Optimizing Immunohistochemistry Dual Staining to Visualize and Understand Protein Co-expression During EMT

Introduction

Immunohistochemistry (IHC) dual staining facilitates the visualization of biomarkers within their original histological context. This makes it a valuable technique for cancer research. The epithelial-mesenchymal transition (EMT) is a natural developmental process that is also important in metastatic events. EMT is one of the changes that cells must undergo to become invasive. This transformation creates a favorable microenvironment for cancer migration and metastasis. Therefore, detecting EMT in human tissue samples is a valuable tool to further the understanding of tumor progression.

Achieving reliable results from this technique depends on two major factors: first, the antibodies must be properly validated to ensure they are binding specifically to their target of interest, and, second, the protocol must be optimized to take into consideration the preferred conditions for each antibody.

Antibodies from Cell Signaling Technology (CST®) recommended for use in IHC are thoroughly validated to ensure specificity for the target protein. Thus, by using CST antibodies, you can have a high degree of confidence in the veracity of the result. This technical note will guide you through the necessary steps for optimizing a dual-staining IHC protocol.

In summary, the optimization steps are as follows:

1. Optimize each antibody with each chromogen to achieve the best signal-to-noise ratio in a single-stain setting.

2. Optimize antibody-chromogen pairing in a dual-stain setting.

The optimization steps we will describe are applicable to any targets you choose. For this example, we selected two targets that are important markers of EMT: E-Cadherin and vimentin.

Single-Stain Dilution Optimization

Before optimizing your dual-staining protocol, it is necessary to determine the optimal dilution for each antibody with each chromogen. Our standard IHC protocol includes an overnight incubation, but to enable completion of the dual-staining procedure in a shorter time, we can adjust the antibody concentration. In this example, we titrated each antibody paired with each chromogen using a 1-hour primary incubation and compared that to the optimal staining result achieved with the standard overnight protocol.

Table 1 lists the different antibodies and detection reagents/chromogen pairs that were tested in our experiment. As depicted, Vimentin (D21H3) XP® Rabbit mAb #5741, hereon referred to as vimentin antibody, was paired with one of the following:

- ImmPRESS®-AP Anti-Rabbit IgG (alkaline phosphatase) Polymer Detection Kit (Vector® Laboratories MP-5401)/ImmPACT® Vector® Red Alkaline Phosphatase (AP) Substrate (Vector® Laboratories SK-5105), hereon referred to as AP Rabbit/Red

- SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114/ SignalStain® DAB Substrate Kit #8059, heron referred to as HRP Rabbit/Brown

E-Cadherin (4A2) Mouse mAb #14472, hereon referred to as E-Cadherin antibody, was paired with one of the following:

- ImmPRESS®-AP Anti-Mouse IgG (alkaline phosphatase) Polymer Detection Kit (Vector® Laboratories MP-5402)/ImmPACT® Vector® Red AP Substrate (Vector® Laboratories SK-5105), heron referred to as AP Mouse/Red

- SignalStain® Boost IHC Detection Reagent (HRP, Mouse) #8125/ SignalStain® DAB Substrate Kit #8059, hereon referred to as HRP Mouse/Brown

Table 1 - Antibodies and detection reagents used in test one (single stain).

Antibody	Detection	Dilution
Vimentin Antibody Vimentin (D21H3) XP® Rabbit mAb #5741	**AP Rabbit/Red** ImmPRESS®-AP Anti-Rabbit IgG (alkaline phosphatase) Polymer Detection Kit (Vector® Laboratories MP-5401)/ImmPACT® Vector® Red Alkaline Phosphatase (AP) Substrate (Vector® Laboratories SK-5105)	1:5
		1:10
		1:25
		1:50
	HRP Rabbit/Brown SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114/SignalStain® DAB Substrate Kit #8059	1:5
		1:10
		1:25
		1:50

Antibody	Detection	Dilution
E-Cadherin Antibody E-Cadherin (4A2) Mouse mAb #14472	**AP Mouse/Red** ImmPRESS®-AP Anti-Mouse IgG (alkaline phosphatase) Polymer Detection Kit (Vector® Laboratories MP-5402)/ImmPACT® Vector® Red Alkaline Phosphatase (AP) Substrate (Vector® Laboratories SK-5105)	1:5
		1:10
		1:25
		1:50
	HRP Mouse/Brown SignalStain® Boost IHC Detection Reagent (HRP, Mouse) #8125/ SignalStain® DAB Substrate Kit #8059	1:5
		1:10
		1:25
		1:50

As illustrated in **Figure 1**, the optimal results for vimentin antibody were achieved at 1:10 using both AP Rabbit/Red and HRP Rabbit/Brown. Optimal results for E-Cadherin antibody were obtained at 1:25 with both AP Mouse/Red and HRP Mouse/Brown.

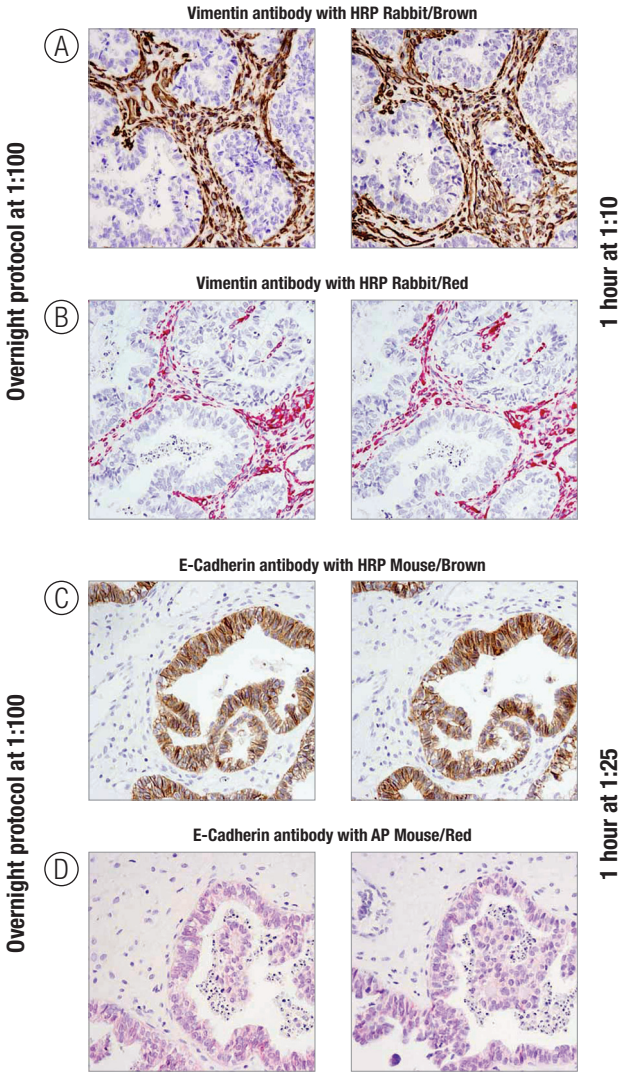

Figure 1: IHC single-stain dilution optimization of formalin-fixed, paraffin-embedded (FFPE) ovarian carcinoma tissue. We were able to achieve antibody staining at 1 hour that compares favorably to the overnight protocol. Vimentin antibody at 1:100 overnight or 1 hour at 1:10 compares favorably with (**A**) HRP Rabbit/Brown and (**B**) AP Rabbit/Red. E-Cadherin antibody at 1:100 overnight or 1 hour at 1:25 compares favorably with (**C**) HRP Mouse/Brown and (**D**) AP Mouse/Red.

Dual-Stain Optimization

The next step in developing the protocol is to test combinations of each optimal antibody/chromogen pairing in order to determine which order of staining will result in the best signal.

Typically, one would test all of the possible antibody/chromogen pairing options (as shown in **Table 2**). Based on previous work, we have determined that our preference is to use DAB (HRP Mouse or Rabbit/Brown) as the second chromogen. As such, we did not test all four conditions in this experiment, but recommend that you optimize this in your lab.

Table 2 - Summary of antibody and chromogen pairing options.

Apply First		Apply Second	
Antibody 1	**Chromogen 1**	**Antibody 2**	**Chromogen 2**
Vimentin Antibody	AP Rabbit/Red	E-Cadherin Antibody	HRP Mouse/Brown
Vimentin Antibody	HRP Rabbit/Brown	E-Cadherin Antibody	AP Mouse/Red
E-Cadherin Antibody	HRP Mouse/Brown	Vimentin Antibody	AP Rabbit/Red
E-Cadherin Antibody	AP Mouse/Red	Vimentin Antibody	HRP Rabbit/Brown

To perform this step, primary antibodies were mixed and added to the tissue samples simultaneously. The detection steps were performed sequentially. First, AP detection reagent was applied, followed by development with Vector® Red. After the washing step, HRP detection reagent was applied, followed by DAB. **Table 3** summarizes these pairings.

The dual-stain pairings were then compared to the optimal single-stain conditions. **Figure 2** shows each optimized single stain alongside the corresponding dual stain for both tests.

At this point in the optimization work, it may be necessary to adjust the development times of the chromogens in order to achieve balanced signals. Note that extreme alterations may require retitration of the primary antibody. For this work, we developed DAB (HRP Mouse or Rabbit/Brown) for 1 minute and Vector® Red (AP Mouse or Rabbit/Red) for 3 minutes.

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Table 3 - Summary of antibody and chromogen pairs in test two (dual stain).

Antibody	Dilution		Antibody	Dilution	First Detection	Second Detection
Vimentin Antibody Vimentin (D21H3) XP® Rabbit mAb #5741	1:10	+	**E-Cadherin Antibody** E-Cadherin (4A2) Mouse mAb #14472	1:25	**AP Rabbit/Red** ImmPRESS®-AP Anti-Rabbit IgG (alkaline phosphatase) Polymer Detection Kit (Vector® Laboratories MP-5401)/ImmPACT® Vector® Red Alkaline Phosphatase (AP) Substrate (Vector® Laboratories SK-5105)	**HRP Mouse/Brown** SignalStain® Boost IHC Detection Reagent (HRP, Mouse) #8125/ SignalStain® DAB Substrate Kit #8059
E-Cadherin Antibody E-Cadherin (4A2) Mouse mAb #14472	1:25	+	**Vimentin Antibody** Vimentin (D21H3) XP® Rabbit mAb #5741	1:10	**AP Mouse/Red** ImmPRESS®-AP Anti-Mouse IgG (alkaline phosphatase) Polymer Detection Kit (Vector® Laboratories MP-5402)/ImmPACT® Vector® Red Alkaline Phosphatase (AP) Substrate (Vector® Laboratories SK-5105)	**HRP Rabbit/Brown** SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114/ SignalStain® DAB Substrate Kit #8059

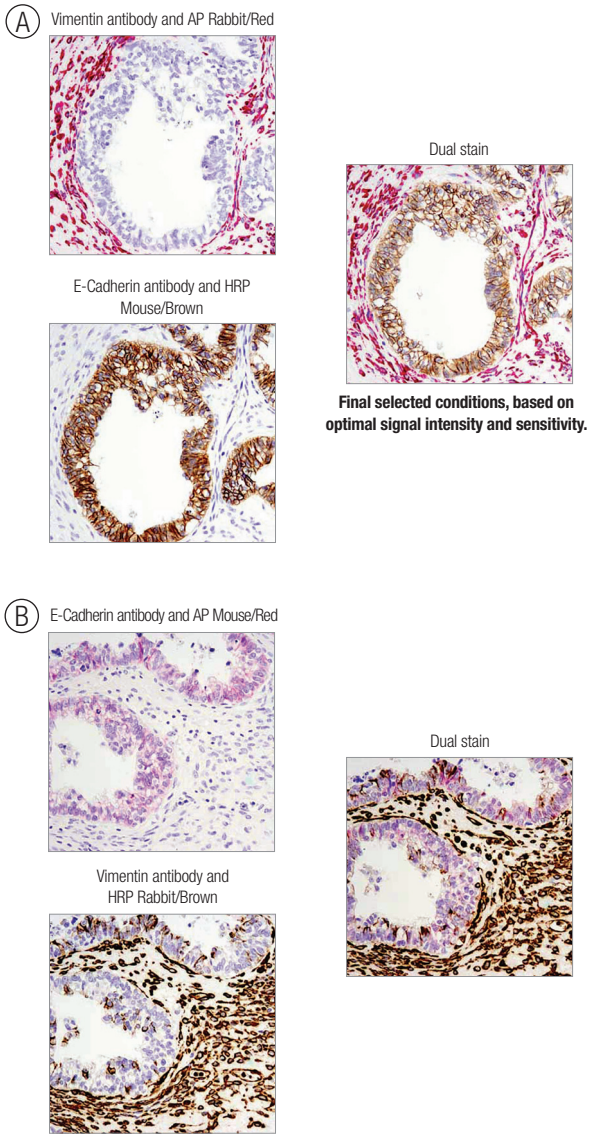

Figure 2: IHC dual-stain panel optimization shows (**A**) vimentin antibody and AP Rabbit/Red tested with E-Cadherin antibody and HRP Mouse/Brown and (**B**) E-Cadherin antibody and AP Mouse/Red tested with vimentin antibody and HRP Rabbit/Brown, single stains (left panels) and dual stain (right panels).

Final Conditions

Once all parameters are optimized, the final conditions can be tested on the tissues of your choice. Our final conditions included vimentin antibody at a dilution of 1:10, AP Rabbit/Red developed for 3 minutes, E-Cadherin antibody at a dilution of 1:25, and HRP Mouse/Brown developed for 1 minute. We chose to apply these conditions to a human tumor array, including lung, ovarian, and stomach carcinomas, as shown in **Figure 3**.

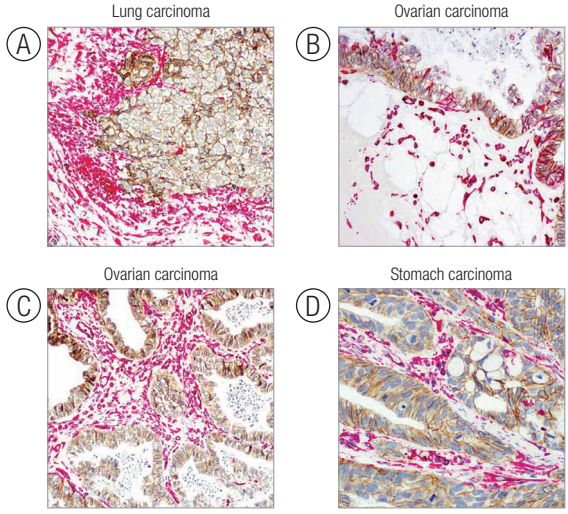

Figure 3: IHC final conditions applied to human tumor array of (**A**) lung carcinoma, (**B**) ovarian carcinoma, (**C**) ovarian carcinoma, and (**D**) stomach carcinoma.

When performing IHC assays, it is important to use antibodies that have been properly validated for IHC. This provides confidence that the antibodies are specific for your target of interest. This is especially important for dual-staining IHC experiments, because there are additional experimental variables that can yield inconclusive results. It is important that your primary antibody provide trusted, consistent results from the beginning.

CST scientists use a variety of methods to validate each IHC-recommended antibody. Moreover, the detection systems and enzyme substrates used for the experiment are rigorously optimized to deliver the high sensitivity, low background, and extreme clarity required to differentiate multiple epitopes simultaneously.

This example shows two targets that are important markers of EMT: E-Cadherin, an epithelial target, and vimentin, a mesenchymal target. Given their combined role in cancer progression, simultaneous detection of each in tissue sections provides insights into tumor invasiveness and potential for metastasis.

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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 using our full catalog of nearly 1000 antibodies approved for IHC, please contact technical support by visiting **www.cellsignal.com/support**.

Ordering Information

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