2024.04.15 – by Dylan Husmann

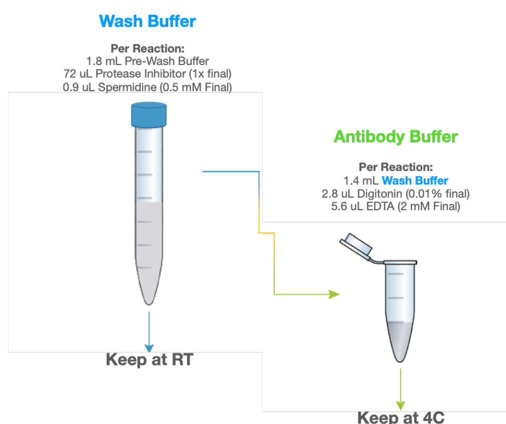
Validation of Antibodies for CnR by Flow Cytometry

Experimental Design: Use at least 3 conditions:

- **IgG Negative Control** (untreated cells incubated with IgG negative control): Diagnostic control to ensure staining is working properly. Typically done with 1 replicate for this condition.
- **Control** (untreated cells incubated with primary antibody/antibodies of interest) : Critical condition that informs the amount of target-specific antibody binding. Typically done with 2 replicates for this condition.
- **Knockout/Knockdown** (cells treated with sg/sh/si-mediated knockdown of antibody target, incubated with primary antibody/antibodies of interest): Critical condition that informs the amount of target-specific antibody binding. Typically done with 2 replicates for this condition.

Starting Material: ~500,000 cells (or nuclei- see *Troubleshooting*) **per replicate**

1. Gather kit reagents stored at 4˚C and -20˚C needed: **Pre-Wash Buffer, Digitonin, Spermidine, Primary Antibody, Secondary Antibody.** Place on ice to thaw/equilibrate.
2. Dissolve 1 protease inhibitor tablet (Roche) in 2 mL water for a **25X Protease Inhibitor** stock. After buffer prep, the remaining 25X stock can be stored for 12 weeks at -20˚C.
3. Prepare **Wash Buffer** by combining Pre-Wash Buffer, 25X Protease Inhibitor, and 1M Spermidine as outlined in Table 1. Store final buffer at RT.
4. To a new tube labeled **Antibody Buffer**, add **Wash Buffer**, 5% Digitonin, and EDTA as in Table 1. Place on ice.



COMPONENT	[FINAL]	1 RXN	8 RXN	16 RXN
Wash Buffer - store at room temperature.				
Pre-Wash Buffer	-	1.8 mL	14.4 mL	28.8 mL
25X Protease Inhibitor	1X	72 µL	576 µL	1.15 mL
1 M Spermidine	0.5 mM	0.9 µL	7.2 µL	14.4 µL
Antibody Buffer - store on ice				
Wash Buffer	-	**1.4 mL**	**11.2 mL**	**22.4 mL**
5% Digitonin	**0.01%**	**2.8 µL**	**22.4 µL**	**44.8 µL**
0.5 M EDTA	**2 mM**	**5.6 µL**	**44.8 uL**	**89.6 uL**

5. Count starting cells and confirm integrity via trypan blue. Harvest 500,000 cells per reaction and spin down in 1.7 mL tubes at 600 x g for 3 min at RT. Remove supernatant.
6. Resuspend cells in 200 µL per reaction RT **Wash Buffer** by gentle yet thorough pipetting. Spin at 600 x g, 3 min, RT. Pipette very carefully to remove supernatant – if you are unable to remove it without disturbing cells, decanting may work better.
7. **Repeat** the previous step **once.**
8. Resuspend cells in 100 µL per reaction cold **Antibody Buffer**.
9. Add 1 µg primary antibody (or manufacturer's recommendation) to each reaction. For negative control reaction, add 1µL **IgG Negative Control Antibody (Alternatively, if lacking IgG negative control, can use no primary antibody for negative control).**
10. Gently vortex to mix. Incubate **4 hours - overnight** on a **nutator** at 4˚C with tube caps elevated. Do **NOT** rotate, as this will cause sample to dry out.
11. Remove tubes from 4˚C incubation and quick spin to collect liquid.
12. Resuspend cells in 200 µL per reaction cold **Antibody Buffer** by gentle yet thorough pipetting. Spin at 600 x g, 3 min, 4C.
13. **Repeat** the previous step **twice** for **a total of 3 washes with Antibody Buffer.**
14. Resuspend cells in 100 µL per reaction cold **Antibody Buffer**.
15. Add 0.5 µg secondary antibody (flurophore conjugated to species-matched to primary antibody) to each reaction. Treat IgG negative Control sample the same as all other samples here.
16. Gently vortex to mix. Incubate **2 hours** on a **nutator** at 4˚C with tube caps elevated. Do **NOT** rotate, as this will cause sample to dry out.
17. Remove tubes from 4˚C incubation and quick spin to collect liquid.
18. Resuspend cells in 200 µL per reaction cold **Antibody Buffer** by gentle yet thorough pipetting. Spin at 600 x g, 3 min, 4C.
19. **Repeat** the previous step **twice for a total of 3 washes with Antibody Buffer**
20. Resuspend in desired buffer for flow cytometry and immediately process cells. Can mix a very small amount of cells (~5% of total) from **IgG Negative Control** and **Control+Primary antibody** to set voltage range for laser + filters of interest.
21. Antibody Evaluation: A suitable antibody should show **significant reduction of signal in the Knockdown** vs **Control** samples (No antibody control should show much, much lower signal than either the Control or Knockdown samples.)

Troubleshooting: **High cytoplasmic signal:** If you have reason to believe your antibody has significant **cytoplasmic signal** in addition to high **on-target nuclear signal**, the above protocol likely won't work as you will end up with both cytoplasmic and nuclear signal when looking at cells by Flow.

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Solution: **Perform nuclear isolation** as recommended in Epicypher CnR Kit. Once isolated nuclei have been obtained, perform this protocol just as you would do for cells. If nuclear isolation is necessary for testing your antibody, you should strongly consider performing nuclear isolation for eventual Cut&Run assays using this antibody.