SOP340704: TOP1 Immunoassay Quality Control, Data Analyses, and Reporting

Effective Date: 2/28/2024

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 History

1. Approvals

Technical Reviewer: Yvonne A. Evrard Date: 2-28-14

NCTVL Approval: Jiuping Ji Date: 3-4-14

IQC Approval: Katherine V. Ferry-Galow Date: 3-4-14

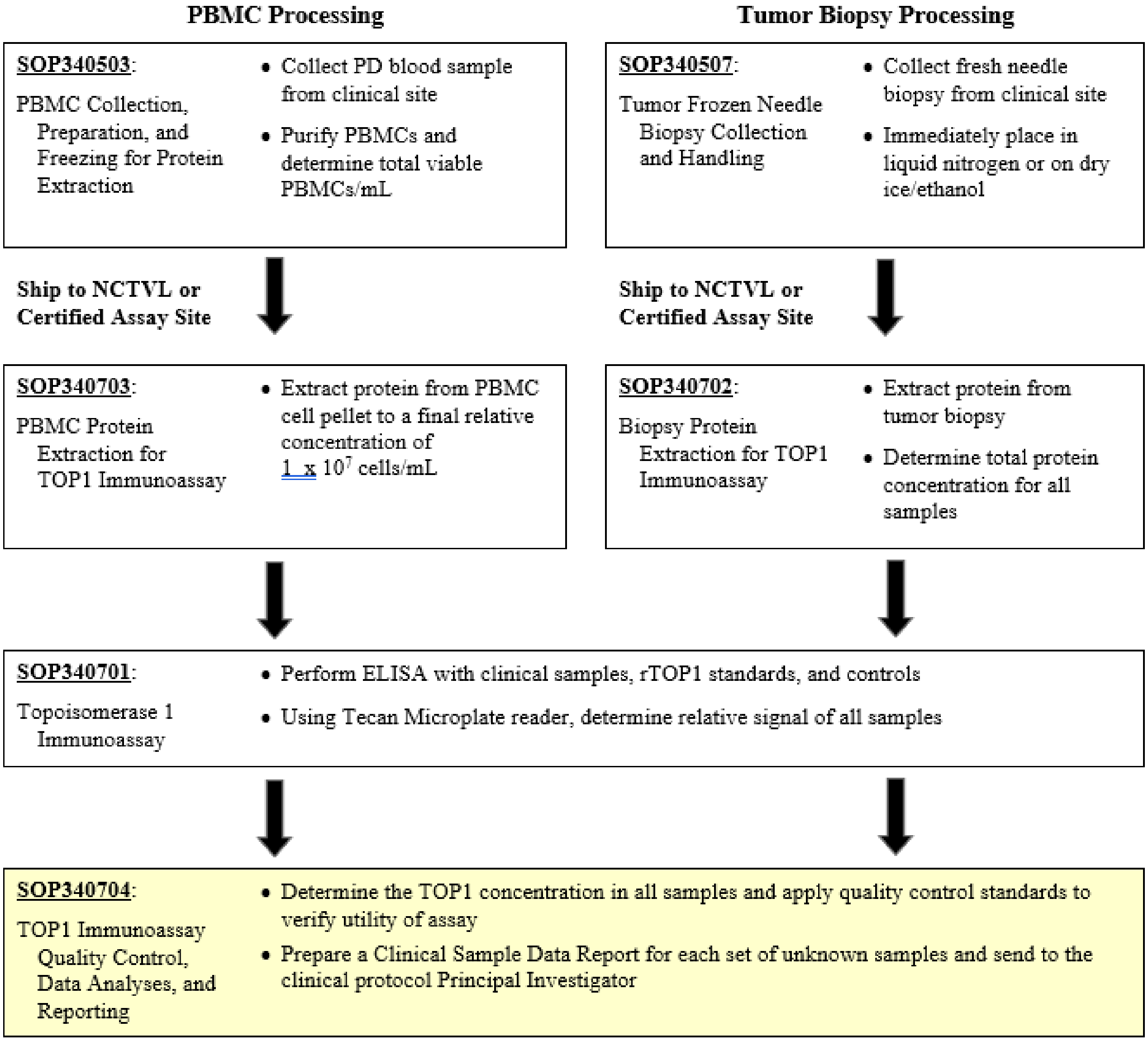
LHTP Approval: Ralph E. Parchment Date: June 6, 2014

DCTD OD Approval: Joseph E. Tomaszewski Date: 06/11/2014

2. Change History

Revision	Approval Date	Description	Originator	Approval
--	10/17/2012	New Document. Previous data analyses were macro-based and included in SOP340701. The macro has been retired and replaced with Excel and GraphPad Prism to allow for a more user-friendly and adaptable analysis package.	KFG, YAE	KFG
A	2/20/2013	Remove internal URL references. Prepare for community launch of assay.	YAE	KFG
B	2/28/2014	Percent CV criteria for background wells removed from SOP and the Data Analysis Excel was updated with this change (ver. 003). Definitions of clinical reporting abbreviations added. Minimal reportable PBMC sample corrected to match SOP340703.	KFG, YAE	KFG

OVERVIEW OF IMMUNOASSAY SAMPLE PROCESSING



PBMC Processing

SOP340503:

PBMC Collection, Preparation, and Freezing for Protein Extraction

- Collect PD blood sample from clinical site
- Purify PBMCs and determine total viable PBMCs/mL

Ship to NCTVL or Certified Assay Site

SOP340703:

PBMC Protein Extraction for TOP1 Immunoassay

- Extract protein from PBMC cell pellet to a final relative concentration of 1 x 10^7 cells/mL

Tumor Biopsy Processing

SOP340507:

Tumor Frozen Needle Biopsy Collection and Handling

- Collect fresh needle biopsy from clinical site
- Immediately place in liquid nitrogen or on dry ice/ethanol

Ship to NCTVL or Certified Assay Site

SOP340702:

Biopsy Protein Extraction for TOP1 Immunoassay

- Extract protein from tumor biopsy
- Determine total protein concentration for all samples

SOP340701:

Topoisomerase 1 Immunoassay

- Perform ELISA with clinical samples, rTOP1 standards, and controls
- Using Tecan Microplate reader, determine relative signal of all samples

SOP340704:

TOP1 Immunoassay Quality Control, Data Analyses, and Reporting

- Determine the TOP1 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

To standardize an enzyme-linked immunosorbent assay (ELISA) method for quantifying topoisomerase 1 (TOP1) levels as a pharmacodynamic (PD) measure of TOP1 inhibitors.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the TOP1 Immunoassay during clinical trials. The goal of the SOP and associated training is to ensure consistency in TOP1 measurement 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
IP = Insufficient Protein
LHTP = Laboratory of Human Toxicology and Pharmacology
LLQ-RLU = Lower Limit of Quantitation for assay in RLU units
LLQ = Lower Limit of Quantitation for assay in pg/mL units
NCTVL = National Clinical Target Validation Laboratory
NR = Not Reportable
PBMC = Peripheral Blood Mononuclear Cell
PD = Pharmacodynamic
QC = Quality Control
RLU = Relative Light Units
SD, STDEV = Standard Deviation
SOP = Standard Operating Procedure
TOP1 = Topoisomerase 1
UA = Unanalyzable
ULQ = Upper Limit of Quantitation

4.0 INTRODUCTION

The TOP1 Immunoassay has been developed to measure the effect of TOP1 inhibitors on TOP1 levels in a variety of biospecimen types, including peripheral blood mononuclear cells (PBMCs) and tissue/tumor biopsies. An enzyme-linked immunosorbent assay (ELISA) type is used to first capture TOP1 protein from total protein extracts on plates coated with a TOP1 capture monoclonal antibody. The captured protein is then detected using a TOP1 polyclonal detection antibody followed by an HRP-conjugate to allow chemiluminescent readout and quantitation of TOP1 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 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 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 state the TOP1 readout value or define the sample as unquantifiable or unanalyzable.

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 for the assay is being used.

6.0 MATERIALS AND EQUIPMENT REQUIRED

6.1 PC
6.2 Microsoft Excel 2003, 2007, or 2010
6.3 GraphPad Prism, Version 5 or 6 (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 SOP340701 (TOP1 Immunoassay)
6.4.2 Lot-specific ranges for High-, Mid-, and Low-C tumor control samples

6.5 Template files supplied during training and available upon request:

6.5.1 TOP1 immunoassay analysis Excel workbook template Version 003: SOP340704_TOP1_template.xslt
6.5.2 TOP1 immunoassay analysis GraphPad Prism template Version 001: SOP340704_TOP1_template.pzf

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

7.0 OPERATING PROCEDURES

7.1 A copy of the Tecan Microplate reader output and the Unknown Sample Calculation Table from SOP340701 will be needed. In addition, the lot-specific ranges for the High-, Mid-, and Low-C tumor control samples used in this assay run will be needed for quality control analysis. **Important**: The TOP1 immunoassay analysis Excel workbook template used in this SOP is based on the 96-well sample designations in the Plate Map from SOP340701.

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 TOP1 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 Layout QC (Excel, "Tecan Raw Data" and "Plate Layout QC")

7.4.1 Open the Excel Template (SOP340704_TOP1_template.xslt) and save as an Excel document with a unique file name (e.g., SOP number + run date + unique plate identifier). Record the file name in the Batch Record (Appendix 1, Section 2A).

7.4.2 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 the data on this worksheet.

7.4.3 If the recommended TOP1 Plate Map from SOP340701 and illustrated on top of the worksheet titled "Plate Layout QC" was used, copy and paste just 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 - N28). To maintain the background colors, select "Match Destination Formatting" after pasting (not required).

7.4.3.1 If the 96-well plate was set-up in a different orientation than that outlined in SOP340701 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 TOP1 color-coded Plate Map layout.

7.4.3.2 Once data has been pasted into the TOP1 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.

7.4.4 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.

7.4.5 Indicate in the Batch Record if the recommended TOP1 Plate Map was used or if cells had to be copy and pasted individually to the "Plate Layout QC" worksheet (Appendix 1, Section 2B). Also specify the reason for the deviation from the plate layout map in the Batch Record.

7.5 Background Well QC (Excel, "Assay QC")

7.5.1 On the worksheet titled "Assay QC," all values from the “Plate Layout QC” are auto-filled to the top of the worksheet and formulas within this worksheet will automatically perform QC determination for the background wells. A total of 9 wells are used for background determination; the corner well (A12) is not used for background level calculation.

7.5.2 A ± 2 SD cut-off is applied to the initial 9-well dataset to identify outliers; if a background well RLU value is ≥ 2 SD from the mean, the cell will be highlighted in red.

7.5.3 To cleanly remove outliers from the background dataset without error, do the following:

7.5.3.1 **First**, copy and paste the RLU values for all wells that are in red (≥ 2 SD from mean) into cells B24 - F24 to document those that will be eliminated as outliers.

7.5.3.2 **Second**, delete those values that were copied and pasted into cells B24 - F24 from the background dataset cells (cells B18 – F19).

Important:

- The mean and SD values will update as outliers are deleted and this may result in loss of the red coloring; use the copy and pasted values as the primary reference for which cells to delete.
- Upon deletion of outliers from the initial background dataset, some wells may be identified as ≥ 2 SD from the new mean. **Do not** delete any further background wells, the ± 2 SD criteria should only be applied once to the dataset.

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

7.6.1 Formulas within the “Assay QC” worksheet 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 Low Standard QC and LLQ Assignment:

- In order to use the 97.7 to 195.3 pg/mL range of the standard curve, the mean RLU readout of the 97.7 pg/mL standard must be ≥ 3 SD above the mean RLU readout of the background; this value is referred to as the LLQ-RLU.
- If the 97.7 pg/mL standard fails, then the mean RLU readout of the 195.3 pg/mL standard must be ≥ 3 SD above the mean RLU readout of the background.
- If the 195.3 pg/mL standard also fails, the **Assay Fails QC** (cells H29).
- The lowest passing standard is assigned as the LLQ (pg/mL) for the assay (cell J24).

7.6.3 Signal-to-background (S/B) ratio QC and ULQ Assignment:

- The ratio for the lowest passing standard (97.7 or 195.3 pg/mL) RLU readout to the mean RLU readout of the background must be ≥ 1.1. If not, the **Assay Fails QC** (cell H33).
- The ratio of the highest standard RLU readout (12,500 pg/mL) to the mean RLU of the background must be ≥ 20. If not, the **Assay Fails QC** (cell H35).
- If the high standard passes QC, it is assigned as the ULQ (pg/mL) for the assay (cell J25).

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

7.7.1 Open the GraphPad Prism template file (SOP340704_TOP1_template.pzf) and save it with a unique file name mirroring the matched Excel analysis file. Record the file name in the Batch Record (Appendix 1, Section 2A).

- The GraphPad template file has already been set-up with all required criteria for data analyses by non-linear regression and transformation. Numerical values of "1" have been assigned as place holders in the data table. See GraphPad set-up in Appendix 2.

7.7.2 On the Excel worksheet titled "GraphPad Prism" copy cells D8 - G38 from the section "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 Log_{10} 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, 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 TOP1 levels (background subtracted) in pg/mL 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.

7.8 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 pg/mL TOP1 ranges (top and bottom end) supplied with the **specific lot** of control reagents used for the assay (cells C13 - D15).

7.8.2 Formulas within the worksheet will report the QC determination for the control samples with the following criteria:

- At least 2 of 3 controls must have a CV of < 20% for the replicate wells. If a control lysate has ≥ 20% CV (column H) and is listed as "Fail" in column I, go to SOP Step 7.8.5 and perform a Dixon's test for outliers.
- At least 2 of 3 controls must fall within the defined TOP1 pg/mL range provided for the **specific lot** of critical reagent (cells E13 - E15).

7.8.3 If either of these criteria are not met, the **Assay Fails QC** (cell F13). 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 lysate 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.

7.8.5 Dixon's Test Outliers on Triplicate Replicates of Control Samples (see Appendix 3 for more details)

Note: While the replicate values can be deleted on the "Control QC" Excel worksheet, the Excel worksheet titled "GraphPad Prism" and the GraphPad Prism file itself will serve as the source for unedited, transformed sample TOP1 values.

7.8.5.1 If a sample has a CV ≥ 20% (column H) 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 (C19 – E19). ***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."

7.8.5.2 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." Delete that value from the replicate data (columns C - E) and state in the "Notes" column "Outlier removed."

7.8.5.3 If one replicate is rejected as an outlier, the remaining two repeats must still meet the 20% CV rule. If not, the entire control (row) fails QC. Delete the mean value for that control (column F) and state "Control Fails %CV QC" in the "Notes" column.

7.8.5.4 If the %CV is ≥ 20%, but none of the replicates are rejected as an outlier, the entire control (row) fails QC. Delete the mean value for that control (column F) and state "Control Fails %CV QC" in the "Notes" column.

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 < 20%. If all samples Pass %CV QC, proceed to SOP Step 7.9.4.
- If an unknown sample has ≥ 20% 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 (see Appendix 3)

7.9.3.1 If a sample has a CV ≥ 20% (column H) 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 (C30 – E30). ***Note**: The values in columns C - E are auto-filled using an Excel formula; when copy and pasting to the Dixon's calculator, "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 E37 – E38). Delete that value from the replicate data (columns C - E) and state in the "Notes" column "Outlier removed."

7.9.3.3 If the 2 remaining repeat values (when one has been removed) have a %CV ≥ 20%, **OR** none of the replicates are rejected as an outlier when %CV is ≥ 20%:

- First assess if the mean (column F) is < LLQ or > ULQ (SOP Step 7.9.4); if it is, 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 TOP1 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 F30 – F31 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) so that it is not carried over to the TOP1 Determination worksheet 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 lower protein load/well as long as it falls within the dynamic range of the assay. If a sample is < LLQ and there is sufficient sample volume, it can be re-run at a higher protein load/well as long as the dilution of tumor lysate into 1X PBS-Casein in the final assay well is at least 1:20.

7.10 Dilution Linearity QC for Unknown Samples (Excel, "TOP1 Determination")

Note: The calculations set up in the lower portion of the "TOP1 Determination" worksheet for dilution linearity QC assume the Plate Map recommendations from SOP340701 were followed.

If both tumor biopsy and PBMC samples are being run on the same plate, the tumor biopsies should have been loaded first followed by the PBMC samples. For example, pre-dose biopsy dilutions in S1 - S3; post-dose in S4 - S6; and PBMC samples in S7 - S20. Every three tumor biopsy samples (e.g., S1, S2, S3) would represent the 6, 3, and 1.5 µg loads (or 4, 2, and 1 µg loads) from a single patient's tumor biopsy sample.

7.10.1 On the Excel worksheet titled "TOP1 Determination" using the Clinical Sample Calculation Table from the SOP340701 Batch Record, enter the following information:

7.10.1.1 Sample/patient ID (cells C6 - C25). Enter "no sample" if the wells were empty.

7.10.1.2 For tumor biopsy samples, enter the total µg protein loaded (e.g., 6, 3, or 1.5 µg/well) for each sample (cells D6 - D25); for PBMC samples, enter the relative cell number loaded in each well (cells F6 - F25).

- The total volume loaded per well, 100 µL, is calculated from SOP340701: 50 µL unknown sample plus 50 µL loading buffer per well.
- Since mean TOP1 values for samples < LLQ or > ULQ were deleted in SOP Step 7.9.4, they are not included in dilution linearity assessment or TOP1 level determination. If all three dilutions from a single sample are < LLQ or > ULQ, the sample is reported at the assay LLQ or ULQ level (see SOP Steps 7.11.4.2/.5.2).

7.10.2 On the bottom half of the "TOP1 Determination" worksheet, formulas will back-calculate the total TOP1 protein in pg/µg total protein (cells D32 - D51) for tumor biopsy samples or total TOP1 in pg/1 x 10^7 PBMCs/well (cells K32 - K51). 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 (e.g., 6, 3, and 1 µg loads) is determined by calculating the percent TOP1 in each dilution relative to the average of the three. Dilutions that are within 85% to 115% of the mean pass dilution linearity QC; any samples falling outside of this range will appear red in the worksheet (cells F32 - F51). Apply dilution linearity pass/fail criteria as follows:

- If **1 of 3 fails** dilution linearity, delete the TOP1 value for that sample (cells D32 - D51) and verify that the remaining 2 samples fall within the acceptable dilution linearity criteria. In the "Notes" column state "Sample failed dilution linearity."
- If **2 of 3 fail** dilution linearity, delete the TOP1 value (cells D32 - D51) that is farthest from 100% and verify that the remaining 2 samples fall within the acceptable dilution linearity. In the "Notes" column state "Sample failed dilution linearity."
- If **3 of 3 fail** dilution linearity OR **the 2 remaining** fail dilution linearity (when one has been removed), in the "Notes" column for all three samples state "All samples failed dilution linearity." 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 7.11.4.3.

7.10.4 For PBMC samples, confirm that the baseline sample is ≥ LLQ.

7.10.4.1 If the sample is ≥ LLQ, no further analysis is needed; proceed to SOP Step 7.11.

7.10.4.2 If the baseline sample is < LLQ, re-analyze only the baseline sample at both 250,000 cells/plate well as outlined in SOP340701 (1:4 dilution of stock lysate) and at 100,000 cells/plate well (1:10 dilution of stock lysate).

7.10.4.3 If upon re-analysis, the baseline sample is ≥ LLQ, report the TOP1 levels for the re-analyzed baseline sample in the Clinical Data Report (Appendix 3) in place of the sample run in this SOP and note the reason and deviation in both Batch Records (Appendix 1, Section 3). Also note the date of the re-run and Plate ID in the Batch Record (Appendix 1, Section 3).

7.10.4.4 If upon re-analysis, the baseline sample is still < LLQ, note in the Batch Record (Appendix 1, Section 3) and Clinical Data Report (Appendix 3) that a re-run was performed, but sample still read < LLQ.

7.11 Clinical Sample Data Report

Important: Decisions regarding levels of pre-dose TOP1 levels needed to measure a drug effect post-dose will be made by the Clinic or PD Laboratory Director/Supervisor and should not be reported in the Clinical Sample Data Report.

7.11.1 For **each patient**, compile the final TOP1 assay results using the Clinical Sample Data Report Template (Appendix 4).

7.11.2 Enter the patient and clinical protocol information.

7.11.3 The following abbreviations should be used for consistency. See details in next sections for when to use these abbreviations.

- < LLQ (< X.XX pg/µg) or > ULQ (> X.XX pg/µg): below or above cut-off for reportable assay results, respectively.
- NA: no biopsy provided;
- NR: not reportable – following descriptors should be added when appropriate
  - QC Fail: assay QC failure;
  - IP: insufficient protein

7.11.4 Report biopsy results to the Clinic using the information on the "TOP1 Determination" worksheet as follows:

7.11.4.1 Unknown samples should be reported as NR, with no value reported, if any of the following are true:

- The assay or sample failed QC and there is insufficient lysate to re-run the unknown (NR, QC Fail).
- A lysate sample had **≤ 0.25 µg/µL** total protein by BCA analysis (NR, IP) (SOP340702; this sample will not have been analyzed by the TOP1 Immunoassay).

7.11.4.2 Unknown samples should be reported as < LLQ or > ULQ, if the average TOP1 levels for **all three** dilutions were < LLQ or > ULQ, and there is insufficient lysate to re-run the unknown sample.

- The GraphPad-transformed Top1 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 "TOP1 Levels" column of the Clinical Sample Data Report (see below).
- For samples where **all three** 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 "TOP1 Determination" worksheet (cells E54-E59) to report the maximum TOP1 concentration in pg/µg 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 * 0.1 mL well volume]/protein load).

Example	**TOP1 Levels pg/µg protein**	**QC Note**
Baseline	31.35	
Post-Dose	< LLQ	4.89 pg/µg; max. possible TOP1 level

- If a sample was > ULQ, use the calculations at the bottom of the "TOP1 Determination" worksheet (cells G54-G59) to report the minimum TOP1 concentration in pg/µg 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 * 0.1 mL well volume]/protein load).

Example	**TOP1 Levels pg/µg protein**	**QC Note**
Baseline	1426.76	
Post-Dose	> ULQ	2,500 pg/µg; min. possible TOP1 level

7.11.4.3 For all other samples, report the TOP1 value as follows:

- For samples where **≥ 2 dilutions passed** the dilution linearity and other QC criteria; report the average TOP1 levels in pg/µg protein (cells E32 – E47).
- If TOP1 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 TOP1 readout value for that dilution and state "*xx µg protein load used; other dilutions < LLQ or > ULQ.*"
- If **3 of 3** OR **the 2 remaining** samples (when one has been removed) fail dilution linearity or other QC criteria, report the TOP1 levels in pg/µg protein for the dilution (i.e., S1, S2, or S3) with the lowest protein load that falls within the range of the assay. In addition, state "*lowest protein load used; sample dilutions non-linear*" in the "QC Note" section.

7.11.5 Report PBMC results to the Clinic using the information on the "TOP1 Determination" worksheet as follows:

7.11.5.1 Unknown samples should be reported as NR, with no value reported, if any of the following are true:

- The assay or sample failed QC and there is insufficient lysate to re-run the unknown: NR, QC Fail.
- A lysate sample had insufficient PBMCs (**≤ 1 x 10^6 PBMCs/mL** (originally <0.3 x 10^6 PBMCs total; SOP340702); this sample will not have been analyzed by the TOP1 Immunoassay: NR, IP.

7.11.5.2 Unknown PBMC samples should be reported as unquantifiable, if the average TOP1 levels for that PBMC sample were < LLQ or > ULQ, and there is insufficient lysate to re-run the unknown sample.

- The Graph-Pad transformed TOP1 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 "TOP1 Levels" column of the Clinical Sample Data Report (see below).
- For samples < LLQ or > ULQ a minimal or maximal 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 (or >ULQ), use the calculations at the bottom of the "TOP1 Determination" worksheet (cells K54-L57) to report the maximum (or minimum) TOP1 concentration in pg/1 x 10^7 cells that *could* have been present in the sample.

      The fold dilution of cells loaded per well from 1 x 10^7 cells is used for this calculation. The worksheet calculation is ([LLQ or ULQ value * 0.1 mL well volume]/ [1 x 10^7 cells / relative cell number in well]).

Example	**TOP1 Levels pg/1E7 PBMCs**	**QC Note**
C1, D1 4h	< LLQ	1954 pg/1E7 PBMCs; max. possible TOP1 level
C1, D18, 6h	> ULQ	250,000 pg/1E7 PBMCs; min. possible TOP1 level

7.11.5.3 For all other PBMC samples, report TOP1 in pg/1 x 10^7 cells (K32 – K51).

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 (Appendix 1) and Clinical Sample Data Report (Appendix 4) 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 (Appendix 4) for each patient should be sent to the clinical protocol Principal Investigator.

APPENDIX 1: BATCH RECORD

Certified Assay Operator: ____

Certification Number: ____________________

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. Name of saved TOP1 Excel data analysis workbook

____

Name of saved TOP1 GraphPad Prism file

____

B. Plate Map Set Up QC

☐ Recommended TOP1 Plate Map used from SOP340701

☐ Alternative plate map used; cells copy and pasted individually to the Plate Layout QC worksheet

Reason: ____

3. Notes, including any deviations from the SOP:

If assay fails QC, state the specific reason for assay failure and notify the Laboratory Director/Supervisor.

4. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: ____________________________________ (PRINT)

____________________________________ (SIGN)

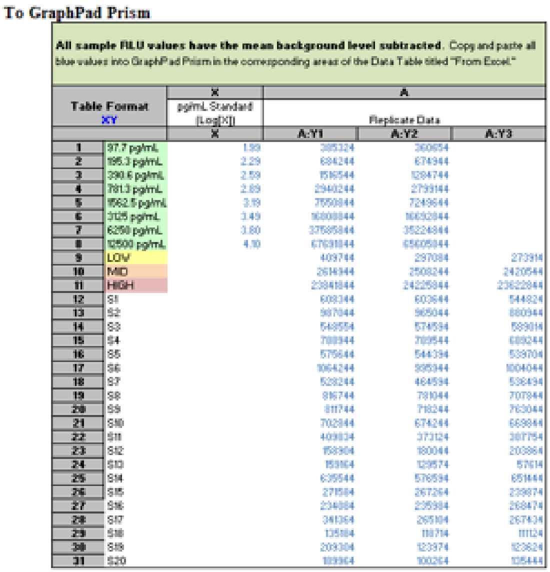
Date: ____________________

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 TOP1 immunoassay analysis GraphPad Prism template (SOP340704_TOP1_template.pzf) supplied during training has 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 sub-columns; plot the Mean only.
    b. Retitle the X and Y headers as shown in the GraphPad image below. 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.”

“GraphPad Prism” Excel worksheet



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		
		pg/mL Standard (Log[X])	Replicate Data		
		X	A:Y1	A:Y2	A:Y3
1	97.7 pg/mL	1.99	385324	360654	
2	195.3 pg/mL	2.29	684244	674944	
3	390.6 pg/mL	2.59	1516544	1284744	
4	781.3 pg/mL	2.89	2940244	2799144	
5	1562.5 pg/mL	3.19	7550844	7249644	
6	3125 pg/mL	3.49	16808844	16692844	
7	6250 pg/mL	3.80	37585844	35224844	
8	12500 pg/mL	4.10	67691844	65605844	
9	LOW		409744	297084	273914
10	MID		2614944	2508244	2420544
11	HIGH		23841844	24225844	23622844
12	S1		608344	603644	544824
13	S2		987044	965044	880944
14	S3		548554	574594	589014
15	S4		788944	789544	689244
16	S5		575644	544394	539704
17	S6		1064244	995944	1004044
18	S7		528244	464594	536494
19	S8		816744	781044	707844
20	S9		811744	718244	763044
21	S10		702844	674244	669844
22	S11		409834	373124	387754
23	S12		158904	180044	203864
24	S13		159164	129574	57614
25	S14		635544	576594	651444
26	S15		271584	267264	239874
27	S16		234084	235984	268474
28	S17		341364	265104	267434
29	S18		135184	118714	111124
30	S19		209304	123974	123624
31	S20		189964	100264	135444

➔

GraphPad Prism Data Table

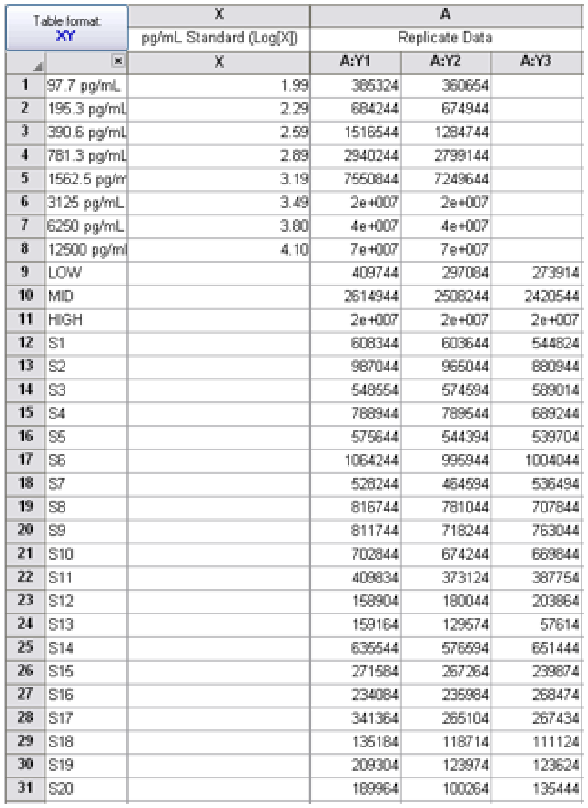


Table format: XY		X	A		
		pg/mL Standard (Log[X])	Replicate Data		
		X	A:Y1	A:Y2	A:Y3
1	97.7 pg/mL	1.99	385324	360654	
2	195.3 pg/mL	2.29	684244	674944	
3	390.6 pg/mL	2.59	1516544	1284744	
4	781.3 pg/mL	2.89	2940244	2799144	
5	1562.5 pg/m	3.19	7550844	7249644	
6	3125 pg/mL	3.49	2e+007	2e+007	
7	6250 pg/mL	3.80	4e+007	4e+007	
8	12500 pg/ml	4.10	7e+007	7e+007	
9	LOW		409744	297084	273914
10	MID		2614944	2508244	2420544
11	HIGH		2e+007	2e+007	2e+007
12	S1		608344	603644	544824
13	S2		987044	965044	880944
14	S3		548554	574594	589014
15	S4		788944	789544	689244
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17	S6		1064244	995944	1004044
18	S7		528244	464594	536494
19	S8		816744	781044	707844
20	S9		811744	718244	763044
21	S10		702844	674244	669844
22	S11		409834	373124	387754
23	S12		158904	180044	203864
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25	S14		635544	576594	651444
26	S15		271584	267264	239874
27	S16		234084	235984	268474
28	S17		341364	265104	267434
29	S18		135184	118714	111124
30	S19		209304	123974	123624
31	S20		189964	100264	135444

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.

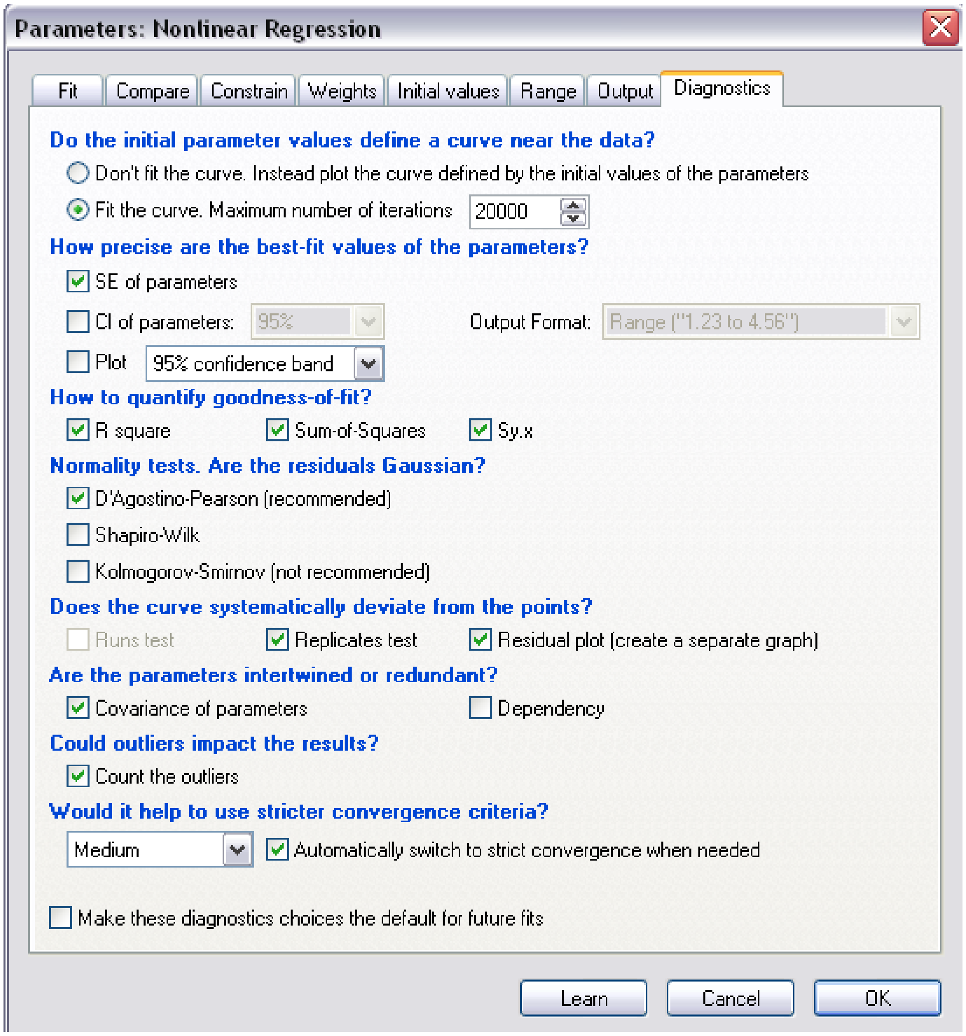
3) The non-linear regression (NLR) parameters window will appear. Tab-by-tab, make the following updates:

   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 $1/Y^2$ (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.

   d. Set up the Diagnostics tab as follows:



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

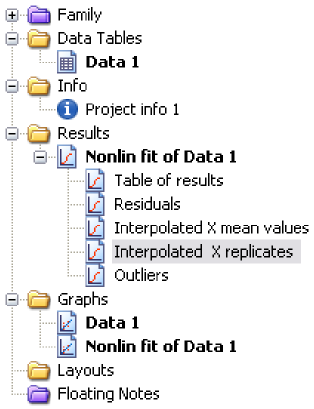
Would it help to use stricter convergence criteria?

Medium ☑ Automatically switch to strict convergence when needed

☐ Make these diagnostics choices the default for future fits

   e. Click OK.

4) A set of Nonlin fit Results sheets will be added to the GraphPad Prism navigator (image on right).



- Family
- Data Tables
  - **Data 1**
- Info
  - Project info 1
- Results
  - **Nonlin fit of Data 1**
    - Table of results
    - Residuals
    - Interpolated X mean values
    - Interpolated X replicates
    - Outliers
- Graphs
  - **Data 1**
  - **Nonlin fit of Data 1**
- Layouts
- Floating Notes

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 readout values for the plate repeats in units matching the standard curve.

APPENDIX 3: DIXON’S TEST FOR OUTLIERS

1. If the %CV for a triplicate well repeat is ≥ 20% a Dixon’s test for outliers can be used to determine if one cell can be eliminated as an outlier

2. The Dixon’s test uses the following criteria:

   A. 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 $X_1 < X_2 < X_3$.

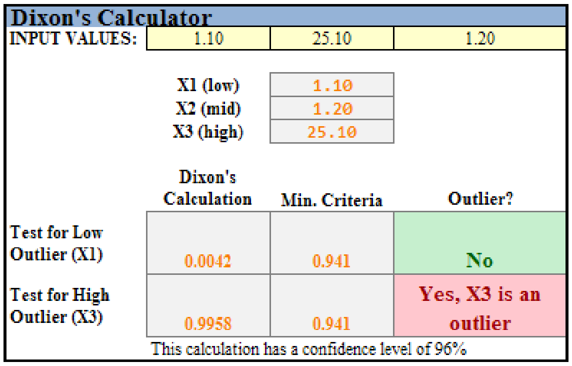
$$X_1 \text{ is an outlier if } \frac{(X_2 - X_1)}{(X_3 - X_1)} > 0.941$$

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

   B. 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.

   C. The TOP1 immunoassay uses the 0.941 confidence limit criterion.

3. 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%

4. 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.”

5. 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.

6. If the %CV is ≥ 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.

APPENDIX 4: CLINICAL SAMPLE DATA REPORT

TOP1 Immunoassay PD Analysis *Page 1 of 2*

Certified Assay Operator (Print): ______________________________

Certification #: ______________________ Today's Date: ________

Trial and Patient Information

Patient ID: Trial Site: ______________________

Clinical Center #: ______________________ CTEP Protocol #: ______________________

Assay Readout

Tumor Biopsy Samples

	Patient/Sample ID	Scheduled Collection Cycle, Day	Actual Collection Date	Time	TOP1 Levels pg/µg protein	QC Note
Baseline						
Post-Dose						

PBMC Samples

	Patient/Sample ID	Timepoint Cycle, Day	Actual Collection Date	Time	TOP1 Levels pg/1E7 PBMCs	QC Note
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						