

DCTD Standard Operating Procedures (SOP)

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National Clinical Target Validation Laboratory (NCTVL)
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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
B	12/23/2019	Minor edits based on NCLN transfer; remove two protein loads, change reporting units, incorporate modified reporting template requirements, update control and sample QC and repeat criteria.	KZ/KFG/JPG/LL	AKS

Please check for revision status of the SOP at <http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm> and be sure to use the current version.

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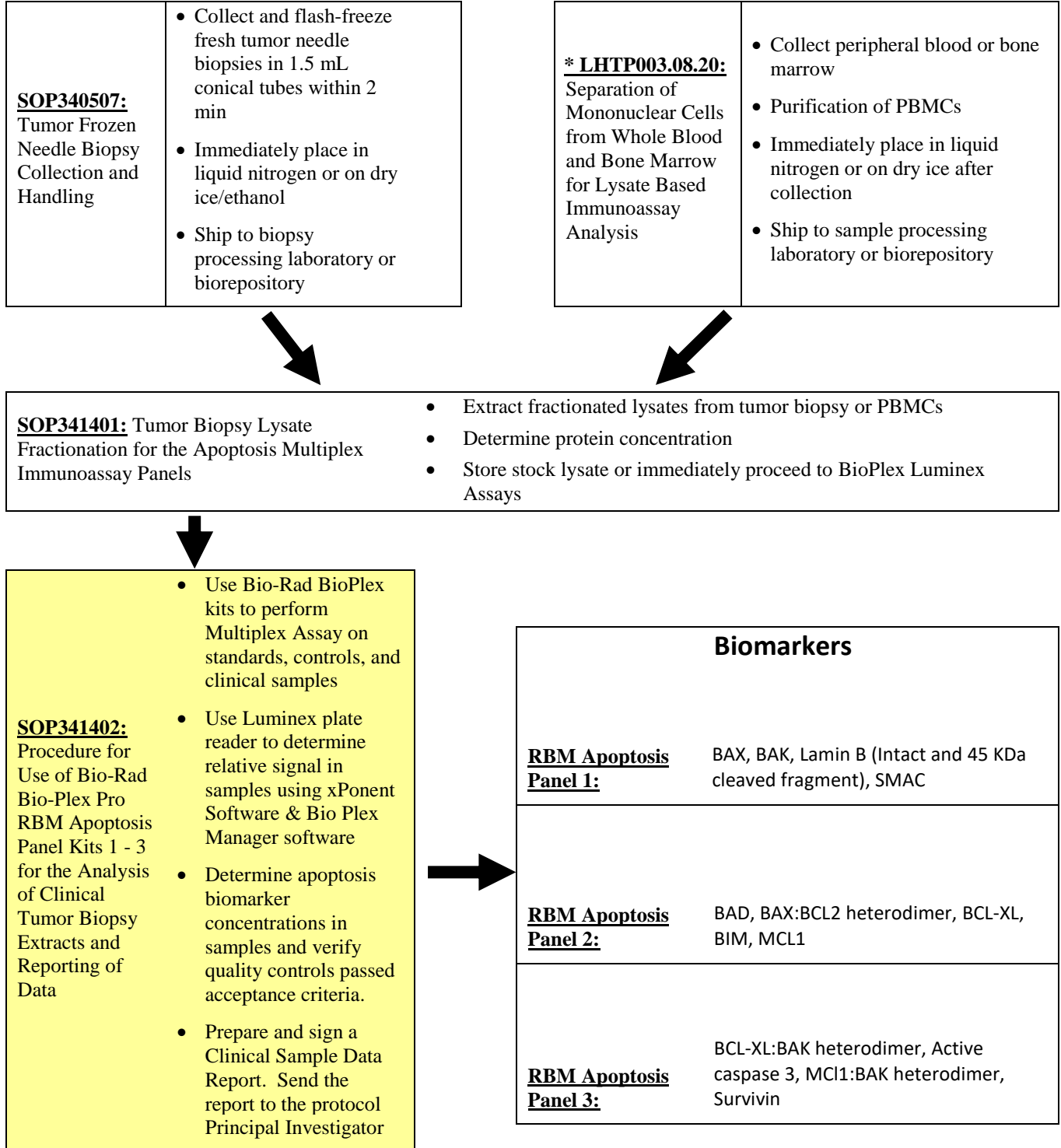
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OVERVIEW OF APOPTOSIS PANEL MULTIPLEX ASSAY



* LHTP003.08.20 will be available after clinical evaluation.

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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 note 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 the cell fraction may change during drug induced apoptosis. Due to limited sampling, only the most informative biomarkers should be prioritized for any necessary repeats.

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
PBMC	=	Peripheral Blood Mononuclear Cell
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

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FLOD = Functional Limit of Detection
 LLQ = Lower Limit of Detection
 ULQ = Upper Limit of Detection

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, Lamin B (intact and 45 kDa fragment), and SMAC; Panel 2 contains BAD, BAX:BCL2 heterodimer, BCL-XL, BIM, and MCL1; and Panel 3 contains active 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 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/lst/literature/10033631.pdf>.

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5.0 ROLES AND RESPONSIBILITIES

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

- 5.1 It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented DCTD training and qualification on this SOP prior to the actual handling and processing of samples from clinical trial patients. The Laboratory Director/Supervisor is responsible for ensuring the Certified Assay Operator running the SOP has sufficient experience to handle and analyze clinical samples.
- 5.2 The Certified Assay Operator responsible for conducting the assay is to follow this SOP and complete the required tasks and associated documentation. The Batch Record ([Appendix 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 5](#)) 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, which can be accessed at the following link: <http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10033631.pdf>. This SOP has been written in accordance with the use of the assay per **Revision A** of the above 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. Refer to BioRad manual for lot specific stability of individual kit components.
 - 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 µL and 10-100 µL) and tips
- 6.8** Multichannel pipettor (30-300 µL) 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** 5 ml serological pipets (VWR Cat#: 53300-421)
- 6.18** Conical bottom wells microplate (VWR Cat#: 82050-678)
- 6.19** Bio-Plex 200 Reader with Sheath Fluid Delivery System
- 6.20** Sorvall Fresco centrifuge, refrigerated (Fisher Scientific)
- 6.21** BioTek 405TS Select Magnetic Washer (BioTek Instruments)
- 6.22** Vortex mixer, digital, 500-3000 rpm (Fisher Scientific, Cat#: 02-215-370)
- 6.23** Sonicator (Ultrasonic Cleaner with Digital Timer and Heater) (VWR Cat# 97043-988)
- 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 references 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, 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) required for processing the samples. For each 10 mL of LDB, add one tablet of cComplete, 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 one concentration within the range of 500 – 125 µg/mL. From the total protein concentration determined by BCA protein assay according to SOP340401, calculate the volume required to prepare a sufficient volume of diluted lysate with sufficient coverage (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, vortex, and centrifuge at 16,000 x g for 5 min at 4°C.
- 7.5.6 Prepare the dilutions of fractionated lysates 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 Milli-Q 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 7.8.10](#)) and bead suspension ([Step 7.8.15](#)).
- 7.6.2 Reconstitute the following lyophilized reagents using UltraPure DNase/RNase-free dH₂O before use according to the table below:

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Reagents	UltraPure 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 or 96-well plate 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 dilution using Standard Diluent 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. It is important for all incubation steps that the lid on the plate shaker remains open to prevent an increase in temperature. 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 Incubate the plate on a 96-well ring magnet for 1 minute.
- 7.8.8 Wash the plate three times with 1X Assay Buffer using the appropriate **Wash** program (see [section 7.9](#) for wash settings).
- 7.8.9 Add 40 μ L of Detection Antibody solution prepared above 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](#)).
- 7.8.10 Do not aspirate after incubation. Prepare 1X SAPE solution by adding 225 μ l of 10X SAPE to 2025 μ l 1X Assay Buffer.
- 7.8.11 Add 20 μ l of diluted SAPE to the required wells on the plate.
- 7.8.12 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](#)).
- 7.8.13 Incubate the plate on a 96-well ring magnet for 1 minute.
- 7.8.14 Wash the plate three times with 1X Assay Buffer using Wash program.
- 7.8.15 After wash step, add 100 μ l/well 1X Assay Buffer using the appropriate **Dispense** program (see [section 7.9](#) for washer settings). Cover plate as in [Step 7.8.4](#) and shake the plate at 850 ± 50 rpm for one minute or up to overnight.
- 7.8.16 Remove the plate seal and read plate (see [Section 7.10](#) for **Bio-Plex Manager** settings). The plate should be read immediately or no more than 2 hr after completion of the assay.

7.9 Plate Washer Settings

7.9.1 Wash Program

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

Wash Step			
Number of Cycles	3		
Aspirate		Dispense	
Number of Cycles	3	Volume	300 ul/well
Travel Rate	3	Flow Rate	5
Delay	0		
<i>Advanced options</i>		<i>Advanced options</i>	
<i>Positioning</i>		<i>Positioning</i>	
Z	11	Z	115
X	0	X	10
Y	0	Y	0
		Start vacuum delay 50 ul/well	
Final Aspirate			
Travel Rate	3		
Delay	0		
<i>Advanced options</i>			
<i>Positioning</i>			
Z	11		
X	0		
Y	0		

7.9.1.2 Select the saved program from the main menu and then press enter on the washer unit. Ensure that the plate cover is removed.

7.9.1.3 Press start to wash the plate once.

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7.9.2 Dispense Program

Dispense	
Volume	100 ul/well
Flow Rate	5
<i>Advanced options</i>	
<i>Positioning</i>	
Z	115
X	10
Y	0

7.9.2.1 Select the saved program from the main menu and then press enter on the washer unit. Ensure that the plate cover is removed.

7.9.2.2 Press start to dispense 100 μ L/well 1X Assay Buffer 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 has warmed up. Perform daily QC procedures using calibration kit (once each day before a plate run), and use a validation kit following the manufacturer's instructions (run at least once a month).

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 menu. To select 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](#)). Controls should be indicated as Unknowns. 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 running 30 samples.

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- 7.10.8 Click **Enter Standards Info** in the Protocol Settings bar. Individual Bio-Rad kit lots may have a different calibration ranges; 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 all checked: **Same regression type for all analytes**; **Same units for all analytes**; and **Same recovery range for all analytes**.
- 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 box is 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. Skip “5.Enter Control Info”, as Controls should have been indicated as Unknown.
- 7.10.15 Click **Enter Sample Info**, and copy the sample information from sample dilution Excel file and paste into Description column.
- 7.10.16 Click **Run Protocol**.
- 7.10.17 Confirm that the **Beads** event is 50 per region and that 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 μ l, 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 the plate, then readout should be exported in “.csv” format. The .csv file can be read by Bio-Plex manager (after conversion to “rbx” format).

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7.11.2 Click standard curve and confirm that the **Log(x) – Log(y)** is selected from **Axis Transformation**. Confirm that 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.

7.12 Data analysis and reporting (Generate summary table)

7.12.1 Click on the top-left corner cell on the Bio-Plex Manager and copy the entire data set (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 lot numbers in the appropriate cells in “**Kit Information**” sheet. Once all the values are entered, the “**Sample Report**” and “**Controls QC**” sheets should organize/analyze/QC the data. If the sample concentration is different from 500 µg/mL, enter sample concentration in “**Summary**” sheet. Final assay results for patients are shown in the “**Summary**” sheet.

7.13 Assay controls and target ranges. Performance characteristics of the 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 represent 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 is not required.

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- 7.13.2 The performance of the kits is established by running a minimum of two levels of controls for each analyte and ensuring that the 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 analytes fall 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 for 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

- 7.14.1.1 The minimum bead count acceptable for individual analyte readout is set at 20. If MFI is assigned to readouts generated from <20 bead counts for both replicates, the affected assay sample fails and must be repeated if possible and necessary; otherwise, the sample should be reported as “not analyzable” for the analyte.

7.14.2 Precision

- 7.14.2.1 All samples and controls must be run with a minimum of two replicates. Any sample with values above LLQ with a >20% CV should be repeated if possible and necessary; otherwise, the sample should be reported as “not analyzable” for the analyte. 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 a single analyte, irrespective of other analytes in the multiplex panel. This means that control values obtained for the 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 against individual target ranges, irrespective of control values for other analytes.

- 7.14.3.2 In order to report data for an analyte, %CV variation between two replicates of the same control must be less than 20%. In addition, data from individual replicates will be calculated for QC acceptability. At least three out of four replicates for the two controls must be within target ranges for the data to be reportable. The failed QC replicate is considered a random error in this case (could be related to instrument readouts).

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7.14.3.3 If only one level of control fails QC, sample data for the analyte can be reported under director's discretion.

7.14.3.4 If both levels of control fail QC, irrespective of duplicate, no results will be reported, and root cause analysis will be executed; problem identified, resolved, and then sample analysis will be repeated. If both levels of control fail again in the repeat run, the samples will be reported as not analyzable.

7.14.3.5 Trends in QC levels should 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.

7.15 Reviewing Results

7.15.1 Review and finalize the Batch Record ([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 Case Report Form and Approval

7.16.1 A Case Report Form should be prepared to summarize the results for each patient's analyzed sample(s) in Medidata Rave.

7.16.2 Reporting units for analytes: pg analyte/ μ g 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

Plate Map - Paired												
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1		B		Pt-2 Cyto Pre		Pt-4 Cyto Pre		Pt-6 Cyto Pre		Pt-8 Cyto Pre	
B	S2		B		Pt-2 Cyto Post		Pt-4 Cyto Post		Pt-6 Cyto Post		Pt-8 Cyto Post	
C	S3		C1		Pt-2 NM Pre		Pt-4 NM Pre		Pt-6 NM Pre		Pt-8 NM Pre	
D	S4		C2		Pt-2 NM Post		Pt-4 NM Post		Pt-6 NM Post		Pt-8 NM Post	
E	S5		Pt-1 Cyto Pre		Pt-3 Cyto Pre		Pt-5 Cyto Pre		Pt-7 Cyto Pre		Pt-9 Cyto Pre	
F	S6		Pt-1 Cyto Post		Pt-3 Cyto Post		Pt-5 Cyto Post		Pt-7 Cyto Post		Pt-9 Cyto Post	
G	S7		Pt-1 NM Pre		Pt-3 NM Pre		Pt-5 NM Pre		Pt-7 NM Pre		Pt-9 NM Pre	
H	S8		Pt-1 NM Post		Pt-3 NM Post		Pt-5 NM Post		Pt-7 NM Post		Pt-9 NM Post	

Plate Map - Three time points												
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1		B		Pt-1 NM Post_1		Pt-3 Cyto Pre		Pt-4 Cyto Post_2		Pt-5 NM Post_1	
B	S2		B		Pt-1 NM Post_2		Pt-3 Cyto Post_1		Pt-4 NM Pre		Pt-5 NM Post_2	
C	S3		C1		Pt-2 Cyto Pre		Pt-3 Cyto Post_2		Pt-4 NM Post_1		Pt-6 Cyto Pre	
D	S4		C2		Pt-2 Cyto Post_1		Pt-3 NM Pre		Pt-4 NM Post_2		Pt-6 Cyto Post_1	
E	S5		Pt-1 Cyto Pre		Pt-2 Cyto Post_2		Pt-3 NM Post_1		Pt-5 Cyto Pre		Pt-6 Cyto Post_2	
F	S6		Pt-1 Cyto Post_1		Pt-2 NM Pre		Pt-3 NM Post_2		Pt-5 Cyto Post_1		Pt-6 NM Pre	
G	S7		Pt-1 Cyto Post_2		Pt-2 NM Post_1		Pt-4 Cyto Pre		Pt-5 Cyto Post_2		Pt-6 NM Post_1	
H	S8		Pt-1 NM Pre		Pt-2 NM Post_2		Pt-4 Cyto Post_1		Pt-5 NM Pre		Pt-6 NM Post_2	

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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 one protein load in duplicate. Sample numbers correspond to those on the Plate Map Design. The protein load utilized for each of the three panels are recorded below:

Panels	Protein Load (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: _____

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APPENDIX 3: LUMINEX TECHNOLOGY PLATFORM

