

Version 2 Last updated 2 October 2020

# ab229433 Human HIF1 $\alpha$ CatchPoint<sup>®</sup> SimpleStep ELISA<sup>®</sup> Kit

For the quantitative measurement of HIF1 $\alpha$  in human cell extracts.

This product is for research use only and is not intended for diagnostic use.

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## 1. Overview

HIF1a *in vitro* CatchPoint® SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of HIF1a protein in human cell extracts.

The CatchPoint® SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. CatchPoint® HRP Development Solution containing the Stoplight Red Substrate is added. During incubation, the substrate is catalyzed by HRP generating a fluorescent product. Signal is generated proportionally to the amount of bound analyte and the intensity is measured in a fluorescence plate reader at 530/570/590 nm Excitation/Cutoff/Emission.

Hypoxia-inducible factor 1-alpha (HIF1 alpha) is a constitutively expressed transcription factor that is degraded under normal oxygen tensions but stabilized when oxygen is limiting (hypoxia). Under hypoxic conditions, stabilized HIF1 alpha translocates to the nucleus and promotes the transcription of a host of genes that enable the cell to adapt to the lack of oxygen. Aspects of the HIF1 alpha mediated hypoxic response include promotion of angiogenesis and the switch from aerobic respiration to anaerobic glycolysis. Many of the HIF1 alpha responsive genes encode proteins that promote glycolysis and/or inhibit oxidative phosphorylation (known as the Warburg effect). An exciting and developing area of current cancer research is examining how HIF-mediated metabolic reprogramming promotes tumor growth and survival.

In most cases, HIF1 alpha will need to be stabilized to be measured (steady state levels of HIF1 alpha in non-hypoxic environments is exceeding low in most cell lines). This can be achieved by (a) creating a hypoxic environment (e.g. using a hypoxia chamber) or (b) by using chemical treatments that mimic hypoxia (e.g. cobalt chloride or deferoxamine). The sample data in this assay protocol was generated using deferoxamine (DFO). DFO is an iron chelator and disrupts the function the prolyl hydroxylases that degrade HIF1 alpha in normoxia. By disrupting the enzymes that degrade HIF1 alpha, DFO increases the abundance of HIF1 alpha protein.

## 2. Protocol Summary

Prepare all reagents, samples, and standards as instructed



Add 50  $\mu$ L standard or sample to appropriate wells



Add 50  $\mu$ L Antibody Cocktail to all wells



Incubate at room temperature for 1 hour



Aspirate and wash each well three times with 350  $\mu$ L 1X Wash Buffer  
PT



Add 100  $\mu$ L of prepared CatchPoint<sup>®</sup> HRP Development Solution to  
each well and incubate for 10 minutes



Read fluorescence at Ex/Cutoff/Em 530/570/590 nm

### 3. Precautions

**Please read these instructions carefully prior to beginning the assay.**

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

### 4. Storage and Stability

**Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

## 5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
- All data, except Typical Standard Curve and Sensitivity were collected using the colorimetric version of this kit (ab171577).

## 6. Materials Supplied

Item	Quantity	Storage Condition
Human HIF1a Capture Antibody 10X	600 µL	+4°C
Human HIF1a Detector Antibody 10X	600 µL	+4°C
Human HIF1a Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 5B	6 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
Cell Extraction Buffer PTR 5X	10 mL	+4°C
Cell Extraction Enhancer Solution 50X	1 mL	+4°C
Stoplight Red Substrate Buffer	12 mL	+4°C
100X Stoplight Red Substrate	120 µL	+4°C
500X Hydrogen Peroxide (H <sub>2</sub> O <sub>2</sub> , 3%)	50 µL	+4°C
Sample Diluent NS*	50 mL	+4°C
SimpleStep Pre-Coated Black 96-Well Microplate	96 Wells	+4°C
Plate Seal	1	+4°C

\*Sample Diluent NS is provided but not necessary for this product.

## 7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Fluorescence microplate reader Ex/Cutoff/Em 530/570/590 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

## 8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.

- The provided Antibody Diluents and Sample Diluents contain protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- The provided Cell Extraction Buffer 5X contains phosphatase inhibitors and protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- The provided Cell Extraction Enhancer Solution 50X may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The Cell Extraction Enhancer Solution 50X can be stored at room temperature to avoid precipitation.
- The incubation times provided in this protocol were optimized for fastest results with good signal. It is possible to increase the signal with longer incubation times; further optimization might be necessary.
- Keep in mind any RFU values shown are relative, NOT absolute. RFU from one plate reader are not comparable to another, especially if different make or model.
- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

## 9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

### 9.1 1X Cell Extraction Buffer PTR:

Prepare 1X Cell Extraction Buffer PTR by diluting Cell Extraction Buffer PTR 5X and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL Cell Extraction Buffer PTR 5X and 200 µL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently. If required protease inhibitors can be added.

Alternative – Enhancer may be added to 1X Cell Extraction Buffer PTR after extraction of cells or tissue. Refer to note in the Troubleshooting section.

### 9.2 1X Wash Buffer PT:

Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

### 9.3 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 5B. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 5B. Mix thoroughly and gently.

#### 9.4 CatchPoint® HRP Development Solution

Just prior to use prepare CatchPoint® HRP Development Solution by diluting the 100X Stoplight Red Substrate and the 500X Hydrogen Peroxide in Stoplight Red Substrate Buffer.

For example, to make 6 mL of the CatchPoint® HRP Development Solution combine 60  $\mu$ L 100X Stoplight Red Substrate and 12  $\mu$ L of 500X Hydrogen Peroxide with 5.928 mL Stoplight Red Substrate Buffer. Mix thoroughly and gently.

## 10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

**10.1** Reconstitute the HIF1 $\alpha$  standard sample by adding 1 mL 1X Cell Extraction Buffer PTR. Mix thoroughly and gently. Hold at room temperature for 10 minutes and mix gently. This is the 200 ng/mL **Stock Standard** Solution.

**10.2** Label 14 tubes, Standards 1– 14.

**10.3** Add 120  $\mu$ L 1X Cell Extraction Buffer PTR into tube number 1 and 150  $\mu$ L of 1X Cell Extraction Buffer PTR into numbers 2-14.

**10.4** Use the Stock Standard to prepare the following dilution series. Standard #14 contains no protein and is the Blank control.

Standards will be added to the plate in step 13.3. If desired all 14 standards can be used for a full standard curve.

Alternatively, to commit fewer wells to the standard curve, select a subset of at least 7 standards plus the blank control. If 7 standards are desired, we recommend standards #4-10.

Standard #	Dilution Sample	Volume to Dilute (μL)	Volume of Diluent (μL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock	180	120	200	120
2	Standard#1	150	150	120	60
3	Standard#2	150	150	60	30
4	Standard#3	150	150	30	15
5	Standard#4	150	150	15	7.5
6	Standard#5	150	150	7.5	3.75
7	Standard#6	150	150	3.75	1.88
8	Standard#7	150	150	1.88	0.94
9	Standard#8	150	150	0.94	0.47
10	Standard#9	150	150	0.47	0.23
11	Standard#10	150	150	0.23	0.12
12	Standard#11	150	150	0.12	0.06
13	Standard#12	150	150	0.06	0.03
14	None	0	150	0	0

## 11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	Range
HeLa (Deferoxamine treated)	50 – 500 µg/mL

### 11.1 Preparation of extracts from cell pellets:

- 11.1.1 Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.
- 11.1.2 Rinse cells twice with PBS.
- 11.1.3 Solubilize pellet at  $2 \times 10^7$  cell/mL in chilled 1X Cell Extraction Buffer PTR.
- 11.1.4 Incubate on ice for 20 minutes.
- 11.1.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.
- 11.1.6 Transfer the supernatants into clean tubes and discard the pellets.
- 11.1.7 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
- 11.1.8 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

### 11.2 Preparation of extracts from adherent cells by direct lysis (alternative protocol):

- 11.2.1 Remove growth media and rinse adherent cells 2 times in PBS.
- 11.2.2 Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer PTR per confluent 15 cm diameter plate).
- 11.2.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes.
- 11.2.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.
- 11.2.5 Transfer the supernatants into clean tubes and discard the pellets.
- 11.2.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
- 11.2.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

### 11.3 Preparation of extracts from tissue homogenates:

- 11.3.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).
- 11.3.2 Homogenize 100 to 200 mg of wet tissue in 500  $\mu$ L – 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly.
- 11.3.3 Incubate on ice for 20 minutes.
- 11.3.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.
- 11.3.5 Transfer the supernatants into clean tubes and discard the pellets.
- 11.3.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
- 11.3.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

## 12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well fluorescence or "edge effects" have not been observed with this assay.
- Ensure plate and all materials are equilibrated to room temperature during use.
- Cover the plate with a plate seal during incubation steps.

## 13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

**13.1** Prepare all reagents, working standards, and samples as directed in the previous sections.

**13.2** Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

**13.3** Add 50 µL of all sample or standard to appropriate wells.

**13.4** Add 50 µL of the Antibody Cocktail to each well.

**13.5** Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

**13.6** Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.

**13.7** Add 100 µL of prepared CatchPoint® HRP Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm. Further optimization of incubation time vs signal strength can be performed if needed.

**13.8** Record the fluorescence at Ex/Cutoff/Em 530/570/590 nm. If using a Molecular Devices' plate reader supported by SoftMax® Pro software, a preconfigured protocol for these CatchPoint® SimpleStep ELISA® Kits is available with all the protocol and analysis settings at [www.softmaxpro.org](http://www.softmaxpro.org)

Mode:	Fluorescence
Instrument settings:	Endpoint
Excitation:	530 nm
Cutoff:	570 nm
Emission:	590 nm
Sensitivity:	6 flashes/read or 200ms
PMT:	Auto
Auto calibrate:	On
Read:	Top
Read Height:	1*

\*For microplate readers with Pre-Read Optimization option, the Read Height as well as Microplate Optimization is recommended before the first read.

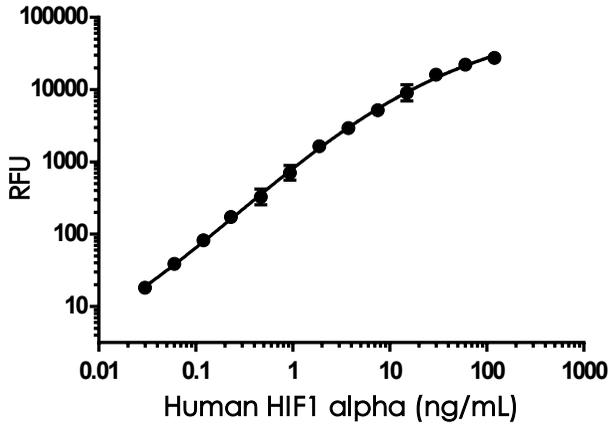
**13.9** Analyze the data as described below.

## 14. Calculations

- 14.1 Preconfigured protocols are available when using SoftMax Pro software from Molecular Devices
  - 14.2 Calculate the average fluorescence value for the blank control (zero) standards. Subtract the average blank control standard fluorescence value from all other fluorescence values.
  - 14.3 **Create a standard curve** by plotting the average blank control subtracted fluorescence value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.
- Δ **Note:** Most fluorescence reader software or graphing software will plot these values and fit a curve to the data. A four-parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4-parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.
- 14.4 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted fluorescence **values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.
  - 14.5 Samples generating fluorescence values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at fluorescence values less than that of the lowest standard should be retested in a less dilute form.

## 15. Typical Data

**Typical standard curve** – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Concentration (ng/mL)	RFU		Mean RFU
	1	2	
0	13	13	13
0.03	31	31	31
0.06	51	53	52
0.12	95	95	95
0.23	187	184	186
0.47	403	286	345
0.94	848	608	728
1.88	1,655	1,654	1,654
3.75	3,343	2,623	2,983
7.5	6,036	4,530	5,283
15	10,917	7,523	9,220
30	17,458	14,824	16,141
60	23,929	20,545	22,237
120	28,964	26,009	27,486

**Figure 1.** Example of human HIF1a standard curve in 1X Cell Extraction Buffer PTR. The HIF1a standard curve was prepared as described in Section 10. Raw data generated on SpectraMax M4 Multi-Mode Microplate Reader is shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

## 16. Typical Sample Values

### SENSITIVITY –

The calculated minimal detectable dose (MDD) is 12 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=22) and adding 2 standard deviations then extrapolating the corresponding concentration.

### RECOVERY –

Three concentrations of HIF1 $\alpha$  recombinant protein were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
50% Cell Culture Media	112	94 – 129
10% FBS	109	102 – 120
5% BSA	97	93 – 106

## LINEARITY OF DILUTION -

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

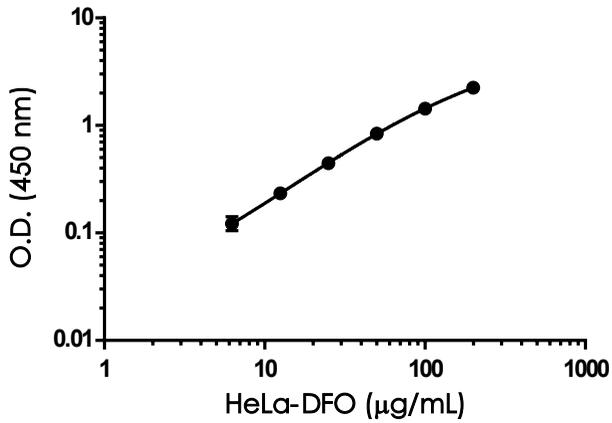
Native HIF1 $\alpha$  was measured in the following biological sample and diluted in a 2-fold dilution series in 1X Cell Extraction Buffer PTR.

Dilution Factor	Interpolated value	50 $\mu$ g/mL HeLa lysate
Undiluted	ng/mL	1.95
	<b>% Expected value</b>	<b>100</b>
2	ng/mL	1.13
	<b>% Expected value</b>	<b>116</b>
4	ng/mL	0.63
	<b>% Expected value</b>	<b>129</b>
8	ng/mL	0.34
	<b>% Expected value</b>	<b>138</b>

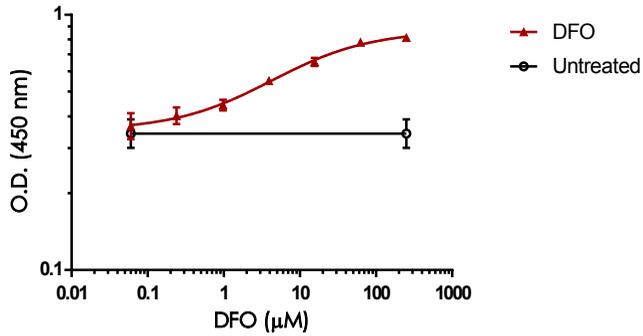
## PRECISION –

Mean coefficient of variations of interpolated values of HIF1 $\alpha$  from three concentrations of HeLa lysates (treated with DFO) within the working range of the assay.

	Intra-Assay	Inter-Assay
n =	5	3
CV(%)	4.3	7.0



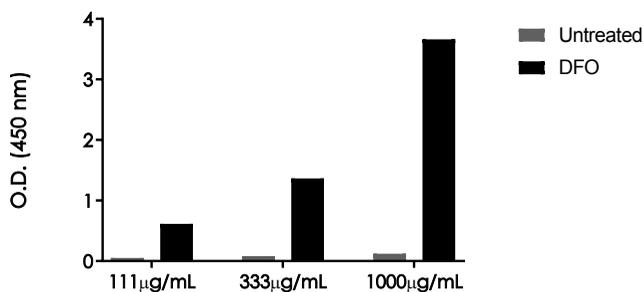
**Figure 2.** Titration of HeLa-DFO extract within the working range of the assay. Background subtracted data from duplicate measurements are plotted. To induce HIF1 alpha protein levels, HeLa cells were treated with 500 µM Deferoxamine (DFO) for 24 hours.



**Figure 3.** Dose-dependent induction of HIF1 alpha in HeLa cells by deferoxamine (DFO). HeLa cells were cultured in 96-well tissue culture plates and were either untreated or exposed to varying dose of DFO for 24 hours. Cells were extracted directly in the culture plate by overlaying culture media with Extraction Buffer PTR (with Extraction Enhancer) such that the final concentration was 1X Extraction Buffer. Extracts were applied to the HIF1 alpha ELISA. Raw data with standard deviation is plotted from triplicate measurements.

## 17. Assay Specificity

This kit recognizes both native and recombinant human HIF1 $\alpha$  protein in cell extracts only.



**Figure 4.** Comparison of HIF1 $\alpha$  expression in HeLa cell extracts (with and without DFO treatment). Background subtracted OD450 nm data from three loading concentrations are shown. In the HeLa cell line, DFO treatment is required to detect HIF1 $\alpha$  protein.

## 18. Species Reactivity

This kit recognizes human HIF1 $\alpha$  protein.

It is not compatible with mouse or rat samples.

Serum and plasma samples have not been tested with this kit.

Please contact our Technical Support team for more information.

## 19. Troubleshooting

Problem	Reason	Solution
<b>Difficulty pipetting lysate; viscous lysate.</b>	Genomic DNA solubilized	Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction.
<b>Poor standard curve</b>	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
<b>Low Signal</b>	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with CatchPoint® HRP Development Solution too brief	Read plate again after longer incubation time
<b>Large CV</b>	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
<b>Low sensitivity</b>	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep Stoplight Red Substrate protected from light.
<b>Precipitate in Diluent</b>	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

# 20. Notes



## Technical Support

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**For all technical or commercial enquiries please go to:**

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