Title:	HIF-1 alpha Immunoassay Tumor Frozen Needle Biopsy Specimen Collection and Handling				Page 1 of 16
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National Clinical Target Validation Laboratory

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Change History

Revision	Approval Date	Description	Originator	Approval
--	12/10/2010	New Document	YZ	JJ
A	4/11/2012	Added critical reagent information, Batch Record, and appendix with directions for receipt and return of critical reagent tubes. Assay transfer version for PADIS to NCTVL.	SRP, YAE	RJK
B	5/29/2012	Sample collection options updated to allow flash freeze biopsy collection if HIF-1 alpha measurement is a secondary clinical objective.	YAE	RJK
C	11/6/2012	New tube type used for collection of tumor biopsies into degassed HIF-1 alpha buffer due to Precellys bead tube breakage following -80°C freezing.	KFG, YAE	KFG
D	6/23/2015	Appendix for participating site preparation of degassed HIF-1 alpha buffer added. 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.

TABLE OF CONTENTS

OVERVIEW OF IMMUNOASSAY SAMPLE PROCESSING 3
1.0 PURPOSE 4
2.0 SCOPE 4
3.0 ABBREVIATONS 4
4.0 INTRODUCTION 5
5.0 ROLES AND RESPONSIBILITIES 5
6.0 MATERIALS AND EQUIPMENT REQUIRED 6
7.0 OPERATING PROCEDURES 7
APPENDIX 1: BATCH RECORD 11
APPENDIX 2: PD SAMPLE SHIPPING MANIFEST 13
APPENDIX 3: PREPARATION OF DEGASSED H-CEB TUBES FOR BIOPSY COLLECTION 14

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 collecting and handling frozen needle tumor biopsies for quantifying 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 the collection and handling of frozen needle tumor biopsies for use in the HIF-1 alpha Immunoassay during clinical trials. The goal of the SOP and associated training is to ensure consistency in tumor needle biopsy collection and handling between 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
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
IA	=	Immunoassay
ID	=	Identification / Identifier
IQC	=	Internal Quality Control
LHTP	=	Laboratory of Human Toxicology and Pharmacology
MW	=	Molecular Weight
NCTVL	=	National Clinical Target Validation Laboratory
PD	=	Pharmacodynamic
PI	=	Protease Inhibitor
PMSF	=	Phenylmethylsulfonyl Fluoride
PSI	=	Pounds Per Square Inch
RT	=	Room Temperature
SOP	=	Standard Operating Procedure

4.0 INTRODUCTION

Specimen handling, shipping, and storage procedures (pre-analytical variables) can have a significant impact on the reliability of biomarker measurements in the laboratory. Following detailed steps for sample collection and handling procedures and recording any deviations from this procedure allows retrospective identification of artifactual changes in biomarker readout and increases the reliability of the data and validity of the analytical results.

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 and/or DCTD PK/PD Support Lab — The Certified Assay Operator and/or DCTD PK/PD Support Lab personnel performs laboratory procedures and specimen collection 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 or (DCTD approval) 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 and/or DCTD PK/PD Support Lab personnel running the SOP has sufficient experience to handle and analyze clinical samples.

5.2 It is the responsibility of the Certified Assay Operator and/or DCTD PK/PD Support Lab personnel to confirm scheduled specimen collection time points, pre-print all labels and data collection sheets in advance, check documentation for accuracy, and verify that the required collection tubes, supplies, and equipment are handled correctly and available for successful collection and handling of biopsy samples.

5.3 The Certified Assay Operator and/or DCTD PK/PD Support Lab personnel responsible for conducting the assay are 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 The responsible personnel are 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 Recommended collection tube (pre-prepared with buffer):

6.1.1 Degassed HIF-1 alpha Biopsy Collection Tubes (1.5-mL Sarstedt tubes containing 250 µL degassed HIF-1 alpha cell extraction buffer with protease inhibitors and PMSF, and 2-hydroxyglutarate (Degassed H-CEB [Complete]; Appendix 3)

6.2 Alternate collection tube (for dry, flash freezing):

6.2.1 2.0-mL Sarstedt o-ring screw cap, skirted tubes (e.g., Fisher Scientific, Cat#: 72.694.006)

6.3 Stop watch, total time in minutes and seconds required

6.4 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.

6.5 Printable microcentrifuge tube labels or BSI labeling system

6.6 81-place freezer boxes (e.g., Fisher Scientific, Cat#: 12-565-182)

6.7 Thermoflask cooler or polystyrene foam container

6.8 Ice bucket

6.9 Liquid nitrogen or dry ice/ethanol bath

6.10 Wet ice

6.11 -80°C freezer (or lower)

7.0 OPERATING PROCEDURES

7.1 Record the name and certification number of the Certified Assay Operator and/or name of the DCTD PK/PD Support Lab personnel performing the SOP, the facility/clinic collecting the specimens, the Patient/Sample ID, and the clinical protocol number in the Batch Record (Appendix 1).

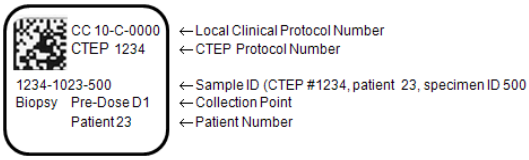
- The Batch Record for this SOP is sufficient for collection of a **single** patient's biopsy samples; if collecting biopsy samples for more than one patient, prepare a separate Batch Record for each patient.

7.2 Dependent on the Clinical Protocol PD collection directions, indicate in the Batch Record (Appendix 1, Section 1) if specimens are being collected into degassed H-CEB (Complete) collection tubes (SOP Section 7.5) or collected by dry flash freezing in 2-mL Sarstedt tubes (SOP Section 7.6).

7.3 Prepare enough pre-printed specimen labels for each biopsy sample to be collected as defined in the Pharmacodynamic/Correlative Study section of the Clinical Protocol; be sure to coordinate with the clinical center if they prepare the labels for sample collection.

If two passes are collected from one tumor, the labels would be identical except that the specimen ID would be followed by a lower case a/b to designate pass number. The specimen ID includes the CTEP protocol number followed by a unique patient identifier and a specimen series ID.

NCI tumor biopsies for PD sampling are series 500 with consecutive numbers identifying the collection time point as defined in the Clinical Protocol. Sample pre-printed label:



7.4 Of the pre-printed labels prepared for each sample, one label will go on each collection tube, one on the Batch Record (Appendix 1), and the last will be given to the research nurse to place into the patient record sheet.

7.5 Recommended Collection Procedure: Degassed Buffer Biopsy Collection Tubes

This collection procedure is preferred if measurement of HIF-1 alpha is a primary objective of a Clinical Protocol.

7.5.1 Preparation for Tumor Needle Biopsy Collection

7.5.1.1 Remove two 1.5-mL degassed H-CEB (Complete) collection tubes (possibility of collecting two passes) from the freezer and place on ice for each patient biopsy to be collected. Tubes should be kept in the dark until arriving at the biopsy collection site.

- Degassed tubes have been flared with nitrogen gas to minimize oxygen seepage into the tube to protect the oxygen-sensitive HIF-1 biomarker in biopsy tissue.
- Do not open degassed tubes until immediately prior to biopsy placement in the tube and then immediately cap the tube to minimize air exchange.

7.5.1.2 Record the expiration date of the degassed H-CEB (Complete) collection tubes used for collection in the Batch Record (Appendix 1, Section 2).

7.5.2 Tumor Needle Biopsy Collection and Handling

7.5.2.1 The research nurse is to notify the laboratory of scheduled PD sample collections, preferably giving at least 24-h notice. Arrive at the biopsy collection site early enough to set up laboratory supplies, collect relevant clinical information, and ensure rapid transport of specimens to the laboratory for placement at -80ºC (or lower) after collection.

7.5.2.2 Bring all necessary lab supplies including: two 1.5-mL degassed H-CEB (Complete) collection tubes (possibility of collecting two passes) on ice in an insulated bucket, plastic disposable tweezers, a liquid nitrogen flask or dry ice/ethanol bath, and one pre-printed specimen label to give to the research nurse for the patient record.

7.5.2.3 Record all available biopsy collection information into the Batch Record (Appendix 1, Section 3A and 3B).

7.5.2.4 The total time elapsed between biopsy collection and placement in degassed buffer is of **key importance** to HIF-1 alpha analysis. The interventional radiologist may eject the biopsy onto a sterile slide (for optimal analyte recovery the slide should be pre-chilled). Start a stop watch (or note the time) at this point (Appendix 1, Section 3A and 3B) and immediately walk the slide to the sample preparation table.

- Preferred collection method is direct ejection into the collection tube.

7.5.2.5 Uncap a chilled degassed H-CEB (Complete) collection tube and using a non-metallic tweezers, pick up the freshly collected needle biopsy and submerge in the 250 µL degassed buffer. Dispose of the tweezers in the appropriate biohazardous waste container(s).

- Indicate if a full or half biopsy is collected in the Batch Record (Appendix 1, Section 3A and 3B).

7.5.2.6 Immediately cap the degassed tube to minimize oxygen seepage and return tube to ice. Stop the stop watch (or note the time) once the biopsy is submerged in buffer/cap is closed. Record the total elapsed time from biopsy collection to placement in degassed buffer in the Batch Record (Appendix 1, Section 3A and 3B).

7.5.2.7 Snap freeze the biopsy and buffer by placing the H-CEB (Complete) collection tube in liquid nitrogen or a dry ice/ethanol bath and record the time of freezing in the Batch Record (Appendix 1, Sections 3A and 3B). **Important**: Be sure the tube remains upright and biopsy is submerged in buffer during the freezing process.

7.6 **Alternate Collection Procedure: Dry, Flash Freezing**

7.6.1 The research nurse is to notify the laboratory of scheduled PD sample collections, preferably giving at least 24-h notice. Arrive at the biopsy collection site early enough to set up laboratory supplies, collect relevant clinical information, and ensure rapid transport of specimens to the laboratory for placement at -80ºC (or lower) after collection.

7.6.2 Bring all necessary lab supplies including: 2-mL Sarstedt tubes (one for each possible biopsy or halved biopsy collected) pre-cooled on dry ice in an insulated bucket non-metallic disposable tweezers, a liquid nitrogen flask or dry ice/ethanol bath, and one pre-printed specimen label to give to the research nurse for the patient record.

7.6.3 Record all pertinent biopsy collection information into the Batch Record (Appendix 1, Section 3A and 3B).

7.6.4 The total time elapsed between biopsy collection and placement into the prechilled tube is of **key importance** to HIF-1 alpha analysis. The interventional radiologist may eject the biopsy onto a sterile slide (for optimal analyte recovery the slide should be pre-chilled). Start a stop watch (or note the time) at this point (Appendix 1, Section 3A and 3B) and immediately walk the slide to the sample preparation table.

- Preferred collection method is direct ejection into the collection tube.

7.6.5 Uncap an empty, prechilled 2-mL Sarstedt tube and using a non-metallic tweezers, pick up the freshly collected needle biopsy with the tweezers at one end, and touch the opposite end of the biopsy to the inner surface of the tube to attach. This should allow it to be dropped into the tube while releasing the tissue from the tweezers without sticking. Dispose of the tweezers in the appropriate biohazardous waste container(s).

- Indicate if a full or half biopsy is collected in the Batch Record (Appendix 1, Section 3.A.a. and 3.B.a.).

7.6.6 Snap freeze the biopsy by placing the tube in liquid nitrogen or a dry ice/ethanol bath and record the time of freezing in the Batch Record (Appendix 1, Sections 3A and 3B).

7.6.7 Calculate the total time elapsed from biopsy collection to biopsy freezing and record the total number of minutes and seconds elapsed in the Batch Record (Appendix 1, Sections 3A and 3B).

7.7 Return to the sample processing laboratory and transfer the frozen biopsy specimen (whether in degassed H-CEB (Complete) collection tubes or dry, flash frozen) to -80°C (or lower) for storage until specimen processing. Record the date and time specimens are placed at -80ºC (or lower; Appendix 1, Section 4).

7.8 Review and finalize the Batch Record and document **ANY** and **ALL** deviations from this SOP in the Batch Record (Appendix 1, Section 5).

7.9 The Laboratory Director/Supervisor should review the Batch Record and sign to affirm the data contained within are correct (Appendix 1, Section 6).

APPENDIX 1: BATCH RECORD

A **separate** Batch Record should be started for each patient sample.

Place PD Specimen Label Here

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

Certified Assay Operator: _____

Certification Number: _____

☐ Check here if DCTD PK/PD Support Lab Personnel (no certification number needed)

Facility/Clinic Collecting Specimens: _____

Clinical Protocol Number: _____

Patient ID: _____

1. Biopsy Collection Method

☐ Collected into degassed H-CEB (Complete) collection tubes

☐ Collected dry, flash frozen in 2-mL Sarstedt tubes

2. H-CEB (Complete) Collection Tubes (if applicable**)**

Reagent Name	**Expiration Date** (5 wk after preparation)
Degassed HIF-1 alpha Biopsy Collection Tubes	/ /

3. Biopsy Collection

A. 1st Pass Biopsy Collection

Specimen ID _____

Biopsy size collected: ☐ Full ☐ Half

	Degassed Buffer			**Dry, Flash Frozen**	
Time biopsy collected	:		(opt)	:	
Time biopsy placed in tube	:		(opt)	:	
Time elapsed from collection to placement in tube	min	sec	**Required**	min	sec
Time placed in liquid nitrogen or a dry ice/ethanol bath	:			:	

B. 2nd Pass Biopsy Collection (if available)

Specimen ID _____

Same or different biopsy site: ☐ Same ☐ Different

Biopsy size collected: ☐ Full ☐ Half

	Degassed Buffer			**Dry, Flash Frozen**	
Time biopsy collected	:		(opt)	:	
Time biopsy placed in tube	:		(opt)	:	
Time elapsed from collection to placement in tube	min	sec	**Required**	min	sec
Time placed in liquid nitrogen or a dry ice/ethanol bath	:			:	

4. Biopsy Storage

Date/time biopsy specimen(s) placed at -80°C (or lower) ____/__/____ ____:____ _____ºC

5. Notes, including any deviations from the SOP:

6. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____(PRINT)

_____(SIGN)

Date: ____/____/_____

APPENDIX 2: PD SAMPLE SHIPPING MANIFEST

From: Phone: E-mail:	**PD Sample Shipping Manifest**	

In Package	Item No	Patient/Specimen ID	Clinical Protocol	Description	Time Point Scheduled	Collection Date	Collection Time
	Example	*1234-1023-500*	*12-C-0000*	*Full biopsy*	*Pre-dose D1*	*06/12/12*	*08:50*
	Example	*1234-1023-501*	*12-C-0000*	*Half biopsy*	*Cycle 1, D8*	*06/20/12*	*16:05*
☐	1					/ /	
☐	2					/ /	
☐	3					/ /	
☐	4					/ /	
☐	5					/ /	
☐	6					/ /	
☐	7					/ /	
☐	8					/ /	
☐	9					/ /	
☐	10					/ /	

Verification of Contents	**Signature**	**Date**
Contents Verified: Collection Laboratory	_____	/ /
Contents Verified: FNLCR PD Central Receiving	_____	/ /
Contents Verified: PD Laboratory	_____	/ /

APPENDIX 3: PREPARATION OF DEGASSED H-CEB TUBES FOR BIOPSY COLLECTION

1. MATERIALS AND EQUIPMENT REQUIRED

- **A.** Sarstedt o-ring screw cap tubes, 1.5-mL (Sarstedt, Cat# 72.692.005)
- **B.** Pipettors (200-1000 µL, 50-200 µL, 2-20 µL) and tips
- **C.** Pasteur pipette, 9 inch, cotton plugged (e.g., Fisher Scientific, Cat#: 22-209-361); sterilized
- **D.** Vacuum filter/storage bottle system, 0.22-µm pore, 500 mL (e.g., Corning, Cat#: 430758)
- **E.** 15-mL polypropylene tube (e.g., VWR, Cat#: 21008-918)
- **F.** 50-mL polypropylene tubes (e.g., VWR, Cat#: 21008-951)
- **G.** Tygon or latex tubing
- **H.** 81-place freezer boxes (e.g., Fisher Scientific, Cat#: 12-565-182)
- **I.** Ice bucket
- **J.** UltraPure DNase⁄RNase-free distilled water (e.g., Life Technologies, Cat#: 10977-015) or Milli-Q water
- **K.** Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P2714 or Roche Diagnostics, Cat#: 11697498001)
- **L.** Phenylmethanesulfonyl fluoride solution, 0.1 M (PMSF; Sigma-Aldrich, Cat#: 93482-50ML-F)
- **M.** Tris, ultra pure (e.g., MP Biomedicals, Cat#: 04819620 or 04819623)
- **N.** Sodium chloride, ReagentPlus grade (e.g., Sigma-Aldrich, Cat#: S9625)
- **O.** Glycerol (e.g., Sigma-Aldrich, Cat#: G5516)
- **P.** EDTA, 0.5 M, pH 8.0 (e.g., Boston BioProducts, Cat#: BM-150)
- **Q.** Magnesium chloride, anhydrous (e.g., Sigma-Aldrich, Cat#: M8266)
- **R.** β-Glycerol phosphate disodium salt, pentahydrate (e.g., Sigma-Aldrich, Cat#: 50020)
- **S.** Sodium fluoride, ACS grade (e.g., Sigma-Aldrich, Cat#: 201154)
- **T.** Triton X-100, non-ionic, aqueous solution, 10% w/v, stored according to manufacturer's direction (e.g., Roche Diagnostics, Cat#: 11332481001)
- **U.** D-2-hydroxypentanedioic acid disodium salt (2R), MW = 192.08 (2-hydroxyglutarate [2HG]; CAS#: 103404-90-6; 3B Scientific Corporation, Cat#: 3B3-053228)
- **V.** Wet ice
- **W.** Bench top dry nitrogen supply with regulator or piped nitrogen dispensed through a filtering apparatus
- **X.** Class II Type A2 biosafety cabinet/tissue culture laminar flow hood
- **Y.** -20°C and -80°C (or lower) freezers
- **Z.** 2°C to 8°C refrigerator

2. Protocol

- **A.** <u>Prepare reagents. Reagents may be prepared ahead of time, but do not add PIs or 2HG to H-CEB until noted in the SOP.</u>
  - a. Protease Inhibitor Cocktail Tablets: Dissolve one PI cocktail tablet in 2 mL ultrapure DNase⁄RNase-free water (25X stock). The 25X stock solution is stable for 1 wk at 2°C to 8°C or 12 wk at -15°C to -25°C. If stored frozen, the material must be prepared as single-use aliquots to prevent repeat freeze-thaw.
  - b. PMSF: Manufacturer's stock solution supplied at 100 mM. Label vial with date of receipt from manufacturer; the expiration date should be considered 6 mo after receipt.

c. 2HG (MW = 192.08): Dissolve 3.84 mg 2HG into 2.0 mL ultrapure DNase⁄RNase-free water to make a 10 mM stock solution. Make 60 µL aliquots and store at -80ºC.

d. HIF-1 alpha Cell Extraction Buffer (H-CEB) **(without** PIs+2HG): Prepare 100 mL of buffer at a time by adding the following reagents to 75 mL ultrapure DNase⁄RNase-free water. Once all reagents have been added, adjust volume to 100 mL with additional ultrapure DNase⁄RNase-free water. Sterile filter the H-CEB **(without** PIs+2HG) and store at 2ºC to 8ºC for ≤ 3 mo.

Reagent	Molecular Weight/ Concentration	Amount Needed	Final Concentration
Tris	121.14	605.7 mg	50 mM Tris
NaCl	58.44	1753.2 mg	300 mM NaCl
Glycerol	100%	10 mL	10% Glycerol
EDTA	0.5 M	0.6 mL	3 mM EDTA
$MgCl_2$	95.22	9.5 mg	1 mM $MgCl_2$
β-Glycerol	306.11	612.2 mg	20 mM β-Glycerol
NaF	41.99	105 mg	25 mM NaF
Triton X-100	10%	10 mL	1% Triton

B. Degas H-CEB (Complete) Buffer

a. H-CEB (Complete) can be prepared, degassed, and stored at -80°C for up to 5 weeks.

The following recipe is for preparation of 7.5 mL degassed buffer in a 15-mL polypropylene tube on ice; scale up as needed. Keep H-CEB (Complete) on ice.

Reagent	Stock Concentration	Amount Needed	Final Concentration
H-CEB (**without** PIs+ 2HG)	--	7050 µL	N/A
PI Cocktail	25X	300 µL	1X PI Cocktail
PMSF	100 mM	75 µL	1 mM PMSF
2HG	10 mM	75 µL	0.1 mM 2HG

b. Label Sarstedt tubes with the preparation date (~20-25 tubes will be used).

c. In a biological safety cabinet with fans turned off to minimize air flow, attach a sufficient length of tygon or latex tubing to the nitrogen gas regulator so that when a Pasteur pipette is attached to the other end of the tubing it moves freely within the cabinet.

d. Place the 15-mL tube of H-CEB (Complete) and the Sarstedt tubes in the cabinet on ice.

e. Attach a sterile Pasteur pipette to the free end of the tubing and turn on the nitrogen valve so that the pressure is no more than 10 PSI. Once the correct PSI has been reached, open the dispensing valve just enough to start nitrogen flowing through the pipette.

f. Place the pipette into the 15-mL tube of freshly made H-CEB (Complete) and slowly increase the nitrogen flow so that the gas bubbles through the H-CEB (Complete) vigorously, but does not splash buffer outside of the tube.

g. Degas the buffer for 3 min and then turn the flow rate down, slowly withdrawing the pipette from the tube while filling the head of the tube with nitrogen. Remove the pipette and immediately cap the 15-mL tube to minimize oxygen seepage back into the tube. Keep tube on ice.

C. 1.5-mL Sarstedt tube preparations

a. Fill volume is dependent on end-use:

i) If degassed buffer will be used to directly collect fresh tissue biopsies, 250 µL degassed buffer will be added to each tube.

ii) If the degassed buffer will be used for extraction of dry, flash frozen biopsies in SOP340910, 300 µL degassed buffer will be added to each tube.

b. Open five (5) 1.5-mL tubes and flare with nitrogen.

- To minimize oxygen seepage into tubes, no more than five (5) 1.5-mL tubes should be prepared at one time between dispensing degassed H-CEB (Complete) and capping.

c. Open the 15-mL tube with degassed H-CEB (Complete) and pipette required volume of degassed buffer into each 1.5-mL tube; immediately recap the 15-mL tube.

d. Quickly place the pipette with low flow nitrogen into the head of each 1.5-mL tube to fill the airspace with nitrogen and screw the cap onto the tube and place on ice.

D. Store the Degassed H-CEB (**with** PIs+2HG) at -80°C until use

a. Place the Sarstedt tubes containing degassed H-CEB (Complete) into an 81-place freezer box and store at -80ºC (or lower) for no longer than 5 weeks from date of preparation.