

DCTD Standard Operating Procedures (SOP)

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Document History

Revision	Approval Date	Description	Originator	Approval
--	6/14/2017	PADIS version LHTP003.04.28 (v. --, 06/12/2014) adapted to DCTD format and for NCTVL laboratory, updates made to the software Bioplex vs. xPonent	AKS/KFG/YZ/JPG	AKS
A	11/09/2018	Revision to add analysis template for Bioplex manager and other minor edits	AKS/JPG/KFG	AKS

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OVERVIEW OF APOPTOSIS PANEL MULTIPLEX ASSAY

SOP340507:

Tumor Frozen Needle Biopsy Specimen Collection and Handling

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



SOP341401: Apoptosis Panel Tumor Extraction

- Extract fractionated lysates from tumor biopsy
- 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 samples relative signal using xPonent Software & Bio Plex Manager software
- Determine the samples apoptosis biomarker concentrations and verify quality controls passed acceptance criteria.
- Prepare and sign a Clinical Sample Data Report. Send the report to the protocol Principal Investigator



Biomarkers

Panel 1: BAX, Lamin B, Intact + 45kDa & Smac/DIABLO

Panel 2: Bad, Bax-Bcl-2, Bcl-xl, Bim & Mcl-1

Panel 3: Bcl-xl-Bak, Caspase-3 Active, Mcl-1-Bak & Survivin

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BORD Standard Operating Procedures (SOP)					
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1.0 PURPOSE

Standardize the method for quantification of analyte levels with multiplex immunoassays using the Bio-Plex 200 platform with Bio-Plex Pro RBM Apoptosis Panel Kits 1 – 3 to determine pharmacodynamic (PD) measurements of drugs targeting apoptosis or downstream biomarkers. The goal of the SOP and associated training is to ensure consistency in the use of the apoptosis multiplex immunoassay panels for the analysis of tumor biopsies and the reporting of data.

It is important to consider that individual biomarker assay data must be interpreted in the context of the fractionated cell lysates, as marker expression may be fraction specific and marker distribution across cell fraction may change during drug induced apoptosis.

2.0 SCOPE

This procedure applies to all personnel involved in using the Bio-Plex Pro RBM apoptosis assays for analysis of tumor biopsy lysates from clinical trials.

3.0 ABBREVIATIONS

BioPlex	=	Proprietary name for Luminex kits produced by Bio-Rad
C	=	Control
Cyto	=	Cytosolic
DCTD	=	Division of Cancer Treatment and Diagnosis
IA	=	Immunoassay
LHTP	=	Laboratory of Human Toxicology and Pharmacology
Luminex	=	Bead based multiplex platform
MFI	=	Mean Fluorescence Units
NCTVL	=	National Clinical Target Validation Laboratory
NucMito	=	Mitochondrial + Nuclear
pAb	=	Polyclonal antibody
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PD	=	Pharmacodynamic
PE	=	Phycoerythrin
PI	=	Protease Inhibitor(s)
QC	=	Quality Control
RBM	=	Myriad RBM, Inc. Austin, TX
RT	=	Room Temperature
SAPE	=	Streptavidin-Phycoerythrin
SOP	=	Standard Operating Procedure
Temp	=	Temperature
FLOD	=	Functional Limit of Detection
LLQ	=	Lower Limit of Detection
ULQ	=	Upper Limit of Detection

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4.0 INTRODUCTION

Cancer cells acquire the ability to evade apoptosis, conferring a survival and growth advantage. Several first-in-class apoptosis inducers are in clinical development to circumvent this advantage. To support pharmacodynamic evaluation of drugs targeting apoptosis, DCTD in collaboration with RBM Myriad, developed a novel multiplex immunoassay panel of 13 biomarkers indicative of the induction, onset, and commitment to apoptosis. The multiplex immunoassays were built on the Bio-Plex platform by grouping 13 biomarkers into three panels. Panel 1 contains Bak, Bax, total lamin-B (intact and 45 kDa fragment), and Smac; Panel 2 contains Bad, Bax–Bcl-2 heterodimer, Bcl-xL, Bim, and Mcl1; and Panel 3 contains active (cleaved) caspase-3, Bcl-xL–Bak heterodimer, Mcl1–Bak heterodimer, and survivin. The multiplexed technique is based on sandwich immunoassays performed on the surface of color-coded 5.5 µm magnetic polystyrene microspheres (Bio-Rad). Capture antibodies are coated on microspheres and reporter antibodies specific to a different epitope are conjugated to biotin. Assay is initiated by mixing samples with beads and reporter antibody and after a wash step phycoerythrin-streptavidin conjugate is added to bind with reporter Ab-biotin. Beads are washed a second time, suspended in a sheath-fluid and counted in Bio-Plex reader to determine mean fluorescence intensity (MFI) (see Appendix 4 for details on bead based multiplexing technology). Details of the assay development, validation and fit-for-purpose experiments have been described [Effect of a Smac Mimetic (TL#2711, Birinapant) on the Apoptotic Program and Apoptosis Biomarkers Examined with Validated Immunoassays Fit for Clinical Use, Srivastava AK, Jaganathan S, Stephen L, Hollingshead MG, Layhee A, *et al*, Clinical Cancer Research, 22(4) 1000-11 (2106)]. Details of Bio-Plex kits for apoptosis panel are available at the following link <http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10033631.pdf>.

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.

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- 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 2](#)) must be completed in *real-time* for each experimental run. Each page of the batch record must have the *protocol number(s)* and *patient ID(s)*, and be *dated and initialed* by the operator.
- 5.3 Digital versions of the sample table in the Batch Record ([Appendix 2, Section 4](#)) 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 tables must be printed and attached to the Batch Record in order to maintain a complete audit trail.
- 5.4 The responsible personnel are to check the DCTD Biomarkers website (<https://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>) to verify that the most recent version of the SOP for the assay is being followed.
- 5.5 This standard operating procedure is a supplement to the Bio-Plex Pro RBM Apoptosis Assay Instruction Manual and can be accessed at the following link: <http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10033631.pdf>. This SOP has been written and references the use of the assay per **Revision A** of the instruction manual.

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6.0 CRITICAL REAGENTS, MATERIALS AND EQUIPMENT REQUIRED

- 6.1** Bio-Plex Kits supplied with all buffers, beads, antibody-conjugates, PE-streptavidin, calibrators, controls (two levels with target ranges) and 96-well plate.
 - 6.1.1 Bio-Plex Pro RBM Apoptosis Panel 1 (BioRad, Cat# 171-WAR1CK)
 - 6.1.2 Bio-Plex Pro RBM Apoptosis Panel 2 (BioRad, Cat# 171-WAR2CK)
 - 6.1.3 Bio-Plex Pro RBM Apoptosis Panel 3 (BioRad, Cat# 171-WAR3CK)
- 6.2** Bio-Rad Bio-Plex Calibration Kit (Cat#: 171-203060)
- 6.3** Bio-Rad Bio-Plex Validation Kit 4.0 (Cat#: 171-203001)
- 6.4** Bio-Rad Bio-Plex 1X sheath fluid in 20L (Cat#: 171-000055)
- 6.5** complete, mini, EDTA-free protease inhibitor cocktail tablets (Roche, Cat# 04693159001)
- 6.6** UltraPure DNase/RNase-free distilled water (e.g., Invitrogen, Cat#: 10977-015)
- 6.7** Pipettors (200-1000 μ L and 10-100 μ L) and tips
- 6.8** Multichannel pipettor (30-300 μ L) and tips
- 6.9** Repeater pipettor (Eppendorf Cat# 4982000322-manual or 486100160-automatic)
- 6.10** Combitips Advance Tips, 1.0mL (Eppendorf Cat# 30089545) and 2.5ml, (Eppendorf Cat# 30089553)
- 6.11** Reagent reservoirs (Fisher Scientific, Cat#: 21-381-27C)
- 6.12** 2.0-mL Sarstedt o-ring screw cap, skirted tubes (Fisher Scientific, Cat#: 72.694.006)
- 6.13** 15-mL polypropylene tubes (e.g., Fisher Scientific, Cat#: 14-959-49B)
- 6.14** 50-mL polypropylene tube (e.g., Becton Dickinson, Cat#: 352098)
- 6.15** Universal Black Microplate Lid with Corner Notch (e.g. VWR 77776-852)
- 6.16** VWR® Black Vinyl Films for Fluorescence and Photoprotection (VWR, Cat#: 89087-692, can be used in place of black lid)
- 6.17** Bio-Plex 200 Reader with Sheath Fluid Delivery System
- 6.18** Sorvall Fresco centrifuge, refrigerated (Fisher Scientific)
- 6.19** BioTek 405TS Select Magnetic Washer (BioTek Instruments)
- 6.20** Vortex mixer, digital, 500-3000 rpm (Fisher Scientific, Cat#: 02-215-370)
- 6.21** Sonicator (Ultrasonic Cleaner with Digital Timer and Heater) (VWR Cat# 97043-988)
- 6.22** 5 ml serological pipets (VWR Cat#: 53300-421)
- 6.23** Conical bottom wells microplate (VWR Cat#: 82050-678)
- 6.24** Incubating Microplate shaker with opaque lid (VWR Cat#:97043-606)
- 6.25** Refrigerator 4°C, -20°C and -80°C freezer
- 6.26** Bio-Plex Data Pro with Bio-Plex Manager Software (v 6.1), (BioRad, Cat# 171-001510)
- 6.27** Microsoft Excel 2007 or higher version and approved Data Analysis Template
- 6.28** Tumor biopsy stock protein lysates processed following SOP341401 (Preparation of Tumor Biopsy Lysates for Apoptosis Panel)

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7.0 OPERATING PROCEDURES

7.1 Record the name and certification number of the Certified Assay Operator and the facility running the assay in the Batch Record ([Appendix 2](#)). Include reference to the clinical protocol number(s), patient ID(s) and 96-well plate ID, if applicable.

7.2 Equipment Information

7.2.1 Record the make, model, and serial numbers of equipment in the Batch Record ([Appendix 2, Section 1](#)).

7.3 Critical Reagents

7.3.1 All Critical Reagents are to be labeled with date of receipt and stored under the specified conditions for no longer than the recommended duration.

7.3.1.1 Storage conditions and expiration dates for all Critical Reagents are provided on the package insert.

7.3.1.2 Do not exchange reagents from one set of qualified Critical Reagents with a set of reagents qualified separately.

7.3.2 Record the date of receipt of kit, lot numbers, and expiration dates for the kits in the Batch Record ([Appendix 2, Section 2](#)).

7.4 Plate Map

7.4.1 Based on the number of patient samples to be analyzed, generate a Plate Map ([Appendix 1](#)) to define the location and replicates of unknown samples, tumor controls, and standards. A single patient's **batched** samples should be contained in one 96-well plate, not split over two, to ensure consistent sample handling.

IMPORTANT: Do not let plate dry out during wash and aspiration steps.

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7.5 Preparation of Unknown Sample Lysates

- 7.5.1 Place the unknown sample stock lysates on ice to thaw. Enter the time sample thaw was initiated in the Batch Record ([Appendix 2, Section 4](#)).
- 7.5.2 Calculate the amount of Lysate dilution buffer (LDB) for preparing the samples. For each 10 mL of LDB, add one tablet of complete, Mini, EDTA-free protease inhibitor. Keep the LDB with PIs on ice or at 2-8°C.
- 7.5.3 Unknown samples will typically be analyzed at two concentrations within the range of 500 – 125 µg/mL. From the total protein concentration determined by BCA protein kit for SOP340401, calculate the volume required to prepare a sufficient volume of diluted lysate for each protein load with sufficient overage (minimum 20 µL volume). Each sample will be loaded in 2 wells at a volume of 30 µL for each panel.
- 7.5.4 Record all sample information and calculations in the Sample Information Table in the Batch Record ([Appendix 2, Section 5](#)).
- 7.5.5 After the samples are thawed, centrifuge them at 16,000g for 5 min at 4°C.
- 7.5.6 Prepare the two dilutions of each fractionated lysate as calculated and keep all samples on ice until loaded into the assay plate.
- 7.5.7 Record the time the sample preparation was completed in the Batch Record ([Appendix 2, Section 4](#)).

7.6 Initial Reagent Preparation

For additional details see the Bio-Plex Instruction Manual

<http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10033631.pdf>

- 7.6.1 Bring 10X Assay Buffer to room temperature and mix by inversion to ensure all salts are in solution. For each plate, prepare 1X Assay buffer by diluting 1 part 10X Assay buffer (60 mL) with 9 parts of dH₂O (540 mL) for use as washing buffer. Save 15 mL of 1X assay buffer in a 50-mL tube for use as diluent for SAPE dilution ([Step 7.8.9](#)) and bead suspension ([Step 7.8.13](#)).
- 7.6.2 Reconstitute the following lyophilized reagents in dH₂O before use according to the table below:

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Reagents	dH ₂ O Volume
Standards mix (S1)	150 µL
Control 1	100 µL
Control 2	100 µL
Blocking Buffer	1.5 mL
Standard diluent	1.0mL
*Detection Antibodies	4.8 mL

* Reconstitute immediately prior to use, see [step 7.8.6](#).

7.6.3 Allow the vials to sit at room temperature for a minimum of 5 min and use the reagents as soon as possible (within 30 min).

7.6.4 Vortex at medium setting.

7.7 Dilution of Standard

7.7.1 Label 8 polypropylene tubes S1 through S8.

7.7.2 Transfer the reconstituted Standards Mix into the tube labeled “S1”.

7.7.3 Prepare working standards at 1:3 according to the table below. This will be sufficient for duplicate standard curves. Vortex each standard at a medium setting for 5 secs before proceeding with the next serial dilution. Change pipet tip at each dilution step.

7.7.4 Label one additional tube “Blank” and pipette 100µL of Standard Diluent into the Blank tube.

Standard	Volume of Standard Diluent	Volume of Standard
S1	-	150 µL
S2	100µl	50 µL of S1
S3	100 µl	50 µL of S2
S4	100 µl	50 µL of S3
S5	100 µl	50 µL of S4
S6	100 µl	50 µL of S5
S7	100 µl	50 µL of S6
S8	100 µl	50 µL of S7

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7.8 Assay Procedure

- 7.8.1 Add 10 μ L of Blocking Buffer to all wells of the plate.
- 7.8.2 Add 30 μ L of the Standard (S1 – S8), Blank, Control 1 or Control 2 or unknown sample dilution to the appropriate wells of the plate following the plate map.
- 7.8.3 Vortex the Capture Beads at medium speed for 10-20 sec. Sonicate the beads for 1 min. Add 10 μ L of the beads to all wells of the plate.
- 7.8.4 The plate should be sealed and covered with a black lid to protect the assay wells from light.
- 7.8.5 Incubate on shaker at 850 ± 50 rpm for 1 hr at RT. Record Start and Stop time for incubation in the Batch Record ([Appendix 2, Section 6](#)).
- 7.8.6 Prepare Detection Antibody 10 minutes before the end of the incubation period by reconstituting the lyophilized powder in 4.8 mL of dH₂O and vortex at medium speed for 10-20 sec.
- 7.8.7 Wash the plate three times (see below for washer settings) with 1x Assay Buffer using the appropriate **Wash** program (see [section 7.9](#) for wash settings).
- 7.8.8 Add 40 μ L of Detection Antibody solution to each well. Cover, as in Step 7.8.4, and incubate at 850 ± 50 rpm for 1 hr. at RT. Record Start and Stop time for incubation in the Batch Record ([Appendix 2, Section 6](#)).
- 7.8.9 Do not aspirate after incubation. Prepare 1X SAPE solution by adding 225 μ l of 10X SAPE to 2025 μ l 1x Assay Buffer.
- 7.8.10 Add 20 μ l of diluted SAPE to the required plate wells.
- 7.8.11 Cover, as in Step 7.8.4, and incubate at 850 ± 50 rpm for 30 min at RT. Record Start and Stop time for incubation in the Batch Record ([Appendix 2, Section 6](#)).
- 7.8.12 Wash the plate three times with 1X Assay Buffer using Wash program.
- 7.8.13 After wash step, add 100 μ l/well 1X Assay Buffer using a multiple channel pipette. Cover plate as in [Step 7.8.4](#) and shake the plate at 850 ± 50 rpm for one minute.
- 7.8.14 Remove the plate seal and read plate (see below for **Bio-Plex Manager** settings).

7.9 Plate Washer Settings

- 7.9.1 Ensure the plate washer is programmed as specified below for all plate washes using the **BioTek 405TS Select CW Magnetic Microplate Washer**.

Plate Washer Parameter	Setting
Default Program	01 - Costar_Flat
Plate Type	96 well plate
Method: Number of Cycles	3
Dispense: Volume	300 μ L/well

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- 7.9.2 Place the plate on the washer unit for 1 minute.
- 7.9.3 Select the plate washer program from the main menu and then press enter on the washer unit. Ensure that the plate cover is removed.
- 7.9.4 Press start to wash the plate once.

7.10 Signal Detection (Bio-Plex Manager Settings)

- 7.10.1 Turn on the Bio-Plex 200 at least 30 min before use.
- 7.10.2 The Bio-Plex 200 should be calibrated each day after the start up procedure is complete and the laser is warmed up. Perform daily QC procedures using calibration kit (once each day before a plate run) and perform validation kit following the manufacturer's instructions (run at least monthly).
- 7.10.3 Create a new protocol, select **File**, then **New Multi-Assay Protocol** from the main menu.
- 7.10.4 Click **Describe Protocol** and enter information about the assay (optional).
- 7.10.5 Click **Select Analytes** and select appropriate Panels (Bio-Plex Pro Apoptosis Panel 1/2/3) from the dropdown. To move all analytes at once, simply click Add All.
- 7.10.6 Make sure the following analytes (bead region in brackets) are selected for the panels.

Panel 1	Bak (74)	Bax (27)	Lamin B (14)	Smac (19)	
Panel 2	Bad (73)	Bcl-2/Bax (42)	Bcl-xl (22)	Bim (12)	Mcl-1 (18)
Panel 3	Active Caspase (57)	Bcl-xl/Bak (47)	Mcl-1/ Bak (54)	Survivin (20)	




- 7.10.7 Click **Format Plate** and format the plate according to the plate layout template (see Plate Map Design in [Appendix 1](#)). Clear the appropriate sample wells (X3 to X39) if running less than 37 samples. For example, clear the wells from X33 to X39 if only run 30 samples.
- 7.10.8 Click **Enter Standards Info** in the Protocol Settings bar. Individual Bio-Rad kit lots may have a different calibration range; it is important to verify and enter correct lot specific Standard concentrations.
- 7.10.9 Select the **Standard Info** Tab. Make sure to select **Logistic – 5PL** in the dropdown list of **Regression Type**, and select **Log(x) – Log(y)** in the dropdown list of **Axis Transformation**, and select **70 – 130%** as Acceptable Recovery Range.
- 7.10.10 Make sure the following check boxes are checked: Same regression type for all analytes, Same units for all analytes, and Same recovery range for all analytes.

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- 7.10.11 Enter the highest concentration of each analyte in the top row (labeled S1) of the table. S1 concentration information is listed in the product data sheet.
- 7.10.12 Make sure **S1** button is selected as **Most Concentration** and **Apply dilution to all analytes** check boxes are checked.
- 7.10.13 Enter a dilution factor of 3 and click **Calculate**. The concentrations for each standard point will be populated for all analytes in the table.
- 7.10.14 Enter standard lot number and expiration date and click **Save** button to Save the protocol.
- 7.10.15 Click **Enter Sample Info** – Copy the sample information from sample dilution Excel file and paste into Description column.
- 7.10.16 Click **Run Protocol**.
- 7.10.17 Confirm the **Beads** event is 50 per region and the check box of **Same run settings across all assays** is checked.
- 7.10.18 Click **Advanced Settings**, confirm that the **Bead Map** is set to 100 region, the sample size is set to 50 µl, and the DD gates are set to 5000 (low) and 25000 (high), and the check box of **Auto save after run** is checked. Click OK.
- 7.10.19 Select **Start**, name and save the rbx file, and begin data acquisition (read plate).
- 7.10.20 When data acquisition is complete, select **Shut Down** and follow the instructions.

7.11 Processing Assay Data for Regression Analysis (Bio-Plex Analysis Settings)

- 7.11.1 Open the rbx file and make sure that the sample information is correct (dilution, sample ID etc.). If Luminex provided software “Xponent” is used for reading plate then readout should be exported in “.csv” format. The .csv file can be read by Bio-Plex manager (after conversion to “rbx” format).
- 7.11.2 Click standard curve and confirm that the **Log(x) – Log(y)** is selected from **Axis Transformation**. Confirm the following check boxes are selected: **Same regression type for all analytes**, **Show Unknown Samples**, **Apply across all analytes**, **Show report after optimization**, and **Same recovery range for all analytes**.
- 7.11.3 In the Report Table screen, select “Multiple Analyte Layout”  and “Show replicates”  and make sure “Organize by Type”  is selected.
- 7.11.4 Go to **Table** options, select **Report table options** and make sure **only** the following items are selected: Type, Well, Description, FI, FI-Bkgd, Conc in Range, Obs Conc, Obs Conc %CV, Exp Conc, Bead Count.
- 7.11.5 Click **Set Number Format** and set the number of decimal places to 4 for **Obs Conc**. Click **OK** to close **Set Column Number Format** window. Click **OK** to close **Report Table Display Options** window.

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- 7.11.6 Click **Set Number Format** and set the number of decimal places to 4 for **Obs Conc.** Click **OK** to close **Set Column Number Format** window. Click **OK** to close **Report Table Display Options** window.

7.12 Data analysis (Generate summary table)

- 7.12.1 Click on the top-left corner cell on the Bio-Plex Manager and copy the entire data (Edit – copy).
- 7.12.2 Open Excel Template (same template for all 3 Panels), go to “**Bioplex Data**” sheet, click A1 cell and paste. Make sure the sheet is empty before pasting data as it can interfere with the downstream calculations. Save the template denoting the panel used and the date of analysis.
- 7.12.3 Enter highest standards (S1), control ranges, LOD values, and Proficiency Panels details in the appropriate cells in “**Kit Information**” sheet.
- 7.12.4 Once all the values are entered, the “**Sample Report**”, “**Proficiency Panels QC**” and “**Controls QC**” sheets should organize/analyze the data.
- 7.12.5 If needed, modify the “**Patient Report**” sheet to add patient ID and protein loads.

7.13 Assay controls and target ranges. Performance characteristics of apoptosis kits must be confirmed or established as described below prior to evaluation of clinical samples.

- 7.13.1 Two controls representing low (or medium) and high levels of each analyte in tumor lysate are included in each apoptosis panel kit for use in each assay run. The analyte levels in each control represents endogenous or spiked protein or a combination of both prepared in a manner to target the desired analytical range of the assay. A third control can be added for additional quality control evaluations but not required.
- 7.13.2 The performance of kits is established by running a minimum of two levels of controls for each analyte and insuring that obtained values fall within the target ranges provided by the manufacturer (Bio-Rad). These target ranges are defined by Bio-Rad in single laboratory and sometimes one or two analyte falls outside the range. This does not mean failure of the assay but indicates that a particular analyte has higher variability and target ranges must be established in individual assay lab for QC evaluation. PADIS has established its own target ranges for select analytes that did not agree with Bio-Rad provided ranges for evaluating day-to-day QC.
- 7.13.3 In order to establish the control range within an individual laboratory for each new lot of kit, the mean and standard deviation for each control should be determined in a minimum of 5 independent assay runs. The assays should be run on a minimum of three separate days. Control ranges are calculated as Mean \pm 3SD to establish the acceptable range for the controls.

7.14 Acceptability Criteria for Patient Sample Reporting

- 7.14.1 Instrumentation Readout

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7.14.1.1 The minimum number of bead counts acceptable for individual analyte readout is set as 20. If MFI is assigned to readouts generated from <20 bead counts for both replicates, the effected assay sample fails and must be repeated.

7.14.2 Precision

7.14.2.1 All sample dilutions (e.g. 125 µg/mL and 500 µg/mL) and controls must be run with a minimum of two replicates. Any sample with values above LLQ with a >20% CV should not be reported. Sample values falling below >LLQ should not be reported.

7.14.3 QC acceptability: for evaluation of controls each analyte in the multiplex is considered as a single analyte, irrespective of other analytes in the multiplex panel. This means that control values obtained for other analytes are independent of each other.

7.14.3.1 Values for an analyte will only be acceptable if a control value for that analyte passes the criteria defined in [Section 7.13](#). Each analyte in the multiplex panel will be evaluated to individual target ranges irrespective of control values for other analytes.

7.14.3.2 Data from individual replicates will be calculated for QC acceptability. For analytes where three out of four control replicates pass, the data is reportable. This rule states that, if all other criteria met, three out of four replicate must pass the QC test. This exception is considered as random error (could be related to instrument readouts).

7.14.3.3 If both levels of control fail QC, irrespective of duplicate, then no results will be reported, and root cause analysis will be executed, problem identified, resolved, and then samples will be repeated.

7.14.3.4 Trends in QC levels could be monitored to document any systematic errors and lot-to-lot variability.

7.14.4 Analytical Sensitivity

7.14.4.1 Only sample values \geq the lot specific, analyte specific LLQ and below the ULQ, defined as the highest standard for that analyte, will be reported.

7.14.4.2 Samples falling below the LLQ will be reported as <LLQ.

7.14.4.3 If a sample is identified in which all dilutions fall above the ULQ, the sample should be repeated at a lower protein load, if possible, and if not, the sample should be reported as >ULQ.

7.14.5 Linearity

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7.14.5.1 Samples may be run at two different protein concentrations between 500 µg/ml and 125 µg/mL and values observed within the assay range would be reported. Linearity refers to the analytical recovery of an analyte at 125 µg/ml samples relative to value of same analyte at 500 µg/mL. The linearity could be affected by the presence of interacting proteins in the multiplex panel. The initial validation identified following acceptable linearity ranges 70%-130%.

7.14.5.2 The linearity of Lamin-B falls out of the range depending on unknown factors in a tumor subtype, therefore, Lamin-B concentrations from the highest protein load falling within the assay range will be reported irrespective of recovery.

7.14.5.3 For samples in which both dilutions fall within the range of the assay and agree within the ranges specified above, the average of calculated value for that analyte should be reported.

7.15 Reviewing Results

7.15.1 Review and finalize the Batch Records ([Appendix 2](#)) and obtain required signatures. Document ANY and ALL deviations from this SOP in the Batch Record ([Appendix 2, Section 7](#)).

7.15.2 The Laboratory Director/Supervisor should review the Batch Record and sample reports and sign the Batch Record ([Appendix 2, Section 8](#)) affirming the data contained within the reports are correct.

7.16 Clinical Sample Report Review and Approval

7.16.1 A Clinical Sample Data Report ([Appendix 3](#)) should be prepared to summarize the results for each patient's analyzed sample(s).

7.16.2 Reporting units for analytes: ng analyte/500µg protein.

7.16.3 Batch records or equivalent experimental records including raw and analyzed data must be reviewed and approved by the laboratory supervisor/director prior to reporting of sample data for clinical samples. Approval by the laboratory supervisor/director indicates their approval that the SOPs have been followed with any incidental deviations fully disclosed, sample data have been verified and the assay and sample data to be reported have met all QC metrics.

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APPENDIX 1: PLATE MAP DESIGN

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	B	B	X8	X8	X16	X16	X24	X24	X32	X32
B	S2	S2	X1 (C1)	X1 (C1)	X9	X9	X17	X17	X25	X25	X33	X33
C	S3	S3	X2 (C2)	X2 (C2)	X10	X10	X18	X18	X26	X26	X34	X34
D	S4	S4	X3	X3	X11	X11	X19	X19	X27	X27	X35	X35
E	S5	S5	X4	X4	X12	X12	X20	X20	X28	X28	X36	X36
F	S6	S6	X5	X5	X13	X13	X21	X21	X29	X29	X37	X37
G	S7	S7	X6	X6	X14	X14	X22	X22	X30	X30	X38	X38
H	S8	S8	X7	X7	X15	X15	X23	X23	X31	X31	X39	X39

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APPENDIX 2: 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/Running Assay:	
Protocol Number(s):	
Patient ID(s):	
Plate ID (optional):	

1. Equipment Information

Luminex Plate reader: Make/Model: _____
 Serial #: _____

BioTek Plate Washer: Make/Model: _____
 Serial #: _____

2. Critical Reagent Information for Bio-Plex Kits

Reagent Information	Panel 1	Panel 2	Panel 3
Lot Number			
Date of Receipt			
Expiration date			

3. Unknown Samples

Unknown samples will be run at two protein loads in duplicate. Sample numbers correspond to those on the Plate Map Design. The two protein loads utilized for each of the three panels are recorded below:

Panels	Protein Load 1 (µg Protein/mL)	Protein Load 2 (µg Protein/mL)
Panel 1		
Panel 2		
Panel 3		

Protocol No.(s): _____
 Patient ID(s): _____

BATCH RECORD: INITIALS _____ DATE: _____

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4. Sample Preparation Elapsed Time

Time Sample Preparation Started:

Time Sample Preparation Completed:

5. Sample Preparation Information Table:

Sample Number	Sample ID	Cyto or NucMito Fraction	Original Concentration (µg/mL)	Sample (µL)	Sample diluent (µL)	Protein Load (µg/mL)
S1						
S2						
S3						
S4						
S5						
S6						
S7						
S8						
S9						
S10						
S11						
S12						
S13						
S14						
S15						
S16						
S17						
S18						

Protocol No.(s): _____

Patient ID(s): _____

BATCH RECORD:

INITIALS _____

DATE: _____

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Enter the sample preparation information (Cont.):

Sample Number	Sample ID	Cyto or NucMito Fraction	Original Concentration (µg/mL)	Sample (µL)	Sample diluent (µL)	Protein Load (µg/mL)
S19						
S20						
S21						
S22						
S23						
S24						
S25						
S26						
S27						
S28						
S29						
S30						
S31						
S32						
S33						
S34						
S35						
S36						
S37						

Protocol No.(s): _____

Patient ID(s): _____

BATCH RECORD: INITIALS _____

DATE: _____

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6. Plate Incubation

Room Temp (°C):		Panel 1		Panel 2		Panel 3	
Process - Plate Incubation	Shaker (850 rpm) Time	Start	Stop	Start	Stop	Start	Stop
Blocking Buffer (10 µL) + Standard, blank, sample or control (30 µL) + Capture Beads (10 µL)	1 hr						
Detection Antibody (40 µL)	1 hr						
SAPE (20 µL)	30 min						

7. Notes, including any deviations from the SOP:

8. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (SIGN)

Date: _____

BATCH RECORD: INITIALS _____ DATE: _____

DCTD Standard Operating Procedures (SOP)

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APPENDIX 3: CLINICAL SAMPLE DATA REPORT

Apoptosis Panel PD Analysis Primary Biomarker:.....

Certified Assay Operator (Print): _____

Certification #: _____ Date: _____

Trial and Patient Information

Patient ID:	Trial Site: _____
Clinical Center #: _____	CTEP Protocol #: _____

Assay Readout: Tumor Samples

		Baseline	Post-Dose
Patient/Sample ID			
Scheduled Collection	Cycle, Day		
Actual Collection	Date		
	Time		

(ng/500ug Protein)

Protein Name	Baseline	Post-Dose	QC Notes

Abbreviations:

FQ, Failed %CV QC; LLQ, assay lower limit of quantitation; NT, not tested due to low protein yield; UA, unanalyzable; ULQ, assay upper limit of quantitation

Additional Information

Raw data available upon request.

To be completed by Laboratory Director/Supervisor

Director/Supervisor

Signature*: _____

Today's Date: _____

*Signature indicates assay results have been reviewed and verified.

Biopsy and Treatment Information (for use by Clinical site)

Site of Biopsy:	Primary Tumor:	_____
Dose Level:		_____
Agent Name(s):	+	_____
Dose and Units:	+	_____

DCTD Standard Operating Procedures (SOP)

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APPENDIX 4: LUMINEX TECHNOLOGY PLATFORM

