ab126584 – Hif1a + GLUT1 Hypoxic Response Human Flow Cytometry Kit

Instructions for Use

For measuring protein levels by flow cytometry: hypoxia-inducible factor 1-alpha (HIF1A) and Transporter GLUT1 (GLUT1) = Solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1).

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

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1. Introduction

Principle: ab126584 is flow cytometry assay kit designed to monitor the hypoxic response by quantifying the protein levels of the HIF1 alpha transcription factor and the HIF1 alpha responsive protein GLUT1. This assay combines the power of single cell analysis obtained from flow cytometry with the specificity of antibody-based immunostaining to quantify protein levels. Cells are harvested, fixed and permeabilized in suspension and targets of interest are detected with antibodies well characterized for specificity and sensitivity. The user provides the fluorescently-labeled secondary antibodies and can choose to detect HIF1 alpha and GLUT1 independently or simultaneously.

Background:

Hypoxia and the cellular response to hypoxic environment are central topics 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 Hypoxic Response Flow Cytometry Kit provides duplexed measurements of the transcription factor HIF1 alpha and the HIF1 alpha responsive protein GLUT1.

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 HIF1 alpha 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.

GLUT1 is a widely expressed cell membrane glucose transporter and is responsible for basal glucose uptake. GLUT1 can transport a range of aldolases including pentoses and hexoses. Stabilization of the HIF1 alpha transcription factor directly leads to increased transcription of GLUT1. In turn, increased expression of GLUT1 in response to hypoxia is thought to provide additional sugars for anaerobic glycolysis. The gene name for GLUT1 is Solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1). The anti-GLUT1 antibody in this assay kit is reactive with Human, Mouse and Rat GLUT1 and recognizes an epitope facing the cytosol.

2. Assay Summary

Harvest cells as a single cell suspension

⇩

Fix cells with 4% paraformaldehyde for 15 minutes and pellet

⇩

Resuspend cells to 90% ice cold Methanol and incubate at -20ºC
for ≥ 30 minutes

⇩

Remove Methanol and rehydrate cells by washing in blocking
buffer; incubate for 30min in blocking buffer.

⇩

Overlay 2X primary antibodies diluted in blocking buffer;
Incubate 1 hour and wash.

⇩

Add secondary antibodies diluted in blocking buffer;
Incubate 1 hour, wash and read on flow cytometer

3. Kit Contents

10X Phosphate Buffered Saline (PBS)	100 mL
10X Blocking Buffer	80 mL
100X HIF1A Primary antibody *(Mouse IgG2b)*	0.12 mL
100X GLUT1 Primary Antibody *(Rabbit)*	0.12 mL

4. Storage and Handling

Upon receipt spin down the contents of the Primary Antibody tubes. Store all components upright at 4°C.   This kit is stable for at least 12 months from receipt.

Additional Materials Required

- Flow cytometer.
- 20% paraformaldehyde.
- Methanol
- Nanopure water or equivalent.
- Centrifuge
- Fluorescently labeled anti-mouse and anti-rabbit secondary antibodies suitable for the available flow cytometer, e.g. anti-mouse-DyLight®488 (ab96879) and anti-rabbit-DyLight®650 (ab96902).

5. Preparation of Reagents

5.1 Prepare 1X PBS by diluting 100 mL of 10X PBS in 900 mL Nanopure water or equivalent. Mix well. Store at room temperature.

5.2 Immediately prior to use prepare 1X Blocking Buffer by diluting 10X Blocking Buffer in 1X PBS.

5.3 Prepare primary antibodies in 1X Blocking Buffer as described in the Assay Procedure section (8.7).

1X Blocking Buffer and primary antibody reagents should be prepared incrementally for the number of samples to be processed on a given day.

6. Sample Preparation

The protocol below describes cell harvesting and staining for flow cytometry using 1.5 mL flip top microtubes. The assay can also be performed using larger tubes or 96-well deep well plates with some minor adjustments to the recommended volumes (at the users' discretion).

Pellet cells at centrifuge speeds of 350 – 500 x g depending on cell size (e.g. 350 x g for larger cells like HeLa and 500 x g for smaller cells like HL-60). Take care not to disturb cell pellet when aspirating wash solutions. <u>Resuspending cell pellets is greatly aided by gently tapping tubes after the supernatant has been aspirated</u>. Make certain that the cell pellets do not dry out at any time.

7.1   Cell culture and treatment conditions are dictated by the experiment at hand. As a general guideline, it is advisable to analyze 10,000 events (cells) on the flow cytometer per sample/data point. Therefore at least 4-10 times that many cells should be harvested for each sample to ensure sufficient material at the end of the staining (some cell loss during the harvest and staining procedure is expected). For the purposes of this protocol, we will assume harvesting and staining 1×10^5 cells/sample.

7.2 For suspension cells, generate a single cell solution by pipetting up and down

7.3 For adherent cells, fully dissociate cells (e.g. trypsin) into single cell suspension. Passaging the cell line the day before the experiment onto a fresh cultured plate may help improve single cell dissociation on the day of the experiment.

7.4 Pellet cells and carefully aspirate supernatant leaving only enough liquid to barely cover cell pellet.

7.5 Resuspend pellet into a suitable volume of 1X PBS to a final concentration of ~0.1-2x10^6 cells per mL (e.g. 400µL for 1x10^5 cells).

7.6 Overlay 20% paraformaldehyde so that the final concentration is 4%, gently mix by inverting the tube and incubate at room temperature for 15 minutes.

7.7 Pellet cells and remove supernatant.
Note: paraformaldehyde is toxic: handle appropriately and dispose of according to local requirements.

7.8 Resuspend the cell pellet by gently tapping the bottom of the tube and add a small volume of 1X PBS. This volume of 1X PBS added should be several times the approximate volume of the cell pellet (e.g. 50µL for 1x10^5 cells). Transfer the tube containing the cells to ice.

7.9 Add 9 volumes of ice cold Methanol to the cells in 1X PBS. Add Methanol slowly and mix well. The end result is cells resuspended in 10% 1X PBS/90% Methanol. Transfer to

-20ºC for at least 30 minutes for permeabilization. Cells are stable in 90% Methanol at -20ºC for at least 6 months.

7. Assay Procedure

We recommend pelleting cells at centrifuge speeds of 350 – 500 x g depending on cell size (e.g. 350 x g for larger cells like HeLa and 500 x g for smaller cells like HL-60). Take care not to disturb cell pellet when aspirating wash solutions. Resuspending cell pellets is greatly aided by gently tapping tubes after the supernatant has been aspirated. Make certain that the cell pellets do not dry out at any time. All steps should be performed at room temperature.

For the purposes of this protocol, we will assume staining $1x10^5$ cells per tube.

8.1 Remove cells from -20ºC and allow to warm to room temperature (~10-20min).

8.2 Invert tubes to mix and then pellet cells at 350 – 500 x g for 5 minutes. Carefully remove the Methanol-containing supernatant and dispose of appropriately. Take care to not disturb the cell pellet when aspirating (spinning down cells in Methanol results in a loose pellet).

8.3 Gently tap the cell pellet to resuspend and add 1 mL of 1X Blocking Buffer to resuspend cells.

8.4 Pellet cells and then aspirate supernatant. Resuspend in 1 mL of 1X Blocking Buffer.

8.5 Repeat step 8.4 for a second wash of the cells. Read step 8.6 before resuspending. After this second wash, the Methanol has been removed and the cells are ready for staining.

8.6 Resuspend the cells 50µL of 1X Blocking Buffer. Incubate for 30 minutes.

8.7 Prepare a 2X primary antibody solution by diluting 100X primary antibodies in 1X Blocking Buffer.

> *8.7.1 It is up to the user whether to measure the HIF1 alpha and GLUT1 staining in parallel samples or duplexed in the same sample. This is a question of the secondary antibodies used. See step 8.11.*

8.8 Overlay 50µL of the 2X Primary Antibody staining solution to the 50µL of cells in 1X Blocking Buffer. This results in a 1X Primary Antibody staining solution on the cells. Incubate 1 hour.

8.9 Tap tubes gently to resuspend if the cells have settled to the bottom of their tubes. Add 1mL 1X Blocking solution. Pellet cells and carefully aspirate supernatant.

8.10 Wash cells a second time by resuspending the cells in 1mL 1X Blocking Buffer. Pellet cells and aspirate supernatant. Tap tube to resuspend cells.

8.11 Add secondary antibodies appropriate for your flow cytometer.

8.11.1 *The HIF1 alpha antibody is a mouse monoclonal and the GLUT1 antibody is a rabbit monoclonal. It is at the users discretion to provide appropriate labeled secondary antibodies to label the primary antibodies included in this kit.*

8.11.2 *Figures 1 and 2 in the sample data section provide recommended secondary antibodies available from Abcam.*

8.12 Incubate 1 hour in the dark.

8.13 Tap tubes gently to resuspend if the cells have settled to the bottom of their tubes. Add 1mL 1X Blocking solution. Pellet cells and carefully aspirate supernatant.

8.14 Wash cells a second time by resuspending the cells in 1mL 1X Blocking Buffer and pelleting. Tap tube to resuspend cells.

8.15 Resuspend cells in 100µL 1X PBS and analyze on flow cytometer.

9   Data Analysis

Specific methods depend on the available flow cytometer. It is important to appropriately establish forward and side scatter gates to exclude debris and cellular aggregates from analysis. Primary antibody should be excluded from all samples of different cell type and treatment condition to determine background fluorescence.

Additional information on flow cytometry analysis can be found in the "Protocols and troubleshooting tips" section at abcam.com.

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.

Figures 1 and 2 show typical flow cytometry results using ab126584. Because confidence in antibody specificity is critical to flow cytometry data interpretation, the primary antibodies in this kit were validated for specificity by fluorescence immunocytochemistry and Western blot (Figures 3 and 4, respectively) using the HeLa and DFO model.
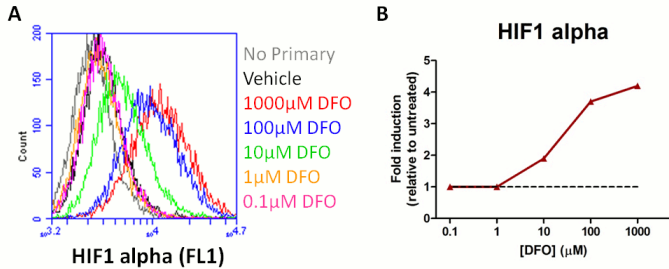
Figure 1. Sample experiment using ab126584 on HeLa cells treated with a titration of DFO: HIF1 alpha readout. *HeLa cells were cultured in standard tissue culture plates and treated with a titration of DFO. After 24 hours of DFO exposure, the cells were fixed and stained as described in the protocol. (A) Flow cytometry histogram showing mean fluorescent intensity of HIF1 alpha staining for untreated (Vehicle) and DFO treated samples. In this experiment anti-mouse-DyLight®488 (ab96879, 1:1000) was used as the secondary antibody and the signal was collected in FL1 . GLUT1 data (Fig. 2) was captured simultaneously in FL4. (B) Plot showing fold induction of HIF1 alpha levels (relative to untreated cells) as a function of DFO concentration (red line). The gray dotted line demarks 1 (the untreated level). DFO concentrations $\geq 10\mu M$ induce HIF1A protein levels in a dose dependent manner.*
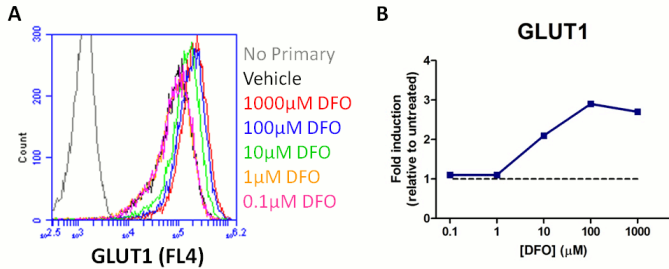
Figure 2. Sample experiment using ab126584 on HeLa cells treated with a titration of DFO: GLUT1 readout. *HeLa cells were cultured, treated and stained as in Fig. 1. (A) Flow cytometry histogram showing mean fluorescent intensity of GLUT1 staining for untreated (Vehicle) and DFO treated samples. In this experiment anti-rabbit-DyLight®650 (ab96902, 1:2000) was used as the secondary antibody and the signal was collected in FL4. HIF1 alpha data (Fig. 1) was captured simultaneously in FL1. (B) Plot showing fold induction of GLUT1 levels (relative to untreated cells) as a function of DFO concentration (blue line). The gray dotted line demarks 1 (the untreated level). DFO concentrations $\geq$10µM induce GLUT1 protein levels in a dose dependent manner.*
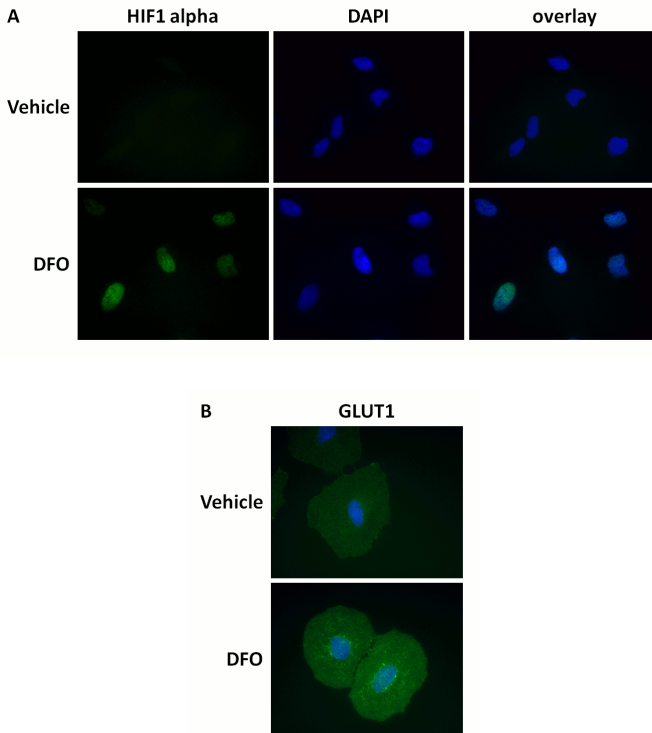
Figure 3. Antibody specificity demonstrated by immunocytochemistry.
Primary antibodies used in this assay kit were validated by staining HeLa cells +/- treatment with 1mM DFO (24h) and imaged by fluorescent microscopy. (A) Using antibodies from this kit, HIF1 alpha staining is absent in untreated cells and induced by DFO treatment. HIF1 alpha localizes to the nucleus (as seen by co-localization with the DNA stain DAPI) as expected. (B) GLUT1 staining labels the cell membrane and the fluorescent intensity increases with DFO treatment.
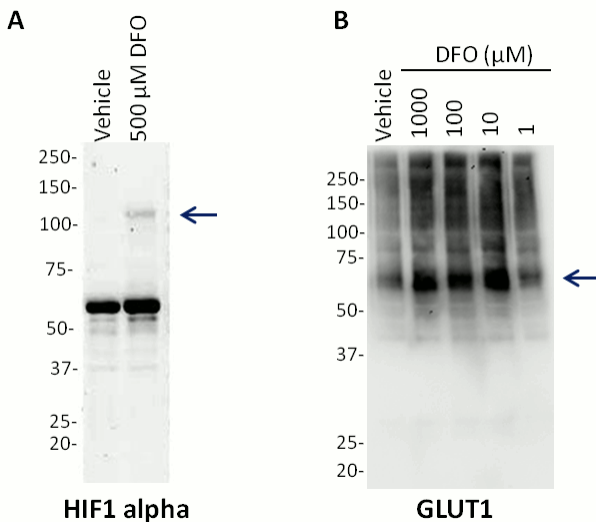
Figure 4. Antibody specificity demonstrated by Western Blot. *Primary antibodies used in this assay kit were validated by Western Blot using HeLa cell lysates that had been treated with a dose titration of DFO as indicated. (A) Using antibodies from this kit, the HIF1 alpha band (indicated by arrow) is absent in untreated cells and induced by DFO. In the Western Blot assay, the HIF1 alpha antibody has a prominent ~60kDa background band; however background signal is absent in In-Cell ELISA (ICE) and immunocytochemistry application (Fig. 1 and Fig. 2). (B) Similarly, GLUT1 levels are increased by DFO treatment in a dose-dependent manner. GLUT1 can be phosphorylated and glycosylated which likely contributes to the "smeary" bands on the Western Blot membrane.*

11   Troubleshooting

Problem	Cause	Solution
Low Signal	Insufficient secondary antibody	Increase concentration of antibody
	Lasers not aligned	Run flow check beads and adjust alignment if necessary
	Second antibody is not compatible with primaries	Use secondary antibodies raised against mouse and rabbit for this kit
High side scatter back-ground	Cells lysed	Samples should be freshly prepared. Do not vortex or shake the sample at any stage. Do not exceed 500 x g for centrifugation
	Bacterial contamination	Ensure sample is not contaminated
Low event rate	Low number of cells	Run 1×10^6 cells/mL
	Cells clumped	Ensure a single cell suspension. Sieve clumps (nylon mesh)
High event rate	High number of cells/mL	Dilute between 1×10^5 cells/mL and 1×10^6 cells/mL

Additional information on flow cytometry analysis and troubleshooting can be found in the "Protocols and troubleshooting tips" section at abcam.com.

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