis buffer containing 0.6% CHAPS, with and without addition of reducing substances, followed by dilution with Sample Diluent or Bicine-CHAPS buffers.

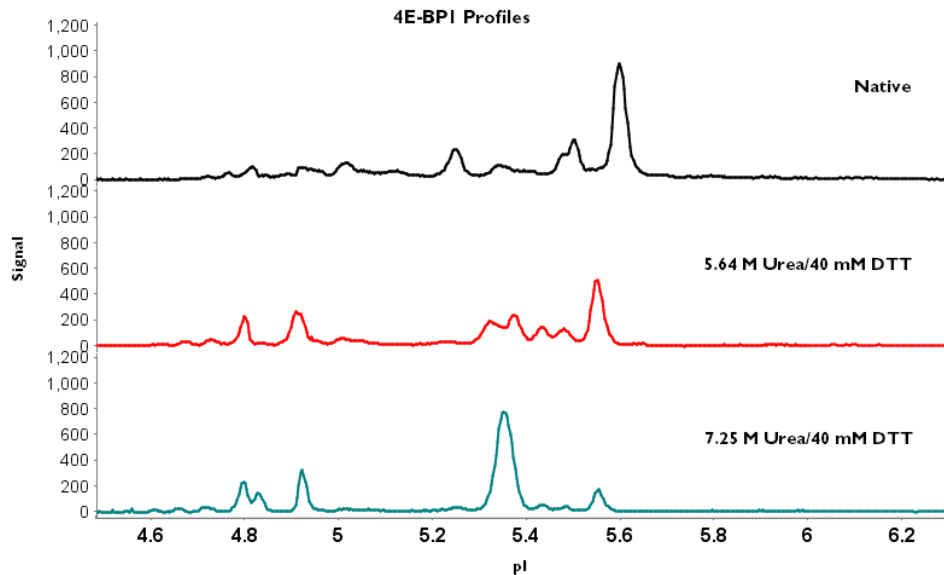
While addition of reducing agents (DTT, TCEP) to urea was beneficial for most applications and resulted in a signal increase, addition of thiourea to urea did not result in any improvement. Interestingly, when urea/thiourea mixtures were used without DTT, a strong reduction in signal was observed.

We also evaluated adding urea/DTT to the lysis buffer directly vs. performing the denaturing procedure post-lysis. The best results were obtained when cell lysates were treated with denaturing solution post-lysis and used directly. Extended incubation with a denaturing lysis buffer during harvest followed by freeze/thaw cycles caused signal deterioration in most applications.

## Comparison of Native and Urea/DTT-Treated Protein Profiles

Native cell lysates previously tested using typical preparation procedures were subjected to a denaturing procedure using 4.03 M urea/40 mM DTT and 7.25 M urea/40 mM DTT for 5-10 minutes at room temperature. Incubation under denaturing conditions was followed by dilution with Sample Diluent, resulting in a final concentration of urea/DTT of <1.5 M and <8 mM respectively. Urea/DTT-treated and native lysate profiles were directly compared using the standard NanoPro assay.

Changes in protein profiles upon specific stimulation/inhibition conditions were assessed in urea/DTT-treated and native lysates. In general, the relations between peaks, identified as phosphorylated and non-phosphorylated forms of the protein, was similar in both states (such as a signal increase/decrease or shift in peak position). At the same time, treatment with urea/DTT caused concentration-dependent changes in many protein profiles.



**Figure 5-1:** Urea/DTT concentration-dependent changes in Anti-4E-BPI (Cell Signaling Technology, 9644) profile after urea/DTT treatment in MCF10A cells.

Changes in peak position, number and signal intensity have also been observed. In general, urea/DTT-treated sample profiles are characterized by fewer peaks, possibly due to the dissociation of protein complexes and a reduction in the number of protein conformations. This results in better peak shape and resolution. The remaining peaks typically shift compared to the peak position in the native assay, and are often in better agreement with predicted pIs. In some cases, a significant increase in signal intensity was observed.

Interestingly, in case of 4E-BPI (Figure 5-1), native lysates present peaks that appear to correspond to the profiles of both the native and urea/DTT-treated protein states. It is known that native states in cellular environments are in dynamic equilibrium with the denatured conformations. Proteins routinely go through unfolding to cross a lipid bilayer, and also prior to proteolysis during protein turnover (6). An assay that can identify both states and indicate the rate of turnover may be very useful.

The effect of urea/DTT treatments on NanoPro assay results varied with different proteins. Table I presents the results for 16 existing applications screened under native and denaturing conditions with high and low urea/DTT concentrations. Both 4.03 M urea/40 mM DTT and 7.25 M urea/40 mM DTT were used for most proteins, with a few targets exposed to wider range of concentrations. The effect of urea-induced denaturation was assessed. It can be speculated that milder denaturing conditions (2-5.5 M urea/40 mM DTT) resulted primarily in complex dissociation, if such complexes were present. Concentrations of 6-7.5 M urea/40 mM DTT caused more complete protein denaturation. A number of targets demonstrated modified profiles, but retained strong signal after treatment with high urea concentrations. But in some cases, exposure to

7.25 M urea/40 mM DTT resulted in a decrease or loss of a signal. Preliminary data indicates this may be due to failure in protein immobilization after more extensive unfolding. Examples of urea-induced denaturation are shown in Figure 5-2 and Figure 5-3.

Target	4.03 M Urea/ 40 mM DTT	7.25 M Urea/ 40 mM DTT	Signal Intensity Level
$\beta$ -2-Microglobulin	No change	No change	Not affected
Thioredoxin I	Fewer peaks, peak height ratio changes	Fewer peaks, peak height ratio changes	Not affected
ALAS1	No change	Fewer peaks	Not affected
EGFR	Not tested	Fewer peaks	Not affected
p-JNK	Not tested	Fewer peaks	Not affected
p-STAT3	Fewer peaks, peak height ratio and position changes	Fewer peaks, peak height ratio and position changes	Not affected
p-STAT5	Fewer peaks, peak height ratio and position changes	Fewer peaks, peak height ratio and position changes	Not affected
Crk-L	Peak position and height ratio changes	Peak position and height ratio changes	Not affected
4E-BP1	Fewer peaks, peak height ratio and position changes	Fewer peaks, peak height ratio and position changes	Not affected
4E-BP2	Minor changes in peak height ratio and position	Minor changes in peak height ratio and position	Not affected
Caspase 3	Fewer peaks, peak height ratio and position changes	Fewer peaks, peak height ratio and position changes	Not affected
ERK1/2	No change	Signal decrease for ERK1/ pERK1 but not for ERK2	Affected at 9 M urea
AKT1	Fewer peaks	Strong signal decrease	Affected at 9 M urea
MEK1	No change	Strong signal decrease	Affected at 9 M urea
MEK2	No change	Strong signal decrease	Affected at 9 M urea
PLC $\gamma$ 1	Change in peak height ratio, resolution improvement	Strong signal decrease	Affected at 9 M urea

**Table 5-1:** Effect of urea/DTT treatments on various protein targets.

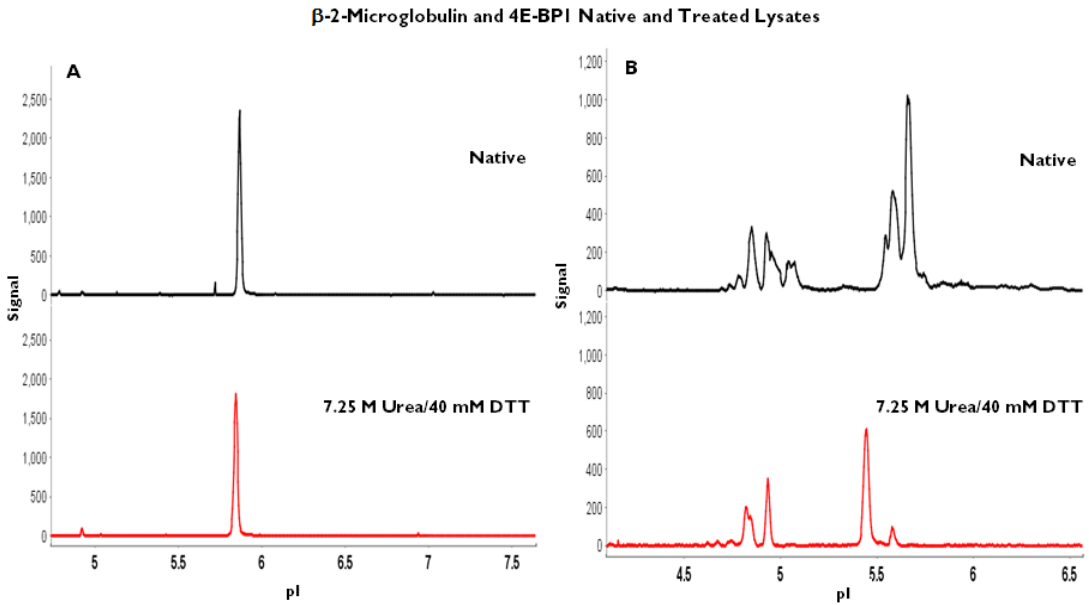


Figure 5-2:  $\beta$ -2-microglobulin (A) and 4E-BPI (B) in native and 7.25 M urea/40 mM DTT treated lysates.

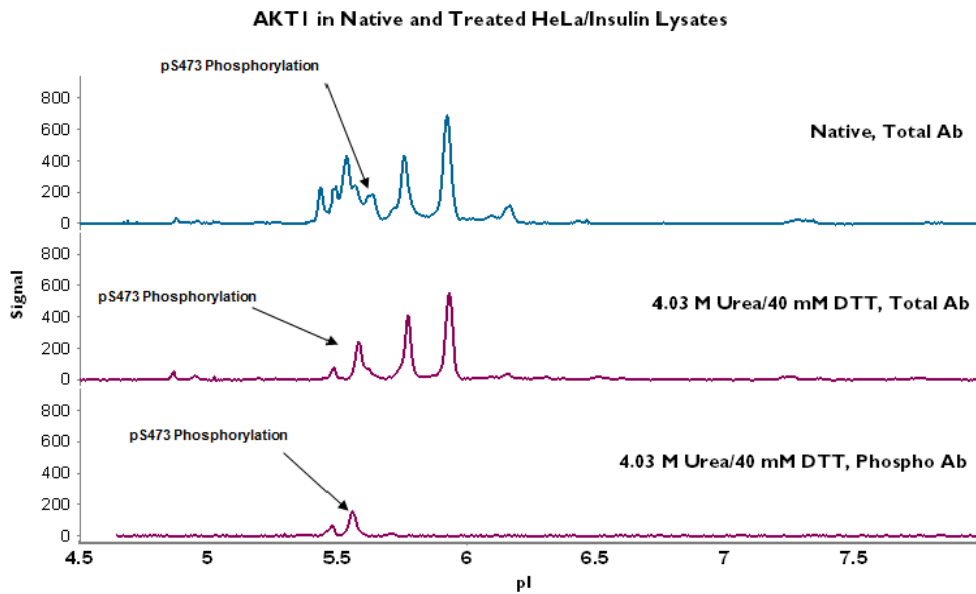


Figure 5-3: AKT1 in native and 4.03 M urea/40 mM DTT treated HeLa/Insulin lysates.

## Optimization of Denaturing Procedure for NanoPro Assays

Denaturing conditions have to be optimized for each particular assay, since all proteins unfold differently. The 4E-BPI protein was used to develop a model assay, and a number of experiments with ERK1/2 and AKT systems were also conducted.

### Sample Preparation

Cell culture, including cell pre-treatment and treatment, lysis buffer and lysis details, lysate storage and assay conditions should be generally the same for native and urea/DTT-treated samples.

### Urea/DTT Concentration

ProteinSimple recommends an initial testing of denaturing conditions in the range of 1.65-7.25 M urea, keeping the reducing agent constant (i.e. DTT at 40 mM). Use only electrophoresis grade urea or higher purity (Sigma-Aldrich, U6504), and make fresh urea solution prior to each experiment. It is always useful to run native samples as a control and observe the effect of different urea/DTT concentrations on a protein profile in relation to the native assay.

### Denaturing Temperature and Incubation Time

Short incubation times (5-10 minutes) at room temperature are sufficient. Signal loss may be observed when extending incubation to over 30 minutes and especially when freezing and thawing proteins with denaturing agents. As a result, ProteinSimple recommends post-lysis treatment with the appropriate amount of urea/DTT dissolved in the Bicine/CHAPS Lysis Buffer (20 mM Bicine, pH 7.6/0.6% CHAPS).

Since urea tends to precipitate easily at high concentrations with variations in room temperature, using a 10.2 M urea stock solution is recommended. Additionally, protein lysates mixed with high concentrations of urea should not be put on ice prior to the addition of diluents due to urea solubility limits.

For example, to achieve a 7.25 M urea/37 mM DTT concentration and carry out a denaturing reaction, mix the lysate sample with 9.67 M urea/50 mM DTT at 1:3 ratio and incubate at room temperature for 5 minutes.

### Dilution of Denatured Lysates

After urea/DTT treatment, lysates should be immediately diluted to the recommended protein concentration (generally in the 0.05-0.20 mg/mL range) with Sample Diluent containing 1X DMSO Inhibitor Mix.

It is necessary to keep urea and DTT concentrations below 3 M and 16 mM respectively before the addition of ampholyte premix. A decrease in signal intensity and distortion of peak shape were observed when the final concentration of urea was more than 1.5 M in the capillary. This indicates that >1.5 M urea may have a negative effect on protein separation and/or immobilization.

## IEF Gradient

Currently, urea/DTT-treated samples are separated using the same IEF gradient and pI Standards as the corresponding native assay. If no corresponding native protocol exists, follow general optimization guidelines for NanoPro assay development.

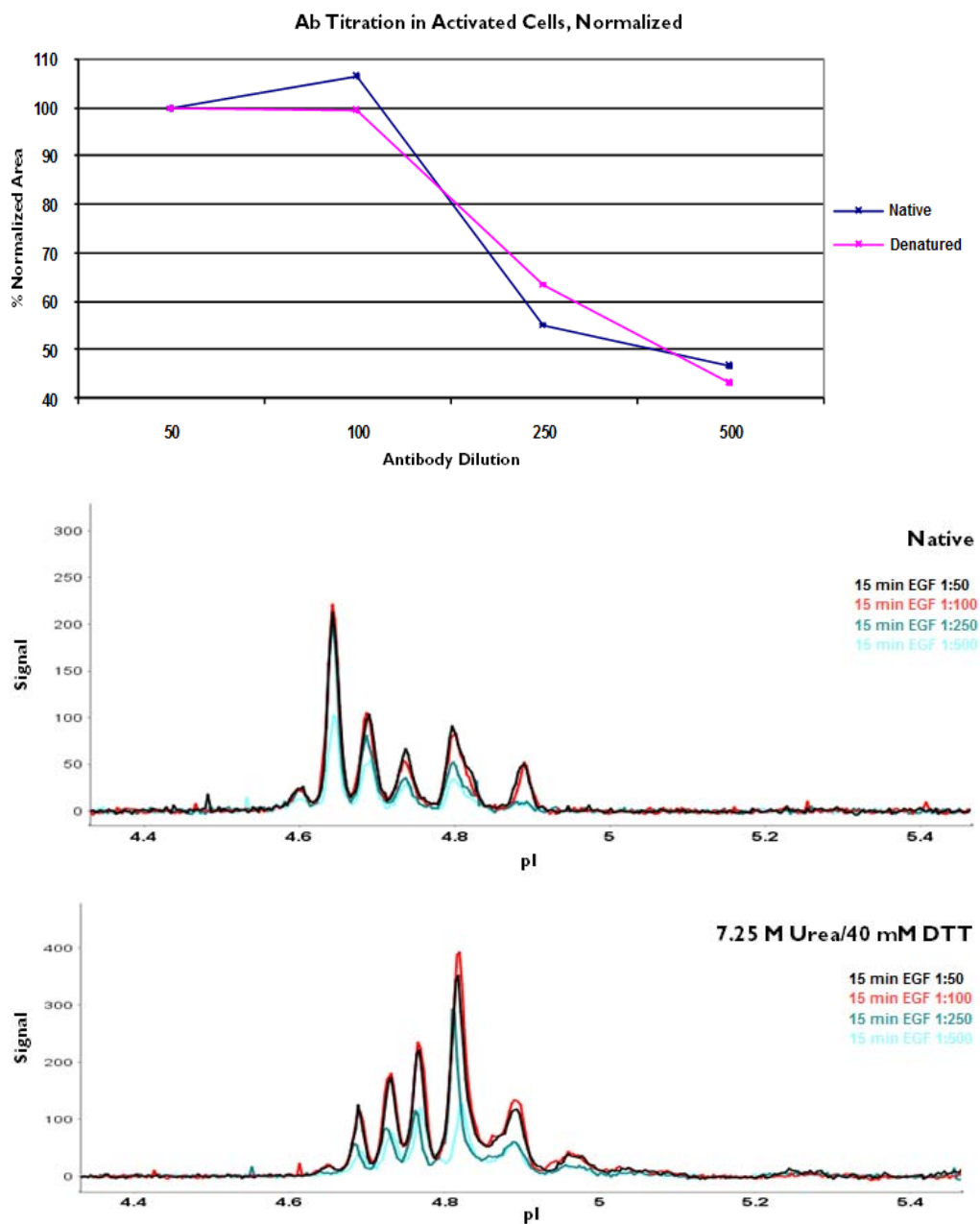
## Choosing an Antibody

In the applications tested so far, antibodies selected for the native NanoPro assay worked for urea/DTT-treated lysates as well. Additional antibody selection should be comprised of the screening process described for the native assay.

## Antibody Incubation Times and Concentrations

Use the primary and secondary antibody dilution ratios and incubation times as described in the ProteinSimple Application Briefs for native proteins as a starting point. Application Briefs can be downloaded at [www.proteinsimple.com/literature](http://www.proteinsimple.com/literature).

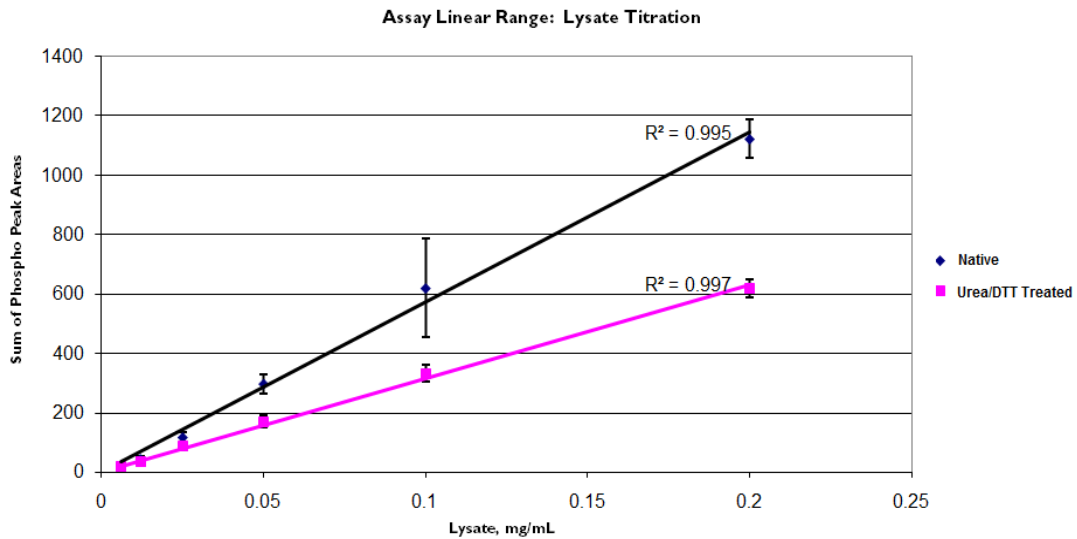
Based on studies of these parameters carried out for ERK1/2 and 4E-BP1, antibody incubation times and dilution curves for native and urea/DTT-treated lysates were similar, with binding equilibrium achieved under the same conditions (Figure 5-4).



**Figure 5-4:** Anti p-4E-BP1 (Cell Signaling Technology, 9459) antibody titration in MCF10a cells (0.2 mg/mL). Native and urea/DTT-treated cell lysates with an incubation time of 2 hours.

## Lysate Titration

Perform lysate titration in the same way as the native assay, generally in the 0.01-0.40 mg/mL range. We observed no difference for the linear range of protein detection in native and urea/DTT-treated samples, indicating similar protein focusing and immobilization efficiency (Figure 5-5).



**Figure 5-5:** K562 lysate titration and detection of 4E-BP1 phosphorylation using anti p-4E-BP1 (Cell Signaling Technology, 9459). 1:50 primary antibody dilution, native and urea/DTT-treated cell lysates, 2-hour incubation.

## UV Immobilization Times

UV immobilization times between 60 and 140 seconds were tested for urea/DTT-treated samples. No significant difference was observed between optimal immobilization times for native and urea/DTT treated lysates (Figure 5-6).

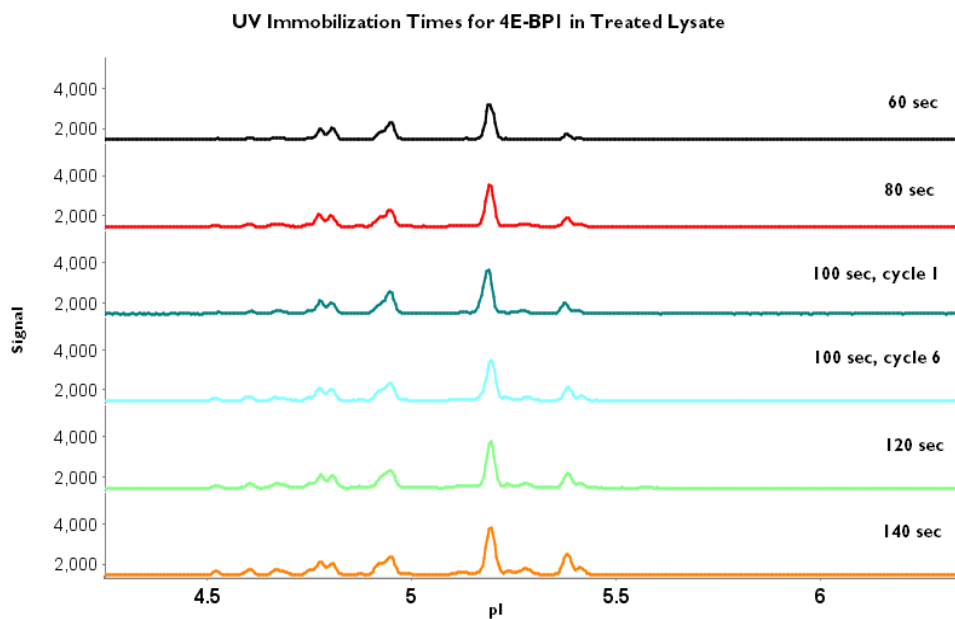


Figure 5-6: UV immobilization times for 4E-BPI protein in urea/DTT-treated cell lysates.

# Recommended Protocol for Optimization of the NanoPro Denaturing Assay

## Items Needed

### Reagents for Denaturing Solution

- Urea, electrophoresis-grade (Sigma-Aldrich, U6504)
- DTT, 7.7 mg, (Pierce, 20291)
- Water, 0.22-micron filtered, deionized (molecular biology grade or better)

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*NOTE: An alternative reducing agent, TCEP-HCl (Pierce, 20490) can also be used at concentrations of 0.1 mM and lower in the denaturing solution.*

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### ProteinSimple Reagents

- Bicine/CHAPS Lysis Buffer
- Sample Diluent containing 1X DMSO Inhibitor Mix

### Materials and Equipment

- Ice and an ice bucket
- Microfuge tubes
- Vortexer
- Pipettors and tips

## Procedure

- I. Prepare a 10.2 M urea stock solution by dissolving 0.22 g of urea in 200  $\mu$ L of Bicine/CHAPS Lysis Buffer (20 mM Bicine, pH7.6/0.6% CHAPS).

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*NOTES:*

*The final volume of the 10.2 M urea stock solution will be 360  $\mu$ L.*

*Prepare a fresh urea stock solution prior to each experiment.*

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2. Prepare a 1 M DTT stock solution by adding 50  $\mu$ L of water to the DTT tube provided by the manufacturer (Pierce).
3. Add 20.4  $\mu$ L of 1 M DTT stock solution prepared in step 2 to the 10.2 M urea stock solution prepared in step 1. This will make a final stock denaturing solution of 9.65 M urea/53 mM DTT.
4. Prepare a Bicine/CHAPS Lysis Buffer/53 mM DTT diluent by adding 20.4  $\mu$ L of 1 M DTT stock solution to 360  $\mu$ L of Lysis Buffer:
5. Prepare sample denaturing solutions by adding 10.2 M urea/53 mM DTT stock denaturing solution (prepared in step 3) to the Bicine/CHAPS Lysis Buffer/DTT diluent (prepared in step 4) using the volumes listed in Table 5-2.

**NOTES:**

To determine the appropriate final concentration of urea for new targets, ProteinSimple recommends testing a range of urea concentrations while keeping the DTT concentration constant at 40 mM.

Lysates are mixed with denaturing solutions at a 1:3 v/v ratio. The recommended final sample denaturing solution concentrations are achieved after mixing the prepared working concentrations and lysate.

Prepare fresh dilutions of the denaturing solution prior to each experiment.

Using a 0 M Urea/40 mM DTT sample denaturing solution generates a native sample as a control. This allows observation of the effects of different urea/DTT concentrations on protein profiles as well as the effects of 40 mM DTT on the sample..

Recommended Final Concentration of Sample Denaturing Solution	Working Concentration of Sample Denaturing Solution	12 M Urea/53 mM DTT	Bicine/CHAPS Lysis Buffer/53 mM DTT
7.25 M Urea/40 mM DTT	9.65 M Urea/53 mM DTT	100 $\mu$ L	0 $\mu$ L
5.64 M Urea/40 mM DTT	7.51 M Urea/53 mM DTT	77.5 $\mu$ L	22.5 $\mu$ L
4.03 M Urea/40 mM DTT	5.37 M Urea/53 mM DTT	55.9 $\mu$ L	44.1 $\mu$ L
1.61 M Urea/40 mM DTT	2.15 M Urea/53 mM DTT	22.5 $\mu$ L	77.5 $\mu$ L
0 M Urea/40 mM DTT	0 M Urea/53 mM DTT	0.0 $\mu$ L	100 $\mu$ L

**Table 5-2: Recommended final and working sample denaturing solution concentrations and preparation volumes.**

6. Thaw lysate sample on ice.

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*NOTE: Lysate concentrations added to the denaturing reaction should be within a range of 5-50  $\mu\text{g}$  total protein.*

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7. Prepare the denaturation reaction by mixing the sample denaturing solution prepared in step 5 with lysate using the volumes listed in Table 5-3. This represents a 3:1 v/v ratio, respectively.

Final Concentration of Sample Denaturing Solution	Working Concentration of Sample Denaturing Solution	Lysate	Total Denaturing Reaction Volume
7.25 M Urea/40 mM DTT	11.25 $\mu\text{L}$	3.75 $\mu\text{L}$	15 $\mu\text{L}$
5.64 M Urea/40 mM DTT	11.25 $\mu\text{L}$	3.75 $\mu\text{L}$	15 $\mu\text{L}$
4.03 M Urea/40 mM DTT	11.25 $\mu\text{L}$	3.75 $\mu\text{L}$	15 $\mu\text{L}$
1.61 M Urea/40 mM DTT	11.25 $\mu\text{L}$	3.75 $\mu\text{L}$	15 $\mu\text{L}$
0 M Urea/40 mM DTT	11.25 $\mu\text{L}$	3.75 $\mu\text{L}$	15 $\mu\text{L}$

**Table 5-3: Recommended working sample denaturing solution and lysate volumes for denaturing reaction.**

8. Incubate for 5 minutes at room temperature.

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**NOTES:**

*Some proteins may require a shorter or longer incubation time. No differences in the degree of denaturing were observed between 5 and 30 minute incubations. However, incubations of longer than 30 minutes may result in decrease signals during NanoPro analysis.*

*To avoid precipitation of urea during sample preparation, do not put lysates with > 3 M concentrations of urea on ice prior to adding Sample Diluent.*

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9. Dilute the denatured sample with Sample Diluent containing 1X DMSO Inhibitor Mix. After urea/DTT treatment, lysates should be diluted to the recommended protein concentration (generally in the 0.05-0.20 mg/mL range).

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**NOTES:**

*The final concentration of urea in the capillary should be < 1.5 M to avoid a decrease in signal intensity and distortion of peak shape.*

*Lysates treated with urea and DTT should not be frozen and reused.*

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10. After dilution, place samples on ice.
11. In a separate tube, mix ampholyte premix and pI standards.
12. Denatured and native samples are prepared the same way by adding diluted sample to the premix containing pI standards using the ratio specified in the assay protocol. Vortex to mix.
13. Analyze the samples using the NanoPro assay.

## References

1. Tanford C., Protein denaturation. C. Theoretical models for the mechanism of denaturation, 1970, *Adv Protein Chem*, 24:1–95.
2. Parker W., and Song P-S., Protein structures in SDS micelle-protein complexes, 1992, *Biophys. J.*, 61, 1435-1439.
3. Bhuyan AK., On the mechanism of SDS-induced protein denaturation, 2010, *Biopolymers*, 93, 2, 186–199.
4. Hua L., Zhoua R., Thirumalaic D., and Bernea BJ, Urea denaturation by stronger dispersion interactions with proteins than water implies a 2-stage unfolding, 2008, *PNAS*, 105, no. 44, 16928–16933.
5. Pace CN., Huyghues-Despointes B., Fu H., Takano K. J., Scholtz M., Grimsley GR., Urea denatured state ensembles contain extensive secondary structure that is increased in hydrophobic proteins, 2010, *Protein Science*, Epub in advance of print 2 March 2010.
6. Dill K.A. and Shortle D., Denatured states of proteins, 991 *Annu. Rev. Biochem.*, 60, 795-825.

