Published in final edited form as:

Methods Mol Biol. 2013 ; 977: 323–338. doi:10.1007/978-1-62703-284-1_26.

Mammalian Two-Hybrid assays for studies of interaction of p300 with transcription factors

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Summary

The two-hybrid system is a powerful genetic assay that allows the interaction between two proteins to be detected in vivo. It was originally described in 1989 and since then it has been one of the main techniques used to identify interactions between proteins from different cellular organisms. Here we describe the methods to study the interaction of p300 with other transcription factors, specifically between p300 and two transcription factors related to hypoxia and inflammation, HIF-1 α and NF- κ B-p65, respectively.

Keywords

Mammalian two-hybrid; p300; protein-protein interaction; Gal4; VP16; transcription

1. Introduction

One of the most important mechanisms utilized by mammalian cells to regulate a variety of cellular and molecular activities, such as transcription, translation, signal transduction and enzyme reaction is protein-protein interaction [396 He, R. 2008;]. The two-hybrid system is one of the various methods used to characterize protein-protein interactions. It was originally described in yeast [403 Fields, S. 1989;], then in bacteria [397 Hu, J.C. 2000; 398 Ladant, D. 2000; 399 Dove, S.L. 2003;] and more recently in mammalian cells [396 He, R. 2008;]. Many eukaryotic transcription factors contain two distinct physical and functional domains: the DNA binding domain (DBD) and the transcription activation domain (TAD) [400 Feng, X. H. 2001;]. The DNA-binding domain specifically binds to a promoter/enhancer element and the transcriptional activation domain directs RNA polymerase II to transcribe the gene downstream of the DNA-binding domain [401 Lee, J. W. 2004;]. Although there are several combinations of DBDs and TADs that could be used in mammalian two-hybrid assays [400 Feng, X. H. 2001;], the most commonly used DNA-binding and transcription activation domains are the Gal4 and Herpes virus VP16, respectively [396 He, R. 2008;]. For the mammalian two-hybrid analysis, at least three different types of constructs are necessary, often called “bait,” “prey,” and “reporter.” The bait plasmid encodes a protein of interest that is fused to the DBD, while the prey plasmid encodes a target protein that is fused to the TAD. These plasmids are then co-introduced into

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appropriate host cells along with the reporter plasmid. Interactions between bait and prey result in association of the DBD with the TAD and the interaction between protein of interest and its potential partner can be measured by the level of reporter gene activity [402 Matsuzawa, S. 2007;]. The genes for the enzymes β -galactosidase, luciferase, and chloramphenicol acetyltransferase (CAT) are commonly used as reporter genes and allow convenient quantitation of multiple samples [400 Feng, X. H. 2001;]. The mammalian two-hybrid system is a straightforward method and overcome the limitations inherent to the yeast two-hybrid system (e.g. non-nuclear proteins can be difficult to detect; hybrid proteins may not be expressed stably in yeast or may not efficiently move into the nucleus; the creation of hybrid proteins may impede the natural folding patterns of the proteins, or obscure their interaction sites; and yeast cells lack complex post-translational modification mechanisms needed by many proteins for proper function) [404 Garcia-Cuellar, M. P. 2009;]. Fig. 1 shows a schematic representation of the Mammalian Two-Hybrid System.

2. Materials

Development of fusion constructs

2.1 Oligonucleotide primers—The underlined sequences indicate restriction sites (for *SgfI* and *PmeI*) and the bold letters indicate start or stop codons.

1. For the C-terminal activation domain (CAD) of HIF-1 α (amino acids 721–826)
 - i. HIF-1 α CAD F:
GCGGCGATCGCCATGGAACATGATGGTTCACCTTTTCAAGCA
 - ii. HIF-1 α CAD R:
AATCGTTTAAACGTTAACTTGATCCAAAGCTCTGAGTAATTCTTCA
AC
2. For the C-terminal activation domain (CAD) of NF- κ B-p65 (amino acids 314–550)
 - a. NF- κ B-p65CAD F:
GTCGGCGATCGCCATGAAGAAGAGTCCTTTCAGCGG
 - b. NF- κ B-p65CAD R: CTAGGTTTAAACGGAGCTGATCTGACTCAGCA
3. For the Zn²⁺-binding cysteine/histidine-rich 1 (CH1) domain of p300 (amino acids 302–418)
 - a. CH1-p300 F: ATGGGCGATCGCCATGGGTCAACAGCCAGCCCCG
 - b. CH1-p300 R:
TTGGGTTTAAACTTTGAGGGGGAGACACACAGGA

2.2 PCR reaction

1. High fidelity DNA polymerase;
2. PCR buffer;
3. dNTPs at 10 mM concentration;

4. MgCl_2 at 50 mM concentration;
5. PCR primers (in detail above, item 2.1) at 10 μM concentration;
6. PCR template;
7. Autoclaved, distilled water;
8. Thermal cycler;
9. Thin-wall PCR tubes.

2.3. DNA purification, Clean-up of PCR / Digestion products

1. QIAquick Gel Extraction Kit (QIAGEN);
2. 96–100% ethanol v/v (for the QIAquick Gel Extraction Kit);
3. 100% Isopropanol (for the QIAquick Gel Extraction Kit);
4. 1.5 or 2.0 ml microcentrifuge tubes;
5. Table top microcentrifuge;
6. Heating block or water bath set at 50°C;
7. Optional: Distilled water or TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 for elution of DNA;
8. Nanodrop Spectrophotometer (Thermo Scientific).

2.4. Digestion of PCR product and Acceptor Flexi Vectors

Digestion of PCR products

1. 5× Flexi Digest Buffer (Promega);
2. Purified PCR product (up to 500 ng);
3. Flexi Enzyme Blend: *SgfI* and *PmeI* from Promega;
4. Nuclease-Free Water to a final volume of 20 μl .

Digestion of Acceptor Flexi Vectors

1. 5× Flexi Digest Buffer (Promega);
2. Acceptor Flexi Vectors pFN10A (ACT) and pFN11A (BIND) (200 ng) (Promega);
3. Flexi Enzyme Blend: *SgfI* and *PmeI* from Promega;
4. Nuclease-Free Water.

2.5. Agarose gel electrophoresis

1. Agarose
2. 50× TAE (Tris-Acetate EDTA) Buffer Stock Solution: to 1 liter of stock solution, add 242g of Tris, 100ml of 0.5M EDTA pH 8.0 (this is prepared by adding 73.2 gram of EDTA in 100 ml 1× PBS), 57.1ml of glacial acetic acid, and add enough

distilled water to bring final volume to 1 liter. To prepare 50ml of a 1× solution, mix one volume of 50× TAE Buffer with 49 volumes of distilled water.

3. 1kb and 100bp ladder;
4. Loading buffer containing tracking dyes;
5. Agarose electrophoresis gel equipment and power supply;
6. Transilluminator (UV light box) and camera (optional).

2.6. Ligation of PCR products and Acceptor Flexi Vectors

1. 2× Flexi Ligase Buffer (Promega);
2. Acceptor Flexi Vector from section 2.4 (50 ng) (Promega);
3. PCR product (approximately 100 ng);
4. T4 DNA Ligase High Concentration (20 units) (Promega);
5. Nuclease-Free Water to a final volume of 20 µl.

2.7. Vector preparation

Transformation and Selection

1. MAX Efficiency DH5α Competent Cells (Invitrogen);
2. Ligation reaction (1 to 5 µl);
3. SOC Medium per liter (Invitrogen): add 2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose, pH 7.0, and bring the final volume to 1 L with ddH₂O;
4. Chilled polypropylene tubes;
5. 37°C shaking and non-shaking incubator;
6. LB agar plates: add 15 g of agarose to 1 L of Luria Bertani (LB) media and autoclave for 20 min, cool to 45 °C, add ampicillin to a final concentration of 100 µg/ml and dispense approximately 25 mL into per 100-mm plate.
7. Ice bucket with ice;
8. 42°C water bath.

Screening for the desired clone

1. Polypropylene tubes;
2. Pipet tips to collect desired colonies;
3. Ampicillin
4. LB Medium: dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 800 ml distilled water. Adjust the pH to 7.0 with 1 N NaOH. Adjust the volume to 1 liter with distilled water. Sterilize by autoclaving.

5. Isopropanol;
6. Ethanol;
7. Glycerol;
8. 37°C shaking incubator.
9. 1.5 or 2.0 ml microcentrifuge tubes (for minipreps);
10. 1.5 ml cryotubes;
11. 50 ml centrifuge tubes (for maxipreps);
12. Flasks (for maxipreps);
13. Table top microcentrifuge (for minipreps);
14. Refrigerated centrifuge with rotor (for maxipreps).

2.8. Tissue culture and Transient Transfection

1. Human fetal osteoblastic cells – hFOB 1.19 (ATCC CRL-11372);
2. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12);
3. Opti-MEM I Reduced Serum Media;
4. Fetal bovine serum (FBS);
5. 100× antibiotic/antimycotic solution (with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml) (Sigma-Aldrich);
6. 100× MEM Non-Essential Amino Acids Solution 10 mM (NEAA) (Invitrogen);
7. 10× trypsin-EDTA solution (Sigma-Aldrich);
8. Phosphate buffered saline (pH 7.4): 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, add double distilled H₂O to 1 liter;
9. Transfection reagent: Attractene (Qiagen);
10. 150 mm tissue culture plates;
11. 48-well tissue culture plates
12. 50 ml conical tubes;
13. Water bath set at 37 °C;
14. Inverted microscope;
15. Hemacytometer with cover glass;
16. Tissue culture incubator;
17. Centrifuge;
18. Biosafety cabinet.

2.9. Mammalian Two-hybrid Plasmids and Dual Luciferase Assay

1. CheckMate / Flexi Vector Mammalian Two-Hybrid System (Promega), which contains the following vectors:

Functional vectors:

- Acceptor Flexi Vectors: pFN10A (ACT), which contains herpes simplex virus VP16 transcriptional activation domain upstream of the cloning site; and pFN11A (BIND), that contains the yeast GAL4 DNA-binding domain upstream of the cloning site;
- Reporter vector pGL4.31 [luc2P/GAL4UAS/Hygro] Vector, which contains a firefly luciferase gene (luc2P).

Positive Control Vectors:

- pBIND-Id and pACT-MyoD, that encode VP16-MyoD and GAL4-Id fusion proteins, respectively.

Negative Control Vectors:

- pBIND and pACT, which lack fusion proteins, and are used as negative controls.
2. Test plasmids (fusion constructs):
 - VP16-CH1-p300, GAL4-CAD-HIF-1 α , GAL4-CAD-NF- κ B-p65
 3. Dual Luciferase Assay Kit (Promega);
 4. Glass test tubes for the Dual Luciferase Assay;
 5. Ultra sensitive tube luminometer.

3. Methods

Development of fusion constructs

3.1 Oligonucleotide primers—*The primers used to construct the fusion plasmids were designed using the Flexi Vector Primer Design Tool, available at Promega website (<http://www.promega.us/techserv/tools/FlexiVectorTool/default.aspx>). According to the manufacturer, to facilitate cloning, the PCR primers used to amplify the protein coding region must append an *SgfI* site and a *PmeI* site to the PCR product. To append these sites, an *SgfI* site in the amino-terminal PCR primer and a *PmeI* site in the carboxy-terminal PCR primer should be incorporated, as shown on the “Materials” section. The primer design tool described above automatically appends the restriction enzyme sites.*

3.2 PCR-mediated amplification—Different PCR reactions were set for each gene of interest (HIF-1 α , NF- κ B-p65 and p300) using the respective primer pair.

1. PCR reaction:
 - Add 5 μ l of 10 $\times$ PCR buffer;

- 1 µl of 10 mM dNTP mixture;
 - 2 µl of 50 mM MgCl₂;
 - 1 µl of each primer at 10 µM each;
 - 3 µl of 100 µg/µl of DNA of PCR template for the genes of interest. For CAD-HIF-1α the template DNA used was the vector HA-HIF-1α-WT obtained from Addgene (Addgene plasmid 18949); for CAD-NF-κB-p65 the template DNA used was the vector NF-κB-p65-CMV (Courtesy of Dr. Albert S. Baldwin, University of North Carolina, Chapel Hill); for CH1-p300 the template DNA used was pCMVb-p300 obtained from Addgene (Addgene plasmid 10717);
 - 1.0 unit (in this case, 0.2 µl) of high fidelity DNA polymerase;
 - Autoclaved distilled water to 50 µl.
2. PCR conditions: after 2 min of the initial denaturation at 94°C, run 30 cycles of the following steps – denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 68°C (1 min per kb) and finish extension at 68°C for 10 min. Samples can be stored at –20°C until further use.
 3. Clean-up of PCR products: using QIAquick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions and elute DNA in buffer EB. This step is important to remove the DNA polymerase and primers.
 4. Determination of yield: To determine the yield, the PCR products should be quantified using UV spectrophotometry at 260 nm (Nanodrop).
 5. Digestion: digest PCR products and Acceptor Flexi Vectors pFN10A (ACT) and pFN11A (BIND) (Promega), using Flexi Enzyme Blend (*SgfI* and *PmeI*) (Promega), according to manufacturer's instructions. Digestion was performed at 37°C for 30 minutes. For the Acceptor Flexi Vectors heat the reaction at 65°C for 20 minutes to inactivate the restriction enzymes. Store on ice until the PCR product and vector are ligated.
 6. Clean-up of digested PCR products: using QIAquick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions and elute DNA in buffer EB. This step is important to remove the small oligonucleotides released by the restriction enzymes. According to the manufacturer's protocol, the digested Acceptor Flexi Vectors do not need to be purified.
 7. 1.0% agarose gel for electrophoresis: subject the digested PCR products and Acceptor Flexi Vectors to 1.0% agarose gel prepared in 1× TAE buffer for electrophoresis, then isolate the band corresponding to the size of the desired gene.
 8. Determination of yield: To determine the yield, the digested PCR products and Acceptor Flexi Vectors should be quantified using UV spectrophotometry at 260 nm (Nanodrop).

9. Ligation: ligate the digested PCR products for p300 (CH1-p300), HIF-1 α (CAD-HIF-1 α) and NF- κ B-p65 (CAD-NF- κ B-p65) to the Acceptor Flexi Vectors (VP16-encoding pFN10A or GAL4-pFN11A). CH1-p300 was ligated to VP16-encoding pFN10A; CAD-HIF-1 α and CAD-NF- κ B-p65 were ligated to GAL4-pFN11A. Ligation was performed for 1 hour at room temperature as recommended (see note 1).

¹Development of fusion constructs

Ligation

Sometimes the ligation between the digested PCR products for p300 (CH1-p300), HIF-1 α (CAD-HIF-1 α) and NF- κ B-p65 (CAD-NF- κ B-p65) and the Acceptor Flexi Vectors (VP16-encoding pFN10A or GAL4-pFN11A) will not work the first time even though the manufacturer's recommendations are followed. In these cases, the pGEM-T Easy Vector system (Promega) should be used. These vectors contain a 3' terminal thymidine to both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases {{407 Mezei, L. M. 1994;}}. The pGEM-T Easy Vector Systems includes a 2 $\times$ Rapid Ligation Buffer for ligation of PCR products. According to the manufacturer, reactions using this buffer may be incubated for 1 hour at room temperature. However, the incubation period may be extended to increase the number of colonies after transformation. Generally, an overnight incubation at 4°C will produce the maximum number of transformants.

Details for ligation using the pGEM-T Easy Vector System:

- 1.1 2 $\times$ Rapid Ligation Buffer (Promega);
- 1.2 pGEM-T Easy Vector (50ng)
- 1.3 PCR Product (insert) (amount to be used should be calculated using the equation below):

$$\frac{\text{ng of vector} \times \text{kbsize of insert}}{\text{kbsize of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Based on the size of our vectors, the calculations were done as follows:

- 1.4 For GAL4-CAD-HIF-1 α :
 - 1.4.1 ng of vector (pGEM-T Easy) = 50 ng
 - 1.4.2 kb size of insert (after digestion with Flexi Enzyme Blend – Promega) = 330 bp
 - 1.4.3 kb size of vector (pFN11A) (after digestion with Flexi Enzyme Blend – Promega) = 6144 kb
 - 1.4.4 Insert:vector molar ratio used was 5:1
- 1.5 For GAL4-CAD-NF- κ B-p65:
 - 1.5.1 ng of vector (pGEM-T Easy) = 50 ng
 - 1.5.2 kb size of insert (after digestion with Flexi Enzyme Blend – Promega) = 729 bp
 - 1.5.3 kb size of vector (pFN11A) (after digestion with Flexi Enzyme Blend – Promega) = 6144 bp
 - 1.5.4 Insert:vector molar ratio used was 5:1
- 1.6 For VP16-CH1-p300:
 - 1.6.1 ng of vector (pGEM-T Easy) = 50 ng
 - 1.6.2 kb size of insert (after digestion with Flexi Enzyme Blend – Promega) = 363 bp
 - 1.6.3 kb size of vector (pFN10A) (after digestion with Flexi Enzyme Blend – Promega) = 5496 bp
 - 1.6.4 Insert:vector molar ratio used was 5:1
- 1.7 Control insert DNA (used as a positive control): 2.0 μ l;
- 1.8 T4 DNA Ligase (3 Weiss units/ μ l) (Promega): 1.0 μ l;
- 1.9 Deionized water to a final volume of 10 μ l.

3.3 Vector preparation

1. Transformation and Selection: Transform MAX efficiency DH5 α competent cells, as recommended by the manufacturer. 50 μ l of MAX Efficiency DH5 α competent cells, and 3 μ l of ligation were used for the transformation. 400 μ l of the transformation mixture was spread on LB agar plates containing 100 μ g/ml of ampicillin. Agar plates were incubated overnight at 37°C. The remaining transformation reaction could be stored at 4°C, since additional cells may be plated out the next day, if necessary (see note 2).
2. Screening of the desired colonies: Screen 8–12 colonies for each fusion construct (VP16-CH1-p300, GAL4-CAD-HIF1 α or GAL4-CAD-NF- κ B-p65). The desired colonies can be selected using pipet tips and placed into polypropylene tubes containing 3 to 5 ml of LB media plus 100 μ g/ml of ampicillin. These selected colonies should be put in a 37°C shaking incubator overnight (12–16h) at 225 rpm;
3. Miniprep: Harvest the bacterial cells by centrifugation at > 8000 rpm (6800 $\times$ g) in a conventional, table-top microcentrifuge for 3 min at room temperature (15–25°C). The DNA purification was done using the QIAprep Spin Miniprep Kit (QIAGEN), according to the manufacturer's recommendations. The DNA was eluted in 30 μ l of Buffer EB;
4. Digestion: Digest the fusion constructs using Flexi Enzyme Blend (*SgfI* and *PmeI*) (Promega) to ensure that both *SgfI* and *PmeI* cleave their recognition sites flanking

²Vector preparation

1. Transformation: The same protocol commented before to transform MAX efficiency DH5 α competent cells can be used if using the pGEM-T Easy Vector System. However, after the transformation LB Agar plates should be prepared for blue/white screening to make it a little easier to select the colonies the next day. White colonies generally contain inserts; however, inserts may also be present in blue colonies.
2. Preparation of LB Agar Plates for Blue / White screening:
Mix 10 μ l 100 mM IPTG with 90 μ l of ddH₂O; Mix 25 μ l X-Gal with 25 μ l ddH₂O; Add 100 μ l SOC Media and spread approximately 250 μ l per plate; Incubate the plates at 37° C and wait 30 to 60 minutes for the mixture to dry before using the plates.
3. Selection and Screening of the desired colonies: the white colonies should if selected if the blue / white screening was performed. Screen 8–12 colonies for each construct (pGEM-T Easy + CAD-HIF-1 α , pGEM-T Easy + CH1-p300, pGEM-T Easy + CAD-NF- κ B-p65). The desired colonies can be selected using pipet tips and placed into polypropylene tubes containing 3 to 5 ml of LB media plus 100 μ g/ml of ampicillin. These selected colonies should be cultured at 37°C in a shaking incubator overnight (12–16h) at 225 rpm.
4. Miniprep: Performed as reviewed in the Methods section (item 3.3.3) using the QIAprep Spin Miniprep Kit (QIAGEN);
5. Digestion: Digest the constructs using Flexi Enzyme Blend (*SgfI* and *PmeI*) (Promega), according to manufacturer's instructions. Digest from here to ligate into the new vector;
6. DNA electrophoresis gel: Subject the fusion constructs to 1.0% agarose gel prepared in 1 $\times$ TAE buffer for electrophoresis to check the band corresponding to the desired size;
7. Maxiprep (large scale, high purity DNA purification): Done as commented on the Methods section (item 3.3.6) using the Qiagen Endofree Plasmid Maxi Kit (QIAGEN);
8. Digestion: Performed as commented before (item 4.2.4);
9. Determination of yield: See Methods section (item 3.3.8);
10. Sequencing: Inserts can be sequenced using the SP6 Promoter Primer and the T7 Promoter Primer, according to the manufacturer's recommendations.

the protein-coding region. Digestion should be performed according to manufacturer's instructions, at 37°C for 30 minutes;

5. DNA electrophoresis gel: Subject the fusion constructs to 1.0% agarose gel prepared in 1× TAE buffer for electrophoresis to check the band corresponding to the desired size. The fragment size for VP16-CH1-p300 digested with the Flexi Enzyme Blend should be 5496 and 363bp; for GAL4-CAD-HIF1α the fragments should be 6144 and 330bp, and for GAL4-CAD-NF-κB-p65, the fragment sizes should be 6144 and 729bp;
6. Maxiprep (large scale, high purity DNA purification): From the minipreps, select one positive colony for each vector and proceed with large scale DNA purification. Inoculate the starter culture (obtained above) of 2–5 ml LB medium containing 100 µg/µl of ampicillin. Incubate for approximately 8 h at 37°C with vigorous shaking (approx. 225 rpm). Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For each fusion construct, inoculate 100 ml medium with 100–200 µl of starter culture. Grow at 37°C for 12–16 h with vigorous shaking (approximately 300 rpm). Harvest the cells by centrifugation at 4 °C for 10 minutes at 4,000 g. Isolate the desired fusion constructs (VP16-CH1-p300, GAL4-CAD-HIF-1α or GAL4-CAD-NF-κB-p65) using a Qiagen Endofree Plasmid Maxi Kit, following the manufacturer's protocol. Dissolve the DNA in a suitable volume (100µl to 1ml) of endotoxin-free Buffer TE, depending on the size of the DNA pellet. Make bacteria freezing stocks of the positive colonies of the fusion constructs using 50% glycerol solution (v/v) in a 1.5 ml cryotube and store at –80°C. Always use the original freezing stock for further testings.
7. Digestion: Digest the fusion using Flexi Enzyme Blend (*SgfI* and *PmeI*) (Promega) constructs to ensure that both *SgfI* and *PmeI* cleave their recognition sites flanking the protein-coding region. Digestion was performed according to manufacturer's instructions, at 37°C for 30 minutes;
8. Determination of yield: To determine the yield, DNA concentration should be determined by UV spectrophotometry at 260 nm (Nanodrop) and quantitative analysis on 1.0% agarose gel prepared in 1× TAE buffer. For reliable spectrophotometric DNA quantification, A260 readings should lie between 0.1 and 1.0. Prepare a 500 µl working solution of 100 µg/µl of DNA for each vector for further use.
9. Sequencing: Since PCR was used to create the original insert, the sequence of the protein-coding region in the initial clone should be verified by DNA sequencing. The T7 promoter primer should be used for sequencing of the fusion constructs.

3.4. Tissue culture and transfection

1. hFOB cell culture: supplement the DMEM/F12 growth media with 10% FBS and 1× penicillin/streptomycin (regular media). Cells should be grown on 150 mm tissue culture plates inside a humidified incubator at 37° C supplied with 5% CO₂.

2. Preparing hFOB cells for transient transfection: hFOBs should be grown until they reach 70–80% of confluency. For each 150 mm plates: rinse cells with 10–12 ml of 1× PBS twice, trypsinize cells with 8 ml of 1× trypsin and incubate for 5 minutes at 37° C incubator. After the 5 minutes incubation, add 12ml of regular media to each 150 mm plate to quench the trypsin. Place detached cells into a screw-top conical tube and spin them at 200 g for 4 minutes. Remove supernatant carefully and do not disturb cell pellet. Resuspend the cells in Opti-MEM media supplemented with 3% FBS and 1× NEAA (transfection media). The amount of media added will depend on the size of the pellet. Perform the cell counting and adjust the cell density (1.6×10^5 cells/ml).

3. Transient transfection:

Table 1 shows recommended combinations of vectors to properly control experiments using the CheckMate™/Flexi Vector System, according to the manufacturer's recommendations. In this case, "X" represents CAD-HIF-1 α or CAD-NF- κ B-p65 (two different experiments will be set up), while "Y" represents CH1-p300. pBIND and pACT are Negative Control Vectors. pBIND-Id and pACT-MyoD are Positive Control Vectors.

The method described here is for transient transfection of hFOB cells in 48-well plates, using the Fast-Forward Protocol. In the Fast-Forward protocol, plating and transfection are performed on the same day. In the Traditional Protocol, cells are plated 24 hours prior to transfection. The DNA is diluted in culture medium without serum and Attractene Transfection Reagent is added to the diluted DNA to produce Attractene-DNA complexes. The cells are seeded and then complexes are added directly to the freshly seeded cells. The detailed steps for the Fast-Forward protocol are explained below, according to the manufacturer's recommendations.

- For each combination showed in table 1 above, dilute 125 ng of DNA of each plasmid (amount recommended for 48-well plates) in serum-free and antibiotic-free Opti-MEM medium to a total volume of 30 μ l per well. For example, if the DNA concentration is 100 μ g/ μ l, dilute 1.25 μ l DNA in 28.75 μ l medium;
- Add 0.5 μ l of Attractene Transfection Reagent per well. Mix by pipetting up and down or vortexing. Centrifuge for a few seconds to remove any liquid from the top of the tube if necessary;
- Incubate the samples for 10–15 minutes at room temperature (15–25°C) to allow transfection complex formation;
- During the 10–15 minutes incubation time, harvest the cells by trypsinization, and suspend in transfection culture medium as described above (item 3.4.2). Count the cells, and adjust the cell density to 1.6×10^5 cells in 1ml per well. The optimal cell density should be determined for each cell line;
- Add 250 μ l of the cell suspension (which contains 4×10^4 cells) to each well of the 48-well plate. Next, add the transfection complexes (30 μ l) to the well. The total volume per well will be 280 μ l. Mix by pipetting up and down twice (or shake the plate);

- Incubate the cells with the transfection complexes under their normal growth conditions (37°C and 5% CO₂) for 18–24 hours. Then, remove the transfection complexes and add fresh culture medium. Perform the Dual Luciferase Assay reading after 24 to 48 hours after the removal of the transfection complexes.

3.5. Dual Luciferase Assay

1. Dual Luciferase Assay Kit Reagent preparation (according to the manufacturer's recommendations):
 - 1× PLB: Add 1 volume of 5× Passive Lysis Buffer (PLB) to 4 volumes distilled water. Mix well. Store at 4°C (1 month);
 - Luciferase Assay Reagent II (LAR II): Resuspend the lyophilized Luciferase Assay Substrate in Luciferase Assay Buffer II. Store at –20°C (1 month) or –70°C (1 year);
 - Stop & Glo Reagent: Add 20µl of 50× Stop & Glo Substrate to 1ml of Stop & Glo Buffer contained in either a glass vial or siliconized polypropylene tube. This will prepare sufficient Stop & Glo Reagent for 10 assays.
2. Passive Cell lysis:
 - Remove growth media from cultured cells;
 - Rinse cultured cells in 1× PBS. Remove all rinse solution. Dispense the recommended volume (for 48-well plate, the recommended volume is 65 µl) of 1× PLB into each well;
 - Passive Lysis: Gently rock/shake the tissue culture plate for 15 minutes at room temperature. Transfer lysate to a tube or vial.
3. Dual Luciferase Assay:

Fig.2 shows an example of the luciferase activity involving the fusion constructs described throughout the text,

The method described here is for Assay with Dual-Injector Luminometer.

 - Before you begin, set the protocol on the machine: Set injectors 1 and 2 to dispense 50 µl of LAR II and Stop & Glo Reagent, respectively. For measurements, use a 1–2 second delay and 5–10 second read time;
 - Predispense enough LAR II Reagent into injector 1 of the luminometer according to the number of samples;
 - Transfer 20 µl PLB Lysate. Mix;
 - Measure firefly luciferase activity;
 - Predispense enough Stop & Glo Reagent into injector 2 of the luminometer according to the number of samples;
 - Measure *Renilla* luciferase activity.

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Figure 1.

Schematic representation of the CheckMate Mammalian Two-Hybrid System. The CheckMate/Flexi Vector System relies upon three plasmids that are co-transfected into mammalian cells. The pFN10A (ACT) Flexi Vector contains a herpes simplex virus VP16 transcriptional activation domain upstream of the cloning site, and the pFN11A (BIND) Flexi Vector contains the yeast GAL4 DNA-binding domain upstream of the cloning site. The pFN11A (BIND) Flexi Vector also expresses the Renilla reniformis luciferase under the control of the SV40 promoter, allowing normalization for differences in transfection efficiency. The third vector, pGL4.31, contains five GAL4 binding sites upstream of a minimal TATA box, which is upstream of a firefly luciferase gene that acts as a reporter for interactions between proteins “X” and “Y”. “X” = CAD-HIF-1 α or CAD-NF- κ B-p65; “Y” = p300. Adapted from CheckMate Mammalian Two-Hybrid System Technical Manual (Promega).

Figure 2.

p65 competes with HIF-1α for p300. hFOB cells were (A) co-transfected with VP16-p300, Gal4-CAD-HIF-1α fusion constructs (125 ng) and p65-CMV (375 ng) for 18 h. Cells were then exposed to Normoxia (N) or DFO-induced Hypoxia (H). Luciferase activity was measured 24 h post-treatment. Statistically significant difference compared to p300 + CAD-HIF-1α + Empty Vector (normoxia) ($p = 0.05$). (B) cotransfected with VP16-p300, Gal4-CAD-p65 fusion constructs (125 ng) and HIF-1α-WT (375 ng) for 18 h. Cells were then exposed to N or H. Luciferase activity was measured 24 h post-treatment. Statistically

significant difference compared to p300 + CAD-p65 + Empty vector (N) ($p < 0.05$). Data are shown as the mean $\pm$ SE of three independent experiments performed in triplicate.

Table 1

Experimental Design for Testing Protein:Protein Interactions

Sample	ACT Vector	BIND vector	pGL4.31
1	pFN10A (ACT)-Y	pFN11A (BIND)-X	+
2	pACT Vector	pFN11A (BIND)-X	+
3	pFN10A (ACT)-Y	pBIND Vector	+
4	pACT Vector	pBIND Vector	+
5	pACT-MyoD Control	pBIND-Id Control	+

Sample 1 is used to test for protein interactions between the X and Y protein-coding regions. Sample 2 is used to test for the background level of reporter activity when one of the protein-coding regions is fused to the GAL4 DNA-binding domain. This also tests for transcriptional activation domain activity of protein X when fused to the GAL4 DNA-binding domain. Sample 3 is used to test for the background levels of reporter activity when one of the protein-coding regions is fused to the activation domain. Sample 4 generally provides the lowest level of background activity of luciferase activity from the reporter plasmid. Sample 5 provides a positive control with interacting proteins fused to the activation and GAL4 DNA-binding domains