Application Note – Phospho-flow

Abwiz Bio

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Phospho-flow: Flow cytometry screening using Abwiz Bio's phosphorylation-specific IgG antibodies

This note provides a detailed protocol for flow cytometry-based screening of a given target's phosphorylation level using one of Abwiz Bio's many phosphorylation-specific rabbit monoclonal antibodies. This protocol has been rigorously optimized and is used by our own scientists for product validation. If you have any questions about this protocol or need help troubleshooting your own experiments, our Ph.D.-level support team is standing by to help you publish great results!

Materials

- Cells, cultured to >90% viability and 80-90% confluency
- T75 cell culture flask (or appropriate cell culture vessel)
- PBS
- 0.5% trypsin solution
- 50 mL falcon tubes
- 16% paraformaldehyde solution
- Ice-cold 100% methanol
- 96-well V-bottom plate
- FACS buffer (1% BSA, PBS, 0.05% sodium azide)
- Phospho-specific IgG antibody (choose from Abwiz Bio's large collection of validated mAbs!)
- Isotype control antibody (e.g. Abwiz Bio Cat. #2141)
- anti-rabbit IgG secondary antibody
- Flow cytometer with 96-well plate reader

Cell treatment and preparation

The cell treatment procedure will vary depending on the cell line and the desired treatment(s) to be used. This representative protocol is for HeLa (adherent) cells.

1. **Cell culturing**. Culture the desired cell line in the appropriate medium to >90% viability. Seed cells the day before in T75 flasks. When cells reach 80-90% confluence, aspirate media to remove.
2. **Cell treatment**. Add the appropriate medium containing the desired amount of treatment additive. Incubate cells at 37°C for the desired length of time.
    a. Treatment conditions will vary based on the phospho target and cell type and may require optimization.
3. **Detach cells**. Aspirate the media supernatant. Wash cells by adding 10 mL of room-temperature PBS to the flask, then aspirate the PBS. Repeat for a total of two PBS washes. Add 1.5 mL of 0.5% trypsin and incubate at 37°C for 1-3 min. Detach cells from the flask by adding 10 mL of PBS and using gentle pipetting. Transfer cell suspension to 50 mL falcon tubes.
4. **Wash cells**. Pellet cells 1,500 x g for 5 min. Aspirate the supernatant and resuspend in 10 mL of PBS. Repeat for a total of two washes. Resuspend the final cell pellet in 2 mL of PBS and count the cells.

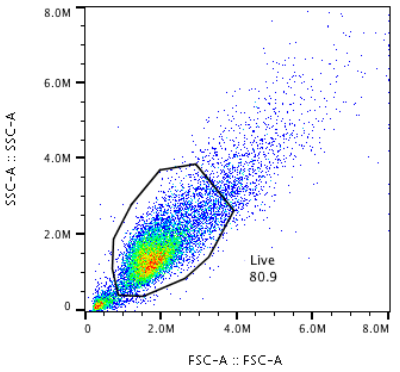
5. **Fix cells**. Add 100 µL of 16% paraformaldehyde per 1 mL of cell suspension. Incubate cells at room temperature for 10 min.
6. **Resuspend cells**. Pellet cells 1,500 x g for 5 min. Aspirate most of the supernatant, resuspending the cell pellet in the remaining 500 µL – 1 mL solution. Add ice-cold 100% methanol to achieve 2-4x10^6 cells/mL density (and >90% final methanol concentration) and vortex to mix. Incubate cells for 20 min at 4°C. Cells can be used immediately for flow analysis or stored at -20°C for up to four months.

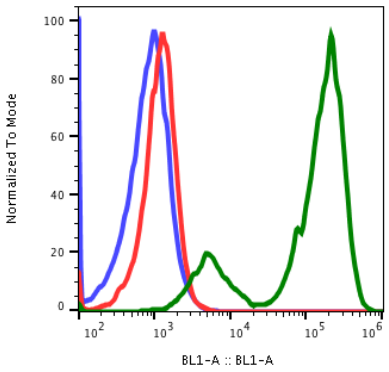
Flow cytometry screening

1. **Cell aliquot and wash**. Aliquot ~1x10^5 cells/well (25µL of 4x10^6 cells/mL suspension) to a 96-well V-bottom plate. Add 200 µL of FACS buffer to each well. Pellet cells 2,000 rpm for 5 min at 4°C. Aspirate supernatant.
2. **Primary antibody incubation**. Prepare primary solutions by diluting antibodies in FACS buffer. Resuspend cells in 100 µL of antibody solution by pipetting. Incubate at 4°C for 1 hour.
    a. The recommended dilution for each of Abwiz Bio's phospho-specific IgG products is posted in the figure legend on the product page. Your experiment may require optimization by titration at multiple concentrations.
    b. Don't forget to include the following controls:
        i. Secondary only – Resuspend cells in FACS buffer only during the primary incubation step
        ii. No secondary – Resuspend cells in FACS buffer only during both the primary and secondary incubation steps
        iii. Isotype control – test at the same concentration as the primary antibody ***For best results, use Abwiz Bio's isotype control rabbit mAb Cat. #2141.***
3. **Wash**. Pellet cells 2,000 rpm for 5 min at 4°C. Aspirate supernatant. Wash by resuspending cells in 200 µL FACS buffer by pipetting. Repeat spin and aspirate supernatant.
4. **Secondary antibody incubation**. Prepare the secondary solution using an anti-rabbit IgG secondary antibody at the dilution recommended by the manufacturer. It may be necessary to titrate the secondary antibody to determine the optimal concentration. Resuspend cells in 100 µL of secondary antibody solution by pipetting gently. Incubate at 4°C for 30 min protected from light.
    a. All Abwiz Bio phospho-specific rabbit mAbs have been tested using DyLight™ 488 Donkey anti-rabbit IgG (Biolegend 406404) secondary antibody at 1:500 dilution.
5. **Wash**. Pellet cells 2,000 rpm for 5 min at 4°C. Aspirate supernatant. Wash by resuspending cells in 200 µL FACS buffer by pipetting. Repeat spin and aspirate supernatant.
6. **Resuspend cells and read plate**. Resuspend cells in 100 µL FACS buffer by pipetting. Read cells on flow cytometer.

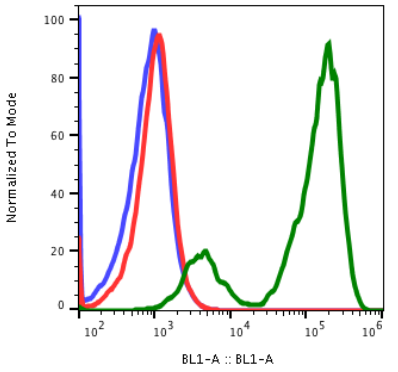
Expected results

Below are representative results from a titration study using Phospho-Slp76 (Y128) rabbit mAb clone Slp76Y128-3F8, Cat. #2136. The antibody was titrated from 1 µg/mL to 1 ng/mL using Ramos cells, serum starved overnight, then untreated or treated using 1mM pervanadate.





SampleID	IgG conc, ug/mL	Count	Median : BL1-A
Pv 3F8	1	12843	148885
Ctrl 3F8	1	10312	1192
Ctrl 2' only	-	13364	845



SampleID	IgG conc, ug/mL	Count	Median : BL1-A
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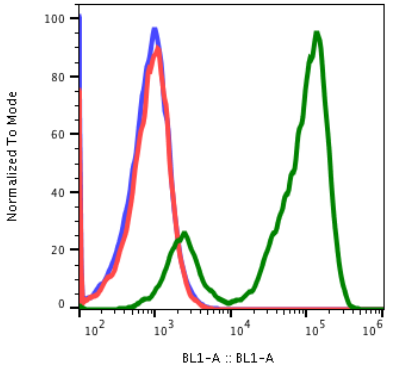

SampleID	IgG conc, ug/mL	Count	Median : BL1-A
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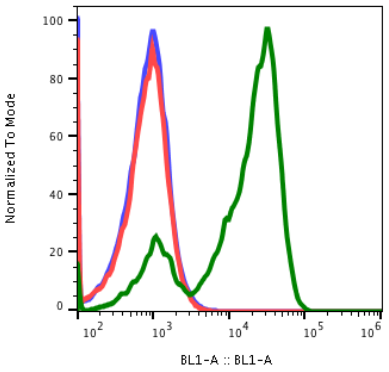

SampleID	IgG conc, ug/mL	Count	Median : BL1-A
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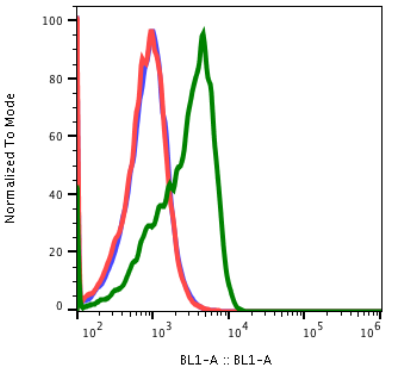

SampleID	IgG conc, ug/mL	Count	Median : BL1-A
Pv 3F8	0.001	13390	2996
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Ctrl 2' only	-	13364	845