Jackson ImmunoResearch LABORATORIES, INC.

Specializing in **Secondary Antibodies** and **Conjugates**

Technical Note:

FabuLight™ - Fab Labeling primary antibodies with fluorophore-conjugated Fab anti-Fc

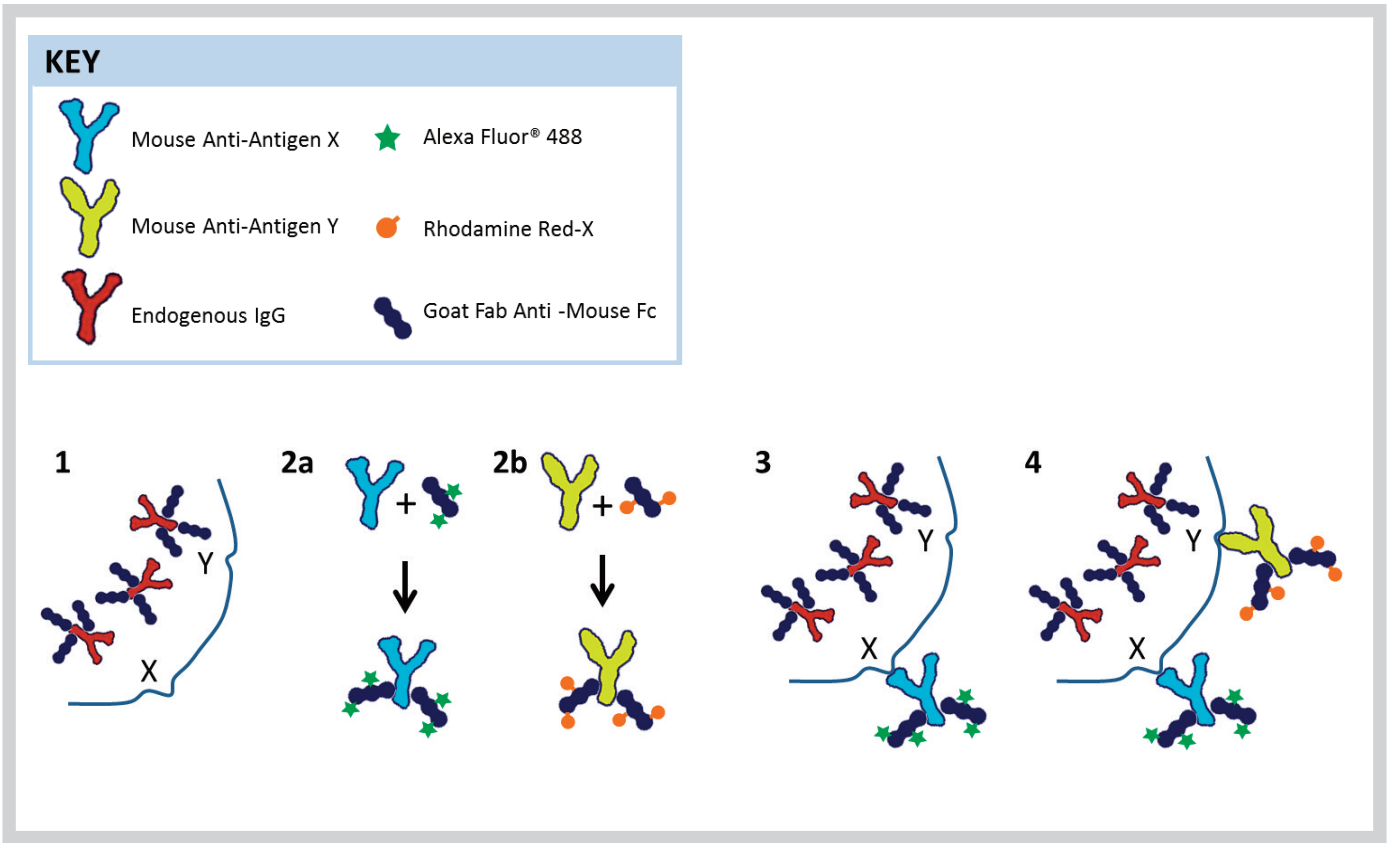
For certain applications it may be convenient to Fab-label primary antibodies prior to incubation with cells or tissue. This can be accomplished by complexing the primary antibody with a dye-conjugated Fab anti-Fc antibody (either anti-IgG, Fcγ specific or anti-IgM, μ chain specific). Because the labeled Fab fragment is monovalent the complex does not precipitate, and binding to the Fc portion of the primary antibody leaves the antigen-binding region active. Fab anti-Fc antibodies are also useful for labeling cell surface immunoglobulins without cross-linking and activating B cells, or for labeling Fc chimeras (fusion proteins).

The Fab-labeled complexes do not provide as bright a signal as sequential incubation with a primary antibody followed by a labeled secondary antibody. The Fab anti-Fc antibodies have not been adsorbed to remove cross-reactivities to other species, so it may be necessary to block endogenous IgG prior to incubation with an unlabeled Fab anti-IgG(H+L) prior to application of a Fab-labeled complex.

Possible applications include flow cytometry, where a Fab-labeled primary antibody allows fewer washes than sequential incubations and washes with primary and labeled secondary antibodies; and immunocytochemistry or immunohistochemistry, where several primary antibodies raised in the same host animal can be sequentially labeled with minimal incubation and wash steps. For all multiple labeling applications, we recommend incubating secondary antibodies sequentially to minimize cross-reactivity, and this is especially important when using Fab-labeled complexes. To avoid displacement of the Fab-primary antibody by a subsequent labeled secondary antibody, a light cross-linking with glutaraldehyde may be necessary, provided that it does not affect antigenicity of subsequent target proteins.

Optimal protocols for each application must be established empirically. Label the less abundant target antigen first for optimal results. Complexing at a 3:1 molar ratio of Fab:primary antibody (equal weight ratios) provides a good degree of labeling of the primary antibody without excessive amounts of unbound Fab. Titrating Fab-labeled complexes vs. their target antigens will minimize the amount of free Fab anti-Fc, thereby minimizing potential cross-talk in a multiple labeling application.

Use of FabuLight - Fab-labeled primaries for labeling two antigens on tissue



1. Include a blocking step if the tissue of interest displays immunoglobulin that could be recognized by the FabuLight, for instance mouse tissue that is to be labeled with a mouse specific FabuLight. Incubate with unconjugated Fab anti-Fc antibody, in this example Fab Goat Anti-Mouse IgG1, Fcγ fragment specific. Fab Goat Anti-Mouse IgG (H+L) could also be used for this step. Wash.

2. Create Fab-primary antibody complexes. In this example Mouse Anti-Antigen X is complexed with Alexa Fluor® 488-conjugated Fab Goat Anti-Mouse IgG1, Fcγ fragment specific; and Mouse Anti-Antigen Y is complexed with Rhodamine Red-X-conjugated Fab Goat Anti-Mouse, IgG1 Fcγ fragment specific.

3. Incubate the sample with Mouse Anti-Antigen X (less abundant target) complexed with Alexa Fluor® 488-conjugated Fab Goat Anti-Mouse IgG1, Fcγ fragment specific. Wash.

4. Incubate the sample with Mouse Anti-Antigen Y (more abundant target) complexed with Rhodamine Red-X-conjugated Fab Goat Anti-Mouse IgG1, Fcγ fragment specific. Wash.

Importance of sequential labeling and titrating primary vs. target antigen

Figures 1-5 show how an optimized multiple labeling method can improve results. The potential for cross-reactive interactions of the FabuLights is minimized by sequentially labeling the less abundant target antigen first, and by titrating Fab-labeled complexes vs. target antigen.

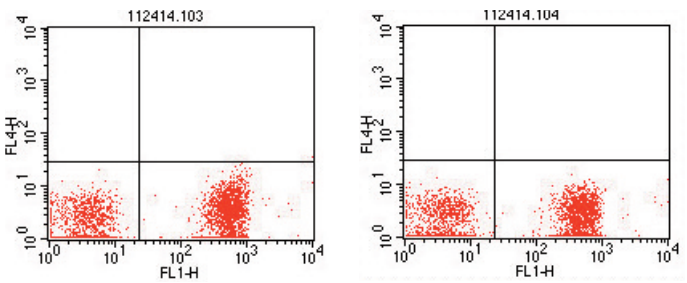
Blood was collected from normal human donors and treated with lysis buffer. Cells were centrifuged, washed, and resuspended in isotonic PBS + 0.5% BSA.

Complexes were formed using Fab anti-Fc:primary at 3:1 molar ratio (equal weight ratios). Alexa Fluor® 488-conjugated Fab Goat Anti-Mouse IgG1, Fcγ fragment specific was complexed with Mouse Anti-Human CD3 (BD #555330)(**AF488/CD3**); and Alexa Fluor® 647-conjugated Fab Goat Anti-Mouse IgG1, Fcγ fragment specific was complexed with Mouse Anti-Human CD19 (BD #555410)(**AF647/CD19**).

Cells were incubated with complexed antibodies under various staining conditions for 30 minutes, washed, and analyzed on a BD FACSCalibur, gated on lymphocytes.

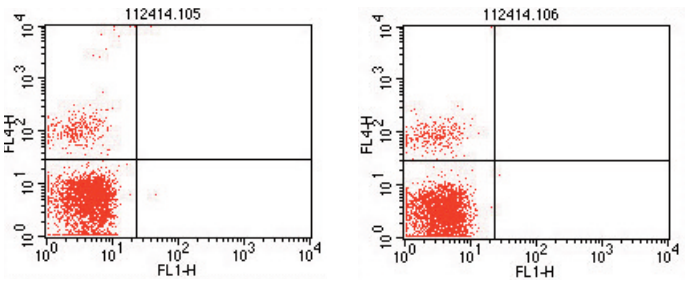
1. **Cells incubated with AF488/CD3 and washed. A large population of T cells is visible in LR quadrant.**

   Left figure *1.0 µg anti-CD3/tube.*
   Right figure *0.5 µg anti-CD3/tube.*



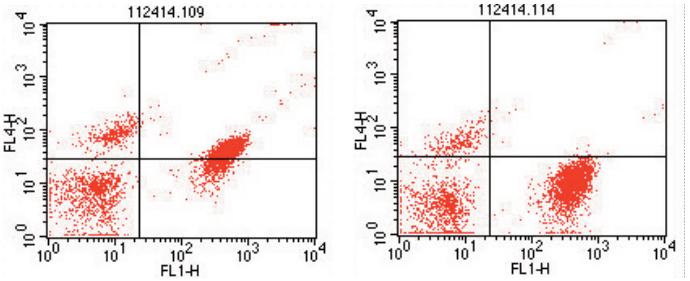
2. **Cells incubated with AF647/CD19 and washed. A smaller population of B cells is visible in UL quadrant.**

   Left figure *1.0 µg anti-CD19/tube*
   Right figure *0.1 µg anti-CD19/tube.*



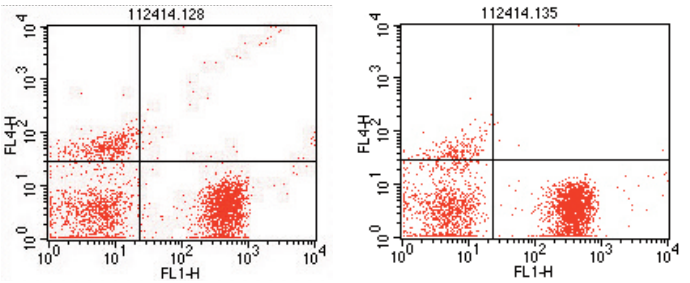
3. Cells incubated with a cocktail of *AF488/CD3* and *AF647/CD19* and washed.

Left figure: *Anti-CD3 and anti-CD19 both at 1 µg of antibody/tube.* Labeled Fab fragments are able to bind to incorrect primary antibody, resulting in unacceptable cross-talk. Most notably, excess AF647-FabuLight has bound to anti-CD3 on T cells, shifting the T cell population into UR quadrant.
Right figure: *Anti-CD3 is at 0.5 µg/tube, anti-CD19 is at 0.1 µg/tube.* Titrated antibodies still result in cross-talk, though T cells have remained in the LR quadrant with insignificant loss of signal.



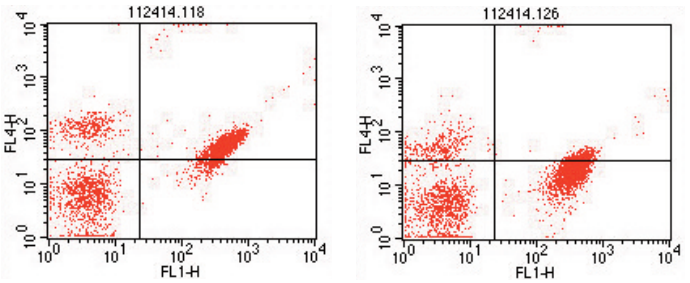
4. Cells incubated with AF647/CD19 (primary to less abundant target used first), washed, then incubated with AF488/CD3 and washed. Note specific labeling of T cells in LR quadrant and B cells in UL quadrant.

Left figure: *Anti-CD3 and anti-CD19 both at 1 µg/tube.*
Right figure: *Anti-CD3 is at 0.5 µg/tube, anti-CD19 is at 0.1 µg/tube.* Titrated antibodies produce cleaner specific labeling with insignificant signal differences.



5. Cells incubated with AF488/CD3 (primary to more abundant target used first), washed, then incubated with AF647/CD19 and washed.

Left figure: *Anti-CD3 and anti-CD19 both at 1 µg/tube.* Excess AF647-FabuLight has bound to anti-CD3 on the T cells, shifting the population into UR quadrant.
Right figure: *Anti-CD3 is at 0.5 µg/tube, anti-CD19 is at 0.1 µg/tube.* T cells have shifted down. Further titration of anti-CD19 can improve this result, but labeling the less abundant target antigen first (as in #4) avoids the problem.



Jackson ImmunoResearch Laboratories Inc.
872 West Baltimore Pike | West Grove | PA USA 19390

Tel: 800-367-5296 Fax: 610-869-0171 Email:cuserv@jacksonimmuno.com www.jacksonimmuno.com