Cellular Response to Hypoxia

The cellular response to hypoxia has become of medical interest, as hypoxia causes broad physiological changes in the cell. Originally identified as a regulator of erythropoietin (EPO) and vascular endothelial growth factor (VEGF), hypoxia-inducible factor-1 alpha (HIF-1 alpha) is a major global regulator of the hypoxic response (Semenza and Wang, 1992; Wang and Semenza, 1993). HIF-1 alpha has subsequently been shown to play a central role in tumor pathology and is a target for treatment and therapy (Reviewed by Tsai and Wu, 2012; Tang and Yu, 2012; Ward et al., 2013; Hu et al., 2013).

HIF-1 alpha is rapidly degraded in oxygenated cells. This occurs within the first 5 minutes of exposure to a normoxic environment, even after growth for over 4 hours in hypoxia (Huang et al., 1996) making it a difficult protein to study in the laboratory environment.

The following protocols have been compiled by Novus from the published literature as well as from Novus Biologicals' extensive experience as a leader in hypoxia research reagents.

Required Equipment and Supplies:

- Tissue culture incubator capable of monitoring and regulating Oxygen levels, or a hypoxic chamber.
- Tissue culture dishes (90 or 150 mm)
- Tissue culture media
- Cell scrapers
- Cell culture grade PBS
- 1x Laemmli Sample Buffer
- Microcentrifuge tubes
- CoCl2 (necessary for CoCl2 treatment only)
- Microprobe sonicator

Hypoxic Protocol

(You will need a tissue culture chamber or hypoxic incubator for this protocol. Standard tissue culture conditions of temperature and humidity still apply).

- Bring tissue culture incubator or chamber to an Oxygen level of 2% or less, by the regulated addition of Nitrogen. If you are using a hypoxic chamber, you will need to consult the operation instructions for the addition of Nitrogen.
- Grow cell line of choice to semi-confluency (70-80%) using the recommended culture media.
    - We use 90 or150 mm tissue culture dishes for speed or sample collection.
- Incubate the cells in the hypoxic incubator or chamber for several time points.
    - We recommend incubating for at least 2 hours and no more than 8 hours.
    - You should have maximum HIF1 alpha induction at 4 hours.
- Immediately remove the samples at their indicated time points to a sterile hood environment. Speed of sample collection is important, as HIF1 alpha is degraded in a normal oxygen environment.
- Aspirate the media from each dish and wash with PBS, aspirate all buffer as quickly as possible.
- Lyse cells following the cell lysis protocol below.

CoCl2 Protocol

- Grow cells in 90 or 150 mm tissue culture dishes to semi-confluency (70-80%).
- Prepare a fresh 100 mM stock solution of CoCl2 in PBS.
- Add the CoCl2 solution to a final concentration of 100-150 µM directly to the tissue culture media in each dish. Mix well.
- Incubate the cells under standard tissue culture conditions (5% C02, 37°C) for 4-8 hours.
- Aspirate the media from each dish and wash with PBS, aspirate all buffer as quickly as possible.
- Lyse cells following the cell lysis protocol below.

Cell Lysing Protocol

- To each aspirated dish, add 1 x Laemmli sample buffer:
    - For 90 mm dishes use 400 µl of sample buffer.
    - For 150 mm dishes use 600 µl of sample buffer
- Scrape cells with a cell scrapper until the sample buffer becomes viscous.
- Collect the buffer with lysed cells into a microcentrifuge tube.
- Sonicate or shear the sample until the viscosity is reduced enough to pipette the lysate.
- Boil samples at 95^{o}C for 5-10 minutes.
- Load samples for gel electrophoresis and Western blot analysis.

Please note that this method for lysing is used to ensure there is little to no protein degradation of the lysate. Once sample buffer is added, protein quantitation is not possible. Optimal loading volumes will need to be determined empirically. If you need to quantitate your sample, Novus Biologicals recommends lysing the cells in RIPA buffer with protease inhibitors. As HIF-1 alpha is rapidly degraded, there are a number of HIF stabilizers that can be added to the sample to inhibit degradation.

- Deferoxamine/Desferrioxamine (DFO/DFX)
    - DFO and DFX are iron chelators that will lower the iron levels in cells (Miller, 1989). HIF1 alpha is known to degrade in the presence of both oxygen and iron (Semenza 2001; Giaccia et al., 2004; Poellinger and Johnson, 2004).
    - When iron or oxygen levels are low (via treatment with an iron chelators-i.e. DFX/DFO) prolyl-4-hydroxylase domain enzymes (PHDs) cannot hydroxylate HIF1 alpha (Ivan et al., 2001; Jaakkola et al., 2001).
- N-acetylcysteine (NAC)
    - Metabolized NAC (SNOAC) has been found to induce a hypoxia-like response in the expression of important genes involved in this pathway (including HIF) in both in vitro an in vivo studies (Palmer et al., 2007).
    - NAC is an antioxidant and has been used to target the hypoxic response in some tumors. Recent studies have shown that NAC prevents HIF1 alpha stabilization in hypoxic conditions, so controls should be used with NAC when investigating (Sceneay et al., 2013).
- Dimethyloxalylglycine (DMOG)
    - DMOG is an ester of N-oxalylglycine and Inhibitor of prolyl-4-hydroxylase domain enzymes that regulate the stability of HIF1 alpha. (Nagel et al., 2011; Jaakkola et al., 2001; Takeda et al., 2007)
    - DMOG stabilizes HIF1 alpha expression at concentrations between 0.1-1.0 mmol/L (Jaakkola et al., 2001).

Under normoxic conditions, HIF1 alpha is expressed at low levels and does not show distinct subcellular localization. However, under hypoxic conditions, HIF1 alpha translocates to the nucleus (Kallio et al., 1998). Detecting HIF1 alpha in a nuclear lysate can provide a more concentrated source of HIF1 protein.

Preparation of Cytoplasmic and Nuclear Extraction Lysates

- Collect cells into PBS by centrifugation (non-adherent cells) or scraping from culture flasks followed by centrifugation (adherent cells).
- Wash cells 2X with ice cold PBS at 1000 x g for 10 minutes.
- Gently resuspend washed pellet in 500 µl Hypotonic Buffer (recipe below). Incubate on ice for 15 minutes.
- Add 25 µl of (10% NP40) and vortex for 10 seconds at highest setting.
- Centrifuge for 10 minutes at 1200 x g at 4^{o}C
- Transfer and save the supernatant – this contains the cytoplasmic fraction. The pellet is the nuclear fraction.
- Resuspend the nuclear pellet in 50 µl Cell Extraction Buffer (recipe below). Incubate on ice for 30 minutes with gentle vortexing every 10 minutes.
- Centrifuge for 30 minutes at 14,000 x g at 4^{o}C. Transfer supernatant (nuclear fraction) to a clean microcentrifuge tube.
- Aliquot and store at -80^{o}C.

Hypotonic Buffer	Cell Extraction Buffer
20 mM Tris-HCl, pH 7.4 10 mM NaCl 3 mM MgCl2	100 mM Tris, pH 7.4 2 mM Sodium Orthovanadate 100 mM NaCl 1 % Triton X-100 1 mM EDTA 10% Glycerol 1 mM EGTA 0.1% SDS 1 mM NaF 0.5% Deoxycholate 20 mM Sodium Diphosphate

HIF Western Blot Protocol

After transferring your gel onto PVDF membrane, place the membrane into Ponceau S solution for a few minutes. Remove Ponceau S solution and rinse the blot with some water. Ensure that your proteins are present and not degraded or smeared on your blot. Clean off Ponceau S solution with TBST until the membrane is clear again. Proceed to blocking step

- Block membrane in 5% non-fat dry milk in TBST (0.1% Tween-20) for 1 hour at room temperature
- Rinse block off and add primary
    - Dilute primary at recommended dilution in 1% non-fat dry milk in TBST
- Incubate your blot with your primary for 1 hour at room temperature or overnight at 4C
- After primary incubation, wash the membrane 3 x 10minutes or 5 x 5 minutes in TBST
- Dilute your secondary antibody (following manufacturers recommendations) in 1% non-fat dry milk in TBST
    - Incubate with secondary for 1 hour at room temperature
- Wash blot 3 x 10minutes or 5 x 5 minutes in TBST
- Place blot in development solution and proceed to imaging

HIF-Specific Western Blot Troubleshooting Tips

My signal is really weak, how can I make it stronger?

Your signal may be weak due to a greater amount of degraded HIF in your samples than non-degraded protein. This can be alleviated by using nuclear extracts instead of whole cell extracts. Since HIF localizes to the nucleus in hypoxic conditions the signal will be enhanced as there will be less degraded HIF in nuclear extracts.

Another way to enhance your signal in Western Blot is to cut the membrane. This should enhance the signal by removing degradation products that may be competing away signal of your antibody from the non-degraded protein.

In immunostaining applications ensure that you are using the appropriate permeabilizing agent to achieve the best staining. For nuclear targets a stronger detergent is often necessary and the lab will recommend Triton X-100 over Tween 20.

HIF ICC Protocol

Grow and treat cells according to the hypoxic/normoxic protocol listed above. Grow your cells directly on your coverslips. (Depending on your cells, you may want to consider coating your coverslips with Poly-L Lysine before beginning your experiment).

- Once cells reach optimal 70-80% confluency, immediately fix cells in 10% formalin for 10 minutes at room temperature.
- Rinse cells with PBS or TBS
- Apply permeabilization buffer to your cells
    - For HIF, we recommend using 0.5% Triton X in TBS for 10 minutes at room temperature
- Wash your cells 3 times with PBST or TBST (0.1% Tween-20)
- Block cells in 1% normal goat serum (or 1% serum from the host species of your secondary) in TBS for 1 hour at room temperature
- Dilute your primary in blocking buffer and incubate overnight at 4C
    - See datasheet for recommended dilution
    - All dilutions listed are recommendations; optimal dilutions must be determined by the end user.
- Wash 3 times with PBST or TBST (0.1% Tween-20)
- Dilute your secondary in blocking buffer for 1 hour at room temperature.
    - Follow recommended dilutions provided by the manufacturer for your secondary.
- Wash 3 times with PBST or TBST (0.1% Tween-20)
- Place your cover slip on a viewing slide
    - Optional: add DAPI nuclear counterstain prior to mounting on slide
- View your cells under a microscope with appropriate filter or laser

Where will my signal be in an immunostaining technique such as IHC or ICC?

HIF-1 alpha can be found at very low levels in the cytoplasm under normoxic conditions. During hypoxia, HIF1a is stabilized and translocates to the nucleus to act as a transcription factor. You should expect to see nuclear staining in your hypoxic samples, but may observe faint cytoplasmic staining in relation to degraded HIF in your samples.

HIF-2 alpha is strongly expressed in the nucleus under hypoxic conditions.

VEGF strongly stains the cytoplasm with additional staining noted on the plasma or nuclear membrane. Spliced variants of VEGF can be secreted.

http://www.ihcworld.com/_protocols/antibody_protocols/hif_1alpha_novus.htm

HIF IHC Protocol

- Deparaffinization and rehydration
    - Prior to staining, tissue sections must be deparaffinized and rehydrated. Incomplete removal of paraffin can cause poor staining of the section.
- Immerse slides in xylene and incubate for 5 minutes. Repeat twice with fresh xylene for another 5 minutes each.
- Immerse slides in 100% ethanol for 5 minutes, and follow with immersion in 95%, 75% and 50% ethanol for 3 minutes each.
- Rinse slides with distilled water for 5 minutes; keep in water until ready to perform antigen retrieval.
    - Heat induced antigen retrieval (HIAR)
        - Most formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining can proceed. Heat induced antigen retrieval can be performed using a steamer, pressure cooker, or a microwave. The retrieval time written in this protocol is based on using a retrieval steamer. The heating time may need to be adjusted if you use a different device and method.
- Fill plastic Coplin jar/container with Antigen Retrieval Buffer.
- Place the Coplin jar/container in steamer.
- Turn on steamer and preheat to 90-100°C. Carefully put slides into the Coplin jar/container and steam for 40 min (95-100°C).
    - If you are using a microwave, perform antigen retrieval for 30 minutes
    - If you are using a pressure cooker, perform antigen retrieval for 10 minutes
- Minimum temperature for antigen retrieval is 87°C
    - Turn off the steamer, remove the Coplin jar, place at room temperature and allow slides to cool for 20 min.
    - Rinse slide by incubation of slide in distilled water for 3 minutes. Repeat this step twice and begin staining procedure.
- Staining
    - Wash sections in dH2O three times for 5 minutes each.
    - Permeablize section in permeabilization buffer (1X PBS with 0.2-0.4% Triton X)
        - Wash tissue slide in permeabilization buffer 3-4 times 5 minutes each
- Block each section with 100-400 µl blocking solution (1X PBST, 5% goat serum or serum of the host species of your secondary) for 1 hour at room temperature.
- Remove blocking solution and add 100-400 µl primary antibody diluted in 1X PBST, 5% goat serum to each section. Incubate overnight at 4°C.
- Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
- Add 100-400 µl biotinylated secondary antibody, diluted in 1X PBST, 5% goat serum. Incubate 30 minutes at room temperature.
- Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.

- Add 100-400 µl Streptavidin-HRP reagents to each section and incubate for 30 minutes at room temperature.
- Wash sections three times in wash buffer for 5 minutes each.
- Perform Peroxidase blocking step (DAB staining only)
    - Apply 3% H2O2 in methanol in the dark to your tissue slide for 10 minutes (longer if needed)
- Add 100-400 µl DAB substrate to each section and monitor staining closely.
- As soon as the sections develop, immerse slides in dH2O.
    - Leaving DAB on the tissue for too long will result in non-specific staining
- Counterstain sections in hematoxylin.
- Wash sections in dH2O two times for 5 minutes each.
- Dehydrate sections.
- Mount coverslips.

Antigen Retrieval Buffer	Prepare Stock Solution
0.01M Citrate Buffer, pH6.0.	A. 0.1M Sodium Citrate; B. 0.1M Citric Acid Make 250mL 1X antigen retrieval buffer each time before use by mixing 20.5mL stock solution A. with 4.5mL stock solution B. and add distilled water to 250mL.

Additional notes

- If you are going to be hypoxically treating (exercise or high altitude treating) animals or tissue samples prior to staining, you should be sure to fix your samples quickly to avoid HIF degradation
    - Wash your tissue samples immediately with cold PBS
    - Within 10 seconds of wash, place your tissue samples in liquid nitrogen (if doing frozen sections) or in 10% formalin (if you are doing paraffin-embedding)
        - If you are doing paraffin-embedding, only fix in formalin for 12-24hours
        - For long term storage of tissue, place in 70% alcohol (do not keep in formalin past 24 hours)
            - You can store your tissue in the 70% alcohol for up to 1 week
- Positive control tissue include:
    - Kidney
    - Lung
    - Brain
    - Heart
- Exposure to hypoxic environment longer than 4 hours can lead to degradation of HIF expression.
- If you are using tumor tissue
    - HIF expression is typically seen in tumors 3mm or larger
    - You should expect to see nuclear staining toward the center with increasing cytoplasmic expression toward the periphery.

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