Conjugation Tricks: Optimization of antibody labeling with novel DNA-based fluorescent labels

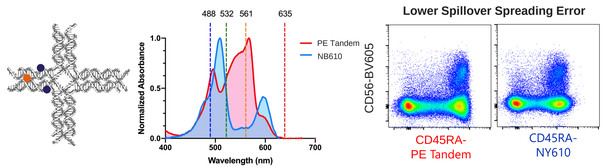
Nicholas Pinkin, Aaron Stroud, Seddon Thomas, Craig LaBoda, Thermo Fisher Scientific, 300 Morris St. Suite 720, Durham, NC 27701

Abstract

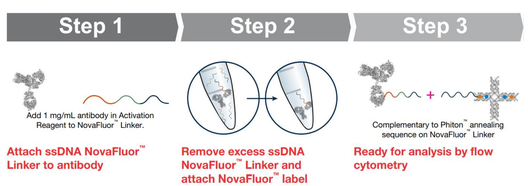
Invitrogen™ NovaFluor™ conjugates enable scientists to take full advantage of their cytometers by utilizing as many channels as possible while minimizing compensation issues due to cross-excitation and spectral spillover. While the number of NovaFluor-antibody conjugates available is rapidly growing, NovaFluor Antibody Conjugation Kits offer researchers the flexibility to create conjugates to any antibody of interest, making them an important tool for researchers studying new targets, lesser-studied species, or looking to fill a spot in their panel with a specific clone-conjugate pairing. Here we present improvements to the protocol to shorten it from three days to one day and discuss various "hacks" to the kit to maximize its utility. While the kits are designed to create 100 µg of a single NovaFluor-antibody conjugate, the conjugation process creates an intermediate antibody-oligo conjugate that can be combined at any scale with any NovaFluor dye sold with the conjugation kits. This allows researchers to create N x N combinations of antibody x NovaFluor dye to rapidly screen different pairings of dye colors and targets without purchasing a kit for every possible combination. We demonstrate for a group of three antibodies and three NovaFluor dyes how nine different combinations can be quickly conjugated and tested at a small scale, allowing the separation index to be tuned to suit the marker. The conjugation kit provides a "one size fits most" protocol that is designed to work for as many targets as possible but may not work ideally for all conjugates. Some antibodies are more sensitive to over-labeling with the oligo that tethers to the NovaFluor dye and perform better with a different degree of substitution. We demonstrate the variables to tweak to control this labeling and show how these changes alter the performance of different antibodies. Exciting new additions to the NovaFluor family may sometimes be available only through a conjugation kit at first, making the conjugation kit the best and fastest route to test new colors in a desired application. With the information presented here, we hope to empower researchers to modify and maximize the utility of NovaFluor conjugation kits and confidently investigate the power of NovaFluor dyes for any antibody of interest.

Introduction

The Invitrogen NovaFluor Platform. Our DNA-based nanostructure acts as a scaffold to arrange fluorophores into FRET networks with engineered spectra. This allows us to create labels with significantly lower spillover across excitation and detection channels, opening detectors for scientists to increase panel complexity and reduce spillover spreading errors.

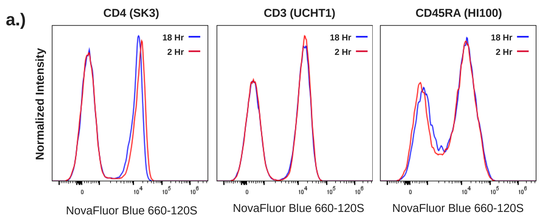


NovaFluor Antibody Conjugation Kits allow researchers to conjugate 100 µg of antibody to any NovaFluor in three simple steps. The first step is a quick, 1-hour activation to enable the antibody to react with a single-stranded DNA (ssDNA) linker that complements a single-stranded extension on the NovaFluor. The antibody-oligo conjugation reaction incubates overnight. Then, the next day the excess linker is removed by precipitation (step 2), which takes about 30 minutes. In the final step, the antibody and NovaFluor are annealed together by simply mixing 1:1 and incubating overnight at 4˚ C.

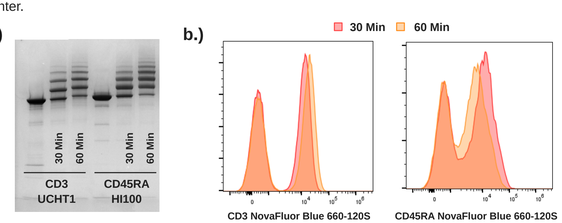


Results

Conjugation in One Day vs. Three. While the NovaFluor Conjugation Kit is simple to perform, the overall procedure takes three days due to two overnight incubations. To reduce the procedure to a single day, we tested the effect of shortening both incubations (steps 1 and 3) to 1-2 hours. **(a.)** The first overnight incubation is the reaction of the activated antibody with the ssDNA linker. We compared the performance of three antibodies (Anti-Hu CD4 (SK3), Anti-Hu CD3 (UCHT1), and Anti-Hu CD45RA (HI100)) using a 2-hour or 18-hour reaction time, followed by conjugation to NovaFluor Blue 660-120S. Comparing the staining on human Peripheral Blood Mononuclear Cells (PBMCs), we observed no loss of performance at the 2-hour reaction time. **(b.)** The second overnight incubation is to anneal the NovaFluor dye to the antibody-oligo intermediate. We compared the performance of three colors annealed to CD4 (SK3) for 18-hours or just 1 hour prior to staining and again observed no difference in performance at the shorter time point. Taken together, these changes enable the entire procedure to be completed in about 5 hours instead of 3 days, allowing functional testing the same day the conjugation is performed.

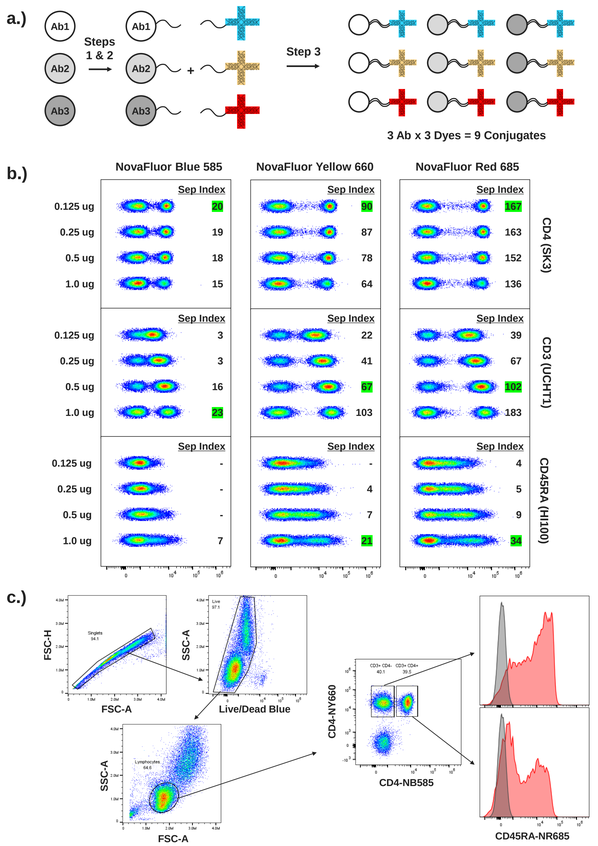


Optimization of Antibody Performance by Tweaking Activation Time. In the first step of the protocol, 100 µg of antibody is added to the activation reagent and allowed to incubate at room temperature for 1 hour, priming it for reaction with the ssDNA linker. A one-hour activation was found to be ideal for a wide variety of clones, but the performance of an antibody can potentially be improved with shorter activation time. To demonstrate, CD3 (UCHT1) and CD45RA (HI100) were activated for 30 or 60 minutes, otherwise following the standard protocol. **(a.)** By PAGE, labeling of the antibody-oligo intermediate is clearly time dependent, showing higher oligo labeling with longer activation time. **(b.)** In flow, CD3 and CD45RA exhibit opposite changes in performance with the shortened activation time, with CD3 getting dimmer and CD45RA getting brighter.



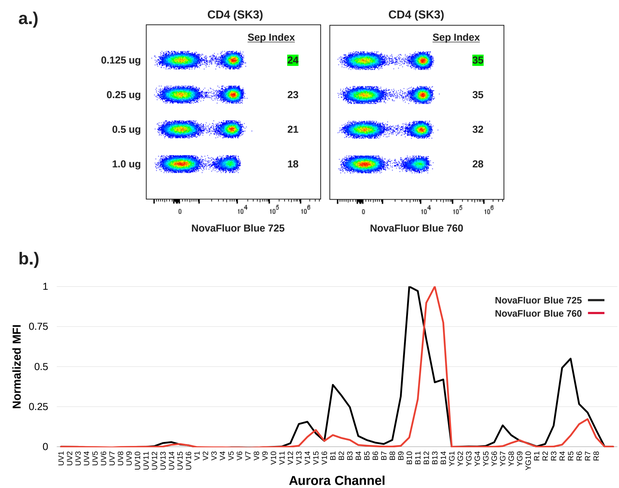
Results

Mixing and Matching Dyes using Multiple Kits. The initial steps of the NovaFluor Conjugation Kit protocol are optimized for 100 µg of antibody, but after purification of the antibody-oligo intermediate, the annealing of the NovaFluor (step 3) can be performed at any scale. **(a.)** A powerful advantage of this is that a small number of NovaFluor Conjugation Kits can be used to screen many combinations of antibodies and NovaFluor dyes to characterize their performance for panel design. As an example, three antibodies with varying expression levels (CD4, CD3, and CD45RA) were converted to their antibody-oligo intermediates using three conjugation kits. Then, 5 µg of each were annealed to three NovaFluors (NF) of varying brightness (NF Blue 585 = low, NF Yellow 660 = medium, NF Red 685 = high). **(b.)** The nine conjugates were titrated on human PBMCs, and their separation indexes were compared to determine the optimal titer (highlighted in green). While all three dyes can be used to resolve CD4 and CD3, only NY660 and NR685 can resolve CD45RA, illustrating the benefit of testing the combinations at a small scale. **(c.)** Using the optimal titers, CD4-NB585, CD3-NY660, and CD45RA NR685 were used in a small panel to compare CD45RA expression levels on different T-cell populations identified by CD3 and CD4.



Results

New Dyes only Available Through Kits. As novel NovaFluor dyes are released, they will first become available through the conjugation kits as their portfolio of antibody conjugates is developed. Two exciting new additions to the NovaFluor catalog, NovaFluor Blue 725 and NovaFluor Blue 760, are currently available only through the NovaFluor Conjugation kit. Their performance **(a.)** on Anti-Human CD4 (SK3) conjugated through the conjugation kit is shown for a four-point titration, as well as **(b.)** their spectral signature on a 5 laser Cytek Aurora.



Conclusions

NovaFluor Conjugation kits are a valuable tool for researchers to create NovaFluor conjugates to any antibody of interest not available in the Thermo Fisher catalog. To simplify the protocol and demonstrate its flexibility, we have demonstrated:

- Simple modifications to the protocol to shorten the procedure form 3 days to 1 day
- Methods to tweak the protocol to optimize the performance for a specific antibody
- A workflow to mix and match antibodies and dyes from multiple kits to optimize a panel on a small scale.
- Performance of two new NovaFluor Blue dyes only available through the conjugation kit

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