Title:	Biopsy Protein Extraction for the HIF-1 alpha Immunoassay				Page 1 of 18
Doc. #:	SOP340910	Revision:	B	Effective Date:	6/23/2015

National Clinical Target Validation Laboratory

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

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Change History

Revision	Approval Date	Description	Originator	Approval
--	5/29/2012	New Document. Version for PADIS to NCTVL assay transfer.	SRP, YAE	RJK
A	11/6/2012	Sample dilution information clarified; degassed buffer tubes defined as critical reagent only when dry flash frozen biopsies are being processed. Requirements for digital sample tables added to SOP Step 5.3.	KFG, YAE	KFG
B	6/23/2015	Statement added to Scope defining the encountered limitations of the assay with clinical specimens. Updated catalog numbers for discontinued items.	YAE	KFG

Please check for revision status of the SOP at

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

and be sure to use the current version.

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OVERVIEW OF IMMUNOASSAY SAMPLE PROCESSING

SOP340902: Tumor Frozen Needle Biopsy Collection and Handling for HIF-1 alpha	• Collect fresh needle biopsy into degassed HIF-1 alpha collection tubes (generally for measurement of HIF-1 alpha as a primary objective) or by dry flash freezing in 2-mL Sarstedt tubes • Immediately place in liquid nitrogen or on dry ice/ethanol • Ship to biopsy processing laboratory

SOP340910: Biopsy Specimen Processing for HIF-1 alpha Immunoassay	• Extract protein from tumor biopsy • Determine protein concentration • Store stock lysate or immediately proceed to immunoassay

SOP340903: HIF-1 alpha Immunoassay	• Perform ELISA with clinical samples, HIF-1 alpha standards, and controls • Using Tecan Microplate reader, determine relative signal of all samples

SOP340904: HIF-1 alpha Immunoassay Quality Control, Data Analyses, and Reporting	• Determine the HIF-1 alpha concentration in all samples and apply quality control standards to verify utility of assay • Prepare a Clinical Sample Data Report for each set of unknown samples and send to the clinical protocol Principal Investigator

1.0 PURPOSE

Standardize the method for preparing lysates from biopsy samples for quantifying of hypoxia inducible factor-1 alpha (HIF-1 alpha) with an enzyme-linked immunosorbent assay (ELISA) for pharmacodynamic (PD) studies.

2.0 SCOPE

This procedure applies to all personnel involved in preparing lysates from biopsy samples for use in HIF-1 alpha Immunoassay during clinical trials. The goal of the SOP and associated training is to ensure consistency in lysate preparation for HIF-1 alpha measurement across samples and clinical sites.

Data generated using Phase I clinical trial samples suggest that this assay may not be sufficiently sensitive to measure HIF-1 alpha decreases in many human biopsy pre- and post-dose pairs (Park, S.R. et al., 2014, Anal Biochem). Analysis of NCI patient samples have established a minimal protein load needed to allow for measurable HIF-1 alpha levels: 60% of patient samples tested with < 6 µg protein/well were <LLQ while only 8% with ≥ 7.5 µg/well were <LLQ. These data are based on 36 patient specimens loaded at 3 different protein concentrations (108 dilutions total) ranging from 2.15-18.7 µg protein/well.

3.0 ABBREVIATONS

2HG	=	2-Hydroxyglutarate
BCA	=	Bicinchoninic Acid
BSA	=	Bovine Serum Albumin
DCTD	=	Division of Cancer Treatment and Diagnosis
DI	=	Deionized
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
HIF-1 alpha	=	Hypoxia Inducible Factor-1 Alpha
H-CEB	=	HIF-1 Alpha Cell Extraction Buffer
HRP	=	Horseradish peroxidase
IA	=	Immunoassay
ID	=	Identification / Identifier
LHTP	=	Laboratory of Human Toxicology and Pharmacology
NCTVL	=	National Clinical Target Validation Laboratory
PADIS	=	Pharmacodynamics Assay Development & Implementation Section
PD	=	Pharmacodynamics
PI	=	Protease Inhibitor
PMSF	=	Phenylmethylsulfonyl Fluoride
RLU	=	Relative Light Unit
RT	=	Room Temperature
SDS	=	Sodium Dodecyl sulfate
SOP	=	Standard Operating Procedure

4.0 INTRODUCTION

The HIF-1 alpha Immunoassay (SOP340903) has been developed to use HIF-1 alpha as a metric for hypoxia in tumor biopsies. An ELISA is used to first capture HIF-1 alpha from total cell extracts on plates coated with a HIF-1 alpha capture monoclonal antibody. The captured protein is then detected using a HIF-1 alpha polyclonal detection antibody followed by a streptavidin-HRP conjugate to allow chemiluminescent readout and quantitation of HIF-1 alpha levels.

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 Director/Supervisor oversees the personnel running SOPs within the laboratory and is responsible for ensuring the personnel are certified and have sufficient experience to handle clinical samples.
Certified Assay Operator	A Certified Assay Operator may be a Laboratory Technician/ Technologist, Research Associate, or Laboratory Scientist who has been certified through DCTD training on this SOP. The Certified Assay Operator works under the guidance of the Laboratory Director/Supervisor. This person performs laboratory procedures and examinations in accordance with the current SOP(s), as well as any other procedures conducted by a laboratory, including maintaining equipment and records, and performing quality assurance activities related to performance.

5.1 It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented DCTD 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 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.

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

5.4 All responsible personnel are to check the DCTD Biomarkers web site (http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm) to verify that the most recent version of the SOP for the assay is being used.

6.0 MATERIALS AND EQUIPMENT REQUIRED

6.1 Precellys Ceramic Bead Collection Tubes, 2.8 mm beads, 2.0-mL reinforced tubes, 50 pack (Precellys, Cat#: KT03961-1-007.2)

6.2 If biopsies were collected dry flash frozen

6.2.1 1.5-mL Sarstedt tubes containing 300 µL degassed HIF-1 alpha Cell Extraction Buffer with protease inhibitors, PMSF, and 2-hydroxyglutarate (Degassed H-CEB [Complete]);

- Instructions for preparation: SOP340902, Appendix 3

6.3 Pipettors (200-1000 µL, 50-200 µL, 2-20 µL) and tips

6.4 Fixed-volume manual pipette, 250 uL (e.g. Hamilton, Cat# 55019-13)

6.5 Multichannel pipettor (5-50 µL and 30-300 µL) and tips

6.6 Sterile, disposable fine tip plastic tweezers (e.g., VWR, Cat#: 12576-934)

- Metal forceps, scalpels, etc., will destabilize HIF-1 alpha and MAY NOT be substituted in specimen handling procedures.

6.7 Reagent reservoirs (Fisher Scientific, Cat#: 13-681-509)

6.8 1.5-mL Sarstedt o-ring screw cap tubes (Sarstedt, Cat#: 72.692.005)

6.9 2-mL Sarstedt o-ring screw cap, skirted tubes (Sarstedt, Cat#: 72.694.006)

6.10 50-mL polypropylene tubes (e.g., VWR, Cat#: 21008-951)

6.11 Vacuum filter/storage bottle system, 0.22-µm pore, 500 mL (Corning, Cat#: 430758)

6.12 Printable microcentrifuge tube labels or BSI labeling system

6.13 0.4-mL 96-well Flat Bottom Bacti Plate (Life Technologies, Cat#: 12-565-361)

6.14 Acetate plate sealers (Fisher Scientific, Cat#: 14-245-18)

6.15 81-place freezer storage boxes (Fisher Scientific, Cat#: 12-565-182)

6.16 Ice bucket

6.17 UltraPure DNase⁄RNase-free distilled water (e.g., Life Technologies, Cat#: 10977-015) or Milli-Q water

6.18 20% sodium dodecyl sulfate (SDS; e.g., Sigma-Aldrich, Cat#: 05030-500ML-F)

6.19 Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P2714 or Roche Diagnostics, Cat#: 11697498001)

6.20 Phenylmethanesulfonyl fluoride solution, 0.1 M (PMSF; Sigma-Aldrich, Cat#: 93482-50ML-F)

6.21 Tris, ultra pure (e.g., MP Biomedicals, Cat#: 04819620 or 04819623)

6.22 Sodium chloride, ReagentPlus grade (e.g., Sigma-Aldrich, Cat#: S9625)

6.23 Glycerol, 100% w/v (e.g., Sigma-Aldrich, Cat#: G5516)

6.24 EDTA, 0.5 M, pH 8.0 (e.g., Boston BioProducts, Cat#: BM-150)

6.25 Magnesium chloride, anhydrous (e.g., Sigma-Aldrich, Cat#: M8266)

6.26 β-Glycerol phosphate disodium salt, pentahydrate (e.g., Sigma-Aldrich, Cat#: 50020)

6.27 Sodium fluoride, ACS grade (e.g., Sigma-Aldrich, Cat#: 201154)

6.28 Triton X-100, non-ionic, aqueous solution, 10% w/v, stored according to manufacturer's direction (e.g., Roche Diagnostics, Cat#: 11332481001)

6.29 BCA Protein Assay Kit (Life Technologies, Cat#: 23227 or 23225)

6.30 Liquid nitrogen or dry ice/ethanol bath

6.31 Sorvall Fresco microcentrifuge, refrigerated (Thermo Scientific)

6.32 Vortex Genie 2 (Daigger, Cat#: EF3030A)

6.33 Precellys®24 Tissue Homogenizer (Precellys, Cat#: EQ03119.200.RD000.0)

6.34 37°C dry incubator, non-humidified

6.35 -80°C freezer

6.36 2°C to 8°C refrigerator

6.37 Frozen needle biopsy specimens collected following SOP340902 (HIF-1 alpha Immunoassay Tumor Frozen Needle Biopsy Specimen Collection and Handling)

7.0 OPERATING PROCEDURES

Important: It is strongly suggested to extract protein from unknown samples using this SOP on the **same day** that the HIF-1 immunoassay (SOP340903) will be run. If protein extraction is performed on a different day than the immunoassay, run (or re-run) the BCA Protein Assay on the day of the immunoassay.

7.1 All reagents for an individual assay are to be prepared for use in one experimental run, and only in the amounts required for the specific assay. All excess reagents are to be discarded following appropriate safety procedures. Process a single patient's samples, **batched**, to ensure consistent sample handling.

7.1.1 Tumor biopsies collected in degassed HIF-1 alpha cell extraction buffer (H-CEB) or dry flash frozen may have limited stability, so biopsies should be extracted as soon as possible, and should be kept at -80ºC (or lower) until the time of processing. Process a single patient's **batched** samples to ensure consistent sample handling.

7.2 Record the name and certification number of the Certified Assay Operator and the facility running the SOP in the Batch Record (Appendix 1). Include reference to clinical protocol number(s), if applicable.

7.3 Record equipment serial numbers that will be used in the assay in the Batch Record (Appendix 1, Section 1A). If needed, prepare H-CEB **(without** PIs+2HG) following the protocol in SOP340902, Appendix 3; you will need ~3 mL/sample.

7.4 Fill in the Sample Information Table in the Batch Record (Appendix 1, Section 2) with the Sample ID. Keep all frozen needle biopsies on dry ice until ready to homogenize.

7.4.1.1 The Sample ID should include the CTEP protocol number followed by a unique patient identifier and a sequential specimen ID (NCI PD tumor biopsies are series 500).

7.5 Label sufficient Precellys ceramic bead collection for all biopsies to be processed and place on ice to chill.

Important: Work quickly, yet carefully, through SOP Steps 7.6 (or 7.7) and 7.8 to minimize the time between thawing of the biopsies and homogenization.

7.6 Preparation of Biopsies Collected into 1.5-mL Sarstedt Tubes Containing Degassed Buffer

7.6.1 If the biopsy was collected following SOP340902 into a 1.5-mL Sarstedt tube containing 250 µL degassed H-CEB (Complete), remove the tube from the freezer and place on ice.

7.6.2 Before the biopsy is fully thawed, work quickly to uncap both the degassed 1.5-mL Sarstedt tube containing the tumor biopsy and a chilled Precellys bead tube. Using a sterile, non-metallic tweezers, transfer the tumor biopsy to the Precellys bead tube, placing it close to the bottom of the tube.

7.6.3 Using a fixed-volume 250-µL pipette, transfer the full 250 µL degassed H-CEB (Complete) remaining in the 1.5-mL Sarstedt tube to the Precellys bead tube, being sure the biopsy is submerged. Immediately cap both the glass vial and the bead tube and return to ice. Dispose of the tweezers in the appropriate biohazardous waste container(s).

7.6.4 Fill in the Sample Information Table in the Batch Record (Appendix 1, Section 2) with degassed H-CEB (Complete) volume for each biopsy.

7.6.5 Quickly proceed to tissue lysis as described in SOP Step 7.8.

7.7 Preparation of Dry, Flash Frozen Biopsies

7.7.1 If the biopsy was collected following SOP340902 and dry, flash-frozen in a 2-mL Sarstedt tube, remove the tube from the freezer and place on ice.

7.7.2 Remove one 1.5-mL Sarstedt containing 300 µL degassed H-CEB (Complete) from the freezer for each specimen to be processed and place on ice to thaw. Record the expiration date in the Batch Record (Appendix 1, Section 1B).

7.7.3 Before the biopsy is fully thawed, work quickly to uncap both the 2-mL Sarstedt tube containing the dry, flash frozen tumor biopsy and a chilled Precellys bead tube. Using a sterile, non-metallic tweezers transfer the tumor biopsy to the Precellys bead tube, placing it close to the bottom of the tube.

7.7.4 Using a fixed-volume 250-µL pipette, pipette 250 µL degassed H-CEB (Complete) from the 1.5-mL Sarstedt to the Precellys bead tube, being sure the biopsy is submerged. Immediately cap the glass vial and the bead tube and return to ice. Dispose of the tweezers in the appropriate biohazardous waste container(s).

7.7.5 Fill in the Sample Information Table in the Batch Record (Appendix 1, Section 3) with degassed H-CEB (Complete) volume for each biopsy.

7.7.6 Quickly proceed to tissue lysis as described in SOP Step 7.8.

7.8 Tissue Lysis

Alternative tissue processing methods using an ultrasonic processor are included in Appendix 2. Be aware that frothing of samples inherent in sonication will introduce oxygen into the buffer and may cause increased variance in HIF-1 alpha readouts. **Important**: If samples will be extracted using ultrasonic processing, biopsies should be collected into conical bottom, o-ringed tubes without beads, but with degassed H-CEB (Complete).

7.8.1 Place the tubes in the Precellys24 Tissue Homogenizer (RT) and process at a 6000 RPM for 15 sec. Wait 15 sec and repeat homogenization a second time. Record the actual homogenizer settings in the Batch Record (Appendix 1, Section 3).

Precellys24 setting: 6000-2x15-015
PAUSE (s)

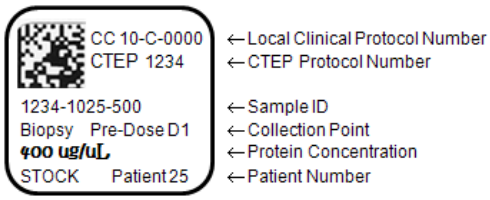
7.8.2 Following homogenization, move samples to ice and add 20% SDS to a final concentration of 0.5% (e.g., add 6.25 µL 20% SDS into 250 µL lysate). Record the final volume SDS added to each sample in the Batch Record (Appendix 1, Section 2).

7.8.3 Immediately place the homogenized biopsy samples on ice and incubate for 20-30 min, vortexing the sample tubes at maximum speed every 5-10 min (at least 3 times). Record the time samples are placed on ice (Appendix 1, Section 3).

7.9 Tumor Lysate Preparation

7.9.1 Clarify all lysates by centrifugation at 13,000 x g for 10-15 min at 2ºC to 8°C. Transfer the cleared lysate into a new 2-mL Sarstedt tube labeled as the stock tumor lysate tube (see sample label). Discard the original tube with beads and any precipitated material in the appropriate waste container.

Protein concentration will be filled in by hand following BCA Protein Assay analysis.



7.10 BCA Protein Assay

7.10.1 Perform BCA protein assay to determine stock tumor lysate protein concentration. Prepare H-CEB (**without** PIs+2HG) for protein assay (recipe SOP340902, Appendix 3). You will need approximately 2.5 mL H-CEB (**without** PIs+2HG) for preparation of standards and background wells and 0.25 mL H-CEB (**without** PIs+2HG) per unknown sample.

7.10.2 Record the date the BCA Protein Assay is run in the Batch Record (Appendix 1, Section 4).

7.10.3 Plate Map for the Protein Assay

7.10.3.1 Use the BCA Protein Assay Plate Map (Appendix 3) for the recommended locations of the standards and unknown samples; the location of the unknown samples should match up with the sample number listed in the Sample Information Table in the Batch Record (Appendix 1, Section 3).

7.10.3.2 Each unknown sample and standard is run in duplicate. A total of 2 dilutions (1:5 and 1:10) for up to 12 different patient samples can be assayed per plate.

7.10.4 Preparation of Bovine Serum Albumin (BSA) Serial Dilutions for the Standard Curve

7.10.4.1 Label seven 1.5-mL Sarstedt tubes, numbered 1 through 7, for the 2000 to 31.3 µg/mL BSA standards.

7.10.4.2 Carefully open the glass ampoule provided with the BCA Protein Assay Kit containing the 2 mg/mL BSA stock.

7.10.4.3 Using the dilution scheme below, pipette the indicated volume of H-CEB (**without** PIs+2HG) into each tube. Add indicated volume of BSA standard to each tube and vortex to mix. Keep samples on ice.

Tube #	**Volume and Source of BSA**	**Volume of H-CEB (without PIs+2HG)**	**Final BSA Conc. (µg/mL)**
1 (H)	200 µL of BSA stock	0 µL	2000
2 (G)	200 µL of BSA stock	200 µL	1000
3 (F)	200 µL of tube # 2	200 µL	500
4 (E)	200 µL of tube # 3	200 µL	250
5 (D)	200 µL of tube # 4	200 µL	125
6 (C)	200 µL of tube # 5	200 µL	62.5
7 (B)	200 µL of tube # 6	200 µL	31.3

7.10.5 Preparation of Tumor Lysates for the BCA Protein Assay

7.10.5.1 For each tumor lysate to be assayed, label two 1.5-mL Sarstedt tubes with the sample number and the lower case letter "a" or "b" (e.g., S1a, S1b). The lower case letters represent the 2 different lysate dilutions to be assayed.

7.10.5.2 If extracts were frozen, thaw on ice and vortex for 5 sec before taking aliquots for protein assay.

7.10.5.3 Using the clarified tumor lysates and the serial dilution scheme below, dilute each tumor lysate 1:5 and 1:10 with H-CEB (**without** PIs+2HG) in labeled 1.5-mL tubes represented by the letters "a" and "b," respectively. This will be sufficient volume for 25 µL of each dilution in duplicate for the BCA Protein Assay. Keep samples on ice.

Lysate Tube	Final Dilution	Volume and Source of Lysate	Volume of H-CEB (**without** PIs+2HG)
a	1:5	18 µL Tumor Lysate	72 µL
b	1:10	30 µL of tube # a	30 µL

7.10.6 BCA Protein Assay Procedure

7.10.6.1 Label the 96-well plate and assemble all samples and standards. Pipette reagents into the plate in the following order:

Wells	Sample/Reagent
B6 to H7	25 µL of each standard into designated duplicate wells
B2 to G5 and B8 to G11	25 µL of each tumor lysate dilution into designated duplicate wells
Remaining wells	25 µL of H-CEB (**without** PIs+2HG); Background Control

7.10.6.2 Prepare BCA Working Reagent as described in the Batch Record and record the lot number for the kit (Appendix 1, Section 4). Pour the BCA Working Reagent into a clean multichannel pipette reservoir.

7.10.6.3 Using a multichannel pipettor, add 200 µL of the BCA Working Reagent to each well, mix by pipetting up and down being careful to prevent bubbles from forming. Change pipette tips between each 96-well plate column.

7.10.6.4 Cover plate with acetate film and incubate in a 37°C incubator for 30 min. At the same time, turn on the Tecan Infinite Microplate Reader so it has at least 30 min to warm up before use. Record the start time for the incubation in the Batch Record (Appendix 1, Section 4).

7.10.6.5 At the end of the 30 min incubation, record the end time in the Batch Record (Appendix 1, Section 4), and immediately read the plate on the Tecan Microplate Reader at 562 nm absorbance.

7.10.7 Determine Protein Concentration

7.10.7.1 Average the absorbance for the background wells A2 - A11 and each duplicate set of standards and prepare a standard curve of average absorbance (minus background) versus µg/mL protein. Attach a copy of the raw data and the graph of the standard curve to the Batch Record (Appendix 1, Section 4).

7.10.7.2 Average the absorbance readings for each duplicate set of unknown samples, and record the average absorbance readout (minus background) for each tumor lysate dilution (a and b) in the Sample Information Table in the Batch Record (Appendix 1, Section 2).

7.10.7.3 Compare the unknown tumor lysate absorbance readouts to the standard curve to determine the protein concentration for each diluted lysate sample. Record the protein concentration in µg/mL for each diluted sample (a and b) on the Sample Information Table (Appendix 1, Section 3). Divide the diluted sample protein concentration by 1000 and record the protein concentration in µg/µL.

7.10.7.4 For each unknown sample dilution (a [1:5] and b [1:10]), back-calculate the protein lysate concentration for dilution (multiply by 5 or 10) and average them to determine the protein concentration corrected for dilution and record it in the Sample Information Table (Appendix 1, Section 2).

7.10.7.5 Write the protein concentration in µg/µL on the label of the 2-mL tumor lysate tube.

7.11 Quality Control (QC) Criteria for Tumor Lysates

7.11.1 A minimal protein concentration of **0.5 µg/µL** is needed for tumor lysate to pass QC. On the Sample Information Table in the Batch Record, indicate if the samples Pass (**≥ 0.5 µg/µL**) or Fail (**< 0.5 µg/µL**) the protein concentration QC (Appendix 1, Section 2).

- If the stock tumor lysate concentration Fails QC (**< 0.5 µg/µL**), the sample is considered unanalyzable and will not be used for HIF-1 alpha evaluation.

7.12 It is strongly suggested to extract protein from unknown samples on the same day that the HIF-1 alpha immunoassay (SOP340903) will be run.

7.12.1 If the HIF-1 alpha immunoassay is not being performed immediately, snap-freeze the clarified tumor lysate on dry ice/ethanol or liquid nitrogen. Store the frozen samples in an 81-place freezer box, batched by patient, at -80°C until analysis. Record the date and time lysate is frozen in the Batch Record (Appendix 1, Section 5).

7.12.2 If a the BCA Protein Assay was run on the lysate on the day of extraction, repeat the BCA Protein Assay to determine protein concentration on the day the samples will be run in the immunoassay.

7.13 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 6).

7.14 The Laboratory Director/Supervisor should review the Batch Record and print and sign their name affirming the data contained within are correct (Appendix 1, Section 7).

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: _____

Certification Number: _____

Facility/Laboratory Running SOP: _____

Clinical Protocol Number: _____

1. Equipment and Reagents

A. Equipment:

Tissue Homogenizer: Make/Model : _____

Serial #: _____

Microplate Reader: Make/Model : _____

Serial #: _____

B. H-CEB (Complete):

Reagent Name	Expiration Date
Degassed H-CEB (Complete) Frozen Aliquots, 300µL	/ /

2. Sample Information Table and BCA Protein Assay Record

Sample No.	Sample /Patient ID	Starting Vol. (µL)	Vol. 20% SDS (µL)	BCA Protein Assay						Conc. QC (Pass/Fail)*
				Tube	Dilution of Stock Lysate	Avg. Abs. (minus background)	Conc. (µg/mL)	Conc. (µg/µL)	Conc. Corrected for Dilution (µg/µL)	
Ex	*1234-1025-500*	*250*	*6.25*	*a*	*1:5*	*xxx*	*168*	*0.168*	*0.85*	*Pass*
				b	*1:10*	*xxx*	*85.7*	*0.086*		
S1				a						
				b						
S2				a						
				b						
S3				a						
				b						
S4				a						
				b						
S5				a						
				b						
S6				a						
				b						
S7				a						
				b						
S8				a						
				b						
S9				a						
				b						
S10				a						
				b						
S11				a						
				b						
S12				a						
				b						

* Stock lysate protein concentration, corrected for dilution, must be ≥ 0.5 µg/µL to pass QC.

BATCH RECORD: INITIALS _____ DATE: _____

3. Tissue Lysis

Homogenize tissue at a setting of _____ RPM for 15 sec; repeat 2 times.

Time samples placed on ice _____ : _____

4. BCA Protein Assay

BCA Working Reagent: **Prepare just before use**. Pipette 21.56 mL of Reagent A and 440 µL of Reagent B into a 50-mL polypropylene tube. Mix by inversion (the solution will turn green).

BCA Protein Assay Kit: Lot#: _____

Date of BCA Protein Assay Run: ____ / ____ / ____

Incubate assay at 37°C for 30 min: Start Time: ____ : ____ Stop Time: ____ : ____

Attach a copy: Raw data and the graph of the standard curve.

5. Stock Lysate Storage

Cell extract frozen in liquid nitrogen or dry ice/ethanol bath: Date _____ Time ____ : ____

Sarstedt tubes placed into -80°C storage Date _____ Time ____ : ____

6. Notes, including any deviations from the SOP:

7. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: ____ / ____ / ____

BATCH RECORD: INITIALS _____ DATE: _____

APPENDIX 2: ALTERNATIVE TISSUE PROCESSING: ULTRASONIC HOMOGENIZATION

These steps can replace SOP Section 8.8 as an alternative biopsy homogenization procedure. The Table below is an example of a head-to-head comparison run by NCI's Pharmacodynamic Assay Development and Implementation Section (PADIS) for the two homogenization procedures.

Table: Comparison of HIF-1 alpha readouts from matched PC3 xenograft lysates processed using the Precellys24 bead homogenizer (Homog.) and an ultrasonic homogenizer (Sonic.).

Sample	Cohort 1		Cohort 2		Cohort 3		Cohort 4	
No.	**Sonic.**	**Homog.**	**Sonic.**	**Homog.**	**Sonic.**	**Homog.**	**Sonic.**	**Homog.**
1	14.6	27.6	35.2	37.7	38.1	37	35.8	21.1
2	39	29	22.2	28.2	28.5	36.4	43.6	32.2
3	31.4	27.9	30.9	27.4	32.4	36.9	31.4	28.4
4	32.3	29.1	28.7	36.6	14.2	29.4	39.3	31.9
5					31	33.7	49.8	39.1
6					13.3	24.7	24.8	36.2
7					31.2	27	42.5	38.7
8					18.5	24.7	17.9	22.9
Mean	29.3	28.4	29.3	32.5	25.9	31.2	35.6	31.3
SD	**10.4**	**0.8**	**5.4**	**5.4**	**9.3**	**5.4**	**10.5**	**6.8**
%CV	**35.4**	**2.7**	**18.5**	**16.7**	**35.8**	**17.3**	**29.5**	**21.7**

Abbreviations: CV, coefficient of variation; Homog., bead homogenization; SD, standard deviation; Sonic., ultrasonic homogenization

1.0 ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

1.1 D-2-hydroxypentanedioic acid disodium salt (2R), MW = 192.08 (2-hydroxyglutarate [2HG]; CAS#: 103404-90-6; 3B Scientific Corporation, Cat#: 3B3-053228)

1.2 Ultrasonic Processor with microtip (e.g., Cole-Parmer Instruments, Model#: CP 130PB-1)

1.3 Disposable fine tip plastic tweezers (e.g., VWR, Cat#: 83009-010)

- Metal forceps, scalpels, etc., will destabilize HIF-1 alpha and MAY NOT be substituted in specimen handling procedures

2.0 CRITICAL REAGENTS

2.1 Record the date of receipt, lot number, and expiration date below. Label reagent with date of receipt and store under the specified conditions for no longer than the recommended duration.

2.1.1 **2HG**: Supplied as a 10 mM stock solution.

Date of Receipt: _____

Lot#: _____ Expiration Date: _____

3.0 ALTERNATIVE HOMOGENIZATION PROCEDURE

Important: If samples will be extracted using ultrasonic processing, biopsies should be collected into conical bottom, o-ringed tubes without beads, but with degassed H-CEB (Complete); recipe in SOP340902, Appendix 3. Be aware that frothing of samples inherent in sonication will introduce oxygen into the buffer and may cause increased variance in HIF-1 alpha readouts.

3.1 Tissue Lysis *Replaces SOP Section 8.8*

3.1.1 Fill in the Sample Information Table in the Batch Record (Appendix 1, Section 2) with Sample ID and degassed buffer volume for each biopsy. Biopsy samples are kept on dry ice and processed **individually** through the homogenization step (Step 3.1.4 below); it is recommended to not process more than 4 samples at one time. Keep all frozen needle biopsies on dry ice until ready to homogenize.

3.1.1.1 The Sample ID should include the CTEP protocol number followed by a unique patient identifier and a sequential specimen ID (NCI tumor biopsies for PD sampling are series 500).

3.1.1.2 Biopsies should be in 1.5-mL reinforced tubes without beads, prefilled with 250 µL degassed H-CEB (Complete).

3.1.2 Place the tube with biopsy in a small beaker with ice to partially thaw. Before fully thawed, mince the still-frozen tissue with fine scissors, vortex the tube at maximum speed for 10 sec, mince the tissue again, and then vortex at maximum speed for 10 sec.

3.1.3 Sonicate the tissue at an output of 02-03 watts for 15 to 30 sec; repeat 3 times. Keep the specimen tube on ice while sonicating and avoid foaming of specimens. Record the sonicator settings in the Batch Record.

3.1.4 Following homogenization, move samples to ice and add 20% SDS to a final concentration of 0.5% (e.g., add 6.25 µL 20% SDS into 250 µL lysate). Record the final volume SDS added to each sample in the Batch Record (Appendix 1, Section 3).

3.1.5 Immediately place the homogenized biopsy sample on ice and incubate for 20-30 min and vortex the sample tube at maximum speed every 5-10 min, 3 times. Record the time each sample is placed on ice.

3.1.5.1 If there are additional biopsies to be homogenized, return to Alternative Homogenization Procedure Step 3.1.2 to process the next sample.

3.2 Return to SOP Section 8.9 to finish processing lysed samples.

APPENDIX 3: BCA PROTEIN ASSAY PLATE MAP

Plate Map for BCA protein assay set up with standards and 12 unknown sample wells (S1-S12) loaded in duplicate. Sample numbers correspond to that listed in the Sample Information in the Batch Record (Appendix 1, Section 2). The 2 different dilutions prepared for each unknown sample (1:5 and 1:10) in SOP Step 8.10.5 are represented by the letters a and b, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
A	x*	H-CEB (**without** PIs+2HG) – Background Control										x
B		S1a		S4a		31.25		S7a		S10a		
C		S1b		S4b		62.5		S7b		S10b		
D		S2a		S5a		125		S8a		S11a		
E		S2b		S5b		250		S8b		S11b		
F		S3a		S6a		500		S9a		S12a		
G		S3b		S6b		1000		S9b		S12b		
H	x					2000						x

B6-H7, BSA standards in duplicate

B2-G5 and B8-G11, 12 unknown samples, two dilutions run in duplicate

Remaining wells, H-CEB (**without** PIs+2HG) will be loaded in all grey-colored wells in example above, but the background RLU reading can be calculated based on A2-A11.

*Readings from the 4 corner wells should not be used to determine background.