

SOP341404: Procedure for Use of Signaling Multiplex Panels 1–3 (Total and Phospho AKT/rpS6 Panels) for the Analysis of Clinical Biopsy and Preclinical Tumor Extracts and Reporting of Data

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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 INFORMATION

1. Approvals

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2. Change History

Revision	Approval Date	Description	Originator	Approval
--	05/21/2019	New Document	WH/CK	AKS
A	02/15/2023	Revised version for inter-lab transfers	WH/LL	AKS/REP

OVERVIEW OF SIGNALING MULTIPLEX PANELS 1-3 (TOTAL AND PHOSPHO AKT/RPS6 PANELS)

SOP340507:

Tumor Frozen Needle Biopsy Specimen Collection, Handling and Shipping for PADIS, Frederick National Laboratory for Cancer Research (FNLCR)

OR

SOP340567:

Tumor Frozen Needle Biopsy Specimen Collection, Handling and Shipment to EET Biobank

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



SOP341210: Tumor Biopsy Protein Extraction for Immunoassays

- Extract total protein from tumor needle biopsies or preclinical tumor specimens
- Determine protein concentration
- Store tumor protein stock lysates or immediately proceed to immunoassay



SOP341404:

Procedure for Use of Signaling Multiplex Panels 1 – 3 (Total and Phospho AKT/rpS6 Panels) for the Analysis of Clinical Biopsy and Preclinical Tumor Extracts and Reporting of Data

- Perform Luminex assays using SOP with clinical samples, standards, and controls
- Using Luminex plate reader, determine relative signals of all samples using xPONENT Software & Bio-Plex Manager data reduction software
- Determine the biomarker concentrations in all samples and apply quality control standards to verify reliability of assay
- Prepare a Clinical Sample Data Report for each set of clinical samples and send to the clinical protocol Principal Investigator



AKT / rpS6 Biomarkers

Signaling Multiplex Panel 1 (Total AKT/rpS6 Panel)	Total AKT1, Total AKT2, Total AKT3, Total rpS6
Signaling Multiplex Panel 2 (Phospho AKT/rpS6 A Panel)	pS473-AKT1, pS474-AKT2, pS472-AKT3, pS235-rpS6
Signaling Multiplex Panel 3 (Phospho AKT/rpS6 B Panel)	pT308-AKT1, pT309-AKT2, pT305-AKT3, pS240/244-rpS6

MEK / ERK Biomarkers

Signaling Multiplex Panel 4 (Total MEK/ERK Panel)	Total ERK1, Total ERK2, Total MEK1, Total MEK2
Signaling Multiplex Panel 5 (Phospho MEK/ERK Panel)	pT202/Y204-ERK1, pT185/Y187-ERK2, pS218/222-MEK1, pS222/226-MEK2

1.0 PURPOSE

Standardize the method for quantification of analyte levels with multiplex immunoassays using the Luminex platform and the Total and Phospho AKT/rpS6 Panels as a pharmacodynamic (PD) measure of drugs targeting the PI3K/AKT pathway, or as potential downstream markers of drug activity.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the Total and Phospho AKT/rpS6 Panels for analysis of tumor protein stock lysates during clinical trials. The goal of the SOP and associated training is to ensure consistency in the use of the signaling multiplex immunoassay panels for the analysis of preclinical samples or tumor biopsies and the reporting of data.

3.0 ABBREVIATIONS

C	=	Control
CV	=	Coefficient of Variation
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked Immunosorbent Assay
FLOD	=	Functional Limit of Detection
LHTP	=	Laboratory of Human Toxicology and Pharmacology
LLQ	=	Lower Limit of Quantification
MFI	=	Median Fluorescence Intensity
NCTVL	=	National Clinical Target Validation Laboratory
PD	=	Pharmacodynamic
PE	=	Phycoerythrin
PI	=	Protease Inhibitor(s)
QC	=	Quality Control
RT	=	Room Temperature
SAPE	=	Streptavidin-Phycoerythrin
SD	=	Standard Deviation
SOP	=	Standard Operating Procedure
Temp	=	Temperature
ULQ	=	Upper Limit of Quantification

4.0 INTRODUCTION

Some of the most common activating mutations in cancer affect the Ras/Raf/MEK/ERK or PI3K/AKT pathways, promoting growth and survival signaling. While there are many drugs targeting these pathways in development or already FDA-approved, it is very common for resistance to develop, frequently by cross-activation of an untargeted pathway. To support pharmacodynamic evaluation of drugs targeting these pathways, DCTD has developed novel isoform-specific signaling multiplex immunoassay panels of 12 biomarkers for AKT and rpS6 (Panels 1–3, described in this document) and 8 biomarkers for MEK and ERK (Panels 4–5, described in SOP341405) to indicate the efficacy of inhibitors by measuring direct inhibition of the enzyme targets and downstream effectors. The Total and Phospho AKT/rpS6 Panels were formatted on the Luminex platform by grouping 12 biomarkers into 3 panels: Signaling Multiplex Panel 1 (Total AKT/rpS6 Panel) detects total levels of AKT1, AKT2, AKT3, and rpS6; Signaling Multiplex Panel 2 (Phospho AKT/rpS6 A Panel) detects pS473-AKT1, pS474-AKT2, pS472-AKT3, and pS235-rpS6; and Signaling Multiplex Panel 3 (Phospho AKT/rpS6 B Panel) detects pT308-AKT1, pT309-AKT2, pT305-AKT3, and pS240/244-rpS6. The multiplexed technique is based on sandwich immunoassays performed on the surface of color-coded 6.5 µm magnetic polystyrene beads (MagPlex, Luminex Corp.). Capture antibodies are coupled to beads and reporter antibodies specific to a different epitope within the target protein are conjugated to biotin. The assay is initiated by mixing samples with beads, washing away unbound sample, mixing analyte-bead complexes with reporter antibody, and after an additional wash step, a streptavidin-phycoerythrin conjugate is added to bind with biotin-conjugated detection antibodies. Beads are washed a third time, suspended in a sheath fluid, and analyzed on a Luminex reader to determine median fluorescence intensity (MFI) values. Details of the assay development, validation, and fit-for-purpose experiments have been published in Herrick et al. *Mol Cancer Ther* 2021;20:749–59.

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 in the 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.

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- 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](#)) and Plate Map Design ([Appendix 2](#)) 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** The sample dilution template is provided as an Excel worksheet, “AKT rpS6 Assay Setup Template”, and calculation results must be copy-pasted into corresponding fields of the Batch Record ([Appendix 1, Section 5](#)) with the sample information. The digital Excel version of the dilution template must be retained with other electronic records to maintain a complete audit trail.
- 5.4** All responsible personnel are to check the DCTD Biomarkers website (<http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>) to verify that the most recent version of the SOP is being used.

6.0 CRITICAL REAGENTS, MATERIALS, AND EQUIPMENT REQUIRED

- 6.1 AKT/rpS6 Panel kits supplied with all buffers, standards and controls, magnetic premixed microparticle cocktails, detection antibodies, Streptavidin-PE and microplates. Refer to product insert for lot specific stability of individual kit components. Discard kits after expiration date.
 - 6.1.1 Total AKT/rpS6 Panel kit (Signaling Multiplex Panel 1)
 - 6.1.2 Phospho AKT/rpS6 A Panel kit (Signaling Multiplex Panel 2)
 - 6.1.3 Phospho AKT/rpS6 B Panel kit (Signaling Multiplex Panel 3)
- NOTE:
 - Performance characteristics for each lot of kits must be confirmed or established as described in [Section 7.13](#) prior to evaluation of clinical samples.
 - DO NOT USE KITS AFTER EXPIRATION DATE.
- 6.2 UltraPure DNase/RNase-free distilled water (e.g., Invitrogen, Cat#: 10977-015)
- 6.3 Roche cOmplete Mini, EDTA-free protease inhibitor cocktail tablets (Sigma, Cat#: 4693159001)
- 6.4 Roche PhosSTOP phosphatase inhibitor cocktail tablets (Sigma, Cat#: 4906837001)
- 6.5 Dry ice
- 6.6 Luminex LX200 Reader with Sheath Fluid Delivery System (Luminex, Cat#: APX10031) or Bio-Plex 200 Reader with Sheath Fluid Delivery System (Bio-Rad, Cat#: 171000201)
- 6.7 Luminex 100/200 Calibration Kit (Luminex, Cat#: LX200-CAL-K25) or Bio-Rad Bio-Plex Calibration Kit (Bio-Rad, Cat#: 171-203060)
- 6.8 Luminex 100/200 Performance Verification Kit (Luminex, Cat#: LX200-CON-K25) or Bio-Rad Bio-Plex Validation Kit 4.0 (Bio-Rad, Cat#: 171-203001)
- 6.9 Luminex Sheath Fluid (Luminex, Sheath Concentrate PLUS Pack, Cat#: 40-50023 or Sheath Fluid PLUS, Cat#: 40-50021) or Bio-Plex 1X Sheath Fluid in 20 L (Bio-Rad, Cat#: 171-000055)
- 6.10 Instrument control software i.e. Luminex xPONENT (v 3.1 or newer; Luminex, Cat#: XPON3.1) or Bio-Plex Manager (v 6.1 or newer; Bio-Rad, Cat#: 171-001510)
- 6.11 Bio-Plex Data Pro with Bio-Plex Manager Software (v 6.1 or newer; Bio-Rad, Cat#: 171-001510)
- 6.12 Pipettors (1-10 µL, 2-20 µL, 10-100 µL and 100-1000 µL) and tips
- 6.13 Multichannel pipettors (10-50 µL and 30-300 µL) and tips (e.g., Rainin Pipet-Lite Multi or Pipet-Lite Adjustable Spacer series)
- 6.14 Repeating pipettor (10-150 µL; e.g., Eppendorf Repeater E3 or Eppendorf Stream) and compatible tips (e.g., Eppendorf Combitips Advanced)
- 6.15 2.0-mL Sarstedt O-ring screw cap, skirted tubes (Fisher Scientific, Cat#: 72.694.006)
- 6.16 1.5-mL snap-cap polypropylene tubes (e.g., Fisher Scientific, Cat#: 21-402-902)
- 6.17 15-mL polypropylene tubes (e.g., Fisher Scientific, Cat#: 14-959-49B)
- 6.18 Universal Black Microplate Lid with Corner Notch (e.g., VWR, Cat#: 77776-852)
- 6.19 Aluminum (or copper) blocks (3) for 1.5 mL tubes (e.g., USA Scientific, Cat#: 9124-8000)
- 6.20 Refrigerated microcentrifuge capable of 10,000 x g (e.g., Sorvall Fresco centrifuge, refrigerated at 4°C)
- 6.21 Microplate shaker (e.g., IKA, Cat#: 003208001)

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- 6.22** Automated plate washer with magnetic separation (e.g., BioTek 405 TS with 96-well flat magnet)
 - 6.23** Vortex mixer, 500–3000 rpm (e.g., Fisher Scientific, Cat#: 02-215-370)
 - 6.24** Bath sonicator/ultrasonic cleaner (e.g., VWR, Cat#: 97043-988)
 - 6.25** Refrigerator (4°C) and freezer (-80°C or colder)
 - 6.26** Microsoft Excel 2007 or higher version
 - 6.27** Excel AKT rpS6 Assay Setup Template
 - 6.28** Excel AKT rpS6 Assay Data Analysis Template with Patient Reporting
 - 6.29** Tumor protein stock lysates processed following SOP341210 (Tumor Biopsy Protein Extraction for Immunoassays)

7.0 OPERATING PROCEDURES

- 7.1 Prior to beginning the assay, refer to the Batch Record and Plate Map Design to review all actions required for successful assay setup ([Appendices 1 and 2](#)).
- 7.2 Record the name of the Certified Assay Operator and the facility running the SOP in the Batch Record ([Appendix 1](#)). Include reference to the clinical protocol number(s) and/or 96-well plate ID, if applicable.
- 7.3 **Equipment and Critical Reagents**
 - 7.3.1 Record the make, model, and serial numbers of equipment in the Batch Record ([Appendix 1, Section 1](#)).
 - 7.3.2 All Critical Reagents are to be labeled with date of receipt and stored under the specified conditions for no longer than the stated duration. Discard critical reagents after expiration date.

NOTE: DO NOT USE ANY CRITICAL REAGENTS AFTER EXPIRATION DATE.

 - 7.3.2.1 Store Total and Phospho AKT/rpS6 Panel kits at 2–8°C.
 - 7.3.2.2 Do not use Total and Phospho AKT/rpS6 Panel kit reagents from more than one lot number when performing an assay.
 - 7.3.3 Record the dates of kit receipt, lot numbers, and expiration dates for the assay critical reagents in the Batch Record ([Appendix 1, Section 2](#)).
 - 7.3.4 If using an untested plate shaker, it is important to perform a trial run of the final bead resuspension ([Step 7.8.20](#)). Due to the nature of the wash buffer, some plate shakers will cause splashing onto the plate sealer that could affect the assay results. The shaker suggested in [Section 6.21](#) does not have this problem within the Clinical PD Biomarker Program laboratories. Perform the trial run using a plate with Sheath Fluid, and if liquid splashes onto the plate sealer, try a different shaker, or lower the shaking speed to 500–600 RPM and increase the time to 3 min for final bead resuspension ([Step 7.8.20](#)). Record any changes in the Notes section of the Batch Record ([Appendix 1, Section 11](#)).
- 7.4 Based on the number of unknown samples* to be analyzed, generate a Plate Map ([Appendix 2](#)) to define the location and replicates of unknown samples, tumor controls, and standards. A single patient's batched unknown samples should be contained in one 96 well plate for each panel (not split over 2) to ensure consistent sample handling.

***NOTE:** "Unknown samples" represent test clinical or preclinical tumor protein stock lysates prepared following SOP341210 throughout the document.

7.5 Initial Reagent Preparation

7.5.1 Sample and Standard Diluent

7.5.1.1 Sample and Standard Diluent are identical for all panels. Determine the volume of Sample and Standard Diluent required for performing the assays following the table below:

Number of Assay Wells	Diluent Volume (mL)
< 64 wells on all 3 panels	10
65–96 wells on all 3 panels	20

7.5.1.2 Add 1 tablet of cOmplete Mini, EDTA-free protease inhibitor cocktail and 1 tablet of PhosSTOP phosphatase inhibitor cocktail to each 10 mL vial of Sample and Standard Diluent provided in the AKT/rpS6 Panel kits; gently agitate on a shaker or end-over-end rotator until the tablets are dissolved. Combine the solutions (if using more than 1 vial) and store on ice. **Inhibitor tablets must be added within 1-hour of use; never save diluent containing inhibitors for later use.**

7.5.1.3 Record Sample and Standard Diluent preparation in the Batch Record ([Appendix 1, Section 3](#)).

7.5.2 1X Wash Buffer

10X Wash Buffer is identical for all panels. Prepare 1X Wash Buffer by bringing 1 vial of 10X Wash Buffer per assay plate to room temperature, inverting to bring any precipitated salts back into solution, and then diluting 1 part 10X Wash Buffer with 9 parts UltraPure DNase/RNase-free distilled water. Record the 1X Wash Buffer preparation in the Batch Record ([Appendix 1, Section 3](#)). Label with the preparation date, store at room temperature, and use within 1 month.

7.6 Preparation of Unknown Samples

IMPORTANT: All unknown samples and dilutions must be thawed, prepared and kept ON ICE until added to the assay plate. Keep unknown samples and dilutions on ice as much as possible and minimize handling to help prevent warming from the air and hands.

7.6.1 Most samples with PI3K/AKT pathway activation are expected to be analyzable at 125 µg/mL, but whenever possible, a small number of representative samples (3–5) should be assayed to determine the appropriate working concentration in the range of 31.25–250 µg/mL before assaying large sets of samples. The assay has been validated for protein concentrations from 31.25 to 250 µg/mL; higher or lower concentrations have not been validated, and therefore should only be used by the testing laboratory after performing its own local validation study.

7.6.2 **Sample Dilution Calculations:** To ensure accurate sample processing with sufficient volume to run desired concentrations on all panels, the Excel AKT rpS6 Assay Setup Template must be used to calculate the volumes and types of buffers needed to process each sample. The table from Excel AKT rpS6 Assay Setup Template is provided in Section 5 of the Batch Record ([Appendix 1, Section 5](#))

7.6.2.1 If running samples at a single protein concentration, select the worksheet for “1 load”, or if running samples at 2 protein concentrations each, select the worksheet for “2 loads”. Following the instructions in the Excel AKT rpS6 Assay Setup Template, input the total protein concentration for each sample (determined from SOP341210) as well as the desired concentration for running them on each panel.

7.6.2.2 Paste the results from Excel AKT rpS6 Assay Setup Template to the corresponding fields of the Sample Preparation Information Table ([Appendix 1, Section 5](#)).

7.6.3 Place the tumor protein stock lysates prepared from SOP 341210 (stored in 2.0 mL Sarstedt O-ring cap tubes) on ice to thaw. Meanwhile, label 1.5 mL snap-cap tubes X1, X2, etc. and store in aluminum tube blocks on ice. Proceed to the next step immediately after thawing.

NOTE: Any appropriately sized tubes or vessels may be used for the sample preparation (e.g., PCR strip tubes with a metal cooling block are also acceptable if they can hold the required volume but must be made from polypropylene).

7.6.4 Centrifuge the samples at 10,000 x g for 5 min at 4°C.

7.6.5 Prepare each lysate dilution with Sample and Standard Diluent (with inhibitors) according to the Excel AKT rpS6 Assay Setup Template and keep all samples on ice until they are loaded onto the assay plates. Immediately snap-freeze any remaining tumor protein stock lysates on dry ice, and place in storage at -80 °C or colder. The remaining tumor protein stock lysates can be analyzed and reported after one freeze thaw cycle. Record the date, freezer ID and location of the remaining tumor protein stock lysate storage in Section 4 of the Batch Record ([Appendix 1, Section 4](#)).

7.7 Preparation of Standards and Controls:

The Standards and Controls are all panel specific. Each kit provides a lyophilized vial of the highest standard to be used in the assay and for further serial dilution to prepare the additional standards. Each kit provides 2 controls, typically a low/medium level control and a high control as cocktails for all analytes in the panel. Prepare the kit controls and high standards for each panel from the lyophilized Control and Standard Cocktails.

IMPORTANT: All standards and controls must be thawed, prepared and kept ON ICE until added to the assay plate. Keep standards and controls on ice as much as possible and minimize handling to help prevent warming from the air and hands.

7.7.1 Reconstitute Standards and Controls from each panel using UltraPure DNase/RNase-free distilled water according to the table below.

Reagent	Water Volume
Standard	150 µL
Control 1	100 µL
Control 2	100 µL

Vortex each vial at medium speed for 5–10 sec, gently tap the vials on the bench or use a wrist flicking motion to bring contents to bottom of vials, then store on ice.

7.7.2 **Perform Steps 7.7.2.1 – 7.7.2.3 for each panel:**

- 7.7.2.1 Label eight 1.5-mL snap-cap polypropylene tubes for standards per panel (T1–T8 for Total AKT/rpS6 Panel, A1–A8 for Phospho AKT/rpS6 A Panel, and B1–B8 for Phospho AKT/rpS6 B Panel) and place in an aluminum tube rack on ice. Alternatively, use PCR strip tubes in an aluminum PCR tube rack on ice.
- 7.7.2.2 Briefly vortex each vial of highest standard for 1–2 sec, bring contents to bottom of vials, then transfer contents of each reconstituted standard to the appropriate tube on ice (T1, A1, or B1). Be sure to transfer the entire contents of each vial without generating foam.
- 7.7.2.3 Prepare working standards with 1:3 serial dilutions according to the table below using Sample and Standard Diluent. Mix thoroughly by pipetting up and down 10 times or by vortexing each standard at high speed for 1 sec before proceeding with the next serial dilution. Pipet carefully (i.e., pipet slowly and ensure no liquid beads on the exterior of the pipet tip) when aspirating and dispensing. Change the pipette tip at each dilution step.

NOTE: An adjustable-spacing multichannel pipettor may be utilized to perform serial dilutions for each panel simultaneously. If a multichannel pipettor is used, ensure the same volume is aspirated in each tip as you perform the dilutions.

Standard	Volume of Standard Diluent	Volume of Standard
T1/A1/B1	-	150 µL
T2/A2/B2	100 µL	50 µL of T1/A1/B1
T3/A3/B3	100 µL	50 µL of T2/A2/B2
T4/A4/B4	100 µL	50 µL of T3/A3/B3
T5/A5/B5	100 µL	50 µL of T4/A4/B4
T6/A6/B6	100 µL	50 µL of T5/A5/B5
T7/A7/B7	100 µL	50 µL of T6/A6/B6
T8/A8/B8	100 µL	50 µL of T7/A7/B7

7.8 Assay Procedure

- 7.8.1 Total AKT/rpS6 Panel, Phospho AKT/rpS6 A and Phospho AKT/rpS6 B Panels should always be performed at the same time for the best results.
- 7.8.2 The manufactured kits are designed for running either 48 wells (half-plate) or 96 wells (full-plate) of a 96-well microplate per assay panel. Each kit includes 2 of each Standard and Control to facilitate the running of 2 half-plates per kit, if desired. Each assay kit only has 1 vial of Sample and Standard Diluent, but 1 vial is sufficient to run a half-plate of all 3 panels. Note: If running a half-plate of each panel they must still be performed on separate microwell plates.
- 7.8.3 Add 40 µL/well of **Blank** (Sample and Standard Diluent) and **panel-specific kit Controls** to the appropriate wells of each plate according to the plate maps [using reverse pipetting*](#).

NOTE: Reverse pipetting is performed through 1) depressing the pipettor plunger completely past the first stop to the second (blowout) stop*; 2) immersing the tip in the liquid, and slowly releasing the plunger to full extension; 3) dispensing by pressing to the first stop; 4) blowing out the small volume of residual liquid to a separate container and properly disposing the used tip. See <https://www.mt.com/us/en/home/library/know-how/rainin-pipettes/how-to-reverse-pipette.html> for details.

* When reverse pipetting these kit reagents, do not go all the way to the second stop because there is not sufficient excess volume.

- 7.8.4** Using a 5-50 µL multichannel pipettor, mix the standards by slowly pipetting up-and-down 2x, then add 40 µL/well of panel-specific **Standards** (T1–T8, A1–A8 and B1–B8) to the appropriate wells of each plate according to the plate maps using reverse pipetting*. Visually confirm that there are equal volumes in each tip when using a multichannel pipettor.

NOTE: For the best accuracy, it is critical to pipet slowly, dispense directly to the bottom edge of the well and ensure there are no liquid droplets on the exterior of the pipet tips.

* When reverse pipetting panel-specific **Standards**, do not go all the way to the second stop because there is not sufficient excess volume.

- 7.8.5** Using a 5-50 µL multichannel pipettor, mix the diluted **unknown samples** by pipetting up-and-down 4x, then add 40 µL/well of **unknown samples** to the appropriate wells of the plates according to the plate maps using reverse pipetting. Since it is recommended that samples should be assayed in all 3 panels at the same time, the sample well locations will typically be identical for each panel and therefore samples can be added to every plate at the same time with a multichannel pipettor. Pipet slowly, visually confirm that equal volume of sample is in each tip when using a multichannel pipettor, and replace tips between samples.

- 7.8.6** Vortex vials of multiplex beads (for Total AKT/rpS6, Phospho AKT/rpS6 A, and Phospho AKT/rpS6 B Panels) for 5 sec, invert to mix, vortex for an additional 5 sec, sonicate for 1 min, vortex for 10 sec, then immediately add 10 µL of beads to every assay well with a repeating pipettor. The multiplex beads for the Total AKT/rpS6 and Phospho AKT/rpS6 A Panels are identical; the multiplex beads for the Phospho AKT/rpS6 B Panels are not identical to the Total AKT/rpS6 and Phospho AKT/rpS6 A Panel multiplex beads.

- 7.8.7** Apply adhesive plate sealers and cover the plates with light blocking black lids.

- 7.8.8** Incubate on a microplate shaker at 850 ± 50 RPM for 2 h at RT. Record the incubation start times and room temperature in the batch record ([Appendix 1, Section 6](#)). If the shaker has a lid, it must be kept open to prevent overheating during the incubation.

- 7.8.9** Before the 2h incubation is completed, prepare 1X Detection Antibody solution by adding 5.5 mL UltraPure DNase/RNase-free distilled water to the vial of lyophilized detection reagent for each panel. Vortex the vial at medium speed for 5–10 sec, then gently tap the vial on the bench or use a wrist flicking motion to bring contents to the bottom of the vial.

- 7.8.10** When the 2 h incubation is complete, stop the shaker, remove plates, and record the stop times in the Batch Record ([Appendix 1, Section 6](#)). Place the Total AKT/rpS6 Panel plate in a plate washer with 96-well flat magnet insert. Wait 1 minute, then wash the plate 3 times with Wash Buffer using the appropriate **Wash** program (see [Section 7.9](#) for plate washer settings). Proceed to the next step immediately, do not let the wells dry out.

- 7.8.11** Following the plate map, immediately add 50 µL of panel-specific 1X Detection Antibody solution to the required plate wells in the washed plate with a repeating pipettor. Replace the plate seal and cover.

- 7.8.12 Immediately repeat Steps 7.8.10–7.8.11 for the Phospho AKT/rpS6 A Panel plate, and then the Phospho AKT/rpS6 B Panel plate.
- 7.8.13 Incubate all 3 plates at 850 ± 50 RPM for 1 h at RT. Record the start times in the Batch Record ([Appendix 1, Section 7](#)).
- 7.8.14 Turn on the Luminex instrument(s) to be used and perform system initialization, confirm that the Calibration & Verification is up to date on each instrument or perform Calibration & Verification according to the manufacturer's protocol if needed, then prepare batches in the instrument control software. Instructions for using xPONENT software to control the instrument can be found in [Section 7.10](#), and instructions for using Bio-Plex Manager software can be found in [Section 7.11](#). Record instrument preparation in ([Appendix 1, Section 8](#))
- 7.8.15 When the 1 h incubation is complete, stop the shaker and record the stop times in the Batch Record ([Appendix 1, Section 7](#)). Wash the Total AKT/rpS6 Panel plate 3 times as in Step 7.8.10. Proceed to the next step immediately, do not let the wells dry out.
- 7.8.16 Following the plate map, immediately add 50 μ L of Total AKT/rpS6 Panel SAPE solution (1X) to the required wells with a repeating pipettor. Replace the plate seal and cover.
- 7.8.17 Immediately repeat steps 7.8.15–7.8.16 for the Phospho AKT/rpS6 A Panel plate with Phospho AKT/rpS6 Panel SAPE solution (1X), and then the Phospho AKT/rpS6 B Panel plate with Phospho AKT/rpS6 Panel SAPE solution (1X).
NOTE: Streptavidin-PE (SAPE) from the Phospho AKT/rpS6 A and Phospho AKT/rpS6 B Panels are identical.
- 7.8.18 Incubate all 3 plates at 850 ± 50 RPM for 30 min at RT. Record the start times in the Batch Record ([Appendix 1, Section 9](#)).
- 7.8.19 When the 30 min incubation is complete, record the stop times in the Batch Record ([Appendix 1, Section 9](#)) and wash the Total AKT/rpS6 Panel plate 3 times as in Step 7.8.10.
- 7.8.20 Immediately after the wash has completed, resuspend the beads in 100 μ L/well of Wash Buffer, do not let the wells dry out. This may be performed with the plate washer using a DISP program. Seal, cover and incubate at 850 ± 50 RPM for 2 min at RT.
- 7.8.21 Replace the next assay plate on the washer magnet. Immediately repeat steps 7.8.19–7.8.20 for the Phospho AKT/rpS6 A Panel plate, and then the Phospho AKT/rpS6 B Panel plate.
- 7.8.22 After 2 min of shaking remove the seals and read plates on Luminex (LX200) instruments (see [Section 7.10](#) if using xPONENT software or [Section 7.11](#) if using Bio-Plex Manager software).

7.9 Plate Washer Settings

NOTE: The following instructions are provided as a starting point. If a laboratory is performing magnetic Luminex assays for the first time, the user should optimize the washing protocol parameters of their specific instrument to minimize bead loss by positioning the aspiration tips away from the aggregated beads and ensuring that > 90% of the wash buffer volume is removed by the final aspiration step.

- 7.9.1 Ensure the plate washer is programmed as specified below for all plate washes using a **BioTek x405 TS Magnetic Microplate Washer**.

NOTE: The Bio-Tek 96-well flat magnet aggregates the beads along the edges of the well. Adjustments to the position of the aspiration tips should be made if using a different magnet.

Plate Washer Parameter	Setting
Default Program	Luminex Wash
Plate Type	96-well plate
Method: Number of Cycles	3
Dispense: Volume	300 µL/well

- 7.9.2 Place the plate on the washer unit for 1 min before starting the wash cycle.
- 7.9.3 From the Main Menu, use the keypad to select RUN→WASH→01 (Costar_Flat), then press Enter.
- 7.9.4 Remove the plate lid and press Start on the keypad to wash the plate.

7.10 Signal Detection (xPONENT Settings)

- 7.10.1 Turn on the Luminex instruments a minimum of 45 minutes before the assay plates will be ready for reading.

NOTE: The plates should be analyzed on Luminex instruments as soon as possible after the final wash and resuspension step. Therefore, at least 2 instruments are required to read the plates within the specified time frame according to the following steps.

- 7.10.2 Rinse the wells of the Maintenance Plate with UltraPure DNase/RNase-free distilled water and dry with Kimwipes. Add UltraPure DNase/RNase-free distilled water to the top well, freshly filtered 70% isopropanol (or ethanol) to the middle well, and freshly filtered 10% bleach to the bottom well.

- 7.10.3 Place the Maintenance Plate in the instrument tray and run the **System Initialization** (xPONENT v. 3) or **Fluidics Prep** (xPONENT v. 4+) routine under the **Maintenance** tab.

NOTE: This step is very important to minimize problems caused by bead aggregation/clumping.

- 7.10.4 The Luminex should be calibrated each week after the system initialization procedure is complete. Perform QC procedures using calibration and performance verification kits following the manufacturer's instructions.

- 7.10.5 If a protocol for the lots of AKT/rpS6 Panel kits being used has been saved to the instrument PC, select the **Batches** tab and click **Create New Batch from an existing protocol**. Enter a name for the batch including the date, run identifier, and panel (Total AKT/rpS6, Phospho AKT/rpS6 A, or Phospho AKT/rpS6 B), select the protocol from the list of available protocols, then click **Plate Layout** on the left and proceed to Step 7.10.7.

If running a new lot of AKT/rpS6 Panel kits for the first time on the instrument PC, select the **Batches** tab and click **Create New Batch from a new Protocol**. Enter a name for the batch including the date, run identifier, and panel (Total, Phospho A, or Phospho B) and enter/select the following settings:

Bead Type: **MagPlex**

Volume: **70 microliters**

DD Gating: 7,500 to 21,000

Reporter Gain: High PMT

- 7.10.6** Click **Next** to open the **Select Analytes** tab. In the order AKT1, AKT2, AKT3, rpS6, click the **Analyte** region number and enter the name for each analyte as described in the lot-specific documentation (see below for an example), and change the **Count** for each analyte to 50. The bead region for each analyte is lot-specific: it is critical that the operator refers to the documentation included with each kit and selects the specified analyte bead regions. Note that the analytes must be entered in the specified order for use with the “AKT rpS6 Assay Template with Patient Reporting” Excel spreadsheet.

AKT1 (25)	AKT2 (45)	AKT3 (52)	rpS6 (66)
-----------	-----------	-----------	-----------

- 7.10.7** Click **Next** to open the **Plate Layout** tab. Add all wells of unknown samples to be assayed to the plate layout as “Unknowns.” If this is the first time running this protocol on the instrument PC, click **Save Prtcl**. In the **Save Protocol** window that appears enter a **Protocol Name** that includes the lot numbers of the Total and Phospho AKT/rpS6 Panels (e.g., “AKT rpS6, Total Lot K21643, Phospho A Lot K21649, Phospho B Lot K21655”) and enter ‘1’ as the **Version**.

NOTE: xPONENT is the software package for control of the Luminex 200 instrument and is used for data acquisition. xPONENT can also be used for data analysis but the Clinical PD Biomarker Program uses the Bio-Rad data analysis software Bio-Plex Manager for all data analysis needs (i.e., standard curve interpolation). The standards, controls and blanks will be changed to their corresponding types during data analysis with Bio-Plex Manager ([Section 7.12](#)).

- 7.10.8** After an assay plate has been washed for the final time and the beads resuspended in Wash Buffer with shaking (Step [7.8.20](#)), remove the plate cover and plate seal. Click **Eject** to eject the plate tray, remove the Maintenance Plate, and insert the assay plate. Click **Retract**, wait until the tray is completely retracted, then click **Run** to begin the instrument analysis. A delay of up to 2 hours between the final wash/resuspension and reading the assay plate is acceptable if necessitated by the availability of instruments. Store the assay plate at 4°C and equilibrate the plate back to room temperature before reading (30 min at room temperature). Record the instrument IDs and reading start times in the Batch Record ([Appendix 1, Section 10](#)).
- 7.10.9** Once the instrument is finished reading the plate, select the appropriate batch and click **Export Results** in xPONENT software and save the output file (.csv format) to a USB drive. The “csv” file generated by xPONENT should not be modified in any way, or the file will be unreadable by Bio-Plex Manager and data could be lost (the raw data handling assures data integrity before analysis by Bio-Plex software).
- 7.10.10** If the instrument will be used again on the same day, remove the assay plate and insert the Maintenance Plate loaded with UltraPure DNase/RNase-free distilled water and 70% isopropanol, then run the **Alcohol Flush** routine under the **Maintenance** tab.


If the instrument will not be used again on the same day, perform the **System Shutdown** routine on the **Maintenance** tab. Once completed, switch off the instrument.

7.11 Signal Detection (Bio-Plex Manager Settings)

- 7.11.1 Turn on the Luminex instruments a minimum of 45 min before the assay plates will be ready for reading.

NOTE: The plates should be analyzed on Luminex instruments as soon as possible after the final wash and resuspension step. Therefore, at least 2 instruments are required to read the plates within the specified time frame according to the following steps.

- 7.11.2 Rinse the wells of the Bio-Plex MCV Plate IV with UltraPure DNase/RNase-free distilled water and dry with Kimwipes. Add UltraPure DNase/RNase-free distilled water, freshly filtered 70% isopropanol (or ethanol), and freshly filtered 10% bleach to the corresponding labeled wells.

- 7.11.3 Place the MCV Plate in the instrument tray and run the **Start Up** routine  on the main toolbar or select the command from the **Instrument** menu.

NOTE: This step is very important to minimize issues caused by bead aggregation/clumping.

- 7.11.4 The Luminex should be calibrated each week after the system initialization procedure is complete. Perform QC procedures using calibration and performance verification kits following the manufacturer's instructions.

- 7.11.5 If a protocol for the lots of AKT/rpS6 Panel kits being used has been saved to the instrument PC, select **File**, then **Open** from the main menu, select the previously saved protocol file (.pbx) and click **Open**, then proceed to Step [7.11.9](#).

If running a new lot of AKT/rpS6 Panel kits for the first time on the instrument PC, create a new protocol by selecting **File**, then **New** from the main menu.

- 7.11.6 Click **Describe Protocol** and enter the lot # of each AKT/rpS6 Panel kit under **Description** (e.g., "AKT rpS6, Total Lot K21643, Phospho A Lot K21649, Phospho B Lot K21655").



- 7.11.7 Click **Select Analytes**, then click **Add Panel**. In the window that appears, click **Add** to enter the region numbers and analyte names in the order AKT1, AKT2, AKT3, rpS6 with region numbers described in the lot-specific documentation (see below for example). Click **Add Continue** after each entry (or **Add** after entering the final analyte, rpS6).




The bead region for each analyte is lot-specific: it is critical that the operator refers to the documentation included with each kit and selects the specified analyte bead regions. Note that the analytes must be entered in the specified order for use with the Excel AKT rpS6 Assay Data Analysis Template with Patient Reporting spreadsheet.

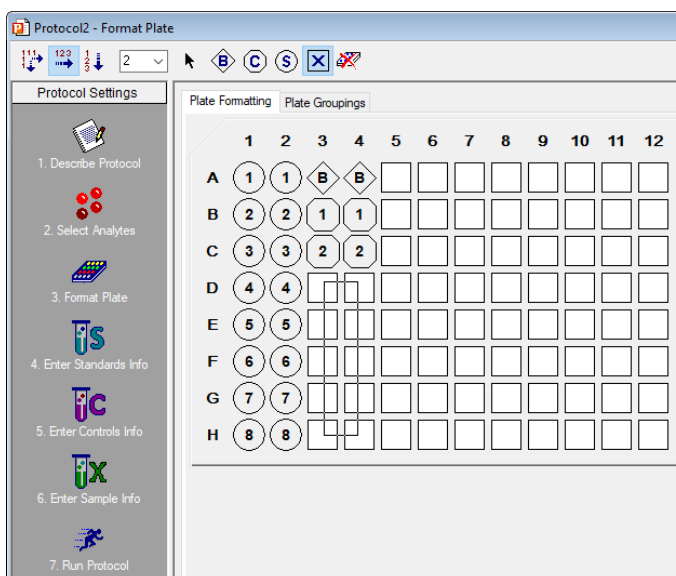
AKT1 (25)	AKT2 (45)	AKT3 (52)	rpS6 (66)
-----------	-----------	-----------	-----------

Enter a **Panel name** with the Total AKT/rpS6 and each Phospho AKT/rpS6 Lot #s (e.g., "AKT rpS6, T Lot K21643, P-A Lot K21649, P-B Lot K21655") and select "Bio-Plex Pro Assay (Magnetic)" from the **Assay** drop-down list. Click **OK** to return to the **Select Analytes** tab.

- 7.11.8 Confirm that the correct **Panel** is selected in the drop-down list, then click **Add All**, ensuring that the correct analyte order is maintained.

- 7.11.9 Select **Format Plate**, then select **Autofill Across** , change the number of replicates in the drop-down box to **2**, then select **Standard**  and click-and-drag

from A1 to H2 on the plate layout to specify the wells as Standards S1 through S8. Also specify the **Blank**  wells, **Control**  wells, and **Unknown Sample**  wells by selecting each respective icon and clicking-and-dragging over the wells specified by the plate map in the Batch Record. When selecting **Unknown Sample** wells, select the wells in pairs of columns as shown in this example:



NOTE: this screen appears slightly different when processing results imported from xPONENT (see Step 7.12.1), but the process for assigning well types, entering standards information, and entering sample names is identical.

- 7.11.10 Click **Enter Standards Info** in the Protocol Settings bar. Select the **Standard Info** Tab. Make sure to select **Logistic – 5PL** in the dropdown list of **Regression Type**, select **Log(x) – Log(y)** in the dropdown list of **Axis Transformation**, and select **70 – 130%** as the Acceptable Recovery Range.
- 7.11.11 Make sure the following check boxes are checked: Same units for all analytes, Same regression type for all analytes, and Same recovery range for all analytes.
- 7.11.12 If panel- and lot-specific standards info has been previously saved to the instrument PC, select it from the **Lot** drop-down list and click **Load**, then proceed to Step [7.11.17](#). Otherwise, enter the panel- and lot-specific highest concentration of each analyte in the top row (labeled S1) of the table. S1 concentration information is listed in the product data sheet.
- 7.11.13 Make sure the **S1** button is selected as **Most Concentrated** and **Apply Dilution to All Analytes** check box is checked.
- 7.11.14 Enter a dilution factor of 3 and click **Calculate**. The concentrations for each standard point will be populated for all analytes in the table.
- 7.11.15 If the standards info was not previously saved, enter the panel name and standard lot number in **Lot**, then enter the **Expiration Date**, and finally click the **Save** button.
- 7.11.16 If running a new lot of AKT/rpS6 Panel kits for the first time on the instrument PC, click **File** then **Save** and save the protocol file (.pbx) with the name “AKT rpS6 T-##### P-A-##### P-B-#####” where T-, P-A-, and P-B- specify the lot numbers of the Total AKT/rpS6, Phospho AKT/rpS6 A, and Phospho AKT/rpS6 B Panels, respectively. This file will be used in future runs (see Step 7.11.5) for either Total AKT/rpS6, Phospho AKT/rpS6 A, or Phospho AKT/rpS6

B Panels, but note that the Standards information must be matched to the panel (see Steps 7.11.12 - 7.11.15).

7.11.17 Click **Enter Sample Info** and copy the sample information from the sample dilution Excel file and paste into the Description column.

7.11.18 Click **Run Protocol**.

7.11.19 Confirm the **Beads** event is 50 per region. Check the box for **Run at High RP1 Target (this is critical)**.

7.11.20 Click **Advanced Settings**, confirm that the **Bead Map** is set to 100 region, the sample size is set to 70 μ L, the DD gates are set to 7,500 (low) and 21,000 (high), and the check box of **Auto Save After Run** is checked. Click **OK**.

7.11.21 Select **Start**, enter a name for the results file including the date, run identifier, and panel (Total AKT/rpS6, Phospho AKT/rpS6 A, or Phospho AKT/rpS6 B). Immediately after an assay plate has been washed for the final time and the beads resuspended in Wash Buffer with shaking (Step [7.8.20](#)), remove the plate cover and plate seal. Click **Eject** to eject the plate tray, remove the Maintenance Plate, and insert the assay plate. Click **Retract** wait until the tray is completely retracted, then begin data acquisition (read plate). A delay of up to 2 hours between the final wash/resuspension and reading the assay plate is acceptable if necessitated by the availability of instruments. Store the assay plate at 4 °C and equilibrate the plate back to room temperature before reading (30 min at room temperature). Record the instrument IDs and reading start times in Batch Record ([Appendix 1, Section 10](#)).


7.11.22 When data acquisition is complete, repeat step 7.11.3 if the instrument will be used again that day; otherwise, select **Shut Down** and follow the instructions.

7.12 Signal Processing (Bio-Plex Analysis Settings)

7.12.1 If xPONENT software was used to read the plate, open Bio-Plex Results Generator, select the appropriate .csv file and click **Generate**. This will convert the .csv file to an .rbx file which is compatible with the Bio-Plex Manager Software.




7.12.2 If xPONENT software was used to read the plate, follow Steps 7.11.9–7.11.15 and 7.11.17 to designate the wells containing Standards, Blanks, Controls, and Unknown Samples and to specify the Standards concentrations.

7.12.3 If Bio-Plex Manager software was used to read the plate, open the .rbx file in Bio-Plex Manager and make sure that the sample information is correct (dilution, sample ID, etc.).

7.12.4 Select the **Report Table Options** icon () and in the window that appears, under the **Display** column, click to uncheck the boxes next to **Std Dev**, **%CV**, **(Obs/Exp) * 100**, and **Dilution**, then click to check the boxes next to **Obs Conc**, **%CV** and **Bead Count**. Select **Set Number Format...**, then in the **Set Column Number Format** window that appears, select **Obs Conc** in the drop-down list and change the number of decimal places to 4, and then select **Conc in Range** and change the number of decimal places to 4. Click **OK** to close the **Set Column Number Format** window and then click **OK** again to close the **Report Table Display Options** window.

7.12.5 Select **Standard Curve**, uncheck **Same Regression Type for All Analytes**, select/check **Show Unknown Samples** and **Show Control Samples**.

In the **Standard Curve** screen, click **Optimize**, set **Regression Type** to **Logistic-5PL**.

- 7.12.6 In the **Report Table** screen, select **Multiple Analyte Layout**  and **Show Replicates** .
- 7.12.7 Export the results layout to MS Excel by clicking the  icon **OR**, if using the Excel AKT rpS6 Assay Data Analysis Template with Patient Reporting, make sure the columns are displayed in the order shown below (or the template will not work), before performing Steps 7.12.8–7.12.12 below.

Type	Well	Description	FI	FI - Bkgd	Conc in Range	Obs Conc	Obs Conc %CV	Exp Conc	Bead Count
------	------	-------------	----	-----------	---------------	----------	--------------	----------	------------

- 7.12.8 Click on the top-left corner cell in Bio-Plex Manager and copy the entire data table (**Edit → Copy**).
- 7.12.9 Open the Excel template (same template for all 3 panels). Make sure “**Bioplex Data, Total**”, “**Bioplex Data, Phospho A**” and “**Bioplex Data, Phospho B**” sheets are empty before pasting data. Go to the “**Bioplex Data, Total**” sheet, click the A1 cell and paste (**Edit → Paste**) the results for Total AKT/rpS6 Panel; go to the “**Bioplex Data, Phospho A**” sheet, click the A1 cell and paste (**Edit → Paste**) the results for Phospho AKT/rpS6 A Panel; then go to the “**Bioplex Data, Phospho B**” sheet, click the A1 cell and paste (**Edit → Paste**) the results for Phospho AKT/rpS6 B Panel. Save the template with a filename denoting the panel(s) used, plate ID(s) and date of analysis.
- 7.12.10 Enter the highest standard (S1) values, control ranges, and LLQ values in the appropriate cells in the “**Kit Information**” sheet.
- 7.12.11 Enter the Assay Operator, Run Date, Panel Kit Lots, Purpose of Run, BioPlex Instrument IDs, and Plate Map in the “**Run Information**” sheet. Specify sample load for the run from the dropdown list in the **Sample Load** field. If the sample load is not the same for every sample, select “variable” and enter the sample load manually for each sample in the “**Summary, Total**” and each “**Summary, Phospho A (or B)**” sheet.
- 7.12.12 Once all the data and information are entered, the “**Controls QC**,” “**Summary**,” “**Summary, Total**,” “**Summary, Phospho A**,” “**Summary, Phospho B**,” “**Sample Report, Total**,” “**Sample Report, Phospho A**,” and “**Sample Report, Phospho B**” sheets should organize/analyze the data.

7.13 Assay Controls and Target Ranges

NOTE: Performance characteristics for each lot of panel 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 protein lysates are included with each AKT/rpS6 Panel kit for use in each assay run. The analyte levels in each control are achieved with recombinant protein prepared in a manner to target the desired analytical range of the assay. An additional control may be added for internal quality control evaluations but is not required.
- 7.13.2 The performance of assays is established by running a minimum of 2 levels of controls for each analyte and ensuring that obtained values fall within the target ranges specified for each control and kit lot. These target ranges are defined by the Clinical PD Biomarker Program in a single laboratory and sometimes 1 or 2 analytes may fall outside of the expected range. This does not necessarily mean

the assay has failed but may indicate that a particular analyte has higher variability and that target ranges must be established in the assay lab for QC evaluation.

- 7.13.3** The control ranges determined by the Clinical PD Biomarker Program for each new lot of kit controls are calculated from a minimum of 5 independent assay runs over a minimum of 3 separate days. Control target ranges are provided to users for the purpose of determining whether an assay run meets the QC acceptability criteria described in [Section 7.14.3](#). An individual laboratory should establish the control ranges for each new lot of kits, by determining the mean and standard deviation for each control in a minimum of 5 independent assay runs. The assays should be run on a minimum of 3 separate days. Control ranges are calculated as $\text{Mean} \pm 3\text{SD}$ to establish the acceptable range for the controls.

7.14 Acceptability Criteria for Patient Sample Reporting

7.14.1 Instrumentation Readout

At least 1 replicate of every standard, control, and unknown sample must have ≥ 20 counted beads, otherwise it fails QC for that analyte. The affected assay sample must be repeated, if possible, otherwise, the sample should be reported as “Not analyzable” for the analyte.

7.14.2 Precision

All standards, controls, and unknown samples must be run with a minimum of 2 replicates. Any sample analyte values $> \text{LLQ}$ with $\text{CV} > 20\%$ should be repeated if possible; otherwise, the sample should be reported as “Not analyzable” for the analyte.

7.14.3 Assay Control QC Acceptability Criteria

For evaluation of controls, each analyte in the multiplex panel is considered as a single assay irrespective of other analytes in the multiplex panel, i.e., control values obtained for each analyte are independent of control values obtained for other analytes. The following criteria are applied to the control values for each analyte:

- 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 based on individual target ranges irrespective of control values for other analytes.
- 7.14.3.2** In order to report data for an analyte, $\% \text{CV}$ variation between 2 replicates of the same control must be less than 20%. In addition, at least 1 replicate of each control must have ≥ 20 counted beads.
- 7.14.3.3** Data from individual replicates will be calculated for QC acceptability. For analytes where 3 out of 4 control replicates are in the expected range, the data is reportable. This rule states that, if all other criteria are met, 3 out of 4 replicates must pass the QC test. This exception is to allow for random instrument error.
- 7.14.3.4** If the above criteria are not met, the assay fails QC for that analyte and no results for that analyte will be reported. A root cause analysis should be executed, the problem identified, resolved, and sample analysis repeated.

- 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 analyte values \geq the lot- and analyte-specific LLQ and \leq ULQ (defined as the highest standard for that analyte) will be reported.
- 7.14.4.2 Sample analyte values $<$ LLQ are reported as $<$ LLQ with the note “re-assay at a higher protein load.”
- 7.14.4.3 If a sample analyte value is $<$ LLQ and cannot be, or has already been re-assayed at a higher protein load, report the value as “ $<$ LLQ”.
- 7.14.4.4 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, otherwise, the sample should be reported as “ $>$ ULQ”.

7.14.5 Linearity

- 7.14.5.1 Samples may be run at 2 different protein concentrations in the range of 31.25 to 250 μ g/mL with values observed within the assay range reported. Linearity refers to the analytical recovery of an analyte value relative to the value obtained when the same sample is assayed at a different concentration in the allowable range (after accounting for the difference in sample concentration/dilution). For example, if a sample ran at 62.5 μ g/mL gives an analyte value exactly equal to $\frac{1}{2}$ of the value obtained when the same sample is assayed at 125 μ g/mL, the dilutional recovery is 100%. All analytes demonstrated dilutional recoveries in the range of 70–130% in the initial analytical validation.
- 7.14.5.2 Dilutional linearity of samples assayed at concentrations $>$ 250 μ g/mL has not been evaluated by the Clinical PD Biomarker Program. If it is desired to run samples at concentrations $>$ 250 μ g/mL, the dilutional linearity should be evaluated and the results, if acceptable, should be interpreted with caution.
- 7.14.5.3 For samples in which both dilutions fall within the recovery range of 70–130%, the average calculated value for that analyte should be reported.
- 7.14.5.4 For samples in which only one dilution falls within the recovery range of 70–130%, only the value for the dilution within range should be reported.

7.15 Reviewing Results

- 7.15.1 Review and finalize the Batch Records ([Appendix 1](#)) and obtain required signatures. Document ANY deviations from this SOP in the Batch Record ([Appendix 1, Section 11](#)).
- 7.15.2 The Laboratory Director/Supervisor should review the Batch Record and sample reports and sign the Batch Record affirming the data contained within the reports are correct ([Appendix 1, Section 12](#)).

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** Results from the Total AKT/rpS6 Panel will be reported in pg analyte/μg protein from the “**Summary, Total**” tab of the Excel AKT rpS6 Assay Data Analysis Template with Patient Reporting.
- 7.16.3** Results from the Phospho AKT/rpS6 A and B Panels will be reported as the ratio of phospho protein:total protein from the “**Summary**” tab of the Excel AKT rpS6 Assay Data Analysis Template with Patient Reporting.
- 7.16.4** Batch Records or equivalent experimental records including raw and analyzed data must be reviewed and approved by the Laboratory Director/Supervisor prior to reporting of sample data for clinical samples. Approval by Laboratory Director/Supervisor 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.

APPENDIX 1: BATCH RECORD

Certified Assay Operator: _____

Facility/Laboratory Running SOP: _____

Protocol number(s): _____

Plate ID: _____

1. Equipment Information

Luminex Plate Reader(s): Make/Model: _____

Serial #(s): _____

BioTek Plate Washer: Make/Model: _____

Serial #: _____

2. Critical Reagent Information

Commercial Product Lots			
Reagent Name	Received	Lot	Expires
Total AKT/rpS6 Panel kit	/ /		/
Phospho AKT/rpS6 A Panel kit	/ /		/
Phospho AKT/rpS6 B Panel kit	/ /		/
Roche cOmplete Mini, EDTA-free	/ /		/
Roche PhosSTOP	/ /		/

NOTE: Do not use kit reagents from more than one lot number when performing an assay.

3. Reagent Preparation

i. Sample and Sample Diluent

Add 1 tablet each of protease and phosphatase inhibitors to every 10 mL of Sample/Standard Dilution Buffer up to 1 hour before use.

☐ cOmplete Mini, EDTA-free added ☐ PhosSTOP added

ii. 1X Wash Buffer

Bring 1 vial of 10X Wash Buffer per assay plate to room temperature, invert to bring any precipitated salts back into solution, and then dilute 1 part 10X Wash Buffer with 9 parts UltraPure DNase/RNase-free distilled water.

1 part 10 X Wash Buffer volume: _____

9 parts UltraPure DNase/RNase-free distilled water volume: _____

4. Sample Preparation Information:

Unknown samples will be run in duplicate. Sample numbers correspond to those on the Plate Map Design. The suggested protein load for unknown samples is 125 µg/mL; however, it is always preferable when assaying new cancer subtypes/samples to perform testing to determine the optimal protein load before analyzing large sets of samples.

Immediately snap-freeze any remaining tumor protein stock lysates on dry ice, and place in storage at -80 °C or colder. The remaining tumor protein stock lysates can be analyzed and reported after one freeze thaw cycle.

Date of remaining tumor protein stock lysates storage: _____

Freezer ID and location for remaining tumor protein stock lysates storage: _____

5. Sample Preparation Information Table:

Use the [Excel AKT rpS6 Assay Setup Template](#) to calculate the sample dilutions. Copy and paste the table on the next page.

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(PASTE THE RESULTS FROM AKT&RPS6 ASSAY SETUP TEMPLATE TO CORRESPONDING FIELDS OF THE TABLE BELOW; Cont. on next page)

[illegible]

[illegible]

- **ALL SAMPLE PREPARATION MUST BE DONE ON ICE.**
- **Prepare Standards, Controls, and unknown samples according to the SOP.**

***NOTE: Stock lysate represents tumor protein stock lysate prepared following SOP341210.**

6. Plate Incubation

- Proceed with loading of blanks, controls, standards, unknown samples and beads according to the Plate Map and SOP.
- The plate must be sealed and covered with an opaque lid to protect the assay wells from light.
- Incubate on shaker at 850 ± 50 RPM for **2 h** at RT. Record the room temperature and start and stop times:

AKT/rpS6 Panel	Start Time	Stop Time
Total	:	:
Phospho A	:	:
Phospho B	:	:
Room Temp (°C):		

7. Add Detection to Plate(s)

- After the 2 h incubation in Step 6, wash plates and add 50 μ L/well of panel-specific 1X Detection Antibodies according to the Plate Maps and SOP. Seal, cover, and incubate at 850 ± 50 RPM, for **1 h** at RT. Record the start and stop times:

AKT/rpS6 Panel	Start Time	Stop Time
Total	:	:
Phospho A	:	:
Phospho B	:	:

8. Prepare Luminex Instruments

- Prepare Luminex instruments during the incubation. Ensure calibration/verification is up to date, the room temperature is within calibration range, and there is sufficient sheath fluid.
 - ☐ Instruments calibrated and temperature is within range
 - ☐ Sheath fluid OK

9. Add SAPE to Plate(s)

- After the 1 h incubation, wash plates and add 50 μ L/well of SAPE (1X) according to the Plate Maps.
- Incubate on shaker at 850 ± 50 RPM for **30 min** at RT. Record start and stop times:

AKT/rpS6 Panel	Start Time	Stop Time
Total	:	:
Phospho A	:	:
Phospho B	:	:

10. Read the Plate(s)

- After the 30 min incubation, wash plates and add 100 μ L of Wash Buffer per well.
- Cover and shake plates at 850 ± 50 RPM for 2 min to resuspend the beads.
- Remove seals and read plates using the appropriate protocol settings according to SOP [Section 7.10](#) (if controlling the instrument with xPONENT software) or [Section 7.11](#) (if controlling the instrument with Bio-Plex Manager). Record the date(s) of the last instrument calibration(s) and the start times below:

AKT/rpS6 Panel	Instrument #	Last Calibration	Reading Start Time
Total			:
Phospho A			:
Phospho B			:

11. Notes, including any deviations from the SOP:

12. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (SIGN)

Date: _____

APPENDIX 2: PLATE MAP DESIGN

Total AKT/rpS6 Panel Plate Map Example

	1	2	3	4	5	6	7	8	9	10	11	12
A	T1	T1	BL	BL	X6	X6	X14	X14	X22	X22	X30	X30
B	T2	T2	C1	C1	X7	X7	X15	X15	X23	X23	X31	X31
C	T3	T3	C2	C2	X8	X8	X16	X16	X24	X24	X32	X32
D	T4	T4	X1	X1	X9	X9	X17	X17	X25	X25	X33	X33
E	T5	T5	X2	X2	X10	X10	X18	X18	X26	X26	X34	X34
F	T6	T6	X3	X3	X11	X11	X19	X19	X27	X27	X35	X35
G	T7	T7	X4	X4	X12	X12	X20	X20	X28	X28	X36	X36
H	T8	T8	X5	X5	X13	X13	X21	X21	X29	X29	X37	X37

Empty Total AKT/rpS6 Panel Plate Map:

	1	2	3	4	5	6	7	8	9	10	11	12
A	T1	T1	BL	BL								
B	T2	T2	C1	C1								
C	T3	T3	C2	C2								
D	T4	T4										
E	T5	T5										
F	T6	T6										
G	T7	T7										
H	T8	T8										

PLATE MAP: INITIALS _____ DATE: _____

Phospho AKT/rpS6 A and B Panel Plate Maps Example:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A/B1	A/B1	BL	BL	X6	X6	X14	X14	X22	X22	X30	X30
B	A/B2	A/B2	C1	C1	X7	X7	X15	X15	X23	X23	X31	X31
C	A/B3	A/B3	C2	C2	X8	X8	X16	X16	X24	X24	X32	X32
D	A/B4	A/B4	X1	X1	X9	X9	X17	X17	X25	X25	X33	X33
E	A/B5	A/B5	X2	X2	X10	X10	X18	X18	X26	X26	X34	X34
F	A/B6	A/B6	X3	X3	X11	X11	X19	X19	X27	X27	X35	X35
G	A/B7	A/B7	X4	X4	X12	X12	X20	X20	X28	X28	X36	X36
H	A/B8	A/B8	X5	X5	X13	X13	X21	X21	X29	X29	X37	X37

Empty Phospho AKT/rpS6 A Panel Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	BL	BL								
B	A2	A2	C1	C1								
C	A3	A3	C2	C2								
D	A4	A4										
E	A5	A5										
F	A6	A6										
G	A7	A7										
H	A8	A8										

PLATE MAP: INITIALS _____ DATE: _____

Empty Phospho AKT/rpS6 B Panel Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	B1	BL	BL								
B	B2	B2	C1	C1								
C	B3	B3	C2	C2								
D	B4	B4										
E	B5	B5										
F	B6	B6										
G	B7	B7										
H	B8	B8										

PLATE MAP: INITIALS _____ DATE: _____

APPENDIX 3: CLINICAL SAMPLE DATA REPORT

Total and Phospho AKT/rpS6 Panels Analysis

Certified Assay Operator (Print): _____

Today's 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			
Total AKT/rpS6 Panel Analytes		Baseline	Post-Dose	QC Notes
		(pg analyte/μg protein)		
Total AKT1				
Total AKT2				
Total AKT3				
Total rpS6				
Phospho AKT/rpS6 A and B Panel Analytes		Baseline	Post-Dose	QC Notes
		Phospho:Total Ratio		
pS473-AKT1:Total AKT1				
pS474-AKT2:Total AKT2				
pS472-AKT3:Total AKT3				
pS235-rpS6:Total rpS6				
pT308-AKT1:Total AKT1				
pT309-AKT2:Total AKT2				
pT305-AKT3:Total AKT3				
pS240/244-rpS6:Total rpS6				

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.

APPENDIX 4: REQUIRED ASSAY BUFFER RECIPES

NOTE: There is sufficient buffer included with the kits but occasionally a user may need more Wash Buffer than is included, due to buffer loss when priming plate washers. Record details in the Batch Record if Wash Buffer is made from scratch ([Appendix 1, Section 11](#)).

1. Wash Buffer – Make fresh daily

Reagent	Stock	Final	Per Liter
PBS*	10X	1X	100 mL
Triton X-100	10%	0.1%	10 mL
ProClin 300	100%	0.05%	0.5 mL
Water			890 mL

* 10X PBS is 10.59 mM KH_2PO_4 , 29.66 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1552 mM NaCl, pH 7.4