The Pathology Company

Leica BIOSYSTEMS

Colocalization Algorithm

User's Guide

MAN-0082, Revision D | 3 August 2015

Colocalization Algorithm User's Guide

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Contents

1 Introduction

This chapter introduces you to the Colocalization algorithm. For general information on using an algorithm, please see the *Aperio Image Analysis User's Guide*.

The primary source for information on creating, testing, and saving image analysis macros is the ***Aperio Image Analysis User's Guide****. That guide also contains details on running image analyses from within eSlide viewers and batch analyses from within eSlide Manager, and viewing and exporting analysis results.*

The Aperio Image Analysis Workstation provides a streamlined image analysis workflow on your local workstation; if you are using that product, please refer to the ***Aperio Image Analysis Workstation User's Guide*** *for instructions on creating, testing, and saving image analysis macros That guide also contains details on running image analyses from within ImageScope, running batch analyses, and viewing and exporting analysis results.*

About This Guide

This guide for this image analysis algorithm discusses how to set the algorithm parameters to suit your image analysis needs. After tuning the parameters, you will save the settings as an algorithm macro. The macro can then be used by you and other users to analyze specific eSlides (digital slide images).

This guide works in concert with the *Aperio Image Analysis User's Guide* or the *Aperio Image Analysis Workstation User's Guide* to present the complete picture of Aperio image analysis.

Prerequisites

The Colocalization algorithm requires that you use Spectrum Release 9 or later or eSlide Manager Release 12 or later.

Because Aperio eSlides are by design high resolution and information rich, for best results you should use a high quality monitor to view them. Make sure the monitor is at the proper viewing height and in a room with appropriate lighting. We recommend any high quality LCD monitor meeting the requirements recommended in the *Aperio ePathology System Requirements*.

Intended Use

Algorithms are intended to be used by trained pathologists who have an understanding of the conditions they are testing for in running the algorithm analysis.

Each algorithm has input parameters that must be adjusted by an expert user who understands the goal of running the analysis and can evaluate the algorithm performance in meeting that goal.

You will adjust (tune) the parameters until the algorithm results are sufficiently accurate for the purpose for which you intend to use the algorithm. You will want to test the algorithm on a variety of images so its performance can be evaluated across the full spectrum of expected imaging conditions. To be successful, it is usually necessary to limit the field of application to a particular tissue type and a specific histological preparation. A more narrowly defined application and consistency in slide preparation generally equates to a higher probability of success in obtaining satisfactory algorithm results.

If you get algorithm analysis results that are not what you expected, please see the "Troubleshooting" section in the *Aperio Image Analysis User's Guide* for assistance.

For research use only. Not for use in diagnostic procedures.

Installing the Algorithm

In most cases you install the algorithm on the eSlide Manager server only. (In fact, Technical Services may install the algorithm for you on your server.) This is because you typically fine-tune and save the algorithm parameters on the eSlide Manager server. For the rare case that you need to fine-tune the algorithm parameters on your local workstation or by using a local image, refer to the *Aperio Image Analysis User's Guide* for installation and use instructions.

The Colocalization Algorithm

In histology and cytology, a variety of staining methods are used to target different types of tissues and cellular structures and for detection of specific proteins. In an H&E stain, for example, Hematoxylin preferentially stains the nucleus, while Eosin stains both nucleus and cytoplasm. In IHC analyses, different stains mark the presence of one or more proteins within the cell.

The Colocalization algorithm calculates the contribution of each stain at every pixel location in the image. For IHC, it determines where specific proteins are present and to what extend the proteins are "colocalized"—that is, whether they occur separately or in combination with each other.

Detecting and measuring the colocalization of multiple proteins is an important part of larger scientific studies, which seek to determine a correlation between the occurrence of these proteins and the outcome of a specific disease treatment.

The Colocalization algorithm classifies each pixel as either part of a single stain or representing a combination of stains based on the separated stains' intensities.

Analysis Steps

1. The first step of colocalization analysis is to calibrate the stain color vectors so that the algorithm can accurately detect the stains. This step is covered in *"Tuning Algorithm Parameters" on page 12*.
2. The next step is to set stain thresholds. This is discussed in *"Thresholding" on page 13*.
3. The final step is to analyze the eSlide to obtain colocalization data (see the example below).

After stain color vectors have been calibrated and staining thresholds set (see the next chapter), you can run the algorithm in analysis mode to determine the percentages and intensities of stains that occur alone in the image and in combination with other stains.

To select the analysis mode for colocalization of stains:

1. Create a macro (as discussed in the next chapter) in which the algorithm parameter **Mark-up Image Type** drop-down list is set to **Co-Localization** and you have selected the analysis mode you want to use from the **Mode** drop-down list.
2. Save the macro (perhaps with a name that reflects the analysis mode you are using).

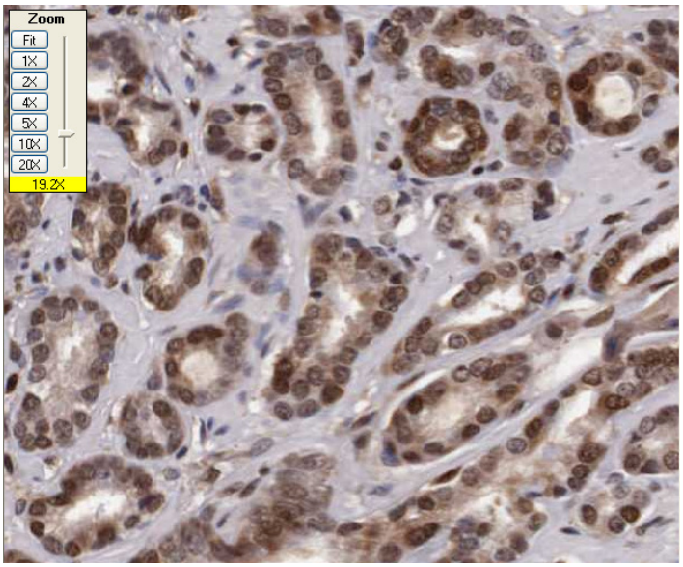
Now you can use the macro to analyze eSlides with the selected colocalization mode. See the examples below.

Cytoplasmic Analysis Example

In the following cytoplasmic example, Hematoxylin was used as the counter-stain with DAB as the cytoplasmic stain.

The objective was to measure only the cytoplasmic component of the DAB staining. Since the DAB stains both cytoplasm and nuclei, this is a difficult task for most algorithms.

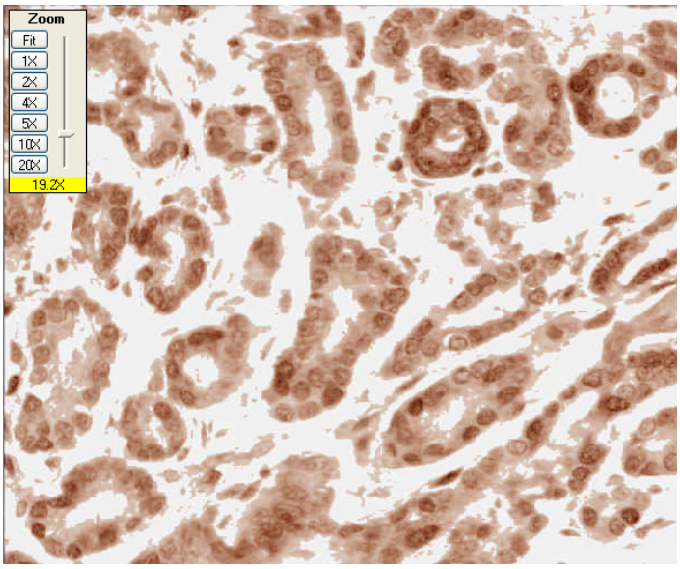
The original image of the eSlide looks like this in the ImageScope main window:



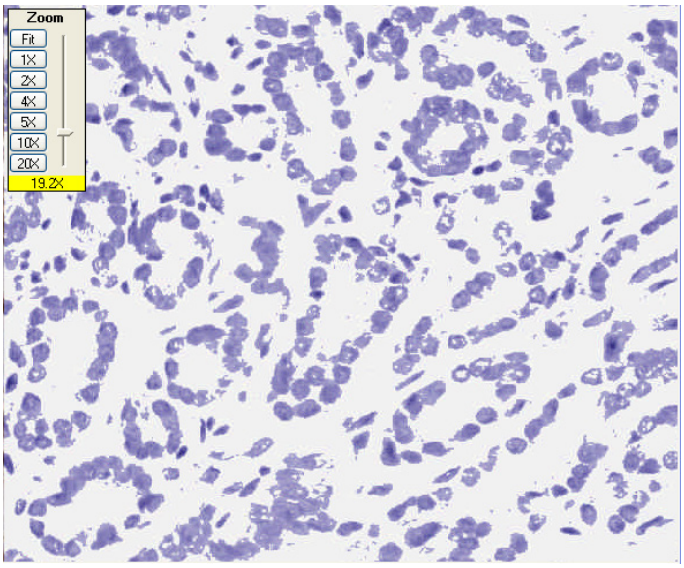
Colocalization separates the two stains and the cytoplasmic component is identified as the area where DAB only is present without Hematoxylin staining—this is the green component in the mark-up image.

The algorithm reports the percentage of the area that is comprised of cytoplasm along with the intensity of the cytoplasm staining.

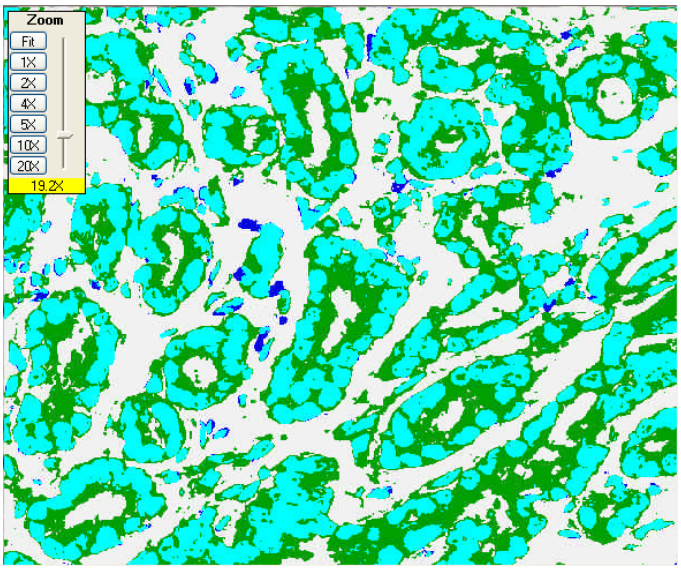
Using the Color 3 deconvolution selection in the Mark-up Image Type drop-down list results in a mark-up image of just the DAB stain (nuclei and cytoplasm):



Using the Color 1 deconvolution selection results in a mark-up image of just the Hematoxylin stain (nuclei):



And running the algorithm in 0 – Colocalization analysis mode results in a mark-up image that shows all the combinations of stains present:



Nuclei – shown in cyan; Cytoplasm–shown in green.

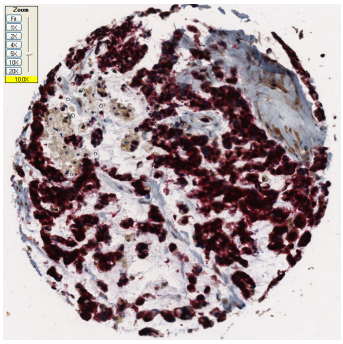
Double Labeling Example

Double-label immunohistochemistry analysis is a special case of the more general colocalization analysis. In the case of double-label analysis, Color 1 represents the counterstain, for which you want information only for where Color 1 occurs by itself, not where it occurs in combination with Color 2 and Color 3. Colors 2 and 3 are used to identify specific protein markers.

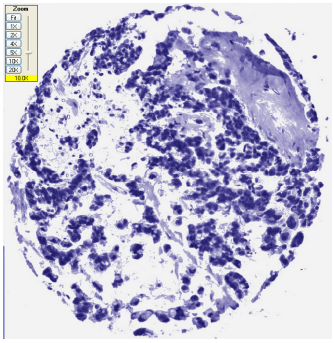
In the following double-labeling example, Fast Red and DAB were used as marker stains with Hematoxylin as the counter-stain.

The Colocalization algorithm separated the stains and reported the percentage of the stained area in which the stains occur separately (Color 2), (Color 3), and together (Colors 2+3). These three states are shown as different colors in the mark-up image.

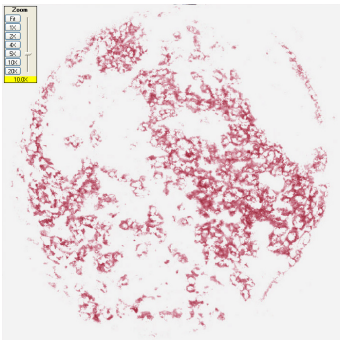
The intensity of each stain was also reported for each of the three states. The intensity information provides a measure of the protein concentration, with darker intensity corresponding to more protein.



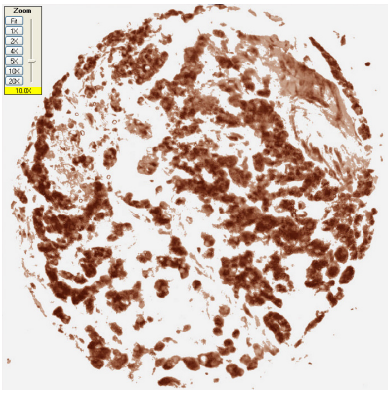
Double-labeled TMA



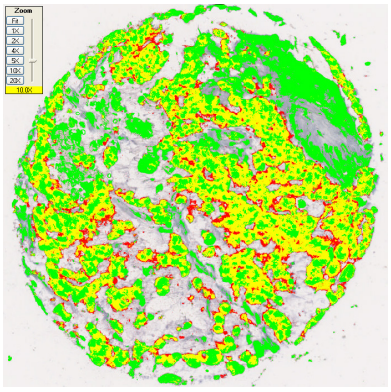
Color 1 – Hematoxylin



Color 2 – Fast Red



Color 3 – DAB



Mark-up Image

Marker	Color	Percent Area
Color 2	Red	10.1
Color 3	Green	50.8
Color 2+3	Yellow	39.1

For More Information

For a quick reference to the Colocalization algorithm input parameters and results, see *"Chapter 2: Tuning Parameters" on page 12*.

See the *Aperio Image Analysis User's Guide* for information on:

- Installing an algorithm
- Opening an eSlide to analyze
- Selecting areas of an eSlide to analyze
- Running the analysis
- Exporting analysis results

For details on using eSlide Manager (for example, for information on running batch analyses), see the *eSlide Manager Operator's Guide*.

For details on using ImageScope to view eSlides and information on using annotation tools to select areas of the eSlide to analyze, see the *ImageScope User's Guide*.

2 Tuning Parameters

This chapter contains information on tuning Colocalization algorithm parameters.

Tuning Algorithm Parameters

To create a macro for the algorithm or to modify an existing macro:

1. In ImageScope, open an eSlide you want to use to tune the algorithm parameters. (Refer to the *ImageScope User's Guide* for instructions.)
2. In ImageScope, open the algorithm and choose to create a new macro or open an existing macro to modify it. (Refer to the *Aperio Image Analysis User's Guide* for instructions.)

   You now see the parameters for the algorithm (these are listed later in this chapter).
3. Now adjust the parameters in the Analysis window as discussed below and move the Algorithm Tuning window on the image to see a mark-up image of the results.

After adjusting the input parameters and selecting the output parameters as discussed in the following sections, move the Algorithm Tuning window to various areas on the eSlide to see an approximation of the analysis results. The results appear in the Annotations window and as a mark-up image in the ImageScope main window.

You can open the ImageScope Annotations window to see the algorithm tuning results displayed numerically.

When saving the algorithm macro by clicking  , you can choose whether to save the macro locally on your workstation or, if connected to eSlide Manager, to save the macro remotely on eSlide Manager. You are asked to supply a name for the macro that will help you identify it in the future. If saving the macro to eSlide Manager, you are also asked to specify which data group the macro will be associated with; only eSlide Manager users who have permission to use that data group will be able to choose that macro for analysis.

Color Calibration

By defining the stain color vectors, you are identifying to the Colocalization algorithm which color identifies which stain.

The default color vector values are as follows:

- **Color 1** – Hematoxylin
- **Color 2** – Eosin
- **Color 3** – DAB

The color vector numbers must be changed if different stains are used. The color for each stain is calibrated separately, using a separate image for each stain in which only that color is present.

If possible, use a separate control slide for each stain you want to analyze.

If this is not possible, look for several areas of the eSlide that are mostly stained with stain of interest and select them by using the ImageScope drawing tools. Pick an area of light staining of only this color. Avoid selecting darker, overstained areas.

See the *eSlide Manager Operator's Guide* for information on logging into eSlide Manager, finding an eSlide, and opening the eSlide in ImageScope.

If you are using only two stains, not three, set the color vector values for the third color to zero.

4. After opening the eSlide in ImageScope, go to the **View** menu and select **Analysis**. You see the Analysis window on which you can select the Colocalization algorithm (see the *Aperio Image Analysis User's Guide* for instructions on selecting an algorithm and tuning its parameters).
5. Use the ImageScope rectangle drawing tool to select the areas of interest (start with representative areas that show Color 1).
6. On the Analysis window, from the algorithm parameters select **Deconvolved Color Channel (1)** from the **Mark-up Image Type** drop-down list.
7. On the ImageScope main window, move the Algorithm Tuning window over one of the areas you annotated in the step above. Note that you can drag the tuning window to other locations in the image, and can change its size by grabbing a corner of the window and pulling it in or out.
8. Go to the **View** menu and select **Annotations**.
9. On the Annotations window, go to the area of the results that shows the OD (optical density) values for Red, Green, and Blue.

Average Red OD	0.540967
Average Green OD	0.630966
Average Blue OD	0.55609

10. Type those values into the Algorithms window corresponding color component lines for Color 1.

Color (1) - Red Component	0.65
Color (1) - Green Component	0.704
Color (1) - Blue Component	0.286

11. The macro is now calibrated for Color 1.

Now repeat these steps for the other colors used, opening an appropriate eSlide for the color and selecting the correct deconvolution choice from the **Mark-up Image Type** drop-down list.

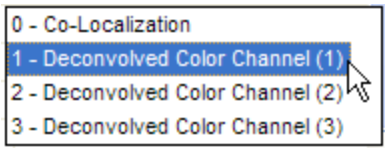
Thresholding

By setting upper and lower stain thresholds, you are selecting feature detection thresholds. For example, if you increase the background lower threshold, you will exclude very dark areas. Change the thresholds to pick up just the range of color you need.

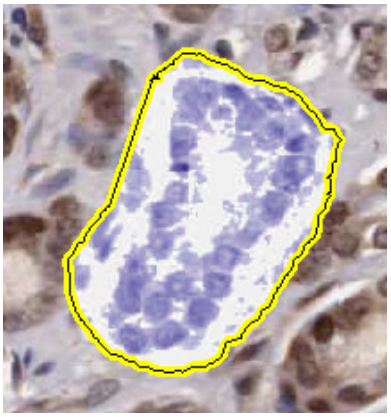
As with the previous calibration step, you will need to open an appropriate eSlide.

For each color being used:

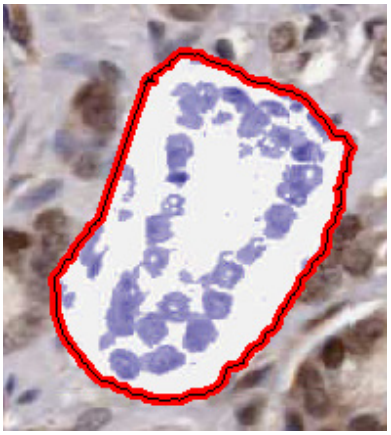
1. Select **Deconvolved Color Channel** for the color you are working with from the **Mark-up Image Type** drop-down list. In this case we select **Deconvolved Color Channel (1)** because we are going to set the thresholds for Color 1:



2. Now set the thresholds for that color to maximize feature detection. For example, in the sample below we want to quantify cytoplasm cells separately from nuclei. Nuclei are stained with Hematoxylin, while nuclei *and* cytoplasm are stained with DAB. Running with **Deconvolved Color Channel (1)** selected and the default, calibrated color vectors results in an image in the tuning window like this:



3. To maximize detection of nuclei, change the upper threshold for Color 1 to a lower number (0 = darkest; 255 = lightest), say from 200 to 180. Now more nuclei and less cytoplasm are detected:



4. Now to minimize picking up background, set the **Mark-up Image Type** to **Deconvolved Color Channel (3)**. (Remember, we aren't using Color 2) and try reducing the upper threshold to limit the amount of background detected.

   If you are not using a color (for example, the slide has been stained only with two stains), then set the unused color thresholds to zero.

Algorithm Parameters

Colocalization algorithm performance is controlled by a set of input parameters, which determine many different types of analysis.

- **View Width** – Width of processing box.
- **View Height** – Height of processing box.
- **Overlap Size** – Size of the overlap region for each view. This should be at least as big as the average object size.
- **Image Zoom** – Zoom level to be used; a higher zoom results in faster algorithm run but less accurate results.
- **Markup Compression Type** – This sets the compression type for the algorithm mark-up image. Choose better compression if you need the image for a special purpose.
- **Compression Quality** – A higher quality takes longer and yields larger files. This selection does not apply to all compression types.
- **Mark-up Image Type** – There are two types of mark-up images – Co-Localization (used for colocalization analysis) and Deconvolved (used to calibrate stain color vectors).
- **Mode** – Choose Colocalization mode or Counter-stain, Double Label mode.

  Double-label immunohistochemistry analysis is frequently used to identify cellular and subcellular colocalization of independent antigens, and is a special case of the more general colocalization analysis. In the case of double-label analysis, Color 1 represents the counterstain, for which you want information only for where Color 1 occurs by itself, not where it occurs in combination with Color 2 and Color 3. Colors 2 and 3 are used to identify specific protein markers. See *"Cytoplasmic Analysis Example" on page 8* for examples of both types of analysis.
- **Color (1) Threshold** – Intensity threshold (upper limit) for color channel 1.
- **Color (1) Lower Threshold** – Intensity threshold (lower limit) for color channel 1.
- **Color (2) Threshold** – Intensity threshold (upper limit) for color channel 2.
- **Color (2) Lower Threshold** – Intensity threshold (lower limit) for color channel 2.
- **Color (3) Threshold –** Intensity threshold (upper limit) for color channel 3.
- **Color (3) Lower Threshold** – Intensity threshold (lower limit) for color channel 3.
- **Color (1) Red Component** – OD (optical density) for color 1 Red (default is Hematoxylin stain).
- **Color (1) Green Component** – OD (optical density) for color 1 Green (default is Hematoxylin stain).
- **Color (1) Blue Component** – OD (optical density) for color 1 Blue (default is Hematoxylin stain).
- **Color (2) Red Component** – OD (optical density) for color 2 Red (default is Eosin stain).
- **Color (2) Green Component** – OD (optical density) for color 2 Green (default is Eosin stain).
- **Color (2) Blue Component** – OD (optical density) for color 2 Blue (default is Eosin stain).
- **Color (3) Red Component** – OD (optical density) for color 3 Red (default is DAB stain).
- **Color (3) Green Component** – OD (optical density) for color 3 Green (default is DAB stain).
- **Color (3) Blue Component** – OD (optical density) for color 3 Blue (default is DAB stain).

- **Clear Area Intensity** – This is the intensity for a clear area on the slide. This value is always 240 for ScanScope generated images.

Algorithm Results

The algorithm results appear in the ImageScope Annotations window (go to the ImageScope **View** menu and select **Annotations**).

The first section of the annotations window displays the algorithm results; the second portion (labeled "Algorithm Inputs") repeat the algorithm input parameters you specified when you ran the algorithm.

The results give information on all the permutations of the colors detected. And the different colors in the mark-up image reflect those data.

Understanding the Results

As an example of interpreting the results listed below, the color magenta in the mark-up image shows all pixels that contain both Color 1 *and* Color 2. The intensities listed under "Percent (1+2) MAGENTA" in the results give the intensity of Color 1 in all areas that contain *both* Color 1 and Color 2 and the intensity of Color 2 in all areas that contain both Color 1 and Color 2.

- **Percent (1) BLUE** – Percent of the analyzed area that contains Color 1. Shown in blue in the mark-up image.
  - **Intensity (1,1)** – Intensity of Color 1 in areas consisting of only Color 1.
- **Percent (1+2) MAGENTA** – Percent of the analyzed area that contains Color 1 and Color 2. Shown in magenta in the mark-up image.
  - **Intensity (1, 1+2)** – Intensity of Color 1 in areas consisting of Color 1 and Color 2.
  - **Intensity (2, 1+2)** – Intensity of Color 2 in areas consisting of Color 1 and Color 2.
- **Percent (2) RED** – Percent of the analyzed area that contains Color 2. Shown in red in the mark-up image.
  - **Intensity (2,2)** – Intensity of Color 2 in areas consisting of only Color 2.
- **Percent (2+3) YELLOW** – Percent of the analyzed area that contains Color 2 and Color 3. Shown in yellow in the mark-up image.
  - **Intensity (2, 2+3)** – Intensity of Color 2 in areas consisting of Color 2 and Color 3.
  - **Intensity (3, 2+3)** – Intensity of Color 3 in areas consisting of Color 2 and Color 3.
- **Percent (3) GREEN** – Percent of the analyzed area that contains Color 3. Shown in green in the mark-up image.
  - **Intensity (3, 3)** – Intensity of Color 3 in areas consisting of only Color 3.
- **Percent (1+3) CYAN** – Percent of the analyzed area that contains Color 1 and Color 3. Shown in cyan in the mark-up image.
  - **Intensity (1, 1+3)** – Intensity of Color 1 in areas consisting of Color 1 and Color 3.
  - **Intensity (3, 1+3)** – Intensity of Color 3 in areas consisting of Color 1 and Color 3.

- **Percent (1+2+3) BLACK** – Percent of the analyzed area that contains Color 1, Color 2, and Color 3. Shown in black in the mark-up image.
  - **Intensity (1, 1+2+3)** – Intensity of Color 1 in areas containing Color 1, Color 2, and Color 3.
  - **Intensity (2, 1+2+3)** – Intensity of Color 2 in areas containing Color 1, Color 2, and Color 3.
  - **Intensity (3, 1+2+3)** – Intensity of Color 3 in areas containing Color 1, Color 2, and Color 3.
- **Overall Intensity (1)** – Overall intensity of Color 1 in the analyzed area.
- **Overall Intensity (2)** – Overall intensity of Color 2 in the analyzed area.
- **Overall Intensity (3)** – Overall intensity of Color 3 in the analyzed area.
- **Total Stained Area (mm^2)** – Total area (in mm2) that is stained.
- **Total Analysis Area (mm^2)** – Total area (in mm2) that was analyzed.
- **Average Red OD** – Average optical density of Red.
- **Average Green OD** – Average optical density of Green.
- **Average Blue OD** – Average optical density of Blue.

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Symbols

- The following symbols may appear on your product label or in this user's guide:

	Manufacturer
	Date of manufacture (year - month - day)
	European Union Authorized Representative
	In vitro diagnostic device
	Serial number
	Catalog number
	Relative humidity range
	Storage temperature range
	Electronic and electrical equipment waste disposal
	The exclamation point within an equilateral triangle is intended to alert you to the presence of important operating and maintenance (servicing) instructions. *Le point d'exclamation dans un triangle équilatéral vise à avertir l'utilisateur qu'il s'agit d'instructions d'utilisation et d'entretien importantes.*
	The lightning flash with arrowhead symbol within an equilateral triangle is intended to alert you to the presence of uninsulated "dangerous voltage" within the product's enclosure that may be of sufficient magnitude to constitute a risk of electric shock to persons. *Le symbole de l'éclair avec la pointe de flèche dans un triangle équilatéral vise à avertir l'utilisateur que le boîtier du produit présente une « tension dangereuse » non isolée d'une amplitude suffisante pour constituer un risque d'électrocution.*
	The flat surface with waves symbol within an equilateral triangle is intended to alert you to the presence of hot surfaces which could cause burn damage. *Le symbole d'une surface plane et de vagues dans un triangle équilatéral vise à avertir l'utilisateur de la présence de surfaces chaudes qui peuvent causer des brûlures.*
	The UV lamp within an equilateral triangle is intended to alert you to the presence of UV light within the product's enclosure that may be of sufficient magnitude to constitute a risk to the operator. *La lampe UV dans un triangle équilatéral vise à avertir l'utilisateur de la présence de rayonnement UV dans le boîtier du produit qui peut être d'une amplitude suffisante pour constituer un risque pour l'utilisateur.*

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