NIH NATIONAL CANCER INSTITUTE

LHTP003.07.25-ADM-01: Addendum to LHTP003.07.25 for Use With γH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation

Effective Date: 3/11/2025

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 INFORMATION

1. Approvals

Technical Reviewer: Angie Dull Date: ANGIE B. DULL -S (Affiliate) Digitally signed by ANGIE B. DULL -S (Affiliate) Date: 2025.05.13 08:47:37 -04'00'

PADIS Approval: Deborah Wilsker Date: DEBORAH F. WILSKER -S (Affiliate) Digitally signed by DEBORAH F. WILSKER -S (Affiliate) Date: 2025.05.13 10:01:38 -04'00'

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LHTP Approval: Katherine V. Ferry-Galow Date: KATHERINE V. FERRY-GALOW -S (Affiliate) Digitally signed by KATHERINE V. FERRY-GALOW -S (Affiliate) Date: 2025.05.13 12:25:17 -04'00'

DCTD OD Approval: Toby Hecht Date: Toby T. Hecht -S Digitally signed by Toby T. Hecht -S Date: 2025.05.13 12:52:28 -04'00'

2. Change History

Revision	Approval Date	Description	Originator	Approval
--	3/11/2025	New Document	LL	TH

OVERVIEW OF IMMUNOFLUORESCENCE ASSAY FOR BIOPSIES

SOP340507:
Tumor Frozen Needle Biopsy Specimen Collection, Handling and Shipping for PADIS, Frederick National Laboratory for Cancer Research (FNLCR)

OR

SOP340567:
Tumor Frozen Needle Biopsy Specimen Collection, Handling and Shipment to EET Biobank

- Collect tumor needle biopsies in 1.5 mL tubes
- Immediately place in liquid nitrogen or on dry ice/ethanol to flash freeze within 2 min of collection
- Ship to biopsy processing laboratory or biorepository

↓

SOP340550:

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

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

↓

Assay Specific Staining SOP

e.g., LHTP003.07.23: γH2AX, pNBS1, pKAP1 IFA Staining with β-Catenin Segmentation for Tumor Biopsy Slides

- Load biopsy and control slides into Bond-RX Processing Module
- Multiplex Bond-RX automated staining of slides with assay specific critical reagents
- Stain slides with DAPI and mount cover slips

↓ **Image with 72h**

LHTP003.07.25:
20X Whole Slide Image Capture of IFA Tumor Biopsy Slides using ZEISS Axioscan 7 Microscope Slide Scanner

AND
LHTP003.07.25-ADM-01
Addendum to LHTP003.07.25 for Use With γH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation

- Capture images of stained biopsy and control slides using a Zeiss AxioScan 7 whole slide image scanner

↓

Assay Specific Image Analysis SOPs

e.g., LHTP003.07.26: Image Analysis of Tumor Biopsy Slides from γH2AX, pNBS1, pKAP1 IFA with β-Catenin segmentation

- Quantitate markers from captured images of stained biopsy and control slides using Indica Labs HALO and HALO AI software

1.0 PURPOSE OF ADDENDUM

To outline assay specific requirements when following LHTP003.07.25 to acquire images from clinical slides stained with γH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation (DDR9). The scan profile selected to acquire images for the DDR9 assay is "20X DDR9_barcode". Images acquired from DDR9 stained slides should follow the recommended exposure ranges, target background intensities and LED lamp intensities detailed in this document. Additionally, the signal to background ratio for all markers should be >2.

2.0 RELATED DOCUMENT

2.1 LHTP003.07.25; 20X Whole Slide Image Capture of IFA Tumor Biopsy Slides using ZEISS Axioscan 7 Microscope Slide Scanner

3.0 ABBREVIATIONS

Cy5	=	Cyanine 5, a far-red fluorescent dye
Cy7	=	Cyanine 7, a near-infrared fluorescent dye
DAPI	=	4',6-Diamidino-2-Phenylindole
DDR9	=	γH2AX, pNBS1, pKAP1 IFA Staining with β-Catenin Segmentation D
DCTD	=	Division of Cancer Treatment and Diagnosis
FFPE	=	Formalin-fixed paraffin-embedded tissue
FITC	=	Fluorescein Isothiocyanate, a green fluorescent dye
IFA	=	Immunofluorescence Assay
LED	=	Light Emitting Diode
LHTP	=	Laboratory of Human Toxicology & Pharmacology
NCLN	=	National Clinical Laboratory Network
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
SOP	=	Standard Operating Procedure

4.0 ASSAY SPECIFIC INFORMATION SUPPLEMENTAL TO LHTP003.07.25

4.1 Clinical slides stained with γH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation should be captured following the steps below.

4.2 SOP Step 7.5: Select "20X DDR9_barcode" as the scan profile.

4.3 SOP 7.9.2.4: Scroll down to autofocus strategies, keep the coarse focus map settings in "20X DDR9_barcode" scan profile as shown below.

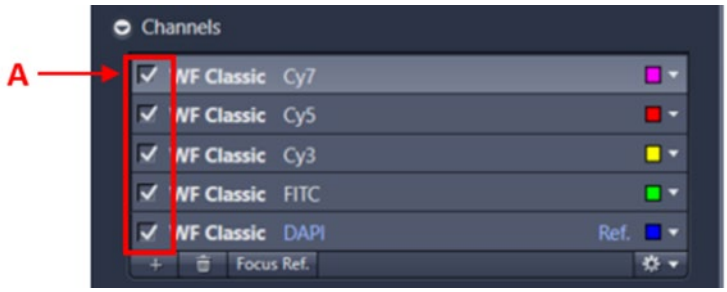
Setting Name	Default setting for "**20X DDR9_barcode**" Profile
Mode	Auto
Quality	Default
Search	Full
Sampling	Default

Setting Name	Default setting for "**20X DDR9_barcode**" Profile
Set Last	4400 µm
Range	400 µm
Step Size	10.52 µm
Set First	4000 µm
Focus Strategy	Fixed Number of Points =6
Sharpness Measure Set	Best

4.4 SOP 7.9.3.4: Scroll down to autofocus strategies, keep the fine focus map settings in "20X DDR9_barcode" scan profile as shown below.

Setting Name	Default setting for "**20X DDR9_barcode**" Profile
Mode	Auto
Quality	Default
Search	Full
Sampling	Fine
Range	120 µm
Step Size	0.52 µm
Sharpness Measure Set	Best
Support Point Distribution Strategy	• "Onion Skin" for xenograft and control tissues • "Fixed Number of Points" for biopsy tissues

4.5 SOP Step 7.9.5.1: Under the Scan Setting section, navigate to the "**Channels**" drop down menu and ensure all channels are selected as shown by arrow (A) below.



4.6 SOP Step 7.9.6: The recommended exposure time ranges, target background intensities and LED lamp intensity settings for each channel in the γH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation assay are shown in the table below. Additionally, the signal to background ratio should be > 2. Check the γH2AX, pNBS1, pKAP1 and β-Catenin staining on the control slides for appropriate signal and to ensure that the staining is not over-exposed. Control slides and clinical patient specimen slides may have different exposures, but all γH2AX control slides and all pNBS1/pKAP1 control slides from the same staining run must have the same exposures and all individual slides from the same patient must have the same exposures. Slides from different patients can have different exposures. All channel settings can be viewed using the "**Compare**" tool in the "**Scan**" section of the Zeiss acquisition profile. See section 7.9.5.1.4 in SOP LHTP003.07.25 for instructions to use the "**Compare**" tool.

Channel	LED Intensity	Exposure Time Range (milliseconds)	Target Background Intensity (Intensity Unit)
DAPI	20%	1-2 ms	n/a
pKAP1 (FITC)	30%	20-30 ms	< 650
β-Catenin (Cy3)	50%	75-150 ms	n/a
pNBS1 (Cy5)	50%	25-50 ms	< 300
γH2AX	50%	10-20 ms	<150