Title:	HIF-1 alpha Immunoassay Quality Control, Data Analyses, and Reporting				Page 1 of 21
Doc. #:	SOP340904	Revision:	B	Effective Date:	6/23/2015

National Clinical Target Validation Laboratory

Applied/Developmental Research Directorate, Leidos Biomedical Research, Inc.

Frederick National Laboratory for Cancer Research

Technical Reviewer:	Yvonne A. Evrard	Date:	3 July 2015
NCTVL Approval:	Jiuping Ji	Date:	9 July 2015
IQC Approval:	Katherine V. Ferry-Galow	Date:	7-14-15
LHTP Approval:	Ralph E. Parchment	Date:	Ralph E. Parchment -S (Affiliate)
DCTD OD Approval:	Toby Hecht	Date:	Toby T. Hecht -S

Change History

Revision	Approval Date	Description	Originator	Approval
--	11/6/2012	New Document. Version for PADIS to NCTVL assay transfer. HIF-1 reporting values as pg/ug total protein per well.	KFG, YAE	KFG
A	9/29/2014	Changed unknown protein loads to 2 per sample at fixed µg loads for assay - based on clinical readiness data.	KFG, YAE	KFG
B	6/23/2015	Statement added to Scope defining the encountered limitations of the assay with clinical specimens.	YAE	KFG

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.

TABLE OF CONTENTS

OVERVIEW OF IMMUNOASSAY SAMPLE PROCESSING 3
1.0 PURPOSE 4
2.0 SCOPE 4
3.0 ABBREVIATONS 4
4.0 INTRODUCTION 5
5.0 ROLES AND RESPONSIBILITIES 5
6.0 MATERIALS AND EQUIPMENT REQUIRED 6
7.0 OPERATING PROCEDURES 7
APPENDIX 1: BATCH RECORD 15
APPENDIX 2: GRAPHPAD PRISM SET-UP 17
APPENDIX 3: DIXON'S TEST FOR OUTLIERS 20
APPENDIX 4: CLINICAL SAMPLE DATA REPORT 21

OVERVIEW OF IMMUNOASSAY SAMPLE PROCESSING

SOP340902: Tumor Frozen Needle Biopsy Collection and Handling for HIF-1 alpha	• Collect fresh needle biopsy into degassed HIF-1 alpha collection tubes (generally for measurement of HIF-1 alpha as a primary objective) or by dry flash freezing in 2-mL Sarstedt tubes • Immediately place in liquid nitrogen or on dry ice/ethanol • Ship to biopsy processing laboratory

SOP340910: Biopsy Specimen Processing for HIF-1 alpha Immunoassay	• Extract protein from tumor biopsy • Determine protein concentration • Store stock lysate or immediately proceed to immunoassay

SOP340903: HIF-1 alpha Immunoassay	• Perform ELISA with clinical samples, HIF-1 alpha standards, and controls • Using Tecan Microplate reader, determine relative signal of all samples

SOP340904: HIF-1 alpha Immunoassay Quality Control, Data Analyses, and Reporting	• Determine the HIF-1 alpha 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 hypoxia inducible factor-1 alpha (HIF-1 alpha) for pharmacodynamic (PD) studies.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the HIF-1 alpha Immunoassay during clinical trials. The goal of the SOP and associated training is to ensure consistency in analysis of HIF-1 alpha measurements across samples and clinical sites. The HIF-1 alpha immunoassay and associated specimen collection and processing procedure are recommended for use in clinical trials in which the drug treatment is associated with an increase in HIF-1 alpha levels.

Data generated using Phase I clinical trial samples suggest that this assay may not be sufficiently sensitive to measure HIF-1 alpha decreases in many human biopsy pre- and post-dose pairs (Park, S.R. et al., 2014, Anal Biochem). Analysis of NCI patient samples have established a minimal protein load needed to allow for measurable HIF-1 alpha levels: 60% of patient samples tested with < 6 µg protein/well were <LLQ while only 8% with ≥ 7.5 µg/well were <LLQ. These data are based on 36 patient specimens loaded at 3 different protein concentrations (108 dilutions total) ranging from 2.15-18.7 µg protein/well.

3.0 ABBREVIATONS

C	=	Control
CV	=	Coefficient of Variation
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
HIF-1 alpha	=	Hypoxia Inducible Factor-1 Alpha
IA	=	Immunoassay
ID	=	Identification / Identifier
IQC	=	Internal Quality Control
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
PADIS	=	Pharmacodynamics Assay Development & Implementation Section
PD	=	Pharmacodynamic
RLU	=	Relative Light Unit
SD, STDEV	=	Standard Deviation
SOP	=	Standard Operating Procedure
UA	=	Unanalyzable
ULQ	=	Upper Limit of Quantitation

4.0 INTRODUCTION

The HIF-1 alpha Immunoassay has been developed to use HIF-1 alpha as a metric for hypoxia in tumor biopsies. First HIF-1 alpha is captured from total cell extracts on plates coated with a HIF-1 alpha capture monoclonal antibody. The captured protein is then detected using a HIF-1 alpha polyclonal detection antibody followed by a streptavidin-HRP conjugate to allow chemiluminescent readout and quantitation of HIF-1 alpha levels. Assay quality control criteria are applied to the background, calibrator and control samples to validate the assay run. Quality control criteria are then applied to the unknown samples and data reporting guidelines are defined.

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 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. This report should state the HIF-1 alpha readout value or define the sample as unquantifiable or unanalyzable.

5.4 The responsible personnel are to check the DCTD Biomarkers web site (http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm) to verify that the latest SOP version is being followed.

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 SOP340903 (HIF-1 alpha Immunoassay)

6.4.2 Lot-specific ranges for High-, Mid-, and Low-C quality control samples

6.5 Template files supplied during training and available upon request:

6.5.1 HIF-1 alpha immunoassay analysis Excel workbook template Version 004: SOP340904_HIF1_alpha_template.xslt

6.5.2 HIF-1 alpha immunoassay analysis GraphPad Prism template Version 004: SOP340904_HIF1_alpha_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 Clinical Sample Calculation Table from SOP340903 will be needed. In addition, the lot-specific ranges for the High-, Mid-, and Low-C samples used in this assay will be needed for quality control analysis. **Important**: The HIF-1 alpha immunoassay analysis Excel workbook template used in this SOP is based on the 96-well sample designations in the Plate Map from SOP340903.

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 HIF-1 alpha 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 (SOP340904_HIF1_alpha_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 HIF-1 alpha Plate Map from SOP340903 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 SOP340903 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 color-coded Plate Map layout.

7.4.3.2 Once data has been pasted into the HIF-1 alpha 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 HIF-1 alpha 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 RLU values from the "Plate Layout QC" are auto-filled to the top of the "Assay QC" worksheet and formulas within this worksheet will automatically perform QC determination for the background wells. A total of 14 wells are used for background determination; the 4 corner wells and 2 wells adjacent to the high standard are not used for background level calculation.

7.5.2 A ± 2 SD criterion is applied to the initial 14-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 - F25 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 - F25 from the background dataset cells (B18 - E21).

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 7.8 to 15.6 pg/mL range of the standard curve, the mean RLU readout of the 7.8 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 7.8 pg/mL standard fails, then the mean RLU readout of the 15.6 pg/mL standard must be ≥ 3 SD above the mean RLU readout of the background.
- If the 15.6 pg/mL standard also fails, the **Assay Fails QC** (cell 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:** Formulas within the worksheet will automatically report the QC determination with the criteria listed below:

- The ratio for the lowest passing standard (7.8 to 15.6 pg/mL) RLU readout to the mean RLU readout of the background must ≥ 1.1. If not, the **Assay Fails QC** (cells H33).
- The ratio of the highest standard RLU readout (1000 pg/mL) to the mean RLU of the background must be ≥ 20. If not, the **Assay Fails QC** (cells 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 (SOP340904_HIF1_alpha_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 - G37 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 HIF-1 alpha 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 HIF-1 alpha 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 automatically report the QC determination for the control samples with following criteria:

- At least one control at each level (Low, Mid, and High) must have a CV of < 20% for the replicate wells (cells G6 - H11).
- At least one control at each level (Low, Mid, and High) and at least 4 of 6 controls overall must fall within the defined HIF-1 alpha pg/mL range provided 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 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.

7.9 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 for more details)

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 (C26 - E26). ***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 E33 - E34). 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 HIF-1 alpha 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 F26 - F27 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 HIF-1 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 µg load/well as long as it falls within the dynamic range of the assay.

7.10 Dilution Linearity QC for Unknown Samples (Excel, "HIF-1 Determination")

Note: The calculations set up in the lower portion of the "HIF-1 Determination" worksheet for dilution linearity QC assume the Plate Map recommendations from SOP340903 were followed. Every two samples (e.g., S1 and S2) would represent two protein loads from a single patient's tumor biopsy sample (e.g., 15 and 7.5 µg/well if the stock lysate were > 1.0 µg/µL, or 7.5 µg/well and a blank [buffer only] if the stock lysate were between 0.5 and 0.99 µg/µL).

7.10.1 On the Excel worksheet titled "HIF-1 Determination" using the Sample Calculation Table from the SOP340903 Batch Record, enter the following information:

7.10.1.1 Sample/patient ID (cells C5 - C20). Enter "no sample" if the wells were empty.

7.10.1.2 Enter the total µg protein loaded (e.g., 15 and 7.5 µg if the stock lysate were > 1.0 µg/µL, or 7.5 and 0 µg if the stock lysate were between 0.5 and 0.99 µg/µL) for each sample (Cells D5-D20).

- The total volume loaded per well, 100 µL, is calculated from SOP340903: 15 µL unknown sample lysate plus 85 µL loading buffer per well.
- Since mean HIF-1 alpha values for samples < LLQ or > ULQ were deleted in SOP Step 7.9.4, they are not included in dilution linearity assessment or HIF-1 alpha level determination. If both dilutions from a single sample are < LLQ or > ULQ, the sample is reported as unquantifiable (SOP Step 7.11.3.2).

7.10.2 On the bottom half of the "HIF-1 Determination" worksheet, formulas will back-calculate the total HIF-1 alpha protein in pg/µg total protein (cells D26 - D41) using the data entered for the 2 dilutions loaded.

7.10.3 Dilution linearity is determined by calculating the percent HIF-1 alpha in each dilution relative to the average. Dilutions that are within 85% and 115% of the mean pass dilution linearity QC; if the sample agreement falls outside of this range the cells will appear red in the worksheet (cells F26 - F41). Apply dilution linearity pass/fail criteria as follows:

- Patients with a paired 7.5 and 15 µg/well load
  - If the two sample dilutions corresponding to a 7.5 and 15 µg/well load fail to agree, in the "Notes" column for both samples, state "Samples fail 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 7.11.3.3.
- Patients with only a 7.5 µg/well load
  - By definition the dilution linearity will fail for the blank well. As long as the sample was >LLQ or <ULQ, report the calculated pg/µg.

7.11 Clinical Sample Data Report

Important: Decisions regarding levels of pre-dose HIF-1 alpha 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 assay HIF-1 alpha assay results using the Clinical Sample Data Report Template (Appendix 4).

7.11.2 Enter the patient and clinical protocol information.

7.11.3 Report results to the Clinic using the information on the "HIF-1 Determination" worksheet as follows:

7.11.3.1 Unknown samples should be reported as unanalyzable (UA), 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.
- A lysate sample had **≤ 0.5 µg/µL** total protein by BCA analysis (SOP340910; this sample will not have been analyzed by the HIF-1 alpha Immunoassay).

7.11.3.2 Unknown samples should be reported as unquantifiable, if the average HIF-1 alpha levels for **both** dilutions were < LLQ or > ULQ and there is insufficient lysate to re-run the unknown sample.

- The GraphPad transformed HIF-1 alpha 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 "HIF-1 alpha 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 "HIF-1 Determination" worksheet (cells D46-E48) to report the maximum HIF-1 alpha 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	**HIF-1 alpha Levels pg/µg protein**	**QC Note**
Baseline	0.77	
Post-Dose	< LLQ	0.04 pg/ug; max. possible HIF-1 alpha level

- o If a sample was > ULQ, use the calculations at the bottom of the "HIF-1 Determination" worksheet (cells F46-G48) to report the minimum HIF-1 alpha 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	**HIF-1 alpha Levels pg/µg protein**	**QC Note**
Baseline	19.17	
Post-Dose	> ULQ	10.0 pg/µg; min. possible HIF-1 alpha level

7.11.3.3 For all other samples, report the HIF-1 alpha value as follows:

- For samples where **both dilutions passed** the dilution linearity criteria, report the average HIF-1 alpha levels in pg/µg total protein (cells E26 – E41).
- If HIF-1 alpha levels for **only 1 dilution** was within the dynamic range of the assay (> LLQ or < ULQ), or only the 7.5 µg protein load was used, report the HIF-1 alpha readout value for that dilution and state "xx µg protein load used."

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 HIF-1 alpha Excel data analysis workbook

_____

Name of saved HIF-1 alpha GraphPad Prism file

_____

B. Plate Map Set Up QC

- ☐ Recommended HIF-1 alpha Plate Map used from SOP340903
- ☐ 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: _____

BATCH RECORD: INITIALS _____ DATE: _____

PAGE LEFT BLANK ON PURPOSE

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 HIF-1 alpha immunoassay analysis GraphPad Prism template (SOP340904_HIF1_alpha_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	7.8 pg/mL	0.89	5620	5364	
2	15.6 pg/mL	1.19	14003	13233	
3	31.3 pg/mL	1.50	24868	24354	
4	62.5 pg/mL	1.80	53878	51910	
5	125 pg/mL	2.10	104486	102087	
6	250 pg/mL	2.40	202475	202733	
7	500 pg/mL	2.70	375588	378169	
8	1000 pg/mL	3.00	650867	654928	
9	LOW 1		46432	39329	
10	LOW 2		37446	39585	
11	MID 1		219294	208396	
12	MID 2		197757	191666	
13	HIGH 1		549343	545892	
14	HIGH 2		515537	517865	
15	S1		60127	54820	61240
16	S2		41725	43779	41126
17	S3		34023	31370	35991
18	S4		41554	43351	41126
19	S5		35991	33938	41554
20	S6		27520	25467	20676
21	S7		112625	103715	117423
22	S8		76052	91124	95577
23	S9		63123	63637	69288
24	S10		102087	98661	103972
25	S11		86842	82131	104743
26	S12		61154	67318	68688
27	S13				
28	S14				
29	S15				
30	S16				

GraphPad Prism Data Table

Table format: XY		X	A		
		pg/mL Standard (Log[X])	Replicate Data		
		X	A:Y1	A:Y2	A:Y3
1	7.8 pg/mL	0.89	5620	5364	
2	15.6 pg/mL	1.19	14003	13233	
3	31.3 pg/mL	1.50	24868	24354	
4	62.5 pg/mL	1.80	53878	51910	
5	125 pg/mL	2.10	104486	102087	
6	250 pg/mL	2.40	202475	202733	
7	500 pg/mL	2.70	375588	378169	
8	1000 pg/mL	3.00	650867	654928	
9	LOW 1		46432	39329	
10	LOW 2		37446	39585	
11	MID 1		219294	208396	
12	MID 2		197757	191666	
13	HIGH 1		549343	545892	
14	HIGH 2		515537	517865	
15	S1		60127	54820	61240
16	S2		41725	43779	41126
17	S3		34023	31370	35991
18	S4		41554	43351	41126
19	S5		35991	33938	41554
20	S6		27520	25467	20676
21	S7		112625	103715	117423
22	S8		76052	91124	95577
23	S9		63123	63637	69288
24	S10		102087	98661	103972
25	S11		86842	82131	104743
26	S12		61154	67318	68688
27	S13				
28	S14				
29	S15				
30	S16				

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.

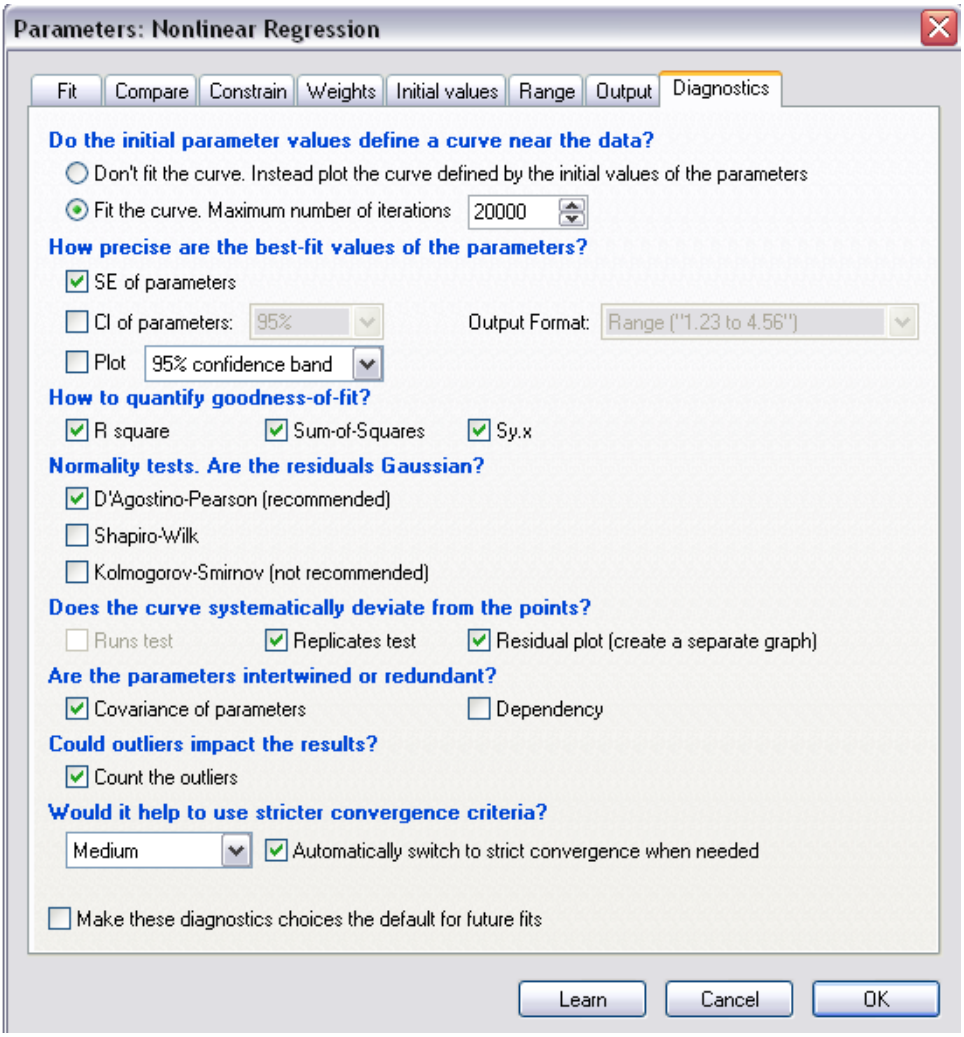
3) The non-linear regression (NLR) parameters window will appear. Tab-by-tab, make the following updates:

   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."

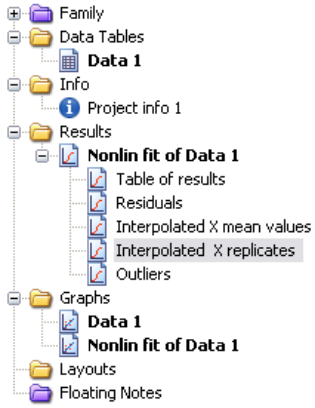
   c. On the Output tab under 'Location of interpolated X values,' select "Y column, maintaining the side-by-side arrangement of replicates." Change Number of digits in output to 2 significant digits

   d. Set up the Diagnostics tab as follows:



   e. Click OK.

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 readout values for the plate repeats in pg/mL. These data are copy and pasted back into the “GraphPad Prism” Excel worksheet.

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 HIF-1 alpha 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

HIF-1 alpha 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

	Patient/Sample ID	Scheduled Collection Cycle, Day	Actual Collection Date, Time	HIF-1 alpha Levels pg/µg protein	QC Note
Baseline					
Post-Dose					

Additional Information

Raw data is 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.

Abbreviations:

LLQ, lower limit of quantitation; QC, quality control; UA, unanalyzable; ULQ, upper limit of quantitation

Biopsy and Treatment Information (for use by Clinical site)

Site of Biopsy: _____ Primary Tumor: _____

Dose Level: _____

Agent Name(s): _____ + _____

Dose and Units: _____ + _____