Methods to Study the Crosstalk Between HIF and NF-κB Signalling in Hypoxia and Normoxia.

Dilem Shakir, Michael Batie, and Sonia Rocha

Abstract
Hypoxia and Inflammation are intensely connected in a functional crosstalk. Within this crosstalk, two major transcription factors take centre stage: HIF and NF-κB. To investigate transcription factor function, an important aspect is its ability to bind DNA. The most appropriate method to study this property in cells is the use of Chromatin Immunoprecipitation followed by qPCR and/or next generation sequencing. This allows identification of potentially directly regulated genes as well as enhancer regions. Here we describe the ChIP-qPCR method in detail, including key aspects important for the success of the technique.

Key words
Hypoxia, Inflammation, HIF, NF-kappaB, ChIP, qPCR.

1 Introduction

Hypoxia is the dysregulation of homeostatic balance between oxygen supply and consumption in cells, leading to insufficient oxygen for regular metabolic activity and survival of multicellular organisms. Hypoxia inducible factors (HIFs) are a transcription factor family that control a specific transcriptional programme in response to decrease in oxygen levels [1]. HIFs typically form heterodimeric complexes composed of oxygen sensitive HIF-α subunits (HIF-1α, HIF-2α or HIF-3α), and a constitutively expressed HIF-1β subunit (gene name, aryl hydrocarbon nuclear translocator (ARNT))[2]. The stability and transcriptional activity of HIF-α subunits is controlled in part by post-translational modifications. Under sufficiently oxygenated conditions (normoxia), HIF-α proteins are degraded by the proteasome following prolyl hydroxylation by 2-oxoglutarate (2-OG) dependent dioxygenases, called prolyl hydroxylases (PHDs) [3]. In normoxia, HIF-α proteins also undergo asparaginyl hydroxylation by another 2-OG dependent dioxygenase, factor inhibiting HIF (FIH), which prevents the recruitment of the HIF coactivators (CBP/p300) in the nucleus [4]. Whereas, under hypoxic conditions, PHDs and FIH have reduced activity due to their dependence on molecular oxygen, thus HIF-α is stabilised and forms a heterodimer with HIF-1β in the nucleus [5]. The HIF complex then binds to the hypoxia response element (HRE) nucleotide sequences on DNA and initiates the transcription of HIF target genes [5,6]. The HIF heterodimer can regulate the expression of wide ranges of genes involved in various physiological functions such as, angiogenesis, erythropoiesis, cell proliferation and survival [7-9]. Dysregulation of this transcription mechanism has been associated with pathological conditions including different cancer types [10-12].

Hypoxia is also associated with another important transcription factor family, Nuclear Factor kappa-light-chain-enhancer of B cells (NF-κB), which is known to have importance in maintaining homeostasis in immune and inflammatory responses [13,14]. Activation of the NF-κB pathway can occur through the stimulation of canonical, non-canonical or atypical pathways [15]. The NF-κB family is composed of five distinct members, including RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52) [15]. All NF-κB proteins have a Rel homology domain (RHD), which enables DNA-binding ability and homo- or hetero-dimer formation [16]. In an unstimulated canonical pathway, inhibitor of kappa B (IκB) holds NF-κB proteins inactive in the cytoplasm. Upon stimulation of the pathway, the upstream kinase complex, IκB Kinase (IKK) phosphorylates the IκBα, which leads to its proteasomal degradation and releases NF-κB to translocate into the nucleus [17].

Besides pro-inflammatory cytokines and pathogen associated molecular stimuli, hypoxia can also lead to activation of the NF-κB pathway. The mechanism of hypoxia-induced inflammation has been extensively described in many studies [18-20]. Also, Tumour Necrosis Factor alpha (TNF-α) is able to stimulate the expression of HIF subunits [21,22]. Apart from sharing common stimuli, both HIF and NF-κB transcription factors are known to have common target genes and regulators [23]. HIF subunits

can have different roles in regulating the transcriptional response of NF-κB. HIF-1α is able to restrict the NF-κB transcriptional activity, whereas HIF-1β is found to be required for full NF-κB activation [24,25]. Various methods have been used to discover the coregulation of target genes by HIF and NF-κB subunits, however a comprehensive analysis on a genome wide scale is still lacking. Genome wide sequencing approaches such as Chromatin Immunoprecipitation (ChIP)-sequencing can be employed to investigate coregulation of NF-κB and HIF target genes in response to hypoxia and inflammation. ChIP assay coupled with quantitative polymerase chain reaction (qPCR) can verify the direct protein-DNA interactions and functional connections between HIF, NF-κB and their target sites in response to different stimuli.

Here we present the ChIP-qPCR method that we have been successfully applying to analyse the crosstalk between HIF and NF-κB signalling pathways in human cell lines. ChIP-qPCR includes multiple steps, which are summarised in Figure 1. Main steps include cell culture and stimulation of endogenous HIFs and NF-κB with hypoxia or TNF-α. After the completion of the treatment, proteins are fixed to the DNA with formaldehyde crosslinking. Then chromatin is extracted with a lysis buffer and fragmented via sonication. After the chromatin fragments have been prepared, appropriate antibodies and protein G-sepharose beads are used to select DNA-bound proteins of interests via immunoprecipitation (IP) and immune complex capture. DNA and protein complexes are detached by reversing the crosslinks and proteins are eliminated by a protein degradation step. After purifying the DNA, qPCR is used to analyse the relative amount of specific genomic DNA sequences enriched for binding by the protein of interest. Although ChIP assays have been a routine molecular technique for identifying DNA-binding proteins, all of its steps, including pre-clearing, washing, and elution needs to be followed carefully to achieve a successful result (Figure 1).

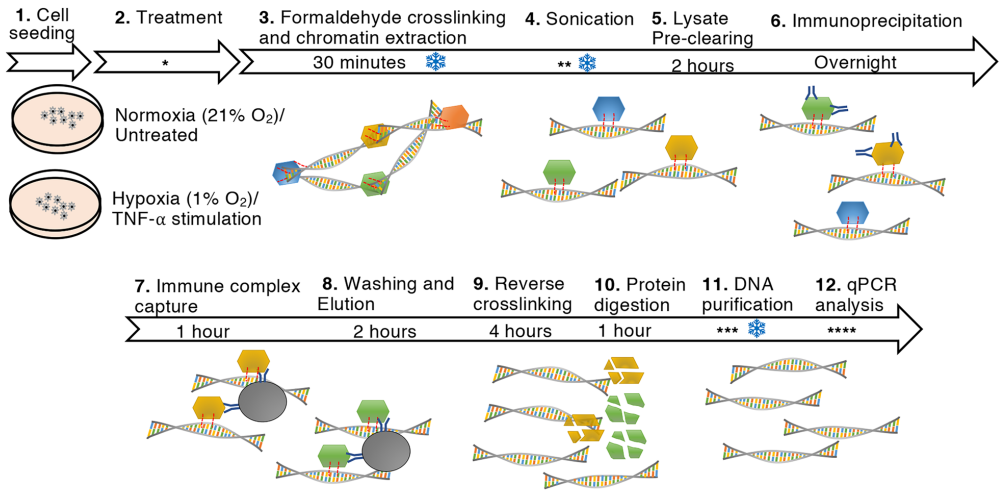

Figure 1: Workflow of ChIP-qPCR.

* Typically 48-72 hours post cell seeding
** Time is dependent on sonication conditions
*** Time is dependent on DNA purification method
**** Time is dependent on the qPCR machine
❄ Samples can be stored at a -80 °C freezer at the end of this step

2 Materials

2.1 Cell Culture (*see* Note 1)

1. Cell culture incubator set to 37°C and 5% CO_2
2. 150 mm cell culture plates
3. Dulbecco's modified eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 10% (v/v) foetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 µg/ml)
4. Dulbecco's phosphate-buffered saline (DPBS)
5. 0.25% Trypsin

2.2 Treatments

Hypoxia stimulation:

1. Hypoxia workstation set to 1% O_2, 5% CO_2, and 37°C

TNF-α stimulation:

1. 10 ng/ml human recombinant TNF-α

2.3 ChIP Assay (*see* Note 2)

1. 36.5% Formaldehyde (*see* Note 3)
2. Phosphate buffer saline (PBS), stored at 4°C.
3. 1.5 M Glycine, filtered to avoid contamination during prolonged storage
4. Scraper
5. Lysis buffer: 1% sodium dodecyl sulfate (SDS), 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl (pH 8.1), 1 protease inhibitor tablet (1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml leupeptin, and 1 mg/ml aprotinin).
6. Dilution buffer: 1% Triton X-100, 2 mM EDTA, 150 mM sodium chloride (NaCl), 20 mM Tris-HCl (pH 8.1)
7. Wash buffer 1: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tri-HCl (pH 8.1), 150 mM NaCl
8. Wash buffer 2: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl
9. Wash buffer 3: 0.25 M (lithium chloride) LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)
10. TE Buffer: 10 mM Tris-HCl (pH 8.1), 1 mM EDTA
11. Elution buffer: 1% SDS, 0.1 M $NaHCO_3$
12. Sheared salmon sperm DNA (ssDNA)
13. Protein G-Sepharose slurry
14. Antibodies (*see* Note 4)
15. 5 M NaCl
16. Proteinase K: 20 µg/µl stock
17. 1 M Tris-HCl (pH 6.5)
18. 0.5 M EDTA (PH 8)
19. 10% Polyoxyethylene laural ether (BRIJ35) solution
20. Benchtop centrifuge
21. Rotating wheel set to 40 rpm
22. Thermomixer
23. Sonicator
24. Vacuum aspirator
25. Nuclease free water
26. QIAquick PCR Purification kit (Qiagen)

2.4 qPCR Analysis

1. 100 µM forward and reverse primers
2. Brilliant II Sybr green kit
3. Mx3005P qPCR platform (Stratagene/Agilent)
4. Mx3005P 96 well plates

5. Mx3005P 96 well plate lids
6. Plate centrifuge
7. Nuclease free water

3 Methods

3.1 Cell culture growth conditions

1. Cells are maintained in 150 mm tissue culture plates containing DMEM and kept in 5% CO_2 and 37°C.
2. For preparing the cells for the experiment, remove culture medium and wash cells with 5 ml PBS. Add 5 ml of pre-warmed trypsin and incubate the cells at 37°C until the cells have detached (*see* Note 5)
3. Resuspend cells by adding 5 ml DMEM and move the 10 ml cell solution to a 15 ml canonical tube. Pipette the cells up and down to have a single cell suspension.
4. Plate cells to a final density of ~$4.5x10^6$ cells into two 150 mm culture plates in a final volume of 15 ml of medium (*see* Note 6).
5. Apply the appropriate treatment for desired length of time prior to chromatin extraction.

3.2 Treatments

TNF-α stimulation:

1. Add 10 ng/ml of human recombinant TNF-α to the cells
2. Mix plates by moving up and down and side to side

Hypoxia stimulation:

1. Put the cells into a hypoxia workstation set to 1% O_2, 5% CO_2 and 37°C

3.3 ChIP-qPCR

All ChIP steps are carried out in 4°C temperature, unless otherwise specified. Samples are kept on ice at all times. Rotating wheel and sonicator are processed in a cold room set to 4°C.

3.3.1 Formaldehyde cross-linking and chromatin extraction

1. Cross-link proteins to DNA by adding formaldehyde drop-wise directly to the cell medium to a final concentration of 1% (i.e. 410 µl of 36.5% formaldehyde into 15 ml of culture medium in a 150 mm plate). Mix by moving the plate up and down and side by side and incubate at room temperature for 10 minutes (*see* Note 7).
2. Stop the cross-linking reaction by adding glycine drop-wise to the cell medium to a final concentration of 0.125 M (i.e. 1.284 ml of 1.5 M glycine into 15.410 ml). Mix by moving the plate up and down and side by side and incubate it at room temperature for 5 minutes.
3. Discard the media and wash twice with cold DPBS by disposing the waste to an appropriate formaldehyde waste container.
4. Add 1 ml of cold DPBS and scrape the cells into a 15 ml canonical tube. Centrifuge at 1,000 rpm for 5 minutes at room temperature.
5. Aspirate out the supernatant carefully and resuspend the pellet in 450 µl lysis buffer. Allow the cells to lyse for 10 minutes on ice.
6. After step 5, lysed cells can be carried on to the sonication procedure or they can be stored in a -80°C freezer for later use.

3.3.2 Sonication

1. Sonicate the HeLa cell samples at 4°C, twenty times for 15 seconds with a 30 seconds gap between each sonication at 50% amplitude (*see* Notes 8).
2. After sonication, spin down the samples at 12,000 rpm for 10 minutes at 4°C and discard the pellet.

3. Reserve 10 µl of the sheared chromatin from each condition, as the input samples in a separate 1.5 ml eppendorf tube. The input samples can be kept in a -20°C freezer until processing with rest of the samples in section 3.3.7 (*see* Note 9).
4. Split the samples into 100 µl aliquots, one for each IP that is intended to run.
5. Dilute each of the 100 µl samples 10-fold by adding 900 µl dilution buffer to each sample.
6. After step 5, samples can be carried on to the pre-clearing procedure or they can be stored in a -80°C freezer for later use.
7. Before continuing to the pre-clearing section, prepare the protein G-sepharose slurry by following the steps in section 3.3.3.

3.3.3 Preparing the Protein G-Sepharose slurry

1. Calculate the total starting volume of the slurry required for all of the samples (i.e. 20 µl per sample), put the required volume into an Eppendorf tube (*see* Note 10).
2. Spin down the beads by centrifugation at 1,000 rpm for 1 minute at room temperature and discard the ethanol without disturbing the beads.
3. Wash the beads by resuspending them in a 500 µl PBS.
4. Centrifuge the solution at 1,000 rpm for 1 minute at room temperature and discard the PBS without disturbing the beads.
5. Resuspend the beads in PBS by adding 50% of the starting volume.
6. Prepare a master mix by adding 2 µg ssDNA per 20 µl protein G-sepharose slurry and proceed to the next section.

3.3.4 Lysate Pre-clearing

1. Add 22 µl of the prepared master mix into each sample (*see* Note 11).
2. Incubate the samples for 2 hours on the rotating platform at 4°C.
3. Centrifuge at 1,000 rpm for 1 minute at 4°C. Then, transfer the supernatant to a new 1.5 ml eppendorf tube and discard the pelleted beads.

3.3.5 Immunoprecipitation (IP)

1. Add BRIJ35 at a final concentration of 0.1% (10 µl of 10% BRIJ35 solution) to each sample and appropriate amount of antibody (*see* Note 12).
2. Incubate the samples overnight on the rotating wheel at 4°C.

3.3.6 Immune complex capture, washing and elution

1. Prepare a mastermix containing 30 µl protein G-sepharose slurry and 2 µg ssDNA per sample (follow the steps 1-5 in section 3.3.3 for preparing the protein G-sepharose slurry).
2. Add 32 µl of the prepared mastermix to each sample and incubate for 1 hour on the rotating wheel at 4°C.
3. Centrifuge the samples at 1,000 rpm for 1 minute at 4°C and store the supernatant in a -80°C freezer (*see* Note 13).
4. Re-suspend pelleted beads in 1 ml wash buffer 1 and incubate the samples for 5 minutes on the rotating wheel at 4°C.
5. Centrifuge the samples at 1,000 rpm for 1 minute at 4°C and discard the supernatant without disturbing the beads.
6. Re-suspend pelleted beads in 1 ml wash buffer 2 and incubate them for 5 minutes on the rotating wheel at 4°C.
7. Repeat step 5 and re-suspend the pelleted beads in 1 ml wash buffer 3.
8. Incubate the samples for 5 minutes on the rotating wheel at 4°C and repeat step 5.
9. Wash the beads twice by re-suspending them in 1 ml TE buffer.
10. Repeat step 5 and re-suspend the pelleted beads with 100 µl elution buffer and incubate them for 1 hour at room temperature on a rocker.
11. Spin down the beads at 1,000 rpm for 1 minute at room temperature.
12. Transfer the supernatant to a new eppendorf tube and discard the pelleted beads.

3.3.7 Reverse Crosslinking and DNA Purification

1. Thaw 10 µl of the input samples reserved after the sonication (section 3.3.2, step 3) and add 90 µl nuclease free water.
2. For reverse crosslinking, add 0.2 M NaCl into each IP and input samples (i.e. 4 µl of 5 M NaCl stock in 100 µl of each sample). Incubate them for 4 hours on a thermomixer pre-set to 65 °C and 500 rpm.
3. For digesting the proteins, prepare a master mix to add the following into each 100 µl IP and input samples: 20 µg Proteinase K, 40 mM Tris-HCl (pH 6.5), 10 mM EDTA (pH 8) (i.e. 1 µl Proteinase K, 4 µl 1 M Tris-HCl, 2 µl 0.5 M EDTA). After adding 7 µl of the mastermix into the tubes, incubate them for 1 hour on a thermomixer pre-set to 45 °C and 500 rpm.
4. Purify DNA using a QIAquick PCR Purification kit (Qiagen) following manufacturer's instructions, eluting the samples in 50 µl nuclease free water (see Note 14).
5. Eluted DNA can be stored in a -80ºC freezer for later use.

3.3.8 qPCR Analysis

1. Prepare a mastermix to add the following into each qPCR reaction well: 3.9 µl nuclease free water, 0.6 µl of 10 µM prepared primer mix (5 µM forward and 5 µM reverse) and 7.5 µl Sybr green.
2. Put 3 µl of the purified DNA samples to the 96-well plate reaction wells and add 12 µl of the prepared mastermix (*see* Note 15)
3. Close the plate and centrifuge briefly using a plate centrifuge to collect the reaction at the bottom of the wells.
4. Program the qPCR instrument as follows: 10 minutes 95 °C (1 cycle); 30 seconds 95 °C, 1 minute 60 °C, 1 minute 72°C (50 cycles); 1 minute 95°C, 30 seconds 55°C, 30 seconds 95°C (1 cycle).
5. Data obtained for the ChIP should be normalised to the input sample DNA (*see* Note 16).

4 Notes

1. All cell culture procedure should be performed in a tissue culture hood with sterile practice. All material should be sprayed with 70% ethanol before placing into the tissue culture hood. DMEM and trypsin are stored at 4ºC fridge and should be warmed to 37ºC in a water bath before use. Although, DMEM is used here for the human cervical cancer (HeLa) cells, different cell lines may need to be maintained in a different medium.
2. Throughout the ChIP-qPCR assay, always use the low protein binding and DNase/RNase free eppendorf tubes. Use filter tips and nuclease free water for preparing solutions to prevent cross contamination.
3. Formaldehyde is a highly toxic and flammable chemical. Thus, it should be used with appropriate safety measures, such as protective gloves, and a laboratory coat. All procedure that includes the formaldehyde should be carried on in a tissue culture hood. Waste should be disposed according to regulations for hazardous waste.
4. Selection and the concentration of the ChIP antibodies are one of the most critical factors that will contribute to the success of the ChIP assay. In order to check for the protein binding specificity of the antibodies, western blot method can be applied after the last step of the section 3.3.6.
5. Detachment of the HeLa cells from the tissue culture plate takes 3-5 minutes incubation in trypsin. This should be visible by eye, which can also be confirmed microscopically.
6. The number of cells stated is for lysis 48 hours post cell seeding. This number should be optimised for experiments with different timescales and/or cells lines, with aim of reaching 90% confluency at the time of lysis.
7. If doing a hypoxia treatment, add formaldehyde in the hypoxia chamber then incubate for 10 minutes at room temperature inside the tissue culture hood.
8. Sonication step needs to be optimised to successfully fragment the DNA to 200-500bp. These conditions are dependent on cell line, concentration of chromatin in the lysate and the sonicator used. The indicated settings are specifically based on the sonication of 90% confluent HeLa cells in a 150 mm plate in 450 µl ChIP lysis buffer via Sonics Vibra Cell #VCX130 sonicator.

9. Cut the end of the pipette tip before using the protein G-sepharose slurry as a narrow ending pipette tip can harm the beads. Protein G-sepharose slurry comes in 50% ethanol, thus it is important to remove the ethanol before using it.
10. The input sample represents the amount of chromatin used in the ChIP without any specific selection of DNA fragments through IP. This sample will be used to do the final analysis in the last section of the experiment.
11. Mix the mastermix gently by pipetting up and down before adding to each sample to ensure beads are evenly distributed. Remember to cut the pipette tip before while using the mastermix as otherwise it can harm the beads.
12. Apart from using antibodies to detect the proteins of interests, a non-specific antibody (IgG) is used as a negative control. IgG needs to be from the same species as the specific antibody is used to account for non-specific binding.
13. The supernatant can be used to determine IP efficiency (e.g. how much of the protein of interest is captured and how much of it is lost following the IP and immune capture steps). These can be investigated by the western blot method.
14. Alternative methods can be used to purify the DNA such as magnetic bead or ethanol precipitation-based DNA purification procedures.
15. Typically input DNA is diluted to 2% with nuclease water prior to using it in qPCR.
16. When analysing the data, first of all, normalise the IgG (negative control) and IP samples to the input sample. Then, in order to determine if there is significant enrichment of protein of interest binding to a specific DNA sequence, IgG is compared to the IP (specific antibody IP) within a treatment. Also, input normalised IP signal can be compared across different treatments to find out if the binding is affected by the treatments.

References

1. Smith TG, Robbins PA, Ratcliffe PJ (2008) The human side of hypoxia-inducible factor. Br J Haematol 141 (3):325-334. doi:10.1111/j.1365-2141.2008.07029.x
2. Rocha S (2007) Gene regulation under low oxygen: holding your breath for transcription. Trends Biochem Sci 32 (8):389-397. doi:10.1016/j.tibs.2007.06.005
3. Salceda S, Caro J (1997) Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 272 (36):22642-22647. doi:10.1074/jbc.272.36.22642
4. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 16 (12):1466-1471. doi:10.1101/gad.991402
5. Jiang BH, Rue E, Wang GL, Roe R, Semenza GL (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem 271 (30):17771-17778. doi:10.1074/jbc.271.30.17771
6. Semenza GL (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol (1985) 88 (4):1474-1480. doi:10.1152/jappl.2000.88.4.1474
7. Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL (2005) Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 105 (2):659-669. doi:10.1182/blood-2004-07-2958
8. Semenza G (2002) Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol 64 (5-6):993-998. doi:10.1016/s0006-2952(02)01168-1
9. Jiang BH, Zheng JZ, Leung SW, Roe R, Semenza GL (1997) Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. J Biol Chem 272 (31):19253-19260. doi:10.1074/jbc.272.31.19253
10. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL (2000) The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. The American journal of pathology 157 (2):411-421

11. Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, Semenza GL (2000) Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. Cancer 88 (11):2606-2618
12. Volm M, Koomagi R (2000) Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Res 20 (3A):1527-1533
13. Zhang Q, Lenardo MJ, Baltimore D (2017) 30 Years of NF-kappaB: A Blossoming of Relevance to Human Pathobiology. Cell 168 (1-2):37-57. doi:10.1016/j.cell.2016.12.012
14. Cummins EP, Taylor CT (2005) Hypoxia-responsive transcription factors. Pflugers Arch 450 (6):363-371. doi:10.1007/s00424-005-1413-7
15. Perkins ND (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol 8 (1):49-62. doi:10.1038/nrm2083
16. Wan F, Lenardo MJ (2009) Specification of DNA binding activity of NF-kappaB proteins. Cold Spring Harb Perspect Biol 1 (4):a000067. doi:10.1101/cshperspect.a000067
17. Adhikari A, Xu M, Chen ZJ (2007) Ubiquitin-mediated activation of TAK1 and IKK. Oncogene 26 (22):3214-3226. doi:10.1038/sj.onc.1210413
18. Culver C, Sundqvist A, Mudie S, Melvin A, Xirodimas D, Rocha S (2010) Mechanism of hypoxia-induced NF-kappaB. Mol Cell Biol 30 (20):4901-4921. doi:10.1128/MCB.00409-10
19. Fitzpatrick SF, Tambuwala MM, Bruning U, Schaible B, Scholz CC, Byrne A, O'Connor A, Gallagher WM, Lenihan CR, Garvey JF, Howell K, Fallon PG, Cummins EP, Taylor CT (2011) An intact canonical NF-kappaB pathway is required for inflammatory gene expression in response to hypoxia. J Immunol 186 (2):1091-1096. doi:10.4049/jimmunol.1002256
20. Melvin A, Mudie S, Rocha S (2011) Further insights into the mechanism of hypoxia-induced NFkappaB. [corrected]. Cell Cycle 10 (6):879-882. doi:10.4161/cc.10.6.15157
21. van Uden P, Kenneth NS, Webster R, Muller HA, Mudie S, Rocha S (2011) Evolutionary conserved regulation of HIF-1beta by NF-kappaB. PLoS Genet 7 (1):e1001285. doi:10.1371/journal.pgen.1001285
22. van Uden P, Kenneth NS, Rocha S (2008) Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. Biochem J 412 (3):477-484. doi:10.1042/BJ20080476
23. Bandarra D, Rocha S (2013) Tale of two transcription factors: NF-κB and HIF crosstalk. OA Molecular & Cell Biology 1 (1):1-7
24. Bandarra D, Biddlestone J, Mudie S, Muller HA, Rocha S (2015) HIF-1alpha restricts NF-kappaB-dependent gene expression to control innate immunity signals. Dis Model Mech 8 (2):169-181. doi:10.1242/dmm.017285
25. D'Ignazio L, Shakir D, Batie M, Muller HA, Rocha S (2020) HIF-1beta Positively Regulates NF-kappaB Activity via Direct Control of TRAF6. Int J Mol Sci 21 (8). doi:10.3390/ijms21083000

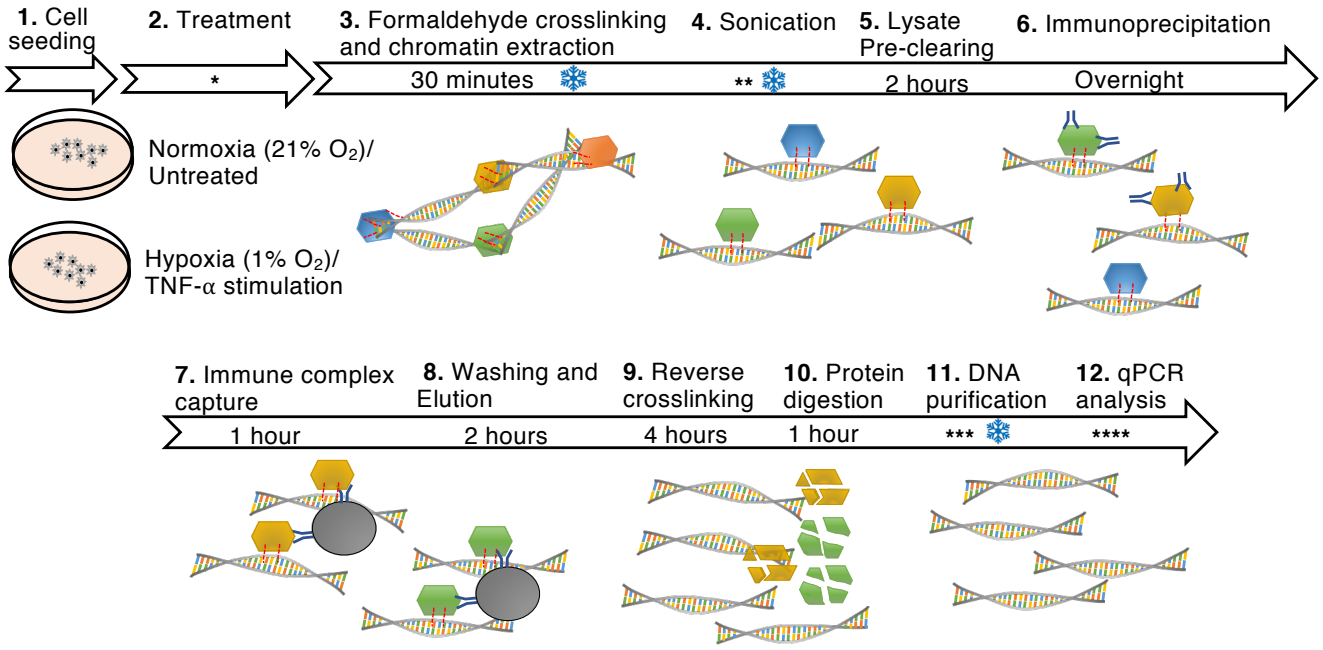

Figure 1: Workflow of ChIP-qPCR.

* Typically 48-72 hours post cell seeding

** Time is dependent on sonication conditions

*** Time is dependent on DNA purification method

**** Time is dependent on the qPCR machine

❄ Samples can be stored at a -80 ºC freezer at the end of this step