SOP340545: Image Extraction and Analysis of Tumor Biopsy Slides from γH2AX, pNBS1 IFA with β-Catenin Segmentation

Effective Date: 4/19/2019

Please check for revision status of the SOP at
http://dctd.cancer.gov/drug-discovery-development/assays/validated-biomarker-assays
and be sure to use the current version.

TABLE OF CONTENTS

OVERVIEW OF IMMUNOFLUORESCENCE ASSAY FOR BIOPSIES....3
1.0 PURPOSE....4
2.0 SCOPE....4
3.0 ABBREVIATIONS....4
4.0 INTRODUCTION....4
5.0 ROLES AND RESPONSIBILITIES....5
6.0 MATERIALS AND EQUIPMENT REQUIRED....6
7.0 OPERATING PROCEDURES....7
8.0 QUALITY CONTROL, DATA ANALYSIS AND REPORTING....34
APPENDIX 1: BATCH RECORD....39
APPENDIX 2: CALIBRATOR/CONTROL SLIDES AND CLINICAL SLIDES....40
APPENDIX 3: CLINICAL SAMPLE DATA REPORT....43
APPENDIX 4: EXAMPLE OF CLINICAL SAMPLE DATA REPORT....45

VERSION INFORMATION

1. Approvals

Technical Reviewer:	Angie Dull	Date:	Angie B. Dull -S (Affiliate) Digitally signed by Angie B. Dull -S (Affiliate) Date: 2019.04.23 11:07:07 -04'00'
Technical Reviewer:	Debbie Wilsker	Date:	Deborah F. Wilsker -S (Affiliate) Digitally signed by Deborah F. Wilsker -S (Affiliate) Date: 2019.04.25 12:16:41 -04'00'
NCTVL Approval:	Jiuping Ji	Date:	Jiuping Ji -S (Affiliate) Digitally signed by Jiuping Ji -S (Affiliate) Date: 2019.04.29 14:14:04 -04'00'
IQC Approval:	Katherine V. Ferry-Galow	Date:	Katherine V. Ferry-galow -S (Affiliate) Digitally signed by Katherine V. Ferry-galow -S (Affiliate) Date: 2019.04.30 15:46:39 -04'00'
LHTP Approval:	Ralph E. Parchment	Date:	Ralph E. Parchment -S (Affiliate) Digitally signed by Ralph E. Parchment -S (Affiliate) Date: 2019.05.03 10:22:12 -04'00'
DCTD OD Approval:	Toby Hecht	Date:	Toby T. Hecht -S Digitally signed by Toby T. Hecht -S Date: 2019.05.09 08:13:48 -04'00'

2. Change History

Revision	Approval Date	Description	Originator	Approval
A	4/19/2019	Significant revision due to incorporation of use of new Definiens build, incorporation of revised reporting requirements (analysis per pass, minimum cell requirements)	DW/AD/KFG/DK	REP
--	04/27/2016	New Internal Use Document	DW/AD/KFG/DK	JJ

OVERVIEW OF IMMUNOFLUORESCENCE ASSAY FOR BIOPSIES

SOP340507:
Tumor Frozen Needle Biopsy Specimen Collection and Handling

- Collect and freeze tumor needle biopsies for use in biomarker assays

↓

SOP340550:
Tumor Frozen Needle Biopsy Preparation for Pharmacodynamic Immunofluorescence Assays Utilizing Murine Testis and/or Jejunum Control

- NBF fix and paraffin embed tumor needle biopsies and positive control sample
- Section biopsies for use in IFA
- Stain slides by H&E for standard histology analysis

↓

SOP340543:
γH2AX, pNBS1 IFA Staining with β-Catenin Segmentation for Tumor Biopsy Slides

- Load biopsy and control slides into Bond-RX Processing Module
- Multiplex Bond-RX automated staining of slides with γH2AX-FITC antibody, pNBS1-DIG antibody with Anti-DIG-AF647and β-Catenin-AF546 antibody
- Stain slides with DAPI and mount cover slips

↓

Image within 72 h

SOP340544:
Whole Slide Image Capture of Tumor Biopsy Slides from γH2AX, pNBS1 IFA with β-Catenin Segmentation

- Capture images of γH2AX, pNBS1, β-Catenin-stained biopsy and control slides using Aperio ScanScope FL

↓

SOP340545:
Image Extraction and Analysis of Tumor Biopsy Slides from γH2AX, pNBS1 IFA with β-Catenin Segmentation

- Quantitate captured images of γH2AX, pNBS1, β-Catenin-stained biopsy and control slides using Definiens Tissue Studio analysis software.

1.0 PURPOSE

To standardize immunohistochemical methods to detect and quantify histone H2AX phosphorylated at serine 139 (γH2AX) and NBS1 phosphorylated at serine 343 (pNBS1) using β-Catenin tumor segmentation for pharmacodynamic (PD) evaluation of DNA damage repair status for formalin-fixed paraffin-embedded (FFPE) tissue biopsy sections. The goal of the SOP and associated training is to ensure consistency of biomarker measurements between operators and clinical sites.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the γH2AX, pNBS1 IFA with β-Catenin Segmentation for Tumor Biopsy Slides from patients participating in clinical trials. This SOP outlines the recommended procedure for image extraction and analysis from whole slide image capture of stained, paraffin-embedded tumor biopsy sections.

3.0 ABBREVIATIONS

Ab	=	Antibody
DAPI	=	4',6-Diamidino-2-Phenylindole
DCTD	=	Division of Cancer Treatment and Diagnosis
FFPE	=	Formalin-fixed paraffin-embedded tissue
γH2AX	=	Histone H2AX Phosphorylated at Serine 139
H&E	=	Hematoxylin and Eosin
IFA	=	Immunofluorescence Assay
LHTP	=	Laboratory of Human Toxicology & Pharmacology
NCTVL	=	National Clinical Target Validation Laboratory
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
pNBS1	=	NBS1 phosphorylated at serine 343
QC	=	Quality Control
ROI	=	Region of Interest
SOP	=	Standard Operating Procedure
SVS	=	Scientific Visualization Studio

4.0 INTRODUCTION

The γH2AX, pNBS1 IFA with β-Catenin segmentation is an immunohistochemistry-based staining assay developed to quantify γH2AX and pNBS1 using β-Catenin staining. Staining with β-Catenin provides tumor area masking and is used, together with pathologist annotation, to define the areas in which γH2AX and pNBS1 are quantitated.

5.0 ROLES AND RESPONSIBILITIES

Laboratory Director/Supervisor	The Laboratory Director/Supervisor directs laboratory operations, supervises technical personnel and reporting of findings, and is responsible for the proper performance of all laboratory procedures. The Laboratory Director/Supervisor oversees the personnel who follow the SOPs within the laboratory and is responsible for ensuring the personnel are certified and have sufficient experience to handle clinical samples.
Certified Assay Operator	A 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 Certified Assay Operator works under the guidance of the Laboratory Director/Supervisor. This person performs laboratory procedures and examinations in accordance with the current SOP(s), as well as 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.

5.3 The Certified Assay Operator responsible for conducting the assay is to follow this SOP with associated addendum 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.

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 software

6.2 Aperio ImageScope software

6.3 Definiens Architect DDR BUILD; Tissue Studio Analysis software

6.4 Microsoft Excel 2010 or higher software

6.5 Whole slide images of stained clinical sample and control slides imaged according to SOP340544

6.6 SOP340545 Excel Master Template

6.7 PC

7.0 OPERATING PROCEDURES

7.1 The Batch Record (Appendix 1) for image analysis should be completed to document image extraction and analysis for a single patient's data.

7.1.1 Record the Facility/Laboratory Name, Assay Operator performing the image extraction and quantitation and the date(s) of analysis in the Batch Record (Appendix 1, Section 1).

7.1.2 Record the Patient ID, CTEP/Protocol number and Slide numbers in the Batch Record (Appendix 1, Section 1).

7.1.3 Comments on extraction and analysis of individual slides or images can be made in the comments section of the Batch Record (Appendix 1, Section 2).

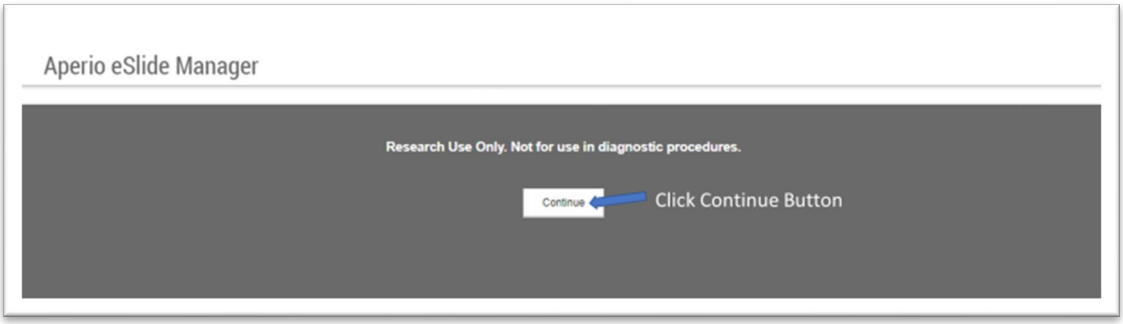
7.2 Annotate and extract slide images located on the Aperio eSlide Manager database

Note: See Appendix 2 for calibrator/control and clinical slide section orientation.

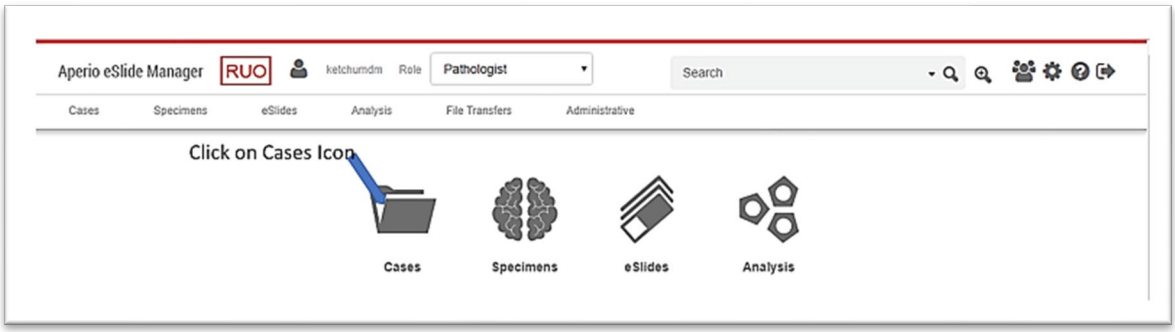
7.2.1 Retrieve each clinical slide within the clinical eSlide Manager database and open individual images using Aperio ImageScope, following these steps (also depicted below): Login into Aperio eSlide Manager (A), and click **Continue** button (B). Click Cases (folder) icon (C) and select the project of interest by clicking on the file folder next to the project number (D). Click on the brain icon next to the patient of interest (E), and open the digital slide by clicking on the slide image (F). The slide image should have opened in ImageScope (example shown in G).

A.

B.



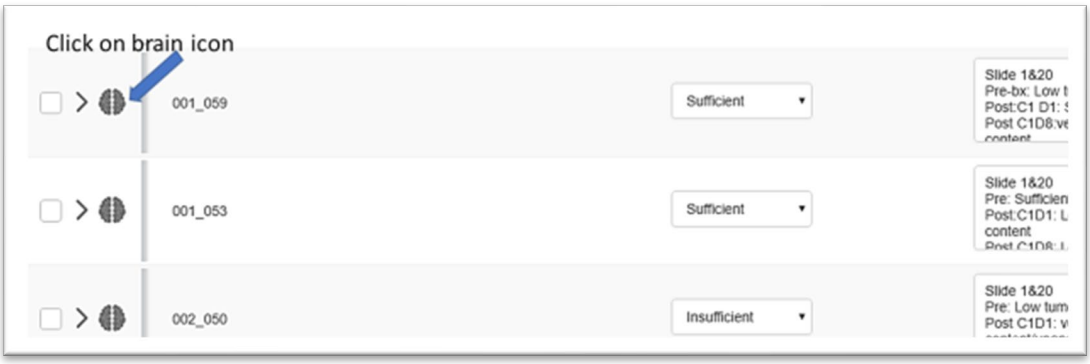
C.


Aperio eSlide Manager
RUO
ketchumdm
Role
Pathologist
Search
Cases
Specimens
eSlides
Analysis
File Transfers
Administrative
Click on Cases Icon
Cases
Specimens
eSlides
Analysis

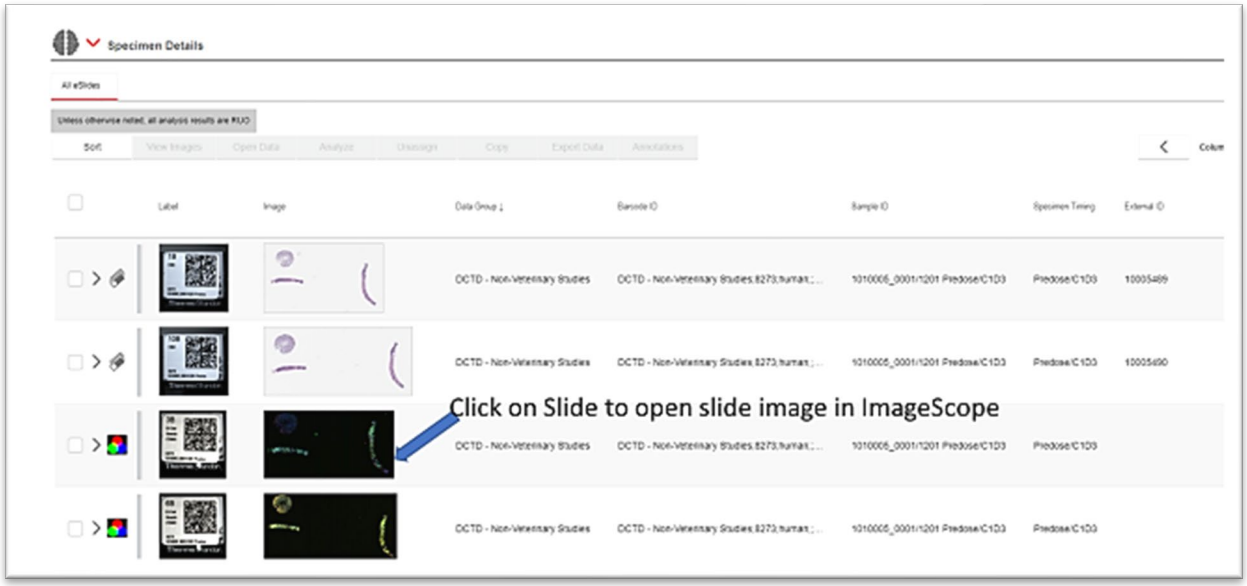
D.


DCTD - Non-Veterinary Studies 9350 human
DCTD - Non-Veterinary Studies 7998 human
Click the folder icon for project of interest
DCTD - Non-Veterinary Studies 7977 human

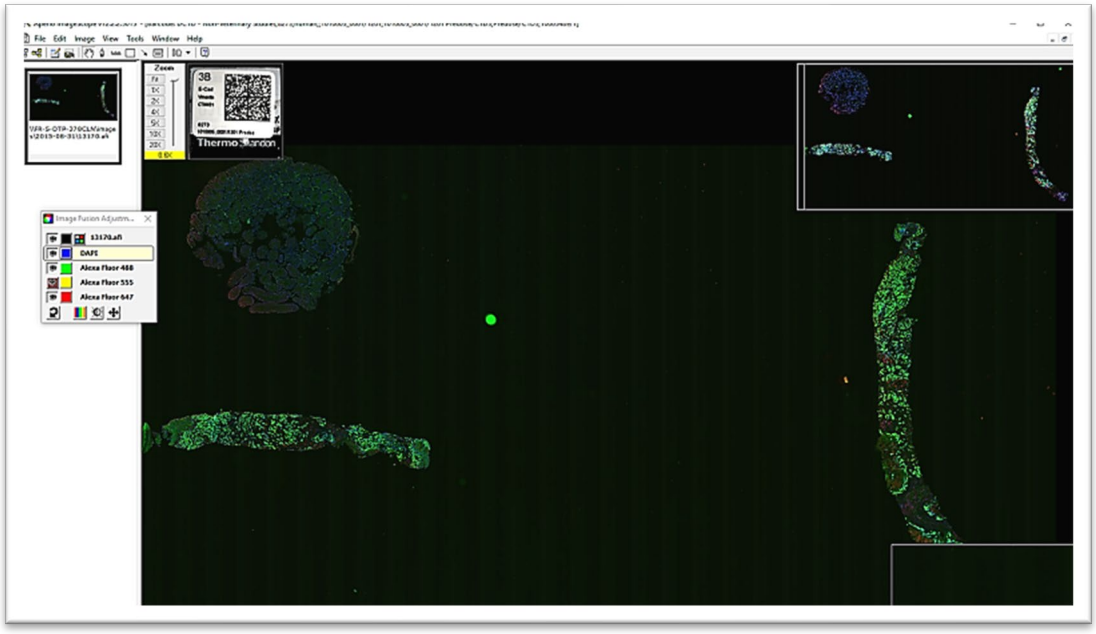
E.


Click on brain icon
001_059
Sufficient
001_053
Sufficient
002_050
Insufficient

F.


Specimen Details
Click on Slide to open slide image in ImageScope

G.



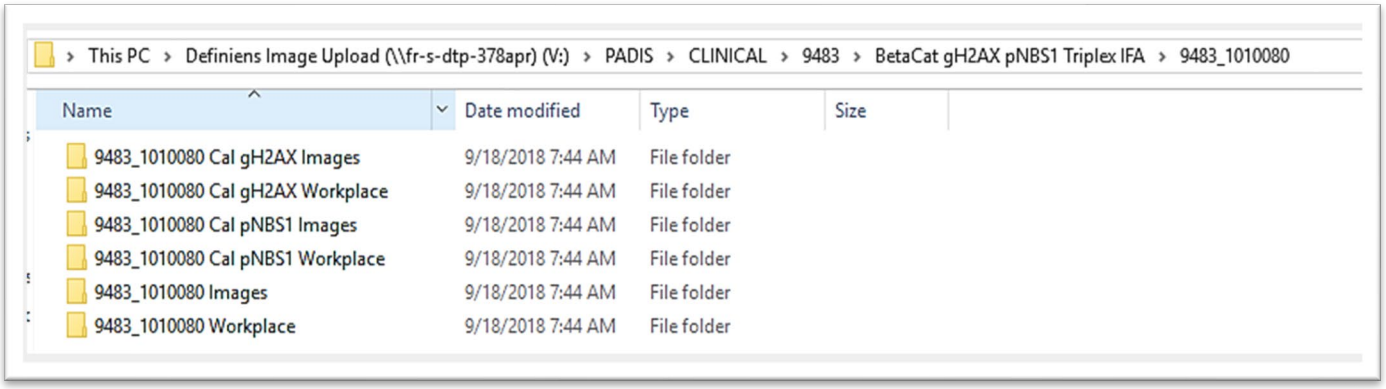
7.3 For each patient, create a Header Folder on the Definiens Drive.

Clinical data within NCTVL at FNLCR must be stored in the CLINICAL folder: Definiens Image Upload > PADIS > CLINICAL > specific Trial folder-> BetaCat gH2AX pNBS1 Triplex IFA. The suggested naming convention for this Header Folder is: CTEP#_patient ID#. *For example: Definiens Image Upload Y: PADIS\...\ 9483_1010080.* Under the Header Folder, 6 subfolders should be created: one patient workplace, two Calibrator marker workplaces, and three folders for the images. For details on how to create each subfolder see the sections below, and note the figure after section 7.3.3 as an example of subfolder structure.

7.3.1 Create one patient workplace subfolder, where the Definiens analysis data will be stored. This folder should be named CTEP#_Patient ID Workplace (for example: 9483_1010080 Workplace). The workplace subfolder is created in Definiens Architect XD. The operator is prompted in Definiens Architect to name a folder and to designate the location the folder should be placed. Assign the Workplace folder to the appropriate header folder.

7.3.2 Create two subfolders for the calibrators. These should be named CTEP#_Patient ID# Calibrator Marker (gH2AX or pNBS1) Workplace (for example: 9483_1010080 Calibrator gH2AX Workplace). It is critical to specify which calibrator marker is being analyzed in the subfolder title. For the calibrator slides only, separate folders are generated for each marker. The operator is prompted in Definiens Architect to name a folder and to designate the location the folder should be placed. Assign the Workplace folder to the appropriate header folder.

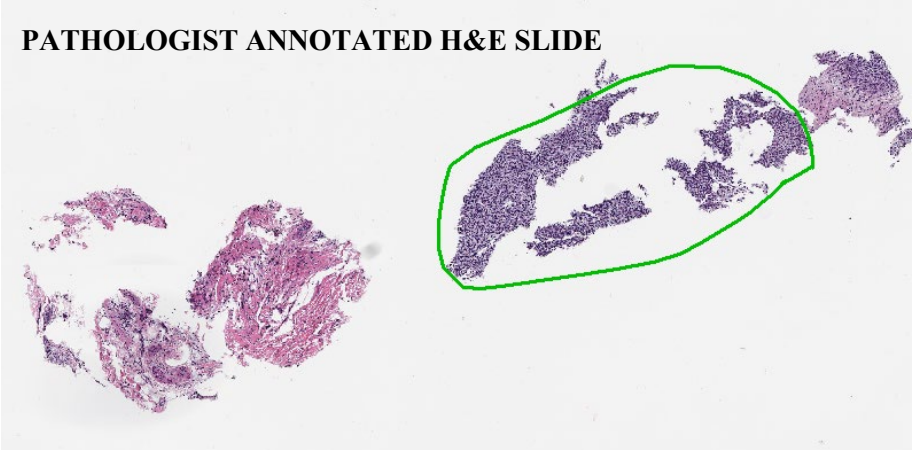
7.3.3 Create three subfolders for extracted images: 1) patient images (e.g. 9483_1010080 Images), 2) calibrator gH2AX images (e.g. 9483_1010080 Cal gH2AX images), and 3) calibrator pNBS1 images (e.g. 9483_1010080 Cal pNBS1 images). The image extraction subfolders should be made when the header folder is made. When the same calibrators are used for slides from multiple patients on the same staining run, the calibrator images can be stored in just one patient folder to save memory space. If the images are not stored in the patient header folder, their location should be noted in the Excel Spreadsheet on the Definiens Data sheet under the data.



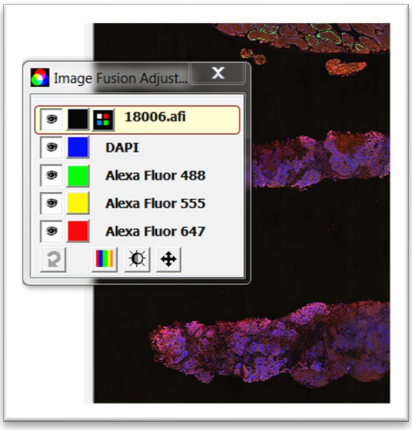
7.4 General requirements for the extraction of images

7.4.1 For clinical tissue sections on all clinical slides analyzed, capture regions of interest (ROI) representing images covering the entire analyzable area, using the nearest adjacent pathologist-annotated H&E slide image as a guide (see figure below as an example of an annotated H&E slide). It is not necessary to extract images from all possible ROIs. For

most clinical biopsies 8-10 optimal ROIs spanning across the length of the analyzable areas of the tissue is recommended. For lower tumor content biopsies and those with sparse tumor areas throughout the biopsy, it may be necessary to extract additional fields in order to achieve the requirement of 3000 cells analyzed per two slides required to report data for an individual tissue. The maximum number of images to be acquired per tissue per slide is 15. Overlap in ROI selection should be avoided. Verify that the regions of interest are in clear focus on the stained slides. Annotate and extract images from the Aperio scan representing **only** the analyzable area for the clinical tissue sections; avoid edges, necrotic areas, large gaps in the tissue, normal tissue areas or areas where the tissue has folded onto itself.



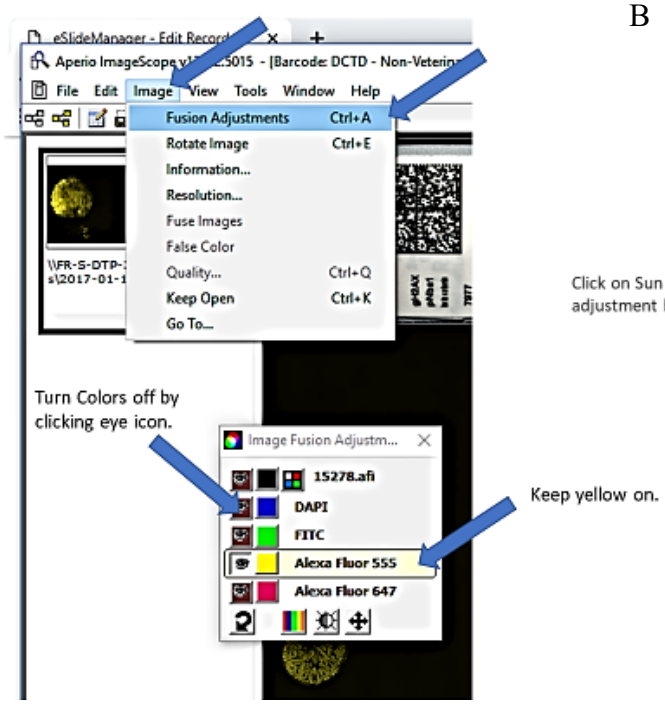
7.4.2 Tissues visualized in Aperio ImageScope software are given "false" color assignments: γH2AX signal is assigned green (Alexa Fluor 488), pNBS1 signal is assigned red (Alexa Fluor 647), β-Catenin signal is assigned yellow (Alexa Fluor 546) and DAPI signal is assigned blue, as depicted below.



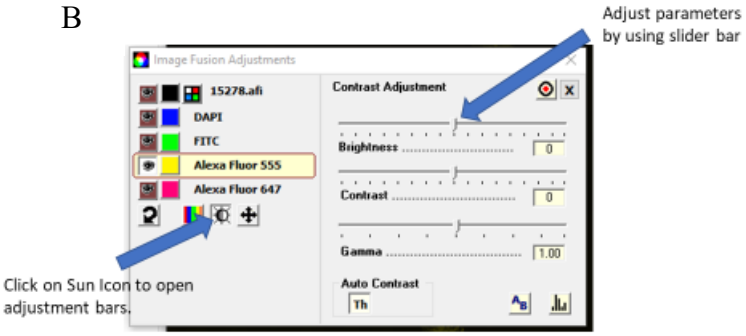
7.4.3 β-Catenin has been shown to permit delineation of tumor tissue from surrounding tissues. All tumor areas extracted for analysis from the pathologist-annotated area should be confirmed to be positive for β-Catenin. The intensity profile of the β-Catenin signal can be adjusted to optimize visualization.

To adjust the β-Catenin signal, follow these steps (also depicted below): Click the Image tab, select "Fusion Adjustment" from the drop-down menu, and the "Image Fusion Adjustment" dialog box will appear (A). On the dialog box turn off all colors but yellow (β-Catenin signal) by clicking each eye-icon next to the colored box. Click on the sun icon to open the adjustments for brightness, contrast, and gamma (B). Change the parameters by using the slider bar until the desired intensity is achieved, and then click on the eye button at the top to turn all the colors back on. **The intensity of the measured biomarkers γH2AX and pNBS1 should not be adjusted.**

A



B

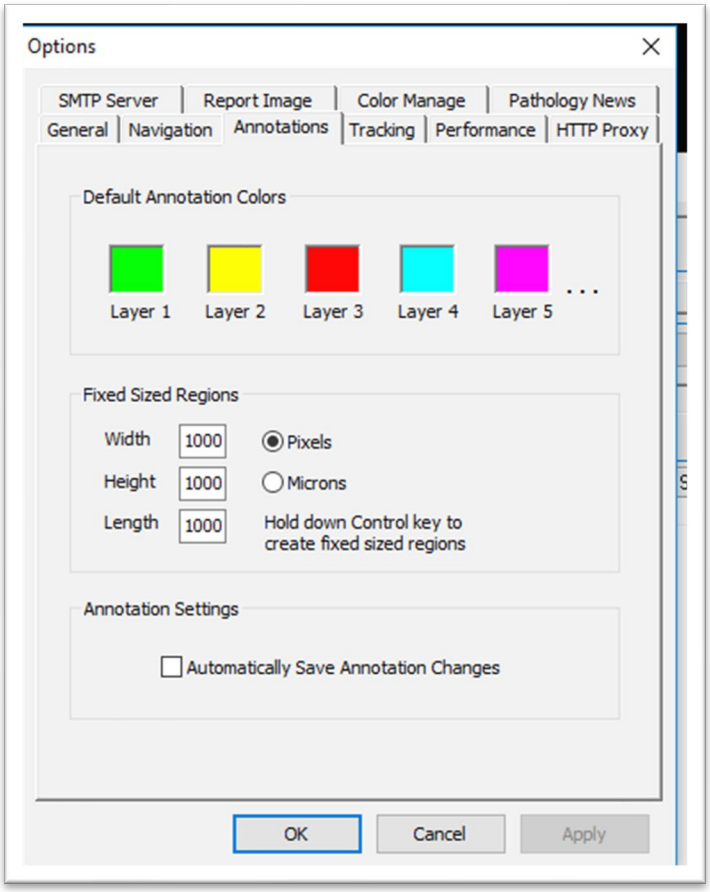


7.4.4 For control slides, a minimum of **six** non-overlapping fields are required for **pNBS1** and **γH2AX** calibrator tissues. Avoid regions on the edge of the tissue and regions of necrosis (as indicated by rounded up DAPI stained nuclei). For calibrator material only (NOT FOR CLINICAL SLIDES), ROI selection is **guided by where the marker is present in the calibrator tissue**. ***For Calibrator slides only****:* The intensity of the measured biomarkers γH2AX and pNBS1 can be adjusted for better visualization. Extractions of the negative and positive control tissues are not necessary as they are qualitative controls.

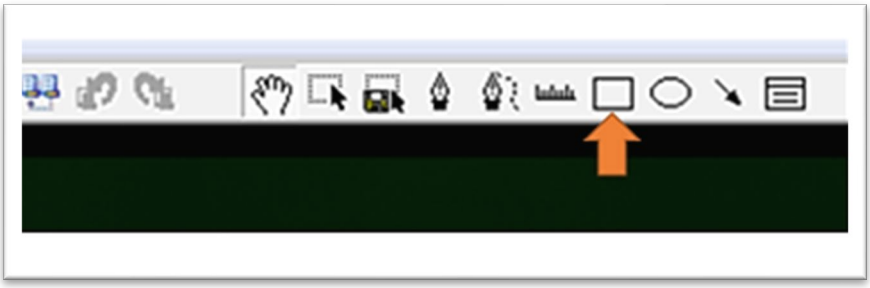
7.5 Image Annotation

Prior to image extraction, select and annotate the image fields to be extracted.

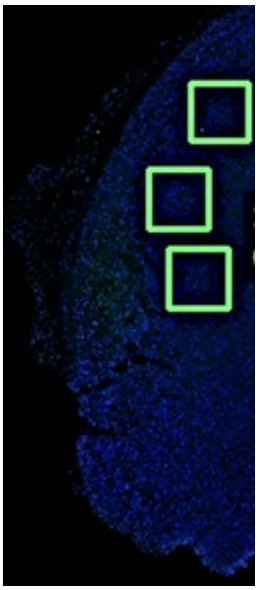
7.5.1 The rectangle tool is used to draw boxes over the target areas. The size of the rectangle can be automated by selecting: Tools > Options > Annotations (as shown below). In the height tab type 1000, and in the width tab type 1000. Check the box that indicates pixels. This is equivalent to approximately 3.10 x 10^5 μm^2. The dimensions of the annotated regions should be kept fixed and should not overlap.



7.5.2 Holding down the control button on the keyboard prior to clicking the rectangle tool (shown below) will automatically create a 1000 x 1000 box, which will allow for faster field selection.



7.5.3 Place the box over the ROI and let go of the control button. The box will stay on the location that it was placed. Repeat this process to create several ROIs, as shown below.



7.5.4 Annotated ROIs are named in the Aperio Annotation - Detail View display. Within Annotation - Detail View in the "Layer Region" section, type the desired name in the "Text" box for every ROI (see figure A below). Make sure to save the annotations by clicking the save button on the top left of the Annotations - Detailed View display. This will save the annotations with the names. The annotated text will then appear on the Aperio image overlay, as shown below (see figure B).

7.5.4.1 Each ROI is named in Aperio for the patient as follows:

Project# (5 digits)_Patient# (4 digits) *space* tissue ID (4 digits) *space* Slide *space* Slide# (3 digits) *space* timepoint (4 digits) *space* Image ID (2 digits) (e.g. 07977_0039 500a Slide 007 C1D1 03).

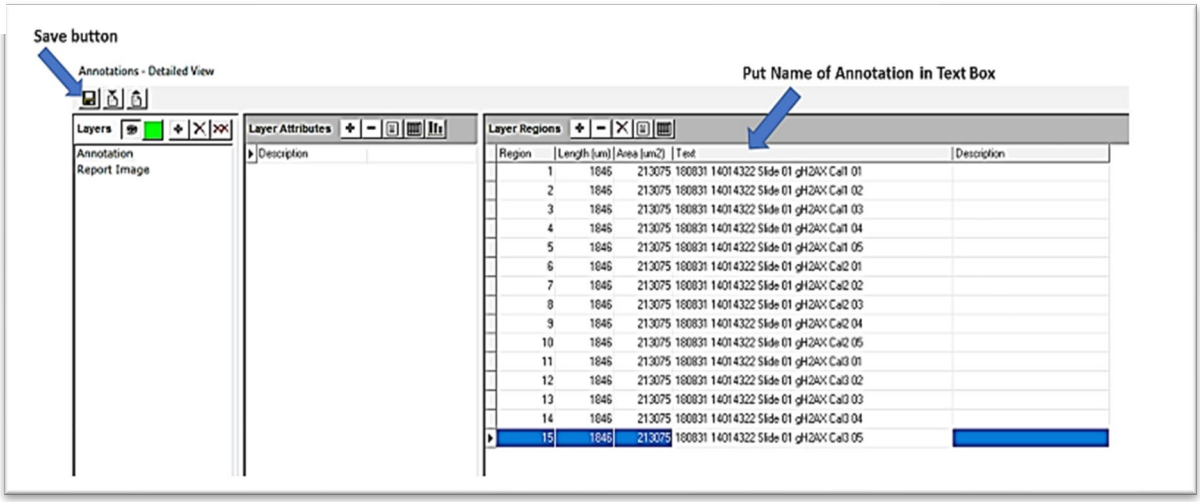
7.5.4.2 Annotation names for calibrators are as follows:

Date (yy/mm/dd) (6 digits) *space* Lot# (8 digits) *space* Slide *space** Slide# (2 digits; *exception: no space after the word "slide" if slide# is 3 digits, for example Slide103) *space* Marker (5 digits) *space* Cal# *space* Image# (2 digits) (e.g. 180831 14014320 Slide 03 gH2AX Cal2 04). Note that the Aperio Image ID can be used instead of the date; however, if the Aperio Image ID is only 5 digits, 2 spaces instead of one are required after the ID.

The 3 levels of Calibrators will be named as Cal1, Cal2, and Cal3, which will indicate the following:

Cal1 = Cal Low
Cal2 = Cal Mid
Cal3 = Cal High

A


Save button
Annotations - Detailed View
Put Name of Annotation in Text Box
Layers
Annotation
Report Image
Layer Attributes
Description
Layer Regions
Region
Length (um)
Area (um2)
Text
Description
1 1846 213075 180831 14014322 Slide 01 gH2AX Cal1 01
2 1846 213075 180831 14014322 Slide 01 gH2AX Cal1 02
3 1846 213075 180831 14014322 Slide 01 gH2AX Cal1 03
4 1846 213075 180831 14014322 Slide 01 gH2AX Cal1 04
5 1846 213075 180831 14014322 Slide 01 gH2AX Cal1 05
6 1846 213075 180831 14014322 Slide 01 gH2AX Cal2 01
7 1846 213075 180831 14014322 Slide 01 gH2AX Cal2 02
8 1846 213075 180831 14014322 Slide 01 gH2AX Cal2 03
9 1846 213075 180831 14014322 Slide 01 gH2AX Cal2 04
10 1846 213075 180831 14014322 Slide 01 gH2AX Cal2 05
11 1846 213075 180831 14014322 Slide 01 gH2AX Cal3 01
12 1846 213075 180831 14014322 Slide 01 gH2AX Cal3 02
13 1846 213075 180831 14014322 Slide 01 gH2AX Cal3 03
14 1846 213075 180831 14014322 Slide 01 gH2AX Cal3 04
15 1846 213075 180831 14014322 Slide 01 gH2AX Cal3 05

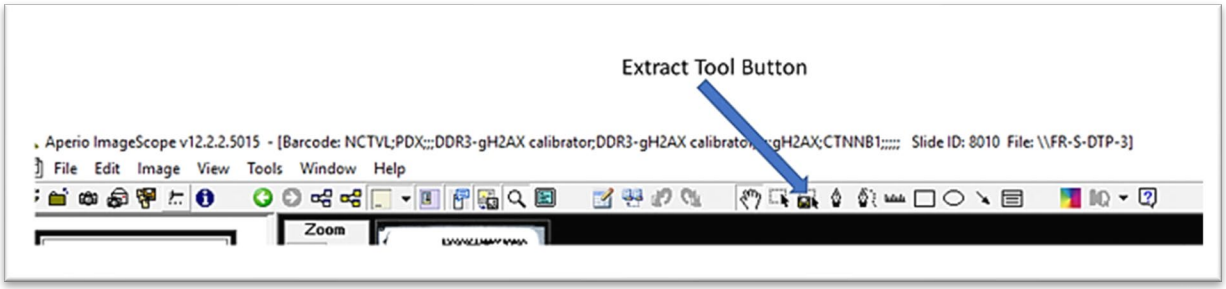
B


180831 14014322 Slide 01 gH2AX
Cal1 04
180831 14014322 Slide 01 gH2AX
Cal1 01
180831 14014322 Slide 01 gH2AX
Cal1 02
180831 14014322 Slide 01 gH2AX
Cal2 02
Cal2 05
Cal2 04
180831 14014322 Slide 01 gH2AX
Cal3 05
Cal3 04
Cal3 02

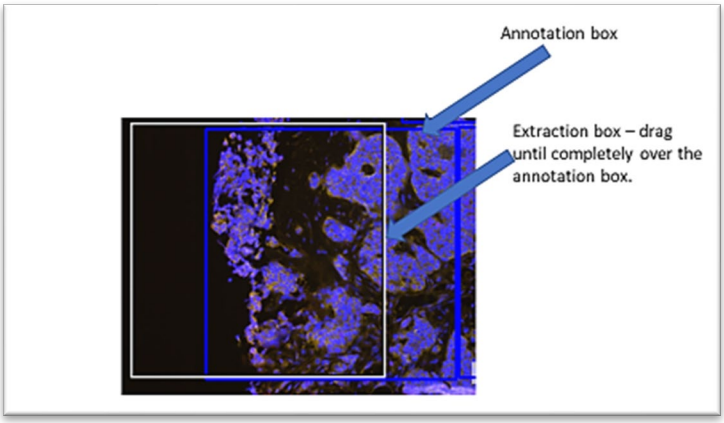
7.6 Extraction of Annotated Images

7.6.1 Hold down the control button on the keyboard and click the extract image tool button, as depicted below (A). An extraction box will appear (shown as a white square in B). Still holding down the control button, drag the extraction box until it completely overlaps with the annotation box (shown as a blue square in B). Let go of the control button and the extraction box will stay in place.

A



B



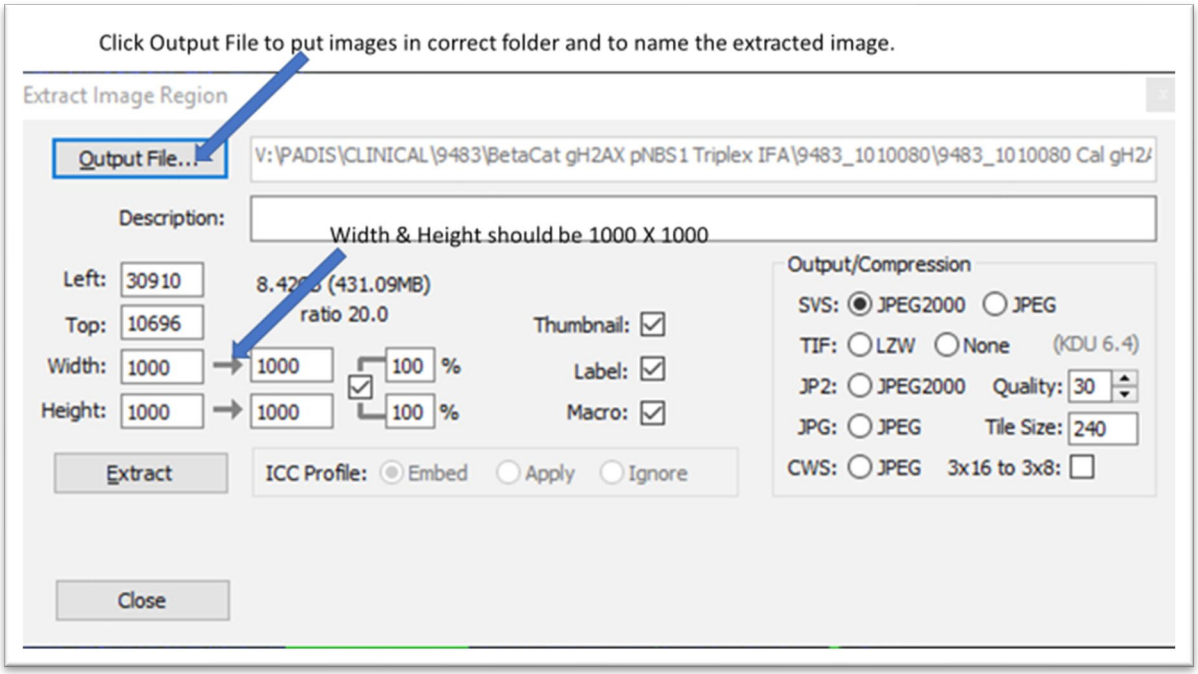
7.6.2 An "Extract Image Region" dialog box will appear. In the dialog box set the width and height of the extracted image as 1000, as shown below (A).

7.6.3 Each annotated ROI is extracted as an SVS file (JPEG2000 file) and saved to the Definiens drive.

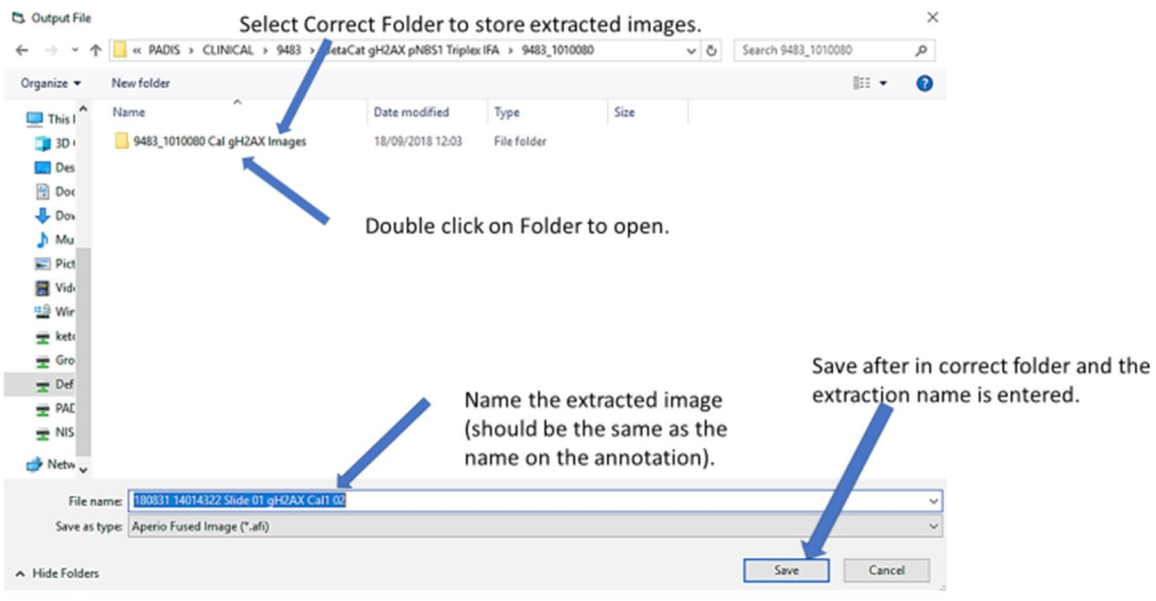
Select "Output File" in the dialog box and give the image to be extracted a name matching the annotation name as shown below (B). Save the image to the appropriate Header folder on the Definiens drive (see section 7.3).

7.6.4 Press "Extract" in the dialog box (C). Once the region is extracted, press "Close" to exit the dialog box. Repeat the process for all the annotated ROIs to be extracted for the same sample.

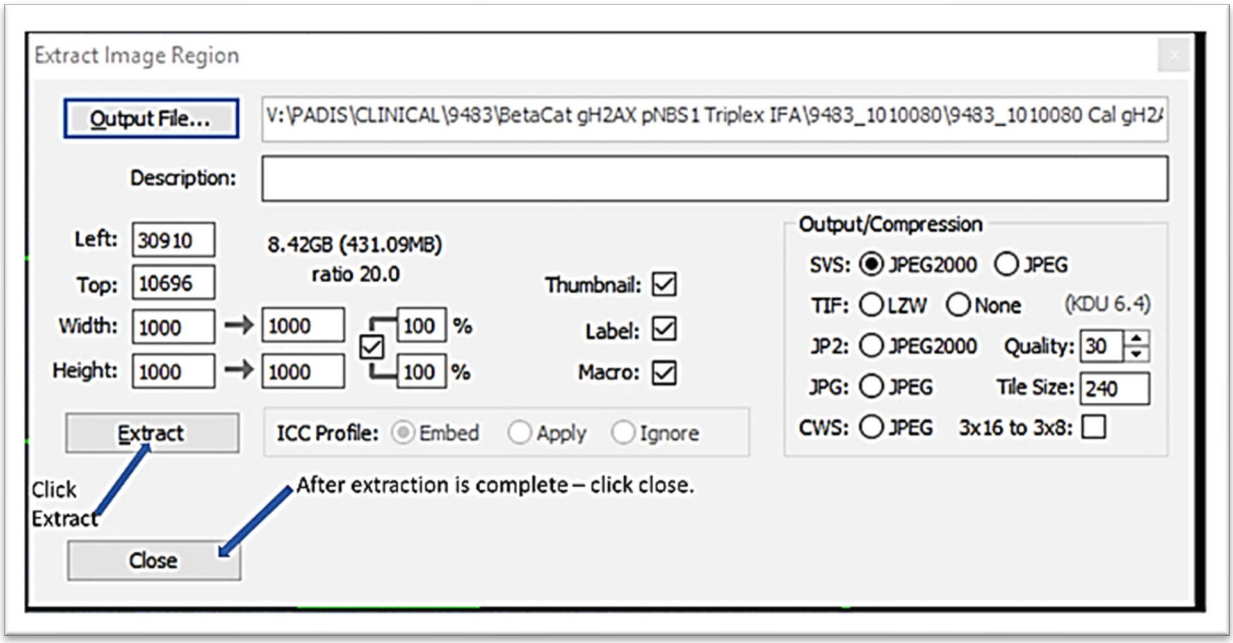
A


Click Output File to put images in correct folder and to name the extracted image.
Extract Image Region
Output File...
V:\PADIS\CLINICAL\9483\BetaCat gH2AX pNBS1 Triplex IFA\9483_1010080\9483_1010080 Cal gH2A
Description:
Width & Height should be 1000 X 1000
Left: 30910
Top: 10696
Width: 1000
Height: 1000
8.42 (431.09MB)
ratio 20.0
1000
1000
100 %
100 %
Thumbnail:
Label:
Macro:
Output/Compression
SVS: JPEG2000 JPEG
TIF: LZW None (KDU 6.4)
JP2: JPEG2000 Quality: 30
JPG: JPEG Tile Size: 240
CWS: JPEG 3x16 to 3x8:
Extract
ICC Profile: Embed Apply Ignore
Close

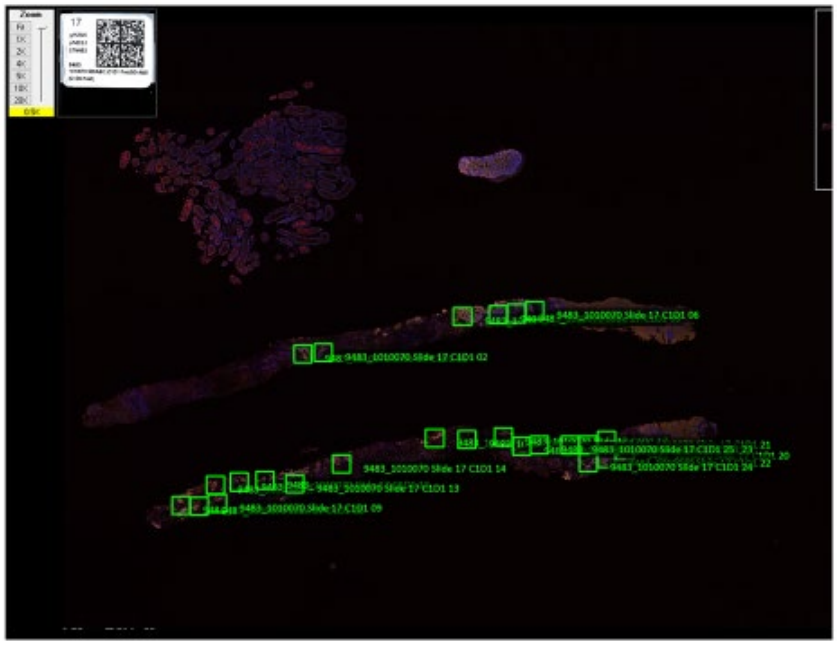
B


Output File
Select Correct Folder to store extracted images.
« PADIS › CLINICAL › 9483 › BetaCat gH2AX pNBS1 Triplex IFA › 9483_1010080
Search 9483_1010080
Organize
New folder
Name
Date modified
Type
Size
9483_1010080 Cal gH2AX Images
18/09/2018 12:03
File folder
Double click on Folder to open.
Save after in correct folder and the extraction name is entered.
Name the extracted image (should be the same as the name on the annotation).
File name: 180831 14014322 Slide 01 gH2AX Cal1 02
Save as type: Aperio Fused Image (*.afi)
Hide Folders
Save
Cancel

C



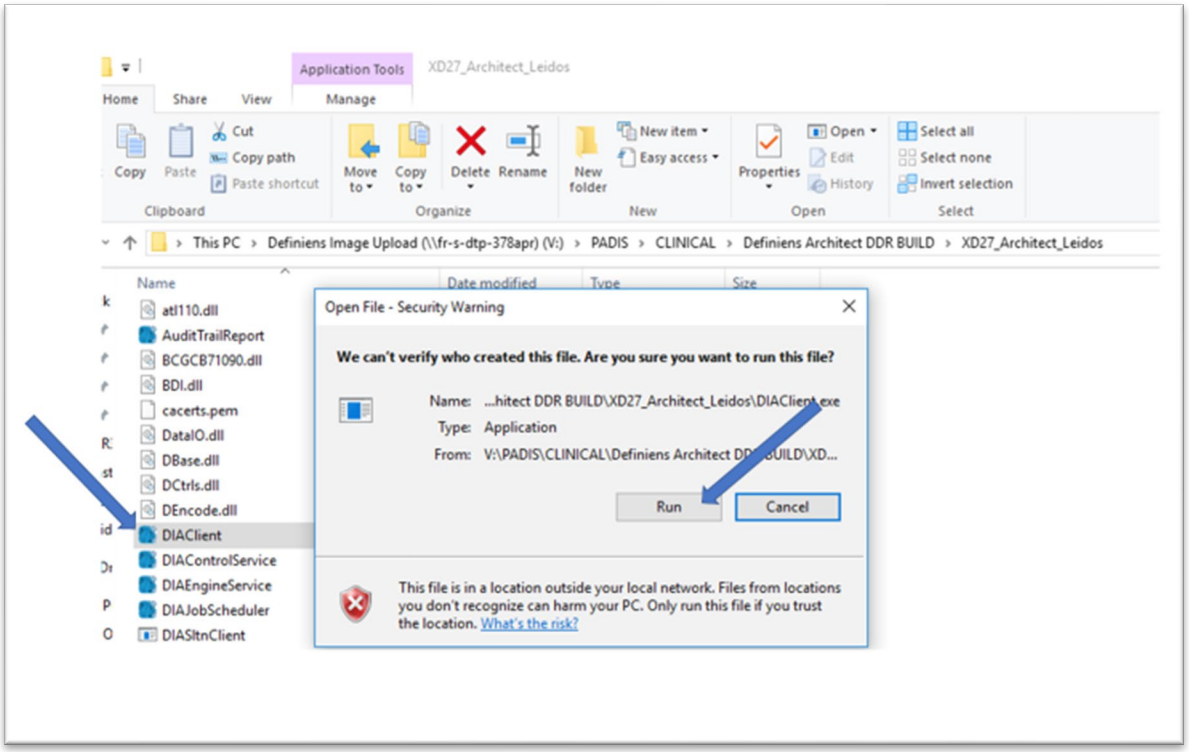
7.6.5 When you have finished extracting all the images acquired for that tissue, zoom out to view the entire tissue and use the Snipping Tool on your PC to copy and save a snapshot of the annotated image. Do this for each timepoint. An example snapshot of the ROIs for two passes of C1D1 is shown below.



7.6.6 After completing and saving image extractions, close the ImageScope software and log out of the Aperio clinical image database.

7.7 Image Analysis

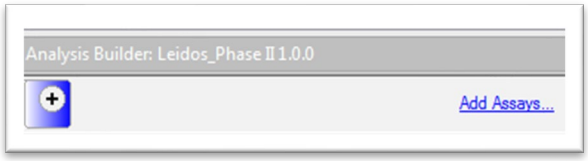
7.7.1 Open Definiens Architect DDR Build. Go to Definiens Image upload (within NCTVL at FNLCR this is located at: \PADIS\CLINICAL\DEFINIENS ARCHITECT DDR BUILD) and open XD27_Architect Leidos folder. Double click on DIA Client. There will be a pop-up box to Run the file, as shown below.



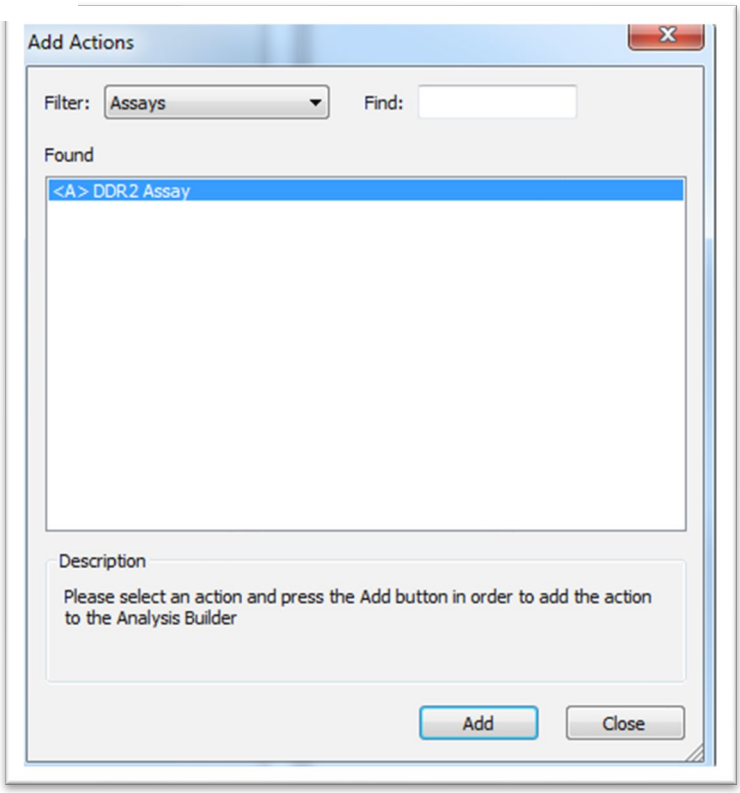
7.7.2 Go to the Configure tab and click on "Add assays" (as shown below, A), and another window will open (B). Select DDR2 assay and click "Add" (C). Close the Add Actions window.

A

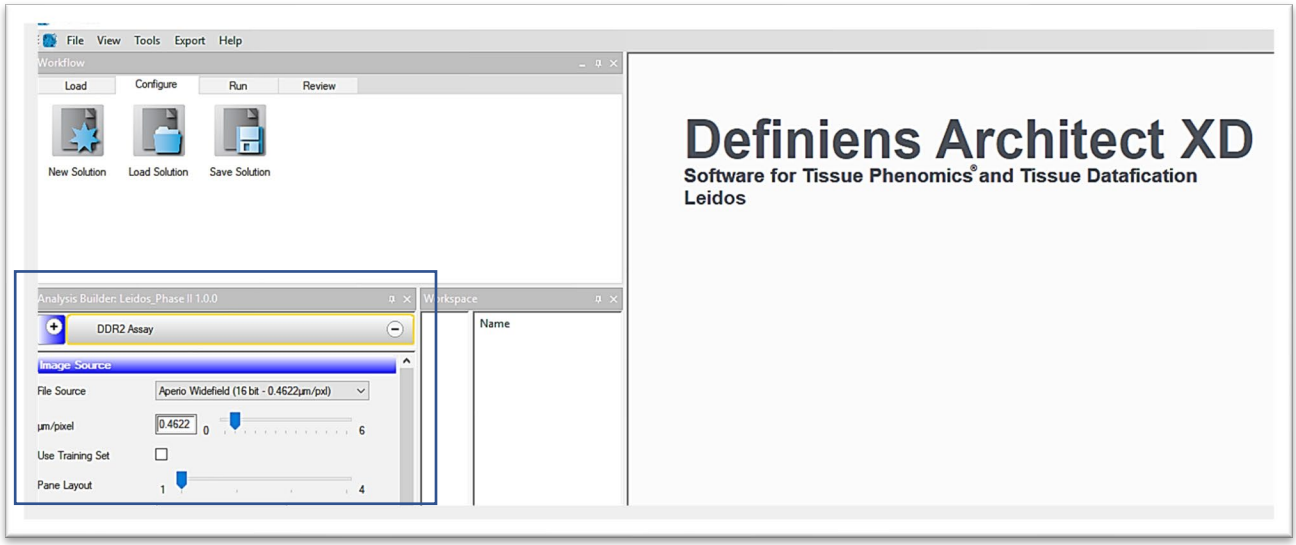
B



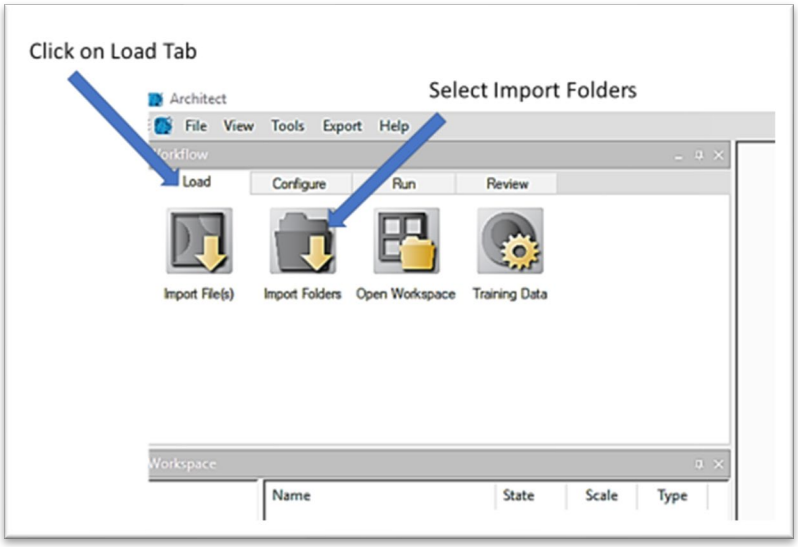
C



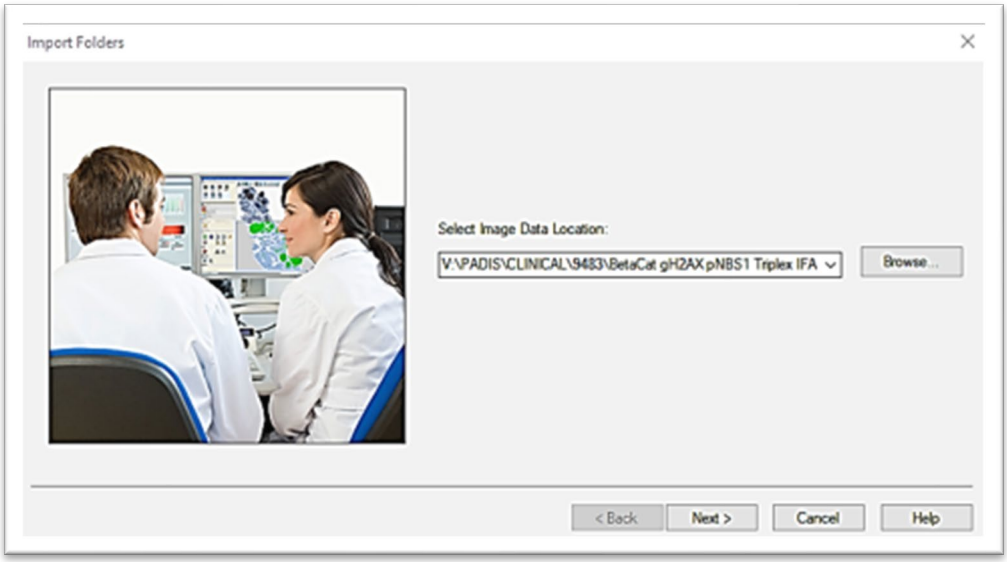
7.7.3 If the assay was correctly added, the *Analysis Builder: Leidos_Phase II 1.0.0* -box will appear toward the bottom left hand corner of the screen, as shown below.



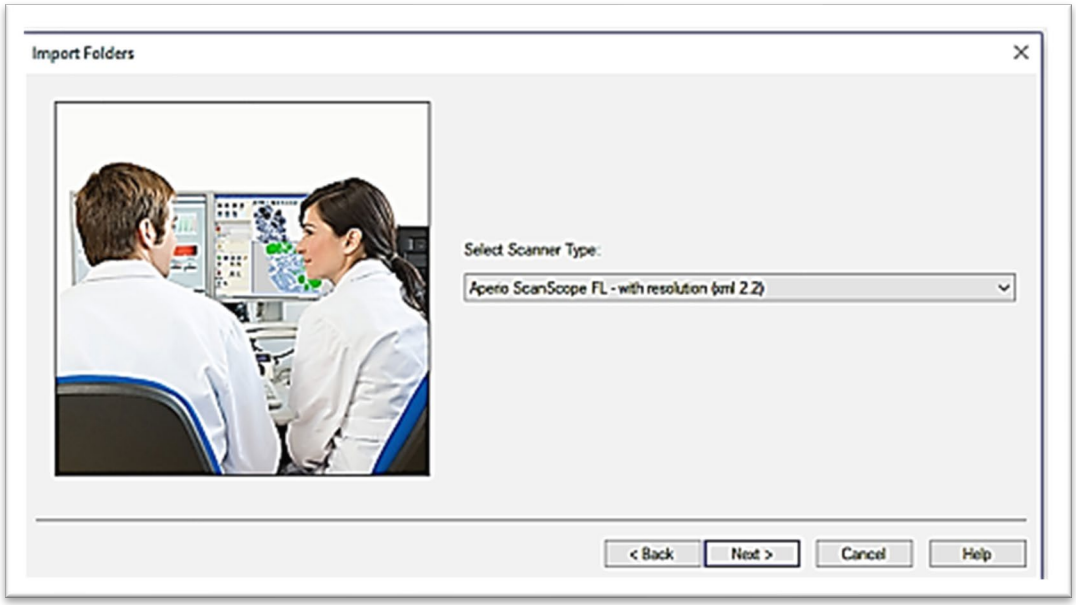
7.7.4 Click on the "Load" tab, and select "Import Folders", as shown below.



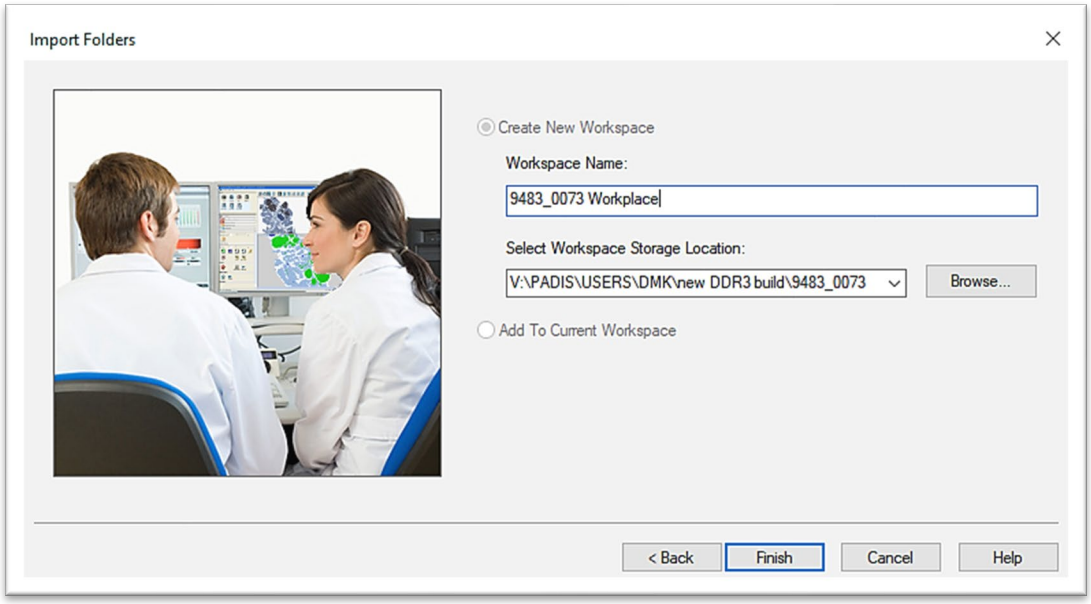
7.7.5 Under *Select Image Data Location*, browse and select the specific patient slide folder containing the SVS images to be analyzed from the Definiens drive (must be Definiens drive and NOT a local drive or the program will not analyze the data or generate a report) and then press "Next". A screen shot of the import image dialog box is provided below. **NOTE**: Each calibrator slide must be analyzed separately from patient specimens and each patient must be analyzed individually in a customized manner due to morphological differences. Therefore, load only images from one patient or from one calibrator.



7.7.6 Under *Select Scanner Type* choose "Aperio Scan Scope FL-with resolution" from the drop-down menu and press Next, as shown below.



7.7.7 Under *Workspace Name* type the workspace analysis folder name (as shown below). There should be separate workspaces for clinical slides and for calibrator slides. For clinical tissues, the workspace should be named CTEP#_Patient ID# Workspace (e.g., 7977_1010012 Workspace). For calibrator tissues, the workspace should be named CTEP#_patient ID *space* Calibrator *space* Marker name Workplace (e.g., 7977_1010012 Calibrator gH2AX Workplace). Under *Select Workspace Storage Location*, browse and select the same header folder on Definiens network location where the SVS images are stored, then press Finish.

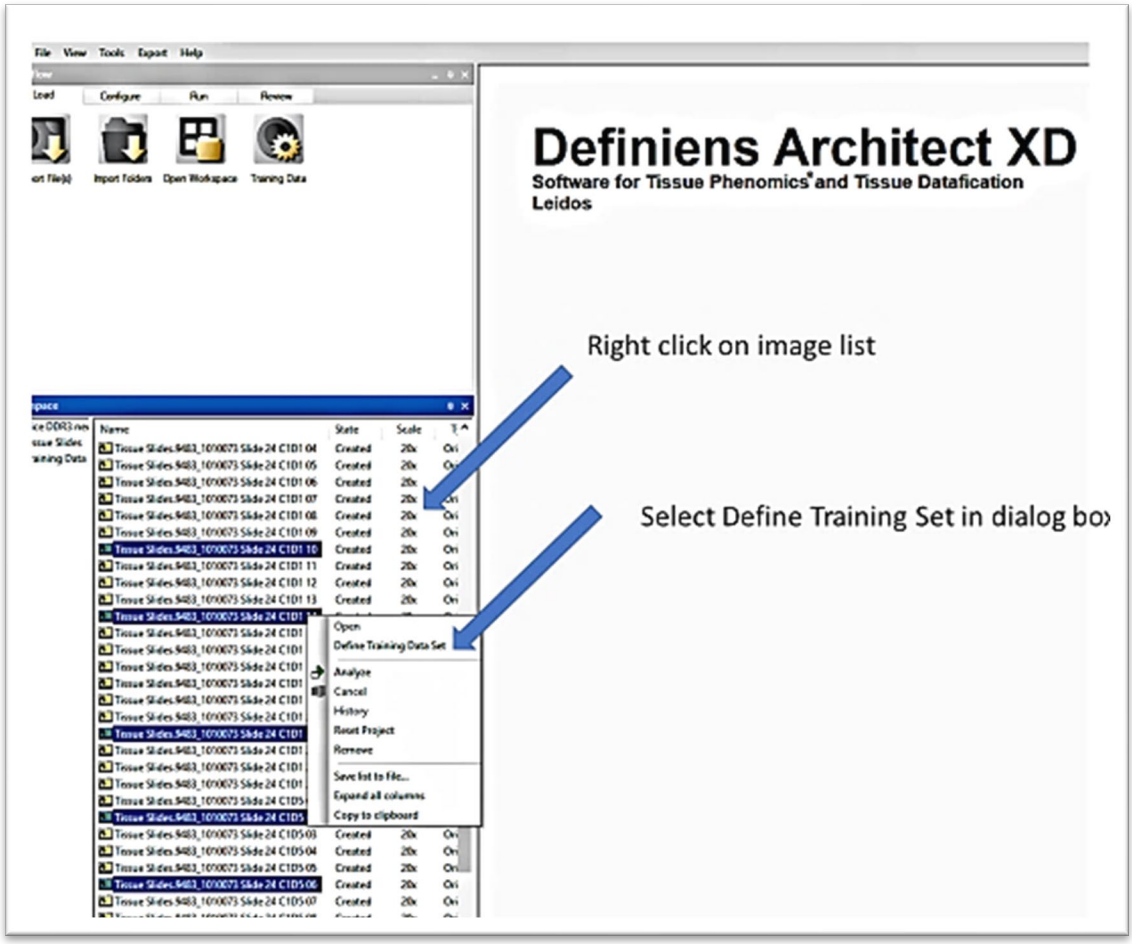


7.7.8 All image files in the Definiens folder should be downloaded into the Definiens Workspace.

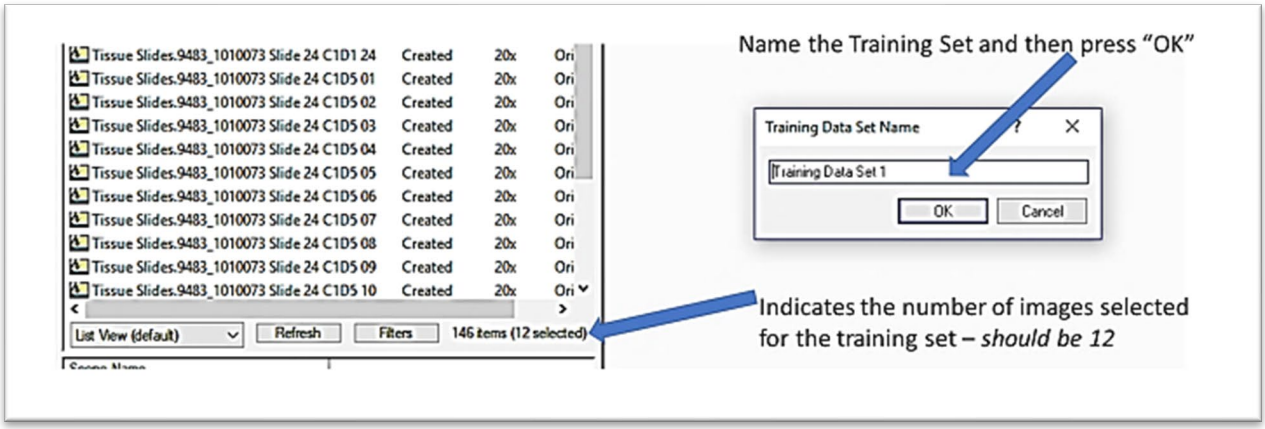
7.7.9 Define a Training Set from the loaded image list in the Workspace.

The training set will be used to teach the Definiens software to segment and threshold all images. A maximum of 12 images can be selected for the training set. To select multiple images, hold the control key on the keyboard and, using the mouse, click on the images from the image list that are to be included in the training set. Make sure to include images from all timepoints. Right click on the list of images, and a dialog box will appear (as shown below in A). Select "Define Training Set", and another dialog box will appear asking for the Training Data Set name (B). Enter the name of the training set and click "OK". This will load the Training Image Set. ***Using the maximum number of images (12) for training is important to get the best results for tissue segmentation and marker thresholding.***

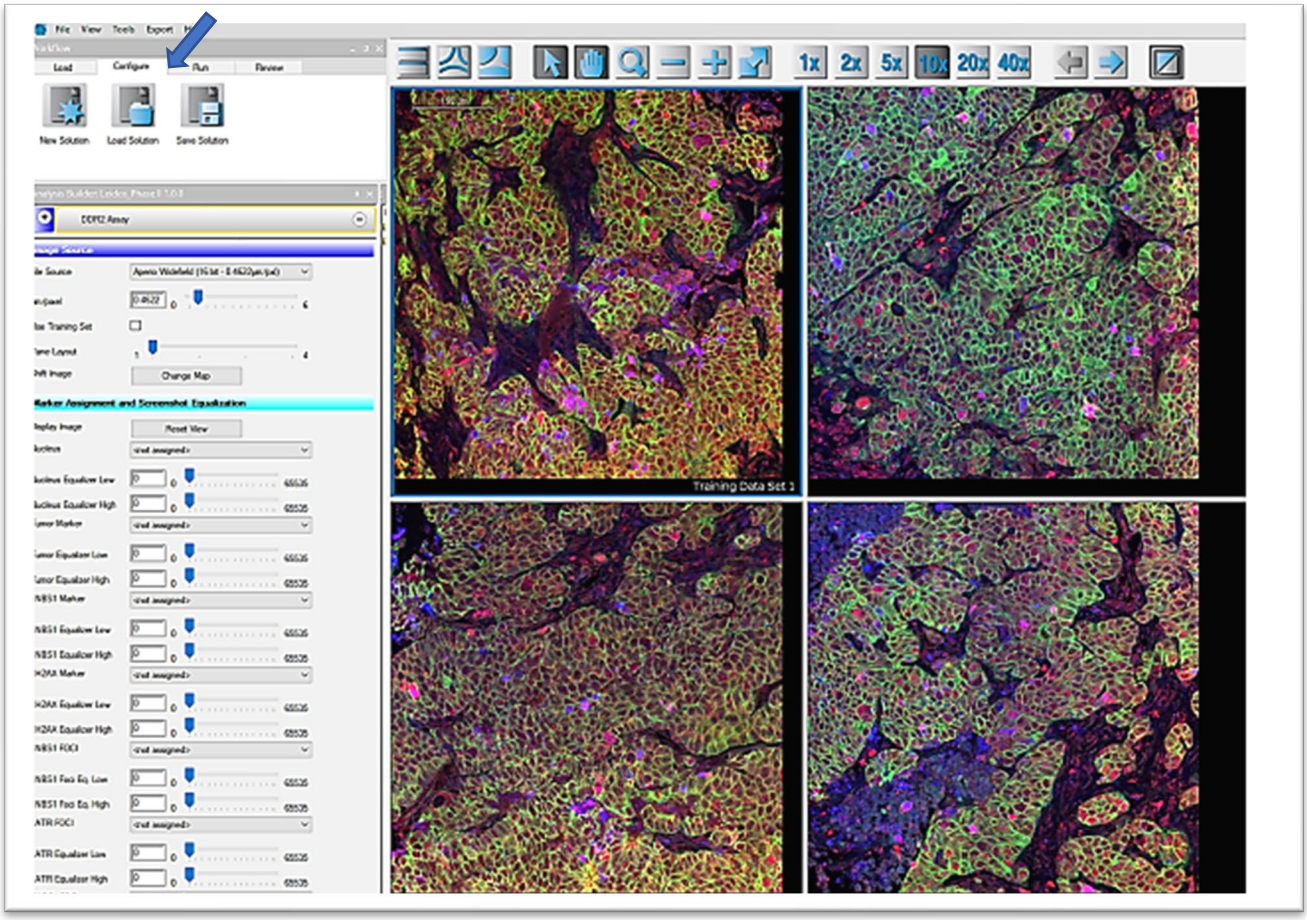
A



B



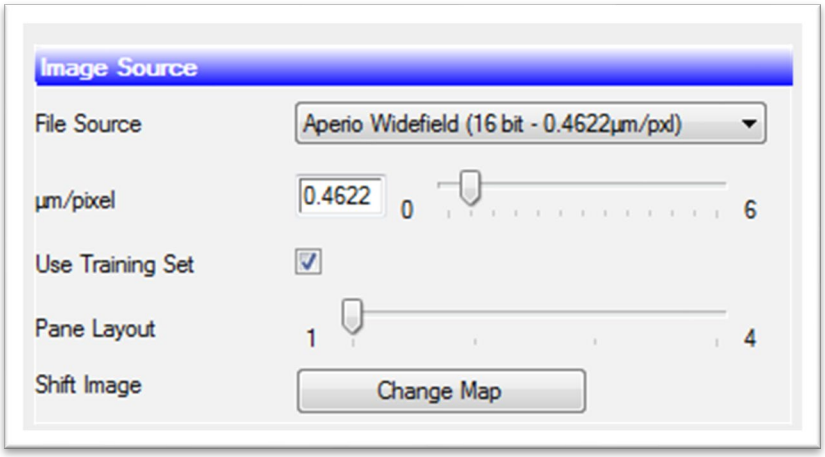
7.7.10 When the images have loaded, click on the Configure tab as shown below.



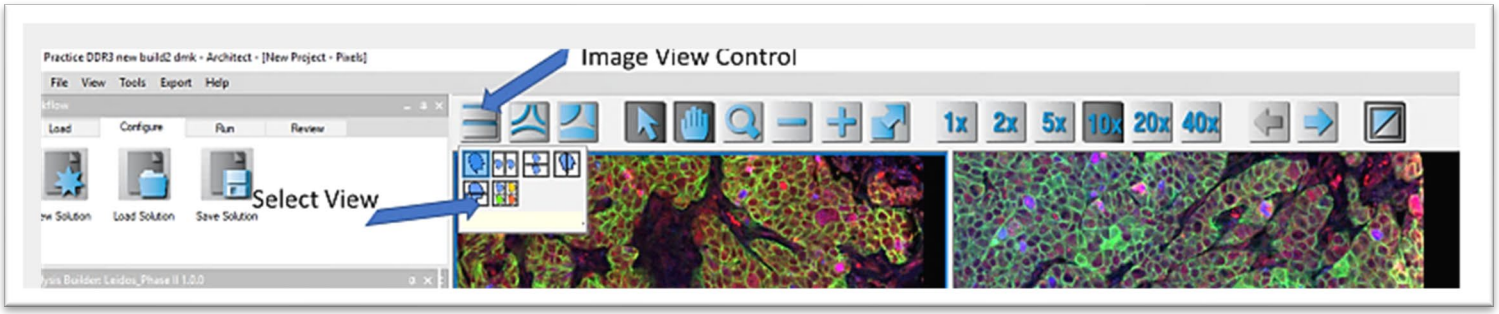
7.7.11 Set the *Image Source* settings, as shown below in A. Select the File Source as Aperio Widefield (16 bit - 0.4622um/pxl). Enter the correct resolution µm/pixel if different than 0.4622. **Check box next to "Use Training Set".**

7.7.11.1 The Pane Layout can be set between 1- 4. However, if you select to view more than one pane, adjusting the zoom will result in only one image being zoomed. If you wish to view more than one pane, you can do this by clicking *Image View Control* above the image view (as shown below, B) and selecting a multi map view.

A



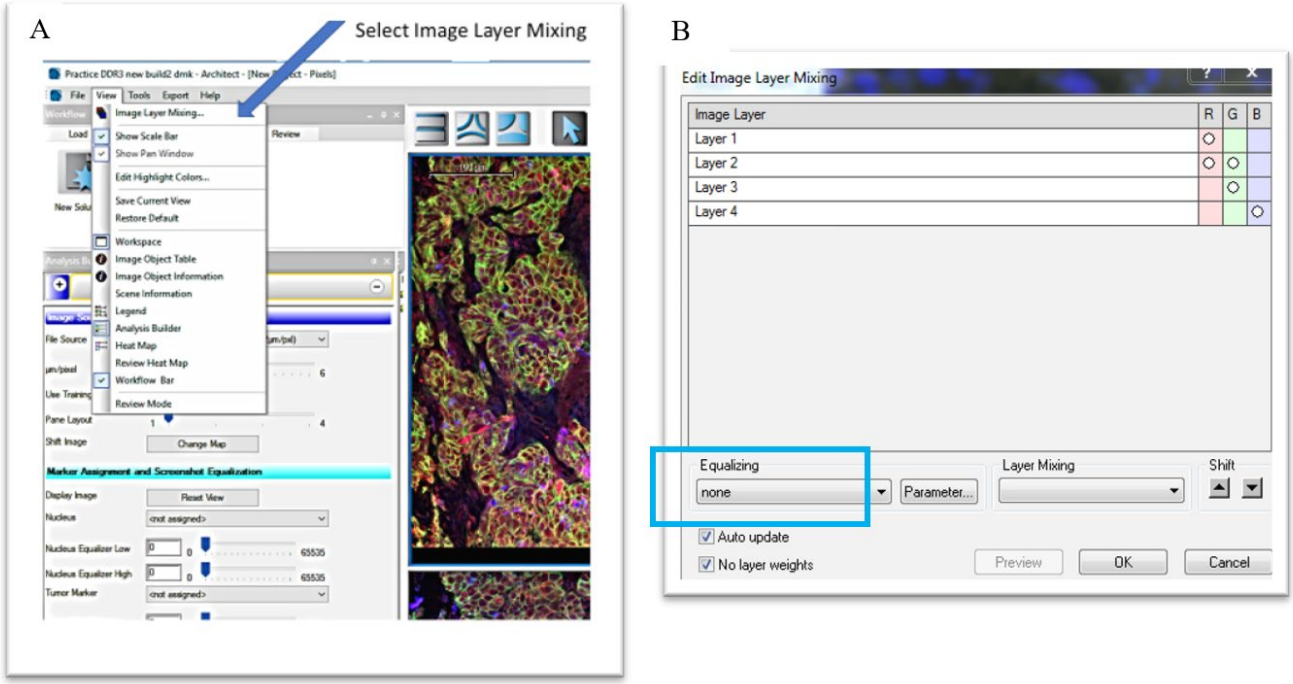
B



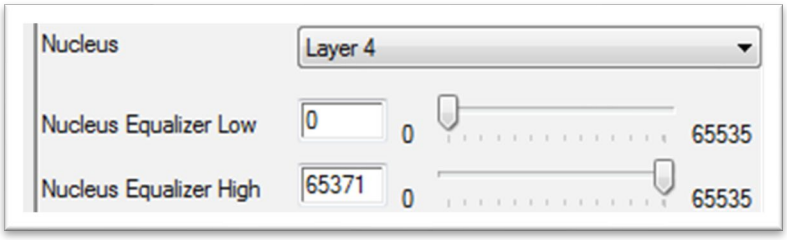
7.7.12 Adjust the image layer mixing to assign colors to markers.

Go to View, and select "Image Layer Mixing" as shown below (A). *Edit Image Layer Mixing* pop-up box will appear (as shown below, B). Select "none" from the *Equalizing* drop-down menu. Assign correct colors to each marker: Layer 1 (pNBS1) – red; Layer 2 (B-Cat) – red and green; Layer 3 (gH2AX) – green; and Layer 4 (DAPI) – blue. To select the color, click on the color box, and a circle will appear when the color is selected. Click "OK" to accept changes.

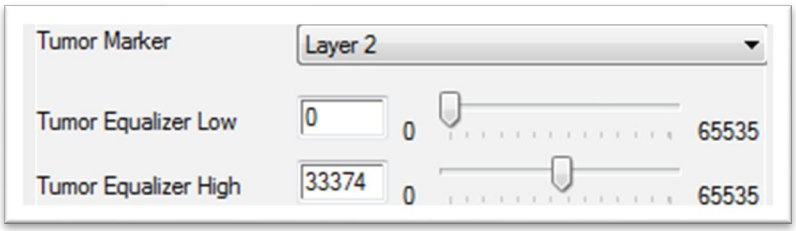
7.7.13 Set the *Marker Assignment and Screenshot Equalization* settings.



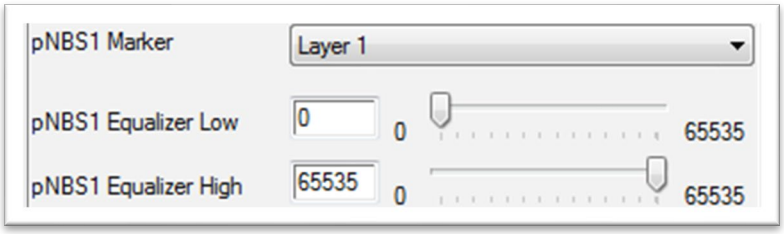
7.7.13.1 Assign **Layer 4** to Nucleus and adjust the Nucleus Equalizer Low and High, as depicted below. The intensity range should be 0-65535. If the nuclear signal appears weak, a shorter range may be needed.



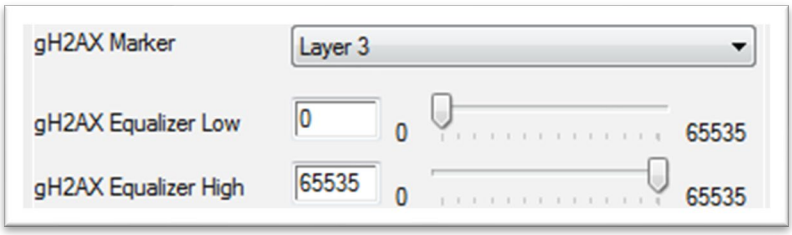
7.7.13.2 Assign **Layer 2** to Tumor Marker and adjust the Tumor Equalizer Low and High to best visualize the β-Catenin signal, as depicted below. If the tumor signal appears weak, a shorter range may be needed.



7.7.13.3 Assign **Layer 1** to pNbs1, as depicted below. Adjust equalizer to the full intensity range (low=0; high=65535).

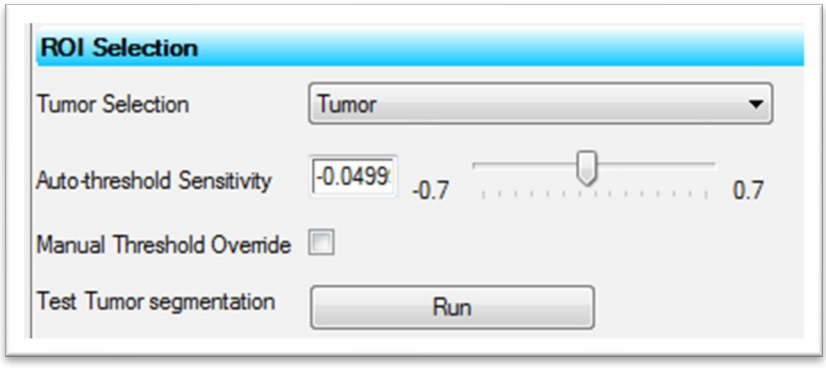


7.7.13.4 Assign **Layer 3** to gH2AX, as depicted below. Adjust equalizer to full intensity range (low=0; high=65535).

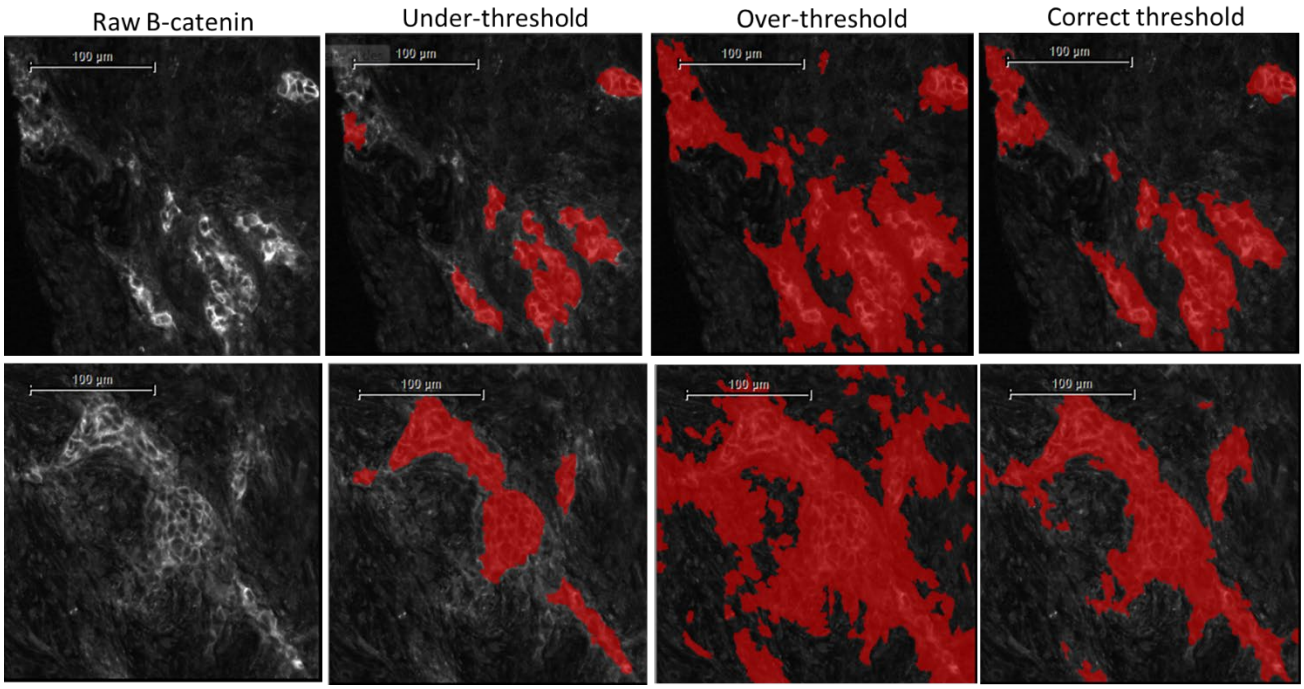


7.7.14 Set thresholds at *ROI Selection*

7.7.14.1 Select "Tumor" from *Tumor selection* drop down menu, as depicted below. The images will change to the β-Catenin images automatically. Adjust the *Auto-threshold Sensitivity* -slider until the β-Catenin signal is masked. This will take some time for the first selection, but additional adjustments are faster. It is not necessary to click Run, as the mask will automatically update when you adjust the auto-threshold sensitivity. The larger the number the more sensitive the thresholding.

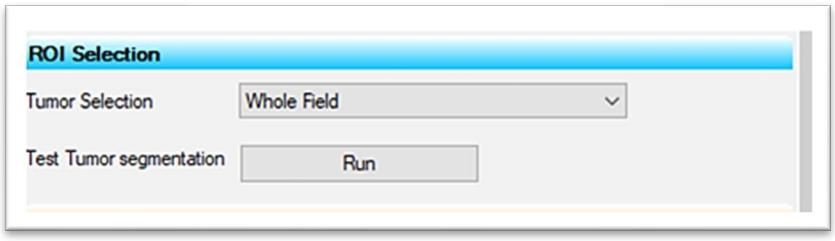


7.7.14.2 Examples of under-thresholded, over-thresholded and correctly thresholded image masks for β-Catenin are shown below.



7.7.14.3 Evaluation of calibrator tissue and clinical tissues that <u>do not</u> require β-Catenin segmentation

Calibrator slides should be analyzed separately from clinical slides. The **calibrator tissues** within the biomarker control slides have little β-Catenin positivity and do not require tissue segmentation. For segmentation of calibrator tissue, the user can select Whole Field from the drop-down in the ROI Selection (see below) or raise the auto-threshold sensitivity until the whole tissue is masked.

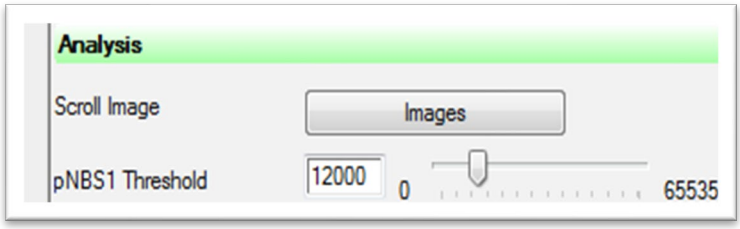


7.7.15 *Nuclear Segmentation* tab

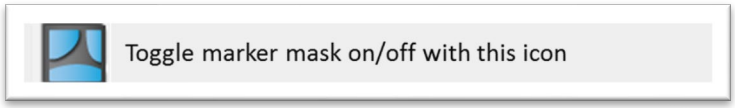
Nuclear segmentation is automated so there are no adjustments to be made. You can click on Run to view the nuclear segmentation, but it is not necessary.

7.7.16 *Analysis* Tab: pNBS1

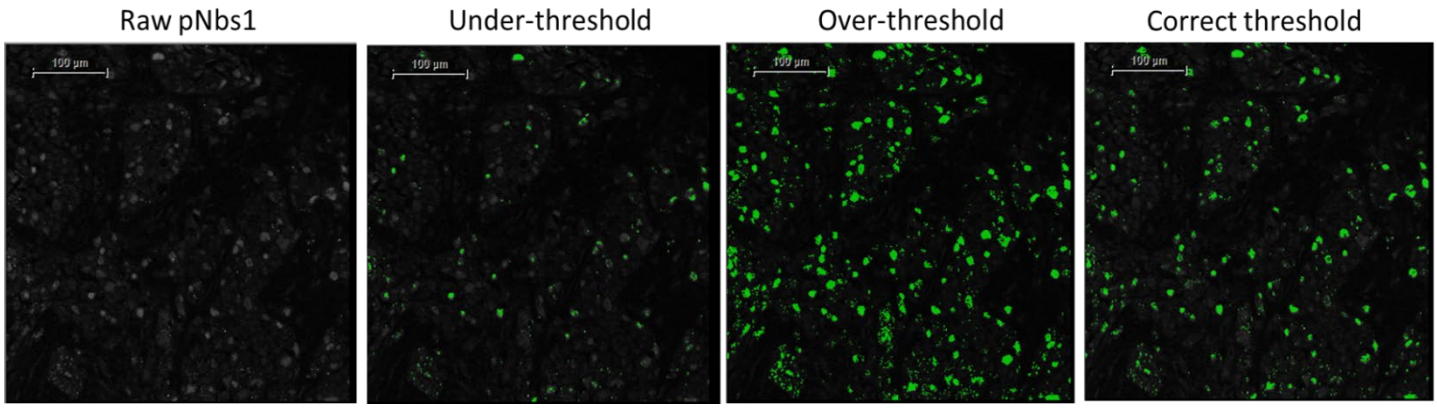
Adjust the pNbs1 threshold, as shown below. Values can be typed in or the slider can be used. The pNbs1 image will open when you adjust the threshold.



The marker mask can be toggled on and off by clicking the show/hide classification of objects icon, as shown below.

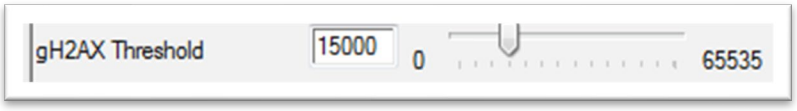


Examples of under-thresholded, over-thresholded and correctly thresholded image masks for pNBS1 are shown below.

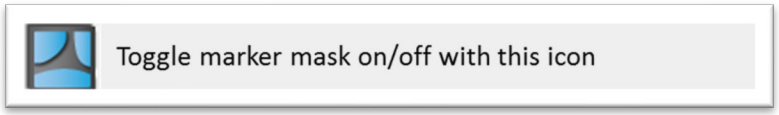


7.7.17 *Analysis* Tab: γH2AX

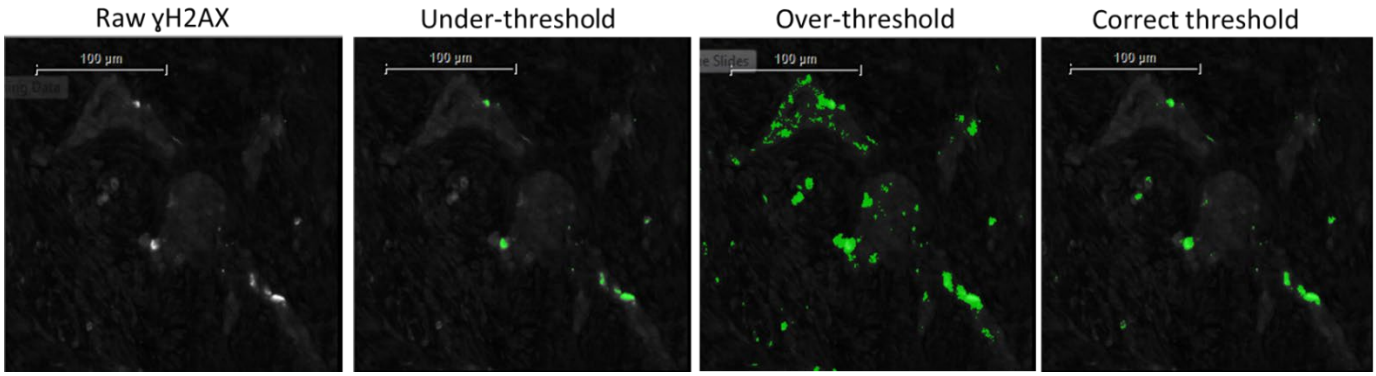
Adjust the γH2AX threshold, as shown below. Values can be typed in or the slider can be used. You **do not** need to check the "Apply foci size" for these markers. The larger the value the less sensitive the thresholding.



The marker mask can be toggled on and off by clicking the show/hide classification of objects icon, as shown below.

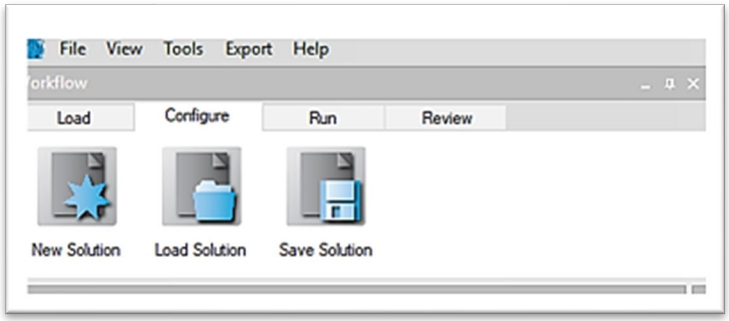


Examples of under-thresholded, over-thresholded and correctly thresholded image masks for γH2AX are shown below.



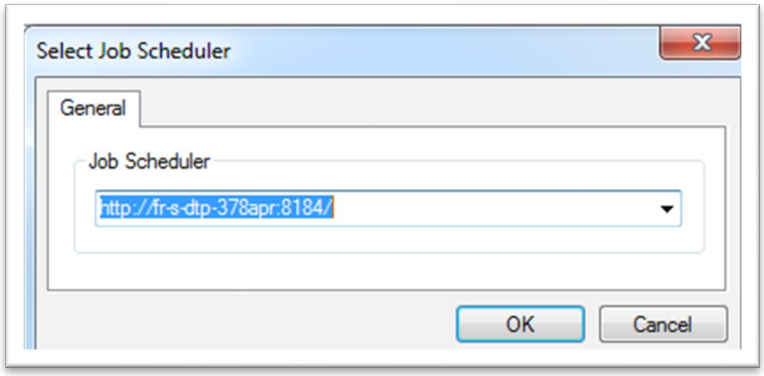
7.7.18 *Save Solution*

Go to the *Configure* tab and click "Save Solution". Save solution to the folder containing the current workspace.

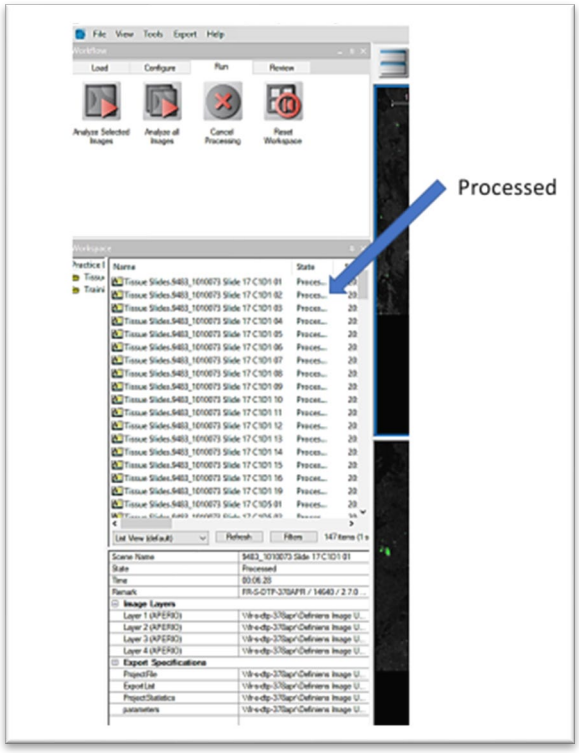


7.8 Data analysis

7.8.1 Assign Job Scheduler. Go to File>Configure Analysis. Within NCTVL at FNLCR, select **http://fr-s-dtp-378apr:8184/** from the drop-down and click OK, as shown below.

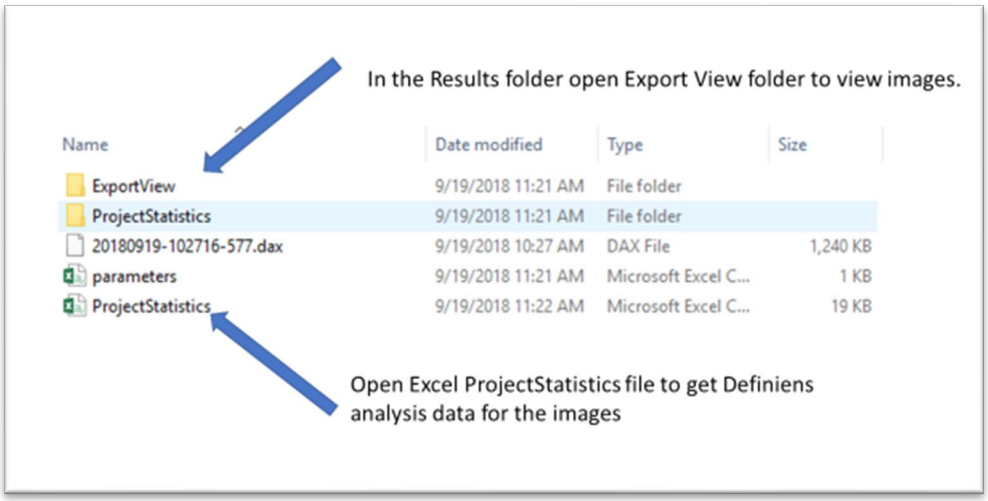


7.8.2 Go to *Run* tab and click on "Analyze all Images". When the status of the last file changes from "Processing" to "Processed" the quantitation is complete.

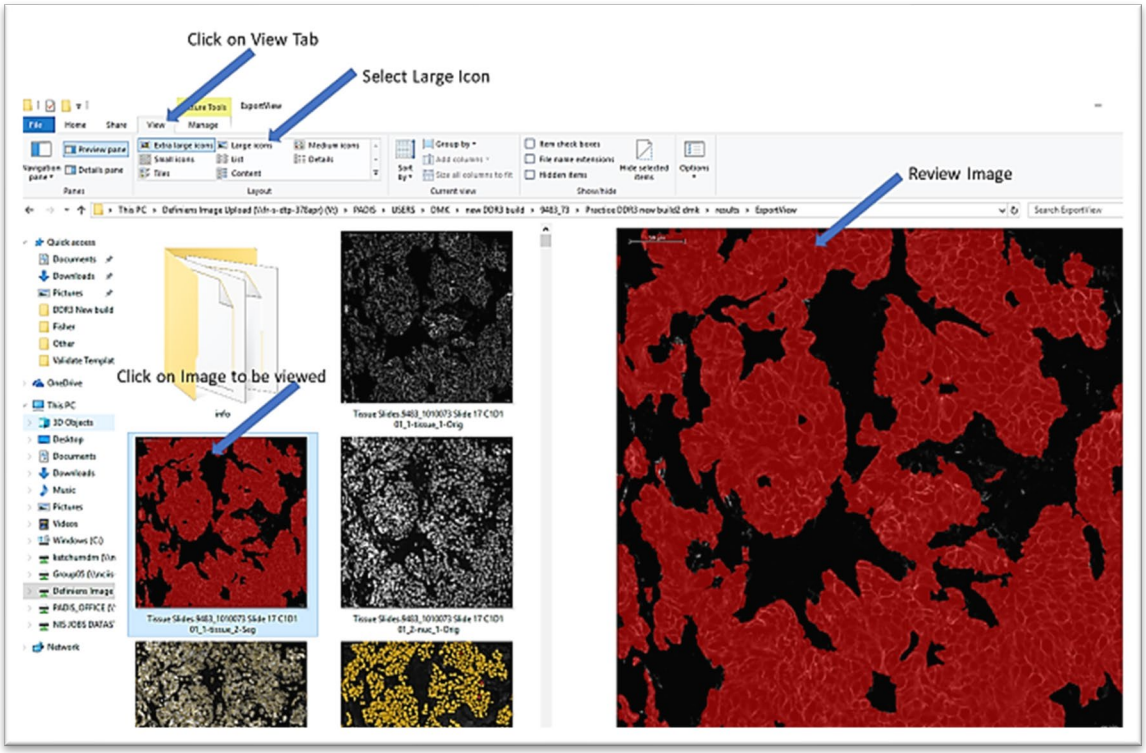


7.9 View Data Analysis and Image Screenshots

7.9.1 The Definiens images are located in the header folder in the Workplace subfolder. In the Workplace subfolder open the results folder (as shown below), and there open the Excel "Project Statistics" file. Nuclear counts are located in Column D, %NAP pNbs1 in column F and %NAP gH2AX in column H.



7.9.2 To view the image screenshots, open ExportView (see above), select View tab as shown below, and select to view icons as “Extra Large”.



7.9.3 For QC purposes, all the analysis parameters are saved in the results folder named “parameters”.

7.9.4 *Note: As discussed in more detail in Section 8.2.2, all screenshots must be inspected, including comparison of masked areas to gray-scale images, to determine that thresholding and segmentation were correctly applied. If it is apparent that the marker mask is detecting inappropriate signal (i.e. collagen, autofluorescence, tissue folds, debris or other factors that interfere with nuclear detection) individual fields can be removed from analysis. However, no more than 20% of the images can be removed from an analysis. If more than 20% of the images have an issue, the training set may not have been representative of all the images and the analysis will have to be rerun.*

8.0 QUALITY CONTROL, DATA ANALYSIS AND REPORTING

8.1 Biomarker Specific Control Slide QC and Data Analysis

8.1.1 Variability in sample processing and staining is monitored by employing biomarker specific control slides. At least one γH2AX and one pNBS1 biomarker control slide are included in each staining run and should be evaluated to confirm that acceptable levels of the Cal1 (Cal Low), Cal2 (Cal Mid) and Cal3 (Cal High) for both biomarkers fall into the range acceptable as determined for each lot of calibrator slides. A slide layout for each biomarker specific control slide is shown in Appendix 2.

8.1.1.1 Additional controls included on the control slides are evaluated qualitatively. The current γH2AX and pNBS1 control slides include a section of murine testis and jejunum.

8.1.1.2 The jejunum should be very low or negative for both γH2AX or pNBS1. If minimal staining for γH2AX is observed, it should be limited to the crypt cells.

8.1.1.3 The mouse testis should be negative for pNBS1 in the nuclei throughout. Some non-nuclear staining may be observed in the center of a small subset of tubules. The spermatogonia, spermatocytes, and spermatids should be intensely stained for γH2AX.

8.1.2 Open the Clinical Specimen Analysis Excel Template for the calibrators. The template is located on the Definiens drive at: \PADIS\Solutions\SOP340545 gHAX_pNBS1_BCat_New Build Master Template. The template will be used to analyze the clinical and calibrator data.

8.1.3 In the Excel spreadsheet generated by Definiens in the Calibrator Workplace subfolder open in the results folder > open the Excel "Project Statistics" file. Nuclear counts are located in Column D, %NAP pNbs1 in column F and %NAP gH2AX in column H. Copy the spreadsheet and paste into the appropriate Cal "Marker" raw sheet in the Master Template.

8.1.4 The acceptable levels for the calibrator slides will be determined by lot. At least one of each Cal1, Cal2 and Cal3 on each of the two calibrator slides should pass QC to proceed to the clinical analysis.

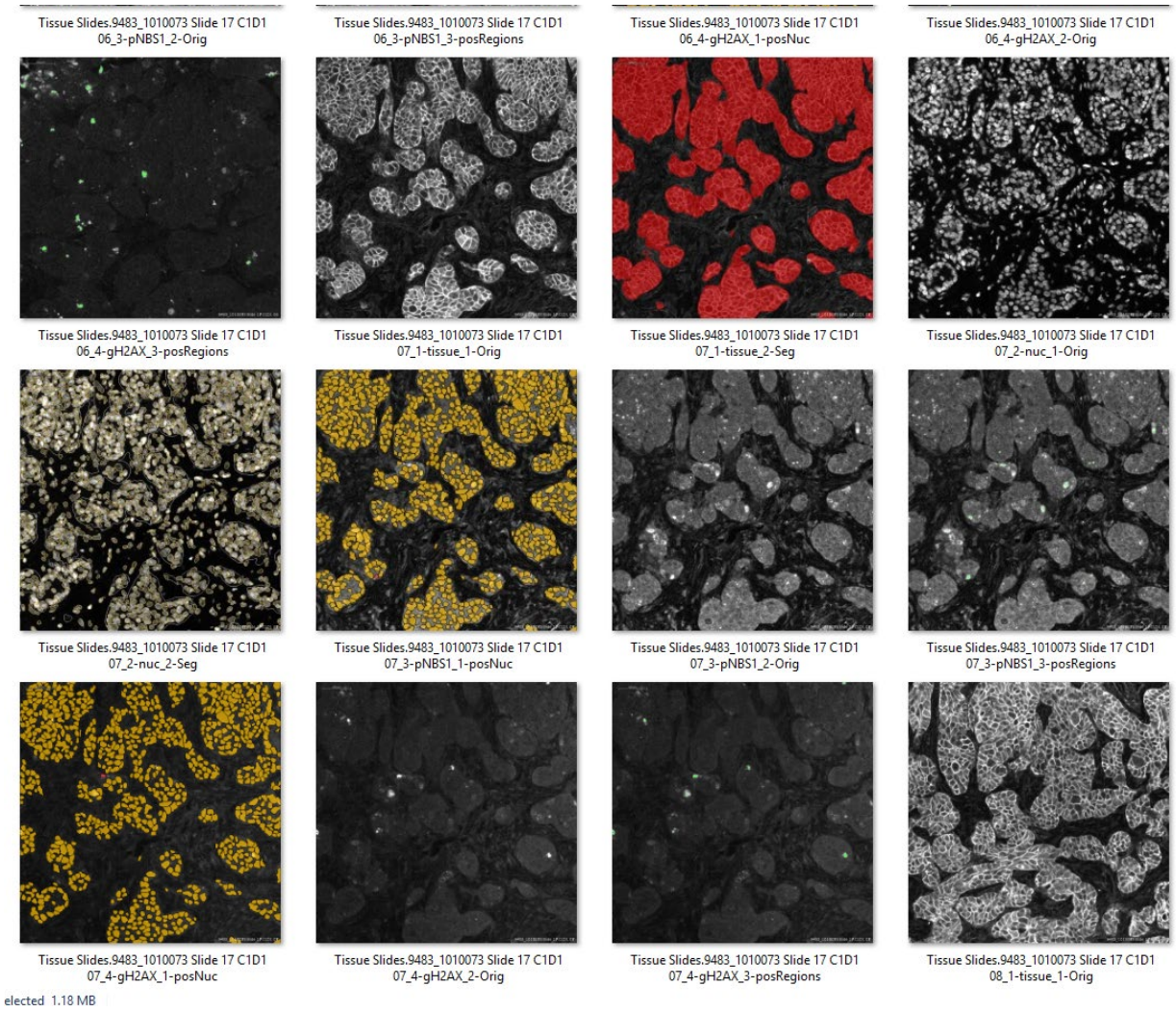
8.1.5 In some cases, there may be an isolated calibrator slide failure that is slide specific. If all controls pass on the remaining slides, and the failure to achieve acceptable %NAP on the control slide is thought to be a slide failure and not an assay failure, the Laboratory Director/Supervisor may allow exceptions to QC. In this event, an additional control slide should be run with all the same critical reagents within 7 days to confirm that the failure was isolated to that individual slide.

8.2 Clinical Slide QC and Data Analysis

8.2.1 As described in SOP340550, murine testes and jejunum tissues are fixed and processed in parallel with the clinical biopsies. The testes and jejunum controls on these slides serve as controls for both proper tissue fixation and staining of γH2AX and pNBS1 and are assessed qualitatively as described above in Section 8.1.1. If these criteria do not pass for staining of γH2AX and pNBS1, the clinical slide should not be evaluated, and the potential source of the error should be evaluated.

8.2.2 QC of Screenshots

Images for Quality Control evaluation of screenshots of clinical tissue ROI can be viewed in the Export View folder in the Results folder. Images should be displayed as extra large icons. See images below.



8.2.3 Visually inspect every screenshot and compare masked areas to gray-scale images to determine that thresholding and segmentation were correctly applied for each image. The following criteria should be met: 1) the β-Catenin segmentation should accurately reflect that only β-Catenin positively stained tumor tissue is included in the analysis, and 2) the marker threshold should accurately capture marker positive cells. If tissue segmentation is not correct due to the marker threshold being too high or low, , or if more than 20% of the images would need to be removed from the final analysis the training set may not have been representative of all the images and the analysis may need to be run again. For the second training set select images with suboptimal thresholding. If it is apparent that the marker mask is detecting signal other than tumor cell specific expression (i.e., collagen, autofluorescence, tissue folds, any features that interfere with nuclear detection) ***those fields should be noted for removal from analysis.***

8.2.4 Note Shortcut: *If after reviewing the screenshots it is determined 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 existing training set*

or create new training set. Go back to the configure tab and work down through the script again.

8.2.5 In order to report data for each individual clinical biopsy pass, the sum of cells from two non-adjacent slides must be ≥3000 cells. Although data can be reported from more than two slides, it is required that each tissue reaches this cell number across two non-adjacent slides in order to ensure that data is reported from tissues of adequate quality.

8.2.6 How to use the SOP340545 γH2AX_pNBS1_βCat New Build Master template

1. Open SOP340545 γH2AX_ pNBS1_ βCat New Build Master template and save it as SOP340545 gH2AX_pNBS1_BCat New Build Analysis CTEP#_Patient#, i.e. "SOP340545 gH2AX_pNBS1_BCat New Build Analysis 7977_1010056" in the header folder.
2. Copy data from the Definiens Architect DDR Build for the Specimens. This is located in the header folder > CTEP Workplace folder > results folder > Project Statistics Excel file.
3. Paste the copied Definiens Architect DDR data into the Excel Template on the "**Definiens Data**" Sheet.
4. On the "Definiens Data" sheet, images that need to be removed from the final analysis due to tissue, staining or other problems, should be highlighted (bright yellow). All images should be reviewed before running the template macro. Images are located in Header folder > CTEP Workplace folder > results folder > ExportView folder. If more than a few images are removed, a comment should be made right below the pasted data why the images were removed from the analysis. This page is not part of the macro analysis but serves as a means verify and to audit data. *(Suggestion: when multiple images must be removed, paste a screen shot of an example image.*)
5. Copy the data from the Definiens Data Sheet including the column headers and paste it into "Raw Data 1" sheet. *Don't copy the comments made on the Definiens Data sheet.*
6. In "Raw Data 1" sheet remove all highlighted rows and make sure the images were named correctly before using the Macro. *Most macro failures are due to image naming errors.*
7. Copy the data from the Definiens Architect DDR Build Data Analysis for each calibrator (gH2AX & pNBS1). These are located in the header folder > Marker Workplace folder > results folder > Project Statistics Excel file.
8. Paste the appropriate data into the Excel template sheets "Cal gH2AX raw" or "Cal pNBS1 raw". Make sure the images were correctly named.
9. Go to the Developer tab and select the Macro "DDR3_NEWBuild". When the macro has completed, it will go to the "Lookup" sheet.
10. Go to the "Cal Analysis & Summary" sheet and type the required information into the "Enter Information" boxes for each Marker Calibrator. In the appropriate boxes put the calibrators' **limits**, **exposure time**, and **threshold** information (the adjacent table will indicate if the calibrator slides passed QC for each calibrator level). At the top of the page, in the yellow box, put the **date** the slides were imaged.
11. Check all pages for errors. For example, the "Lookup" sheet has totals. Compare the totals to the number of raw data images analyzed. Make sure that on the "Template Analysis" and "Cal Analysis & Summary" sheets the data is present in all cells that should have it contain data.

12. Check the "DDR3 Summary" sheet to make sure that the requirement of 3000 cells for 2 slides per pass is met. The table "2 Slide Max Nuclei Value/Pass" should say "Pass" for each biopsy pass. If there is a "fail" for a biopsy pass, the data from the pass cannot be used in the final data report. Make a comment on the "Definiens Data" sheet why the data will be removed and highlight the data selected for removal. Copy the Definiens data and paste it into the "Raw Data 1" sheet. Remove all highlighted data. Run the macro and review the data.
13. On the "Data Report" sheet, type in the name of the operator and the date of the report. Indicate if the calibrator slides passed QC (Pass or Fail). Review the data report of accuracy and completeness. If needed, make comments in the Additional information section.
14. The Data Report must be finalized. The supervisor needs to sign the data report, which indicates that the supervisor reviewed the report and found it to be complete and accurate.
15. **Image Naming Requirements for Specimens (NOT CALIBRATORS):**
    - Project# (5 digits)_Patient#(4 digits) *space* tissue ID (4 digits) *space* Slide *space* Slide# (3 digits *space* timepoint* (4 digits) *space* Image ID (2 digits)

      For example, if the project is 7977, patient is 39, timepoint is pre, slide is 5, image is 8, and the specimen ID is 500a, the image should be named: 07977_0039 500a Slide 005 C1D0 08
    - *Timepoints must be indicated with 4 characters. For example:
      - Pre-dose/Screening of the first cycle = C1D0
      - 2 hr post dose of the first cycle = D1H2
      - 4 hr post dose of the first cycle = D1H4
      - Day 1 post dose of the first cycle = C1D1
      - Day 8 post dose of the first cycle = C1D8

16. **Image Naming Requirements for Calibrator tissue (2 choices):**

    A. Aperio Image ID (5-6 digits) *double space (*if 5 digit Aperio image ID is used) Lot# (8 digits) *space* Slide (slide) Slide# (2 digits) *space* Marker (5 digits) *space* Image # (2 digits).

    Cal1 = Cal Low
    Cal2 = Cal Mid
    Cal3 = Cal High

    spaces ↓↓ ↓ ↓↓ ↓ ↓

    example: 55553 14014320 Slide 03 gH2AX Cal2 04

    B. Date of Staining (6 digits – yy/mm/dd) *space* Lot# (8 digits) *space* Slide *space* Slide# (2 digits) *space* Marker (5 digits) *space* Cal# *space* Image # (2 digits)

    spaces ↓ ↓ ↓↓ ↓ ↓

example: 180831 14014320 Slide 03 gH2AX Cal2 04

17. Other information:

    A. Complete name by Definiens: Tissue Slides.180831 14014320 Slide 03 pNBS1 Cal3 01

    B. If the slide number for the calibrator is 3 digits instead of 2 digits, do not put a space after the word Slide (for example, slide number 105 becomes Slide105).

    C. Definiens DDR3 New Build Analysis program will add the words "Tissue Slide." before the name that was given to the extracted image. (Tissue Slide. MUST appear in front of the name for the Macro to work correctly.)

8.2.7 In the Batch Record enter the name and location of the header folder (Appendix 1, Section 1). All associated files should be located under the header folder CTEP number, and patient ID. The related calibrator files can be copied and placed in the header folder.

8.2.8 Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 1, Section 2.)

8.2.9 Complete, review and finalize the Batch Record (Appendix 1) and obtain required signatures (Appendix 1, Section 3).

8.2.10 The Data Report in the Excel Master Template should be completed by the operator including entering the operator's name, date of the report, indicate if the calibrator slides passed/failed QC. The report should be reviewed by the operator for accuracy and completeness. The supervisor needs to sign and date at the bottom of the Data Report (Appendix 3, Appendix 4). The supervisor indicates by signing the report that it the data report has been reviewed and was found to be accurate.

APPENDIX 1: BATCH RECORD

A. Image Extraction and Analysis

Facility/Lab Name:		Date:	
Assay Operator:			
CTEP #:		Patient ID:	
Slide #'s:		Calibrator Lot #s:	
Name of Header Folder:			
Location of Header Folder:			

	γH2AX (Calibrator)	pNBS1 (Calibrator)	γH2AX (Patient)	pNBS1 (Patient)
Thresholds:				

B. Comments:

C. Laboratory Director/Supervisor Review of Batch Record

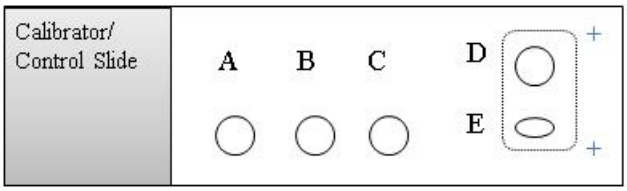
Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

APPENDIX 2: CALIBRATOR/CONTROL SLIDES AND CLINICAL SLIDES

A. Calibrator/Control Slides

1. Biomarker specific control slides are required for each clinical Bond-RX run. The layouts of the biomarker specific control slides used for this SOP are shown below.

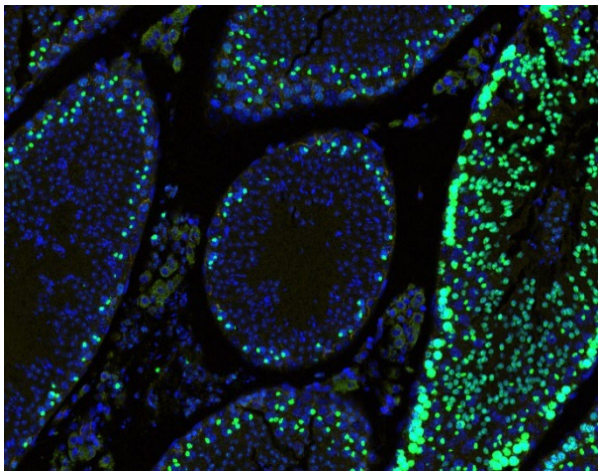


Sections & Tissue – Biomarker Control slide	
Cal-Low (A)	Xenograft (Vehicle-treated)
Cal-Mid (B)	Xenograft (Treatment A)
Cal-High (C)	Xenograft (Treatment B)
Tissue Control (D)	Mouse testes
Tissue Control (E)	Mouse jejunum

2. Controls included on the control slides are evaluated qualitatively.

   a) The jejunum should be very low or negative for γH2AX and pNBS1.

   b) The mouse testis should be negative for pNBS1 in the nuclei throughout. Some non-nuclear staining may be observed in the center of a small subset of tubules. The spermatogonia, spermatocytes, and spermatids should be intensely stained for γH2AX. An image of the testis control showing intense staining for γH2AX is shown below.



3. γH2AX & pNBS1 Calibrator Section Criteria

a) Calibrator sections are generated from NBF-fixed, paraffin-embedded mouse xenograft tumor quadrants derived from vehicle and drug treated animals representing three different levels of biomarker expression (Calibrators [Cal]: (1) Low, (2) Mid, and (3) High). The calibrators serve as a visual reference standard for drug-effect on target.

b) The specifications for acceptable biomarker levels in the calibrators are determined by control slide lot.

Calibrators Summary

Image Date: 11/29/2018

Tissue Slides.1MOCH2 11142018 Slide S0 gH2AX

Cal	1	2	3	4	5	6	7	8	9	10
	01	02	03	04	05	06	07	08	09	10
1	3	2	2	2	2	2				
2	1	1.5	0.1	2	2	3.8928558				
3	14.07568	13.08081	14.8457	14.21045	12.48007	8.3908417				

Tissue Slides.1Moch2 11142018 Slide B5 pNBS1

Cal	1	2	3	4	5	6	7	8	9	10
	01	02	03	04	05	06	07	08	09	10
1	0.39994	0.119638	0.148782	0.01	0.9	1				
2	4	5	5	6	7	2				
3	16.36455	22.08838	26.26723	22.90328	31.74066	25.573417				

Tissue Slides.1MOCH2 11142018 Slide S0 gH2AX

Cal	1	2	3	4	5	6	7	8	9	10
	01	02	03	04	05	06	07	08	09	10
1	1238	1251	1196	1207	1143	1203				
2	1260	1343	1264	1177	1043	1378				
3	1433	1385	1448	1546	1404	1295				

Tissue Slides.1Moch2 11142018 Slide B5 pNBS1

Cal	1	2	3	4	5	6	7	8	9	10
	01	02	03	04	05	06	07	08	09	10
1	1193	1067	1135	1087	1093	1177				
2	1073	1104	1133	987	853	1059				
3	1081	1081	1096	1073	1120	1094				

γH2AX

	Avg %NAP	SD	Pass/Fail	%NAP Limits		Total # Nuclei
Low	2.166667	0.408248	Fail	<	1.0	7238
Mid	1.748809	1.269936	Fail	Low> <High	NA	7465
High	12.84726	2.341372	Pass	≥	10.0	8511

	Enter Information	Threshold
γH2AX Lot #	11142018	6500
Limits		Exposure
Low	1	250
Mid	NA	
High	10	

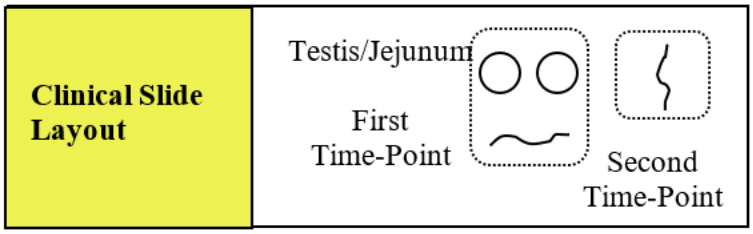
pNBS1

	Avg %NAP	SD	Pass/Fail	%NAP Limits		Total # Nuclei
Low	0.429727	0.423913	Pass	<	1.0	6752
Mid	4.833333	1.722401	Pass	Low> <High	NA	6209
High	24.15625	5.109686	Pass	≥	10.0	6545

	Enter Information	Threshold
pNBS1 Lot #	11142018	7000
Limits		Exposure
Low	1	1600
Mid	NA	
High	10	

B. Clinical Slides

1. Clinical samples for this assay will be frozen needle biopsies collected according to SOP340507, embedded and sectioned according to SOP340550, and stained for γH2AX, pNBS1, and B-Cat according to SOP340543. A representative clinical slide layout is shown below. In some cases, there maybe two passes embedded for each time point (see this layout in SOP340550).



2. Two or three slides are pre-stained with H&E and are used to evaluate the tissue quality prior to γH2AX, pNBS1, and B-Cat staining.
3. Typically, slides submitted for IFA staining represent every third or, in smaller slides sets, every second section from the range of slides determined to be optimal based on visual inspection and H&E evaluation.
4. Backup slides are available for use if the first slide set does not meet QC criteria for biomarker staining and analysis.

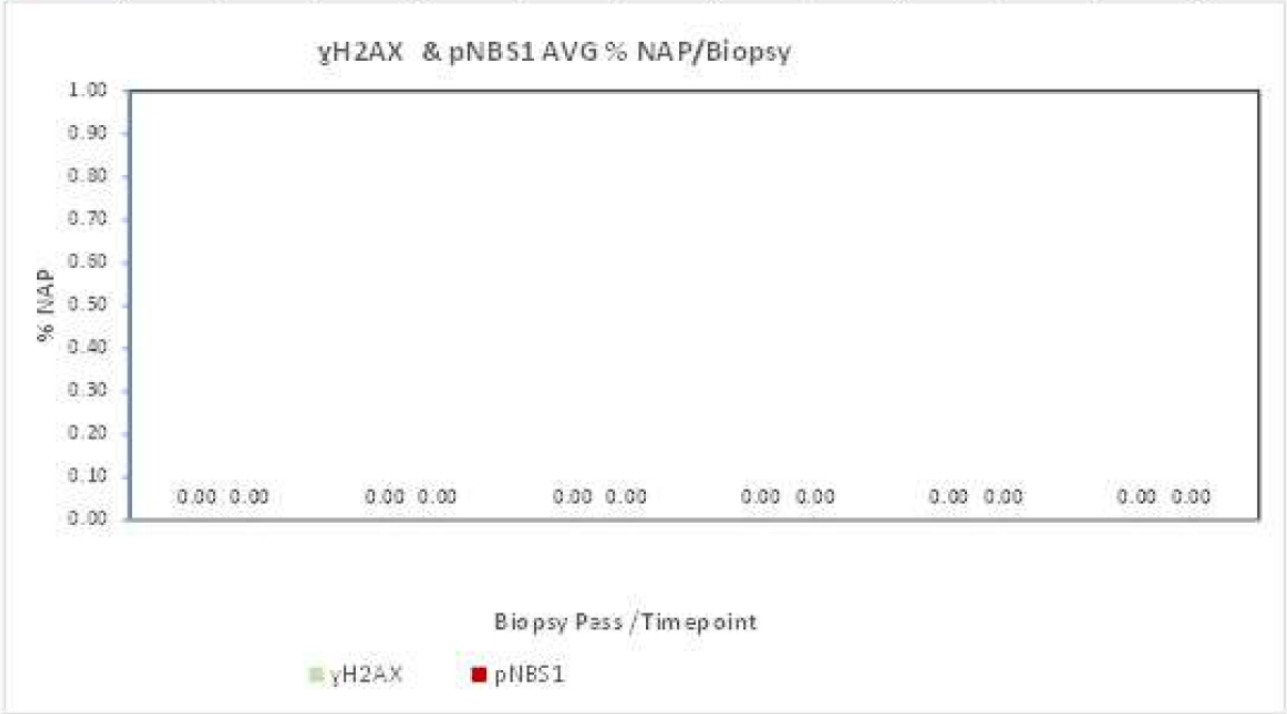
APPENDIX 3: CLINICAL SAMPLE DATA REPORT

DCTD (SOP*340545*)Clinical Sample Data Report

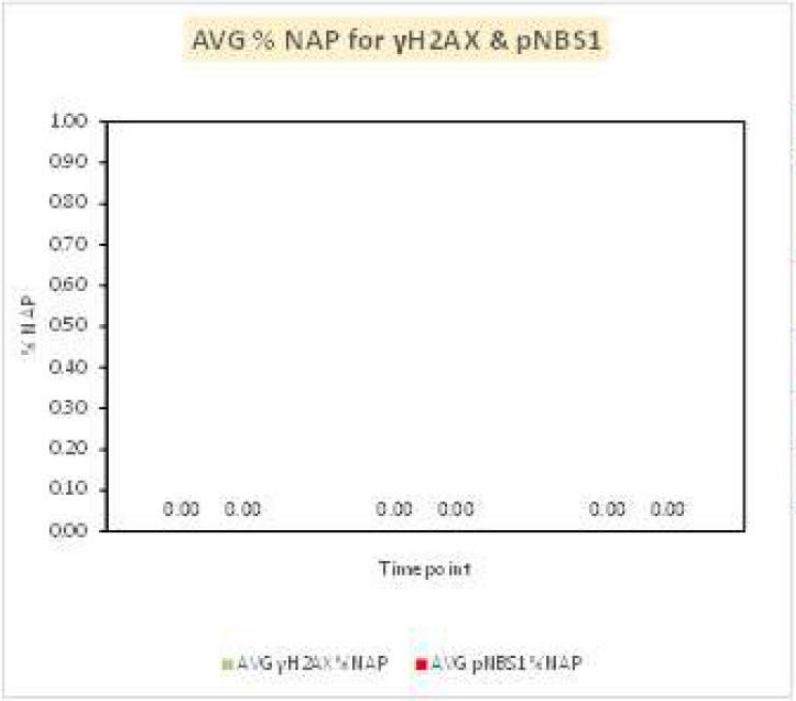
γH2AX and pNBS1 PD Analysis (DDR3)

Assay Operator:								Date:				
Patient ID:			#VALUE!					Protocol #:		#VALUE!		
Specimen IDs:							Timepoints:					

	γH2AX %NAP						pNBS1 %NAP					
Timepoint												
Slide Number	Pass 1	Pass 2	Pass 1	Pass 2	Pass 1	Pass 2	Pass 1	Pass 2	Pass 1	Pass 2	Pass 1	Pass 2
AVG												
SD												
Total # Nuclei												



DCTD (SOP*340545*)Clinical Sample Data Report



	Timepoints		
AVG γH2AX %NAP			
AVG pNBS1 %NAP			
SD γH2AX %NAP			
SD pNBS1 %NAP			
Total # Nuclei	0	0	0

Quality Controls: _____

Pass or Fail

Additional Information:

Director/Supervisor*: _____ **Date:** _____

*Signature indicates the assay results have been reviewed and verified.

Facility Reporting: _____

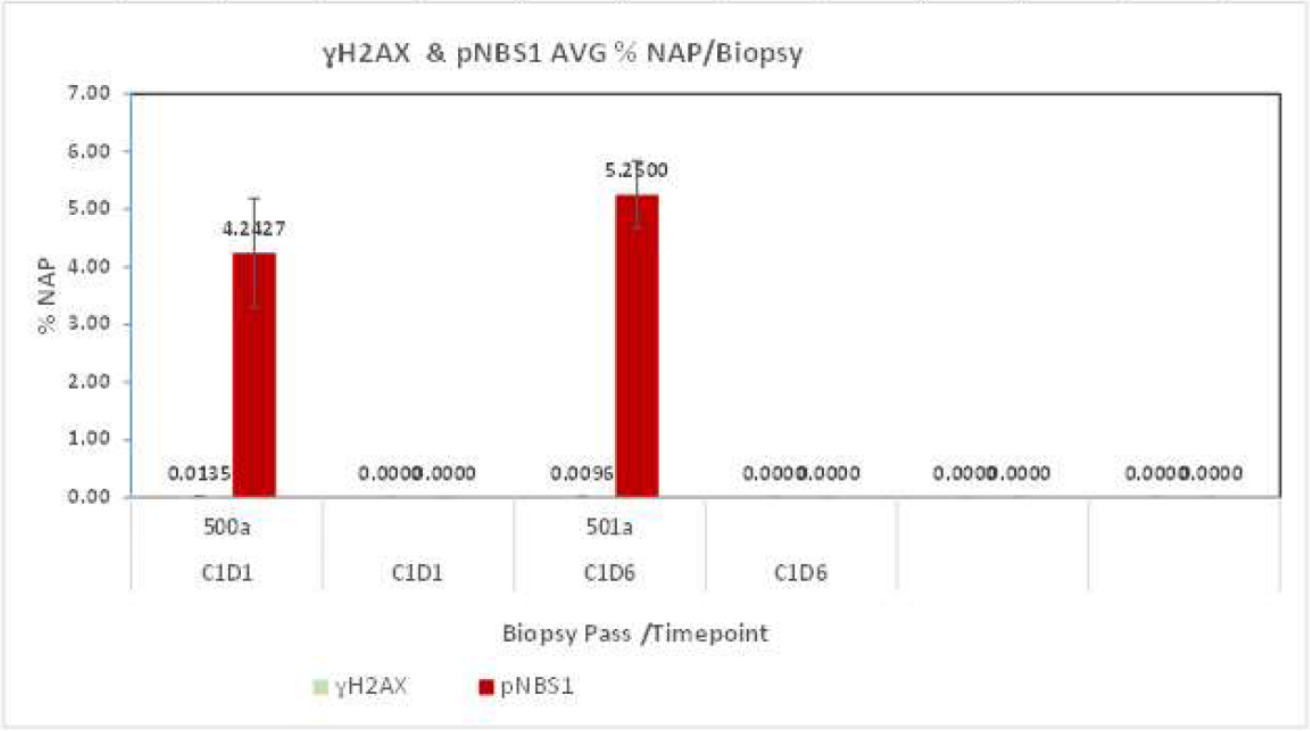
APPENDIX 4: EXAMPLE OF CLINICAL SAMPLE DATA REPORT

DCTD (SOP*340545*)Clinical Sample Data Report

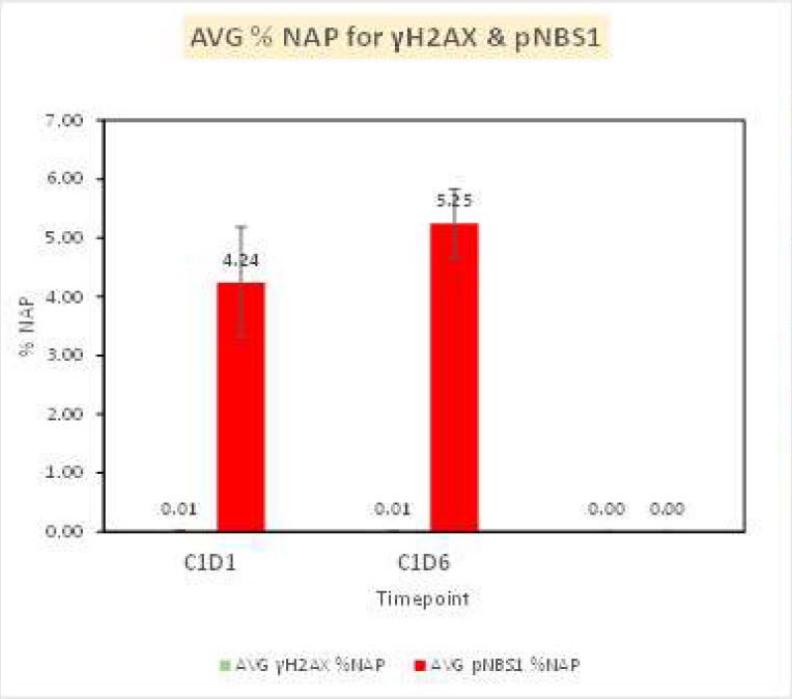
γ H2AX and pNBS1 PD Analysis (DDR3)

Assay Operator:	Jesse Smith						Date:		9/1/2018		
Patient ID:	9483_0052						Protocol #:		9483		
Specimen IDs:	500a	501a					Timepoints:	C1D1	C1D6		

γH2AX %NAP							pNBS1 %NAP					
Timepoint	C1D1		C1D6				C1D1		C1D6			
Slide Number	Pass 1	Pass 2	Pass 1	Pass 2	Pass 1	Pass 2	Pass 1	Pass 2	Pass 1	Pass 2	Pass 1	Pass 2
	500a		501a				500a		501a			
19	0.01		0.01				3.26		5.81			
23	0.00		0.01				4.33		4.64			
27	0.02		0.01				5.14		5.30			
AVG	**0.01**		**0.01**				**4.24**		**5.25**			
SD	0.0111		0.0014				0.9409		0.5856			
Total # Nuclei	35922		31579				35922		31579			



DCTD (SOP*340545*)Clinical Sample Data Report



	Timepoints		
	C1D1	C1D6	
AVG γH2AX %NAP	0.01	0.01	
AVG pNBS1 %NAP	4.24	5.25	
SD γH2AX %NAP	0.011	0.001	
SD pNBS1 %NAP	0.941	0.586	
Total # Nuclei	35922	31579	0

Quality Controls: (mid gH2AX failed)
Pass or Fail

Additional Information:

**calibrator tissue quality problem for the mid calibrator not a stain/run failure

Director/Supervisor*: J. Jones **Date:** #######

*Signature indicates the assay results have been reviewed and verified.

Facility Reporting: NCTVL