



ab129733 –

Hif1a + BNIP3 Hypoxia

Human In Cell ELISA

Kit (IR)

Instructions for Use

For measuring in high throughput hypoxia-inducible factor 1-alpha (HIF1A) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3).

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

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1. Introduction

Principle: ab129733 is an In-Cell ELISA (ICE) assay kit that uses quantitative immunocytochemistry to measure HIF1 alpha and BNIP3 protein levels in cultured cells. Cells are fixed in a microplate and targets of interest are detected with highly specific, well-characterized antibodies. Relative protein levels are quantified using IRDye®-labeled Secondary Antibodies and IR imaging using a LICOR® Odyssey® or Aeries® system.

Background:

Hypoxia and the cellular response to hypoxic environment is a central topic in studies of metabolism, cancer progression and development and stem cells. A key player is the transcription factor HIF1 alpha (hypoxia inducible factor 1 alpha) which is stabilized at the protein level in response to decreased oxygen tension. HIF1 alpha then promotes transcription of a number of factors that alters cellular physiology. This Hypoxia ICE assay kit provides duplexed measurements of the transcription factor HIF1 alpha and the HIF1A responsive protein BNIP3.

HIF1 alpha is a constitutively expressed transcription factor that is degraded under normal oxygen tensions but stabilized (at the protein level) when oxygen is limiting (hypoxia). Under hypoxic conditions, stabilized HIF1A promotes the transcription of a host of genes that

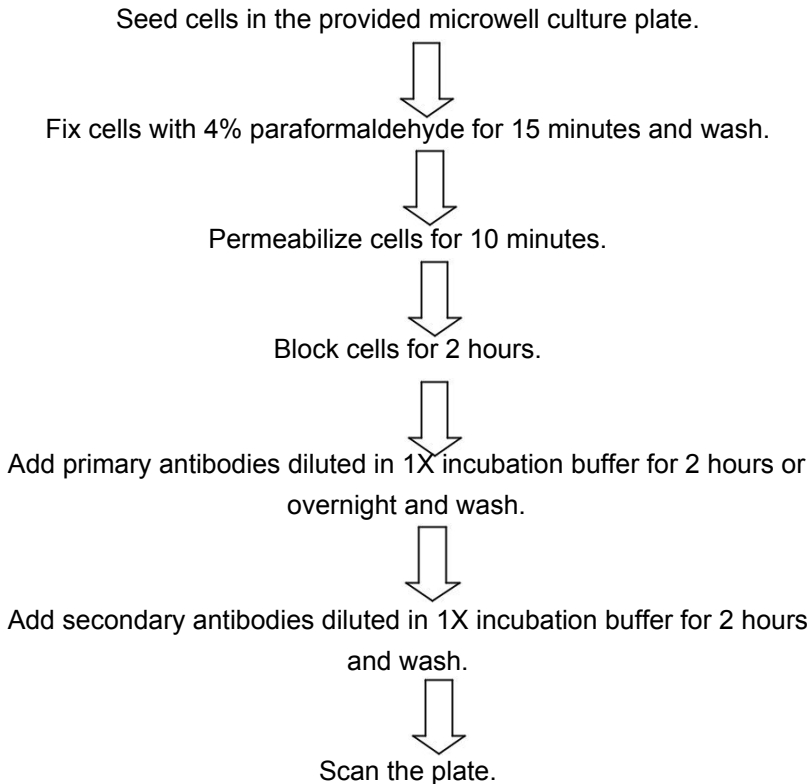
enable the cell to adapt to the lack of oxygen. A key aspect of the hypoxic response is the switch from aerobic respiration to anaerobic glycolysis and many of the HIF1 alpha responsive genes encode proteins that promote glycolysis and/or inhibit oxidative phosphorylation. Stabilization of the HIF1 alpha protein levels can be detected quickly after the onset of hypoxia, whereas changes in the levels of HIF1 alpha responsive proteins take longer to manifest. The anti-HIF1 alpha antibody used in this assay is specific for Human HIF1 alpha protein.

BNIP3 is a hypoxia-inducible member of the BCL-2 family of apoptosis regulators. Stabilization of the HIF1 alpha transcription factor directly leads to increased transcription of BNIP3. BNIP3 appears to have a role in hypoxia-induced cell death and is associated with mitochondria. The anti-BNIP3 antibody in this assay kit is reactive with Human and Rat BNIP3.

In-Cell ELISA (ICE) technology is used to perform quantitative immunocytochemistry of cultured cells with a near-infrared fluorescent dye-labeled detector antibody. The technique generates quantitative data with specificity similar to Western blotting, but with much greater quantitative precision and higher throughput due to the greater dynamic range and linearity of direct fluorescence detection and the ability to run 96 samples in parallel. This method rapidly fixes the cells in situ, stabilizing the in vivo levels of proteins, and thus essentially eliminates changes during sample handling, such as

preparation of protein extracts. Finally, the HIF1 alpha and BNIP3 signals can all be normalized to cell amount, measured by the provided Janus Green whole cell stain, to further increase the assay precision.

2. Assay Summary



3. Kit Contents

Item		Quantity
10X Phosphate Buffered Saline (PBS)		100
100X Triton X-100 (10% solution)	500	
400X Tween – 20 (20% solution)		2
10X Blocking Buffer		10
100X HIF1a Primary antibody (<i>Rabbit</i>)	120	
100X BNIP3 Primary Antibody (<i>Mouse</i>)	120	
1000X IRDye®-labeled Secondary Antibody cocktail	24	
Janus Green Stain		17

4. Storage and Handling

Upon receipt spin down the contents of the IRDye®-labeled Secondary Antibody tube and protect from light. Store all components upright at 4°C. This kit is stable for at least 6 months from receipt.

5. Additional Materials Required

- A LI-COR® Odyssey® or Aeries® infrared imaging system.
- 96 or 384-well amine coated plate(s).
- 20% paraformaldehyde.
- Nanopure water or equivalent.
- Multi and single channel pipettes.
- 0.5 M HCl (optional for Janus Green cell staining procedure).
- Optional humid box for overnight incubation step.
- Optional plate shaker for all incubation steps.

6. Preparation of Reagents

- 6.1 Equilibrate all reagents to room temperature.
- 6.2 Prepare 1X PBS by diluting 45 mL of 10X PBS in 405 mL Nanopure water or equivalent. Mix well. Store at room temperature.
- 6.3 Prepare 1X Wash Buffer by diluting 0.625 mL of 400X Tween-20 in 250 mL of 1X PBS. Mix well. Store at room temperature.
- 6.4 Immediately prior to use prepare 4% or 8% Paraformaldehyde Solution in PBS. To make 4% Paraformaldehyde combine 2.4mL of 20% Paraformaldehyde with 9.6mL 1X PBS; to make 8%

Paraformaldehyde combine 4.8mL of 20% Paraformaldehyde with 7.2mL 1X PBS.

Note – Paraformaldehyde is toxic and should be prepared and used in a fume hood. Dispose of paraformaldehyde according to local regulations.

- 6.5 Immediately prior to use prepare Permeabilization Solution by diluting 150 μ L of 100X Triton X-100 in 15 mL of 1X PBS.
- 6.6 Immediately prior to use prepare 1X Blocking Buffer by diluting 5 mL 10X Blocking Buffer 45 mL of 1X PBS.

7. Sample Preparation

Note: The protocol below is described for a 96-well plate. If performing assay on a 384-well plate, adjust volumes

accordingly. This assay has been optimized for use on adherent cells. For suspension cells, assay optimization will be required. Make certain that the microplate does not dry out at any time before or during the assay procedure.

- 7.1 Seed adherent cells directly into an amine coated plate and allow them to attach for >6 hours or overnight. It is advised to seed in a 100 μ L volume of the same media used to maintain the cells in bulk culture. The optimal cell seeding density is cell type dependent. The goal is to seed cells such that they are just reaching confluency (but not over-confluent) at the time of fixation. As an example, HeLa cells may be seeded at ~10,000 cells per well 48 hours before fixation.
- 7.2 The attached cells can be treated if desired with a drug of interest.

7.3 Fix cells by adding a final concentration of 4% Paraformaldehyde Solution. This can be achieved by one of two means:

7.3.1 Add an equi-volume of 8% Paraformaldehyde Solution to the culture volume (e.g. add 100 μ L 8% Paraformaldehyde to a well with 100 μ L media).

- or -

7.3.2 Gently remove/dump to culture media from the wells and replace with 100 μ L 4% Paraformaldehyde Solution.

7.4 Incubate for 10 minutes at room temperature.

7.5 Gently aspirate or dump the Paraformaldehyde Solution from the plate and wash the plate 3 times briefly with 1X PBS. For each wash, rinse each well of the plate with 200 μ L of 1X PBS. Finally, add 100 μ L of 1X PBS to the wells of the plate. The plate can now be stored at 4°C for several days. Cover or seal the plate during incubation. For prolonged storage supplement 1X PBS with 0.02% sodium azide.

Note – The plate should not be allowed to dry at any point during or before the assay. Both paraformaldehyde and sodium azide are toxic, handle with care and dispose of according to local regulations.

8 Assay Procedure

It is recommended to use a plate shaker (~200 rpm) during incubation steps. Any step involving removal of buffer or solution should be followed by blotting the plate gently upside down on a paper towel before refilling wells. Unless otherwise noted, incubate at room temperature.

During development of this assay we have not observed problems with edge effects. However if edge effects are of concern, the perimeter wells of the plate can be used as control wells (primary antibody omitted). Regardless, it is required to leave at minimum one well from which the primary antibodies are excluded to determine background levels of the assay.

- 8.1 Remove 1X PBS and add 100 μ L Permeabilization Solution to each well of the plate. Incubate 10 minutes at room temperature.
- 8.2 Wash plate 1 time with 200 μ L of 1X PBS solution.
- 8.3 Add 200 μ L of 1X Blocking Buffer and incubate for 2 hours at room temperature.
- 8.4 Prepare 1X Primary Antibody Solution by diluting 100X HIF1A Primary and 100X BNIP3 Primary Antibody into appropriate volume of 1X Blocking Buffer (i.e. 120 μ L of the 100X HIF1A Primary + 120 μ L of the 100X BNIP3 Primary Antibody + 11.76 mL of incubation buffer).

- 8.4.1 Because the HIF1 alpha antibody is a rabbit monoclonal and the BNIP3 antibody is a mouse monoclonal, they can be multiplexed using the provided cocktail of IRDye®-labeled species specific secondary antibodies when using a LI-COR infrared imager.
- 8.4.2 If the user prefers to use the HIF1 and BNIP3 antibodies individually that may be done as well. There is sufficient secondary antibody cocktail to use each of the primary antibodies individually.
- 8.5 Remove Blocking/Permeabilization Solution and add 100 μ L 1X Primary Antibody Solution to each well of the plate. Incubate for 2 hours at room temperature or overnight at 4°C. *Note – To determine the background signal it is essential to omit primary antibody from at least one well containing cells for each experimental condition.*
- 8.6 Remove 1X Primary Antibody Solution and wash the plate 3 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 200 μ L of 1X Wash Buffer. **Do not remove the last wash until step 8.9.** *Note: if the primary antibody was incubated at 4°C then allow the plate to warm to room temperature before proceeding.*
- 8.7 Prepare 1X Secondary Antibody Solution by diluting 12 μ L 1000X IRDye®-labeled Secondary Antibody

cocktail into 12 mL 1X Blocking Buffer. Protect labeled antibodies from light.

- 8.8 Remove the 1X Wash Buffer and add 100 μ L 1X Secondary Antibody Solution to each well of the plate. Incubate 2 hours at room temperature in the dark.
- 8.9 Remove Secondary Antibody Solution and wash 3 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 200 μ L of 1X Wash Buffer.
- 8.10 Wash 2 times with 1X PBS, using 200 μ L for each well.
Do not remove the last wash.
- 8.11 Wipe the bottom of the plate and the scanner surface with a damp lint-free cloth to clean and scan the plate on the LI-COR® Odyssey® system. Collect data in both the 700 and 800 channels according to manufacturer's instructions. The optimal focus off-set for typical amine plates is 3.9. *Note – The absolute value of the IR signal is dependent on the 800 channel intensity setting. Value 8.5 is recommended for initial scanning. Adjust as needed so that neither signal is saturated in any well.*
- 8.12 The HIF1 alpha signal (Rabbit) is IRDye680® and the BNIP3 signal is IRDye800®.
- 8.13 Remove last 1X Wash and add 100 μ L of Janus Green Stain per well. Incubate plate for 5 minutes at room temperature.

Note – The IR signal should be normalized to the Janus Green Stain intensity to account for differences in cell seeding density.

- 8.14 Remove dye, wash plate 5 times in water or until excess dye is removed.
- 8.15 Remove last water wash, blot to dry, add 100 μ L of 0.5 M HCl and incubate for 10 minutes.
- 8.16 Measure using a LI-COR® Odyssey® scanner in the 700 nm channel or measure OD595 nm using a standard microplate spectrophotometer.

9 Data Analysis

- 9.1 Background subtraction. Determine the raw signal intensity (Integrated Intensity) values for the IR700 and IR800 channels for the wells that lacked primary antibody. Subtract the mean IR700 and IR800 background values from all other IR700 and IR800 experimental values.
- 9.2 Janus Green normalization. Divide the background subtracted IR intensities (from 9.1) by the Janus Green value of the corresponding well. The result is the “normalized intensity”.

10 Assay Performance and Specificity

Assay performance and specificity were tested using HeLa cells treated with a dose titration of deferoxamine (DFO). DFO is an iron chelator that disrupts the degradation of HIF1 alpha and thus causes stabilization and increased levels of HIF1 alpha protein.

Figure 1 shows typical results using this kit. Because confidence in antibody specificity is critical to ICE data interpretation, the primary antibodies in this kit were validated for specificity by fluorescence immunocytochemistry and Western blot (Figures 2 and 3, respectively) using the HeLa and DFO model.

In Figures 1-3, note that HIF1 alpha is undetectable in untreated cells but is stabilized following DFO treatment to detectable levels. In contrast, BNIP3 is constitutively expressed and DFO treatments increase expression over basal levels.

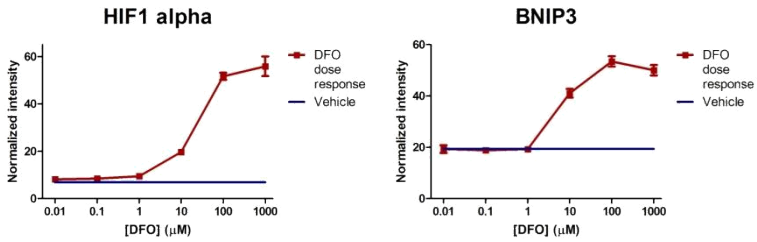


Figure 1. Sample experiment using the kit on HeLa cells treated with a titration of DFO. HeLa cells were seeded to an amine coated 96-well microplate and the following day treated with a titration of DFO. After 24h of DFO exposure, the cells were fixed and stained as described in the protocol and the normalized data is presented here +/- SD (as described in the protocol and data analysis sections). HIF1 alpha results are on the left: DFO concentrations $\geq 10\mu\text{M}$ induce HIF1A protein levels in a dose dependent manner. Similarly, BNIP3 levels (right) are increased with $\geq 10\mu\text{M}$ DFO.

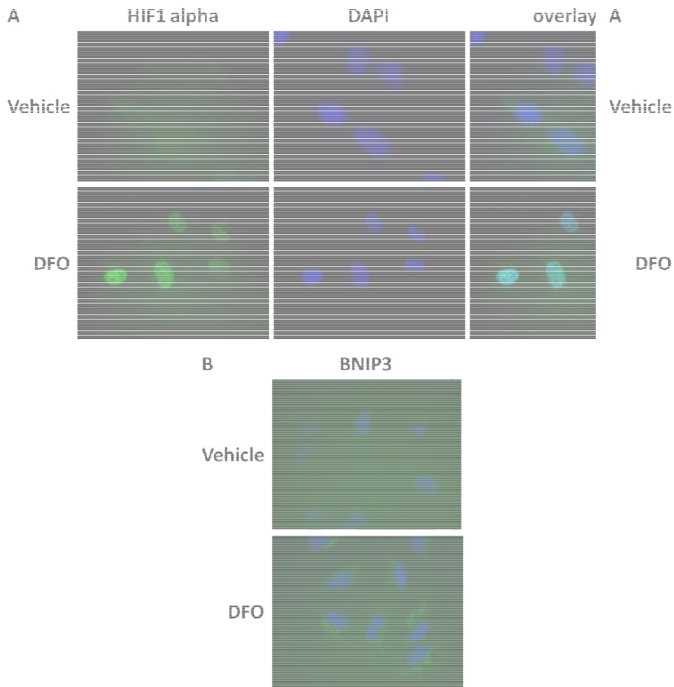


Figure 2. Antibody specificity demonstrated by immunocytochemistry.

Primary antibodies used in this assay kit were validated by staining HeLa cells +/- treatment with 1mM DFO (24h) and imaged by fluorescent

microscopy. (A) HIF1 alpha staining is absent in untreated cells and induced by DFO treatment. HIF1 alpha localizes to the nucleus (as seen by co-localization with the DNA stain DAPI) as expected. (B) BNIP3 staining is nearly undetectable in untreated HeLa cells but is induced by DFO treatment. BNIP3 appears to have a mitochondrial staining pattern in the DFO-treated samples.

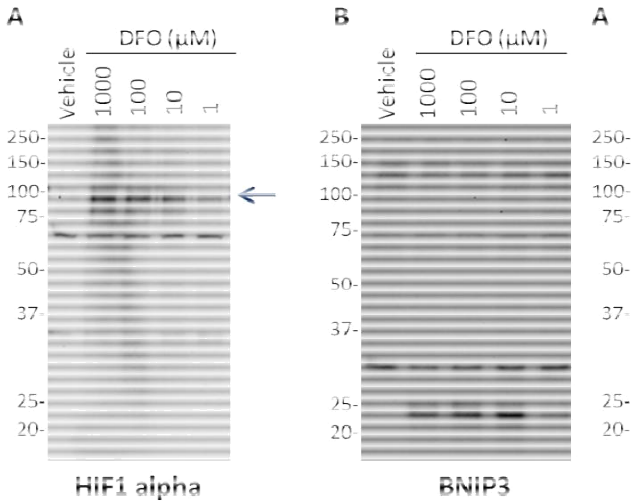


Figure 3. Antibody specificity demonstrated by Western Blot. Primary antibodies used in this assay kit were validated by Western Blot using HeLa cell lysates that had been treated with a dose titration of DFO as indicated.

(A) The HIF1 alpha band (indicated by arrow) is absent in untreated cells and induced by DFO. (B) Similarly, BNIP3 levels are increased by DFO treatment in a dose-dependent manner.

Reproducibility - ICE results provide accurate quantitative measurements of antibody binding and hence cellular antigen concentrations. The coefficient of the intra-assay of variation of this assay kit with HeLa cells is typically <6% for both analytes.

Coefficient of variation				
		replicates	HIF1	BNIP3
Vehicle		6	5%	4%
DFO (µM) dose response	1000	3	3%	1%
	100	3	2%	3%
	10	3	2%	4%
	1	3	2%	3%
	0.1	3	3%	1%
	0.01	3	3%	3%

Table 1. Coefficient of variation for the experiment described in Figure 1.

11 Frequently Asked Questions

11.1 How many cells do I seed per well?

The cell seeding density is cell type dependent and depends both on the cell size and the abundance of the target protein. The cell seeding will likely need to be determined experimentally by microscopic cell density observation of serially diluted cells. For adherent cells, prepare serial dilution of the cells in a plate and allow them to attach prior to observation. The goal is to have cells that are just

confluent at the time of fixation. Overly confluent cells may have compromised viability and tend to not adhere as well to the plate. Under-seeded cells may yield too low a signal, depending on the analyte. Keep in mind that drug treatments or culture conditions may affect cell density/growth.

11.2 Do I have to use an amine-coated microplate?

We have tested black wall amine and cell culture treated microplates and found that amine coated plates improve reproducibility and specificity in comparison to standard plates. In addition, multiple cell types appear to have the most favorable growth and even seeding on amine plates. The assay performance is only guaranteed with amine plates.

11.3 I grow my cells in 15% FBS, will this interfere with the cell fixation?

Culture media containing up to 15% fetal serum does not interfere with the cell fixation and cross-linking to the plate.

11.4 How do I measure the assay background?

It is essential to omit primary antibody in at least one well (3 wells recommended) to provide a background signal for the experiment which can be subtracted from all measured data. This should be done for each experimental condition.

11.5 Is Janus Green normalization necessary?

Janus Green is a whole-cell stain that is useful to determine if a decrease in IR intensity in a well is due to a relevant down-regulation or degradation of the target analyte OR if it is a function of decreased cell number (e.g. due to cytotoxic effect of a treatment). As such it is not a required readout, but is useful in analysis to determine a normalized intensity value (section 9.2).

12 Troubleshooting

Problem	Cause	Solution
Low Signal	Too brief incubation times	Ensure sufficient incubation times
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Insufficient cells	Increase seeding density of cells; goal is newly confluent cells at time of fixation
High CV	Plate is insufficiently washed	Review the manual for proper washing. If using a plate washer, check that all ports are free from obstruction
	Contaminated wash buffer	Prepare fresh wash buffer
	Artifacts creating increased signal on IR	Troughs used for multichannel pipetting could be dirty. Clean bench and troughs
	Edge effects	Do not use the edges of the plate. Incubate in a humid box
	Variable cell seeding	Plate cells with care and normalize with Janus Green

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