Optimization of Mouse CD8 and FoxP3 Dual Staining on the Leica® BOND™

Introduction and Background

Immunohistochemistry (IHC) is an invaluable laboratory technique that allows scientists to label cellular proteins in tissue samples using the interaction between a primary antibody/target antigen and detection reagents. For decades, IHC has routinely been used at the bench, and many scientists are proficient with manual staining methods.

Application of these methods using an autostainer allows increased workload throughput while maintaining consistency, reproducibility, and quality of staining. However, each protocol must be optimized to obtain accurate results. This is especially important when performing an IHC experiment with dual staining, as there are several experimental variables in the procedure.

One critically important consideration to ensure successful IHC is to work with antibodies that have been specifically validated for use in their intended application. Cell Signaling Technology (CST®) performs numerous validation and protocol optimization steps for each of our antibodies to ensure they are specific and sensitive to the target of interest.

Here, we outline the steps we perform to optimize sequential dual staining of formalin-fixed, paraffin-embedded (FFPE) mouse tissue on an autostainer using the following two rabbit monoclonal primary antibodies:

- FoxP3 (D6O8R) Rabbit mAb #12653, hereon referred to as FoxP3
- CD8α (D4W2Z) XP® Rabbit mAb (Mouse Specific) #98941, hereon referred to as CD8α

CD8α is a marker for cytotoxic T cells, while FoxP3 is a marker for regulatory T cells. These two cell types are important players in the tumor microenvironment, and the presence or absence of these cells can be a useful readout when investigating immunotherapy drugs.

The use of rabbit antibodies with mouse tissue offers a tremendous advantage over mouse monoclonal antibodies. There will be no complicating nonspecific staining from the secondary antibody, which is the case when using mouse monoclonal antibodies with mouse tissue. However, because we are using two primary antibodies of the same species, optimization of a stripping step to remove residual primary antibody in between staining with each antibody/chromogen pair is essential for successful detection of each target.

Primary Antibody Optimization

To optimize the primary antibodies, FoxP3 and CD8α, each was titrated in SignalStain® Antibody Diluent #8112 and paired with each chromogen, DAB and AP-Red. DAB, or mixed DAB Refine, is part of the Bond™ Polymer Refine Detection (Leica® Biosystems DS9800) kit. AP-Red is part of the Bond™ Polymer Refine Red Detection (Leica® Biosystems DS9390) kit. Antibodies were tested at the following dilutions: 1:100, 1:200, 1:400, and 1:800.

These initial titration conditions were selected based on the optimal dilutions from the manual protocol. We expect that the optimal primary antibody dilutions will be similar with each chromogen, but if this is not the case, more dilution points must be tested.

See **Table 1** for a summary of the experimental set-up. Optimal dilutions per antibody/chromogen pair are indicated.

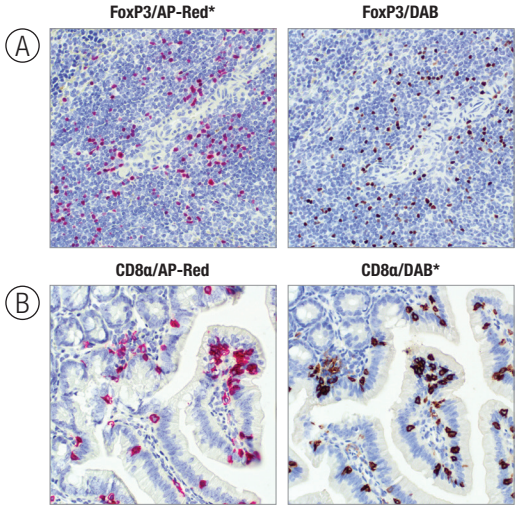
Table 1 - Experimental set-up for primary antibody optimization.

Primary Antibody	Chromogen	Titration	Optimal Dilution
CD8α (D4W2Z) XP® Rabbit mAb (Mouse Specific) #98941[3]	DAB (mixed DAB refine)[1]	1:100-1:800	1:400
	AP-Red (mixed Red refine)[2]	1:100-1:800	1:200
FoxP3 (D6O8R) Rabbit mAb #12653[3]	DAB (mixed DAB refine)[1]	1:100-1:800	1:400
	AP-Red (mixed Red refine)[2]	1:100-1:800	1:200

1: Included in Bond™ Polymer Refine Detection (DS9800).
2: Included in Bond™ Polymer Red Refine Detection (DS9390).
3: Primary antibodies diluted in #8112 SignalStain® Antibody Diluent.

As shown in **Figure 1**, IHC staining was sharper and more localized when DAB was paired with CD8α and AP-Red was paired with FoxP3.

Based on our results, we paired AP-Red with FoxP3 and DAB with CD8α. The combination of FoxP3 with AP-Red is consistent with prior knowledge that AP-Red tends to perform better with nuclear antibody targets. Even so, we recommend that this be confirmed experimentally.



*Optimal Antibody/Chromogen Pairing per Antibody

Figure 1: Initial Chromogen Pairing. (**A**) IHC images using FFPE mouse spleen stained with FoxP3 at 1:200 paired with AP-Red and 1:400 paired with DAB. As shown, the combination of FoxP3/AP-Red yields sharper staining. (**B**) IHC images using FFPE mouse small intestine stained with CD8α at 1:200 paired with AP-Red and 1:400 paired with DAB. As shown, both combinations provide appropriate staining.

Optimizing Primary Antibody Stripping Conditions

After we have optimized each primary antibody, we must ensure that we successfully remove the first antibody completely before application of the second antibody to the tissue sample for dual staining. This is achieved by performing a stripping step in between the application of each antibody/chromogen pair.

To confirm the efficacy of the antibody stripping protocol, after removal of the first antibody we omitted the application of the second antibody and applied only the second detection reagent and chromogen. This way, any residual antibody remaining after the antibody stripping step could be visualized.

The steps to test for successful primary antibody removal are as follows:

1. Apply the first antibody without chromogen or hematoxylin.
2. Perform a stripping protocol with either Bond™ Epitope Retrieval 1 (Leica® Biosystems AR9961) (ER1; citrate buffer) for 10 minutes at 100°C or Bond™ Epitope Retrieval 2 (Leica® Biosystems AR9640) (ER2; EDTA buffer) for 20 minutes at 100°C.
3. Apply the AP-Red protocol with SignalStain® Antibody Diluent #8112 only (ie, no primary antibody should be applied at this step).

In our experiment, we saw more effective stripping when using ER2 for 20 minutes.

Figure 2 depicts the IHC images of each primary antibody after stripping with each buffer. Note that primary antibody is still detected with the use of ER1 citrate buffer after the stripping step, whereas using ER2 EDTA and a longer incubation time resulted in more complete primary antibody removal. For this reason, we chose the ER2 EDTA buffer for 20 minutes for our optimized primary antibody stripping step.

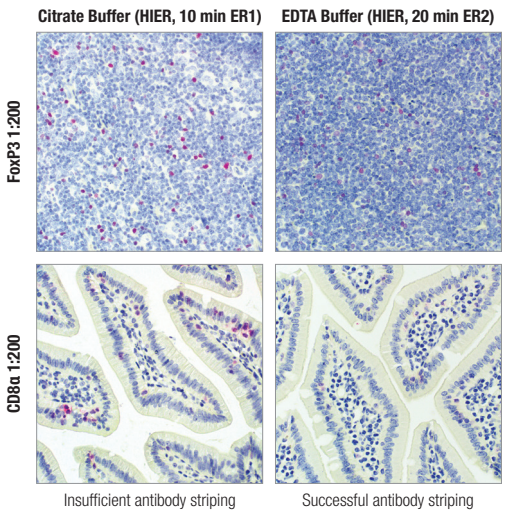


Figure 2: Primary Antibody Stripping Optimization Images. This figure depicts the IHC images from the primary antibody stripping optimization step, using FoxP3 and CD8α antibodies with both the ER1 citrate buffer (AR9961) and ER2 EDTA buffer (AR9640). Note that, with ER1 citrate buffer, primary antibody can still be detected after the stripping step, suggesting that it is not fully removed by this buffer. Alternately, ER2 buffer results in more complete antibody stripping and is, therefore, the better option.

Optimizing Antibody Staining Order

These results dictate the staining order for this combination of antibodies. Some residual FoxP3 staining remains after the antibody stripping step, as such the FoxP3 antibody must be placed second. If the results of the stripping test do not require that one antibody be placed second, one would test the order of antibody staining in order to determine the best sequence of antibody application. To do this, the optimal antibody/chromogen pairing is tested in both the first and second positions. Staining results are compared, and the sequence that yields the most robust signal for each marker should be selected.

Final Conditions/Optimized Protocol

In summary, the following steps are necessary to optimize an automated dual-staining protocol using FFPE tissue:

1. Optimize each antibody with each chromogen.
2. Perform a matrix for the stripping test of each chromogen with each antibody (primary antibody > no chromogen > strip > no primary > second chromogen).
3. Optimize order of staining.

Using the optimized conditions from the previous steps, we compared a single stain with each primary antibody used individually to the optimized dual-staining protocol to ensure that the signal:noise ratio and sensitivity of detection was retained (**Figure 3**). The steps for the dual-staining assay are as follows:

1. De-wax the FFPE tissue samples.
2. Perform the initial antigen retrieval, using ER2 EDTA for 20 minutes at 100°C.
3. Perform the first step of the autostain, starting with CD8α/DAB 1:200.
4. Remove primary antibody between protocols, using ER2 EDTA buffer for 20 minutes at 100°C, as determined previously.
5. Perform the second step of the autostain, using FoxP3/AP-Red 1:200.

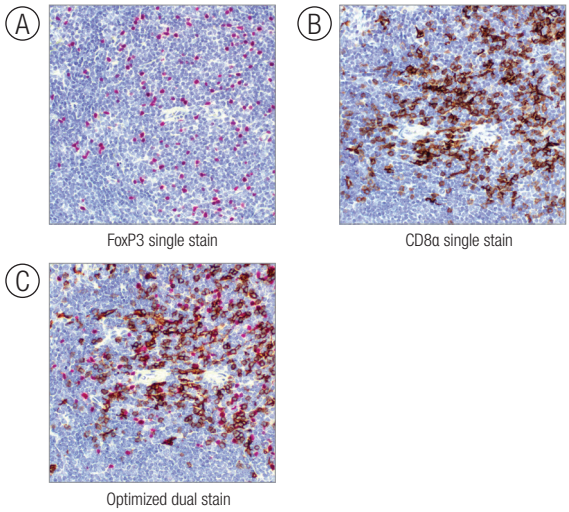


Figure 3: Optimized Dual-Staining Protocol. Comparison of the single-stain images of (**A**) FoxP3 (#12653) and (**B**) CD8α (#98941) to the optimized dual stain (**C**).

This comparison demonstrates that results obtained for each antibody in the dual staining assay are equivalent to those achieved when each antibody is used individually (**Figure 3**).

The steps outlined in this protocol serve as a guide to optimize dual staining using a variety of tissue samples and primary antibody combinations (**Figure 4**).

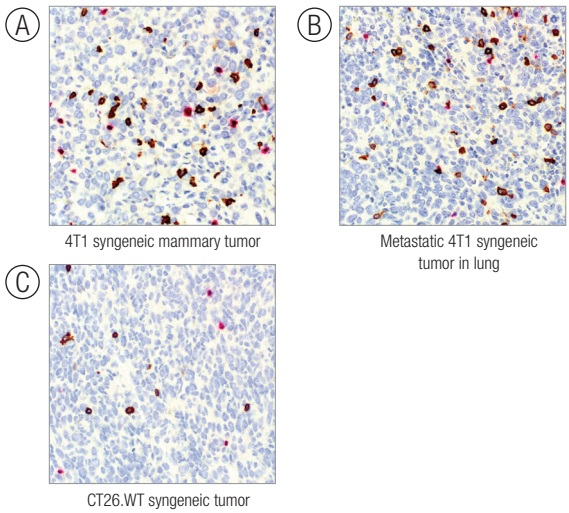


Figure 4: Optimized Dual-Staining Assay on Syngeneic Tumor Models. IHC images from the application of the optimized dual-staining assay on (**A**) 4T1 syngeneic mammary tumor, (**B**) metastatic 4T1 syngeneic tumor in lung, and (**C**) CT26.WT syngeneic tumor.

Conclusion

Here, we described in detail the steps required to optimize an automated dual-staining protocol using FFPE tissue.

There are several key takeaway points for this protocol. Primary antibodies that are specifically validated for IHC must be used. While there is a large selection of antibodies available to choose from, antibodies from CST are rigorously validated for IHC using appropriate controls and multiple tissues. It is also advantageous to use rabbit antibodies when working with mouse tissue in order to avoid nonspecific staining from secondary antibodies. CST has a portfolio of IHC-validated, mouse-reactive antibodies raised in rabbit that may be used for this purpose. Furthermore, when performing dual staining with two antibodies raised in the same species, it is critically important to confirm the complete removal of the first primary antibody via an optimized stripping step before the addition of the second primary antibody/chromogen pair. This ensures that all signal from the second application of the detection reagent is associated only with the second primary antibody.

Lastly, it is best practice to compare the results obtained with the combination of antibodies to those obtained with each antibody individually to ensure that similar performance of each antibody is achieved.

Technical Support

At CST, providing exceptional customer service and technical support are top priorities. Our scientists work at the bench daily to produce and validate our antibodies, so they have hands-on experience and in-depth knowledge of each antibody's performance. In the process, these same scientists generate valuable reference information that they use to answer your questions and help troubleshoot your experiment by phone or email.

For questions about how to customize your protocol, please contact technical support by emailing support@cellsignal.com, visiting **www.cellsignal.com/support**, or calling 1-877-678-8324.

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