NIH NATIONAL CANCER INSTITUTE

SOP341401: Tumor Biopsy Lysate Fractionation for the Apoptosis Multiplex Immunoassay Panels

Effective Date: 12/23/19

Please check for revision status of the SOP at

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

and be sure to use the current version.

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Version History

1. Approvals

Technical Reviewer:	Apurva K. Srivastava	Date:	Apurva K. Srivastava -S (Affiliate) Digitally signed by Apurva K. Srivastava -S (Affiliate) Date: 2020.01.03 13:46:57 -05'00'
NCTVL Approval:	Jiuping Ji	Date:	Jiuping Ji -S (Affiliate) Digitally signed by Jiuping Ji -S (Affiliate) Date: 2020.01.06 10:04:15 -05'00'
IQC Approval:	Katherine V. Ferry-Galow	Date:	Katherine V. Ferry-galow -S (Affiliate) Digitally signed by Katherine V. Ferry-galow -S (Affiliate) Date: 2020.01.06 16:30:32 -05'00'
LHTP Approval:	Ralph E. Parchment	Date:	Ralph E. Parchment -S (Affiliate) Digitally signed by Ralph E. Parchment -S (Affiliate) Date: 2020.02.05 20:02:55 -05'00'
DCTD OD Approval:	Toby Hecht	Date:	Toby T. Hecht -S Digitally signed by Toby T. Hecht -S Date: 2020.02.14 15:56:45 -05'00'

2. Change History

Revision	Approval Date	Description	Originator	Approval
--	5/12/2010	New Document	AKS, SJ	AKS
A	5/16/2014	Format for DCTD style, define critical reagents, expand Batch Record, and define assay steps.	YAE, KFG, JPG	AKS
B	03/18/2016	Modifications made to BCA assay steps and plate layout to match to terminology and BCA workflow used across other DCTD protein-based pharmacodynamic assays. Appendix added for guidelines to process preclinical tumors.	KFG, YAE	AKS
C	12/23/2019	Modifications made to add correct biopsy tube number, clarification on shaking step, clarify steps for handling xenograft tumor quarters (Appendix 4), add section for PBMCs fractionation, and clarify %CV criteria for BCA assays.	JPG, KFG, LL, KZ	AKS

OVERVIEW OF APOPTOSIS PANEL MULTIPLEX ASSAY

SOP340507: Tumor Frozen Needle Biopsy Collection and Handling	• Collect and flash-freeze fresh tumor needle biopsies in 1.5 mL conical tubes within 2 min • Immediately place in liquid nitrogen or on dry ice/ethanol • Ship to biopsy processing laboratory or biorepository

*** LHTP003.08.20:** Separation of Mononuclear Cells from Whole Blood and Bone Marrow for Lysate Based Immunoassay Analysis	• Collect peripheral blood or bone marrow • Purification of PBMCs • Immediately place in liquid nitrogen or on dry ice after collection • Ship to sample processing laboratory or biorepository

SOP341401: Tumor Biopsy Lysate Fractionation for the Apoptosis Multiplex Immunoassay Panels	• Extract fractionated lysates from tumor biopsy or PBMCs • Determine protein concentration • Store stock lysate or immediately proceed to BioPlex Luminex Assays

SOP341402: Procedure for Use of Bio-Rad Bio-Plex Pro RBM Apoptosis Panel Kits 1 - 3 for the Analysis of Clinical Tumor Biopsy Extracts and Reporting of Data	• Use Bio-Rad BioPlex kits to perform Multiplex Assay on standards, controls, and clinical samples • Use Luminex plate reader to determine relative signal in samples using xPonent Software & Bio Plex Manager software • Determine apoptosis biomarker concentrations in samples and verify quality controls passed acceptance criteria. • Prepare and sign a Clinical Sample Data Report. Send the report to the protocol Principal Investigator

	Biomarkers
RBM Apoptosis Panel 1:	BAX, BAK, Lamin B (Intact and 45 KDa cleaved fragment), SMAC
RBM Apoptosis Panel 2:	BAD, BAX:BCL2 heterodimer, BCL-XL, BIM, MCL1
RBM Apoptosis Panel 3:	BCL-XL:BAK heterodimer, Active caspase 3, MCl1:BAK heterodimer, Survivin

* LHTP003.08.20 will be available after clinical evaluation.

1.0 PURPOSE

Standardize the method for preparing cytosolic and combined nuclear/mitochondrial cell fraction lysates from frozen needle tumor biopsies and frozen PBMCs isolated from peripheral blood and bone marrow to enable quantification of analyte levels with multiplex immunoassays using Luminex platform in pharmacodynamic (PD) studies of BH3 mimetics and/or other chemotherapeutic agents.

2.0 SCOPE

This procedure applies to all personnel involved in the use of apoptotic proteins as PD markers during clinical trials and in the preparation of samples for the analysis of protein levels in the apoptotic panel by the multiplex immunoassays. The goal of the SOP and associated training is to ensure consistency in preparation of nuclear and cytosolic cell fractions from clinical samples for use in the apoptosis multiplex panel immunoassays. General guidelines for applying this procedure to preclinical samples is provided in Appendix 4.

3.0 ABBREVIATIONS

BH3	=	Bcl-2 Homology Domain
BSA	=	Bovine Serum Albumin
Cyto	=	Cytosolic
DCTD	=	Division of Cancer Treatment and Diagnosis
IA	=	Immunoassay
LHTP	=	Laboratory of Human Toxicology and Pharmacology
MIM	=	Cyto extraction buffer
NCTVL	=	National Clinical Target Validation Laboratory
NucMito	=	Nuclear + Mitochondrial
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PBMC	=	Peripheral Blood Mononuclear Cell
PBS	=	Phosphate Buffered Saline
PD	=	Pharmacodynamic
PI	=	Protease Inhibitors
QC	=	Quality Control
RBM	=	Myriad RBM, Inc., Austin, TX
SAPE	=	Streptavidin-Phycoerythrin
SD	=	Standard Deviation
SOP	=	Standard Operating Procedure
Temp	=	Temperature
WR	=	Working Reagent

4.0 INTRODUCTION

The Apoptosis Multiplex Immunoassay has been developed to measure the levels of biomarkers in the apoptosis panel(s) using the Luminex platform (Millipore). The assays first capture proteins of interest from fractionated cell extracts on antibody-coated magnetic beads. The captured proteins are then detected using biotinylated detection antibodies followed by a streptavidin-phycoerythrin (SAPE) conjugate to allow fluorescence read-out and quantification.

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. 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.
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 and reports to 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 and works under the guidance of the Laboratory Director/Supervisor.

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 table in the Batch Record (Appendix 1, Section 3), Plate Map (Appendix 2), and BCA Assay Calculations (Appendix 3) can be created for logging sample information as long as all column information exactly matches the table in the Batch Record. A copy of the completed, digital sample table 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 SOP version is being used.

6.0 CRITICAL REAGENTS, MATERIALS AND EQUIPMENT REQUIRED

6.1 PADIS/IQC Critical Reagents
- **6.1.1** PhosSTOP, phosphatase inhibitor cocktail tablets (Roche, Cat#: 04906837001)
- **6.1.2** cOmplete, mini, EDTA-free protease inhibitor cocktail tablets (Roche, Cat# 04693159001).

6.2 Pipettors (100-1000 µL, 50-200 µL and 2-20 µL) and tips
6.3 Fine-tipped stainless steel scissors (e.g., Noyes Spring Scissors, Fine Science Tools, Cat#: 15012-12)
6.4 1.5-mL Sarstedt o-ring screw cap, conical tubes (Sarstedt Cat#: 72.703.416)
6.5 2.0-mL Sarstedt o-ring screw cap, skirted tubes (Fisher Scientific, Cat#: 72.694.006)
6.6 50-mL polypropylene tubes (e.g., VWR, Cat#: 21008-951))
6.7 0.4-mL 96-well flat bottom plate, clear (e.g., Nunc, Cat#: 260836)
6.8 Printable microcentrifuge tube labels
6.9 Cryogenic marker
6.10 81-place freezer storage boxes (e.g., Fisher Scientific, Cat#: 12-565-182)
6.11 Dry ice
6.12 Ice bucket

6.13 UltraPure DNase/RNase-free distilled water (e.g., Life Technologies, Cat#: 10977-015)
6.14 EDTA, 0.5 M, pH 8.0 (e.g., Boston BioProducts, Cat#: BM-150)
6.15 HEPES (Sigma-Aldrich, Cat#: H7523)
6.16 Sucrose (Sigma-Aldrich, Cat#: S7903)
6.17 CHAPS, non-ionic, powder (Sigma-Aldrich, Cat# C3023)
[Prepare a 30% stock solution w/v in UltraPure DNase/RNase-free distilled water and store at 2°C-8°C for up to 1 y]
6.18 Phosphate Buffered Saline, 10X, pH 7.2 (PBS; Invitrogen, Cat#: 70013-072)
[Dilute 1:10 in UltraPure DNase/RNase-free distilled water to prepare 1X PBS]
6.19 Triton X-100, non-ionic, aqueous solution, 100% w/v, stored according to manufacturer's direction (e.g., Sigma-Aldrich, Cat#: T8787)
[Prepare a 20% w/v working solution in UltraPure DNase/RNase-free distilled water; dissolve for 2 hours at 25°C -28°C; store at 2°C-8°C in dark for up to 6 mo]
6.20 BCA Protein Assay Kit (Thermo Scientific Pierce, Cat#: 23227 or 23225)

6.21 PRO200 Homogenizer, 120 V (Pro Scientific, Cat#: 01-01200)
6.22 Multi-Gen 7 motor unit adapter (Pro Scientific, Cat#: 07-07200)
6.23 Multi-Gen homogenizer generator, 5 mm (Pro Scientific, Cat#: 02-05075)
6.24 Sorvall Legend Microcentrifuge, refrigerated (Fisher Scientific)
6.25 Vortex mixer, digital, 500-3000 rpm (Fisher Scientific, Cat#: 02-215-370)
6.26 Standard orbital shaker, model 1000 (VWR International, Cat#: 89032-088)
6.27 Infinite® 200 Microplate Reader (Tecan US) with instrument included i-control microplate reader software (alternative: Magellan data analysis software)
6.28 -20°C and -80°C freezers
6.29 4°C refrigerator

6.30 Microsoft Excel 2003 or newer
6.31 Frozen needle biopsy samples processed following SOP340507 (Tumor Frozen Needle Biopsy Sample Collection and Handling)

7.0 OPERATING PROCEDURES

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.

7.2 A maximum of 5 biopsy specimens can be processed in one extraction run. Clinical specimens from patients with pre- and post-dose biopsies should be run with 4 biopsy samples from 2 patients.

7.2.1 Tumor biopsies and PBMCs have limited stability at -80°C; however, the stability of biomarkers of Apoptosis Panel in human tumor biopsies and PBMCs has not yet been determined. Therefore, process tumor biopsies and PBMCs as soon after collection as possible. **Batch** samples from a single patient to ensure consistent sample handling.

7.3 Record the name and certification number of the Certified Assay Operator, the facility running the SOP, and the clinical protocol number in the Batch Record (Appendix 1).

7.4 Critical Reagent

7.4.1 Record the lot number, concentration, and expiration date for the Critical Reagent in the Batch Record (Appendix 1, Section 1). Store as indicated below. Label with date of receipt and store under the specified conditions for no longer than the recommended duration.

7.4.1.1 **PhosSTOP, phosphatase inhibitor cocktail tablets**: Supplied as individual tablets; each tablet is sufficient for 10 mL of prepared buffer according to the SOP. Store at 2-8°C until manufacturer's expiration date or date provided on IQC shipping manifest.

7.4.1.2 **cOmplete, mini, EDTA-free protease inhibitor cocktail tablets**: Supplied as individual tablets; each tablet is sufficient for 10 mL of prepared buffer according to the SOP. Store at 2-8°C until manufacturer's expiration date or date provided on IQC shipping manifest.

7.5 Record model and serial numbers of the equipment to be used in the Batch Record (Appendix 1, Section 2A). Prepare the buffers listed in the Batch Record (Appendix 1, Section 2B).

7.6 Tissue Lysis for Needle Biopsies

7.6.1 Fill in the Sample Information Table in the Batch Record (Appendix 1, Section 3A) with Patient/Sample ID for each biopsy to be processed. Keep biopsy samples submerged in dry ice (not resting on top). Process biopsies individually through the homogenization step and ice incubation (Step 7.6.7; do not process more than 5 specimens at one time).

7.6.2 Determine the total volume of mitochondrial isolation medium (MIM) (**with** inhibitors) and Buffer-A (**with** inhibitors) needed for all samples using the calculations in the Batch Record (Appendix 1, Section 3B).

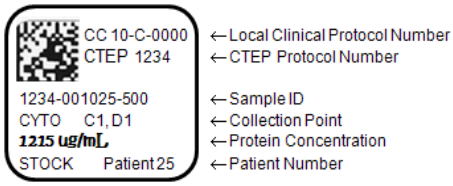
7.6.2.1 Round both volumes up to nearest 10-mL volume and prepare by dissolving one Protease Inhibitor (PI) cocktail tablet and one PhosSTOP tablet into each 10 mL; referred to as "**with** inhibitors".

7.6.2.2 Both buffers should be chilled on ice for 30 min prior to addition of tablets.

7.6.3 During processing, both a cytosolic ("Cyto") and a nuclear+mitochondrial ("NucMito") lysate fraction will be isolated.

7.6.3.1 For each sample, label two (2) 2-mL Sarstedt tubes (for stock lysate fractions) and four (4) 1.5-mL Sarstedt tubes (for BCA Assay) with the Patient/Sample ID; label half of the tubes for each size as "Cyto" and half as "NucMito." Keep tubes on ice so they are pre-chilled when needed in SOP.

- Example of a tube label for a Cyto fraction 2-mL Sarstedt tube. Protein concentration will be filled in using a cryogenic marker.



7.6.4 Move **<u>one</u>** tube containing an 18-g frozen needle biopsy to wet ice and add 350 µL pre-chilled MIM (**with** inhibitors); biopsies should have been frozen in 1.5-mL conical bottomed tubes (Sarstedt 72.703.416).

7.6.5 Immediately mince the still-frozen tissue with fine scissors in the tube, keeping tube on ice. Note: between samples, clean scissors with sterile water and wipe with Kimwipe.

7.6.6 Place the biopsy tube in a small beaker with wet ice and immediately homogenize the biopsy with the PRO200 homogenizer with Multi-Gen adaptor and 5 mm generator at a medium setting (3) for 5 sec. The tube should remain on ice throughout the homogenization process. Record the actual homogenizer setting in the Batch Record (Appendix 1, Section 3C).

7.6.7 Place samples in an ice/water bath and incubate on a standard orbital shaker for a minimum of 10 min (speed could be set at 200 rpm). Record the start time of the incubation <u>for the first sample</u> in the Batch Record (Appendix 1, Section 3C).

7.6.7.1 If more than one biopsy is to be homogenized, fill a 50-mL polypropylene tube with ~20 mL sterile water, immerse the end of the 5 mm grinder in the water, and run at medium setting (3) for 2-3 sec. Wipe the grinder with a clean Kimwipe and return to Step 7.6.4 to process the next sample.

7.6.8 Quickly homogenize additional samples and place them on ice. **<u>The maximum time on ice for any sample should be 20 min</u>**, the last sample processed will have the shortest incubation time (10 min). Record the stop time for incubation in the Batch Record (Appendix 1, Section 3C).

7.6.9 Follow **steps 7.6.10-7.6.15** to **Collect Cytosolic ("Cyto") Fraction**

7.6.10 Centrifuge total cell lysate in a Sorvall Fresco microcentrifuge at 16,000 x g for 30 min at 2-8ºC.

7.6.11 Carefully, transfer the supernatant (without disturbing the pellet) into the pre-chilled, 2-mL Sarstedt tube labeled "Cyto" and keep lysate on ice.

- **Important**: The pellet contains the membrane, mitochondrial, and nuclear (NucMito) cell fractions, keep the tube with pellet on ice for processing according to Step 7.6.16.

7.6.12 To each Cyto stock lysate, add 17.5 µL 20% Triton X-100 and 21 µL 10% CHAPS. Mix by pipetting up and down 5-8 times and minimize creation of bubbles. Discard excess 10% CHAPS working solution.

7.6.13 At this point, begin Steps 7.6.17 and 7.6.18; while the NucMito pellet is centrifuging, complete Steps 7.6.14 and 7.6.15 below.

7.6.14 For each Cyto stock lysate, transfer a 20 µL aliquot into one of the 1.5-mL Sarstedt tubes labeled "Cyto" and add 80 µL 1X PBS; label the tube "1:5" for BCA Protein Assay (Section 8.0). In the second 1.5-mL Sarstedt tube labeled "Cyto", prepare a 1:10 dilution by mixing 10 µL of the lysate with 90 µL 1X PBS and label it "1:10".

You should now have two tubes labeled "SampleID-Cyto-1:5" and "SampleID-Cyto-1:10".

7.6.14.1 If this is the second time a sample is being assayed, a different dilution factor may be needed.

7.6.14.2 Verify the dilutions made for the BCA Protein Assay in the Sample Information Table (Appendix 1, Section 3A).

7.6.14.3 If the BCA Protein Assay will not be performed immediately, the 1.5-mL tube aliquots can be snap-frozen on dry ice and stored at -80ºC for up to 5 d before analysis. If the BCA assay will be performed the same day, store the samples at 2-8ºC.

7.6.15 Snap-freeze Cyto stock lysates on dry ice, and store at -80ºC. Record the date and time stock lysate is frozen in the Batch Record (Appendix 1, Section 4).

7.6.16 Follow **steps 7.6.17-7.6.24** to **Lyse and Collect Nuclear+Mitochondrial ("NucMito") Fraction**

7.6.17 Wash the NucMito pellet by adding 350 µL MIM (**with** inhibitors). Pipette up and down 5 times with the 100-1000 µL pipette.

7.6.18 Centrifuge the sample in Sorvall Fresco microcentrifuge at 16,000 x g for 10 min at 2-8°C. Without disturbing the pellet, remove and discard the supernatant; save the pellet.

7.6.19 Wash the pellet again with 350 µL MIM (**with** inhibitors), pipetting up and down to mix. Centrifuge at 16,000 x g for 10 min at 2-8°C. Without disturbing the pellet, remove the supernatant and discard; save the pellet.

7.6.20 Resuspend the pellet in 350 µL Buffer-A (**with** inhibitors) and vortex for 10 sec at maximum speed on the Digital Vortex Mixer (3000 rpm).

7.6.21 Place samples in an ice/water bath and incubate at 2-8ºC for 45 min on a standard orbital shaker with shake speed set at 4. Vortex samples every 20 min for 10 sec while samples are shaking.

7.6.22 Clarify lysates by centrifugation in a Sorvall Fresco microcentrifuge at 16,000 x g for 10 min at 2-8ºC. Transfer the NucMito stock lysate into the pre-chilled, 2-mL Sarstedt tube labeled "NucMito" and keep lysate on ice. Discard the original tube with any precipitated material in an appropriate waste container.

7.6.23 For each NucMito stock lysate, transfer a 20 µL aliquot into one of the 1.5-mL Sarstedt tubes labeled "NucMito" and add 80 µL 1X PBS; label the tube "1:5" for BCA Protein Assay (Section 8.0). In the second 1.5-mL Sarstedt tube labeled "NucMito," prepare a 1:10 dilution by mixing 10 µL of the lysate with 90 µL 1X PBS and label it "1:10".

You should now have two tubes labeled "SampleID- NucMito-1:5" and "SampleID-NucMito-1:10".

7.6.23.1 If this is the second time a sample is being assayed, a different dilution factor may be needed.

7.6.23.2 Verify the dilutions made for the BCA Protein Assay clinical sample aliquots in the Sample Information Table (Appendix 1, Section 3A).

7.6.23.3 If the BCA Protein Assay will not be performed immediately, the 1.5-mL tube aliquots can be snap-frozen on dry ice and stored at -80ºC for up to 5 d before analysis. If the BCA assay will be performed the same day, then store the samples at 2-8 ºC.

7.6.24 Snap-freeze the NucMito stock lysate on dry ice, and store at -80ºC. Record the date and time stock lysate is frozen in the Batch Record (Appendix 1, Section 4).

7.6.25 Proceed to Section **8.0 BICINCHONINIC ACID (BCA) PROTEIN ASSAY** for protein concentration measurement

7.7 Cell Lysis for PBMCs

7.7.1 Fill in the Sample Information Table in the Batch Record (Appendix 1, Section 3A) with Patient/Sample ID for PBMC sample to be processed. Keep cell pellets submerged in dry ice (not resting on top). Process PBMC samples individually through the homogenization step and ice incubation (Step 7.7.6; do not process more than 5 specimens at one time).

7.7.2 Determine the total volume of mitochondrial isolation medium (MIM) (**with** inhibitors) and Buffer-A (**with** inhibitors) needed for all samples using the calculations in the Batch Record (Appendix 1, Section 3B).

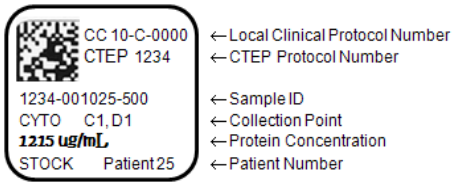
7.7.2.1 Round both volumes up to nearest 10-mL volume and prepare by dissolving one Protease Inhibitor (PI) cocktail tablet and one PhosSTOP tablet into each 10 mL; referred to as "**with** inhibitors".

7.7.2.2 Both buffers should be chilled on ice for 30 min prior to addition of tablets.

7.7.3 During processing, both a cytosolic ("Cyto") and a nuclear+mitochondrial ("NucMito") lysate fraction will be isolated.

7.7.3.1 For each sample, label two (2) 2-mL Sarstedt tubes (for stock lysate fractions) and four (4) 1.5-mL Sarstedt tubes (for BCA Assay) with the Patient/Sample ID; label half of the tubes for each size as "Cyto" and half as "NucMito". Keep tubes on ice so they are pre-chilled when needed in SOP.

- Example of a tube label for a Cyto fraction 2-mL Sarstedt tube. Protein concentration will be filled in using a cryogenic marker.



7.7.4 Move **one** tube containing a PBMC pellet to wet ice and add 250 µL of pre-chilled MIM (**with** inhibitors) per 20 million PBMCs; cell pellets should have been frozen in 1.5-mL conical bottomed tubes (Sarstedt 72.703.416).

7.7.5 Place the sample tube in a small beaker with wet ice and immediately homogenize the biopsy with the PRO200 homogenizer with Multi-Gen adaptor and 5 mm generator at a medium setting (3) for 5 sec. The tube should remain on ice throughout the homogenization process. Record the actual homogenizer setting in the Batch Record (Appendix 1, Section 3C).

7.7.6 Place samples in an ice/water bath and incubate on a standard orbital shaker for a minimum of 10 min (shaker speed could be set at 200 rpm). Record the start time of the incubation for the first sample in the Batch Record (Appendix 1, Section 3C).

7.7.6.1 If more than one biopsy is to be homogenized, fill a 50-mL polypropylene tube with ~20 mL sterile water, immerse the end of the 5 mm grinder in the water, and run at medium setting (3) for 2-3 sec. Wipe the grinder with a clean Kimwipe and return to Step 7.6.4 to process the next sample.

7.7.7 Quickly homogenize additional samples and place them on ice. **The maximum time on ice for any sample should be 20 min**, the last sample processed will have the shortest incubation time (10 min). Record the stop time for incubation in the Batch Record (Appendix 1, Section 3C).

7.7.8 Follow **steps 7.7.9-7.7.14** to **Collect Cytosolic ("Cyto") Fraction**

7.7.9 Centrifuge total cell lysate in a Sorvall Fresco microcentrifuge at 16,000 x g for 30 min at 2-8ºC.

7.7.10 Carefully, transfer the supernatant (without disturbing the pellet) into the pre-chilled, 2-mL Sarstedt tube labeled "Cyto" and keep lysate on ice.

- **Important**: The pellet contains the membrane, mitochondrial, and nuclear (NucMito) cell fractions, keep the tube with pellet on ice for processing according to Step 7.7.15.

7.7.11 To each Cyto stock lysate, add 12.5 µL 20% Triton X-100 and 15 µL 10% CHAPS. Mix by pipetting up and down 5-8 times and minimize creation of bubbles. Discard excess 10% CHAPS working solution.

7.7.12 At this point, begin Steps 7.7.16 and 7.7.17; while the NucMito pellet is centrifuging, complete Steps 7.7.13 and 7.7.14.

7.7.13 For each Cyto stock lysate, transfer a 20 µL aliquot into one of the 1.5-mL Sarstedt tubes labeled "Cyto" and add 80 µL of 1X PBS; label the tube "1:5" for BCA Protein Assay (Section 8.0). In the second 1.5-mL Sarstedt tube labeled "Cyto," prepare a 1:10 dilution by mixing 10 µL of the lysate with 90 µL 1X PBS and label it "1:10".

You should now have two tubes, labeled "SampleID-Cyto-1:5" and "SampleID-Cyto-1:10".

7.7.13.1 If this is the second time a sample is being assayed, a different dilution factor may be needed.

7.7.13.2 Verify the dilutions made for the BCA Protein Assay in the Sample Information Table (Appendix 1, Section 3A).

7.7.13.3 If the BCA Protein Assay will not be performed immediately, the 1.5-mL tube aliquots can be snap-frozen on dry ice and stored at -80ºC for up to 5 d before analysis. If the BCA assay will be performed the same day, store the samples at 2-8ºC.

7.7.14 Snap-freeze Cyto stock lysates on dry ice, and store at -80ºC. Record the date and time stock lysate is frozen in the Batch Record (Appendix 1, Section 4).

7.7.15 Follow **steps 7.7.16-7.7.23** to **Lyse and Collect Nuclear+Mitochondrial ("NucMito") Fraction**

7.7.16 Wash the NucMito pellet by adding 250 µL MIM (**with** inhibitors). Pipette up and down 5 times with the 100-1000 µL pipette.

7.7.17 Centrifuge the sample in Sorvall Fresco microcentrifuge at 16,000 x g for 10 min at 2-8°C. Without disturbing the pellet, remove and discard the supernatant; save the pellet.

7.7.18 Wash the pellet again with 250 µL MIM (**with** inhibitors), pipetting up and down to mix. Centrifuge at 16,000 x g for 10 min at 2-8°C. Without disturbing the pellet, remove the supernatant and discard; save the pellet.

7.7.19 Resuspend the pellet in 250 µL Buffer-A (**with** inhibitors) and vortex for 10 sec at maximum speed on the Digital Vortex Mixer (3000 rpm).

7.7.20 Place samples in an ice/water bath and incubate at 2-8ºC for 45 min on a standard orbital shaker with shake speed set at 4. Vortex samples every 20 min for 10 sec while samples are shaking.

7.7.21 Clarify lysates by centrifugation in a Sorvall Fresco microcentrifuge at 16,000 x g for 10 min at 2-8ºC. Transfer the NucMito stock lysate into the pre-chilled, 2-mL Sarstedt tube labeled "NucMito" and keep lysate on ice. Discard the original tube with any precipitated material in an appropriate waste container.

7.7.22 For each NucMito stock lysate, transfer a 20 µL aliquot into one of the 1.5-mL Sarstedt tubes labeled "NucMito" and add 80 µL 1X PBS; label the tube "1:5" for BCA Protein Assay (Section 8.0). In the second 1.5-mL Sarstedt tube labeled "NucMito," prepare a 1:10 dilution by mixing 10 µL of the lysate with 90 µL 1X PBS and label it "1:10".

You should now have two tubes labeled "SampleID- NucMito-1:5" and "SampleID-NucMito-1:10".

7.7.22.1 If this is the second time a sample is being assayed, a different dilution factor may be needed.

7.7.22.2 Verify the dilutions made for the BCA Protein Assay clinical sample aliquots in the Sample Information Table (Appendix 1, Section 3A).

7.7.22.3 If the BCA Protein Assay will not be performed immediately, the 1.5-mL tube aliquots can be snap-frozen on dry ice and stored at -80ºC for up to 5 d before analysis. If the BCA assay will be performed the same day, then store the samples at 2-8 ºC.

7.7.23 Snap-freeze the NucMito stock lysate on dry ice, and store at -80ºC. Record the date and time stock lysate is frozen in the Batch Record (Appendix 1, Section 4). For protein concentration measurement, proceed to Section **8.0 BICINCHONINIC ACID (BCA) PROTEIN ASSAY**.

8.0 BICINCHONINIC ACID (BCA) PROTEIN ASSAY

8.1 Record the date of the BCA Protein Assay run in the Batch Record (Appendix 1, Section 5).

8.2 Plate Map for the Protein Assay

8.2.1 Use the BCA Protein Plate Map in Appendix 2 for the recommended locations of the standards and unknown samples; the location of the unknown samples should match with the sample number listed in the Sample Information Table in the Batch Record (Appendix 1, Section 3A).

8.2.1.1 Each unknown sample and standard is run in duplicate.

8.2.1.2 Each unknown sample will have one Cyto and one NucMito fraction for analysis and each fraction will have two dilutions (1:5 and 1:10), as prepared according to Steps **7.6.14 and 7.6.23** or **7.7.13 and 7.7.22**.

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

8.3.1 Label eight (8) 1.5-mL Sarstedt tubes, lettered A through H, for the Blank (A) and the 1000 to 15.6 µg/mL BSA standards.

8.3.2 Carefully open the glass ampoule provided with the BCA Protein Assay Kit containing the 2 mg/mL (2000 µg/mL) BSA stock and transfer it to a 1.5-mL Sarstedt tube labeled as "BSA Stock.".

8.3.3 Using the dilution scheme below, pipette the indicated volume of 1X PBS into each tube A-H. Add indicated volume of BSA standard to each tube and vortex to mix. Keep samples on ice. Standards are stable for 10 d at -20°C.

Tube #	Volume and Source of BSA	Volume of Diluent, 1X PBS	Final BSA Conc. (µg/mL)
H	500 µL of 2000 µg/mL BSA Stock	500 µL	1000
G	500 µL of tube # H	500 µL	500
F	500 µL of tube # G	500 µL	250
E	500 µL of tube # F	500 µL	125
D	500 µL of tube # E	500 µL	62.5
C	500 µL of tube # D	500 µL	31.3
B	500 µL of tube # C	500 µL	15.6
A	0 µL	500 µL	Blank

8.4 Preparation of Tumor Lysates for the BCA Protein Assay

8.4.1 If the tumor lysate dilutions prepared for the BCA assay are frozen, thaw on ice, vortex for 5 sec, and return on ice.

8.4.2 For each unknown sample, two dilutions (1:5 and 1:10) of the Cyto and NucMito fractions were prepared in Steps **7.6.14 and 7.6.23** or **7.7.13 and 7.7.22**. Ensure the tubes are labeled with the correct sample number, fraction name, and dilution factor from the Sample Information Table (Appendix 1, Section 3A).

8.5 BCA Protein Assay Procedure

8.5.1 Label a 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 1X PBS; Background Control

8.5.2 Prepare BCA Working Reagent by pipetting 21.56 mL of Reagent A and 440 µL of Reagent B into a 50-mL polypropylene tube and record the lot number for the kit (Appendix 1, Section 5). Pour the BCA Working Reagent into a clean multichannel pipette reservoir

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

8.5.4 Cover plate with acetate film and incubate in a 37°C incubator (without CO_2 and without humidity) for 30 min. Record the date and start time for the incubation in the Batch Record (Appendix 1, Section 5). At the same time, turn on the Tecan Infinite Microplate Reader so it has at least 30 min to warm up before use.

8.5.5 At the end of the 30 min incubation, record the end time in the Batch Record (Appendix 1, Section 5), cool the plate for 5 min at ambient temperature and then immediately read the plate on a Microplate Reader at 562 nm absorbance.

8.6 BSA Standards: RLU Read-out Quality Control (QC)

8.6.1 Using the Tecan exported RLU readings, calculate the mean RLU with standard deviation (SD) and percent coefficient of variation (%CV) for background wells A2 - A11 and each duplicate set of standards (example in Appendix 3).

8.6.2 The mean RLU of the lowest BSA standard must be > 3 SD above the mean background RLU value. In the event that the 15.6 µg/mL standard is ≤ 3 SD above the mean background RLU value but the 31.3 µg/mL standard is > 3 SD, the reportable assay range becomes 31.3 µg/mL – 1000 µg/mL.

8.6.3 The **Assay Fails QC** and must be rerun if:

- The mean RLU of the 15.6 µg/mL standard and the 31.3 µg/mL standard is ≤ 3 SD above the mean background RLU value.
- The mean RLU of the 1000 µg/mL standard is ≥ 2.5
- Any assay standard duplicate has a %CV of ≥ 10%.

8.7 Unknown Samples: RLU Read-out Replicate QC

8.7.1 Calculate the mean RLU with SD and %CV for all Cyto and NucMito fraction samples (example in Appendix 3) and record them in the Sample Information Table (Appendix 1, Section 3A).

8.7.2 A **Sample Fails QC** and must be re-run if:

- Any fraction has a mean RLU value < the mean RLU of the lowest BCA assay standard which is either the 15.6 or the 31.3 µg/mL standard.
- The %CV of both the 1:5 and the 1:10 dilution is ≥ 10%

8.7.3 If the %CV of only the 1:5 dilution sample is ≥ 10%, the 1:10 dilution read-out can be used to calculate the sample protein concentration.

8.7.4 If the mean RLU value of the 1:5 Cyto fractions dilution is > the 1000 µg/mL standard, 1:10 dilution read-out can be used to calculate the sample protein concentration.

8.7.5 If the mean RLU value of the 1:5 NucMito fractions dilution is > the 1000 µg/mL standard, 1:10 dilution read-out can be used to calculate the sample protein concentration.

8.7.6 For failed samples, write "Failed QC" for the final protein concentration in the Sample Information Table (Appendix 1, Section 3A).

8.7.7 For samples that fail because the mean RLU falls outside of the absorbance readings for the standard curve, the sample dilution should be adjusted and rerun.

8.8 Determine Protein Concentration

8.8.1 If the BSA standards passed QC, prepare a standard curve of mean RLU (minus background) versus µg/mL protein. Plot the linear regression line for the standard curve and display the equation for the line and correlation coefficient (example in Appendix 3, Section 1). Attach a copy of the raw data and the graph of the standard curve to the Batch Record.

8.8.2 For all unknown Cyto and NucMito fraction samples that passed QC, use the mean RLU value for each duplicate set of unknown samples, and record the mean RLU readout (minus background) for each tumor fraction dilution (1:5 and 1:10) in the Sample Information Table in the Batch Record (Appendix 1, Section 3A).

8.8.3 Compare the unknown tumor fraction RLU 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 (1:5 and 1:10) on the Sample Information Table (Appendix 1, Section 3A).

8.8.4 For each unknown sample dilution (1:5 and 1:10), back-calculate the protein lysate concentration for dilution (multiply by 5 or 10) and average them to determine the average stock lysate concentration corrected for dilution, and record it in the Sample Information Table (Appendix 1, Section 3A). If the %CV of the protein lysate concentrations calculated from 1:5 and 1:10 dilutions is > 20%, the sample must be rerun.

8.8.5 Write the protein concentration in µg/mL on the label of the 2-mL **stock lysate** tube

8.9 Apoptosis Panel Immunoassay QC Criteria for Tumor Lysates

8.9.1 Tumor lysates will be loaded in the apoptosis panel immunoassays based on total protein concentration, and the final biomarker levels in each unknown sample will be back-calculated based on the µg lysate loaded in each well.

8.9.2 Unknown samples will be analyzed at up to two concentrations in the range of 125 – 500 µg/mL. A minimal protein concentration of **500 µg/mL** is desired, however, protein concentrations down to a minimum concentration of **125 µg/mL** are considered analyzable for some analytes in the Apoptosis Assay Panels. Samples with protein concentrations below **125 µg/mL fail QC** and are not analyzable in the Apoptosis Assay Panels.

8.10 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).

8.11 The Laboratory Director/Supervisor should review the Batch Record and sign to affirm the data contained within are correct (Appendix 1, Section 7).

APPENDIX 1: BATCH RECORD

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

Certified Assay Operator: _____

Certification Number: _____

Facility/Laboratory Running SOP: _____

Clinical Protocol Number: _____

1. Critical Reagents

Reagent Name	Date Received	Lot Number	Provided Concentration	Expiration Date
PhosSTOP, phosphatase inhibitor cocktail tablets			Tablet form	
cOmplete, mini, EDTA-free protease inhibitor cocktail tablets			Tablet form	

2. Equipment and Preparation of Reagents

A. Equipment

Homogenizer Make/Model : _____

_____

Serial #: _____

Microplate reader Make/Model : _____

_____

Serial #: _____

B. Reagents

Buffers should be prepared based on volumes needed to complete all the steps, with at least 10% excess volume of buffer to ensure adequate volume to complete the study.

a. Mitochondrial Isolation Medium (MIM) (**without** inhibitors): Prepare 100 mL of buffer at a time by adding the reagents listed below to 50 mL ultrapure DNase⁄RNase-free water. Once all reagents have been added, adjust volume to 100 mL with additional ultrapure DNase⁄RNase-free water. Store at -20°C for 30 d.

Reagent	Molecular Weight/ Concentration	Amount Needed
EDTA	0.5 M	4 mL
HEPES	238.3	238 mg
Sucrose	342.3	10,260 mg

b. Buffer-A (**without** inhibitors): Prepare 100 mL of buffer at a time by adding the reagents listed below to 50 mL ultrapure DNase⁄RNase-free water. Once all reagents have been added, adjust volume to 100 mL with additional ultrapure DNase⁄RNase-free water and adjust pH to 7.4 with 1N HCl and 1N NaOH. Store at -20°C for 30 d.

Reagent	Molecular Weight/ Concentration	Amount Needed
EDTA	0.5 M	0.2 mL
CHAPS	614.9	615 mg
Triton X-100	10%	5 mL
PBS	10X	10 mL

c. 10% CHAPS: In a Sarstedt tube, prepare a 300 µL working solution of 10% CHAPS by diluting 100 µL 30% CHAPS into 200 µL ultrapure DNase⁄RNase-free distilled water (1:3 dilution).

3. Protein Extraction for Frozen Needle Biopsies

A. Sample Information Table

No.	Patient/Sample ID	Fraction	Determine Protein Concentration: BCA Assay								
			Mean RLU	SD	%CV	Mean RLU (minus background)	Conc. Diluted Lysate (µg/mL)	Dil'n Factor	Corrected for Dilution (µg/mL)	Avg. Conc. Corrected for Dilution (µg/mL)	Conc. QC Pass/ Fail
Ex:	*1234-001025-500*	*Cyto*	*0.393*	*0.003*	*0.72*	*0.301*	*168.9*	*5*	*844*	*845*	*Pass*
			0.259	*0.005*	*1.91*	*0.167*	*84.5*	*10*	*845*		*Pass*
		NucMito	*0.288*	*0.054*	*18.78*	--	--	*5*	--	*551*	*Fail*
			0.212	*0.009*	*4.35*	*0.120*	*55.1*	*10*	*551*		*Pass*
S1		Cyto						5			
								10			
		NucMito						5			
								10			
S2		Cyto						5			
								10			
		NucMito						5			
								10			
S3		Cyto						5			
								10			
		NucMito						5			
								10			
S4		Cyto						5			
								10			
		NucMito						5			
								10			
S5		Cyto						5			
								10			
		NucMito						5			
								10			

B. <u>Calculations for MIM (**with** inhibitors) and Buffer-A (**with** inhibitors)</u>

For biopsies:

Total Vol. MIM (**with** inhibitors) = (___ biopsies + 2) * 1.05 mL = _____ mL

Total Vol. Buffer-A (**with** inhibitors) = (___ biopsies + 2) * 0.35 mL = _____ mL

For PBMCs:

Total Vol. MIM (**with** inhibitors) = (___ PBMC samples + 2) * 0.75 mL= _____ mL

Total Vol. Buffer-A (**with** inhibitors) = (___ PBMC samples + 2) * 0.25 mL= _____ mL

C. Tumor Biopsy or Cell Lysis

Homogenize frozen needle biopsies or cell pellets, **one at a time**, at a setting of _____.

Incubate lysates at 2-8ºC on a standard orbital shaker for 10 min.

No.	Incubation Start Time	Incubation Stop Time
S1	:	N/A
S5 (or last sample analyzed)	N/A	:

4. Biopsy Stock Lysate Storage

"Cyto" fractions frozen on dry ice	Date	/ /	Time	:
"Cyto" tubes placed into -80°C storage	Date	/ /	Time	:
"NucMito" fractions frozen on dry ice	Date	/ /	Time	:
"NucMito" tubes placed into -80°C storage	Date	/ /	Time	:

5. BCA Protein Assay

BCA Working Reagent: **Prepare immediately 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.

6. Notes, including any deviations from the SOP:

7. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: _____

APPENDIX 2: BCA PROTEIN ASSAY PLATE MAP

Plate Map for BCA protein assay with standards and up to 5 unknown sample wells (S1-S5) loaded in duplicate; each unknown sample will have one "Cyto" and one "NucMito" fraction for analysis. Sample numbers correspond to that listed in the Sample Information in the Batch Record (Appendix 1, Section 2). Each unknown sample is diluted 1:5 and 1:10 for the protein assay.

Representative plate map layout for 5 unknown samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	x*	1X PBS – Background Control										x
B		S1-Cyto 1:5		S2-NucMito 1:5		15.6		S4-Cyto 1:5		S5-NucMito 1:5		
C		S1-Cyto 1:10		S2-NucMito 1:10		31.3		S4-Cyto 1:10		S5-NucMito 1:10		
D		S1-NucMito 1:5		S3-Cyto 1:5		62.5		S4-NucMito 1:5				
E		S1-NucMito 1:10		S3-Cyto 1:10		125		S4-NucMito 1:10				
F		S2-Cyto 1:5		S3-NucMito 1:5		250		S5-Cyto 1:5				
G		S2-Cyto 1:10		S3-NucMito 1:10		500		S5-Cyto 1:10				
H	x				x	1000		x				x

B6-H7	BSA standards in duplicate
B2-G5 and B8-G11	5 unknown samples, two dilutions run in duplicate
Remaining wells	1X PBS 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 and wells adjacent to high standard should not be used to determine background.

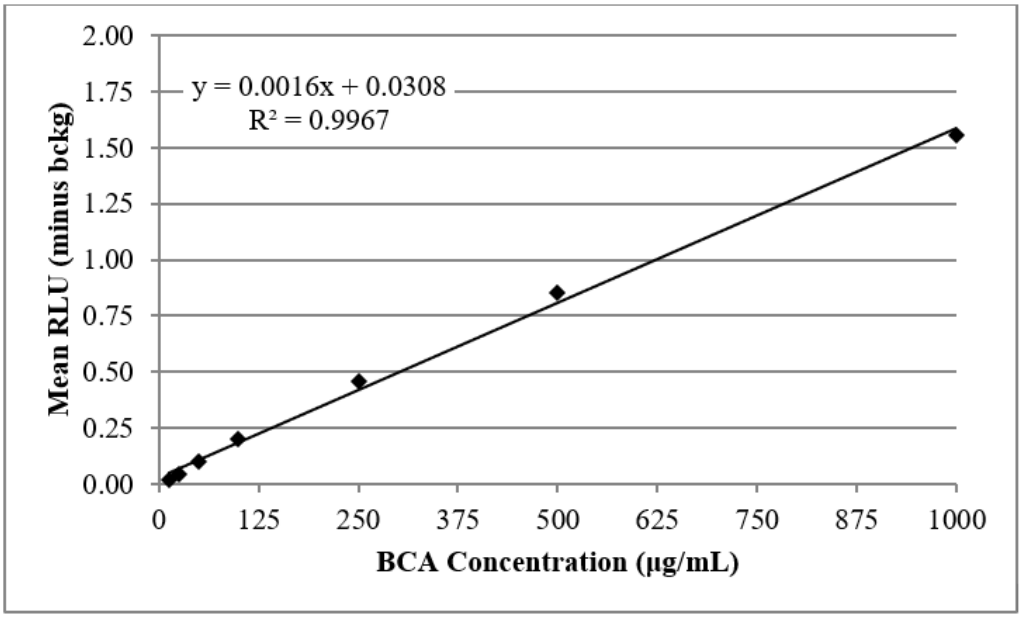
APPENDIX 3: BSA STANDARD CURVE ANALYSIS

1. Example of Standard Curve Calculations and Graph

Plot the standard curve and display the logarithmic trend line in order to get the equation for the standard curve.

	Mean RLU	SD	% CV	Mean RLU (minus background)*	QC
Background	0.092	0.001	0.2	--	
15.6 µg/mL	0.112	0.003	0.2	0.020	Low Standard > Background Mean RLU + 3 SD (= 0.095) if 15.6 µg/mL does not meet this criteria, the lowest standard becomes 31.3 µg/mL provided it meets this criteria.
31.3 µg/mL	0.135	0.008	6.1	0.043	
62.5 µg/mL	0.192	0.005	2.4	0.100	
125 µg/mL	0.292	0.010	3.6	0.200	
250 µg/mL	0.552	0.013	2.3	0.460	
500 µg/mL	0.945	0.011	1.1	0.853	
1000 µg/mL	1.648	0.014	0.8	1.556	High Standard < 2.5

* Used to plot standard curve

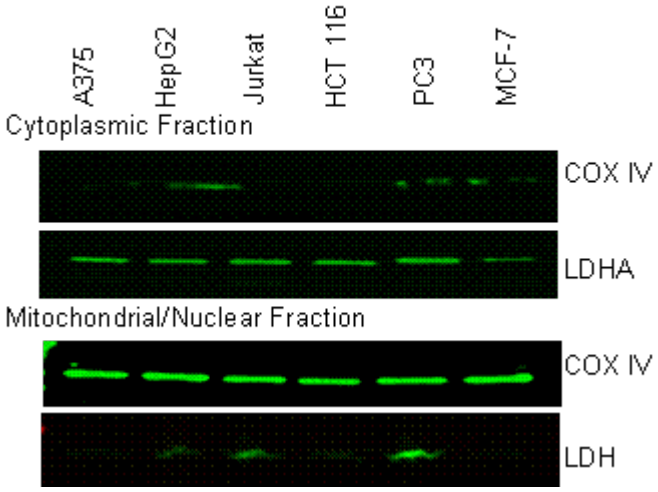


2. Example of Calculations in Sample Information Table (Appendix 1, Section 3A)

No.	Patient/Sample ID	Fraction	Determine Protein Concentration: BCA Assay								
			Mean RLU	SD	%CV	Mean RLU (minus background)	Conc. Diluted Lysate (µg/mL)	Dilution Factor	Corrected for Dilution (µg/mL)	Avg. Conc. Corrected for Dilution (µg/mL)	QC Pass/Fail
S1	1234-AEBJ2-1A	Cyto	0.393	0.003	0.72	0.301	168.9	5	844	845	Pass
			0.259	0.005	1.91	0.167	84.5	10	845		Pass
		NucMito	0.288	0.054	18.78	~~0.196~~	~~102.6~~	5	~~513~~	551	Fail
			0.212	0.009	4.35	0.120	55.1	10	551		Pass
S2	1234-AEBJ2-2A	Cyto	0.376	0.001	0.38	0.284	158.3	5	791	771	Pass
			0.243	0.003	1.16	0.151	75.1	10	751		Pass
		NucMito	0.264	0.002	0.81	0.172	88.3	5	441	474	Pass
			0.205	0.009	4.50	0.113	50.8	10	508		Pass

3. Sample Western blot of isolated Cyto and NucMito fractions.

Cytosolic (Cyto) and nuclear+mitochondrial (NucMito) fractions isolated from HCT 116 (colorectal), A375 (melanoma), PC3 (prostate), and MCF7 (breast) xenografts were blotted and then probed with antibodies for lactate dehydrogenase (LDH), which is localized in the cytoplasm, and the mitochondrial marker cytochrome c oxidase IV (COX IV).



APPENDIX 4: PROCESSING PRECLINICAL TUMOR QUADRANTS

For application of this procedure to xenograft tumors for preclinical applications, the use of tumor quadrants is recommended. If xenograft tumor is not provided in Sarstedt tube (#72.703.416), the tumor sample should be transferred frozen (on dry ice) to above tube before starting extractions.

An increased volume of both MIM and Buffer A are required, and should be adjusted depending on the approximate size of the tumor quadrants. The volumes below are provided as a general guideline; however, the volumes may need to be further optimized for each tumor model. All "Cyto" fractions should be reconstituted in buffers containing exact ratio of detergents as specified in Section 7.6.12.

Approximate Tumor Quadrant Size	MIM Volume (µL)	Buffer-A Volume (µL)
Small (<3 mm in diameter)	350	350
Medium/Average (~3 – 5 mm in diameter)	500	350
Large (>5 mm in diameter)	700	500