# Protocol: Hypoxic and Simulated-Hypoxic Cell Lysate Preparations

To achieve hypoxic conditions, cells may be exposed in a chamber or incubator to an $O_2$ level of ~2% ($pO_2$), alternatively hypoxic conditions may be induced chemically by treating cells with heavy metals (e.g., $CoCl_2$)

**Equipment and Materials:**

Tissue culture incubator capable of regulating $O_2$ percentage

Small probe sonicator

Heat block (at 95°C) or boiling water bath

Tissue culture dishes (90-150 mm)

Tissue culture media

Cell scrapers

Microcentrifuge tubes (1.5 mL)

Cell culture grade 1XPBS

1x Laemmli Sample Buffer: 2% SDS, 2.5% 2-mercaptoethanol (βME), 25% glycerol, 0.01% bromophenol blue, 62.5 mM Tris HC, pH 6.8

$CoCl_2$: sterile 100mM $CoCl_2$ in distilled water.

**Methods:**

**Hypoxic Cell Lysate Preparation**

1. Grow adherent cells, following standard tissue culture procedures and under normoxic (~18-21% $O_2$) conditions, to a semi-confluent density of 70-80%.

   Note: Cell culture density is critical for hypoxic response. In overly dense cultures, cells are unresponsive to hypoxic treatment or gene expression changes may be atypical, in contrast low density cultures yield low-concentration lysates and result in excessive cellular stress.

2. Under sterile conditions in a biosafety hood, remove half of the media from semi-confluent cultures leaving sufficient media to just cover the cells.

   Note: Removal of excess media allows for a faster cellular response to hypoxic conditions.

3. Prepare the incubator for hypoxic conditions according with manufacturer specifications, some require Nitrogen flow for at least 10 min to ensure hypoxic conditions.
4. Incubate cultures for 4 hours under hypoxic conditions (~2% $O_2$), and under normoxic conditions for control lysates. Standard cell culture conditions for temperature (37$^oC$) and humidity (95%), still apply.

   Note: Treatment period is critical to elicit an optimal response to hypoxia, incubation periods shorter than 4 hours elicit a suboptimal response, while longer treatment periods of over 4 hours lead to excessive cellular stress.

5. Harvest cells and prepare lysates:

   Note: The process of cell harvesting and lysing should be expedited to decrease exposure of HIF proteins to ambient $O_2$ and reduce their degradation. While direct treatment of cell cultures with sample buffer allows for rapid cell lysate preparation, this method precludes protein quantification. Lysing cells in RIPA buffer allows the quantification of proteins, but specific stabilizers of HIF proteins should be used (e.g., protease inhibitors, Deferoxamine/Desferrioxamine, Dimethyloxalylglycine).

   Working with one culture dish at a time under sterile conditions, remove media.

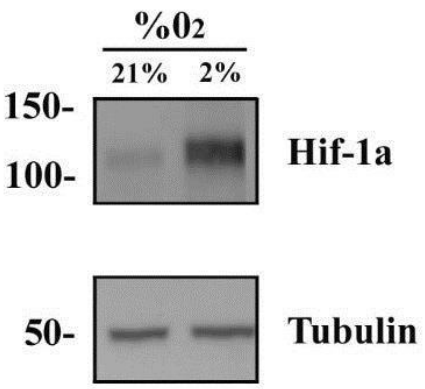
   a. Rinse with room temperature 1XPBS, remove 1XPBS efficiently to avoid excessive dilution of the final cell lysate.
   b. Lyse cells by the addition of 1x Laemmli Sample Buffer (e.g., use 400µL for 90 mm culture dish or 660 µL for 150 mm culture dish).
   c. Use a cell scraper to distribute the 1x Laemmli Sample Buffer over the entire surface of the culture dish and to lift cells away from the dish.
   d. Transfer the viscous cell lysate to a microcentrifuge tube and cap promptly.

   Note: For the processing of cell cultures maintained for 4 hours at normal oxygen level, protein degradation is not a concern, cell lysate preparation does not need to be expedited

6. Sonicate the cell lysates 3 times for periods of ~20 seconds each to decrease viscosity prior to the separation of proteins by gel electrophoresis.
7. Centrifuge sonicated cell lysate at 14,000 rpm for 10minutes to precipitate and eliminate any insoluble material (remove pellet).
8. Denature samples at 95$^oC$ for 10 minutes.
9. Allow samples to cool down to room temperature and spin quickly to collect all the material.
10. Save samples at -20$^oC$ or at -80$^oC$ for short and long term storage, respectively.

Note: While samples from hypoxic treated cells are stable, repeated freeze-thaw cycles should be avoided.

Positive controls for WB applications: HeLa Hypoxic/Normoxic Cell Lysate/ https://www.novusbio.com/products/hela-lysate_nbp2-36452




Western Blot: HeLa Hypoxic / Normoxic Tissue Lysate [NBP2-36452] - Western blot analysis of HIF-1 alpha antibody (NB100-134) in HeLa hypoxic and normoxic lysates (NBP2-36452). Tubulin loading control is shown below.

**Simulated-Hypoxic Cell Lysate Preparation**

1. Grow adherent cells, following standard tissue culture procedures and under normoxic conditions, to a semi-confluent density of 70-80%.
2. Under sterile conditions in a biosafety hood, add 10 µL of 100mM $CoCl_2$ per 10 mL media. For control cell cultures add 10 µL of sterile water per 10 mL media.
3. Incubate cultures for 4 hours with $CoCl_2$ and water for controls under normoxic conditions (~18-21% $O_2$).

   Note: Treatment period is critical to elicit an optimal response with $CoCl_2$, incubation periods shorter than 4 hours elicit a suboptimal response, while longer treatment periods of over 4 hours lead to excessive cellular stress.

4. Harvest cells and prepare lysates:

   Working with one culture dish at a time under sterile conditions, remove media.

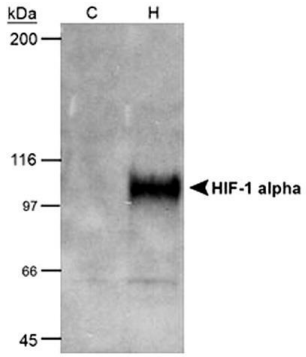
   a. Rinse with room temperature 1XPBS, remove 1XPBS efficiently to avoid excessive dilution of the final cell lysate.
   b. Lyse cells by the addition of 1x Laemmli Sample Buffer (e.g., use 400µL for 90 mm culture dish or 660 µL for 150 mm culture dish).
   c. Use a cell scraper to distribute the 1x Laemmli Sample Buffer over the entire surface of the culture dish and to lift cells away from the dish.
   d. Transfer the viscous cell lysate to a microcentrifuge tube and cap promptly.
5. Sonicate cell lysates 3 times for periods of ~20 seconds each to decrease viscosity prior to the separation of proteins by gel electrophoresis.
6. Centrifuge sonicated cell lysate at 14,000 rpm for 10 minutes to precipitate and eliminate any insoluble material (remove pellet).
7. Denature samples at 95°C for 10 minutes.

8. Allow samples to cool down to room temperature and centrifuge quickly to collect all the material.
9. Save samples at -20°C or at -80°C for short and long term storage, respectively.

Note: While samples from cells treated with $CoCl_2$ are stable, repeated freeze-thaw cycles should be avoided.

Positive controls for WB applications: COS-7 Nuclear Hypoxic Induced Cell Lysate: https://www.novusbio.com/products/cos-7-lysate_nb800-pc26

HeLa Hypoxic (CoCl2)/Normoxic Lysate: https://www.novusbio.com/products/hela-lysate_nbp2-36450l




Western Blot: COS-7 Nuclear Hypoxic Induced Cell Lysate [NB800-PC26] - WB analysis of 50ug nuclear lysate of COS7 cells which were left untreated (C) or were treated with Cobalt Chloride / $CoCl_2$ (H) before preparation of lysates (catalog# NB800-PC26). The blot was developed using HIF-1 alpha antibody (clone H1alpha67; catalog# NB100-105).