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National Clinical Target Validation Laboratory (NCTVL)

Applied Developmental Directorate, Leidos Biomedical Research, Inc.

Frederick National Laboratory for Cancer Research

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DCTD OD Approval: Toby Hecht TTH Date: 8/10/16

Change History

Revision	Approval Date	Description	Originator	Approval
--	6/4/2012	New Document.	AKS, YAE	AKS
A	2/7/2014	Defined minimal lysate concentration. Expanded description for clinical dilution preparation; preparation changed to only prepare 2 different dilutions for each clinical specimen. MET readout changed from pM/µg to pmol/µg. Defined room temperature.	YAE, KFG	AKS
B	7/5/2016	Removed reference to pY1235 MET IA, changes to critical reagent list, change to recommended initial protein loading, other minor edits in preparation for community transfer.	KFG	AKS

Please check for revision status of the SOP at

http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm

and be sure to use the current version.

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OVERVIEW OF MET IMMUNOASSAY SAMPLE PROCESSING

SOP340507:
Tumor Frozen Needle Biopsy Specimen Collection and Handling

- Collect and flash-freeze fresh tumor needle biopsies within 2 min
- Immediately place in liquid nitrogen or on dry ice/ethanol

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SOP341201: Preparation of Tumor Biopsy Lysates for MET Immunoassay(s)

- Extract protein from tumor biopsy
- Determine protein concentration
- Store stock lysate or immediately proceed to immunoassay

↓

SOP341203:
Intact MET Immunoassay

- Perform ELISA with clinical samples, standards, and controls
- Using Tecan Microplate reader, determine relative signal of all samples

→ Only samples with a readout of **≥ 200 fmol/mg from the Intact MET Immunoassay**, should be assessed with the phospho-MET immunoassay

↓

SOP341206:
Dual Phospho-Y1234/Y1235 MET Immunoassay

SOP341205:
Phospho-Y1356 MET Immunoassay

- Purpose: to measure increased pMET or target inhibition in overexpressed/amplified disease conditions
- Perform ELISA with clinical samples, standards, and controls
- Using Tecan Microplate reader, determine relative signal of all samples

Listed in recommended order. Mono-pMET assays is only run if dual-pMET assay is positive.

↓

SOP341208: MET Immunoassay(s) Quality Control, Data Analyses, and Reporting

- Determine the MET concentration in all samples and apply quality control standards to verify utility of assay
- Prepare a Clinical Sample Data Report for each set of unknown samples and send to the clinical protocol Principal Investigator

1.0 PURPOSE

Standardize an enzyme-linked immunosorbent assay (ELISA) method for quantifying increases in dual phosphorylated MET levels (phospho-Y1234 and/or -Y1235), or target inhibition in overexpressed/ amplified disease conditions, as a pharmacodynamic (PD) measure of chemotherapeutic agents.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the dual phospho-Y1234/Y1235 MET Immunoassay during clinical trials. A baseline concentration of 200 fmol/mg intact MET is needed to measure phospho-MET in tumor biopsy extracts at baseline. This minimal intact MET requirement is based on preclinical studies indicating that 50% to 60% (100 - 120 fmol/mg) of total MET is phosphorylated and the sensitivity of the phospho-MET assays. The goal of the SOP and associated training is to ensure consistency of dual phospho-Y1234/Y1235 MET measurement between clinical sites.

3.0 ABBREVIATIONS

C	=	Control
Capture Antibody Coated Strips	=	Nunc Maxisorp 8-well white strips, pre-coated with affinity-purifiedMET goat pAb
DCTD	=	Division of Cancer Treatment and Diagnosis
Dual Phospho-Y1234/Y1235	=	Phosphorylated Tyrosine 1234 and/or Tyrosine 1235 on the MET Protein
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
HGF	=	Hepatocyte Growth Factor
HRP	=	Horse Radish Peroxidase
IA	=	Immunoassay
LHTP	=	Laboratory of Human Toxicology and Pharmacology
MET goat pAb	=	Human HGF R/c-MET antibody; affinity-purified polyclonal goat IgG
NCTVL	=	National Clinical Target Validation Laboratory
pAb	=	Polyclonal antibody
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PBS	=	Phosphate Buffered Saline
PBS-Casein	=	Phosphate Buffered Saline/Casein Block and Diluent
PI	=	Protease Inhibitor(s)
PD	=	Pharmacodynamic
RLU	=	Relative Light Units
SOP	=	Standard Operating Procedure
SPB	=	Sample Preparation Buffer
Temp	=	Temperature

4.0 INTRODUCTION

The Dual Phospho-Y1234/Y1235 MET Immunoassay has been developed to measure chemotherapeutic effects resulting in increased pMET levels or target inhibition in overexpressed/amplified disease conditions in tumor biopsy samples. The amount of intact MET measured in SOP341203 (Intact MET Immunoassay) will serve as a denominator reading to determine the fraction of dual phospho-Y1234/Y1235 MET in the samples. An ELISA is used to first capture MET from total cell extracts on plates coated with a purified goat polyclonal antibody reactive to the extracellular domain of MET. The captured protein is then detected using a biotin conjugated rabbit monoclonal antibody against dual phospho-Y1234/Y1235 MET followed by addition of a poly-HRP conjugate to allow chemiluminescent readout and quantitation of dual phospho-Y1234/Y1235 MET levels.

5.0 ROLES AND RESPONSIBILITIES

Laboratory Director/Supervisor	The Laboratory Director/Supervisor, directs laboratory operations, supervises technical personnel and reporting of findings, and is responsible for the proper performance of all laboratory procedures. Oversees the personnel running SOPs within the laboratory and is responsible for ensuring 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 and reports to the Laboratory Director/Supervisor. This person performs laboratory procedures and examinations in accordance with the current SOP(s), as well as any other procedures conducted by a laboratory, including maintaining equipment and records and performing quality assurance activities related to performance.

5.1 It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented DCTD training and qualification on this SOP prior to the actual handling and processing of samples from clinical trial patients. The Laboratory Director/Supervisor is responsible for ensuring the Certified Assay Operator running the SOP has sufficient experience to handle and analyze clinical samples.

5.2 The Certified Assay Operator responsible for conducting the assay is to follow this SOP and complete the required tasks and associated documentation. The Plate Map Design (Appendix 1) and Batch Record (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 Digital versions of the sample table in the Batch Record (Appendix 2, Section 3) can be created for logging sample information as long as all column information exactly matches the table in the Batch Record. A copy of the completed, digital sample tables must be printed and attached to the Batch Record in order to maintain a complete audit trail.

5.4 All responsible personnel are to check the DCTD Biomarkers web site (http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm) to verify that the most recent SOP version of the SOP for the assay is being used.

6.0 CRITICAL REAGENTS, MATERIALS, AND EQUIPMENT REQUIRED

6.1 PADIS/IQC-supplied Critical Reagents:

- **6.1.1** Capture Antibody Coated Strips; 12 Capture Antibody Coated Strips per 96-well frame
- **6.1.2** MET standards (concentration provided by lot number)
- **6.1.3** Tumor Lysate Control samples, 50 µg/mL (custom preparations of Low, Mid, and High MET level controls)
- **6.1.4** Biotin-conjugated phospho-Y1234/Y1235 MET rabbit monoclonal antibody, clone D26 (custom conjugated preparation of Cell Signaling, Cat#: 3077)
- **6.1.5** Streptavidin poly-HRP conjugate, 0.5 mg/mL (500 µg/mL; Thermo Scientific Pierce, Cat#: 21140)
- **6.1.6** SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific Pierce, Cat#: 37070)
- **6.1.7** Phosphate Buffered Saline/Casein Block and Diluent, 5X concentrate (5X PBS-Casein; BioFx, Cat#: PBSC-1000-01)

6.2 Tween 20 non-ionic, aqueous solution, 10% w/v (Roche Applied Science, Cat#: 11332465001) or Surfact-Amps Purified Detergent Solution, 10% (w/v) (Thermo Scientific, Cat# 28320)
6.3 PhosSTOP, phosphatase inhibitor tablets (Roche Applied Science, Cat#: 04906837001)
6.4 Pipettors (200-1000 µL and 10-100 µL) and tips
6.5 Multichannel pipettor (30-300 µL) and tips
6.6 Reagent reservoirs (Fisher Scientific, Cat#: 21-381-27C)
6.7 Nunc Maxisorp Immuno-Module, 8-well white strips, framed (Thermo Scientific, Cat#: 437591)
6.8 2.0-mL Sarstedt o-ring screw cap, skirted tubes (Fisher Scientific, Cat#: 72.694.006)
6.9 15-mL polypropylene tubes (e.g., Fisher Scientific, Cat#: 14-959-49B)
6.10 50-mL polypropylene tube (e.g., Becton Dickinson, Cat#: 352098)
6.11 Adhesive plate sealers (Edge BioSystems, Cat#: 48461)
6.12 Ice bucket
6.13 UltraPure DNase⁄RNase-free distilled water (e.g., Invitrogen, Cat#: 10977-015)
6.14 BioStab Antibody Stabilizer (Sigma-Aldrich, Cat#: 55514)
6.15 Triton X-100, non-ionic, aqueous solution, 10% w/v, stored according to manufacturer's direction (Roche Applied Science, Cat#: 11332481001)
6.16 10X Phosphate Buffered Saline, pH 7.2 (PBS; Invitrogen, Cat#: 70013-072)
6.17 Convertible Tabletop Impulse Sealer, SECO (VWR International, Cat#: 58606-008)
6.18 Sorvall Fresco centrifuge, refrigerated (Fisher Scientific)
6.19 BioTek ELx405 Select, ELx405 Select CW or ELx405 Microplate Washer (BioTek Instruments)
6.20 Vortex mixer, digital, 500-3000 rpm (Fisher Scientific, Cat#: 02-215-370)
6.21 Orbital microplate shaker with temperature control (VWR International, Cat#: 12620-930); able to maintain 25°C (± 3°C)
6.22 Non-humidified, fixed temperature incubator able to maintain 25°C (± 3°C)
6.23 Infinite® 200 Microplate Reader, Infinite M200 Pro Microplate Reader or Tecan Genios Pro (same instrument settings; Tecan US)
6.24 -20°C and -80°C freezer
6.25 4°C refrigerator
6.26 Microsoft Excel 2003, 2007, or 2010
6.27 Tumor biopsy stock protein lysates processed following SOP341201 (Preparation of Tumor Biopsy Lysates for MET Immunoassay)

7.0 OPERATING PROCEDURES

Important: Only samples with ≥ **200 fmol/mg <u>intact MET</u>** will be assayed in the phospho-MET immunoassays.

7.1 Prior to beginning the assay, refer to the Plate Map Design and Batch Record to review all actions required for successful assay setup (Appendices 1 and 2).

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

7.3 Critical Reagents

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

- Storage conditions and expiration dates for all Critical Reagents are provided on the package insert.
- Do not exchange reagents from one set of qualified Critical Reagents with a set of reagents qualified separately.

7.3.2 Record the date of receipt, lot numbers, stock/supplied reagent concentration, recommended working dilution/concentration, and expiration dates for the Critical Reagents in the Batch Record (Appendix 2, Section 1).

7.3.2.1 **Capture Antibody Coated Strips**: Nunc Maxisorp 8-well white strips adsorbed with affinity-purified MET goat pAb provided in a 96-well format in sealed foil pouches.

7.3.2.2 **MET Standards:** Affinity purified-recombinant wild type MET protein from 293E cells in Sample Preparation Buffer (SPB).

7.3.2.3 **Tumor Lysate Controls (C):** 50 µg/mL stock xenograft extracts prepared in a buffered solution containing PhosSTOP and protease inhibitors.

7.3.2.4 **D26-Biotin Conjugate**: PADIS/IQC custom antibody stock solution. Biotin-conjugated phospho-Y1234/Y1235 MET rabbit monoclonal antibody, clone D26.

7.3.2.5 **Streptavidin Poly-HRP conjugate**: Supplied as a 0.5 mg/mL (500 µg/mL) stock solution.

7.3.2.6 **PBS-Casein Block and Diluent (PBS-Casein):** 5X stock solution qualified from the manufacturer. Use to prepare Phospho-Assay Buffer and Sample Preparation Buffer (SPB) as instructed in Appendix 2, Section 2B.

7.3.2.7 **Chemiluminescent Substrate Solutions**: Stock solutions (Peroxide and Pico Luminol/Enhancer Solutions) qualified from the manufacturer. Protect from light.

7.4 Plate Map and Buffer Preparation

7.4.1 Based on the number of patient samples to be analyzed, generate a Plate Map (Appendix 1) to define the location and replicates of unknown samples, tumor controls, and MET standards. A single patient's **batched** samples should be contained in one 96-well plate, not split over two, to ensure consistent sample handling. A minimum of 8 Capture Antibody Coated Strips will be used per experiment to accommodate the standards, controls, and a minimum of one patient's samples.

Important: The data analysis template (SOP341208) is based on the well designations in the Plate Map (Appendix 1). To prevent user errors, load the plate according to the Plate Map well designations.

7.4.2 Once the number of wells is known, determine the amount of reagents required for the assay using the Batch Record in Appendix 2. Once these calculations are complete, check that sufficient reagents and supplies are on hand to complete the assay.

7.4.3 Record the make, model, and serial numbers of equipment in the Batch Record (Appendix 2, Section 2A) and prepare the buffers outlined in the Batch Record (Appendix 2, Section 2B).

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

7.5 Plate Preparation

7.5.1 Take out a foil ziplock pouch containing Capture Antibody Coated Strips from the refrigerator and let come to ambient temperature for 30 min before opening the pouch. Strips are stored as a 96-well frame with 12 capture antibody coated 8-well strips per frame.

7.5.2 Cut open the pouch just below the heat seal and be sure the color of the desiccant capsule is blue. If desiccant has turned pink, discard strips and use a new pouch as there has been air leakage into the pouch.

7.5.3 If fewer than 12 Capture Antibody Coated Strips are needed according to the Plate Map, remove the unneeded coated strips from the 96-well frame and replace them with uncoated Maxisorp 8-well white strip. The strip in column 12 of the Plate Map (Appendix 1) should be replaced with an uncoated Maxisorp 8-well white strip.

7.5.3.1 Place any unused coated strips back in the foil ziplock pouch containing the desiccant capsule and close the pouch. When resealing the foil ziplock pouch, be sure to remove as much air as possible.

7.5.3.2 Seal the foil ziplock pouch with an Impulse-Sealer set on medium heat, or setting of 4, until indicator light turns off (use manufacturer's instructions).

7.5.3.3 Record the number of remaining coated strips on the package and return to 2ºC to 8°C. Strips in resealed pouches can be used for up to 6 mo from the preparation date if stored under these conditions.

7.5.4 In case strips accidentally get removed from the frame, use a marker to label each Capture Antibody Coated Strip with the corresponding column number from the Plate Map (Appendix 1).

7.6 Preparation of MET Standards, Unknown Sample Lysates, and Tumor Controls

7.6.1 Preparation of MET standards, run in triplicate

7.6.1.1 Place the MET standard stock solution in a water/ice bath to thaw, vortex briefly to mix. Label eight 1.5-mL Sarstedt tubes, numbered 1 through 8, for the MET standards. Prepare all standards on ice.

7.6.1.2 Use the calculations in the Batch Record (Appendix 2, Section 3A) to prepare 1 mL of the 40 pM standard in tube #1.

7.6.1.3 Prepare the remaining standards by serial dilution as outlined with final concentrations of 20 to 0.31 pM (tube #2-8) in Sample Preparation Buffer (SPB). Once thawed, keep standards on ice and use within 2 h.

7.6.2 Unknown biopsy sample lysates, run in triplicate

7.6.2.1 Place the unknown sample stock lysates on ice to thaw. Record the time samples are removed from freezer, the sample/patient IDs and stock lysate concentration in the Batch Record (Appendix 2, Section 3B). Each unknown biopsy lysate will take up 2 sample spots (e.g., S1 and S2). Once thawed, keep lysates on ice and use within 1 h.

7.6.2.2 For each unknown sample, prepare two different dilutions (50 and 100 µg/mL) in SPB for analysis using the directions in the Batch Record (Appendix 2, Section 3B). While each well will have a 100 µL loading volume, S1 triplicate wells will hold 50 µg/mL and S2 100 µg/mL total protein in each well.

- Wells should never contain > 200 µg/mL total protein in the MET Immunoassays.
- Samples with total protein concentration of < **250 µg/mL** (0.25 µg/µL) should not be used in the MET Immunoassays and will be reported as unanalyzable in the Clinical Sample Data Report.

7.6.2.3 Record the volume stock lysate and SPB used as well as the final concentration of each **diluted lysate** in Appendix 2, Section 3B. Clearly label 1.5-mL tubes with the sample number (e.g., S1, S2, etc.). Excess **diluted lysate** will be discarded.

7.6.2.4 For each sample number (S1, S2, etc.), verify that the tube number matches the actual protein concentration (50 and 100 µg/mL) of tumor lysate in the Batch Record (Appendix 2, Section 3B).

7.6.3 Preparation of tumor lysate control samples, run twice on plate in duplicate.

7.6.3.1 For one 96-well plate, retrieve one each High-, Mid-, and Low-Control (C) tumor lysate control vials from the -80°C freezer. Place in a water/ice bath to thaw and label a 1.5-mL Sarstedt tube for each diluted control sample. Thawing may take 30-45 min.

7.6.3.2 Use the dilution scheme in the Batch Record (Appendix 2, Section 3C) to prepare a 1:5 dilution of each control stock solution in SPB and label the tubes accordingly. Once thawed, keep controls on ice and use within 1 h. Excess control lysate will be discarded.

7.7 MET Protein Capture

7.7.1 Using the Plate Map Design (Appendix 1) and the Sample Calculation Table (Appendix 2, Section 3B) as a guide, add 100 µL of each sample into the corresponding well of the 96-well plate. Samples prepared include MET standards (SOP Step 7.6.1), unknown samples (SOP Step 7.6.2), and control samples (SOP Step 7.6.3).

Order	Sample/Reagent and Volume
1	100 µL of specified concentrations of MET standards into designated triplicate wells. Load the lowest concentration first.
2	100 µL of each unknown sample into designated triplicate wells (S1-S12)
3	100 µL each of tumor controls (Low-C, Mid-C, and High-C) into both sets of designated duplicate wells
4	100 µL of SPB into each of the designated background wells

7.7.2 Cover the plate with an adhesive seal and incubate for 1 h ± 5 min with 650 rpm shaking on the orbital microplate shaker with the temperature set at 25°C (± 3°C). Record the incubation conditions in the Batch Record (Appendix 2, Section 4).

7.8 Dual Phospho-Y1234/Y1235 MET Detection

7.8.1 Using the calculations in Appendix 2, Sections 5A, prepare a sufficient amount of D26-biotin conjugate working solution in Phospho-Assay Buffer ≤ 30 min before washing the plate (next step) that has been incubating with samples. D26-biotin conjugate working solution should be stored in a fixed temperature incubator at 25°C and used within 30 min of preparation.

7.8.2 After the 1-h plate incubation is complete, aspirate the plate and wash the wells 4 times with 350 µL 1X Wash Buffer per well. Record the time samples were removed from the wells in the Batch Record (Appendix 2, Section 4).

For the ELx405, ELx405 Select and ELx405 Select CW Microplate Washers, the settings are:

METHOD	
Number of Cycles:	**4**
Soak/Shake:	**No**
DISPENSE	
Dispense Volume:	**350 µL/well**
Dispense Flow Rate:	**06**
Dispense Height:	**120 (15.240 mm)**
Horizontal DISP POS:	**00 (0.000 mm)**
Bottom Wash First:	**No**
Prime Before Start:	**No**
ASPIRATE	
Aspirate Height:	**031 (3.937 mm)***
Horizontal ASPR POS:	**-40 (-1.829 mm)***
Aspiration Rate:	**05 (6.4 mm/sec)**
Aspirate Delay:	**0000 MSec**
Crosswise ASPIR:	**No**
Final Aspiration:	**Yes**
Final Aspirate Delay:	**1000 MSec**

*Recommended initial setting, adjust Aspirate Height and Horizontal ASPR Pos to optimize complete aspiration for an individual unit and plate type following the manufacturer's recommendations.

7.8.3 After the wash, tap the plate on a paper towel to remove residual Wash Buffer. Proceed immediately to the next step; do not allow the plate to dry out.

7.8.4 Add 100 µL of the D26-biotin conjugate working solution per well using a multichannel pipettor, cover the plate with an adhesive seal, and incubate for 1 h ± 5 min with 650 rpm shaking on the orbital microplate shaker with the temperature set at 25°C. Discard residual working solution and record the incubation conditions in the Batch Record (Appendix 2, Section 5B).

7.8.5 Just before the 1-h incubation with D26-biotin conjugate working solution is complete, prepare a sufficient amount of streptavidin poly-HRP conjugate for the assay. See Appendix 2, Section 6A for conjugate preparation and record the time the conjugate is prepared. Poly-HRP conjugate can be stored at 25°C (± 3°C) for up to 30 ± 5 min before use.

7.8.6 After the 1-h D26-biotin conjugate incubation is complete, aspirate and wash the wells 4 times with 350 µL of 1X Wash Buffer (same wash program as SOP Step 7.8.2). Tap plate on a paper towel to remove residual liquid and proceed immediately to the next step.

7.8.7 Add 100 µL of streptavidin poly-HRP conjugate per well using a multichannel pipettor. Cover the plate with an adhesive seal and incubate for 30 ± 5 min with 650 rpm shaking on the orbital microplate shaker with the temperature set at 25°C. Record the incubation conditions in the Batch Record (Appendix 2, Section 6B).

7.9 Signal Detection

7.9.1 Turn on the Tecan Microplate Reader at least 30 min before use. For chemiluminescence readings, the plate reader should be set to the following reading parameters:

Shaking duration:	**5 sec**
Mode:	**linear**
Amplitude:	**1 mm**
Attenuation:	**OD1**
Integration Time:	**100 ms**
Settling Time:	**60 ms**

7.9.2 Just before the streptavidin poly-HRP conjugate incubation is finished, prepare the Chemiluminescent Substrate as outlined in the Batch Record, be sure to note the time of preparation (Appendix 2, Section 7A). This must be made up immediately before use, kept at RT in the dark, and at a sufficient volume for the assay.

7.9.3 After the 30-min poly-HRP conjugate incubation is complete, aspirate and wash the wells 4 times with 350 µL of 1X Wash Buffer (same wash program as SOP Step 7.8.2). Tap plate on a paper towel to remove residual liquid and proceed immediately to the next step.

7.9.4 Add 100 µL of the freshly made Chemiluminescent Substrate to each well using a multichannel pipettor. Cover the plate with an opaque lid to protect it from light and place on the shaker for 1 min with shaking at 650 rpm and temperature set to 25°C. Record the time of addition to wells (Appendix 2, Section 7B).

7.9.5 Luminescence reading should be done within 15 min of substrate addition. Record the time of the relative light unit (RLU) reading in the Batch Record (Appendix 2, Section 7B).

- If the signal is too high (no read-out, invalid read-out), report sample as "out of range - high."

7.9.6 Save the resulting readings in an Excel file to a secure computer; recommended to label with a unique file name (e.g., SOP number + "Tecan" + run date + unique plate identifier). Print a paper copy of the raw data for inclusion with the Batch Record.

7.10 Review and finalize the Batch Records (Appendix 2) and obtain required signatures. Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 2, Section 8).

7.11 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 2, Section 9).

7.12 Proceed to SOP341208 for Quality Control and Data Analyses and preparation of the Sample Data Report to send to the clinical protocol Principal Investigator.

APPENDIX 1: PLATE MAP DESIGN

	1	2	3	4	5	6	7	8	9	10	11	12*
A	SPB Only †				0.31 pM			SPB Only				
B	High-C	S1	S3	S5	0.63 pM			S7	S9	S11	Low-C	
C					1.25 pM							
D	Medium-C				2.5 pM						Medium-C	
E		S2	S4	S6	5.0 pM			S8	S10	S12		
F	Low-C				10.0 pM						High-C	
G					20.0 pM							
H	SPB Only				40.0 pM			SPB Only				
	Control Samples	Unknown Samples (S), Triplicate			MET Standards, Triplicate			Unknown Samples (S), Triplicate			Control Samples	

* The strip in column 12 of the Plate Map should be replaced with an uncoated Maxisorp 8-well white strip.

† RLU readings from the 4 corner background wells and wells adjacent to the highest standard will not be used to determine background variability.

- Each numbered column represents a single 8-well Capture Antibody Coated Strips; alternating colored column headers indicate each strip.
- Background control wells are loaded with Sample Preparation Buffer (SPB) only (no sample).
- S1 through S12 are unknown sample wells in triplicate, grouped by column. This allows for up to 6 tumor biopsy samples (3 paired samples) to be analyzed per plate at 2 protein dilutions.

  Important: The Plate Map design and sample well designation, illustrated above are assumed for the proper format of the Tecan output data file that will be analyzed (described in SOP341208: MET Quality Control and Data Analysis). If an alternate Plate Map is used or fewer unknown samples are run, manual adjustment of the Tecan data file will be required, as outlined in the SOP.

  Note: If samples S7-S12 are not being run, the column 11 control lysate strip can be run in column 8; manual adjustments to the Tecan output data will be required in SOP341208.

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APPENDIX 2: BATCH RECORD

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

Certified Assay Operator: _____

Certification Number: _____

Facility/Laboratory Running SOP: _____

Plate ID (optional): _____

1. Critical Reagents

Be sure the lot numbers and recommended working solution concentrations on each of the reagents match those cited in the product insert accompanying the reagents. Reagents from one pack **should not** be exchanged with reagents from another pack.

Reagent Name	Date Received	Lot Number	Provided Reagent	Recommended Dilution/Conc. for Working Solution	Expiration Date
Capture Antibody Coated Strips	/ /		N/A	N/A	/ /
MET Standards	/ /		pM	40 pM	/ /
Tumor Lysate Controls	/ /		50 µg/mL	10 µg/mL	/ /
D26-Biotin Conjugate	/ /		100 µg/mL	µg/mL	/ /
Poly-HRP Conjugate	/ /		500 µg/mL	µg/mL	/ /
5X PBS-Casein	/ /		N/A	N/A	/ /
Chemiluminescent Substrate	/ /		N/A	N/A	/ /

2. Equipment and Preparation of Reagents

A. Equipment

BioTek Plate Washer: Make/Model: _____

Serial #: _____

Microplate Reader Make/Model: _____

Serial #: _____

BATCH RECORD: INITIALS _____ DATE: _____

B. Buffers

Sufficient buffer volume should be prepared to complete all steps for all 96-well plates being prepared in the experimental run. Always prepare at least 10% excess volume of buffer to ensure adequate volume to complete the run.

a. 1X Wash Buffer: To prepare 1 L of buffer, pipette 100 mL 10X PBS and 10 mL 10% Tween 20 (0.1% final) into 890 mL UltraPure DNase/RNase-free distilled water. Store at ambient temperature for up to 5 d.

Reagent	Source	Lot Number	Expiration Date
10% Tween 20 or Surfact-Amps Purified Detergent Solution			

b. Phospho-Assay Buffer: In a 50-mL polypropylene tube, add 5 mL 5X PBS-Casein and 25 µL 10% Triton X-100 (0.01% final) to 20 mL UltraPure DNase/RNase-free distilled water. Make fresh for each assay and keep at RT.

c. Sample Preparation Buffer (SPB): In a 15-mL polypropylene tube, dissolve a PhosSTOP tablet in 7.9 mL UltraPure DNase/RNase-free distilled water and then add 2 mL 5X PBS-Casein and 100 µL 10% Triton X-100 (0.1% final). SPB for use with unknown samples, standards and controls should be kept on ice (2-8°C). If made fresh, chill on ice for at least 20 min before use. Can be stored at -20°C for up to 1 mo, thaw on ice before use.

Reagent	Lot Number	Expiration Date
PhosSTOP, phosphatase inhibitor cocktail tablets		

3. Preparation of MET Standards (A) and Unknown Samples (B)

A. Recombinant MET Standards (SOP Step 7.6.1)

Calculations for preparation of 40 pM MET standard in 1 mL from provided **STOCK** solution. e.g., PADIS/IQC-supplied MET standard **STOCK** Lot#: 090710-S2 is supplied at 492 pM.

$$\left(\frac{40\text{ pM} * 1\text{ mL}}{\text{Conc. of MET standard } \textbf{STOCK}\text{ (pM)}}\right) * 1000\ \mu\text{L/mL} = \underline{\text{XX}}\ \mu\text{L MET standard } \textbf{STOCK} \text{ solution in 1 mL final}$$

$$\left(\frac{40\text{ pM} * 1\text{ mL}}{\underline{\quad\quad}\text{ pM}}\right) * 1000\ \mu\text{L/mL} = \underline{\quad\quad}\ \mu\text{L MET standard } \textbf{STOCK} \text{ solution in 1 mL final}$$

Use the calculations above to prepare the 40 pM standard in tube #1. Serial dilution of the MET standards is used to prepare the remaining tubes with final concentrations ranging from 20 to 0.31 pM in SPB. 100 µL of each standard will be added per plate well without further dilution. Label tubes with final concentration of standard.

Tube # (Plate Row)	**Vol. and Source of Concentrated Standard**	**Vol. SPB**	**Resulting Diluted Conc. of Standard**
1 (H)	_____ µL **STOCK** solution	_____ µL (bring to 1 mL)	40 pM
2 (G)	500 µL of tube #1	500 µL	20 pM
3 (F)	500 µL of tube #2	500 µL	10 pM
4 (E)	500 µL of tube #3	500 µL	5 pM
5 (D)	500 µL of tube #4	500 µL	2.5 pM
6 (C)	500 µL of tube #5	500 µL	1.25 pM
7 (B)	500 µL of tube #6	500 µL	0.63 pM
8 (A)	500 µL of tube #7	500 µL	0.31 pM

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B. Unknown Sample Calculation Table (SOP Step 7.6.2)

Unknown samples will be run in triplicate, 100 µL sample/well (preparing 0.5 wells extra). Sample numbers correspond to those on the Plate Map Design in Appendix 1. A pre-dose and post-dose sample from the same patient would have the same Patient ID, but different Sample ID numbers. Tumor lysate samples are prepared according to SOP341201.

Time Unknown Samples Removed From Freezer: _____ : _____

Sample No.	Sample/Patient ID	Protein Conc. Stock Lysate (µg/mL)	Diluted Lysate†		
			Vol. Lysate (µL)	Vol. SPB (350 µL - Vol. Lysate)	Final conc. of diluted lysate (µg/mL)
S1					50 µg/mL
S2					100 µg/mL
S3					50 µg/mL
S4					100 µg/mL
S5					50 µg/mL
S6					100 µg/mL
S7					50 µg/mL
S8					100 µg/mL
S9					50 µg/mL
S10					100 µg/mL
S11					50 µg/mL
S12					100 µg/mL

†For each unknown sample, perform the following calculation to determine the total volume stock tumor lysate needed to make each of 2 different lysate dilutions (50 and 100 µg/mL) in SPB at a final volume of 350 µL. This is sufficient volume to run each dilution in triplicate preparing 0.5 wells extra; final diluted volumes will be loaded at 100 µL/well.

- For each unknown sample, record volumes for preparation of the **Diluted Lysate** sample in table above.

$$\frac{\text{(50 or 100) µg/mL } \textbf{Diluted Lysate}}{\text{XX.X µg/mL Conc. Stock Lysate}} * 350\ \mu L = \text{Add } \underline{\text{XX.X}}\ \mu L \text{ Stock Lysate (bring to 350 µL with SPB)}$$

- The volume of stock lysate used to make the **Diluted Lysate** should always be **≥ 2 µL**. If the calculated volume is less, make a 1:10 dilution (2 µL stock lysate into 18 µL SPB) and then use 10x more volume. Notate "1:10" in parenthesis next to the volume used if a 1:10 dilution was needed to load **≥ 2 µL**.
    - For example, if the calculated volume to use from the stock lysate was 1.2 µL, after making the 1:10 dilution of the stock lysate, 12 µL would be used to prepare the **Diluted Lysate** in the table above.

C. Tumor Lysate Controls (SOP Step 7.6.3)

The High-, Mid-, and Low-C tumor lysate dilutions (10 µg/mL) are prepared in SPB Buffer (2-8°C) using individual stock lysates (50 µg/mL); each stock lysate has a different level of MET protein. 100 µL of each dilution will be placed into plate wells without further dilution.

Control Tube Dilution	Vol. and Source of Tumor Lysate Control	Vol. SPB Buffer (2-8°C)
High-C (1:5)	**100** µL of High-C Stock	400 µL
Mid-C (1:5)	**100** µL of Mid-C Stock	400 µL
Low-C (1:5)	**100** µL of Low-C Stock	400 µL

4. Plate Incubation (SOP Steps 7.7.2 and 7.8.2)

Add 100 µL unknown samples, controls, and MET standards to the 96-well plate, cover plate, and incubate at 25°C for 1 h ± 5 min shaking at 650 rpm.

Start Time: ____ : ____ Stop Time: ____ : ____ Incubation Temp: _____ °C

5. Detection Antibody: D26-Biotin Conjugate (SOP Steps 7.8.1 and 7.8.4)

A. Preparation of D26-Biotin Conjugate Working Solution

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/(1000 µL/mL) = 11 mL. Prepare **D26-Biotin Conjugate working solution** using the following calculations.

a. Recommended working concentration of D26-Biotin Conjugate = _____µg/mL

e.g., PADIS/IQC-supplied D26-Biotin Conjugate recommended working concentration for Lot# 011210 is 0.2 µg/mL.

$$\frac{\text{Working Concentration}}{\text{100 µg/mL D26-Biotin Conjugate STOCK}} * 11\text{ mL} * 1000\text{ µL/mL} = \underline{XX}\text{ µL D26-Biotin Conjugate STOCK}$$

$$\frac{\underline{\quad}\text{ µg/mL}}{\text{100 µg/mL D26-Biotin Conjugate STOCK}} * 11\text{ mL} * 1000\text{ µL/mL} = \underline{\quad}\text{ µL D26-Biotin Conjugate STOCK}$$

b. Place the following in a 15-mL polypropylene tube:

11 mL Phospho-Assay Buffer
___ µL D26-Biotin Conjugate

Preparation Time: _____ : _____

B. Addition of Prepared D26-Biotin Conjugate Working Solution

Add 100 µL of the **D26-Biotin Conjugate Working Solution** to each well and incubate at 25°C for 1 h ± 5 min shaking at 650 rpm.

Start Time: ____ : ____ Stop Time: ____ : ____ Incubation Temp: _____ °C

6. Reporter: Streptavidin Poly-HRP Conjugate (SOP Steps 7.8.5 and 7.8.7)

A. Preparation of Streptavidin Poly-HRP Conjugate

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/(1000 µL/mL) = 11 mL. Prepare **Poly-HRP Conjugate working solution** using the following calculations:

a. Recommended working concentration of Poly-HRP Conjugate = _____µg/mL

e.g., PADIS/IQC-supplied Poly-HRP conjugate recommended working concentration for Lot# LK151842 in the phospho-MET immunoassays is 0.2 µg/mL.

$$\frac{\text{Working Concentration} * 11\text{ mL}}{500\ \mu\text{g/mL Poly-HRP Conjugate } \textbf{STOCK}} * 1000\ \mu\text{L/mL} = \underline{\text{XX.X}}\ \mu\text{L Poly-HRP Conjugate } \textbf{STOCK}$$

$$\frac{\_\_\_\_\ \mu\text{g/mL} * 11\text{ mL}}{500\ \mu\text{g/mL Poly-HRP Conjugate } \textbf{STOCK}} * 1000\ \mu\text{L/mL} = \_\_\_\_\ \mu\text{L Poly-HRP Conjugate } \textbf{STOCK}$$

b. Place the following in a 15-mL polypropylene tube:

11 mL Phospho-Assay Buffer
___ µL Poly-HRP Conjugate

Preparation Time: ____:____

B. Addition of Poly-HRP Conjugate Working Solution

Add 100 µL of the **Poly-HRP Conjugate Working Solution** to each of the washed wells and incubate at 25°C for 30 ± 5 min shaking at 650 rpm.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

7. Chemiluminescent Substrate (SOP Step 7.9.2-7.9.5)

A. Preparation of Substrate Solution

Calculate volume of substrate required for the experimental run. For one 96-well plate, prepare 110 wells: (100 µL/well*110)/(1000 µL/mL) = 11 mL. Prepare the following in a 15-mL polypropylene tube wrapped with aluminum foil. Mix by inversion 5 to 8 times and store at 25°C in the dark until use.

5.5 mL Pico Stable Peroxide (50 µL/well*110)/(1000 µL/mL)
5.5 mL Pico Luminol/Enhancer (50 µL/well*110)/(1000 µL/mL)

Time of Substrate Preparation: _____:_____

B. Substrate Solution Incubation and RLU Reading Times

Time of Substrate Addition to Wells: _____:_____

Time RLU Reading is Captured: _____:_____

8. Notes, including any deviations from the SOP:

9. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: _____

BATCH RECORD: INITIALS _____ DATE: _____