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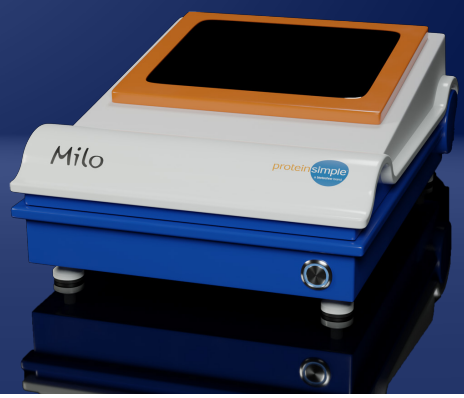
APPLICATION NOTE

IMPROVING ANTIBODY PERFORMANCE ON MILO WITH THE SIGNAL ENHANCEMENT REAGENT



MILO PERFORMS SINGLE-CELL WESTERN ANALYSIS

As the world's first Single-Cell Western platform, Milo performs immunodetection of protein targets in ~1000 single cells per run, offering unparalleled insight into population heterogeneity at the protein level.[1] In fact, publications that feature Milo data frequently appear in some of the world's premier scientific journals, including *Nature* and *Science*.[2] As an open platform, Milo is not restricted to any particular antibody. While many antibodies that have been validated for applications like Simple Western and traditional Western also work on Milo (see the Milo Antibody Database for a list of previously validated antibodies on Milo), other antibodies produce weak signal or no signal at all. To improve the performance of these antibodies, exchanging the Lysis/Run buffer with a 0.25 M glycine-HCl solution, called the Signal Enhancement Reagent, before the UV capture step may increase the signal intensity, or even produce signal for targets that did not have detectable signal with default assay conditions. In this Application Note, we show you how it may be possible to optimize the signal of poorly behaving antibodies with the Signal Enhancement Reagent on Milo.

MATERIALS AND METHODS

UPDATING THE MILO CONTROLLER SOFTWARE

Before using the Signal Enhancement Reagent, update the Milo Controller Software, which is the software on the Milo instrument that executes the lysis, electrophoresis, and UV capture of a loaded chip. Updating the software version to 2.3.0 (Beta) includes the ability to program up to 4 custom scripts, which makes swapping out the Lysis/Run Buffer before the UV capture step easier.

Stepwise instructions for updating the Milo Controller Software are described here.

1. Obtain the Install Guide and download the Milo Controller Software to a USB flash drive from the website: https://www.proteinsimple.com/scout/downloads/
2. Insert the USB flash drive into the USB slot on the right side of the back of Milo.
3. Follow the installer instructions.
4. Power cycle Milo when the installation is complete.
5. Confirm that the software version number is 2.3.0.

NAME	VENDOR	PART NUMBER
10^6/mL NALM Cells (included in the Milo Training Kit)	ProteinSimple	035-119
scWest Kit	ProteinSimple	K600
Milk-Free Antibody Diluent	ProteinSimple	043-524
Glycine-HCl for preparation of the Signal Enhancement Reagent	Sigma-Aldrich	G2879-500G
Rabbit anti-β-Actin Antibody	Cell Signaling Technology	8457
Mouse anti-β-Actin Antibody	Cell Signaling Technology	3700
Rabbit anti-β-Actin Antibody	Cell Signaling Technology	4970
Mouse anti-β-Tubulin Antibody	Genscript	A01717
Rabbit anti-β-Tubulin Antibody	Novus Biologicals	NB600-936
Goat anti-GAPDH Antibody	Novus Biologicals	NB300-320
Rabbit anti-AKT1 Antibody	Cell Signaling Technology	2938S
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A-11055
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Thermo Fisher Scientific	A-31572
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific	A-31571

TABLE 1. Reagents used in this study. All primary antibodies were diluted 1:10 in Milk-Free Antibody Diluent. All secondary antibodies were diluted 1:20 in Milk-Free Antibody Diluent.

The reagents used in this study are listed in **TABLE 1**.

PREPARING THE SIGNAL ENHANCEMENT REAGENT

To prepare the Signal Enhancement Reagent, add glycine-HCl to diH_2O at a final concentration of 0.25 M. The expected pH should be around 2. The Signal Enhancement Reagent should be stored at room temperature and it is stable for at least two weeks. Each Milo chip requires 15 mL of the reagent.

PROGRAMMING THE MILO CONTROLLER SOFTWARE TO USE THE SIGNAL ENHANCEMENT REAGENT

The new Milo Software Controller allows users to create up to 4 scripts for custom lysis, electrophoresis, and UV capture settings. To program the Milo Controller Software for use with the Signal Enhancement Reagent, two scripts can be created, one to perform the lysis/electrophoresis steps only, and the other to perform the UV capture step only. This allows for a simpler workflow for the buffer to be exchanged between electrophoresis and UV capture steps. Example instructions on how to do so are included here, but the settings (e.g. lysis and electrophoresis times) may vary depending on your cell type and target of interest.

1. Create two assay scripts on Milo, one for lysis/electrophoresis only, and the other for UV capture only, as shown in **FIGURE 1**.

- With the **Setup** tab selected, input appropriate **Lysis** and **Electrophoresis** settings and input "0 s" for **UV Capture** settings.
- Press the **Scripts** tab and then press **Populate 1** to save the lysis/electrophoresis assay.
- Select the **Setup** tab to input "0 s" for **Lysis** and **Electrophoresis** settings and input "240 s" for UV capture. Electrophoresis voltage settings can be left at "240 V".
- Then, select the **Scripts** tab again, and press **Populate 2** to save the UV capture assay.

2. To use these new assays, while in the **Scripts** tab, press the **Disabled** button. It will change to **Enabled** and 4 buttons will appear in the top left over the **Run** settings (**FIGURE 1**).

3. To run a saved assay, press on the appropriate numbered button to load the saved run settings. **FIGURE 1** shows the run settings when **1** is pressed.

4. Follow the standard procedure for preparing your chip and settling cells on the chip. *Refer to the product insert of the scWest Kit for directions on how to load the chip.*

5. Load the lysis/separation assay by pressing the **1** button. Then, place the chip in Milo and add Lysis/Run Buffer, quickly close the lid, and press the **Run** button.

6. When separation is complete, open Milo and remove the entire electrophoresis chamber with the chip. Carefully pour out the Lysis/Run Buffer in an appropriate waste stream. The chip should remain in the chamber due to surface tension.

7. Place the electrophoresis chamber back in Milo. Add 15 mL of the Signal Enhancement Reagent and close the lid. Then, run the UV capture assay by pressing the **2** button and then press the **Run** button.

8. Once the UV capture is complete, proceed with the standard procedure for chip washing and immunoprobing.

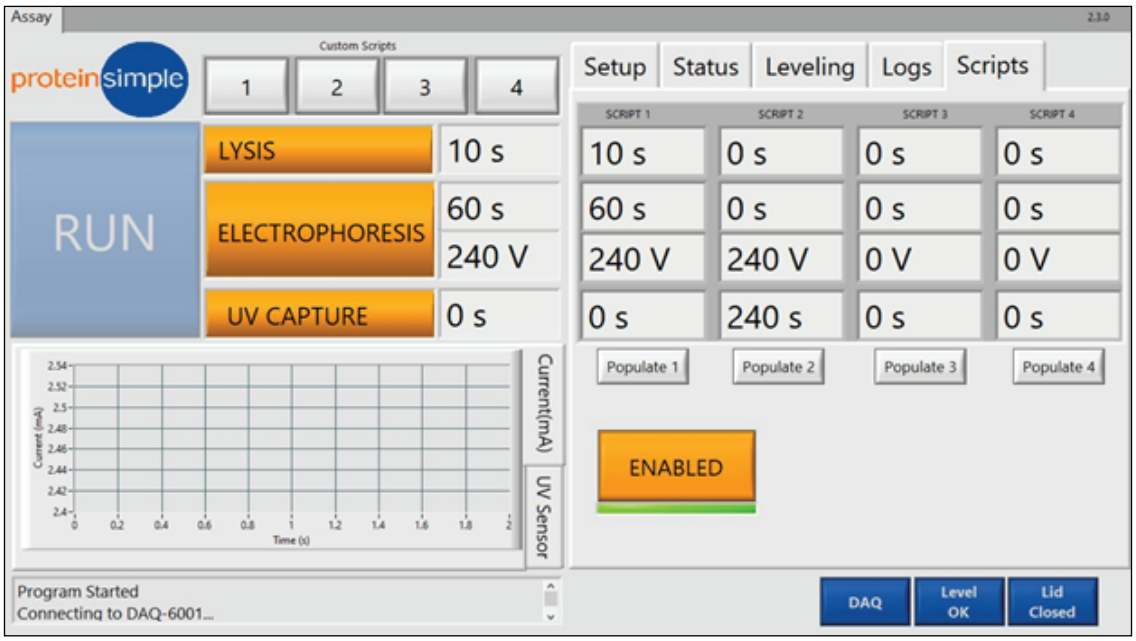


FIGURE 1. The new Milo Controller Software allows users to program up to 4 custom scripts on Milo. The Signal Enhancement Reagent is added after the lysis and electrophoresis steps and before the UV capture step. Thus, Scripts 1 and 2 shown here can be used in tandem to easily allow for the buffer exchange that occurs after the lysis and electrophoresis steps and before the UV capture step.

PUTTING THE SIGNAL ENHANCEMENT REAGENT TO THE TEST

The β-Actin antibody from Cell Signaling Technology (PN 8457) is an antibody that has been validated on other applications, including Western blot, immunofluorescence, and flow cytometry, but it does not produce a detectable signal under default assay conditions on Milo. By adding the Signal Enhancement Reagent before the UV capture step, as described in the Materials and Methods above, a robust signal appeared that was easily detectable by Scout™ software (**FIGURE 2**). Therefore, this is a great example of how the Signal Enhancement Reagent generates a strong signal where otherwise no signal was detectable under default assay conditions. It should be noted, however, that this trend does not necessarily apply to all antibodies.

Some antibodies may produce a detectable signal on Milo, but the signal they produce is weak or diffuse, which makes data analysis with Scout a challenge. When this occurs, the Signal Enhancement Reagent may improve the antibody signal, as was shown here for another β-Actin antibody from Cell Signaling Technology (PN 3700) (**FIGURE 3**). It should also be noted here, however, that the Signal Enhancement Reagent does not improve the signal of all antibodies.

Default assay conditions

Signal Enhancement Reagent

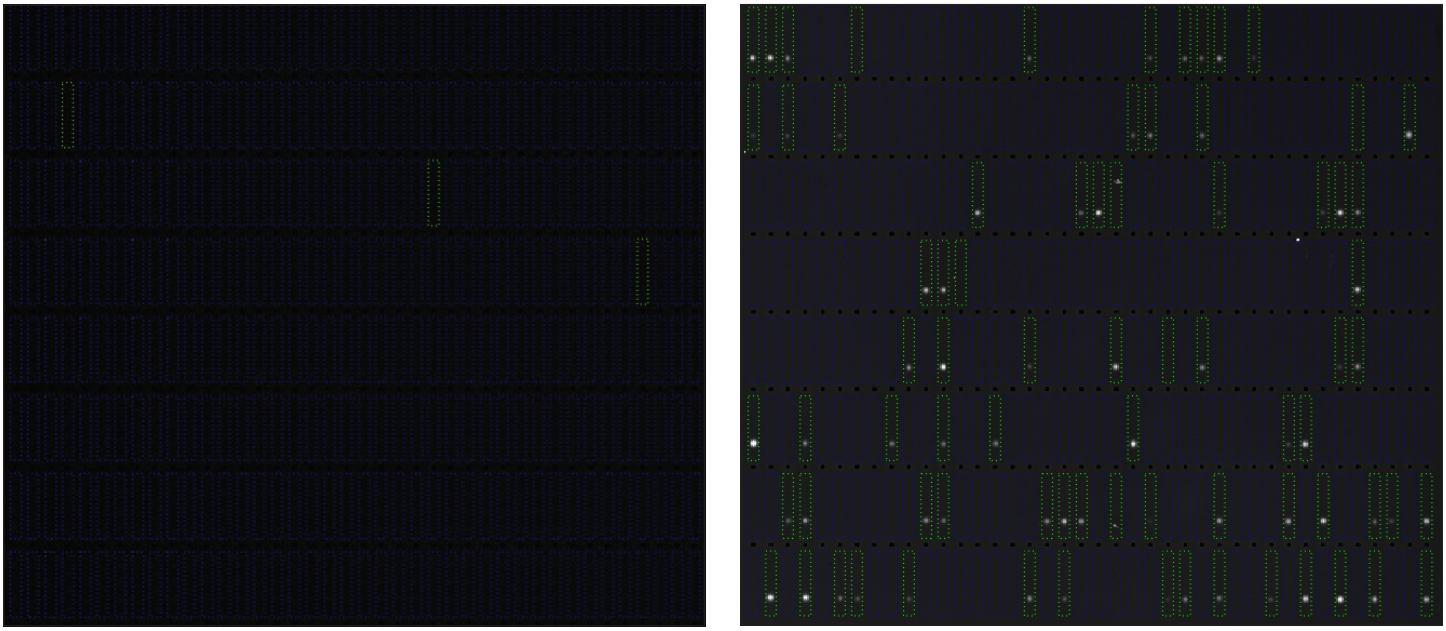


FIGURE 2. Use of the Signal Enhancement Reagent (right) results in a signal for β-Actin (Cell Signaling Technology, PN 8457) while no signal was detectable with the same antibody under default assay conditions (left). The Scout image contrast range was 0 – 65494 for the image on the left and 41 – 65535 for the image on the right.

Default assay conditions

Signal Enhancement Reagent

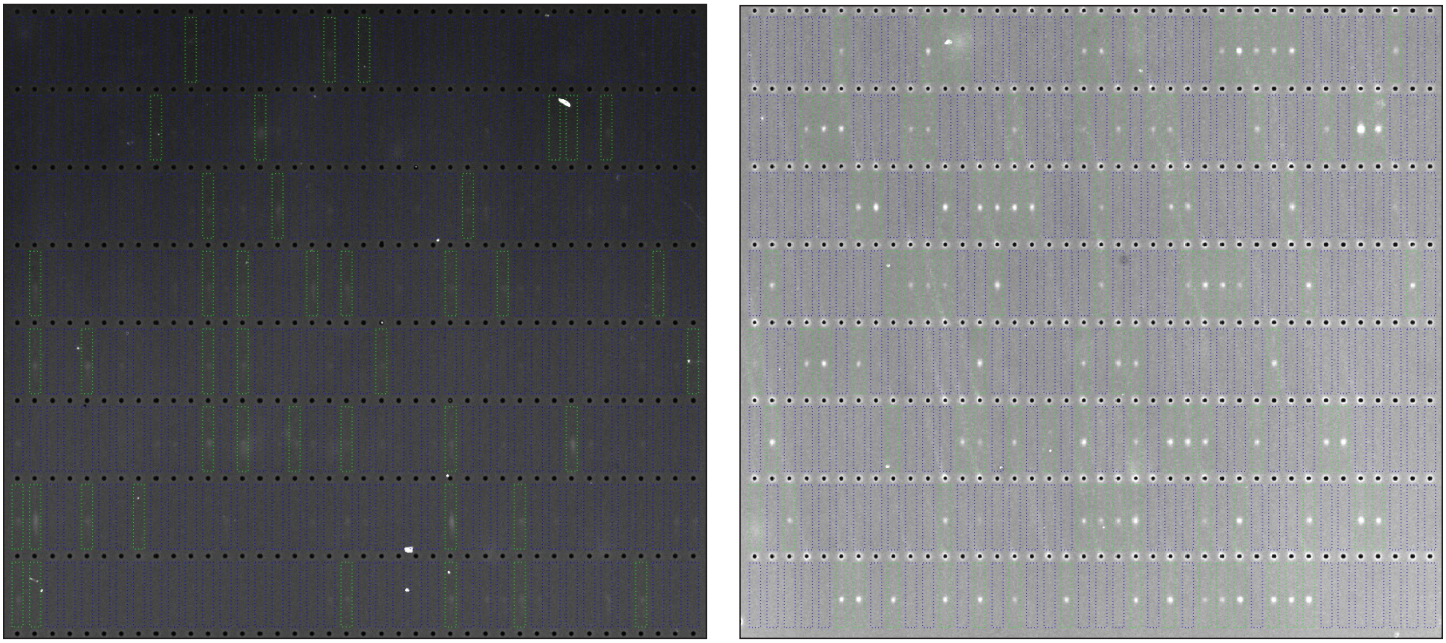


FIGURE 3. Use of the Signal Enhancement Reagent (right) results in an increase in signal intensity for β-Actin (Cell Signaling Technology, PN 3700) that is more intense than when the same antibody was used under default assay conditions (left). The Scout image contrast range was 12 – 10742 for the image on the left and 133 – 10863 for the image on the right.

Finally, we compared the peak area, signal to noise ratio (SNR), and background between default assay conditions and the Signal Enhancement Reagent for a panel of 7 antibodies (FIGURE 4). As demonstrated visually in FIGURE 2, a clear signal in terms of peak area and SNR for β-Actin 3 (Cell Signaling PN 8457) emerged in the presence of the Signal Enhancement Reagent, whereas no peaks were visible under default assay conditions (FIGURE 4). For β-Actin 2 (Cell Signaling PN 4970) under default assay conditions, no clear peaks were detected by Scout software when the SNR threshold was set to the default of 3, though faint peaks were visible on the chip. By contrast, in the presence of the Signal Enhancement Reagent, these peaks were more visible on the chip and became readily detectable by Scout software in terms of both peak area and SNR (FIGURE 4). For all other antibodies tested here, the SNR and peak area improved when the Signal Enhancement Reagent was used compared with the default assay conditions (FIGURE 4).

Due to the high biological variability that can exist in a sample of ~1000 or more cells, the error bars representing the standard deviations are large, and they overlap between the two assay conditions tested in some cases. However, the mean values for peak area and SNR are consistently increased in the presence of the Signal Enhancement Reagent. For all 7 antibodies, the fold increases in the peak area and SNR when the Signal Enhancement Reagent was used in place of the default assay conditions are shown in TABLE 2. Here, antibodies that have a fold increase of ∞ did not produce detectable signal under default assay conditions with Scout software when the SNR threshold was set to three. While we observed variability in background signal between default assay conditions and the Signal Enhancement Reagent, this depended on the antibody, suggesting that there was no clear correlation between Signal Enhancement Reagent and increased background signal (FIGURE 4).

TARGET	ANTIBODY VENDOR	PART NUMBER	PEAK AREA FOLD INCREASE	SNR FOLD INCREASE
GAPDH	Novus Biologicals	NB300-320	15.37	11.39
β-Tubulin 1	Genscript	A01717	8.83	4.66
β-Tubulin 2	Novus Biologicals	NB600-936	1.65	3.83
β-Actin 1	Cell Signaling Technology	3700	3.09	2.41
β-Actin 2	Cell Signaling Technology	4970	∞	∞
β-Actin 3	Cell Signaling Technology	8457	∞	∞
AKT1	Cell Signaling Technology	2938S	1.98	1.48

TABLE 2. Fold increase in peak area and SNR with the Signal Enhancement Reagent. The default assay conditions for β-Actin 2 resulted in no clear detectable peaks when SNR threshold was set to default value of 3 in Scout software; however, peaks with weak signal (having an SNR less than 3) were visible on the chip.

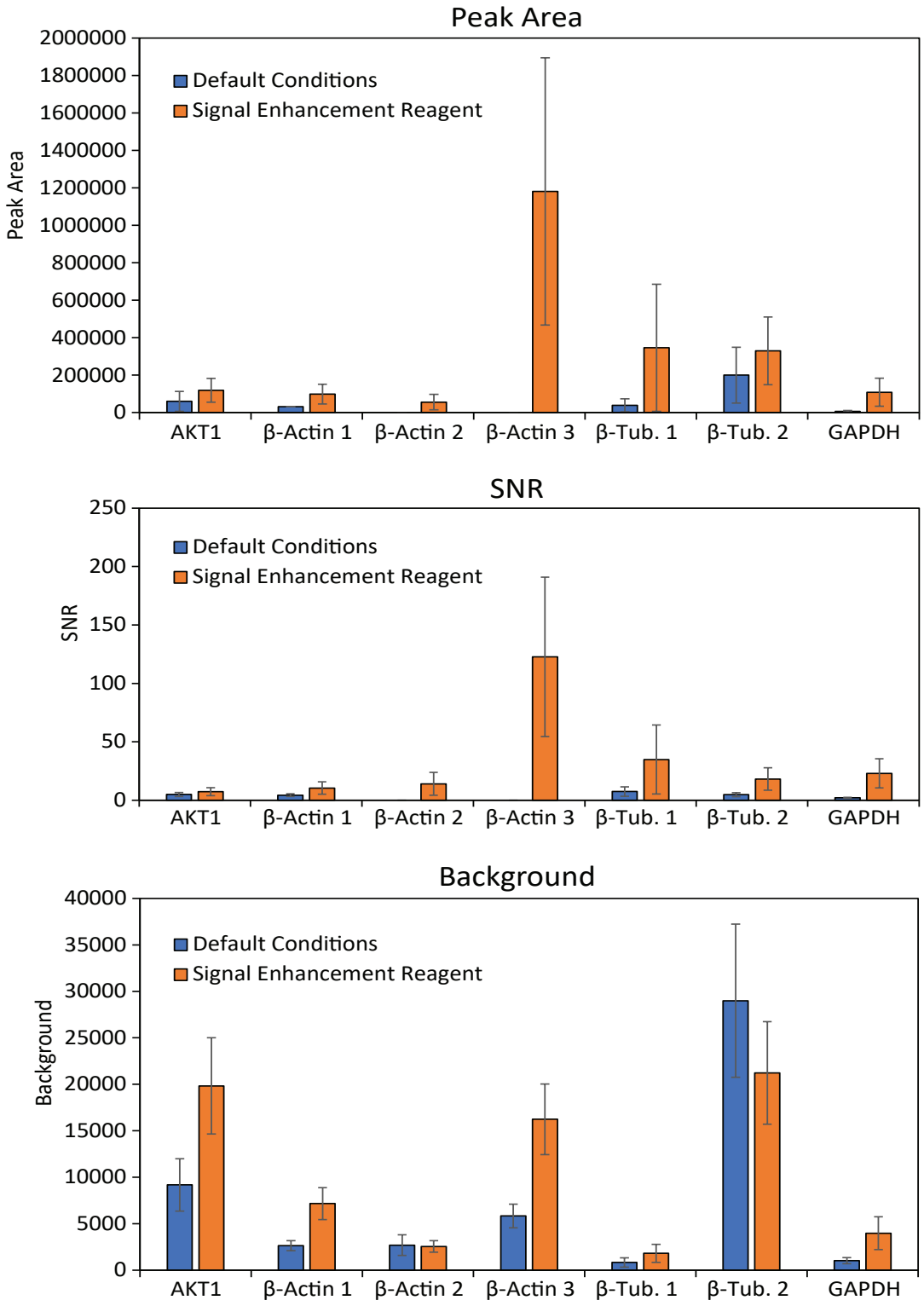
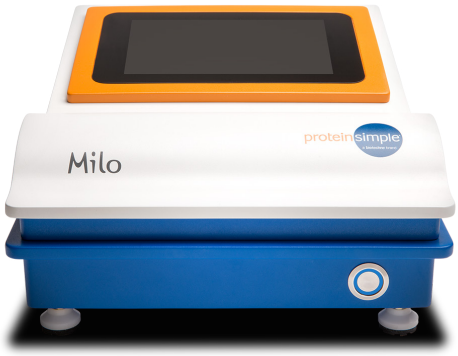


FIGURE 4. A comparison of background, peak area, and SNR between default assay conditions and assay using the Signal Enhancement Reagent. β-Actin 1, β-Actin 2, and β-Actin 3 correspond to Cell Signaling Technology PNs 3700, 4970, and 8457, respectively. β-Tubulin 1 and β-Tubulin 2 correspond to Genscript PN A01717 and Novus Biologicals PN NB600-936, respectively. The default assay conditions for β-Actin 2 resulted in no clear detectable peaks when SNR threshold was set to the default threshold of 3 in Scout software; however, peaks with weak signal were visible on the chip. Error bars represent the standard deviations of the means.

INCREASE THE ODDS OF FINDING THE RIGHT ANTIBODY

Many antibodies that work on Simple Western and traditional Western blots also work on Milo. When probing a new target, the Milo Antibody Database is a useful resource for identifying antibodies that have previously been validated on Milo, which may save time and effort when screening new antibodies. Antibodies for targets that are not listed in the database may work well on Milo, but you might find that some antibodies produce little or no signal, even if they have been validated for other applications like flow cytometry or traditional Western blot. To help you optimize poorly performing antibodies, we provide this protocol, which involves exchanging the Lysis/Run Buffer with the Signal Enhancement Reagent before the UV capture step. The Signal Enhancement Reagent may improve signal strength or generate signal where previously no signal could be detected. However, it should be noted that the Signal Enhancement Reagent does not improve the function of all antibodies, but it increases the likelihood that you will find an antibody that works for you.



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