Assays to Study Hypoxia-Inducible Factor Prolyl Hydroxylase Domain 2 (PHD2), a Key Human Oxygen Sensing Protein

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Summary/Abstract

Molecular oxygen is essential for all multicellular lifeforms. In humans, the hypoxia-inducible factor (HIF) prolyl hydroxylase domain-containing enzymes (PHDs) serve as important oxygen sensors by regulating the activity of HIF, the master regulator that mediates cellular oxygen homeostasis, in an oxygen-dependent manner. In normoxia, PHDs catalyse the prolyl hydroxylation of HIF, which leads to its degradation and prevents cellular hypoxic response to be triggered. PHDs are current inhibition targets for the potential treatments of a number of diseases. In this chapter, we discuss *in vitro* and cell-based methods to study the modulation of PHD2, the most important human PHD isoform in normoxia and mild hypoxia. These include the production and purification of recombinant PHD2, the use of mass spectrometry to follow PHD2-catalysed reactions, and the studies of HIF stabilisation in cells by immunoblotting.

Key Words

Hypoxia-inducible factor (HIF), Prolyl hydroxylase domain (PHD), PHD2, Oxygen homeostasis, Hypoxic response, Recombinant protein, Matrix-assisted laser desorption/ionisation (MALDI), Enzyme assay, Mass spectrometry, Immunoblotting

1. Introduction

The ability to monitor and maintain cellular oxygen homeostasis is crucial to all metazoan organisms. In humans, this important task is regulated by the hypoxia-inducible factor (HIF), a transcription factor that manages the cellular response to low oxygen concentrations [1,2]. In hypoxic conditions, the α-isoform of HIF heterodimerises with the β-isoform in the nucleus to change the expression (directly and indirectly) of over 100 genes. The activation of cellular hypoxic response allows hypoxic cells to adapt to the deprivation of oxygen and maintain homeostasis.

HIFα is continuously produced in the cells. Hence, to prevent the unnecessary triggering of hypoxic response under normoxic conditions, several mechanisms exist to control the activity of HIF. Two of the most important regulatory components of the human hypoxic response pathway are enzyme mediated. The enzymes involved are factor-inhibiting HIF (FIH) and HIF prolyl hydroxylase domain-containing enzymes (PHDs) [3–5]. FIH and PHDs belong to the non-haem Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenase superfamily. In the presence of oxygen, these enzymes catalyse the hydroxylation of HIFα. FIH catalyses the hydroxylation of an asparagine residue (Asn803 in human HIF1α) in a region of HIFα that is known as the C-terminal transactivation domain [6]. This inhibits the binding of HIF to its transcriptional co-activator, and hence prevents the transcription of HIF-activated genes. PHDs, in contrast, catalyse the prolyl hydroxylation of HIFα. In human HIF1α, PHDs catalyse the hydroxylation of two proline residues (Pro402 and Pro564) [7,8]. Pro402 locates in a region of HIF1α that is known as the N-terminal oxygen-dependent degradation domain (NODD), while Pro564 is located at the C-terminal oxygen-dependent degradation domain (CODD). The prolyl hydroxylation of CODD and/or NODD promotes the binding of HIFα to the von Hippel–

Lindau protein, which signals the degradation of HIFα via the ubiquitin-proteasome pathway. Hence, PHDs regulate HIF at the level of protein stability.

There are increasing amount of evidence that shows PHDs as the main oxygen sensors of the cells. In humans, there are three PHD isoforms (PHD1-3) and they work complementarily with each other [9,10]. PHD2 is the most abundant isoform in most cell types, and it is considered the important oxygen sensor in normoxia and mild hypoxia [11]. PHD1 was found to play an important role in the regulation of HIF2α [12], and PHD3 is strongly induced in hypoxia and during reoxygenation [13]. PHDs are being investigated as inhibition targets for the potential treatments for a range of diseases [14]. For example, a number of PHD inhibitors are currently undergoing clinical trials for the treatment of anaemia [15].

In 2007, Hewitson and co-workers published an excellent set of instructions on the purification and assays of PHD2 [16]. As the work from Hewitson et al. is almost 15 years old, we believe an up-to-date guide that reflects the improvement in PHD assays would benefit the studies of this important oxygen sensor. Herein, we provide detailed protocols to study the modulation of PHDs by using PHD2 as a model. Three areas will be covered. These include the recombinant production and purification of PHD2, the study of PHD2 kinetics and inhibition by using matrix-assisted laser desorption/ionisation (MALDI)-mass spectrometry (MS), and the detection of cellular HIF protein stabilisation by immunoblotting as a measure of PHD activity.

2. Materials

Prepare all solutions using ultrapure water (Type 1 water) unless otherwise specified. Reagents can be stored at 4 °C unless otherwise specified.

2.1 Reagents and Solutions for Recombinant PHD2 Production and Purification

2.1.1 Cloning and Expression Vector Construction

1. Synthetic gene fragment encoding $PHD2_{181\text{-}426}$ (*see* **Note 1** for DNA sequence)
2. Vector pNIC28-Bsa4 (Addgene plasmid # 26103)
3. *Escherichia coli* XL10-Gold ultracompetent cells
4. NEBuffer r2.1
5. NEBuffer r3.1
6. NEB deoxynucleotide (dNTP) solution set
7. Restriction enzyme BsaI-HFv2
8. T4 DNA Polymerase
9. GeneJet Plasmid Miniprep Kit
10. LB agar powder
11. 2YT medium
12. SOC medium
13. Kanamycin sulfate powder
14. Sucrose

2.1.2 *Production of Recombinant* $PHD2_{181\text{-}426}$

1. *E. coli* BL21 (DE3) competent cells
2. LB agar powder
3. 2YT medium
4. SOC medium
5. Kanamycin sulfate powder
6. Isopropyl β-D-1-thiogalactopyranoside (IPTG, dioxane-free)

2.1.3 *Purification of Recombinant* $PHD2_{181\text{-}426}$

2.1.3.1 Purification by Immobilised Metal Affinity Chromatography (IMAC)

1. 5 mL HisTrap HP column
2. Binding buffer: Contains 5 mM imidazole, 500 mM NaCl and 50 mM HEPES (pH 7.8)
3. Wash buffer: Contains 30 mM imidazole, 500 mM NaCl and 50 mM HEPES (pH 7.8)
4. Elution buffer: Contains 500 mM imidazole, 500 mM NaCl and 50 mM HEPES (pH 7.8)
5. DNAse I
6. Bradford reagent

2.1.3.2 Apo-Protein Preparation

1. 0.5 M ethylenediaminetetraacetic acid (EDTA; pH 8.0)
2. 20 mM ammonium acetate (pH 7.0)

2.1.3.3 Purification by Size Exclusion Chromatography

1. HiPrep 16/60 Sephacryl S-100 HR HP column
2. Size exclusion buffer: 150 mM NaCl and 50 mM Tris (pH 7.5)

2.2 Reagents and Solutions to Study PHD2 Activity

1. Apo-$PHD2_{181\text{-}426}$
2. $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$
3. 2-Oxoglutaric acid disodium salt
4. Sodium ascorbate
5. Peptidyl substrate (19-mer peptides mimicking CODD or NODD) (*see* **Note 2** for amino acid sequences)
6. 50 mM Tris-HCl (pH 7.5)

7. α-Cyano-4-hydroxycinnamic acid (CHCA)
8. Acetonitrile
9. Dimethyl sulfoxide (DMSO)

2.3 Reagents and Solutions for HIF immunoblotting

2.3.1 Urea-SDS Lysis buffer (see **Note 3***)*

1. 8 M Urea: Add 4.8048 g of urea in 10 mL of water.
2. 20% SDS: Add 20 g of SDS in 100 mL of water.
3. 1 M Tris-HCl (pH 7.5): Dissolve 15.76 mg of Tris-HCI in 100 μL of water.
4. 1 M 1,4-Dithiothreitol (DTT): Dissolve 100 mg of DTT in 1 mL of water
5. Prepare lysis buffer by mixing 10 mL of 8 M urea, 1 mL of glycerol, 0.5 mL of 20% SDS, 100 μL of 1M Tris-HCl (pH.7.5) and 11.6 μL of 1 mM DTT.
6. Aliquot into 1 mL microtubes and store at -20°C.

2.3.2 SDS Polyacrylamide Gel

1. 1.5 M Tris (pH 8.8): Dissolve 18.165 g of Tris base in about 80 mL of water. Mix and adjust the pH with HCI. Make up to 100 mL with water.
2. 0.5 M Tris pH 6.8: Dissolve 6 g of Tris base in about 80 mL of water. Mix and adjust the pH with HCI. Make up to 100 mL with water.
3. 10% sodium dodecyl sulfate (SDS): Add 1 g of SDS in 10 mL of water.
4. 10% ammonium persulfate (APS): Add 1 g of APS in 10 mL of water.
5. Resolving gel solution (16 mL, enough for 2 gels): Prepare a mixture containing 8.05 mL of water, 3.6 mL of 40% Acrylamide/Bis solution (29:1), 4 mL of 1.5 M Tris pH 8.8 and 160 μL of 10% SDS. Once the resolving gel is ready to be casted,

add 160 μL of 10% APS and 16 μL of tetramethylethylenediamine (TEMED) into this mixture.

6. Stacking gel solution (5 ml, enough for 2 gels): Prepare a mixture containing 3.15 mL of water, 0.5 mL of 40% Acrylamide/Bis solution (29:1), 1.25 mL of 1.5M Tris pH 8.8 and 50 μL 10% SDS. Once the stacking gel is ready to be casted, add 160 μL of 10% APS and 16 μL of TEMED into this mixture.

2.3.3 Other Buffers and Solutions

1. 10x running buffer: Dissolve 30.2 g of Tris base, 144 g of glycine and 10 g of SDS in 800 mL of water. Make up to 1 L with water. Dilute 1:10 (100 mL of 10x running buffer + 900 mL water) to make 1x running buffer.
2. 20x transfer buffer: Dissolve 24.2 g of Tris base, 150.14 g of glycine in about 800 mL of water. Make up to 1 L with water. To make 1x transfer buffer, add 100 mL of methanol, 50 mL of 20x transfer buffer and 0.25 mL of 20% SDS into about 800 mL of water. Make up to 1 L with water. Chill the 1x transfer buffer on ice before use.
3. Washing buffer (1x PBS-Tween): To make 10x PBS-Tween, dissolve 80 g of sodium chloride (NaCl), 2 g of potassium chloride (KCl), 11.5 g of sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) and 2 g of potassium dihydrogen phosphate (KH2PO4) in about 900 mL of water. Add 10 mL of Tween-20 and make up to 1 L with water. Dilute 1:10 to make 1x PBS-Tween.
4. Blocking solution (5% milk in PBS-Tween): Dissolve 2 g of skimmed milk in 40 mL of washing buffer (1x PBS-Tween). Prepare fresh.
5. Phosphate-buffered saline (PBS)

6. Sample buffer (2x): Add 5 mL of 20% SDS, 5 mL of glycerol, 300 μL of 0.1% bromophenol blue and 4 mL of 1 M Tris-HCl pH 6.8 into 30 mL of water. Make up to 50 mL with water. This can be stored at room temperature. Add appropriate volume of 2-mercaptoethanol to a final concentration of 5% before use.
7. PVDF membranes
8. Blotting papers (cut to a slightly larger size than the size of the gel)
9. Ponceau S stain
10. Primary antibodies for HIF1α and HIF2α. We typically use mouse anti-human HIF1α clone 54 antibody (available commercially from BD Transduction) and mouse anti-human HIF2α clone EP190B antibody (available commercially from various antibody suppliers).
11. Anti-mouse HRP-conjugated secondary antibodies
12. Chemiluminescent substrate
13. Clean forceps (for handling membrane)

3. Methods

3.1 Recombinant PHD2 Production and Purification

Full-length PHD2 contains a N-terminal MYND (myeloid, Nervy, and DEAF-1)-type zinc finger domain and a C-terminal catalytic domain. For *in vitro* activity and binding assays, a truncated PHD2 that contains only the C-terminal catalytic domain (residues 181-426) is used. Synthetic gene encoding $PHD2_{181-426}$ (*see* **Note 1** regarding the DNA sequence) is inserted into a pNIC28-Bsa4 vector to allow for their overexpression in *Escherichia coli* BL21 (DE3) via a T7 promoter. The resulting recombinant $PHD2_{181-426}$ protein is produced with an N-terminal polyhistidine tag, which allows for its purification by immobilised metal-affinity

chromatography (IMAC). The purified protein is incubated with EDTA to remove any metals to afford apo-$PHD2_{181-426}$, which is then further purified by size exclusion chromatography.

3.1.1 *Cloning of Recombinant Plasmid Encoding $PHD2_{181-426}$*

3.1.1.1 Preparation of Gene Fragment Insert to Generate Cohesive Ends

1. Resuspend synthetic gene fragment encoding $PHD2_{181-426}$ in water to a final concentration of 80 ng/µL and incubate at 50 ºC for 20 minutes.
2. Mix 5 µL of the resuspended gene fragment with 1 µL NEBuffer r2.1, 0.25 µL dCTP solution and 3.5 µL water. Add 0.25 µL of T4 DNA polymerase to the mixture. Mix by gently pipetting up and down once.
3. Incubate mixture at 22 °C for 30 minutes to generate the cohesive ends.
4. Incubate mixture at 75 °C for 20 minutes to inactivate the T4 DNA polymerase.
5. The T4 DNA polymerase-treated gene fragment can be stored at -20 °C until further use if needed.

3.1.1.2 Preparation of Vector (Cutting and Cohesive Ends Generation)

1. Mix 5 µg pNIC28-Bsa4 (adjust volume according to DNA concentration) with 1 µL NEBuffer r3.1, 3 µL BasI-HFv2 and water (to a total volume of 100 µL). BsaI-HFv2 should be added last after all the other components are mixed. Mix by gently pipetting up and down once.
2. Incubate mixture at 50 °C for 3 hours to cut open the vector.
3. Purify the DNA using the PCR purification column. Elute the DNA with 50 µL elution buffer.

4. Mix 50 μL of the eluted DNA with 10 μL NEBuffer r2.1, 2.5 μL dGTP solution and 35 μL water. Add 2.5 μL of T4 DNA polymerase to the mixture. Mix by gently pipetting up and down once.
5. Incubate mixture at 75 °C for 20 minutes to inactivate the T4 DNA polymerase.
6. The BsaI-HFv2 and T4 DNA polymerase-treated vector can be stored at -20 °C until further use if needed.

3.1.1.3 Annealing and Transformation

1. Prepare a LB-agar plate with 50 μg/mL kanamycin and 5% sucrose. Sterilise one 500 mL Erlenmeyer flask containing 100 mL of 2-YT medium.
2. In a sterile microcentrifuge tube, mix 1 μL of the treated vector with 2 μL of the treated insert. Incubate at room temperature for 10 minutes.
3. Thaw an aliquot of *E. coli* XL10-Gold Ultracompetent Cells on ice.
4. Transfer the treated vector-treated insert mixture on ice. Add 40 μL of the thawed competent cells to the mixture. Mix by gently pipetting up and down once. Incubate on ice for 20 minutes.
5. Transfer to a water bath to incubate for 45 seconds, and then immediate return to ice for 1 minute.
6. Add 200 μL of SOC medium to the mixture, and incubate at 37 °C for 1 hour
7. Plate the entire transformation mix onto the LB-agar plate (with 5% sucrose and 50 μg/mL kanamycin). Incubate the plates at 37 °C overnight. Colonies should appear after overnight incubation.
8. Inoculate one colony to a culture containing 100 mL 2-YT medium supplemented with 30 μg/mL kanamycin. Incubate the flask in a shaking incubator at 37 °C with shaking at 180 rpm for 14 hours.

9. Use the GeneJet Plasmid Miniprep Kit to purify the recombinant plasmid by following the manufacturer's instructions. Use 5 mL of the overnight culture for purification. Elute the plasmid with 50 µL water. Store the purified plasmid DNA at -20 °C until further use.

3.1.2 Production and Purification of Recombinant PHD2$_{181\text{-}426}$

3.1.2.1 Production of Recombinant PHD2$_{181\text{-}426}$

1. Prepare a LB-agar plate with 50 µg/mL kanamycin. Sterilise one 500 mL Erlenmeyer flask containing 100 mL of 2-YT medium, and twelve 2 L Erlenmeyer flasks each containing 600 mL of 2-YT medium.
2. Thaw an aliquot of *E. coli* BL21 (DE3) competent cells on ice.
3. In a sterile microcentrifuge tube, mix 1 µL of the recombinant plasmid with 35 µL of the thawed competent cells. Mix by gently pipetting up and down once. Incubate on ice for 20 minutes.
4. Transfer to a water bath to incubate for 45 seconds, and then immediate return to ice for 1 minute.
5. Add 200 µL of SOC medium to the mixture, and incubate at 37 °C for 1 hour
6. Plate the entire transformation mix onto the LB-agar plate (with 50 µg/mL kanamycin). Incubate the plates at 37 °C overnight. Colonies should appear after overnight incubation.
7. Inoculate one colony to a culture containing 100 mL 2-YT medium supplemented with 30 µg/mL kanamycin. Incubate the flask in a shaking incubator at 37 °C with shaking at 180 rpm for 14 hours. This generates the overnight "starter culture".
8. Prewarm the twelve 2 L Erlenmeyer flasks containing the 2-YT medium in a shaking incubator at 37 °C for 30 minutes. Add freshly prepared kanamycin to a

final concentration of 30 μg/mL. Inoculate each 2 L flask with 7 mL of the starter culture and incubate the cultures in a shaking incubator at 37 °C with shaking at 180 rpm.

9. Check the optical density at 600 nm wavelength (OD) periodically. When the OD reaches ~0.6 (typically around 3 hours), add freshly prepared IPTG to each of the flasks to a final concentration of 0.2 mM. Further incubate the flasks in a shaking incubator at 28 °C with shaking at 180 rpm for 16 hours.
10. Harvest the cells by centrifugation at 7000 × *g* for 20 minutes. Discard the supernatant and freeze the cell pellet at -80 °C until further use.

3.1.2.2 Initial Purification by IMAC

1. Use an ÄKTA chromatography system to equilibrate a 5 mL HisTrap HP with binding buffer by following the manufacturer's instructions.
2. Remove the cell pellet from the freezer and resuspend the cell pellet in binding buffer (typically 5 mL of buffer for gram of cells). Supplement the mixture with ~5 mg DNAse I.
3. Lyse the cells on ice using a sonicator with three 15 seconds bursts at 60% amplitude. A rest time of 45 seconds should be followed in between the bursts.
4. Remove the cell debris by centrifugation at 30000 × *g* for 20 minutes. Filter the supernatant with a 0.45 μm filter and apply the filtered supernatant to the equilibrated HisTrap column.
5. Wash the column with 15 column volumes of wash buffer. Elute the protein with elution buffer to collect 1 mL fractions. The starting and ending point of the elution phase can be tracked by monitoring absorbance at 280 nm.

6. Check the fractions by using SDS-PAGE. Concentrate the fractions containing PHD2$_{181-426}$ by spin concentration using by a spin concentrator with a molecular weight cut-off (MWCO) limit of 10 kDa. Concentrate protein to ~20 mg/mL. Use Bradford reagent to estimate protein concentration as the presence of imidazole in the buffer would compromise the results obtained by direct spectrophotometry techniques such as the NanoDrop.

3.1.2.3 Apo-Protein Preparation

1. Prepare 500 mL of 0.5 M EDTA at pH 8.0 and 500 mL of 20 mM ammonium acetate at pH 7.0. EDTA is only soluble at high pH so raise the pH value of the solution first to dissolve all the EDTA solid before adjusting the pH back to pH 8.0.
2. Dilute the IMAC-purified protein to a concentration of <1 mg/mL with the EDTA and ammonium acetate solution in a 40:60 (v/v) ratio (*see* **Note 4**). Incubate overnight at 4 °C.

3.1.2.4 Final Purification by Size Exclusion Chromatography

1. Use an ÄKTA chromatography system to equilibrate a HiPrep 16/60 Sephacryl S-100 HR HP with the size exclusion buffer by following the manufacturer's instructions.
2. Concentrate the EDTA-treated protein to 1 mL. Inject the concentrated protein using a 1 mL sample loop onto the ÄKTA chromatography system.
3. Elute the protein with size exclusion buffer to collect 1 mL fractions. The starting and ending point of the elution phase can be tracked by monitoring absorbance at 280 nm.
4. Use SDS-PAGE to check the purity of the proteins. Concentrate the proteins to the desired concentration (typically ~50 mg/mL) and aliquot into clean microcentrifuge

tubes. Store at -80 °C until further use. NanoDrop Spectrophotometer can be used to measure protein concentration.

3.2 PHD2 Activity Assay

In vitro PHD2 activity assays typically include apo-$PHD2_{181\text{-}426}$, Fe(II), 2OG, peptidyl substrate and ascorbate in 50 mM Tris (pH 7.5). The Fe(II) and ascorbate solutions should be freshly prepared to avoid oxidation. The Fe(II) stock solution is prepared in acid to avoid oxidation to Fe(III). Typically, 19-mer peptides mimicking the CODD and NODD regions of HIF1α are used as substrates (*see* **Note 2** for amino acid sequence). Due to the limited solubility of the CODD and NODD peptides, stock solutions are prepared in DMSO. Potential inhibitors can also be added to the assay (typically prepared in DMSO especially if the inhibitor molecules contain aromatic moieties). It is important to maintain the DMSO concentration to <5% in the final reaction as a high DMSO concentration may disrupt the structure and activity of the protein. Reactions are typically conducted in a microcentrifuge tube.

3.2.1 Setting Up the PHD2 Reaction

1. Prepare a stock solution of 250 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ in 20 mM HCl. This solution should be freshly prepared every time.
2. Prepare a stock solution of 1 mM 2OG in 50 mM Tris (pH 7.5). This stock solution can be aliquoted and store at -20 °C for future use.
3. Prepare a stock solution of 5 mM CODD or NODD peptide in DMSO. This stock solution can be aliquoted and store at -20 °C for future use.
4. Prepare a stock solution of 50 mM ascorbate in 50 mM Tris (pH 7.5).
5. Immediately before the reaction, dilute the Fe(II) stock solution in ultrapure water to a concentration of 1 mM (no buffer solution should be used) by mixing 1 μL of

the Fe(II) stock solution with 250 μL of ultrapure water in a microcentrifuge tube. Mix using a vortex mixer.

6. The reaction mixture should contain 1 μM apo-PHD2$_{181-426}$, 10 μM Fe(II), 60 μM 2OG, 50 μM peptidyl substrate and 500 μM ascorbate in 50 mM Tris (pH 7.5). The reaction volume is 100 μL. Based on the concentration of the protein stock solution, calculate the appropriate amount of 50 mM Tris solution that is needed for the reaction. Add the Tris solution to a microcentrifuge tube. Pre-warm the solution at 37 °C using an Eppendorf ThermoMixer.
7. In the same microcentrifuge tube, add 6 μL of the 2OG stock solution, 1 μL of the peptide stock solution and 1 μL of the ascorbate solution. Mix using a vortex mixer. Immediately before the reaction, add 1 μL of the 1 mM Fe(II) stock solution and mix using a vortex mixer. Immediately after this, add the appropriate amount of the apo-PHD2$_{181-426}$ to initiate the reaction. Mix gently by pipetting up and down once and immediately return the microcentrifuge tube to the Eppendorf ThermoMixer and incubate at 37 °C with a shaking rate of 1300 rpm.
8. After the appropriate incubation time (depending on the desired application), the reaction can be quenched by add 100 μL of 200 mM HCl to the reaction mixture. The quenched reaction could be stored at -20 °C until analysis.

3.2.2 Monitoring Peptide Hydroxylation By MALDI-MS

MALDI-MS has been extensively used to measure the molecular weight of peptide analytes. As PHD2 catalyses the hydroxylation of peptidyl substrates, MALDI-MS could be used to follow the turnover of the substrate as the product will have an addition 16 Da when compared to the substrate.

MALDI allows the ionisation of non-volatile biomolecules such as peptides, including the 19-mer HIF-α peptidyl substrates, by using a laser beam [17]. The sample was first mixed with an acidic-based matrix (e.g., CHCA). Soft ionisation of the analytes (peptidyl substrates) occurs as a result of a laser beam striking of the analyte-matrix mixture. The analytes are usually ionised by being protonated or deprotonated by the matrix. The ionised sample then get accelerated into the gas phase and travel the flight tube to reach the detector where there are detected by linear or reflective mode.

3.2.3 Sample Preparation for MALDI-TOF Measurements

1. Freshly prepare a saturated CHCA solution by dissolving 10 mg of CHCA in 50% acetonitrile (ACN) in ultrapure water with 0.1% trifluoroacetic acid (TFA).
2. Spot 0.6 μL of the saturated CHCA solution onto the MALDI sample plate. Immediate add 0.6 μL of the reaction mixture to the same spot (1:1, v/v ratio). Mix by pipetting up and down. Alternatively, spot 0.6 μL CHCA onto the MALDI sample plate and let to air-dry. Then, add 0.6 μL of reaction mixture to the dried matrix and let to air-dry. Finally, add another 0.6 μL CHCA to the same spot (sandwich method). A third method involves mixing 0.6 μL of the saturated CHCA solution with 0.6 μL of the reaction mixture in a 0.2 mL centrifuge tube. After mixing, spot 1.2 μL of the mixture onto the target plate.
3. Allow the samples to completely air-dry for co-crystallisation of the reaction mixture and the matrix.
4. Analyse the samples with a MALDI-TOF mass spectrometer.
5. Select the detection mode on the mass spectrometer. The Negative Reflective (RN) mode is used for the detection of both HIF-α and hydroxy-HIF-α peptides

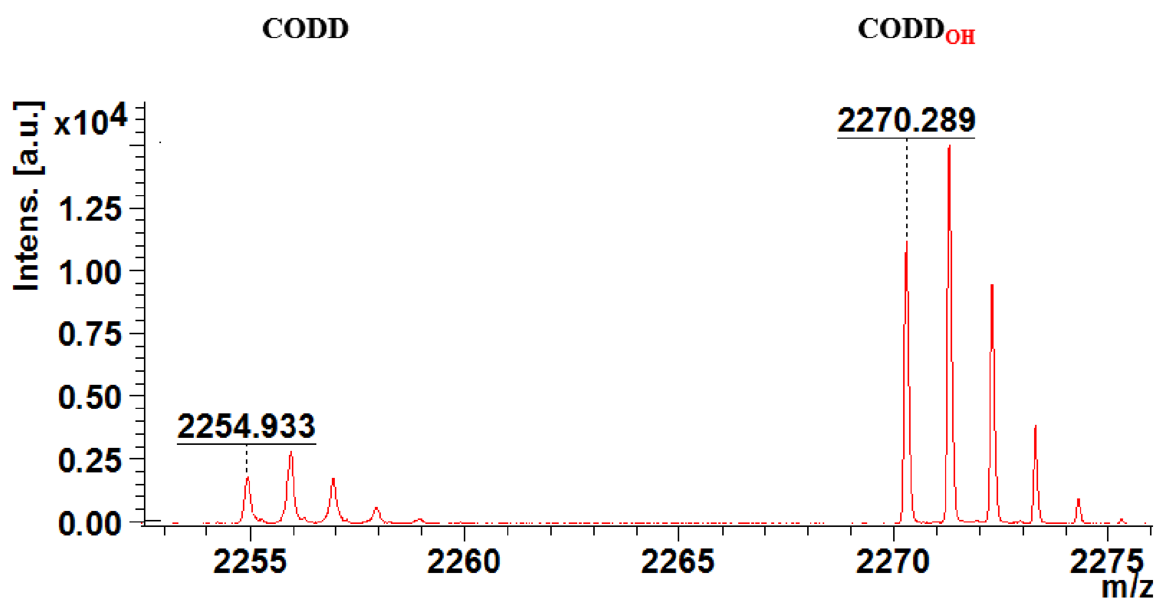


Figure 1: *MALDI mass spectrum of PHD2*$_{181\text{-}426}$*-catalysed hydroxylation of the 19-mer HIF1α CODD peptide (sequence: DLDLEMLAPYIPMDDDFQL). The peptide was best detected in Negative Reflective (RN) mode. MALDI-TOF-MS: m /z calculated for non-hydroxylated CODD [M-H]*$^-$ *2254.56, found 2254.21. The hydroxylated CODD is observed as +16 Da to that of the non-hydroxylated CODD.*

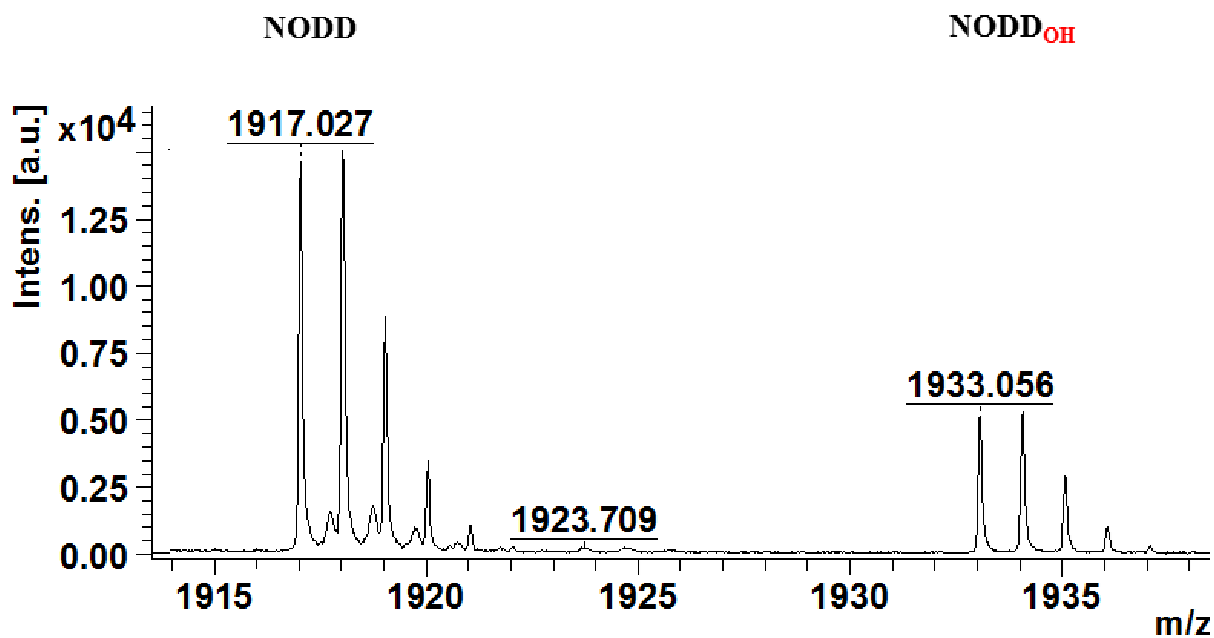


Figure 2: *MALDI mass spectrum of PHD2*$_{181\text{-}426}$*-catalysed hydroxylation of the 19-mer HIF1α NODD peptide (sequence: DALTLLAPAAGDTIISLDF) as measured and analysed by MALDI-TOF. The peptide was best detected in Negative Reflective (RN) mode. MALDI-TOF-MS: m /z calculated for non-hydroxylated NODD [M-H]*$^-$ *1917.18, found 1916.96. The hydroxylated NODD is observed as +16 Da to that of the non-hydroxylated NODD.*

3.3 HIF Immunoblotting as a Measure of PHD Activity in Cells

Direct detection of the endogenous cellular activity of each PHD isoforms is difficult without manipulating the cells, hence an indirect method based on the stabilisation of HIFα subunits are typically used as a measure of cellular PHD activity. Given that PHD2 is the most abundant isoform (relative to PHD1 and PHD3) in most cell lines under normoxia, the induction of both the HIF1α and HIF2α proteins are good indicators for PHD2 inhibition [9]. It is important to note that the inhibition of two or all three isoforms of PHDs will often result in a stronger induction of HIFα proteins, depending on the relative expression levels of each PHD isoforms in the cell lines used.

3.3.1 Cell Seeding and Treatment

1. Seed cells in 12-well plates at least 16 hours before treatment, with expected confluency of around 80% to 90% the next day. (see **Note 5**)
2. Treat cells with the desired small molecule inhibitors. (see **Note 6**)

3.3.2 Cell Harvesting

1. Remove cell culture medium using micropipettes.
2. Wash cells once with 1 mL of PBS.
3. Tilt plate for 10 seconds and remove any excess PBS using micropipettes.
4. Add 30 μl of lysis buffer per well and scrap cells off the wells using P200 micropipettes. Note that the lysate will be viscous.
5. Repeat step 4 for all other wells containing cells. (*see* **Note 7**)
6. Add appropriate amount of sample buffer into microtubes. Transfer the cell lysate into each microtubes using micropipettes and vortex to mix.
7. Centrifuge the lysate briefly to remove any bubbles.

8. Boil samples on a heat block at 95 °C for 5 mins.
9. Vortex vigorously and centrifuge at maximum speed for 3 to 5 mins.
10. Repeat step 9 until cell lysate is no longer viscous.
11. Freeze lysate at -20°C or proceed to gel electrophoresis immediately.

3.3.3 Preparation of SDS Polyacrylamide Gels

1. Assemble the glass plates on the gel casting cassettes.
2. Place a comb accordingly and mark on the glass plate 1 cm below the teeth of the comb to indicate the level for resolving gel.
3. Prepare resolving and stacking gel solutions without APS and TEMED.
4. Add APS and TEMED into the resolving gel solution and mix gently. Transfer the solution into the glass plates up to the level marked in step 2.
5. Add 500 μl of water or isopropanol to the top of the resolving gel solution in the glass plates as an overlay solution. This is to ensure an even interface between the resolving gel and the stacking gel.
6. Allow the resolving gel to polymerise (around 30 to 45 mins).
7. Once polymerised, pour off the overlay solution. Additional solution can be carefully dried off using blotting papers.
8. Add APS and TEMED to the stacking gel solution. Mix well and transfer to the top of the resolving gel to the brim of the glass plates. Place the comb gently between the glass plates.
9. Allow the stacking gel to polymerise (around 30 to 45 mins).
10. Store gels at 4°C if not used immediately by wrapping the gels (in glass plates with combs unremoved) with wet paper towels soaked in water.

3.3.4 SDS polyacrylamide gel electrophoresis

1. Assemble the gel in glass plates in the electrophoresis gel tank. Fill tank with running buffer and remove the comb gently.
2. Rinse each well of the gel with running buffer gently using micropipettes.
3. Load the appropriate volume of cell lysate containing sample buffer into each well. Load the appropriate volume of protein ladder on each side of the gel.
4. Run the electrophoresis at a constant voltage of 180 V for 45 to 50 mins or until the protein ladder is separated accordingly.
5. Remove the glass plates containing gel and submerge it in a container containing the transfer buffer.

3.3.5 Electrophoretic Transfer

1. Wet the blotting paper and transfer sponge with transfer buffer.
2. Activate the PVDF membrane by adding methanol briefly and before rinsing with water and keeping in transfer buffer.
3. Separate the glass plates in a container containing the transfer buffer. Gently shake to separate the gel from the glass plates but ensure that the gel remains on top of one of the glass plates (for transferring the gel onto the transfer sandwich).
4. Prepare the transfer sandwich layer by layer starting from the bottom as indicated below:

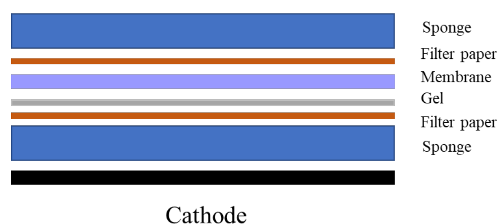


Figure 3*: Assembly of the sandwich layers for wet-transfer*

5. Gently remove any air bubbles between each layer of the transfer sandwich with a roller. Any bubbles formed may affect the transfer of the proteins to the membrane.
6. Place the sandwich into a transfer cassette and fill the transfer tank with ice-cold transfer buffer and set it to run at 100 V for 1 h.
7. After transfer, remove each layer carefully and transfer the membrane using a clean forcep into a container with the protein side up (*see* **Note 8**).

3.3.6 Total Protein Staining with Ponceau S

1. Completely submerge the membrane with Ponceau S solution for 1 min.
2. Remove the Ponceau S staining solution. Destain the membrane in PBS with gentle rocking. Capture the image once the background stain becomes clearer to reveal the total protein in each lane (see image below).

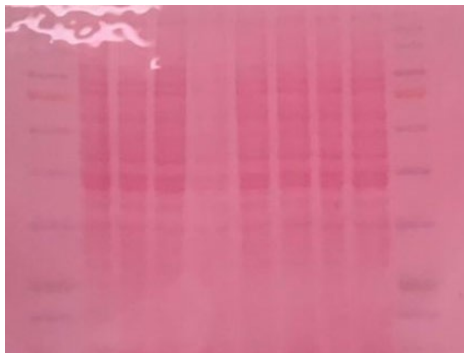


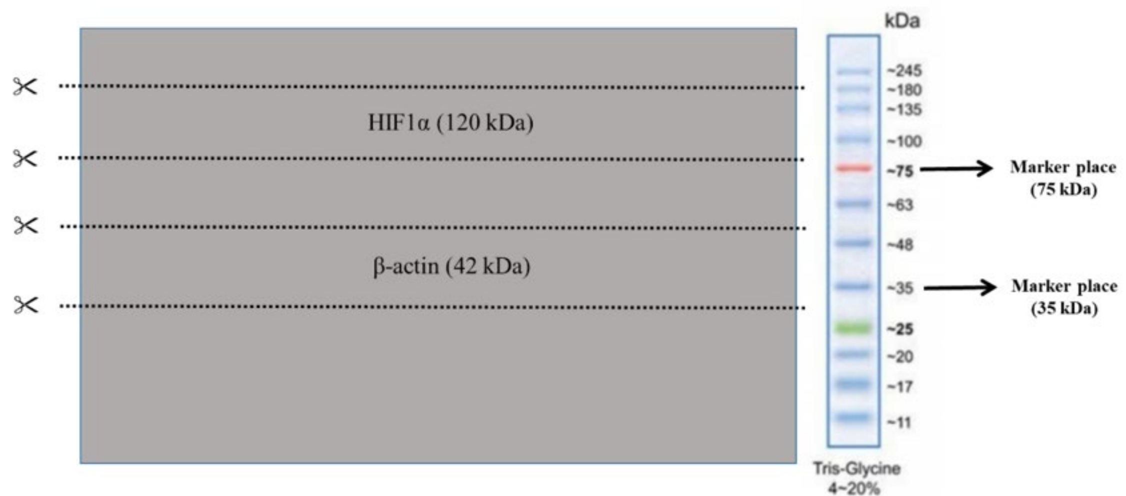
Figure 4*: An example of a gel after staining with Ponceau S solution.*

3. After the desired image is captured, further destain with PBS until the red stain is completely removed.

3.3.7 Blocking and Antibody Incubation

1. Cut the blot according to the size of the target protein. For HIF1α and HIF2α, the suggested cuts based on their expected sizes are indicated below (detection of β-actin protein level is suggested here as another loading control):

A



B

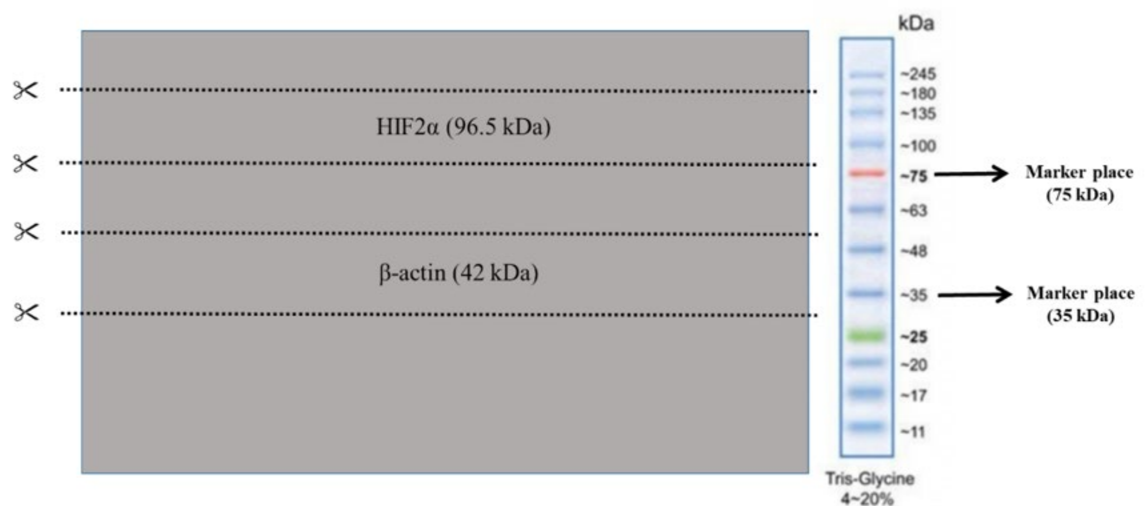


Figure 5*: Trimming of the membrane after the transfer of separated proteins from SDS-PAGE gel. Suggested trimming of membrane for the detection of HIF1α (****A****) and HIF2α (****B****) proteins are indicated by dotted lines. Trimmed membrane for the detection of b-actin protein will be used as loading control for each membrane.*

2. After cutting, label each strip of the membrane accordingly with a pencil.

3. Block each strip of membrane in the blocking solution for 5 mins (up to overnight at 4°C). (*see* **Note 9**)
4. After blocking, incubate each strip of membrane in the desired primary antibodies at the desired incubation time with gentle rocking to ensure uniform exposure (*see* **Note 10**). For overnight incubation, incubate the membrane with gentle rocking at 4°C. (*see* **Note 11**)
5. Remove the primary antibodies and rinse 3 times with washing buffer. (*see* **Note 12**).
6. Incubate the membrane in the appropriate secondary antibody for at least 1 h with gentle rocking.
7. Remove the secondary antibodies and wash 5 times with washing buffer for 5 mins each time.
8. Drip dry each strip of membrane and gently place it between two paper towels. Ensure that the membrane is semidry.
9. Place each strip of membrane on a transparency paper and add the appropriate volume of chemiluminescence HRP substrate. Place another transparency paper on top of the membrane and capture the image using a chemiluminescence imaging system. (*see* **Note 13**)

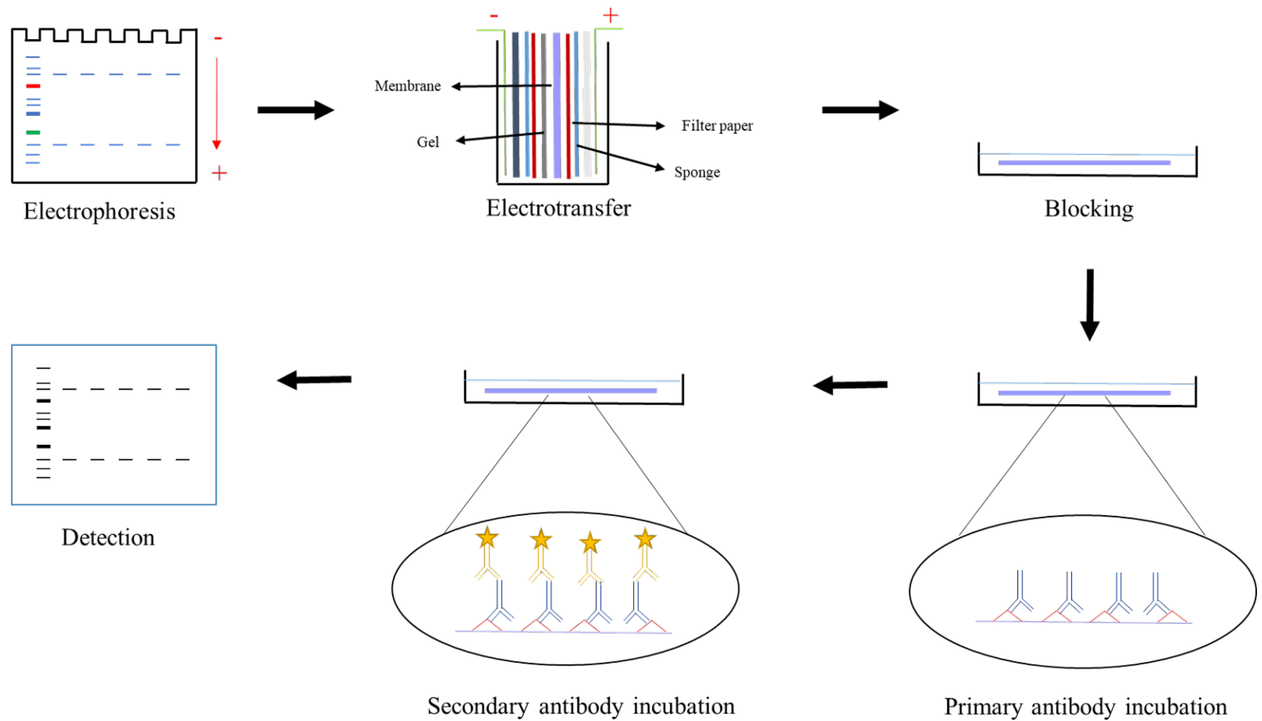


Figure 6*: An overview of immunoblotting. Cell lysates containing proteins are first separated based on size using SDS-PAGE. The separated proteins are then transferred from the gel to the membrane. The membrane is blocked and incubated with the appropriate primary and secondary antibodies for the protein of interest. Finally, the detection step is performed by the addition of a chemiluminescent substrate.*

4. Notes

1. Synthetic gene sequence for $PHD2_{181\text{-}426}$. Text in italic indicates the sequence required for cloning to the vector pNIC28-Bsa4 : *tacttccaatccatg* CCC AAC GGG CAG ACG AAG CCC CTG CCG GCG CTG AAG CTG GCG CTC GAG TAC ATC GTG CCG TGC ATG AAC AAG CAC GGC ATC TGT GTG GTG GAC GAC TTC CTC GGC AAG GAG ACC GGA CAG CAG ATC GGC GAC GAG GTG CGC GCC CTG CAC GAC ACC GGG AAG TTC ACG GAC GGG CAG CTG GTC AGC CAG AAG AGT GAC TCG TCC AAG GAC ATC CGA GGC GAT AAG ATC ACC TGG ATC GAG GGC AAG GAG CCC GGC TGC GAA ACC ATT GGG CTG CTC ATG AGC AGC ATG GAC GAC CTG ATA CGC CAC TGT AAC GGG AAG CTG GGC AGC TAC AAA ATC AAT GGC CGG ACG AAA GCC ATG GTT GCT TGT TAT CCG GGC AAT GGA ACG GGT TAT GTA CGT CAT GTT GAT AAT CCA AAT GGA GAT GGA AGA TGT GTG ACA TGT ATA TAT TAT CTT AAT AAA GAC TGG GAT

GCC AAG GTA AGT GGA GGT ATA CTT CGA ATT TTT CCA GAA GGC AAA GCC CAG TTT GCT GAC ATT GAA CCC AAA TTT GAT AGA CTG CTG TTT TTC TGG TCT GAC CGT CGC AAC CCT CAT GAA GTA CAA CCA GCA TAT GCT ACA AGG TAC GCA ATA ACT GTT TGG TAT TTT GAT GCA GAT GAG AGA GCA CGA GCT AAA GTA AAA TAT CTA ACA GGT GAA AAA GGT GTG AGG GTT GAA CTC AAT AAA CCT TCA GAT TCG GTC GGT AAA GAC GTC TTC TAG *cagtaaaggtggata*

2. HIF1α CODD peptide: DLDLEMLAPYIPMDDDFQL

   HIF1α NODD peptide: DALTLLAPAAGDTIISLDF

3. We find that protein extraction using Urea-SDS lysis buffer gave the best signal for HIF proteins. Alternatively, other lysis buffer (such as RIPA buffer) may be used.
4. Some proteins may precipitate upon the addition of EDTA and/or during incubation. This, unfortunately, cannot be avoided in order to obtain apo-proteins.
5. The cells referred to here are human cancer cell lines typically used to investigate cellular activities of PHD inhibitors. These cell lines express functional VHL protein. Alternatively, human cell lines without functional VHL and thus, stabilise HIF proteins constitutively (such as renal cell carcinoma cell line, RCC4) can be used to assess for HIF1α hydroxylation status using commercially available HIF1α hydroxyproline-specific antibodies [18]. HIF1α hydroxyasparagine-specific antibody can also be used to investigate inhibition of FIH when HIF1α induction is observed [18,19]. However, this antibody is not yet commercially available.
6. We find that HIF proteins are typically induced maximally after 5 to 8 hours of PHD inhibitor treatment or hypoxia (0.5% O_2). This may differ depending on cell type and inhibitor. If no previous studies have reported the induction of HIF proteins in your

chosen cell type, we recommend to first test the induction level and treatment duration using IOX2, a well characterised and specific small molecule inhibitor of the PHDs [20].

7. Ensure consistency in cell harvesting across all the wells. More than 1 well can be harvested at the same time.
8. All handling of membrane should always be done using clean forceps to avoid or minimise transferring of contaminants to the membrane.
9. We find that blocking of 5 mins at room temperature is sufficient for most antibodies.
10. We find that an incubation time of 1 to 2 hours is sufficient for primary antibodies of HIF proteins.
11. At this point (after the blocking step), any steps can be paused by keeping the blots in washing buffer at room temperature (or at 4°C if overnight).
12. We find that primary antibodies for HIF proteins can be reused multiple times (even up to 10 times). After the incubation of blots, store the primary antibodies at 4°C (if overnight) or -20°C (for long term storage).
13. Immunoblotting is a semi-quantitative method. However, quantification of the signal can be done using a densitometry software called ImageJ [21]. Normalisation of signal can be done either against the β-actin signal or using total protein stain (Ponceau S).

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