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LHTP003.8.1.1: γH2AX Immunofluorescence Assay Antibody Qualification and Laboratory Proficiency Testing

Effective Date: 9/3/2013

Please check for revision status of the SOP at

http://dctd.cancer.gov/drug-discovery-development/assays/validated-biomarker-assays

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Version History

1. Approvals

Technical Reviewer:	Lihua Wang	Date: 9-3-2013
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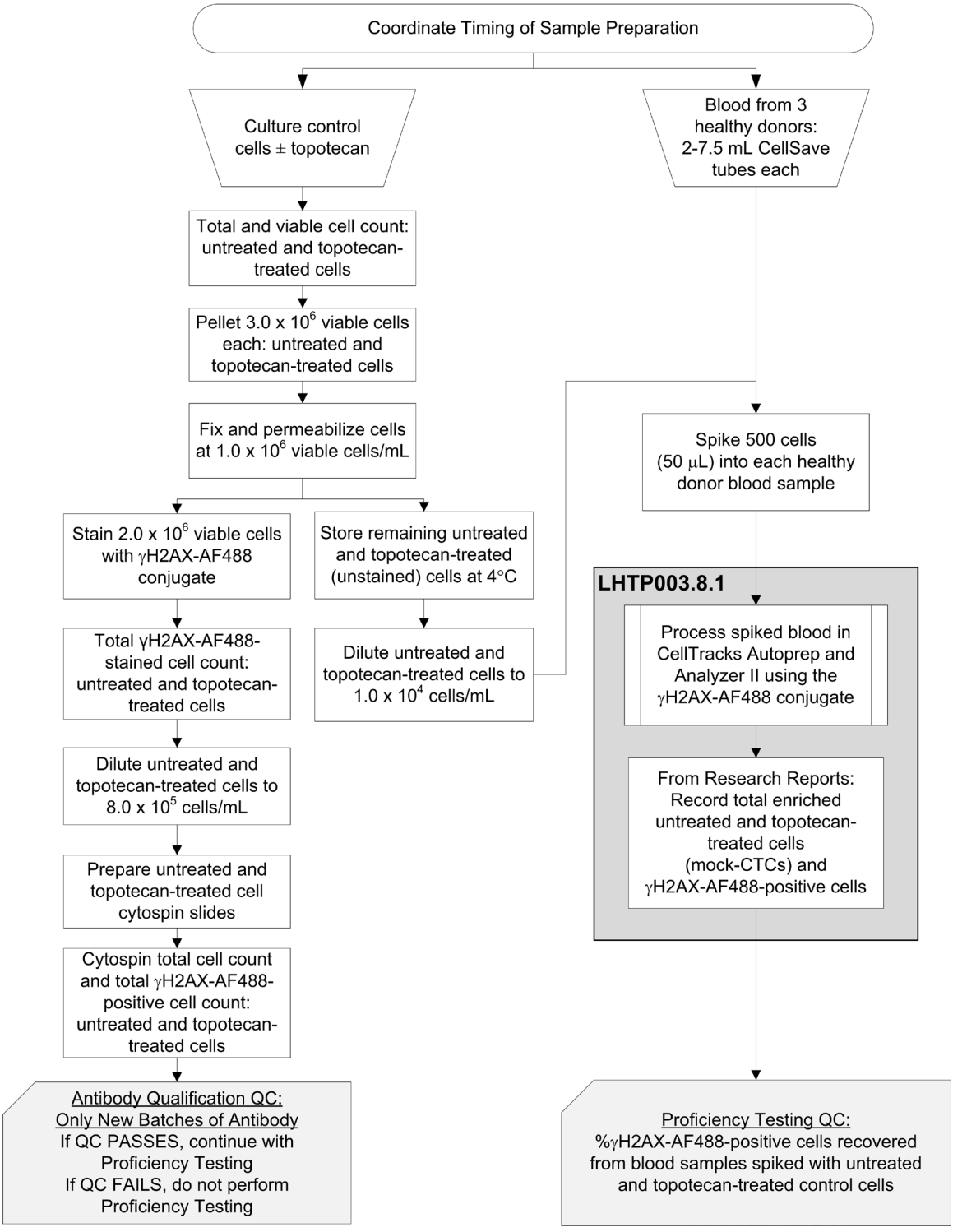
DCTD OD Approval:	Joseph E. Tomaszewski	Date: Tomaszewski, Joseph (NIH/NCI) [E] Digitally signed by Tomaszewski, Joseph (NIH/NCI) [E] DN: cn=Tomaszewski, Joseph (NIH/NCI) [E], o=Office of the Director, ou=OD, DCTD, NCI, email=tomaszej@mail.nih.gov, c=US Date: 2013.09.18 14:01:19 -05'00'

2. Change History

Revision	Approval Date	Description	Originator	Approval
--	7/07/2010	New Document	YAE	RJK
A	12/23/2010	Updates based on first training course. Detailed cell number calculations added to Batch Record.	LW, TP	RJK
B	9/13/2011	Preparation instructions for γH2AX-AF488 working solution updated, preparation of topotecan working solution added, and percent acceptable γH2AX-positive nuclei updated for topotecan-treated cells. Cell concentration in SOP Step 7.9.7 modified to improve automated %NAP determination.	LW, TP	RJK
C	4/6/2012	Dilutions for cell fixation and staining clarified and calculations added to Batch Record. References to LHTP003.8.1 updated (no longer γH2AX-specific). Change SOP Section 7.9 and 7.15 to perform γH2AX staining of spiked blood samples in the CellSearch system on-line; allows LHTP003.8.1 to be run without modification.	YAE, LW	RJK
D	9/3/2013	Details for image capture and manual counting protocol added for cytospin data. Antibody acceptance criteria updated.	TP, SML, LW, KFG	RJK

OVERVIEW OF ANTIBODY QUALIFICATION AND PROFICIENCY TESTING



1.0 PURPOSE

Standardize the method for qualification of new batches of the γH2AX monoclonal antibody conjugated to Alexa Fluor 488 (γH2AX-AF488) and ensure all certified trainees and qualified antibodies pass laboratory proficiency testing.

2.0 SCOPE

All new batches of γH2AX-AF488 conjugated antibody must be qualified following this SOP before they can be used with clinical samples following SOP LHTP003.8.1 "Immunofluorescence Assay for Circulating Tumor Cells Using the CellSearch System." Proficiency testing must be performed during initial laboratory set up, any time a new Certified Assay Operator is added to the laboratory, and each time a new batch of γH2AX-AF488 conjugated antibody is qualified. The goal of the SOP and associated training is to ensure consistency of γH2AX measurement in circulating tumor cells (CTCs) between clinical sites.

3.0 ABBREVIATIONS

AF488	=	Alexa Fluor 488
COA	=	Certificate of Analysis
CTC	=	Circulating Tumor Cell
DAPI	=	4',6-Diamidino-2-Phenylindole
DCTD	=	Division of Cancer Treatment and Diagnosis
DI	=	Deionized
EMEM	=	Eagle's Minimum Essential Medium
FBS	=	Fetal Bovine Serum
FITC	=	Fluorescein Isothiocyanate
γH2AX	=	Histone H2AX phosphorylated at serine 139
IFA	=	Immunofluorescence Assay
LHTP	=	Laboratory of Toxicology and Pharmacology
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PBS	=	Phosphate Buffered Saline
QC	=	Quality Control
RT	=	Room Temperature (25°C ± 3°C)
SOP	=	Standard Operating Procedure
T25 flask	=	25 cm^2 Cell Culture Flask

4.0 INTRODUCTION

The Immunofluorescence Assay (IFA) for CTCs is an immunohistochemistry-based staining assay developed to quantify CTCs positive for a biomarker of interest using a qualified antibody conjugate. The assay uses a qualified primary antibody conjugated to FITC (or similar) dye as the reporter for immunofluorescent identification of cells. CTCs are enriched from whole blood and isolated using the CellSearch System, an automated system for the purification and enumeration of CTCs with the capability of detecting a single CTC in 7.5 mL of whole blood.

5.0 ROLES AND RESPONSIBILITIES

Laboratory Director/Supervisor	The Laboratory Director/Supervisor, directs laboratory operations, supervises technical personnel, reporting of findings, and is responsible for the proper performance of all laboratory procedures. The Laboratory Director/Supervisor oversees the personnel running SOPs within the laboratory and is responsible for ensuring this person(s) is certified and has sufficient experience to handle clinical samples.
Laboratory Scientist	A Laboratory Scientist should have at least a PhD and 3 years or an MS and 5 years of experience in assay validation and have worked in a clinical testing laboratory setting. Laboratory Scientists supervise Laboratory Technicians, review SOP results, perform assay steps requiring specialized scientific skills, and, in the absence of the Laboratory Supervisor, is responsible for the proper performance of all laboratory procedures. The Laboratory Scientist is also responsible for monitoring Batch Record preparation and following-up on any issues that arise during SOP performance that qualify as a deviation.
Certified Assay Operator	A Certified Assay Operator **must be** a 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, in accordance with the current SOP(s), performs laboratory procedures and examinations and any other procedures conducted by a laboratory, including maintaining equipment and records and performing quality assurance activities related to performance.

5.1 It is the responsibility of the Laboratory Director/Supervisor to ensure that all Certified Assay Operators meet the criteria for the role defined as Laboratory Scientist.

5.2 It is the responsibility of the Laboratory Director/Supervisor to ensure that the Certified Assay Operator has received training by Veridex, LLC in the use, operation, and maintenance of the CellSearch System, including the CellTracks AutoPrep System and the CellTracks Analyzer II. Veridex certification is **required** for performance of this SOP. In addition the Certified Assay Operator must have documented DCTD training and certification on this SOP prior to the actual handling and processing of specimens from clinical trial patients.

5.3 It is the responsibility of the Laboratory Director/Supervisor of the specimen collection laboratory to confirm scheduled specimen collection time points, print all labels in advance, check documentation for accuracy, and verify that the required collection tubes, supplies, and equipment are available for successful isolation and preparation of CTCs.

5.4 Certified Assay Operators following this SOP are required to be certified in working safely with bloodborne pathogens in research laboratories in accordance with OSHA Bloodborne Pathogen Standard (29 CFR 1910.1030).

5.5 It is the responsibility of the Certified Assay Operator to ensure timely transport and processing of the samples, enter and review all of the required collection and processing data, and archive all data sheets in the appropriate files.

5.6 The Certified Assay Operator responsible for carrying out 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*** each time a new batch of γH2AX-AF488 antibody is qualified and/or laboratory proficiency testing is verified, with each page ***dated and initialed***.

5.7 The Certified Assay Operator is to check the DCTD Biomarkers Web site (http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm) to verify that the latest SOP version is being followed.

6.0 MATERIALS AND EQUIPMENT REQUIRED

6.1 Critical Reagent

6.1.1 Custom Millipore order (PADIS item number CUS500-AX): Monoclonal anti-phospho Ser139 H2A.X, clone JBW301 (Millipore, Cat#: 05-636) conjugated to Alexa Fluor 488 (Invitrogen, Cat#: A-20000), 1 mg/mL. Store at 2ºC to 8ºC, protected from light.

- Request Certificate of Analysis and Western blot showing mono-specificity of the conjugate for the **exact** lot of γH2AX antibody used for the supplied conjugate.
- Millipore contact: Antonia Shtereva, Product Manager
  Bulk, Custom and OEM Antibodies, EMD Millipore, 290 Concord Road
  Billerica, MA 01821. Tel: 951-265-4621.

6.2 Pipettors (100-1000 µL, 50-200 µL, 2-20 µL) and tips
6.3 Electronic pipettor
6.4 1-, 5-, 10-, and 25-mL pipettes, sterile, individually wrapped (Fisher Scientific, Cat#: 13-675-15C, 13-675-22, 13-675-20, and 13-668-2)
6.5 1.5-mL Sarstedt o-ring screw cap tubes (Fisher Scientific, Cat#: 72.692.005)
6.6 15-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352097)
6.7 50-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352098)
6.8 Vacuum filter/storage bottle system, 0.22-µm pore, 500 mL (e.g., Corning, Cat#: 431097)
6.9 Cell culture flask, 25 cm^2, vent cap (T25; e.g., Corning, Cat#: 3289)
6.10 CellSave Preservative Tubes, 100-pack (Veridex, Cat#: 7900005)
6.11 Kimwipes (Fischer Scientific, Cat#: 06-666A)
6.12 Solvent-resistant laboratory marker
6.13 Hemocytometer and cover slips
6.14 Deionized (DI) water, sterile
6.15 10X phosphate buffered saline (PBS; Invitrogen, Cat#: 70013-073) [Dilute 1:10 in DI water to prepare 1X PBS for use in assay]
6.16 McCoy's 5a Medium Modified, 500-mL bottles (ATCC, Cat#: 30-2007) for HT-29 cells
6.17 Gentamicin sulfate, 50000 mg/L (BioWhittaker, Cat#: 17-518)
6.18 Fetal bovine serum, 500-mL bottles (FBS; Gemini Bio-Products, Cat#: 100-106) [Store at -20°C as 50-mL aliquots in 50-mL polypropylene tubes]
6.19 Trypan blue, 0.4% solution (Sigma-Aldrich, Cat#: T8154)
6.20 Trypsin-EDTA Solution, 1x (0.25% Trypsin/0.53 mM EDTA, ATCC, Cat# 30-2101) or Trypsin-Versene mixture (Lonza, Cat#: 17-161E)
6.21 Topotecan hydrochloride (Hycamtin, GlaxoSmithKline or equivalent; order by prescription through a clinical center). Dissolve in DI water to make a 10 mM stock solution, aliquot, and store at -80°C.
6.22 100% ethanol – histology grade, filtered using 0.22-µm pore size [dilute in DI water to prepare 70% ethanol for use in assay]
6.23 BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences, Cat#: 554714) [contains BD Cytofix/Cytoperm solution and 1X BD Perm/Wash Buffer]
6.24 Bond Wash Solution 10X Concentrate (Leica Microsystems, Cat#: AR9590) [Dilute 1:10 in DI water to prepare 1X Wash Solution for use in assay]
6.25 ProLong Gold antifade reagent with DAPI (Invitrogen, Cat#: P36931)

6.26 HT-29 human colorectal adenocarcinoma (ATCC, Cat#: HTB-38) cells

6.27 Blood from 3 healthy human donors, two tubes per donor with a minimum of 7.5 mL volume per collection

6.28 Automated cell counter (optional; e.g., Cellometer Auto T4 instrument [Nexcellom])

6.29 Table-top centrifuge with a swing-bucket rotor

6.29.1 Sorvall Legend RT centrifuge (Fisher Scientific) with a Swing Bucket Rotor (Fisher Scientific, Cat#: 75-006-434) and manufacturer-recommended tube adaptors

6.30 Cytocentrifuge (200 x *g* speed required) and slides with cytology funnels.

6.30.1 Shandon Cytospin 4 cytocentrifuge (Fisher Scientific, Cat#: A78300002) and Shandon cytology funnel with white filter cards (Fischer Scientific, Cat#: A78710003)

6.31 Fluorescent microscope with 200X objective

6.32 DAPI (e.g., Chroma A4 filter, BP 360/40) and FITC (e.g., Chroma L5 filter, BP 480/40) filter sets

6.33 Monochrome CCD camera

6.34 Class II Type A2 biosafety cabinet/tissue culture hood

6.35 37°C tissue culture incubator, humidified, 5% CO_2

6.36 -20°C freezer

6.37 4°C refrigerator

6.38 SOP LHTP003.8.1 (Immunofluorescence Assay for Circulating Tumor Cells Using the CellSearch System) available on the DCTD Biomarkers Web site.

7.0 OPERATING PROCEDURES

7.1 Prior to beginning antibody qualification and/or laboratory proficiency testing, carefully examine the planned timing of healthy blood donor collection and cell line culture confluence.

7.1.1 Patient blood samples must be processed within 96 h of collection in CellSave Preservative Tubes.

7.1.2 Two 25 cm^2 cell culture flasks (T25 flasks) of cells should be ~80% confluent on the day the SOP is run.

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

7.3 Document the reason for running the protocol in the Batch Record (Appendix 1, Section 1).

Note: SOP Steps 7.10 - 7.12 are unique to antibody qualification; all other steps are required for both antibody qualification and laboratory proficiency testing.

7.3.1 Antibody Qualification:

7.3.1.1 If a new batch of γH2AX-AF488 conjugate antibody is being qualified, Millipore should provide a Western blot showing mono-specificity of the γH2AX antibody using the **exact** lot of γH2AX antibody used to make the conjugate (Appendix 2).

7.3.1.2 Qualification of a new batch of antibody **also** requires completion of laboratory proficiency testing, and can be performed with the same untreated and topotecan-treated HT-29 cell samples.

7.3.2 Laboratory Proficiency Testing:

7.3.2.1 Must be performed during initial laboratory set up, any time a new certified assay operator is added to the laboratory, and each time a new batch of γH2AX-AF488 conjugate antibody is qualified.

7.3.2.2 The proficiency panel for this test is 3 healthy donor blood samples spiked with untreated or topotecan-treated HT-29 cells and then assayed using the CellSearch System as if they were clinical samples.

7.3.2.3 Laboratory proficiency testing can only be performed using a qualified batch of γH2AX-AF488 conjugate.

7.3.2.4 A proficiency testing log (sample in Appendix 3) should be maintained along with the Batch Records to document laboratory compliance.

7.4 In the Batch Record, record the date the γH2AX-AF488 conjugate being used in the assay was received as well as the lot number of the γH2AX antibody used to make the conjugate (Appendix 1, Section 2). If a qualified antibody is being used and only laboratory proficiency testing is being performed, record the date of antibody qualification. All reagents are to be labeled with date of receipt and stored under the specified conditions for no longer than the recommended duration.

7.5 Healthy Donor Blood Collection

7.5.1 Two (2) CellSave tubes containing at least 7.5-mL of blood will be needed from 3 different healthy donors for proficiency testing (6 tubes total).

7.5.1.1 Two identical, pre-printed specimen labels should be prepared for each healthy donor. Labels should include donor number (e.g., Donor 1, 2, and 3), collection date, and space to write the actual collection time.

7.5.2 Blood sample collection laboratory personnel should ensure that the phlebotomist is using the recommended CellSave Preservative Tube to draw the blood samples; if necessary, supply the phlebotomist with the correct tubes. A minimum of 7.5 mL of blood should be collected into the tubes for each blood draw.

7.5.3 Be sure the phlebotomist or sample collection laboratory personnel place the pre-printed labels onto the freshly collected CellSave tubes for identification.

7.5.4 Blood samples should be mixed immediately by gently inverting the tube 8 times to prevent clotting.

7.5.5 Write the actual time of blood sample collection on the pre-printed label. Record the date and time the blood was collected and verify that 2 tubes were collected for each donor, 6 tubes total, in the Batch Record (Appendix 1, Section 3).

7.5.6 Samples should be kept at RT (25°C ± 3°C); **do not** place on ice or refrigerate. Patient blood samples are stable for 96 h in CellSave Preservative Tubes at RT.

7.6 Cell Line Preparation

7.6.1 HT-29 cells maintained in tissue culture should be regularly split to maintain overall cell line health. Cells should be cultured for no longer than 30 passages before using a fresh vial of cells.

7.6.2 Use sterile technique to prepare culture media; media can be prepared ahead of time. Prepare McCoy's 5a medium for HT-29 cells.

7.6.2.1 For one 500-mL bottle of medium, thaw a 50-mL aliquot of FBS in a 37°C water bath, then move to the tissue culture hood and clean the outside of tube with 70% ethanol.

7.6.2.2 Using a disposable filter unit and sterile technique, filter 500 mL medium with 50 mL FBS and 0.5 mL of 10 µg/mL gentamicin sulfate.

7.6.2.3 Label bottle with lot number and expiration dates (if applicable) of media components and date of preparation. Store at 2ºC to 8ºC until use.

7.6.3 Pre-warm medium in a 37°C water bath and 1X PBS and trypsin at RT for 15 to 20 min.

7.6.4 Record the date and current passage number of the cells in the Batch Record and verify that two T25 flasks are ~80% confluent (Appendix 1, Section 4A).

7.6.5 Prepare 1 mL of a 100 µM topotecan working solution by adding 10 µL of a 10 mM topotecan stock solution into 990 µL sterile 1X PBS in a sterile eppendorf tube. Cap and invert tube 5 to 8 times to mix.

7.6.6 In a 15-mL propylene tube, add 100 µL of 100 µM topotecan working solution into 10 mL fresh medium (1 µM final concentration).

7.6.7 In one T25 flask, aspirate old medium, add 10 mL fresh medium + 1 µM topotecan, and label it "topotecan-treated." Record the time that topotecan is added in the Batch Record (Appendix 1, Section 4B). Label the other flask "untreated." Incubate flasks for 2 h at 37ºC in 5% CO_2.

7.6.8 Wash the cells, twice with RT 1X PBS (10 mL), noting the first wash time in the Batch Record (Appendix 1, Section 4B).

7.6.9 Aspirate PBS, add 0.5 mL RT trypsin to each flask, and incubate for 2 to 10 min at 37°C. Record the time trypsin is added to the flasks in the Batch Record (Appendix 1, Section 4C) until cells disperse. Monitor this process under an inverted microscope. To avoid generating cell sheets or clumps, do not agitate the cells by tapping or shaking the flask while waiting for the cells to detach.

7.6.10 Add 8 mL culture medium containing 10% FBS to each flask and pipette cell suspension into 15-mL polypropylene tubes labeled as "untreated" or "topotecan-treated." Record the time culture medium is added to the flasks in the Batch Record (Appendix 1, Section 4C).

7.6.11 Gently pellet cells by centrifugation in a swing-bucket rotor at 200 x *g* for 5 min. Carefully remove the trypsin solution and resuspend each cell pellet in 5 mL 1X PBS.

7.7 Total and Viable Cell Count

7.7.1 Immediately after resuspending the cell pellets, prepare a 1:5 dilution of the untreated and topotecan-treated samples by transferring 20 µL of each cell suspension into a labeled microtube containing 60 µL 1X PBS and 20 µL of 0.4% trypan blue. Gently mix by pipetting (100 µL final volume).

7.7.2 Determine the total and viable cell counts for both untreated and topotecan-treated cells using a hemocytometer (or automated cell counter) and record the following information for both the untreated and topotecan-treated samples in the Batch Record (Appendix 1, Section 5A; see Appendix 4 for information on hemocytometer use):

- Total cell count in each hemocytometer corner square that was counted
- Total viable cell count in each hemocytometer corner square that was counted
- Dilution factor
- The calculated total cells/mL, total cells, viable cells/mL, total viable cells, and percent viability

7.7.3 Cells must be ≥ 85% viable to be used for antibody qualification and laboratory proficiency testing. Indicate whether the untreated and topotecan-treated samples **Pass** or **Fail** percent viability QC in the Bath Record (Appendix 1, Section 5A).

7.7.3.1 If either the untreated or topotecan-treated cells **Fail** percent viability QC (< 85% viable), both sets of cells should be discarded. A fresh culture of cells will need to be used for antibody qualification and/or proficiency testing and a new Batch Record should be started.

7.7.4 Using the calculations in Appendix 1, Section 5B, aliquot 3.0 x 10^6 viable cells for both the untreated and topotecan-treated lines into labeled 15-mL polypropylene tubes for permeabilization and fixation; excess cells can be discarded.

7.7.5 Gently pellet the 3.0 x 10^6 viable cells for each cell treatment by centrifugation in a swing-bucket rotor at 200 x *g* for 5 min.

7.8 Cell Permeabilization and Fixation

7.8.1 Resuspend each cell pellet in 3 mL BD Cytofix/Cytoperm for a final concentration of 1.0 x 10^6 viable cells/mL and incubate for 20 min at 2°C to 8°C. Record the start and end times in the Batch Record (Appendix 1, Section 6).

7.8.2 While cells are fixing, prepare 1.1 mL of a 5 µg/mL γH2AX-AF488 working solution as outlined in Appendix 1, Section 7A (preparing 10% extra). Gently pipette up and down to mix and store at 2°C to 8°C, or on ice, in the dark until use.

Note: 5 µg/mL is the recommended initial test concentration for the γH2AX-AF488 antibody. Use the calculations in Appendix 1, Section 7A if additional concentrations need to be tested.

7.8.3 Gently pellet the fixed cells by centrifugation in a swing-bucket rotor at 500 x *g* for 5 min. Wash fixed cells with 3 mL 1X BD Perm/Wash Buffer and pellet the cells in a swing-bucket rotor at 500 x *g* for 5 min. Carefully remove the buffer and wash and pellet the cells a second time. **Note**: 1X BD Perm/Wash Buffer is used in washing steps to keep cells permeabilized.

7.8.4 Resuspend each fixed and permeabilized cell pellet in 3 mL 1X BD Perm/Wash Buffer for a final concentration of 1.0 x 10^6 viable cells/mL.

7.8.5 For γH2AX-AF488 staining (next step), label two (2) 15-mL polypropylene tubes as "untreated" and "topotecan-treated." Transfer 2 mL of the 1.0 x 10^6 viable cells/mL untreated and topotecan-treated cell suspensions into the correctly labeled tube.

7.8.5.1 Save the remaining 1 mL of 1.0 x 10^6 viable cells/mL suspensions in their original 15-mL polypropylene tubes at 2°C to 8°C for CellSearch analysis in SOP Step 7.14.

7.9 γH2AX-AF488 Staining

7.9.1 Using the 2 mL of aliquoted cells (2.0 x 10^6 cells), pellet the cells in a swing-bucket rotor at 500 x *g* for 5 min. Carefully remove the buffer.

7.9.2 Resuspend each pellet in 0.5 mL of the γH2AX-AF488 working solution. Incubate for 30 min at 2°C to 8°C in the dark. Record the start and end time of γH2AX-AF488 staining in the Batch Record (Appendix 1, Section 8A).

7.9.3 Wash γH2AX-AF488 stained cells with 1 mL 1X BD Perm/Wash Buffer and then pellet the cells at 500 x *g* for 5 min. Carefully remove the buffer and resuspend the cell pellet in an additional 1 mL 1X BD Perm/Wash Buffer.

7.9.4 Total Cell Count of γH2AX-AF488–Stained Cells

7.9.4.1 Prepare a 1:10 dilution of γH2AX-AF488–stained untreated and topotecan-treated cells by transferring 10 µL of cell suspension into a labeled microtube containing 90 µL 1X PBS (no trypan blue). Gently mix by pipetting.

7.9.4.2 Determine the total cells/mL for both untreated and topotecan-treated cells using a hemocytometer (see Appendix 4 for information on hemocytometer use). Record the cell counts and calculate the total cells remaining in the cell suspensions in the Batch Record (Appendix 1, Section 8A).

7.9.5 Dilute γH2AX-AF488–Stained Cell Suspension to 8.0 x 10^5 cells/mL

Note: Selection of the set of calculations to use in Appendix 1, Section 8B for preparation of the **8.0 x 10^5 cells/mL** suspension is based on the 'Total cells/mL' determined in SOP Step 7.9.4. Complete the appropriate portion of the Batch Record (Appendix 1, Section 8B) for untreated and topotecan-treated cells based on the total cells/mL (either ≤ or > 8.0 x 10^5 cells/mL).

7.9.5.1 For samples with a total cell count of **≤ 8.0 x 10^5 cells/mL** complete Appendix 1, Section 8Ba:

- Calculate the total volume of 1X BD Perm/Wash Buffer needed to resuspend the cell pellet ('Total Cells in remaining 0.99 mL') to a final concentration of **8.0 x 10^5 cells/mL**. A minimum of 0.4 mL of each 8.0 x 10^5 cells/mL cell suspension will be needed for the SOP.
- Pellet the cells in a swing-bucket rotor at 500 x *g* for 5 min and then resuspend the cell pellets in the calculated volume of 1X BD Perm/Wash Buffer.

7.9.5.2 For samples with a total cell count of **> 8.0 x 10^5 cells/mL** complete Appendix 1, Section 8Bb:

- Calculate the total volume of 1X BD Perm/Wash Buffer to add to the current cell suspension (0.99 mL) for a final concentration of **8.0 x 10^5 cells/mL**.
- Add the calculated volume of 1X BD Perm/Wash Buffer to the cell suspension and, if necessary, use a larger tube to get even mixing of cells.

7.9.5.3 Record the date and time in the Batch Record that the cells are resuspended/diluted to 8.0 x 10^5 cells/mL (Appendix 1, Section 8C).

7.10 Prepare Cytospin Slides

Note: SOP Steps 7.10 - 7.12 are required for antibody qualification. If a qualified antibody is already being used, skip to SOP Step 7.13 for laboratory proficiency testing.

7.10.1 Clearly label one cytospin slide "untreated" and one cytospin slide "topotecan-treated." Close cytology funnel slide support firmly and place into the rotor of the cytocentrifuge, being sure to balance the samples.

7.10.2 Use a 1000-µL pipette and load 0.3 mL of each 8.0 x 10^5 cells/mL suspension onto the correct slide funnel. Be sure that each sample is deposited onto the cell deposition area

on the bottom of the sample chamber and does not drip down the sides of the funnel. Save the remaining cell suspension for laboratory proficiency testing.

7.10.3 Set the cytocentrifuge to spin the cytology slides at no greater than 200 x *g* for 5 min (if using a Shandon Cytospin 4 system, 600 rpm is recommended). Record the speed of the cytocentrifuge and time the cytospin run begins and ends in the Batch Record (Appendix 1, Section 9A).

7.10.4 Once the cytocentrifuge has stopped, remove the cytology funnels one at a time. Detach the cytology funnels from each slide, being careful not to smear the slides.

7.10.5 Use a solvent-resistant laboratory marker trace a circle around the deposited cell spots; leave about a 3 mm space between the cells and the line.

7.10.6 Place the slides face up on a paper towel to air dry at RT for 2 min.

7.10.7 Using a 1000-µL pipette, place no more than two drops of Prolong Gold antifade reagent with DAPI onto the cytospin cell spot and cover with a cover slip. Record the date and time in the Batch Record (Appendix 1, Section 9B).

7.11 γH2AX-AF488–Stained Cytospin Slide Image Capture

7.11.1 Turn on the microscope, camera, and fluorescent illuminator. Wait at least 2 min for the fluorescent light source to warm up before imaging.

7.11.2 Images should be captured with a monochrome CCD camera and saved as a merge of the DAPI and FITC channels (example in Appendix 5). Set the camera settings to default or manually adjust the gain/gamma so there is no signal enhancement. For cooled cameras, allow the camera to reach operating temperatures before imaging.

7.11.3 Start your desired image acquisition software.

7.11.4 Place the slide on the slide stage and examine each cytospin cell spot on the fluorescent microscope at 200X final magnification using the DAPI filter.

7.11.5 Image capture guidelines (minimum of 200 nuclei needed for QC):

7.11.5.1 With the DAPI channel, identify a representative region on the slide where stained cells occupy the majority of the field of view with little to no cell overlap/clumping.

7.11.5.2 Still on the DAPI channel, use the auto-exposure function on the camera to get the DAPI exposure time for the representative region; record the exposure time in the Batch Record (Appendix 1, Section 10). The DAPI exposure should result in cells that are visible but not overly intense. *It is recommended to adjust the DAPI exposure to 75% of the auto-exposure in most cases; however, different equipment may require alternate settings.*

7.11.5.3 Acquire an image from the DAPI channel.

7.11.5.4 Switch to the FITC channel and use the auto-exposure function on the camera to get the FITC exposure time; record the exposure time in the Batch Record (Appendix 1, Section 10). The FITC exposure should result in cells that are visible and intense, but the majority should not be saturated. *It is recommended to adjust the FITC exposure to 150% of the auto-exposure in most cases; however, different equipment may require alternate settings.*

7.11.5.5 Acquire an image from the FITC channel.

7.11.5.6 Using the image acquisition software, merge the DAPI (blue) and FITC (green, γH2AX-AF488) channels into a single image and save as a TIFF file for further analysis (example in Appendix 5). Make sure no lookup table (LUT) enhancement is saved with the image.

7.11.6 Using the DAPI and FITC exposure times determined (Appendix 1, Section 10), image a minimum of 3 fields per treated and untreated control samples as described in SOP Step 7.11.5. A minimum of 200 nuclei from each are needed per treated and untreated samples.

7.12 Percent γH2AX-AF488–Positive Nuclei

7.12.1 View images in RGB 8-bit (20x fields, 1x zoom). For each field, count DAPI-and γH2AX-AF488 (FITC)-positive cells as outlined below. Repeat with additional fields until a minimum of 200 DAPI-positive nuclei are scored for each sample.

7.12.1.1 Count the total number of DAPI-positive nuclei and record in the Batch Record (Appendix 1, Section 11).

- DAPI (nuclei) signal will be in blue.
- Properly prepared slides should have intact cells with spherical nuclei and minimal evidence of cell lysis or apoptosis.

7.12.1.2 Count the total number of scored DAPI-positive nuclei (previous step) that have γH2AX-AF488–positive staining and record in the Batch Record (Appendix 1, Section 11).

- γH2AX-AF488 signal will be in green
- Only γH2AX-AF488 (green) signal that overlaps with DAPI (blue, nuclei) are counted as positive for γH2AX.
- Cells are only considered positive or negative as a binary function (yes/no); for cells to be considered positive, they must have obvious green staining of the nucleus.

7.12.2 Calculate the percent γH2AX-AF488–positive nuclei for both the untreated and topotecan-treated samples and record in the Batch Record (Appendix 1, Section 11).

7.13 Antibody Qualification QC

7.13.1 Based on the calculated percent γH2AX-AF488–positive nuclei (Appendix 1, Section 12) and the following QC criteria, indicate **Pass** or **Fail** for the untreated and topotecan-treated cells in the Batch Record (Appendix 1, Section 12A):

- Untreated HT-29 cells should have < 8% γH2AX-AF488–positive nuclei. A value of zero is considered acceptable.

- Topotecan-treated HT-29 cells should have ≥ 17% γH2AX-AF488–positive nuclei.
- The ratio of % γH2AX-AF488–positive nuclei for the topotecan-treated HT-29 cells to the untreated HT-29 cells should be ≥ 4.0.

7.13.2 If both of these QC criteria are met, the new batch of γH2AX-AF488 conjugate antibody is now considered a qualified reagent. Indicate **PASS** in the Batch Record (Appendix 1, Section 12B).

IMPORTANT: The qualified antibody has to pass laboratory proficiency testing before it can be used to analyze clinical samples. Proceed to SOP Step 7.14 to complete proficiency testing.

7.13.3 If the either of the QC criteria are **not** met, indicate **FAIL** in the Batch Record (Appendix 1, Section 12B). Do not proceed further with the SOP.

7.13.3.1 If this is the first time antibody qualification has failed, rerun the SOP with a fresh preparation of HT-29 cells and a new Batch Record.

7.13.3.2 If antibody qualification fails a second time, this lot of antibody should be labeled as failed QC, and a new lot of antibody should be ordered.

7.14 Spike Untreated and Topotecan-Treated Cells Into Healthy Donor Blood

Important: A qualified lot of γH2AX-AF488 conjugate antibody is required for laboratory proficiency testing. Newly qualified γH2AX-AF488 conjugate antibodies must also pass laboratory proficiency testing.

7.14.1 Label 15-mL AutoPrep tubes (included with the CTC kit). For each set of 2 CellSave tubes from each healthy donor (3 donors, 6 tubes total), label one 15-mL AutoPrep tube "untreated" and the other "topotecan-treated" to identify which cell sample will be spiked into the tube and include the donor number on all tubes. Blood samples should be at RT (25°C ± 3°C); **do not** place on ice or refrigerate.

7.14.2 Using a 10-mL pipette, transfer 7.5 mL of blood from the CellSave tube to the corresponding labeled 15-mL AutoPrep tube, and then add 6.5 mL Dilution Buffer (also included with the CTC kit). Be sure the buffer has reached room temperature before addition.

7.14.3 With the remaining (unstained) 1 mL of 1.0 x 10^6 viable cells/mL untreated and topotecan-treated cell suspensions from SOP Step 7.9.5.1, prepare a **1.0 x 10^4 cells/mL** suspension of both untreated and topotecan-treated cells in 1X BD Perm/Wash Buffer. Use the calculations in the Batch Record (Appendix 1, Section 13A), and record the date and time of preparation.

7.14.4 Spike 50 µL of the **1.0 x 10^4 cells/mL** untreated or topotecan-treated cells (500 ± 50 cells) into the pre-labeled AutoPrep tubes of healthy donor blood and buffer. Record the time cells are spiked into the blood samples in the Batch Record (Appendix 1, Section 13B). In addition, verify **each** donor has 1 untreated and 1 topotecan-treated spiked blood sample (Appendix 1, Section 13B).

7.15 Spiked Sample CTC Enumeration

Note: Untreated and topotecan-treated cells serve as mock-CTCs during enumeration.

7.15.1 Follow **LHTP003.8.1** (Immunofluorescence Assay for Circulating Tumor Cells Using the CellSearch System) beginning at SOP Step 7.5.

7.15.2 Attach the LHTP003.8.1 Batch Record and copies of the CellTracks Analyzer II Research Reports for each sample to the Batch Record (Appendix 1, Section 14).

7.16 Proficiency Testing QC

7.16.1 Using the CellTracks Analyzer II Research Reports from the cell line-spiked blood samples, complete Appendix 1, Section 15A in the Batch Record by recording the total number of cells and γH2AX-AF488–positive cells enriched from each spiked blood sample.

7.16.2 For each blood sample, calculate the percent γH2AX-positive cells and enter this in the Batch Record (Appendix 1, Section 15A).

7.16.3 Using the following the QC criteria indicate **Pass** or **Fail** for each untreated and topotecan-treated cell-line–spiked blood sample in the Batch Record (Appendix 1, Section 15A).

- Blood samples spiked with untreated cells should have < 3% isolated γH2AX-AF488–positive cells (a value of zero is acceptable).
- Blood samples spiked with topotecan-treated cells should have ≥ 10% isolated γH2AX-AF488–positive cells.

7.16.4 If both of these QC criteria are met for all 3 healthy donors, indicate **PASS** for overall laboratory proficiency testing in the Batch Record (Appendix 1, Section 15B), and the laboratory and/or new operator can analyze clinical samples following SOP LHTP003.8.1 using a qualified antibody.

7.16.5 If the above QC criteria are **not** met for any of the healthy donor samples, indicate **FAIL** for overall laboratory proficiency testing in the Batch Record (Appendix 1, Section 15B).

7.16.5.1 If this is the first time proficiency testing has failed, rerun the SOP a second time with a fresh preparation of cells and a new Batch Record.

7.16.5.2 If the proficiency testing fails a second time, a new batch of antibody will need to be ordered or if this is a new operator, additional training may be needed.

7.17 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 16).

7.18 A second certified Veridex user should review the Batch Record and sample reports and sign the Batch Record affirming that all antibody qualification and/or laboratory proficiency testing criteria are met (Appendix 1, Section 17).

7.19 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 18).

APPENDIX 1: BATCH RECORD

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

Certified Assay Operator: _____

Veridex certification number: _____

DCTD certification number: _____

Date: ____/____/_____

Facility/Laboratory Running SOP: _____

1. Reason(s) for Running SOP (SOP Step 7.2)

A. Qualifying a New Batch of γH2AX-AF488 Conjugate

☐ YES ☐ NO

B. Reason(s) for Laboratory Proficiency Testing

☐ Initial Laboratory Set-up

☐ New Batch of γH2AX-AF488 Conjugate (replied YES above)

☐ Antibody Requalification (6 month interval)

☐ New Certified Assay Operator (should be same operator as listed above)

☐ Other: _____

2. Antibody Information (SOP Step 7.3)

Date γH2AX-AF488 conjugate received: _____

Lot # γH2AX antibody in conjugate: _____

Stock concentration of γH2AX antibody (ug/mL) _____

If already qualified, date of antibody qualification: _____

3. Healthy Donor Blood Collection (SOP Step 7.6.5)

	Collection Date MM/DD/YY	Collection Time	Verify 2 Tubes of Blood Collected
Donor 1	/ /	:	☐ Yes
Donor 2	/ /	:	☐ Yes
Donor 3	/ /	:	☐ Yes

4. Cell Line Preparation (SOP Step 7.7)

A. HT-29 Cell Line Information

Cell Passage Number: _____

Verify 2 flasks of cells are ~80% confluent: ☐ Yes

Date: _____ / / _____

B. Topotecan Treatment

		Untreated Cells	Topotecan-Treated Cells
Time 1 µM topotecan added:		N/A	:
Time 1st PBS wash:		:	:

C. Cell Harvest

Time trypsin added: _____ : _____

Time culture medium added: _____ : _____

5. Total and Viable Cell Count (SOP Step 7.8)

A. Total and Viable Cell Count

(with trypan blue)	Untreated Cells				Topotecan-Treated Cells			
Hemocytometer area (Appendix 4):	A	B	C	D	A	B	C	D
a. Total cells per hemocytometer square counted:								
b. Viable cells per hemocytometer square counted:								
c. Hemocytometer dilution factor:	1: ___				1: ___			

$$\text{CELLS/ML} = \left(\frac{\text{Cells in A+B+C+D}}{4} * 10{,}000\right) * \text{dilution factor}$$

d. Calculated ***Total*** CELLS/ML:	CELLS/ML	CELLS/ML
e. ***Total Cells*** in remaining 4.98 mL: (CELLS/ML (d.) * 4.98 mL)	***Total Cells***	***Total Cells***
f. Calculated ***Viable*** CELLS/ML:	CELLS/ML	CELLS/ML
g. ***Total Viable Cells*** in remaining 4.98 mL: (***Viable*** CELLS/ML (f.) * 4.98 mL)	***Total Viable Cells***	***Total Viable Cells***
h. Percent viability: ([***Total Viable Cells*** (g.)/***Total Cells*** (e.)]*100)	%	%
i. Percent viability (h.) must be ≥ **85%** to Pass QC:	☐ Pass ☐ Fail	☐ Pass ☐ Fail

B. Aliquot 3.0 x 10^6 ***Viable Cells***

	Untreated Cells	Topotecan-Treated Cells
j. Divide 3.0 x 10^6 cells by concentration of 'Calculated ***Viable*** CELLS/ML (f.)' to get Volume Cell Suspension to use for Permeabilization and Fixation	mL	mL

6. Cell Permeabilization and Fixation (SOP Step 7.9.1)

BD Cytofix/Cytoperm incubation: Start Time: _____ : _____

End Time: _____ : _____

7. γH2AX-AF488 Conjugate Cell Staining (SOP Step 7.9.2 and 7.10.2)

Place the γH2AX-AF488 conjugate **STOCK** on ice. For two cell preparations (untreated and topotecan-treated), prepare 1.1 mL (0.55 mL/tube * 2; preparing 10% extra) γH2AX-AF488 Working Solution using the following calculations:

A. Calculate the required amount of γH2AX-AF488 conjugate **STOCK to prepare 1.1 mL of** γH2AX-AF488 Working Solution using the calculation below.

Initial test concentration of γH2AX-AF488 Working Solution is 5 µg/mL; use the following calculations if another concentration needs to be tested.

$$\frac{\text{Working Conc. of γH2AX-AF488 conjugate}}{\text{γH2AX-AF488 conjugate STOCK}} * 1.1\text{ mL} * 1000\ \mu\text{L/mL} = \underline{\text{XX}}\ \mu\text{L γH2AX-AF488 STOCK}$$

$$\frac{\text{µg/mL}}{\text{___ µg/mL γH2AX-AF488 conjugate STOCK}} * 1.1\text{ mL} * 1000\ \mu\text{L/mL} = \text{____}\ \mu\text{L γH2AX-AF488 STOCK}$$

B. γH2AX-AF488 Working Solution

1.1 mL	1X BD Perm/Wash Buffer
___ µL	γH2AX-AF488 conjugate **STOCK**

γH2AX-AF488 incubation: Start Time: _____ : _____

End Time: _____ : _____

8. γH2AX-AF488–Stained Cells

A. Total Cell Count of γH2AX-AF488–Stained Cells (SOP Step 7.10.4)

(no trypan blue)	Untreated Cells				Topotecan-Treated Cells			
Hemocytometer area:	A	B	C	D	A	B	C	D
a. Total cells per hemocytometer square counted:								
b. Hemocytometer dilution factor:	1: ___				1: ___			
CELLS/ML = ($\frac{\text{Cells in A+B+C+D}}{4}$ * 10,000) * dilution factor								
c. Calculated total CELLS/ML:	CELLS/ML				CELLS/ML			
d. ***Total Cells*** in remaining 0.99 mL: (CELLS/ML (c.) * 0.99 mL)	***Total Cells***				***Total Cells***			

B. Resuspend γH2AX-AF488–Stained Cells at 8.0 x 10^5 cells/mL (SOP Step 7.10.5)

Note: Complete only the portions that apply. For example, for the untreated and topotecan-treated cells, one sample may be calculated using the ≤ **8.0 x 10^5 cells/mL** section (a) and the other using the > **8.0 x 10^5 cells/mL** section (b).

	Untreated Cells	Topotecan-Treated Cells
a. **≤ 8.0 x 10^5 CELLS/ML**		
Divide '***Total Cells*** in remaining 0.99 mL' by 8.0 x 10^5 cells/mL	$\frac{\textit{Total Cells}}{8.0 \times 10^5 \text{ cells/mL}}$	$\frac{\textit{Total Cells}}{8.0 \times 10^5 \text{ cells/mL}}$
Equals Vol. 1X BD Perm/Wash Buffer Used to Resuspend Pellet	mL	mL
b. **> 8.0 x 10^5 CELLS/ML**		
Divide '***Total Cells*** in remaining 0.99 mL' by 8.0 x 10^5 cells/mL	$\frac{\textit{Total Cells}}{8.0 \times 10^5 \text{ cells/mL}}$	$\frac{\textit{Total Cells}}{8.0 \times 10^5 \text{ cells/mL}}$
Equals Final Vol. Needed	mL	mL
Minus Starting Vol.	- 0.99 mL	- 0.99 mL
Equals Vol. 1X BD Perm/Wash Buffer to Add to Suspension	mL	mL

C. Date and Time γH2AX-AF488–Stained Cells Resuspended/Diluted to 8.0 x 10^5 cells/mL

Date ___/___/___ Time _____:_____

9. Cytospin Slide Preparation (SOP Step 7.11)

Note: If a qualified antibody is already being used, skip to Appendix 1, Section 13.

A. Cytocentrifuge

Speed (200 x *g* max.): _____

Spin Start Time _____ : _____ Spin End Time _____ : _____

B. ProLong Gold Antifade Reagent with DAPI and Cover Slips Added

Date ___ / ___ / ___ Time _____ : _____

10. DAPI and FITC Channel Exposure Times

DAPI exposure time: _____

FITC (γH2AX-AF488) exposure time: _____

11. Percent γH2AX-AF488–Positive Nuclei (SOP Step 7.12)

	Untreated Cells	**Topotecan-Treated Cells**
Total DAPI-positive nuclei scored (minimum 100 nuclei)		
Total DAPI-positive–scored cells that have γH2AX-AF488–positive nuclei		
% γH2AX-AF488–positive nuclei ([DAPI-positive/γH2AX-positive]*100)	**%**	**%**

12. Antibody Qualification Criteria (SOP Step 7.13)

A. Antibody QC Criteria

	Untreated Cells	**Topotecan-Treated Cells**
% γH2AX-AF488–positive nuclei to pass QC	**< 8%**	**≥ 17% AND Ratio of ≥ 4.0.**
Pass/Fail	☐ Pass ☐ Fail	☐ Pass ☐ Fail

B. Overall Antibody Qualification

Both untreated and topotecan-treated cell line samples need to pass QC using the new batch of γH2AX-AF488 conjugate.

Check One: ☐ **PASS** ☐ **FAIL**

13. Spiked Healthy Blood Samples (SOP Step 7.14)

A. Prepare 1.0 x 10^4 cells/mL untreated and topotecan-treated samples as follows:

10 µL 1.0 x 10^6 cells/mL untreated or topotecan-treated cells
990 µL 1X BD Perm/Wash Buffer.

Date ____/____/____ Time _____:_____

B. Spike Blood Samples

Time _____:_____

Verify cells are spiked into each sample	Untreated Cells	Topotecan-Treated Cells
Donor 1	☐ Yes	☐ Yes
Donor 2	☐ Yes	☐ Yes
Donor 3	☐ Yes	☐ Yes

14. Attach the following documents (SOP Step 7.15)

- LHTP003.8.1 Batch Record for Healthy Donor Spiked Samples
- CellTracks Analyzer II Research Reports for Each Sample From Each Healthy Donor

15. Laboratory Proficiency Testing Criteria (SOP Step 7.16)

A. Individual Spiked Sample QC Criteria

	Spike Sample	Total Cells (mock-CTCs)	Total γH2AX-AF488–Positive Cells	% γH2AX-AF488–Positive Cells	Pass/Fail*
Donor 1	Untreated cells:			%	☐ Pass ☐ Fail
	Topotecan-treated cells:			%	☐ Pass ☐ Fail
Donor 2	Untreated cells:			%	☐ Pass ☐ Fail
	Topotecan-treated cells:			%	☐ Pass ☐ Fail
Donor 3	Untreated cells:			%	☐ Pass ☐ Fail
	Topotecan-treated cells:			%	☐ Pass ☐ Fail

*** Pass criteria**: Untreated cells: < 3% γH2AX-AF488–positive nuclei
Topotecan-treated cells: ≥ 10% γH2AX-AF488–positive nuclei

B. Overall Laboratory Proficiency Testing

All spiked donor samples need to pass QC in order for laboratory to pass the proficiency testing:

Check One: ☐ **PASS** ☐ **FAIL**

16. Notes, including any deviations from the SOP:

17. Second Certified Operator Review of Clinical Samples

Certified Assay Operator: _____ (PRINT)

_____ (SIGN)

Veridex certification number: _____

Date: ____/____/____

18. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: ____/____/____

APPENDIX 2: COMPANY-SUPPLIED WESTERN BLOT

1. Text accompanying the γH2AX Western blot provided within the Certificate of Analysis (COA) specifically states that the blot is a "representative blot from a previous lot" of antibody (see below). According to Millipore, the text is part of a standard COA template document. If a blot from the exact lot of antibody used is specifically requested, Millipore replaces the "representative blot" with the blot from the exact lot; however, the text in the template document is not changed.

2. Millipore should send an e-mail verifying that the blot in the COA is from the exact lot of γH2AX antibody used to create the conjugate, and listed on the COA (as requested with order). Be sure you receive and save this e-mail along with the COA.

upstate
cell signaling solutions
Certificate of Analysis

10 Old Barn Road • Lake Placid, NY 12946
Technical Support: T: 800 548-7853 • F: 518 523-4513
email: techserv@upstate.com
Sales Department: T: 800 233-3991 • F: 781 890-7738
Licensing Dept.: 800 310-4659
www.upstate.com

Anti-phospho-Histone H2A.X (Ser139), clone JBW301
(mouse monoclonal IgG_1)
Catalog # 05-636
Lot # 23760

Immunogen: KLH-conjugated, synthetic peptide (C-KATQA[pS]QEY) corresponding to amino acids 134-142 of human histone H2A.X. The immunizing sequence has 8 identical amino acids in yeast and mouse. Clone JBW301.

Specificity: Recognizes Histone H2A.X phosphorylated at Ser139, Mr 14kDa.

Species Cross-reactivity: Human. A broad species cross-reactivity is expected based on conservation of sequence homology.

Storage and Stability: Stable for 2 years at -20°C from date of shipment. For maximum recovery of product, centrifuge the vial prior to removing the cap.

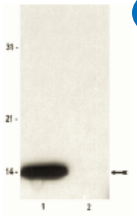
Formulation: **200µg** of protein G purified mouse IgG_1 in **200µl** of 0.1M Tris-glycine, pH 7.4, 0.15M NaCl, 0.05% sodium azide before the addition of glycerol to 30%. Liquid at -20°C.

FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS

Quality Control Testing

Immunoblot Analysis: 0.05-1µg/ml of this lot detected phosphorylated histone H2A.X (Ser139) in acid extracted histone lysates from Jurkat cells treated with 0.5µM staurosporine (Catalog # 19-123).

Immunocytochemistry: 2µg/ml of this antibody detected phosphorylated histone H2A.X in Jurkat cells treated with 0.5µM staurosporine for 4-6 hours.



Immunoblot Analysis
Representative blot from a previous lot. Staurosporine-treated (lane 1), and untreated (lane 2) Jurkat cell lysates were resolved by electrophoresis, transferred to nitrocellulose and probed with anti-phospho-Histone H2A.X (Ser139) (0.05µg/ml). Proteins were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a chemiluminescence detection system. Arrow indicates phosphorylated H2A.X (14kDa).

APPENDIX 3: SAMPLE LABORATORY PROFICIENCY TESTING LOG

Each laboratory should maintain a proficiency testing log outlining the date each proficiency test was run, name of Certified Assay Operator, reason for the test, and whether it passed or failed. Batch Records for the proficiency tests recorded in the log should be stored in a secure location.

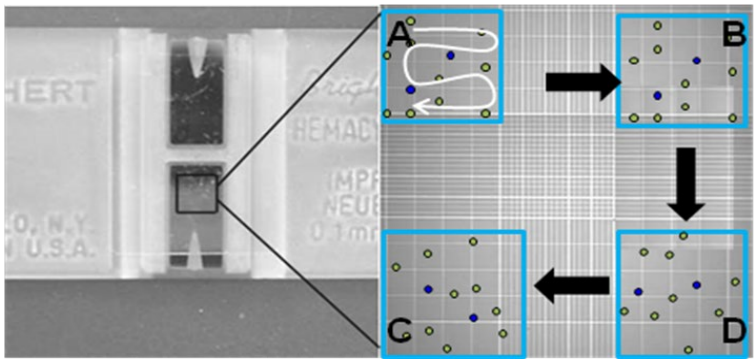
Laboratory Proficiency Testing Log

Date*	Certified Assay Operator	Reason for Proficiency Test†	Pass/Fail
01/01/2012	*Jane A. Doe*	*(1) Initial laboratory set-up*	*Pass*

* Date entered in the log should correspond to a specific Batch Record (Appendix 1).

† Reasons for proficiency testing: (1) initial laboratory set-up, (2) new batch of qualified γH2AX-AF488 antibody, (3) new Certified Assay Operator in the laboratory, (4) other – include explanation.

APPENDIX 4: HEMOCYTOMETER CELL COUNT



1. Prepare hemocytometer chamber and cover glass for use by cleaning with 70% ethanol and wiping dry with Kimwipes.

2. Prepare a known dilution of the cell sample to be counted. If doing a viability count, prepare a 1:5 dilution of the cell sample as follows: 20 µL sample + 60 µL 1X PBS + 20 µL 0.4% trypan blue).

3. Place cover glass squarely on top of hemocytometer and transfer 10 to 20 µL of the cell dilution under the cover glass on one side of hemocytometer and allow cells to settle.

4. Using the 20X objective, locate the upper left square of one grid (A).

5. When counting cells follow these general guidelines:

A. The middle of the triple lines separating each corner square (A-D) is the boundary line. Cells that touch the upper or left boundaries are included, but cells that touch the lower or right boundaries are excluded.

B. Optimal cell counts should be 30-150 cells/corner area, do additional 1:5 dilutions for high density samples

C. If greater than 10% of particles are clusters of cells, try to disperse cells in original cell suspension and repeat the count.

6. For total cells/mL:

A. Count all cells in each corner area (A - D) of the hemocytometer using a snake-like motion as indicated in corner A. Record all 4 counts (A, B, C, and D) in the Batch Record.

7. For viability counts:

A. Count total cells (white and blue cells) in the same manner. Record all 4 counts (A, B, C, and D) in the Batch Record.

B. Count viable cells (white cells only) in the same manner. Record all 4 counts (A, B, C, and D) in the Batch Record.

8. Total cells and total viable cells/mL can be determined using the following equation:

$$\text{cells/mL} = \left(\frac{\text{Total Cells in A+B+C+D}}{4} * 10{,}000 \right) * \text{dilution factor}$$

APPENDIX 5: REPRESENTATIVE IMAGE OF γH2AX-POSITIVE CELLS

The image below is a merge of the DAPI (blue) and FITC (green) channels into a single image. Nuclei demonstrating obvious γH2AX positivity are circled.