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National Clinical Target Validation Laboratory

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

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Change History

Revision	Approval Date	Description	Originator	Approval
--	5/29/2012	New Document. Version for PADIS to NCTVL assay transfer.	SRP, YAE	RJK
A	11/6/2012	Plates and adhesive sealers removed from critical reagent list. Critical Reagent storage conditions removed from SOP; to be supplied with the reagent shipping manifest. Requirements for digital sample tables added to SOP Step 5.3.	YAE, KFG	KFG
B	9/29/2014	Define room temperature. Thermoconductive plate holder used for 25°C incubation steps, all incubations in fixed-temperature incubators. Changed unknown protein loads to 2 per sample at fixed µg loads for assay - based on clinical readiness data.	YAE, KFG	KFG
C	6/23/2015	Statement added to Scope defining the encountered limitations of the assay with clinical specimens. Updated catalog numbers for discontinued items.	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.

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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

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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

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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

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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-1α) 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 HIF-1α immunoassay performance 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

2HG	=	2-Hydroxyglutarate
BSA	=	Bovine Serum Albumin
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
HIF-1 alpha	=	Hypoxia Inducible Factor-1 Alpha
H-CEB	=	HIF-1 Alpha Cell Extraction Buffer
HRP	=	Horseradish Peroxidase
IA	=	Immunoassay
ID	=	Identification / Identifier
LHTP	=	Laboratory of Human Toxicology and Pharmacology
NCTVL	=	National Clinical Target Validation Laboratory
PADIS	=	Pharmacodynamics Assay Development & Implementation Section
PBS	=	Phosphate Buffered Saline
PD	=	Pharmacodynamic
PI	=	Protease Inhibitor
PMSF	=	Phenylmethylsulfonyl Fluoride
RLU	=	Relative Light Unit
SD	=	Standard Deviation
SDS	=	Sodium Dodecyl Sulfate
SOP	=	Standard Operating Procedure

4.0 INTRODUCTION

The HIF-1 alpha Immunoassay has been developed to use HIF-1 alpha as a metric for hypoxia in tumor biopsies. An ELISA is used to first capture HIF-1 alpha protein 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.

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 assay is to follow this SOP and complete the required tasks and associated documentation. The Plate Map Design (Appendix 1) and Batch Record (Appendix 2) must be completed in ***real-time*** for each experimental run, with each page ***dated and initialed,*** and placed with the clinical sample information.

5.3 Digital versions of the sample tables in the Batch Record (Appendix 2, Sections 5) can be created for logging sample information as long as all column information exactly matches the tables in the Batch Record. A copy of the completed, digital sample tables must be printed and attached to the Batch Record in order to maintain a complete audit trail.

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

6.0 CRITICAL REAGENTS, MATERIALS, AND EQUIPMENT REQUIRED

6.1 PADIS/IQC-Supplied Critical Reagents

6.1.1 DUOSet IC ELISA Kit, for detection of human/mouse total HIF-1 alpha (R&D Systems, Inc., Cat#: DYC1935-5 or DYC1935E); manufacturer instructions removed

6.1.2 HIF-1 alpha tumor lysate controls (custom preparation)

6.1.3 D-2-hydroxypentanedioic acid disodium salt (2R), MW = 192.08 (2-hydroxyglutarate [2HG]; CAS#: 103404-90-6; 3B Scientific Corporation, Cat#: 3B3-053228)

6.1.4 LumiGLO Chemiluminescent Substrate Solutions (KPL, Cat#: 54-61-02)

6.2 Pipettors (200-1000 µL, 50-200 µL, 2-20 µL) and tips

6.3 Pipettor, 10 µL single-volume and tips

6.4 Multichannel pipettors, 8-well (50-300 µL, 5-50 µL) and tips

6.5 Reagent reservoirs (Fisher Scientific, Cat#: 13-681-509)

6.6 1.5-mL Sarstedt tubes, o-ring screw cap, conical bottom (Sarstedt, Cat#: 72.692.005)

6.7 15-mL polypropylene tubes (e.g., VWR, Cat#: 21008-918)

6.8 50-mL polypropylene tubes (e.g., VWR, Cat#: 21008-951)

6.9 Reacti-Bind White Opaque 96-well Plate (Fisher Scientific, Cat#: 15042)

6.10 Acetate plate sealers (Fisher Scientific, Cat #: 14-245-18)

6.11 Ice bucket

6.12 UltraPure DNase/RNase-free distilled water (e.g., Life Technologies, Cat#: 10977-015)

6.13 20% sodium dodecyl sulfate (SDS; e.g., Sigma-Aldrich, Cat#: 05030-500ML-F)

6.14 Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P2714 or Roche Diagnostics, Cat#: 11697498001)

6.15 Phenylmethanesulfonyl fluoride solution, 0.1 M (PMSF; Sigma-Aldrich, Cat#: 93482-50ML-F)

6.16 Tris, ultra pure (e.g., MP Biomedicals, Cat#: 04819620 or 04819623)

6.17 Sodium chloride, ReagentPlus grade (e.g., Sigma-Aldrich, Cat#: S9625)

6.18 Glycerol, 100% w/v (e.g., Sigma-Aldrich, Cat#: G5516)

6.19 EDTA, 0.5 M, pH 8.0 (e.g., Boston BioProducts, Cat#: BM-150)

6.20 Magnesium chloride, anhydrous (e.g., Sigma-Aldrich, Cat#: M8266)

6.21 β-Glycerol phosphate disodium salt, pentahydrate (e.g., Sigma-Aldrich, Cat#: 50020)

6.22 Sodium fluoride, ACS grade (e.g., Sigma-Aldrich, Cat#: 201154)

6.23 10X Phosphate Buffered Saline, pH 7.2 (PBS; e.g., Life Technologies, Cat#: 70013-073)

6.24 Triton X-100, non-ionic, aqueous solution, 10% w/v (e.g., Roche Diagnostics, Cat#: 11332481001)

6.25 Tween 20, non-ionic, aqueous solution, 10% w/v (Roche Diagnostics, Cat#: 11332465001 or Life Technologies, Cat#: 28320)

6.26 Albumin, bovine serum (BSA; Sigma-Aldrich, Cat#: A7030)

6.27 Sorvall Fresco microcentrifuge (Thermo Scientific)

6.28 BioTek ELx405 Select or ELx405 Microplate Washer (BioTek Instruments)

6.29 Non-humidified, fixed temperature incubator able to maintain 25°C (± 3°C)

6.30 Biocision CoolSink 96F Themoconductive Plate for Flat Bottom Plates, (VWR, Cat#: 95045-476)

6.31 Tecan Infinite M200 or M1000 Microplate Reader (Tecan US)

6.32 -80°C freezer

6.33 2°C to 8°C refrigerator

6.34 Biopsy sample extracts prepared following SOP340910 (Biopsy Specimen Processing for HIF-1 alpha Immunoassay)

7.0 OPERATING PROCEDURES

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

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

7.3 **PADIS/IQC-Supplied**Critical Reagents

Important: While the DUOSet IC ELISA Kit from R&D Systems is being used, modifications have been made to the assay process in order to create a validated method for use on first-in-human clinical samples. Discard the manufacturer's instructions and follow the SOP as written.

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

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

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

7.3.2.1 **DUOSet IC ELISA Kit – HIF-1 alpha Standard**: Provided as a lyophilized powder in the manufacturer-supplied vial from a qualified Kit lot. According to the manufacturer, the standard is purified recombinant human HIF-1 alpha (amino acid residues 575-826).

7.3.2.2 **DUOSet IC ELISA Kit – HIF-1 alpha Capture Ab**: Provided as lyophilized powder in the manufacturer-supplied vial from a qualified Kit lot.

7.3.2.3 **DUOSet IC ELISA Kit – HIF-1 alpha Detection Ab**: Provided as lyophilized powder in the manufacturer-supplied vial from a qualified Kit lot.

7.3.2.4 **DUOSet IC ELISA Kit – Streptavidin-HRP Conjugate**: Supplied as a 1-mL stock solution in the manufacturer-supplied vial from a qualified Kit lot.

7.3.2.5 **Tumor Lysate Controls**: Lysates are prepared from a human-origin cancer cell lines cultured in vitro spiked with lysate from a transient transfectant of HIA-1 alpha to meet pre-determined criteria for High, Mid, and Low analyte levels. Each stock solution is 0.67 µg/µL (10 µg protein in 15 µL load volume).

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

7.3.2.7 **2HG**: Supplied as a 10 mM stock solution.

7.4 **Plate Map and Reagent Preparation**

7.4.1 Based on the number of patient samples to be analyzed, generate a Plate Map (Appendix 1) to define the location and replicates of clinical samples, control samples, and HIF-1 alpha standards. A single patient's **batched** samples should be contained on one 96-well plate, not split over two, to ensure consistent sample handling.

Important: The data analyses template (SOP340904) is based on the 96-well sample designations in the Plate Map (Appendix 1). To prevent user errors, always load the plate according to the plate map well designations.

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

7.4.3 Record serial numbers of equipment in the Batch Record (Appendix 2, Section 2A). Prepare the Wash Buffer, Reagent Diluent, and H-CEB (**without** PIs+2HG) as outlined in the Batch Record (Appendix 2, Section 2B). Do not prepare H-CEB (**Complete**) until stated in the SOP.

7.4.4 If reconstituting HIF-1 alpha capture or detection Ab from a new Kit, use the calculations in the Batch Record (Appendix 2, Section 2C) to prepare the stock solutions.

7.4.4.1 After reconstitution, aliquots can be stored at 2°C to 8°C for up to 2 wk or -80ºC in single use aliquots for up to 3 mo, but must be used before the Kit expiration date.

7.4.5 All 25°C incubation steps for the HIF-1 alpha assay will be carried out in fixed-temperature incubators. Each 96-well plate will be placed on a CoolSink thermoconductive plate during these incubation steps.

7.4.5.1 Place a sufficient number of CoolSink thermoconductive plates inside each incubator **at least 1h prior** to the initiation of each incubation step. For each assay plate, one thermoconductive plate will be placed in a 25°C incubator.

7.4.5.2 The plates should be placed horizontally inside the incubator in direct contact with the incubator bottom or shelf and should not be stacked. The assay plate will be placed and carefully centered onto a prewarmed thermoconductive plate inside the incubator for each step.

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

7.5 **Plate Preparation**

7.5.1 Use the calculations in the Batch Record (Appendix 2, Section 3A) to prepare 11 mL **HIF-1 alpha Coating Solution** for the assay. This is sufficient for one 96-well plate (preparing enough for 110 wells).

7.5.1.1 If more than one 96-well plate is to be coated, pool the aliquots and then dilute appropriately. This will ensure that all plates are exposed to identical coating antibody. Discard excess diluted antibody.

7.5.2 Add 100 µL of the **HIF-1 alpha Coating Solution** per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate at 2°C to 8°C for 16 ± 1 h. Record the coating antibody incubation conditions in the Batch Record (Appendix 2, Section 3B).

7.5.3 Following incubation with the **HIF-1 alpha Coating Solution**, aspirate the plate using a plate washer (for the BioTek Plate Washer, use the *Aspirate* program). Immediately wash the plate 3 times with 400 µL Wash Buffer, aspirating the plate between each wash and being sure no residual liquid remains.

For the BioTek Microplate Washer, the settings are:

METHOD	**ELx405 Select**	**ELx405**
Number of Cycles:	3	3
Soak/Shake:	Yes	Yes
Soak Time:	5 sec	5 sec
Shake Before Soak:	No	No
Prime After Soak:	No	No
DISPENSE		
Dispense Volume:	400 µL/well	400 µL/well
Dispense Flow Rate:	06	06
Dispense Height:	120 (15.240 mm)	120 (15.240 mm)
Horizontal DISP POS:	00 (0.000 mm)	00 (0.000 mm)
Bottom Wash First:	No	No
Prime Before Start:	No	No
ASPIRATE		
Aspirate Height:	028 (3.556 mm)*	028 (3.556 mm)*
Horizontal ASPR POS:	-40 (-1.829 mm)*	-30 (-1.372 mm)*
Aspiration Rate:	05 (6.4 mm/sec)	05 (6.4 mm/sec)
Aspirate Delay:	1000 MSec	1000 MSec
Crosswise ASPIR:	No	No
Final Aspiration:	Yes	Yes
Final Aspirate Delay:	1000 MSec	1000 MSec

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

7.5.4 After the wash, tap the plate on paper towels to remove residual buffer. Proceed immediately to the next step; do not allow the plate to dry out.

7.5.5 Add 300 µL of Reagent Diluent to each well. Cover the plate with an acetate sheet and incubate in a fixed-temperature incubator at 25°C for 1 to 2 h on the prewarmed CoolSink thermoconductive plate. Record the blocking incubation conditions in the Batch Record (Appendix 2, Section 4).

7.6 Preparation of HIF-1 alpha Standards, Lysate Controls, and Unknown Sample Lysates

7.6.1 Preparation of HIF-1 alpha standards; run in duplicate

7.6.1.1 Use the calculations in the Batch Record (Appendix 2, Section 5.A.a.) to prepare a 100 ng/mL HIF-1 alpha standard stock solution in Reagent Diluent.

7.6.1.2 Label eight 1.5-mL tubes, numbered 1 through 8, for the HIF-1 alpha standards. Prepare the HIF-1 alpha standards by serial dilution with final concentrations of 1000 to 7.8 pg/mL in Reagent Diluent as outlined in the Batch Record (Appendix 2, Section 5.A.b.). Use a 10 µL single-volume pipettor when preparing the first standard.

7.6.1.3 Standards will be added directly to the 96-well plate with no further dilution.

7.6.1.4 Keep diluted standards on ice until use. Diluted standards should be used within 1 h. Only make enough standards for the assay and discard any excess stock or diluted standards.

7.6.2 Preparation of lysate controls; run twice on plate in duplicate

7.6.2.1 For one 96-well plate, retrieve one each High-, Mid-, and Low-Control (C) lysate control vials from the -80°C freezer and thaw on ice; vortex and mix by inverting 5-8 times before use. If more than one 96-well plate is being run, pool the lysate controls from the same lot prior to loading.

7.6.2.2 Controls will be loaded as-is at 15 µL/well and will be diluted 6.7-fold with Reagent Diluent once loaded into the 96-well plate.

7.6.2.3 Keep samples on ice until use. Only thaw enough control lysate for the assay and discard any excess.

7.6.3 Preparing Dilutions of Unknown Biopsy Lysates

7.6.3.1 Samples with total protein concentration of <**0.5 µg/µL** will not be used in the HIF-1 alpha immunoassay and will be reported as unanalyzable in the Clinical Sample Data report (SOP340904).

7.6.3.2 Place all stock biopsy lysates to be assayed on ice. Based on the protein concentration for the **stock tumor lysate** (µg/µL), prepare one of the following Lysates Stocks in H-CEB (Complete) on ice for use in the HIF-1 alpha immunoassay.

Important: Pre- and post-treatment biopsies from a single patient should be prepared with matched protein concentrations (matched to sample with lowest protein concentration).

7.6.3.3 For unknown stock lysates with a protein concentration of **≥1.0 µg/µL**:

- Perform the following calculation to prepare 2 different **Working Lysate** dilutions (1.0 and 0.5 µg/µL) in H-CEB (**Complete**).

$$\frac{\text{(1.0 or 0.5) µg/µL } \textbf{Working Lysate} \quad * \quad 60\ \mu L}{\text{XXX µg/µL Conc. Stock Lysate}} = \underline{\text{XX}}\ \mu L \text{ Vol. Stock Lysate to use}$$

- This is sufficient volume to run each dilution in triplicate (+1 well extra) giving a final concentration of 15 and 7.5 µg/well when loaded at 15 µL/well.
- For example (see Appendix 1 Plate Map and Appendix 2, Section 5B.a.), S1 wells would be loaded with 15 µL 1.0 µg/µL protein lysate each and the S2 triplicate wells would be loaded with 15 µL 0.5 µg/µL protein lysate each.
- Clearly label each tube with the sample number (e.g., S1, S2).
- Record volume stock lysate and H-CEB (**Complete**) used to prepare each **Working Lysate** in the Batch Record (Appendix 2, Section 5B.a.).

7.6.3.4 For unknown stock lysates with a protein concentration of **between 0.50 and 0.99 µg/µL**:

- Perform the following calculation to prepare a **Working Lysate** dilution of 0.5 µg/µL in H-CEB (Complete).

$$\frac{\text{0.5 µg/µL } \textbf{Working Lysate} \quad * \quad 60\ \mu L}{\text{XXX µg/µL Conc. Stock Lysate}} = \underline{\text{XX}}\ \mu L \text{ Vol. Stock Lysate to use}$$

- This is sufficient volume to run the dilution in triplicate (+1 well extra) giving a final concentration of 7.5 µg/well when loaded at 15 µL/well.
- In addition, prepare a second tube with 60 µL of H-CEB (Complete). This will be loaded in triplicate wells paired with the 0.5 µg/µL sample. For example (see Appendix 1 Plate Map and Appendix 2, Section 5B.a.), S3 wells would be loaded with 15 µL protein lysate each and the S4 triplicate wells would be loaded with 15 µL H-CEB (Complete). This is **essential** to ensure the 96-well plate data analysis Excel in SOP340904 reports the specimen levels correctly.
- Clearly label each tube with the sample number (e.g., S3, S4).
- Record volume stock lysate and H-CEB (**Complete**) used to prepare each **Working Lysate** in the Batch Record (Appendix 2, Section 5B.a.).

7.6.3.5 In labeled 1.5-mL tubes, add sufficient H-CEB (**Complete**) to the calculated volume of stock lysate to create the Working Lysate dilutions with a final volume to 60 µL each.

7.6.3.6 Keep samples on ice until use. Only aliquot enough of each clinical sample for the assay. Flash freeze remaining stock lysate in liquid nitrogen or dry ice/ethanol bath and return to -80°C freezer.

7.7 HIF-1 alpha Protein Capture

7.7.1 Following incubation with Reagent Diluent (SOP Step 7.5.5), aspirate and wash the plates 3 times with 400 µL of Wash Buffer using a plate washer (same wash program as SOP Step 7.5.3).

7.7.2 After the wash, tap the plate on paper towels to remove residual buffer. Proceed immediately to the next step; do not allow the plate to dry out.

7.7.3 Immediately add 85 µL of Reagent Diluent Columns 1-5 and 8-12 **ONLY** of the Reacti-Bind assay plate to each well using a multichannel pipettor. Each well will hold a final volume of 100 µL after sample addition.

Important: **DO NOT** add Reagent Diluent to Columns 6 and 7 of the Reacti-Bind assay plate, these will contain 100 µL HIF-1 alpha standards.

7.7.4 Use the Plate Map Design (Appendix 1) and the Sample Calculation Table (Appendix 2, Section 5B.a.) as a guide to set up the 96-well plate for incubation with HIF-1 alpha standards (SOP Step 7.6.1), tumor cell controls (SOP Step 7.6.2), and clinical samples (SOP Step 7.6.3). Pipette reagents in the following order; **do not deviate** from order of addition:

Order	Sample/Reagent and Volume
1	100 µL of HIF-1 alpha standards into designated duplicate wells (columns 6-7); contains 0 µL Reagent Diluent. Load the lowest concentration first.
2	15 µL of each clinical sample into designated triplicate wells
3	15 µL each of tumor controls (Low-C, Mid-C, and High-C) into both sets of designated duplicate wells
4	15 µL of additional Reagent Diluent into each of the Reagent Diluent Only well

7.7.5 Cover the plate with an acetate sheet and incubate at 2°C to 8°C for 16 ± 1 h. Record the date, start time, and incubation temperature in the Batch Record (Appendix 2, Section 6).

7.8 HIF-1 alpha Detection (next day)

7.8.1 Place a sufficient volume of Reagent Diluent for preparation of the HIF-1 alpha detection Ab working solution and the streptavidin-HRP conjugate working solution at ambient temperature for one hour prior to proceeding with the HIF-1 alpha Detection.

7.8.2 Using the calculations in Appendix 2, Sections 7A, prepare a sufficient amount of HIF-1 alpha detection Ab working solution in Reagent Diluent before washing the plate (next step) that has been incubating with samples.

7.8.3 After the 16-h incubation is complete, aspirate and wash the wells 3 times with 400 µL of Wash Buffer (same wash program as SOP Step 7.5.3). Record the date and stop time samples were removed from the wells in the Batch Record (Appendix 2, Section 6).

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

7.8.5 Add 100 µL of the HIF-1 alpha detection Ab working solution per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate for 2 h in a fixed temperature incubator at 25°Con a prewarmed CoolSink thermoconductive plate. Discard residual working solution and record the incubation conditions in the Batch Record (Appendix 2, Section 7B).

7.8.6 Just before the 2-h incubation with HIF-1 alpha detection Ab working solution is complete, prepare a sufficient amount of streptavidin-HRP conjugate working solution for the assay. See Appendix 2, Section 8A for conjugate preparation and record the time of preparation. Keep streptavidin-HRP conjugate working solution in the dark at ambient temperature.

7.8.7 Once the 2-h HIF-1 alpha detection Ab incubation is complete, aspirate and wash the wells 3 times with 400 µL Wash Buffer (same wash program as SOP Step 7.5.3). Tap plate on paper towel to remove residual liquid and proceed immediately to the next step.

7.8.8 Add 100 µL of the streptavidin-HRP conjugate working solution per well using a multichannel pipettor. Cover the plate with an acetate sheet and incubate in the dark for 30 min in a fixed temperature incubator at 25°C on a prewarmed CoolSink thermoconductive plate. Discard residual working solution and record the incubation conditions in the Batch Record (Appendix 2, Section 8B).

7.9 **Signal Detection**

7.9.1 Move the LumiGLO Solutions to ambient temperature and turn on the Tecan Microplate Reader at least 30 min before use. For luminescence optical density readings, the plate reader should be set to the following reading parameters:

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

7.9.2 Just before the streptavidin-HRP conjugate incubation is finished, prepare the ambient LumiGLO Chemiluminescent Substrate as outlined in Appendix 2, Section 9A, being sure to note the time of preparation. This must be made up immediately before use, kept in the dark, and at a sufficient volume for the assay.

7.9.3 After the 30 min streptavidin-HRP conjugate incubation is complete, aspirate and wash the wells 3 times with 400 µL Wash Buffer (same program as SOP Step 7.5.3). Tap plate on paper towel to remove excess buffer and proceed immediately to the next step.

7.9.4 Add 100 µL of the freshly made Substrate Solution per well with a multichannel pipettor and avoid bright light. Record the time of addition to wells (Appendix 2, Section 9B).

7.9.5 The first chemiluminescence reading should be taken within 2 min of substrate addition. Record the time of the initial relative light unit (RLU) reading in the Batch Record (Appendix 2, Section 9B).

7.9.5.1 If the signal is too high from the initial reading, wait 5 min and read the plate again at the same instrument setting, repeat until the RLU signal is on scale. Record time the final RLU reading is taken in Appendix 2, Section 9B.

7.9.6 Save the resulting readings in an Excel file to a secure computer; recommended to label the file with the date and a unique assay identifier (Plate ID). Print a paper copy of the raw data for inclusion with the Batch Record.

7.10 Review and finalize the Batch Records (Appendix 2) and obtain required signatures. Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 2, Section 10).

7.11 The Laboratory Director/Supervisor should review the Batch Record and sample reports and sign the Batch Record affirming the data contained within are correct (Appendix 2, Section 11).

7.12 Proceed to SOP340904 for Quality Control and Data Analyses and preparation of the Sample Data Report to send to the clinical protocol Principal Investigator.

APPENDIX 1: PLATE MAP DESIGN

	1	2	3	4	5	6	7	8	9	10	11	12
A	Reagent Diluent Only*					7.8 pg/mL		Reagent Diluent Only				
B	High-C	S1	S3	S5	S7	15.6 pg/mL		S9	S11	S13	S15	Low-C
C						31.2 pg/mL						
D	Mid-C					62.5 pg/mL						Mid-C
E		S2	S4	S6	S8	125 pg/mL		S10	S12	S14	S16	
F	Low-C					250 pg/mL						High-C
G						500 pg/mL						
H	Reagent Diluent Only					1000 pg/mL		Reagent Diluent Only				
	Control Samples	Unknown Samples, Triplicate				HIF-1 alpha Standards, Duplicate		Unknown Samples, Triplicate				Control Samples

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

- S1 through S16 are unknown sample (S) wells in triplicate, grouped by column. If fewer samples are run, fill the empty sample wells with Reagent Diluent and ignore for data analyses.
- Background control wells are loaded with Reagent Diluent only (no sample).
- Document the sample/patient IDs and other pertinent information in the Sample Calculation Table in the Batch Record (Appendix 2, Section 5B.a.).

  Important: This Plate Map design and well designation is assumed for the format of the Tecan output file that will be used in SOP340904: HIF-1 alpha Quality Control and Data analyses. Manual adjustment of the output well data is outlined in the SOP if a different Plate Map is used.

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APPENDIX 2: BATCH RECORD

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

Certified Assay Operator: _____

Certification Number: _____

Facility/Laboratory Running SOP: _____

Clinical Protocol Number: _____

Plate ID (optional): _____

1. Critical Reagents

The critical reagents are listed below; complete the table as designated. Be sure the lot numbers on each of the reagents match those cited in the product insert accompanying the reagents. Reagents from one kit **should not** be exchanged with reagents from another.

Reagent Name	Date Received	Lot Number	Provided Reagent	Recommended Dilution/Conc. for Working Solution	Expiration Date
DUOSet IC ELISA Kit	/ /		N/A	N/A	/ /
• Total HIF-1 alpha Standard	/ /		ng lyophilized powder	N/A	/ /
• HIF-1 alpha Capture Ab	/ /		µg lyophilized powder	µg/mL	/ /
• HIF-1 alpha Detection Ab	/ /		µg lyophilized powder	ng/mL	/ /
• Streptavidin-HRP Conjugate	/ /		1 mL	1:	/ /
Control Lysates (High, Mid, and Low)	/ /		N/A	N/A	/ /
LumiGLO Chemiluminescent Substrate Solutions	/ /		N/A	N/A	/ /
2HG	/ /		10 mM	0.1 mM	/ /

BATCH RECORD: INITIALS _____ DATE: _____

2. Equipment and Preparation of Reagents

A. Equipment

Plate Washer Make/Model: _____

Serial #: _____

Microplate Reader Make/Model: _____

Serial #: _____

B. Reagents

Buffers should be prepared based on volumes needed to complete all the steps with the number of 96-well plates in the experimental run. Always prepare at least 10% excess volume of buffer to ensure adequate volume to complete the study (scale-up appropriately for additional plates).

a. Wash Buffer (1X PBS-0.05% Tween): To prepare 2 L of buffer pipette 200 mL 10X PBS and 10 mL 10% (w/v) Tween 20 into 1790 mL ultrapure DNase/RNase-free water. For each 96-well plate, ~1.6 L will be needed (preparing enough for 110 wells). Keep at RT for up to 2 wk.

b. Tween 20, 10%: Manufacturer's solution supplied at 10% (w/v). Store at 2-8°C and discard following manufacturer's assigned expiration date.

Lot#: _____ Expiration Date: _____

c. Reagent Diluent (Wash Buffer-5% BSA): Prepare 80 mL of buffer by adding 4.0 g BSA to 80 mL Wash Buffer. Keep at 2ºC to 8°C. Discard unused buffer at end of assay run.

d. H-CEB (**without** PIs+2HG): Prepare 10 mL of buffer by adding the following reagents to 7.5 mL ultrapure DNase/RNase-free water. Once all reagents have been added, adjust volume to 10 mL with additional ultrapure DNase/RNase-free water. Sterile filter the H-CEB and store at 2ºC to 8ºC for no longer than 3 mo.

Reagent	Molecular Weight/ Concentration	Amount Needed	Final Concentration
Tris	121.14	60.57 mg	50 mM Tris
NaCl	58.44	175.32 mg	300 mM NaCl
Glycerol	100%	1.0 mL	10% Glycerol
EDTA	0.5 M	0.06 mL	3 mM EDTA
$MgCl_2$	95.22	0.95 mg	1 mM $MgCl_2$
β-Glycerol	306.11	61.22 mg	20 mM β-Glycerol
NaF	41.99	10.5 mg	25 mM NaF
Triton X-100	10%	1.0 mL	1% Triton

e. Protease Inhibitor Cocktail Tablets: Dissolve one PI cocktail tablet in 2 mL ultrapure DNase/RNase-free water ddH_20 (25X stock). The 25X stock solution is stable for 1 wk at 2°C to 8°C or 12 wk at -15°C to -25°C. If stored frozen, the material must be prepared as single-use aliquots to prevent repeat freeze-thaw.

Lot#: _____ Expiration Date: _____

f. PMSF: Manufacturer's stock solution supplied at 100 mM. Label vial with date of receipt from manufacturer; the expiration date should be considered 6 mo after receipt.

Lot#: _____ Expiration Date: _____

g. H-CEB (Complete): 1 mL H-CEB (Complete) is sufficient to prepare all unknown sample dilutions. **Note**: If H-CEB (**with** PIs+2HG) is already prepared in the laboratory, simply add SDS to final concentration of 0.5%.

Reagent	Stock Concentration	Amount Needed	Final Concentration
H-CEB (**without** PIs+2HG)	stock	915 µL	N/A
PI Cocktail	25X	40 µL	1X PI Cocktail
PMSF	100 mM	10 µL	1 mM PMSF
2HG	10 mM	10 µL	0.1 mM 2HG
SDS	20%	25 µL	0.5% SDS

C. Reconstitution of DUOSet IC Kit Antibodies

a. HIF-1 alpha capture Ab **STOCK** solution preparation (500 µg/mL)

Supplied HIF-1 alpha capture Ab = _____µg lyophilized powder

$$\frac{\text{XX µg lyophilized powder}}{\text{Final concentration HIF-1 alpha capture Ab STOCK}} * 1000\ \mu L/mL = \text{XX µL 1X PBS}$$

$$\frac{\text{\_\_\_\_\_µg}}{\text{500 µg/mL HIF-1 alpha capture Ab STOCK}} * 1000\ \mu L/mL = \text{\_\_\_µL 1X PBS}$$

Add the calculated volume 1X PBS to HIF-1 alpha capture Ab vial included in the Kit.

b. HIF-1 alpha detection pAb **STOCK** solution preparation (5.0 µg/mL)

Supplied HIF-1 alpha detection pAb = _____µg lyophilized powder

$$\frac{\text{XX µg lyophilized powder}}{\text{Final concentration HIF-1 alpha pAb STOCK}} * 1000\ \mu L/mL = \text{XX µL Reagent Diluent}$$

$$\frac{\text{\_\_\_\_\_µg}}{\text{5.0 µg/mL HIF-1 alpha pAb STOCK}} * 1000\ \mu L/mL = \text{\_\_\_µL Reagent Diluent}$$

Add the calculated volume Reagent Diluent to HIF-1 alpha pAb vial included in the Kit.

3. Capture Antibody: HIF-1 alpha Coating Ab

A. Preparation of **HIF-1 alpha Coating Solution**

Place **STOCK** HIF-1 alpha capture Ab on ice. For one 96-well plate (preparing 110 well volumes): (100 µL/well*110)/(1000 µL/mL) = 11 mL. Prepare **HIF-1 alpha Coating Solution** using the following calculations:

a. Recommended working concentration of HIF-1 alpha capture Ab = _____µg/mL

e.g., PADIS/IQC-supplied HIF-1 alpha capture Ab **STOCK** recommended working concentration for the DUOSet item Lot# KQE0411012 is 4 µg/mL.

$$\frac{\text{Working Concentration} \quad * 11 \text{ mL}}{\text{HIF-1 alpha capture Ab STOCK}} * 1000 \text{ µL/mL} = \underline{\text{XX}} \text{ µL HIF-1 alpha capture Ab STOCK}$$

$$\frac{\text{\_\_\_\_\_ µg/mL} \quad * 11 \text{ mL}}{500 \text{ µg/mL HIF-1 alpha capture Ab STOCK}} * 1000 \text{ µL/mL} = \text{\_\_\_\_\_ µL HIF-1 alpha capture Ab STOCK}$$

b. Place the following in a 15-mL polypropylene tube and mix by inversion 5 to 8 times.

11 mL	1X PBS
____ µL	HIF-1 alpha capture Ab **STOCK**

B. Incubation Conditions for Coating Plate

Add 100 µL **HIF-1 alpha Coating Solution** to each well, and incubate at 2°C to 8°C for 16 ± 1 h.

Date: ___/___/___ Start Time: ____:____ Incubation Temp: _____°C

Date: ___/___/___ Stop Time: ____:____

4. Block Step

Following the washing step after plate coating, add 300 µL Reagent Diluent to each well and incubate at 25°C for 1 to 2 h on a prewarmed CoolSink thermoconductive plate.

Incubation conditions for blocking plate:

Date: _____/___/_____ Incubation Temp: _____°C

Start Time: _____:_____ Stop Time: _____:_____

5. Preparation of HIF-1 alpha Standards (A) and Unknown Samples (B)

A. HIF-1 alpha Standards

a. Prepare HIF-1 alpha standard stock solution (100 ng/mL)

The DUOSet IC Kit includes vials of lyophilized HIF-1 alpha standard. Reconstitute one (1) vial to 100 ng/mL using the following calculations; reconstituted standard should be discarded within 1 h.

Supplied HIF-1 alpha standard = _____ng lyophilized powder

e.g., PADIS/IQC-supplied HIF-1 alpha standard for DUOSet item Lot# 1248512 is 90 ng of lyophilized powder.

$$\frac{\text{XX ng lyophilized powder}}{\text{Final concentration HIF-1 alpha standard } \textbf{STOCK}} * 1000\ \mu L/mL = \text{XX } \mu L \text{ Reagent Diluent}$$

$$\frac{\text{\_\_\_\_\_ng}}{\text{100 ng/mL HIF-1 alpha standard } \textbf{STOCK}} * 1000\ \mu L/mL = \text{\_\_\_\_}\mu L \text{ Reagent Diluent}$$

Add the calculated volume Reagent Diluent to HIF-1 alpha standard vial included in the Kit.

b. Serial dilution of HIF-1 alpha standards for reference curve

Serial dilutions of the HIF-1 alpha standards are used to generate a reference curve ranging from 1000 to 7.8 pg/mL. Label tubes with final concentration of standard. Discard unused stock solution.

Tube # (Plate Row)	Vol. and Source of Concentrated Standard	Vol. Reagent Diluent	Resulting Conc. of Diluted Standard
Stock	HIF-1 alpha standard Stock Solution	N/A	100 ng/mL (100,000 pg/mL)
1 (H)	10 µL of Stock Solution	1000 µL	1000 pg/mL
2 (G)	300 µL of tube #1	300 µL	500 pg/mL
3 (F)	300 µL of tube #2	300 µL	250 pg/mL
4 (E)	300 µL of tube #3	300 µL	125 pg/mL
5 (D)	300 µL of tube #4	300 µL	62.5 pg/mL
6 (C)	300 µL of tube #5	300 µL	31.3 pg/mL
7 (B)	300 µL of tube #6	300 µL	15.6 pg/mL
8 (A)	300 µL of tube #7	300 µL	7.8 pg/mL

B. Unknown Samples

a. Sample Calculation Table

Each unknown sample is run as two separate samples on the Plate Map either a 1.0 and 0.5 µg/µL sampel set or a 0.5 µg/µL and buffer-only set – depending on stock lysate concentration. 15 µL Working Lysate is loaded per well, in triplicate, for each sample. Sample numbers correspond to those on the Plate Map Design in Appendix 1.

Sample No.	**Patient/Sample ID**	**Stock Lysate Conc.** xx µg/µL Lysate	**Working Lysate Conc.** 1.0 and 0.5 µg/µL or 0.5 µg/µL and 0	**Vol. Stock Lysate** (µL)	**Vol. H-CEB (Complete)** 60 µL – Vol. of Stock Lysate Used
S1	*Example*	*3.8 µg/µL*	*1.0*	*15.8*	*44.2*
S2	*1234-001005-501*		*0.5*	*7.9*	*52.1*
S3	*Example*	*0.64 µg/µL*	*0.5*	*44.8*	*15.2*
S4	*1234-001005-502*		*0*	*0*	*60*
S1		µg/µL			
S2					
S3		µg/µL			
S4					
S5		µg/µL			
S6					
S7		µg/µL			
S8					
S9		µg/µL			
S10					
S11		µg/µL			
S12					
S13		µg/µL			
S14					
S15		µg/µL			
S16					

6. Plate Incubation

Add clinical samples, tumor controls, and HIF-1 alpha standards to the 96-well plate, cover plate, and incubate at 2°C to 8°C for 16 ± 1 h.

Date: __/__/__ Start Time: __:__ Incubation Temp: ____°C

Date: __/__/__ Stop Time: __:__

7. Detection Antibody: HIF-1 alpha detection Ab

A. Preparation of HIF-1 alpha Detection Ab Working Solution (100 µL/well)

Place **STOCK** HIF-1 alpha detection Ab on ice. For one 96-well plate (preparing 110 well volumes): (100 µL/well*110)/(1000 µL/mL) = 11 mL. Prepare **HIF-1 alpha detection Ab working solution** using the following calculations.

Note: The 5.0 µg/mL **STOCK** solution prepared in Appendix 2, Section 2C is expressed as 5000 ng/mL in the calculations below.

a. Recommended working concentration of HIF-1 alpha detection Ab = _____ng/mL

e.g., PADIS/IQC-supplied HIF-1 alpha detection Ab **STOCK** recommended working concentration for the DUOSet item Lot# KKR0311012 is 100 ng/mL.

$$\frac{\text{Working Concentration} * 11\text{ mL}}{\text{5000 ng/mL HIF-1 alpha detection Ab STOCK}} * 1000\ \mu\text{L/mL} = \underline{\text{XX}}\ \mu\text{L HIF-1 alpha detection Ab STOCK}$$

$$\frac{\text{\_\_\_\_\_ ng/mL} * 11\text{ mL}}{\text{5000 ng/mL HIF-1 alpha detection Ab STOCK}} * 1000\ \mu\text{L/mL} = \text{\_\_\_\_\_}\ \mu\text{L HIF-1 alpha detection Ab STOCK}$$

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

11 mL	Reagent Diluent
____ µL	HIF-1 alpha detection Ab **STOCK**

B. Addition of Prepared HIF-1 alpha Detection Ab Working Solution

Add 100 µL of the **HIF-1 alpha detection Ab working solution** to each well and incubate for 2 h at 25°C on a prewarmed CoolSink thermoconductive plate.

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

8. Reporter: Streptavidin-HRP Conjugate

A. Preparation of Streptavidin-HRP Conjugate Working Solution (100 µL/well)

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

a. Recommended dilution of Streptavidin-HRP Conjugate **STOCK** = 1: _____

e.g., PADIS/IQC-supplied Streptavidin-HRP Conjugate **STOCK** recommended dilution for Kit Lot# 1260918 is 1:200.

$$\frac{11\text{ mL}}{\text{Recommended dilution of Streptavidin-HRP Conjugate STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L Streptavidin-HRP Conjugate STOCK}$$

$$\frac{11\text{ mL}}{\frac{\quad}{\text{(dilution factor)}}} * 1000\ \mu\text{L/mL} = \_\_\_\_\_\ \mu\text{L Streptavidin-HRP Conjugate STOCK}$$

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

11 mL	Reagent Diluent
__ µL	Streptavidin-HRP Conjugate **STOCK**

Preparation Time: ____:____

B. Addition of Streptavidin-HRP Conjugate Working Solution

Add 100 µL of the **streptavidin-HRP conjugate working solution** to each of the washed wells and incubate in the dark at 25°C for 30 min on a prewarmed CoolSink thermoconductive plate.

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

9. Chemiluminescent Substrate

A. Preparation of Substrate Solution

Calculate volume of substrate required for the experimental run. For one 96-well plate, prepare enough for110 wells in a 15-mL polypropylene tube wrapped with aluminum foil. Mix by inversion 5 to 8 times and store at ambient in the dark until use.

5.5 mL LumiGLO Substrate A	(50 µL/well*110)/(1000 µL/mL)
5.5 mL LumiGLO Substrate B	(50 µL/well*110)/(1000 µL/mL)

Time of Substrate Preparation: ____:____

B. Substrate Solution Incubation and RLU Reading Times

Time of Substrate Addition to Wells: ____:____

Time Initial RLU Reading is Captured: ____:____

Time Final RLU Reading is Captured (opt): ____:____

BATCH RECORD: INITIALS _____ DATE: _____

10. Notes, including any deviations from the SOP:

11. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: _____

BATCH RECORD: INITIALS _____ DATE: _____