Optimize Goat and Sheep Primary Antibody Performance on Milo Using Milk-Free Antibody Diluent

Introduction

The Milo™ Single-Cell Western™ system is an open platform that allows you to use any sample or western-blot validated primary antibody for maximum user flexibility. Most commercial primary antibodies are raised in rabbit, mouse, or goat requiring the use of fluorescently-labeled anti-rabbit, anti-mouse, and anti-goat secondary antibodies to detect signal with Milo. However, non-specific binding can sometimes be observed when using anti-goat and anti-sheep secondary antibodies with milk-containing buffers used for blocking or diluting antibodies in an immunoassay, negatively impacting assay results.

In this technical note, we demonstrate how diluting antibodies with Milk-Free Antibody Diluent when probing scWest chips with goat or sheep primary antibodies delivers improved assay performance. Goat antibodies diluted with Milk-Free Antibody Diluent exhibit increased peak area, increased signal-to-noise ratios (SNR), and decreased background levels. Therefore, we recommend diluting both primary and secondary antibodies in Milk-free Antibody Diluent whenever you're using goat or sheep primary antibodies in any probing cycle for optimal results. Antibody Diluent 2 should still be used when your experiment will not utilize goat or sheep primary antibodies in any probing cycle. Both diluents are shipped with all scWest kits for your convenience.

Materials and Methods

CELLS, ANTIBODIES, AND REAGENTS

Jurkat, Clone E6-1 peripheral blood T lymphocytes (ATCC) were maintained in RPMI-1640 media supplemented with 10% FBS (ThermoFisher) and incubated at 37 °C, 5 CO_2. Samples were probed for GAPDH using a goat anti-GAPDH primary antibody (Sigma, PN SAB2500450, lot 6377C3) and signal detected using donkey anti-goat Alexa Fluor 647 secondary antibody (Invitrogen, A21447).

MILO RUN SETTINGS AND IMMUNOASSAY CONDITIONS

Samples were run using a Standard scWest Kit (PN K600). The Jurkat suspension cells were diluted in 1X Suspension Buffer to 100K/mL concentration and cells were loaded onto a rehydrated Standard scWest chip. Cells were allowed to settle for 5 minutes before they were lysed for 10 seconds and then separated for 75 seconds at 240 V before UV capturing the protein into the gel for 4 minutes.

The scWest chip was then probed using a 3-chamber probing fixture in order to compare antibodies diluted in different diluents on the same chip. Goat anti-GADPH primary antibody was diluted 1:10 in either Milk-Free Antibody Diluent (PN 043-524) or Antibody Diluent 2 (PN 042-203) and applied to separate chambers for 2 hours at room temperature. Donkey anti-goat Alexa Fluor 647 secondary antibody was diluted 1:20 in either diluent and applied to the appropriate chamber for 1 hour at room temperature. Chips were imaged using an InnoScan 710 microarray scanner and .tiff files analyzed using Scout Software.

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Anti-goat and anti-sheep secondary antibodies can sometimes non-specifically bind to milk-containing blocking buffers or antibody diluents in immunoassays. Using a model system consisting of a goat primary antibody and donkey anti-goat Alexa Fluor 647 secondary antibody, we compared background signal, target peak area and target signal-to-noise ratio for Milk-Free Antibody Diluent and Antibody Diluent 2. Sheep antibodies have considerable homology with goat antibodies and thus anti-sheep secondary antibodies exhibit similar behavior to anti-goat secondary antibodies (data not shown).

A decrease in background was observed with the Milk-Free Diluent, indicating a reduction in non-specific antibody binding (**Figure 1**). Median background signal decreases from approximately 3000 to 1950 when Antibody Diluent 2 is replaced with Milk-Free Diluent for a 1.5X improvement.

Developing a successful immunoassay depends, in part, on finding a good balance between blocking non-specific binding while still allowing for specific antibody to target binding. To ensure we were still getting efficient detection of the GAPDH protein, we analyzed the target signal or peak area generated using Milk-Free Diluent compared to Antibody Diluent 2 for given antibody dilutions. There was a clear increase in the peak area generated with the Milk-Free Diluent immunoassay system indicating that there was more specific binding by the anti-goat secondary antibody (**Figure 2**). Median GAPDH signal was around 84,600 using Antibody Diluent 2 while signal increased to approximately 120,000 using Milk-Free Diluent for an increase of approximately 1.4X.

We then looked at the signal to noise ratio (SNR) to determine whether a decrease in background combined with an increase in peak area signal would translate to an improved SNR. Median SNR for Antibody Diluent 2 was approximately 9 which increased to 16.5 with Milk-Free Antibody Diluent for an overall SNR improvement of approximately 1.8X (**Figure 3**).

Conclusion

When developing a new immunoassay, selecting a good primary antibody to probe for your target of interest is of utmost importance. Often times this means screening through a collection of primary antibodies that may have been raised in different host species for optimal performance. Optimizing antibody diluents is another variable that can play a role in tuning assay specificity.

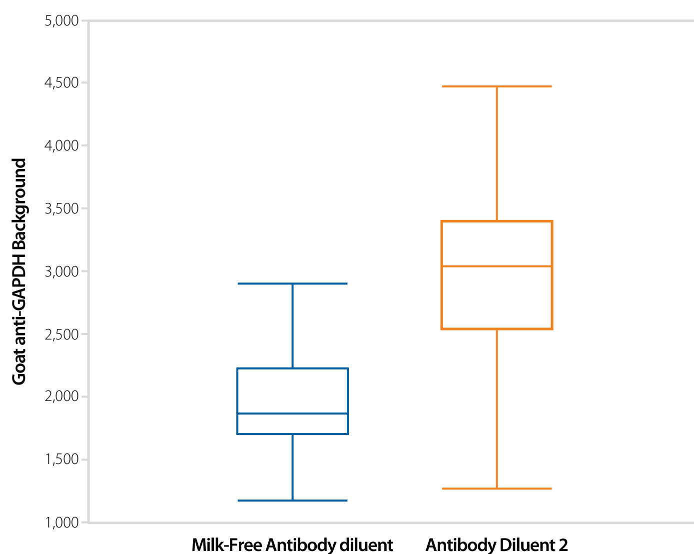

FIGURE 1. Background signal decreases when Milk-Free Antibody Diluent is used, demonstrating a reduction in non-specific antibody-binding.

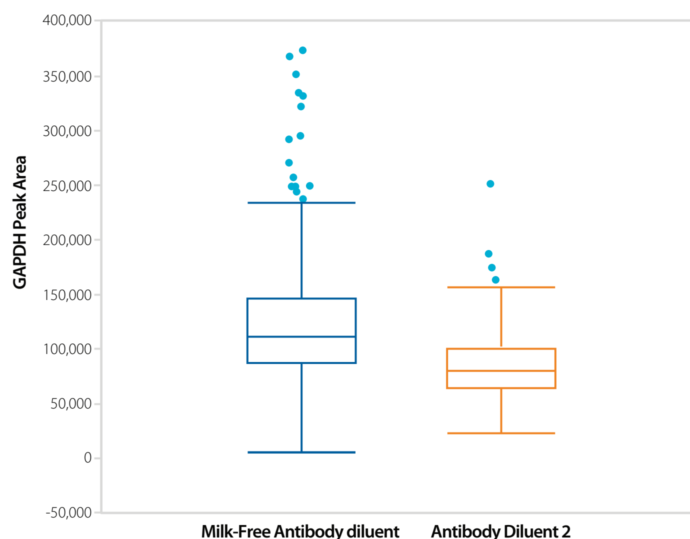

FIGURE 2. Target peak area increases when Milk-Free Diluent is used instead of Antibody Diluent 2.

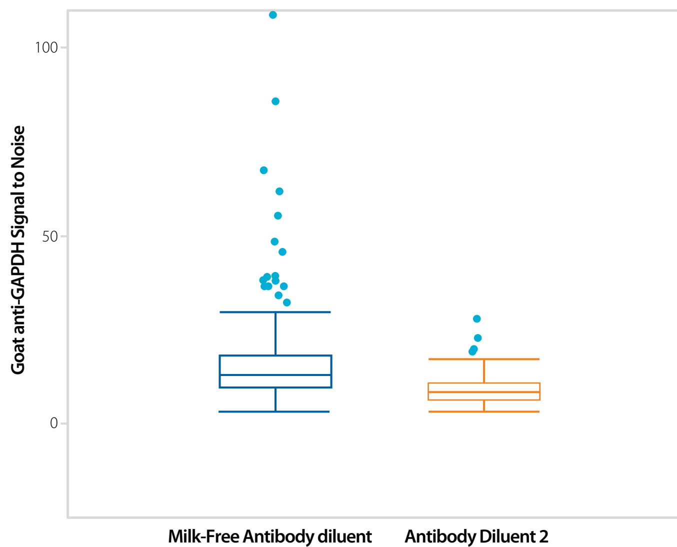

FIGURE 3. Peak signal to noise ratio (SNR) increases when Milk-Free Antibody Diluent is used, which translates into an increase in assay sensitivity.

We recommend diluting primary and secondary antibody cocktails in Milk-Free Antibody Diluent if a goat or sheep primary antibody will be used in any probing cycle on Milo. The same antibody diluent should be used for both primary and secondary antibody probing and across all probing cycles. Utilizing Milk-Free Antibody Diluent in the appropriate situation can deliver an increase in peak area, increase in the SNR, and a decrease in overall assay background compared to antibodies diluted in Antibody Diluent 2. Continue using Antibody Diluent 2 if no goat or sheep primary antibodies will be used in any probing cycle in your experiment.

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