Title:	Image and Data Analyses of Tumor Biopsy Slides from γH2AX Immunofluorescence Assay				Page 1 of 24
Doc. #:	SOP340534	Revision:	C	Effective Date:	1/13/2015

National Clinical Target Validation Laboratory

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

Frederick National Laboratory for Cancer Research

Technical Reviewer:	Yvonne A. Evrard	Date:	Jan 13, 2015
NCTVL Approval:	Jiuping Ji	Date:	1/26/15
IQC Approval:	Katherine V. Ferry-Galow	Date:	1/20/15
LHTP Approval:	Ralph E. Parchment	Date:	Ralph E. Parchment -S (Affiliate) Digitally signed by Ralph E. Parchment -S (Affiliate) DN: c=US, o=U.S. Government, ou=HHS, ou=NIH, ou=People, 0.9.2342.19200300.100.1.1=00123115933, cn=Ralph E. Parchment -S (Affiliate) Date: 2015.02.25 09:19:55 -05'00'
DCTD OD Approval:	Joseph E. Tomaszewski	Date:	03/23/2015

Change History

Revision	Approval Date	Description	Originator	Approval
C	1/13/2015	Updates made based on change to minimum image per slide for clinical biopsy sections. References in Excel template updated to accommodate new image requirements.	KFG, YAE	KFG
B	9/22/2013	New calibrator/control slide set-up used. Update of analysis Excel to accommodate these changes and apply built-in QC criteria and report data as mean ± SD. Removed pre-defined calibrator ranges; these will be determined by lot and supplied with the critical reagent.	KFG, YAE	JJ
A	4/27/2011	Updates to macro scripts including version numbers, macro toolbar and capture menu. New macro scripts require the use of Image-Pro 7.0 or higher.	WHY	JJ
--	12/29/2010	Image and data analyses section split into a separate SOP from SOP340533 Revision A. Update macro and Excel template information.	WHY	JJ

Please check for revision status at

http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm

and be sure to use the current version.

TABLE OF CONTENTS

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

Ship to Certified Assay Site

SOP340522:

Tumor Frozen Needle Biopsy Preparation for the γH2AX IFA

- NBF fix and paraffin embed tumor needle biopsies and positive control sample
- Section biopsies for use in γH2AX IFA
- Stain slides by H&E for standard histology analysis

Ship to Certified Assay Site

Calibrator/Control Slides

(PADIS/IQC-Provided Critical Reagent)

SOP340523:

γH2AX IFA for Tumor Biopsy Slides

- Load biopsy slides into Bond-Max Processing Module
- Bond-Max automated staining of slides with biotinylated-γH2AX monoclonal primary antibody as the detector and a streptavidin, Alexa Fluor 488 conjugate as the reporter
- Stain slides with DAPI and mount cover slips

Ship to Certified Assay Site

Image within 18 to 72 h

SOP340533:

Image Capture of Tumor Biopsy Slides From γH2AX IFA

- Capture images of γH2AX-stained biopsy slides from a single patient (1 Bond-Max slide tray) using a fluorescent microscope and Plan Apo 20x objective with ≥ 0.7 NA.

SOP340534:

Image and Data Analyses of Tumor Biopsy Slides From γH2AX IFA

- Quantitate captured images of γH2AX-stained biopsy slides using Image-Pro software, custom macros, and a data analyses Excel

1.0 PURPOSE

Standardize an immunohistochemical method for detecting and quantifying histone H2AX phosphorylated at serine 139 (γH2AX) staining in formalin-fixed, paraffin-embedded human tissue biopsies for pharmacodynamic studies of chemotherapeutic DNA-damaging agents.

2.0 SCOPE

This procedure applies to all personnel involved in image and data analyses of slides stained using the γH2AX Immunofluorescence Assay (IFA) for Tumor Biopsy Slides (SOP340523). This SOP outlines the recommended procedure for image and data analyses of γH2AX-stained, paraffin-embedded tumor biopsy sections. The goal of the SOP and associated training is to ensure consistency of γH2AX measurement between clinical sites.

3.0 ABBREVIATIONS

CalCon	=	Calibrator/Control
DAPI	=	4',6-Diamidino-2-Phenylindole
DCTD	=	Division of Cancer Treatment and Diagnosis
γH2AX	=	Histone H2AX Phosphorylated at Serine 139
H&E	=	Hemotoxylin and Eosin
ID	=	Identification/Identifier
IFA	=	Immunofluorescence Assay
LHTP	=	Laboratory of Human Toxicology & Pharmacology
NA	=	Numerical Aperture
NBF	=	Neutral Buffered Formalin
NCTVL	=	National Clinical Target Validation Laboratory
%NAP	=	Percent Nuclear Area Positive for γH2AX
QC	=	Quality Control
SD	=	Standard Deviation
SOP	=	Standard Operating Procedure
Strp488	=	Alexa Fluor 488-Streptavidin Conjugate

4.0 INTRODUCTION

The γH2AX IFA is an immunohistochemistry-based staining assay developed to quantify the nuclear DNA damage marker, γH2AX. The assay uses a biotinylated-γH2AX monoclonal antibody as the detector and an Alexa Fluor 488-streptavidin conjugate (Strp488) as the reporter for immunostaining.

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 image analysis and quality control techniques.

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 Web site (http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm) to verify that the most recent version of the SOP for the assay is being used.

6.0 MATERIALS AND EQUIPMENT REQUIRED

6.1 Image Information Table from SOP340533 (Appendix 1, Section 2) for all images being analyzed

6.2 Image-Pro Pro 7.0 or higher (lower versions of Image-Pro are not supported and may not work with the macro)

6.3 PC (Parallels for Mac not supported)

6.4 Microsoft Excel 2003, 2007, or 2010

6.5 The following files will be provided to DCTD Certified Assay Operators during the training course:

6.5.1 A self-extracting zip file entitled "gH2AX qIFA macro v091611.exe" will include required files for image capture and analysis including the macro files gH2AXCaptureControl_ v011211.bas, ListandExcelSorter_for_gH2AXImageAssay.bas, and qIFA_gH2AX_ v072007.ipm).

6.5.2 "SOP340534_gH2AX_IFA_Data_Template.xltx" ver. 003 Microsoft Excel template for data analyses

7.0 OPERATING PROCEDURES

Reminder: A minimum of 8 non-overlapping analyzable images (captured images that lack necrotic regions, gaps, or folded tissue) are needed for a single patient in order to proceed with image analysis for the γH2AX IFA and to be confident in the image quantitation.

7.1 Image analysis should be completed for images from all sections of a single slide tray (clinical slides from a single patient and two calibrator/control [CalCon] slides) captured in SOP340533.

7.1.1 Record the name and certification number of the Certified Assay Operator performing the image analysis, the facility running the SOP, the Patient ID, and the clinical protocol/CTEP number in the Batch Record (Appendix 1).

7.1.2 Record the name of the Header Folder where the *.tif images captured in SOP340533 are stored in the Batch Record (Appendix 1, Section 1).

7.1.3 Use the Image Information Table from the Batch Record of SOP340533 for the capture order of the images as well as the reference Bond Slide ID number.

7.2 If image and data analyses are being performed on a different PC than image capture, be sure the Image-Pro software is installed and all DCTD-provided macros have been unzipped and loaded onto the PC.

7.2.1 Macro installation instructions can be found in SOP340533 (Appendix 3, Section 1A); the first time the macro is run on a computer the instruction in SOP340533 (Appendix 3, Section 1B) should be followed before proceeding.

7.2.2 If using software other than Image-Pro, the specifications for the macro scripts that are used for image capture and analysis are outlined in SOP340533 (Appendix 3, Section 3).

7.3 Protocol for Image Quantitation

7.3.1 Open the "SOP340534_gH2AX_IFA_Data_Template.xltx" Excel template **(*.xltx)** workbook for data analyses and save as an Excel workbook (***.xls**) in the Header Folder.

7.3.1.1 The naming convention for the data analyses Excel workbook should include the SOP number and the remainder of the file name should match the Header Folder created in SOP340533 (e.g., ***SOP340534_CTEP1234_2010-10-24_1.xls).***

7.3.1.2 Record the name of the Excel workbook in the Batch Record (Appendix 1, Section 1).

7.3.2 Be sure the data analyses Excel workbook is open to "Sheet1" and all other Excel workbooks are closed as the macro may overwrite data in them.

7.3.3 If not already open, open the Image-Pro software and in the macro toolbar that pops up select **Analyze Images**. In the pop-up window that opens, browse and select the Header Folder where the images captured in SOP340533 are stored and click OK.

7.3.4 In the next window, leave everything at the default settings and click OK. The macro will create a new folder called "Masks" inside the Header Folder.

7.3.4.1 The macro-processed images for quantitation will be stored in the Masks Folder with the same file name as the *.tif files preceded by "***M_**."

7.3.4.2 Examples of an original captured image and a macro-processed image are shown in Appendix 2, Section 1A and 1B, respectively. A sorted macro-processed image is also created (file name preceded by "SL_") and stored in the Masks Folder (sample sorted image in Appendix 2, Section 1C).

7.3.5 In the **Open File Location** window, select the following files and then click **OK**.

1st field	Select the Header Folder as the location of images to be processed (location of *.tif images from microscope).
2nd field	Select the Masks Folder (within the Header Folder) as the location to store the processed images.
3rd field	Leave as the default: qIFA_gH2AX_v072007.ipm

7.3.6 In the next window, **Excel & Image Formatting**, the specified Row and Column numbers where the data will be pasted into "Sheet1" of the data analyses Excel workbook will be listed; leave these in the default setting. Change the field for **Image Format** to ***.tif** and click **OK**.

7.3.7 All *.tif images in the Header Folder will be processed; processing by the macro will take approximately 30 to 60 sec/image. Once the run is complete, a message saying, "**I made it!**" will appear in the **Output Window**. **Note:** Image processing speed can be increased by minimizing the Image-Pro window so that the program does not have to generate a digital image on the computer screen with each *.tif processed.

7.3.8 The data are exported to the open "Sheet1" of the data analyses Excel workbook.

7.3.8.1 Output data are grouped by imaged slide and then by tissue section. See a map of "Sheet1" in Appendix 3, Section 1.

7.3.8.2 Row 7 of "Sheet1" will contain a representative name for each section, the average percent nuclear area positive (%NAP) for γH2AX, and standard deviation (SD) for each set of images from one tissue section. The raw data for each tissue section are listed below the averaged data (Appendix 3, Section 2).

7.3.8.3 Visually inspect the data to ensure data for a single section are grouped underneath the appropriate heading. This is a quality assurance step to ensure the **Next→**, **Next Sample→,** or **Next Slide→** option was selected with each image captured in SOP340533.

8.0 DATA ANALYSES AND ASSAY QUALITY CONTROL

8.1 The data saved on "Sheet1" are automatically sorted and organized into subsequent worksheets in the data analyses Excel workbook. Rows 4 and 5 in "Sheet1" (see Appendix 3, Sections 1 and 2) indicate the slide capture order based on the **recommended image capture order** in SOP340533.

8.1.1 If you captured slides in a different order than that recommended in SOP340533, the data will need to be manually copied and pasted into the correct column. Do not drag and drop as Excel maintains the cell linkages and this will not allow for proper sorting of the data. We suggest opening a new clean workbook and copying cells from the original macro data into the correct cells in the new workbook to minimize mistakes.

8.2 Quality control (QC) **Pass/Fail** criteria are applied in the worksheet using built-in formulas and formatting. Pass/Fail criteria are first determined for the entire slide tray by analyzing the CalCon sections in SOP Step 8.3 and then for individual clinical sections and slides in SOP Step 8.4.

8.2.1 If the CalCon slide passes QC and a minimum of 8 non-overlapping images are acquired from the pre- and post-dose sections from a single patient's batched clinical slides , the clinical data for the slides can be reported.

8.2.2 Appendix 4 contains a flowchart that can be followed while determining whether γH2AX slide data pass QC criteria.

8.3 Calibrator/Control Slides QC (Excel, "Calibrator-Control QC")

8.3.1 Data on the "Calibrator-Control QC" worksheet is auto-filled from "Sheet1" (see Appendix 3, Section 3).

8.3.2 QC for analyzable fields requires that each CalCon section contain ≥ 3 analyzable images. Pass/Fail for each CalCon section is based on the number of images captured and is assigned in cells H4 – H13.

8.3.2.1 If **only one** CalCon section for a level fails field QC, the slide still **Passes QC**. However, delete the average %NAP for the section that Failed field QC (cells F4 – F13).

8.3.2.2 If **both** images for any of the CalCon sections fails field QC, then the entire CalCon slide CalCon. Go to SOP Step 8.3.8.

8.3.3 Enter the specific Lot# for the CalCon slide used in the assay in cell D16.

8.3.4 For each calibrator tissue (Cal-Low (A), -Mid (B), and -High (C)) enter the %NAP threshold (min or max) supplied with the **specific lot** of CalCon slide used for the assay (cells C20 – C21 and D19).

8.3.5 For the Cal-Mid (B) and Cal-High (C) enter the provided fold-over Cal-Low (A) needed to pass QC (cells E20 - E21; e.g., if Cal-Mid is required to be 10x over Cal-Low, enter 10 in cell E20).

CalCon Sections	Acceptable Average %NAP Range*	Description
Cal-Low (A)	Max %NAP threshold, set by lot	Xenograft (Vehicle-treated)
Cal-Mid (B)	Min %NAP threshold and min fold over Cal-Low, set by lot	Xenograft (Treatment A)
Cal-High (C)	Min %NAP threshold and min fold over Cal-Low, set by lot	Xenograft (Treatment B)
Positive Control (D)	Intensely stained; > 10% NAP	Mouse testes
Negative Control (E)	≤ 1% NAP	Mouse jejunum

* Check product insert supplied with CalCon slides for %NAP ranges of the lot number being used. For example, CalCon Slide Lot#1300120 specifications are for Cal-Low (A) to have ≤ 2% NAP, Cal-Mid (B) to have > 8% NAP and be ≥ 10x Cal-Low (A), and for Cal-High (C) to have > 20% NAP and be ≥ 25x Cal-Low (A).

8.3.6 QC for CalCon threshold and fold-difference criteria:

- The mean %NAP for each calibrator tissue must fall within the provided lot-specific ranges (entered in SOP Step 8.3.4-8.3.5).
- The mean %NAP for the positive control section must be > 10% and the negative control ≤ 1% NAP.
- Pass/Fail for each CalCon section is assigned in cells H19 – H23.

8.3.7 If **ALL** CalCon levels pass QC, cell G26 will state "**CalCon Slide Passes QC.**" Proceed to QC of clinical slides in SOP Step 8.4.

8.3.8 If any of the CalCon levels **Fail QC** because either they have < 3 analyzable images or fall outside of the defined thresholds, the **Assay Fails QC** (cell G26).

Do the following:

8.3.8.1 Recapture images for the entire slide tray following SOP340533 as follows:

(1) Delete the Header Folder, and all files within it.

(2) Begin a new Batch Record for the new images and note in the original Batch Record that the CalCon slide **Failed QC** and samples were rerun. Keep Batch Records together.

(3) Begin with SOP340533 Step 7.3 (Protocol for Image Capture) and repeat image capture and then image and data analyses using SOP340534 and a new Batch Record.

8.3.8.2 If **ALL** CalCon levels now pass, the **CalCon Slide Passes QC** (cell G26), proceed to SOP Step 8.4 with ONLY the new image data.

8.3.8.3 If the CalCon slides fail a second time, then the entire **Slide Tray** and **Assay Fail QC** (cell G26).

- **Do not** analyze clinical slides and **do not** fill out a Sample Data Report**.**
- Indicate **Assay Failed QC** in the new Batch Record and label the first page of the Batch Record with "**Assay Fails QC**."
- The patient biopsy will need to be rerun using a Backup slide set and a new CalCon slide following SOP340523.

Note: If the **Control sections Pass (positive and negative), but the Calibrators Fail**, γH2AX can still be reported, but should not be considered quantitative. This should be reported in the deviations in the Batch Record (Appendix 1, Section 3) and on all Clinical Data Reports for samples analyzed as: "*Calibrators out of range, biomarker readout values should not be interpreted in relation to efficacy*."

8.4 Clinical Slide QC, by Slide (Excel, "Individual Slide QC")

8.4.1 Data on the "Individual Slide QC" worksheet are auto-filled from "Sheet1." Each clinical slide contains two biopsies (pre- and post-dose), as well as a positive control section, images captured for clinical slides are grouped by slide on the worksheet (see Appendix 3, Section 4).

8.4.2 At the top of the worksheet, enter the Patient ID (cell E2), BondMax slide tray number (cell G2), and the date of the BondMax run (cell J2).

8.4.3 Quality control (QC) **Pass/Fail** criteria are applied in the worksheet using built-in formulas and formatting with the following criteria:

8.4.3.1 The positive control section on each clinical slide should be intensely stained for γH2AX with > 10% NAP and ≥ 3 analyzable images.

- If either of these criteria is not met, the corresponding cell in column K for the positive control section will state "**Slide Fails QC**."
- The biopsy sections on that slide will not be used for further analysis.

8.4.3.2 There must be ≥ 8 analyzable images for the entire slide set of the clinical biopsies.

- If a biopsy section has < 8 analyzable images, cell D4 (post-dose) or cell F4 (pre-dose) will state "**Slide Set Fails QC.**"

8.4.3.3 Each clinical section must have > 1% NAP in order to be above the minimum cut-off for reportable assay results (negative control [jejunum] ≤ 1%).

- If a biopsy section has ≤ 1%NAP, column K will state "**Below Assay Cut-off**" for that tissue section.

8.5 Overall Clinical Slide Set QC (Excel, "Slide Set QC")

8.5.1 Data on the "Slide Set QC" worksheet are auto-filled from "Calibrator-Control QC" and "Individual Slide QC" worksheets (see Appendix 3, Section 5).

8.5.1.1 If the CalCon slide Failed QC, the **Assay Fails QC** (cell K4), %NAP values for the CalCon slide will not be reported and the clinical slides should not be analyzed. Follow the instructions in SOP Step 8.6.1.

8.5.1.2 For clinical slides, if the CalCon slide passes QC, but the positive control section on a clinical slide fails QC (column K), no values are reported for either the pre- or post-dose biopsy from that slide (column K and cells E6 – F13).

8.5.1.3 If one clinical biopsy fails QC, the %NAP for the other biopsy will still be reported.

8.5.2 At least 8 sufficient and non-overlapping images must be collected for the pre-dose and post-dose biopsy slide sets to pass QC. If **< 8** images for a slide set are captured, Column K will indicate "Slide Set Fails QC" and **no data** should be filled out in the Clinical Sample Data Report for those slides.

8.5.2.1 Data from the failed slides should be discarded. A Backup set of slides for the patient and a new CalCon slide should be stained for γH2AX following SOP340523.

8.5.2.2 Inquiries can be directed to the Laboratory Director/Supervisor to allow exceptions to Failed QC. If, after expert review, a patient's slides that failed QC are permitted to pass, be sure to record this decision in the Batch Record as a deviation (Appendix 1, Section 3), complete a Clinical Sample Data Report, and note the deviation on the Report.

8.5.3 If **≥ 8 each** pre- and post-dose images from a single patient's batched slide set are successfully analyzed from the slides that Pass QC, prepare a Clinical Data Report as described in the next section. Read-outs from all slides that passed QC will be graphed as progressive slides through the biopsy on the worksheet (see Appendix 3, Section 5).

8.6 Clinical Sample Data Report; by Patient (Excel, "Slide Set QC," Appendix 5)

8.6.1 Only prepare a Clinical Sample Data Report if all of the CalCon sections **Passed QC** (SOP Step 8.3; "Slide Set QC" cell K4) and **at least 8** images from the clinical slides from each biopsy **Pass** QC are successfully analyzed (SOP Step 8.5; "Slide Set QC" cells E6 – F13).

One Clinical Sample Data Report (Appendix 5) is prepared per patient.

8.6.1.1 If the Control sections Pass (positive and negative), but the Calibrators Fail, γH2AX can still be reported, but should not be considered quantitative. This should be noted in the deviations in the Batch Record (Appendix 1, Section 3) and on all Clinical Data Reports for samples analyzed in this manner as calibrators out of range, cannot report biomarker readout values in relation to efficacy.

8.6.2 Data should be reported to the hundredth decimal (e.g., 5.53%, 1.06%). The clinic will make final decisions on use of these data.

8.6.3 The following abbreviations should be used for consistency:

- < LLQ (< 1%): value below minimum cut-off for reportable assay results
- NA, no biopsy provided;
- NR: not reportable – following descriptors should be added when appropriate
    - QC Fail, not reportable due to assay or slide QC failure;
    - TQ, not reportable due to insufficient or poor tissue quality

8.6.4 Report the average %NAP for the CalCon slide sections ("Slide Set QC" worksheet reference cells C5 – C9) on the Clinical Sample Data Report.

8.6.5 For each clinical slide (up to 8 slides/patient), use the Image Information Table from SOP340533 to report the **Specimen ID** and **Bond Slide ID Number** for each.

8.6.6 For clinical slides/sections that **Passed QC**:

8.6.6.1 Record the average %NAP for the pre- and post-dose samples in the row for the correct Bond Slide ID ("Slide Set QC" worksheet, cells E6 – F13).

8.6.6.2 Record the overall average %NAP ± SD for the entire set of passing slides ("Slide Set QC" worksheet reference cells E14 – F15).

8.6.7 Reporting <LLQ values:

8.6.7.1 If the **overall mean %NAP** for a slide set is < LLQ (<1%) – (reference cells E14-F14):

- Do not report the values for individual slides
- State "< LLQ (<1%)" instead of a %NAP value for the overall mean on the Clinical Sample Data Report.

8.6.7.2 If the **overall mean % NAP** for a slide set is > LLQ (reference cells E14-F14), but some of the individual slides are < LLQ, reported as "Below Assay Cut-off" in cells K10 – K25:

- Report the overall mean as calculated on the Clinical Sample Data Report.
- Report all individual section %NAP values, even if <LLQ, on the Clinical Sample Data Report.

8.6.8 For clinical slides/sections that Fail QC:

8.6.8.1 If a clinical slide pre- or post-dose biopsy failed QC due to a failure to capture the minimum required 8 analyzable images (reference cells K10 – K25, "Slide Set Fails QC") no value will be reported for either biopsy sample on that slide.

8.6.8.2 State "NR" instead of a %NAP value on the Clinical Sample Data Report for each of the clinical slides analyzed for that patient slide set. If the inability to capture an image was due to tissue quality, state "NR, TQ."

8.6.8.3 If an individual individual slide fails to yield any analyzable images, no value will be reported for that slide.

- State "NR" instead of a %NAP value on the Clinical Sample Data Report. If the inability to capture an image was due to tissue quality, state "NR, TQ."

8.7 Depending on reporting requirements at the individual Clinical sites, the "Slide Set QC" worksheet can be printed and attached to the Batch Record. A copy of the "Slide Set QC" worksheet could also be submitted with the Clinical Data Report to the Clinical PI.

8.8 Once all of the images have been processed, create a second folder within the Header Folder called "**TIFS**," and move all original *.tif images from the main Header Folder to this subfolder.

8.9 Once γH2AX data are acquired for a patient, any remaining "Backup" slides and embedded tissue can be used per institutional guidelines.

8.10 Review and finalize the Batch Record (Appendix 1) and obtain required signatures. Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 1, Section 3).

8.11 The Laboratory Director/Supervisor should review the Batch Record and Clinical Sample Data Report for each patient and date and sign both affirming the data contained within the reports are correct (Appendix 1, Section 4).

8.12 The **signed** Clinical Sample Data Report for each patient should be sent to the clinical protocol Principal Investigator along with the attached copy of the "Slide Set QC" worksheet.

APPENDIX 1: BATCH RECORD

The Batch Record should contain information for one slide tray, and therefore a **single** patient's batched clinical slides.

NOTE*:* Record times using **military time** (24-h designation); for example, specify 16:15 to indicate 4:15 PM.

Certified Assay Operator: _____

Certification Number: _____

Facility/Laboratory Running Image Analysis: _____

Patient ID: _____

Clinical Protocol/CTEP Number: _____

1. File Names for Image Analysis

Clear and consistent labeling of folders and files is essential for easy data retrieval.

Name of the Header Folder: _____

Final name of "SOP340534_gH2AX_IFA_Data_Template" Excel workbook: _____

Final storage location of Header Folder: _____

2. Attach copies of the "Individual Slide QC" and "Slide Set QC" worksheets from the SOP340534_gH2AX_IFA_Data_Template Excel workbook.

3. Notes, including any deviations from the SOP:

4. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: _____

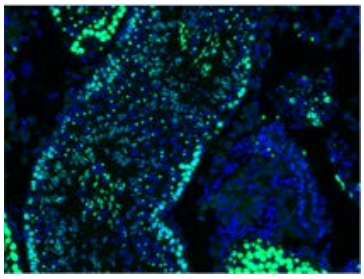
BATCH RECORD: INITIALS _____ DATE: _____

THIS PAGE LEFT BLANK ON PURPOSE

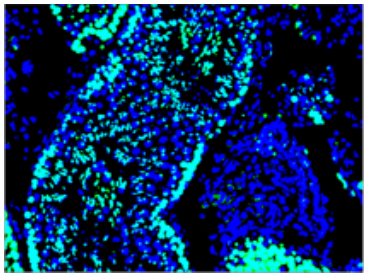
APPENDIX 2: IMAGE CAPTURE EXAMPLES

1. Captured fluorescent images:

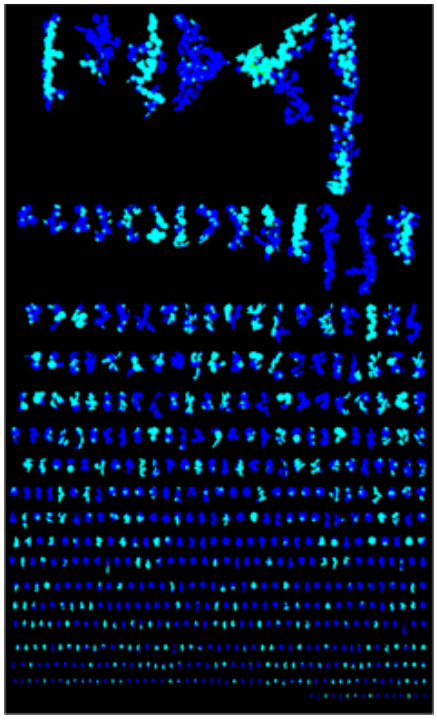
A. An example of the original *.tif image of a positive control section following image capture.



B. An example of a macro-processed image of a positive control section stored in the Masks folder. Image file name is preceded by "M_."



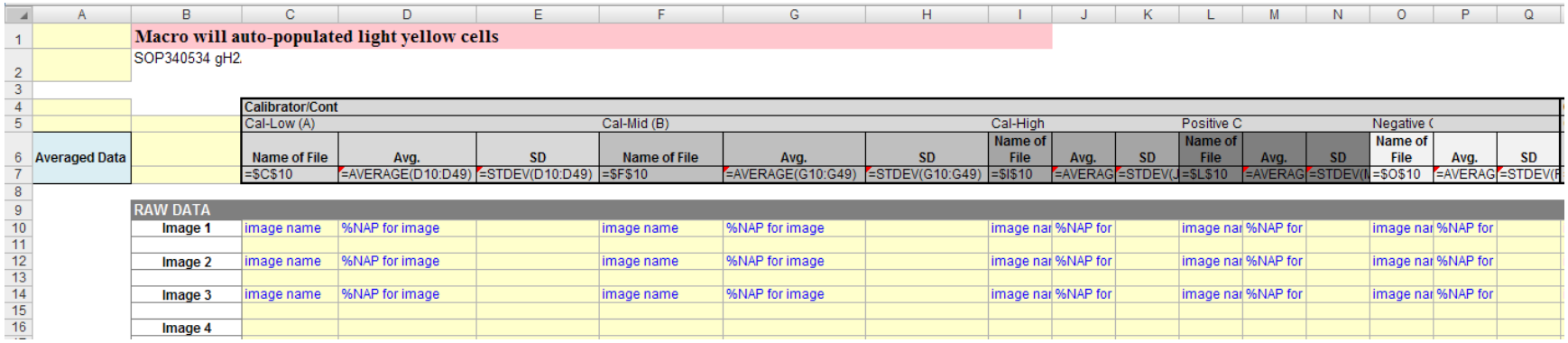
C. An example of a macro-processed image sorted by size and stored in the Masks folder. Image file name is preceded by "SL_."



APPENDIX 3: "SOP340534 gH2AX IFA DATA TEMPLATE" EXCEL WORKBOOK

1. Map of "Sheet1" Image Quantitation Output

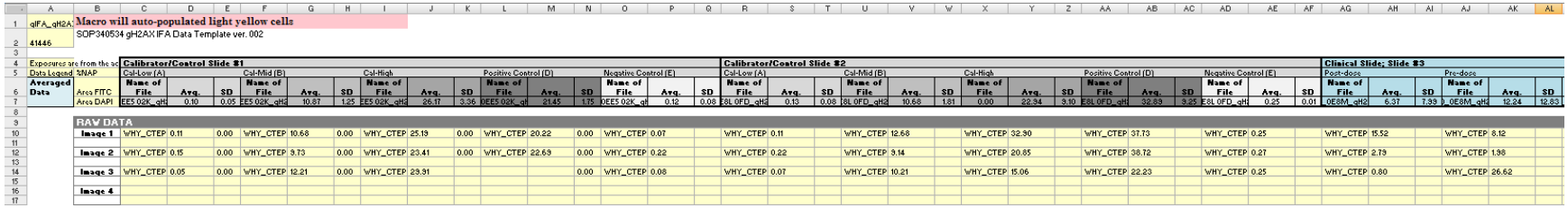
Formulas are displayed in Row 7 to demonstrate how the %NAP values are generated from the imported image data. Blue text in row 8 indicates the capture order as recommended in SOP340533. Blue text in rows 10-14 indicate the type of data that are imported to "Sheet1" by the macro.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1		**Macro will auto-populated light yellow cells**															
2		SOP340534 gH2															
3																	
4			Calibrator/Cont														
5			Cal-Low (A)			Cal-Mid (B)			Cal-High			Positive C			Negative (		
6	Averaged Data		Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD
7			=$C$10	=AVERAGE(D10:D49)	=STDEV(D10:D49)	=$F$10	=AVERAGE(G10:G49)	=STDEV(G10:G49)	=$I$10	=AVERAG	=STDEV(J	=$L$10	=AVERAG	=STDEV(I	=$O$10	=AVERAG	=STDEV(F
8																	
9		RAW DATA															
10		Image 1	image name	%NAP for image		image name	%NAP for image		image nai	%NAP for		image nai	%NAP for		image nai	%NAP for	
11																	
12		Image 2	image name	%NAP for image		image name	%NAP for image		image nai	%NAP for		image nai	%NAP for		image nai	%NAP for	
13																	
14		Image 3	image name	%NAP for image		image name	%NAP for image		image nai	%NAP for		image nai	%NAP for		image nai	%NAP for	
15																	
16		Image 4															

2. "Sheet1" Worksheet

Raw data collected during image acquisition and quantitation from each image of each section are saved into "Sheet1" of the data analyses Excel workbook when the macro is run. The average %NAP and SD for each section is automatically calculated in row 7; the file name for the first image captured is carried as an identifier throughout the workbook. *Additional slide data will be displayed in progressive columns to the right.* The macro also exports data into cells A1-A2, A4-A5, and B6-B7; these fields can be ignored.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL
1	gIFA_gH2A	**Macro will auto-populated light yellow cells**																																			
2	41446	SOP340534 gH2AX IFA Data Template ver. 002																																			
3																																					
4	Exposures are from the ac	**Calibrator/Control Slide #1**																**Calibrator/Control Slide #2**																**Clinical Slide; Slide #3**				
5	Data Legend: %NAP	Cal-Low (A)			Cal-Mid (B)			Cal-High			Positive Control (D)			Negative Control (E)				Cal-Low (A)			Cal-Mid (B)			Cal-High			Positive Control (D)			Negative Control (E)			Post-dose			Pre-dose		
6	Averaged Data	Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD		Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD	Name of File	Avg.	SD		Name of File	Avg.	SD	Name of File	Avg.	SD
7	Area FITC	EE5 02K_gH	0.10	0.05	EE5 02K_gH2	10.87	1.25	EE5 02K_gH3	26.17	3.36	DEE5 02K_gH	21.45	1.75	DEE5 02K_gH	0.12	0.08		E8L 0FD_gH2	0.13	0.08	E8L 0FD_gH2	10.68	1.61	E8L 0FD_gH	0.00	22.94	3.10	E8L 0FD_gH	32.83	3.25	E8L 0FD_gH	0.25	0.01	0E8M_gH2	6.37	7.33	0_0E8M_gH2	12.24	12.83
8	Area DAPI																																				
9		RAW DATA																																			
10		Image 1	WHY_CTEP	0.11	0.00	WHY_CTEP	10.68	0.00	WHY_CTEP	25.19	0.00	WHY_CTEP	20.22	0.00	WHY_CTEP	0.07			WHY_CTEP	0.11		WHY_CTEP	12.68		WHY_CTEP	32.30		WHY_CTEP	37.73		WHY_CTEP	0.25			WHY_CTEP	15.52		WHY_CTEP	8.12	
11																																					
12		Image 2	WHY_CTEP	0.15	0.00	WHY_CTEP	3.73	0.00	WHY_CTEP	23.41	0.00	WHY_CTEP	22.69	0.00	WHY_CTEP	0.22			WHY_CTEP	0.22		WHY_CTEP	9.14		WHY_CTEP	20.85		WHY_CTEP	38.72		WHY_CTEP	0.27			WHY_CTEP	2.79		WHY_CTEP	1.98	
13																																					
14		Image 3	WHY_CTEP	0.05	0.00	WHY_CTEP	12.21	0.00	WHY_CTEP	29.31				0.00	WHY_CTEP	0.08			WHY_CTEP	0.07		WHY_CTEP	10.21		WHY_CTEP	15.06		WHY_CTEP	22.23		WHY_CTEP	0.25			WHY_CTEP	0.80		WHY_CTEP	26.62	
15																																					
16		Image 4																																			
17																																					

3. "Calibrator-Control Slide QC" Worksheet

Data from "Sheet1" for the CalCon slide are auto-populated into the worksheet in cells D4 – G8. Formulas in column C auto-calculate the total number of images captured for a given section. The Assay Operator enters lot-specific threshold levels (yellow cells) for the calibrator sections in the bottom half of the worksheet. Built-in formulas in the worksheet assign Pass/Fail criteria to the CalCon sections. Each section is required to have ≥ 3 analyzable images captures (cells C4 – C13) and meet the threshold limits established for the slides (cells C19 – E23). If any section fails QC, the CalCon slide and therefore the slide tray Fails QC (reported in cell G26). Conditional formatting in the Excel worksheet is applied to highlight assigned Pass/Fail designations (column H).

Proper application of the QC criteria requires that images for the slide were captured in the **recommended image capture order** in SOP340533.

Protected worksheet except for light yellow cells.

QC for Analyzable Fields (≥ 3); cells auto-filled from Sheet1

Capture order per SOP30533	Number of Fields Captured/Section	Name of Sample	Avg. %NAP	SD:	Analyzable field QC: Pass/Fail
Cal-Low (A) - #1	3	WHY_CTEP 1234 002050 20130405__0(	0.10	0.05	Pass
Cal-Low (A) - #2	3	WHY_CTEP 1234 1010035 20130307___	0.13	0.08	Pass
Cal-Mid (B) - #1	3	WHY_CTEP 1234 002050 20130405__0(	10.87	1.25	Pass
Cal-Mid (B) - #2	3	WHY_CTEP 1234 1010035 20130307___	10.68	1.81	Pass
Cal-High (C) - #1	3	WHY_CTEP 1234 002050 20130405__0(	26.17	3.36	Pass
Cal-High (C) - #2	3	WHY_CTEP 1234 1010035 20130307___	22.94	9.10	Pass
Positive Control (D) - #1	2	WHY_CTEP 1234 002050 20130405__0(		1.75	Fail
Positive Control (D) - #2	3	WHY_CTEP 1234 1010035 20130307___	32.89	9.25	Pass
Negative Control (E) - #1	3	WHY_CTEP 1234 002050 20130405__0(	0.12	0.08	Pass
Negative Control (E) - #2	3	WHY_CTEP 1234 1010035 20130307___	0.25	0.01	Pass

Enter Cal-Low (A), -Mid (B), and -High (C) thresholds (yellow cells) provided with the critical reagent lot being used.

Calibrator/Control Slide Lot#:	13000120

Capture order per SOP30533	%NAP Min Threshold	%NAP Max Threshold	Fold Difference From Cal-Low	Avg. %NAP	Calculated fold Above Cal-Low	Threshold QC: Pass/Fail
Cal-Low (A)	0.0	2.0	N/A	0.12	N/A	Pass
Cal-Mid (B)	8.0	N/A	10.0	10.78	91.0	Pass
Cal-High (C)	20.0	N/A	25.0	24.55	207.3	Pass
Positive Control (D)	10.0	100.0	N/A	32.89	N/A	Pass
Negative Control (E)	0.0	1.0	N/A	0.19	N/A	Pass

Assay QC measures met?	Cal/Con Slide Passes QC

4. "Individual Slide QC" Worksheet

Data from "Sheet1" for the clinical slides are auto-populated into the worksheet in cells D8 – F31. Formulas in column C auto-calculate the total number of images captured for a given section. Columns G-J sort the data by slide type based on the tissue type selected (Sample or Control) in the Capture Menu (SOP340533). Conditional formatting in the Excel worksheet is applied to highlight assigned Pass/Fail designations (column K). The SOP requires that a minimum of 8 images across a slide set are captured else it is reported as "Slide Set Fails QC" (cells D4, F4). Any data points **≤ 1% NAP** are considered below the minimum cut-off for reportable assay results and are identified as "Below the Assay Cut-off."

Proper application of the QC criteria requires that images for the slide were captured in the **recommended image capture order** in SOP340533.

Protected worksheet except for light yellow cells.

Patient ID: XXX Slide Tray#: 0 Bond-Max Date: WW1900

QC for Analyzable Fields (≥ 8/slide set) and Positive Control Sections (> 10% NAP)

≥ 8 Images/Slide Set QC	Post-Dose Slide Set QC	Pass	Pre-Dose Slide Set QC	Pass

Capture order per SOP30533	Number of Fields Captured for Section	All Slides File Name	All Slides Avg. %NAP	All Slides SD:	Control Avg. %NAP	Control SD	Sample Avg. %NAP	Sample SD	Tissue Section QC	Notes and Deviations
Slide 3: Post-dose	3	WHY_CTEP 7998 Patien	1.33	0.12			1.33	0.12	Pass	
Slide 3: Pre-dose	3	WHY_CTEP 7998 Patien	0.42	0.19			0.42	0.19	Below Assay Cut-off	
Positive Control	3	WHY_CTEP 7998 Patien	20.65	2.68			20.65	2.68	Pass	
Slide 4: Post-dose	2	WHY_CTEP 7998 Patien	1.28	0.10			1.28	0.10	Pass	
Slide 4: Pre-dose	3	WHY_CTEP 7998 Patien	0.11	0.06			0.11	0.06	Below Assay Cut-off	
Positive Control	3	WHY_CTEP 7998 Patien	19.15	6.08			19.15	6.08	Pass	
Slide 5: Post-dose	3	WHY_CTEP 7998 Patien	1.33	0.12			1.33	0.12	Pass	
Slide 5: Pre-dose	3	WHY_CTEP 7998 Patien	0.15	0.04			0.15	0.04	Below Assay Cut-off	
Positive Control	3	WHY_CTEP 7998 Patien	22.48	6.18			22.48	6.18	Pass	
Slide 6: Post-dose	3	WHY_CTEP 7998 Patien	1.33	0.12			1.33	0.12	Pass	
Slide 6: Pre-dose	2	WHY_CTEP 7998 Patien	0.09	0.00			0.09	0.00	Below Assay Cut-off	
Positive Control	3	WHY_CTEP 7998 Patien	30.21	4.72			30.21	4.72	Pass	
Slide 7: Post-dose	3	WHY_CTEP 7998 Patien	1.33	0.12			1.33	0.12	Pass	
Slide 7: Pre-dose	0	WHY_CTEP 7998 Patien								
Positive Control	3	WHY_CTEP 7998 Patien	24.09	3.72			24.09	3.72	Pass	
Slide 8: Post-dose	2	WHY_CTEP 7998 Patien	1.28	0.10			1.28	0.10	Pass	
Slide 8: Pre-dose	3	WHY_CTEP 7998 Patien	0.20	0.02			0.20	0.02	Below Assay Cut-off	
Positive Control	3	WHY_CTEP 7998 Patien	26.97	8.30	26.97	8.30			Pass	
Slide 9: Post-dose	3	WHY_CTEP 7998 Patien	1.33	0.12			1.33	0.12	Pass	
Slide 9: Pre-dose	3	WHY_CTEP 7998 Patien	0.09	0.08			0.09	0.08	Below Assay Cut-off	
Positive Control	3	WHY_CTEP 7998 Patien	34.06	6.36	34.06	6.36			Pass	
Slide 10: Post-dose	0	0								
Slide 10: Pre-dose	0	0								
Positive Control	0	0							Slide Fails QC	

5. "Slide Set QC" Worksheet

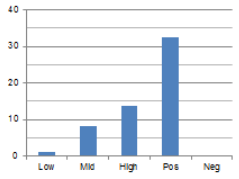
Data from the "Calibrator-Control QC" and "Individual Slide QC" worksheets are auto-filled (cells K4 – L25) and %NAP is plotted for the CalCon slide (bar graph) and the clinical samples (scatter plot). The %NAP for samples or slides that failed QC are not carried over to this worksheet. The graph represents the %NAP for progressive sections through a single patient's biopsy.

If < 3 each of the pre- or post-dose sections from a single patient's batched slide set Pass QC (cells E6 – F13), do not fill out a Clinical Sample Data Report.

Protected worksheet. Print a copy and attach to the Batch Record

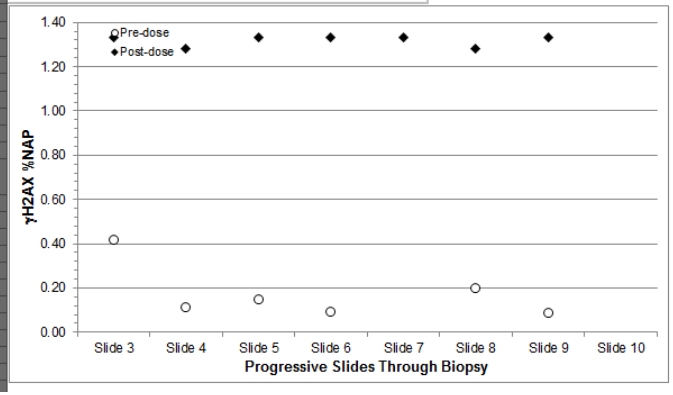
Summary of IFA Data *Samples <LLQ are still used to determine the average Slide Set read-out

Calibrators/Controls Plotted:	%NAP
Low	0.96
Mid	8.29
High	13.80
Pos	32.42
Neg	0.01



Clinical Samples Plotted: Sections Through Biopsy	%NAP Pre-dose	%NAP Post-dose
Slide 3	0.42	1.33
Slide 4	0.11	1.28
Slide 5	0.15	1.33
Slide 6	0.09	1.33
Slide 7	#N/A	1.33
Slide 8	0.20	1.28
Slide 9	0.09	1.33
Slide 10	#N/A	#N/A
Overall Mean	<LLQ (<1%)	1.32
Overall SD		0.03

Patient ID:	XXX
Bond-Max Date:	1/0/1900
Calibrator/Control Slide Lot#:	XXX

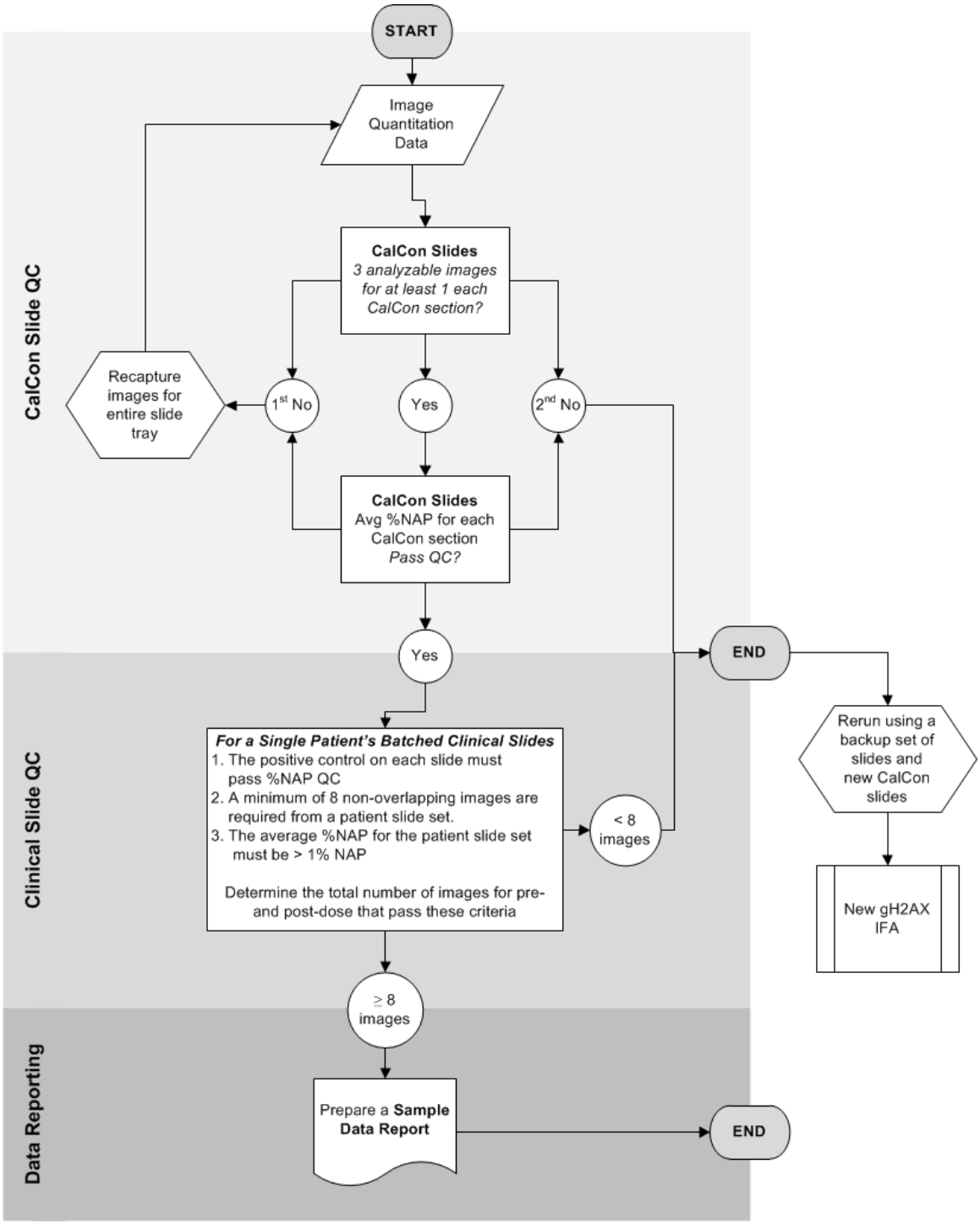


Data below are auto-filled from "Calibrator-Control QC" and "Individual Slide QC" worksheets and used to generate summary section to the left

Slide recommended capture order	File Name	Pass/Fail*	Avg. %NAP*
Avg: Cal-Low	WHY_CTEP 7998 Patient 56____0_0_0_0DM	CalCon Slide Passes QC	0.96
Avg: Cal-Mid	WHY_CTEP 7998 Patient 56____0_0_0_0DM		8.29
Avg: Cal-High	WHY_CTEP 7998 Patient 56____0_0_0_0DM		13.80
Avg: Pos	WHY_CTEP 7998 Patient 56____0_0_0_0DM		32.42
Avg: Neg	WHY_CTEP 7998 Patient 56____0_0_0_0DM		0.01
Slide 3: Post-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DN	Pass	1.33
Slide 3: Pre-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DN	Below Assay Cut-off	0.42
Slide 4: Post-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DN	Pass	1.28
Slide 4: Pre-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DN	Below Assay Cut-off	0.11
Slide 5: Post-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DN	Pass	1.33
Slide 5: Pre-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DN	Below Assay Cut-off	0.15
Slide 6: Post-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DN	Pass	1.33
Slide 6: Pre-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DN	Below Assay Cut-off	0.09
Slide 7: Post-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DM	Pass	1.33
Slide 7: Pre-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DM		
Slide 8: Post-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DM	Pass	1.28
Slide 8: Pre-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DM	Below Assay Cut-off	0.20
Slide 9: Post-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DM	Pass	1.33
Slide 9: Pre-dose	WHY_CTEP 7998 Patient 56____0_0_0_0DM	Below Assay Cut-off	0.09
Slide 10: Post-dose	0	Slide Failed QC	
Slide 10: Pre-dose	0	Slide Failed QC	

APPENDIX 4: QUALITY CONTROL FLOWCHART

General flowchart of QC pass/fail criteria outlined in SOP Step 8.0 for a single patient's batched slides from one Bond-Max slide tray.



APPENDIX 5: CLINICAL SAMPLE DATA REPORT

γH2AX IFA PD Analysis

Page 1 of 2

Certified Assay Operator (Print): _____

Certification #: _____ Today's Date: _____

Trial and Patient Information

Patient ID: _____ Trial Site: _____

Clinical Center #: _____ CTEP Protocol #: _____

Assay Readout

Date Slides Processed in Bond-Max: _____ Slide Tray #: _____

Average %NAP		**Average %NAP**	
Cal-Low (A)		Positive Control (D)	
Cal-Mid (B)		Negative Control (E)	
Cal-High (C)			

Assay Quality Control (QC) measures met? (Pass/Fail): _____

		Average %NAP	
	Specimen IDs:		
Slide No.	**Bond Slide ID**	**Pre-dose**	**Post-dose**
1			
2			
3			
4			
5			
6			
7			
8			
	Overall Average %NAP ± SD	±	±

Graph of slides across biopsy attached.

Designations: NA, no biopsy provided; < LLQ (<1%), below minimum cut-off for reportable assay results; NR, not reportable; QC Fail, not reportable due to assay or slide QC failure; TQ, not reportable due to insufficient or poor tissue quality

Additional Information *Page 2 of 2*

Images are available for review upon request.

To be completed by Laboratory Director/Supervisor

Complete the following table. Signature indicates assay results have been reviewed and verified.

	Average %NAP		**Average %NAP**
Cal-Low (A)	_____	Positive Control (D)	_____
Cal-Mid (B)	_____	Negative Control (E)	_____
Cal-High (C)	_____		

Assay Quality Control (QC) measures met? (Pass/Fail): _____

Director/Supervisor Signature*: _____

Today's Date: _____

*Signature indicates assay results have been reviewed and verified.

Biopsy and Treatment Information (for use by Clinical site)

Site of Biopsy: _____ Primary Tumor: _____

Dose Level: _____

Agent Name(s): _____ + _____

Dose and Unit(s): _____ + _____