

DCTD Standard Operating Procedures (SOP)

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Doc. #:	SOP341208	Revision:	A	Effective Date: 7/5/2016

National Clinical Target Validation Laboratory (NCTVL)

Applied Developmental Directorate, Leidos Biomedical Research, Inc.

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Change History

Revision	Approval Date	Description	Originator	Approval
--	2/7/2014	New Document.	KFG, YAE	KFG
A	7/5/2016	Change to recommended initial protein load, removed reference to pY1235 MET IA, added additional standard to pY1356 (1.56 pM), additional edits for 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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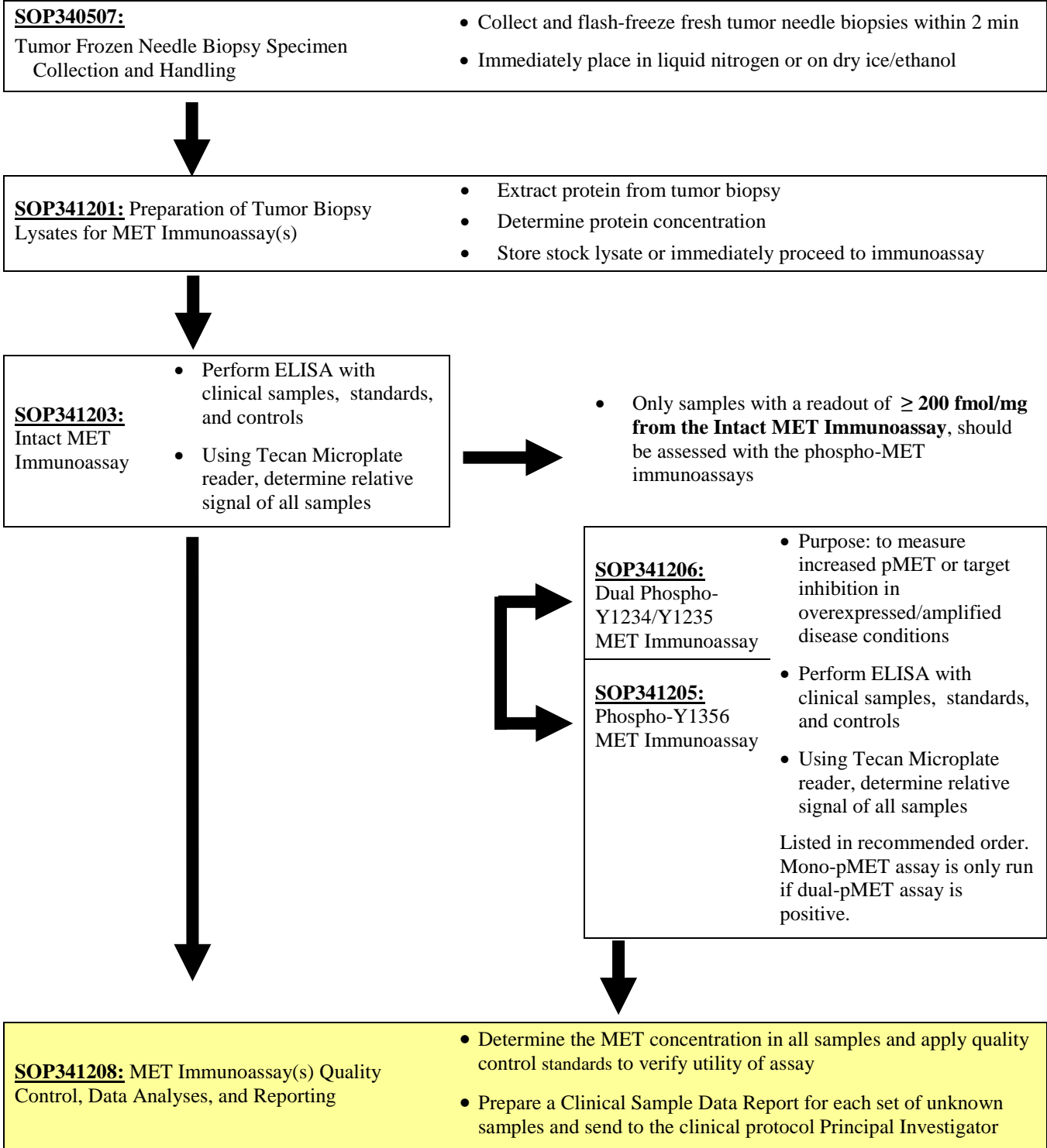
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OVERVIEW OF IMMUNOASSAY SAMPLE PROCESSING



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1.0 PURPOSE

Standardize an enzyme-linked immunosorbent assay (ELISA) method for quantifying MET and phospho-MET assay readouts for pharmacodynamic (PD) studies of chemotherapeutic agents.

2.0 SCOPE

This procedure applies to all personnel involved in the analysis of MET levels by the MET Immunoassays (SOP341203, SOP341206, or SOP341205) during clinical trials. The goal of the SOP and associated training is to ensure consistency in analysis of MET measurements across samples and clinical sites.

3.0 ABBREVIATIONS

C	=	Control
CV	=	Coefficient of Variation
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
IA	=	Immunoassay
ID	=	Identification / Identifier
IQC	=	Internal Quality Control
LHTP	=	Laboratory of Human Toxicology and Pharmacology
LLQ	=	Lower Limit of Quantitation for assay in pM units
NCTVL	=	National Clinical Target Validation Laboratory
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PD	=	Pharmacodynamic
RLU	=	Relative Light Unit
SD, STDEV	=	Standard Deviation
SOP	=	Standard Operating Procedure
UA	=	Unanalyzable
ULQ	=	Upper Limit of Quantitation
UQ	=	Unquantifiable

4.0 INTRODUCTION

The MET Immunoassays (SOP341203, SOP341205, and SOP241206) have been developed to measure the effect of MET inhibitors and/or other chemotherapeutic agents on MET levels in tumor biopsies. An ELISA is used to first capture MET from total cell extracts on plates coated with a MET capture monoclonal antibody. The captured protein is then detected using a MET polyclonal detection antibody followed by a streptavidin-HRP conjugate to allow chemiluminescent readout and quantitation of MET levels.

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5.0 ROLES AND RESPONSIBILITIES

Laboratory Director/Supervisor The Laboratory Director/Supervisor, directs laboratory operations, supervises technical personnel and reporting of findings, and is responsible for the proper performance of all laboratory procedures. The Director/Supervisor oversees the personnel running SOPs within the laboratory and is responsible for ensuring the personnel are certified and have sufficient experience to handle clinical samples.

Certified Assay Operator A Certified Assay Operator may be a Laboratory Technician/Technologist, Research Associate, or Laboratory Scientist who has been certified through DCTD training on this SOP. The Certified Assay Operator works under the guidance of the Laboratory Director/Supervisor. This person performs laboratory procedures and examinations in accordance with the current SOP(s), as well as any other procedures conducted by a laboratory, including maintaining equipment and records, and performing quality assurance activities related to performance.

- 5.1 It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented DCTD training and qualification on this SOP prior to the actual handling and processing of samples from clinical trial patients. The Laboratory Director/Supervisor is responsible for ensuring the Certified Assay Operator running the SOP has sufficient experience to handle and analyze clinical samples.
- 5.2 The Certified Assay Operator responsible for conducting the data analysis and quality control metrics is to follow this SOP and complete the required tasks and associated documentation. The Batch Record ([Appendix 1](#)) must be completed in *real-time*, with each page *dated and initialed*, and placed with the clinical sample information.
- 5.3 Digital versions of the Clinical Sample Data Report ([Appendix 4](#)) can be created as long as all column information exactly matches. A copy of the completed, digital Report must be printed and attached to the Batch Record in order to maintain a complete audit trail.
- 5.4 Neither the Certified Assay Operator nor the Laboratory Director/Supervisor will interpret the assay readout data in terms of drug effect in the Clinical Sample Data Report ([Appendix 4](#)). This report should simply state the MET readout value or define the sample as unquantifiable or unanalyzable.
- 5.5 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.

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6.0 MATERIALS AND EQUIPMENT REQUIRED

- 6.1** PC
- 6.2** Microsoft Excel 2003, 2007, or 2010
- 6.3** GraphPad Prism, Version 5.0 or 6.0 (GraphPad Software, Inc.)
- 6.4** Data needed from immunoassay results to be analyzed:
 - 6.4.1** Plate Map well assignments, Sample Calculation Table, and Tecan plate readout from the MET Immunoassay being analyzed
 - 6.4.2** Lot-specific ranges for High-, Mid-, and Low-C control samples
- 6.5** Template files supplied during training and available upon request:
 - 6.5.1** MET immunoassay analysis Excel workbook template Version 003: SOP341208_Intact_Dual_MET_template.xslt
 - 6.5.2** MET immunoassay analysis Excel workbook template Version 003: SOP341208_Mono_Phospho_MET_template.xslt
 - 6.5.3** MET immunoassay analysis GraphPad Prism template Version 001: SOP340208_Intact_Dual_MET_template.pzf
 - 6.5.4** MET immunoassay analysis GraphPad Prism template Version 002: SOP340208_Mono_Phospho_MET_template.pzf

* If software differ from those recommended above, the Certified Assay Laboratory must prove their comparability or equivalence to those recommended.

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7.0 OPERATING PROCEDURES

- 7.1** A copy of the Tecan Microplate reader output and the Sample Calculation Table from the MET Immunoassay being analyzed (SOP341203 or SOP341206, or SOP341205) will be needed. In addition, the lot-specific ranges for the High-, Mid-, and Low-C samples used in this assay run will be needed for quality control analysis. **Important:** The MET immunoassay analysis Excel workbook template used in this SOP is based on the 96-well sample designations in the Plate Map from immunoassay SOP.
- 7.2** Record the name and certification number of the Certified Assay Operator and the facility running the SOP in the Batch Record (Appendix 1). In addition, indicate the date of the original MET immunoassay run and Plate ID (if available) in the Batch Record.
- 7.3** Record the version of GraphPad Prism and name of original Tecan data file to be analyzed (Appendix 1, Section 1).

Important: If at any time the **Assay Fails QC**, do not continue with the analysis. State in the Batch Record (Appendix 1, Section 3) the reason for assay failure and notify the Laboratory Director/Supervisor of the assay failure. Rerun the assay with fresh reagents. Batch Records for any assay that fails QC should be maintained per laboratory procedures to track assay and laboratory performance.

7.4 Plate Map Quality Layout QC (Excel, “Tecan Raw Data” and “Plate Layout QC”)

- 7.4.1** In the Batch Record, indicate which MET Immunoassay the data being analyzed are from: SOP341203, SOP341206, or SOP341205 (Appendix 2, Section 2A).
- 7.4.2** Open the Excel Template file for use with the assay being analyzed and save as an Excel document with a unique file name (e.g., SOP number + run date + unique plate identifier). Record the Excel template used and the unique file name in the Batch Record (Appendix 1, Section 2B).
- For the Intact and Dual pY1234-1235 MET immunoassays (SOP341203 or SOP341206) use SOP341208_Intact_Dual_MET_template.xslt.
 - For the mono-phospho pY1356 MET immunoassay (SOP341205) use SOP341208_Mono_Phospho_MET_template.xslt.
- 7.4.3** Paste the exported Tecan data into the worksheet titled “Tecan Raw Data.” This is considered the raw, source data for data analysis; do not edit or modify this worksheet.
- 7.4.4** If the recommended MET Plate Map from the immunoassay SOP and illustrated on top of the worksheet titled “Plate Layout QC” was used, simply copy and paste the RLU readings from the “Tecan Raw Data” worksheet into the blank plate cells provided at the bottom of the “Plate Layout QC” worksheet (cells C21 - M28). To maintain the background colors, select “Match Destination Formatting” after pasting (not required).
- 7.4.4.1** If the 96-well plate was set-up in a different orientation than that outlined in immunoassay SOP and illustrated on top of the worksheet titled “Plate Layout QC” (not recommended), cell-by-cell copy and paste the data from the “Tecan Raw Data” worksheet into the appropriate wells on the “Plate Layout QC” worksheet such that they line up with the MET color-coded Plate Map layout.
- 7.4.4.2** Once data has been pasted into the MET Plate Map QC section of the “Plate Layout QC” worksheet, **do not drag and drop data** from one cell to another. Cells are linked throughout the Excel worksheets and dragging and dropping will break these links.

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7.4.5 If any sample wells were blank (i.e., no sample loaded), delete RLU readings from those cells on the Plate Map QC section of the “Plate Layout QC” worksheet.

7.4.6 Indicate in the Batch Record if the recommended MET Plate Map was used or if cells had to be copy and pasted individually to the “Plate Layout QC” worksheet (Appendix 1, Section 2C). Also specify the reason for the deviation from the plate layout map in the Batch Record.

7.5 Background Wells (Excel, "Assay QC")

7.5.1 On the worksheet titled "Assay QC," all values from the “Plate Layout QC” are auto-filled at the top of the worksheet and formulas within the worksheet will automatically perform QC determination for the standard curve. A total of 10 wells are averaged for background determination; the 4 corner wells and 2 adjacent to the high standard are not used for background level calculation.

7.5.2 The mean background RLU value is used for standard curve determination in the next section and is subtracted from all samples before analyses in GraphPad.

7.6 Standard Curve QC (Excel, “Assay QC”)

7.6.1 The Excel worksheet “Assay QC” will automatically perform QC determination for the low and high standards with the criteria listed below. If any QC section states “**Assay Fails QC,**” do not continue with the analysis; notify the Laboratory Director/Supervisor of the assay failure. State in the Batch Record (Appendix 1, Section 3) the reason for assay failure, and go to SOP Step 7.11. Rerun the assay with fresh reagents.

7.6.2 Signal-to-background (S/B) ratio QC and LLQ and ULQ Assignment: Formulas within the worksheet will automatically report the QC determination with the criteria listed below:

7.6.2.1 In order to use the range of the standard curve between the two lowest standards for MET level determination, the ratio of the mean RLU readout for the lowest standard to the mean RLU readout of the background must be at least 1.5.

- If the lowest standard fails the minimum requirement, then the ratio of the mean RLU readout for the second lowest standard to the mean RLU readout of the background must be at least 1.5.
- If the second lowest standard also fails, then the **Assay Fails QC** (cell I29).
- The lowest passing standard is assigned as the LLQ (pM) for the assay (cell J24).

7.6.2.2 The ratio of the highest standard RLU readout to the mean RLU of the background must be at least 50. If not, the **Assay Fails QC** (cells I35).

- If highest standard passes QC, it is assigned as the ULQ (pM) for the assay (cell J25).

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7.7 Non-Linear Regression and Data Transformation (GraphPad and Excel, “GraphPad Prism”)

- 7.7.1** Open the GraphPad Prism template file and save it with a unique file name, preferably mirroring the matched Excel analysis file. Record the file name in the Batch Record (Appendix 1, Section 2A). For information on the GraphPad file set-up, see [Appendix 2](#).
- For the Intact and Dual pY1234-1235 MET immunoassays (SOP341203 or SOP341206) use SOP341208_Intact_Dual_MET_template.pzf.
 - For the mono-phospho pY1356 MET immunoassays (SOP341205) use SOP341208_Mono_Phospho_MET_template.pzf.
- 7.7.2** On the Excel worksheet titled “GraphPad Prism” copy cells D8 - G33 from the section titled “To GraphPad Prism” and paste it into the GraphPad Data Table titled “From Excel” (Data Tables > From Excel). Delete any remaining “1” values in the GraphPad Prism file from unused sample rows.
- All standard, control, and unknown sample RLU values (Replicate Data columns “A:Yn”) have the mean background level subtracted.
 - The values for the expected standard ranges (column “X”) are expressed as a Log₁₀ value for non-linear regression analysis.
- 7.7.3** Go to the “Table of results” sheet (Results > Nonlin fit of From Excel > Table of results) and verify that the “R square (weighted)” value in row 17 is ≥ 0.96 .
- If the R square is < 0.96 , look at the “Outliers” sheet (Results > Nonlin fit of From Excel > Outliers) to see if one of the low or high standard replicates was ruled an outlier by GraphPad. If so, on the “From Excel” sheet, right click on the cell that is an outlier and select “Exclude value” (Ctrl-E can also be used). Review the R square value, if it now passes, document the exclusion as a deviation in the Batch Record (Appendix 1, Section 3).
 - If no outliers were identified or if the R square is still < 0.96 , the **Assay Fails QC**. State in the Batch Record (Appendix 1, Section 3) the reason for assay failure, and go to SOP Step 7.11. Rerun the assay with fresh reagents.
- 7.7.4** Before proceeding, print the following four tables and graphs from GraphPad:
- Data Tables > From Excel
 - Results > Table of results
 - Results > Transform of Nonlin fit of From Excel
 - Graph > From Excel
- 7.7.5** Go to the results sheet titled “Transform of Nonlin fit of Data” (Results > Transform of Nonlin fit of From Excel). These values have been transformed to represent MET levels (background subtracted) in pM rather than RLU.
- 7.7.6** Copy the values in the columns A:Y1, A:Y2, and A:Y3 and paste into the Excel worksheet titled “GraphPad Prism” under the section titled “From GraphPad” (light yellow cells). To maintain the background colors, simply select “Match Destination Formatting” after pasting (not required).
- 7.7.7** Save and close the GraphPad Prism file.

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7.8 Tumor Control Lysate QC (Excel, “Control QC”)

- 7.8.1** On the Excel worksheet titled “Control QC,” for each control sample (Low, Mid, and High) enter the pM MET ranges (top and bottom end) supplied with the **specific lot** of control reagents used for the assay (cells C16 - D21).
- 7.8.2** Formulas within the worksheet will report the QC determination for the control samples with the following criteria:
- At least one control at each level (Low-, Mid-, and High-C) must have a CV of < 20% for the replicate wells (cells H6 - H11).
 - At least one control at each level and at least 4 of 6 controls overall must fall within the defined MET pM range supplied for the specific lot of critical reagent (cells E16 - E21).
- 7.8.3** If any of these criteria are not met, the **Assay Fails QC** (cell G16). State in the Batch Record (Appendix 1, Section 3) the reason for assay failure, and go to SOP Step 7.11. Rerun the assay with fresh reagents.
- 7.8.4** For any control samples out of specification, bring the data to the Laboratory Director/Supervisor to review along with recent assay runs to be sure there is not a broader problem with critical reagents or assay equipment.

7.9 Unknown Sample Replicate QC and LLQ/ULQ QC (Excel, “Unknown QC”)

- 7.9.1** Replicate values on the “Unknown QC” worksheet are auto-filled from the “GraphPad Prism” worksheet.
- 7.9.1.1** If during GraphPad Prism analysis all replicate values for a sample are removed (blank cells), review the RLU readings for this sample compared to the lowest and highest calibrator and state in the “Notes” column if the sample was < LLQ or > ULQ, respectively, based on this comparison.
- 7.9.1.2** In the “Notes” column, specify “no sample” for those rows where blank cells simply indicate no sample was loaded.
- 7.9.2** Formulas in the “Unknown QC” worksheet will automatically perform %CV QC determination for the unknown sample replicate wells with the criteria listed below:
- Triplicate repeats for each sample must have a CV < 15%. If all samples Pass %CV QC, proceed to SOP Step 7.9.4.
 - If an unknown sample has $\geq 15\%$ CV (column H) and is listed as “Fail” in column I, go to SOP Step 7.9.3 and perform a Dixon’s test for outliers.
- 7.9.3** Dixon’s Test Outliers on Triplicate Replicates of Unknown Samples ([Appendix 3](#))
- 7.9.3.1** If a sample has a CV $\geq 15\%$ (column H; highlighted in red) and is listed as “Fail” in column I, copy and paste the triplicate repeat values* (columns C - E) into the Dixon’s calculator input cells (C22 - E25). ***Note:** The values in columns C - E are auto-filled using an Excel formula; when copy and pasting to the Dixon’s calculator, you will have to “Paste Special” as “Values Only.”
- 7.9.3.2** If either the lowest (X1) or highest (X3) value from the triplicate repeats can be eliminated, the Dixon’s calculator will state “Yes, Xn is an Outlier” (cells E29 - E30). Delete that value from the replicate data (columns C - E) and state in the “Notes” column “Outlier removed.”

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7.9.3.3 If the 2 remaining repeat values (when one has been removed) have a %CV \geq 15%, **OR** none of the replicates are rejected as an outlier when %CV is \geq 15%:

- First determine if the value is $<$ LLQ or $>$ ULQ per SOP Step 7.9.4; if either is true, delete the mean for that dilution (column F), and state “Sample $<$ LLQ or $>$ ULQ.”
- If sample is within the dynamic range of the assay, delete the mean for that dilution (column F), and state “Sample Fails %CV QC.”

7.9.4 Review the average MET levels (column F) and identify any values that are $<$ LLQ or $>$ ULQ (highlighted in blue). The assay LLQ and ULQ values have been carried over in cells F22 - F23 from the “Assay QC” worksheet.

7.9.4.1 If the average value for an unknown sample is $<$ LLQ or $>$ ULQ, delete the mean value for that dilution (column F) and in the “Notes” column state “Sample $<$ LLQ” or “Sample $>$ ULQ.”

7.9.4.2 If a sample is $>$ ULQ and there is sufficient sample volume, it can be re-run with fresh reagents at a 2-fold lower protein load/well. If a sample is $<$ LLQ and there is sufficient sample volume, it can be re-run with fresh reagents at a 2-fold higher protein load/well. The maximum allowed protein load for the MET immunoassays is 200 μ g/mL.

7.10 Dilution Linearity QC for Unknown Samples (Excel, “MET Determination”)

Note: The calculations set up in the lower portion of the “MET Determination” worksheet for dilution linearity QC assume the Plate Map recommendations from SOP340903 were followed. Every two samples (e.g., S1, S2) would typically represent the 50 and 100 μ g/mL sample loads from a single patient’s tumor biopsy sample.

7.10.1 On the Excel worksheet titled “MET Determination” using the Clinical Sample Calculation Table from the SOP341203, SOP341205, or SOP341206 Batch Record, enter the following information:

7.10.1.1 Sample/patient ID (cells C6 – C17). Enter “no sample” if the wells were empty.

7.10.1.2 For tumor biopsy samples, enter the total μ g/mL protein loaded (e.g., 50 or 100 μ g/mL) for each sample (cells D6 – D17).

- The average analyte readout in pM is carried over from the ‘Unknown QC’ worksheet in cells E6 – E17. **Note:** Mathematically 1 pM = 1 fmol/mL and this conversion is used to calculate the final MET units, fmol/mg protein load (fmol/ μ g * 1000 μ g/mg).
- Since mean MET values for samples $<$ LLQ or $>$ ULQ were deleted in SOP Step 7.9.4, they are not included in dilution linearity assessment or MET level determination.

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7.10.2 On the bottom half of the “MET Determination” worksheet, formulas will automatically back-calculate the total MET protein in fmol/mg total protein (cells D23 – D34) for each dilution. Sample/Patient ID information is auto-filled dependent on sample data entered at the top of the worksheet.

7.10.3 Dilution linearity for the tumor biopsy dilutions (50 and 100 µg/mL loads) is determined by calculating the percent MET in each dilution relative to the average of the two. Values between 80% and 120% pass dilution linearity QC; if the samples fail dilution linearity, they will be highlighted in red in the worksheet (cells F23 – F34).

Apply dilution linearity criteria as follows:

- If the calculated values fail dilution linearity, delete the MET value for the lowest protein load (typically 50 µg/mL), in the “Notes” column for the samples state “*Dilution non-linear*”.
- If there is sufficient lysate, re-run the unknown in a new assay; if there is insufficient lysate, report results as described in SOP Step 7.11.3.3.

7.11 Clinical Sample Data Report

Important: Decisions regarding levels of pre-dose MET levels needed to measure a drug effect post-dose will be made by the Clinic or pharmacodynamic Laboratory Director/Supervisor and should not be reported in the Clinical Sample Data Report.

7.11.1 For **each patient**, compile the final MET assay results using the Clinical Sample Data Report Template ([Appendix 4](#)).

7.11.2 Fill in the patient and clinical protocol information and indicate which MET assay the reporting results are for.

7.11.3 If a pre- or post-dose sample was not collected, state “No Sample” in the Patient/Sample ID column, indicate the schedule collection time, and leave the rest of the cells blank for that sample.

7.11.4 Unknown samples should be reported as unanalyzable (UA), with no value reported, if any of the following are true:

- If the assay failed QC and there is insufficient lysate to re-run the unknown, state note “UA” in the MET level column and “Assay Failed QC” in the QC notes section.
- If both dilutions of a sample failed %CV QC, note “UA” in the MET level column and “FQ” in the QC notes section, the abbreviation for “failed %CV QC”
- If a lysate sample had $\leq 250 \mu\text{g/mL}$ ($0.25 \mu\text{g}/\mu\text{L}$) total protein by BCA analysis and was not analyzed in the MET assay, note “UA” in the MET level column and “NT” in the QC notes section, the abbreviation for “not tested due to low protein yield.”

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7.11.5 Unknown samples should be reported as unquantifiable, if the average MET levels for **all three** dilutions were < LLQ or > ULQ, and there is insufficient lysate to re-run the samples.

- The GraphPad-transformed MET readout value for samples < LLQ or > ULQ should not be reported as these are outside the dynamic range of the assay. Instead state if the sample was < LLQ or > ULQ in the “MET Levels” column of the Clinical Sample Data Report (see below).
- For samples where **both** dilutions were < LLQ or > ULQ a maximal or minimal possible concentration will be reported to the Clinic in the “QC Note” section of the Clinical Sample Data Report as follows:

- If a sample was < LLQ, use the calculations at the bottom of the “MET Determination” worksheet (cell D39) to report the maximum MET concentration in fmol/mg that could have been present in the sample.

The calculation for the **highest protein load** for the unknown sample should be used as the LLQ assignment. The worksheet calculation is (LLQ value [fmol/mL]/protein load [$\mu\text{g/mL}$] * 1000 $\mu\text{g/mg}$).

<i>Example</i>	MET Levels fmol/mg protein	QC Note
Baseline	43.02	
Post-Dose	< LLQ	3.10 fmol/mg; max. possible MET level

- If a sample was > ULQ, use the calculations at the bottom of the “MET Determination” worksheet (cell F38) to report the minimum MET concentration in fmol/mg that *could* have been present in the sample

The calculation for the **lowest protein load** for the unknown sample should be used for ULQ assignment. The worksheet calculation is (ULQ value [fmol/mL]/protein load [$\mu\text{g/mL}$] * 1000 $\mu\text{g/mg}$).

<i>Example</i>	MET Levels fmol/mg protein	QC Note
Baseline	464.94	
Post-Dose	> ULQ	800 fmol/mg; min. possible MET level

7.11.6 For all other samples, report the MET value as follows:

- For samples where **both dilutions passed** the dilution linearity and other QC criteria; report the calculated average MET levels in fmol/mg protein (cells E23 – E33).
- If MET levels for **only 1 dilution** was within the dynamic range of the assay (> LLQ or < ULQ), and there is insufficient lysate to re-run the unknown sample; report the MET readout value for that dilution and state “*xx μg protein load reported; other dilution < LLQ or > ULQ.*”
- If the **both samples failed dilution linearity** or other QC criteria, report the MET levels in fmol/mg protein for the 50 $\mu\text{g/mL}$ dilution. In addition, state “*Highest protein load reported; sample dilutions non-linear*” in the “QC Note” section.

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- 7.12** Review and finalize the Batch Records (Appendix 1) and obtain required signatures. Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 1, Section 3).
- 7.13** The Laboratory Director/Supervisor should review the Batch Record and Clinical Sample Data Report for each patient and date and sign both affirming the data contained within the reports are correct (Appendix 1, Section 4).
- 7.14** The **signed** Clinical Sample Data Reports for each patient should be sent to the clinical protocol Principal Investigator.

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APPENDIX 1: BATCH RECORD

Certified Assay Operator: _____

Certification Number: _____

Date: ____ / ____ / ____

Facility/Laboratory Running SOP: _____

Data Immunoassay Run: ____ / ____ / ____

Plate ID (optional): _____

1. Software

GraphPad Prism Version: _____

Name of original Tecan data file: _____

2. Plate Map QC

A. MET Immunoassay being analyzed:

- SOP341203: Intact MET
- SOP341206: Dual pY1234/Y1235 MET
- SOP341204: pY1235 MET

B. Excel template used and name of saved MET Excel data analysis workbook

- SOP341208_Intact_Dual_MET_template.xslt
- SOP341208_Mono_Phospho_MET_template.xslt

Unique file name, Excel: _____

Unique file name, GraphPad: _____

C. Plate Map Set Up QC

- Recommended MET Plate Map used from MET Immunoassay indicated above
- Alternative plate map used; cells copy and pasted individually to the Plate Layout QC worksheet

3. Notes, including any deviations from the SOP:

If assay fails QC, state the specific reason for assay failure.

4. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: _____

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BATCH RECORD:

INITIALS _____

DATE: _____

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APPENDIX 2: GRAPHPAD PRISM SET-UP

While a GraphPad Prism Template document is supplied, below are the steps used to set-up the document from a blank Project File. The MET immunoassay analysis GraphPad Prism templates supplied during training (SOP340208_Intact_Dual_MET_template.pzf and SOP340208_Mono_Phospho_MET_template.pzf) have all data tables and graphs set-up with numerical values of “1” assigned as place holders in the data table.

1) GraphPad Prism Non-Linear Regression and Unknown Sample Transformation

- a. Open GraphPad Prism and create an XY graph type with the Y axis showing 3 replicate values in side-by-side subcolumns; plot the Mean only.
- b. Retitle the X and Y headers as shown in the GraphPad images below. Two GraphPad templates will be needed if all 3 MET assays will be assessed: one for the Intact and Dual pY1234/Y1235 MET assays (image on left below) which uses a standard curve comprised of 8 standards ranging from 0.31 – 40 pM and one for the mono-phospho pY1356 MET assay (image on right below), which has a standard curve comprised of 8 standards ranging from 1.56 – 200 pM.

GraphPad Prism template **for Intact and Dual pY1234/Y1235 MET** Immunoassays

Table format: XY		X	A		
		pM Standard (Log[X])	Replicate Data		
▲	x	X	A:Y1	A:Y2	A:Y3
1	0.31 pM	1.00	1	1	1
2	0.63 pM	1.00	1	1	1
3	1.25 pM	1.00	1	1	1
4	2.5 pM	1.00	1	1	1
5	5.0 pM	1.00	1	1	1
6	10.0 pM	1.00	1	1	1
7	20.0 pM	1.00	1	1	1
8	40.0 pM	1.00	1	1	1
9	LOW 1		1	1	
10	LOW 2		1	1	
11	MID 1		1	1	
12	MID 2		1	1	
13	HIGH 1		1	1	
14	HIGH 2		1	1	
15	S1		1	1	1
16	S2		1	1	1
17	S3		1	1	1
18	S4		1	1	1
19	S5		1	1	1
20	S6		1	1	1
21	S7		1	1	1
22	S8		1	1	1
23	S9		1	1	1
24	S10		1	1	1
25	S11		1	1	1
26	S12		1	1	1
27	Title				

GraphPad Prism template **for mono-phospho pY1356 MET** Immunoassays

Table format: XY		X	Group A		
		pM Standard (Log[X])	Replicate Data		
▲	x	X	A:Y1	A:Y2	A:Y3
1	1.56 pM	1.00	1	1	1
2	3.13 pM	1.00	1	1	1
3	6.25 pM	1.00	1	1	1
4	12.5 pM	1.00	1	1	1
5	25.0 pM	1.00	1	1	1
6	50.0 pM	1.00	1	1	1
7	100.0 pM	1.00	1	1	1
8	200.0 pM	1.00	1	1	1
9	LOW 1		1	1	1
10	LOW 2		1	1	1
11	MID 1		1	1	1
12	MID 2		1	1	1
13	HIGH 1		1	1	1
14	HIGH 2		1	1	1
15	S1		1	1	1
16	S2		1	1	1
17	S3		1	1	1
18	S4		1	1	1
19	S5		1	1	1
20	S6		1	1	1
21	S7		1	1	1
22	S8		1	1	1
23	S9		1	1	1
24	S10		1	1	1
25	S11		1	1	1
26	S12		1	1	1

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- c. Copy and paste the values in blue on the ‘GraphPad Prism’ Excel worksheet onto the Data Table (see image below for set-up). **Note:** In the supplied GraphPad Prism template, the data table is titled “From Excel,” the default name is “Data 1.”

Example of ‘GraphPad Prism’ Excel worksheet from an Intact MET Immunoassay

GraphPad Prism Data Table

To GraphPad Prism

All sample RLU values have the mean background level subtracted. Copy and paste all blue values into GraphPad Prism in the corresponding areas of the Data Table titled "From Excel."

Table Format XY	X		A		
	pM Standard (Log[X])	Replicate Data			
		X	A:Y1	A:Y2	A:Y3
1	0.31 pM	-0.51	691030	236140	178530
2	0.63 pM	-0.20	728780	517550	600500
3	1.25 pM	0.10	1466420	1188220	1159420
4	2.5 pM	0.40	3049820	2779420	2699920
5	5.0 pM	0.70	5840420	5543520	5325320
6	10.0 pM	1.00	11443020	11028020	10832020
7	20.0 pM	1.30	22889020	21731020	21256020
8	40.0 pM	1.60	44858020	42737020	40531020
9	LOW 1		3456120	3414220	
10	LOW 2		3499420	3456587	
11	MID 1		7244020	7367620	
12	MID 2		7361020	7324220	
13	HIGH 1		30123020	30288020	
14	HIGH 2		30426020	30252353	
15	S1		1064720	1003420	1037220
16	S2		2087620	2020220	1971220
17	S3		4044320	4008020	3879220
18	S4		805620	769220	626590
19	S5		877120	917920	819320
20	S6		1896620	1662820	1638120
21	S7		2139920	2202420	2102920
22	S8		4043120	4118020	4065720
23	S9		7919220	7989320	7918720
24	S10		1302120	1149020	1140220
25	S11		2389720	2358120	2295120
26	S12		4400020	4426120	4302720



Table format: XY		X	A		
		pM Standard (Log[X])	Replicate Data		
		X	A:Y1	A:Y2	A:Y3
1	0.31 pM	-0.51	691030	236140	178530
2	0.63 pM	-0.20	728780	517550	600500
3	1.25 pM	0.10	1466420	1188220	1159420
4	2.5 pM	0.40	3049820	2779420	2699920
5	5.0 pM	0.70	5840420	5543520	5325320
6	10.0 pM	1.00	1e+007	1e+007	1e+007
7	20.0 pM	1.30	2e+007	2e+007	2e+007
8	40.0 pM	1.60	4e+007	4e+007	4e+007
9	LOW 1		3456120	3414220	
10	LOW 2		3499420	3456587	
11	MID 1		7244020	7367620	
12	MID 2		7361020	7324220	
13	HIGH 1		3e+007	3e+007	
14	HIGH 2		3e+007	3e+007	
15	S1		1064720	1003420	1037220
16	S2		2087620	2020220	1971220
17	S3		4044320	4008020	3879220
18	S4		805620	769220	626590
19	S5		877120	917920	819320
20	S6		1896620	1662820	1638120
21	S7		2139920	2202420	2102920
22	S8		4043120	4118020	4065720
23	S9		7919220	7989320	7918720
24	S10		1302120	1149020	1140220
25	S11		2389720	2358120	2295120
26	S12		4400020	4426120	4302720

- 2) In GraphPad Prism, on the menu ribbon in the Analysis box, select Analyze. Under the XY Analysis section, select “Nonlinear regression (Curve fit)” and the A-Replicate Data set should be selected in the right-hand window. Click OK.
- 3) The nonlinear regression (NLR) parameters window will appear. Tab-by-tab, make the following updates:
 - a. On the Fit tab, under “Dose response-Stimulation”, select “log (agonist) vs. response -- Variable slope (four parameters).” At the bottom of the tab, under the Fitting Method select “Least squares (ordinary) fit” and under Interpolate check “Interpolate unknowns” with CI set at None.
 - b. On the Weights tab select “Weight by 1/Y² (minimize the relative distances squared)” as the Weighting method and under Replicates select “Consider each replicate Y value as an individual point.”
 - c. On the Output tab under Location of interpolated X values, select “Y column, maintaining the side-by-side arrangement of replicates.” Change Number of digits in output to 2 significant digits.

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d. Set up the Diagnostics tab as follows:

Parameters: Nonlinear Regression

Fit Compare Constrain Weights Initial values Range Output **Diagnostics**

Do the initial parameter values define a curve near the data?

Don't fit the curve. Instead plot the curve defined by the initial values of the parameters

Fit the curve. Maximum number of iterations: 20000

How precise are the best-fit values of the parameters?

SE of parameters

CI of parameters: 95% Output Format: Range ("1.23 to 4.56")

Plot: 95% confidence band

How to quantify goodness-of-fit?

R square Sum-of-Squares Sy.x

Normality tests. Are the residuals Gaussian?

D'Agostino-Pearson (recommended)

Shapiro-Wilk

Kolmogorov-Smirnov (not recommended)

Does the curve systematically deviate from the points?

Runs test Replicates test Residual plot (create a separate graph)

Are the parameters intertwined or redundant?

Covariance of parameters Dependency

Could outliers impact the results?

Count the outliers

Would it help to use stricter convergence criteria?

Medium Automatically switch to strict convergence when needed

Make these diagnostics choices the default for future fits

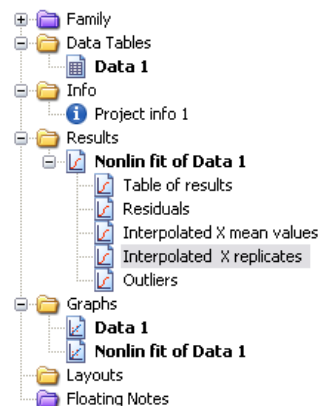
Learn Cancel OK

e. Click OK.

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- 4) A set of Nonlin fit Results sheets will be added to the GraphPad Prism navigator (image on right).
- 5) The “Table of results” sheet will open by default.
- 6) Open the “Interpolated X replicates” sheet and on the menu ribbon in the Analysis box, select Analyze. Under the Transform, Normalize... section, select “Transform” and the A-Replicate Data set in the right-hand window should be selected. Click OK.
- 7) Set up the Transform Parameters with the following items selected: “Standard functions,” “Transform Y values using ‘ $Y=10^{-Y}$,’” and “Transform individual Y values.” Under the New graph section, “Create new graph” should be selected.
- 8) Click OK.
- 9) A new data sheet will be created called “...Transform of Nonlin fit of Data.” This sheet now contains MET values for the plate repeats in pM. These data are copy and pasted back into the “GraphPad Prism” Excel worksheet in SOP Step 7.7.5.



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APPENDIX 3: DIXON'S TEST FOR OUTLIERS

- If the %CV for a triplicate well repeat is $\geq 20\%$ a Dixon's test for outliers can be used to determine if one cell can be eliminated as an outlier
- The Dixon's test uses the following criteria:

- The Dixon's test for outliers is outlined in United States Pharmacopeia (USP) General Chapter <111> "Design and Analysis of Biological Assays" under the "Rejection of Outlying or Aberrant Observations" section. The Dixon's test is used to determine if one (and only one) value from a small set of values can be legitimately rejected from a normally distributed set of data. The Dixon's test can be applied by first arranging the values from the smallest to the largest: triplicate repetitions $X_1 < X_2 < X_3$.

$$X_1 \text{ is an outlier if } \frac{(X_2 - X_1)}{(X_3 - X_1)} > 0.941$$

$$X_3 \text{ is an outlier if } \frac{(X_3 - X_2)}{(X_3 - X_1)} > 0.941$$

- The cutoff in USP <111> for rejection of outliers from three values is $P > 0.976$, which gives a 98% confidence level for a one-sided test and a 96% confidence level for a two-sided test. Because an outlier can be either high or low in the triplicate samples, a two-sided approach is suggested. Either the 0.976 or 0.941 confidence limit can be selected as the decision criterion as long as the confidence level is indicated and applied objectively to the data set.
- The MET Immunoassay uses the 0.941 confidence limit criterion.

- A calculator is provided in the Excel Data Analysis worksheets so that the triplicate repeat numbers only need to be entered into the input cells (light yellow below) and the Dixon's calculations are performed automatically. Example of the highest value being eliminated using the Dixon's calculator:

Dixon's Calculator			
INPUT VALUES:	1.10	25.10	1.20
X1 (low)	1.10		
X2 (mid)	1.20		
X3 (high)	25.10		
	Dixon's Calculation	Min. Criteria	Outlier?
Test for Low Outlier (X1)	0.0042	0.941	No
Test for High Outlier (X3)	0.9958	0.941	Yes, X3 is an outlier
This calculation has a confidence level of 96%			

- If either the lowest (X1) or highest (X3) value from the triplicate repeats can be eliminated, the Dixon's calculator will state "Yes, Xn is an Outlier." Delete that value from the replicate data and state in the "Notes" column "Outlier removed."
- If one replicate is rejected as an outlier, the remaining two repeats must still meet the 20% CV rule. If not, the entire sample fails QC. Delete the mean value so that it is not used for further analysis and state "Sample Fails %CV QC" in the "Notes" column.
- If the %CV is $\geq 20\%$, but none of the replicates are rejected as an outlier, the entire sample fails QC. Delete the mean value so that it is not used for further analysis and state "Sample Fails %CV QC" in the "Notes" column.

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APPENDIX 4: CLINICAL SAMPLE DATA REPORT

Report all MET PD results for a single patient on a single Clinical Sample Data Report to PI.

MET Immunoassay PD Analysis

Certified Assay Operator (Print): _____
 Certification #: _____ 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		

MET (fmol/mg)

	Baseline	Post-Dose	QC Notes
Intact MET			
Dual pY1234/Y1235 MET			
pY1356 MET			

Abbreviations:

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

Additional Information

Raw data available upon request.

To be completed by Laboratory Director/Supervisor

Director/Supervisor Signature*: _____
 Today's Date: _____

*Signature indicates assay results have been reviewed and verified.

Biopsy and Treatment Information (for use by Clinical site)

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