Title:	γH2AX Immunofluorescence Assay for Tumor Biopsy Slides				Page 1 of 30
Doc. #:	SOP340523	Revision:	E	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:	Donna Butcher	Date:	1/22/15
NCTVL Approval:	Jiuping Ji	Date:	1/26/15
IQC Approval:	Katherine V. Ferry-Galow	Date:	1/29/15
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=001231593, cn=Ralph E. Parchment -S (Affiliate) Date: 2015.02.25 09:04:37 -05'00'
DCTD OD Approval:	Joseph E. Tomaszewski	Date:	03/23/2015

Please check for revision status at

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

and be sure to use the current version.

Change History

Revision	Approval Date	Description	Originator	Approval
E	1/13/2015	Modified dewaxing procedure added for slides that are dipped in paraffin after microtomy, additional minor edits.	KFG, DB	KFG
D	4/28/2014	DAPI staining returned to a modified off-line procedure due to variability in automated staining. Recommended DAPI working concentration has been changed. BondMax staining protocol (Appendix 2, Section 4A) has been updated for these changes.	WHY, KFG	KFG
C	9/22/2013	Update antibody preparation calculations and residual volume increased. DAPI staining has been added to the BondMax automated procedure. Shipping instructions have been added in case slides are stained at one certified site and imaged at a second site. Details on manual IF staining of slides have been removed as the ability to meet SOP QA/QC criteria cannot be controlled.	YAE, DB, KFG	JJ
B	12/29/2010	Update SOP following in-house assay runs of patient samples. Critical Reagents identified, updated and detailed handling provided.	WHY	JJ
A	2/01/2010	Add appendices, including Batch Record, update calibrator/control slide information, prepare for Web. Separate γH2AX slide staining (SOP340523) and image capture and quantitation (SOP340533) SOPs.	YAE	JJ
--	10/22/2008	New Document	WHY	JJ

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

Calibrator/Control Slides

(PADIS/IQC-Provided Critical Reagent)

Ship to Certified Assay Site

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 Analysis 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 the use of the γH2AX Immunofluorescence Assay (IFA) for tumor biopsies from patients participating in clinical trials. This SOP outlines the recommended procedure for staining of paraffin-embedded tumor biopsy sections using the automated Leica Microsystems Bond-Max™ Autostainer. The goal of the SOP and associated training is to ensure consistency of γH2AX measurement between operators and clinical sites.

3.0 ABBREVIATIONS

Ab	=	Antibody
CalCon	=	Calibrator/Control
DAPI	=	4',6-Diamidino-2-Phenylindole
DCTD	=	Division of Cancer Treatment and Diagnosis
DI	=	Deionized
ER	=	Epitope Retrieval
γH2AX	=	Histone H2AX Phosphorylated at Serine 139
H&E	=	Hematoxylin and Eosin
HIER	=	Heat-Induced Epitope Retrieval
ID	=	Identification/Identifier
IFA	=	Immunofluorescence Assay
LHTP	=	Laboratory of Human Toxicology & Pharmacology
NA	=	Numerical Aperture
NCTVL	=	National Clinical Target Validation Laboratory
PBS	=	Phosphate-Buffered Saline
QC	=	Quality Control
SOP	=	Standard Operating Procedure
Strp488	=	Alexa Fluor 488-Streptavidin Conjugate
UPI	=	Unique Pack Identifier

4.0 INTRODUCTION

The γH2AX IFA is an immunohistochemistry-based staining assay developed to quantify the nuclear DNA damage marker, histone 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 operation of the Bond-Max System.

5.3 Digital versions of the Slide Information Table in the Batch Record (Appendix 1, Section 3) can be created for logging sample information as long as all column information exactly matches the tables in the Batch Record. A copy of the completed, digital sample tables must be printed and attached to the Batch Record in order to maintain a complete audit trail.

5.4 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.

5.5 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 PADIS/IQC-Supplied Critical Reagents

6.1.1 Anti-phospho-histone H2AX (Ser139), clone JBW301, biotin conjugate with Certificate of Analysis (γH2AX Ab; Millipore, Cat#: 16-193)
6.1.2 Streptavidin, Alexa Fluor® 488 conjugate; (Strp488; Invitrogen, Cat#: S11223
6.1.3 DAPI dihydrochloride, FluoroPure™ grade (Invitrogen, Cat#: D21490).
6.1.4 Calibrator/control slides

6.2 Pipettors (100-1000 µL, 50-200 µL, 2-20 µL, 0.2-2 µL) and tips
6.3 50-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352098)
6.4 Premium cover glasses, approx. 50 mm x 22 mm (e.g., Fisher Scientific, Cat#: 12-548-5E; Thermo Scientific; Cat#: 12440S)
6.5 Kimwipes (e.g., Fischer Scientific, Cat#: 06-666A)
6.6 Slide mailer/folder (e.g., Leica Microsystems, Cat#: 3802617)

6.7 Sterile-filtered, molecular biology grade deionized (DI) water (e.g., Invitrogen, Cat#: 10977-015)
6.8 10X phosphate-buffered saline (PBS; e.g., Invitrogen, Cat#: 70013-073) [Dilute 1:10 in DI water to prepare 1X PBS for use in assay.]
6.9 Anhydrous ethanol, histology grade (Fisher Scientific, Cat#: A405-20 [Filtered using 0.22 µm pore size before use.]) ACS/USP Grade can be purchased and used without filtration (Pharmco-AAPER, Cat#: 111000200PL05)
6.10 Xylene, ACS grade (e.g. EMD Millipore, Cat# XX0055-3)
6.11 ProLong® Gold antifade reagent (Invitrogen, Cat#: P36930)

6.12 Bond-Max Autostainer (Leica Microsystems, Cat#: 21.0051.110)
6.13 Bond Dewax Solution (Leica Microsystems, Cat#: AR9222)
6.14 Bond Epitope Retrieval Solution 1 (Leica Microsystems, Cat#: AR9961)
6.15 Bond Open Container – 10 pack; 30 mL (Leica Microsystems, Cat#: OP309700); alternate container sizes are listed in Appendix 2, Section 1
6.16 Bond Research Detection Kit (Leica Microsystems, Cat#: DS9455)
6.17 Bond Primary Antibody Diluent (Leica Microsystems, Cat#: AR9352)
6.18 Bond Universal Covertiles, 100 Pack (Leica Microsystems, Cat#: S21.2001.110)
6.19 Bond Wash Solution 10X Concentrate (Leica Microsystems, Cat#: AR9590)
6.20 Bond Universal Slide Labels and Printing Ribbon kit (Leica Microsystems, Cat#: S21.4564.A)
6.21 Tissue-Tek® Slide Staining Dish White (Sakura, Cat#: 4457)
6.22 Tissue-Tek® 24-Slide Holder with Detachable Handle (Sakura, Cat#: 4465)
6.23 -80°C and -20°C freezers
6.24 2°C -8°C refrigerator

6.25 Clinical slides prepared following SOP340522 with paraffin-embedded pre- and post-dose biopsy samples and a testis positive control sample on each slide

7.0 OPERATING PROCEDURES

7.1 Record the name and certification number of the Certified Assay Operator and the facility running the SOP in the Batch Record (Appendix 1).

7.2 If slides were shipped from a separate site, save the clinical shipping manifest for the laboratory record and attach a copy to this Batch Record.

7.3 Record the unique Patient/Sample ID for the slides being assayed. This SOP and its associated Batch Record are sufficient for one patient's slide run on a single Bond-Max slide tray.

7.4 Prior to beginning the assay, read the SOP and ensure sufficient materials and reagents are in stock to run the SOP. All reagents are to be prepared for use in one experimental run, and only in the amounts required for the specific assay.

7.4.1 The 30-mL Open Containers used in this SOP are for use under the assumption that 3 slide trays will be processed in every Bond-Max run. If a single slide tray is run on a regular basis, please read Appendix 2, Section 1 for modifications.

7.5 Critical Reagents

7.5.1 Record the date of receipt, lot numbers, stock/supplied reagent concentration, recommended working concentration, recommended dilution and expiration dates for the critical reagents in the Batch Record (Appendix 1, Section 1A).

7.5.2 All Critical Reagents are to be labeled with the date of receipt and stored under the specified conditions for no longer than the recommended duration.

- Storage conditions and expiration dates for all Critical Reagents are provided on the shipping manifest that accompanies the critical reagent shipment.
- If the critical reagents are purchased directly from the manufacturer, Certified Assay Sites must qualify the reagents prior to use in the Assay. Lot-to-lot differences, particularly for primary antibodies, are expected due to general reagent quality differences in manufacturer-supplied reagents.

7.5.3 **Anti-γH2AX biotin conjugate (γH2AX Ab)** as a stock solution in 70% storage buffer (0.02 M phosphate buffer, pH 7.6, 0.25M NaCl, 0.1% sodium azide) and 30% glycerol provided by the manufacturer. The stock concentration is approximately 1 mg/mL. Actual concentration of the material as well as the recommended working concentration of the material will be provided by lot.

7.5.4 **Strp488 conjugate** as a stock solution in 50% glycerol/1X PBS. Store frozen at -20°C for up to 3 mo.

7.5.5 **Calibrator/control (CalCon) slides** store in a desiccator at 2°C to 8°C away from volatile chemicals. Can be stored indefinitely.

7.5.6 **DAPI stock solution** as a 14.3 mM (5 mg/mL) solution in DI water. Aliquots can be stored at -20°C for up to 1 y; thawed aliquots can be stored at 4°C for up to 3 mo. Light sensitive; protect all solutions from light.

7.6 If not already done, program the following information into the Bond-Max System prior to experimental setup:

7.6.1 Facility or laboratory running the assay should be added to the "Doctors List" (Appendix 2, Section 2).

7.6.2 Any new antibodies and Bond Open Containers (Appendix 2, Section 3).

7.6.3 The γH2AX staining protocol (Appendix 2, Section 4A). In addition, verify that the Bond-Max pre-programmed Dewax and HIER protocols match those listed in Appendix 2, Section 4B and 4C, respectively.

7.6.4 If a new Research Detection Kit is being used, scan the bar code to open the **Add Reagent** dialog box. Select the name of the reagent from the **Reagent name** drop-down list (select "Wash Buffer" for the Open Container) and in the expiration selection put a future date (suggest 1 yr after today's date).

7.7 Calibrator/Control (CalCon) and Clinical Slides

7.7.1 Two CalCon slides are required for each Bond-Max run and will be placed in the first and last position of the slide tray.

7.7.2 Clinical samples for this assay will be frozen needle biopsies collected according to SOP340507 and formalin-fixed and paraffin-embedded and sectioned according to SOP340522. One slide tray in the Bond-Max System should contain a single patient's slides; a maximum of 3 slide trays can be run in one experimental run.

7.8 Preparation of Reagents

7.8.1 During reagent preparation, be sure to note the lot number/serial number, expiration dates, and dates of preparation as indicated in the Batch Record (Appendix 1, Section 1B). All reagents are to be labeled with date of receipt and stored under the specified conditions for no longer than the recommended durations.

Note: Some of the following reagents may be prepared ahead of time.

7.8.2 <u>1X Bond Wash Solution</u>

7.8.2.1 Make 1 L of 1X solution by adding 100 mL Bond 10X Wash Solution to 900 mL DI water. Mix the solution until it is homogenous, and label the bottle as "1X Bond Wash Solution" with the lot number and preparation date. Store Bond 1X and 10X Wash Solutions at 2 ºC to 8°C out of direct sunlight. 1X Bond Wash Solution can be used for 4 mo.

7.8.2.2 When ready for use, 1X Bond Wash Solution can be poured into the bulk container marked "Wash Buffer" located within the Bond-Max Processing Module.

7.8.3 Research Detection Kit

7.8.3.1 Add 30 mL of 1X Bond Wash Solution to the 30-mL Open Container in the kit and record the UPI number for the Open Container in the Batch Record (Appendix 1, Section 1C). Note: This container is required to be loaded with the Research Detection Kit, but the solution is not used in the actual BondMax run.

7.9 Make sure that all required bulk reagent containers have sufficient volumes before starting the Bond-Max staining procedure. The bulk reagents containers should be at least a quarter full.

7.9.1 The bulk reagents include: 1X Bond Wash Solution, Bond Dewax solution (if on-line dewax will be utilized – see SOP Step 7.11.3), anhydrous ethanol, DI water, and Bond Epitope Retrieval (ER) Solution 1.

7.9.1.1 When not in use, the bulk reagents, 1X Bond Wash Solution, and ER Solution 1 containers are stored in a 2ºC to 8°C refrigerator, and the other bulk reagent containers are stored in the Bond-Max bulk reagent cavity.

7.9.1.2 Pre-warming the solutions that were stored in the refrigerator is not required; temperature does not adversely affect staining.

7.9.2 **Visually inspect all solutions for assay** to ensure there is no cloudiness or precipitate present. If they are cloudy or have a precipitate, discard the solutions and clean the bottles with a mild bleach solution. Rinse the containers thoroughly with water before reuse.

7.10 Preparation of Antibody and Ancillary Working Solutions

7.10.1 Label two Titration Containers or Open Containers for the assay working solutions as follows: “Marker” and “Strp488.” The Container size is dependent upon number of slides to be stained in a run; refer to Appendix 2, Section 1 for Container volumes. The Container labels correspond to the steps programmed into the staining protocol (Appendix 2, Section 4).

7.10.2 Record the lot number and expiration date of the Bond Primary Antibody Diluent and the UPI numbers of the Bond Open Containers in the Batch Record (Appendix 1, Section 1B and 1C).

7.10.3 Perform the calculations in Appendix 1, Section 2 to prepare the antibody working solutions as follows:

7.10.3.1 γH2AX Ab Working Solution

- The γH2AX Ab Working Solution should be prepared fresh using Bond **Primary Antibody Diluent**. This will be used as "Marker" in the staining protocol.
- To be sure there is sufficient volume for all of the slides to be stained, perform the calculations in the Batch Record (Appendix 1, Section 2A).
- Briefly warm the γH2AX Ab supplied Critical Reagent vial and then pipette the calculated volumes of γH2AX Ab and Bond Primary Antibody Diluent into the ”Marker” Container; record the preparation date and time in the Batch Record (Appendix 1, Section 2A)**.**

7.10.3.2 Strp488 Working Solution

- The Strp488 Working Solution should be prepared fresh using Bond **Primary Antibody Diluent**. This will be used as "Strp488" in the staining protocol.
- To be sure there is sufficient volume for all of the slides to be stained, perform the calculations in the Batch Record (Appendix 1, Section 2B).
- Briefly warm the Strp488 supplied Critical Reagent vial, and then pipette the calculated volumes of Strp488 and Bond Primary Antibody Diluent into the "Strp488" Container; record the preparation date and time in the Batch Record (Appendix 1, Section 2B).

7.10.4 It is strongly recommended to always use fresh working solutions. Working antibody solutions can be stored at 2ºC to 8°C and used for up to 5 d after preparation. If you use a stored Working Solution, note this in the deviations section (Appendix 1, Section 5).

7.11 Protocol for Slide Staining in Bond-Max Processing Module

7.11.1 System Setup for Bond-Max Run

7.11.1.1 **Turn on** the computer and **open** the Bond software by clicking on the Bond icon, then **turn on** the Bond-Max Processing Module.

7.11.1.2 In the Bond software, select the **Slide Setup Screen,** and then select the **Add Case** button. In the **Add Case** window, change the fields as follows and then click **OK**:

Field	Fill in
Case ID	Date of sample processing (e.g., 2010-10-24)
Patient Name	If a single patient's slides are run on the tray, enter their CTEP#-Patient ID (e.g., 1234-0001001); else, leave blank.
Case Comments	Add comments as needed
Doctor	Facility or laboratory running assay (from drop-down list)
Dispense Volume	150 µL
Preparation Protocol	N/A (Use *Dewax if slides are NOT paraffin dipped and off-line dewaxed)

7.11.2 Add Slides to Bond-Max Run

7.11.2.1 While still in the **Slide Setup Screen**, click the **Add Slide** button, and in the **Add Slide** window, change the fields as follows:

Field	Fill in / Select
Tissue Type	Test tissue
Dispense Volume	150 µL
Staining Mode	"Single" and "Research"
Process	IHC
Marker	gH2AX Ab
Staining Protocol	Tissue_Section_gH2AX_Strp488
Preparation Protocol	N/A (Use *Dewax if slides are NOT paraffin dipped and off-line dewaxed)
HIER Protocol	*HIER 10 min with ER 1

7.11.2.2 For each new slide, a **Bond Slide ID Number** will be assigned automatically and listed in the upper left-hand corner of the window—this Bond Slide ID Number should be entered, along with all patient and tissue information on the Slide Information Table in the Batch Record (Appendix 1, Section 3).

7.11.2.3 For additional slides, click the **Add Slide** button at the bottom of the window. A maximum of 3 slide trays can be run in the Processing Module at one time, yielding 6 CalCon slides and up to 24 clinical slides when filled.

7.11.2.4 Once all slides are entered, click **Close.** Record the total number of slides and trays to be processed in Appendix 1, Section 3.

7.11.2.5 Select the **Print Labels** button at the bottom of the screen to print the labels for the slides. Select **This Case** and click **OK.** If a label does not print correctly, right-click on the label and select **Print Label**.

7.11.2.6 Affix the printed Bond labels to the slides in the tray; be sure they are aligned squarely with the inside edges of the slide so that the Processing Module can scan the information.

7.11.3 Off-line Dewax of Paraffin Dipped Slides

7.11.3.1 This procedure should be followed for all Tumor Biopsy Slides and CalCon slides that have been dipped in paraffin to prolong stability.

- For slides that have not been dipped in paraffin, such as those prepared prior to the release of SOP340522 Rev. D, the dewaxing procedure should be carried out on the Bond-Max Autostainer using the program detailed in Appendix 2, Section 4B.

7.11.3.2 Prepare the reagents for the off-line dewaxing procedure in Tissue-Tek Staining dishes and place both the clinical slides and 2 CalCon slides (per patient slide set) in a Tissue-Tek slide rack. Deparaffinize, rehydrate, and rinse slides as follows:

Number of Containers	Volume and Reagent	Incubation Time
4	200 mL Xylenes	10 min each
4	200 mL Anhydrous ethanol	3 min each
3	200 mL 95% Ethanol	3 min each
3	200 mL DI water	2 min each
1	1X Bond Wash Buffer	Final wash

7.11.3.3 Record the time of initiation of the dewaxing procedure, check off the appropriate box to acknowledge the completion of each incubation step, and record the time the slide rack is placed in the Bond Wash Buffer in the Batch Record (Appendix 1, Section 2A).

7.11.3.4 Remove one slide from the Bond Wash and place it in the appropriate position on a Bond Slide Tray. The first and last slide of each Bond-Max slide tray will be a CalCon slide. One slide tray should contain a single patient's slides; a maximum of 3 slide trays with 8 patient slides and 2 CalCon slides in each tray can be run in one experimental run.

7.11.3.5 Hold a covertile at about a 20° angle above the slide, placing the wicking end of the covertile on the bottom of the frosted end of the slide.

7.11.3.6 Using a transfer pipette gently apply 1X Bond Wash to the tip of the covertile and continue flush while carefully lowering the covertile onto the slide.

7.11.3.7 If bubbles are introduced, remove covertile and repeat application with a fresh covertile.

7.11.3.8 Obtain next slide from the Tissue-Tek container of Bond Wash, place onto the Bond Slide Tray and repeat above covertile application process.

7.11.3.9 Load full Bond Slide Tray onto the BondMax autostainer to begin staining.

7.11.4 Add and Load Reagents for Bond-Max Run

7.11.4.1 Go back to the Bond main menu and select the Reagent icon. Using the hand-held scanner, scan the Research Detection Kit and antibody working solution Containers to enter them into the Processing Module software inventory list.

- If you are using an Open Container or Research Detection Kit that is already in the Reagent list, after scanning the Container/vial the BondMax interface will report the remaining volume (inventory) in that container. If this is **sufficient volume** for your current run, proceed to the next step.
- Otherwise, click "**Refill**" in the pop-up window before placing the containers in the Processing Module. Note: 30-mL Open Containers can only be refilled to a 90-mL volume maximum.

7.11.4.2 Place the Open Containers containing the: "Marker" and "Strp488" working solutions into a reagent tray, then slide the reagent tray into a reagent tray slot at the front of the machine and lock into position. These containers can also be added to the tray containing the Research Detection Kit.

7.11.4.3 Place the Research Detection Kit with the "Wash Buffer" Open Container into a second slot and lock into position; the Wash Buffer Container needs to be placed into the first position of the reagent tray. The Processing Module will scan the reagent container bar codes to verify loading.

7.11.4.4 Place the slide trays into the front of the Processing Module in their corresponding slots until locked in, and then press the **Load/Unload** button on the front of each slot to initiate scanning of the slide labels. Tray 3 will load into the Processing Module closest to the reagent trays.

Note: Once slides are loaded into the Processing Module, the staining procedure needs to be started within 15 min or new slide labels will need to be assigned.

7.11.4.5 Once scanned, go to the computer screen and ensure that all of the labels were read correctly. If a slide label was not read correctly, right-click the corresponding slide and manually select the **Bond Slide ID** in the window.

7.11.5 Once all slides and reagent containers have been scanned, the **Play** button (triangle) will activate on the **System Status Screen** on the computer. Click the **Play** button on the screen to start processing the slides. **Note**: If the **Play** button does not light up, recheck that all trays are loaded correctly and that all containers have been scanned in. An error message will be displayed on the screen. Right-click on the error message and investigate as necessary.

7.11.6 The Bond software will generate a **Batch Number** for the run; record this number as well as the time the run started and the estimated time to completion in the Batch Record (Appendix 1, Section 4A).

Note: If the Bond Universal Covertiles are sticking to the slides during the staining procedure (they normally slide back and forth), it is likely that there is contamination in one of the bulk reagent solutions. Discard slides and all solutions. Clean bulk reagent bottles with a mild bleach solution and then rinse thoroughly with water before reuse.

7.12 Completion of Bond-Max Staining Run

7.12.1 Allow the Prolong Gold Antifade Reagent to equilibrate to ambient temperature; using a heat source to warm the vial is not recommended. If the solution appears cloudy, discard according to your institution's safety guidelines and retrieve a fresh vial. Prolong Gold should be discarded 6 mo after opening.

7.12.2 Just prior to slide staining completion, prepare two 250-mL Tissue-Tek staining dishes:

7.12.2.1 Fill the first staining dish with 200 mL DI water only. Place a Tissue-Tek 24-slide holder into this diH2O staining dish.

7.12.2.2 In second staining dish, prepare the DAPI Working Solution by adding 10 µL of the DAPI Stock Solution to 200 mL DI water, and mix thoroughly. Protect the solution from light by covering the entire dish with aluminum foil. Record the time of Working Solution preparation in the Batch Record (Appendix 1, Section 4B).

7.12.3 At the completion of the BondMax staining run, push the Load/Unload Button to unlock the slide trays; remove the trays from the Processing Module and note the time the run is completed and slide trays are removed in the Batch Record (Appendix 1, Section 4B). **Note:** Once the slides are removed from Processing Module, protect from light.

7.12.4 One slide at a time, remove the Bond Universal Covertile and immediately place it in the 24-slide holder immersed in the DI water staining dish.

7.12.5 Once all the slides are immersed in the DI water containing staining dish, transfer the rack to the DAPI Working Solution staining dish.

7.12.6 Incubate the slides for 50 min at ambient temperature in the dark (cover entire dish with aluminum foil) and gently agitate every 15 min. Record the DAPI staining start time in the Batch Record (Appendix 1, Section 4B).

7.12.7 During the incubation time, fill three additional 250-mL staining dishes with 200 mL DI water each.

7.12.8 After the 50 min DAPI incubation step, remove the slide rack from the DAPI Working Solution and place it into a staining dish containing fresh DI water for 5 min. Record the time slides are removed from the DAPI Working Solution in the Batch Record (Appendix 1, Section 4B).

7.12.8.1 Repeat the DI water wash process two additional times using a fresh DI water staining dish. Confirm the completion of each wash step in the Batch Record (Appendix 1, Section 4B).

7.12.9 One slide at a time:

7.12.9.1 Transfer the slides to a paper towel, and use a Kimwipe to wick away any residual liquid, taking care not to touch the tissue or let it dry out.

7.12.9.2 Using a 1000-µL pipette, place no more than two drops of Prolong Gold Antifade Reagent onto the sections and cover with a cover slip.

7.12.10 Record the time and date all slides have been cover slipped in the Batch Record (Appendix 1, Section 4B). Place the slides in a slide book, lying flat in a safe location. Allow the slides to cure overnight in the dark at ambient temperature.

7.13 Slides should be stored in the dark at 2ºC to 8°C and imaged **18 to 72 h** after cover slipping by following SOP340533.

7.14 Review and finalize the Batch Record and document **ANY** and **ALL** deviations from this SOP during the slide staining process in the Batch Record (Appendix 1, Section 5).

7.15 The Laboratory Director/Supervisor should review the Batch Record and sample reports and sign the Batch Record affirming the data contained within the reports are correct (Appendix 1, Section 7).

7.16 Clean-up

7.16.1 If this is the last experimental run of the day, be sure to **turn off** the Bond-Max Processing Module; this will ensure the lines are cleaned at the beginning of each new day when the module is turned back on. Empty the waste containers as needed.

7.16.2 Store ER Solution 1 and 1X Wash Solution bulk reagent bottles at 2ºC to 8°C. The rest of the bulk reagent containers can remain inside the body of the Bond-Max Processing Module.

7.16.3 Bond Open Containers can be rinsed and used 3 times (90 mL total) for the **same** reagent. It is recommended to always use fresh working solutions, but working antibody solutions can be stored at 2ºC to 8°C and used for up to 5 d after preparation.

7.16.4 Place the Bond Universal Covertiles into anhydrous ethanol for 10 min to clean. Remove from ethanol and dry with a Kimwipe for reuse. If cracked or damaged, discard.

7.16.5 Make sure all Bond-Max daily maintenance procedures have been completed. For overall maintenance, clean the bulk reagent bottles with a mild bleach solution every 3-6 mo; rinse thoroughly with water before reuse. Additionally, at least once per month perform Cleaning and Maintenance as outlined in the Leica Bond User Manual (Section 12, pages 207-243).

8.0 OPTIONAL: SHIP TO CERTIFIED ASSAY SITE FOR ANALYSIS

If the IFA will be performed at a separate certified assay site, ship the slides as follows:

IMPORTANT: Include a copy of the Batch Record for all samples being shipped with the Shipping Manifest.

8.1 Send an e-mail to the certified assay site prior to shipping to advise recipient of scheduled shipping time. Be sure to request and receive a confirmation e-mail prior to shipping.

8.2 Generate a shipping list containing all the specimen records using the Shipping Manifest template as shown in Appendix 2. In the Batch Record, verify that all slides in the slide box are from a single patient(Appendix 1, Section 6).

8.2.1 A Shipping Manifest may contain more than one patient's samples, but a single patient's slide box should contain only a single patient's slides and be clearly labeled.

8.3 **Verify** that the contents of the package match the Shipping Manifest.

8.4 Print and attach the shipping address onto the outside of the shipping container.

8.5 Record the shipping date, time, tracking number, and shipping information in the Batch Record (Appendix 1, Section 6).

8.6 Ship the specimens **with a copy of** the Shipping Manifest and copies of the completed Batch Records for all patient specimens. Retain copies of the completed Shipping Manifest and Batch Records in your records.

8.7 E-mail the certified assay site shipment notification. State "*Protocol Name* PD Specimen Shipment" in the subject line and reference the tracking number and shipping information in the e-mail.

PAGE LEFT BLANK ON PURPOSE

APPENDIX 1: BATCH RECORD

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 Assay: _____

Patient ID: _____

Clinical Protocol Number: _____

1. Reagents

A. Critical Reagents

Critical Reagents supplied for the SOP are listed below. Be sure that the lot number on each of these critical reagents matches those cited in the product insert accompanying the reagents. Reagents from one pack **should not** be exchanged with reagents from another pack.

Reagent Name	Date Received	Lot Number	Stock Reagent Conc'n	Recommended Working Conc'n	Recommended Dilution	Expiration Date
γH2AX-Biotin Conjugate	/ /				1 :	/ /
Strp488 Conjugate	/ /				1 :	/ /
DAPI	/ /		14.3 mM (5 mg/mL)	0.25 µg/mL	1 : 20,000	/ /
CalCon Slides	/ /			N/A		/ /

B. Reagent Log

	Stock Solution		*Working Solution*	
Reagent	Lot#	Expiration Date	Concentration	Preparation Date
10X Bond Wash Solution		/ /	1X Solution	/ /
Bond Primary Antibody Diluent		/ /	N/A	N/A

C. UPI Open Container Log

Open Container labeled "Wash Buffer" _____

Open Container labeled "Marker" _____

Open Container labeled "Strp488" _____

2. Off-line Dewaxing of Paraffin Dipped Slides

Were slides dipped in paraffin to prolong stability? ☐ Yes ☐ No

A. Off-line Dewax Reagent Applications

Record the times and acknowledge the reagent applications step below:

Step	Time
Time that Off-line Dewax Procedure Began	:
Four, 10 min Xylene Incubations Completed	☐ 1 ☐ 2 ☐ 3 ☐ 4
Four, 3 min Anhydrous Ethanol Incubations Completed	☐ 1 ☐ 2 ☐ 3 ☐ 4
Three, 3 min 95% Ethanol Incubations Completed	☐ 1 ☐ 2 ☐ 3
Three, 2 min DI H_2O Rinses Completed	☐ 1 ☐ 2 ☐ 3
Time Slides Placed in 1X Bond Wash Solution	:

3. Preparation of Working Solutions

The calculations below are for **30-mL Containers**; change the residual volume to **300 or 1000 µL** if using a different Container size (Appendix 2, Section 1).

A. γH2AX Ab Working Solution

Recommended dilution of ***γH2AX Ab* STOCK** = 1: _____

e.g., Recommended dilution of ***γH2AX Ab* STOCK** Lot# DAM1460180 is 1:100.

Total number of slides to be stained: _____ x 300 µL/slide	=	µL
Plus corresponding residual volume	+ 1500	µL
Total Vol. needed for staining	**=**	**µL**
Vol. γH2AX Ab (**Total Vol.**/recommended dilution)	- _____	µL*
*Vol. Bond Primary Antibody Diluent **(Total Vol. – *Vol. γH2AX Ab*)**	=	**µL***

Preparation Date: ____/__/____ Time: _____:_____

B. Strp488 Working Solution

Recommended dilution of ***Strp488* STOCK** = 1: _____

e.g., Recommended dilution of ***Strp488* STOCK** Lot# 425913is 1:100.

Total number of slides to be stained: ___ x 150 µL/slide	=	µL
Plus corresponding residual volume	+ 1500	µL
Total Vol. needed for staining	**=**	**µL**
Vol. Strp488 Solution (**Total Vol.**/ recommended dilution)	- _____	µL*
* Vol. Bond Primary Antibody Diluent **(Total Vol. – *Vol. Strp488*)**	=	**µL***

Preparation Date: ____/__/____ Time: _____:_____

4. Slide Information Table:

Number of Slide Trays Loaded into Processing Module: _____

Fill in the Bond Slide ID Number, clinical protocol/CTEP#, and Patient/Sample ID for each slide in the corresponding slide tray position. A maximum of 3 slide trays can be used per run in the Bond-Max Processing Module. The **first and last** slide in the slide tray should be a CalCon slide.

Slide Tray	Slide Position	Bond Slide ID Number	Clinical Protocol/ CTEP#	Patient/Sample ID* and Slide Number	Notes on Slides
Ex:		*05C9*	*12-C-0000/1234*	*1234-1025-500*	
Ex:		*1002*	*N/A*	*CalCon slide: 12001836*	
1	**1**			CalCon slide	
1	**2**				
1	**3**				
1	**4**				
1	**5**				
1	**6**				
1	**7**				
1	**8**				
1	**9**				
1	**10**			CalCon slide	

* For CalCon slides, use the Slide ID in place of the Patient ID.

3. Slide Information Table (cont.)

Slide Tray	Slide Position	Bond Slide ID Number	Clinical Protocol/ CTEP#	Patient/Sample ID* and Slide Number	Notes on Slides
2	**1**			CalCon slide	
2	**2**				
2	**3**				
2	**4**				
2	**5**				
2	**6**				
2	**7**				
2	**8**				
2	**9**				
2	**10**			CalCon slide	
3	**1**			CalCon slide	
3	**2**				
3	**3**				
3	**4**				
3	**5**				
3	**6**				
3	**7**				
3	**8**				
3	**9**				
3	**10**			CalCon slide	

* For CalCon slides, use the Slide ID in place of the Patient ID.

BATCH RECORD: INITIALS _____ DATE: _____

4. Staining of Slides

A. Slide Staining in Bond-Max Processing Module

Date: _____

Bond Batch Number: _____

Start Time: _____

Est. Time to Completion: _____

B. DAPI Staining and Cover Slip Application

Prepare DAPI Working Solution by diluting 10 µL DAPI stock (5 mg/mL) into 200 mL DI water in a 250-mL staining dish. Discard excess Working Solution at end of the assay run.

	Time
Slide Trays Removed From Processing Module	:
DAPI Working Solution Prepared	:
DAPI Working Solution Added to Slides	:
DAPI Working Solution Removed	:
Three, 5 min DI Water Washes Completed	☐ 1 ☐ 2 ☐ 3
ProLong Gold Antifade Reagent With Cover Slips Added	:

5. Notes, including any deviations from the SOP:

6. Shipping to Certified Assay Site

Verify a single patient's slides are in slide box: ☐ Yes ☐ No

Date and time samples shipped: _____

Tracking information: _____

Attach copy of Shipping Manifest

7. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: _____

PAGE LEFT BLANK ON PURPOSE

APPENDIX 2: BOND-MAX PROCESSING MODULE

1. Modifications to SOP for running a single slide tray in Bond-Max System

The SOP is written as if the Certified Assay Operator is running 3 full slide trays each run.

If a single slide tray is regularly run, a Bond Titration Container with Insert (Titration Kit below) or 7-mL Bond Open Containers can be used in place of the 30-mL Bond Open Container for antibody preparation (see table below for volumes and ordering information).

When using a Bond Titration Container with Insert, be sure to scan the bar code on the titration container when programming the Bond-Max System, and clearly label each container as gH2AX Ab or Strp488. The Bond Container Insert should be discarded after use, but the Bond Titration Container can be reused multiple times.

Product	Max. Vol. (mL)	Dead Space (mL)	Actual Vol. (mL)
SOP as written: Bond Open Containers, 30 mL	30	1.5	28.5
Bond Titration Kit (Containers and Inserts; Leica Microsystems, Cat#: OPT9049)	6	0.3	5.7
Bond Open Containers, 7 mL (Leica Microsystems, Cat#: OP79193)	7	1.0	6.0

2. Add the facility or laboratory running the assay to the "Doctors List"

Select "Doctors list..." from the System Configuration Menu. Assign the name of the facility or laboratory running the assay to the Name field and be sure to set the "Preferred" option so the name is available in the drop-down menu when creating new cases.

3. Register new antibodies and Open Containers in the Bond-Max System

A. On the Reagent Screen, add "gH2AX Ab" and "Strp488" to the reagent list as follows:

Field	γH2AX Antibody	Strp488 Antibody
Name:	*gamma H2AX ab*	*Strp488*
Abbreviated name:	*gH2AX Ab*	*Strp488*
Type:	*Primary*	*Ancillary*
Single/double stain	*Single*	*N/A*
Default Staining protocol:	*Tissue_Section_gH2AX_Alexa488*	*N/A*
Default HIER protocol:	*HIER 10 min with ER1*	*N/A*
Default enzyme protocol:	*- - -	*N/A*
Preferred	*Selected*	*Selected*

B. Scan the new Open Container, Titration Kit Container, or Research Detection Kit Container bar codes to open the Add Reagent dialog box. Select gH2AX Ab from the Reagent name drop-down list and label the Container with the antibody name for easy identification. Repeat this procedure with a second Container for Strp488. The Containers will not need to be entered again until a new Container, and therefore new bar code, is used.

4. Staining protocols

Create the following staining protocol (A), Tissue_Section_gH2AX_Strp488, on the Bond-Max Processing Module. Protocols B and C are pre-programmed protocols on the Bond-Max Processing Module and will be used for the γH2AX IFA.

A. **Staining Protocol**: "Tissue_Section_gH2AX_Strp488" (protocol entered by user)

Solution	Temperature °C	Time*
Wash Buffer†	Ambient	0
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	0
Marker	Ambient	30 min
Marker	Ambient	30 min
Bond Wash Solution	Ambient	5 min
Bond Wash Solution	Ambient	5 min
Bond Wash Solution	Ambient	0
Strp488	Ambient	30 min
Bond Wash Solution	Ambient	5 min
Bond Wash Solution	Ambient	5 min
Bond Wash Solution	Ambient	0

*A time of zero indicates that the solution is applied, but that minimal time elapses before the next application.

† The Bond-Max Processing Module requires one established solution be used from its reagent selection list. For the Research Detection Kit, 1X Bond Wash Solution is placed into a 30-mL Open Container and is used in this protocol.

B. **Preparation Protocol**: "*Dewax" (using Processing Module preset protocol)
Note: This protocol is only used if slides are NOT paraffin dipped/dewaxed following the off-line procedure.

Solution	Temperature °C	Time
Bond Dewax Solution	72	30 sec
Bond Dewax Solution	72	0
Bond Dewax Solution	Ambient	0
100% Ethanol	Ambient	0
100% Ethanol	Ambient	0
100% Ethanol	Ambient	0
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	5 min

C. **HIER Protocol:** "*HIER 10 min with ER 1" (using Processing Module preset protocol)

Solution	Temperature °C	Time
Bond ER1 Solution	Ambient	0
Bond ER1 Solution	Ambient	0
Bond ER1 Solution	100	10 min
Bond ER1 Solution	(Cool-down phase)	12 min
Bond Wash Solution	35	0
Bond Wash Solution	35	0
Bond Wash Solution	35	0
Bond Wash Solution	Ambient	3 min

APPENDIX 3: SAMPLE SHIPPING MANIFEST

Ship From: Contact Name: Tel: E-mail:	**Shipping Manifest**	**Ship To:** Attn: Tel: E-mail:

Shipping Date: **Carrier:**

In Package	Item No.	Patient/Sample ID	Clinical Protocol/CTEP#	Item/Description
☒	*Example*	*1234-1025-500 and -501*	*12-C-0000/ 1234*	*Patient slide set stained according to SOP340523*
☐	1			
☐	2			
☐	3			
☐	4			
☐	5			
☐	6			
☐	7			
☐	8			
☐	9			
☐	10			