Validation and Fitness Testing of a Quantitative Immunoassay for HIF1α in Biopsy Specimens

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Abstract (#C3)

HIF1α is an important marker of hypoxia in human tumors that is altered in a number of cancers, but a reproducible method to determine changes in HIF1α protein expression in human tumor biopsy specimens has not been available. HIF1α is being used as a pharmacodynamic marker in clinical trials in the NCI (CTEP# 8610, 9534). However, an important limitation for studying the response of HIF1α to cancer therapeutic agents is the lability of the protein, in the presence of oxygen, upon collection of the sample. We have devised a method of specimen collection, handling, and extraction that preserves and stabilizes HIF1α levels in tumor biopsies. Employing this specimen-handling method allowed validation of a two-site immunoassay for HIF1α quantitation in solid-tissue extracts, such as tumor biopsies. Intra-assay variability was less than 10%, and inter-assay variability was less than 20%. Accuracy, assessed by spike recovery, was 99 +/- 7%. HIF1α readings declined linearly with decreasing sample load over a range of 1 to 10 µg protein per well. Fitness for purpose was demonstrated by quantifying a reduction in HIF1α protein levels following topotecan treatment of a xenograft model. HIF1α was also demonstrated to be upregulated under low oxygen tension culture conditions in DU145 human prostate cancer cells. The HIF1α immunoassay is currently being transferred to the NCI's National Clinical Target Validation Laboratory for use in support of NCI-sponsored early clinical trials. This research has been funded with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E.

Materials and Methods

- The assay is a two-site chemiluminescence readout enzyme immunoassay. The assay uses a purified monoclonal antibody to HIF1α (R&D Systems, 841689) as the capture reagent and a biotin-labeled goat anti-HIF1α polyclonal antibody (R&D Systems, 841690) as a probe, followed by streptavidin conjugated to horseradish peroxidase (HRP) (R&D Systems, 890803) as the reporter. Prior to the addition of tumor lysate, the extracts are assayed for total protein using the BCA assay (Pierce), and then diluted in Reagent Diluent (5% BSA in Wash Buffer – 1X PBS, pH 7.2–7.4 with 0.05% vol/vol Tween) to a concentration of 1 µg/µL for the assay. 10 µL of specimens diluted in Reagent Diluent are added to wells containing 90 µL of Reagent Diluent (10 µg/well). All specimens are assayed in duplicate or triplicate. Capture antibody and specimen incubation are 16 +/- 1 hr at 2–8°C.
- Standard is purified recombinant human HIF1α (amino acid residues 575-826; R&D Systems, 841691) diluted in Reagent Diluent. Assay dynamic range is approximately 7.8 to 1,000 pg/mL. The assay controls are produced from HIF1α-transfected HEK293 (1 µg/µL) (OriGene Technology, Inc., Cat # LY419880) and PC3 cell extract (1 µg/µL). The PC3 cells are harvested after washing in cold PBS containing protease inhibitor and lysed in cell extraction buffer (CEB), and processed as for cell extracts. The PC3 extracts are assayed for total protein using the BCA assay (Pierce) and then diluted to a concentration of 1 µg/µL for the assay. High-control is a mixture of 0.5 µg of HEK293 and 9.5 µg of PC3 per well. Medium-control is a mixture of 0.125 µg of HEK293 and 9.875 µg of PC3 per well. Low-control is PC3 10 µg per well. Dilutions are made in Reagent Diluent to achieve reads in the desired range of the assay, and expected ranges of assay readouts for the controls are provided for each lot. High-controls will read 683–869 pg/ml HIF1α, while mid-range controls will read in the range of 205–313 pg/ml and low controls will read between 41 and 69 pg/ml HIF1α.

Degassing buffer:

1. Cell extraction buffer was prepared on ice with protease inhibitor.
2. 400 ul of CEB with protease inhibitor was added to the microfuge tube with O-ring screw cap and kept on ice.
3. Nitrogen gas was gently bubbled through the buffer at the bottom of the microfuge tube using a pipette tip until the bubble replaced the air above the buffer. It takes about 5–10 sec to replace air in the tube with nitrogen gas.
4. The vial was sealed immediately by closing the cap tightly.
5. The buffer was shipped to NCTVL on ice for biopsy collection.
6. The buffer can be stored for a month at 2–8°C.

Biopsy sample collection:

1. Mix the biopsy collection tube containing degassed buffer by inverting several times.
2. Prepare 3–4 vials for collecting samples at a time.
3. Keep the vials upright in ice bath.
5. Place needle biopsy into the vial by placing the tip of the biopsy needle **into the buffer** in the cone at the bottom of the vial, then ejecting the tissue core.
6. Seal the vial immediately by closing the cap tightly.
7. Flash-freeze the vials for storage.

Specimen Collection and Processing Are Critical to Successful Development of HIF1α Immunoassay

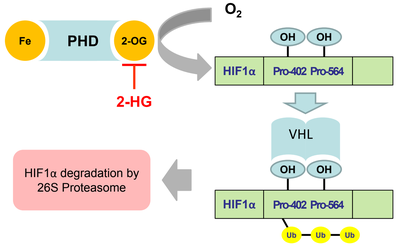
Specimen and control samples should be processed as follows:

- Make protease inhibitor cocktail by dissolving 1 tablet into 1 mL water
- Prepare cell extraction buffer (CEB) on ice:
  - Fresh PMSF (100 mM stock solution, Sigma) 1:100
  - Protease inhibitor (Roche) 1:50

Components	MW	For 500 ml CEB
50 mM Tris	121.14	3028.5 mg
300 mM NaCl	58.44	8766 mg
10% Glycerol	92.09	50 ml
3 mM EDTA	0.5 M liquid	3 ml
1 mM $MgCl_2$	95.22	47.5 mg
20 mM beta Glycerol	306.11	3061.2 mg
25 mM NaF	41.99	524.75 mg
1% Triton		5 ml
100 µM 2-hydroxyglutarate	192.08	9.604 mg

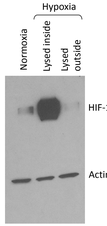
- Add PMSF 1:1000 and Protease Inhibitor 1:50 to required volume.

2-Hydroxyglutarate (2-HG) as a PHD Inhibitor by Competitively Inhibiting 2-Oxo(or Keto)glutarate (α-KG), a Substrate of PHD



HIF1α Expression in Western Blots (PC3 Cells)

Collection of cells in degassed buffer, plus processing of specimens in the hypoxia chamber yielded HIF1α.



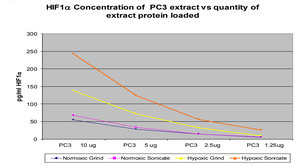
The critical step is the collection of specimen and freeze-down in hypoxic conditions. After that the specimen is stable and can be handled in a manner consistent with PADIS/NCTVL operations.

Log phase PC3 cells were cultured.
Lane 1: Normoxia
Lanes 2 and 3: Hypoxia (1%) overnight
Lane 2: **Preconditioned PBS wash and cells were immediately lysed in RIPA inside the hypoxia chamber**
Lane 3: After overnight hypoxia, cells were:

- Scraped in preconditioned PBS
- Centrifuged at 3,000 g for 2 min (Sarstedt tube)
- Aspirate supernatant inside chamber
- Pellet flash frozen in dry ice
- Pellet lysed in RIPA
- Protein - Western Blotting - HIF1α

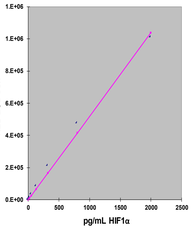
HIF1α Expression in Immunoassay (PC3 Cells)

- Discrepancy between quantitative ELISA readout and Western blot:
  - Western says no HIF1α in normoxic condition, and ELISA says it's there, but hypoxic is 3X higher. The Western says the differential is much greater than 3X.
- Tissue processing a critical factor:
  - Sonication is better than grinding with a homogenizer.
- The data suggest that stabilization of HIF1α during the assay is also critical.



	Average readout HIF1α pg/ug protein	Average readout Actin pg/ug protein	pg HIF1α/ng Actin	Molecules HIF1α /cell
Normoxic	6.41	9.15	0.70	926
Hypoxic	23.86	10.44	2.29	2856

Assay Variability & Accuracy by Spike Recovery



LLQ= 3.2 pg/mL
%CV at zero = 3.4

HIF1α Spike Recovery

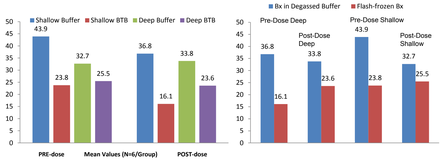
OBSERVED pg/mL	EXPECTED pg/mL	% RECOVERY
1904	2001	95.2
564	501	88.9
165	126	112.6
2002	2000	100
506	500	101
2083	2006	103.8
536	506	105.9
126	131	96.2
115	125	92
2002	2006	99.8
491.5	506	97.1
		Mean 99.30%
		SDEV 6.60%

	Mean (pg/mL HIF1α)	SDEV	CV%
Intra-assay Variability 8 samples	27.9	1.9	6.5
Inter-ssay Variability 16 samples	29.3	2.0	6.7

Comparison of DU-145 Intratumoral HIF1α Levels in PMA-treated Mice

DU145 xenografts staged to 250 or >600 mg size. Treatment was phorbol 12-myristate 13-acetate [PMA] IP, QDx3. Specimen collection was by biopsy of live nude mice (N=6/treatment).

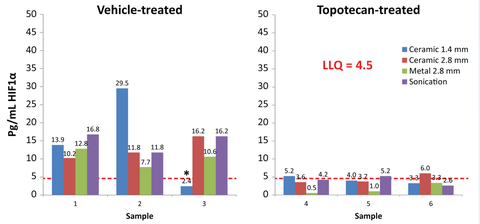
Deep vs. shallow biopsies and degassed buffer followed by flash-frozen biopsy (Buffer) vs. flash-frozen biopsy alone (BTB) were compared.



There was no detectable effect of PMA injection on recovered HIF1α levels. However, the degassed collection buffer in the collection vials yielded a significant increase of recovered HIF1α levels compared to the BTB standard method (unpaired 2-tailed t-test = 0.004).

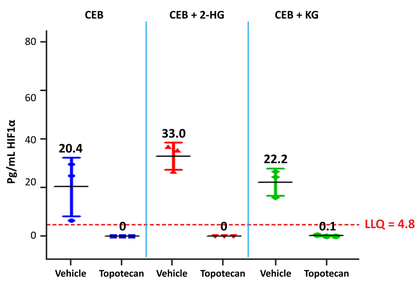
Bead Homogenizer vs. Sonication (10 µg load)

Note that topotecan treatment significantly reduced HIF1α expression vs. vehicle

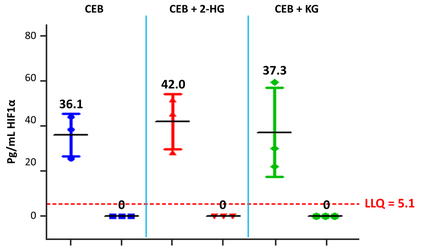


* Less than 10 µg of protein was loaded due to the diluted sample concentration to <1 µg/mL.

CEB vs. CEB + 2-HG (100 µM) vs. CEB + α-KG (100 µM) (10 µg load)



CEB vs. CEB + 2-HG (100 µM) vs. CEB + α-KG (100 µM) (10 µg load) Second Experiment

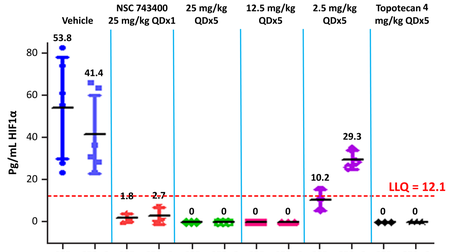


These studies were all performed with archived specimens collected by the BTB standard method

Experiment Design

- Animal: A375 (human melanoma) nu/nu mouse xenografts
- Treatment:
  - Vehicle (G1)
  - Indenoisoquinoline (NSC 743400) 25 mg/kg QD x1 IV (G2)
  - NSC 743400 25 mg/kg QD x5 IV (G3)
  - NSC 743400 12.5 mg/kg QD x5 IV (G4)
  - NSC 743400 2.5 mg/kg QD x5 IV (G5)
  - Topotecan 4 mg/kg QDx5 IP (G6)
- Tumor collection: 1 hr post–dose 5
- Entire tumor was collected, divided into 4 pieces, and flash-frozen.
- Each tumor piece was divided into 4 pieces and was lysed in degassed buffer.
- Different methods of homogenization were compared between sonication and bead homogenization with 3 different beads (ceramic 1.4 mm, ceramic 2.8 mm, metal 2.8 mm).
- Different lysis buffers were compared (CEB vs. CEB + 2-HG 100 µM vs. CEG + α-Ketoglutarate 100 µM).
- 10 µg of protein was loaded in each well.

Topotecan and NSC 743400 Significantly Reduced HIF1α levels: CEB vs. CEB + 2-HG Buffer



- 3 of 4 mice per group assayed
- Sampling time: 1 hr post–dose 5

Conclusions

- HIF1α immunoassay was developed and shown to be useful in recovering HIF1α levels in xenograft models.
- Alternative methods of homogenization were evaluated: Sonication and homogenization with ceramic beads were superior to homogenization with metallic beads or tissue grinding.
- Degassed collection buffer yielded a significant increase of recovered HIF1α levels compared to the standard method of fresh-freeze only.
- Alternative collection buffers show a trend for improved yield with the addition of 2-hydroxyglutarate.
- Topotecan and indenoisoquinoline NSC 743400 significantly reduced the amount of HIF1α in A375 xenografts.

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