Simple Western Size Assay Development Guide

ProteinSimple
3040 Oakmead Village Drive
Santa Clara, CA 95051
Toll-free: (888) 607-9692
Tel: (408) 510-5500
Fax: (408) 510-5599
email: support@proteinsimple.com
web: www.proteinsimple.com

Simple Western Size Assay Development Guide

P/N 042-889

Revision 1, March 2014

For research use only. Not for use in diagnostic procedures

Patents and Trademarks

Table of Contents

Preface

The Simple Western Size Assay Development Guide sums up ProteinSimple's experience in developing immunoassays, and includes all the details you'll need as far as assay development principles go for each of our Simple Western Size instruments. With all these tips and tricks, we know you'll be developing your own assays in no time!

This guide gets updated regularly, so to make sure you have the latest on assay development, visit www.proteinsimple.com/literature to download the most recent version.

For ordering and general information on any of the ProteinSimple assay reagents in this guide, please call (888) 607-9692 or visit www.proteinsimple.com.

Chapter 1:

General Information

Chapter Overview

- Introduction to Simple Western Size Assays
- Safety
- Customer Service and Technical Support

Introduction to Simple Western Size Assays

Simple Western assays are automated, capillary-based immunoassays. That means no gels, no transfer devices, no blots, no film, and no manual analysis!

Just like traditional Western blot analysis, your protein samples are separated, immobilized and probed with target specific antibodies (Figure 1-1). Simple Western systems automate all the steps for you, letting you cut through the variability and straight to highly reproducible quantitation for the proteins in your sample.

The Process

Load Matrices
The capillary is filled with separation matrix and stacking matrix.

Load Sample
Sample containing SDS, DTT, and fluorescently labeled MW markers are loaded into the capillary.

Separate
Voltage is applied across the capillary to separate proteins in the sample.

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

Immunoprobe
The capillary is rinsed and immunoprobed for specific proteins with an HRP-labeled antibody. Luminol and peroxide are loaded into the capillary to catalyze chemiluminescent light generation, which is captured by a CCD camera.

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

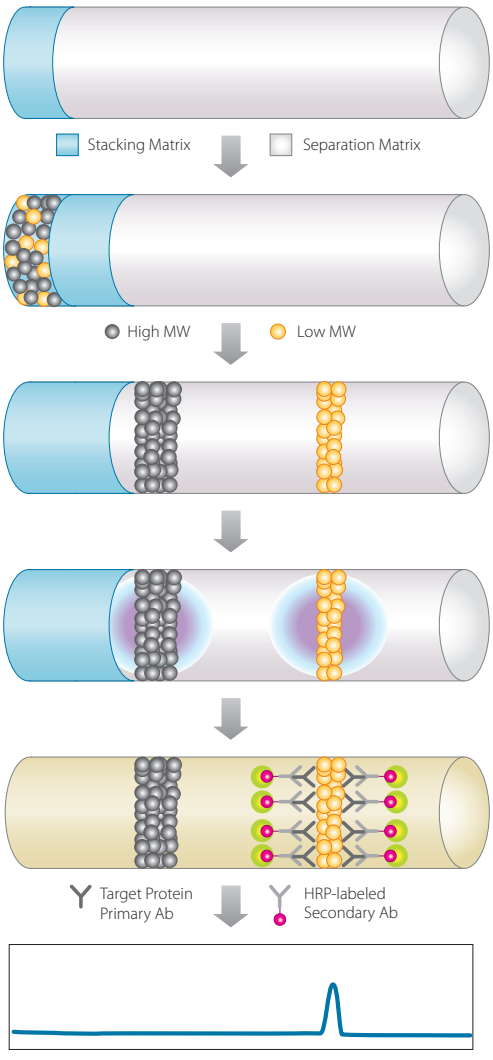

Figure 1-1: *The Simple Western Size Assay process. All steps happen in a single capillary.*

Simple Western systems capture your data as a chemiluminescent image of the capillary. Compass software then analyzes the images, processes all the data, and gives you the option to view your data as an electropherogram or as a lane view (Figure 1-2).

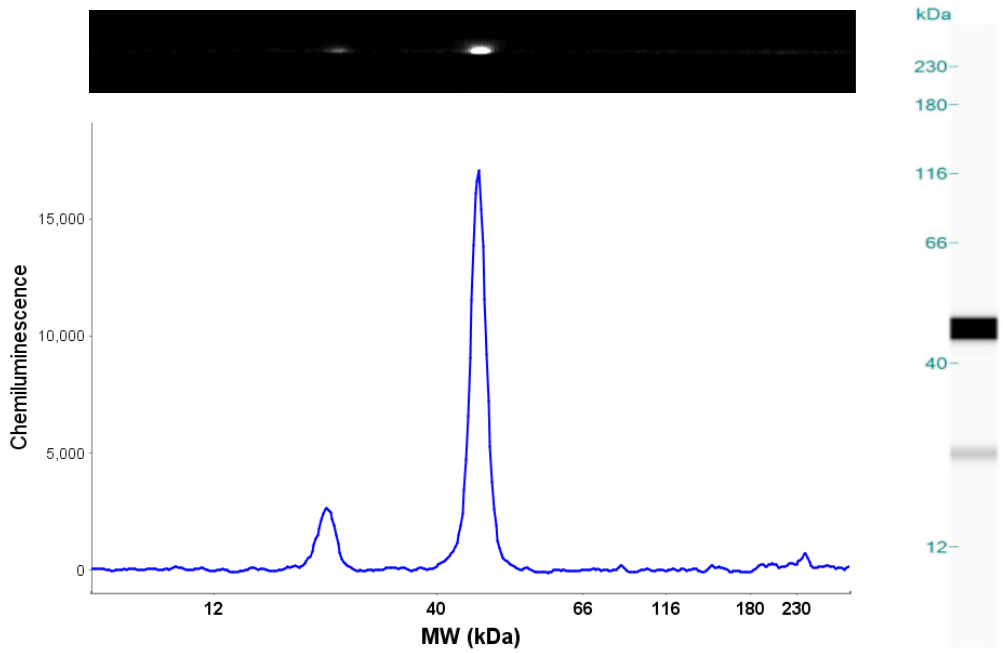

Figure 1-2: *Simple Western Size Assay sample data. Chemiluminescent image of separated proteins in the capillary (top), electropherogram (bottom left) and lane view (right).*

Safety

User Attention Notifications

To help you get the most from your Simple Western size assays, we've added some attention phrases to guide you through this manual:

NOTE	Points out useful information.
IMPORTANT	Indicates information necessary for proper operation of the instrument.
CAUTION	Cautions you about potentially hazardous situations that could result in injury to you or damage to the instrument.
!WARNING!	Warns you that serious physical injury can result if the listed precautions aren't followed.

Chemical Hazards

!WARNING! CHEMICAL HAZARD

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

- Read and understand the Safety Data Sheets (SDSs) 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 SDS.
- 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 SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Safety Data Sheets

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

SDSs provide you with safety information needed to store, handle, transport and dispose of the chemicals safely. We recommend updating laboratory SDS records periodically.

Safety Data Sheets for ProteinSimple reagents are available online at 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

Telephone
(408) 510-5500
(888) 607-9692 (toll free)

Fax
(408) 510-5599

E-mail
support@proteinsimple.com

Web
www.proteinsimple.com

Address
ProteinSimple
3040 Oakmead Village Drive
Santa Clara, CA 95051
USA

Chapter 2:

Getting Started

Chapter Overview

- Simple Western Size Assay Development Overview
- Choose a Model System
- Prepare Stock Lysates
- Prepare Simple Western Samples
- Screen Antibodies
- Suggested Protocol for Your Initial Experiment
- Suggested Plate Templates for Your Initial Experiment
- Data Acquisition and Interpretation

Simple Western Size Assay Development Overview

Developing assays on a Simple Western system is really similar to developing other immunoassays like ELISA and Western blot. We recommend you follow the process outlined in this chapter when you're initially developing a Simple Western Size Assay (Figure 2-1).

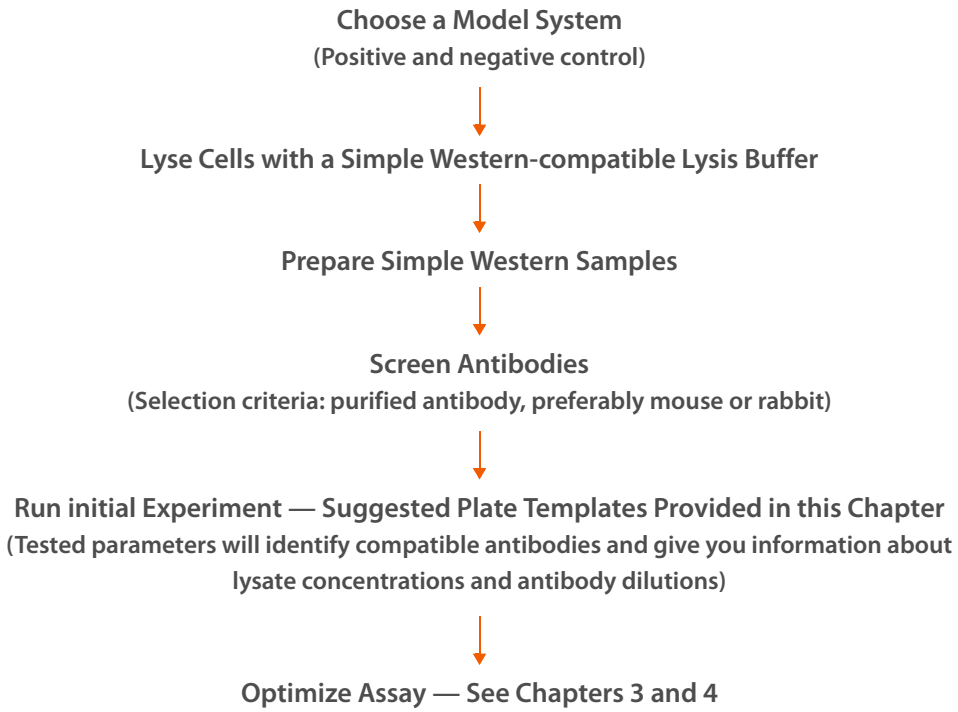

Figure 2-1: *Simple Western Size Assay development process.*

Choose a Model System

General Guidelines

- **Get started with an easily accessible model system.** It's best to perform assay development with readily available material such as lysates from cultured cell lines, even if your end goal is analysis of precious biological samples. The assay can be transferred to your precious biological samples after your optimized assay conditions are defined.

- **Work with a model system of choice**. The choice of the right biological system depends on your protein of interest. Choose a cell model system where treatment will result in a strong biological response. Using positive and negative control samples (high and low expression of the target/modification of interest) during assay development will give you confidence in the peaks detected and preliminary information about the assay and baseline signals that will guide the later steps of your assay development.

Prepare Stock Lysates

Prepare your stock lysates with a Simple Western-compatible lysis buffer that will efficiently extract your protein. See Chapter 3, *"Sample Preparation Optimization"* and Appendix A, *"Reagent and Lysis Buffer Compatibility"* for more information.

Total protein concentration of your stock lysates should be ≥ 2 mg/mL to start off with. If your protein of interest is a low expressing protein, make the lysates as concentrated as possible to maximize the amount of protein that you can load into the capillary. You can adjust total protein concentrations once the assay is optimized.

You can determine the total concentration of your lysate by using protein assay kits like the BCA Protein Assay Kit (Pierce, P/N 23225) or the Bradford Protein Assay Kit (Pierce, P/N 23200).

Prepare Simple Western Samples

NOTES:
For best results, store Wes plates at room temperature for at least 24 hours after you get them to make sure components that precipitate out during shipping go back into solution.

Store Running Buffer II at room temperature after you've received it, and be sure to check for precipitation before starting an experiment. To remove precipitation, put the bottle in a 37 °C environment for at least 1 hour and invert the bottle 3 times every 20 minutes to mix, or let it sit out at room temperature for 24 hours and invert the bottle every few hours to mix. Then invert the bottle 5 times so the solution is uniform.

Protein signal should change with concentration and as a response to treatment. So using two different lysate concentrations in your initial experiment will give you preliminary information about the linearity of your assay.

When conditions for a traditional Western blot are known:

For Simon, Sally, and Peggy, load the same amount of protein in your initial Simple Western experiment that you would use for a traditional Western blot. For Wes, Sally Sue, and Peggy Sue, you can use 5X less protein than you normally would for a traditional Western blot.

EXAMPLE: A traditional Western blot where you'd load 20 µg of protein in a 10 µL volume per lane. Simple Western Size Assays need 5 µL of sample, so to load the equivalent of 20 µg and 5 µg, prepare samples with these concentrations:

Sample Info	Simon, Sally and Peggy	Wes, Sally Sue and Peggy Sue
Protein/well	20 µg, 5 µg	4 µg, 1µg
Volume	5 µL	5 µL
Concentration	4 mg/mL, 1 mg/mL	0.8 mg/mL, 0.2 mg/mL

For a novel assay where traditional Western blot conditions aren't known:

For Simon, Sally and Peggy, prepare the samples so you load the maximum concentration possible for the high concentration sample, and dilute the sample 1:4 for the lower concentration. On Wes, Sally Sue, and Peggy Sue, you should load 0.8 mg/mL and 0.2 mg/mL lysate.

EXAMPLE: If your lysate has a stock concentration of 4 mg/mL, sample required for 1 well:

Simon, Sally and Peggy	Wes, Sally Sue and Peggy Sue
High concentration: • Add 4 µL of stock lysate to 1 µL of 4X Master Mix • Final concentration = 3 mg/mL	**High concentration:** • Dilute lysates to 1 mg/mL using 0.1X Sample Buffer • Add 4 µL diluted lysate to 1 µL of 5X Master Mix • Final concentration = 0.8 mg/mL
Low concentration: • Dilute lysates to 1 mg/mL using 0.1X Sample Buffer • Add 3 µL of diluted lysate to 1 µL of 4X Master Mix • Final concentration = 0.75 mg/mL	**Low concentration:** • Dilute lysates to 0.25 mg/mL using 0.1X Sample Buffer • Add 4 µL diluted lysate to 1 µL of 5X Master Mix • Final concentration = 0.2 mg/mL

Its a good idea to include a "no lysate" control sample to assess antibody background noise when possible. For those samples, just use 0.1X Sample Buffer in place of your lysate sample.

For your initial experiment, prepare your samples using the default sample preparation protocol in Table 2-1

Simon, Sally and Peggy	Wes, Sally Sue and Peggy Sue
1. Prepare 1 M DTT: • Add 50 µL of H_2O to the lyophilized DTT 2. Prepare 4X Master Mix — add to the lyophilized fluorescent standards tube: • 22 µL of H_2O • 20 µL of 10X Sample Buffer • 8 µL of 1 M DTT 3. Dilute lysate: • Dilute lysates in 0.1X Sample Buffer to 1.33X of the desired final concentration 4. Add diluted lysate to Master Mix: • Mix 1 part 4X Master Mix with 3 parts lysate 5. Heat samples at 95 ℃ for 5 minutes	1. Prepare 400 mM DTT: • Add 40 µL of H_2O to the lyophilized DTT 2. Prepare 5X Master Mix — add to the lyophilized fluorescent standards tube: • 20 µL of 10X Sample Buffer • 20 µL of 400 mM DTT 3. Dilute lysate: • Dilute lysates in 0.1X Sample Buffer to 1.25X of the desired final concentration 4. Add diluted lysate to Master Mix: • Mix 1 part 5X Master Mix with 4 parts lysate 5. Heat samples at 95 ℃ for 5 minutes

Table 2-1: *Simple Western Size Assay default sample preparation protocol.*

NOTE: Sample denature conditions can be adjusted based on the properties of your protein. Please see Chapter 3, "Sample Preparation Optimization" for more options on how to prepare your sample.

Screen Antibodies

Choosing an Antibody

Most antibodies are compatible with Simple Western Size Assays, but you may need to test alternative antibodies to get optimal results. For example, depending on the quantitative needs for your assay, you may want to screen for the antibody that will give you the best linear dynamic range.

The ProteinSimple Antibody Database (www.proteinsimple.com/antibody/antibodies.html) is a great resource with information on hundreds of Simple Western-validated antibodies. You can also learn about the experiences other scientists in the ProteinSimple community have had with your protein of interest.

If your target of interest isn't listed in the antibody database yet, we suggest you select antibodies that are validated to screen with multiple applications (for example, one that you could use for Western blot, IHC, IP, IF, and ELISA). We also prefer purified antibodies over antibodies in ascites fluid or serum.

When running an assay where traditional Western blot conditions are known:

You can use your Western blot-validated antibody with 1-2 antibodies chosen specifically for Simple Western Size Assays, or screen at least 2-3 antibodies chosen specifically for Simple Western Size Assays.

For novel assays where traditional Western blot conditions aren't known:

Screen at least 2-3 antibodies in your initial experiment.

For a new assay where Western blot parameters aren't known, or for an assay where the primary antibody is used at a ~1:1000-1:6000 dilution with overnight incubation at 4 ℃, use the parameters in Table 2-2 for your initial experiment.

Reagent or Parameter	Simon, Sally and Peggy	Wes, Sally Sue and Peggy Sue
Primary Antibody	Purified rabbit or mouse antibody that works for several applications. For a new assay, screen 2-3 antibodies.	
Antibody Diluent	Antibody Diluent PLUS	Antibody Diluent II
Antibody Dilution	1:50, 1:150	1:50, 1:150
Primary Antibody Incubation Time	2 hr	30 min
Secondary Antibody	Simon, Peggy or Sally rabbit or mouse RTU secondary antibody	Wes, Peggy Sue or Sally Sue rabbit or mouse RTU secondary antibody
Secondary Antibody Incubation Time	1 hr	30 min

Table 2-2: *Experimental parameters for assays you don't currently run using Western blot.*

If your primary antibody has high enough affinity that it is diluted > 1:6000 for Western blot, you can adjust primary antibody dilutions for Simple Western Size Assays to 1:150 and 1:450.

Suggested Protocol for Your Initial Experiment

For your initial experiment, run the default protocol conditions in Table 2-3. For Sally, Sally Sue, Peggy, and Peggy Sue, you can test multiple conditions in one experiment. Suggested additional parameters you may want to consider testing are listed with the plate templates in the next section.

Protocol Step	Simon	Sally and Peggy	Wes	Sally Sue and Peggy Sue
Separation Matrix	120 sec	120 sec	200 sec	150 sec
Stacking Matrix	12 sec	12 sec	15 sec	15 sec
Sample	8 sec	8 sec	9 sec	9 sec
Separation Time	40 min	40 min	25 min	40 min
Separation Voltage	250 V	250 V	375 V	250 V
Matrix Removal	140 sec	140 sec	230 sec	230 sec
Blocking Time	15 min	23 min	30 min	30 min
Primary Antibody	1 hr	1 hr	30 min	30 min
Secondary Antibody	1 hr	1 hr	30 min	30 min
Detection	30 sec, 60 sec, 120 sec, 240 sec, 480 sec, 960 sec	30 sec, 60 sec, 120 sec, 240 sec, 480 sec, 960 sec	15 sec, 30 sec, 60 sec, 120 sec, 240 sec, 480 sec	15 sec, 30 sec, 60 sec, 120 sec, 240 sec, 480 sec

Table 2-3: *Suggested assay protocol for your initial experiment.*

Suggested Plate Templates for Your Initial Experiment

We chose all the experimental conditions for the following plate templates based on traditional Western blot conditions, where 10 µg of protein in a 5 µL volume are loaded per well and the membrane is exposed to primary antibody diluted 1:1000, overnight at 4 ℃. We've included some examples of plate template set ups on different Simple Western systems for you here.

Simon

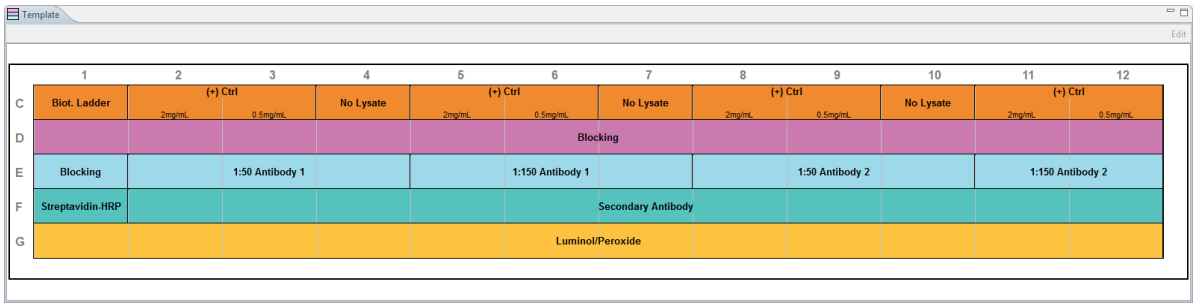

Figure 2-2: *Suggested Compass plate template for your initial Simon experiment. You can test one sample (positive control), two sample concentrations, two antibodies, and two antibody dilutions using default assay conditions. The row that contains sample is in orange, blocking buffer in purple, primary incubation in light blue, secondary incubation in teal, and detection reagents in gold.*

Cycle	Stacking/Sample Load Time	Primary Row	Primary Ab Incubation
1	12 sec/8 sec	C	2 hr

Table 2-4: *Suggested Compass assay protocol for your initial Simon experiment.*

Wes

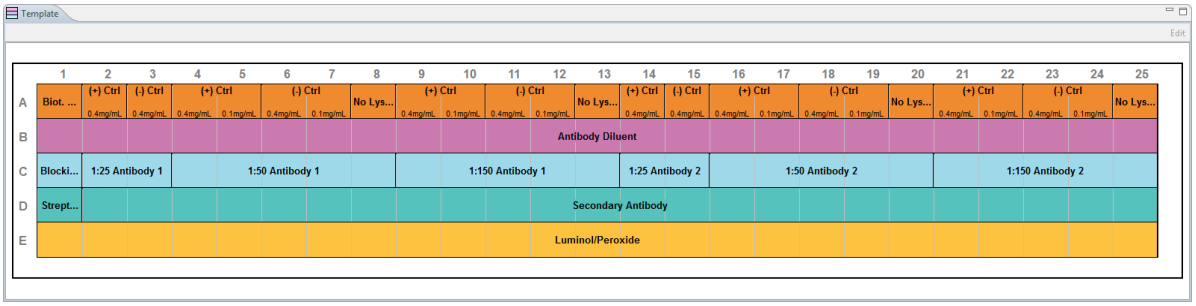

Figure 2-3: *Suggested Compass plate template for your initial Wes experiment. You can test two samples (a positive and negative control), two sample concentrations, two antibodies, and three antibody dilutions using default assay conditions. The row that contains sample is in orange, blocking buffer in purple, primary incubation in light blue, secondary incubation in teal, and detection reagents in gold.*

Cycle	Stacking/Sample Load Time	Primary Row	Primary Ab Incubation
1	15 sec/9 sec	C	30 min

Table 2-5: *Suggested Compass assay protocol for your initial Wes experiment.*

Sally and Peggy

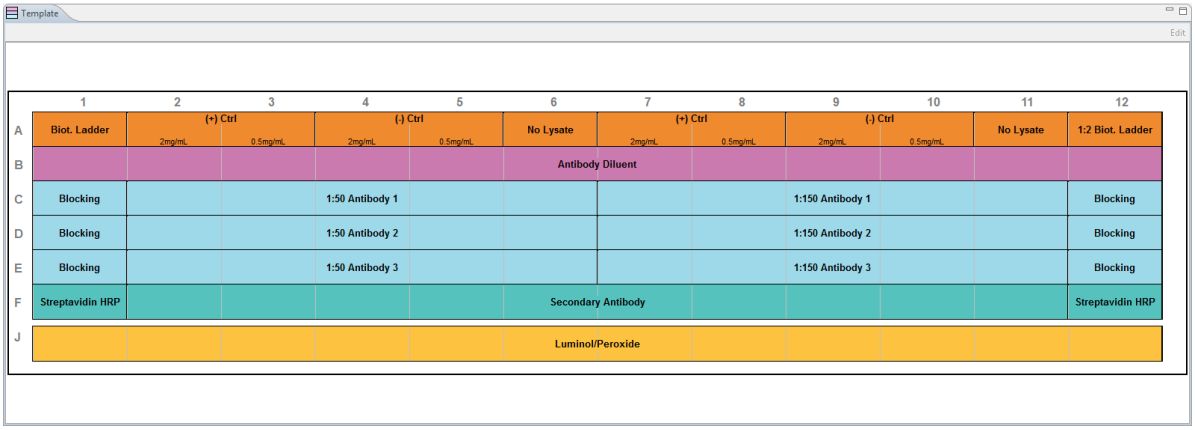

Figure 2-4: *Suggested Compass plate template for your initial Sally or Peggy experiment. You can test two samples (positive and negative control), two sample concentrations, three antibodies, and two antibody dilutions. Multiple stacking matrix and sample loads can also be tested if your assay needs higher resolution or more sensitivity instead. The row that contains sample is in orange, blocking buffer in purple, primary incubation in light blue, secondary incubation in teal, and detection reagents in gold.*

Cycle	Stacking/Sample Load Time	Primary Row	Primary Ab Incubation
1	12 sec/8 sec	C	2 hr
2	12 sec/8 sec	D	2 hr
3	12 sec/8 sec	E	2 hr
4	15 sec/10 sec	C	2 hr
5	15 sec/10 sec	D	2 hr
6	15 sec/10 sec	E	2 hr

Table 2-6: *Suggested Compass assay protocol for your initial Sally or Peggy experiment.*

Sally Sue and Peggy Sue

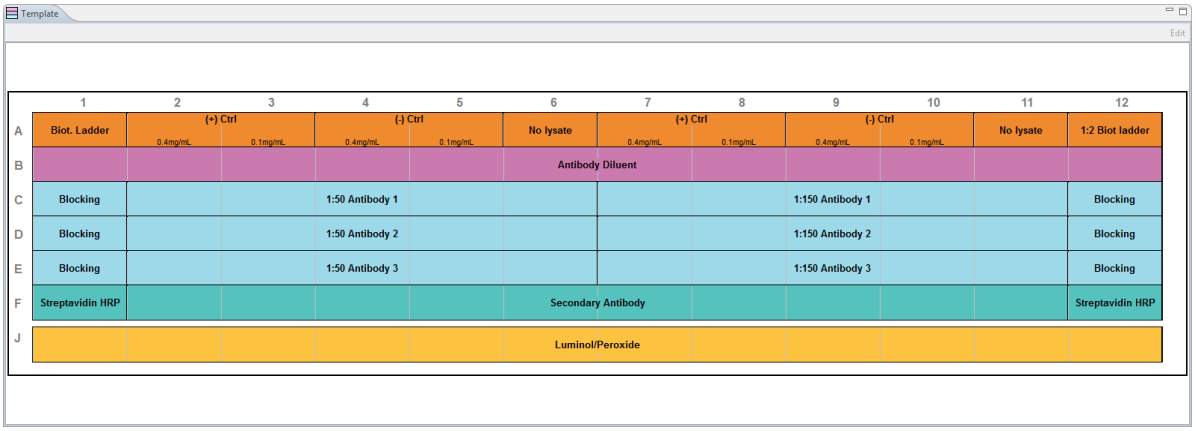

Figure 2-5: *Suggested Compass plate template for your initial Sally Sue or Peggy Sue experiment. You can test two samples (positive and negative control), two sample concentrations, three antibodies, and two antibody dilutions. If this is an assay that requires more resolution, multiple stacking and sample load ratios can be tested. The row that contains sample is in orange, blocking buffer in purple, primary incubation in light blue, secondary incubation in teal, and detection reagents in gold.*

Cycle	Stacking/Sample Load Time	Primary Row	Primary Ab Incubation
1	15 sec/9 sec	C	0.5 hr
2	15 sec/9 sec	D	0.5 hr
3	15 sec/9 sec	E	0.5 hr
4	12 sec/6 sec	C	0.5 hr
5	12 sec/6 sec	D	0.5 hr
6	12 sec/6 sec	E	0.5 hr

Table 2-7: *Suggested Compass assay protocol for your initial Sally Sue or Peggy Sue experiment.*

Data Acquisition and Interpretation

Data acquisition on Simple Western systems couldn't be easier — Compass software does it all for you. Data is acquired during the run, and at the end of each experiment/cycle Compass analyzes the data and then displays it in the Analysis Screen. You'll wan to confirm that the fluorescent standards peaks, registration peak (not applicable to Wes), and biotinylated ladder peaks are correctly identified before starting your review of the analyzed data.

Look for reproducible peaks — protein peaks at the same molecular weight (MW) between different antibody dilutions, different protein concentrations, and different antibodies if multiple antibodies were screened. Signal changes between your positive and negative control should also agree with the expected biology.

NOTE: Expected biology may not match observed data if the assay is significantly outside its linear dynamic range.

After the initial screening experiment, you can improve the signal and resolution of your assays. Routinely optimized conditions include:

- Stacking load time and sample load time
- Lysate concentration
- Primary antibody concentration and incubation time

Details on these variables and other assay development considerations can be found in Chapter 4, *"Simple Western Size Assay Optimization"*.

Chapter 3:

Sample Preparation Optimization

Chapter Overview

- Buffer Compatibility for Stock Lysates
- Preparing Samples for Simple Western Size Assays

Buffer Compatibility for Stock Lysates

When you choose a lysis buffer, it's important to consider the extraction efficiency of the buffer as well as its compatibility with Simple Western Size Assays.

We offer two lysis buffers that are optimized for Simple Western Size Assays:

- **ProteinSimple Bicine/CHAPS Lysis Buffer:** can be used for most cytoplasmic and nuclear proteins.
- **ProteinSimple RIPA Lysis Buffer:** a more stringent lysis buffer for proteins that are more difficult to extract.

NOTE: For Peggy or Peggy Sue, we strongly recommend you use a ProteinSimple lysis buffer if you want to use the sample lysate for both size and charge experiments.

If you would rather use your own lysis buffer, please see the lysis buffer compatibility tables in Appendix A, *"Reagent and Lysis Buffer Compatibility"*.

Preparing Samples for Simple Western Size Assays

It's best to use our standard protocol for preparing Simple Western samples, but you can adjust sample preparation depending on the properties of your protein of interest.

Preparing Samples in Commercial Sample Buffers

If your samples are already in a sample buffer like Laemlli or LDS, first check whether your sample buffer contains ~1% SDS at its working concentration. If it does, dilute the lysates with a 1X concentration of the same sample buffer and prepare samples without ProteinSimple Sample Buffer as described in Table 3-1. Otherwise, prepare your samples using our standard sample preparation protocol.

Simon, Sally and Peggy	Wes, Sally Sue and Peggy Sue
1. Prepare 1 M DTT: • Add 50 µL of H_2O to the lyophilized DTT 2. Prepare 10X Master Mix — add to the lyophilized fluorescent standards tube: • 12 µL of H_2O • 8 µL of 1 M DTT 3. Add 1 part 10X Master Mix to 9 parts lysate 4. Heat samples at 95 ℃ for 5 minutes	1. Prepare 800 mM DTT: • Add 20 µL of H_2O to the lyophilized DTT 2. Prepare 10 X Master Mix — add to the lyophilized fluorescent standards tube: • 10 µL of H_2O • 10 µL of 800 mM DTT 3. Add 1 part 10X Master Mix to 9 parts lysate 4. Heat samples at 95 ℃ for 5 minutes

Table 3-1: *How to prepare samples using a non-ProteinSimple sample buffer.*

If you see significant migration differences between the biotinylated ladder and your prepared samples, reconstitute the Biotinylated Ladder in the same sample buffer.

EXAMPLE: Reconstituting the Biotinylated Ladder with Invitrogen 4X NuPage LDS Sample Buffer:

Simon, Sally and Peggy	Wes, Sally Sue and Peggy Sue
1. Prepare 1X NuPage LDS Sample Buffer + DTT: • 12.5 µL of 4X NuPage LDS Sample Buffer • 2 µL of prepared 1 M DTT • 35.5 µL of H_2O 2. Resuspend lyophilized Biotinylated Ladder with 20 µL of 1X NuPage LDS Sample Buffer + DTT 3. Heat at 95 ℃ for 5 minutes	1. To the Biotinylated Ladder, add: • 2 µL of prepared 400 mM DTT • 5 µL of 4X NuPage LDS Sample Buffer • 13 µL of H_2O 2. Heat at 95 ℃ for 5 minutes

Preparing Immunoprecipitation Samples

Follow your current protocol for binding your protein of interest to the antibody-conjugated immunoprecipitation beads.

You've got two options for preparing IP samples for Simple Western Size Assays:

1. Elute protein in 1X Master Mix (preferred):
    a. Add 1X Master Mix to your protein:bead complex
    b. Heat the beads to elute the protein
    c. Spin beads down and load the IP sample/supernatant onto the sample plate
2. Elute protein in 1X ProteinSimple Sample Buffer:
    a. Add 1X ProteinSimple Sample Buffer to your protein:bead complex
    b. Heat the beads to elute the protein
    c. Mix supernatant/IP sample with 4X or 5X Master Mix
    d. Heat samples again at 95 ℃ for 5 minutes to denature and reduce fluorescent standards
    e. Load IP sample onto the sample plate

Preparing Samples with More SDS and DTT

Sometimes your protein of interest may need additional SDS or DTT to fully denature or reduce the protein. If this is the case with your protein, check the buffer compatibility tables Appendix A, *"Reagent and Lysis Buffer Compatibility"* for compatible reagent ranges.

For Simon, Sally, and Peggy, you should add the additional DTT or SDS in place of the H_2O used to reconstitute the 4X Master Mix.

EXAMPLE: Using 80 mM of DTT instead of the default 40 mM DTT:

1. Prepare 1 M DTT:
   - Add 50 µL of H_2O to the lyophilized DTT
2. Prepare 4X Master Mix — add to lyophilized fluorescent standards tube:
   - 14 µL of H_2O
   - 20 µL of 10X Sample Buffer
   - 16 µL of 1 M DTT

For Wes, Sally Sue, and Peggy Sue, you should prepare a higher concentration DTT stock so the additional DTT or SDS can be added in some of the volume usually reserved for DTT.

EXAMPLE: Adding an additional 1% of SDS to the current formulation, if stock SDS is a 20% solution:

1. Prepare 800 mM DTT:
   - Add 20 µL of H_2O to the lyophilized DTT
2. Prepare 5X Master Mix — add to lyophilized fluorescent standards tube:
   - 20 µL of 10X Sample Buffer
   - 10 µL of 800 mM DTT
   - 8 µL of 20% SDS
   - 2 µL of H_2O

Preparing Samples with an Additional Component

In cases where your protein needs an additional component like 1 M urea for complete denaturation, supplement the sample by adding the urea in place of H_2O for Simon, Sally, and Peggy. For Wes, Sally Sue, and Peggy Sue, add the component in the volume usually reserved for DTT.

Preparing Samples with an Alternate Reducing Agent

If your protein is more efficiently reduced with a different reducing agent, prepare the Master Mix with your reducing agent of choice in place of DTT.

EXAMPLE: Prepare Wes 5X Master Mix with 200 mM β-mercaptoethanol instead of 40 mM DTT. If β-mercaptoethanol comes as a 2 M stock:

1. Add to the lyophilized fluorescent standards tube:
   - 20 µL of 10X Sample Buffer
   - 20 µL of 2 M β-mercaptoethanol
   - Pipette up and down to resuspend

Preparing Non-Reduced Samples

When you need to use non-reducing conditions, replace the DTT normally added to the 4X or 5X Master Mix or the Biotinylated Ladder with H_2O.

Reagent	Simon, Sally and Peggy	Wes, Sally Sue and Peggy Sue
Biotinylated Ladder	Resuspend the Biotinylated Ladder with 20 µL of Ladder Resuspension Buffer.	Resuspend the Biotinylated Ladder with 18 µL H_2O + 2 µL of 10X Sample Buffer.
Fluorescent Master Mix	Resuspend the Fluorescent Master Mix with 20 µL of 10X Sample Buffer + 30 µL of H_2O.	Resuspend the Fluorescent Master Mix with 20 µL of 10X Sample Buffer + 20 µL of H_2O.

Table 3-2: *How to prepare non-reduced samples.*

Alternate Denaturing Conditions

Your protein may need different heating conditions to fully denature or, like membrane proteins, may need denaturation at lower temperatures to prevent protein aggregation. In these cases, just adjust the time and/or temperature to optimize the sample denaturation conditions for your protein.

Examples of alternate denaturation conditions:

1. No heat (RT), 20 min
2. 37 ℃, 20 min
3. 70 ℃, 20 min
4. 95 ℃, 10 min

Chapter 4:

Simple Western Size Assay Optimization

Chapter Overview

- Simple Western Separation Optimization
- Simple Western Immunoassay Optimization
- Using Immunoassay Reagents from Other Vendors

Simple Western Separation Optimization

Sample and Stacking Matrix Load Time

Our Simple Western default size assay protocol is optimized to balance resolution and sensitivity for most assays. You can also make adjustments to assay parameters to improve resolution or sensitivity, but this may affect how fast your sample migrates. You'll want to adjust your separation time to make sure you use the effective separation length of the capillary. See "Separation Time" on page 32 for more details.

Recommended assay parameter ranges:

Parameter	Simon, Sally and Peggy	Wes	Sally Sue and Peggy Sue
Separation Time	40-50 min	25-31 min	40-50 min
Stacking Matrix Load Time	6-21 sec	6-21 sec	6-21 sec
Sample Load Time	4-13 sec	3.6-12.6 sec	4-13 sec
Stacking Time: Sample Time Ratio (Default)	1.5:1	1.7:1	1.7:1

Table 4-1: *Parameter ranges we recommend for maximizing signal or resolution.*

If you'd like to improve resolution, increase the ratio of the stacking matrix load time to the sample load time to approximately 2:1 to improve efficiency (Figure 4-1).

If you'd like to improve sensitivity, our first choice is to increase the protein concentration of the sample in the assay plate. But, if you're limited by your stock lysate concentration, try increasing the sample and stacking matrix load time so there is more sample into the capillary (Figure 4-1). Keep the stacking matrix load to sample load ratio the same as the recommended range in Table 4-1.

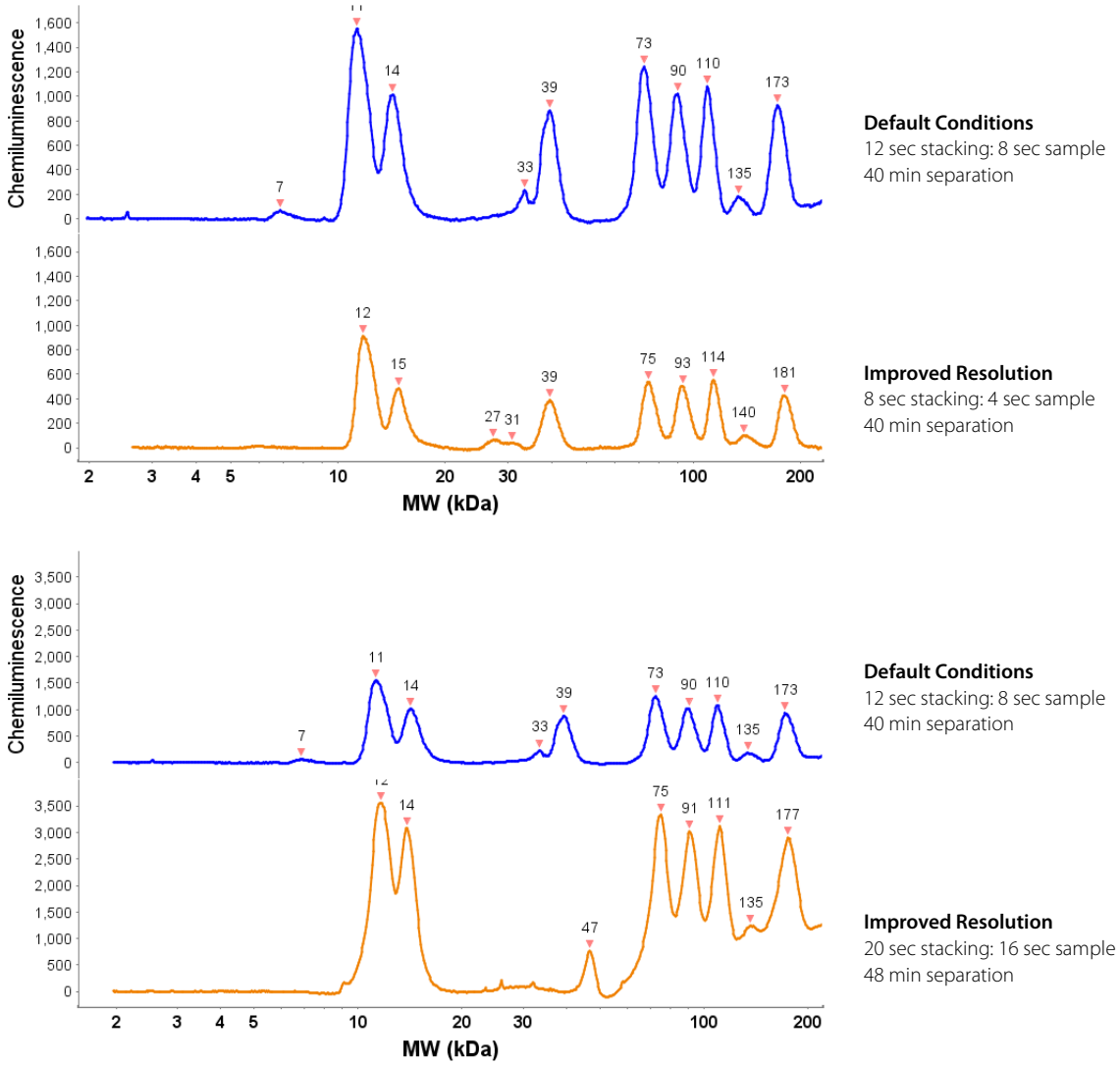

Figure 4-1: *Stacking and sample load times can be optimized to improve your resolution (top) and signal (bottom). Data generated by Sally.*

For actual examples of stacking matrix and sample load times that we've found to improve resolution and sensitivity see Table 4-2.

Assay Type	Simon, Sally and Peggy	Wes	Sally Sue and Peggy Sue
Default Conditions	12 sec stacking load 8 sec sample load 40 min separation time	15 sec stacking load 9 sec sample load 25 min separation time	15 sec stacking load 9 sec sample load 40 min separation time
Increased Resolution	10 sec stacking load 5 sec sample load 40 min separation time	12 sec stacking load 6 sec sample load 25 min separation time	12 sec stacking load 6 sec sample load 40 min separation time
Increased Sensitivity	15 sec stacking load 10 sec sample load 45 min separation time	20 sec stacking load 12 sec sample load 28 min separation time	20 sec stacking load 12 sec sample load 45 min separation time

Table 4-2: *Stacking matrix and sample load times that can improve resolution or sensitivity.*

NOTE: You may need to dilute the Biotinylated Ladder by an additional factor of 2 to keep the signal in the linear range when running conditions that increase sensitivity.

For Simon, Sally, and Peggy, you'll need to resuspend the Biotinylated Ladder in 40 µL of Ladder Resuspension Buffer + DTT.

For Wes, Sally Sue, and Peggy Sue, you'll need to resuspend the Biotinylated Ladder with 32 µL H_2O + 4 µL 10X Sample Buffer + 4 µL prepared 400 mM DTT.

Separation Time

Protein migration in the capillary can be affected by a few things: components in the buffer, the amount of protein loaded in the capillary, and the stacking and sample load times. To compensate for these effects, just increase or decrease your separation time (Figure 4-2).

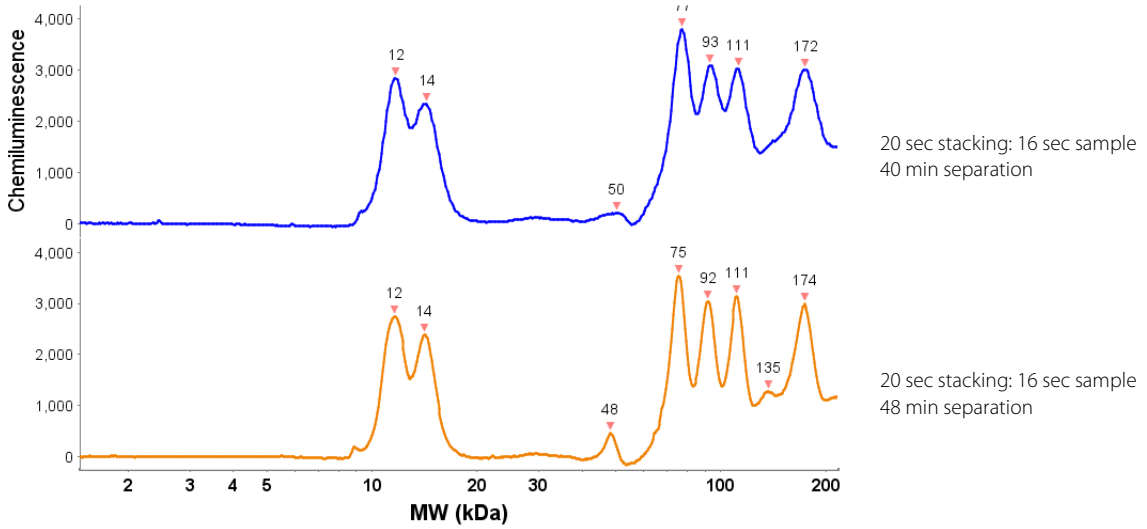

Figure 4-2: *How changing the separation time improves resolution when loading higher amounts of stacking and sample into the capillary. Data generated by Sally.*

Simple Western Immunoassay Optimization

Determining the Linear Dynamic Range

An assay is linear when changes in signal are directly proportional to changes in the amount of your protein in the sample. You should titrate the lysate to determine the limit of detection (LOD) of your assay at lower sample concentrations, and when the protein signal saturates at higher sample concentrations (Figure 4-3) to define the range where your assay signal is linear.

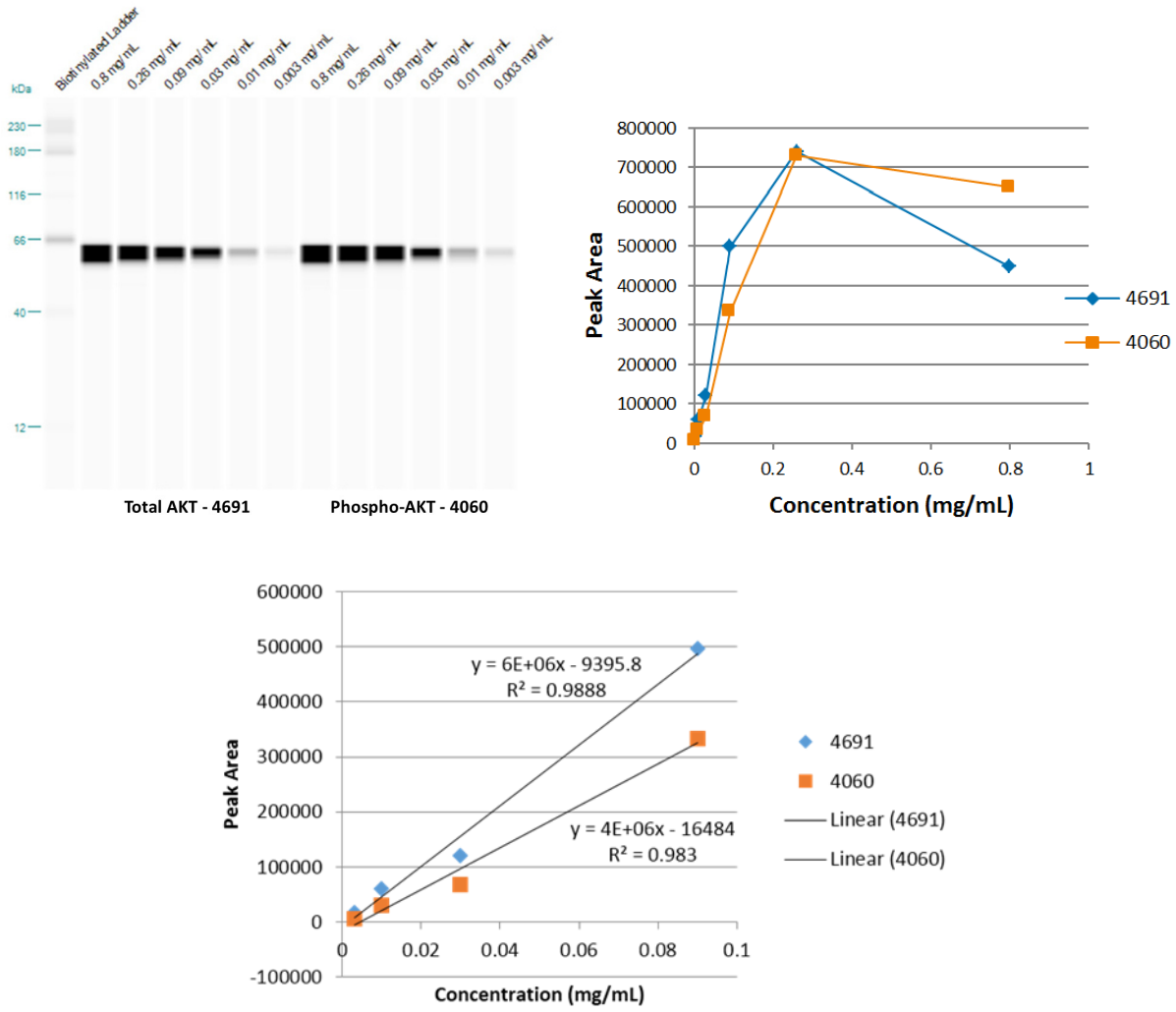

Figure 4-3: *Example lysate titration on Wes (top left) showing the lane view of Jurkat lysate when probed with 1:50 Total AKT (CST4691) and 1:50 phospho-AKT (CST4060). Peak area from lysate titration (top right). The signal isn't linear above 0.26 mg/mL, indicating the max of the linear dynamic range. The signal drops at 0.8 mg/mL because the luminol is being used up at really fast rate (luminol depletion). Linear regression analysis confirms the assay is linear from 0.003- 0.09 mg/mL (bottom).*

Optimizing Antibody Dilution and Incubation Time

Optimizing an antibody for a Simple Western Size Assay is really similar to ELISA. In both cases, your antibody conditions must be optimized to ensure the antibody is at saturating concentrations and signal changes are only due to changes in protein amount (Figure 4-4).

When optimizing an antibody, it's best to analyze baseline uncorrected data in order to assess assay peak area to the baseline.

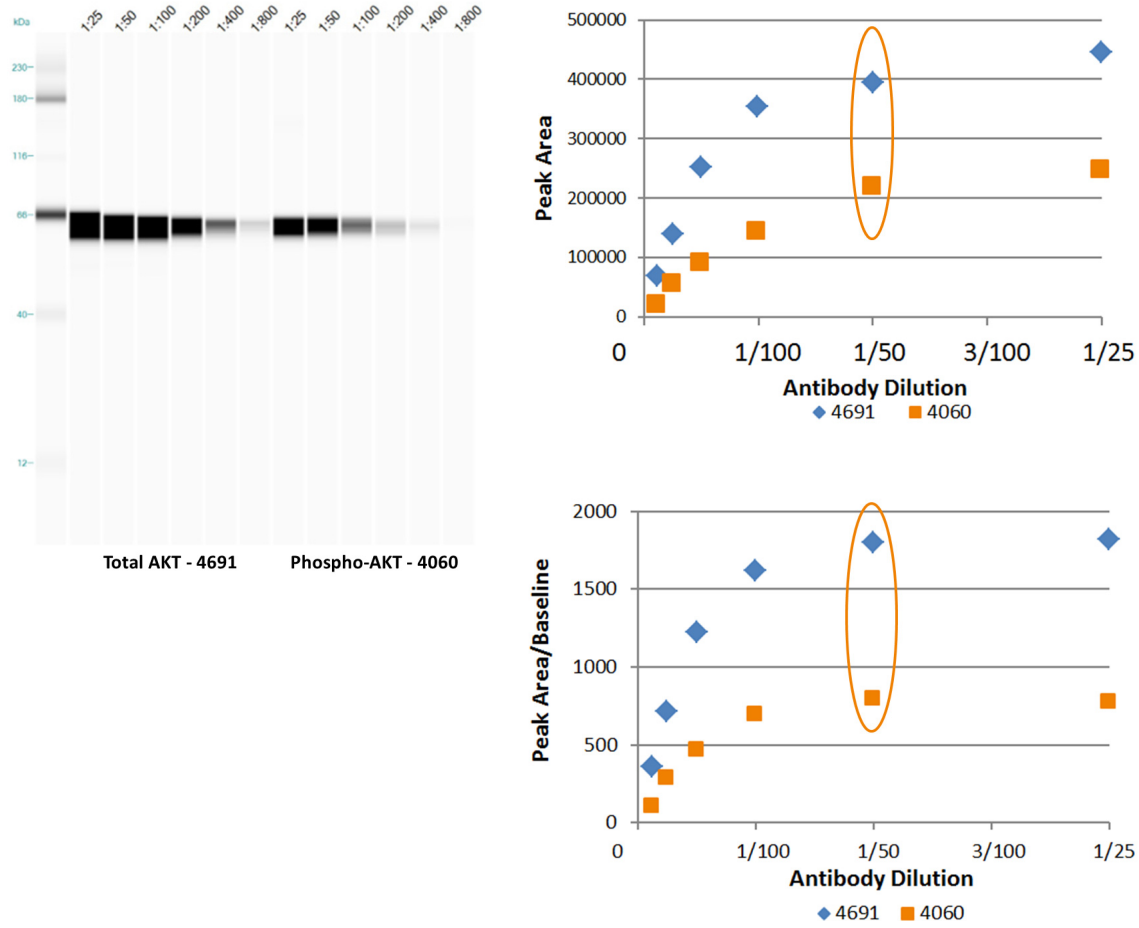

Figure 4-4: *Example antibody titration on Wes (left). Total AKT (CST4691) and Phospho-AKT (CST4060) were serially diluted 1:2 with dilutions ranging from 1:25-1:800. Chemiluminescent signal/peak area graph (top right). The area is still slightly increasing with higher concentrations of antibody. Chemiluminescent signal to baseline ratio graph (bottom right). Both antibodies saturate around 1:50-1:100 indicating the optimal dilution to use the antibody.*

Depending on the antibody, there may be cases where using a higher concentration of antibody actually isn't preferred because the background of the assay increases at a faster rate than the signal (Figure 4-5).

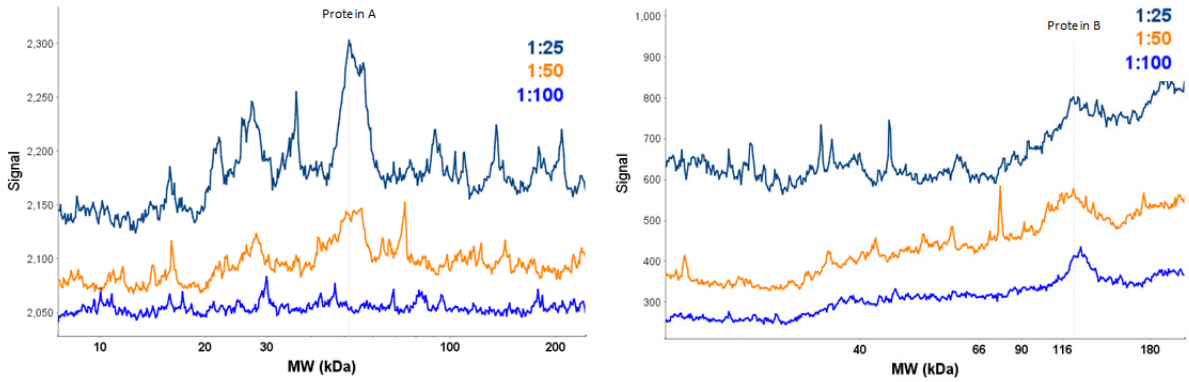

Figure 4-5: *Example antibody titration for two different protein models. The non-baseline corrected electropherogram data for Antibody A shows that assay signal increases faster than the noise (left), so a 1:25 dilution of antibody A is optimal. Non-baseline corrected electropherogram data for Antibody B shows that noise increases at a faster rate than the assay signal (right), so here a 1:100 dilution of Antibody B is optimal.*

You can also optimize primary antibody incubation time to maximize signal. Again, depending on the antibody, there may be cases where a longer incubation doesn't improve assay signal because the antibody is already at saturation (Figure 4-6).

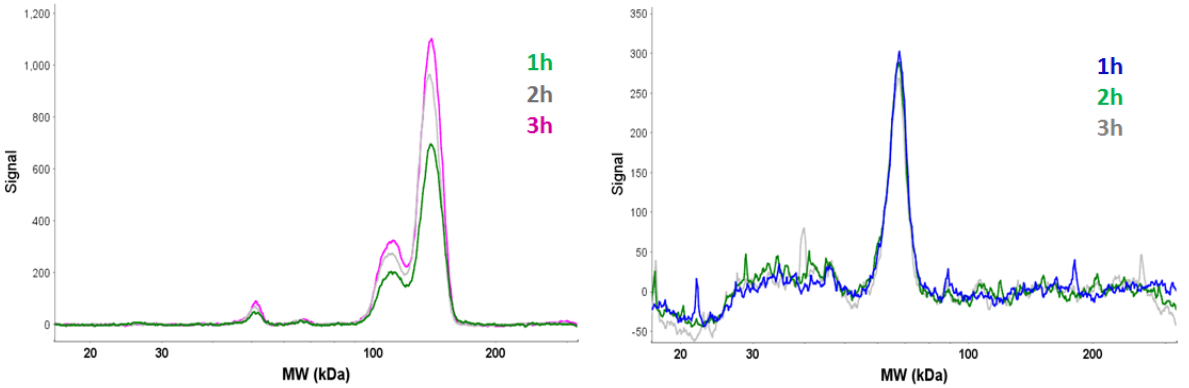

Figure 4-6: *Example of different primary antibody incubation times for two different protein models on Sally. The signal for Antibody A hasn't reached steady state yet at 1 hr (left), so a longer incubation can improve signal strength. For Antibody A, the optimal antibody incubation time is 2-3 hrs. The signal for Antibody B reaches steady state at 1 hr (right), so there's no significant benefit in incubating the primary antibody longer.*

Accelerating Antibody Binding Kinetics (Simon, Sally, and Peggy)

Increasing the antibody concentration is a really common way to drive an antibody to steady state faster. Another method you can use to speed up antibody binding kinetics is volume exclusion, or macromolecular crowding (Figure 4-7).

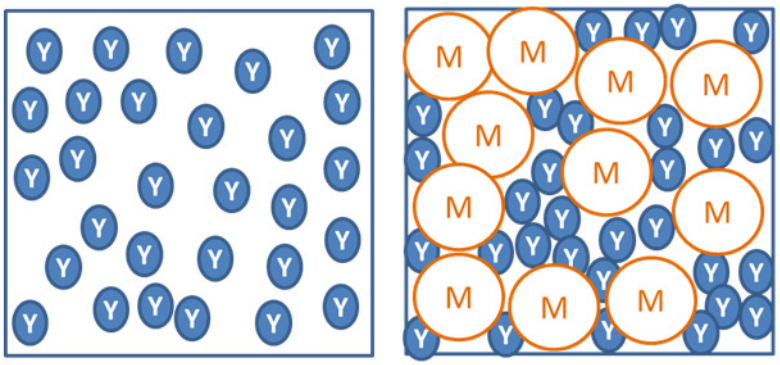

Figure 4-7: *The concept of local concentration increase through volume exclusion. M represents the macromolecule added, and Y is the antibody molecule.*

Volume exclusion works especially well for primary antibodies with low background and should be used with Antibody Diluent Plus (Figure 4-8).

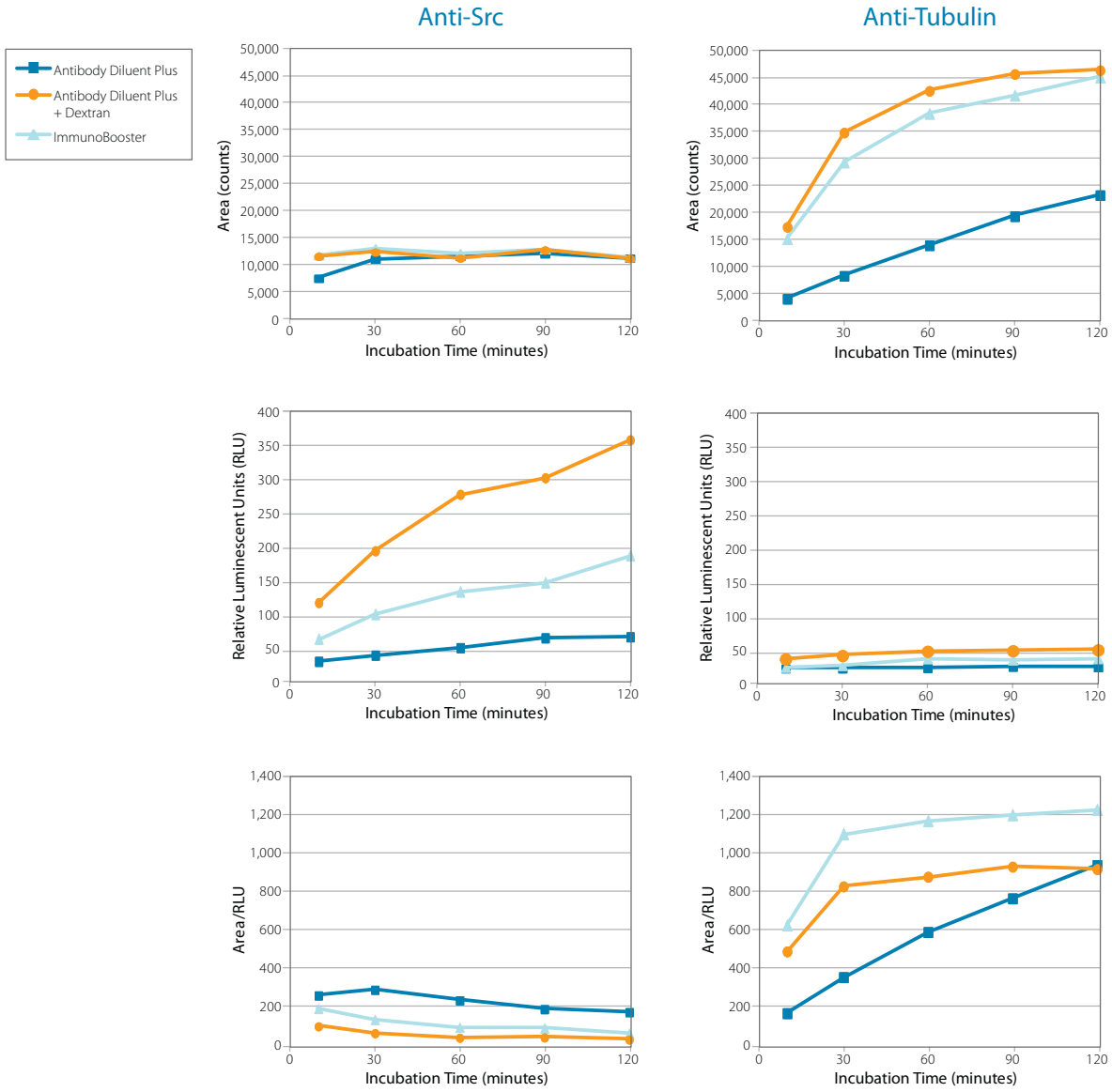

Figure 4-8: *Effects of volume exclusion on antibody binding kinetics. The binding kinetics of two different antibodies are shown: Anti-Src (CST 2109) exhibits fast antibody binding kinetics while Anti-Tubulin (CST 2125) exhibits slower antibody binding kinetics.*

There are two molecules you can use for volume exclusion in Simple Western Size Assays:

Dextran

Materials:

- Antibody Diluent Plus (ProteinSimple, P/N 041-808)
- Dextran, MW 150,000 (Sigma, P/N D4876)

Procedure:

1. Add 0.5 g of Dextran to 5 mL of Antibody Diluent Plus.
2. Dissolve overnight via end-over-end rotation until Dextran is in solution.
3. Dilute your primary antibody using Antibody Diluent Plus + Dextran.

ImmunoBooster Reagent

Materials:

- ImmunoBooster ELISA Antibody Diluent (Bioworld Consulting Lab, P/N IBE-25)
- Rapid Western Blocking Solution, 5X (Bioworld Consulting Lab, P/N RWW-100)

Procedure:

1. Add 0.25 mL of Rapid Western Blocking Solution (5X) to 10 mL of ImmunoBooster ELISA Antibody Diluent (1:40).
2. Dilute your primary antibody using the ImmunoBooster ELISA Antibody Diluent + Rapid Western Blocking Solution.

You can find more information on using macromolecular crowding to optimize Simple Western Size Assays in the *Spring 2013 Simply Speaking Newsletter.*

Chemi Exposure Time

Taking multiple chemi exposures is just like taking multiple film exposures on a traditional Western blot.

Chemi exposures with Simple Western Size Assays are serial, not cumulative, so if a protocol contains a 30 sec chemi exposure and a 60 sec chemi exposure, by the end of the 60 sec chemi exposure 90 sec will have elapsed. For a protocol with six chemi exposures (30 sec, 60 sec, 120 sec, 240 sec, 480 sec, 960 sec), a little over 15 minutes have elapsed before the camera starts taking the 960 sec chemi image.

With each exposure, luminol is consumed for signal generation. So if you want to improve your assay signal, just remove the shorter chemi images so data collection for the longer chemi images starts before the luminol/peroxide begins decaying.

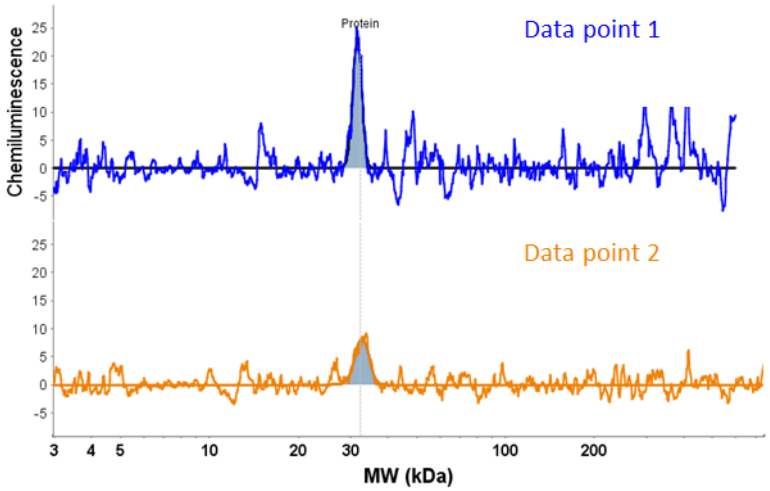

Figure 4-9: *Example of a low expression Simon assay where deleting the shorter chemi exposures results in better assay sensitivity. Antibody dilution, sample load time and stacking load time are the same between data points. Data point 1 (blue) was taken from a protocol with only three chemi exposures: 30 sec, 480 sec, and 960 sec. Data point 2 (orange) was taken from a protocol with six chemi exposures: 30 sec, 60 sec, 120 sec, 240 sec, 480 sec, and 960 sec.*

Using Immunoassay Reagents from Other Vendors

ProteinSimple reagents were extensively screened and optimized for Simple Western Size Assays, and we strongly recommended you use them as they'll help simplify new assay development. But, you can also use reagents from other vendors if needed with additional assay development.

Antibody Diluent/Blocking Buffer

When you're testing a new antibody diluent/blocking buffer, assess the chemiluminescent signal compared to the baseline. Some diluents will be very good at blocking non-specific antibody binding but may also block specific binding of the antibody to your target protein. It's best to test at least two antibodies: one known to have a high background and one known to have a clean background.

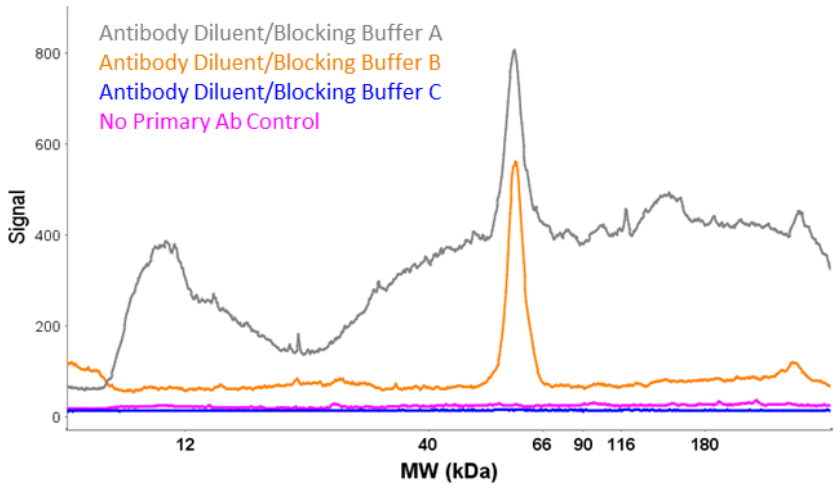

Figure 4-10: *Example electropherograms comparing different antibody diluents/blocking buffers on a known dirty antibody without baseline subtraction in Compass. A no primary antibody control was run to get baseline background with just sample and secondary antibody. Antibody diluent/blocking buffer A has high signal but also has the highest baseline, increasing assay noise without improving the overall signal:baseline ratio. Antibody diluent/blocking buffer C cleans up the baseline really well but also blocks the signal. Between the three diluents tested, we consider antibody diluent/blocking buffer B best because it significantly blocks the baseline noise while still maintaining a strong signal.*

The antibody diluent/blocking buffer also shouldn't contain components, like 5% dry milk, that can clog the manifold. Data with no signal or a smear can often mean your manifold is clogged.

Secondary Antibody

We recommend you first check the Antibody Database (www.proteinsimple.com/antibody/) to see which secondary antibodies have been successfully used with Simple Western Size Assays.

If the secondary antibody you want to use isn't listed in the database yet, screen HRP-conjugated secondary antibodies. If you're screening secondary antibodies for more general use and not for one specific protein target, test at least four or five protein models to choose the best HRP-conjugated secondary antibody. You should also titrate the lysate to get a better understanding of how the secondary antibody noise will affect the assay LOD.

Affinity purified whole IgGs that are HRP-labeled work best for Simple Western Size Assays. Dilute the secondary antibody in the antibody diluent for your system and titrate the antibody dilution, starting with a 1:100 dilution. If you're using Simon, Sally, or Peggy, incubate the secondary antibody for 1 hr. If you're using Wes, Sally Sue, or Peggy Sue, incubate the secondary antibody for 30 minutes for your initial experiment.

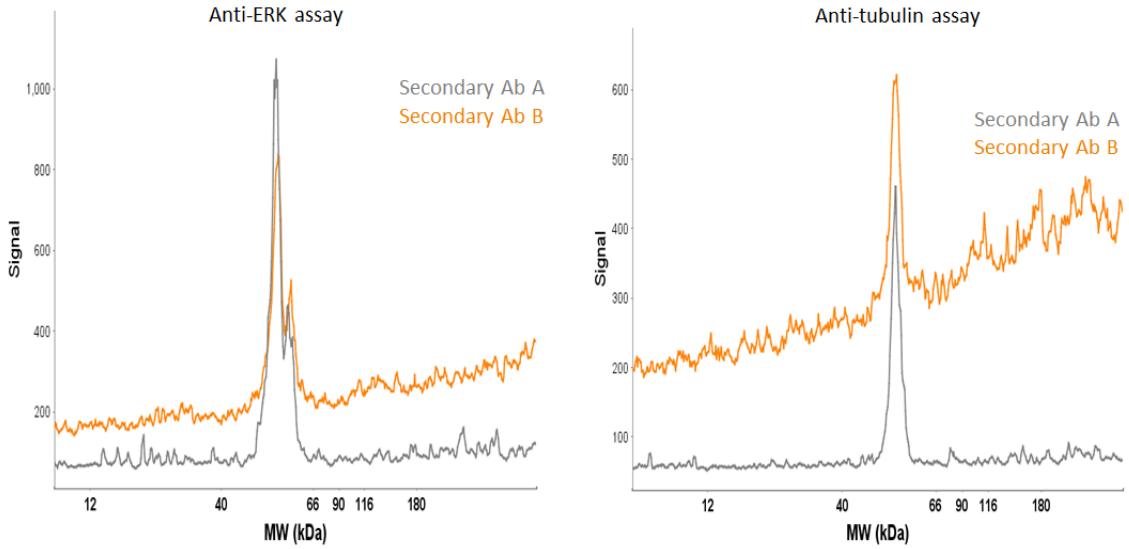

Figure 4-11: *Comparison of two different secondary antibodies in two protein models. HUT78 lysate was used close to LOD (0.2 mg/mL on Sally). Secondary antibody A has lower baselines that translate to a better signal:noise ratio for both models.*

Luminol/Peroxide

If you're testing different luminol/peroxides for general use and not for one specific protein target, assess the baseline and signal for at least four or five protein models. You should compare the signal at different chemi exposures to understand the enzymatic conversion kinetics of luminol to light. If the kinetics of the conversion is very quick, the initial light output at shorter chemi exposures will be significant, but subsequent chemi exposures will not necessarily see a gain in signal if the luminol has been depleted.

You'll also want to assess the reproducibility of the assay when testing different sources of luminol/peroxide, since extremely fast kinetics can have more variability from experiment to experiment and between high and low expressing models.

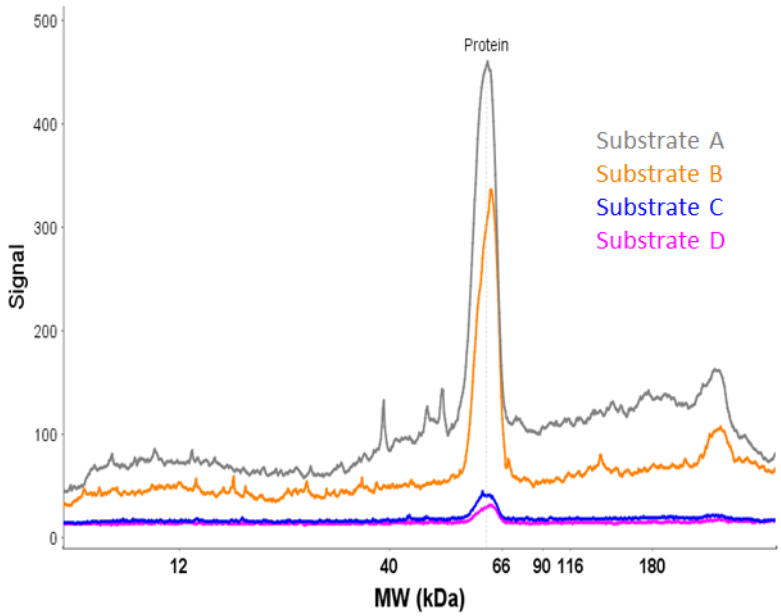

Figure 4-12: *Comparison of four different substrates (luminol/peroxide mixtures) on Sally. 1 mg/mL of K562 lysates were loaded in each capillary. Substrate A and B have better signal:noise compared to substrate C and D.*

Chapter 5:

Additional Protocols and Applications

Chapter Overview

- 1- Incubation Step Protocol
- Multiplexing
- Loading Controls

1- Incubation Step Protocol

A default Simple Western Size Assay contains two incubation steps after the blocking steps (primary antibody and secondary antibody). A 1-incubation step protocol is a protocol where there is only one incubation step after the blocking step. You can run a 1-incubation step protocol to shorten assay time when:

1. Your primary antibody is HRP-labeled
2. Your analyte is a human IgG molecule that can be detected with an anti-human-HRP antibody
3. Your analyte is a biotinylated protein that can be detected with ProteinSimple Streptavidin-HRP

Remember, you get more signal amplification with each incubation step, so a 1-incubation step protocol may be less sensitive than a 2-incubation step protocol.

We suggest using these conditions for your initial experiment:

Condition	Simon, Sally and Peggy	Wes, Sally Sue and Peggy Sue
Antibody Diluent	Antibody Diluent PLUS	Antibody Diluent II
Detection Antibody Dilution	1:50, 1:100 for primary Ab-HRP 1:100, 1:200 for anti-human-HRP Ready-to-use Streptavidin-HRP	1:50, 1:100 for primary Ab-HRP 1:100, 1:200 for anti-human-HRP Ready-to-use Streptavidin-HRP
Detection Antibody Incubation Time	2 hr	30 min

Table 5-1: *1- incubation step protocol conditions we recommended to get started.*

To run a 1-incubation step protocol in Compass, delete the second incubation step in the Assay Screen. Just select the Secondary Antibody row in the Layout tab and click **X** to delete the row (Figure 5-1).

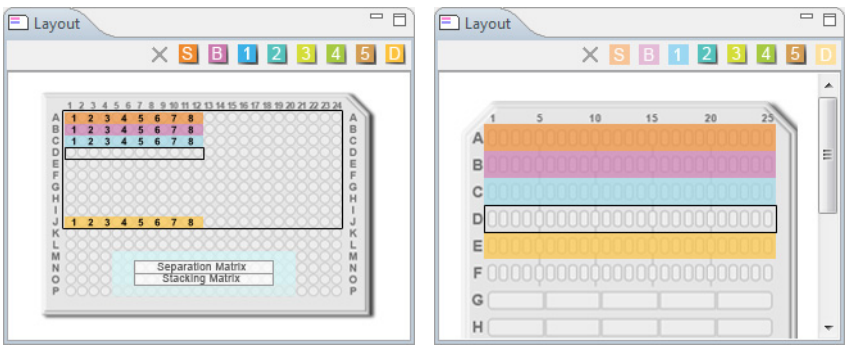

Figure 5-1: *Example of a 1-incubation protocol plate layout for Sally, Sally Sue, Peggy, Peggy Sue (left) and Wes (right).*

Multiplexing

You can detect multiple proteins in one capillary if the proteins migrate at distinct molecular weights. Primary antibody backgrounds are cumulative, so you'll want to optimize the dilution for each antibody individually and screen for the cleanest antibody before multiplexing. Choosing primary antibodies from the same host species also simplifies transfer from individual assays to a multiplexed assay. You also want to keep stacking matrix load times, sample load times, and antibody incubation times consistent between the two assays.

When you're developing multiplexed assays, titrate the lysate to determine the linear dynamic range for both assays at the same lysate concentration. An example is shown in Figure 5-2.

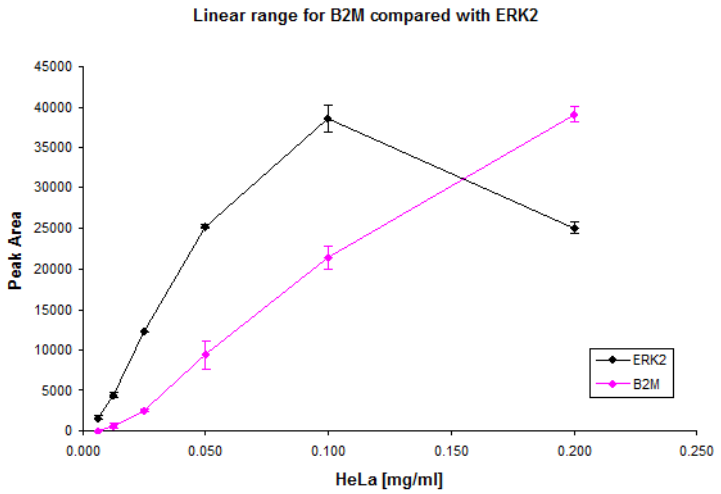

Figure 5-2: *Example HeLa lysate titration for an ERK2 and B2M assay. The ERK2 assay is no longer linear at 0.2 mg/mL, even though B2M is. To multiplex the two assays, your sample concentration will need to be < 0.1 mg/mL for results to be quantitative.*

After conditions for both assays are optimized, you can multiplex the assays by combining the primary antibodies in one well.

EXAMPLE: Multiplexing Protein A and Protein B. If α-Protein A should be diluted 1:50 and α-Protein B should be diluted 1:100, then to prepare enough antibody for 10 wells on Wes (10 µL per well) add:

- 2 µL α-Protein A
- 1 µL α-Protein B
- 97 µL Antibody Diluent II

When your primary antibodies are from different host species, mix the secondary antibodies together at a ratio that maximizes the linear dynamic range of all the assays.

If you're mixing the ProteinSimple RTU Primary Mouse with the ProteinSimple RTU Primary Rabbit Antibody, test these ratios:

- 90% α-mouse HRP/10% α-rabbit HRP
- 75% α-mouse HRP /25% α-rabbit HRP
- 50% α-mouse HRP /50% α-rabbit HRP
- 25% α-mouse HRP /75% α-rabbit HRP
- 10% α-mouse HRP /90% α-rabbit HRP

If you need to mix a non-mouse and non-rabbit HRP with the ProteinSimple RTU Anti-Mouse/Anti-Rabbit Secondary Antibody, dilute the non-mouse/non-rabbit HRP secondary antibody directly into ProteinSimple RTU Anti-Mouse/Anti-Rabbit Secondary Antibody.

If you need to mix two non-ProteinSimple secondary antibodies, dilute the secondary antibodies into the antibody diluent that should be used with your ProteinSimple instrument.

Loading Controls

Loading controls are critical for meaningful quantitative analysis because they guarantee data reliability. Normalizing your data against a biological or system control corrects for potential sources of error.

Running loading controls in adjacent capillaries in the same run or multiplexed in the same capillary is the best way to correct your data. The same considerations apply for multiplexing a loading control with your protein of interest as they are for any multiplexed assay: develop each assay separately by selecting antibodies with the lowest background and then determine the linear range for both assays before multiplexing them.

There are two types of loading controls that you can use to control for different sources of variability in your data:

Source of Variability	Biological Loading Control	System Control
Varying cell numbers used for samples	Yes	No
Varying protein concentration between samples	Yes	No
Varying extraction efficiencies between samples	Yes	No
Differences in environmental conditions (day to day, lab to lab)	Yes	Yes
Instrument to instrument differences	Yes	Yes
Differences in detection reagents (lot to lot)	Yes	Yes

Table 5-2: *Types of loading controls used to control for different sources of data variability.*

Biological Loading Control

A biological loading control is an endogenous protein found in the lysate that does not respond to treatment, so it has consistent expression levels between different biological samples. Commonly used biological loading controls include actin, β-tubulin, and GAPDH. If you're preparing samples from a specific compartment of the cell (nuclear extracts for example), pick a biological loading control protein that's also present in that subcellular fraction.

Differences in loading control signal indicate differences in the total amount of protein present from sample to sample. This can be caused by inaccurate total protein concentration determination or the variability that's often introduced with manual pipetting during sample preparation. Normalizing the data for your protein of interest against a biological loading control will correct for these errors.

System Control (Wes, Sally Sue, and Peggy Sue)

We offer System Controls for improved comparison of data from lab to lab and instrument to instrument. Our System Control is a protein that's added to the 5X Fluorescent Standard so it's present in equal amounts in every sample. And because it is present in equal amount in every sample, data from different instruments can be normalized and compared as shown in the example in Figure 5-3.

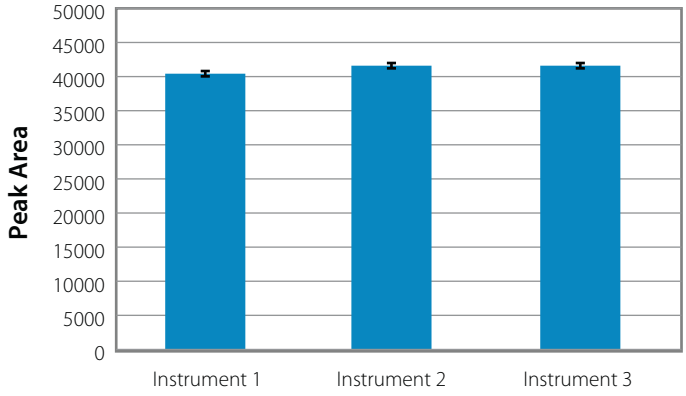

Figure 5-3: *Peak area values of CDK6 from three different instruments after normalization with the ProteinSimple System Control. The inter-instrument CV following normalization is ≤ 3%.*

We have two options for running a System Control with your assay. The Standard Pack 1 in the Master Kit includes a 29 kDa System Control protein in the 5X Fluorescent Standard. For proteins that migrate close to 29 kDa, try the Standard Pack 2 (P/N PS-ST02) which has a 180 kDa alternative in the 5X Fluorescent Standard.

To detect the System Control protein in your sample, all you need to do is add the System Control antibody to your primary antibody mixture. The System Control antibody is offered as a 10X System Control Mouse Primary Antibody (P/N 042-191) or a 10X System Control Rabbit Primary Antibody (P/N 042-196). Choose the one that matches the host species of the primary antibody for your target protein. If you're multiplexing with both rabbit and mouse primary antibodies, choose the System Control primary antibody species based on the secondary antibody that makes up the majority of your secondary antibody mixture.

The System Control protein produces a readily identifiable peak in the electropherogram that you can use to normalize your data in Compass.

Normalizing Data in Compass

Normalizing data to a loading control is simple and fast in Compass software. In the Peak Names menu of the Analysis window, just check the **Control Area** box to select the loading control peak. You then have the option of selecting a reference sample that all samples will be normalized against, or designating a control area that all the samples will be normalized to. For inter-instrument comparisons, normalize to a consistent control area in the analysis settings.

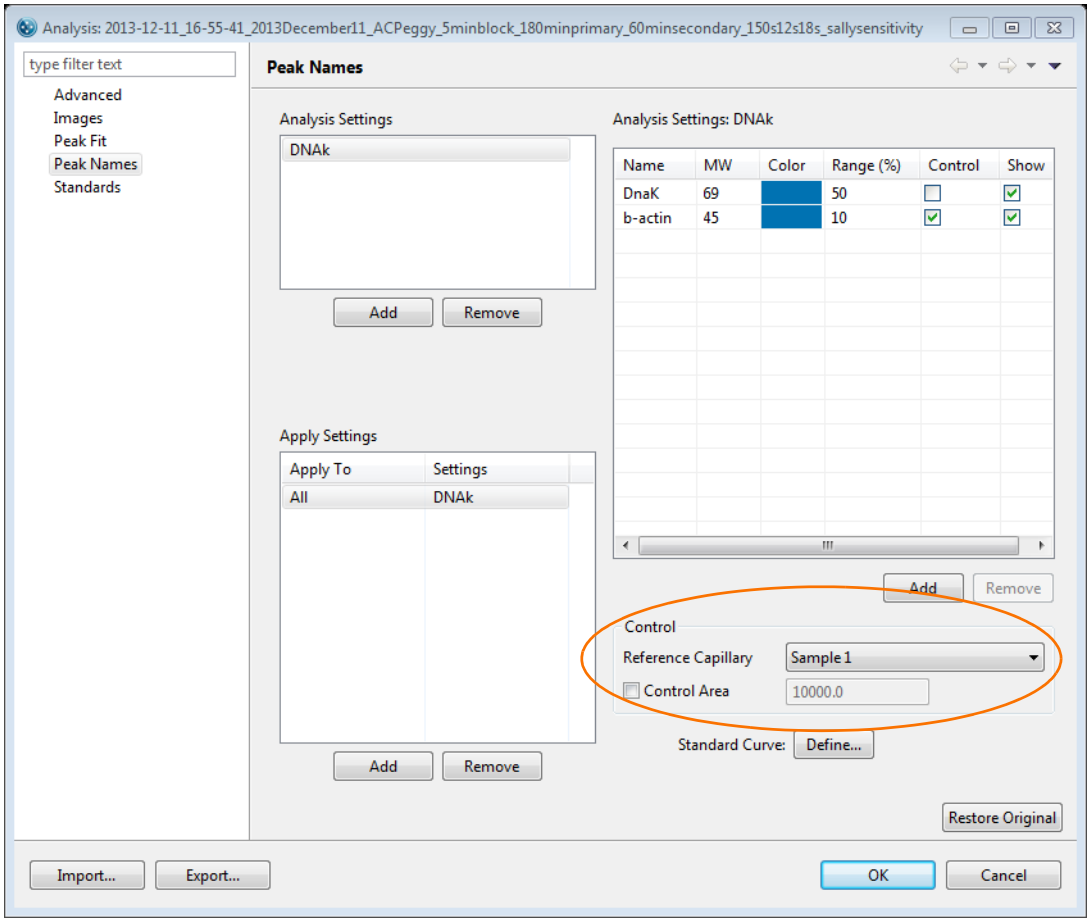

Figure 5-4: *Example of how to normalize data to loading control proteins in Compass. In this example, β-actin is the biological loading control selected in the Peak Names menu and Sample 1 is the sample selected to normalize all the data against.*

Chapter 6:

Troubleshooting

Chapter Overview

- Troubleshooting Flowchart
- Incorrect or Varying Molecular Weight
- Signal Burn-Out
- Separation Issues
- No Chemiluminescent Signal

Troubleshooting Flowchart

Compass analyzes your data at the end of each run, but if it doesn't automatically find your peak of interest, this troubleshooting flowchart walks you through what to do:

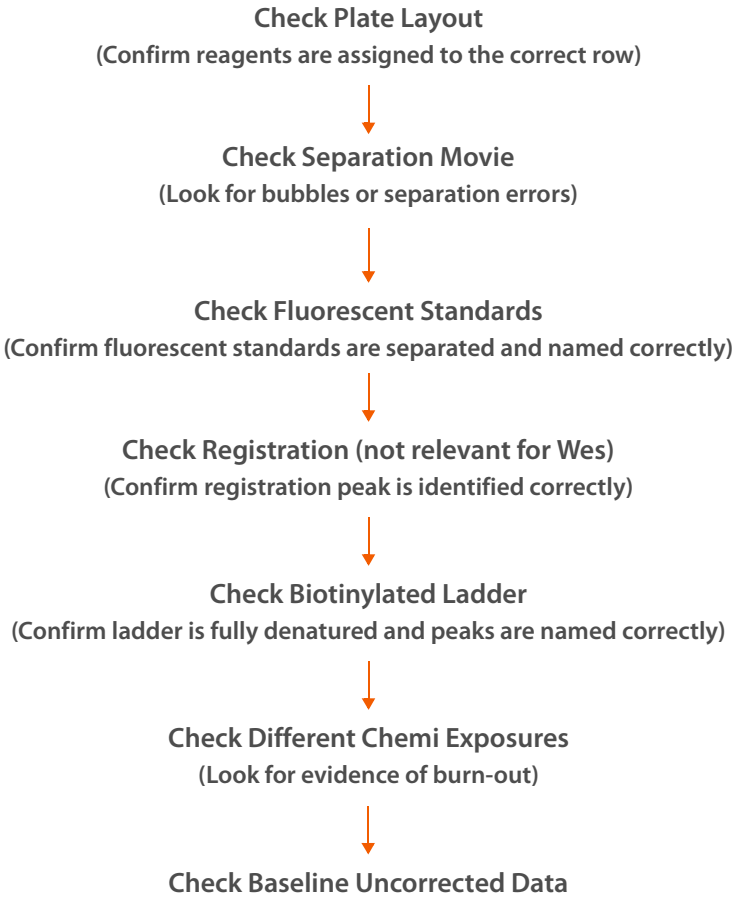

Figure 6-1: *Data analysis troubleshooting steps for Simple Western Size Assays.*

Incorrect or Varying Molecular Weight

Just like all other SDS-based size separation protein analysis techniques, the molecular weight Compass reports is the apparent molecular weight. This value can be influenced by properties of your protein that affect SDS binding and its interactions with the separation matrices.

You'll get a different apparent molecular weight depending on the technique you use. This is due to formulation differences between the separation matrices used for each technique. So, observing a biological event that correlates with the behavior you'd expect from your protein is the best way to confirm peak identity.

If you find unexpected differences in molecular weight between samples, first check the fluorescent standards (all systems) and registration (all systems except Wes) to confirm they're assigned properly. If that seems fine, check the biotinylated ladder to make sure it's both completely denatured and that all peaks are assigned correctly. Sometimes, if Compass thinks Ldr 40 is Ldr 12, you will need to expand the x-axis to properly assign the standards. Just click **View**, then select **View Region** and check the **Full Range** box to see the entire capillary.

You should also compare how the fluorescent standard in the biotinylated ladder migrates with the fluorescent standards in your sample. If your sample lysis buffer affects protein migration, you will see differences in how the fluorescent standards migrate. To avoid this, resuspend your biotinylated ladder in the sample buffer you prepared your sample with. See Chapter 3, *"Sample Preparation Optimization"* for details on how to do this.

Insufficient denaturing of the biotinylated ladder happens either because the resuspension buffer wasn't used correctly or the ladder wasn't heated for the recommended times and/or temperatures (Figure 6-2).

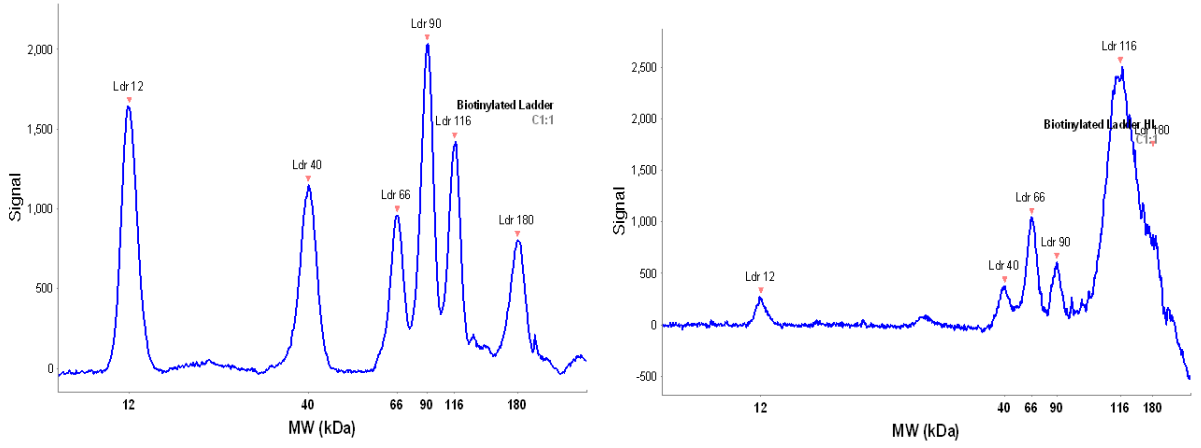

Figure 6-2: *Example of biotinylated ladder that's been completely denatured (left) and one that hasn't (right).*

The lysis buffer and protein concentration loaded in the capillary can also cause non-linear compression of the separation matrix. When this happens, you'll see a step-wise migration of you protein (Figure 6-3). The migration will correlate with the amount of lysis buffer/protein loaded in the capillary. You can avoid this by either preparing your stock lysates at a higher total protein concentration (which minimizes the amount of lysis buffer loaded) or using a lower protein concentration (if the linear dynamic range of your assay allows it).

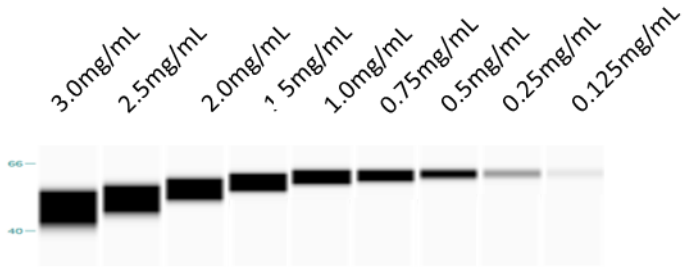

Figure 6-3: *Step-wise migration of a protein caused by buffer effects or high protein concentration.*

Protein glycosylation can also affect protein migration. When you need to confirm migration shifts are caused by protein glycosylation, use one of these kits to deglycosylate your protein:

1. PNGase F kit (New England BioLabs P/N P0704S)
2. Deglycosylation Mix kit (New England BioLabs P/N P6039S)

Follow the instructions that come with the kit to optimize the deglycosylation assay. Then run your deglycosylated samples on the Simple Western Size Assay. It's also a good idea if you include a buffer only and a buffer + enzyme only (no sample) to control for kit-related background noise.

Signal Burn-Out

Signal burn-out happens when protein is loaded at such high levels in the capillary that the peroxidase substrate depletes at an extremely fast rate, to the point where the luminol becomes the limiting factor in the peroxidase reaction.

You can find out if your assay is burning out by looking at different chemiluminescence exposure times. Exposure times are sequential, so longer exposures can have less or no signal if the local concentration of luminol is being used up.

The y-axis in an electropherogram reports signal/time, so the data from each exposure should have a similar signal/time coefficient. This coefficient decreases when luminol becomes depleted (Figure 6-4). Less extreme cases of luminol depletion are seen when your peak of interest decreases in height with longer chemi exposure times. More significant cases of burn-out are seen as a signal dip at longer exposures. This will happen at the molecular weight where the peak was detected using shorter exposures (Figure 6-4).

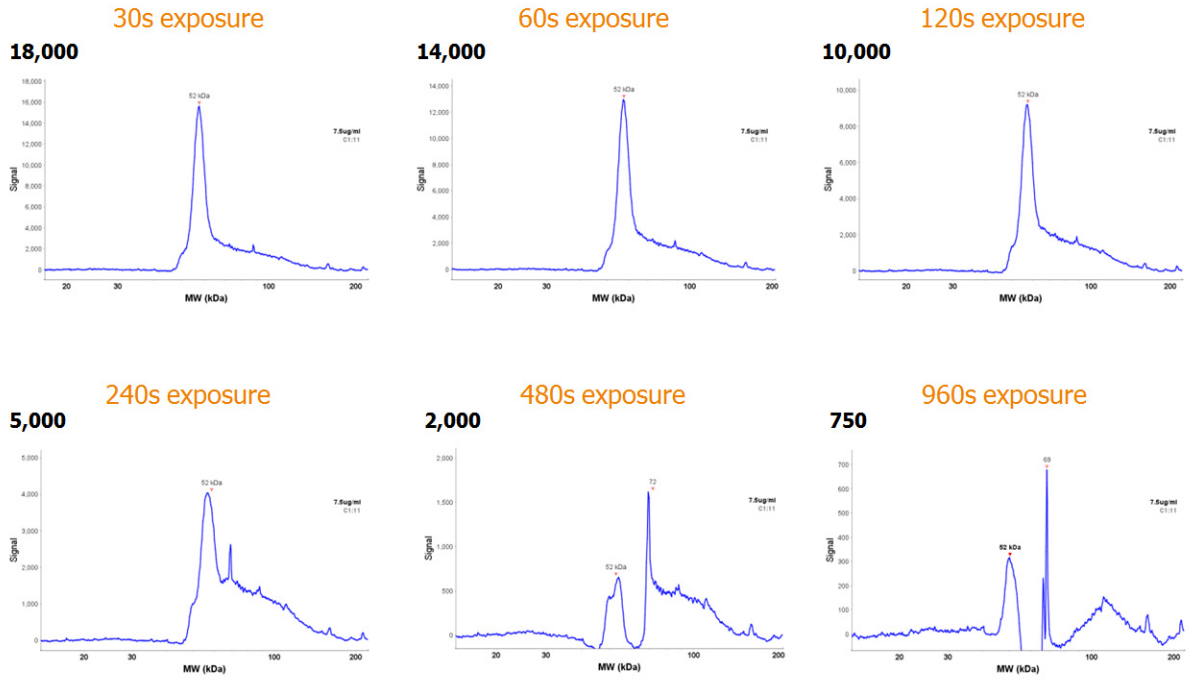

Figure 6-4: *Comparing different exposure images to pinpoint assay burn-out. The y-axis max is noted in black at the top of each axis. At the 240 sec exposure, the signal/time coefficient has decreased by half, indicating luminol depletion. By 480 sec the signal is non-existent due to depletion of the local luminol/peroxide. To avoid data bias, only the data collected at 30-120 sec exposures is considered quantitative.*

An assay has extreme burn-out when your peak of interest is only observed at lower protein concentrations but not at higher protein concentrations. (Figure 6-5).

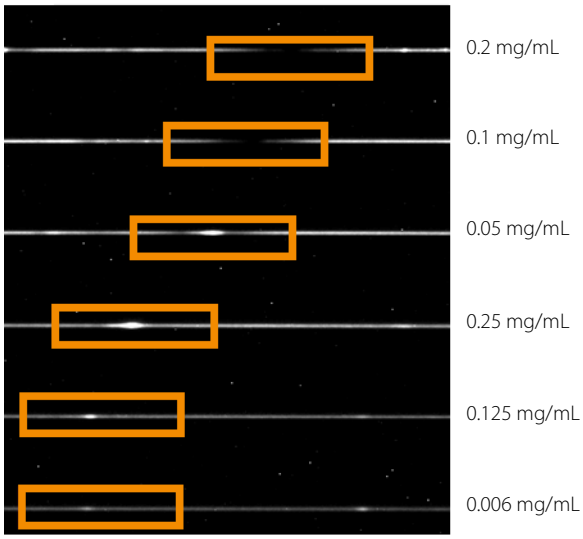

Figure 6-5: *Example of extreme burn-out at high lysate concentrations. A HeLa lysate was serially diluted 1:2. Burn-out is indicated by the lack of detected protein signal at the two highest concentrations and the recovery of signal at lower lysate concentrations.*

If you experience burn-out, lower the protein concentration in the assay but don't lower the primary antibody concentration. If you keep the primary antibody concentration the same, you'll avoid any negative impact on assay linearity, sensitivity, and reproducibility.

Separation Issues

Increased Assay Variability

You may see higher data CVs than expected for Simple Western Size Assays when there are inconsistent levels of Separation Matrix between wells in an assay plate. To avoid the variability that can sometimes happen with pipetting issues, we recommend using a positive displacement pipette or a repeat pipette to add Separation Matrix into your sample plate.

Leaving the lid off the assay plate too long allows the Separation Matrix to start evaporating, causing sample loading variability (Figure 6-6) and/or stacking inefficiencies. Depending on the degree of loading variability, you'll either see increased assay variability or a complete loss of usable data.

Figure 6-6: *Example of fluorescent standards in the separation movie from a plate that was centrifuged with the lid left off. This image was taken after a 60 min separation using a 12 sec stacking load time: 8 sec sample load time. The migration of both the samples and the standards is really variable, and there's significant loss in standard resolution.*

Poor Separation and/or Reproducibility on Wes, Sally Sue or Peggy Sue

Wes plates and Running Buffer II have to be stored properly once you get them to avoid precipitation of cold-sensitive components. To make sure you get the best assay performance, store Wes plates at room temperature for at least 24 hours after they're received. Running Buffer II should to be warmed at 37 ℃ for at least 1 hour or sit out at room temperature for 24 hours after it's been received. See Chapter 4, *"Simple Western Size Assay Optimization"* for more detailed recommendations on removing precipitation from the Running Buffer II.

If you use Wes plates or Running Buffer II before they've warmed up for the time we recommend, you'll most likely see very poor sample separation or decreased data reproducibility (Figure 6-7) depending on how many precipitants were still in the Running Buffer.

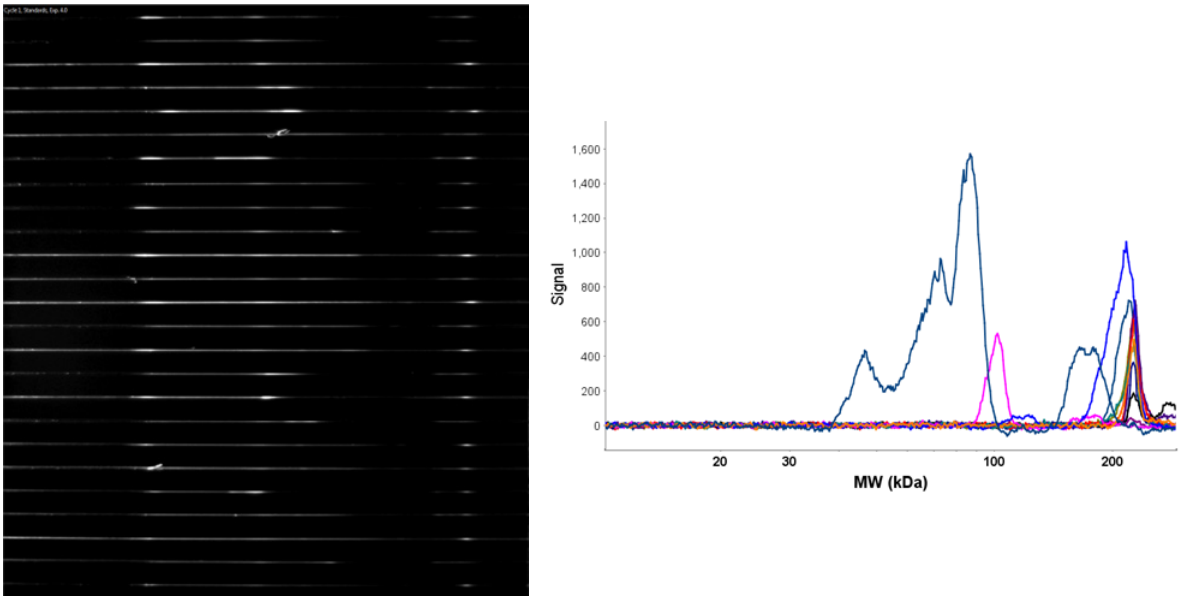

Figure 6-7: *Fluorescent standards in the separation movie and chemi data from 25 capillaries with Biotinylated Ladder for an assay run two hours after the Wes Master Kit was received.*

Bubbles in the Capillary

Always centrifuge the plate after you've loaded reagents to remove any bubbles from the sample, Separation Matrix, and Stacking Matrix. You should also always store the Separation Matrix at room temperature. When this reagent is chilled, gases are more soluble. So once it's in the capillary, bubbles will form due to outgassing.

Bubbles in a capillary will look like small fluorescent dots migrating ahead of the fluorescent standards when you view the separation movie (Figure 6-8).

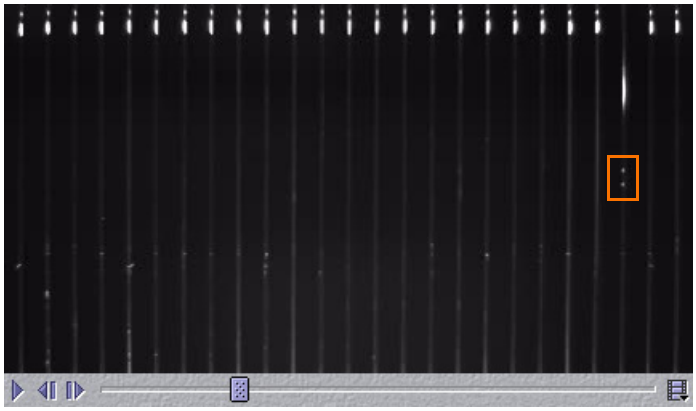

Figure 6-8: *Example of a bubble (orange box) in a Wes capillary shown in the fluorescent standard image during the separation movie.*

Bubbles cause the fluorescent standard to migrate faster than the standards in neighboring capillaries. Depending on how much faster the fluorescent standards migrate, you'll either see a loss in peak resolution (to the point where a peak becomes a smear) or your sample will run off the capillary completely. And unfortunately both result in unusable data.

No Sample Separation

You should always store the Separation Matrix at room temperature. When it's chilled, sometimes during a run you'll see a phenomenon where samples enter the capillary but they don't separate (Figure 6-9).

You'll also see a complete lack of protein separation if there isn't any Separation Matrix loaded on your assay plate. When this happens, the capillary will just completely fill with fluorescent material at the beginning of the separation. To rescue your experiment: stop the run and start the instrument cleanup protocol. While that's running, you can prepare more sample (most of the sample from your original plate will already be in the capillaries). Then transfer the blocking buffer, primary antibody, secondary antibody, and luminol/peroxide to a new assay plate and add fresh sample, Separation Matrix, and Stacking Matrix. You can then use the new plate to restart your run.

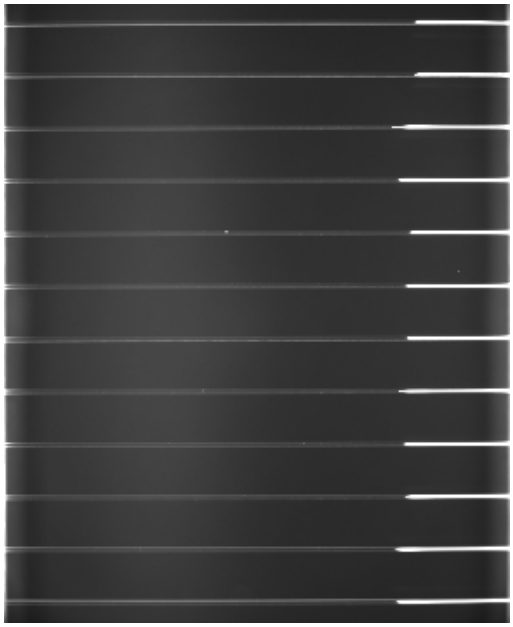

Figure 6-9: *Example of fluorescent standards in the separation movie after 40 minutes of separation from a plate that was centrifuged at 4 °C. Samples entered the capillary but didn't separate.*

Fluorescent Standards Don't Separate

If the Matrix Removal Buffer and Running Buffer are accidentally switched, you'll see fluorescent standards in the capillary that don't separate (Figure 6-10). To rescue your experiment: quickly stop the run, start the instrument cleanup protocol and the put the correct reagent in the trough/reagent cup.

Figure 6-10: *Example of fluorescent standards in the separation movie during an experiment where the Matrix Removal Buffer and Running Buffer were switched. The fluorescent standards don't separate.*

No Chemiluminescent Signal

If you don't see any signal at the end of your experiment:

1. First check to see if there's any biotinylated ladder signal.
    a. If there's no signal for the biotinylated ladder or samples, check your plate map layout and make sure secondary antibody/streptavidin-HRP and luminol/peroxide were assigned to the right plate row.
    b. If there's signal for the biotinylated ladder but no signal for any of your samples, make sure the correct secondary antibody was used for each of your primary antibodies.
2. If you're missing chemiluminescent signal in one capillary only, check the registration peak (all systems except Wes) and the fluorescent standards to make sure they're present in that capillary and assigned correctly.
3. If there's no fluorescent standard in a capillary but you can see the capillary in the separation movie/chemi image in Compass, check your plate to confirm that there was sample added to every sample well during the plate preparation.

Appendix A:

Reagent and Lysis Buffer Compatibility

Appendix Overview

- Simple Western Reagent Compatibility
- Simple Western Commercial Lysis Buffer Compatibility

Simple Western Reagent Compatibility

All results compared against ProteinSimple Bicine/CHAPS lysis buffer.

Buffering Reagents

Reagent	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
Bicine	20-50 mM	20-50 mM	No effect	No effect	No effect	No effect
TrisCl, pH 7.5	10-50 mM	10-50 mM	No effect	No effect	No effect	No effect
HEPES, pH 8.0	10-50 mM	10-50 mM	No effect	No effect	No effect	No effect
Sodium Phosphate (NaH2PO4/ Na2HPO4)	10 - 30 mM	10 - 30 mM	No effect	No effect	No effect	No effect

Table A-1: *Buffering reagent compatibility.*

Dyes

Dye	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
Bromophenol Blue	0.001-0.01%	0.001-0.01%	No effect	No effect	No effect	No effect
Phenol Red	0.005-0.01%	0.005-0.01%	No effect	No effect	No effect	No effect

Table A-2: *Dye compatibility.*

Salts

Salt	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
NaCl	0-700 mM	0-150 mM	Signal decrease > 150 mM, especially at higher MW range.	Resolution loss > 150 mM.	180/230 kDa standard resolution loss > 150 mM.	MW sizing affected at high MW region due to 180/230 kDa standard resolution loss > 150 mM.
NH_4Cl	0-300 mM	0-100 mM	Signal loss at high MW > 100 mM. Stronger impact in Separation Matrix II.	No effect	180/230 kDa standard resolution loss > 100 mM.	MW sizing affected at high MW region due to 180/230 kDa standard resolution loss > 100 mM.
$MgCl_2$	0-10 mM	0-10 mM	No effect	No effect	No effect	No effect

Table A-3: *Salt compatibility.*

Reducing Agents

Agent	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
DTT	40-80 mM	40-80 mM	No effect	No effect	No effect	No effect
βME	40-400 mM	40-400 mM	No effect	No effect	No effect	No effect
TCEP	0.5-20 mM	< 0.5 mM	Loss of high MW signal > 0.5 mM. Effect only observed in Separation Matrix II.	No effect	180/230 kDa runs as multiple peaks. Can be correctly identified < 0.5mM. Effect only observed in Separation Matrix II.	MW sizing affected if 180/230 kDa peak not correctly identified.

Table A-4: *Reducing agent compatibility.*

Denaturing Agents

Agent	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
Urea	0-8 M	0- 1 M	Signal decrease in high MW region > 4 M.	No effect	Resolution loss and migration shift. 180/230 kDa standard runs as multiple peaks > 1 M. Stronger effect in Separation Matrix I.	Affects sizing due to 180/230 kDa standard. Manual peak assignment may be required. Prepare biotinylated ladder with the same sample buffer.
Urea/ Thiourea	1 M Urea/ 0.2 M Thiourea 5 M Urea/ 1 mM Thiourea	≤ 1 M Urea/ 0.2 M Thiourea	Signal decrease in high MW region ≥ 2M Urea/ 0.4 M Thiourea.	Resolution loss ≥ 2M Urea/ 0.4 M Thiourea.	Resolution loss and migration shift. 180/230 kDa standard runs as multiple peaks > 1 M. Stronger effect in Separation Matrix I.	Affects sizing due to 180/230 kDa standard. Manual peak assignment may be required. Prepare biotinylated ladder with the same sample buffer.

Table A-5: *Reducing agent compatibility.*

Detergents

Detergent	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
Triton X-100	0-2%	0-0.25%	Signal loss > 0.5%. Stronger effect in Separation Matrix II.	Slight resolution loss ≥ 1%.	Resolution loss and migration shift > 0.25%. Stronger effect in Separation Matrix II.	Affects sizing at HMW > 0.5% due to loss of resolution for 180/230 kDa standard.
NP40	0-2%	0-0.5%	Signal loss > 0.5%. Stronger effect in Separation Matrix II.	Resolution loss > 1%.	Resolution loss and migration shift ≥ 1%.	Affects sizing > 0.5%.
Igepal CA 630	0-1%	0-0.1%	No effect	No effect	1 kDa standard resolution loss and migration shift.	Affects sizing for small proteins because 1 kDa standard shift. Add to biotinylated ladder for reproducible sizing.
C7BZO	0-2%	0-0.5%	Signal loss > 1%.	Resolution loss > 1%.	180/230 kDa standard signal loss > 1%. 1 kDa standard shoulder visible.	Affects sizing at high MW > 0.5% due to 180/230 kDa standard resolution loss.
CHAPS	0.6-2%	0.6-2%	No effect	No effect	Resolution loss and migration delay of standards.	Prepare biotinylated ladder in same sample buffer.
SDS	1-2%	1-2%	No effect	No effect	1 kDa standard splits ≥ 2%.	Affects sizing if 1 kDa inconsistently identified due to peak splitting. Manual assignment may be required.

Detergent	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
Sodium Deoxycholate	0-1.2%	0-0.6%	Signal decrease ≥ 0.6%.	No effect	1 kDa standard splits or smears > 0.6%.	Affects sizing due to 1 kDa standard smear.
LDS	0-2%	0-0.5%	Signal decrease ≥ 0.5%.	Resolution loss ≥ 0.5%.	1kD standard splits ≥ 2%.	Affects sizing if 1 kDa inconsistently identified due to peak splitting. Manual assignment may be required.

Table A-6: *Detergent compatibility.*

Fixative

Fixative	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
Formaldehyde	0-2%	0-0.1%	Signal decrease ≥ 0.1%.	Resolution loss ≥ 0.1%.	Resolution loss and migration shift > 0.1%.	Affects sizing due to resolution loss and migration shift of standards > 0.1%.

Table A-7: *Fixative compatibility.*

Viscosity/Density Additives

Additive	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
Glycerol	0-20%	0-20%	No effect	No effect	No effect	No effect
Sucrose	0-300 mM	0-300 mM	No effect	No effect	No effect	No effect
PEG MW 20,000	0-5%	0-0.05%	Signal loss ≥ 1%.	Resolution loss ≥ 1%.	Complete resolution loss ≥ 1%.	Affects sizing due to standards resolution loss. Affect minimized at ≤ 0.05%.
PEG MW 4400-5000	0-10%	0-2.5%	Significant signal loss ≥ 2.5%.	Resolution loss ≥ 2.5%.	Resolution loss and migration shift > 2.5%.	Affects sizing due to standards resolution loss and migration shift > 0.25%.

Table A-8: *Viscosity and density additive compatibility.*

Miscellaneous Reagents

Fixative	Test Range	Compatible Range	Chemi Signal	Chemi Resolution	Fluorescent Signal	MW Sizing
EDTA	0-40 mM	No effect	No effect	No effect	No effect	No effect
Imidazole	0-100 mM	0-100 mM	No effect	No effect	180/230 kDa standard migrates slower.	Prepare biotinylated ladder in same sample buffer.

Table A-9: *Miscellaneous reagent compatibility.*

Simple Western Commercial Lysis Buffer Compatibility

All results compared against ProteinSimple Bicine/CHAPS lysis buffer.

Buffer	Usage	Vendor & P/N	Range Tested	Chemi Signal	Chemi Resolution	Fluorescent Standards/MW Sizing	Recommendations
I-PER*	Insect cells	Pierce #89802	50-100%	No effect	No effect	No effect	Samples can be used undiluted.
Syn-PER*	Synaptic proteins	Pierce #87796	50-100%	No effect	No effect	No effect	Samples can be used undiluted.
NE-PER* (cytoplasmic)	Nuclear & cytoplasmic proteins	Pierce #78833	50-100%	No effect	No effect	No effect	Samples can be used undiluted.
NE-PER* (nuclear)	Nuclear & cytoplasmic proteins	Pierce #78833	50-100%	No effect	No effect	No effect	Samples can be used undiluted.
MEM-PER*	Membrane proteins	Pierce #89826	3-100%	Signal decrease > 25%.	No effect	1 kda standard splits > 25%.	May require manual assignment of fluorescent standards. For best signal and resolution, dilute 1:2 in 0.1X sample buffer.
T-PER	Tissue proteins	Pierce #78510	50-100%	Slight signal decrease ≥ 50%.	No effect	No effect	For best signal, dilute 1:2 in 0.1X sample buffer.
N-PER*	Neuronal proteins	Pierce #87792	50-100%	Slight signal decrease ≥ 50%.	No effect	No effect	For best signal, dilute 1:2 in 0.1X sample buffer.
M-PER*	Mammalian cells	Pierce #78501	50-100%	No effect	No effect	No effect	Samples can be used undiluted.
Y-PER*	Yeast protein	Pierce #78991	50-100%	Signal decrease ≥ 50%.	Resolution loss ≥ 50%.	180/230 kDa standard resolution loss ≥ 50%.	For best signal & resolution, dilute 1:2 in 0.1X sample buffer.
B-PER*	Bacterial cells	Pierce #78243	50-100%	No effect	No effect	No effect	Samples can be used undiluted.
RIPA*	General whole cell lysis buffer	Thermo-Scientific #89900	50-100%	No effect	No effect	Slight resolution loss for 180/230 kDa > 50%.	For best resolution, dilute 1:2 in 0.1X sample buffer.

Buffer	Usage	Vendor & P/N	Range Tested	Chemi Signal	Chemi Resolution	Fluorescent Standards/MW Sizing	Recommendations
Laemmli*	General whole cell lysis buffer	BioRad #161-0737	3-100%	No effect	No effect	1 kDa standard splits > 12.5%. Non-linear compression causes peaks to shift > 50%.	For best resolution and MW sizing, dilute 1:8 in 0.1X sample buffer. Prepare biotinylated ladder in same sample buffer for consistent sizing.
ACK*	General whole cell lysis buffer	Life Technologies #A10492-01	50-100%	No effect	No effect	No effect	Samples can be used undiluted.
NuPage*	General whole cell lysis buffer	Invitrogen #NP0007	3-100%	Signal decrease > 25%.	Resolution loss > 25%.	1 kDa standard splits > 25%.	For best signal and resolution, dilute 1:4 in 0.1X sample buffer.
RIPA	General whole cell lysis buffer	Cell Signaling #9806S	0-100%	Slight signal decrease at high MW region.	No effect	180/230 kDa signal decrease. Non-linear compression causing 1 kDa and 29 kDa standards to run closer.	For best signal and resolution, dilute 1:2 in 0.1X sample buffer. Prepare biotinylated ladder in same sample buffer for consistent sizing.
Cell Lysis Buffer	General whole cell lysis buffer	Cell Signaling #9803S	0-100%	Slight signal decrease at high MW region.	No effect	180/230 kDa signal decrease. Non-linear compression causing 1 kDa and 29 kDa standards to run closer.	For best signal and resolution, dilute 1:2 in 0.1X sample buffer. Prepare biotinylated ladder in same sample buffer for consistent sizing.
SDS Lysis Buffer	General whole cell lysis buffer	Millipore #20-163	0-100%	No effect	No effect	Significant signal decrease for 1 kDa standard and registration due to peak splitting.	For best signal and resolution, dilute 1:2 in 0.1X sample buffer. Prepare biotinylated ladder in same sample buffer for consistent sizing.
IP Lysis Buffer	General whole cell lysis buffer	Pierce #87787	0-100%	Slight signal decrease at high MW region.	No effect	Non-linear compression causing 1 kDa and 29 kDa standards to run closer.	For best signal and resolution, dilute 1:2 in 0.1X sample buffer. Prepare biotinylated ladder in same sample buffer for consistent sizing.

Buffer	Usage	Vendor & P/N	Range Tested	Chemi Signal	Chemi Resolution	Fluorescent Standards/MW Sizing	Recommendations
Cellytic MT	General whole cell lysis buffer	Sigma #C3228	0-100%	No effect	No effect	Improved fluorescent standard signal and resolution.	Samples can be used undiluted.
Various Commercial Lysates	N/A	Cell Signaling (various lysis buffers)	0-100%	N/A	N/A	Signal decrease of all fluorescent standards, especially 1 kDa.	May require manual assignment of fluorescent standards. For best signal and resolution, dilute 1:2 in 0.1X sample buffer. Prepare biotinylated ladder in same sample buffer for consistent sizing.

**Only tested in Separation Matrix II.*

Table A-10: *Lysis buffer compatibility.*

Appendix B:

Sample-Specific Lysis Protocols

Appendix Overview

- Cell Lines — Guidelines and Preparation
- Tissue Homogenization Protocol
- Fine Needle Aspirate (FNA) — Guidelines and Preparation
- Peripheral Blood Mononuclear Cells (PBMC) - Guidelines and Preparation

Cell Lines — Guidelines and Preparation

We offer two cell lysis kits that contain all the reagents necessary to lyse cells. See Chapter 3, *"Sample Preparation Optimization"* for buffer selection considerations.

Kit Components

- Bicine/CHAPS or RIPA Lysis Buffer, 10 mL
- Cell Wash Buffer, 100 mL
- 50X DMSO inhibitor Mix, 200 µL
- 25X Aqueous Inhibitor Mix, 400 µL

Additional Consumables/Equipment Needed

- Microfuge tubes
- Cell scrapers
- Pipettes and tips
- Ice bucket
- Liquid nitrogen or dry ice
- Microfuge centrifuge
- Vortexer

Important Information

- All steps should be performed on ice or at 4 ℃
- Make sure Cell Wash Buffer is ice-cold
- Pre-chill microfuge tubes on ice

Lysis Buffer Preparation

NOTE: Make lysis buffer right before lysing cells by adding DMSO and Aqueous Inhibitors to Bicine/CHAPS or RIPA Buffer.

- Dilute DMSO inhibitor 1:50 into lysis buffer.
- Dilute Aqueous Inhibitor 1:25 into lysis buffer
- Ideally, lysate total protein concentration should be ≥ 3 mg/mL

Recommended Volumes of Lysis Buffer

Number of Cells	Lysis Buffer Volume
Less than 10^5	10-20 µL
10^5-10^6	20-50 µL
More than 10^6	50-100 µL

Table B-1: *Lysis buffer volumes per number of cells. Optimized volumes will be cell size dependent.*

Procedure for Lysis of Adherent Cells

Procedure for 10cm Dish

1. Wash cells 2X with ice-cold Cell Wash Buffer.
2. After the second wash, tilt the dish to aspirate the wash buffer completely from plate.
3. Add 400-500 µL of ice-cold Lysis Buffer to plate, and swirl around to ensure good coverage.
4. Incubate 5 minutes.
5. Scrape cells off plate with a cell scraper and transfer to pre-chilled microfuge tube.
6. Pipette 5X up and down.
7. Incubate 30 minutes, vortexing briefly every 5 minutes.
8. Centrifuge for 15 minutes at 14,000 x g, 4 ℃.
9. Immediately transfer supernatant to a clean microfuge tube and aliquot.

10. Snap-freeze aliquots on dry ice or in liquid nitrogen.
11. Store at -80 ℃.

Procedure for Lysis of Suspension Cells

1. Collect cells by centrifugation for 5 minutes at 500 x g.
2. Wash cells 2X by resuspending in ice-cold Cell Wash Buffer and centrifuging for 5 minutes at 500 x g each time.
3. Completely remove supernatant by aspiration.
4. Add ice-cold Lysis Buffer.
5. Incubate 5 minutes.
6. Pipette 5X up and down.
7. Incubate 30 minutes, vortexing briefly every 5 minutes.
8. Centrifuge for 15 minutes at 14,000 x g, 4 ℃.
9. Immediately transfer supernatant to a clean microfuge tube and aliquot.
10. Snap-freeze aliquots on dry ice or in liquid nitrogen.
11. Store at -80 ℃.

Tissue Homogenization Protocol

Recommended Materials

- Homogenizer (VWR or equivalent)
- Pestles/1.5 mL microtube combo (VWR 47747-366)
- Dry ice or liquid nitrogen
- Hammer

Important Information

- This protocol is intended for use with fresh tissue samples
- Minimize time the samples are not on dry ice

Protocol

1. Upon sample collection, remove blood vessels and connective tissue.
2. Keep tissue frozen on dry ice.
3. Determine tissue weight:
   a. Weigh empty vial.
   b. Chill the vial on ice.
   c. Place tissue in the weighed vial using a spatula that has been pre-chilled using dry ice or liquid nitrogen.
   d. Weigh sample + vial.

NOTE: Minimize the time the sample is not on dry ice.

---

4. Prepare tissue samples.

   For tissues pieces > 20 mg:

   a. Place tissue in an aluminum pouch.
   b. Immerse the entire pouch in liquid nitrogen.
   c. Pulverize the tissue with hammer on a metal plate pre-chilled on dry ice.
   d. Transfer tissue powder into a microcentrifuge tube.

   For tissue pieces 5-20 mg:

a. Proceed directly to the next step (sample lysis, step 5).

5. Homogenize the sample.

NOTE: Sample processing should be performed in a 1.5 mL tube.

Add 5-10X µL ice cold lysis buffer of choice containing freshly added phosphatase and protease inhibitors/mg tissue (for example if the tissue sample is 5 mg, add 20-25 µL ice cold lysis buffer).

- The exact volume of Lysis Buffer used will depend on the tissue type.
- For ideal homogenization efficiency with a hand held pestle system, the lysis buffer volume should not exceed 100 µL in a 1.5 mL microcentrifuge tube.

6. Grind tissue with dedicated pestle until sample homogeneity is reached.
   - Periodically place the sample on dry ice to maintain low temperature.
7. Lyse samples for another 20 minutes.
   - Leave tubes on ice and gently flick the tubes every 5 minutes OR place samples on a rotator at 4 ℃.
8. Spin samples at 16,000 x g in microcentrifuge for 20 minutes at 4 ℃.
9. Transfer supernatant to a fresh, chilled microcentrifuge tube.
10. Determine lysate concentration using an assay like Bradford or BCA.
11. Flash freeze sample on dry ice or liquid nitrogen.
    - Optional/recommended: aliquot lysates prior to flash freezing.
12. Store samples at -80 ℃ until ready to use.

Fine Needle Aspirate (FNA) — Guidelines and Preparation

This FNA procedure was provided by 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 lumps or masses where a thin, hollow needle is inserted into the mass to extract cells. FNAs are minimally invasive, but produce a comparatively low cell number. Simple Western technology allows protein analysis in these small FNA samples.

Sample Procurement and Processing

Actual animal protocols and human subjects protocols for 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 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. Empty contents of the syringe into 4 mL of cold PBS immediately after acquiring aspirate material.

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 processing of the sample for research. During this time, it is important to keep the cell suspension strictly on ice. Addition of phosphatase or protease inhibitors to the RPMI may further improve sample stability, however investigators 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 successful lysis.

The FNA preparation procedure was optimized to achieve sample protein concentrations of 2-5 mg/mL, approximately equivalent to lysing 1 million cells in 20 µL of lysis buffer.

Hemoglobin Contamination

The iron in hemoglobin acts as a catalyst for the chemiluminescence reaction that causes luminol to glow. Blood contamination of samples may result in an antibody-independent, luminol-dependent hemoglobin peak.

There are several commercially available methods to lyse red blood cells and remove the hemoglobin from a sample of interest. One of the most common methods used for clinical specimens is treatment of samples with PharmLyse™ (BD Biosciences, 555899). While PharmLyse is compatible with the Simple Western Size Assay and does remove the hemoglobin peak, it does significantly increase the variability between samples. We recommend you remove the red blood cells only when the hemoglobin peaks resolve in the same region as you protein of interest.

Effect of Residual Cell Media on Simple Western Size Assay Performance

After sample collection, spin down cells and remove the RPMI (Cellgro RPMI 1640, 10-041-CV). Take care to completely remove the RPMI from the sample to avoid variable salt content between samples — 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. It also eliminates variability associated with the sample lysis since samples can be lysed simultaneously and batch analyzed regardless of their collection date.

Tissue Origin

Also consider the specimen that is collected for successful sample preparation. For example, FNA samples drawn from lung tissue often have a very viscous supernatant after spinning the cells down, 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.

FNA Preparation Procedures

Sample Procurement and Freezing

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

1. Investigator (for preclinical specimens) or cytopathologist (for clinical specimens) should perform at least 10 passes through the tissue and collect 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.

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

---

3. Divide the FNA suspension equally into three 1.5 mL tubes. Perform the optional PharmLyse step here if required using the protocol that follows.
4. Spin 1.5 mL tubes at 5000 x 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 Buffer

EXAMPLE: 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 x g for 10 minutes at 4 °C.
7. Collect supernatant with P200 filter tip. This is the lysate.
8. Aliquot 5-10 µL into individual microfuge tubes and snap freeze in liquid nitrogen. Store at -80 °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 x g for 5 minutes at 4 °C.
3. Discard supernatant as described in step 5 of "Sample Procurement and Processing" on page 81.
4. Resuspend cells in 500 µL of 1X PharmLyse.
5. Incubate for 10 minutes at room temperature.
6. Add 750 µL sterile PBS.
7. Spin at 5000 x g for 5 minutes at 4 °C.
8. Remove supernatant and follow protocol from step 6 of "Sample Procurement and Processing" on page 81.

References

Human FNA

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

Mouse FNA

4. Assessment of gefitinib- and CI-1040-mediated changes in epidermal growth factor receptor signaling in HuCCT-1 human cholangiocarcinoma by serial fine needle aspiration, M Hidalgo, M L Amador, A Jimeno, H Mezzadra, P Patel, A Chan, M E Nielsen, A Maitra, and S Altiok, *Mol Cancer Ther.*, Jul 2006; 5(7): 1895-903.

5. Evaluation of the novel mitotic modulator ON 01910.Na in pancreatic cancer and preclinical development of an ex vivo predictive assay, A Jimeno, A Chan, G Cusatis, X Zhang, J Wheelhouse, A Solomon, F Chan, M Zhao, S C Cosenza, M V Ramana Reddy, M A Rudek, P Kulesza, R C Donehower, E P Reddy, and M Hidalgo, *Oncogene*, Jan 2009; 28(4): 610-8.

Peripheral Blood Mononuclear Cells (PBMC) - Guidelines and Preparation

This PBMC procedure was provided by Fernando Shahijanian and Dr. Holden Maecker, Stanford University, Human Immune Monitoring Center, Palo Alto, CA.

PBMC Preparation Procedures

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. Add Ficoll (GE Healthcare, 17-1440-02). 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 x 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.

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.

---

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 x 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, count cells. 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.

NOTE: It is recommended to store vials at -80 °C for 24 hours prior to transferring them to a liquid nitrogen freezer for long-term storage.

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 x 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 x g.
9. Aspirate the supernatant from the cells and resuspend in 1 mL PBS.
10. If needed, count cells using a Vi-CELL® 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.

NOTE: 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.

Lysis

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 Buffer

EXAMPLE: 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 of ice cold Lysis Buffer.

---

2. Resuspend cells in 1 mL of PBS.
3. Centrifuge cells in PBS for approximately one minute at 500 x g.
4. Aspirate PBS supernatant and add 30-50 µL of Bicine/CHAPS Lysis Buffer containing inhibitors to each sample.

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 x 10^5-10 x 10^6 cells.

---

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 x g for 10 minutes at 4 °C.
7. Transfer supernatant to fresh tubes for flash freezing in liquid nitrogen or dry ice. Aliquots of 5-10 µL 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.