

In-Cell ELISA (ICE) Assay Platform

Rev.0

Monitoring the HIF-mediated hypoxic response in cells: a high-throughput, quantitative cell-based assay.

Introduction:

ICE:

In-Cell ELISA (ICE), also known as Fixed-Cell ELISA or In-Cell Western, is a high-throughput, quantitative, and very reproducible method to determine protein levels in cultured cells. Adherent or suspension cells are seeded, treated, fixed, and assayed in 96- or 384-well microplates. In-well duplexing is possible with a LI-COR® Infrared imager and IRDyes® or alternatively single analytes can be measured per well using a plate reader if the LI-COR® instrument is not available. Inter-well normalization can be achieved using a control protein readout (e.g. tubulin) or with a whole cell stain (e.g. Janus Green). Given the flexibility, throughput, and reproducibility, ICE is a powerful platform for characterizing the protein composition of cells, and for examining alterations in protein levels induced by drugs or changes in cellular homeostasis. More information and resources for ICE applications are available on the MitoSciences website (www.mitosciences.com/in-cell-elisa.html).

Hypoxic response:

This application note demonstrates the utility of the ICE technique for examining the HIF1A (hypoxia inducible factor alpha) mediated hypoxic response in cultured cells. HIF1A is a constitutively expressed transcription factor that is degraded under normal oxygen tensions but stabilized when oxygen is limiting (hypoxia) (see [1] for a comprehensive review). Under hypoxic conditions, stabilized HIF1A promotes the transcription of a host of genes that enable the cell to adapt to the lack of oxygen. A key aspect of the hypoxic response is the switch from aerobic respiration to anaerobic glycolysis and many of the HIF1A responsive genes encode proteins that promote glycolysis and/or inhibit oxidative phosphorylation [e.g. 2,3]. An exciting and developing area of current cancer research is examining how HIF-mediated metabolic reprogramming promotes tumor growth and survival [4,5].

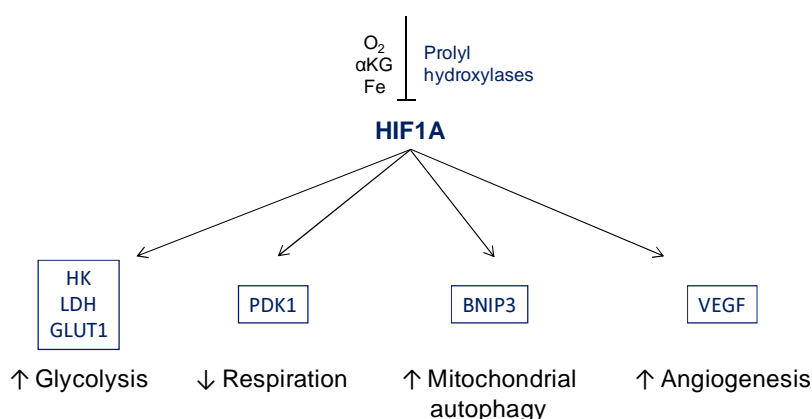


Figure 1. HIF1A-mediated transcriptional response to hypoxia. Prolyl hydroxylases (with cofactors oxygen, alpha-ketoglutarate and iron) target the transcription factor HIF1A protein for degradation. In the absence of oxygen (or alpha-ketoglutarate or iron), HIF1A is stabilized and promotes transcription of multiple genes that mediate the hypoxic response, including a shift to glycolytic energy production.

In this experiment, we investigate the kinetics of HIF1A stabilization and the induction of HIF1A responsive proteins in response to pseudo-hypoxia using the ICE technique. HeLa cells were treated with a time-course (2, 6, and 24 hours) and dose-response (1 mM – 10 nM) of deferoxamine (DFO). DFO is an iron chelator that disrupts the degradation of HIF1A, a process that requires iron as a cofactor. Table I lists the readouts measured in this experiment; all of the readouts as well as ICE assay kits are available from MitoSciences.

Cat. #	Target	Name	Function
MS779	HIF1A	Hypoxia inducible factor 1	Transcription factor
MSR05	GLUT1	Glucose transporter 1	Promote glucose transport for glycolysis
MSP49	PDK1	Pyruvate dehydrogenase kinase 1	Phosphorylates pyruvate dehydrogenase, inhibiting TCA cycle
MSR23	LDH	Lactate dehydrogenase	Converts pyruvate to lactate, the final step in glycolysis
MSR10	HK2	Hexokinase II	Generates glucose-6P, the first step in glycolysis

Table I. Hypoxia related readout assayed in this experiment. HIF1A is stabilized under conditions that mimic hypoxia; GLUT1, PDK1, LDH, and HK2 are known to be HIF1A-responsive genes.

Methods:

Cell culture and treatments: HeLa cells were cultured in standard DMEM media supplemented with 10% FCS and seeded at 10,000 cells per 100 μ L per well in 96 well plates. The following day, triplicate wells were treated by overlaying a 6-point, 10-fold dose response of deferoxamine such that the final concentration spanned from 1 mM to 10 nM. 18 hours and 22 hours later, additional overlays were made to the appropriate wells such that at the time of fixation for ICE, cells had been treated for 2, 6, or 24 hours. Treatments at each time-point were done so that all wells were continually exposed to the same volume of media. All treatments were performed in triplicate using the plate layout in Figure 2.

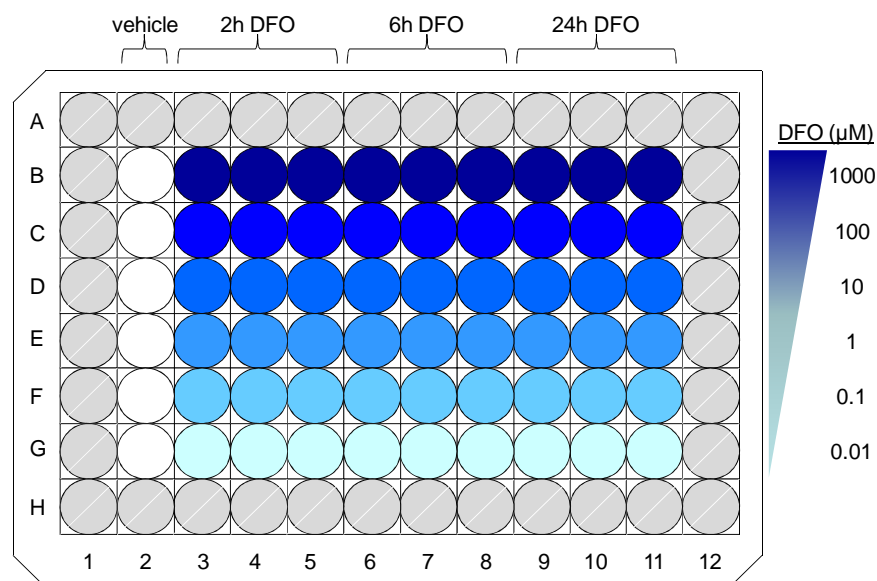


Figure 2. Plate set-up. HeLa cells were seeded to all wells of a 96-well plate. Wells B2-G10 were treated with the indicated dilution titration of DFO for the indicated period of time. The remaining wells were vehicle treated. After fixation, wells B2-G11 were stained with the appropriate primary and secondary antibodies. Perimeter wells (gray) were stained with secondary antibody only and used to determine plate background.

ICE assay and analysis: At the end of treatment, the incubation medium was gently decanted and then 100 μ L of 4% paraformaldehyde was slowly added to fix cells. The plates were subsequently washed with PBS, permeabilized with 0.1% Triton X-100 and blocked. Primary antibodies were incubated overnight at 4 $^{\circ}$ C and the following day plates were washed with PBST, exposed to secondary antibodies, washed with PBST, and scanned on the LI-COR $^{\circ}$ Odyssey $^{\circ}$ infrared imager. Then, plates were stained with 0.3% Janus Green, washed thoroughly with water, destained with HCl, and read at OD595 on a SpectraMax plate reader. All reagents required to perform ICE are available from MitoSciences (MS922).

Analysis: The average background intensity for each secondary antibody on each plate was determined from the signal of wells stained without primary antibody; this value was then subtracted from the remaining wells that were stained with primary antibody. The resulting value was then divided by the Janus Green total cell stain to account for differences in cellular amount (due to seeding imperfection or to effect of treatment); the result is the normalized value. %CV was determined for each dose and time-point by dividing the standard deviation of the triplicate values by the mean value (Table 2). Graphs were generated by plotting the normalized value for each dose and time-point relative to the Vehicle treated wells.

Results:

1. Protein detection and quantification.

Plate images and quantification for the HIF1A and GLUT1 readouts are shown in Figures 3 and 4. Because the HIF1A and GLUT1 antibodies are from mouse and rabbit, respectively, they can be duplexed when using the LI-COR $^{\circ}$ Odyssey $^{\circ}$ system and the data for Figures 3 and 4 were generated using two different IR-conjugated secondary antibodies on the same plate. Results for PDK1, LDH and HK2 are presented in Figure 5.

Note that HIF1A induction is detected after 2 hours of DFO treatment and that levels continue to increase to 24 hours of DFO exposure. For GLUT1, PDK1, LDH, and HK2, increased protein levels are not observed until 6 hours and are significantly increased at 24 hours.

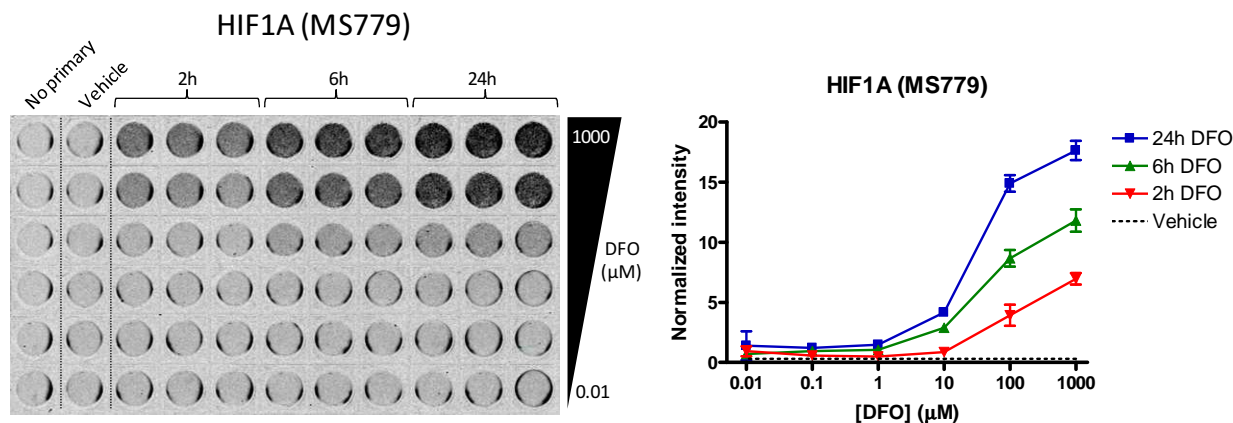


Figure 3. DFO induces HIF at 2 hours. Plate image (left) and graphical representation (right) of HIF1A induction following a deferoxamine (DFO) time-course and dose-response in HeLa cells. Error bars represent standard deviation of triplicate measurements.

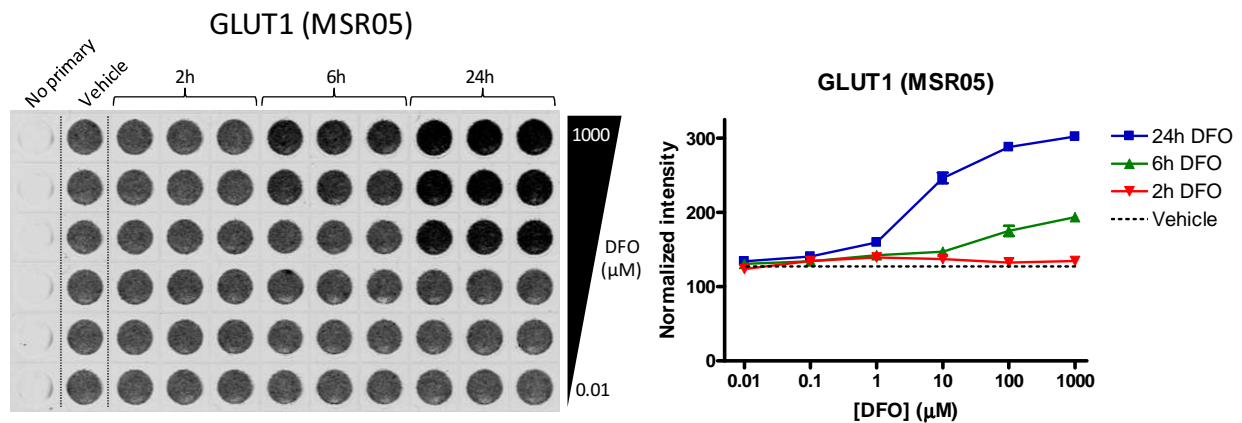


Figure 4. DFO induces GLUT1. Plate image (left) and graphical representation (right) of GLUT1 induction following a deferoxamine (DFO) time-course and dose-response in HeLa cells. Error bars represent standard deviation of triplicate measurements.

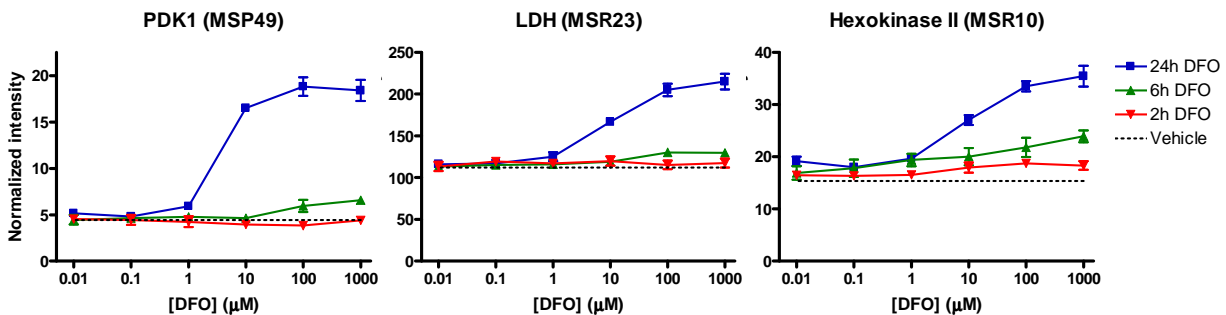


Figure 5. DFO induces PDK1, LDH and Hexokinase II. DFO time and dose-dependent induction of PDK1, LDH, and HK2 in HeLa cells. Error bars represent standard deviation of triplicate measurements.

II. Janus Green normalization

Following antibody detection (by IR scanner or plate reader), the microplates are stained with Janus Green. Janus Green is a total cell stain that is used to account for differences in cellular material between wells (due to seeding imperfection or to effect of treatment). Janus Green data from two sample plates is shown in Figure 6. Note that at 24h, high doses of DFO cause a relative reduction in cell number compared to the 2 and 6 hour DFO treatments and Vehicle treated cells.

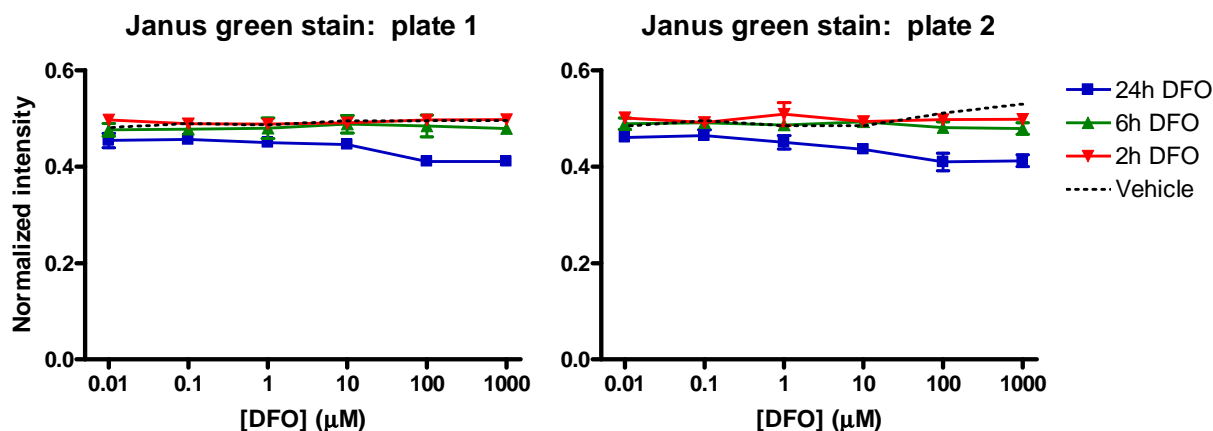


Figure 6. Total cell stain by Janus Green. Two HeLa cell plates from the DFO dose response and time-course experiment were stained with Janus Green (MS930). Error bars represent standard deviation of triplicate measurements.

III. Coefficient of Variation

Table 2 includes the coefficient of variation (%CV) values for each of the triplicate measurements in this experiment. Average CV across GLUT1, PDK1, LDH and HK2 readouts is 4%. Note that CVs are higher for the HIF1A readout at low doses of DFO and early time-points when HIF1A levels are extremely low. In contrast, once HIF1A has been induced to detectable levels the CV values are $\leq 8\%$.

DFO (μM)	HIF1A (MS779)			GLUT1 (MSR05)			PDK1 (MSP49)			LDH (MSR23)			HK2 (MSR10)		
	2h	6h	24h	2h	6h	24h	2h	6h	24h	2h	6h	24h	2h	6h	24h
1000	7%	8%	5%	0%	3%	1%	1%	2%	6%	5%	3%	4%	4%	5%	6%
100	22%	8%	5%	1%	4%	0%	2%	11%	5%	5%	3%	4%	4%	8%	3%
10	23%	6%	2%	1%	4%	3%	5%	8%	1%	5%	3%	0%	5%	8%	4%
1	31%	10%	14%	3%	4%	1%	14%	3%	6%	3%	2%	4%	3%	6%	1%
0.1	25%	13%	12%	2%	2%	2%	12%	4%	9%	3%	3%	1%	4%	9%	4%
0.01	44%	29%	85%	2%	3%	4%	2%	12%	7%	5%	2%	1%	2%	8%	5%

Table 2. Table of CV values for all readouts. CV value for each DFO dose and time-point determined from triplicate measurements (CV<10% highlighted yellow).

Conclusions:

The experiments detailed here allow several basic conclusions, all of which can be extended by additional experiments (e.g. more detailed time-courses, different hypoxic conditions, HIF inhibitor screens, biochemical activity assays, etc).

- Acute accumulation of HIF1A through reduced degradation.** Elevated HIF1A protein levels can be detected as early as 2 hours following treatment with DFO and increasing levels of HIF1A are detected up to 24 hours (Fig. 1). At all time-points, HIF1A levels are dependent of DFO concentration with 1mM DFO resulting in the greatest accumulation of HIF1A. Notably, essentially no HIF1A is detected in the absence of DFO or at DFO concentrations of less than 1 μM . This is consistent with the expectation that DFO inhibits the degradation of HIF1A protein resulting in a rapid increase in HIF1A levels.

- **Increase in GLUT1, PDK1, LDH and HK2 protein levels lags HIF1A induction.** GLUT1, PDK1, LDH and HK2 are all detected in the absence of DFO, but are induced to greater levels following DFO treatment. In all cases, increased protein levels are not detected until the 6 hour time-point and are more significant at the 24 hour time-point. A lag between an increase in HIF1A and responsive proteins is expected because HIF must form an active transcription factor, translocate to the nucleus and then target genes must be transcribed and translated before the proteins are detected.
- **Differences in the induction of HIF1A responsive proteins.** While GLUT1, PDK1, LDH and HK2 are all induced by DFO treatment, their individual responses vary. For example, PDK1 is induced to >4 times its steady state level whereas GLUT1, LDH and HK2 are induced to 2-2.5 times their steady state level. There is also a kinetic difference: GLUT1 and HK2 are already appreciably increased at 6 hours, whereas PDK1 and LDH require a longer time (significant increases at 24 hours).
- **A robust and flexible assay system.** The ICE platform is an ideal high-throughput microplate assay capable of in-well duplexing. ICE assays are reliable (CV values for this experiment averaged 4%) and inherently flexible as readouts can be selected from a large offering of MitoSciences-validated antibodies (www.mitosciences.com/in-cell-elisa.html). Sample applications of the described HIF ICE analytes include:
 - Screen for inhibitors of HIF1A stabilization: simultaneously assay the induction of HIF1A and responsive protein(s) following a hypoxic stimulus.
 - Profile tumor cell line panels to compare levels of HIF1A protein under normoxic or hypoxic conditions.
 - Kinetic studies of HIF1A stabilization and/or downstream target induction.

References:

1. Semenza, G.L. Regulation of Oxygen Homeostasis by Hypoxia-Inducible Factor 1. *Physiology* 24, 97-106 (2008).
2. Kim J., Tchernyshyov I., Semenza G.L., Dang C.V. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metabolism* 3, 177-185 (2006).
3. Papadopoulos I., Cairns R.A., Fontana, L., Lim A.L., Denko N.C. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metabolism* 3, 187-197 (2006).
4. Denko, N.C. Hypoxia, HIF1 and glucose metabolism in the solid tumor. *Nature Reviews Cancer* 8, 705-713 (2008).
5. Semenza, G.L. HIF-1: upstream and downstream of cancer metabolism. *Current Opinion in Genetics and Development* 20, 51-56 (2010).

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

Sample product offerings from MitoSciences for In-Cell ELISA:

ICE-validated primary antibodies to Glycolysis and Krebs Cycle targets:


✓ Cat. No.	Description	Pathway
 MSR11	Glucose 6 phosphate dehydrogenase (G6PD) polyclonal antibody	Glycolysis Pentose Phosphate
 MSR05	Glucose transporter 1 (GLUT1) monoclonal antibody	Glycolysis
 MSR06	Glucose transporter 4 (GLUT4) monoclonal antibody	Glycolysis
 MS751	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody	Glycolysis
 MSR10	Hexokinase II (HK2) monoclonal antibody	Glycolysis
 MSR23	Lactate dehydrogenase (LDH) monoclonal antibody	Glycolysis
 MSR07	Phosphoglycerate kinase (PGK1) polyclonal antibody	Glycolysis
 MSP03	Pyruvate dehydrogenase (PDH) subunit E1 alpha monoclonal antibody	Glycolysis Krebs Cycle
 MSP05	Pyruvate dehydrogenase (PDH) subunit E2 monoclonal antibody	Glycolysis Krebs Cycle
 MSR01	Pyruvate dehydrogenase E1 α P $\text{Ser}232$ polyclonal antibody	Glycolysis Krebs Cycle
 MSR02	Pyruvate dehydrogenase E1 α P $\text{Ser}293$ polyclonal antibody	Glycolysis Krebs Cycle
 MSR03	Pyruvate dehydrogenase E1 α P $\text{Ser}300$ polyclonal antibody	Glycolysis Krebs Cycle
 MSP49	Pyruvate dehydrogenase kinase isoform 1 (PDK1) monoclonal antibody	Glycolysis Krebs Cycle
 MSR15	Pyruvate kinase isoenzymes M1/M2 monoclonal antibody	Glycolysis
 MSR16	Pyruvate kinase isoform M2 monoclonal antibody	Glycolysis

ICE support products:

Secondary Antibodies

Data	Cat. No.	Name	Amount
	MS923	Goat anti-mouse IRDye800, IgG (H+L)	12 x 96 tests
	MS924	Goat anti-rabbit IRDye680, IgG (H+L)	12 x 96 tests
	MS925	Goat anti-mouse IRDye680, IgG1 isotype specific	12 x 96 tests
	MS926	Goat anti-mouse IRDye800, IgG2a isotype specific	12 x 96 tests
	MS927	Goat anti-mouse IRDye800, IgG2b isotype specific	12 x 96 tests
	MS928	Goat anti-mouse HRP, IgG (H+L)	12 x 96 tests
	MS929	Goat anti-rabbit HRP, IgG (H+L)	12 x 96 tests

Whole Cell Stain

Data	Cat. No.	Name	Amount
	MS930	Janus Green cell normalization stain	2 x 96 tests

Support Pack

Cat. No.	Name	Amount
MS922	In-Cell ELISA (In-Cell Western) Support Pack (Includes 5x tissue-culture treated 96-well black/clear imaging plates.)	5 x 96 tests