

Enzyme Substrates

A palette of colors for immunohistochemistry

Vector Laboratories is an industry leader in the development and manufacture of enzyme substrates for protein and nucleic acid detection and visualization applications.

Choose from 18 different enzyme substrate kits for immunohistochemistry (IHC):

- 13 substrate kits that react with horseradish peroxidase (HRP)
- 5 substrate kits that react with alkaline phosphatase (AP)

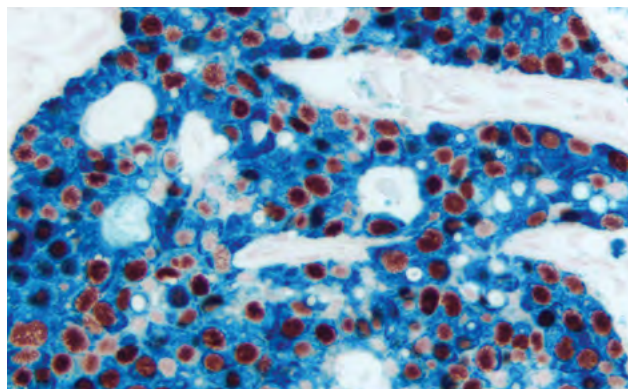
These 18 kits provide **9 different color tones** for single antigen staining and enable 42 combinations for double staining applications.

For single or multiple antigen labeling, enzyme substrate selection plays a crucial role in IHC staining outcomes. Featured in this document are key considerations regarding substrate choice to aid you during IHC assay development. The information and insights provided here should help you achieve the most optimal and reliable staining results, and avoid common pitfalls encountered in an often neglected and disregarded segment of the IHC workflow.

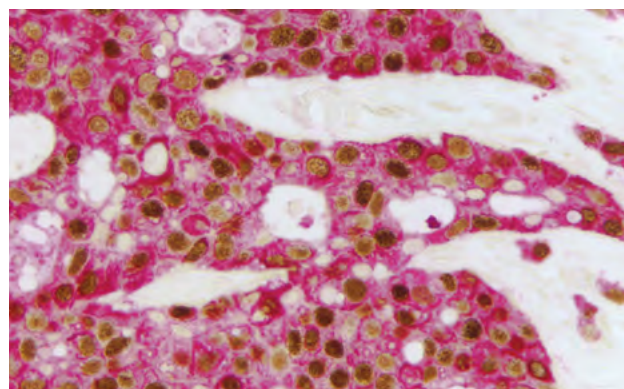
KEY CONSIDERATIONS WHEN CHOOSING AN ENZYME SUBSTRATE

- Enzyme
- Color
- Sensitivity
- Appearance
- Stability of Working solution
- Ease of Use
- Multiple Antigen Labeling (multiplex IHC)

A different substrate, or combination of substrates for double labeling, may yield more information and/or greater clarity of antigen detection.



Breast Carcinoma – Double label • Estrogen Receptor (m), ImmPRESS® Universal HRP Reagent, Vector® NovaRED™ HRP substrate (red) • Cytokeratin 8/18 (m), VECTASTAIN® Universal ABC-AP Kit, Vector Blue AP substrate (blue).






















Breast Carcinoma – Double label • Estrogen Receptor (m), ImmPRESS® Universal HRP Reagent, DAB HRP substrate (brown) • Cytokeratin 8/18 (m), VECTASTAIN Universal ABC-AP Kit, Vector Red AP substrate (magenta).

Are you using the best substrate choice for your IHC?

Enzyme Substrates

Enzyme Substrates for Immunohistochemistry

Vector Laboratories' portfolio of enzyme substrates and the corresponding microscopy characteristics for each substrate are featured in the chart below. While the information provided here focuses on their use for IHC, it should be noted that many of these substrates are suitable for use in other protein and nucleic acid detection and visualization applications including ELISAs, ELISpots, blotting and *in situ* hybridization. Further procedural information regarding use of the substrates is provided at www.vectorlabs.com.

Substrate	Color	Catalog Number	Microscopy					Mounting	Contrast in Pigmented Tissue	Multiple Labeling	Heat Resistant*
			Bright-field	Darkfield	Electron	Fluorescence	Spectral Imaging				
Peroxidase											
DAB	 Brown	SK-4100	•	•	•		•	Non-aqueous or Aqueous		•	•
DAB +Ni	 Gray-Black	SK-4100	•	•	•		•	Non-aqueous		•	
ImmPACT® DAB	 Brown	SK-4105	•	•	•		•	Non-aqueous or Aqueous		•	•
ImmPACT DAB EqV	 Brown	SK-4103	•	•	•		•	Non-aqueous or Aqueous		•	•
Vector VIP	 Purple	SK-4600	•	•	•		•	Non-aqueous	•	•	
ImmPACT VIP	 Purple	SK-4605	•	•	•		•	Non-aqueous	•	•	
Vector SG	 Blue-Gray	SK-4700	•	•	•		•	Non-aqueous or Aqueous	•	•	
ImmPACT SG	 Blue-Gray	SK-4705	•	•	•		•	Non-aqueous or Aqueous	•	•	
Vector NovaRED	 Red	SK-4800	•	•	•		•	Non-aqueous	•	•	
ImmPACT NovaRED	 Red	SK-4805	•	•	•		•	Non-aqueous	•	•	
AEC	 Red	SK-4200	•				•	Aqueous	•	•	
ImmPACT AEC	 Red	SK-4205	•				•	Aqueous	•	•	
ImmPACT AMEC Red	 Red	SK-4285	•				•	Aqueous	•	•	
TMB	 Blue	SK-4400	•				•	Non-aqueous			
Alkaline Phosphatase											
Vector Red	 Magenta	SK-5100	•				•	•	Non-aqueous or Aqueous	•	•
ImmPACT Vector Red	 Magenta	SK-5105	•				•	•	Non-aqueous or Aqueous	•	•
Vector Blue	 Blue	SK-5300	•				•	•	Non-aqueous or Aqueous	•	•
Vector Black	 Brown-Black	SK-5200	•						Non-aqueous		
BCIP/NBT	 Indigo	SK-5400	•				•	•	Non-aqueous or Aqueous		•

Key Considerations when Choosing an Enzyme Substrate

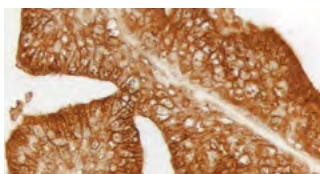
1) Enzyme

- Select a substrate that matches the enzyme incorporated in the detection system. If you intend to use an HRP detection system, then use an HRP substrate that will react with that detection system.
- For IHC, HRP is the most widely used enzyme detection methodology, probably due to the fast reaction kinetics and dense precipitate that is deposited at the site of antigen localization.
- Note that an HRP substrate will not react with an AP based detection system, and an AP substrate will not react with an HRP detection system. This reaction specificity of the substrate for the target enzyme provides opportunities in multiple labeling applications. Additionally, should persistent endogenous enzyme activity be problematic in certain specimens, changing to a different enzyme detection system is likely to circumvent this issue.

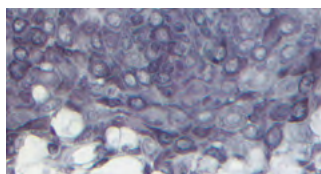
2) Color

- Consider the inherent characteristics of the tissue specimen that will be used for the IHC assay, as there may be advantages of one colored substrate over another. To avoid possible false positive interpretation of specific staining, it is imperative that a good contrast exists between the target antigen stain and the surrounding tissue specimen.
- In situations with pigmented tissue such as melanomas or other brown or black tissue deposits, a purple, red or blue colored substrate would be recommended over a traditional brown DAB substrate.

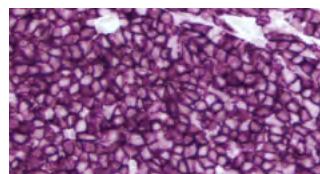
Peroxidase Substrates



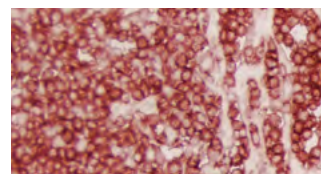
Brown - ImmPACT DAB ([SK-4105](#)), ImmPACT DAB EqV ([SK-4103](#)), DAB ([SK-4100](#)).



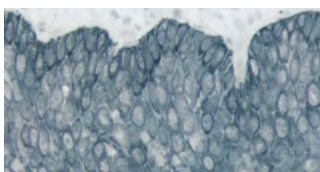
Gray-Black - DAB-Ni ([SK-4100](#)).



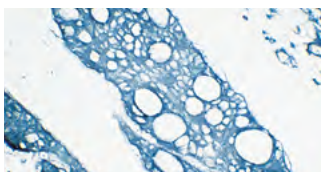
Purple - ImmPACT VIP ([SK-4605](#)), Vector VIP ([SK-4600](#)).



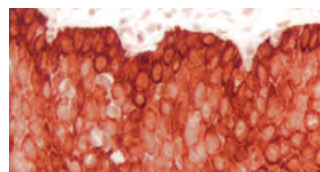
Red - ImmPACT AMEC Red ([SK-4285](#)).



Blue-Gray - ImmPACT SG ([SK-4705](#)), Vector SG ([SK-4700](#)).



Blue - TMB ([SK-4400](#)).

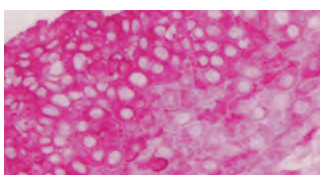


Red - ImmPACT NovaRED ([SK-4805](#)), Vector NovaRED ([SK-4800](#)).

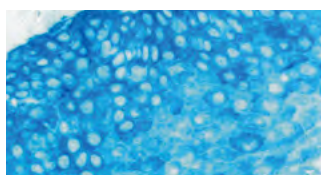


Red - ImmPACT AEC ([SK-4205](#)), AEC ([SK-4200](#)).

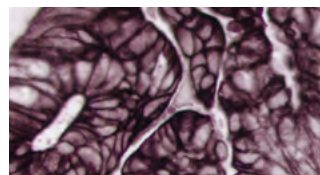
Alkaline Phosphatase Substrates



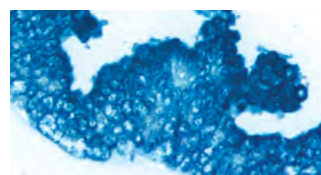
Magenta - ImmPACT Vector® Red ([SK-5105](#)), Vector Red ([SK-5100](#)).



Blue - Vector Blue ([SK-5300](#)).



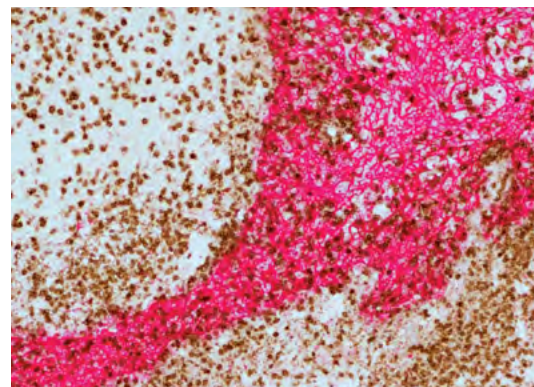
Brown-Black - Vector Black ([SK-5200](#)).



Indigo - BCIP/NBT ([SK-5400](#)).

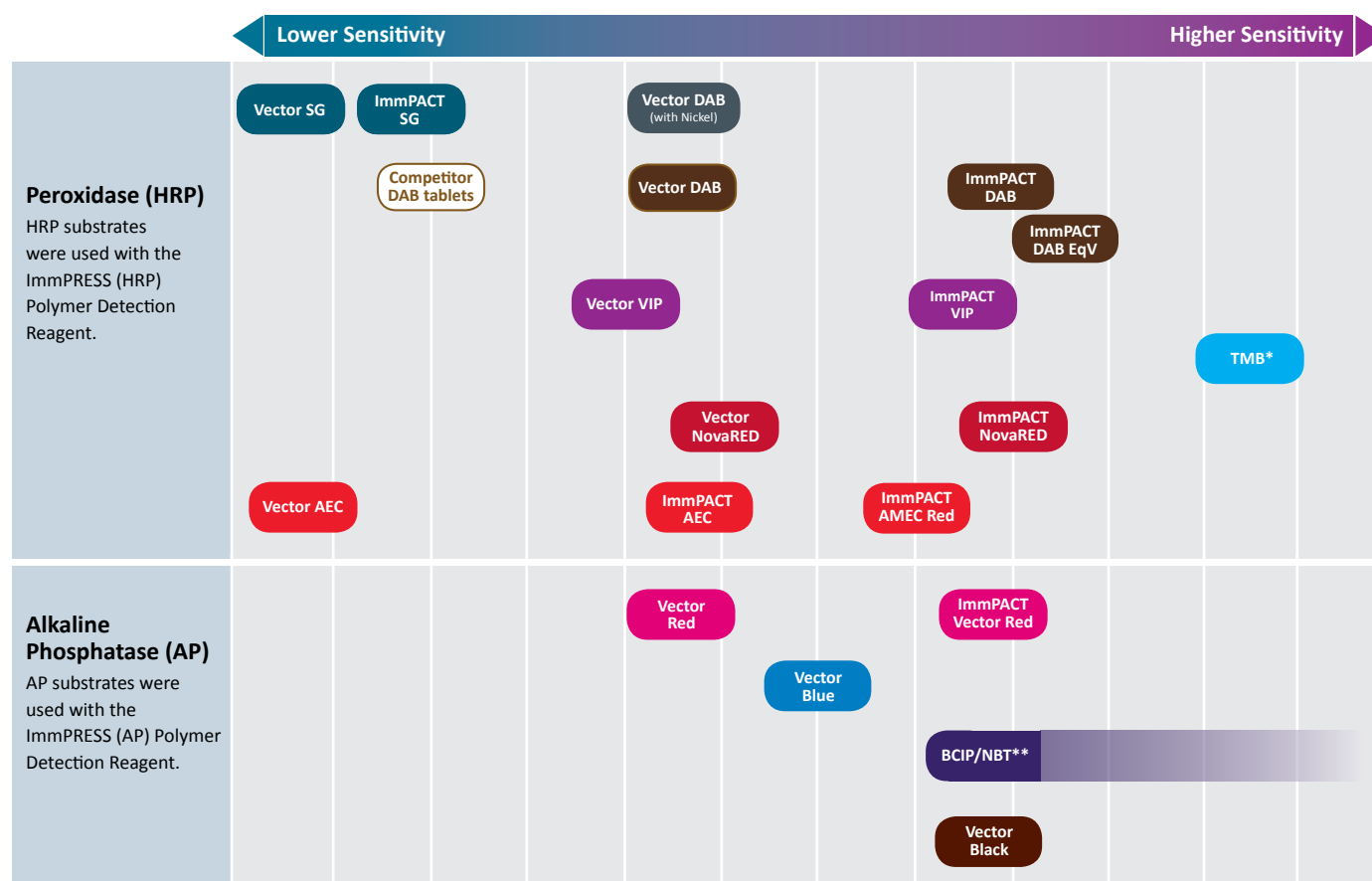
3) Sensitivity

- Not all substrates have equivalent sensitivity and indeed some formulations of the same substrate are more sensitive than others.
- In staining applications that require maximum sensitivity, it is recommended that a more sensitive substrate be used. Examples of these would be in gene knock-in studies or where target antigens are weakly expressed or have unknown expression in a disease state or treated specimen.
- For maximum sensitivity, we recommend the ImmPACT substrate formulations.



Human tonsil - Double Label: • CD3 (ImmPACT DAB, brown) and AE1/AE3 cytokeratin (ImmPACT Vector Red (magenta) using ImmPRESS Duet Kit ([MP-7714](#))

Relative Sensitivity of Substrates in Immunohistochemistry

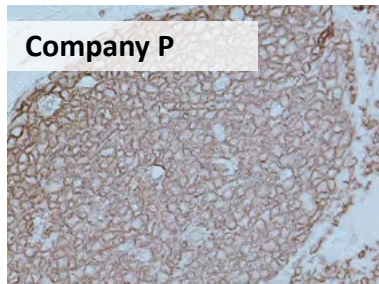
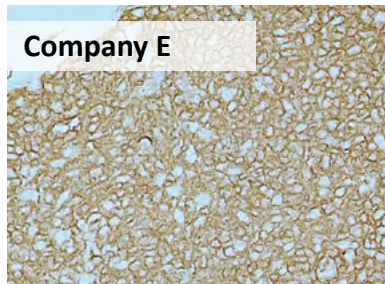
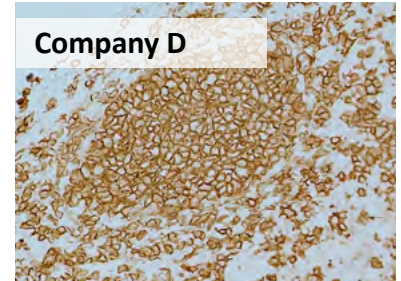
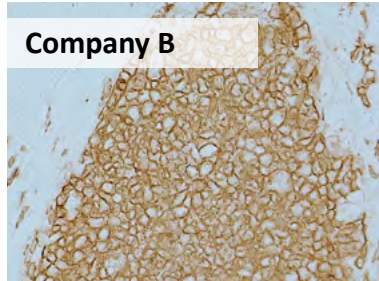
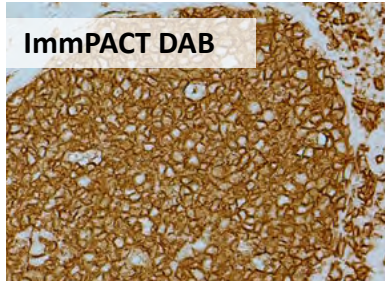


* Reaction product deposition not discreet or reliable for IHC.

**Longer incubation times increase sensitivity.

DAB (HRP) Substrate Sensitivity Comparisons

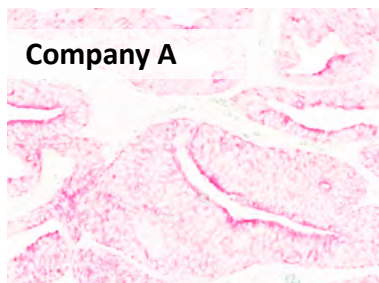
Human tonsil (FFPE serial sections): Stained for CD20 (1:300 dilution), detected with ImmPRESS HRP anti-mouse IgG ([MP-7402](#)). The only reagent variable was the DAB substrate used for color development.



Overall Results: In parallel assays on serial sections from the same tissue block with only the DAB substrate being substituted, Vector ImmPACT DAB appeared to generate the most robust, crisp staining intensity (highest sensitivity) without appreciable background compared with DAB from the other sources.

Red (AP) Substrate Sensitivity Comparisons

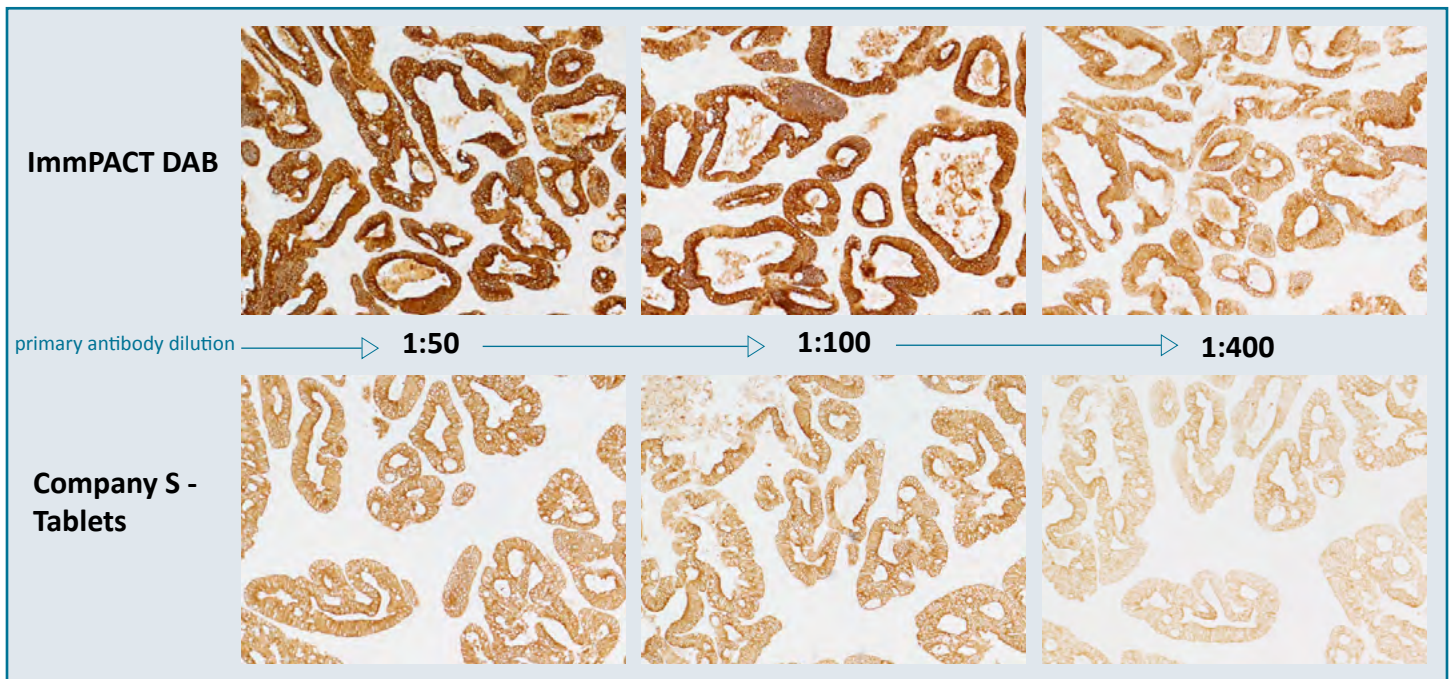
Human Prostate (FFPE serial sections): Stained for Cytokeratin (1:250 dilution), detected with ImmPRESS-AP anti-mouse IgG ([MP-5402](#)). The only reagent variable was the AP substrate for color development.



Overall Results: In parallel assays on serial sections from the same tissue block with only the AP red substrate being substituted, ImmPACT Vector Red appeared to generate the most robust, crisp staining intensity (highest sensitivity) without appreciable background compared with AP red substrates from the other sources.

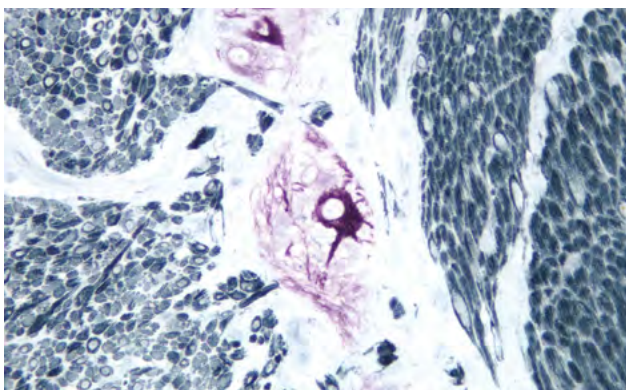
3) Sensitivity (continued)

- It may be possible for some target antigens to realize a 2x to 4x further dilution of the primary antibody, compared with a less sensitive substrate, and still obtain appropriate staining intensity.
- Further dilution of key IHC workflow components would be a significant cost savings over time.

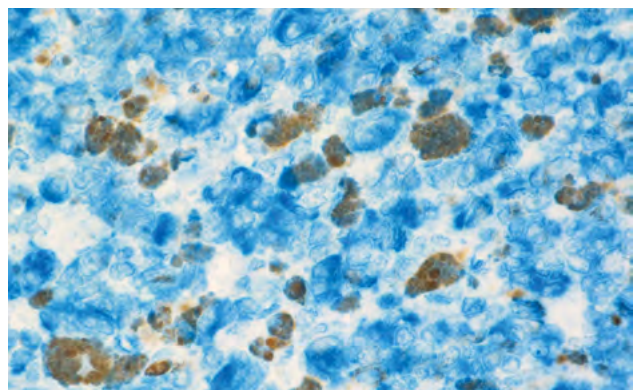


4) Appearance

- HRP substrates, in general, generate sharper, more dense reaction product (precipitate) compared with AP substrates. This characteristic may be helpful if the target is located intracellularly, expressed in a punctate manner, or in situations that require highly delineated localization.
- AP substrate precipitates are more diffuse and translucent. These characteristics may be preferred when target antigens are expressed broadly and to allow visualization of underlying tissue structure.



Small Bowel: • Neurofilament 200 kDa (m), ImmPRESS HRP Reagent, Vector VIP HRP substrate (purple) • Desmin (m), ImmPRESS HRP Reagent, Vector SG HRP substrate (blue-gray). Note: Heavy, dense color deposition of the HRP substrates with sharp localization of the respective target antigens.

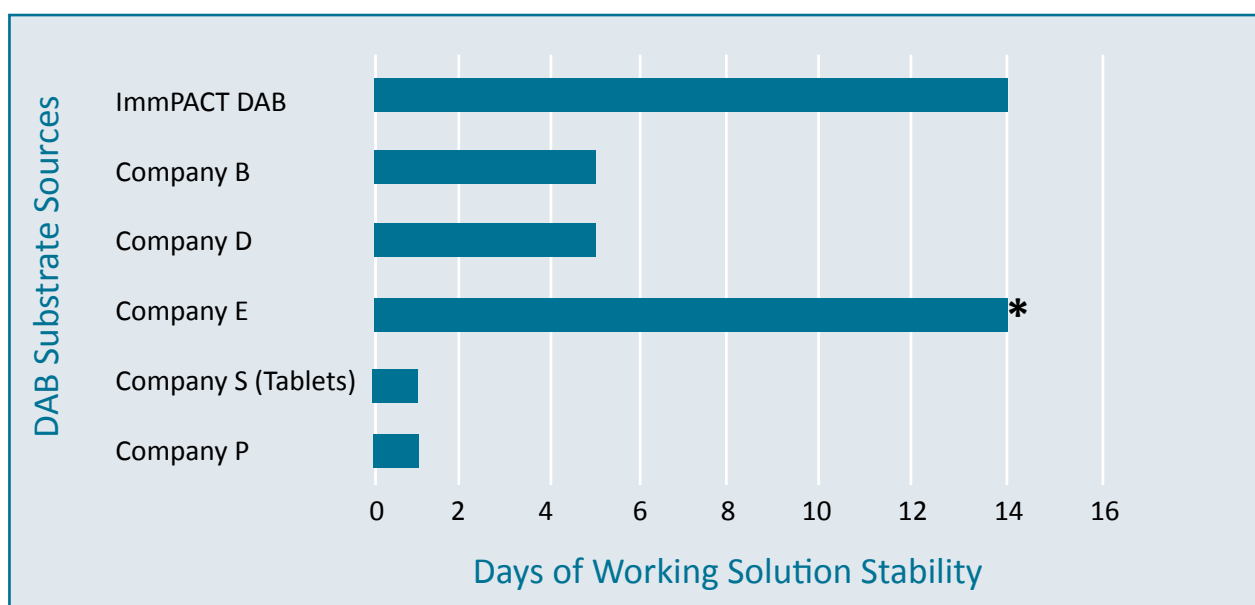


Melanoma: Vimentin (rm), ImmPRESS-AP Reagent, Vector Blue AP substrate (blue). Note color contrast with brown pigments in tissue. The more translucent appearance of the AP substrate offers insight into the underlying tissue architecture.

5) Stability of Working Solution

- Using an enzyme substrate with a more stable, longer working solution time line would be advantageous particularly for labs that run large numbers of slides and utilize considerable volumes of IHC reagents that are applied either manually and/or on automated staining platforms.
- Benefits of using a substrate with an extended working solution shelf-life would be realized with decreased labor requirements by not having to make the substrate each day, increased convenience, minimizing waste and potentially increasing assay reproducibility by eliminating a variable.

DAB Stability Comparison



* Background staining increases over time, reducing signal to noise ratio.

6) Ease of Use:

- Tablet and powdered formats of substrates are time-consuming and inconvenient to make and expose the end-user to potentially hazardous conditions through inhalation or spillage when measuring.
- Liquid and liquid concentrated enzyme substrates are superior formats that overcome the shortcomings of tablet and powdered formats.
- All substrates from Vector Laboratories are presented as liquid formats.



Key Considerations when Choosing an Enzyme Substrate

7) Multiple Antigen Labeling

- When applying a second or third substrate for multiple antigen labeling on the same tissue section, the order of labeling, use of control sections, and additional blocking steps are important to obtain optimal results.
- This chart ensures that distinct colors are visible after the labeling reactions are completed using an optimized multiple labeling protocol.
- Refer to the “IHC Multiplexing Guide - Discovery through color” for full application details.

Enzyme Substrate Combinations

Recommended combinations of substrates and the recommended order in which they should be used.

Second Substrate First Substrate		Alkaline Phosphatase			Peroxidase					
		ImmPACT Vector Red & Vector Red (magenta) SK-5105 & SK-5100	Vector Blue (blue) SK-5300	BCIP/NBT (indigo) SK-5400	ImmPACT VIP & Vector VIP (purple) SK-4605 & SK-4600	ImmPACT DAB, ImmPACT DAB EqV & DAB (brown) SK-4105 , SK-4103 , SK-4100	DAB-Ni (gray-black) SK-4100	ImmPACT NovaRED & Vector NovaRED (red) SK-4805 & SK-4800	ImmPACT SG & SG (blue-gray) SK-4705 & SK-4700	ImmPACT AEC, ImmPACT AMEC Red & AEC (red) SK-4205 , SK-4285 , SK-4200
Alkaline Phosphatase	ImmPACT Vector Red & Vector Red (magenta) SK-5105 & SK-5100		—	—	—	+	+	—	+	—
	Vector Blue (blue) SK-5300	+		—	+	+	+	+	+	+
	BCIP/NBT (indigo) SK-5400	+	—		+	+	+	+	+	+
Peroxidase	ImmPACT VIP & Vector VIP (purple) SK-4605 , SK-4600	—	+	—		+	+	—	+	—
	ImmPACT DAB, ImmPACT DAB EqV & DAB (brown) SK-4105 , SK-4103 , SK-4100	+	+	+	+		—	—	+	+
	DAB-Ni (gray-black) SK-4100	+	—	—	+	+		+	—	—
	ImmPACT NovaRED & Vector NovaRED (red) SK-4805 , SK-4800	—	+	+	—	+	+		+	—
	ImmPACT SG & SG (blue-gray) SK-4705 , SK-4700	+	—	—	+	+	—	—		+
	ImmPACT AEC, ImmPACT AMEC Red & AEC (red) SK-4205 , SK-4285 , SK-4200	—	—	—	—	+	—	—	+	

+ Indicates good contrast

— Indicates incompatibility of substrates for various reasons

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vectorlabs.com/substrates