

DCTD Standard Operation Procedures (SOP)

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Doc. #:	SOP340548	Revision:	A	Effective Date: 6/16/2020

National Clinical Target Validation Laboratory

Applied/Developmental Research Directorate, Leidos Biomedical Research, Inc.

Frederick National Laboratory for Cancer Research

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**Please check for revision status at**  
<https://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>  
**and be sure to use the current version.**

Change History

Revision	Approval Date	Description	Originator	Approval
--	8/01/2017	New Document	TN/KFG/DK	JJ
A	6/16/2020	Preparation for posting to public website, updated SOP340549 to SOP340550 throughout the document; Updated jejunum control information; Updated ROI naming conventions and final reporting information. Other editorial and screenshot updates throughout the document.	KFG/DK/LL	JJ

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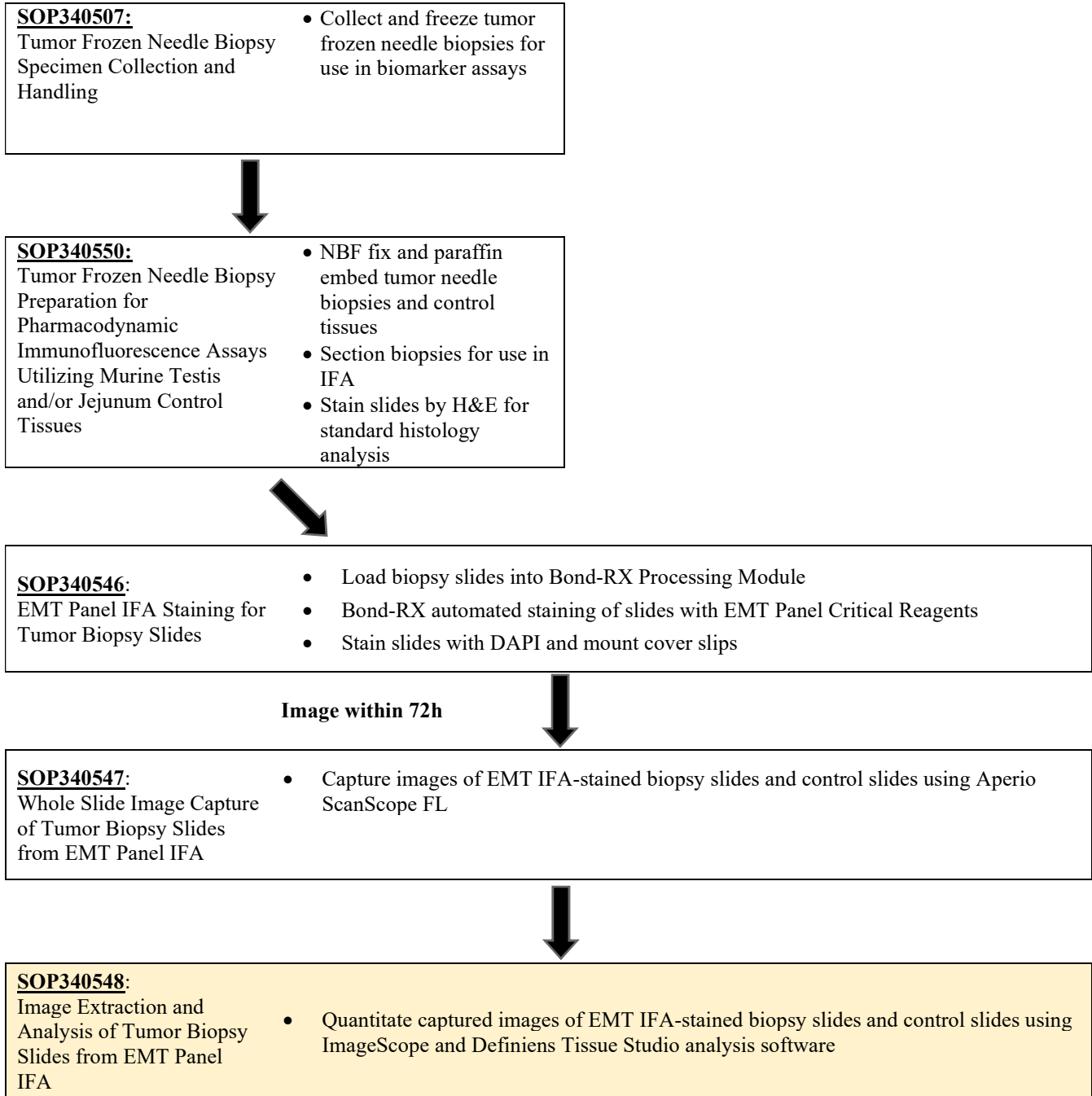
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**OVERVIEW OF EMT PANEL IMMUNOFLUORESCENCE ASSAY FOR BIOPSIES**



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### 1.0 PURPOSE

The purpose of this SOP is to standardize an immunohistochemical method for detecting and quantifying epithelial-mesenchymal transition (EMT) markers in formalin-fixed, paraffin-embedded tumor biopsy sections to support clinical trial pharmacodynamic (PD) studies. The EMT Panel includes E-Cadherin and Vimentin, with  $\beta$ -Catenin for tumor segmentation. The goal of the SOP and associated training is to ensure consistency of measurements, and to standardize the reporting of PD data for EMT Immunofluorescence Assay (IFA) measurements.

### 2.0 SCOPE

This procedure applies to all personnel involved in the use of the EMT Panel IFA for tumor biopsies from patients participating in clinical trials. This SOP outlines the recommended procedure for image extraction and quantitation of EMT-stained, paraffin-embedded tumor biopsy sections.

### 3.0 ABBREVIATIONS

Ab	=	Antibody
AF488	=	Alexa Fluor® 488
AF546	=	Alexa Fluor® 546
AF647	=	Alexa Fluor® 647
CTNNB1	=	$\beta$ -Catenin
DAPI	=	4',6-Diamidino-2-Phenylindole
DCTD	=	Division of Cancer Treatment and Diagnosis
EMT	=	Epithelial to mesenchymal transition
FFPE	=	Formalin-fixed paraffin-embedded tissue
FNLCR	=	Frederick National Laboratory for Cancer Research
H&E	=	Hematoxylin and Eosin
ID	=	Identification/Identifier
IFA	=	Immunofluorescence Assay
LHTP	=	Laboratory of Human Toxicology and Pharmacology
NCTVL	=	National Clinical Trial Validation Laboratory
NA	=	Numerical Aperture
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
QC	=	Quality Control
ROI	=	Region of Interest
RT	=	Room Temperature
SOP	=	Standard Operating Procedure
SVS	=	Scientific Visualization Studio

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### 4.0 INTRODUCTION

The EMT Panel IFA was developed to quantitatively evaluate epithelial (E-Cadherin positive), mesenchymal (Vimentin positive) and transitional (colocalization of Vimentin and E-Cadherin) phenotypes in tumor tissue sections. In some cancers, high levels of Vimentin and low levels of E-Cadherin have been associated with a poor prognosis.

This assay is designed to detect, quantify, and analyze changes in the amounts of cellular markers Vimentin, E-Cadherin, and their transitional overlap in biopsied tissue in support of PD clinical trials. The tissue biopsies are collected at different time-points, usually pre-dose and post-dose. The assay detects E-Cadherin and Vimentin using monoclonal antibodies that are directly conjugated to Alexa Fluor immunostaining reporters. To define the area of the tumor tissue within which markers are quantitated, a pathologist annotates the viable tumor areas within each biopsy. In addition,  $\beta$ -Catenin staining is used to better define tumor cells from surrounding normal tissue, such as stroma, which also expresses significant levels of Vimentin. **It is imperative to segment tumor regions using  $\beta$ -Catenin to preclude measuring Vimentin from normal mesenchyme.**

Detailed information regarding the development, validation and application of this assay has been described in: Navas, T. et al, Clinical evolution of epithelial-mesenchymal transition in human carcinomas. Cancer Res. Nov. 15, 2019.

### 5.0 ROLES AND RESPONSIBILITIES

**Laboratory Director/Supervisor** The Laboratory Director/Supervisor, directs laboratory operations, supervises technical personnel, reporting of findings, and is responsible for the proper performance of all laboratory procedures. The Laboratory Director/Supervisor oversees the personnel running SOPs within the laboratory and is responsible for ensuring this person(s) is certified and has sufficient experience to handle clinical samples.

**Certified Assay Operator** The Certified Assay Operator may be a Laboratory Technician/Technologist, Research Associate, or Laboratory Scientist who has been certified through DCTD training on this SOP. The Assay Operator works under the guidance of the Laboratory Director/Supervisor. This person, in accordance with the current SOP(s), performs laboratory procedures and examinations and any other procedures conducted by a laboratory, including maintaining equipment and records and performing quality assurance activities related to performance.

**5.1** It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented training and qualification on this SOP prior to the actual handling and processing of samples from clinical trial patients. The Laboratory Director/Supervisor is responsible for ensuring the Certified Assay Operator running the SOP has sufficient experience to handle and analyze clinical samples.

**5.2** The Certified Assay Operator for this SOP should be well versed and comfortable with light microscopy techniques, slide scanning techniques, as well as have familiarity with the Definiens Tissue Studio Software for image analysis.

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- 5.3** The Certified Assay Operator responsible for conducting the assay is to follow this SOP and complete the required tasks and associated documentation. The Batch Record ([Appendix 1](#)) must be completed in *real-time* for each experimental run, with each page *dated and initialed*, and placed with the clinical sample information.
- 5.4** All responsible personnel are to check the DCTD Biomarkers website (<https://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>) to verify that the most recent version of this SOP is being used.

## 6.0 MATERIALS AND EQUIPMENT REQUIRED

- 6.1** Aperio eSlide Manager Leica Biosystems software
- 6.2** Aperio ImageScope software (version 12.4.2.5010 or higher)
- 6.3** Definiens Architect XD 64 version 2.7.0; Tissue Studio Analysis software version 4.4.3
- 6.4** EMT Solution version 2.7
- 6.5** Microsoft Excel software (Excel for Office 365 or higher – with Developer ribbon enabled)
- 6.6** Computer operating system - Windows 10 or higher
- 6.7** Whole slide images of stained clinical sample and control slides that are imaged according to SOP340547 and stained according to SOP340546
- 6.8** SOP340548 Microsoft Excel EMT Master Template version B
- 6.9** Workstation with at least 32.0 GB memory, 64 bit operating system, 4 cores, 8 logical processors, and speed of 3.1 GHz. Must be capable of running at least 2 UHD monitors.

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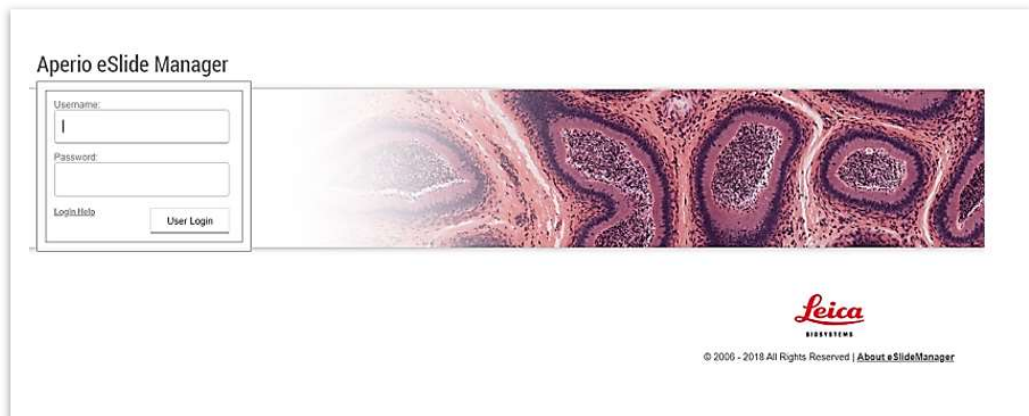
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**7.0 OPERATING PROCEDURES**

- 7.1 The Batch Record ([Appendix 1](#)) for image analysis should be completed to document image extraction and analysis for each patient from a Bond-RX staining run.
  - 7.1.1 Record the Facility/Lab Name, Assay Operator performing the image extraction and quantitation, and the date(s) of analysis.
  - 7.1.2 Record the Patient ID, Tissue ID(s), CTEP Protocol#, Calibrator Lot#, Calibrator Image ID(s), and Patient Slide# in the Batch Record.
  - 7.1.3 After completing Definiens analysis, record marker thresholds for the calibrator and patient images in the Batch Record. Record also the name of the header folder and its location (the EMT Master Template /Data Report must be saved in the header folder).
  - 7.1.4 Comments on extraction and analysis of individual slides or images can be made in the **Comments** section of the batch record.
  - 7.1.5 The Batch Record must be signed and dated before the record is submitted.
- 7.2 **Preparation for annotation and extraction of slide images located on the Aperio eSlide Manager database.**

*Note: See [Appendix 2](#) for control and clinical slide section orientation.*

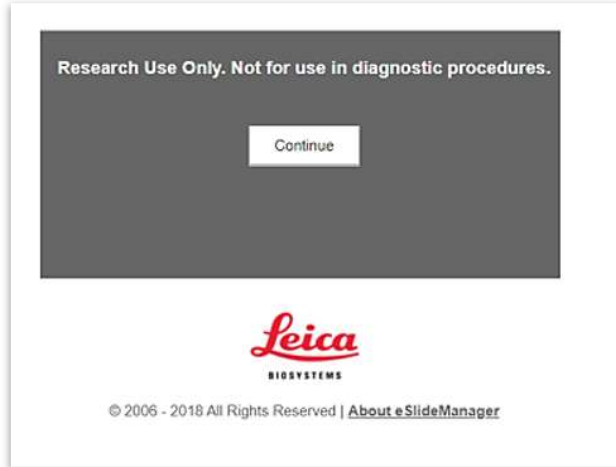
- 7.2.1 Initiate Aperio ImageScope.
- 7.2.2 Access each clinical slide within the clinical eSlide Manager database, and open individual images using Aperio ImageScope following these steps and referring to the associated screenshots shown below: 1) Login into Aperio eSlide Manager; 2) Click **Continue** button; 3) Click the **Cases** icon; 4) Click the file icon next to the case of interest; 5) Click on the brain icon next to the patient of interest; 6) Open the digital slide by clicking once on the slide image. The slide image should be opened in ImageScope.
  - 1) Login with username and password.



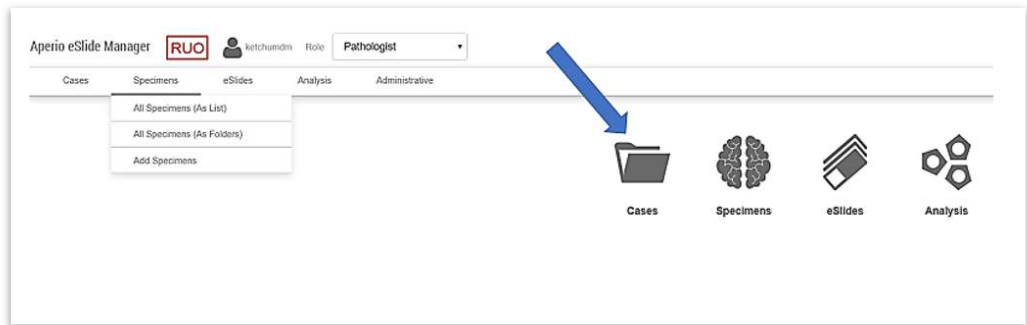
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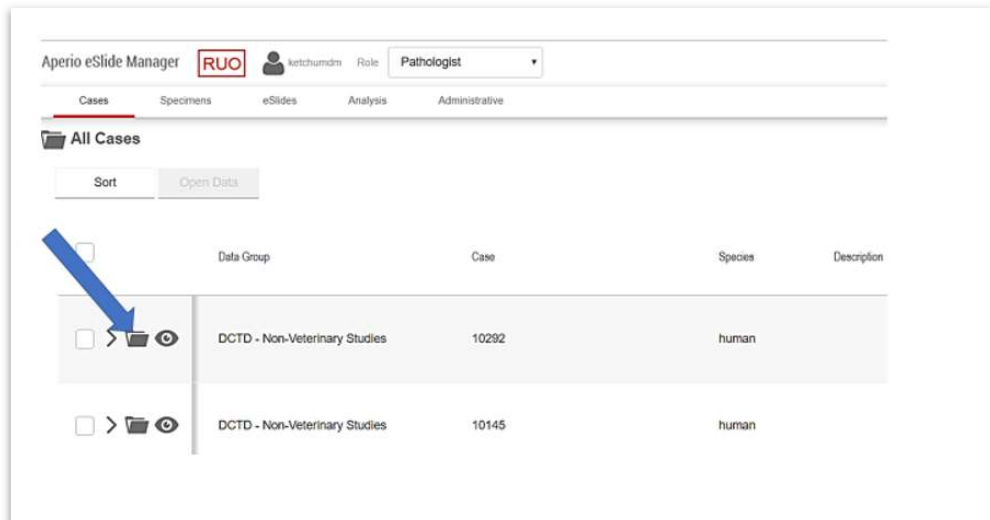
2) Click Continue button.



3) Click the Cases icon.



4) Click the file icon next to the case of interest.

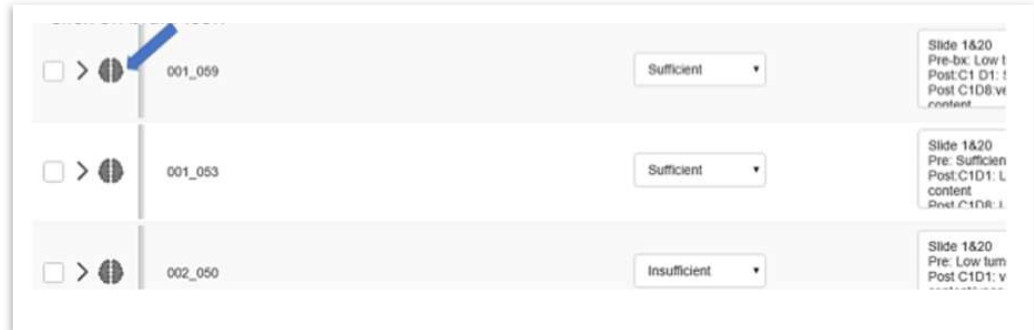




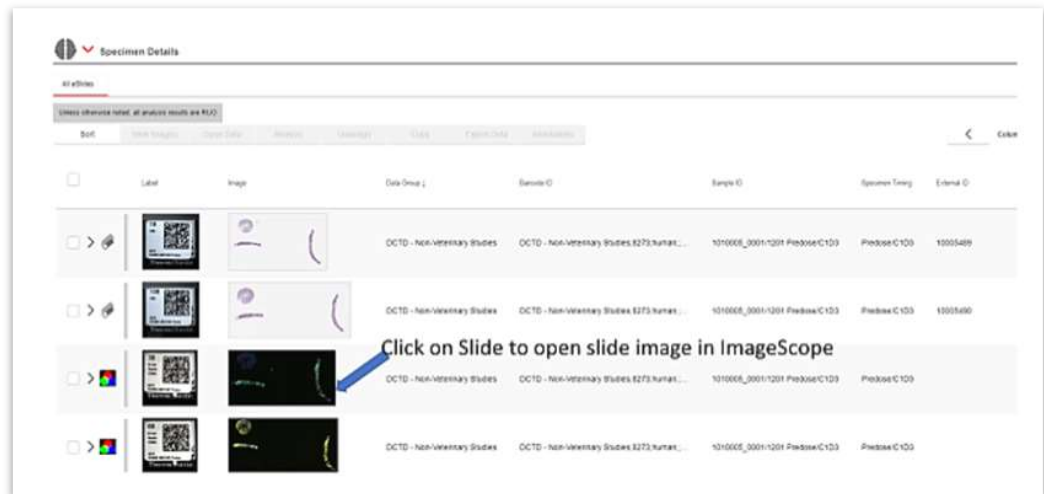
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5) Click the brain icon.



6) Click on slide image to open digital slide in ImageScope.

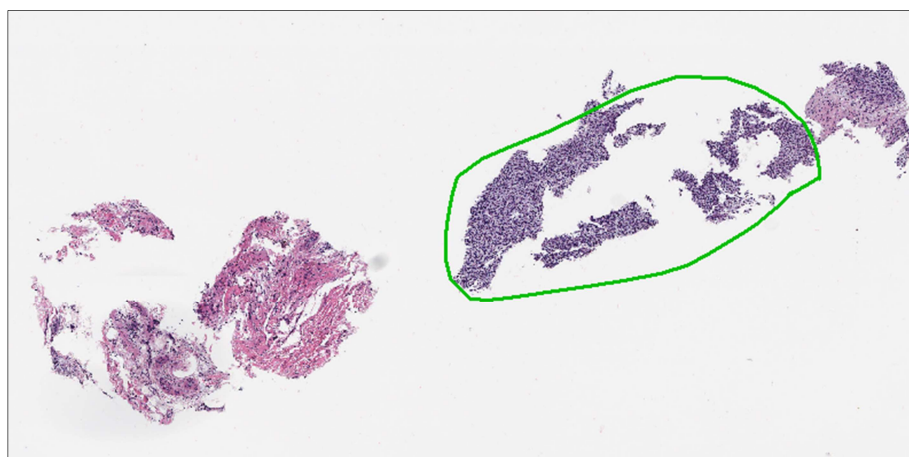


- 7.2.3 Clinical data within NCTVL at FNLCR must be stored in the CLINICAL folder. For each patient, create one header folder on the Definiens Drive: Definiens Image Upload > PADIS > CLINICAL > Specific Trial folder > EMT. The suggested naming convention for this header folder is: CTEP protocol number\_patient ID. Example: **Definiens Image Upload Y: PADIS\CLINICAL\9510\EMT\9510\_1010001**. See [Appendix 6, Section 1](#) for a recommended list of header folder contents.
- 7.2.4 For each EMT control slide, create a folder within the specific CTEP/patient header folder. Name the folder as “EMT Cal CTEP protocol number\_patient number(s)” (more than one patient may be run with a single control slide). Example: EMT Cal Images 9659\_25\_30\_31.
- 7.2.5 Tissues visualized in Aperio ImageScope software are given a “false” color assignment for each biomarker: **E-Cadherin** signal is assigned **green** (Alexa Fluor 488), **Vimentin** signal is assigned **red** (Alexa Fluor 660), **β-Catenin** signal is assigned **yellow** (Alexa Fluor 546) and **DAPI** signal is assigned **blue**.
- 7.2.6 General requirements for the annotation and extraction of images from clinical slides:

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- 7.2.6.1 For clinical tissues, select as many regions of interest (ROIs) as necessary to adequately cover the entire analyzable area, up to **30 images** per tissue/slide, using the pathologist annotated H&E image as a guide. In addition, ROIs should be in clear focus on the stained slides. If large areas of a tissue are not in focus, the tissue cannot be analyzed, and the slide should be re-scanned. ROIs should cover **only** the analyzable areas for the clinical tissues, and avoid areas with necrotic or normal cells, large gaps, tissue folds, nonspecific staining or debris.

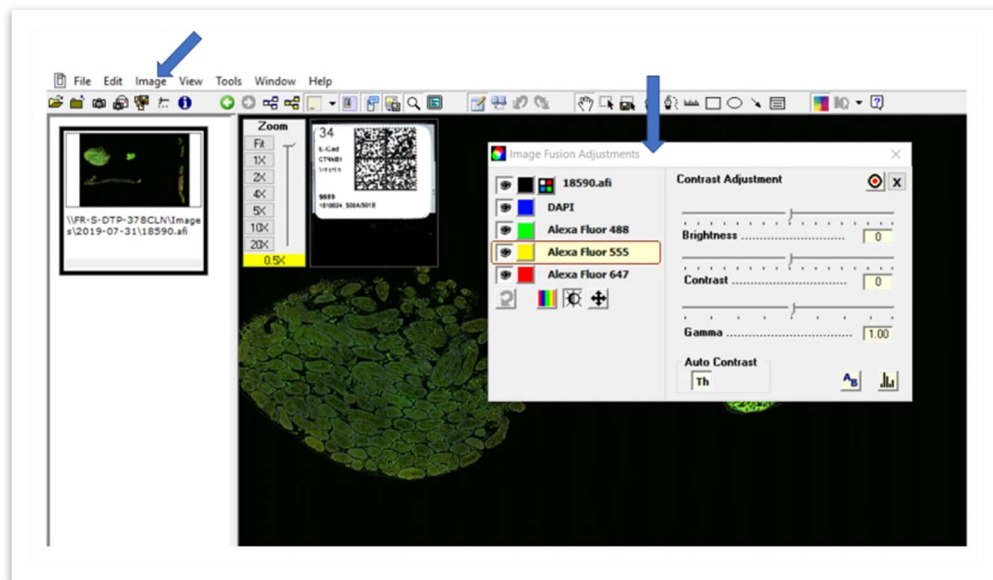
The number of ROIs to be examined per sample will depend on the total area annotated by the pathologist as containing tumor cells and the size of the viable tumor regions. An example of a pathologist's annotated H&E image is shown below. ROI annotations should not overlap. A minimum of at least **2 nonadjacent slides** must be analyzed for each patient. Each biopsy pass must have at least 3000 analyzable nuclei over 2 slides for the data to be reported.



- 7.2.6.2  $\beta$ -Catenin has been shown to permit delineation of tumor tissue from surrounding stroma and normal tissue in many carcinoma biopsies and in some sarcoma biopsies (see published reference in [Section 4.0](#)). All ROIs extracted for analysis from the pathologist-annotated areas should be confirmed to be positive for  $\beta$ -Catenin.

The intensity profile of the  $\beta$ -Catenin signal can be adjusted to optimize visualization. To adjust the  $\beta$ -Catenin signal, follow these steps, and refer to image below: Click the **Image** from toolbar, then select **Fusion Adjustment** from the drop-down menu. In the pop-up box, turn off all colors except yellow by clicking on eye icons next to the colored boxes, then adjust brightness, contrast, and gamma until the desired intensity is achieved. After the adjustment, turn all colors back on by clicking on the eye button on the top.

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7.2.6.3 The intensity of the measured biomarkers Vimentin and E-Cadherin should not be adjusted for patient images.

7.2.6.4 For EMT control slides, select at least 5 different ROIs with a maximum of 10 ROIs per control tissue, and avoid regions of necrosis (as indicated by rounded up nuclei). In addition, extract one glass only or tumor with glass field to facilitate tumor segmentation by DAPI. **For control materials only (NOT FOR CLINICAL SLIDES), ROI selection is guided by where the marker is present in the control tissue.** Vimentin (Fluor 647) and E-Cadherin (Fluor 488) intensity can be adjusted to help guide ROI selection only for control images.

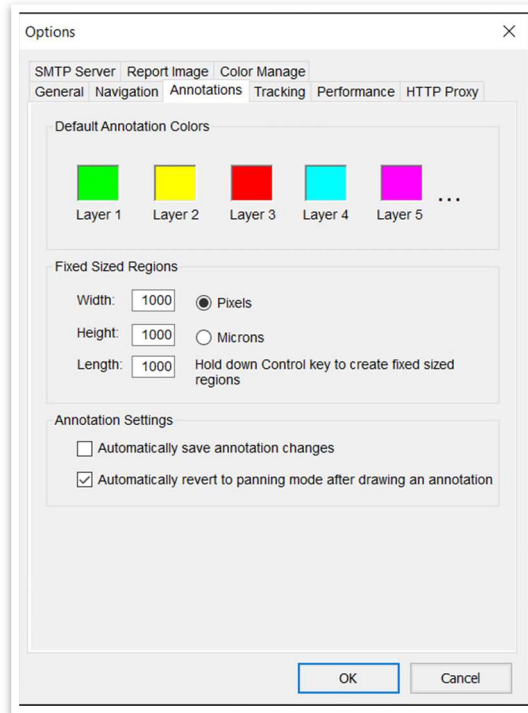
### 7.3 Image Annotation

7.3.1 Prior to image extraction, the image fields to be extracted are selected and annotated.

7.3.2 The rectangle tool, as shown below, is used to draw boxes over the target areas. The size of the rectangle can be automated by selecting: **Tools > Options > Annotations**. Use 1000 for the height, width and length fields.

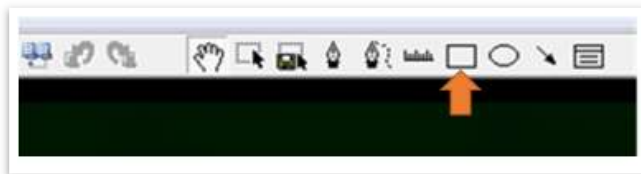
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Simultaneously holding down the keyboard control (Ctrl) button and clicking the rectangle tool (shown below) will automatically create a 1000 x 1000 annotation box, which will allow for faster field selection. If the placement of the annotation box needs to be adjusted, hold down the keyboard Ctrl button and move the box to the desired position.

*Tool bar with retangle tool.*



Each annotated ROI is named in ImageScope as follows: Project (5 digits), (underscore), Patient ID (4 digits), (space), Specimen ID (4 digits), (space), Slide, (space), Slide# (3 digits), (space), timepoint (5 digits), (space), ROI# (2 digits). For any number with less digits than required, add zeros in front of the number to make up for the requirement. Example: 09659\_0029 502D Slide 015 C1D08 01.

The annotated ROIs are named by typing the desired text in the **Text** box in the **Layer Regions** of the **Annotations - Detailed View** window following these steps and referring to images below: 1) Open the **Detail View** window by selecting **View > Annotations** (make sure that the **View Standard Toolbar** is selected). If the **Summary View Annotation** window is open, select the **Details** button to switch to **Detailed View**.

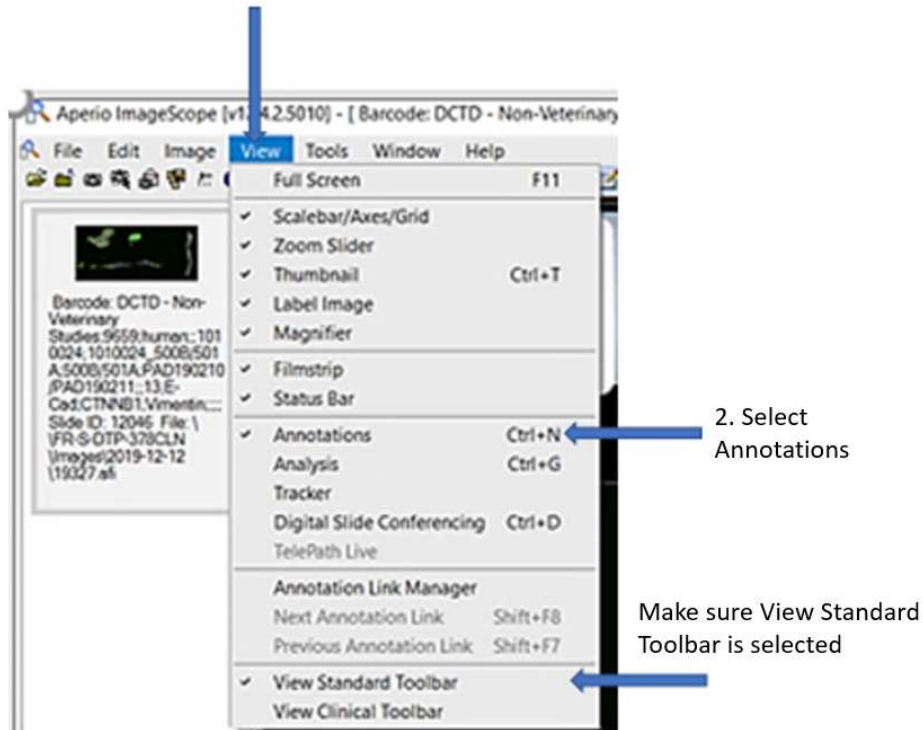
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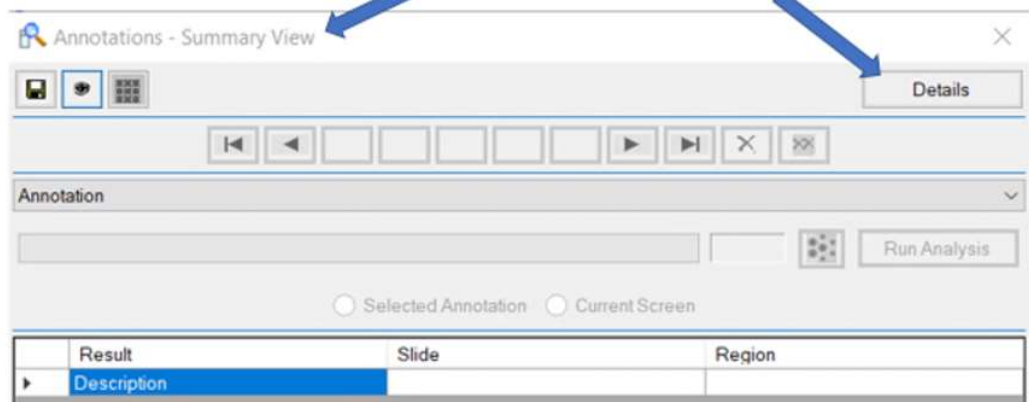
2) In the **Layer Regions** of the **Detailed Annotation** window, widen the **Text** selection and enter the appropriate annotation names; 3) Save the annotations by clicking on the Save button. See an example screenshot of annotated image below.

1) Open Annotation Detailed View box.

1. Click on View tab



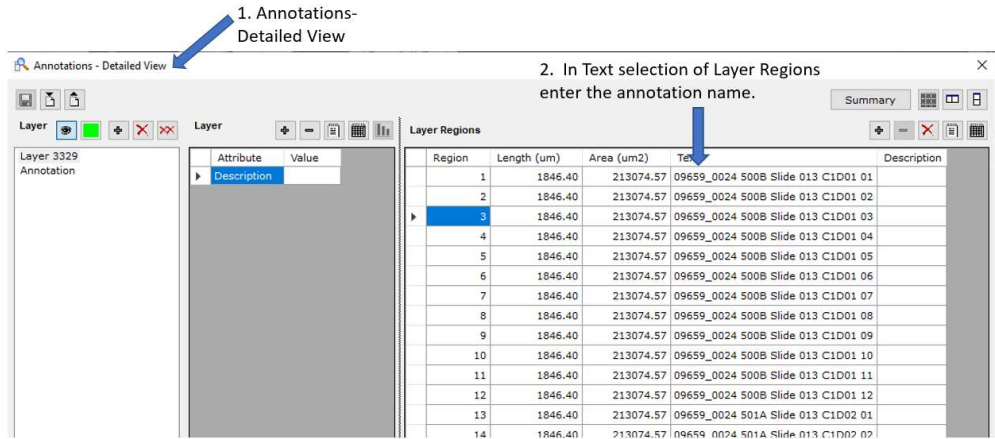
1. If annotation box is in Summary View. Click "Detail" button.



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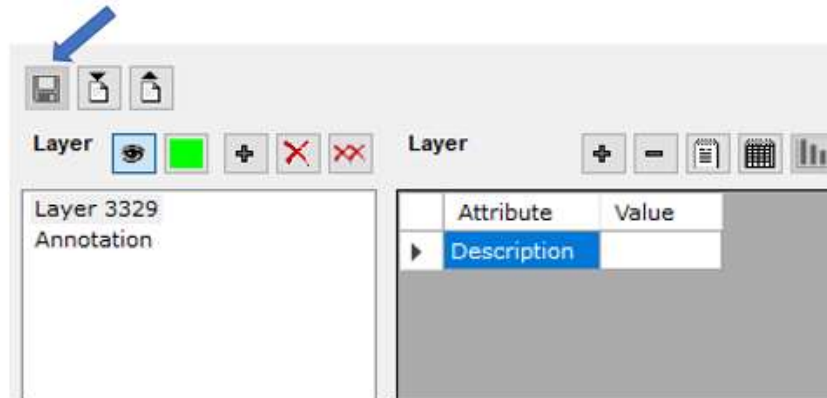
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2) Enter annotation names in the Layer Region of the Annotation Detailed View box.



3) Save the annotations.

Save Annotations by clicking Floppy disc button



4) An example screenshot of pre-treatment tissue with annotations.



### 7.4 Image Extraction

7.4.1 Each annotated ROI is extracted as an SVS file (JPEG2000 file) and saved to the Definiens drive, as described below.

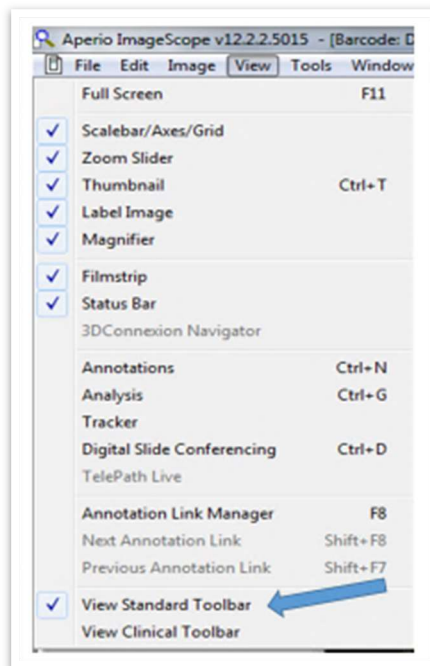
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7.4.2 Select a region of the tumor and zoom in with a magnification of at least 4X. Use the **Extract Tool** button (as shown below) to place the extraction box over the annotated region of interest.



7.4.3 If the Extraction image tool button is not located on the toolbar, go to the View tab and select the **View Standard Toolbar** from the menu, as shown below.



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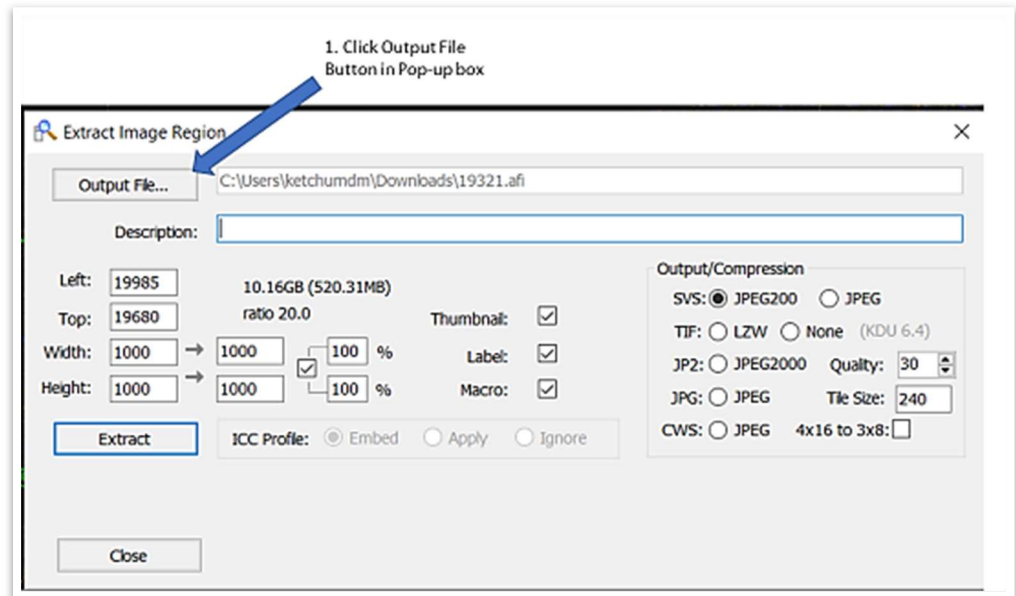
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**7.4.4** An **Extract Image Region** dialog box will appear (as shown below in [section 7.4.5.1](#)).

7.4.4.1 **Note:** The dimensions of the extracted regions do not need to be kept fixed since the E-Cadherin or Vimentin marker area will be normalized to the total tissue area or the total number of cells or nuclei in the extracted ROI. However, to be consistent and to reduce variability, 1000 x 1000 box size will be used.

**7.4.5** To name the file, select **Output File** in the dialog box (as shown in step 1 below) and save the image to the appropriate header folder on the Definiens drive. Each ROI is given a file name, and each file should be put in the header folder > CTEP protocol\_patient ID Extracted Images (as shown in steps 2-4 below). File name for each image should follow the annotation naming criteria. After naming the image, click **Save** button, then press **Extract** button in the dialog box. Once the region is extracted, press **Close** to exit the dialog box (as shown in steps 5-6 below). Move on to the next region to be extracted.

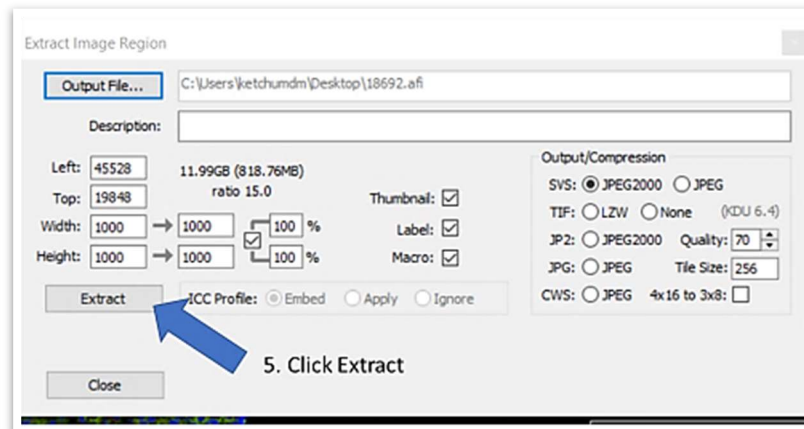
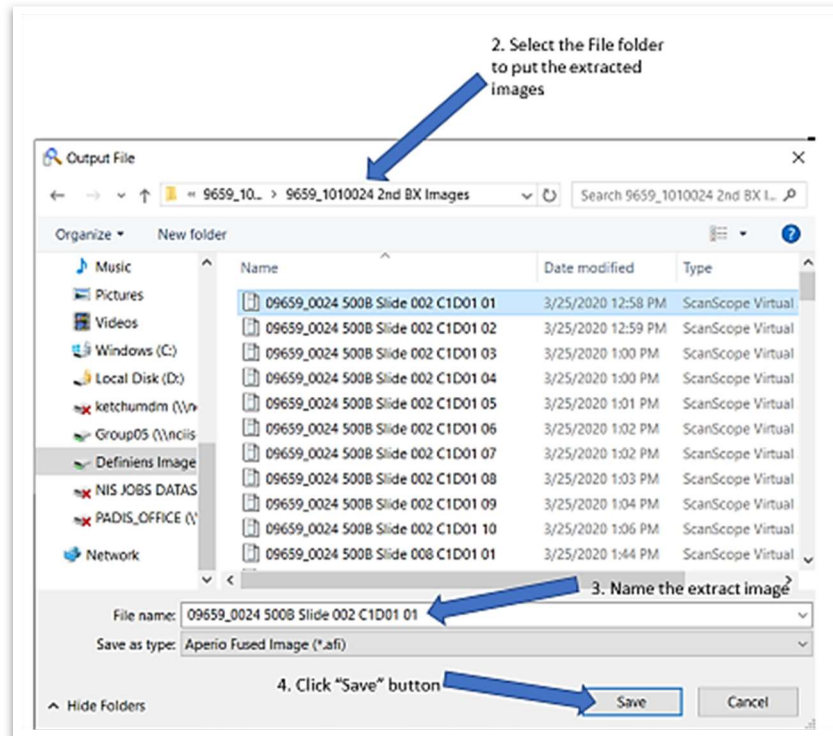
7.4.5.1 **Note:** It is important to strictly follow the naming convention as detailed for annotation naming in [section 7.3.2](#). The exact nomenclature will enable proper export of Definiens data, and calculations within the EMT Master Template file.





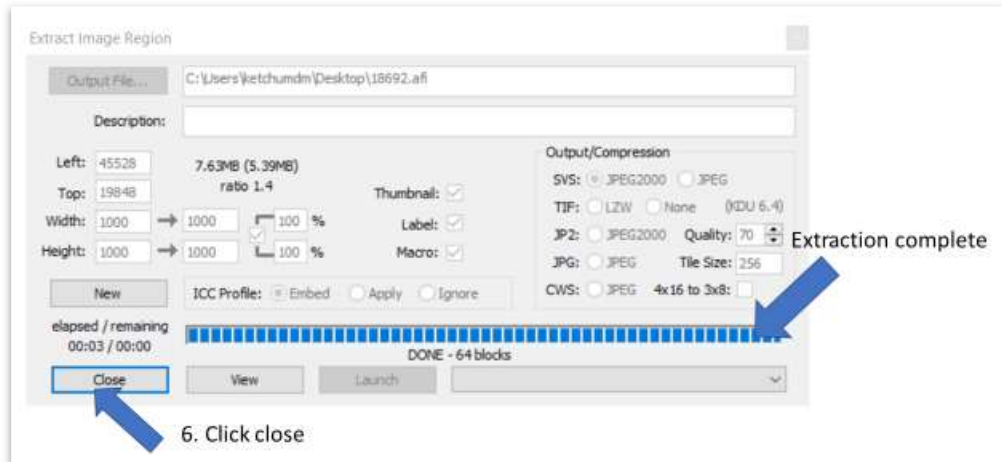
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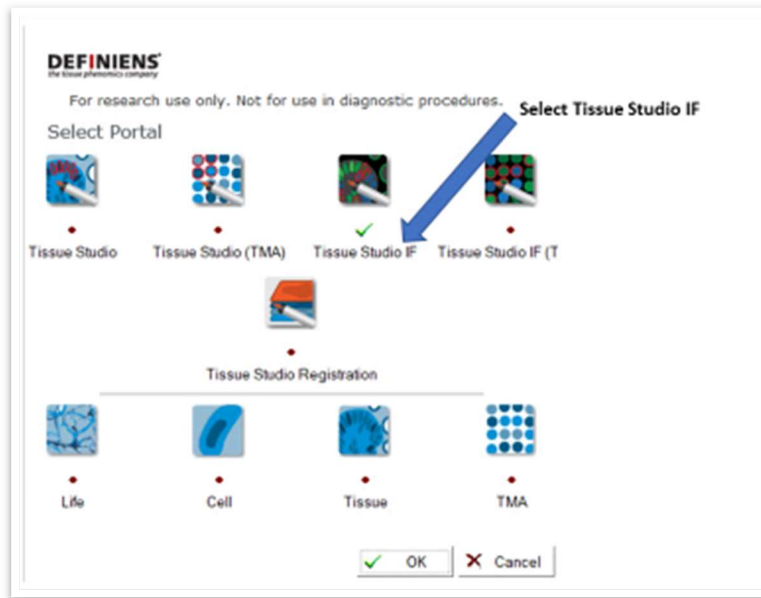


**7.4.6** When image extraction is complete for all images acquired per patient (pre- and post-dose tissues), zoom out to view the entire tissue and use the snapshot tool to save a snapshot of each annotated image. Save the snapshots of the biopsy tissue in the header folder.

**7.4.7** After image extraction is completed, close the ImageScope software and log off the Aperio eSlide Manager database.

**7.5 Initial Definiens Image Analysis**

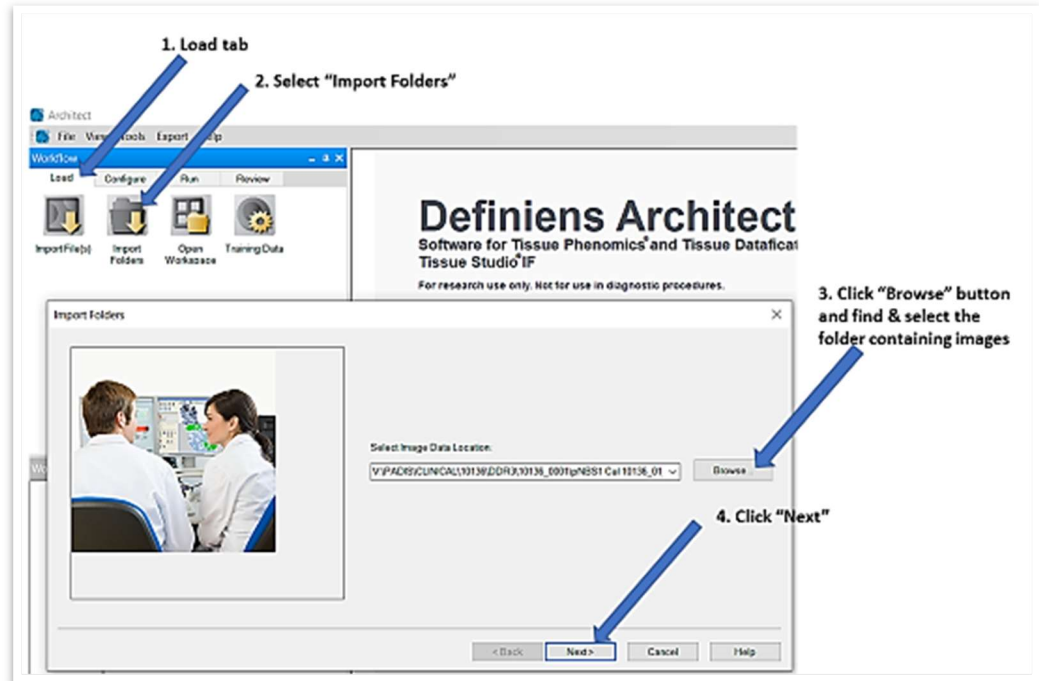
**7.5.1** Open Definiens Tissue Studio software (Architect XD version 2.7.0 -Tissue Studio IF version 4.4.3).



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To import the folder containing the extracted Aperio images, select **Import Folder** option in the **Load** tab as shown below. Use the **Browse** option to select the folder containing the extracted SVS images to be analyzed.

**7.5.2** A screenshot of the **Import Folder** dialog box is provided below. Calibrator tissue must be analyzed separately from patient specimens, and biopsies from each patient must be analyzed individually due to morphological differences. Therefore, load only images from one patient or from a calibrator.

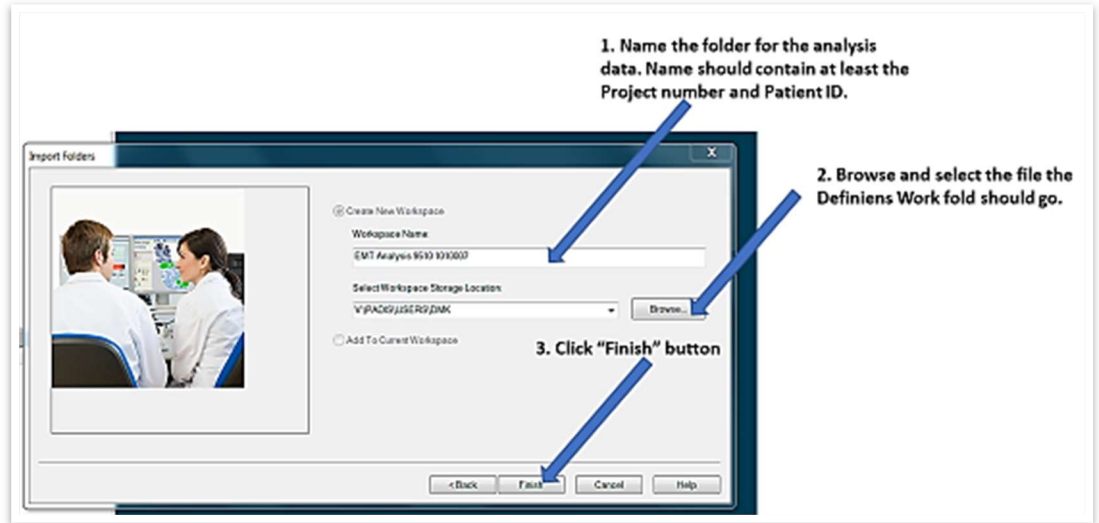


**7.5.3** In the next screen, from the drop-down menu under **Select Scanner Type**, choose **"Aperio Scan Scope FL-with resolution"**. Press **Next**.

**7.5.4** In **Workspace Name** field on the next screen, create a workspace analysis folder name as shown below. The workspace name must contain the CTEP protocol number and the patient ID. In **Select Workspace Storage Location**, browse and select folder where the SVS images are stored. Press **Finish**.

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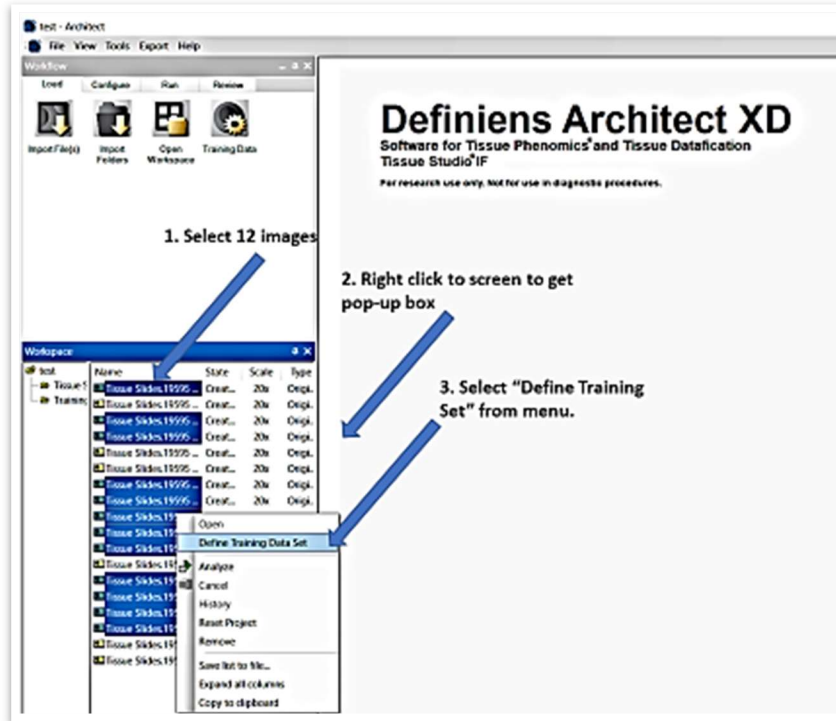
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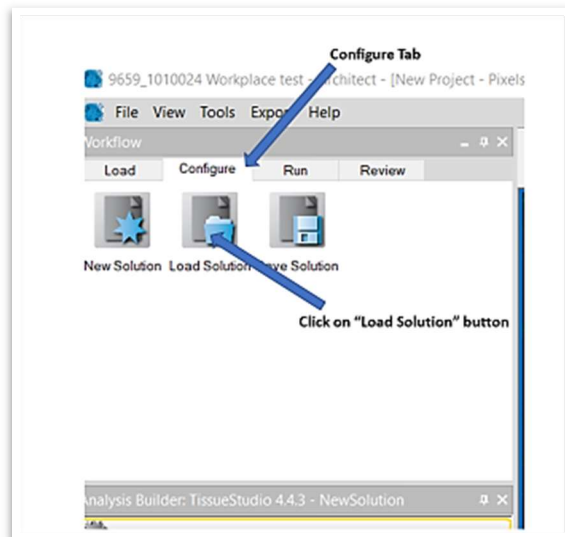
**7.5.5** Select and highlight 12 images from the imported image file and define them as a training set. Make sure to include images from all timepoints. To select images, hold down the Ctrl key on the keyboard and left click until 12 images have been chosen. The selected images will be highlighted in blue, as shown below. Load the training image set by right clicking on the screen and selecting **Define Training Data Set** on the pop-up menu. Using the maximum number of images (12) and including images from all the time points for the training set is important in order to get the best results for tissue segmentation and marker thresholding.

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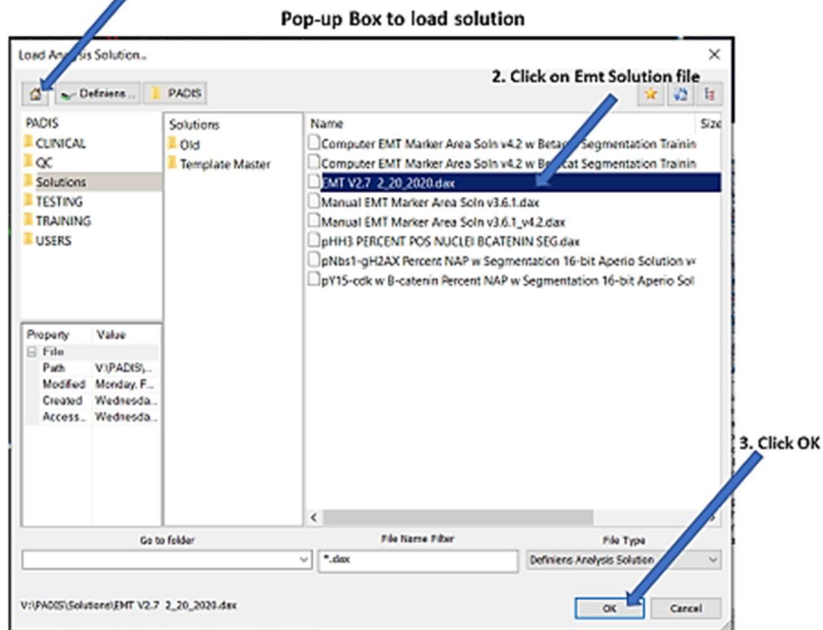


7.5.6 Under the **Configure** tab (shown below), select **Load Solution**. In the pop-up **Load Analysis Solution** box, browse to Definens Image Upload "fr-s-dtp-378apr" > PADIS > Solutions folder, select file EMT V2.7 2- \_20\_2020.dax and click **OK** (refer to images below). This Definens solution is the default setting for analysis of control tissues and clinical samples for EMT IFA PANEL. It is designed to segment the tumor tissue and exclude normal tissue that could interfere with or dilute the analysis.



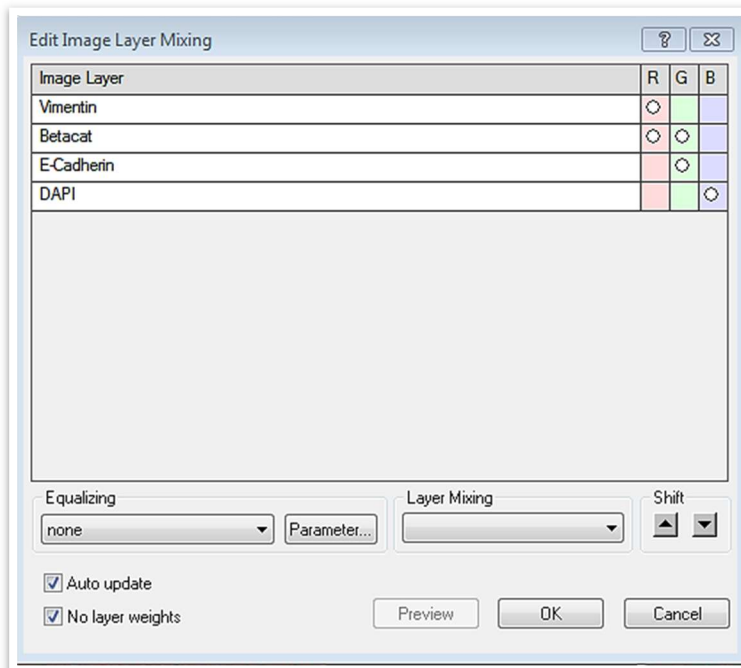
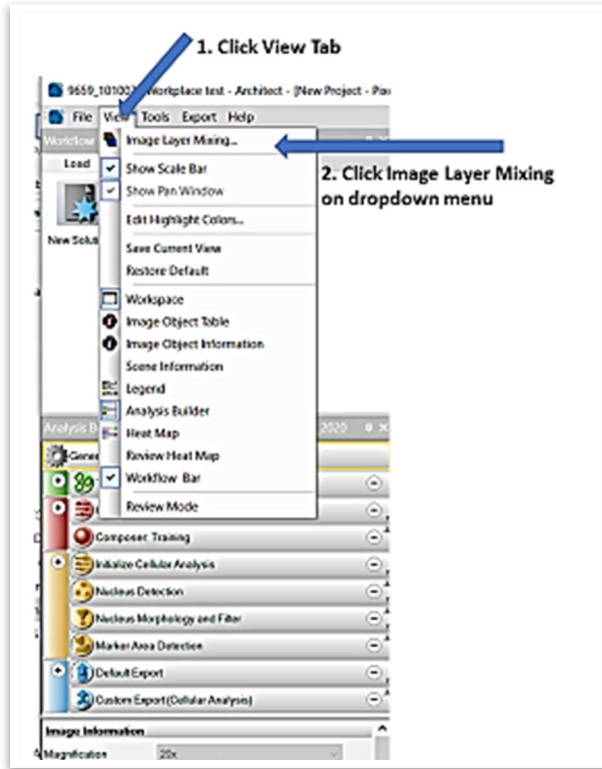
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1. Click browser button to locate solution



7.5.7 After loading the solution, adjust the image layer mixing to assign colors to layers by going to **View > Image Layer Mixing**. In the **Edit Image Layer Mixing** popup box, assign correct colors to each marker as indicated below and then click **OK** to accept changes.

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**7.5.8** In the Analysis Builder menu, confirm General Settings for the solution are correct as outlined below.

<b>Under Image Information Settings:</b>	
Magnification	20X
Magnification (um/pixel)	0.4622
Bit depth	16-bit
Edit names	Make sure layers are assigned correctly: Layer 1: Vimentin Layer 2: $\beta$ -Catenin Layer 3: E-Cadherin Layer 4: DAPI

- To check the assigned layers, click the Edit Names button in General Settings, as shown below.



**7.5.9** Tissue Segmentation Detection

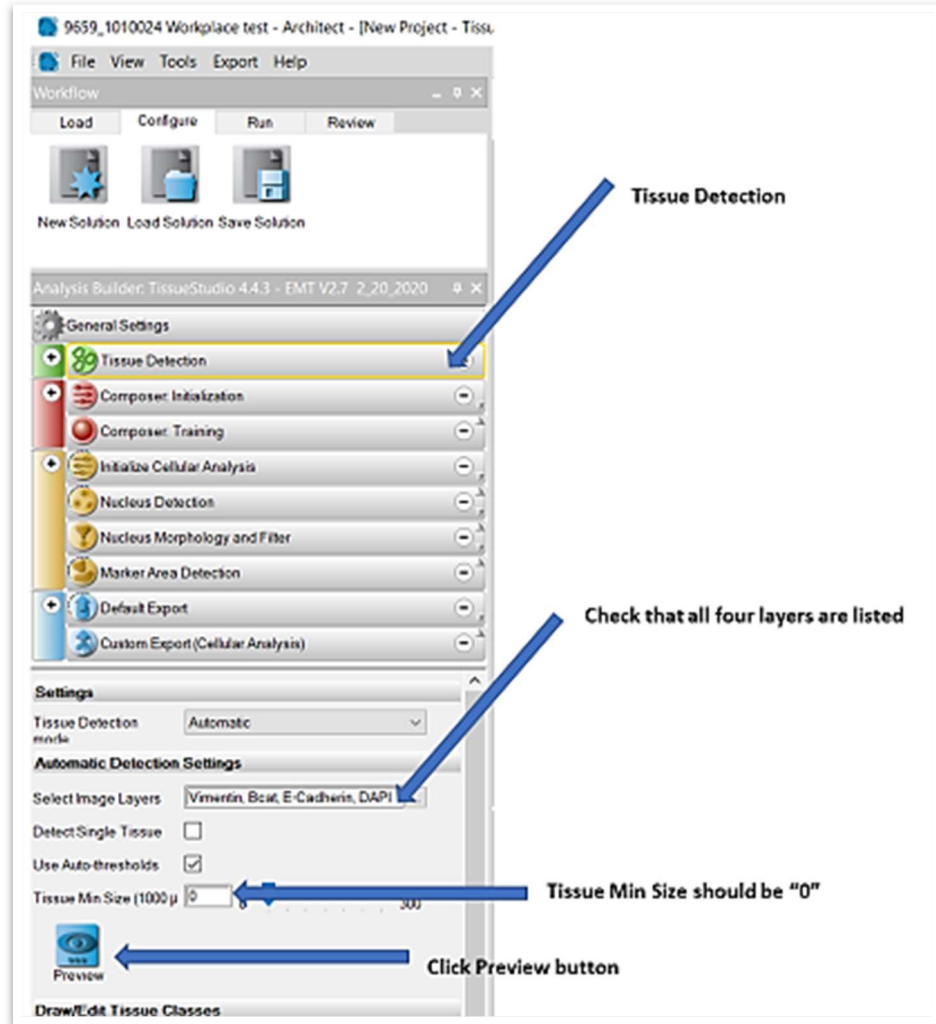
In the **Analysis Builder** menu, select **Tissue Detection** as shown below. Verify that in the **Select Image Layers**, the selected image layers are Vimentin, Bcat, E-Cadherin, DAPI. Verify that **Tissue Min Size** is 0. Select the **Preview** button. (When finished, the images will appear blue, as shown below.)

- 1) Analysis Builder (Screenshot) with Tissue Detection selected.



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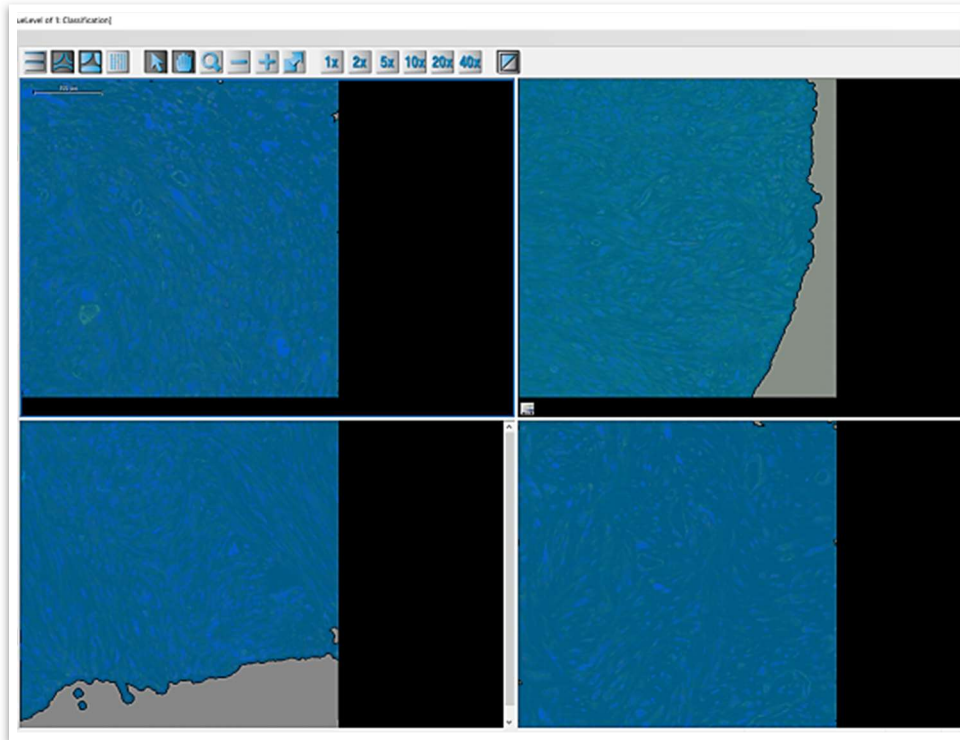
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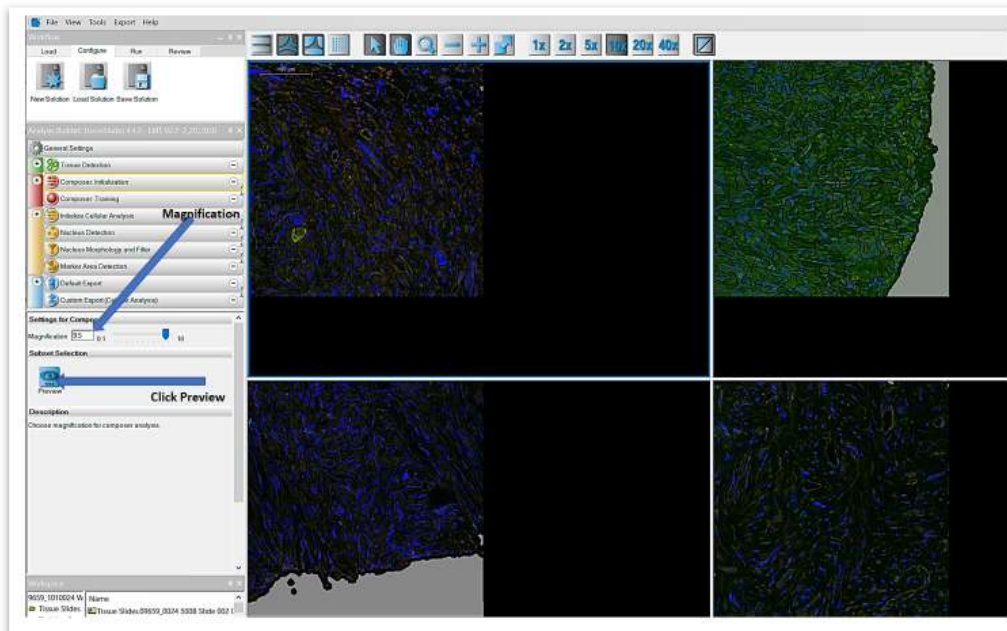
2) Completed image “Preview” screenshot.

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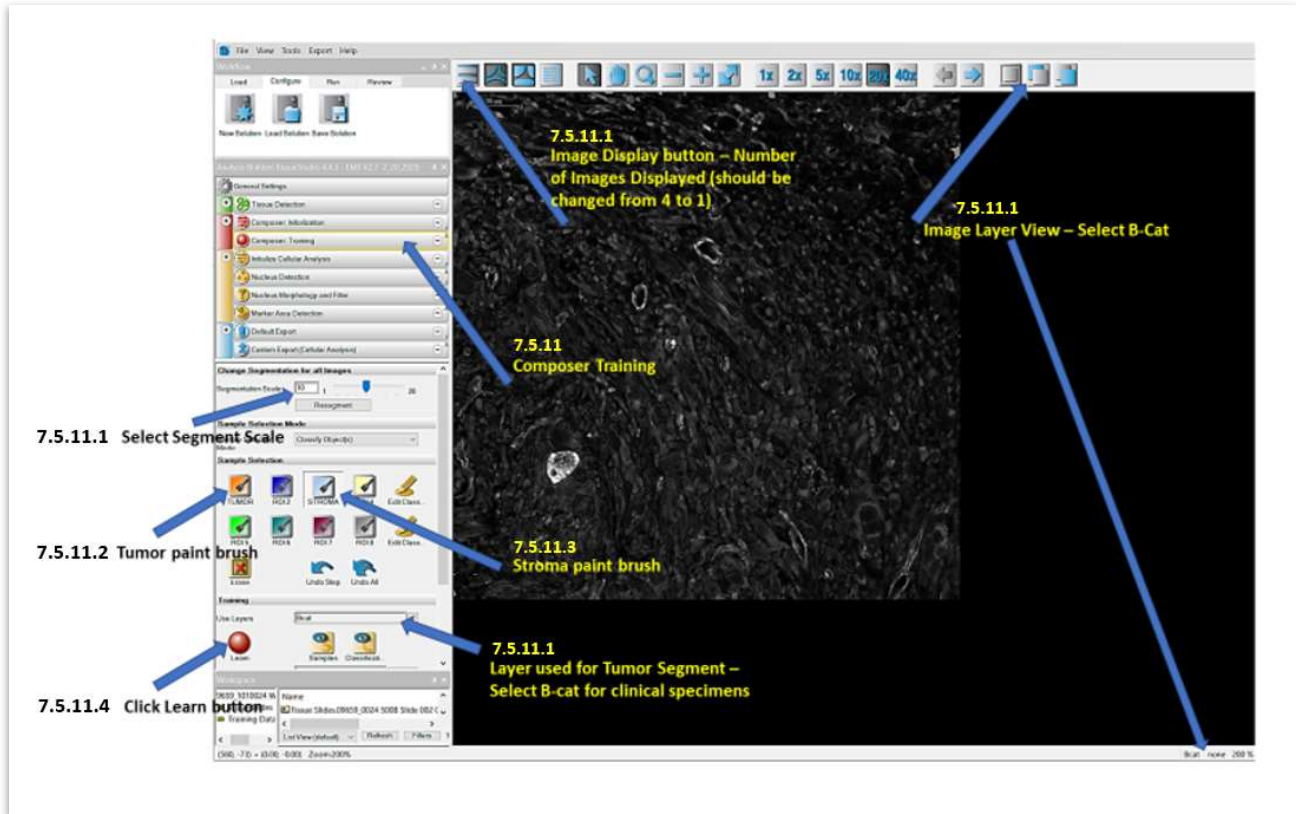


**7.5.10** In the **Analysis Builder** menu, select “Composer: Initialization” (shown below). Click the **Preview** button (shown below). The finished images will contain multiple colors and segmentation. Make sure magnification is set at 9.5.



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7.5.11 Next on the **Analysis Builder** menu select “Composer: Training”, as shown below.

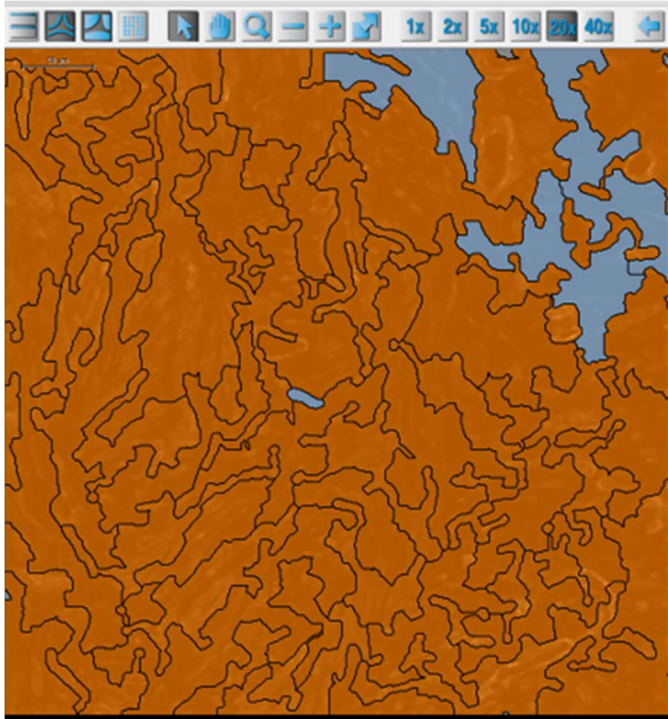


7.5.11.1 Under **Change Segmentation for all Images**, change the **Segmentation Scale** to a value in the range of 3 to 12 and press the **Resegment** button (refer to image in section 7.5.11 above). Make sure that under **Training** on the **Use Layers** drop-down menu, Bcat is selected. The segmentation scale may need to be lower if the tumor tissue has much stroma within the tumor tissue. Image layer view should be changed to B-cat, and the image display will change from displaying 4 images to 1 image.

7.5.11.2 Select the **Tumor** paintbrush button under **Sample Selection** (refer to image in section 7.5.11 above). Manually select unequivocal areas that are tumor, based on  $\beta$ -Catenin positive staining. This will color tumor area in orange and non-tumor area in gray. Shortcut for segmentation: when tumor paintbrush is selected, dragging the brush across large areas of tumor will quickly designate the tissue rather than selecting each segment individually. Do this for each of the images included in the training set. A minimum of one training set (12 images) must be segmented.

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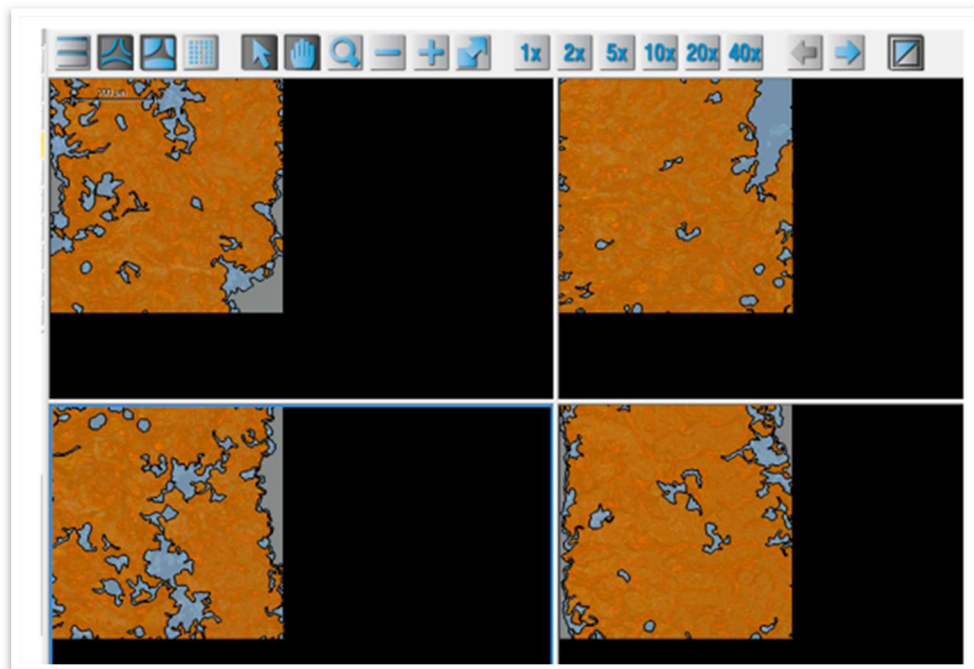
7.5.11.3 If any segments were mistakenly included and need to be removed, click **Erase** button, and then click back on the segment to be deselected. **Stroma** or **Glass** can be designated in the same way using the **Stroma** paint brush (refer to image in section 7.5.11).

7.5.11.4 After all the images have been segmented, press the **Learn** button. Review the training set images for proper tumor segmentation. The user may have to adjust the segmentation scale (value range 3-12) to properly segment the image.

The image below shows the tumor segmentation based on  $\beta$ -Catenin signal. Tumor regions are shown in orange and non-tumor are shown in gray.

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**7.5.12** Evaluation of calibrator tissue and clinical tissues that do not require  $\beta$ -Catenin segmentation

7.5.12.1 Tissues in the **control slides** do not require tissue segmentation. Tumor tissue is distinguished from non-tumor **based on DAPI positivity only**.

*In addition to control tissue, this approach may be used on **clinical specimens that have high tumor content**, where all of the 20 extracted ROIs contain all tumor tissue, as documented in the pathologist review.*

7.5.12.2 Under **Composer: Training** select the **DAPI layer**. Make sure the image layer view is on the DAPI mask.

7.5.12.3 Change the segmentation scale to “3-12” and re-segment the images based on this value.

7.5.12.4 Select the **Tumor** paintbrush button under **Sample Selection** and manually select DAPI positive regions.

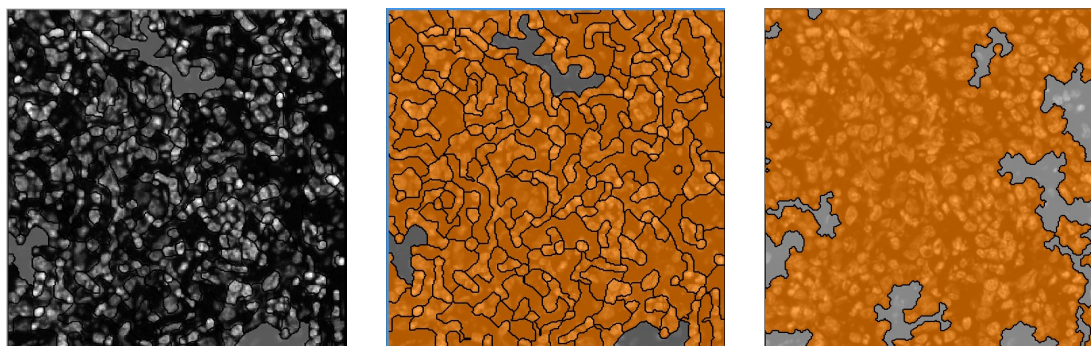
7.5.12.5 To distinguish stroma/non-tumor regions use the **Stroma** paintbrush to select areas of glass where there are no nuclei present. Only 1 area of glass/or area with no nuclei need to be selected per image in training set.

7.5.12.6 Select other areas with nuclei and designate them as tumor. This may be done most easily by visualizing the DAPI channel only during selection of tumor and glass.

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7.5.12.7 Press **Learn** button. The segmentation scale (value range 3-12) may need to be adjusted to pick up areas of glass/no nuclei in order to properly segment the image.

7.5.12.8 The image below demonstrates how the tissue is segmented based on DAPI signal. The raw DAPI image is shown on the left, the middle image shows the segmented regions denoted as tumor shown in orange and non-tumor shown in gray, and on the right is a representative image showing the tumor segmentation image created after pressing **Learn**.

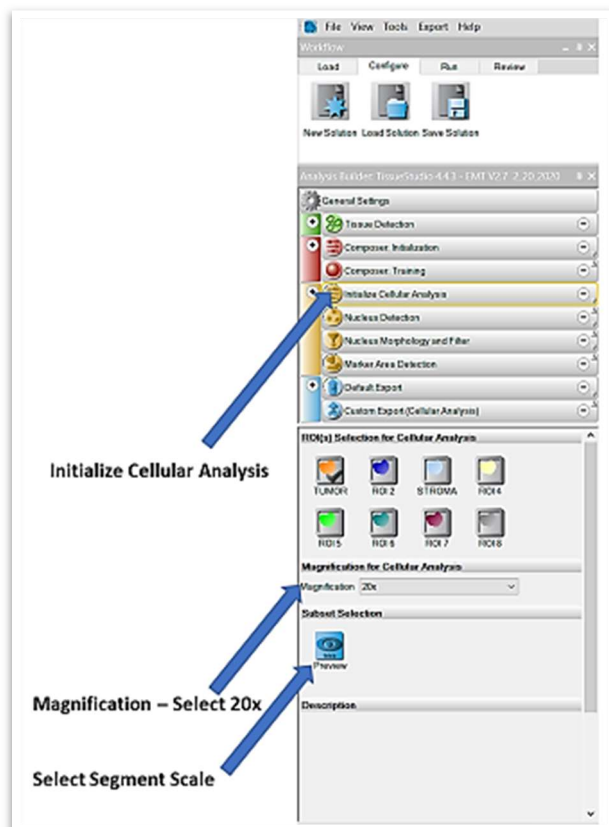


### 7.5.13 Initialize Cellular Analysis

7.5.13.1 In the **Analysis Builder**, select **Initialize Cellular Analysis**. Under **Initialize Cellular Analysis**, enter the following:

- 1) If not already checked, under **ROI(s) Selection for Cellular Analysis**, select **TUMOR**.
- 2) Under **Magnification for Cellular Analysis**, choose 20x for Magnification.
- 3) Press the Preview button.

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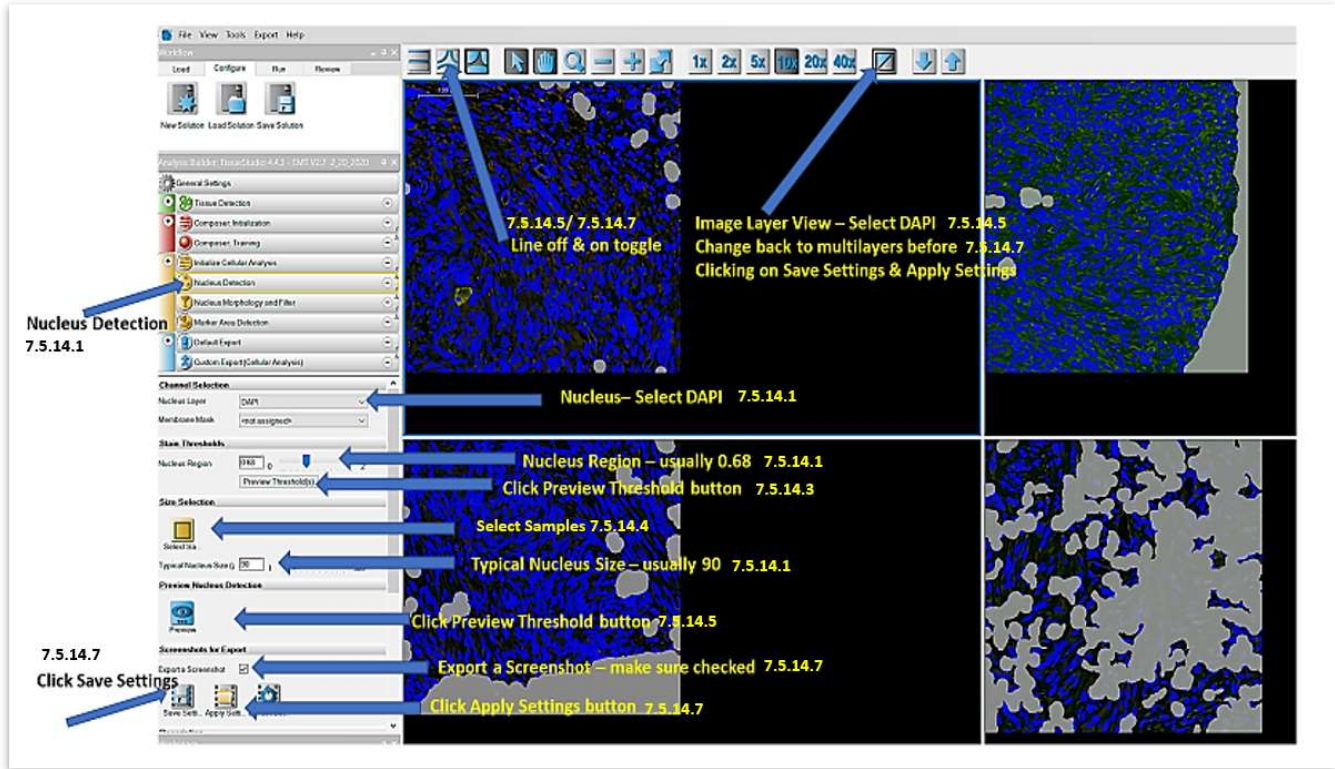


### 7.5.14 Nucleus Detection

7.5.14.1 Next on the **Analysis Builder** menu, select **Nucleus Detection** (shown below), and enter the following:

<b>Nucleus Layer</b>	DAPI (selected from drop-down menu)
<b>Membrane</b>	<not assigned>
<b>Nucleus Region</b>	Enter a value in the range of 0.1 to 0.98 (Usually set for 0.68)
<b>Typical Nucleus Size (<math>\mu\text{m}^2</math>)</b>	Enter a value in the range of 10 to 220 (Usually set for 90)

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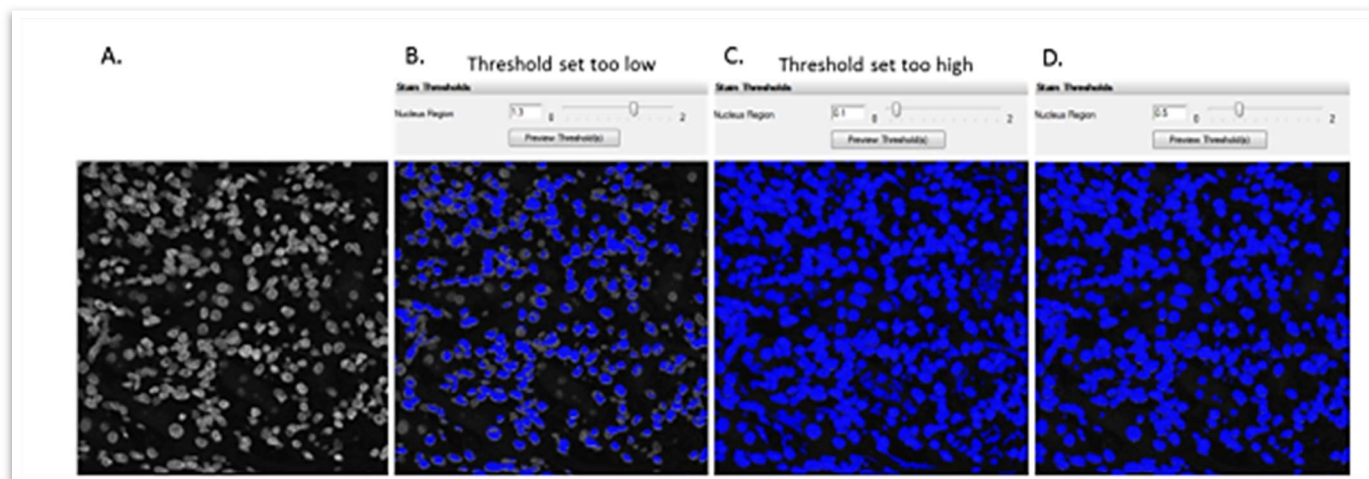


Note: Numbers in the image indicate associated SOP steps.

- 7.5.14.2 For nucleus region and size, a suggested range of values are provided based on clinical feasibility testing of human FFPE tumor biopsies. Examples of images with proper and improper thresholding are shown below (section 7.5.14.3).
- 7.5.14.3 When setting the threshold, make sure the entire nucleus is covered by the blue mask and that regions in between the nuclei are not covered by the blue mask, though some overlap in regions between cells is acceptable. Below are examples of the raw image (A), an under thresholded image (1.3, B), over thresholded image (0.1, C), and properly thresholded image (0.5, D).



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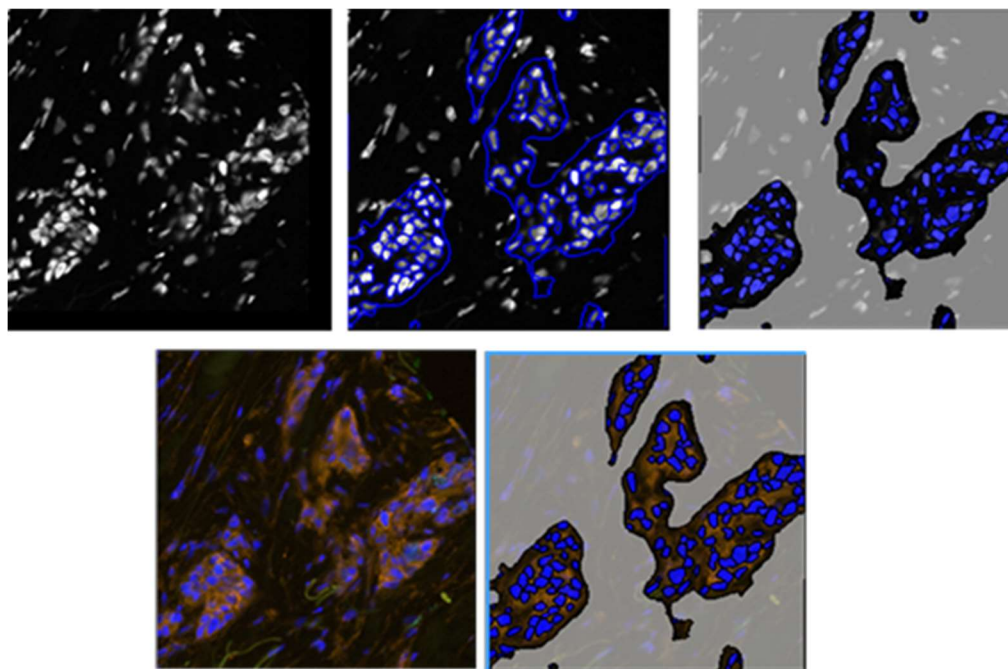
- 7.5.14.4 For nucleus size, a range of 10 to 220 is suggested. The average nuclear size can be approximated by using the **Select Samples** button (shown below), and then choosing 10-12 representative nuclei to get an average size; for best results, choose mid to large sized cells, avoiding the smallest or overlapping cells.



- 7.5.14.5 Before selecting preview, **switch the training set images to DAPI single layer view**. The accuracy of the nuclear segmentation can be assessed in this channel. The DAPI mask and nuclear outline masks should be toggled off and on to assess the accuracy of nuclear segmentation in the DAPI layer after pressing **Preview**.
- 7.5.14.6 The threshold measures the DAPI level, so if shadows of missed cells are present, keep the size constant and change the threshold by slowly increasing or decreasing the threshold until the faint cells are captured. On the other hand, if one cell is divided into two, keep the threshold constant and change the size incrementally.
- 7.5.14.7 Once the nuclear segmentation is accurate, switch the toggle to multi-layer view to view the DAPI segmentation within the  $\beta$ -Catenin staining. **Make sure that the multi-layered mixing view is selected, and the lines are off, prior to exporting a screenshot by pressing Save Settings and then Apply Settings (The Export a screenshot box must be checked)**. The multi-layer view will allow the user to check the  $\beta$ -Catenin segmentation in the screenshots after analysis is completed to QC the image set.

*The image below shows tumor segmentation based on  $\beta$ -Catenin signal. DAPI images are shown with tumor segmentation overlay (top images), and bottom images show the tumor segmentation with overlay of DAPI and  $\beta$ -Catenin signals to confirm that the  $\beta$ -Catenin segmentation is correct.*

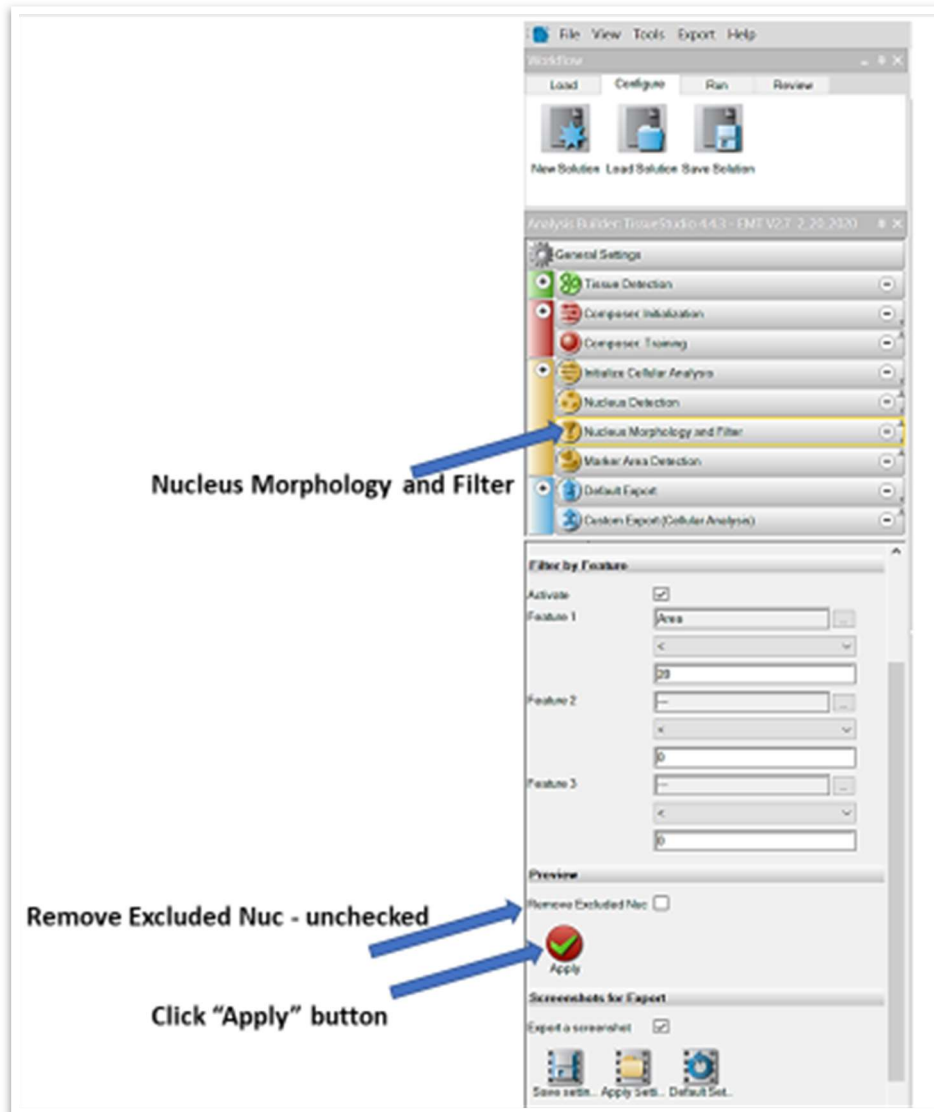
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**7.5.15 Nucleus Morphology Filter** (Switch images from multilayer mixing view back to DAPI single layer view).

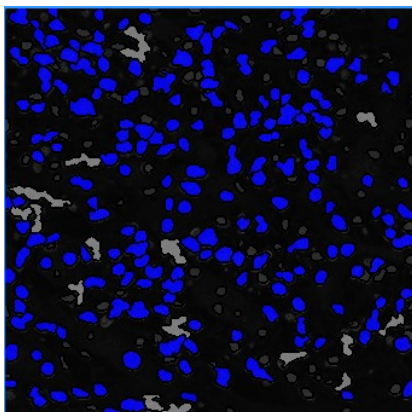
- 7.5.15.1 In the **Analysis Builder** menu, select **Nucleus Morphology and Filter**: In the **Filter by Feature** section, check the box next to **Activate**, if not already checked. In **Feature 1**, Area < than 20 should be selected. **Features 2 & 3** are not used.
- 7.5.15.2 If not present, **Area** can be found by clicking the button with “...” then under **Object features** → **Geometry** → **Extent**.

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7.5.15.3 Press **Apply** button. Make sure the **Remove Excluded Nuc** box is **not** checked. Image below shows the resulting segmented nuclei after application of the nuclear morphology filter. Nuclei shown in blue will be included in analysis, whereas nuclei shown in gray will not be included in further analysis.

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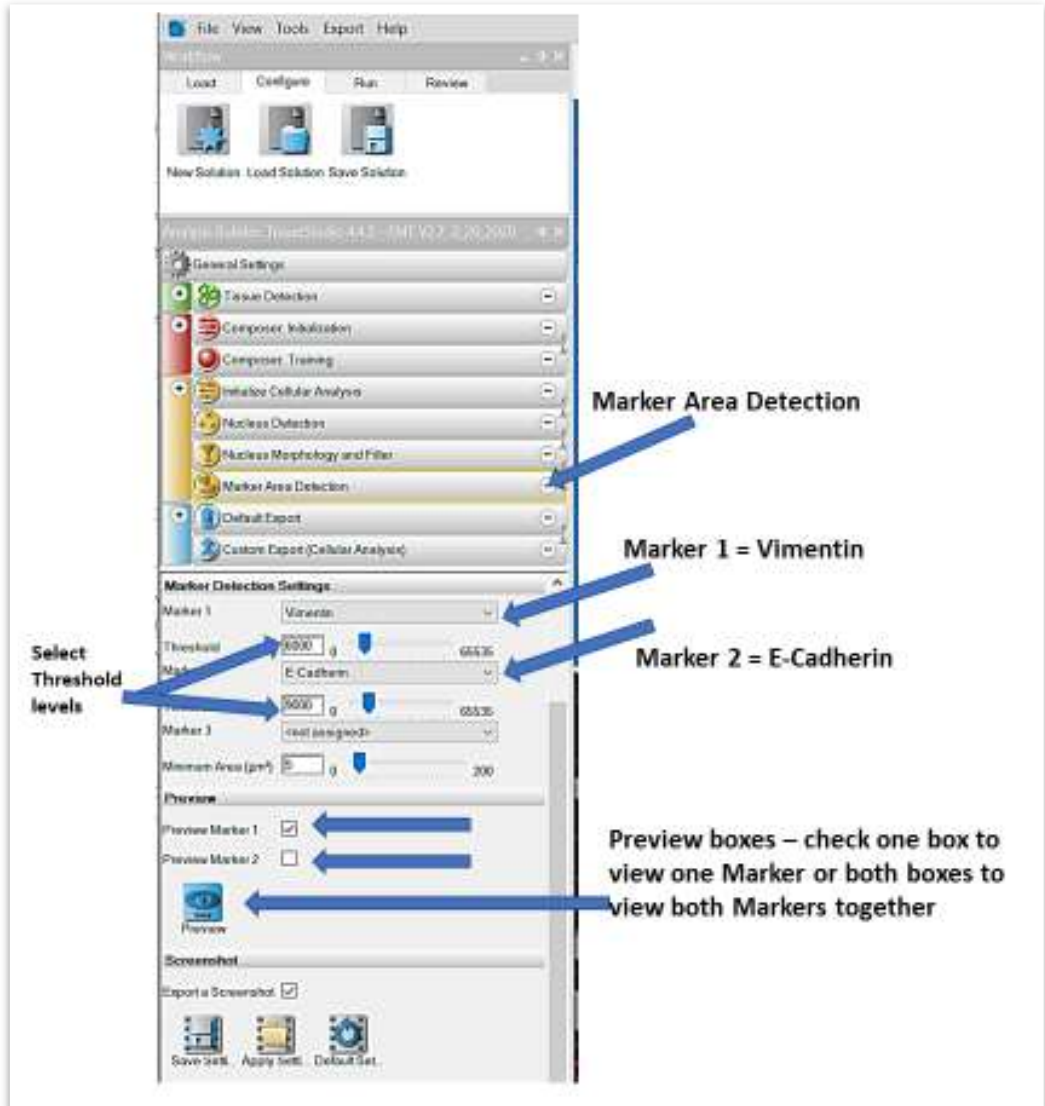
7.5.15.4 Export a screenshot in the DAPI single layer view by first pressing **Save Settings** and then **Apply Settings**. Keep **Fill Mask** on when saving settings.

### 7.5.16 Marker Area Detection

7.5.16.1 In the **Analysis Builder** menu, select **Marker Area Detection** and define the following features for clinical and control slides (refer to image below):

<b>Marker 1</b>	Vimentin
<b>Threshold</b>	5000-20,000
<b>Marker 2</b>	E-Cadherin
<b>Threshold</b>	5000-20,000
<b>Marker 3</b>	<not assigned>
<b>Minimum Area (um<sup>2</sup>)</b>	5

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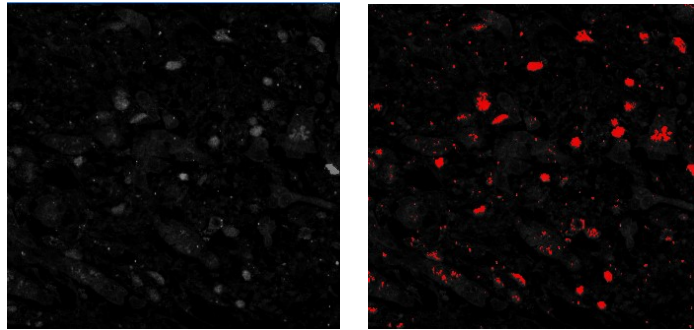
7.5.16.2 Check **Preview Marker 1**, Press **Preview**.

7.5.16.3 View the marker mask with the **Fill** option clicked and the **Outline** option turned off (icons shown below).



7.5.16.4 Check within the Vimentin image layer whether the RED marker mask colocalizes with the Vimentin RGB layer image. The marker mask for Vimentin image layer should cover the regions of positive nuclear signal and be negative on regions of background, as shown below (Left - Image; Right - Marker Mask).

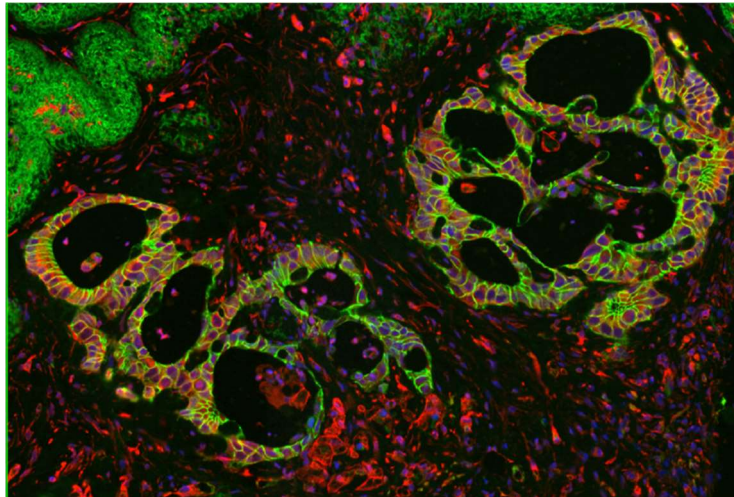
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*To improve thresholding, toggle off mask, and place cursor over a place with true but weak signal. The intensity value will be displayed next to the selected area.*

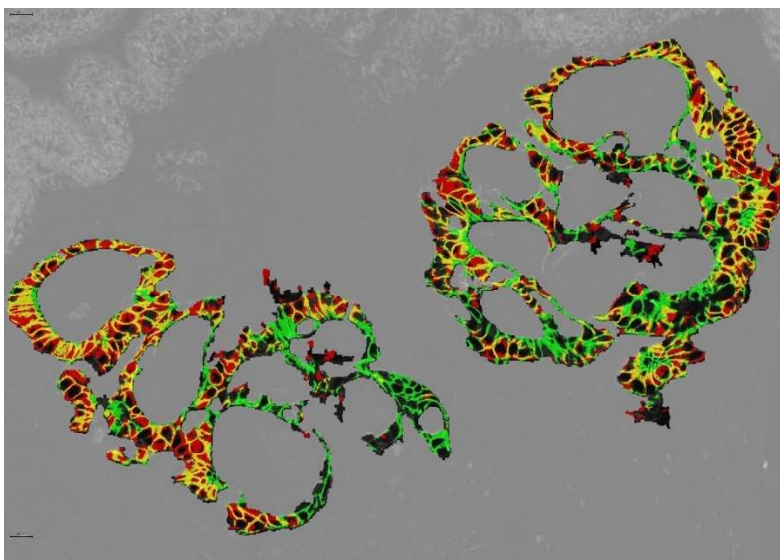
7.5.16.5 A representative preview RGB image from the Aperio Scan is shown in the top panel below. A representative Definiens marker mask image showing E-Cadherin in green, Vimentin in red and co-localizing areas in yellow is shown in the panel below.

***RGB Image from Aperio Scan***



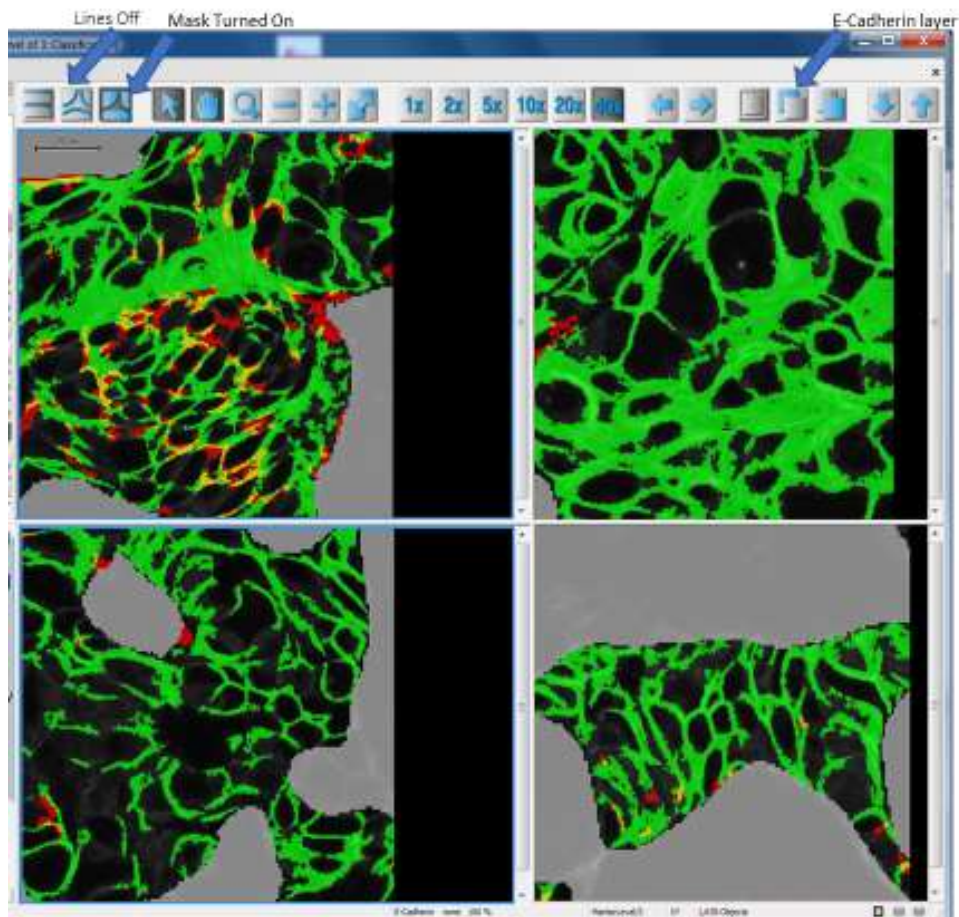
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*Definiens marker mask image*



- 7.5.16.6 If necessary, adjust thresholds until the marker mask colocalizes with the layered marker staining.
- 7.5.16.7 Check **Preview Marker 2**, and press **Preview**. Check within the E-Cadherin image layer whether any green marker mask colocalizes with the E-Cadherin RGB layer image. If necessary, adjust the threshold, preview, and repeat until the correct threshold is found. If any changes are made to the initial values while adjusting marker thresholds based on the marker layer staining, these new values should be noted in the Batch Record.
- 7.5.16.8 Check **Preview Marker 1** and **2**, then press **Preview**. Representative preview images are shown below.

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7.5.16.9 **Save Settings** and then **Apply Settings**. When saving settings, keep **Fill** mask on with E-Cadherin (green) single layer selected. *Exception:* if the tumor is primarily composed of Vimentin (red) keep the Vimentin single layer with mask on when saving settings.

### 7.5.17 Export a Screenshot

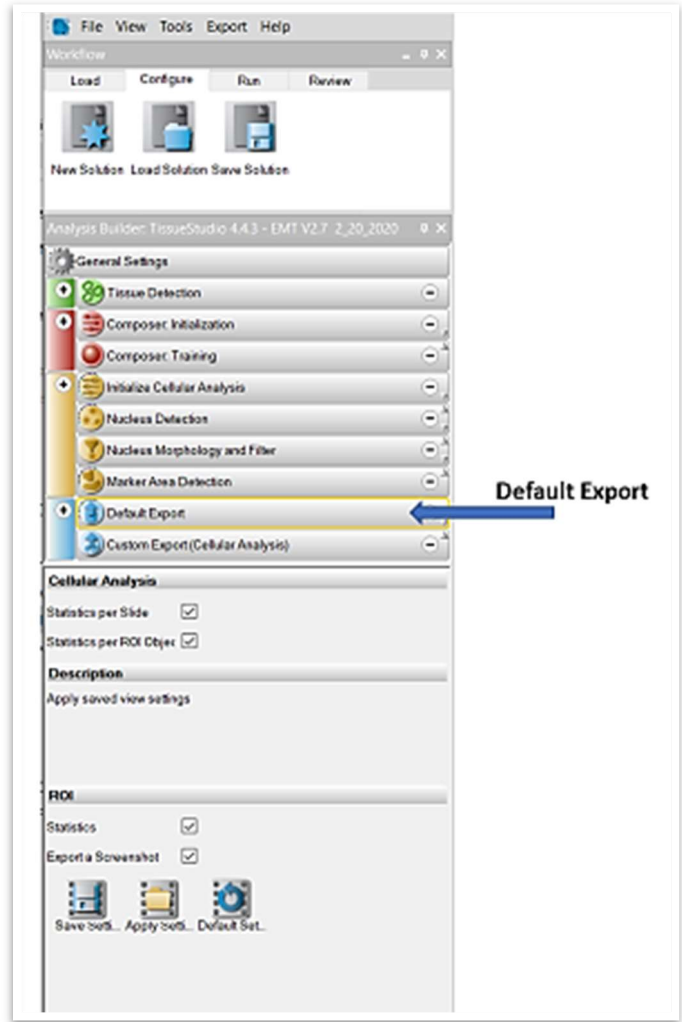
7.5.17.1 In the **Analysis Builder** menu, select **Default Export**.

7.5.17.2 Under **Default Export**, press **Save Settings** and **Apply Settings**. See the list of settings for all Definiens screenshots in [Appendix 6, Section 2](#).

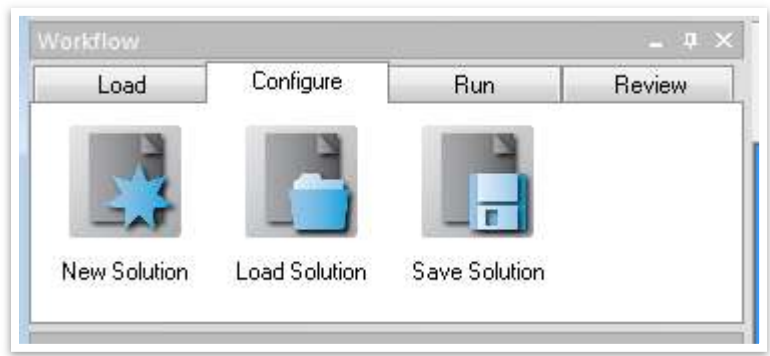


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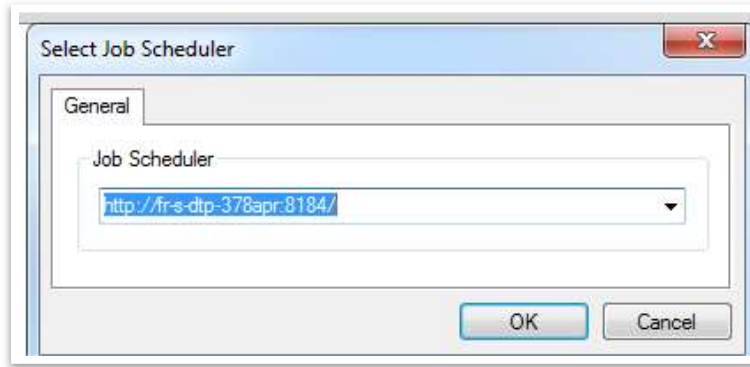
7.5.17.2 Select **Save Solution** (shown below) and save the solution in the header folder.



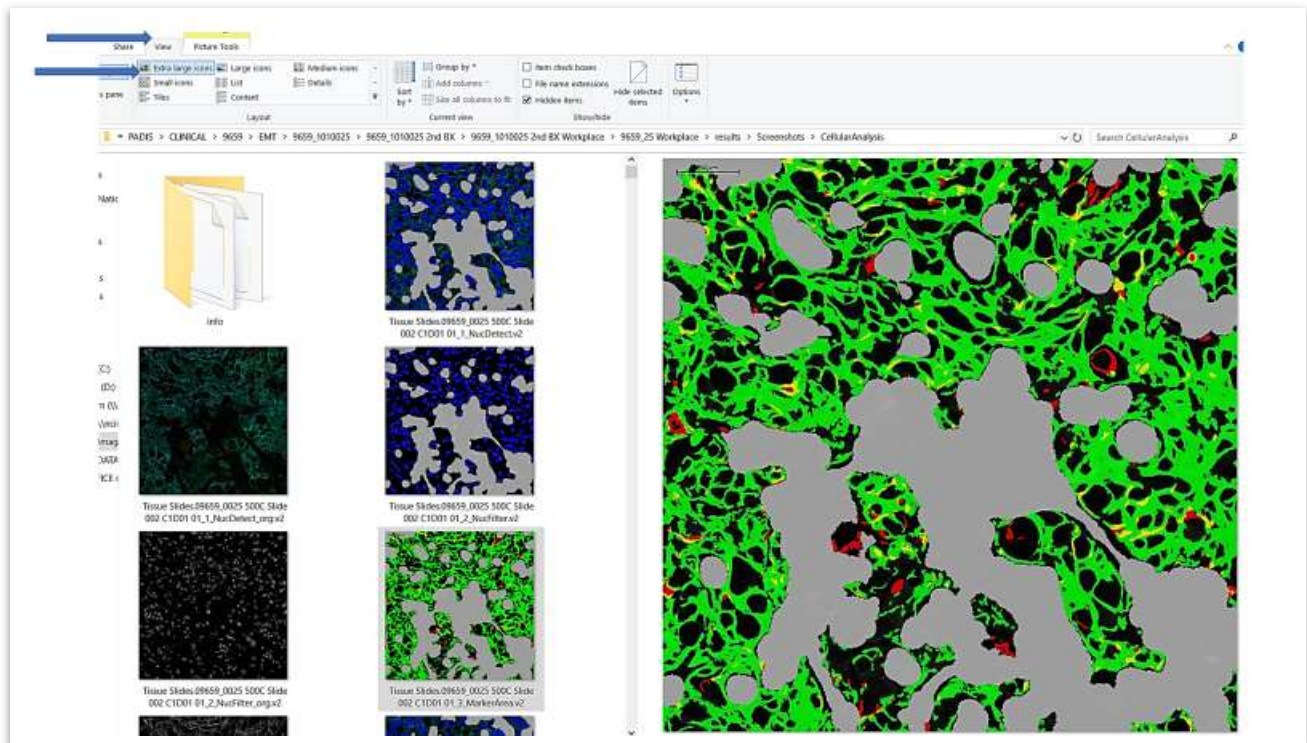
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### 7.5.18 Run Definiens Analysis

7.5.18.1 Click the **File** tab and select **Configure Analysis**. A job scheduler box will appear with a drop-down menu. From the menu, select “http://fr-s-dtp-378apr:8184”. Press **OK** to close box.



7.5.18.2 Press the **Run** Tab and select **Analyze All Images** from the sub-menu. When the status of the last file changes from **Processing** to **Processed**, the quantitation is complete.

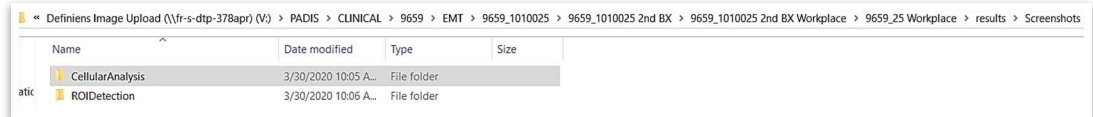


### 7.5.19 Reviewing the Results

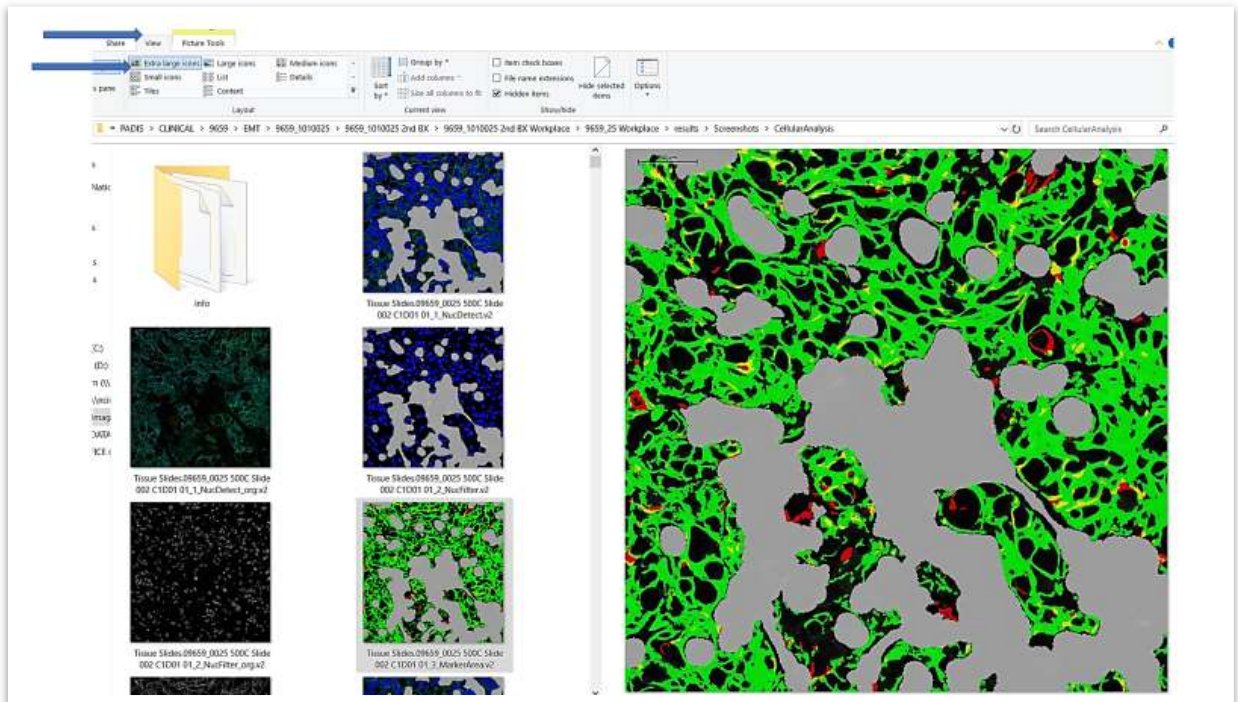
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7.5.19.1 Review screenshots in the Result > Screenshots > Cellular Analysis folder (as shown below).



7.5.19.2 In file explorer, view the images by selecting the **View** tab and **Extra Large Icons** option. Once the Cellular Analysis folder in the results folder is opened, **visually inspect each of the EMT masked/gray-scale images** to ensure each image was analyzed appropriately. Vimentin should have a red color mask and E-Cadherin should have a green color mask. Make sure normal tissue is not included tumor segmentation. Normal tissue will stain positive for Vimentin; therefore the tumor segmentation should be as accurate as possible.



7.5.19.3 If there are segmentation or thresholding issues with more than 20% of the images, the workplace should be reset, and a new training set should be selected and analyzed\* (refer to [section 8.2.1.1](#) for detailed screenshot QC criteria). Exceptions to the 20% rule can be made, but it must be listed as a deviation with a reason in the **Comments** section of the batch record ([Appendix 1](#)).

\*Shortcut: If, after reviewing the screenshots, it is determined that a new training set or different thresholds are needed and the Definiens Architect is still open, the workspace can be reset. Click on the Run tab. Select “Reset workspace”, select

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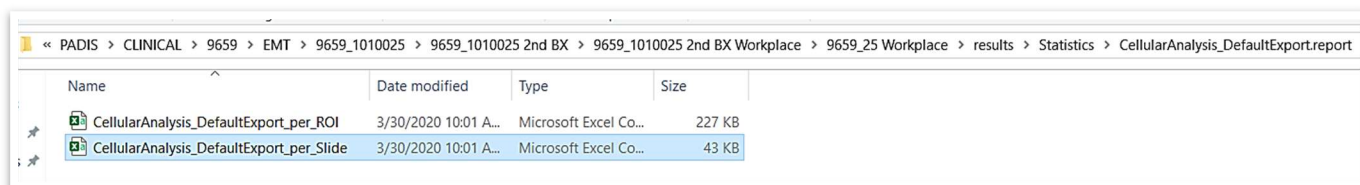
existing training set or create new training set. Go back to the configure tab and work down through the script again ([section 7.5.6](#)).

## 8.0 QUALITY CONTROL, DATA ANALYSIS AND REPORTING

**8.1** Initiate Data Analysis Template (see [Appendix 7](#) for EMT Master Template Naming Instructions).

**8.1.1** Open the clinical specimen analysis Excel template *SOP340548 EMT Master Template* (available upon request). The template will be used to analyze the clinical and calibrator data.

**8.1.2** In the **Workplace** folder go to results > Statistics > CellularAnalysis\_DefaultExport.report > CellularAnalysis\_DefaultExport-per\_Slide (Excel file – see below). Copy all the raw data, excluding the headers, for the clinical specimens from the Definiens Tissue Studio analysis sheet and paste the data into the **EMT Master Template** on the **Original Raw Data** sheet. Copy all the raw data for the control slides and paste the raw data onto the **CAL Original Data** sheet. Save the **EMT Master Template** and give it a new name with the patient ID and protocol number.

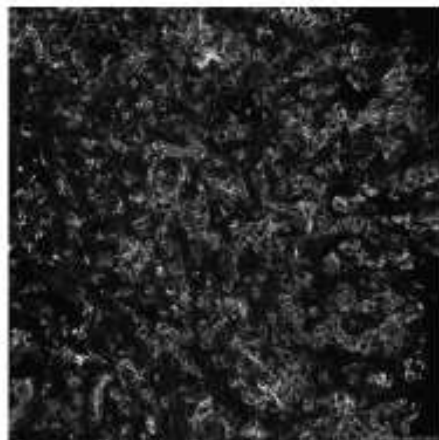


**8.2** Quality Control Inspection of all Images

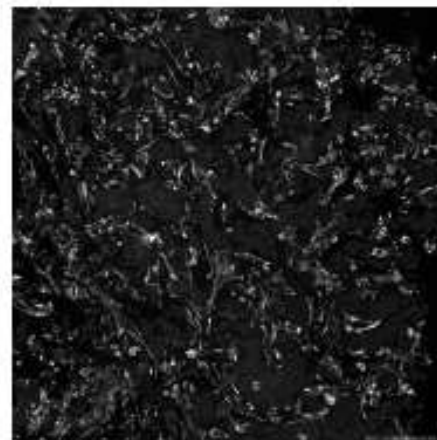
**8.2.1** Quality Control Evaluation of Screenshots

**8.2.1.1** Within the Definiens analysis open the results > Screenshots > Cellular Analysis. Visually inspect every screenshot for the clinical tissue to ensure the following criteria are met: 1) The  $\beta$ -Catenin segmentation reflects accurately that only  $\beta$ -Catenin positively stained tumor tissue is included in the analysis (refer to section [7.5.14.7](#) for example of correct  $\beta$ -Catenin segmentation); 2) The nuclear segmentation accurately depicts DAPI positive nuclei (refer to section [7.5.14.3](#) examples of under, over and properly thresholded nuclei). ***If either the tissue or nuclear segmentation is not accurate, the analysis should be refined and re-run;*** and 3) Ensure that the marker threshold accurately captures marker positive cells. Examples of correctly and incorrectly segmented tissue are shown below. The criteria for acceptable segmentation are that the image has a maximum of **-/+ 10%** of the tumor area and/or of the marker area over or under thresholded. If the marker mask is detecting signals other than tumor cell specific expression (i.e., collagen, auto fluorescence, tissue folds, any features that interfere with nuclear detection), ***those images should be noted for removal from analysis.***

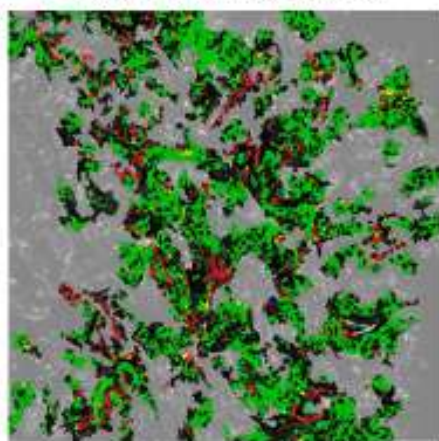
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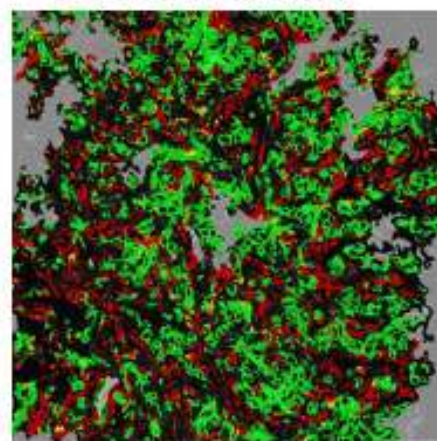
E-Cadherin (Gray Scale)



Vimentin (Gray Scale)



Correct Segmentation



Incorrect Segmentation

- 8.2.1.2 After reviewing the images as stated above, the data for the images to be removed from the final analysis should be highlighted in the **Original Raw Data** sheet of the EMT Master Template. **Do not remove the data from the Original Raw Data sheets. If more than a few images are to be removed, it is good practice to make a note as to why the images were not included in the analysis.**
- 8.2.1.3 Copy the **Original Raw Data** sheet, with the highlighted rows indicating any image data to be removed, and paste the data into the **Raw Data2pass** sheet. Delete all highlighted rows from the **Raw Data2pass** sheet.
- 8.2.1.4 Review the control slide images from Definiens. If, after reviewing the images, there are images that are deemed to be outliers for the control tissue, highlight the row of data that is to be removed in the **CAL Original Data** sheet. **Do Not Remove the data from the CAL Original Data Sheet.**

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8.2.1.5 Copy the data from **CAL Original Data** sheet and paste it on the **CAL Data** sheet. Delete all highlighted rows from the **CAL Data** sheet.

**8.2.2** Select the **Developer** tab and click on **Macro**. In the drop-down menu, select **EMT2Pass**. After the run is done, save the EMT Master Template. *(If the Developer tab is not visible, it will need to be enabled.)*

**8.2.3** Check all pages for errors. For example, the **Lookup 2pass** sheet shows the total image number. Compare the total image number to the number of raw data images analyzed. Make sure that on the **Template 2pass** and **Cal Summary** sheets the data is present in all cells that should contain data.

### 8.3 EMT Control Slide QC

**8.3.1** Variability in sample processing and staining is monitored by employing assay specific control slides. At least one EMT control slide should be included in each clinical staining run, and it should be evaluated to confirm that biomarker levels fall into the acceptable range. The Control Slide layout and tissue description are provided in [Appendix 2](#).

**8.3.2** The acceptable range for biomarker levels for the tissue control slides is determined by lot, and will be set for Log<sub>10</sub> of V:E (Vimentin Area : E-Cadherin Area) for all the tissues on the control slide. All tissues (epithelial, mesenchymal and the E/M mix control) on the control slide should pass QC in order to proceed to reporting of clinical analysis data. A **minimum of 5 ROIs** from each control tissue should be analyzed.

**8.3.3** The lot number and the image ID of the tissue control slides will be part of the image name, thus will be captured on the EMT Master Template.

**8.3.4** If the control slides meet the defined criteria above, proceed to the evaluation of the clinical slide QC. If the control slides do not meet the criteria above, the assay fails QC. Check for errors in data acquisition, labeling and analysis of the QC slides. If no errors are found, the assay should be rerun using a backup clinical slide set and fresh control slides.

**8.3.5** In some cases, there may be an isolated control tissue failure that is tissue specific. If all other control tissues pass on the control slide, and the failure to achieve the expected Log<sub>10</sub> of V:E for one tissue is thought to be tissue specific and not an assay failure, the Laboratory Director/Supervisor may allow exceptions to the QC.

### 8.4 EMT Clinical Slide QC

**8.4.1** The clinical slide layout and tissue description is provided in [Appendix 2](#).

**8.4.2** Each clinical slide typically contains two to three time point biopsies with 1-2 passes for each time point, and a positive fixation control for the EMT tissue (mouse jejunum). The positive control should be assessed qualitatively on each slide.

**8.4.3** As described in SOP340550, murine jejunum tissue is fixed and processed in parallel with the clinical biopsies. This tissue serves as a control for proper tissue fixation and staining for the EMT Panel IFA. E-Cadherin staining is positive in the epithelial cells lining the villi. β-Catenin stains all the cells that comprise both the crypts and the villi. Vimentin staining should be negative for all the cells in jejunum. *Note: murine testis*

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*tissue is also included for tissues processed under SOP340550, however there are no requirements for this tissue for the EMT panel IFA.*

**8.4.4** If the expected staining patterns for E-Cadherin, Vimentin and/or  $\beta$ -Catenin are not confirmed by qualitative analysis of the murine jejunum section on the clinical slide, the slide should not be evaluated, and the potential source of the problem should be investigated.

**8.4.5** A minimum of **6 ROIs** for each biopsy tissue from at least **two non-adjacent slides** for each patient with a minimum of **3000 nuclei/tissue** for 2 slides are required to report quantitative clinical slide data. If this number of nuclei is not achieved for a clinical tissue, additional slides can be run and analyzed.

**8.5** Finalizing Data Report and Batch Record

**8.5.1** Completion of Data Report

8.5.1.1 Before completing the sample **Data Report** make sure there are no errors on the EMT Master Template by reviewing **all** generated sheets.

8.5.1.2 Complete the **Data Report** sheet by filling in the name of the operator and the date of the report. Make sure the calibrator data passed. The limits for Cal 1 & Cal 3 and the Vimentin & E-Cadherin image exposure times, and Definiens Architect Analysis Vimentin & E-Cadherin thresholds must be entered manually on the **CAL Summary** sheet. The calibrator pass/fail status will automatically be displayed on both **CAL Summary** and **Data Report** sheets as long as the limits are entered.

8.5.1.3 Review the nuclei number for each pass to determine if the minimum of 3000 cells / 2 slides were met. If less than 3000 cells were analyzed, **DO NOT REPORT THE DATA** for that pass.

8.5.1.4 In the **Comments** section of the **Data Report** list any deviations or problems that occurred.

8.5.1.5 Review **Data Report** for accuracy and completeness.

8.5.1.6 The **Data Report** is not complete until the supervisor signs the report. By signing the **Data Report**, the supervisor or reviewer indicates that the **Data Report** is both complete and accurate. Save the completed EMT Master Template in the appropriate header folder. The **Data Report** can be signed electronically; if, instead of the electronic version, a paper copy of the **Data Report** is signed, scan the signed paper copy. The scanned **Data Report** must be kept in the header folder.

8.5.1.7 See [Appendix 3](#) for blank **Data Report**. See [Appendix 4](#) for an example of a completed **Data Report**. [Appendix 5](#) shows the calculations used to generate the **Data Report**.

**8.5.2** Completion of Batch Record

8.5.2.1 Make sure to complete the Batch Record, including filling in name of the facility/lab analyzing the images, date of the analysis, name of the operator

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performing the analysis, CTEP protocol number, patient ID, slide numbers, tissue (pass) IDs, calibrator lot number and image ID ([Appendix 1](#)).

- 8.5.2.2 Record, for both the calibrators and clinical tissues, the threshold settings for Vimentin and E-Cadherin used in the Definiens image analysis ([Appendix 1](#)).
- 8.5.2.3 Record the names and the location of the header folder.
- 8.5.2.4 Document ALL deviations from this SOP in the **Comments** section of the Batch Record ([Appendix 1](#)).
- 8.5.2.5 Review finalized Batch Record for accuracy and completeness and obtain required signature with date.



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**APPENDIX 1: BATCH RECORD**

**1. Image Extraction and Analysis**

Facility/Lab Name:		Date(s):		
Assay Operator:		Patient ID and Tissue ID(s):		
CTEP Protocol#:		Calibrator Lot#:		
Calibrator Image ID(s):		Thresholds: (Calibrator)	Vimentin	
			E-Cadherin	
Patient Slide #:		Thresholds: (Patient)	Vimentin	
			E-Cadherin	
Name of Header Folder*:				
Location of Header Folder:				

\* EMT Master Template /Data Report must be kept in Header Folder

**2. Comments:**

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

*(signature indicates that the information in the batch record has been reviewed and verified)*

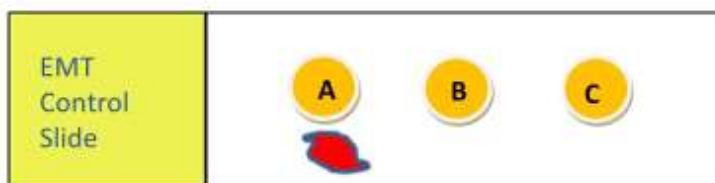
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## APPENDIX 2: CONTROL SLIDES AND CLINICAL SLIDES LAYOUT

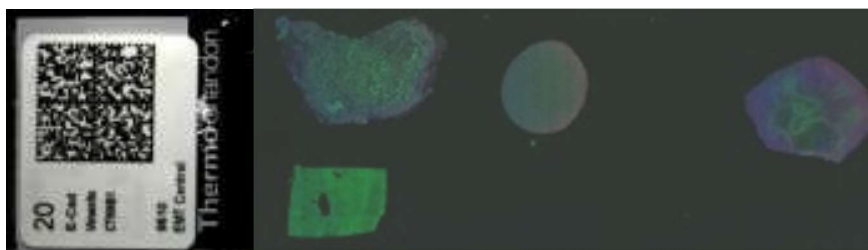
### 1. Control Slides

- a) Day-to-day variability in sample processing and staining is monitored by employing a control slide run with each clinical staining and analysis for the EMT panel IFA. **One control slide is required for each set of patient slides and should be run on the same tray of a Bond-RX run as the patient slides.**

Control slide layout



Example of Control Slide

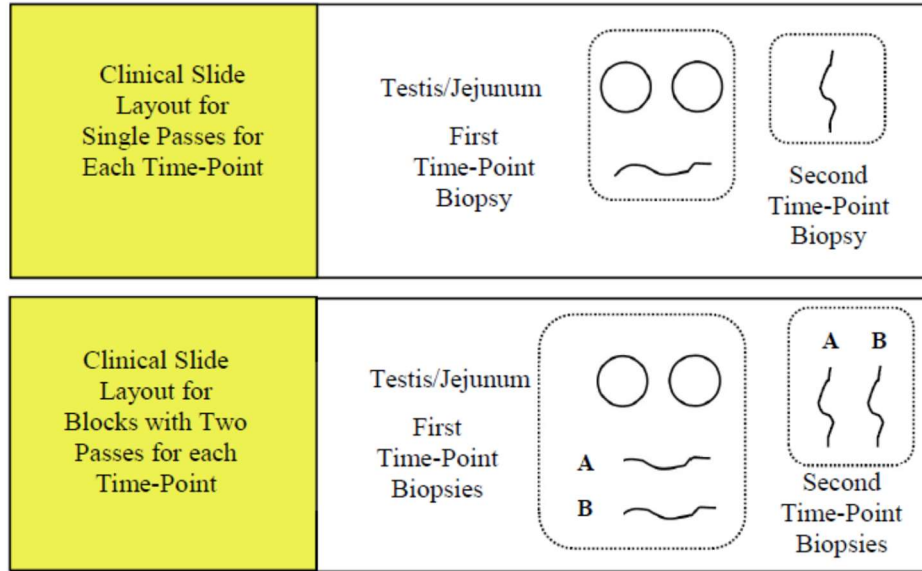


- b) Control sections are generated from NBF-fixed, paraffin-embedded, untreated xenograft tissues or cell pellets from **A.** HT29, **B.** SUM-149-PT and **C.** MDA-MB231. Liver control tissue (red) is included for orientation.
- c) The positive staining control for E-Cadherin (AF488) and  $\beta$ -Catenin (AF546) is the HT29 tissue (A). The positive control for Vimentin staining is the MDA-MB231 tissue (C). The SUM-149-PT tissue (B) represents a mixture of epithelial and mesenchymal cells and should therefore have staining for both the E-Cadherin and Vimentin biomarkers.
- d) The negative/low control specimen for Vimentin is the HT29 tissue, while the negative/low control for E-Cadherin is the MDA-MB231 tissue.  $\beta$ -Catenin is present in all control tissues and there is no designated negative control for  $\beta$ -Catenin.
- e) Acceptable ranges for  $\text{Log}_{10}$  of V:E will be set for all the tissues on the tissue control slide and determined by lot. All control tissues on the slide must pass QC to proceed to the clinical analysis. A **minimum of 5 images** from each calibrator tissue should be analyzed (one image represents one data point).
- f) Representative data and image of the control slide can be seen above.

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**2. Clinical Slides**

- a) The layout for the clinical slides with 1 or 2 passes is shown below. The first time point biopsy is processed and blocked together with a mouse jejunum (testis is also often included as it is a control for related pharmacodynamic assay panels). The second time point biopsy is processed and blocked in a separate block without any other tissues.



- b) The EMT positive fixation control specimen is mouse jejunum. In a tissue cross-section, E-Cadherin staining is positive in the epithelial cells lining the villi, and  $\beta$ -Catenin stains all the cells that comprise both the crypts and the villi. Vimentin staining should be negative for all the cells in jejunum.
- c) Clinical samples for this assay will be frozen needle biopsies collected per SOP340507, embedded and sectioned per SOP340550, and stained for the EMT Panel per SOP340546.
- d) Backup slides are available for use if the first slide set does not meet QC criteria for the EMT Panel.

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**APPENDIX 3: BLANK DATA REPORT GENERATED IN THE TEMPLATE MASTER**  
*(AREAS COLORED IN YELLOW MUST BE COMPLETE FOR THE FINAL DATA REPORT)*

**DCTD (NCTVL) Clinical Sample**  
 Data Report - EMT-IFA -(SOP340548v.B )

Assay Operator: \_\_\_\_\_ Date: \_\_\_\_\_

Patient ID: #VALUE! Protocol: #VALUE!

Specimen IDs: \_\_\_\_\_

Avg:Log10(V/E)	SD	Pass/Fail
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!

**Log10 (V:E) Sample Data Summary**

Timepoint	SpecimenID	Mean Vimentin Area (µm <sup>2</sup> ) per 1000 cells for	+ SD	Mean E-Cadherin Area (µm <sup>2</sup> ) per 1000 cells for	+ SD	Mean Log10 (V:E)	+ SD	Total # of Nuclei Analyzed

Vimentin & Cadherin Area/1000 cells

Mean Log10 (V:E)

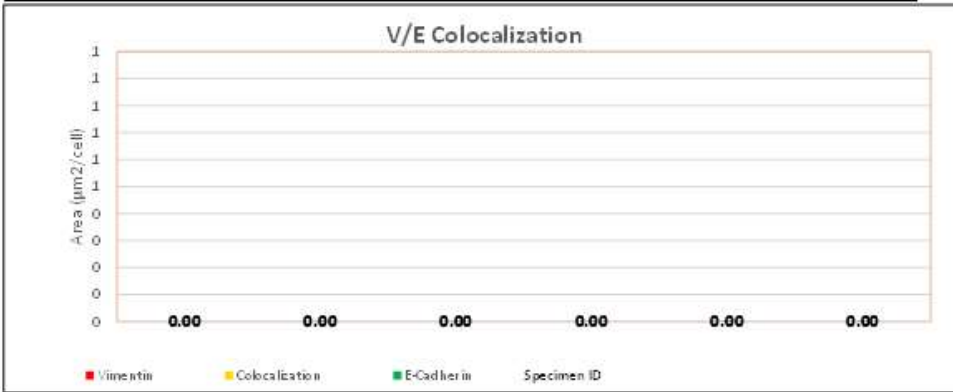
1

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Patient ID:  Protocol:

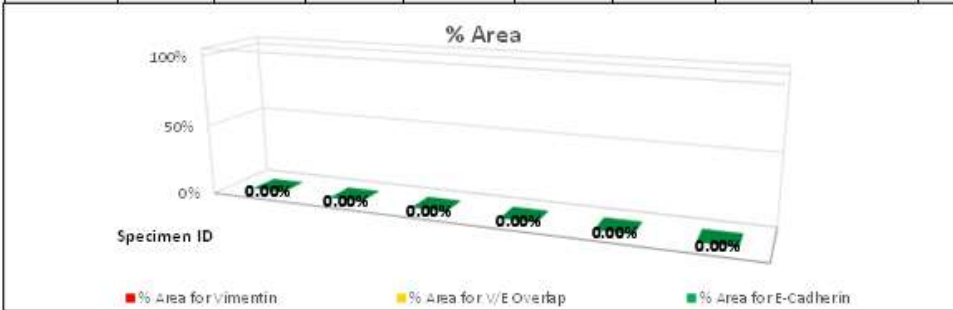
**V/E Colocalization Sample Data Summary**

Timepoint	Specimen ID	Vimentin Only Area (Mean $\mu\text{m}^2/\text{cell}$ )	+ SD	E-Cadherin Only Area (Mean $\mu\text{m}^2/\text{cell}$ )	+ SD	V/E Colocalization Area (Mean $\mu\text{m}^2/\text{cell}$ )	+ SD	Total # of Nuclei Analyzed



**Areas Sample Data Summary**

Timepoint	Specimen ID	Total Area Analyzed	% Area for Vimentin	% Area for E-Cadherin	% Area for V/E Overlap	Vimentin Area for total Nuclei	E-Cadherin Area for total Nuclei	V/E Area for total Nuclei

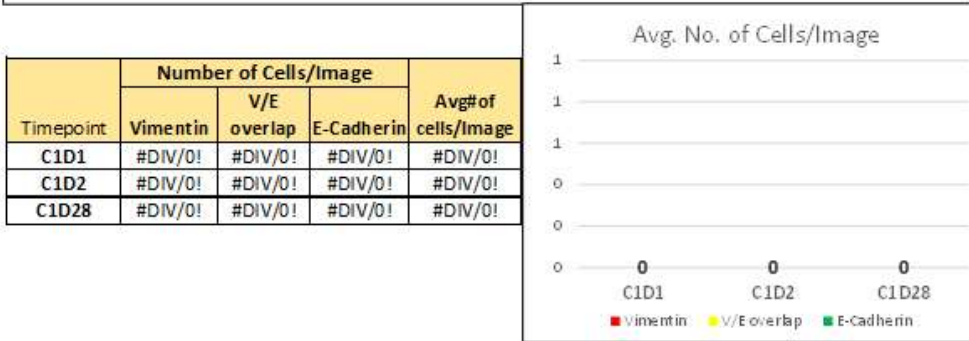
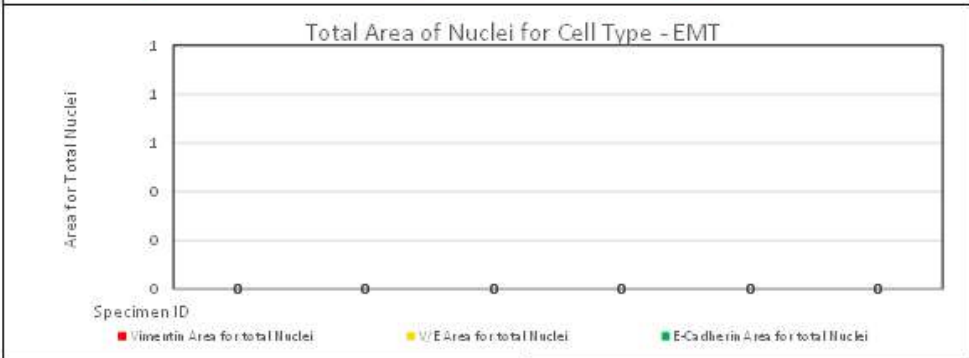
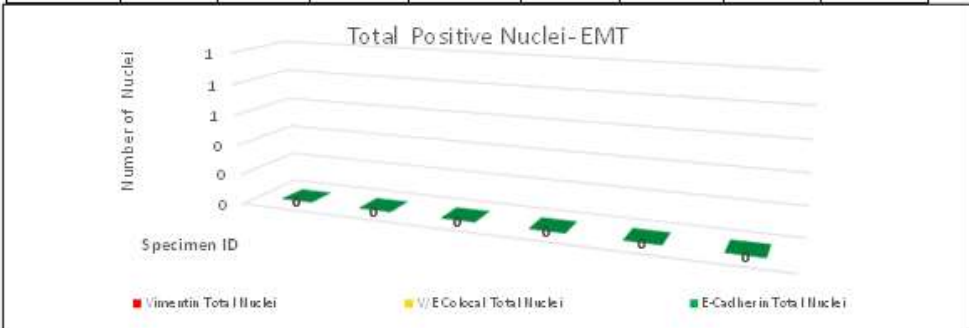


Patient ID:

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**Total Number of Positive Nuclei - Data Summary**

Timepoint	Specimen ID	Total # of Nuclei Analyzed	% Area for Vimentin	Vimentin % Area X Total # Nuclei	E-Cadherin % Area	E-Cadherin % Area X Total # Nuclei	V/E Colocalization % Area	V/E Colocalization % Area X Total # Nuclei



Timepoint	Number of Cells/Image			Avg# of cells/Image
	Vimentin	V/E overlap	E-Cadherin	
C1D1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
C1D2	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
C1D28	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!



DCTD Standard Operation Procedures (SOP)

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**APPENDIX 4: EXAMPLE OF A COMPLETED DATA REPORT GENERATED IN THE EMT TEMPLATE MASTER**

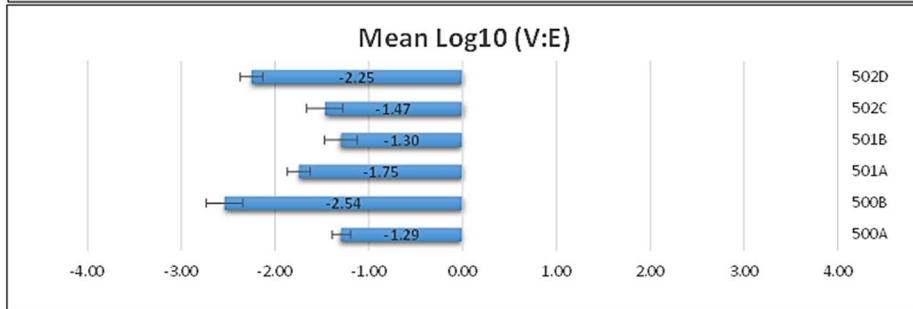
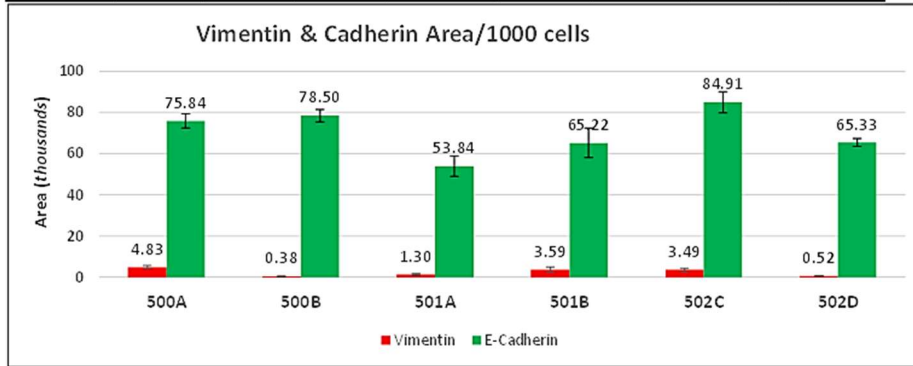
**DCTD (NCTVL) Clinical Sample  
Data Report - EMT-IFA -(SOP340548v.B)**

Assay Operator: S. S. Smith Date: 4/7/2020  
 Patient ID: 09659\_0020 Protocol: 09659  
 Specimen IDs: 500A 500B 501A 501B 502C 502D

Avg:Log10(V/E)	SD	Pass/Fail
-4.8735	0.0284	Pass
0.3113	0.0712	Pass
1.7534	0.4671	Pass

**Log10 (V:E) Sample Data Summary**

Timepoint	Specimen ID	Mean Vimentin Area (µm <sup>2</sup> ) per 1000 cells for	+ SD	Mean E-Cadherin Area (µm <sup>2</sup> ) per 1000 cells for	+ SD	Mean Log10 (V:E)	+ SD	Total # of Nuclei Analyzed
C1D01	500A	4829.4	789.9	75835.6	3459.7	-1.2923	0.1000	27960
	500B	378.0	103.2	78500.6	3031.0	-2.5370	0.1960	45822
C1D02	501A	1300.1	374.6	53838.0	4985.2	-1.7463	0.1231	12665
	501B	3589.1	1268.4	65222.2	7126.2	-1.2958	0.1731	6702
C1D28	502C	3492.0	873.0	84908.1	5123.9	-1.4699	0.1935	6000
	502D	522.7	53.0	65326.8	1928.0	-2.2522	0.1201	26091





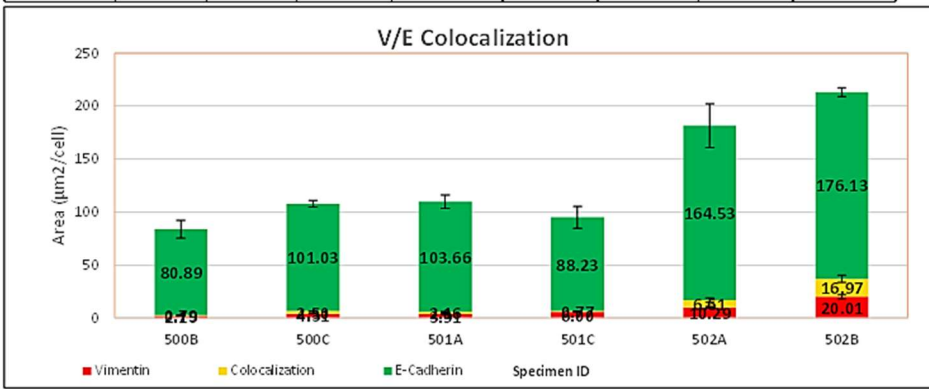
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Patient ID: 09659\_0025 Protocol: 09659

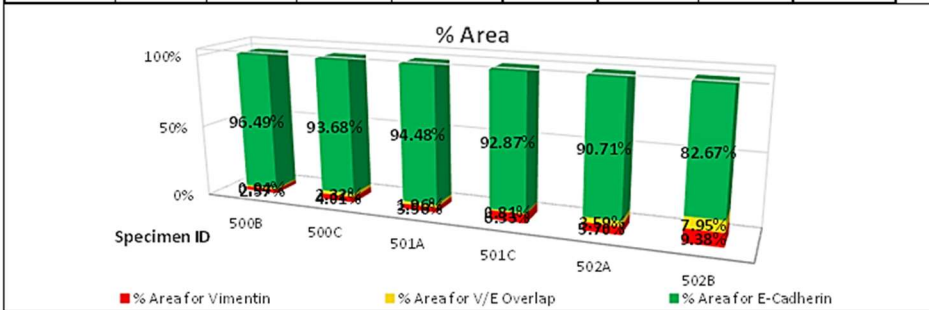
V/E Colocalization Sample Data Summary

Timepoint	Specimen ID	Vimentin Only Area (Mean $\mu\text{m}^2/\text{cell}$ )	+ SD	E-Cadherin Only Area (Mean $\mu\text{m}^2/\text{cell}$ )	+ SD	V/E Colocalization Area (Mean $\mu\text{m}^2/\text{cell}$ )	+ SD	Total # of Nuclei Analyzed
C1D01	500B	2.152	0.152	80.892	8.298	0.793	0.164	38230
	500C	4.314	0.227	101.028	3.212	2.498	0.067	25538
C1D02	501A	3.910	0.542	103.658	6.323	2.164	0.449	31349
	501C	5.996	0.516	88.231	10.188	0.772	0.168	20542
C1D28	502A	10.292	0.734	164.528	20.583	6.612	1.984	13747
	502B	20.009	1.911	176.131	4.239	16.967	3.305	13519



Areas Sample Data Summary

Timepoint	Specimen ID	Total Area Analyzed	% Area for Vimentin	% Area for E-Cadherin	% Area for V/E Overlap	Vimentin Area for total Nuclei	E-Cadherin Area for total Nuclei	V/E Area for total Nuclei
C1D01	500B	252	2.57%	96.49%	0.94%	328.05	12295.98	119.30
	500C	324	4.01%	93.68%	2.32%	342.67	7972.73	197.26
C1D02	501A	329	3.56%	94.48%	1.96%	364.63	9879.85	205.19
	501C	285	6.33%	92.87%	0.81%	433.22	6358.88	55.23
C1D28	502A	544	5.70%	90.71%	3.59%	261.05	4155.81	165.47
	502B	639	9.38%	82.67%	7.95%	420.07	3720.23	366.03



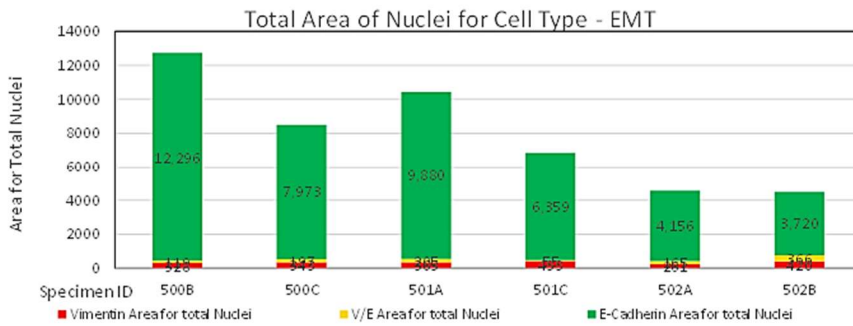
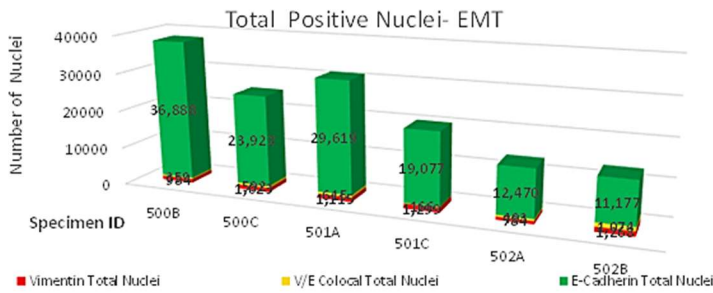
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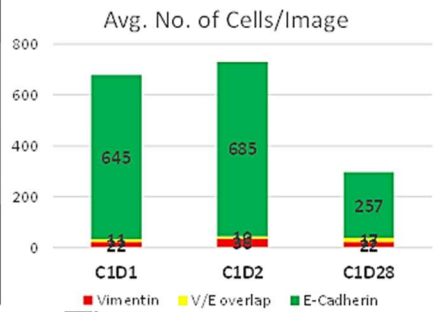
Patient ID: 09659\_0025 Protocol: 09659

Total Number of Positive Nuclei - Data Summary

Timepoint	Specimen ID	Total # of Nuclei Analyzed	% Area for Vimentin	Vimentin % Area X Total # Nuclei	E-Cadherin % Area	E-Cadherin % Area X Total # Nuclei	V/E Colocalization % Area	V/E Colocalization % Area X Total # Nuclei
C1D01	500B	38230	2.57%	984	96.49%	36888	0.94%	359
	500C	25538	4.01%	1023	93.68%	23923	2.32%	592
C1D02	501A	31349	3.56%	1115	94.48%	29619	1.96%	615
	501C	20542	6.33%	1299	92.87%	19077	0.81%	166
C1D28	502A	13747	5.70%	784	90.71%	12470	3.59%	493
	502B	13519	9.38%	1268	82.67%	11177	7.95%	1074



Timepoint	Number of Cells/Image			Avg# of cells/Image
	Vimentin	V/E overlap	E-Cadherin	
C1D1	22	11	645	678
C1D2	36	10	685	731
C1D28	22	17	257	296



Frederick National Laboratory for Cancer Research

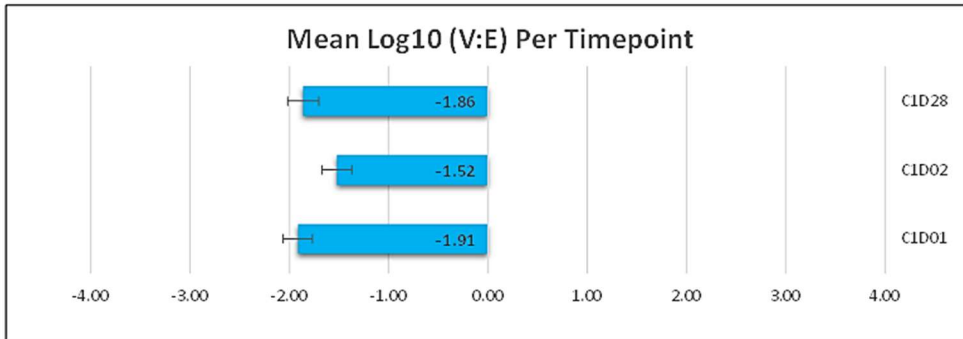
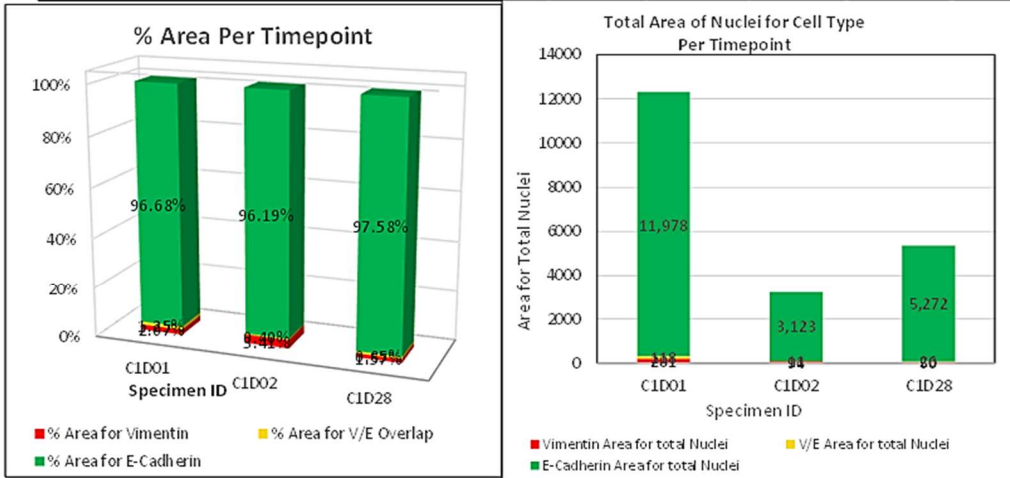


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Patient ID:  Protocol:

**Areas Timepoint Data (passes combined) Summary**

Timepoint	Total # of Nuclei Analyzed	Total Area Analyzed	% Area for Vimentin	% Area for E-Cadherin	% Area for V/E Overlap	Vimentin Area for total Nuclei	E-Cadherin Area for total Nuclei	V/E Area for total Nuclei	Mean Log <sub>10</sub> (V:E)
C1D01	73782	236.39	2.07%	96.68%	1.25%	201.20	11977.73	118.07	-1.91
C1D02	19367	185.15	3.41%	96.19%	0.40%	94.14	3123.14	10.55	-1.52
C1D28	32091	229.24	1.57%	97.58%	0.85%	50.35	5272.06	26.09	-1.86



Comments:

Supervisor:

electronic signature



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## APPENDIX 5: SAMPLE DATA REPORT CALCULATIONS

### A. $\text{Log}_{10}(\text{V:E})$ – Calculated for each ROI, and for both the mean per slide and the mean of all imaged slides combined for each time-point pass.

1. Calculate the Total Area ( $\text{um}^2$ ) per nuclei (cell) for Marker 1 (Vimentin) and Marker 2 (E-Cadherin).  
Total Area = Total Area of Marker/ # of nuclei evaluated (includes colocalization)
2. Total Area for each Mark of 1000 cells = Total Area for Marker X 1000 cells. (For total areas that are less than one, the number one will be recorded and used in the calculations for  $\text{Log}_{10}(\text{V:E})$ .)
3.  $\text{Log}_{10}(\text{V:E}) = \text{Log}_{10}(\text{Total Area of 1000 cells Marker1/ Total Area for 1000 cells Marker2})$
4. Calculate the mean and the mean standard deviation of the Total Area of Each Marker and for the Total  $\text{Log}_{10}$  for all imaged slides.
5. Add the total number of nuclei (cells) analyzed for each time-point pass.

### B. V/E Colocalization – Calculated for each ROI, and for both the mean per slide and the mean of all imaged slides combined for each time-point pass

1. Calculate the V/E Overlap Area ( $\text{um}^2$ ) per cell = Overlap Area/total # nuclei
2. Calculate Marker 1 & Marker 2 Area per cell = (Marker area – Overlap area)/ total # nuclei
3. Calculate the mean and the mean standard deviation for V/E Overlap only, the Vimentin only and E-Cadherin only areas per cell.

### C. Area % for Vimentin only, E-Cadherin only, and V/E Overlap only - Calculated for both the mean per slide and the mean of all imaged slides combined for each time-point pass

1. Calculate % Area for Vimentin only, E-Cadherin only, and V/E overlap only per slide.  
% Marker Area per slide = (Mean Area for a Marker for each slide/ Mean of the sum of the total area for each slide) x 100%
2. Calculate % Area for Vimentin only, E-Cadherin only, and V/E overlap for all slides per time-point pass  
% Total Area for Timepoint pass = (Mean of Area for Marker for each time-point pass/ Mean of the sum of the total area for each time-point) x 100%

### D. Area for Vimentin only, E-Cadherin only, and V/E Overlap for total nuclei - Calculated for both the mean per slide and the mean of all imaged slides combined for each time-point pass

1. Calculate Total Area for each Marker for all nuclei for each slide pass = Mean of the Area for Marker for each slide pass x Sum of the total # nuclei for each slide pass
2. Calculate Average Area for each marker for all nuclei for each time-point pass = Mean of the Marker Area from all slides for each time-point x all nuclei from all slides for each time-point pass

### E. Total Number Cells Positive for Vimentin only, E-Cadherin only, and V/E Overlap

1. Total number of Positive Cells for each Marker per time-point = Mean % Area for the Marker for all Sides passes x the sum of the total # nuclei for each time-point pass.

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**F. Total Area of Nuclei for each Cell Type (Vimentin, E-Cadherin, and V/E Overlap)**

1. Marker Overlap Area ( $\text{um}^2$ ) per cell = Marker Overlap Area/# nuclei  
 Marker Area ( $\text{um}^2$ ) per cell = (Marker Area – Overlap Area)/# nuclei
2. Average Marker Area Overlap/Nuclei for each slide/timepoint/tissue  
 Average Marker Area Vimentin/Nuclei for each slide/timepoint/tissue
3. Sum Avg Marker Areas/Nuclei for each pass

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**APPENDIX 6: LIST OF HEADER FOLDER CONTENTS & LIST OF SETTINGS (EXPORT A SCREENSHOT) IN DEFINIENS**

1. List of files/folders that should be placed in the Header Folder.

The screenshot shows a Windows File Explorer window with the following path: This PC > Definiens Image Upload (\\fr-s-dtp-378apr) (V:) > PADIS > CLINICAL > 9659 > EMT > 9659\_1010030. The file list is as follows:

Name	Date modified	Type	Size
EMT 9659_30 Extracted Images	8/9/2019 8:55 AM	File folder	
EMT 9659_pt25_30 Cal Slide 36 Extracted images	8/7/2019 2:26 PM	File folder	
EMT Cal 9659_pt 30 workplace	8/23/2019 3:37 PM	File folder	
EMT Cal Workspace_pt 25_30	8/21/2019 10:56 A...	File folder	
extra	12/9/2019 12:13 PM	File folder	
9659_1010030 SOP340548 EMT Data Report 8-22-19	8/22/2019 1:22 PM	Microsoft Excel M...	642 KB

2. List of settings for Exported Screenshots in Definiens.

- a) Nucleus Detection – Mix Layer, Mask on, Lines off
- b) Nucleus Detection – DAPI Single Layer, Mask on, Lines on
- c) Marker Area Detection – E-Cadherin single layer, Mask on, Lines off
- d) Default Export – Save and Apply as is.

3. Other reminders

- 6-20 images per clinical tissue
- 3000 nuclei/tissue per 2 slides for clinical tissues
- <3000 nuclei/tissue per 2 slides cannot be reported
- A minimum of 5 images per tissue per calibrator slide

Spreadsheet (Template) can analyze up to 2 passes for 3 timepoints each with 30 images per tissue per slide. Six is the maximum number of slides that can be run in the template.

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**APPENDIX 7: NAMING ANNOTATED ROI**

1. Naming guidelines for clinical ROIs, as specified below, must be followed to ensure that the template works correctly.

Example of extraction naming: 09659\_0029 502D Slide 015 C1D08 01

- a. First part of the name is the project number. The project number must be 5 digits in length; enter a “0” in front of a 4-digit project number. Abbreviate project numbers longer than 5 digits. In the above example, the project number is 9659, and a zero was added to make it 5 digits.
- b. After the project put an underscore, and then the patient number. The patient number must be 4 digits in length; for numbers less than 4 digits, enter zeros before the number. In the above example, the patient number is 29, and two zeros were added in front of the number to make it 4 digits.
- c. After the patient number put a space, and then the specimen ID. The specimen ID is 4 digits in length. In the above example, the specimen ID is 502D.
- d. Next, leave a space and write the word “Slide”. After the word slide, leave a space and put the slide number. The slide number must be 3 digits long; for slide numbers less than 3 digits, add zeros before the number to make it 3 digits. In the example above, the slide number is 15, and a zero was added before the number to make it 3 digits long.
- e. After the slide number, leave a space and put the timepoint. The timepoint must be 5 digits; use zero to make the timepoint 5 digits long. In the above example, the timepoint is cycle 1 day 8, and a zero was added before the 8 to make 5 digits.
- f. After the timepoint, leave a space and put the 2 digit image ID. If the image ID is less than 2 digits long, add a zero in front of the number. In the above example, the image ID is 1, and a zero was added in front of the 1 to make it 2 digits long.

2. Naming rules for Calibrator ROIs, as specified below, must be followed:

Example of extraction naming: 00019382 19000096 EMT Cal1 01

- a. The name starts with the image ID. The image ID must be 8 digits in length; for image IDs less than 8 digits long, add zeros before the number. In the example above, the image ID is 19382, and zeros were added to make the number 8 digits in length.
- b. Leave a space after the image ID and put the lot number. The lot number must be 8 digits in length. In the above example the lot number is 19000096.
- c. After the lot number leave a space and type the word “EMT”.
- d. After EMT, leave a space and put Cal1, Cal2, or Cal3.
- e. After the Cal number, leave a space, and then put the image ID. The image ID is 2 digits; for image IDs fewer than 2 digits, put a zero in front of the number.

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