Cell-Based ELISA

Human/Mouse Total HIF-1α Immunoassay

Catalog Number KCB1935

An ELISA-based assay using fluorogenic substrates to measure total HIF-1α in the context of a whole cell.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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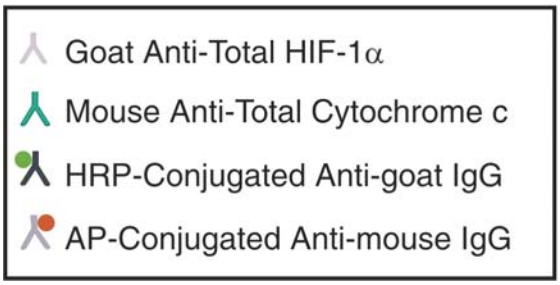
PRINCIPLE OF THE ASSAY

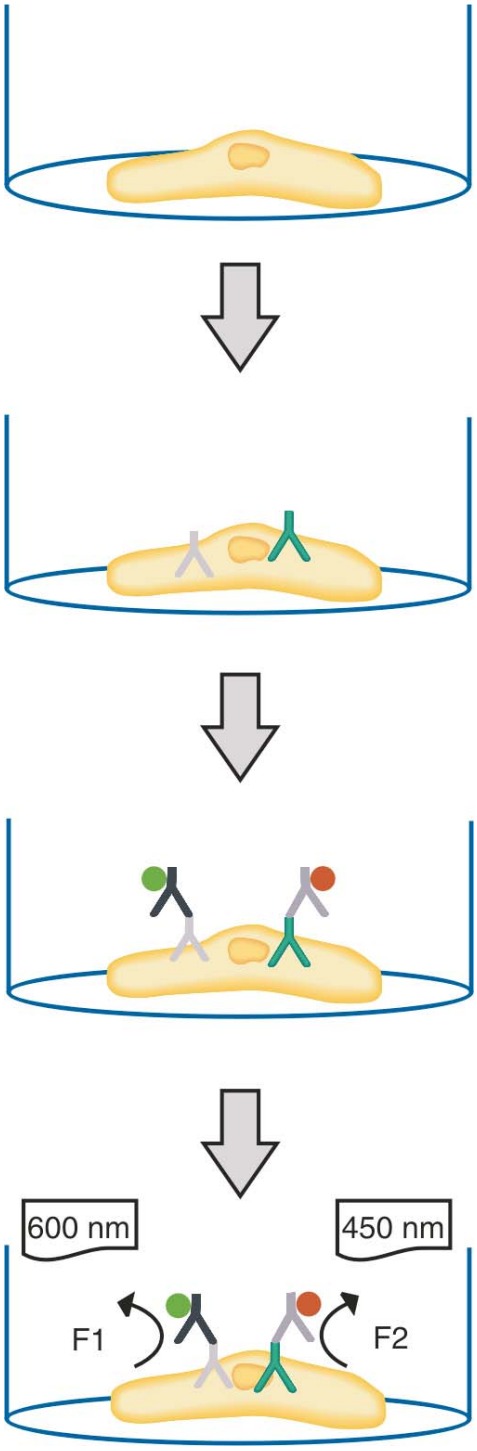
This Cell-Based ELISA contains the components required to measure total HIF-1α in the context of a whole cell. This simple and efficient cell-based assay eliminates the need to prepare cell lysates and uses as little as 10,000 cells per well. Cells are grown in 96-well plates and treated as desired. Following treatment, cells are fixed and permeabilized in the wells. The target protein amount is measured using a double immunoenzymatic labeling procedure. The cells are simultaneously incubated with two primary antibodies: an antibody specific for the target protein and a normalization antibody that is specific for Cytochrome c, a housekeeping protein. The primary antibodies are derived from different species. Two species-specific secondary antibodies labeled with either horseradish peroxidase (HRP) or alkaline phosphatase (AP), and two spectrally distinct fluorogenic substrates for either HRP or AP are used for detection. The fluorescence of the target protein is normalized to that of the housekeeping protein in each well for the correction of well-to-well variations. This two-wavelength assay results in accurate analysis of HIF-1α with good reproducibility.

TECHNICAL HINTS AND LIMITATIONS

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Cell-Based ELISA should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- When mixing or reconstituting protein solutions, always avoid foaming.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- A thorough and consistent wash technique is essential for proper assay performance. To minimize cell loss during the wash steps, avoid dispensing liquid directly onto the cell surface. Instead, gently dispense the liquid down the wall of the cell culture wells, always using the same side of the wells. Empty the wells by decanting and remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- To avoid cross-contamination, change pipette tips between additions of each reagent and/or sample. Also, use separate reservoirs for each reagent.
- It is recommended that all samples and controls be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8° C.

ASSAY OVERVIEW





1. **Seed cells in a 96 well plate. Treat cells as desired. Fix, permeabilize, and block cells.**

2. **Add primary antibodies (goat anti-total HIF-1α and mouse anti-total Cytochrome c).**

3. **Add secondary antibodies (HRP-conjugated anti-goat IgG and AP-conjugated anti-mouse IgG).**

4. **Add fluorogenic substrates F1 and F2 and measure fluorescence.**

MATERIALS PROVIDED

Store the unopened kit at 2-8° C. Do not use past the kit expiration date. This kit contains sufficient materials to run one 96-well plate.

Microplate (Part 607582) - One 96-well cell culture clear-bottom black microplate and cover for use as a vessel in the assay.

Total HIF-1α Antibody (Part 965571) - 1 vial of lyophilized goat anti-total HIF-1α antibody.

Total Cytochrome c Antibody (Part 965572) - 1 vial of lyophilized mouse anti-total Cytochrome c antibody.

HRP-conjugated anti-goat IgG (Part 893280) - 110 µL of HRP-conjugated donkey anti-goat IgG secondary antibody.

AP-conjugated anti-mouse IgG (Part 893297) - 110 µL of AP-conjugated donkey anti-mouse IgG secondary antibody.

Substrate F1 Concentrate (Part 893232) - 50 µL of a sensitive and stable fluorogenic substrate for horseradish-peroxidase (HRP).

F1 Diluent (Part 893233) - 10 mL of a solution for diluting the Substrate F1 Concentrate.

Substrate F2 (Part 893234) - 8 mL of a sensitive and stable fluorogenic substrate for alkaline phosphatase (AP).

Blocking Buffer (Part 893235) - 35 mL of 10% fetal bovine serum in diluent with preservatives.

Wash Buffer (5X) (Part 893236) - 60 mL of a buffered surfactant with preservatives.

Plate Sealers - 4 adhesive strips.

OTHER SUPPLIES REQUIRED

- 37% formaldehyde (Molecular Biology Grade, Sigma; Catalog # F8775). Refer to MSDS prior to use.
- 30% H_2O_2 (Sigma, Catalog # H1009). Refer to MSDS prior to use.
- 1X PBS (Irvine Scientific, Catalog # 9240).
- Deionized or distilled water.
- Pipettes and pipette tips.
- Multi-channel pipette for washing.
- Cell culture incubator.
- Microfuge tubes.
- Orbital shaker.
- Fluorescence plate reader with two channels: excitation 540 nm / emission 600 nm and excitation 360 nm / emission 450 nm.

REAGENT PREPARATION

Total HIF-1α Antibody - Prepare a 100X working concentration of the antibody by reconstituting with 110 µL of 1X PBS. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer for up to 60 days.*

Total Cytochrome c Antibody - Prepare a 100X working concentration of the antibody by reconstituting with 110 µL of 1X PBS. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer for up to 60 days.*

Primary Antibody Mixture** - Immediately before use, add 100 µL of the Total HIF-1α Antibody and 100 µL of the Total Cytochrome c Antibody to 9.8 mL of Blocking Buffer. 100 µL of the primary antibody mixture is required per well. **If a full plate is not being assayed, adjust volumes accordingly.**

Secondary Antibody Mixture** - Immediately before use, add 100 µL of the HRP-conjugated antibody and 100 µL of the AP-conjugated antibody to 9.8 mL of Blocking Buffer. 100 µL of the secondary antibody mixture is required per well. **If a full plate is not being assayed, adjust volumes accordingly.**

Substrate F1 - Add the contents of the Substrate F1 Concentrate vial (50 µL) to the 10 mL of F1 Diluent in the brown bottle. Store Substrate F1 at 2-8° C for up to 60 days.*

1X Wash Buffer - Add 60 mL of Wash Buffer (5X) to 240 mL of 1X PBS to prepare 1X Wash Buffer. Store at 2-8° C for up to 60 days.*

SOLUTION PREPARATION

4% Formaldehyde (for adherent cells) - Add 1.3 mL of 37% formaldehyde to 10.7 mL of 1X PBS to prepare 4% formaldehyde. **If a full plate is not being assayed, adjust volumes accordingly.**

8% Formaldehyde (for non-adherent cells) - Add 2.6 mL of 37% formaldehyde to 9.4 mL of 1X PBS to prepare 8% formaldehyde. **If a full plate is not being assayed, adjust volumes accordingly.**

Quenching Buffer - Add 200 µL of 30% H_2O_2 to 9.8 mL of 1X Wash Buffer to prepare 0.6% H_2O_2. **If a full plate is not being assayed, adjust volumes accordingly.**

*Provided this is within the expiration date of the kit.

**Once prepared, the primary and secondary antibody mixtures cannot be stored. Prepare only enough as needed to run the assay.

GENERAL ASSAY PROCEDURE

A. Culture, Stimulate, Fix, and Block Cells

1. Seed 100 µL of 10,000-20,000 adherent cells into each well of the black 96-well microplate with clear bottom and incubate overnight at 37° C in a cell culture incubator.

   Note: *The cell number used is dependent upon the cell line and the relative amount of protein phosphorylation. Optimal cell numbers should be determined by each laboratory for each assay.*

2. Grow and treat the cells as desired.

3. Fix cells by replacing the medium with 100 µL of 4% formaldehyde in 1X PBS. Add the plate cover and incubate for 20 minutes at room temperature. Alternatively, apply a plate sealer and store the plate containing the fixed cells at 2-8° C for up to 2 weeks.

   Warning: *Formaldehyde is highly toxic. Confine vapors to a chemical hood and wear appropriate gloves and eye protection when using this chemical. Refer to MSDS prior to use.*

4. Remove the formaldehyde solution and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

5. Remove the Wash Buffer and add 100 µL of Quenching Buffer. Add the plate cover and incubate for 20 minutes at room temperature.

6. Remove the Quenching Buffer and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

7. Remove the Wash Buffer, and add 100 µL of Blocking Buffer. Add the plate cover and incubate for 1 hour at room temperature.

B. Binding of Primary and Secondary Antibodies

1. Remove the Blocking Buffer and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

2. Add 100 µL of the Primary Antibody Mixture to each well. Cover with a plate sealer and incubate for 16 hours at 2-8° C. In cells known to generate high amounts of total HIF-1α, a 2-3 hour primary antibody incubation is sufficient; however, for maximum sensitivity, an overnight incubation is recommended.

   Note: *Depending on the experimental design (refer to the Calculation of Results section), some wells may be incubated with Primary Antibody Mixture and some with only the Blocking Buffer as the negative controls (secondary antibody alone).*

3. Remove the Primary Antibody Mixture and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

4. Add 100 µL of the Secondary Antibody Mixture to each well. Cover with a plate sealer and incubate for 2 hours at room temperature.

   Note: *The Secondary Antibody Mixture is added into each well including the negative control wells.*

C. Fluorogenic Detection

1. Remove the Secondary Antibody Mixture from each well and wash the cells 2 times with 200 µL of 1X Wash Buffer, followed by 2 washes with 200 µL of 1X PBS. Each wash step should be performed for 5 minutes with gentle shaking.

2. Remove the 1X PBS from the plate and add 75 µL of Substrate F1 to each well. Incubate for 20-60 minutes at room temperature. Protect the plate from direct light. A pink or rosy color should develop in the wells.

   Note: *It is critical to add Substrate F1 into each well and incubate for 20-60 minutes for fluorescence development prior to the addition of Substrate F2. Adding Substrate F1 and Substrate F2 simultaneously into the wells will result in the inhibition of fluorescence development.*

3. Add 75 µL of Substrate F2 to each well and incubate for an additional 20-40 minutes at room temperature. Protect the plate from direct light.

4. Read the plate using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm. Then read the plate with excitation at 360 nm and emission at 450 nm. The readings at 600 nm represent the amount of total HIF-1α in the cells, while readings at 450 nm represent the amount of total Cytochrome c in the cells.

PROCEDURE FOR NON-ADHERENT CELLS

This protocol has been validated for use with non-adherent cells by culturing and fixing cells as follows.

1. Seed 100 µL of suspension cells into each well of the black 96-well microplate in serum-free or normal growth media at the desired cell density (seeding cells at a density of 0.2 to 1.0 x 10^6 cells/mL is recommended).

2. Incubate the plate at 37° C. Depending on the cell line and treatment, the typical incubation time is 0.5-16 hours.

3. Treat the cells as desired. Prepare the treatment media containing a 5X final concentration of treatment. Add 25 µL of 5X treatment media to the wells and bring the total volume up to 125 µL. Mix solution by gently agitating the plate and incubate according to your treatment protocols.

4. Centrifuge the plate at 500 x g for 3 minutes at 4° C and remove media.

5. Fix the cells by adding 100 µL of 8% formaldehyde in 1X PBS. Apply the plate cover and incubate for 20 minutes at room temperature. Alternatively, apply a plate sealer and store the plate containing the fixed cells at 2-8° C for up to 2 weeks.

6. Continue with section A, step 4 of the General Assay Procedure on page 6.

CALCULATION OF RESULTS

Control wells with no primary antibody (secondary antibody alone) should be included in each experiment. The fluorescence (RFUs) from these wells is the background fluorescence and is subtracted from all sample wells. The total HIF-1α fluorescence at 600 nm in each well is normalized to that of the total Cytochrome c fluorescence at 450 nm. The normalized duplicate readings for each sample are then averaged.

KIT PERFORMANCE DATA

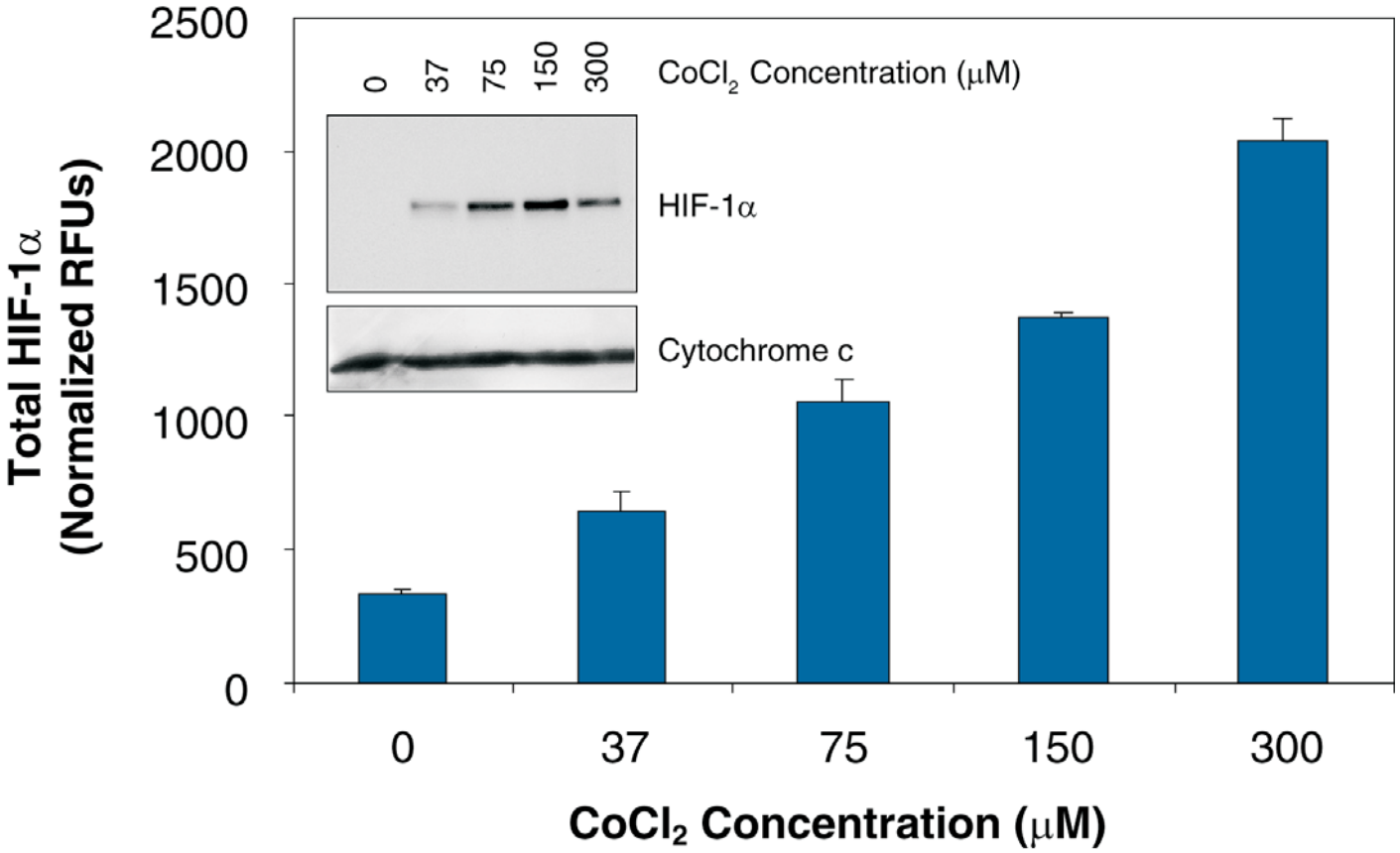


Figure 1: Dose response of $CoCl_2$-induced HIF-1α protein expression. MCF-7 human breast cancer cells were cultured in 96-well plates and treated with the indicated amounts of $CoCl_2$, a hypoxia mimetic, for 8 hours. After fixation of cells in the wells, HIF-1α levels were determined using this Total HIF-1α Cell-Based ELISA Kit. Values represent the mean ± the range of duplicate determinations. Analysis of HIF-1α and Cytochrome c (the normalization house-keeping protein) by Western blotting using the antibodies supplied in this kit is also shown (inset).

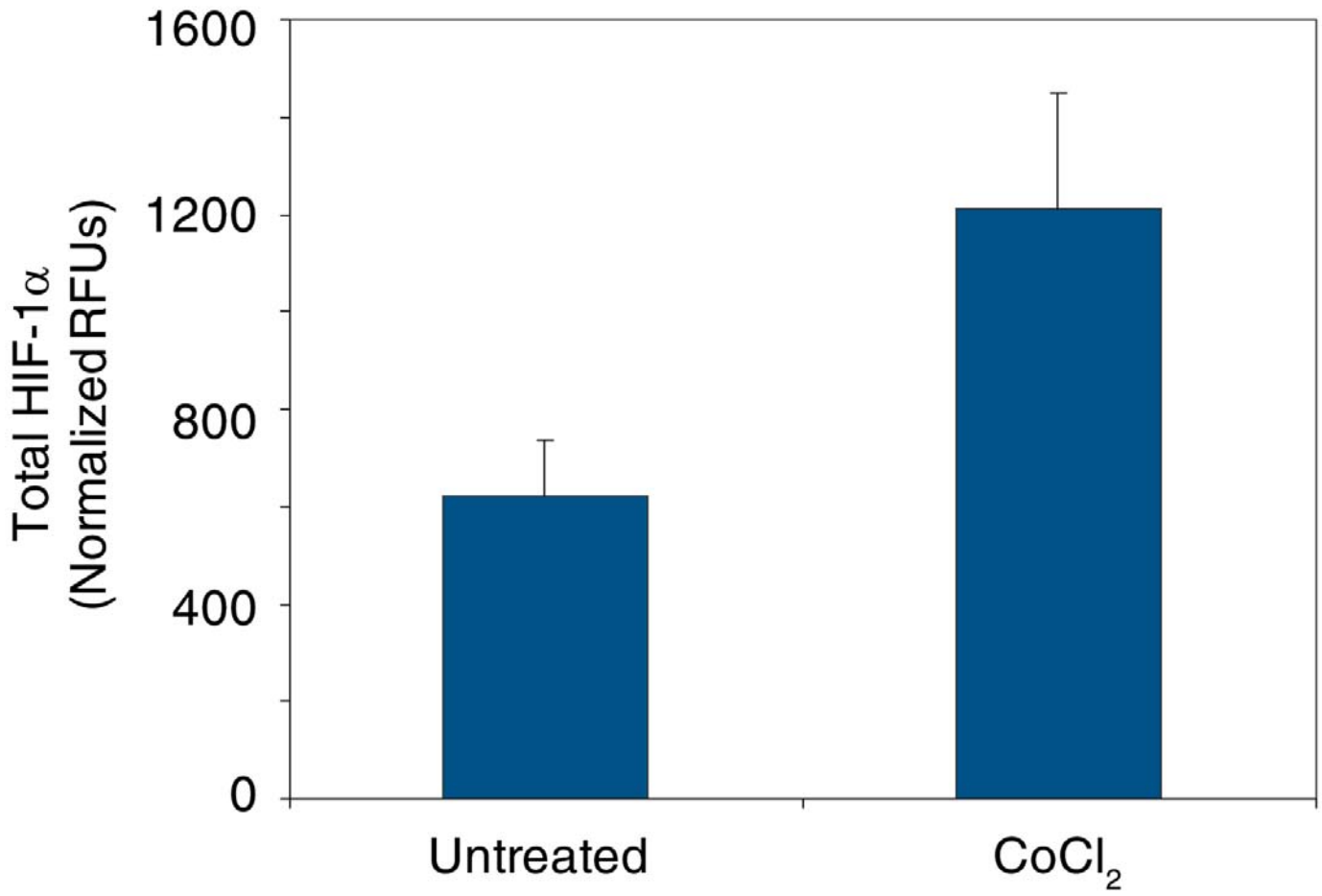
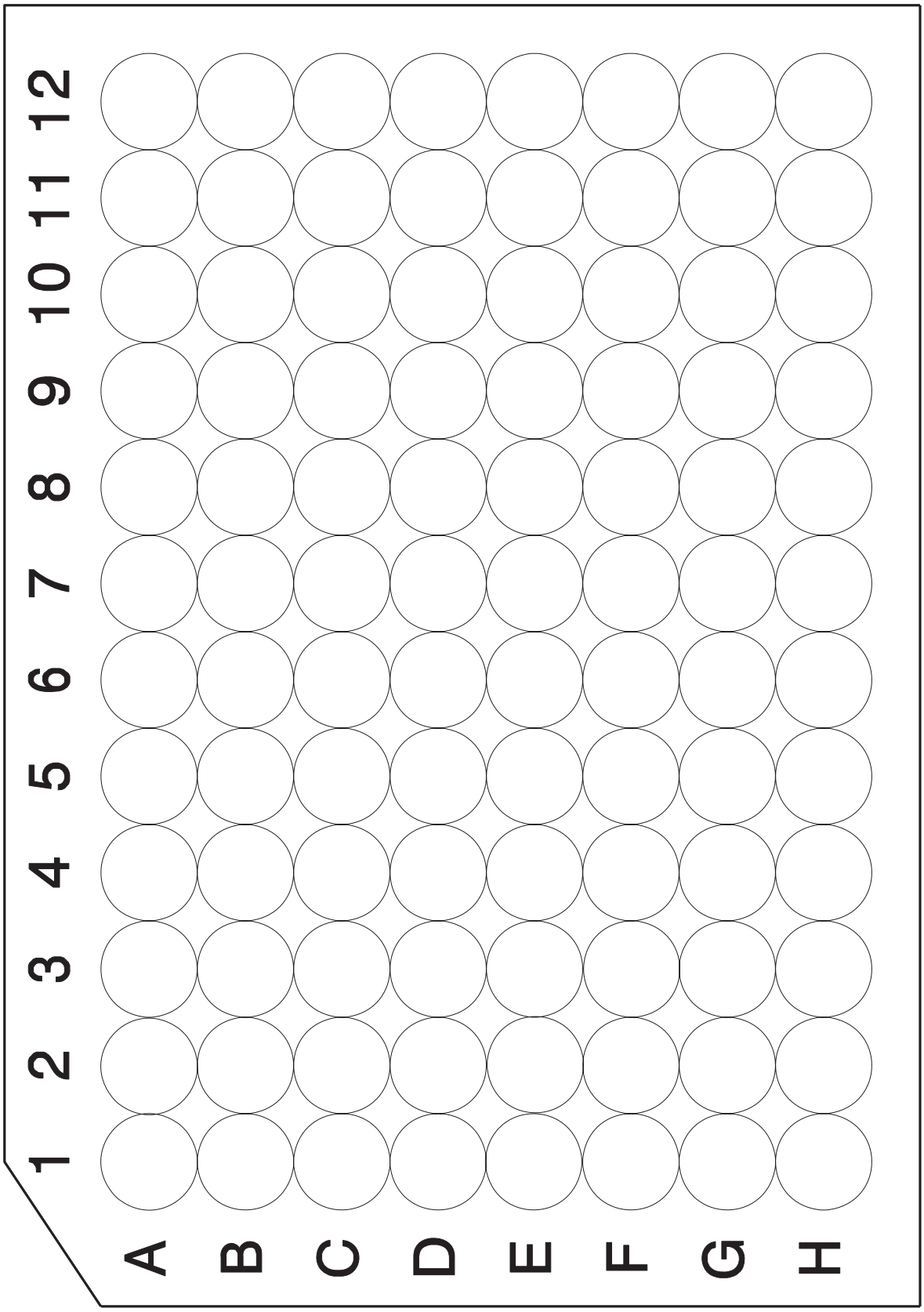


Figure 2: Measurement of $CoCl_2$-induced HIF-1α protein expression in mouse cells. Balb/3T3 mouse embryonic fibroblasts were cultured in 96-well plates and treated with 150 μM of $CoCl_2$, a hypoxia mimetic, for 16 hours. After fixation of cells in the wells, HIF-1α levels were determined using this Total HIF-1α Cell-Based ELISA Kit. Values represent the mean ± the range of duplicate determinations.

PLATE LAYOUT

Use this plate layout as a record of samples assayed.



NOTES

This product is covered by one or more of the following U.S. Patents: 5,882,914; 6,020,462; 6,222,018 and foreign equivalents.