User Guide for Milo

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User Guide for Milo

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ProteinSimple's Single-Cell Western technology is covered by issued and pending patents in the U.S. and other countries. For more information please see www.proteinsimple.com/Intellectual_Property.html.

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Chapter 1:

Let's Get Started

Chapter Overview

- Welcome
- Single-Cell Western Assays on Milo
- Overview of Single-Cell Western Protocol

Welcome

Congratulations on bringing Milo into your lab! We welcome you as a new user and are excited to be a part of your work as you discover new biological insights and truly understand the uniqueness of each single-cell within your complex samples.

To help you get the most from Milo, we've added some attention phrases to guide you through the user guide:

NOTE	Points out useful information
IMPORTANT	Indicates information necessary for proper operation of Milo
CAUTION	Cautions you about potentially hazardous situations that could result in injury to you or damage to Milo
	Indicates information that can give you confidence that your assay is running correctly

Single-Cell Western Assays on Milo

This user guide will provide you with the information you need to partition cells from a single-cell suspension into individual microwells on a scWest chip and to perform Single-Cell Western (scWestern) analysis on isolated cells. The user guide explains all steps needed including: single-cell capture, cell imaging for confirmation of cell isolation, cell lysis, SDS-PAGE on individual cell lysates, photo-immobilization of separated protein bands, antibody probing, target protein imaging, and archiving of scWest chips.

A Single-Cell Western assay is a multiplexed western blot on ~1,000+ single-cells in parallel. Single-Cell Western assays measure protein expression in each analyzed single-cell.

Single-Cell Western assays take place on an scWest chip. Milo automates the key assay steps required to run an scWest chip including cell lysis, SDS-PAGE separation of each single-cell lysate and immobilization of separated protein bands. One sample is loaded per scWest chip which yields 1,000+ Single-Cell Westerns per chip.

The scWest chips described in this protocol use a modified photoactive polyacrylamide gel that covalently binds protein bands into the gel after electrophoretic separation, eliminating the protein transfer and immobilization step of conventional blotting procedures. This technique allows users to directly measure protein expression with single-cell resolution rather than having to rely on RNA-based measurements to study gene expression. The technique is compatible with commercially available antibodies and is well suited for analysis of intracellular protein targets that cannot be easily studied with flow cytometry. In addition to providing protein expression information at single-cell resolution to understand cell subpopulations, the Single-Cell Western workflow also saves time over conventional Western blotting techniques. The scWest chips can also be archived for future re-probing of precious cellular samples.

Overview of Single-Cell Western Protocol

Steps		Time
Cell and reagent preparation		
1.	Dilute 10X Suspension Buffer to 1X Suspension Buffer Rehydrate scWest chip in 1X Suspension Buffer (gel face up)	10+ minutes (may be performed during cell preparation)
2.	Dilute 5X Wash Buffer to 1X Wash Buffer	5 minutes
3.	Create single-cell suspension in 1X Suspension Buffer	15–30 minutes
4.	Dilute cells to 10,000-100,000 cells/mL in 1X Suspension Buffer	5 minutes
Cell loading and QC		
5.	Pipette cells onto scWest chip (gel face up)	1 minute
6.	Let cells settle	5–20 minutes
7.	Wash 1-3X with 1 mL 1X Suspension Buffer	5 minutes
8.	Inspect scWest chip on brightfield microscope to confirm cell isolation (gel face up)	5 minutes
Electrophoresis and photo-immobilization		
9.	Enter desired run parameters into Milo	1 minute
10.	Place scWest chip into Milo (gel face up)	1 minute
11.	Pour Lysis/Run Buffer into buffer reservoir	
12.	Close lid and run cell lysis, separation, and protein capture	5–6 minutes
Antibody probing		
13.	Remove chip and place in Petri dish (gel face up); quick-wash with 15 mL of 1X Wash Buffer	1 minute
14.	Add fresh 1X Wash Buffer, wash on shaker	2x10 minutes
15.	Prepare primary antibody solution	5 minutes
16.	Add primary antibodies to scWest chip (gel face down) using probing chamber	1 minute
17.	Incubate primary antibodies (gel face down)	1–2 hours*
18.	Remove chip and place in Petri dish (gel face up) with 15 mL 1X Wash Buffer	1 minute

"Overview of Single-Cell Western Protocol", *continued.*

	Steps	Time
19.	Add fresh 1X Wash Buffer, wash on shaker	3x10 minutes
20.	Prepare secondary antibody solution	5 minutes
21.	Add secondary antibodies to scWest chip (gel face down) using probing chamber	1 minute
22.	Incubate secondary antibodies (gel face down)	30–60 minutes*
23.	Remove chip and place in Petri dish (gel face up) with 15 mL 1X Wash Buffer	1 minute
24.	Add fresh 1X Wash Buffer, wash on shaker	3x15 minutes
25	Rinse chip with DI water to remove salts	1 minute
26	Dry slide using microarray slide spinner	3–5 minutes
27.	Scan on microarray scanner (gel orientation depends on scanner)	8–35 minutes
Total		**~4–6.5 hours**

*Antibody incubation concentration/times should be optimized by the user.

Chapter 2:
What You'll Need

Chapter Overview

- Required Consumables and Reagents
- How to Choose scWest Chips Based on Cell Size
- Required Equipment
- Compatible Microarray Scanners
- Recommended Consumables & Equipment

Required Consumables and Reagents

Product Name	Company	Part Number	Details
scWest Kits • 10X Suspension Buffer • 5X Wash Buffer • Antibody Diluent 2 • Lysis/Run Buffer • 8 scWest chips	ProteinSimple	• K500 • K600 • K700 • K800	• Small scWest kit for small cells • Standard scWest kit • Large scWest kit for large cells • scWest calibration kit for unknown cell diameter
Primary antibodies	Various		
Fluorescently-conjugated secondary antibodies	Various		
Trypsin or other dissociation agent	Various		
10-cm Petri dishes (2 per scWest chip)	Various		
15-cm Petri dishes (1 per scWest chip)	Various		

Table 2-1: *Required consumables and reagents.*

How to Choose scWest Chips Based on Cell Size

Each scWest kit contains enough reagents for 8 scWest chip runs. One sample can be run on each scWest chip.

The K600 kit chips are appropriate for most cell types including HeLa, 293T, CHOK1, MCF-7, and other epithelial lines. The K500 kit is suitable for small cells, such as lymphocytes and other immune cells, while the K700 kit is suitable for larger than average cells. If the cells are of unknown diameter, calibration chips in the K800 kit are recommended to establish an optimal well diameter for your sample.

Cell Type	Diameter (µm)	scWest Chip
Dendritic Cell	7	Small
Lymphocyte	7	
Neutrophil	8	
Monocyte	9	
HT29	11	Standard
Jurkat	12	
PC12	12	
HEK293	13	
U87	13	
COLO-205	13	
CHO	14	
HUVEC	15	
A431	16	
K562	17	
Hela	18	
HepG2/C3A	18	
NIH/3T3	18	
SF-21	18	
U2OS	20	
Aveolar Macrophage	21	Large
COS-7	25	

Table 2-2: *Choosing scWest chips based on cell sizes.*

Required Equipment

- Milo
- Antibody incubation fixture and sponges (included with Milo, reorder PN A200) or Three-Plex Antibody Probing Fixture (included with Milo, reorder PN A300)
- Metal tweezers for scWest chip handling (included with Milo)
- Benchtop vortexer
- Benchtop shaker
- 500 mL beaker/bottle for preparing 1X Suspension Buffer
- 500 mL beaker/bottle for preparing 1X Wash Buffer
- Inverted brightfield microscope capable of 10X magnification
- Microarray scanner (see list of compatible scanners on page 8)
- Computer for data analysis using Scout software (Windows 7, Windows 8, Windows 10, or Mac OSX with 16 GB RAM or higher)
- Scout Software (download at proteinsimple.com/scout/downloads/)

Compatible Microarray Scanners

Please visit our website for a current list of third party microarray scanners that are compatible for scanning scWest chips.

Recommended Consumables & Equipment

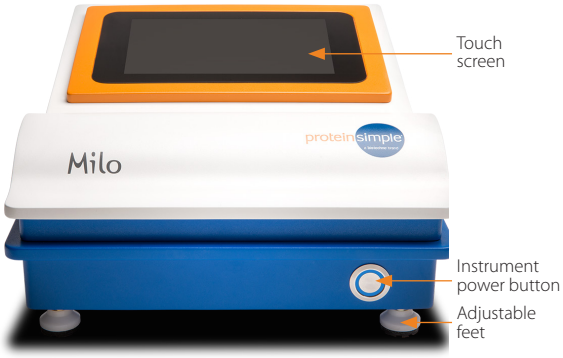
- Aluminum foil
- Microarray slide spinner (various suppliers)

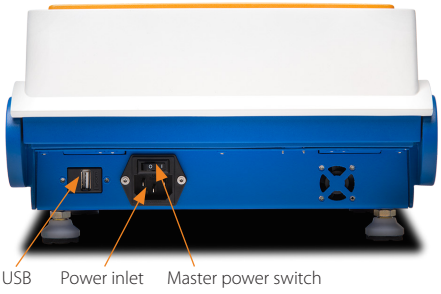
Chapter 3:
Milo

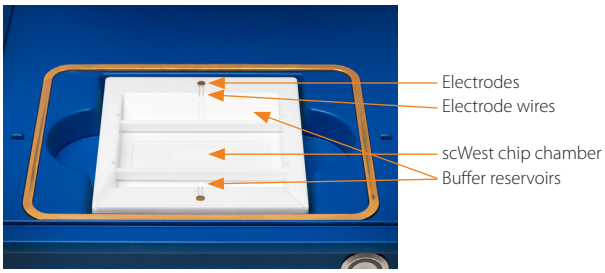
Chapter Overview

- Milo System Components
- Installation Requirements
- System Label
- Powering Milo On and Off
- Electrical Specifications
- Environmental Specifications
- Lamp Specifications

Milo System Components







Installation Requirements

Install Milo on a standard laboratory bench surface away from equipment that may create excessive vibration or shaking (e.g. large centrifuge). The bench surface must be flat and stable enough to accommodate leveling of Milo to +/- 0.2 degrees using the instrument's adjustable feet and internal electronic level.

System Label

A system label is also located on Milo's bottom panel. It includes the ProteinSimple location, system model, power requirements, serial number and certification markings.

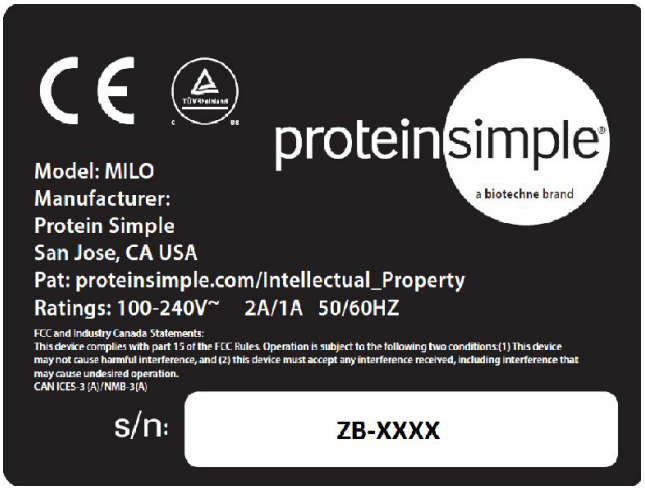


Figure 3-1: *Milo system label.*

Powering Milo On and Off

To avoid damaging Milo when powering on and off, please follow the guidelines below:

To turn on the instrument:

1 Make sure the power switch on the back of the instrument and the round power button at the front of the instrument are both in the off position.

2 Plug in the power cable.

3 Flip the power switch on the back of the instrument to ON.

4 Press the power button on the front of the instrument so that it is illuminated.

Avoid plugging in the power cord while the power switch on the back of the instrument is in the ON position. This can cause damage to the electronics or corrupt the software.

To turn off the instrument:

1 Press the power button on the front of the instrument to OFF so that it is no longer illuminated. The power switch on the back of the instrument can remain in the ON position as long as the instrument is still plugged in.

To unplug the instrument:

1 Press the power button on the front of the instrument to OFF so that it is no longer illuminated.

2 Flip the power switch on the back of the instrument to OFF.

3 Unplug the power cord.

Unplugging the instrument while the power switch on the back of the instrument is still ON can cause damage to the electronics or corrupt the software.

Physical Specifications

Description	Specification
Depth	13.25 in (34 cm), allow ~18 in (~46 cm) total for ventilation and cable clearance at rear
Width	11 in (28 cm)
Height (lid closed)	6.5 in (17 cm)
Height (lid open)	12 in (31 cm)
Weight	<12 lb (<5.5 kg)

Table 3-4: *Milo dimensions and weight.*

Electrical Specifications

Description	Specification
Power	100-240 VAC input, 50/60 Hz 2A/1A Maximum rated input current
HV circuit rating	300 V, 2.5 A max

Table 3-5: *Milo voltage and current specifications.*

Environmental Specifications

Description	Specification
Operating temperature range	10–30 °C
Operating humidity range	10–90% RH (non condensing)
Pollution degree	2
Altitude	2000 m

Table 3-6: *Milo environmental specifications.*

Lamp Specifications

Description	Specification
UV Rating	240–400 nm, < 20 mW/cm^2

Table 3-7: *Milo lamp specifications.*

Chapter 4:

Experimental Setup

Chapter Overview

- How to Set Up Your Experiment
- Antibody Selection Guidelines
- Antibody Diluent Selection Guidelines
- Milo Three-Plex Probing Fixture for Antibody Screening

How to Set Up Your Experiment

One sample should be run per scWest chip. Triplicate chips can be run to increase the number of cells analyzed and ensure repeatability of quantitation results.

Antibody Selection Guidelines

Unlabeled primary antibodies (IgG isotype recommended) should be selected against each target of interest. For targets that have less than a 50 kDa molecular weight difference, primary antibodies must be from distinct host species (e.g., rabbit and mouse). For targets that have greater than a 50 kDa molecular weight difference, primary antibodies from the same host species (e.g., both rabbit) may be used. However, if two primary antibodies from the same host species are used, it is recommended to probe sequentially.

Secondary antibodies against each primary antibody host species should be chosen to have spectrally-distinct fluorescent dyes that match the laser and filter specifications of the microarray scanner that will be used to image the scWest chip.

Example antibody/dye configuration for a two-target experiment:

- **Primary antibodies:** Rabbit anti-Target 1 polyclonal antibody, mouse anti-Target 2 monoclonal antibody
- **Secondary antibodies:** Donkey anti-rabbit IgG conjugated to Cyanine 3, donkey anti-mouse IgG conjugated to Cyanine 5
- **Readout:** Using a two-laser scanner capable of Cyanine 3/Cyanine 5 imaging

Antibody Diluent Selection Guidelines

If goat or sheep primary antibodies will be used in any probing cycle, dilute the primary and secondary antibodies in Milk-Free Antibody Diluent (P/N 043-524). If goat or sheep primary antibodies will not be used, dilute all antibodies in Antibody Diluent 2 (P/N 042-203). scWest kits ship with both Antibody Diluent 2 (P/N 042-203) and Milk-Free Antibody Diluent (P/N 043-524). If the incorrect antibody diluent is used, non-specific antibody binding may be observed.

Milo Three-Plex Probing Fixture for Antibody Screening

A Three-Plex Antibody Probing Fixture can be used to speed up your assay development time by screening multiple antibodies against targets on a single scWest chip. The Three-Plex Probing Fixture allows for three different antibody cocktails to be applied to three different regions of the chip simultaneously. Test up to 12 antibodies simultaneously (using 4 colors and 3 regions) or test different concentrations for the same antibody—all on the same chip. For more information on how to use the Three-Plex Antibody Probing Fixture, please refer to the Milo Three-Plex Antibody Probing Fixture Quick Reference Guide located at proteinsimple.com/milo.

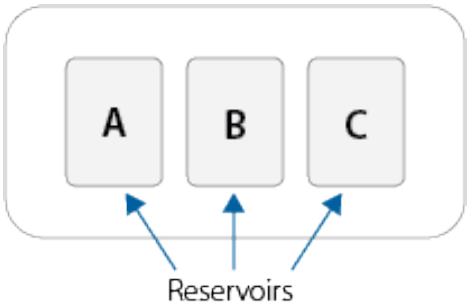


Figure 4-1: *Three-Plex Probing Fixture, containing three reservoirs where different antibody solutions can be applied.*

Chapter 5:

Single-Cell Western Protocol

Chapter Overview

- Single-Cell Western Workflow
- Cell and Reagent Preparation
- Cell Loading and Quality Control
- Loading and Running Milo
- Antibody Probing
- Imaging scWest Chips

Single-Cell Western Workflow

The following step-by-step instructions summarize the basic steps needed to perform a Single-Cell Western with Milo (Figure 5-1).

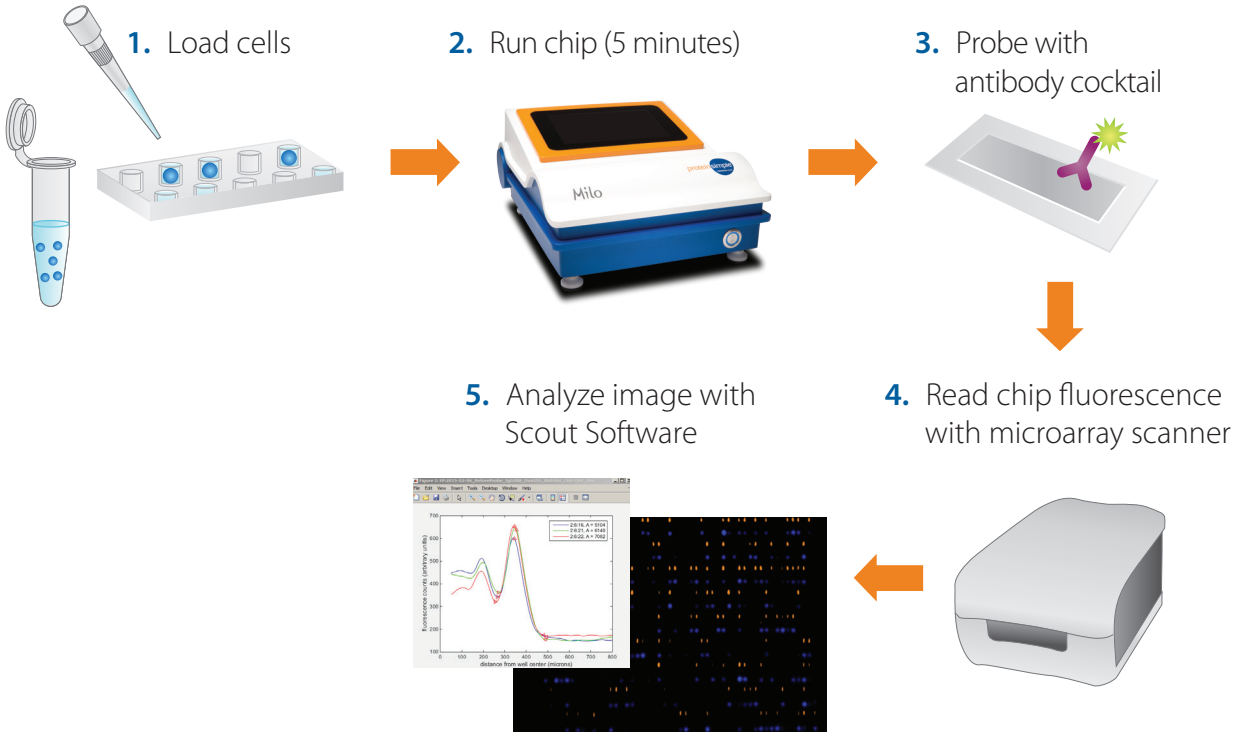


Figure 5-1: *Add 1 mL of a single-cell suspension onto an scWest chip. Individual cells settle into microwells patterned into the pre-cast polyacrylamide gel. Milo lyses the cells, does rapid (~1 min) SDS-PAGE on each single-cell lysate and immobilizes the proteins in the gel. Probe with conventional antibodies in the probing fixture and image chip fluorescence. Scout Software analyzes images to extract data.*

Cell and Reagent Preparation

A. Rehydrating scWest Chips

1 Determine how many scWest chips are needed for the experiment. One scWest chip is required for each sample to be analyzed.

NOTE: *scWest chips are currently available in four formats as listed in "Table 2-1: Required consumables and reagents." on page 6, If the cells are of unknown diameter, use the scWest calibration chip to determine the optimal microwell diameter. See the scWest Calibration Chip Quick Reference Guide for more information.*

2 Remove the correct number of scWest chips from the cannister and place each in a separate clean Petri dish with the gel facing up. If the logo is on the left and the barcode on the right, the gel is facing up (Figure 5-2).The gel also faces upwards when the "ProteinSimple" logo and barcode are legible. Take care not to touch the gel surface of the chip. Powder-free gloves are recommended. If using nitrile or powdered gloves, wash gloved hands with soap and water before handling chips to minimize chip fouling.

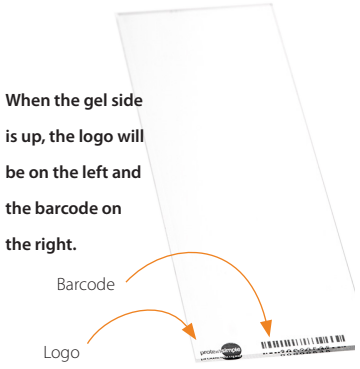


Figure 5-2: *scWest gel orientation.*

3 Prepare 1X Suspension Buffer by adding 3 mL 10X Suspension Buffer to 27 mL DI water (30 mL total volume needed per chip).

4 Add 15 mL of 1X Suspension Buffer into each Petri dish to cover each scWest chip (Figure 5-3). Equilibrate all chips in 1X Suspension Buffer for >10 minutes (up to a maximum of 4 hours) at room temperature before use. This can be done prior to the single-cell suspension preparation, so that the chips are rehydrated by the time the single-cell suspension is prepared.

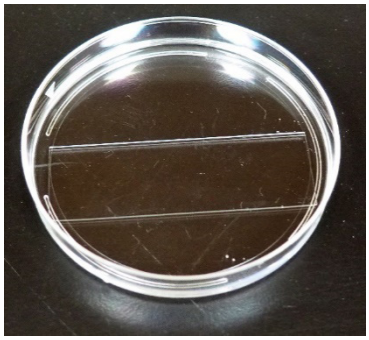


Figure 5-3: *scWest chip rehydrating in 15 mL of 1X Suspension Buffer.*

B. Preparing Wash Buffer

1 Prepare 1X Wash Buffer by adding 40 mL of 10X Wash Buffer to 160 mL DI water for a final volume of 200 mL. Excess 1X Wash Buffer can be stored at room temperature for future uses.

C. Preparing Cell Samples

1 Create a single-cell suspension using standard methods.

2 Centrifuge and wash the cell pellet with 5 mL 1X Suspension Buffer.

3 Centrifuge and re-suspend the cells in 1 mL 1X Suspension Buffer.

4 Count the cells using a hemocytometer and dilute to 100,000 cells/mL in 1X Suspension Buffer. Each scWest chip requires 1 mL of cell suspension containing 10,000–100,000 cells/mL.

Cell Loading and Quality Control

D. Loading Cells on the scWest Chip

1 Carefully aspirate the 1X Suspension Buffer from the Petri dish in which the scWest chip has been rehydrating.

NOTES:

Tilt the Petri dish so that the 1X Suspension Buffer pools to one side, then aspirate from the dish.

Remove excess droplets in the Petri dish without disturbing any 1X Suspension Buffer that remains beneath the scWest chip. If the chip is not flat when the cell suspension is added, the cells may not settle evenly across the chip. If air becomes trapped under the chip, additional 1X Suspension Buffer can be pipetted underneath the chip to re-balance it.

2 Pipette 1 mL of the single-cell suspension drop-wise on top of the scWest chip in the Petri dish. The cell suspension should pool on top of the scWest chip and be sufficient volume to cover the gel surface without spilling over the chip (Figure 5-4).

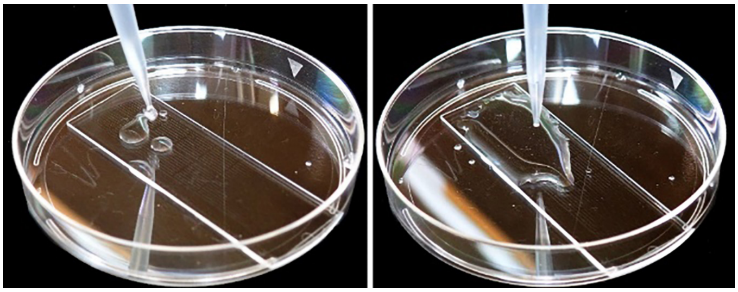


Figure 5-4: *Cells in 1X Suspension Buffer are steadily added (left) and form a pool on top of the scWest chip (~50% of cell suspension shown added to chip on the right). The cell suspension should entirely cover the chip after complete addition (not shown). If the cell suspension does not cover the entire chip, additional 1X Suspension Buffer can be added (<1 mL).*

3 Allow the cells to settle for 5–20 minutes at room temperature. Cell settling time should be optimized by the user and can be monitored by brightfield microscopy (Figure 5-5). Cells in microwells should appear out of focus with respect to cells that are floating or on the surface of the gel.

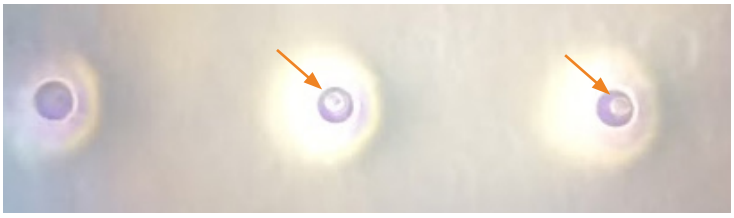


Figure 5-5: *Brightfield image of three wells. The well on the left is empty, while the central well and well on the right each contain one cell (arrows).*

NOTE: *Avoid excessive movement of the scWest chip as it can disturb cell settling. If the cell solution spills over the edge of the chip, continue incubation and check well occupancy after the desired settling time. If occupancy is low, the chip can be reloaded with an additional 1 mL of cell suspension. See "Milo Troubleshooting and FAQ" on page 41.*

4 Gently tilt the scWest chip/Petri dish at a ~45° angle so the cell solution pools at the bottom of the chip (Figure 5-6).

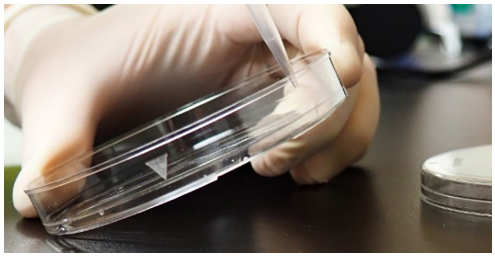


Figure 5-6: *Correct angle of scWest chip and Petri dish when collecting excess cells and performing washes.*

5 Aspirate the pooled cell solution from the Petri dish.

6 Still holding the Petri dish/scWest chip at ~45°angle, gently pipette 1 mL 1X Suspension Buffer on the top, short edge of the scWest chip to wash unsettled cells off of the gel surface.

7 Aspirate the pooled 1X Suspension Buffer from the wash step.

8 Repeat the washing step 1–2 more times as necessary to remove unsettled cells from the surface of the gel. Confirm that minimal cells remain on the gel surface using brightfield microscopy.

9 Estimate well occupancy by counting the number of wells in one block of a scWest chip that contain single cells and the number of wells that contain multiple cells. Count in a serpentine pattern through the block's 10 rows of 40 wells (Figure 5-7). Multiply the results from one block by 16 to get an estimate for the whole chip.

Optimal settling is achieved when a minimum of 10% of wells contain single cells and 2% or fewer wells contain multiple cells. Typical single-cell occupancies range from 10–25%. See "Milo Troubleshooting and FAQ" on page 41.

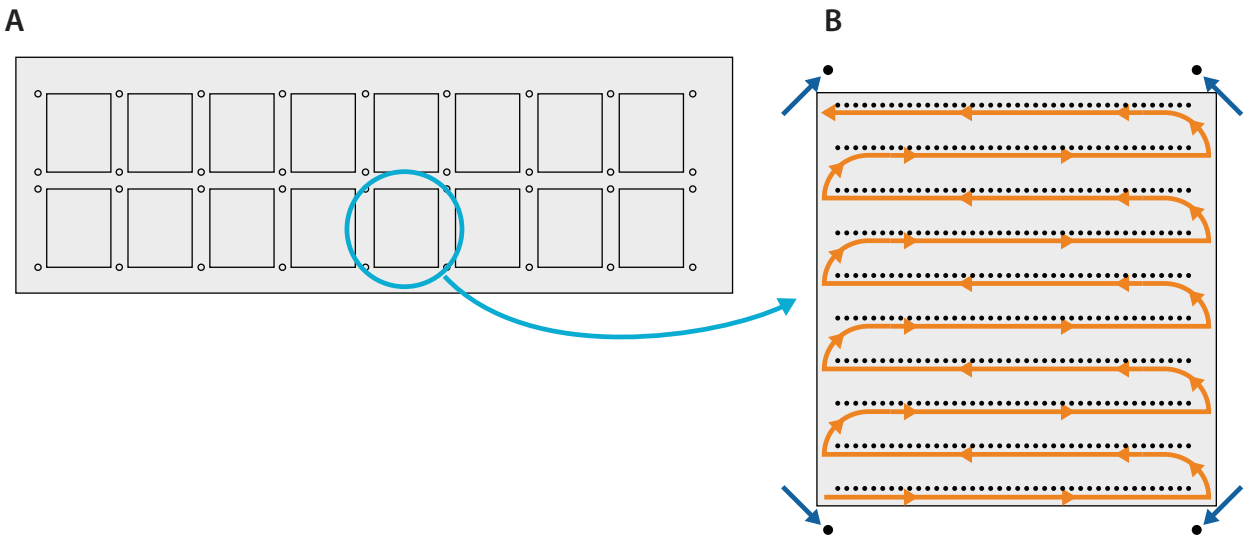


Figure 5-7: *Schematic of scWest chip architecture. A: The scWest chip is a 2x8 array of 400-well blocks separated by alignment markers (indicated by arrows). Each block contains 400 microwells arrayed in a 10-row by 40-column well pattern. B: Typical layout of one block of an scWest chip (not to scale). Determine occupancy by first identifying an alignment marker (arrows) at the corner of a block, then counting the number of cells in each well of a block. This can be done rapidly by scanning across the block one row at a time in a serpentine fashion, as shown by the orange arrow.*

10 Proceed to the next step within 10 minutes to prevent the scWest chip from drying out. If the scWest chip does dry out, gently add 1–2 mL 1X Suspension Buffer to the surface of the scWest chip until the surface is covered, allow to sit for 1 minute, then gently tilt and aspirate the 1X Suspension Buffer before proceeding to the next step.

Proceed to the next step when optimal cell settling is achieved and minimal unsettled cells remain on the surface of the gel.

Loading and Running Milo

E. Inserting the scWest chip into Milo, setting run parameters and running Milo

!WARNING! SHOCK HAZARD

When operating, the pogo pin terminals may contain voltage up to 300 V at hazardous current levels. Do not directly touch the pogo pins. Never override the safety interlocks. The buffer solution is conductive. If spillage of buffer solution occurs outside of the sample area, immediately remove power to the unit and wait 30 seconds before opening the lid and cleaning the system.

CAUTION

Exposure to UV radiation can cause permanent damage to the eyes and skin. The UV transilluminator emits light in the range of 240–400 nm using a UV source which is not user serviceable/replaceable. This product is provided with dual redundant safety interlocks which prevent the UV source from operating when the lid is opened.

Never override the interlocks and operate the unit with the lid open.

1 Make sure the back power switch is off. Plug Milo into a power outlet using the provided power cord. Avoid plugging in the power cord while the power switch on the back of the instrument is in the ON position as this can cause damage to the electronics or corrupt the software.

2 Turn on the master power switch at the back of the instrument.

3 Power on the instrument by pushing the power button on the front right of the instrument. Wait for the user interface to load on the touch screen (Figure 5-8). Avoid powering off the unit while it is starting up. Wait until the control screen is visible before powering down again.

Figure 5-8: *The power button for the instrument is on the bottom right side. Push to power on the instrument and touchscreen.*

4 Make sure the instrument is level by selecting the **Leveling** tab on the touchscreen interface. If the instrument is not completely leveled, adjust all four instrument feet until both electronic levels read a value of 0 ± 0.2 degrees. It is easiest to adjust the level one axis at a time left to right first, then back to front (Figure 5-9).

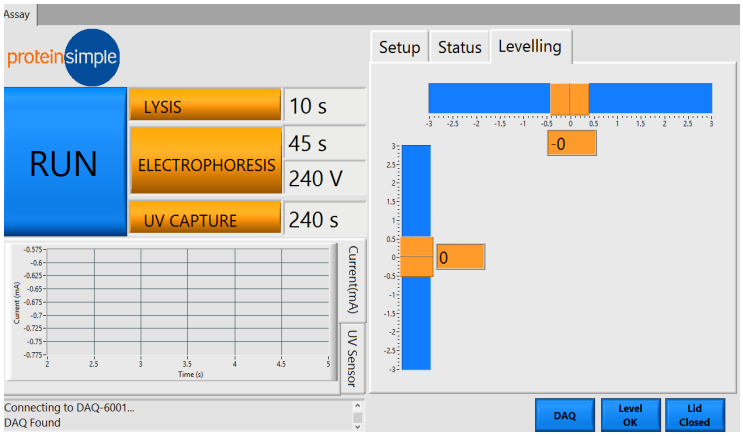


Figure 5-9: *Ensure the instrument is level and that the electronic levels both read 0 ± degrees on the Leveling tab.*

5 Enter the desired lysis (Figure 5-10) and electrophoresis (Figure 5-11) parameters using Milo's touchscreen. Recommended initial values are given in Table 5-1. For more detailed recommendations on electrophoresis time, please refer to the Tech Note entitled "Selecting an Electrophoresis Time for Multiplexed Single-Cell Western Assays" found on the ProteinSimple website. Enter the times in MM:SS format on the right side of the software interface. Values on the left side of the software interface will be used in the assay run.

IMPORTANT

DO NOT press the Run button at this step.

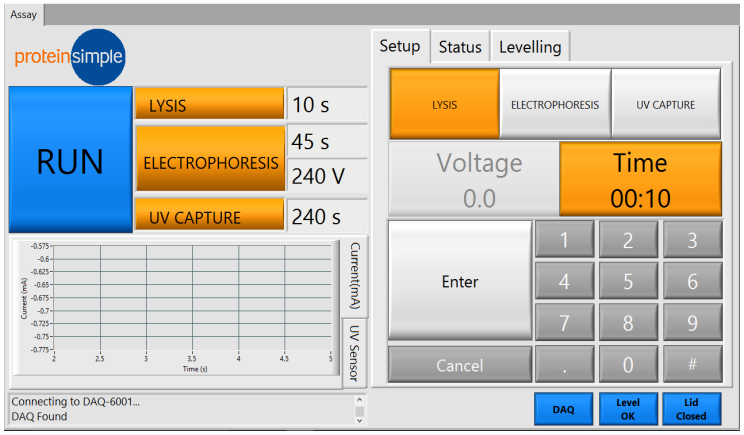


Figure 5-10: To set the lysis time, press the **Lysis** *button above the number pad, enter the desired length of time in seconds, then press* **Enter**.

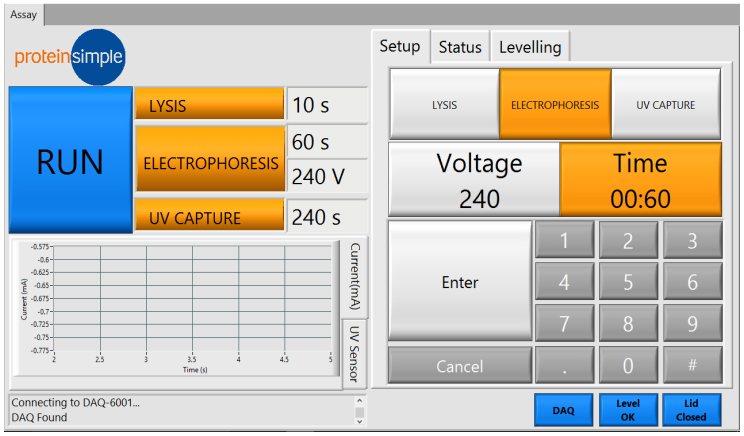


Figure 5-11: To set the electrophoresis run parameters, press the **Electrophoresis** *button above the number pad, enter the desired length of time (in minutes and/or seconds), and press* **Enter**. *Confirm that the Voltage is 240 V and that UV Capture is 240 sec.*

Target MW	Electrophoresis Time
10–30 kDa	45 seconds
30–80 kDa	60 seconds
80–175 kDa	90 seconds

Table 5-1: General guidelines for selecting an electrophoresis time. For more detailed recommendations on electrophoresis time, please refer to the Tech Note entitled "Selecting an Electrophoresis Time for Multiplexed Single-Cell Western Assays" found on the ProteinSimple website.

Recommended run parameters:

- **Lysis Time:** 10–15 seconds. Start with 10 seconds and adjust as needed.
- **Electrophoresis Time:** 45–90 seconds. Table 5-1 provides recommended electrophoresis times for varying target molecular weights. For more detailed recommendations on electrophoresis time, please refer to the Tech Note entitled "Selecting an Electrophoresis Time for Multiplexed Single-Cell Western Assays" found on the ProteinSimple website.
- **Electrophoresis Voltage:** 240 V.
- **UV Capture:** 4 minutes (240 seconds).

See "Milo Troubleshooting and FAQ" on page 41 for more information on modifying Milo run parameters.

6 Open Milo's lid and verify that the electrophoresis cell is well-seated in the recessed area (Figure 5-12).

Figure 5-12: *Electrophoresis cell inside Milo.*

7 Pipette 300 µL of Lysis/Run Buffer on one end of the recessed region of the electrophoresis cell. Avoid/remove air bubbles. Remove the scWest chip from the Petri dish using the tweezers. Gently squeezing the top and bottom of the Petri dish or pushing down on the Petri dish with the tweezers can allow the dish to bow enough to facilitate putting the tweezers under the chip. Carefully place the scWest chip in the electrophoresis cell with the gel side up and the bar code legible, starting with the end touching the drop of Lysis/Run Buffer and slowly lowering the scWest chip using the flat edge of the supplied tweezers until it is lying flat on a layer of buffer. The Lysis/Run Buffer should wick across the bottom of the chip. Avoid large air bubbles under the scWest chip.

NOTE: *At this step, avoid letting the Lysis/Run Buffer contact the gel layer on the top side of the chip.*

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8 Verify that Milo is ready by confirming the Level OK and DAQ icons on the lower part of the touchscreen are illuminated, the Run button is solid blue and that all run settings are correct.

9 **IMPORTANT: Time-sensitive step!** Pour the remaining aliquot of Lysis/Run Buffer into one of the two buffer reservoirs on the electrophoresis cell (Figure 5-13). Quickly verify that the entire scWest chip is covered by Lysis/Run Buffer. The chip should remain in the bottom of the electrophoresis cell and should not float away during the run.

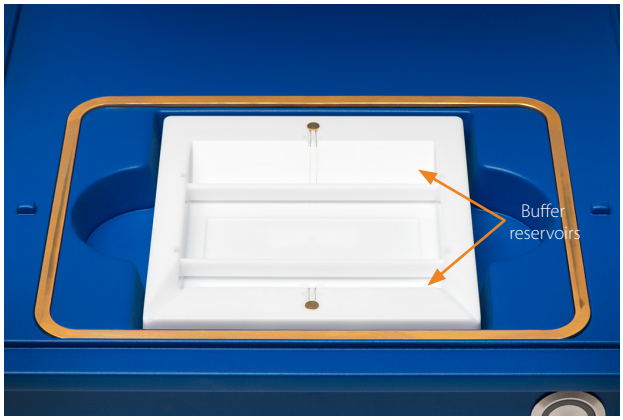

Figure 5-13: *Lysis/Run Buffer should be added to the upper or lower reservoir of the electrophoresis cell. It will spread to cover the scWest chip. Reservoirs are indicated by arrows.*

10 Immediately close Milo's lid and press the **Run** button on the touch screen to initiate the run (Figure 5-14).

IMPORTANT

Lysis will begin immediately after buffer addition so it is important to initiate the run immediately after the Lysis/Run Buffer is added. Delays in starting the run may result in loss of cell lysate and poor run quality.

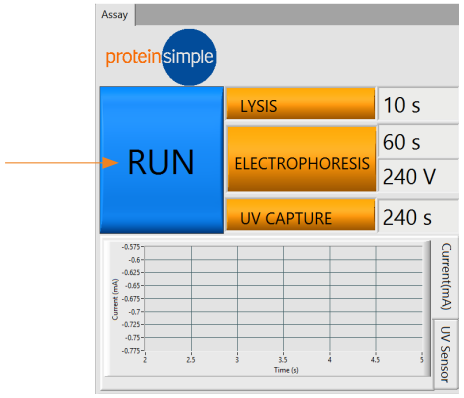

Figure 5-14: *Location of the Run button on Milo.*

11 Monitor the run progress by observing the count-down timers, UV flux and current traces on the left side of the screen. A typical run should take 5–6 minutes (Figure 5-15).

The current during electrophoresis will increase over the course of the run, but should stay in the range of 50–130 mA. If the current is significantly outside this range, check if the correct buffer was used.

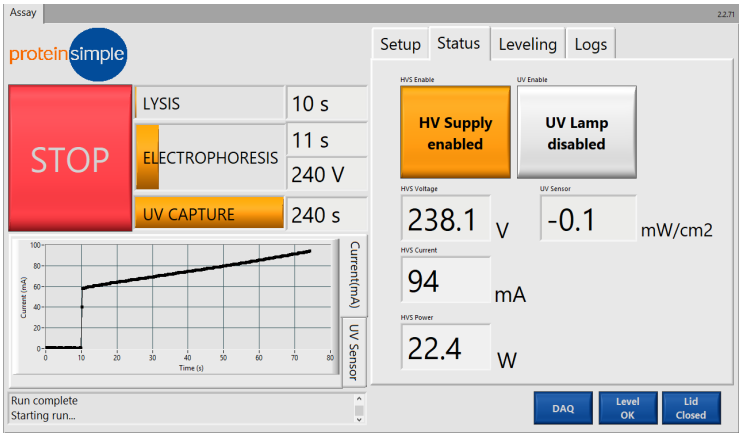

Figure 5-15: *The software interface during a run.*

12 Upon completion of the run, remove the scWest chip from Milo by gently lifting under one edge of the chip with the flat edge of the supplied tweezers. Do not attempt to pinch the chip with the tweezers as this will damage the gel layer. Place the chip in a new 10-cm Petri dish, gel-side up. Always use pristine Petri dishes to avoid introducing dust or debris to the gel surface.

13 After each run, remove the electrophoresis cell from Milo and collect the Lysis/Run Buffer from the electrophoresis cell. Dispose of the Lysis/Run Buffer according to standard biohazardous waste disposal procedures. Use a DI water wash bottle to rinse the electrophoresis cell and remove any residual buffer and cell debris and then return it to Milo so it is ready for the next chip run. All free liquid should be removed from the electrophoresis cell before the next run, though it need not be completely dry to run the next scWest chip.

14 After all scWest chip runs are completed, the electrophoresis cell should be emptied, rinsed with DI water and allowed to dry completely before returning to Milo for storage.

15 The Milo instrument should be left on between uses and can be safely left on for extended periods of time. The unit will go into a power saving mode after a period of inactivity.

F. scWest Chip Washing

1 Pour 15 mL of 1X Wash Buffer into the Petri dish to cover the scWest chip. Swirl to briefly wash the chip. Collect the 1X Wash Buffer for disposal according to standard biohazardous waste disposal procedures (e.g., bleach and drain disposal). Replace with 15 mL of fresh 1X Wash Buffer and incubate for 10 minutes on a shaker. Repeat for a total of two 10 minute washes.

NOTE: *Following the washes, unprobed chips can be stored in 1X Wash Buffer at 4 °C for 2–3 days or immediately probed for targets of interest. If storing chips for longer than 3 days before probing, dry the chips using the drying protocol on page 37 and rehydrate in 1X Wash Buffer for >10 minutes before probing.*

If probing immediately, proceed to the next steps.

Antibody Probing

Intro to Antibody Probing

You can use either a Three-Plex Antibody Probing Fixture or a standard antibody probing fixture, depending on how much of the chip surface you want to probe with your antibody cocktail. A Three-Plex Antibody Probing Fixture requires 35 µL of total antibody volume per fixture and each fixture probes approximately 25% of the chip surface. A standard antibody probing fixture requires up to 80 µL of antibody volume which covers the entire chip surface.

If goat or sheep primary antibodies will be used in any probing cycle, dilute the primary and secondary antibodies in Milk-Free Antibody Diluent (P/N 043-524). If goat or sheep primary antibodies will not be used, dilute all antibodies in Antibody Diluent 2 (P/N 042-203).

The following protocol describes how to use a standard antibody probing fixture. Please refer to the Three-Plex Antibody Probing Fixture Quick Reference Guide located at proteinsimple.com for detailed information on how to use the Three-Plex Antibody Probing Fixture.

G. Primary Antibody Probing

NOTE: *If you are using a Three-Plex Antibody Probing Fixture, see "If Using a Three-Plex Antibody Probing Fixture" on page 33 and refer to the Three-Plex Antibody Probing Fixture Quick Reference Guide.*

1 While the scWest chip is being washed, prepare the primary antibody cocktail solution. When using a standard antibody probing fixture, up to 80 µL of prepared antibody solution is needed to probe each scWest chip. Dilute the primary antibodies to the desired final primary antibody concentration using Antibody Diluent 2 (see Table 5-2). If goat or sheep primary antibodies will be used in any probing cycle, dilute the primary and secondary antibodies in Milk-Free Antibody Diluent. If goat or sheep primary antibodies will not be used, dilute all antibodies in Antibody Diluent. Individual antibody concentrations must be determined by the user, but a suggested starting concentration is 100 µg/mL.

The composition of an example primary antibody cocktail solution in which two primary antibodies are used is shown below:

Component	1 mg/mL Ab 1	1 mg/mL Ab 2	Antibody Diluent*	Total
Dilution	1:10	1:10	NA	NA
Volume	8 µL	8 µL	64 µL	80 µL

Table 5-2: *Composition of an example primary antibody cocktail solution for use with a standard antibody probing fixture. *Milk-Free Antibody Diluent should be used if a goat or sheep primary antibody will be used for probing. Antibody Diluent 2 should be used if no goat or sheep primary antibody will be used.*

2 Place a standard probing fixture in a clean 15 cm Petri dish. Keep the probing fixture clean to ensure uniform probing.

3 After scWest chip washing is complete, dab the edge of the chip on a laboratory wipe to remove excess Wash Buffer. Place the chip into the microarray slide spinner and spin the chip dry for 3-5 seconds. The chip should not be fully dehydrated but there should not be any visible droplets remaining on the surface of the chip which will dilute the antibody solution during probing. Be careful not to touch the gel surface to the laboratory wipe as it can damage or contaminate the gel.

4 Lay the chip down on the empty antibody probing fixture with the gel facing down. Align the chip so that the gel is centered over the probing chamber. The barcode should be just outside the chamber (Figure 5-16).

5 Slowly inject the antibody cocktail into the gap between the chamber and the edge of the chip so that the antibody solution fills the chamber completely. Stop injecting antibody solution once the chamber is filled completely. Excess filling or too rapid filling can cause the antibody solution to spill out of the probing chamber. Avoid/remove air bubbles as they will prevent antibody access to the gel (Figure 5-16).

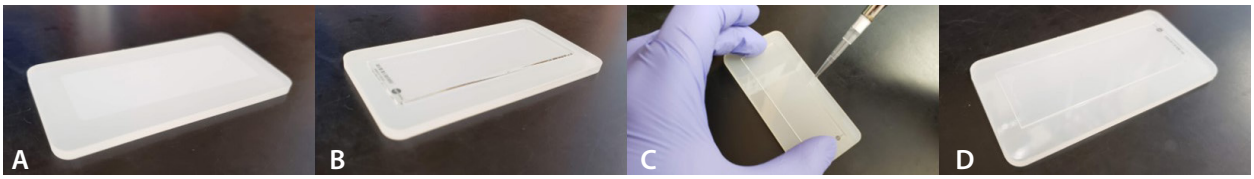

Figure 5-16: *Semi-dry probing protocol on a standard (full-chip) antibody probing chamber. A: Start with a clean, dry probing chamber on your benchtop. B: Gently lower the semi-dry chip onto the antibody probing chamber, gel side down. C: Inject antibody cocktail into the gap between the chip edge and the chamber edge in the middle of the long side of the chip. Antibody solution will wick across the chamber until it is completely filled. D: scWest chip incubating in antibody cocktail solution with no air bubbles.*

6 While probing, cover the incubation fixture with an overturned 15 cm Petri dish. Moisten the sponges provided and place them under the Petri dish to maintain humidity and prevent evaporation of the antibody solution.

7 Incubate the chip for 1–2 hours at room temperature. Antibody incubation times should be optimized by the user.

If Using a Three-Plex Antibody Probing Fixture

1 While the scWest chip is being washed, prepare the primary antibody cocktail solution(s). A total of 35 µL of prepared antibody solution should be loaded in each fixture on a Three-Plex Antibody Probing Fixture. Dilute the primary antibodies to the desired final primary antibody concentration using Antibody Diluent (see Table 5-3). It may be helpful to create 40 µL of total volume per fixture and then load 35 µL per fixture. Individual antibody concentrations must be determined by the user, but a suggested starting concentration is 100 µg/mL.

The composition of an example primary antibody cocktail solution in which two primary antibodies are used in each fixture is shown in Table 5-3.

Chamber on Fixture	Component	1 mg/mL Ab 1	1 mg/mL Ab 2	Antibody Diluent*	Total
A, B, or C	Dilution	1:10	1:10	NA	NA
	Volume	4 µL	4 µL	32 µL	40 µL

Table 5-3: *Composition of an example primary antibody cocktail solution for use with a Three-Plex Antibody Probing Fixture. *Milk-Free Antibody Diluent should be used if a goat or sheep primary antibody will be used for probing. Antibody Diluent 2 should be used if no goat or sheep primary antibody will be used.*

2 Place a Three-Plex Antibody Probing Fixture in a clean 15 cm Petri dish. Keep the probing fixture clean to ensure uniform probing.

3 After scWest chip washing is complete, remove the chip from the wash buffer, dry the chip by spinning it in a slide spinner briefly (3 seconds), place your scWest chip on the Three-Plex Antibody Probing Fixture and probe the chip with your antibody cocktails by following the procedure detailed in the Milo Three-Plex Antibody Probing Fixture Quick Reference Guide located at proteinsimple.com.

H. Washing

1 Remove the scWest chip from the primary antibody incubation fixture (Figure 5-17). If using a standard antibody probing fixture, gently push down on the top left or right corner of the chip with your middle finger. Gently lever tweezers under the diagonally-opposite corner of the scWest chip, using care not to scratch the underlying probing fixture (repeated damage to the probing fixture can result in decreased probing efficiency). Lift the chip up and remove it by pinching the opposite edges of the chip between your thumb and index finger. Place the chip in a clean Petri dish with the gel facing up.

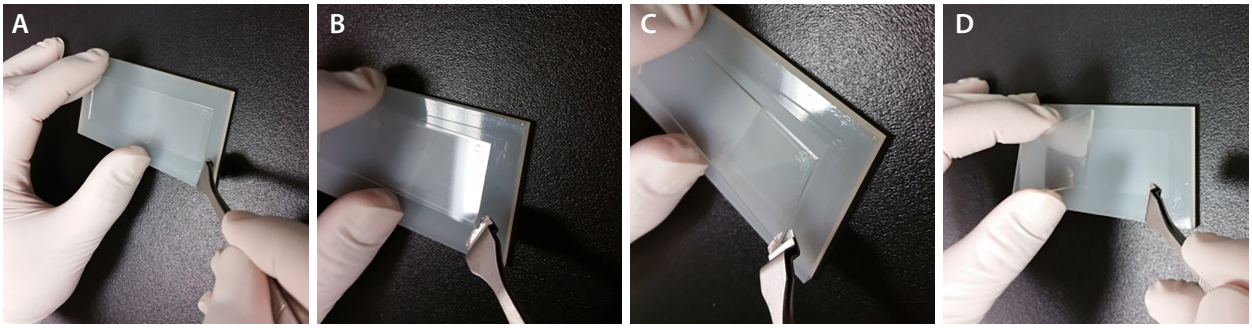

Figure 5-17: *Removing the scWest chip from the primary antibody incubation fixture. A: Gently push down on the top corner of the chip with your index or middle finger. B: Lever the tweezers under the opposite corner of the chip without damaging the probing fixture. C, D: Remove the chip by rotating the tweezers under the corner of the scWest chip and pinching the opposite edges of the chip between your index finger and thumb.*

2 Pour 15 mL of 1X Wash Buffer into the Petri dish to cover the scWest chip. Place on a shaker and wash for 10 minutes.

3 Collect the 1X Wash Buffer for disposal according to standard biohazardous waste disposal procedures (e.g., bleach and drain disposal).

4 Repeat the wash step two additional times for a total of three 10 minutes washes.

I. Fluorescent Secondary Antibody Probing

1 While the scWest chip is in the final wash, prepare the secondary antibody solution. When using the standard antibody probing fixture, a total of 80 µL of prepared secondary antibody solution is needed per scWest chip. Dilute the secondary antibodies to the desired final secondary antibody concentration using Antibody Diluent (see Table 5-4). Use the same Antibody Diluent as was used for primary antibody probing. Individual antibody concentrations must be determined by the user, but a suggested starting concentration for most secondary antibodies is 50 µg/mL.

Component	1 mg/mL Ab 1	1 mg/mL Ab 2	Antibody Diluent*	Total
Dilution	1:20	1:20	NA	NA
Volume	4 µL	4 µL	72 µL	80 µL

Table 5-4: *Composition of an example secondary antibody solution in which two secondary antibodies are used on a standard antibody probing fixture. *The same antibody diluent should be used for primary and secondary antibody probing steps.*

2 Turn the probing fixture over to use the other side for secondary antibody probing.

3 After the wash step is complete, dab the scWest chip on a laboratory wipe to remove excess Wash Buffer. Place the chip into the microarray slide spinner and spin the chip dry for 3-5 seconds. The chip should not be fully dehydrated but there should not be any visible droplets remaining on the surface of the chip which will dilute the antibody solution during probing. Be careful not to touch the gel surface to the laboratory wipe as doing so can damage or foul the gel.

4 Lay the chip down on the empty antibody probing fixture with the gel facing down. Align the chip so that the gel is centered over the probing chamber. The barcode should be just outside the chamber (Figure 5-18).

5 Slowly inject the antibody cocktail into the gap between the chamber and the edge of the chip so that the antibody solution fills the chamber completely. Stop injecting antibody solution once the chamber is filled completely. Excess filling or too rapid filling can cause the antibody solution to spill out of the probing chamber. Avoid/remove air bubbles as they will prevent antibody access to the gel (Figure 5-18).

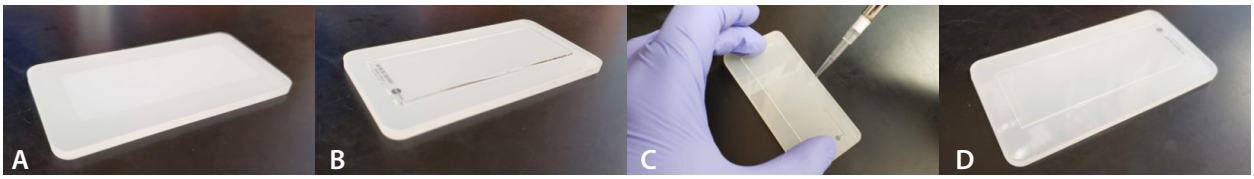

Figure 5-18: *Semi-dry probing protocol on a standard (full-chip) antibody probing chamber. A: Start with a clean, dry probing chamber on your benchtop. B: Gently lower the semi-dry chip onto the antibody probing chamber, gel side down. C: Inject antibody cocktail into the gap between the chip edge and the chamber edge in the middle of the long side of the chip. Antibody solution will wick across the chamber until it is completely filled. D: scWest chip incubating in antibody cocktail solution with no air bubbles.*

6 Cover the incubation fixture with an overturned 15 cm Petri dish. Moisten the sponges provided and place them under the Petri dish to maintain humidity and prevent evaporation of the antibody solution.

7 Incubate the chip for 1 hour at room temperature, protected from light. Antibody incubation times should be optimized by the user.

J. Washing

1 Remove the scWest chip from the secondary antibody incubation fixture using tweezers and place in a clean Petri dish gel side up (Figure 5-19).

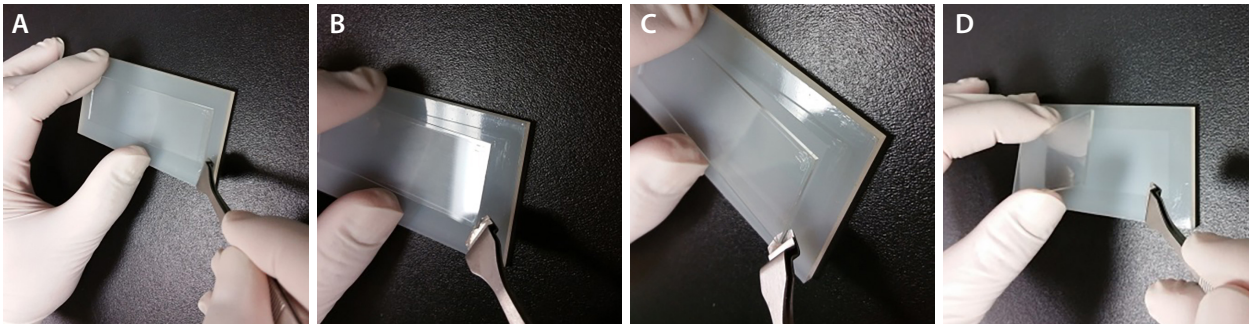

Figure 5-19: *Removing the scWest chip from the secondary antibody incubation fixture. A: Gently push down on the top corner of the chip with your index or middle finger. B: Lever the tweezers under the opposite corner of the chip without damaging the probing fixture. C, D: Remove the chip by rotating the tweezers under the corner of the scWest chip and pinching the opposite edges of the chip between your index finger and thumb.*

2 Pour 15 mL of 1X Wash Buffer into the Petri dish to cover the scWest chip. Place on a shaker and wash for 15 minutes in the dark.

3 Collect the 1X Wash Buffer for disposal according to standard biohazardous waste disposal procedures (e.g., bleach and drain disposal).

4 Repeat the wash step two additional times for a total of three 15 minute washes.

Probed chips can be stored in 1X Wash Buffer at 4 °C for 2–3 days or dried and immediately imaged in a microarray scanner for targets of interest. If storing chips for longer than 3 days before scanning, dry the chips using the drying protocol on page 37.

NOTE: *For best results, scan scWest chips as soon as possible after probing (e.g., within 12–24 hours).*

Imaging scWest Chips

K. Scanning scWest Chips on a Microarray Scanner

1 Remove the scWest chip from the Petri dish and gently dab the glass edge of the chip dry using a laboratory wipe. Be careful not to touch the gel surface.

2 Rinse the chip in DI water to remove salts from the wash buffer. Recommended: repeat DI water wash two additional times for a total of three washes.

3 Recommended: spin the scWest chip with the gel side facing downward/inward for 3 min on an orbital slide spinner.

4 Recommended: air-dry the scWest chip by gently blowing compressed nitrogen or filtered compressed air at the surface of the chip for 1 minute. Make sure to dry the chip evenly and be careful not to damage it by drying too forcefully. Do not use unfiltered air straight from a compressor as particulates will contaminate the gel surface and result in unwanted noise upon scanning.

NOTE: *If compressed air is not available, spin the scWest chip in the orbital slide spinner for 3–5 minutes before scanning. If no orbital slide spinner is available, dry the chip for 3 minutes with filtered air. If neither a slide spinner nor compressed air is available, air dry the slide overnight protected from light and dust in a dry, uncovered Petri dish.*

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5 Turn on the microarray scanner and allow it to warm up for the recommended time (typically 5–30 minutes). Please consult the manual for your microarray scanner for exact times.

6 Place the scWest chip in the scanner face up or face down as appropriate to the type of scanner. Scan at 5 µm resolution at the appropriate wavelengths for the secondary antibodies used. For a list of compatible microarray scanners, please see "Compatible Microarray Scanners" on page 8 .

7 Images for each spectral channel should be saved as single color TIFFs for analysis using Scout Software.

8 Imaged chips can be dried using the drying protocol and archived at room temperature, protected from light, for up to 9 months (see "scWest Chip Drying and Long Term Archiving Protocol" on page 37).

Chapter 6:

scWest Chip Storage and Archiving

Chapter Overview

- scWest Kit Storage
- scWest Chip Drying and Long Term Archiving Protocol

scWest Kit Storage

Upon receipt of a scWest kit, store the Antibody Diluent 2 at 2–8°C protected from light. Store the remaining kit components (scWest chips and buffers) at room temperature protected from light.

scWest Chip Drying and Long Term Archiving Protocol

1 In a Petri dish, rinse the chip in 15 mL of DI water for 10 minutes.

2 Spin the scWest chips dry for 3 min on a slide spinner. If no slide spinner is available, dry the underside of the scWest chip with a laboratory wipe after dabbing away any excess liquid.

3 Place the scWest chip gel side up in a clean, dry Petri dish without the lid and dry in the dark protected from dust at room temperature overnight. Alternatively, air-dry the scWest chip by gently blowing compressed nitrogen or filtered compressed air at the surface of the chip for 1 minute. Make sure to dry the chip evenly and be careful not to damage it by drying too forcefully. Do not use unfiltered air straight from a compressor as particulates will contaminate the gel surface and result in unwanted noise upon scanning.

4 Place the scWest chips in the provided chip holder and store in the dark at room temperature for up to 9 months. Re-hydrate chips in 1X Wash Buffer for >10 minutes before probing. Scanning chips after dry storage should be done without rehydrating the chip(s).

Chapter 7:

Scout Software Basics

Chapter Overview

- Brief Outline of Analysis Protocol Using Scout Software

Brief Outline of Analysis Protocol Using Scout Software

1 Launch Scout Software.

2 Under the File menu, add the first single-color scan image for a single chip (File > Add scan to current chip).

3 Register the scan image to correct for image offsets or rotation using the auto registration feature or by selecting two blocks for registration and clicking on the first well in the first selected registration block and last well in the second selected registration block.

4 The software will automatically identify all the lanes in an image and all the peaks in each lane using default settings. Repeat steps 2-4 for all single-color TIFF images of the scWest chip being analyzed. Copy the chip registration from the current scan if images were all collected at the same time without moving the chip within the scanner.

5 Reject any major unwanted regions of the chip due to chip damage or, if using a Three-Plex Antibody Probing Fixture, regions of the chip located between each probing fixture region that were not probed with any antibody (visible as dark regions). To exclude regions of the chip from analysis, highlight those regions, right click, and mark as "Rejected" [or keyboard shortcut "r"].

6 Optimize peak detection. To adjust the detection settings, select Edit > Scan Properties. Once the peaks are detected properly, continue to the next step.

7 View the Peak Table to see all detected peaks in a spectral channel (Tools > Peak tables > Show/update peak table) and exclude any false positive peaks owing to particulates or dust.

8 Use the Peak Table to tag the correct peaks as your protein target(s) of interest. Peaks that lie outside the expected molecular weight range (migration distance) can be excluded.

9 Once all the appropriate peaks are detected, visualize the heterogeneity of your protein target(s) of interest using the visualization tools in Scout (Tools > Data Visualization Tools) or export the tagged peaks from the Peak Table to .csv or .fcs file formats to visualize your data in your favorite statistical software package.

For more information, please refer to the Scout Software User Guide.

Chapter 8:

Single-Cell Western Troubleshooting and FAQ

Chapter Overview

- Milo Troubleshooting and FAQ

Milo Troubleshooting and FAQ

Question/Problem	Possible Cause/Issue	Solution
How do I determine which scWest chip to use?	Cell size unknown.	The scWest calibration chip can be used to determine which scWest chip size to use.
How do I use the scWest calibration chip?	First scWest chip run.	Refer to the scWest Calibration Chip Quick Reference Guide for more information.
Can I load fewer than 100,000 cells onto each scWest chip?	Low number of cells available.	Lower starting cell numbers can be used either by loading down to 10,000 cells in 1 mL or by decreasing the volume of cell suspension and settling over a smaller area on a dry chip. Optimal cell settling should be confirmed using brightfield microscopy.
My cell suspension did not spread across the entire chip.	Poor chip rehydration.	Add additional (<1 mL) 1X Suspension Buffer to cover the surface of the chip and rehydrate your chip for a longer period of time. ***NOTE:*** scWest chips should be rehydrated in 1X Suspension Buffer for at least 10 min prior to cell settling.
	Chip was gel face-down.	Confirm that chip is gel face-up before adding cells.
How should cell settling time be determined?	Too many or too few cells in microwells.	Optimal cell settling is achieved when a minimum of 10% of wells contain single cells and 2% or fewer wells contain multiple cells. Typical single-cell occupancies range from 10–25%. Increasing settling time will lead to increased occupancy, but may increase the number of wells with more than one cell.

Milo Troubleshooting and FAQ, *continued*

Question/Problem	Possible Cause/Issue	Solution
What if less than 10% of my wells contain a single cell?	Too few cells added.	Increase number of cells added.
	Cell settling time was too short.	Increase cell settling time.
	Cells were too large for chip well size.	Select a scWest chip with larger wells.
	Cells were not in single-cell suspension (e.g., clumped cells).	Triturate cells to create single-cell suspension.
	Chip was gel face down.	Confirm that chip is gel face-up before adding cells.
What if more than 2% of my wells contain two or more cells?	Too many cells added.	Decrease number of cells added.
	Cell settling time was too long.	Decrease cell settling time.
	Cells were too small for chip well size.	Select an scWest chip with smaller wells.
Can phosphorylated proteins be detected using Milo?	N/A	Yes, Milo enables detection of phosphorylated proteins. To preserve phosphorylation states, users can add 1 mM sodium fluoride and 1 mM sodium orthovanadate to the Lysis/Run Buffer just prior to use.
Protein signal is only near well.	Incomplete lysis.	Increase lysis time.
	Electrophoresis too short.	Increase electrophoresis run time.
Protein signal is smeared across the lane starting from well.	Incomplete lysis. Continuous protein injection.	Lysis time should be increased.
	Antibody binding off target to background cell lysate.	Antibody concentrations might be too high and should be decreased. Try a different primary antibody.
Protein signal too close to next well or into next lane.	Electrophoresis time too long.	Decrease electrophoresis time.
Native fluorescent signal weak/not appearing.	Electrophoresis time too long.	Decrease electrophoresis time.
	Photobleaching due to UV exposure.	Decrease UV exposure time to 1 minute.

Milo Troubleshooting and FAQ, *continued*

Question/Problem	Possible Cause/Issue	Solution
Air bubbles while probing with antibodies.	Air bubbles in antibody droplets.	Avoid air bubbles in the antibody droplets when preparing the antibody solution.
	scWest chip laid down poorly in antibody probing fixture.	scWest chips must be lowered slowly in the antibody probing fixture to allow antibody spreading without air bubble formation.
No signal for protein target.	Antibody concentration too low.	Increase primary and/or secondary antibody concentration.
	Poor antibody.	Try a different Western-validated antibody.
	Probing orientation.	Confirm gel is face-down during antibody probing to contact the antibody solution.
Blue "Windows repair screen" shown on Milo touchscreen during instrument start.	Instrument was powered off while booting or the power plug was inserted while the power switch was in the ON position.	Select the restart button on the Milo touchscreen. The instrument should restart after a brief pause.

Chapter 9:

General Information

Chapter Overview

- Compliance
- Safety
- Safety Data Sheets
- Instrument Cleaning and Decontamination
- Customer Service and Technical Support
- Legal Notices

Compliance

NOTE: *This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his/her own expense.*

- This Class A digital apparatus complies with Canadian ICES-003. Cet appareil numérique de la classe A est conforme à la norme'.
- **EN 61010-1:2010 (Third Edition):** Safety requirements for electrical equipment for measurement, control, and laboratory use - Part 1: General requirements (EU)
- **EN 61326-1:2013:** Electrical equipment for measurement, control and laboratory use. EMC Requirements. General requirements (EU)

CE

Safety

As with all experiments, please adhere to general lab safety guidelines including, but not limited to, the following:

- Wear personal protective equipment (PPE) including safety glasses, fully enclosed shoes, long pants, and gloves.
- Know the locations of all safety equipment including fire extinguishers, spill kits, eyewashes/showers, first aid kits, and emergency/injury reporting procedures.

Explanation of Symbols Used

The following safety alert labels are located on Milo or are displayed in the user guide indicating a potential safety hazard.

Symbol	Description
	**CAUTION** Performing or omitting a specific action may result in equipment damage or injury.
	**CAUTION** Performing or omitting a specific action may result in exposure to hazardous ultraviolet (UV) radiation.
	**!WARNING!** Performing or omitting a specific action may result in electrical shock.

Safety Guidelines

!WARNING!
To prevent electric shock, do not remove the covers. No user serviceable parts inside. The unit contains hazardous voltages/currents and should only be opened by a trained service person. To avoid the possibility of electric shock, remove the power cord before servicing.

!WARNING! SHOCK HAZARD
When operating, the pogo pin terminals may contain voltage up to 300 V at hazardous current levels. Do not directly touch the pogo pins. Never override the safety interlocks. The buffer solution is conductive. If spillage of buffer solution occurs outside of the sample area, immediately remove power to the unit and wait 30 seconds before opening the lid and cleaning the system.

!WARNING! CLASS 1 EQUIPMENT
This equipment must be earthed. The power plug must be connected to a properly wired earth grounded socket outlet.

Pluggable Equipment: The socket outlet should be installed near the equipment and be easily accessible.

!WARNING!
If Milo is not used as specified by ProteinSimple, overall safety will be impaired.

!WARNING!
If Milo is damaged and doesn't function properly, stop him safely and contact ProteinSimple Technical Support right away.

CAUTION
Protection may be impaired if this device is used in a manner not specified by the manufacturer.

CAUTION
Exposure to UV radiation can cause permanent damage to the eyes and skin. The UV transilluminator emits light in the range of 240–400 nm using a UV source which is not user serviceable/replaceable. This product is provided with dual redundant safety interlocks which prevent the UV source from operating when the lid is opened.

Never override the interlocks and operate the unit with the lid open.

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 Milo may be listed as hazardous. Warnings are displayed on the labels of all chemicals when hazards exist.

SDSs provide users 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 website.

Instrument Cleaning and Decontamination

Milo can be cleaned by wiping hard surfaces with a soft cloth saturated in 70% ethanol.

NOTE: *Do not spray liquids directly onto the instrument.*

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
3001 Orchard Pkwy
San Jose, CA 95134
USA

Legal Notices

NOTE: *Read the Legal Notices carefully before using Milo and Scout software.*

Milo Disclaimer of Warranty

EXCEPT AS EXPRESSLY PROVIDED IN ANY ProteinSimple SOFTWARE LICENSE AGREEMENT OR QUOTATION, THE PRODUCTS SOLD AND SERVICES PROVIDED BY ProteinSimple ARE PROVIDED ON AN "AS IS" AND "AS AVAILABLE" BASIS WITHOUT WARRANTY OF ANY KIND. ProteinSimple AND ITS SUPPLIERS DO NOT WARRANT THE SECURITY, PRIVACY, OR ACCURACY OF ANY DATA PROVIDED VIA THE PRODUCTS OR SERVICES, AND YOU AGREE THAT THE USE OF ANY SUCH DATA BY YOU IS AT YOUR SOLE RISK. TO THE MAXIMUM EXTENT ALLOWED UNDER APPLICABLE LAW, ProteinSimple AND ITS SUPPLIERS DISCLAIM ANY AND ALL WARRANTIES, WHETHER EXPRESS, IMPLIED OR STATUTORY, INCLUDING, WITHOUT LIMITATION, ANY WARRANTY OF MERCHANTABILITY, TITLE, NON-INFRINGEMENT, OR FITNESS FOR A PARTICULAR PURPOSE.

Scout Software and Authorization Server License Agreement

IMPORTANT — PLEASE READ CAREFULLY THE TERMS OF THIS SCOUT SOFTWARE AND AUTHORIZATION SERVER LICENSE AGREEMENT ("AGREEMENT"). BY CLICKING ON THE "I AGREE" BUTTON, (1) YOU ACKNOWLEDGE THAT YOU HAVE READ, UNDERSTAND, AND AGREE TO BE BOUND BY THIS AGREEMENT AND (2) YOU REPRESENT THAT YOU HAVE THE AUTHORITY TO ENTER INTO THIS AGREEMENT, PERSONALLY OR IF YOU HAVE NAMED A COMPANY AS CUSTOMER, ON BEHALF OF THAT COMPANY (YOU OR ANY SUCH COMPANY, THE "CUSTOMER"), AND TO BIND THE CUSTOMER TO THE TERMS OF THIS AGREEMENT. IF YOU DO NOT AGREE TO ALL TERMS AND CONDITIONS OF THIS AGREEMENT, OR IF YOU DO NOT HAVE SUCH AUTHORITY, YOU SHOULD CLICK ON THE "CANCEL" BUTTON TO DISCONTINUE THE DOWNLOAD OF THE LICENSED SOFTWARE.

1. Definitions

1.1 **"Authorized Use Parameters"** means the following usage restrictions, which restrict the operation of the Licensed Software to a particular set of conditions: Customer shall (a) limit simultaneous use of the Licensed Software to a maximum of ten (10) Authorized Users; and (b) use the Licensed Software only in connection with the accompanying System purchased by Customer pursuant to the System Quotation and located at the Site.

1.2 **"Authorized User"** means one (1) User who initiates the execution of the Licensed Software and/or interacts with or directs the Licensed Software in the performance of its functions. Multiple Authorized Users may work simultaneously with one installation of the Licensed Software, as on a server, or they may each have their own installation on single-user machines, or a mix of these, provided that in all cases the total number of simultaneous Users does not exceed the applicable Authorized Use Parameters.

1.3 "Company" means ProteinSimple.

1.4 "Documentation" means Company's then-current manuals, guides, and on-line help pages, if any, applicable to the Licensed Software and made generally available by Company to its customers.

1.5 "Enterprise" means those organizations that have Internet addresses located at top level and second-level domain names set forth in the System Quotation.

1.6 "Error" means a reproducible error in the Licensed Software that prevents such Licensed Software from operating substantially in accordance with its Documentation.

1.7 "Executable Code" means the fully compiled binary version of Licensed Software that can be executed by a computer and used by an end user without further compilation.

1.8 "Intellectual Property Rights" means all copyrights, trade secrets, patents, patent applications, moral rights, contract rights, and other proprietary rights, but specifically excluding any trademarks or service marks.

1.9 "Licensed Software" means the Scout Software program in Executable Code form, and any Updates that Company makes available to Customer in accordance with this Agreement.

1.10 "Site" means the facility or campus set forth in the System Quotation.

1.11 "System" means the proprietary Milo Single-Cell Western system or any future model or successor thereto that is provided to Customer by Company pursuant to a separate agreement between the parties (the "System Quotation").

1.12 "Update" means those releases of the Licensed Software that Company provides to customers to correct Errors, fix bugs, or create minor improvements, incremental features, or enhancements of existing features which Company designates by a change in the number to the right of the first or second decimal point. Updates do not include those releases of the Licensed Software that provide substantial new features or additional functionality which Company designates by a change in the number to the left of the first decimal point.

1.13 "User" means any individual that has an e-mail address within the Enterprise.

2. License and Restrictions

2.1 License Grant. Subject to the terms and conditions of this Agreement and the payment of the required fees set forth in the System Quotation, Company grants to Customer a nontransferable, nonexclusive, royalty-free, revocable, worldwide license (without the right to sublicense) to (a) install the Licensed Software on any computer located at any Site; (b) use, execute, and display the Licensed Software, in Executable Code form only; and (c) copy the Licensed Software and Documentation, solely as necessary to support Authorized Users; in each of the foregoing, solely in accordance with the Documentation and the Authorized Use Parameters. Customer agrees that it will comply with the Authorized Use Parameters.

2.2 License Restrictions. Customer acknowledges that the Licensed Software and its structure and organization constitute valuable trade secrets of Company. Accordingly, the license granted in this Agreement is subject to the following restrictions: Customer and its Authorized Users (a) may not reverse

engineer, disassemble, decompile, or otherwise attempt to derive the source code of Licensed Software; (b) may not modify, adapt, alter, translate, or create derivative works from the Licensed Software; (c) may not merge the Licensed Software with other software; (d) may not use the Licensed Software in any service bureau or time-sharing arrangement, license, sell, rent, lease, transfer, assign, distribute, host, outsource, disclose, or otherwise commercially exploit or make the Licensed Software or Documentation available to any third party; (e) shall only make that number of exact copies of the Licensed Software and Documentation as delivered by Company that are necessary to support Customer's use of the Licensed Software in accordance with this Agreement; (f) shall include any titles, trademarks, and copyright and restricted rights notices that are included on or in the Licensed Software as delivered by Company on and in any copies of the Licensed Software that it makes; and (g) shall ensure that Customer's use of the Licensed Software does not exceed the scope of the license that Customer has purchased pursuant to this Agreement.

2.3 Open Source Software. Certain items of independent, third-party code may be included in the Licensed Software that are subject to open source licenses ("Open Source Software"). Such Open Source Software is licensed under the terms of the license that accompanies such Open Source Software. Nothing in this Agreement limits Customer's rights under, or grants Customer rights that supersede, the terms and conditions of any applicable end user license for such Open Source Software. In particular, nothing in this Agreement restricts Customer's right to copy, modify, and distribute such Open Source Software that is subject to the terms of such open source licenses.

2.4 Ownership. Company reserves all rights not expressly granted to Customer in this Agreement. Without limiting the generality of the foregoing, Customer acknowledges and agrees that, except as expressly set forth in this Agreement, Company and its suppliers retain all Intellectual Property Rights, title and interest in and to the Licensed Software and Documentation.

3. Support and Maintenance Services

3.1 Services. Subject to Customer's payment of the Services fees, as set forth in the System Quotation, and to the terms and conditions herein, Company will use commercially reasonable efforts to provide to Customer the following support and maintenance services (the "Services") for the Licensed Software: (a) Company will answer technical questions concerning functions and features of the Licensed Software; (b) Company will provide Error verification, analysis and corrective efforts for the Licensed Software; and (c) Company will provide, without charge, Updates of the software released during the term of this Agreement. Customer will be responsible for providing, in a manner consistent with good industry practice, all Services to Users. Customer acknowledges that Company may not be able to correct all reported Errors. Any Update of the Licensed Software will be deemed part of the Licensed Software and Customer will use such Updates in accordance with the requirements and obligations in this Agreement.

3.2 Service Conditions. Company's obligation to provide the Services is conditioned on Customer: (a) notifying Company of any Error within a reasonable period of time; (b) providing Company all information relating to the Error; (c) providing access to the Licensed Software and Customer's facility where the Licensed Software is located and informing Company of any potential hazards which may be encountered while servicing the Licensed Software. Customer may contact Company via telephone at

1-888-607-9692 or e-mail at support@proteinsimple.com during the hours of 8 a.m. (Pacific Time) and 5 p.m. (Pacific Time) Monday through Friday, excluding holidays, to report any Error. A list of standard holidays will be provided to Customer upon request. Company shall have the right to determine in its sole discretion what corrective action Company will perform to support the Licensed Software. Company may subcontract the Services to a third party contractor provided that Company will be responsible for the third party contractor's compliance with this Agreement.

3.3 Service Exclusions. Company will not be obligated to provide the Services if (a) Company determines that an Error is caused by malfunction of any hardware (other than malfunction of the System) or third party software used with the Licensed Software; or (b) Customer has failed to incorporate the latest Update previously released to Customer.

4. Warranty

4.1 Licensed Software Warranty. Company warrants that the Licensed Software, as properly installed, and under normal use, will perform substantially in accordance with its Documentation during the Warranty Period. The "Warranty Period" for the Licensed Software begins on date Customer downloads the Licensed Software and ends twelve (12) months thereafter.

4.2 Remedy. If Customer notifies Company in writing during the Warranty Period of an Error, Company will, at its expense and as its sole obligation for any breach of the foregoing warranty, use commercially reasonable efforts to correct the Error or replace the Licensed Software. Any Error correction or replacement of the Licensed Software will not extend the original Warranty Period. The warranty and the remedies provided above will not apply to the Licensed Software if (a) Company determines that an Error is caused by accident, abuse, misuse, negligence, fire, earthquake, flood, other force majeure event, failure of electrical power, the use of unauthorized products, or unauthorized repairs or modifications; (b) Company determines that an Error is caused during or as a result of delivery; (c) a problem arises from or is based on Company's compliance with Customer's specifications; or (d) Company determines that an Error is caused by malfunction of any hardware (other than malfunction of the System) or third party software used with the Licensed Software.

4.3 Disclaimer. THE WARRANTIES ABOVE ARE EXCLUSIVE AND IN LIEU OF ALL OTHER WARRANTIES, WHETHER EXPRESS, IMPLIED OR STATUTORY, INCLUDING WITHOUT LIMITATION THE IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, TITLE, AND NONINFRINGEMENT.

5. Limitation of Liability. NEITHER COMPANY NOR ITS SUPPLIERS SHALL BE RESPONSIBLE OR LIABLE WITH RESPECT TO ANY SUBJECT MATTER OF THIS AGREEMENT OR TERMS OR CONDITIONS RELATED THERETO UNDER ANY CONTRACT, NEGLIGENCE, STRICT LIABILITY OR OTHER THEORY (A) FOR LOSS OR INACCURACY OF DATA, LOSS OF PROFITS OR COST OF PROCUREMENT OF SUBSTITUTE GOODS, SERVICES OR TECHNOLOGY, OR (B) FOR ANY INDIRECT, INCIDENTAL OR CONSEQUENTIAL DAMAGES INCLUDING, BUT NOT LIMITED TO LOSS OF REVENUES AND LOSS OF PROFITS. COMPANY'S AGGREGATE CUMULATIVE LIABILITY HEREUNDER SHALL NOT EXCEED THE GREATER OF FIVE HUNDRED DOLLARS ($500.00).

6. Term and Termination

6.1 Term of Agreement. The Agreement is effective on the date Customer downloads the Licensed Software and shall remain in effect until terminated by either party as provided in this section.

6.2 Termination For Material Breach. Either party may terminate this Agreement upon written notice if the other party materially breaches this Agreement and fails to cure such breach within thirty (30) calendar days following receipt of written notice from the other party specifying the breach in detail. Notwithstanding the foregoing, Company may immediately terminate this Agreement and all licenses granted hereunder if Customer breaches Section 2 (License and Restrictions) hereof or upon termination of the System Quotation. The foregoing rights of termination are in addition to any other rights and remedies provided in this Agreement or by law.

6.3 Effect of Termination. Upon termination of this Agreement (or termination or expiration of any license granted hereunder), all rights of Customer to use the Licensed Software and Documentation will cease and (a) all license rights granted under this Agreement will immediately terminate and Customer shall promptly stop all use of the Licensed Software and Documentation; (b) all Services will terminate immediately; (c) Customer shall promptly erase all copies of the Licensed Software from Customer's computers, and destroy all copies of the Licensed Software and Documentation on tangible media in Customer's possession or control or return such copies to Company; and (d) upon request by Company, Customer shall certify in writing to Company that it has returned or destroyed such Licensed Software and Documentation. The parties' rights and obligations under Sections 1 (Definitions), 2.4 (Ownership), 4.3 (Disclaimer), 5 (Limitation of Liability), 6 (Term and Termination), and 7 (General) shall survive termination of this Agreement.

7. General

7.1 Assignment. This Agreement and Customer's rights hereunder may not be assigned to any third party by Customer except with the prior written approval of Company. Any attempted assignment of this Agreement or any rights or obligations hereunder will be null and void.

7.2 Governing Law. This Agreement is made in, governed by, and shall be construed in accordance with the laws of the State of California, without regard to any conflicts of law principles that would result in application of laws of any other jurisdiction. The United Nations Convention on Contracts for the International Sale of Goods does not apply to this contract. Any legal action or other legal proceeding relating to this contract or the enforcement of any provision of this contract must be brought in any state or federal court located in Santa Clara County, California. Customer and Company expressly and irrevocably consents and submits to the jurisdiction of such courts.

7.3 Injunctive Relief. Customer acknowledges that the Licensed Software contains valuable trade secrets and proprietary information of Company, that any actual or threatened breach of this Agreement will cause harm to Company for which monetary damages would be an inadequate remedy, and that injunctive relief is an appropriate remedy for such breach.

7.4 Modifications. Company reserves the right to change the terms and conditions of this Agreement or its policies relating to the Licensed Software at any time. Company will notify Customer of any material changes to this Agreement by sending Customer an e- mail to the last e-mail address Customer provided to Company or by prominently posting notice of the changes on Company's website. Any material changes to this Agreement will be effective upon the earlier of thirty (30) calendar days following Company's dispatch of an e-mail notice to Customer or thirty (30) calendar days following Company's post-

ing of notice of the changes on Company's website. These changes will be effective immediately for new users of our Licensed Software. Please note that at all times Customer is responsible for providing Company with its most current e-mail address. In the event that the last e-mail address that Customer has provided Company is not valid, or for any reason Company is not capable of delivering to Customer the notice described above, Company's dispatch of the e-mail containing such notice will nonetheless constitute effective notice of the changes described in the notice. If Customer does not agree with the changes to this Agreement, Customer must notify Company prior to the effective date of the changes that Customer wishes to terminate its license to the Licensed Software. Continued use of the Licensed Software, following notice of such changes, shall indicate Customer's acknowledgement of such changes and agreement to be bound by the terms and conditions of such changes.

7.5 Severability. In the event any provision of this Agreement is held to be invalid or unenforceable, the remaining provisions of this Agreement will remain in full force.

7.6 Waiver. The waiver by either party of any default or breach of this Agreement shall not constitute a waiver of any other or subsequent default or breach.

7.7 Export. Customer agrees not to export, reexport, or transfer, directly or indirectly, any U.S. technical data acquired from Company, or any products utilizing such data, in violation of the United States export laws or regulations.

7.8 Force Majeure. Company shall not be liable, directly or indirectly, for any delay or failure in performance of any obligation under this Agreement, including any delivery obligation, where such delay or failure arises or results from a cause beyond Company's reasonable control, or beyond the reasonable control of Company's suppliers or contractors, including, but not limited to strike, boycott or other labor disputes, embargo, governmental regulation, inability or delay in obtaining materials, acts of God, war, earthquake, fire, or flood. In the event of such force majeure, the time for delivery or other performance will be extended for a period equal to the duration of the delay caused thereby, provided that Company notifies Customer of the nature and duration of such force majeure event.

7.9 Entire Agreement; Notice. This Agreement constitutes the complete agreement between the parties and supersedes all prior or contemporaneous agreements or representations, written or oral, concerning the subject matter of this Agreement. Except as otherwise expressly provided in this Agreement, any modifications of this Agreement must be in writing and agreed to by both parties. Company may provide any notice to Customer by e-mail. Customer may provide notice to Company by sending an e-mail to info@proteinsimple.com or a letter by United States mail to ProteinSimple, 3001 Orchard Parkway, San Jose, CA 95134, or to such other address as Company may specify in writing by posting the new address on the Company website.

7.10 Relationship of the Parties. The parties are acting hereunder as independent contractors and not as partners, agents, fiduciaries, or joint venturers. Neither party has the power or authority represent, act for, bind, or otherwise create or assume any obligation on behalf of the other party.