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National Clinical Target Validation Laboratory (NCTVL)

Applied Developmental Directorate

SAIC-Frederick, Inc.

NCI-Frederick Cancer Research Facility

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Change History

Revision	Approval Date	Description	Originator	Approval
--	10/22/08	New Document	WHY	JJ
A	2/01/10	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
B	12/29/10	Update SOP following in-house assay runs of patient samples. Critical Reagents identified, updated and detailed handling provided.	WHY	JJ

Please check for revision status at

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

and be sure to use the current version.

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Ship to NCTVL or 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 2 slides by H&E for standard histology analysis

Calibrator/Control Slides (DCTD-Provided Critical Reagent)

Ship to NCTVL or Certified Assay Site

SOP340523:
γH2AX IFA for Tumor Biopsy Slides

- Load biopsy slides into Bond-Max Processing Module; maximum of 3 patients, slides loaded so 1 patient per slide tray
- 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

Image within 18 to 72 h

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1.0 PURPOSE

Standardize an immunohistochemical method for detecting and quantifying 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
Cal	=	Calibrator
DAPI	=	4',6-Diamidino-2-Phenylindole
DAB	=	3,3'-Diaminobenzidine
DCTD	=	Division of Cancer Treatment and Diagnosis
DI	=	Deionized
ER	=	Epitope Retrieval
γH2AX	=	Histone H2AX Phosphorylated at Serine 139
H&E	=	Hemotoxylin and Eosin
HIER	=	Heat-Induced Epitope Retrieval
ID	=	Identification/Identifier
IFA	=	Immunofluorescence Assay
LHTP	=	Laboratory of Human Toxicology & Pharmacology
NA	=	Numerical Aperature
NCTVL	=	National Clinical Target Validation Laboratory
PBS	=	Phosphate-Buffered Saline
QC	=	Quality Control
RT	=	Room Temperature
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 H2AX phosphorylated at serine 139 (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.

*Depending on the laboratory, one person may have multiple roles.

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 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, CRITICAL REAGENTS, AND EQUIPMENT REQUIRED

6.1 Anti-phospho-histone H2AX (Ser139), clone JBW301, biotin conjugate with Certificate of Analysis, 1 mg/mL (γH2AX Ab; Millipore, Cat#: 16-193; DCTD-provided Critical Reagent)

6.2 Streptavidin, Alexa Fluor® 488 conjugate; (Strp488; Invitrogen, Cat#: S11223). The DCTD-provided Critical Reagent is supplied as a 1 mg/mL stock solution in 50% glycerol/1X PBS

6.3 DAPI dihydrochloride, FluoroPure™ grade (Invitrogen, Cat#: D21490). The DCTD-provided Critical Reagent is supplied as a 14.3 mM [5 mg/mL] stock solution in DI water.

6.4 Calibrator/control slides (DCTD-provided Critical Reagent)

6.5 Pipettors (100-1000 µL, 50-200 µL, 2-20 µL, 0.2-2 µL) and tips

6.6 50-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352098)

6.7 Fisherfinest premium cover glasses, 50 mm x 22 mm (Fisher Scientific, Cat#: 12-548-5E)

6.8 Kimwipes (e.g., Fischer Scientific, Cat#: 06-666A)

6.9 Slide mailer/folder (e.g., Leica Microsystems, Cat#: 3802617)

6.10 Sterile-filtered, molecular biology grade deionized (DI) water (Invitrogen, Cat#: 10977-015)

6.11 10X phosphate-buffered saline (PBS; Invitrogen, Cat#: 70013-073) [Dilute 1:10 in DI water to prepare 1X PBS for use in assay.]

6.12 Anhydrous ethanol, histology grade (Fisher Scientific, Cat#: A405-20 [Filtered using 0.22 µm pore size before use.])

6.13 ProLong® Gold antifade reagent (Invitrogen, Cat#: P36930)

6.14 Bond-Max Autostainer (Leica Microsystems, Cat#: 21.0051.110)

6.15 Bond Dewax Solution (Leica Microsystems, Cat#: AR9222)

6.16 Bond Epitope Retrieval Solution 1 (Leica Microsystems, Cat#: AR9961)

6.17 Bond Open Container – 10 pack; 30 mL (Leica Microsystems, Cat#: OP309700)

6.18 Bond Research Detection Kit (Leica Microsystems, Cat#: DS9455)

6.19 Bond Primary Antibody Diluent (Leica Microsystems, Cat#: AR9352)

6.20 Bond Slide Labeler Printing Ribbon (Leica Microsystems, Cat#: S21.1912.110)

6.21 Bond Universal Cover-tiles, 100 Pack (Leica Microsystems, Cat#: S21.2001.110)

6.22 Bond Universal Slide Labels (Leica Microsystems, Cat#: S21.2011.110)

6.23 Bond Wash Solution 10X Concentrate (Leica Microsystems, Cat#: AR9590)

6.24 -80°C and -20°C freezers

6.25 4°C refrigerator

6.26 Clinical slides prepared following SOP340522 with paraffin-embedded 1st and 2nd biopsy samples and a positive control sample on each slide

*If instruments and/or reagents differ from those specified above, the Laboratory performing the assay must prove their comparability or equivalence to those recommended using the manufacturer's specifications and experimental validation data.

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). Prior to beginning the assay, read the SOP and ensure sufficient materials and reagents are in stock to run the SOP.

7.1.1 All reagents are to be prepared for use in one experimental run, and only in the amounts required for the specific assay.

7.1.2 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.

7.2 Validated Critical Reagents for this SOP will be provided to clinical sites with Certified Assay Operators trained on the performance of this assay. Each Critical Reagent Pack will contain sufficient material for analysis of paired tumor biopsy samples from 3 patients, with 8 clinical slides and 2 calibrator/control slides per patient. The reagents provided include 1 vial of γH2AX Ab (228 µL/vial), 1 vial of Strp488 conjugate (150 µL/vial), 1 vial of DAPI (10 µL/vial), and 12 calibrator/control slides. DCTD-provided Critical Reagents include a 2-fold surplus, so under optimal conditions, 6 sets of patient samples can be assayed. An Equivalence Pack with known γH2AX levels for laboratory equivalence and system verification is available upon request.

7.3 Check that the lot number of each of these Critical Reagents match the lot number cited in the product inserts provided by DCTD as some Critical Reagent dilutions, specifically for the antibodies, may vary between lots. Record the Lot number and date of receipt in the Batch Record (Appendix 1, Section 1A). Store all reagents as indicated below. All reagents are to be labeled with date of receipt and stored under the specified conditions for no longer than the recommended duration.

7.3.1 **Anti-γH2AX biotin conjugate (γH2AX Ab)** as a 100X (1 mg/mL) stock solution as provided by the manufacturer; Lot#: DAM1460180): Aliquots are prepared so that each is sufficient for up to 6 full slide trays; store frozen at -20°C for up to 3 mo.

7.3.2 **Strp488 conjugate** as a 100X (1 mg/mL) stock solution in 50% glycerol/1X PBS (Lot#: 425913): Aliquots are prepared so that each is sufficient for up to 6 full slide trays; store frozen at -20°C for up to 3 mo.

7.3.3 **Calibrator/control slides:** Lot#: 11001938, 11001939, and 11001940. Store in a desiccator at 2-8°C away from volatile chemicals. Can be stored indefinitely.

7.3.4 **DAPI stock solution** as a 14.3 mM (5 mg/mL) solution in DI water. Aliquots are prepared so that each is sufficient for up to 6 full slide trays; store frozen at -20°C for up to 6 mo. **Note:** DAPI is light sensitive; protect all solutions from light.

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

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

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

7.4.3 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.

7.4.4 If a new Open Container or 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).

7.5 Calibrator/Control and Clinical Slides

7.5.1 Two calibrator/control slides are required for each Bond-Max run and will be the first and last slide in each slide tray.

7.5.2 Clinical samples for this assay will be frozen needle biopsies collected according to SOP340507 and 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 with 8 patient slides and 2 calibrator/control slides in each tray can be run in one experimental run.

7.6 Preparation of Reagents

7.6.1 During reagent preparation, be sure to note the lot number/serial number, expiration dates, and dates of preparation as indicated in the Reagent Log of 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.

7.6.2 1X Bond Wash Solution

7.6.2.1 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 2-8°C out of direct sunlight. 1X Bond Wash Solution can be used for 4 mo.

7.6.2.2 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.

7.6.3 Research Detection Kit

7.6.3.1 Add 30 mL of 1X Bond Wash Solution to the 30-mL Open Container.

7.6.3.2 Add 3 mL of 1X Bond Wash Solution to both the DAB Part 1 and DAB Part 3 containers of the Detection Kit.

7.7 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.

7.7.1 The bulk reagents include: 1X Bond Wash Solution, Bond Dewax solution, anhydrous ethanol, DI water, and Bond Epitope Retrieval (ER) Solution 1.

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

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

7.7.2 **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.

7.8 Preparation of Antibody Working Solutions

7.8.1 Prepare two 30-mL Open Containers for the working antibody solutions by labeling one "γH2AX Ab" and the other "Strp488."

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

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

7.8.3.1 γH2AX Ab Working Solution

- The γH2AX Ab Working Solution should be prepared fresh (1:100 dilution of provided Critical Reagent) using Bond Primary Antibody Diluent.
- 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 γH2AX Ab Bond Open Container; record the preparation date and time in the Batch Record (Appendix 1, Section 2A)**.**

7.8.3.2 Strp488 Working Solution

- The Strp488 Working Solution should be prepared fresh (1:100 dilution of provided Critical Reagent) using Bond Primary Antibody Diluent.
- 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 Bond Open Container; record the preparation date and time in the Batch Record (Appendix 1, Section 2A).

7.8.4 Working antibody solutions can be stored at 2-8°C and reused for up to 5 d after preparation. Return the antibody Critical Reagent vials to -80°C.

7.9 Protocol for Slide Staining in Bond-Max Processing Module

Note: Information on manual staining of slides can be found in Appendix 3.

7.9.1 System Setup for Bond-Max Run

7.9.1.1 If not already on, **turn on** the computer and **open** the Bond software by clicking on the Bond icon, then **turn on** the Bond-Max Processing Module.

7.9.1.2 Place the slides in a Bond-Max Processing Module slide tray in the correct orientation. The first and last slide of each Bond-Max slide tray will be a calibrator/control slide. One slide tray should contain a single patient's slides; a maximum of 3 slide trays with 8 patient slides and 2 calibrator/control slides in each tray can be run in one experimental run.

7.9.1.3 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	Leave blank
Case Comments	Add comments as needed
Doctor	Facility or laboratory running assay (from drop-down list; set up in SOP Step 7.4.1)
Dispense Volume	150 µL
Preparation Protocol	*Dewax

7.9.2 Add Slides to Bond-Max Run

7.9.2.1 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 "Routine"
Process	IHC
Marker	gH2AX Ab
Staining Protocol	Tissue_Section_gH2AX_Strp488
Preparation Protocol	*Dewax
HIER Protocol	*HIER 10 min with ER 1

7.9.2.2 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).

7.9.2.3 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 calibrator/control slides and up to 24 clinical slides when filled.

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

7.9.2.5 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**.

7.9.2.6 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. Add a Bond Universal Cover-tile to each slide, orienting it correctly in the tray (refer to the diagram on the slide tray).

7.9.3 Add and Load Reagents for Bond-Max Run

7.9.3.1 Go back to the Bond main menu and select the Reagent icon. Using the hand-held scanner, scan the Research Detection Kit and 30-mL antibody working solution Open Containers (set up in SOP Step 7.8.3) 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, simply click "**Refill**" in the pop-up window before placing the containers in the Processing Module. Note: 30-mL Open Containers can only be reused 3 times (90-mL volume maximum).

7.9.3.2 Place the Open Containers containing the corresponding working solutions of γH2AX Ab and Strp488 into a reagent tray, then slide the reagent tray into a reagent tray slot at the front of the machine and lock into position.

7.9.3.3 Place the Research Detection Kit with the "Wash Buffer" Open Container into a second slot and lock into position. The Processing Module will scan the reagent container bar codes to verify loading.

7.9.3.4 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.

7.9.3.5 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.

7.9.4 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.

7.9.5 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 Cover-tiles 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.

7.10 Completion of Bond-Max Staining Run

7.10.1 Just prior to slide staining completion, prepare the DAPI Working Solution by adding 1 µL of the DAPI Stock Solution to 25 mL 1X PBS in a 50-mL polypropylene tube, and mix thoroughly. Protect the solution from light. Note the time of preparation in the Reagent Log of the Batch Record (Appendix 1, Section 1B).

7.10.2 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).

7.10.3 Remove the Bond Universal Cover-tiles from the slides, transfer the slides to a paper towel, and wipe away any residual liquid, taking care not to touch the tissue or let it dry out. **Note:** Once the slides are removed from Processing Module, protect from light.

7.10.4 Add 500 µL of DAPI Working Solution to the top of the tissue. All tissue must be covered by the DAPI solution. Incubate the slides for 10 min at RT in the dark (use aluminum foil to create a tent to keep slides in the dark), noting the DAPI start time in the Batch Record (Appendix 1, Section 4B).

7.10.5 After 10 min, use a Kimwipe and wick away the DAPI solution from the sections, taking care not to touch the tissue or let it dry out. Note the time DAPI is removed in the Batch Record (Appendix 1, Section 4B).

7.10.6 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, again noting the time and date 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 RT.

7.11 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).

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

7.13 Clean-up

7.13.1 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.

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

7.13.3 Bond Open Containers can be rinsed and reused 3 times (90 mL total) for the **same** reagent. Working antibody solutions can be stored at 2-8°C and reused for up to 5 d after preparation.

7.13.4 Place the Bond Universal Cover-tiles (SOP Step 7.8.3) into anhydrous ethanol overnight to clean. Remove from ethanol the next morning and dry for reuse. If cracked or damaged, discard.

7.13.5 Make sure all Bond-Max daily maintenance procedures have been completed. In addition, for overall maintenance, clean the bulk reagent bottles with a mild bleach solution every 3-6 mo; rinse thoroughly with water before reuse.

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: _____

Date: _____

Laboratory Director/Supervisor: _____

Date: _____

Facility/Laboratory Running Assay: _____

A maximum of 3 patient slide sets can be run per Batch Record.

1. Reagents

A. Critical Reagents

Critical Reagents validated for this SOP are provided to clinical sites with Certified Assay Operators upon request. Critical Reagents supplied for the SOP are γH2AX Ab, Strp488 conjugate, DAPI, and calibrator/control slides. Make sure that the lot number on each of these critical reagents match those cited in the product insert accompanying the reagents. Record date received and lot number for each reagent in the table below.

Date Received	Reagent Name	Lot Number	Amount Used in Experiment	Amount Remaining
	Anti-γH2AX Biotin Conjugate		µL	µL
	Strp488 Conjugate		µL	µL
	Calibrator/Control Slides		slides	slides
	DAPI		µL	µL

B. Reagent Log

Reagent	*Stock Solution**			*Working Solution*	
	Lot#/Serial#/ UPI#	Expiration Date	Preparation Date	Concentration	Preparation Date/Time
10X Bond Wash Solution			N/A	1X Solution	
DAPI (14.3 mM [5 mg/mL])				572 nM	
Bond Primary Antibody Diluent			N/A	N/A	N/A
Open Container labeled "Wash Buffer"		N/A	N/A	N/A	N/A
Open Container labeled "gH2AX Ab"		N/A	N/A	N/A	N/A
Open Container labeled "Strp488"		N/A	N/A	N/A	N/A

*Stock solutions may not be prepared every time.

2. Preparation of Antibody Working Solutions

A. γH2AX Ab Working Solution (0.01 mg/mL final; SOP Step 7.8.3.1)

Total number of slides to be stained:_____x 300 µL/slide = µL

Plus 800 µL residual volume + 800 µL

Total Vol. needed for staining **= µL**

Vol. γH2AX Ab (1:100 dilution; **Total Vol.**/100) - _____µL*

*Vol. Bond Primary Antibody Diluent **(Total Vol. – *Vol. γH2AX Ab*)** = **µL***

Preparation Date/Time: _____

B. Strp488 Working Solution (0.01 mg/mL final; SOP Step 7.8.3.2)

Total number of slides to be stained: ___x 150 µL/slide = µL

Plus 800 µL residual volume + 800 µL

Total Vol. needed for staining **= µL**

Vol. Strp488 Solution (1:100: **Total Vol.**/100) -_____µL*

* Vol. Bond Primary Antibody Diluent **(Total Vol. – *Vol. Strp488*)** = **µL***

Preparation Date/Time: _____

BATCH RECORD: INITIALS _____ DATE: _____

3. Slide Information Table (SOP Step 7.9.2.2):

Number of Slide Trays Loaded into Processing Module: _____

Total Number of Calibrator/Control Slides (2 per tray): _____

Total Number of Patient Slides
(maximum 3 patients, 8 slides per tray): _____

Fill in the Bond Slide ID Number, clinical protocol/CTEP#, Specimen ID, and Patient 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 each slide tray should be a calibrator/control slide.

Slide Tray	Slide Position	Bond Slide ID Number	Clinical Protocol/ CTEP#	Specimen IDs	Patient ID/ Slide ID*	Notes on Slides
Ex:		*05C9*	*09-C-0000*	*AT12061306 and AT12043306*	*06*	
Ex:		*1002*	*N/A*	*Calibrator/control slide*	*043A*	
1	**1**			Calibrator/control slide		
1	**2**					
1	**3**					
1	**4**					
1	**5**					
1	**6**					
1	**7**					
1	**8**					
1	**9**					
1	**10**					

* For calibrator/control slides, use the Slide ID in place of the Patient ID.

BATCH RECORD: INITIALS _____ DATE: _____

3. Slide Information Table (cont.)

Slide Tray	Slide Position	Bond Slide ID Number	Clinical Protocol/ CTEP#	Specimen IDs	Patient ID/ Slide ID*	Notes on Slides
2	**1**			Calibrator/control slide		
2	**2**					
2	**3**					
2	**4**					
2	**5**					
2	**6**					
2	**7**					
2	**8**					
2	**9**					
2	**10**					
3	**1**			Calibrator/control slide		
3	**2**					
3	**3**					
3	**4**					
3	**5**					
3	**6**					
3	**7**					
3	**8**					
3	**9**					
3	**10**					

* For calibrator/control 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

	Date	Time
Slide Trays Removed From Processing Module		:
DAPI Working Solution Added to Slides		:
DAPI Working Solution Removed		:
ProLong Gold Antifade Reagent With Cover Slips Added		:

5. Notes, including any deviations from the SOP:

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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)	Max. No. Slides
SOP as written: Bond Open Containers, 30 mL	30	0.8	29.2	97
Bond Titration Kit (Containers and Inserts; Leica Microsystems, Cat#: OPT9049)	6	0.8	5.2	17
Bond Open Containers, 7 mL (Leica Microsystems, Cat#: OP79193)	7	0.8	6.2	20

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 (1:100)*	*Strp488*
Abbreviated name:	*gH2AX Ab*	*Strp488*
Type:	*Primary*	*Ancillary*
Single/double stain	*Double*	*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 Bond Open Container bar codes to open the **Add Reagent** dialog box. Select gH2AX Ab from the **Reagent name** drop-down list and label the Open Container with the antibody name for easy identification. Repeat this procedure with a second Open Container for Strp488. The Open Containers will not need to be entered again until a new Open 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
Deionized H_2O	Ambient	5 min
Deionized H_2O	Ambient	5 min
Deionized H_2O	Ambient	5 min

*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 Bond-Max Processing Module preset protocol)

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 Bond-Max Processing Module preset protocol)

Solution	Temperature ^{o}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: OVERVIEW OF MANUAL STAINING OF SLIDES

Reference: Redon CE, Nakamura AJ, Sordet O, Dickey JS, Gouliaeva K, Tabb B, Lawrence S, Kinders RJ, Bonner WM, and Sedelnikova OA. γ-H2AX detection in peripheral blood lymphocytes, splenocytes, bone marrow, xenografts, and skin. In: Didenko VV, ed. *DNA Damage Detection In Situ, Ex Vivo, and In Vivo: Methods and Protocols, Methods in Molecular Biology*. New York, NY: Springer; 2011 [In Press].

1. Materials and reagents needed in addition to those listed in SOP Step 6.0

1.1. Desktop incubator set at 35°C (e.g., Quincy Lab, model 10-140)
1.2. Tween-20 (Roche, Cat#: 11 332 465 001)
1.3. Coplin jars, or similar slide staining containers (e.g., Electron Microscopy Sciences, Cat#: 70315)
1.4. Water bath set at 72˚C and 100˚C

2. Operating Procedure for Manual Staining

Note: Temperature control is important. A 0.05% Tween-20/1X PBS solution may be substituted for the Bond Wash Solution and Bond Primary Antibody Diluent.

2.1. Read through the manual staining protocol and preheat all solutions at designated temperatures so steps can occur sequentially. Time and temperature settings are important for reproducibility.

2.2. Reagent Preparation

2.2.1. Prepare a 05% Tween/1X PBS solution for slide washes.

2.2.2. Preheat all solutions as outlined in the following steps.

2.2.3. Slides can be rinsed in ethanol, Wash Solution, and ER Solution 1 using coplin jars. Dewax Solution and antibody solutions should be applied to the top of the sections and then carefully blotted off before the next slide wash.

2.3. Deparaffinization:

2.3.1. Apply Dewax Solution preheated to 72°C to the slides for 30 sec followed by 2 rinses with Dewax Solution at 72°C. Note: a standard xylene dewax protocol can also be used.

2.3.2. Rinse slides 3 times with ethanol at RT and then twice with Wash Solution at RT.

2.3.3. Incubate slides in fresh Wash Solution for 5 min at RT.

2.4. Antigen Rescue

This step can be performed using solutions preheated in water baths, the Bond-Max Processing Module, or a similar instrument that can hold solutions at defined temperatures.

2.4.1. Rinse the slides 2 times in a coplin jar with ER Solution 1.

2.4.2. Add fresh ER Solution 1 and heat solution to 100°C. Once at 100°C, hold the temperature for 10 min.

2.4.3. Using a 35°C water bath, cool the slides to 35°C in the ER Solution 1 for 12 min.

2.4.4. Rinse slides 3 times with 35°C Wash Solution, and then add fresh Wash Solution and incubate 3 min at 35°C.

2.5. Staining with γH2AX Ab

2.5.1. The γH2AX Ab working concentration is 10 µg/mL in Primary Antibody Diluent.

2.5.2. Rinse slides 3 times with Wash Solution.

2.5.3. Slides are then incubated twice with γH2AX Ab working solution; 30 min each, 35°C. Use sufficient antibody solution to cover all sections (~200 µL per section).

2.5.4. Wash slides 2 times with Wash Solution, 5 min each.

2.6. Developing with Strp488 conjugate

2.6.1. The Strp488 conjugate working concentration is 10 µg/mL in Primary Antibody Diluent.

2.6.2. Slides are incubated with Strp488 conjugate working solution; 30 min each, 35°C. Use sufficient antibody solution to cover all sections (~200 µL per section).

2.6.3. Wash slides 2 times with Wash Solution, 5 min per wash at RT.

2.6.4. Wash slides 3 times with DI water, 5 min per wash at RT.

2.6.5. Wash slides 2 times with 0.05% Tween-20/1X PBS, 5 min per wash at RT.

2.7. Return to SOP Step 7.10 using the manually stained slides in place of the Bond-Max stained slides from the Processing Module.