

SOP341405: Procedure for Use of Signaling Multiplex Panels 4–5 (Total and
Phospho MEK/ERK Panels) for the Analysis of Clinical Biopsy and Preclinical
Tumor Extracts and Reporting of Data

Effective Date: 2/15/2023

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

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2. Change History

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--	04/15/2019	New Document	CK	AKS
A	02/15/2023	Revised version for inter-lab transfers	WH/LL	AKS/REP

OVERVIEW OF SIGNALING MULTIPLEX PANELS 4-5 (TOTAL AND PHOSPHO MEK /ERK PANELS)

SOP340507:

Tumor Frozen Needle Biopsy Specimen Collection,
Handling and Shipping to
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 conical 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



SOP341405: Procedure for
Use of Signaling Multiplex
Panels 4–5 (Total and
Phospho MEK/ERK
Panels) for the Analysis of
Clinical Biopsy and
Preclinical Tumor Extracts
and Reporting of Data

**NOTE: The Total and
Phospho MEK/ERK
Panels require an
additional denaturation
step and overnight
incubation as compared to
Total and Phospho
AKT/rpS6 Panels
(Signaling Multiplex
Panels 1–3).**

- Perform Luminex assays with 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 QC standards to verify acceptability 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 Total and Phospho MEK/ERK Panels as a pharmacodynamic (PD) measure of drugs targeting the MEK/ERK 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 MEK/ERK 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
DB (6 M)	=	Denaturation Buffer (6 M)
DCTD	=	Division of Cancer Treatment and Diagnosis
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	=	Pharmacodynamics
PE	=	Phycoerythrin
PI	=	Protease Inhibitor(s)
QC	=	Quality Control
RT	=	Room Temperature
SAPE	=	Streptavidin-Phycoerythrin
SD	=	Standard Deviation
SDB (0 M)	=	Sample Dilution Buffer (0 M)
SOP	=	Standard Operating Procedure
Temp	=	Temperature
ULQ	=	Upper Limit of Quantification
USD (1.5 M)	=	Urea/ Thiourea Sample Dilution Buffer (1.5 M)
USD (1 M)	=	Urea/ Thiourea Sample Dilution Buffer (1 M)

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 pathways. 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 (described in SOP341404 Signaling Multiplex Panels 1–3) and 8 biomarkers for MEK and ERK (described in this document) to indicate the efficacy of inhibitors by measuring direct inhibition of the enzyme targets and downstream effectors. The Total and Phospho MEK/ERK Panels were formatted on the Luminex platform by grouping 8 biomarkers into 2 panels: Signaling Multiplex Panel 4 (Total MEK/ERK Panel) measures total levels of ERK1, ERK2, MEK1, and MEK2; Signaling Multiplex Panel 5 (Phospho MEK/ERK Panel) measures pT202/Y204-ERK1, pT185/Y187-ERK2, pS218/222-MEK1, and pS222/226-MEK2. 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.

- 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 MEK ERK 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 web site (<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 Total and Phospho MEK/ERK Panel kits (Custom Manufactured) are supplied with all buffers, standard and control cocktails, magnetic premixed microparticle cocktails, Biotin antibody cocktails, Streptavidin-Phycoerythrin and microplates. Refer to the product insert for lot specific storage and stability information for individual kit components. Discard kits after expiration date.

6.1.1 Human Total MEK/ERK Panel kit (Signaling Multiplex Panel 4); referred as Total MEK/ERK Panel for the remainder of the document

6.1.2 Human Phospho MEK/ERK Panel kit (Signaling Multiplex Panel 5); referred as Phospho MEK/ERK Panel for the remainder of the document

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 Thiourea, $\geq 99\%$ pure (e.g., Sigma, Cat#: T8656)

6.4 Pierce no-weigh format DTT (dithiothreitol) (Thermo Fisher, Cat#: A39255)

6.5 Roche cOmplete Mini, EDTA-free protease inhibitor cocktail tablets (Sigma, Cat#: 4693159001)

6.6 Roche PhosSTOP phosphatase inhibitor cocktail tablets (Sigma, Cat#: 4906837001)

6.7 Dry ice

6.8 Pipettors (1-10 μ L, 2-20 μ L, 10-100 μ L and 100-1000 μ L) and tips

6.9 Multichannel pipettors (10-50 μ L and 30-300 μ L) and tips (e.g., Rainin Pipet-Lite Multi or Pipet-Lite Adjustable Spacer series)

6.10 Repeating pipettor (10-150 μ L; e.g., Eppendorf Repeater E3 or Eppendorf Stream) and compatible tips (e.g., Eppendorf Combitips Advanced)

6.11 2.0-mL Sarstedt O-ring screw cap, skirted tubes (e.g., Fisher Scientific, Cat#: 72.694.006)

6.12 1.5-mL snap-cap polypropylene tubes (e.g., Fisher Scientific, Cat#: 21-402-902)

6.13 15-mL polypropylene tubes (e.g., Fisher Scientific, Cat#: 14-959-49B)

6.14 Tubes and Flat Caps, strips of 8 (made of polypropylene) (e.g., Thermo Fisher, Cat#: AB1182)

6.15 Universal Black Microplate Lids (2) with Corner Notch (e.g., VWR, Cat#: 77776-852)

6.16 Aluminum (or copper) blocks (3) for 1.5 mL tubes (e.g., USA Scientific, Cat#: 9124-8000)

6.17 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)

6.18 Luminex 100/200 Calibration Kit (Luminex, Cat#: LX200-CAL-K25) or Bio-Rad Bio-Plex Calibration Kit (Bio-Rad, Cat#: 171-203060)

- 6.19** 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)
- 6.20** 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)
- 6.21** 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)
- 6.22** Refrigerated microcentrifuge capable of 16,000 x g (e.g., Sorvall Fresco centrifuge, refrigerated at 4°C)
- 6.23** Two microplate shakers, one at room temperature and one at 4°C (e.g., IKA, Cat#: 003208001)
- 6.24** Automated plate washer with magnetic separation (e.g., BioTek 405 TS with 96-well flat magnet)
- 6.25** Vortex mixer, 500–3000 rpm (e.g., Fisher Scientific, Cat#: 02-215-370)
- 6.26** Bath sonicator/ultrasonic cleaner (e.g., VWR, Cat#: 97043-988)
- 6.27** Refrigerator (4°C) and freezer (-80°C or colder)
- 6.28** Microsoft Excel 2007 or higher version
- 6.29** Excel MEK ERK Assay Setup Template
- 6.30** Excel MEK ERK Assay Data Analysis Template with Patient Reporting
- 6.31** 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 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 reagents after expiration date.

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

 - 7.3.2.1 Store Total and Phospho MEK/ERK Panel kits at 2–8°C.
 - 7.3.2.2 Reconstituted solutions containing urea/thiourea must be made fresh the day they are used and discarded after the assay procedure in accordance with proper waste management procedures.
 - 7.3.2.3 Do not use Total and Phospho MEK/ERK Panel kits reagents from more than one lot number when performing an assay.
 - 7.3.2.4 All reagents must be stored according to the manufacturer’s instructions.
 - 7.3.3 Record the dates of kit receipt, lot numbers, and expiration dates for the assay 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 6.23](#) 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, assay controls, and standards. A single patient’s batched 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

NOTE: The reagent preparation described below will only use the Sample Dilution Buffer, Denaturation Buffer, and Tris Buffered Saline from *one panel's kit* to run *both* panels. If running half a plate for each panel, the remaining unprepared reagents may be used on a later date to run another half plate; if running full plates, the reagent preparation below will provide sufficient buffer in most cases, otherwise additional buffer can be prepared in the same way, as needed. Record reagent preparation in the Batch Record ([Appendix 1, Section 3](#)).

7.5.1 Sample Dilution Buffer (0 M): Add 1 tablet of cOmplete Mini, EDTA-free protease inhibitor cocktail and 1 tablet of PhosSTOP phosphatase inhibitor cocktail to *each* of 2 x 10 mL vials of Sample Dilution Buffer (0 M) provided in the Total and Phospho MEK/ERK Panel kits; gently agitate until the tablets are dissolved and store on ice. **Inhibitor tablets must be added within 1-hour of use; never save buffer containing inhibitors for later use.**

7.5.2 1M DDT: Immediately before use, make 100 μ L of 1 M DTT by adding 50 μ L of UltraPure DNase/RNase-free distilled water to each of 2 x 7.7 mg DTT tubes (Pierce “No-Weigh” DTT) at room temperature and mix well by pipetting until dissolved. Combine the solutions into a single tube.

NOTE: The DTT pellet will require forceful agitation with a pipet tip to dissolve.

7.5.3 Denaturation Buffer (6 M): Prepare Denaturation Buffer (6 M) by adding 761 mg of thiourea, 3.5 mL of 1X Tris Buffered Saline and 50 μ L of 1 M DTT to the lyophilized reagent bottle. Vortex for 30 sec, then shake at room temperature until dissolved. Do not put the Denaturation Buffer (6 M) on ice, or it will precipitate.

NOTE: It will take a minimum of 30 minutes for the solids to go into solution. This solution must be made on the day it will be used.

7.5.4 Urea/ Thiourea Sample Dilution Buffer (1.5 M): Make Urea/ Thiourea Sample Dilution Buffer (1.5 M) by adding 385 mg thiourea, 9.5 mL of Sample Dilution Buffer (0 M) with inhibitors, and 25 μ L of 1 M DTT to the lyophilized reagent bottle that contains urea. Vortex for 10 sec, shake at room temperature until dissolved, then store on ice.

7.6 Preparation of Unknown Samples

NOTE: Unknown sample preparation involves several key steps following the Excel MEK ERK Setup Template (Example shown below). First, the samples must be **diluted** to a standard concentration of 1 mg/mL (or 0.5 mg/mL if the starting concentration is between 0.5 mg/mL and 1 mg/mL) following the **Initial Dilution** section. Then, diluted unknown samples must be **denatured** in an equal volume of Denaturation Buffer (6 M) following the **Denaturation** section. After the denaturation, samples must be **diluted** again to the desired final concentration in Sample Dilution Buffer (0 M) and Urea/Thiourea Sample Dilution Buffer (1.5 M) following **Dilution to High Concentration** and/or **Dilution to Low Concentration** sections. Finally, to complete the denaturation process, every sample must be **frozen** and **thawed** before loading on the assay plate.

Sample Information						Initial Dilution			Denaturation	Dilution to High Concentration			Dilution to Low Concentration		
						Dilute samples with Sample Diluent			Add, mix, wait 10'	Add to Denatured Samples (same tube)			Combine in a new tube		
Sample #	Identifier	Protein Conc.	Pairs of Replicates	T	P	READ: Conc. of Initial Protein Dilution	ADD: Sample Diluent (0 M)	ADD: Stock Lysate	ADD: Denaturation Buffer (6 M)	SELECT: High Protein Conc.	ADD: Sample Diluent (0 M)	ADD: Sample Diluent (1.5 M)	SELECT: Low Protein Conc.	ADD: Sample Diluent (1.5 M)	ADD: High Protein Concentration Sample
						(µg/mL)	(µL)	(µL)	(µL)	(µg/mL)	(µL)	(µL)	(µg/mL)	(µL)	(µL)
X1, X2	Sample 1	1100	2	X	X	1000	3.41	34.09	37.5	125.00	75.0	150.0	62.50	100.0	100

IMPORTANT: All tumor protein stock lysates and dilutions must be thawed, handled, and prepared ON ICE. Keep tumor protein stock lysates and dilutions on ice as much as possible and minimize handling to help prevent warming from air and hands.

- 7.6.1** Most samples with MEK/ERK pathway activation are expected to be analyzable at a final concentration of 125 µg/mL, but whenever possible, a small number of representative samples (3–5) should be assayed to determine the appropriate working concentrations in the range of 31.25–250 µg/mL before assaying large sets of samples. The assay has been validated for final protein concentrations up 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** **Samples must have a starting total protein concentration ≥ 0.5 mg/mL to be assayed using this method.** Samples with starting total protein concentrations between 0.5 mg/mL and 1 mg/mL cannot be assayed at final concentrations > 125 µg/mL.
- 7.6.3** **Sample Dilution Calculations:** The Total MEK/ERK Panel and Phospho MEK/ERK Panel should always be performed at the same time for the best results. To ensure accurate sample processing with sufficient volumes to run the desired concentrations on both panels, the Excel MEK ERK Assay Setup Template should be used to calculate the volumes of buffers needed to process each sample. The table from the Excel MEK ERK Assay Setup Template is provided in Section 5 of the Batch Record ([Appendix 1, Section 5](#)).

7.6.3.1 Following the instructions in the Excel MEK ERK Assay Setup Template, input the total protein concentrations for each tumor protein stock lysate (determined from SOP341210) as well as the desired assay concentration(s) for their analysis on each panel. Use the calculated volumes from this template to perform the sample processing steps described in this section.

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

7.6.4 Place the tumor protein stock lysates to be assayed (stored in 2.0 mL Sarstedt O-ring cap tubes) on ice to thaw. Meanwhile, label 1.5 mL snap-cap tubes with the specimen number (X1, X2, etc.) and store in aluminum tube holder 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 but must be made from polypropylene). However, the use of individual tubes for each sample will aid in the following steps when initial or final protein concentrations vary between samples (see Section 7.6.7).

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

7.6.6 Initial Dilution: On ice, using the prelabeled snap-cap tubes, dilute each tumor protein stock lysate to 1 mg/mL (or 0.5 mg/mL if the starting concentration is between 0.5 mg/mL and 1 mg/mL) in Sample Dilution Buffer (0 M) according to the Excel MEK ERK Assay Setup Template and/or the Sample Preparation Information Table ([Appendix 1, Section 5](#)). Immediately snap-freeze any remaining tumor protein stock lysates on dry ice, and place in storage at -80 °C or colder.

7.6.7 Denaturation

Add an equal volume of Denaturation Buffer (6 M) to each diluted sample with a repeating pipettor according to the table in the Excel MEK ERK Assay Setup Template and/or the Sample Preparation Information Table ([Appendix 1, Section 5](#)). Cap all the vials, vortex briefly, then immediately start a 10 min timer and incubate on ice.

NOTE: The volume of buffers to add to each sample will vary depending on the starting and final protein concentrations of the sample and the # of “Pairs of Replicates” selected. Arrange the diluted samples in groups to make it easier to efficiently add the correct volumes to the correct tubes with a repeating pipettor. For example, if half of the samples require 25 µL of Denaturation Buffer (6 M) and half require 50 µL, they should be grouped so that you can efficiently add Denaturation Buffer to the first half and then to the second half without any chance of adding the wrong volume.

7.6.8 Final Dilution

7.6.8.1 Approximately 2 minutes before the timer is up, prepare repeating pipettor(s) to add the specified volumes of Sample Dilution Buffer (0 M) and Urea/ Thiourea Sample Dilution Buffer (1.5 M). Uncap all the tubes.

7.6.8.2 As soon as the 10 minutes are up, add Sample Dilution Buffer (0 M) to each tube with a repeating pipettor according to the Excel MEK ERK

Assay Setup Template and/or the Sample Preparation Information Table ([Appendix 1, Section 5](#)). Next, add Urea/ Thiourea Sample Dilution Buffer (1.5 M) to each tube with a repeating pipettor according to the MEK/ERK Assay Setup Template and/or the Sample Preparation Information Table ([Appendix 1, Section 5](#)).

7.6.8.3 For those samples that will be run at 2 final concentrations in the range of 31.25–250 µg/mL, take the specified volume of high concentration sample (prepared in step 7.6.8.2) and combine in a separate tube with the specified volume of Urea/ Thiourea Sample Dilution Buffer (1.5 M) according to the Excel MEK/ERK Assay Setup Template and/or the Sample Preparation Information Table ([Appendix 1, Section 5](#)).

7.6.8.4 **The final urea/thiourea concentration in all samples must be 1.5 M.**

7.6.9 Immediately snap-freeze the denatured and diluted samples on dry ice. Samples will freeze in approximately 10 minutes unless the volume is significantly greater than 200 µL. Record the sample preparation and storage date, as well as the freezer ID and location of sample storage in the Batch Record ([Appendix 1, Section 4](#)).

7.6.10 When ready to perform the assay, place the denatured/diluted samples on ice to thaw and immediately proceed to the next section.

7.7 Preparation of Standards and Controls

Each Total and Phospho MEK/ERK Panel kit provides a lyophilized vial of the highest concentration 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.

NOTE: Buffers must be prepared fresh for the remaining assay steps if the denatured and diluted samples were prepared prior to the day the assay is performed.

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 air and hands.

7.7.1 Label eight 1.5-mL snap-cap polypropylene tubes T1–T8 and eight tubes P1–P8 for the Total and Phospho MEK/ERK Panel standard curves, respectively. Place in aluminum cold blocks on ice to chill.

NOTE: As with sample dilutions, PCR strip tubes made of polypropylene are acceptable.

7.7.2 The Standard and Control vials in some lots of Total and Phospho MEK/ERK Panel kits may contain lyophilized pellets that have stuck to the side of the tube or the underside of the cap. Visually check each vial and make a note in section 11 of the Batch Record ([Appendix 1, Section 11](#)) for each Standard or Control that has the pellet stuck to the side or under the cap.

- 7.7.3** Remove the caps from the Standard, High Control, and Low Control for each panel (6 vials in total) and line the vials up on ice with the caps nearby. Segregate the vials/caps with pellets that are on the side or under the caps. Using a repeating pipettor with a 500 μ L tip at maximum ejection speed, add 25 μ L of Sample Dilution Buffer (0 M) to each vial or cap with lyophilized pellet, then immediately add 25 μ L of Denaturation Buffer (6 M) to each vial or cap with lyophilized pellet and start a 10 min timer.

NOTE: It's important to label the caps of the vials to avoid mix-up and cross-contamination, e.g., "TS," "TH," and "TL" for Total Standard, Total High, and Total Low controls, respectively; "PS," "PH," and "PL" for the Phospho Standard, Phospho High, and Phospho Low controls, respectively.

- 7.7.4** Working rapidly in order to complete the step within 2–3 minutes, use a pipettor set to 40 μ L to address the misplaced lyophilized pellets: 1) dislodge any pellets stuck to the side of the tube by pipetting the denaturation solution over the pellet repeatedly, or mechanically dislodge with the pipet tip and dissolve the pellet by repeated pipetting up-and-down; 2) dissolve any pellets stuck to the underside of tube caps by repeated pipetting up-and-down and stirring with the pipet tip, then slowly transfer the cap contents to the matching tube. Be careful not to leave any contents behind in the tip or cap. If a pellet sticks to the pipet tip, continue to stir and pipet until it eventually dissolves. Replace the pipet tip after each Standard/Control and be careful not to create foam when pipetting.
- 7.7.5** Re-cap all vials and vortex on high speed for 5 sec, carefully invert capped tubes and vortex for 10 sec, turn tubes right-side-up and vortex an additional 5 sec, and finally spin down the tubes in a benchtop microcentrifuge for 5 sec to pool contents at bottom of tubes and then place the tubes back on ice. There are typically 4–5 minutes left on the denaturation timer at this point.
- 7.7.6** During the 10-minute incubation, prepare Urea/ Thiourea Sample Dilution Buffer (1 M) by combining 1 mL of Urea/ Thiourea Sample Dilution Buffer (1.5 M) with 500 μ L of Sample Dilution Buffer (0 M) in a 2 mL Sarstedt tube and place on ice.
- 7.7.7** Prepare a repeating pipettor with a 1 mL tip set to 150 μ L with maximum ejection speed. Approximately 30 sec before the 10 minute incubation is complete, remove caps from all the vials and set nearby. When the 10 minute incubation is completed add 150 μ L of Urea/ Thiourea Sample Dilution Buffer (1 M) to each vial. Recap the vials, vortex at high speed for 5 sec and place back on ice.
- 7.7.8** Prepare working standards with 1:2.5 serial dilutions starting with the 1X standards (T1, P1) according to the tables below using Urea/ Thiourea Sample Dilution Buffer (1.5 M). This will be sufficient for duplicate standard curves. Mix thoroughly by pipetting 10 times before proceeding with the next serial dilution. Change the pipet 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.

NOTE: The standard values below are only for demonstration purposes; the user must always use the lot-specific information included with each kit.

Total MEK/ERK Panel Standard	Total [ERK1] (ng/mL)	Total [ERK2] (ng/mL)	Total [MEK1] (ng/mL)	Total [MEK2] (ng/mL)	Vol. Std (μL)	Volume of USD (1.5 M) (μL)
T1	200	500	1500	125	Reconstituted T1	
T2	80.00	200.00	600.00	50.00	80 of T1	120
T3	32.00	80.00	240.00	20.00	80 of T2	120
T4	12.80	32.00	96.00	8.00	80 of T3	120
T5	5.12	12.80	38.40	3.20	80 of T4	120
T6	2.05	5.12	15.36	1.28	80 of T5	120
T7	0.82	2.05	6.14	0.51	80 of T6	120
T8	0.33	0.82	2.46	0.20	80 of T7	120

Phospho MEK/ERK Panel Standard	Phos [ERK1] (ng/mL)	Phos [ERK2] (ng/mL)	Phos [MEK1] (ng/mL)	Phos [MEK2] (ng/mL)	Vol. Std (μL)	Volume of USD (1.5 M) (μL)
P1	50	150	1000	100	Reconstituted P1	
P2	20.00	60.00	400.00	40.00	80 of P1	120
P3	8.00	24.00	160.00	16.00	80 of P2	120
P4	3.20	9.60	64.00	6.40	80 of P3	120
P5	1.28	3.84	25.60	2.56	80 of P4	120
P6	0.51	1.54	10.24	1.02	80 of P5	120
P7	0.20	0.61	4.10	0.41	80 of P6	120
P8	0.08	0.25	1.64	0.16	80 of P7	120

7.8 Assay Procedure

7.8.1 The Total and Phospho MEK/ERK Panels should always be performed at the same time for the best results.

7.8.2 The manufactured Total and Phospho MEK/ERK Panel kits are designed for running either 48 wells (half-plate) or 96 wells (full-plate) of a 96-well microplate per assay panel.

NOTE: If running half a plate of each panel, they must still be performed on separate microwell plates.

7.8.3 Add 40 μL/well of **Blank** (USD 1.5 M) 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 pipetting up-and-down 2x, then add 40 µL/well of panel-specific **Standards** (T1-T8 or P1-P8) 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 these kit reagents, 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 thawed, denatured/diluted **unknown samples** by pipetting up-and-down 4x, then add 40 µL/well **unknown samples** to the appropriate wells of the plates according to the plate maps using reverse pipetting. Since it is recommended that samples always be assayed in both the Total and Phospho MEK/ERK Panels at the same time, the sample well locations will typically be identical for each panel and therefore can be added to both plates at the same time with a multichannel pipettor. Visually confirm that equal volumes of sample are in each tip when using a multichannel pipettor, and replace tips between samples.
- 7.8.6** Vortex a vial of magnetic premixed microparticle cocktail (1x) for 5 sec, invert, 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 microparticle cocktail is identical for the Total and Phospho MEK/ERK Panels and can be added to each plate at the same time.
- 7.8.7** Apply adhesive plate sealers and cover the plates with light blocking black lids.
- 7.8.8** Incubate on a microplate shaker set at 850 ± 50 RPM overnight (16–18 h) at 4°C. Record the incubation start times 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. To reduce condensation on the shaker, it is helpful to keep one shaker at 4°C at all times.
- 7.8.9** After 16–18 h of incubation, prepare volumes of 1X Biotin-Ab Cocktail(s) for each panel that are sufficient to add 50 µL to each assay well, while allowing for 300 µL excess volume to account for dead volume in the repeating pipettor tips, by making a 1:100 dilution of panel-specific Human Premixed Biotin-Ab Cocktail (100X) in Detection Buffer for each panel. The table below provides 3 examples for plates of different sizes. Mix the 1X Biotin-Ab Cocktail(s) well with gentle inversion. Record the actual volumes used in the Batch Record ([Appendix 1, Section 7](#)).

# Wells	1X Biotin-Ab Vol. (mL)	100X Biotin-Ab Cocktail Vol. (µL)	Detection Buffer Vol. (mL)
48 (half plate)	2.7	27	2.673
72 (3/4 plate)	3.9	39	3.861
96 (full plate)	5.1	51	5.049

NOTE: The 100X Biotin-Ab Cocktail(s) are *panel-specific*. The detection buffer is identical for both panels.

- 7.8.10 Stop the shaker and record the stop times in the Batch Record ([Appendix 1, Section 6](#)). Place the Total MEK/ERK Panel plate in a plate washer with a 96-well flat magnet insert. Wait 1 minute to allow the beads to settle, 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 μ L of panel-specific 1X Biotin-Ab Cocktail solution to the required wells 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 MEK/ERK Panel plate.
- 7.8.13 Incubate both plates at 850 ± 50 RPM for 1 h at RT (typically around 20–21°C). Record the start times and room temperature in the Batch Record ([Appendix 1, Section 7](#)).
- 7.8.14 Turn on the Luminex instrument(s) to be used and perform system initialization. Ensure that the Calibration and Verification are up to date, the room temperature is within calibration range, and that there is sufficient sheath fluid. The Calibration and Verification should be performed according to the manufacturer’s protocol if needed, then prepare the batches in the instrument control software. Instructions for using xPONENT to control the instrument can be found in [Section 7.10](#), and instructions for using Bio-Plex Manager can be found in [Section 7.11](#). Note the completion of all instrument checks in the Batch Record ([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 MEK/ERK 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 Streptavidin-PE (1X) solution to the required wells with a repeating pipettor. Replace the plate seal and cover.

NOTE: Streptavidin-PE is identical for both panels.
- 7.8.17 Immediately repeat steps 7.8.15–7.8.16 for the Phospho MEK/ERK Panel plate.
- 7.8.18 Incubate both plates at 850 ± 50 RPM for 30 min at RT (typically around 20–21°C). 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 MEK/ERK 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. 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 Immediately repeat steps 7.8.19–7.8.20 for the Phospho MEK/ERK Panel plate.
- 7.8.22 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). Plates should be read within 2 hours after the final wash and resuspension step.

7.9 Plate Washer Settings

NOTE: The following instructions are given 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. Both plates can be read on a single instrument 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 Total and Phospho MEK/ERK 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 or Phospho MEK/ERK), 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 Total and Phospho MEK/ERK 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 or Phospho MEK/ERK) and enter/select the following settings:

Bead Type: **MagPlex**

Volume: **70 microliters**

DD Gating: **8000 to 16500**

Reporter Gain: **Default**

- 7.10.6** Click **Next** to open the **Select Analytes** tab. In the order ERK1, ERK2, MEK1, MEK2, click the **Analyte** region number and enter the name for each analyte as described in the lot-specific documentation (see below for 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 refer to the documentation included with each kit and select the specified analyte bead regions.

NOTE: The analytes must be entered in the specified order for use with the Excel MEK ERK Assay Data Analysis Template with Patient Reporting.

ERK1 (75)	ERK2 (13)	MEK1 (18)	MEK2 (22)
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- 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 lots of the Total and Phospho MEK/ERK Panels, e.g., “MEK ERK, T Lot 1594216, P Lot 1594215” 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 (see [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 has 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. ANY modifications will make the file 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, instead 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, both plates can be read on a single instrument 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 Total and Phospho MEK/ERK 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.






- 7.11.6** If running a new lot of Total and Phospho MEK/ERK Panel kits for the first time on the instrument PC, create a new protocol by selecting **File**, then **New** from the main menu. Click **Describe Protocol** and enter the lot # of each MEK/ERK Panel kit under **Description**, e.g., “MEK ERK, T Lot 1594216, P Lot 1594215”
- 7.11.7** Click **Select Analytes**, then click **Add Panel** and in the window that appears click **Add** to enter the region number and analyte name in the order ERK1, ERK2, MEK1, MEK2 with region numbers described in the lot-specific documentation (see below for example). Click **Add Continue** after each entry (or **Add** when entering the final analyte, MEK2).

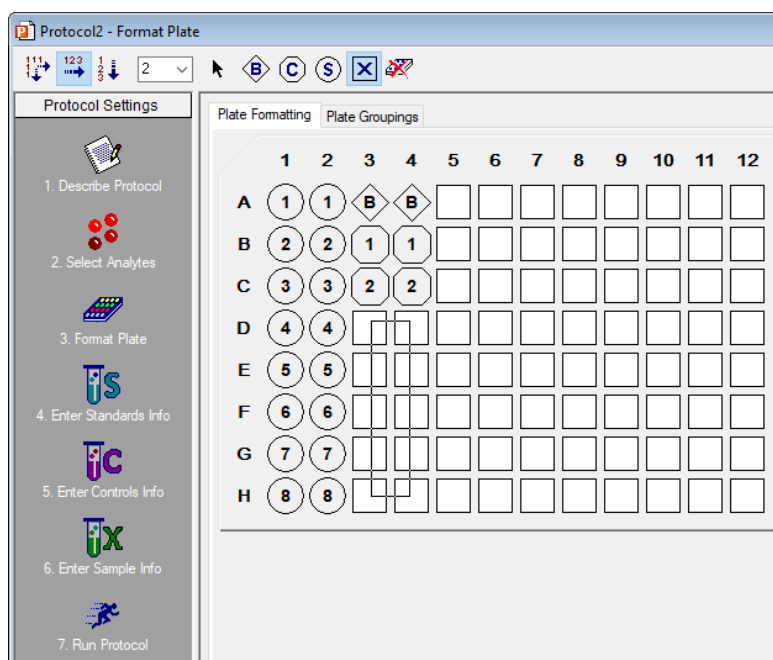
The bead region for each analyte is lot-specific: it is critical that the operator refer to the documentation included with each kit and select the specified analyte bead regions.

NOTE: The analytes must be entered in the specified order for use with the Excel MEK ERK Assay Data Analysis Template with Patient Reporting

ERK1 (75)	ERK2 (13)	MEK1 (18)	MEK2 (22)
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Enter a **Panel name** with the Total and Phospho MEK/ERK Panel Lot #s (e.g., “MEK ERK, T Lot 1594216, P Lot 1594215”) 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 like 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.
- 7.11.13** If the Standards info was not previously saved, 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 lot-specific product data sheet.
- 7.11.14** Make sure **S1** button is selected as **Most Concentrated** and **Apply dilution to all analytes** check box is checked.
- 7.11.15** Enter a dilution factor of 2.5 and click **Calculate**. The concentration for each standard point will be populated for all analytes in the table.
- 7.11.16** Enter the panel name and standard lot number in **Lot**, then enter the **Expiration Date**, and finally click the **Save** button.

If running a new lot of Total and Phospho MEK/ERK Panel kits for the first time on the instrument PC, click **File** then **Save** and save the protocol file (pbx) with the name “MEK ERK T-##### P-#####” where T- and P- specify the lot numbers of the Total and Phospho MEK/ERK Panels, respectively. This file will be used in future runs (see Step 7.11.5) for either Total or Phospho MEK/ERK Panel, but note that the Standards information must be matched to the panel (see Steps 7.11.12–7.11.16).

- 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.
- 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 8000 (low) and 16500 (high), and the check box **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 or Phospho MEK/ERK). 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** and 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 the 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....** 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, 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-4PL** or **Logistic-5PL** according to the regression type pre-determined for each kit lot.

NOTE: The regression type is specific to each kit lot and will be provided in the kit documentations or will need to be determined when validating a new kit lot.

7.12.6 In the **Report Table** screen, select **Multiple Analyte Layout**  and **Show Replicates** .

7.12.7 Export the results layout to Microsoft Excel by clicking the  icon, or, if using the Excel MEK/ERK 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
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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 both panels). Make sure “**Bioplex Data, Total**” and “**Bioplex Data, Phospho**” 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 MEK/ERK Panel; go to the “**Bioplex Data, Phospho**” sheet, click the A1 cell and paste (**Edit → Paste**) the results for Phospho MEK/ERK 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 Start Date, Run End Date, Panel Kit Type (Total or Phospho MEK/ERK), Panel Kit Lots, Purpose of Run, and 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 “varied” and enter the sample load manually for each sample in the “**Summary, Total**” and “**Summary, Phospho**” sheets.

7.12.12 Once all the data and information are entered, the “**Controls QC**,” “**Summary**,” “**Summary, Total**,” “**Summary, Phospho**,” “**Sample Report, Total**,” “**Sample Report, Phospho**” sheets should organize/analyze the data.

7.13 Assay Controls and Target Ranges

NOTE: Performance characteristics for each lot of assay 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 the tumor protein lysates are included with each 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 ranges 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 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 panels 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 panels 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 $\mu\text{g/mL}$ 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 run at 62.5 $\mu\text{g/mL}$ gives an analyte value exactly equal to $\frac{1}{2}$ of the value obtained when the same sample is assayed at 125 $\mu\text{g/mL}$, 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 $\mu\text{g/mL}$ has not been evaluated by the Clinical PD Biomarker Program. If it is desired to run samples at concentrations $>$ 250 $\mu\text{g/mL}$, 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 MEK/ERK Panel will be reported in pg analyte/μg protein from the “**Summary**” tab of the Excel MEK ERK Assay Data Analysis Template with Patient Reporting.
- 7.16.3** Results from the Phospho MEK/ERK Panel will be reported as the ratio of phospho protein:total protein from the “**Summary**” tab of the Excel MEK ERK 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 the 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: _____

Plate ID(s): _____

Protocol number(s): _____

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
Human Total MEK/ERK Panel Kit	/ /		/ /
Human Phospho MEK/ERK Panel Kit	/ /		/ /
Roche cComplete Mini, EDTA-free	/ /		/ /
Roche PhosSTOP	/ /		/ /

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

3. Initial Reagent Preparation:

i. Sample Dilution Buffer (0 M)

Add 1 tablet each of protease and phosphatase inhibitors to every 10 mL of Sample Dilution Buffer (0 M). Gently agitate until the tablets are dissolved and store on ice.

☐ cComplete Mini, EDTA-free added ☐ PhosSTOP added

ii. 1 M DDT

Add 50 µL of UltraPure DNase/RNase-free distilled water to each of 2 x 7.7 mg DTT tubes (Pierce “No-Weigh” DTT). Mix well by pipetting until dissolved.

☐ 50 µL UltraPure DNase/RNase-free distilled water added to each DDT tube

iii. Denaturation Buffer (6 M)

Add 761 mg of thiourea, 3.5 mL of 1X Tris Buffered Saline and 50 µL 1 M DTT to the lyophilized reagent bottle. Vortex for 30 sec, then shake at room temperature until dissolved. Do not put the Denaturation Buffer (6 M) on ice, or it will precipitate. **This solution must be made on the day it will be used.**

☐ 761 mg of thiourea added

☐ 3.5 mL of 1X Tris Buffered Saline added

☐ 50 µL 1 M DTT added

iv. Urea/ Thiourea Sample Dilution Buffer (1.5 M)

Add 385 mg thiourea, 9.5 mL of Sample Dilution Buffer (0 M) with inhibitors, and 25 μ L 1 M DTT to the lyophilized reagent bottle. Vortex for 10 sec, shake at room temperature until dissolved, then store on ice.

- ☐ 385 mg of thiourea added
- ☐ 9.5 mL of Sample Dilution Buffer (0 M) with inhibitors added
- ☐ 25 μ L 1 M DTT added

4. **Sample Preparation Information:**

- Unknown samples will be run in duplicate. Sample numbers correspond to those on the Plate Map Design. The initial suggested protein load for unknown samples is 125 μ g/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.
- Sample preparation requires four steps: (1) An initial dilution to normalize sample concentrations, (2) a denaturation step, (3) dilution to the final concentration, and (4) one freeze/thaw cycle. The final concentration of urea/thiourea in all diluted samples must be 1.5 M.
- Prepare Standards, Controls, and unknown samples according to the SOP. If the unknown samples were prepared and stored on a different day, record that date below.

Date of sample preparation and storage: _____ (if not today)

Freezer ID and location for sample storage: _____

5. **Sample Preparation Information Table:**

Use the Excel MEK ERK Assay Setup Template to calculate the sample dilution and denaturation volumes. Copy and paste the table data on the next page:

(PASTE THE RESULTS FROM MEK/ERK 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: Protein Concentration represents tumor protein stock lysate prepared following SOP341210.**

6. Initial 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 a black lid to protect the assay wells from light.
- Incubate on shaker at 850 ± 50 RPM overnight (16–18 h) at 4°C . Record start and stop times:

MEK/ERK Panel	Start Time	Stop Time
Total	:	:
Phospho	:	:

7. Prepare 1X Biotin-Ab Cocktail(s) and Add to Plate(s)

- Prepare 1X biotin-Ab cocktails according to the SOP. Record volumes used:

MEK/ERK Panel	1X Biotin-Ab Vol. (mL)	100X Biotin-Ab Cocktail Vol. (μL)	Detection Buffer Vol. (mL)
Total			
Phospho			

- After the 16–18 h incubation, wash plates and add 50 μL/well of panel-specific 1X Biotin-Ab cocktail according to the Plate Maps and SOP. Seal, cover, and incubate at 850 ± 50 RPM, for **1 h** at RT. Record the room temperature, start and stop times:

MEK/ERK Panel	Start Time	Stop Time
Total	:	:
Phospho	:	:

Room temperature: _____

8. Prepare Luminex Instruments

- Prepare Luminex instruments during the Biotin-Ab 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 µL/well of Streptavidin-PE (1X) according to the Plate Maps.
- Incubate on shaker at 850 ± 50 RPM for **30 min** at RT. Record start and stop times:

MEK/ERK Panel	Start Time	Stop Time
Total	:	:
Phospho	:	:

10. Reading the Plate

- After the 30 min incubation, wash plates and add 100 µL 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 start times below:

MEK/ERK Panel	Instrument #	Last Calibration	Reading Start Time
Total			:
Phospho			:

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 MEK/ERK 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 MEK/ERK 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 MEK/ERK Panel Plate Map Example:

	1	2	3	4	5	6	7	8	9	10	11	12
A	P1	P1	BL	BL	X6	X6	X14	X14	X22	X22	X30	X30
B	P2	P2	C1	C1	X7	X7	X15	X15	X23	X23	X31	X31
C	P3	P3	C2	C2	X8	X8	X16	X16	X24	X24	X32	X32
D	P4	P4	X1	X1	X9	X9	X17	X17	X25	X25	X33	X33
E	P5	P5	X2	X2	X10	X10	X18	X18	X26	X26	X34	X34
F	P6	P6	X3	X3	X11	X11	X19	X19	X27	X27	X35	X35
G	P7	P7	X4	X4	X12	X12	X20	X20	X28	X28	X36	X36
H	P8	P8	X5	X5	X13	X13	X21	X21	X29	X29	X37	X37

Empty Phospho MEK/ERK Panel Plate Map:

	1	2	3	4	5	6	7	8	9	10	11	12
A	P1	P1	BL	BL								
B	P2	P2	C1	C1								
C	P3	P3	C2	C2								
D	P4	P4										
E	P5	P5										
F	P6	P6										
G	P7	P7										
H	P8	P8										

PLATE MAP: INITIALS _____ DATE: _____

APPENDIX 3: CLINICAL SAMPLE DATA REPORT

Total and Phospho MEK/ERK 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 MEK/ERK Panel Analytes		Baseline	Post-Dose	QC Notes
		(pg analyte/μg protein)		
Total MEK1				
Total MEK2				
Total ERK1				
Total ERK2				
Phospho MEK/ERK Panel Analytes		Baseline	Post-Dose	QC Notes
		Ratio		
pS218/222-MEK1:Total MEK1				
pS222/226-MEK2:Total MEK2				
pT202/Y204-ERK1:Total ERK1				
pT185/Y187-ERK2:Total ERK2				

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: ASSAY BUFFER RECIPES

NOTE: Always use buffers from the manufactured kits; the recipes are listed here for reference and should only be made from scratch if absolutely necessary. Record details of any supplemental buffer preparation in the Batch Record ([Appendix 1, Section 11](#)).

TBS is 100 mM Tris-HCl, 150 mM NaCl, pH 7.8

10X PBS is 10.59 mM KH₂PO₄, 29.66 mM Na₂HPO₄·7H₂O, 1552 mM NaCl, pH 7.4

1. Wash Buffer: Make fresh daily.

Reagent	Stock	Final	Per Liter
PBS	10X	1X	100 mL
Tween-20	10%	0.05%	5 mL
ProClin 300	100%	0.05%	0.5 mL
Water	--	--	894.5 mL

2. Sample Dilution Buffer (0 M): Store at -20°C in 10 mL aliquots. Stable for ≥ 1 month.

Reagent	Stock	Final	Per 10 mL
TBS	--	--	9 mL
Triton X-100	10%	1%	1 mL
BSA	--	1%	100 mg
EDTA	0.5 M	1 mM	20 µL
cOmplete	1 Tablet	--	1 ea
PhosStop	1 Tablet	--	1 ea

3. Denaturation Buffer (DB, 6M): Must be made fresh each day.

Reagent	Stock	Final	Per 5 mL
TBS	--	--	3.5 mL
Urea	--	4 M	1.2 g
Thiourea	--	2 M	0.761 g
DTT	1 M	10 mM	50 µL

4. Urea/ Thiourea Sample Dilution Buffer USD (1.5 M): Must be made fresh each day.

Reagent	Stock	Final	Per 10 mL
Sample Dilution Buffer (0M)	--	--	9.5 mL
Urea	--	1 M	0.6 g
Thiourea	--	0.5 M	0.385 g
DTT	1 M	2.5 mM	25 µL

5. Detection Buffer: Store at -20°C in 5 mL aliquots. Stable for ≥ 1 month.

Reagent	Stock	Final	Per 10 mL
TBS	--	--	9.6 mL
BSA	--	1%	100 mg
FBS	100%	4%	0.4 mL

6. SAPE Buffer: Store at -20°C in 5 mL aliquots. Stable for ≥ 1 month.

Reagent	Stock	Final	Per 10 mL
TBS	--	--	10 mL
BSA	--	1%	100 mg
SDS	20%	0.02%	10 µL
Tween 20	10%	0.025%	25 µL