T.C.
DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

Molecular cloning and production of recombinant hODF extracellular domain

MUHAMMET MEMON

MOLECULAR BIOLOGY AND GENETICS MASTER'S PROGRAM

MASTER OF SCIENCE THESIS

İZMİR-2019

TEZ KODU: DEU.IBG.MSc-2016850003

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Supervising Faculty Member: Assoc. Prof. M. Kasım DİRİL

Bu araştırma TÜBİTAK 1007 Kamu Kurumları Araştırma ve Geliştirme Projelerini Destekleme Programı tarafından 115G073 nolu proje ile desteklenmiştir.

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Dokuz Eylül University İzmir International Biomedicine and Genome Institute Department of Genomics and Molecular Biotechnology, Molecular Biology and Genetics Graduate Grogram Master of Science student Muhammet MEMON has successfully completed his Master of Science thesis titled "Molecular cloning and production of recombinant hODF extracellular domain" on the date of April 3, 2019.

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ABBREVIATIONS

hODF……Human Osteoclast Differentiation Factor

NFAT…….....Nuclear Factor of Activated T cells

NF-KB ……...……Nuclear Factor-Kappa B

OPG…….……... Osteoprotegerin

RANK.……Receptor Activator of Nuclear Factor-KB

TRAF..... Tumor Necrosis Factor Receptor-Associated Factor

NFATc1……...….... Nuclear Factor of Activated T Cells, Calcineurin-dependent 1

PCR……...…….…Polymerase Chain Reaction

TAE Buffer…….....Tris Acetic Acid EDTA

RD……...restriction digest

FastAP……. Fast Alkaline Phosphatase

CIP……...……Calf Intestine Phosphatase

SDS PAGE…….....Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

MCSF……...……...…...……Macrophage Colony Stimulating Factor

MMP…….…...…….……matrix metalloproteinase

CRDs…….. cysteine-rich domains

CLC MWB……...……CLC Main Work Bench

E. coli ……. *Escherichia coli*

HRV3C…….…. Human Rhino Virus 3C

NTA…….……...Nitrilotriacetic acid

SPR…… Surface Plasma Resonance

ELISA……Enzyme-linked immunosorbent assay

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MOLECULAR CLONING AND PRODUCTION OF RECOMBINANT hODF EXTRACELLULAR DOMAIN

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ABSTRACT

Human osteoclast differentiation factor (hODF) is a membrane protein expressed by osteoblasts. Its proteolytic cleavage releases a truncated ecto-domain composed of its extracellular domain between amino acids 143-317. hODF extracellular domain, binds to and activates its receptor RANK on osteoclast membrane. Activated RANK triggers an intracellular signaling pathway which leads to activation and nuclear localization of NF-KB and transcription of osteoclastogenic genes. This eventually results in differentiation and activation of osteoclasts, eliciting a bone remodeling response. To study osteoclastogenesis in vivo and in vitro requires a significant amount of hODF protein which can be used in downstream experiments such as RAW264.7 osteoclastogenesis assay and in vitro binding assays. Therefore, we decided to express and purify recombinant hODF extracellular domain. Recombinant hODF DNA was generated from human cellular genomic DNA and cloned into several expression vectors by PCR based molecular cloning methods. After trial and optimization of different purification strategies, GST tagged hODF was expressed in bacterial cells and purified by glutathione–agarose beads. hODF was released from the beads by proteolytic cleavage by HRV3C protease and results were analyzed by SDS-PAGE. The protocol we developed enabled us to produce sub-milligram quantities of hODF with higher than %70 purity.

Keywords: hODF, RANK, Osteoclastogenesis, Recombinant Protein Production

REKOMBİNANT hODF EKSTRASELLÜLER DOMAİN'İN MOLEKÜLER KLONLANMASI VE ÜRETİMİ

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ÖZET

İnsan osteoklast değişim faktörü (hODF), osteoblastlar tarafından üretilen bir membran proteinidir. Proteolitik olarak kesilmesiyle 143-317 amino asitlerden oluşan kesik-ekstraselüler domainini açığa çıkarır. hODF ekstraselüler domaini osteoklast membranındaki reseptörü olan RANK'e bağlanır ve aktive eder. Aktive edilmiş RANK, NF-KB'nin aktivasyonuna ve hücre çekirdeğine lokalize olmasına sebep olan hücre içi sinyal yolağını tetikler ve osteoklastojenik genlerin transkripsiyonuna sebep olur. Bu nihayetinde osteoklastların farklılaşması ve aktivasyonu ile sonuçlanır ki bu da kemikleri yeniden biçimleme yanıtıdır. Osteoklast oluşumunu *in vivo* ve *in vitro* olarak çalışabilmek için yeteri miktarda hODF proteinine ihtiyaç duyulur ki ilerki basamaklarda RAW264.7 osteoklast oluşum deneyinde ve in vitro bağlanma testlerinde kullanılabilsin. Bu yüzden biz rekombinant hODF ekstraselüler domainini ekspresyonuna ve saflaştırılmasına karar verdik. Rekombinant hODF, insan hücrelerinden elde edilen genomik DNA kullanılarak amplifiye edildi ve moleküler klonlama yöntemleriyle birkaç ekspresyon vektörlerine klonlandı. Farklı saflaştırma statejilerinin denenmesi ve optimize edilmesinden sonra, GST tag edilmiş hODF bakterilerin içinde ekspres edilip glutatyon-agaroz boncuklarıyla saflaştırıldı. hODF, HRV3C proteazı ile boncuklardan ayırıldı ve sonuçlar SDS-PAGE ile analiz edildi. Geliştirdiğimiz protokol ile yaklaşık milligram miktarlarında ve %70'ten daha fazla saflıkta hODF üretildi.

Anahtar Kelimeler: hODF, RANK, Osteoklast oluşumu, Rekombinant Protein Üretimi

1. INTRODUCTION AND PURPOSE

Osteoclasts are an important class of bone cells that have roles in dissolving the bone's organic and inorganic components thereby helping in bone remodeling. They differentiate from hematopoietic cells such as macrophages. It is very difficult to isolate fully differentiated osteoclasts as they are very rare and found in bone. Mincing limbs of chicken, mouse, or rabbit and using it as a primary culture, isolating osteoclast precursors and co-culturing osteoblasts and bone marrow stromal cells, isolating human peripheral blood mononuclear cells (PBMCs) from peripheral blood are some commonly used methods (Marino, Logan, Mellis, & Capulli, 2014) to extract and culture osteoclasts. However, all of these methods are arduous and time consuming.

To circumvent this problem, RAW264.7 murine macrophages are routinely utilized. These cells can be easily cultured in vitro and readily differentiated to mature osteoclasts by addition of the human osteoclast differentiation factor (hODF) extracellular domain to their culture medium which is normally expressed by osteoblasts of the bones (Nguyen & Nohe, 2017).

The aim of this project is the production of the hODF extracellular domain using the *Escherichia coli* (*E. coli*) host expression system. The DNA coding for the extracellular domain of hODF was amplified from human genomic DNA and cloned into a protein expression vector using molecular cloning techniques.

2. GENERAL INFORMATION

Bone, serves as a support for muscles, contains bone marrow which is the factory of hematopoietic cells, protects vital organs, serves as a storage for essential ions, such as calcium and phosphate (Boyce & Xing, 2007). Trabeculae and cortical bone constantly undergo breakdown and remodeling where pockets of bone are removed by osteoclasts and rebuilt by osteoblasts. At any given time point, there are at least a million of these microscopic remodeling foci (**Figure *1***). The main function of this process, which is highly regulated, is to remove worn out parts and replace them with new bone (Boyce & Xing, 2007). These processes are controlled by three key regulatory proteins, RANK/hODF/OPG. These key proteins are regulated by osteotropic hormones and cytokines that either increase (transforming growth factor-β (TGF-β) and estrogens) or decrease (glucocorticoids, inflammatory cytokines e.g.

interleukin-1 (IL-1), parathyroid hormone (PTH), prostaglandin E2 (PGE2), vitamin D3) the OPG/hODF ratio (Stejskal et al., 2001).

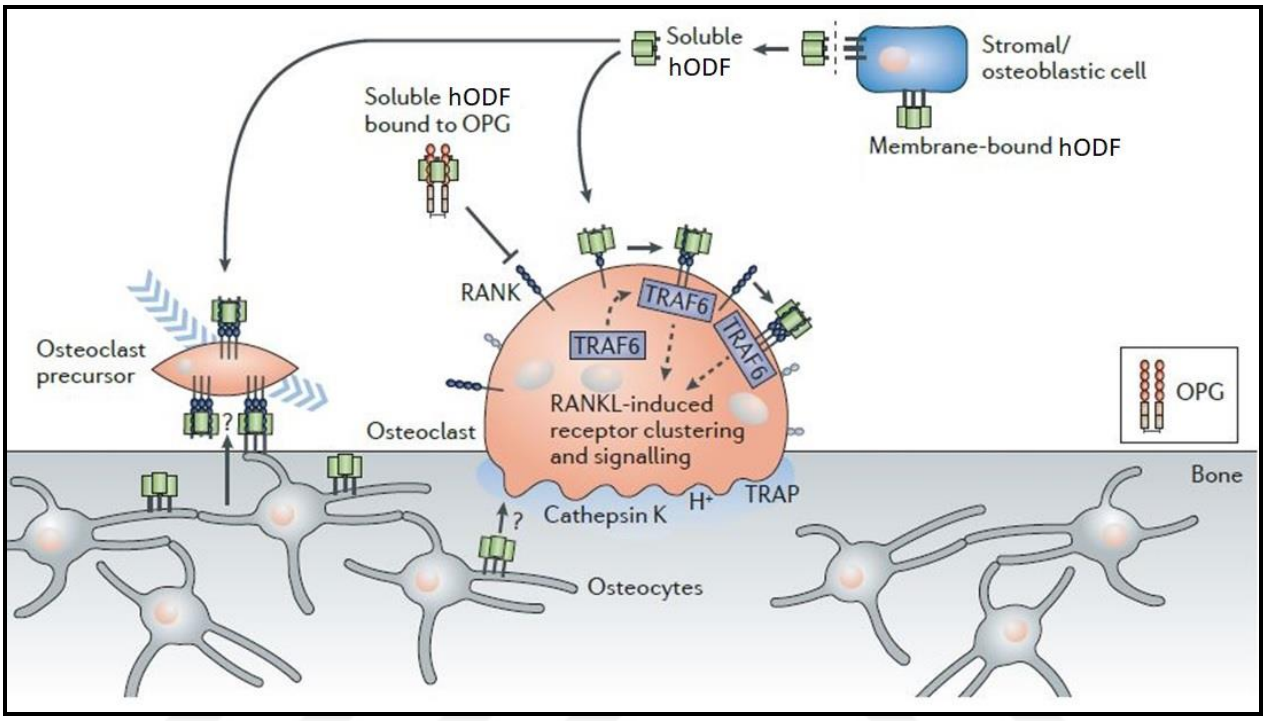


Figure 1. Role of OPG/RANK/hODF pathway in bone turnover. Several factors including cytokines, hormones and growth factors are needed for the maturation of hematopoietic stem cells to osteoclast precursors. One of these factors, hODF, can be found as a membrane bound protein in stromal/osteoblastic cells or as a soluble form. The soluble form is generated by the cleavage of the transmembrane protein which is also known as shedding. Binding of hODF to RANK recruits other monomers of RANK. This in return, fuses osteoclast precursors, forms multinucleated osteoclasts, and calls in TRAF 6, an adapter protein. TRAF 6 triggers signaling pathways that initiate transcription of osteoclastogenic genes. (Udagawa et al., 1999).Osteoprotegerin (OPG) is a decoy receptor that binds to and inhibits interaction of hODF with its receptor RANK, which results in apoptosis of the osteoclasts (adapted from Trouvin & Goëb, 2010).

2.1 Osteoclast Differentiation Factor Function and Biology

Human Osteoclast Differentiation Factor (hODF), also known as TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), and tumor necrosis factor ligand superfamily member 11 (TNFSF11) is encoded as the *TNSF11* gene in humans at chromosome 13 at 13q14.11 band. It is required in osteoclast differentiation and activation, mammary gland development during pregnancy, dendritic cell survival, and T and B cell maturation.(Becherer et al., 1999; Ikeda, Kasai, Utsuyama, & Hirokawa, 2001; Schlöndorff, Lum, & Blobel, 2001; Takayanagi, 2007)

hODF is a ~35 kDa sized, type II membrane protein (Nelson, Warren, Wang, Teitelbaum, & Fremont, 2012) and is known to affect the immune system, regulate body temperature, regulate bone remodeling and regeneration. It is expressed in a variety of tissues and organs such as prostate, pancreases, adrenal gland, osteoblast, mammary gland epithelial cells, skeletal muscle, liver, colon, thymus. Research shows that although it is involved in osteoclastogenesis, bone tissue shows low expression whereas other tissues like thymus, lungs, and lymph nodes show high expression. (Wada, Nakashima, Hiroshi, & Penninger, 2006)

hODF is expressed on the cell membrane of several cells such as, T-lymphocytes, bone marrow stromal cells, and osteoblasts and shows its activity by binding to RANK, its TNF family receptor (Xiong et al., 2018), which is expressed on monocyte-macrophage osteoclast-precursors. Endothelium is another source for hODF and OPG. When RANK is activated it stimulates cell survival and angiogenesis (Ikeda et al., 2001; Mueller & Hess, 2012). In response RANK activates downstream signaling pathways, c-Jun N-terminal kinase, NF-κB, p38 mitogen-activated protein kinase, and nuclear factor of activated T cells c1 which causes the differentiation, activation, and survival of osteoclasts (Hikita et al., 2006) (**Figure *2***). RANK and hODF deficient mice show lack of osteoclasts therefore cause osteopetrosis. In contrast, downregulation of OPG, a natural inhibitor and decoy receptor of hODF, causes severe osteoporosis.

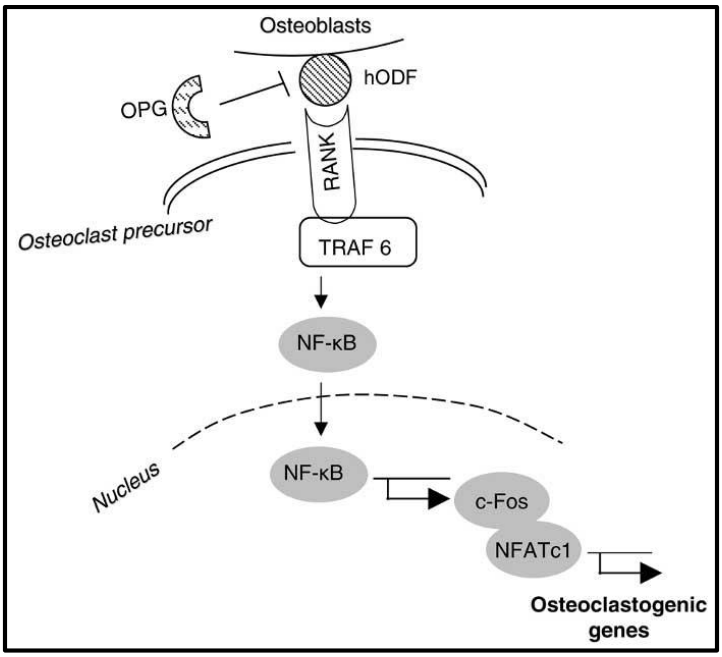


Figure 2. hODF downstream signaling pathway during osteoclastogenesis (adapted from Boyce and Xing, 2007). Under normal physiology, hODF, produced by osteoblasts, binds to RANK on the surface of

osteoclast precursors and recruits the adaptor protein TRAF6, which activates and translocates NF-KB to the nucleus. NF-KB increases c-Fos expression and c-Fos interacts with NFATc1 to initiate the transcription of osteoclastogenic genes. OPG inhibits the initiation of the process by binding to hODF.

Although hODF is coded by a single gene it has three different isoforms as a result of alternative splicing. In humans two of these isoforms are type II homotrimeric transmembrane bound glycoprotein one consisting of 317 amino acids and the other 270 amino acids which differ by the latter containing a shorter intracellular domain. The third type of isoform consists of 243 amino acids and lacks both a transmembrane and intracellular domain which acts as a soluble ligand. (Wright, McCarthy, Middleton, & Marshall, 2009). The cell membrane bound isoforms can also undergo cleavage, also known as ectodomain shedding, a process where hODF membrane-anchored protein is cleaved with a matrix metalloproteinase (**Figure 3**) (Hikita et al., 2006; Lynch et al., 2005).

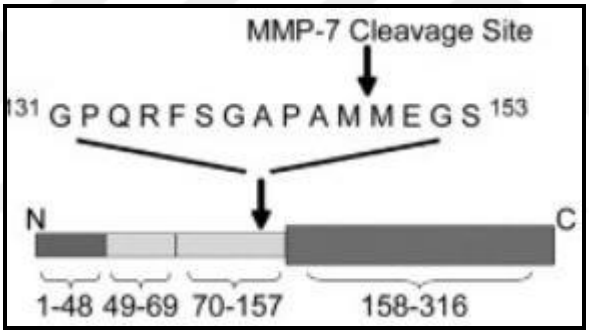


Figure 3. Shedding of hODF. Matrix metalloproteinase 7 (MMP-7) cleaves hODF at its stalk region between 145Met and 146Met residues. This cleavage changes hODF into its soluble form, a process also known as shedding. Numbers 1–316 represent the amino acids in full-length hODF (1–48, cytoplasmic region; 49–69, transmembrane region; 70–157, stalk region; and 158–316, active ligand moiety). (2005; Lynch et al., 2005).

Additional to bone remodeling, it has been shown that osteoclasts also function as immunomodulators in pathologic states such as rheumatoid arthritis, post-menopausal osteoporosis, tooth loss, and cancer metastasis (**Figure 4**). Therefore, understanding the mechanism of action of the osteoclast differentiation factor (ODF) would provide insight on possible target therapeutic formulations.

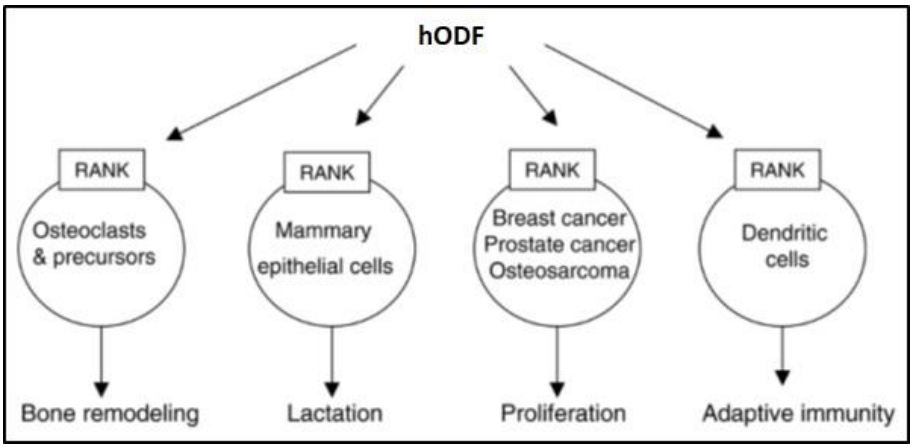


Figure 4. Role of RANK/hODF signaling system. Under normal physiological conditions, hODF, regulates the differentiation of osteoclast precursors into multinucleated osteoclasts, stimulates lactation in mammary epithelial cells, triggers survival in dendritic cells of the adaptive immune system (Boyce & Xing, 2007).

2.2 hODF Structure

hODF is composed of a short N-terminal cytoplasmic region, a trans membrane domain and a connecting C-terminal receptor binding and stalk extracellular domain (**Figure 5**A). Membrane bound hODF activates RANK through cell to cell contact. hODF is also found in a truncated soluble form which forms by its cleavage during inflammatory osteolysis (Nelson et al., 2012; Schlöndorff et al., 2001).

RANK, hODF's cognate cell surface signaling receptor is a ~67 kDa type I transmembrane protein, contains four cytokine bind domains that are rich in cysteine domains (CRD), a transmembrane and a TRAF-binding cytoplasmic motif region. (**Figure 5**B) Binding of hODF to CRDs triggers trimerization (**Figure 5**D). Just by itself, RANK lacks intrinsic enzymatic activity. But when a RANK trimer forms, it calls in signaling adapters and other TNF-associated factors which as a response, turn on osteoclastogenic pathways (Armstrong et al., 2002; Wong et al., 1998).

OPG, a natural decoy receptor, binds to hODF and prevents it from binding to RANK and inhibts osteoclastogenic pathways. It is a ~55 kDa monomer which couples up with itself to form a disulfied-linked homodimer (**Figure 5**) where each monomer contains two death domains and a heparin binding basic motif at its C-terminal . The dimerization increases its ability to bind to hODF which could explain how it inhibts osteoclastogenesis (Schneeweis, Willard, & Milla, 2005). Although rare, but when OPG acquires mutation it loses its ability to bind to hODF, therefore allowing bone resorption and osteoperosis (Walsh & Choi, 2014).

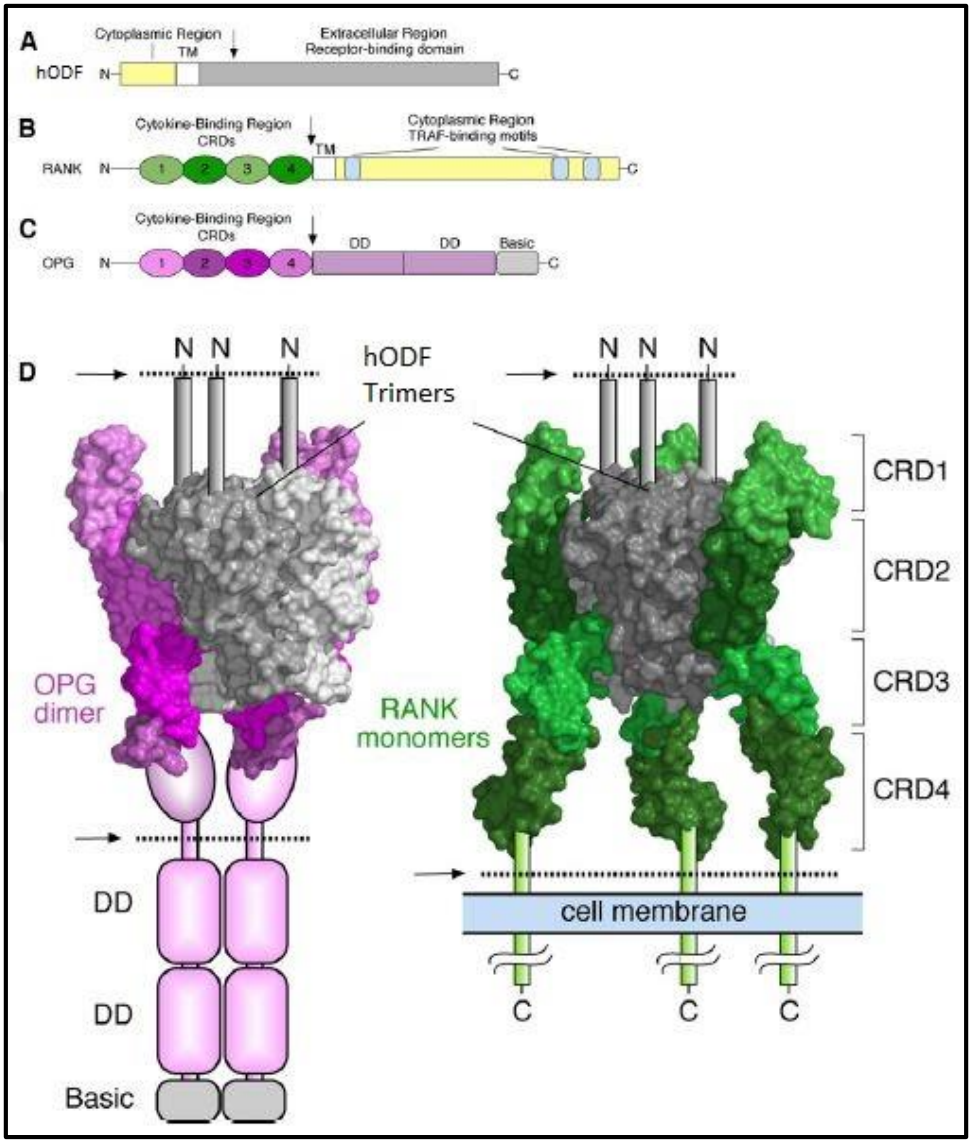


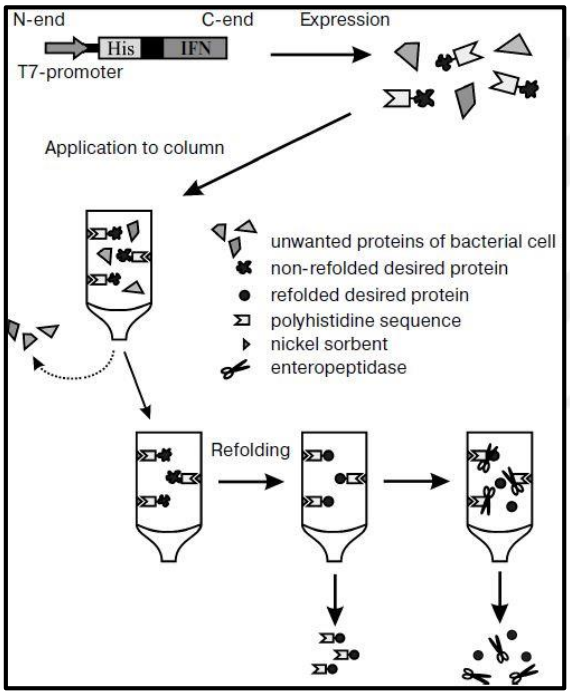
Figure 5. hODF, RANK, and OPG structure. A. hODF domain structure. The extracellular receptor-binding domain is located at the C-terminal region. B. RANK domain structure. In RANK, the cysteine rich domains (CRD) are followed by a transmembrane domain (TM) and a cytoplasmic tail containing TNFR-associated factor (TRAF)-binding motifs. C. OPG domain structure. In OPG, the CRDs are followed by two death domain-related regions (DD). D. OPG/hODF and RANK/hODF complexes. Arrows show where proteins were truncated for crystallographic studies. (adapted from Nelson et al., 2012)

2.3 Recombinant Protein Production and Purification

Recombinant proteins can be expressed in prokaryotic and eukaryotic cells. If the protein does not have any post-translational modifications, such as glycosylation, phosphorylation, and acylation, then bacteria are the best choice for protein production. As for recombinant production in yeast cells, since they are able to glycosylate to a certain extent, they could be used for recombinant production to a certain extent. They are cheaper to produce and maintain. Producing

recombinant protein using a mammalian system is expensive and arduous. Yet if the protein's activity requires extensive glycosylation, then a mammalian system would need to be used to produce it (Overton, 2014).

Protein purification contains a series of sophisticated steps intended to segregate a group of proteins or a single protein from the total protein and other macromolecules in the cell. Purification helps in characterizing the functions, structure, and interactions of the target protein. Protein size, physical-chemical properties, binding affinity, and biological activity are properties of proteins that could be used to purify protein.



Proteins could be purified in large quantities to use for further applications such as nutritional proteins, biopharmaceuticals, commercial enzymes. They also could be analytically purified, in small amounts, to study the structure, biological activity, post-translational modifications, and function. There are various purification methods which differ according to surface features, size and shape, net charge, and biological properties. Preliminary steps include extraction, precipitation and differential solubilization, and ultracentrifugation.

Figure 6. Affinity purification of histidine tagged proteins. Proteins can be purified using different fusion tags. A hexahistidine could be added to the N-terminal or C-terminal of the target protein. Nickel-bound beads have an affinity to the histidine residues which results in capture of the fusion protein by the immobilized glutathione. Then, the column is washed to clear any unwanted proteins. Finally, the fusion protein is eluted by using high concentrations of imidazole, which is a derivative of histidine yet has a higher affinity to the beads. Therefore, when washed with imidazole the fusion protein gets eluted. Also, when a proteolytic site is added in between the hexahistidine tag and the protein of interest, this can be used to cleave the tag and release the protein from the beads (Hikita et al., 2005; Kosobokova, Skrypnik, & Kosorukov, 2016).

One of these methods is purification using histidine fusion tags. An example of the steps for obtaining a histidine tagged protein is given on Figure 6. The histidine residues serve as an affinity tag which has an affinity to chelating metal ions that have been immobilized on a carrier such as

nitrilotriacetic acid (NTA). Some fusion tags allow the restoration of the protein conformation and activity directly on the column (Kosobokova et al., 2016) and therefore could be used if necessary.

3. MATERIALS and METHODS

3.1 Type of Research

The type of research carried out in this study is experimental.

3.2 Time and Place of Research

Experimental procedures were carried out at Diril Lab at İzmir Biomedicine and Genome Center between June 2018-April 2019.

3.3 Working Material

3.3.1 Primers

We designed primers (Table 1) using CLC Main Work Bench.

Table 1. Primers. For cloning hODF into pET28a(+), pGEX6P1, pcDNA, colony screening. Red colored sequences are restriction sites; blue colored sequences represent the extra bases required by the restriction enzymes.

Primer Name	Sequence	Purpose of Use
KD_51	ATGGAGAAAGCGATGGTGGATGGC	143-317 a.a hODF,Amplif.,FWD
KD_53	ATAA**CTCGAG**ATCTATATCTCGAACTTTAAAAGCCCC	143-317 a.a hODF,Cloning,REV, XhoI Overhang
KD_96	TGGGAACCAGATGGGATGTCGGTGGCA	Human ODFExon4 Rev.Primer_Overlapping Exon5
KD_97	**CCATCTG**GTTCCCATAAAGTGAGTCTG	Human ODF Exon5 Forw.Pri. Overlapping Exon4
KD_138	AGCGGATAACAATTTCACACAGG	M13/pUC Forward Sequencing Primer in LacZ gene
KD_153	GGGCTGGCAAGCCACGTTTGGTG	pGEX6P1 colony PCR screening, FWD
KD_154	CCGGGAGCTGCATGTGTCAGAGG	pGEX6P1 colony PCR screening, REV
KD_217	GATC**CTCGAG**GAGAAAGCGATGGTGGATG	Cloning of Human ODF (143-317 aa) into PGEX-6-P1 + XhoI restriction site added
KD_218	GATC**CATATG**GAGAAAGCGATGGTGGATG	Human ODF Forw.Pri_Cloning to pET28a+ NdeI
KD_219	GATC**CTCGAG**TCAATCTATATCTCGAACTTTA	Human ODF Rev.Pri_Cloning to pET28a (and pGEX6P1) + XhoI
KD_344	GATC**CTCGAG**GCCACCATGGAAACCCCA	Cloning 6His tagged huODF into pcDNA3, XhoI overhang
KD_345	TGTGATGATGATGATGATGTCCGGTGGTATCTGG	Cloning 6His tagged huODF into pcDNA3, fusion
KD_346	CCAGATACCACCGGACATCATCATCATCATCACA	Cloning 6His tagged huODF into pcDNA3, fusion

3.3.2 Chemicals

All chemicals used in this study were analytical grade purity and they were purchased from Merck, Sigma, and Gibco.

3.3.3 Enzymes and Kits

The enzymes and kits (Table 2) used in this study are as below.

Table 2. Enzymes and Kits Table

Enzyme/Kit	**Vendor**	**Catalogue #**
FastAP Thermosensitive Alkaline Phosphatase	Thermo Scientific	EF0654
Phusion High-Fidelity Polymerase	NEB	M0530S
Taq DNA Polymerase	Ampliqon	A111103
Pierce Universal Nuclease for cell lysis	Thermo Scientific	88700
FastDigest Restriction Enzymes 1. ScaI 2. XhoI 3. EcoRV 4. HindIII 5. NdeI	Thermo Scientific	1. FD0434 2. FD0694 3. FD0303 4. ER0501 5. FD0583
CutSmart Restriction Enzyme BamHI HF	NEB	R3136S
Pierce HRV3C protease solution kit	Thermo Scientific	88946
Lysozyme	Sigma	L6876-1G
T4 Ligase	Thermo Scientific	EL0014
GeneJet PCR Purification	Thermo Scientific	K0701
GeneJet Plasmid MiniPrep Kit	Thermo Scientific	K0502

3.3.4 Commonly used software

The software and websites (Table 3) used in this study are as below.

Table 3. Software and Websites

Software	**Purpose of Use**	**Company/Web page**
Adobe Photoshop CS 8.0.1	Editing gel pictures	Adobe Systems Inc.
CLC Main Workbench 7	Manipulation of DNA sequences in silico	Qiagen
National Center for Biotechnology Information web site	Obtaining DNA, RNA, AA sequences, literature review	www.ncbi.nlm.nih.gov

Ensembl Genome Browser	Obtaining DNA, RNA, AA sequences	www.ensembl.org
UniProt	Protein characteristics	www.uniprot.org
RCSB Protein Databank	Protein 3D structure	www.rcsb.org
Image Lab 5.2.1	Gel imaging software	Bio-Rad

3.3.5 Special Instruments

The instruments (Table 4) used in this study are as below.

Table 4. Special Instruments

Instrument Name	Purpose of Use	Vendor
MicroCL_17R centrifuge	Table top high speed centrifuge for purifying by centrifugation	Thermo Fisher Scientific
SimpliAmp Thermal Cycler	PCR, amplifying DNA	Applied Biosystems
Miniprotean Tetra System	SDS PAGE	Bio-Rad
OWL EasyCast B1A	Electrophoresis	Thermo Fisher Scientific
MaxQ4000	Orbital shaker for bacteria incubation	Thermo Fisher Scientific
Centrifuge 5810R	To obtain bacterial pellet	Eppendorf
NanoDrop2000 Spectrophotometer	Measure purified DNA concentration	Thermo Fisher Scientific
Gel Doc™ XR+ System	Agarose and SDS PAGE Imaging	Bio-Rad
Avanti J-26XPI Rotor JA-25.50	Protein purification	Beckman Coulter

3.3.6 Commonly used buffers, solutions, and media

The commonly used buffers, solutions, and media (Table 5) in this study is as below.

Table 5. Used Buffers and Media

Buffer/Media	Components
LB medium, 1lt	10g tryptone, 5g yeast extract, 5gNaCl, 1ml 1N NaOH (autoclaved)
LB plates, 1lt	10g tryptone, 5g yeast extract, 5g NaCl, 15g agar (autoclave in a flask, cool down to 40-45°C, add antibiotics and pour into petri dishes)
50X TAE electrophoresis buffer, 1lt	242g Tris base in water, adding 57.1mL glacial acetic acid, and 100mL of 500mM EDTA (pH 8.0) solution

10X PBS	1.37M NaCl, 27mM KCl, 43mM Na2HPO4, 14mM NaH2PO4
SDS-PAGE running buffer	246mM Tris, 1.92M Glycine, 10%SDS
SDS-PAGE 4X Stacking gel buffer	0.4% SDS, 0.5M Tris-Cl pH 6.8
SDS-PAGE 4X Separating gel buffer	0.4% SDS, 1.5M Tris-Cl pH 8.8
6X SDS-PAGE sample buffer	375 mM Tris-HCl, 9% SDS, 50% glycerol, 9% betamercatoethanol and 0.03% bromophenol blue
6X SDS-PAGE sample buffer	0.05% bromophenol blue, 0.05% xylene cyanol,30% glycerol
Coomassie blue SDS-PAGE staining solution	0.1% (w/v) Coomassie 250G, 10% acetic acid, 25% methanol in ddH2O
SDS-PAGE destaining solution	10% acetic acid, 25% methanol in ddH2O
HOTSHOT DNA Alkaline Lysis Reagent	25 mM NaOH, 0.2 mM disodium EDTA and a pH of 12 is prepared by dissolving the salts in water without adjusting the pH.
HOTSHOT DNA Neutralizing Reagent	40 mM Tris-HCl and a pH of 5 is prepared by dissolving Tris-HCl (not Tris base) in water without adjusting the pH
GST binding buffer	50 mM TrisCl pH 8.0, 100mM NaCl, 0.5mM PMSF, 2 mM DTT

3.3.7 Bacterial Strains

All cloning experiments are done with DH5a or Top10 *E. coli* cells, which were used for cloning a desired gene/plasmid. BL21, a different strain of *E. coli* was used for protein production. Specifically, this strain can be used for induction of lac, tac, trc, it is protease deficient which makes this strain useful for bacterial protein expression. DH5a, Top10, BL21 strain *E. coli* cells were used for cloning and protein production purposes.

3.3.8 Mammalian cell lines

HEK293 (Human embryonic kidney) and HCT116 (Human colorectal carcinoma) cell genomic DNA were used as a template in PCR reactions.

3.3.9 Vectors

The vectors below are expression vectors specifically used for expressing high amounts of recombinant protein. We used three types in this study;

i) for expression and purification of histidine tagged proteins in *E. coli:* pET28a(+) (Figure 7) ii) for expression and purification of GST tagged fusion proteins in *E. coli:* pGEX6P1 (Figure 8) iii) for expression, secretion and purification of histidine tagged proteins in mammalian cells: pcDNA3.1 (Figure 9). Each vector has its own technique at purifying the target protein.

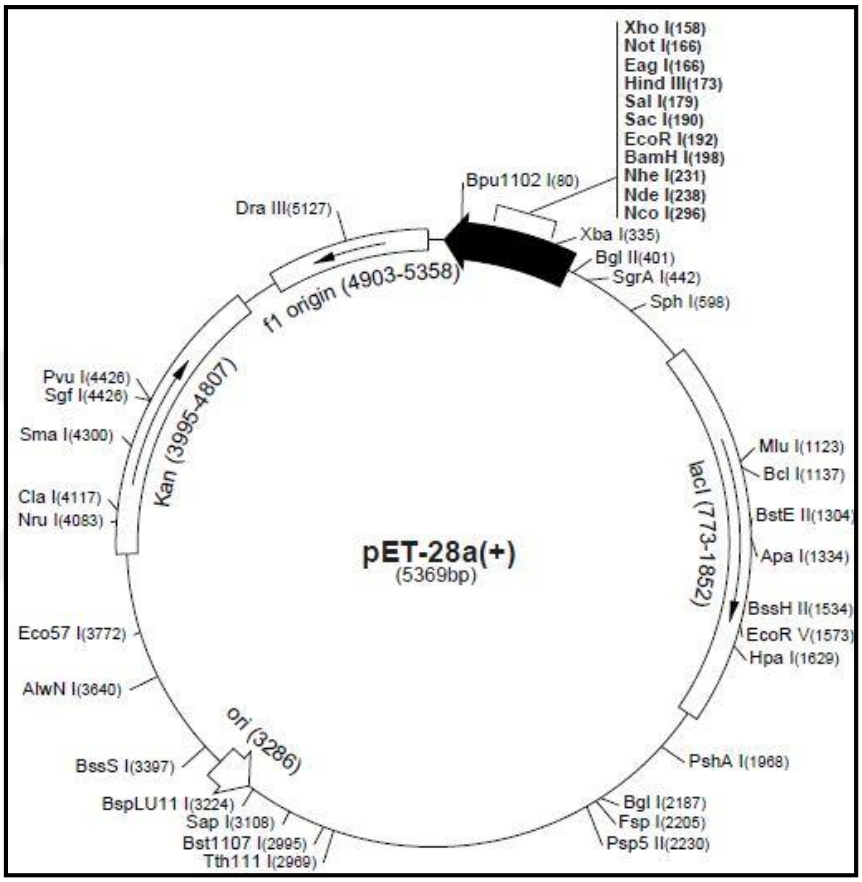


Figure 7. pET28a(+) vector map. This vector has a T7 promoter, hexahistidine tags at the N and C-terminal of the fusion protein which shows affinity to nickel beads. Within the fusion protein, the hexahistidine and the protein of interest should be in the same reading frame.

pET28 has a hexahistidine tag that has affinity against Ni-NTA beads. Imidazole is used for elution.

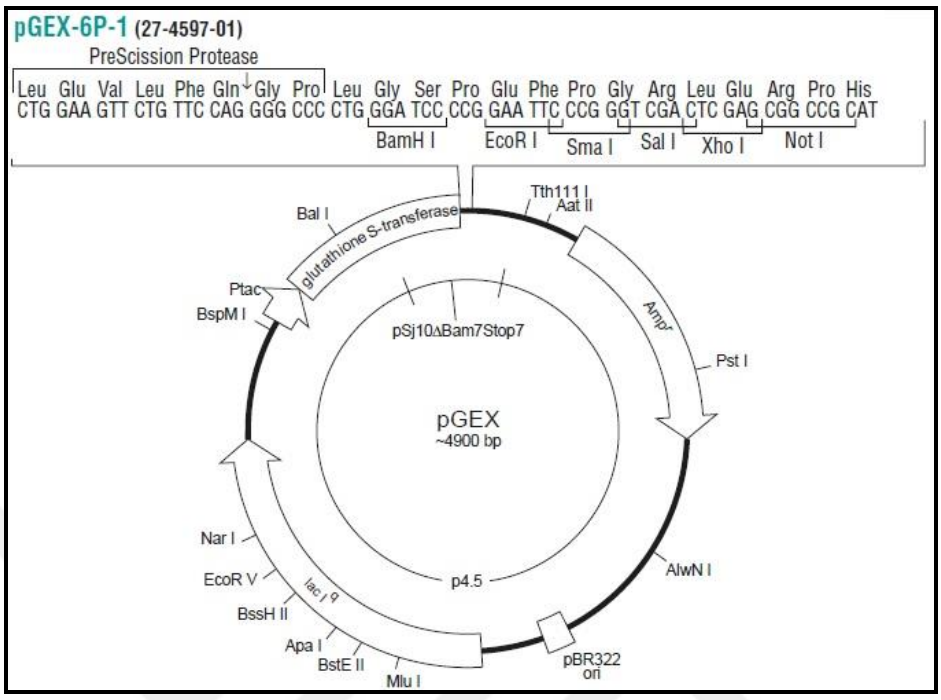


Figure 8. pGEX6P1 vector map. It is a pBR322 based expression vector. (Thermo Fisher Scientific, 2015) that contains a GST tag right before the multiple cloning site which has an affinity to Glutathione linked sepharose beads (Harper & Speicher, 2011) It could be directly eluted with the tag protein or the tag can be cleaved off using a protease called PreScission.

As for pGEX6P1 vector, it has a protease site called PreScission Protease which allows the protein to be cleaved and eluted without the GST tag.

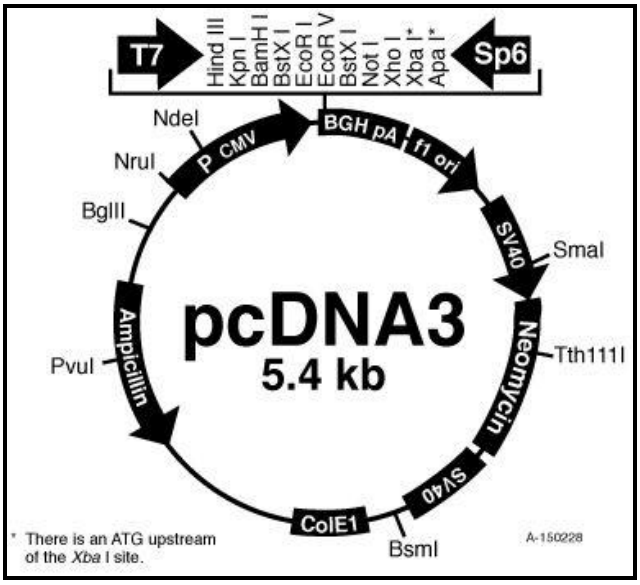


Figure 9. pcDNA3 vector map. This is a mammalian expression vector. A leader sequence preceding hODF was cloned in the same reading frame. This enables the protein to be translocated to the secretory pathway. This enables the fusion protein to be glycosylated and secreted.

For mammalian production of hODF, DNA sequences encoding a signal peptide (for secretion to the extracellular milieu) and a hexahistidine tag (for purification with Ni-NTA beads) need to be added to the 5' site of hODF gene in the same reading frame.

3.4 Research Variables

This study does not have any variables

3.5 Data Collection Tools/Methods

3.5.1 Chemical Competent BL21/Dha5a/TOP10 Bacterial Stock Preparation

The following reagents need to be prepared: 1L of LB, 1L of 0.1M $CaCl_2$, and 10 ml of filtered 10% Glycerol/0.1M $CaCl_2$. 1 uL of bacteria from previous competent cell batch should be taken and transferred into a microcentrifuge tube containing 1000uL of LB without antibiotics. It is then diluted using antibiotic-free LB 10^6 times in sterile microcentrifuge tubes. 100uL of the final dilution is plated on to an antibiotic-free LB agar plate and incubated at 37ºC overnight.

Next day, a single colony is picked up and inoculated in 7.5mL antibiotic-free LB media. It is incubated overnight at 37ºC/250rpm. Next day, two flasks containing pre-warmed 500 ml of antibiotic-free LB media is inoculated with 2.5ml of the mini-culture for each flask. After 1hr of incubation, the OD600 value is checked every 20 minutes. When the OD600 reaches 0.15-0.3 cultures are transferred to pre-chilled 500ml bottles. Make sure the bottles are kept on ice. Bottles are centrifuged at 2750g for 20 min.

The supernatant is discarded and each pellet is resuspended in 250ml of ice-cold 0.1M $CaCl_2$. After 1 h incubation on ice and cells are centrifuged at 2750g for 20 minutes. The supernatant is discarded and pellet is resuspended, gently, in 250mL of ice-cold 0.1M $CaCl_2$. Keep the resuspension on ice for 4 hours.

Afterwards centrifuge at 2750g for 20 minutes and the supernatant is discarded and each pellet is resuspend in 4mL ice-cold 10% glycerol + 0.1M CaCl2. Aliquots of 100uL were prepared and stored at - 80°C.

3.5.2 HOTSHOT Genomic DNA Purification

DNA was purified from HCT116 cell lines using the Hot Sodium Hydroxide and Tris (HOTSHOT) Method (Truett et al., 2000). The alkaline lysis reagent is pH of 12, 25 mM NaOH

and 0.2 mM disodium EDTA. The Neutralization reagent is of pH of 5, 40 mM Tris-HCl. ~25mg of cells/tissue are collected and 75 µL of the alkaline reagent are added to the samples and it is heated to 95°C for 10 min to 1 h. After heating, samples are cooled down to 4°C and 75 µL of neutralizing reagent is added to each sample. One to five microliters of the final preparation are used per each 10-µL PCR volume.

3.5.3 Molecular Cloning

For all molecular biology experiments, protocols derived from Sambrook and Russell (2001), Molecular Cloning, were used.

3.5.3.1 Polymerase Chain Reaction (PCR)

For cloning applications which require high fidelity PCR, Q5 High Fidelity Polymerase (NEB) was used. Taq polymerase (Fermentas) was used for all other applications. All primers were purchased from Sentegen and they ranged in size of 20-40 nucleotides. Annealing temperatures 5°C below the Tm of the primers have been used as a starting point and optimized when necessary. Template DNA was denatured at 98°C for 2-10 minutes prior to PCR cycles. Extension times ranged from 30 seconds to 4-5 minutes depending on the length of the DNA fragment to be amplified and the enzyme used. For instance, when amplifying with Taq polymerase, 1-minute extension time is required per 1000 nucleotides.

A Typical PCR Reaction Mixture		
Amount	**Component**	**Final Conc.**
1 ng–1 µg	template DNA	
0.25µl	forward primer 100µM	0.5 µM
0.25µl	reverse primer 100µM	0.5 µM
1µl	dNTP mix 10mM	200 µM
10µl	5X PCR buffer	1x
0.25µl	Q5 High-Fidelity polymerase 0.5 Unit	0.02 U/µl
50µl	ddH2O	

Temp Time Cycle
98°C 1 min Initial denaturation
98°C 10 sec Denaturation
60°C 30 sec Annealing
72°C 30sec-4 min Extension
72°C 2-5min Final extension
4°C ∞ Storage

Here in this study we first amplified exon 4 and 5 separately (table 6).

Table 6. PCR conditions and cycles for amplifying exon 4 and 5 fragments

Amplifying Exon 4/5	
Amount	**component**
10ul	HF Buffer

PCR Cycle	
°C	**t**

1ul	10mM dNTP	98°C	1min	
0.25+0.25ul	KD_51+KD_96	98°C	10sec	35x
	KD_53+KD_97	65°C exon 4 56°C exon 5	30sec	
0.5ul	template	72°C	30sec	
0.5ul	Phusion polymerase	72°C	2mins	
37.5ul	ddH20	10°C	∞	

After amplification of exon 4 and 5 in separate PCR reactions, we used a 2-stage PCR to fuse the exons and simultaneously introduce restriction sites at the ends of the fused PCR fragment (Table 7).

Table 7. 2-Stage PCR and Restriction Cloning

2-stage PCR reaction	
Amount	**Component**
10ul	5X HF Buffer
1ul	10mM dNTP
0.25+0.25ul	KD_218,KD_219
	KD_217,KD_219
0.5ul+0.5ul	Exon 4 and 5 PCR product
0.5ul	Phusion polymerase
37 ul	ddH20

PCR Cycle		
°C	**t**	
98°C	1min	
98°C	10sec	10x
53°C	30sec	
72°C	30sec	
98°C	10sec	30x
65°C	30sec	
72°C	30sec	
72°C	2mins	
10°C	∞	

3.5.3.2 Restriction Digest

Restriction digestions were done either for analysis (i.e. colony screen) or for cloning of DNA. All restriction enzymes and their reaction buffers were purchased from NEB and Thermo Scientific. Reaction mixtures were incubated at 37°C 2-16 hours.

A Typical Restriction Digest		
Plasmid DNA	**PCR Product**	**Component**
2 µl (up to 1 µg)	1 ng–1 µg	template DNA
2µl	2µl	10 X FD Buffer
1µl	1µl	FD Enzyme
15µl	17µl	ddH2O
20 µl	30 µl	Total

Next, pET28a and pGEX6P1 vectors were digested for 5 hours using the appropriate restriction enzyme, CIP treated for 1 hour, and purified. Afterwards, pET28a and KD218/219 PCR product

were ligated. In a separate reaction pGEX6P1 KD217/219 were ligated (Figure 12, 14). Ligation reaction was conducted at 22ºC for 15 minutes. Competent cells were transformed and spread on Petri dishes, and dishes were incubated at 37^{O}C overnight.

3.5.3.3 Ligation

For a typical ligation reaction, prior to ligation, vector and insert DNA were restriction digested overnight. To prevent self-ligation, ends of vector DNA were dephosphorylated using 5 units of alkaline phosphatase (NEB) for 15 minutes at 37°C. 100ng of vector DNA and 3X equimolar ratio of insert DNA were used. DNA was ligated at 16°C for 4 hours.

A typical ligation reaction	
Vector DNA (i.e. 5kb pcDNA)	100ng
Insert DNA (i.e. 1.5kb PCR product)	100ng
T4 DNA ligase(NEB)	200units
10X Ligation Buffer(NEB)	1µl
ddH2O	up to 10µl

3.5.3.4 Transformation of chemically competent E. coli

Prior to heat shock transformation, aliquots of competent cells were thawed on ice and mixed with a 2µl completed ligation reaction. After incubation on ice for 15 min, heat shock was applied by incubating the cells at 42°C water bath for 60 seconds. Cells were immediately placed on ice and spread on an ampicillin containing LB plate. As for plates with LB-Kanamycin, first, cells were incubated at 37°C gentle shaking in LB medium for 1 h to enhance recovery, then, spread on the LB kanamycin plate. LB plates were incubated at 37°C overnight, 16-18 hours, until colonies appeared.

3.5.3.5 Screening colonies for a positive clone using colony PCR

After overnight incubation of the LB plates at 37°C, the colonies that appeared on the plate were all transferred to 100 uL of LB with their respective antibiotic. These single colonies were incubated at 37°C for 2-3 hours by shaking at 250 rpm. Afterwards, 0.5 uL of each colony was used as a template for the colony PCR mentioned below (Table 8).

Table 8. A typical colony PCR screening condition and cycle

Colony PCR	
amount	**component**
2ul	10xBuffer

Cycle	
ºC	**t**
95^{o}C	1min

0.4ul	10mM dNTP
0.2+0.2ul	Primers
0.5ul	Template
0.05ul	Taq
37.5ul	ddH20

95°C	10sec	35x
50 °C	30sec	
72°C	1.5min	
72°C	3mins	
10°C	∞	

3.5.4 Expression of recombinant proteins

For bacterial expression of recombinant proteins, genes cloned into pET28a or pGEX6P1 vectors have been used. These vectors introduce an N-terminal hexahistidine or GST tag to the cloned DNA, respectively. All proteins were expressed in BL21 strain *E. coli* cells.

A 10ml LB medium, containing 100µg/ml ampicillin (pGEX) or 25µg/ml kanamycin (pET28), was inoculated with the respective glycerol stock of BL21 cells. Cells were grown 16 hours at 37°C with 200rpm shaking. Next day in the morning, 2x5 ml of this starter culture was used to inoculate a 2x250ml LB medium with antibiotics. Cells were grown further 1-2 hours until they reached an OD600 value of 0.6-0.8. Cells were induced to express the recombinant protein by addition of 100uM final concentration of IPTG to the culture medium. Protein expression was generally performed at 30°C for 4 hours at 160 rpm shaking speed. An aliquot of cells before and after the induction were taken for the analysis of protein extracts by SDS-PAGE, in order to check the inducibility of the target protein expression. When induction was over, bacterial cells were pelleted by spinning down at 5000rcf for >20 minutes at 4°C. Afterwards, freeze cells at -20ºC, overnight.

Pellet was resuspended in 8 ml of binding buffer (50 mM TrisCl pH 8.0, 100mM NaCl, 0.5mM PMSF, 2 mM DTT) by pipetting. Transfer into a 2x5ml microfuge tubes. Add lysozyme to 100ug/ml final concentration with the tip of a spatula. Bacterial resuspension was mixed and incubated further 20 minutes on ice for the digestion of the cell walls. Cells were lysed by sonicating 3-4 times (each 30 seconds) on ice with a SONOPLUS sonicator from Bandelin using 50% duty cycle and 60% power settings. A nuclease could be added during this step to break the viscosity of the extract; however, it is not essential. Add 10 Units of Pierce Universal Nuclease per tube, 1mM final concentration of $MgCl_2$, and Triton X-100 was added to a final concentration of 1% and cell extracts were incubated for 30 minutes at 4°C on a rotating wheel. Finally, cell lysate was cleared from debris by spinning down the resuspension at 25000rcf. 100uL aliquot of the cell

lysate and pellet were taken after this step and analyzed by SDS-PAGE in order to check the solubility of the protein.

3.5.5 Purification of recombinant proteins using Ni-NTA beads

In the case of histidine tagged proteins, cleared cell lysate (8ml) was transferred to a 15ml conical tube and 0.2-1ml of PBS washed 100 uL bed volume of Ni-NTA bead slurry was added. For fusion protein to bind to Ni-NTA beads add additional DTT to bring to 1mM, add to 5% glycerol, add to 250mM NaCl, add to 10mM imidazole. These additions will bring Triton-X concentration to 0.5%. For binding of the recombinant proteins to the affinity matrices, conical tubes with lysates were incubated 2 hours at 4°C on the rotating wheel. Afterwards, spin down 15 minutes at 700g at +4ºC. Beads were washed 3 times 10 minutes with wash buffer (25mM Imidazole, no Triton-X), centrifuged 2 minutes 700g at +4ºC, and supernatant was discarded.

Next, prepare the elution buffer (PBS, 250mM NaCl, 1mM DTT, 5% glycerol, 500mM Imidazole. Conduct four separate elutions, with the 1st Elution using 200uL elution buffer, 2nd elution using 400uL elution buffer, 3rd elution using 1M Imidazole, PBS, and 4th elution using 10mM EDTA, PBS.

After the washing steps, purity was analyzed by SDS-PAGE. Histidine tagged proteins were eluted by incubating the beads with 250-500mM imidazole, 60 min at 4°C on the rotating wheel. Also keep post elution beads after adding 200uL 2xSB.

3.5.6 Purification of Recombinant proteins using Glutathione Beads

Cleared cell lysate (8ml) was transferred to a 15ml conical tube and 0.2-1ml of PBS washed 100 uL bead volume of glutathione bead slurry was added. Add to additional 1mM EDTA. For binding of the recombinant proteins to the affinity matrices, conical tubes with lysates were incubated 2 hours at 4°C on the rotating wheel. Spin down 5 minutes at 1000rpm. Beads were washed 3 times 10 minutes with 10ml binding buffer + 1% Triton-X 100. Transfer the beads into a 1.5 ml microfuge tube. Wash once with binding buffer without triton. Wash once with 500 ul ddH2O. After this point the fusion protein could be eluted using reduced glutathione or the tag could be cleaved using a protease such as PreScission and HRV3C (Protease Human Rhino Virus 3C).

3.5.7 Protease Cleavage

A commonly used strategy for the purification of recombinant proteins is using a protease cleavable tag and releasing the protein of interest after affinity purification using the tag. Both pGEX6P1 and pET28a plasmids have specific protease cleavage sites for this purpose.

For protease cleavage of hODF from GST tag, HRV3C protease has been used. After affinity purification of the recombinant fusion protein (GST-hODF), beads are washed once with 1X HRV3C reaction buffer for buffer exchange. This wash buffer is removed by the help of a syringe and needle to avoid aspiration of beads. Next, 200 ul of HRV3C reaction buffer is added over the 100 ul bead volume of beads. A 10 ul aliquot of the bead slurry is taken to observe the protein on the beads prior to cleavage. 2 units of HRV3C protease is added to the bead slurry and beads are mixed well and incubated at 4-5 ºC overnight. (i.e. 16 hrs). Next morning, the supernatant that contains cleaved proteins is recovered and analyzed. Additional 50 ul reaction buffer was added and afterwards vortexed and recovered again. Cleavage products are combined in a single tube. Wash the beads with 500 ul ddH2O and add 100ul 1X SB to release all the protein that remained bound to the beads.

3.5.8 SDS-PAGE Analysis

SDS-PAGE (Polyacrylamide gel electrophoresis) is a widely used biochemical technique for separation and analysis of protein samples. A mini protean II gel casting apparatus (Bio-Rad) has been used for preparation of 8-12% gels according to the (Table 9) below.

Table 9. Preparation of SDS gels

Stacking gel		Separating Gel			
Percentage	4.5%	Percentage	8%	10%	12%
Acrylamide/bis-acrylamide	1.5ml	Acrylamide/bis-acrylamide	2.7ml	3.3ml	4.0ml
4X stacking gel buffer	2.5ml	4X separating gel buffer	2.5ml	2.5ml	2.5ml
10%(w/v) APS	50µl	10%(w/v) APS	50µl	50µl	50µl
ddH_2O	5.9ml	ddH_2O	4.8ml	4.2ml	3.5ml
TEMED	15µl	TEMED	5µl	5µl	5µl

Proteins samples in 1X SDS-PAGE sample buffer were denatured by heating at 95°C for 5-10 min. 10-40µl of the sample was loaded per well. Proteins were separated by running them at 150V until separating gel was reached, and further on at 200-250V for 1-1.5 h. For analysis, gels

were stained 20-30 min in Coomassie blue staining solution and destained 30 min to overnight in destaining solution.

3.5.9 Bradford Assay

The Bradford assay was done to measure the concentration of the protein in a sample. A standard curve needs to be prepared using known concentrations of BSA. First, Bradford dye is diluted 1:5 with ddH2O. 200ul of the diluted dye is added into microfuge tubes. 10ul of the standard or test sample is mixed with the dye. For the standard curve, a total of 0.5, 1, 1.5, 2, 3, 4, 5 ug of BSA is added into the tubes. Then the standard/sample is incubated 5-10 minutes at room temperature, and 200 ul is transferred into the wells of a 96-well plate. The absorbance is measured at 595nm.

4. RESULTS

Our workflow for this study has been summarized as below (Figure 10). After analyzing the human ODF DNA sequence, we designed primers for amplifying hODF exons. Our previous efforts in cloning the full length human ODF cDNA from a human cell line had failed as we could not find a suitable cell line that expresses it in high levels. Therefore, we decided to use the genomic DNA for cloning purposes. Fortunately, the entire extracellular domain of the hODF is coded by the exons 4 and 5 of the hODF gene, with additional 7 nucleotides from exon 3. Briefly, we amplified the exon 4 and 5 separately using genomic DNA isolated from HCT116 cell line. We then fused the two exons by PCR and ligated them into the expression vectors. Chemically competent bacterial cells were transformed with the ligation reactions and cells were spread on LB plates with antibiotics; ampicillin or kanamycin, depending on the plasmid used. We screened the antibiotic resistant colonies and verified the presence of the correct insert by different methods such as PCR, restriction digestion and Sanger sequencing. After identifying the right colony, we induced expression of the fusion protein. Finally, we purified and analyzed our protein of interest and optimized its expression in order to obtain higher expression levels and higher purity.

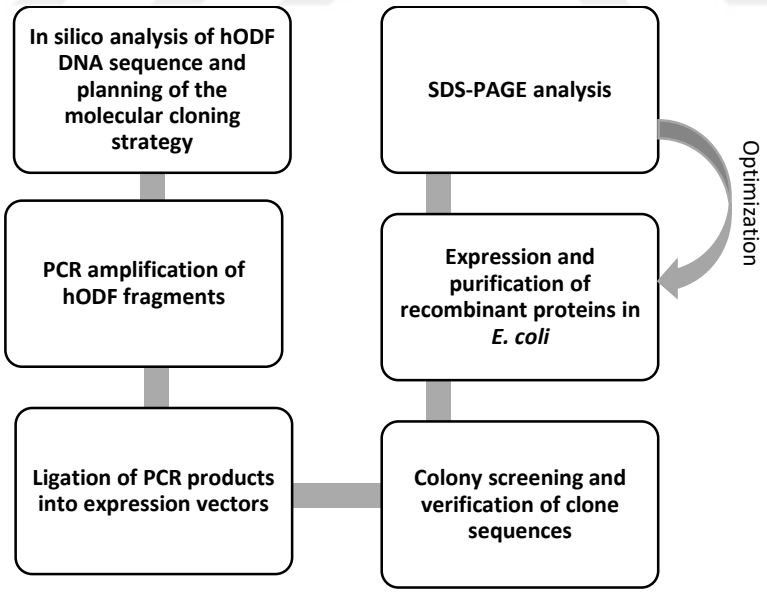


Figure 10. Workflow of recombinant production of hODF

4.1 PCR amplification of human ODF gene fragments

Exon 4 and exon 5 were separately amplified using KD51/KD96 and KD97/KD53 primer pairs (Table 7, Figure 11). KD 51 oligonucleotide primer adds additional 7 nucleotide overhang to the

exon 4 fragment. These are part of exon 3 and they were included on the upstream of primer KD 51, as they code for the first two amino acids of the extracellular domain (Figure 11).

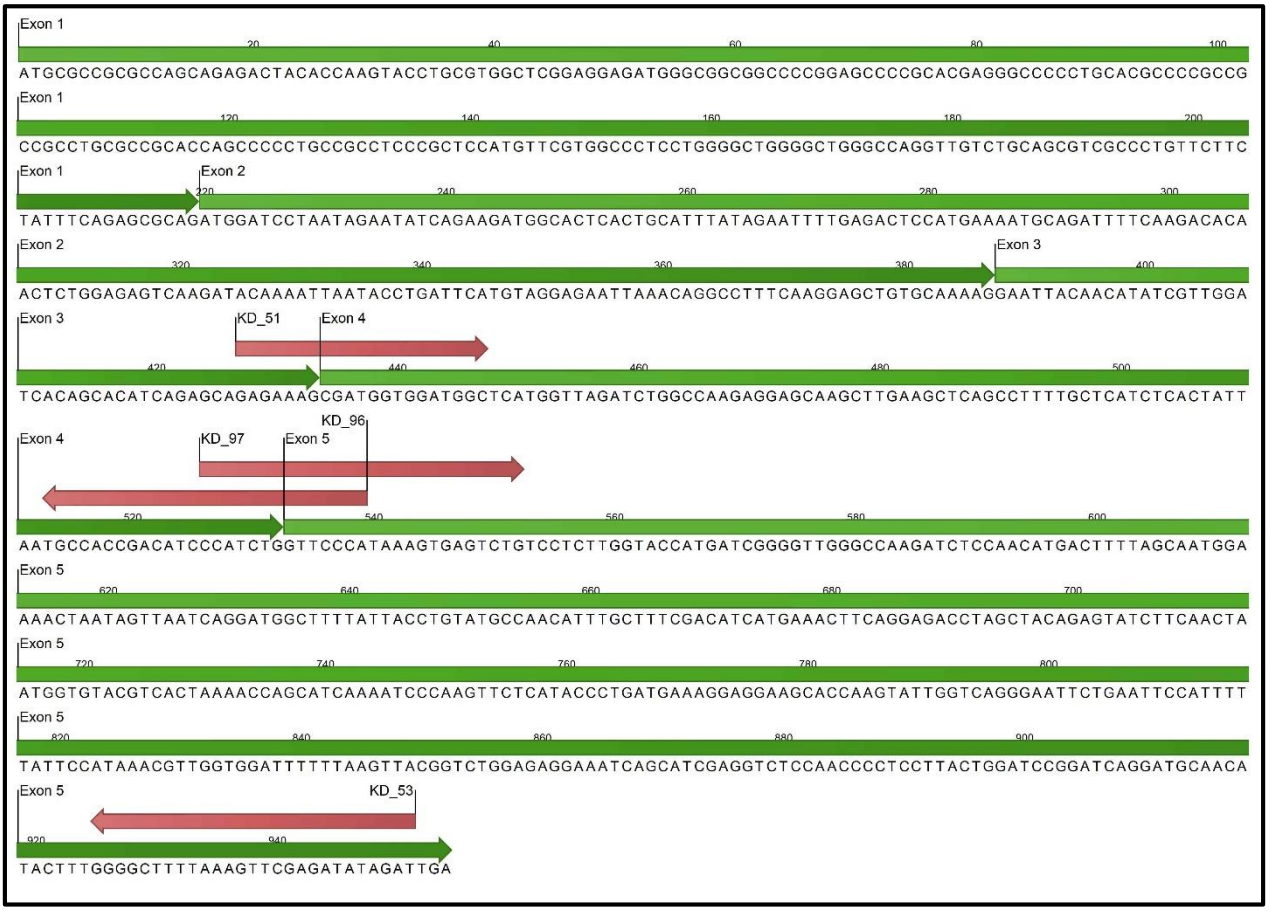


Figure 11. hODF cDNA. CLC MWB representations of Exon 4/5 PCR fragment and primers used to amplify exon 4 (KD51/96) and exon 5 (KD97/53) and fuse the PCR fragments using a 2 step PCR and outermost primers (KD51/53).

KD96 and KD97 include 5' overhangs that overlap with each other in order to fuse the two fragments in a subsequent fusion PCR reaction (Figure 11). After confirming the amplicon sizes by agarose gel electrophoresis (Figure 12) each exon fragment was purified using GeneJet PCR cleanup kit. Afterwards the concentrations were measured using Thermo Scientific™ NanoDrop 2000c.

4.2 Fusion of exon 4 and 5 fragments by PCR

Exon 4 and 5 PCR products have overlapping sequences introduced by KD_96 and KD_97 primers. A 2-Stage PCR program was used to fuse the two exon fragments through their overlapping sequences. During the first stage, the annealing temperature is calculated using the

overlapping sequences and primers are not added. As for the 2nd stage, addition of the outermost primers, KD217/219 (pGEX6P1) and KD218/219 (pET28a) amplify the fused exon4/5 PCR products and simultaneously introduce the restriction site overhangs. KD217/219 primers introduce XhoI overhangs at both ends while KD218/219 pair introduces 5' NdeI and 3' XhoI overhangs analyzed on an agarose gel (Figure 12).

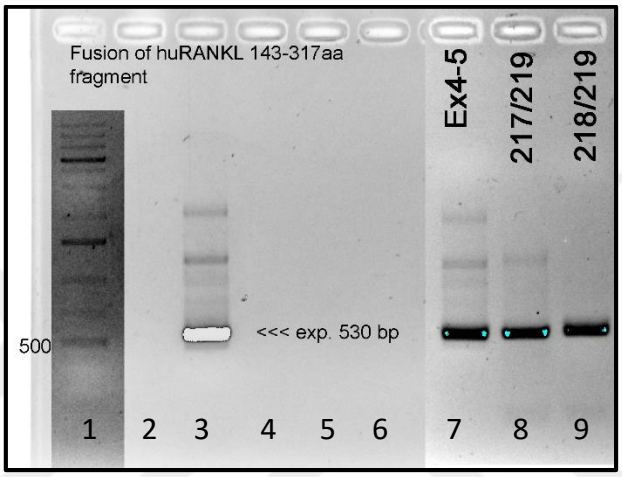


Figure 12. PCR fusion of hODF fragments. Lane 1 MWM, lane 3 fused exon 4 and 5, lane 7 positive control, lane 8 XhoI/XhoI restriction site added fragment, lane 9 XhoI/NdeI restriction site added fragment. Lanes 2, 4, 5, 6 are empty.

Restriction sites that will be used to clone into the plasmid are added together with the primers. The first 10x cycles are conducted at 53 ^{O}C annealing temperature so exon 4 and exon 5 fragments' overlapping parts can bind to each other and prime the synthesis of the full exon 4/5 fragment. Next, primer pairs from both ends are added and the amplification proceeds an additional 30 cycles (Figure 12, Table 8). By this strategy we fused exon 4 and 5 and simultaneously introduced restriction sites to the ends of the DNA fragments that are later used for restriction cloning purposes (Table 8).

4.3 Screening for positive colonies by colony PCR

Colonies were picked from the Petri dishes and were inoculated on a 96-well plate which contains 100uL LB-media containing 100 ug/ml ampicillin and incubated for 1-2 hours at 37^{O}C, 200rpm. Colony PCR is screening for the colony that contains the engineered plasmid. A sample of the colony serves as the DNA template. (Table 9) If there are many colonies and low efficiency of positive clones, colonies can be first "pooled" to ease screening. For preparation of pools we

mix 8 colonies/pool and 10uL/colony. If the pool contains a positive clone, it can be further screened on colony by colony basis. This saves time and reagents such as PCR components.

Here in this study we ligated hODF into three different expression vectors, pET28a, pGEX6P1, and pc3DNA-6His. Insert specific or vector backbone specific primers are used to screen the colonies.

4.3.1 Cloning of pET28a-hODF

pET28a is a widely used expression vector. Its hexahistidine affinity tag is short and therefore usually does not interfere with the expressed protein's conformation and activity. Yet, if one does not want any extra amino acids, the hexahistidine tag can be cleaved off using the thrombin cleavage sites. (Figure 13). Therefore, pET28a vector was our first choice for the production of the recombinant hODF protein. Cloning and screening primers for the pET28a-hODF are depicted below.

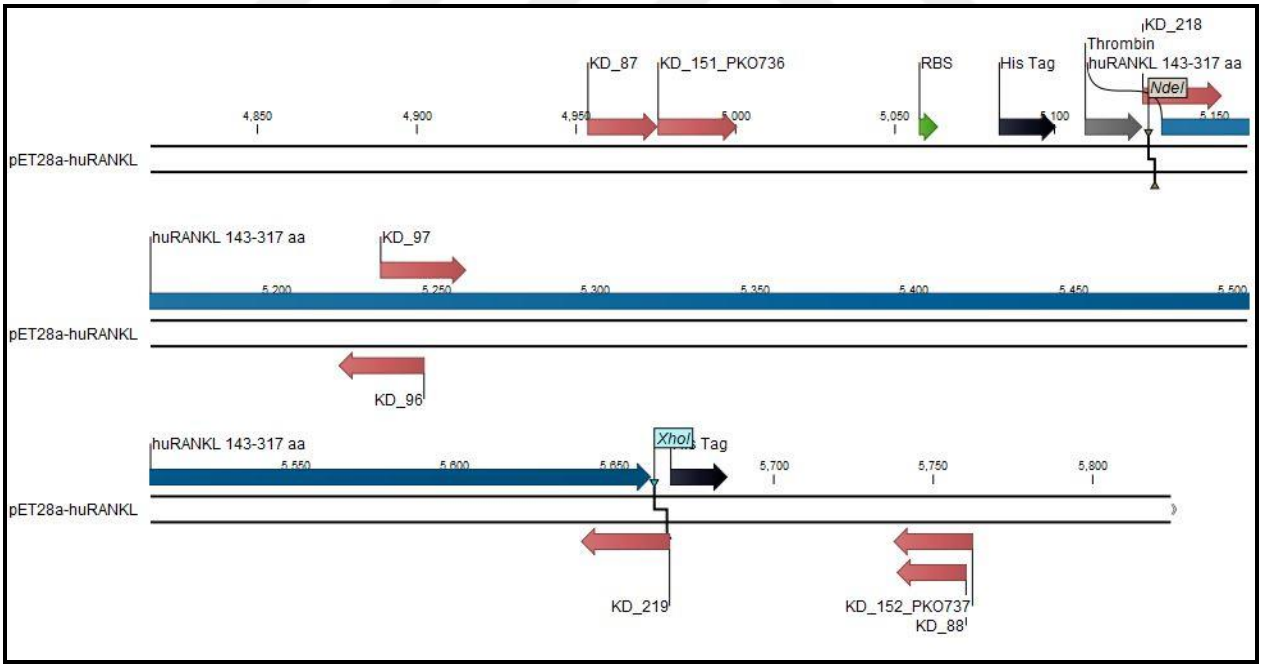


Figure 13. pET28a(+)-hODF plasmid map. Figure depicts the vector and insert specific primers that are used for molecular cloning and colony screening. The vector has N and C-terminal hexahistidine tags. It also contains a protease cleavage site after the N-terminal hexahistidine. We added restriction sites on the fragment using primer KD_218 for introducing NdeI and primer KD_219 for introducing XhoI.

pET28a(+)-hODF was screened (Figure 14) for positive colonies using primer pair KD151/KD152. The primers are vector specific primers and they check the insert size. As two different restrictions sites had been used during the ligation, the chances of reverse orientation cloning are minimal. Colony #4 was sent for Sanger sequencing after confirming by restriction analysis (Figure 20). A miniprep and glycerol stock was prepared with the positive colony.

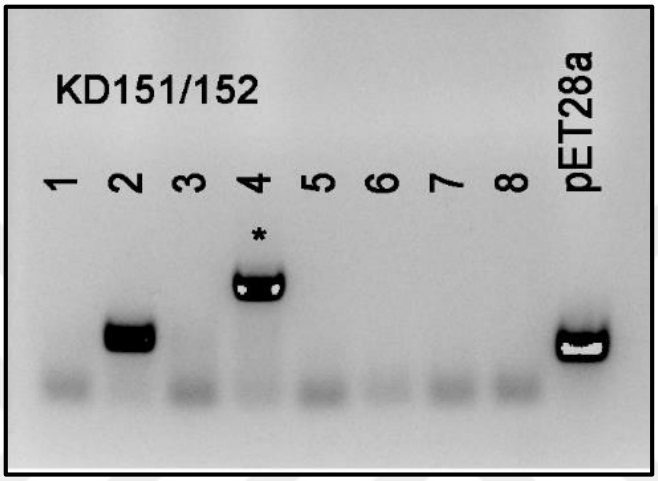


Figure 14. pET28a (+)-hODF Colony PCR. Screened with vector specific primer pair KD151/KD152. An empty pET28 vector is used as a negative control on lane 9. Colony #4 has the expected band size. Colony #2 has a shorter than expected insert, probably self-ligation of the vector.

4.3.2 Cloning of pGEX6P1-hODF

In case our efforts of expression and purification of histidine tagged hODF failed, we also simultaneously cloned hODF into the pGEX6P vector using the fused PCR product and the primers mentioned below (Figure 15)

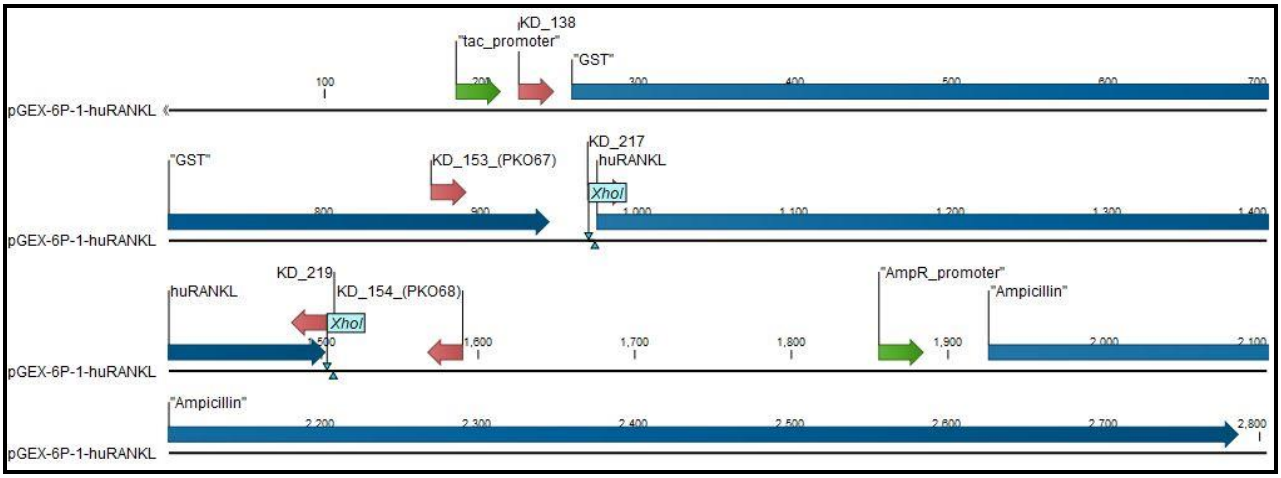


Figure 15. pGEX6P1-hODF plasmid map. We designed vector specific KD138/153/154 and insert specific KD219 primers for molecular cloning and colony screening. This vector also contains a 26kDa GST

tag. The multiple cloning sites is where hODF is cloned which proceeds the GST tag. hODF should be in the same reading frame of the GST protein. Since hODF was cloned into the vector using a single restriction site, XhoI, an additional screening which checks if the fused fragment was inserted in the correct orientation.

This cloning strategy utilizes the XhoI restriction site on both ends of the cloned DNA fragment. Therefore, self-ligation and recircularization of the plasmid DNA is more likely despite the CIP treatment. Since we had many colonies with most turning out to be negative clones (data not shown), we pooled them in groups of 8 and screened each pool (Figure 16). We further screened each colony in positive pools. (Figure 17)

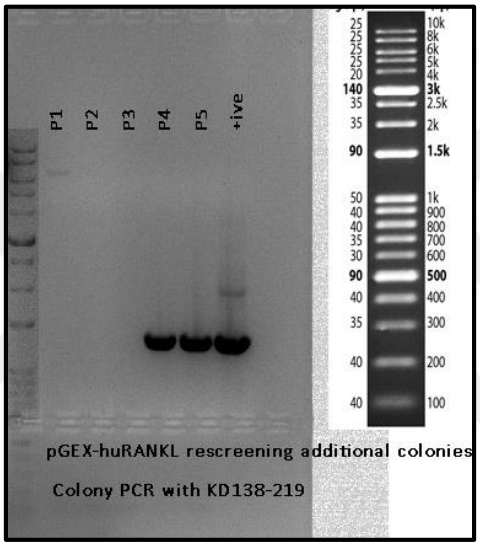


Figure 16. pGEX6P1-hODF colony PCR of pools. KD138-219 vector/insert specific primers were used for screening. Pools 1-3 showed no amplification, pools 4 and 5 were found to be positive and therefore screened further. Positive control refers to a past clone that was verified before however contained a point mutation and therefore not used further for expression. It serves as a positive control of size and PCR reaction.

Pool 4 and 5 turned out to include positive clones. We further screened individual colonies from pool 4 and 5 with vector/vector specific primers, KD153 and KD154, (Figure 17). These primers check the insert size. As for verifying the insert orientation, vector/insert specific primers, KD138 and 219 primer pair was used(Figure 15 and 17).

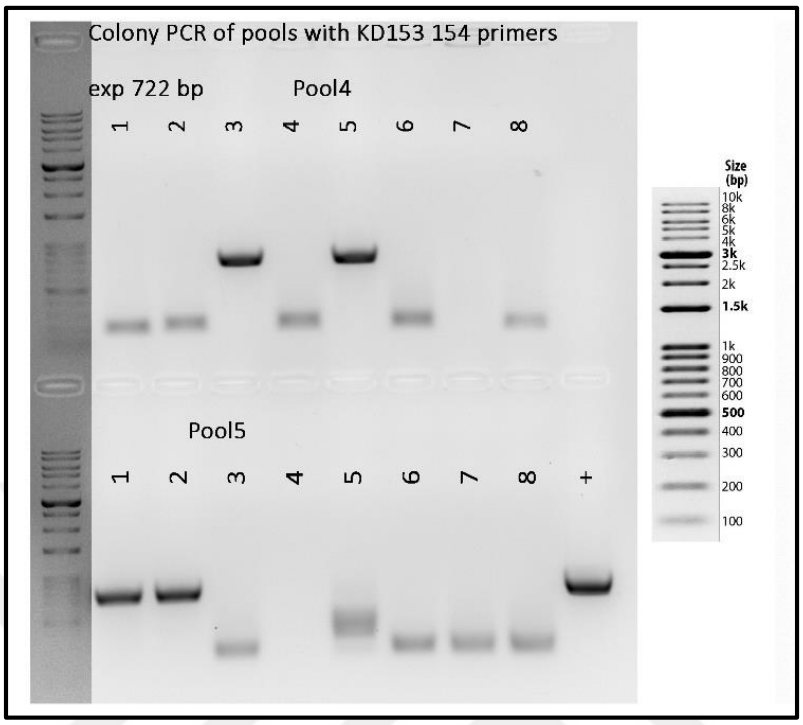


Figure 17. pGEX6P1-hODF colony PCR of pools 4-5 using KD153-154 primers. Individual colonies from pools 4 and 5 were screened Colonies 4.3, 4.5, 5.1 and 5.2 had the correct insert size and were further screened. Other colonies had no insert or had incorrect insert size (5.5) and were therefore not chosen.

Colonies 4.3, 4.5, 5.1, and 5.2 are positive and are to be screened further with insert/vector specific primers (Figure 18).

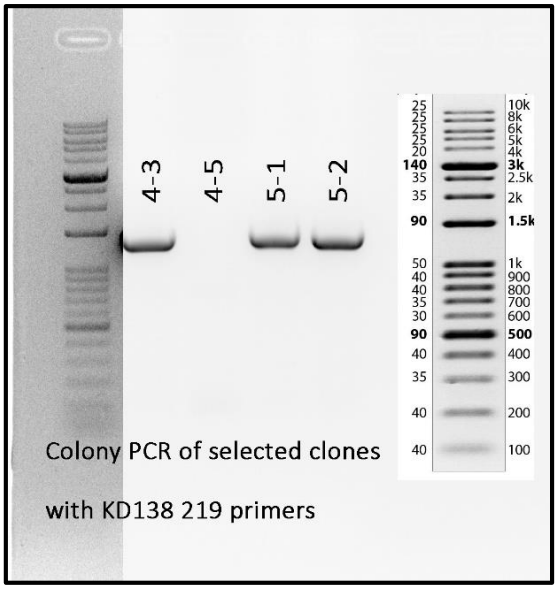


Figure 18. Colony PCR of Colony# 4.3, 4.5, 5.1, and 5.2 with KD138-219 vector/insert specific primers. Presence of the correct insert in the correct orientation results in an expected band size of ~1300 bp. Colonies 4.3, 5.1, 5.2 were found to be positive.

Upon screening the above mentioned colonies, 4.5 did not show an amplification. This might be due to the probability of the insert ligating in the opposite orientation. Therefore, we chose one of the positive clones, clone 5.2, and prepared a mini-prep. We further verified the clone via restriction digest (Figure 20) and Sanger sequencing (Figure 23).

After verification of the sequence, we re-transformed BL21 bacterial cells and prepared BL21 glycerol stocks to be used for subsequent protein expression. BL21 cells are preferred for protein expression as they lack proteases in their cytoplasm which make them useful for intact protein purification.

4.3.3 Cloning of the pcDNA3.1-hODF

The extracellular domain of hODF contains two glycosylation sites. A bacterial system is limited in glycosylating. Therefore, the hODF it produces, most likely, would not be glycosylated. This could dramatically reduce hODF activity. In order to introduce glycosylation sites, a mammalian expression system would be necessary to use. Therefore, as a backup strategy, we cloned the hODF gene into a mammalian expression vector, pcDNA3.1.

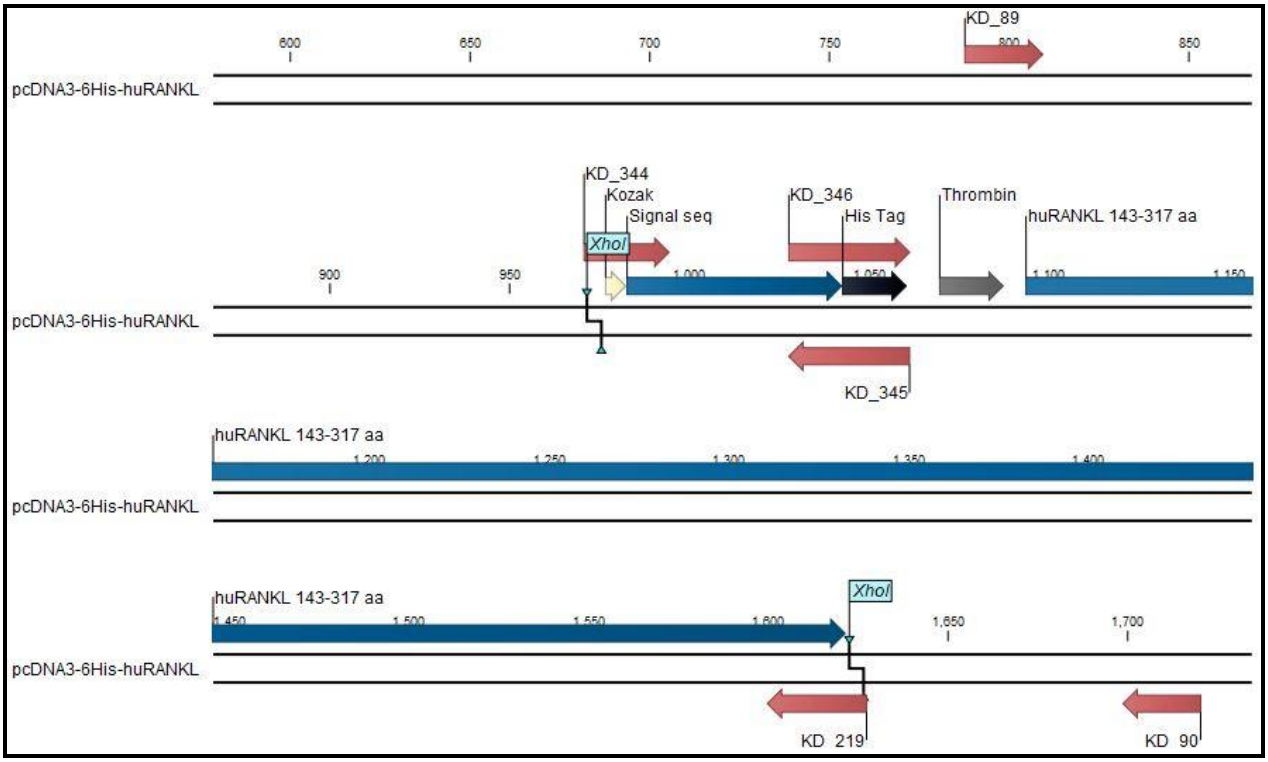


Figure 19. pcDNA3-hexahistidine-hODF plasmid map. This vector contains a Kozak sequence for the translation to start. A signal sequence was cloned into the vector to signal the polypeptide to be transported through the secretory pathway and a N-terminal hexahistidine tag was cloned to purify the fusion protein. Colonies are screened using primers KD219/KD89, which if amplified, it means the insert is inserted in the correct orientation. The vector is also screened with KD219/90 which if amplified, it shows that the insert has been inserted in the reverse orientation. For both reactions 51ºC was used as their annealing temperature.

The designed pcDNA3-6His-hODF plasmid (Figure 19), contains a leader sequence (ETPAQLLFLLLLWLPDTTG) from human immunoglobulin light chain gene for secretion into the extracellular medium. A hexahistidine tag comes after this sequence for purification. The leader peptide coding DNA segment was amplified from an antibody expression construct (pUC57-AEC) utilizing primers KD_344 and KD_345 and hexahistidine-hODF fragment amplified from pET28a-hODF construct using primers KD_346 and KD_219 (Figure 20A).

Afterwards these fragments were fused using 2-stage PCR (Table **7**). The first stage allows the fragments to fuse. The PCR parameters include 5 cycles, 56 ºC annealing, and 30 second extension. The second stage allows the fused fragments to be amplified using the outermost primer pair. The PCR parameters for the second stage are 35 cycles, 66ºC annealing temperature, 30 seconds of extension, KD344/219 primer pairs, 1 ul of the leader sequence, and 1 ul of the 6His-hODF fragment (Figure 20B).

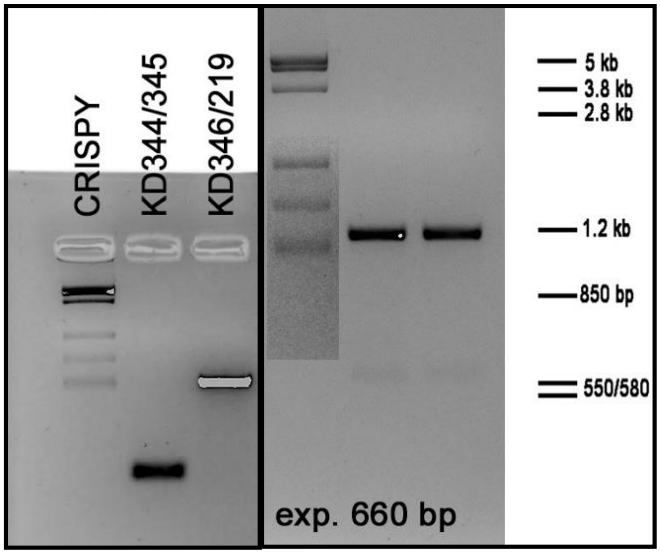


Figure 20. A. (Left) PCR amplification of hODF for pcDNA cloning. CRISPY is a custom made DNA ladder. KD344/345 and KD346/219 primer pair amplified DNA fragments. **B. (Right) Restriction digestion and fusion of pcDNA3 plasmid and 6His-hODF fragment.** Both the vector and fragment were digested using XhoI.

Below, upon ligation of hODF into pcDNA3.1 we screened the colonies with gene specific primers. As the results indicate, colony 10 and 11 seem to have an insert. Therefore, both colonies were screened using insert specific primers. Colony 11 has the insert in the correct orientation. Therefore, we prepared a miniprep and glycerol stock using colony 11 (Figure 21).

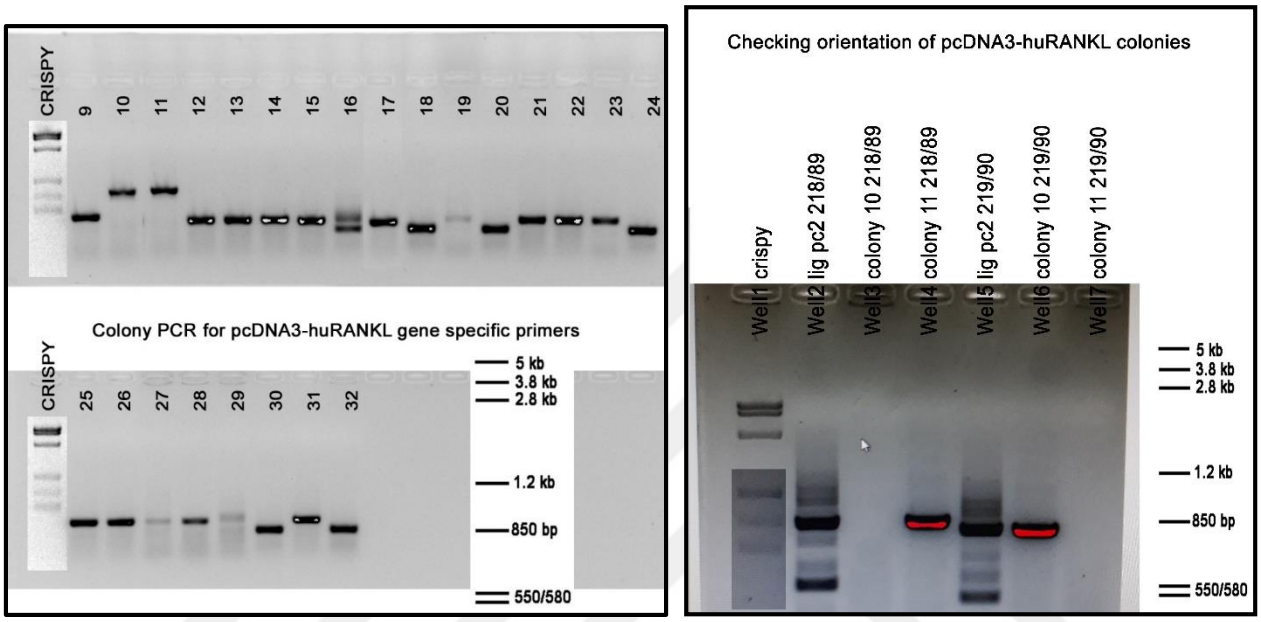


Figure 21. pcDNA3-hexahistidine-hODF Colony PCR. CRISPY is a custom made DNA marker whose band sizes are visible on bottom right insert. **A.** (Left) Colonies 10 and 11 were positive **B.** (Left) Positive colonies were further screened with KD 219/89 primer pair at 51ºC for if the insert is in the correct orientation and screened with primer pair KD 219/90 at 51ºC for if the insert is in the reverse orientation.

Due to time constraints, pcDNA3.1-hODF plasmid was not used further in this thesis study.

4.4 Restriction Analysis of Vectors

After screening the colonies with either vector specific or vector/insert specific primers, to further confirm the plasmid clone's identity, a restriction analysis was done (Figure 22).

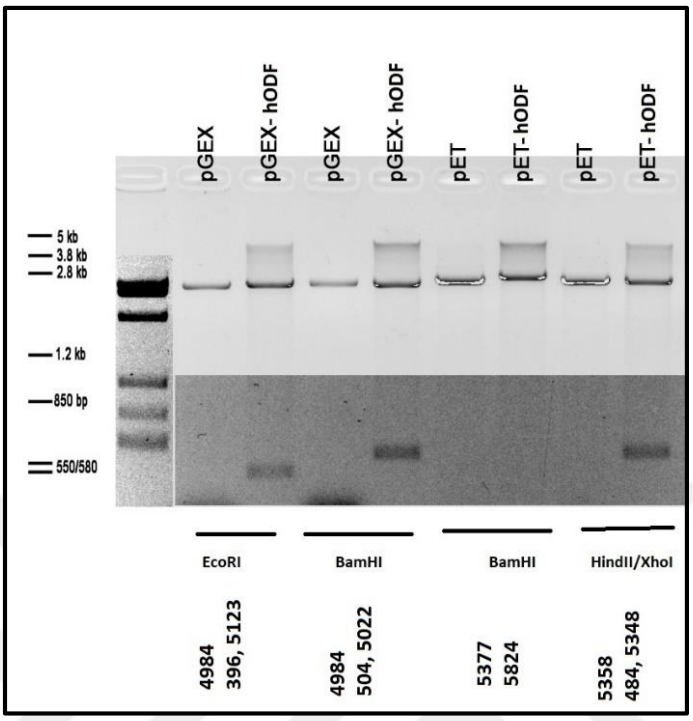


Figure 22. A. Restriction analysis of pET28a-hODF and pGEX6P1-hODF. pGEX empty vector and hODF cloned vector is restriction digested using EcoRI, BamHI. Also pET28a empty and hODF cloned vectors are digested using BamHI, HindIII/XhoI. Expected band sizes are annotated on the image.

The restriction patterns are as above. They confirm the vector identity of pGEX6P1, pET28a empty vectors and also the vectors with the hODF insert.

Although restriction analysis confirms their identity, to be sure of any point mutations we sent the plasmids for Sanger sequencing (Figure 23). Also due to time constraints, we decided to discontinue using the mammalian expression vector, pcDNA3.1 and continue only with pET28a and pGEX6P1 vectors. The induction control of the 6His fusion protein results (Figure 25) showed that significant amount hexahistidine-hODF protein was produced. Unfortunately, other proteins were purified as well. This reduces the purity and therefore requires and additional method to completely purify the fusion protein, such as size exclusion chromatography. To evade this extra step we decided to continue with the pGEX6P1 vector.

4.5 Verification of pGEX6P1-hODF Sequence by Sanger Sequencing

The Sanger sequencing showed that the sequenced sample and our reference sequence had 100% similarity. Therefore, we decided to continue with this vector and start protein expression with *E. coli*.

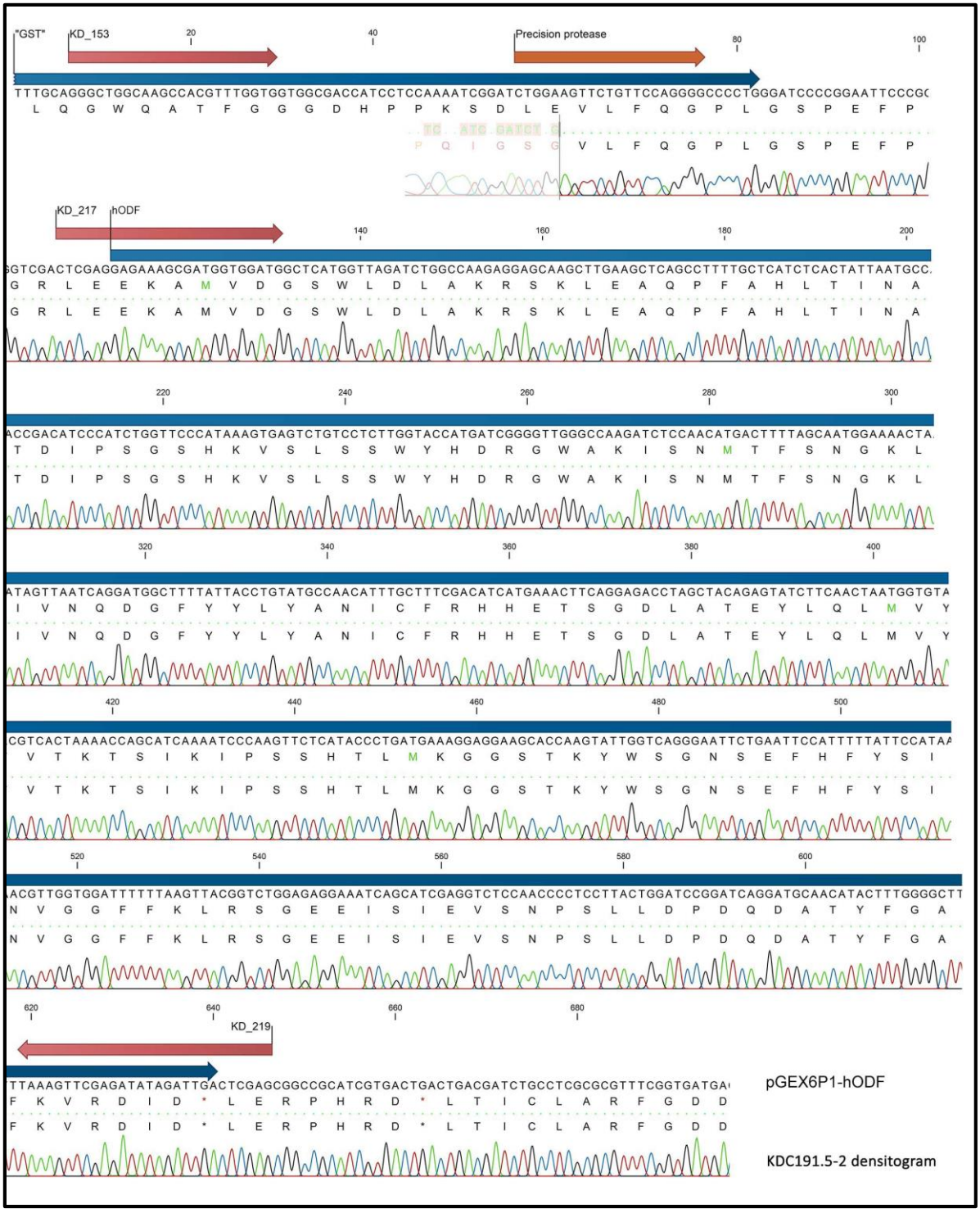


Figure 23. Confirmation of the pGEX6P1-hODF sequence. KDC191.5-2, pGEX6P1-hODF, clone was Sanger sequenced using primer KD_153. The results showed 100% match with our reference sequence. Therefore, this clone was selected for further protein expression studies.

4.6 Induction of Expression

To produce the recombinant proteins in higher purity and yield, BL21 strain *E. coli* cells were used. These cells are ideal for protein expression as they lack some of the proteases and don't express the recombinant protein prior to IPTG induction. Plasmid clones for pET28a-hODF and pGEX6P1-hODF were used to transform BL21 cells and glycerol stocks were prepared for long term storage and usage.

Glycerol stocks were used to inoculate a starter overnight culture. Next morning, starter cultures were used to inoculate the higher volumes of LB-AMP media for protein expression. Cells were grown until OD reached 0.6-0.8 density. Protein expression was induced with 1mM IPTG and checked via SDS PAGE analysis (Figure 24). The Coomassie Blue stained gel shows that we were able to induce expression of GST (a), GST-hODF (b) and 6His-hODF (c) proteins.

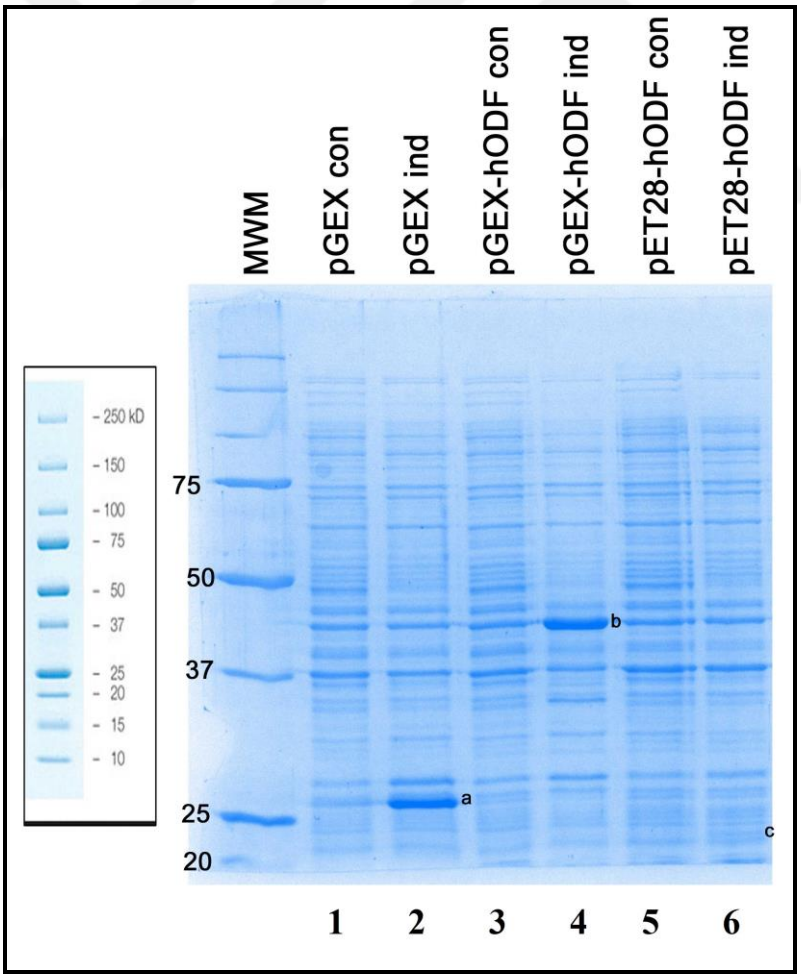


Figure 24. Coomassie Blue staining of GST-hODF and hexahistidine-hODF induction. 10 ul of bacterial culture was loaded per well. 15% separating gel was used. 1st and 2nd lane were loaded with cells

carrying empty pGEX6P1 vector, uninduced control and induced. 3rd and 4th lanes have pGEX6P1-hODF uninduced control and induced. 5th and 6th lanes pET28a hODF uninduced control and induced cells. Lane 2 shows induction of GST expression with empty vector (a). Lane 4 show the induction of GST-hODF fusion protein (b). Lane 6 shows the induction of hexahistidine-hODF fusion protein (c). a is the 26 kDa GST protein, b is the 46 kDa GST-hODF fusion protein, and c is the ~20kDa hexahistidine-hODF fusion protein.

Our next step would be to either directly elute the fusion protein or cleave the protein from the beads. Since hexahistidine tag is a small moiety, we considered that it has minimal effects on protein conformation, solubility, and activity. And therefore we decided not to cleave the fusion protein. As for the large GST tag protein, since it could have an effect on the protein conformation and activity, we decided to cleave off hODF.

4.7 pET28a-hODF Protein Purification and Quantification

The gel's wells were loaded as below:

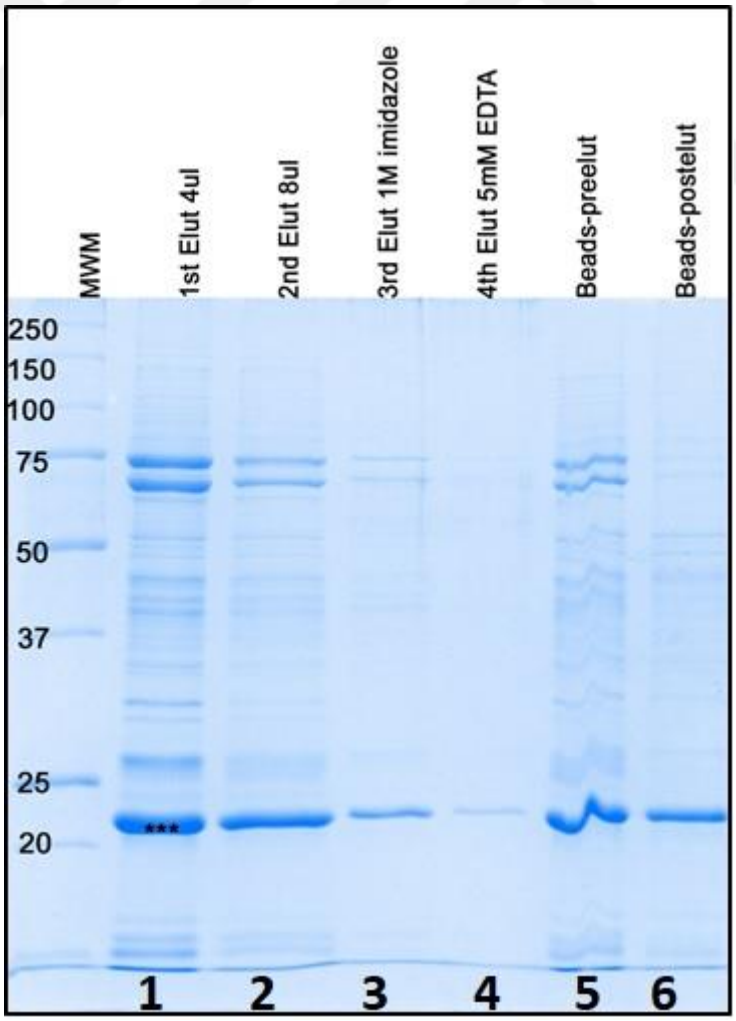


Figure 25. SDS PAGE Analysis of 6His-hODF expression and purification. 6His-hODF expression and purification with different elution conditions. For the 1st elution 4uL/200uL, 2nd elution 8uL/400uL elution buffer, 3rd elution 1M imidazole, 4th elution 5mM EDTA was used. 5th and 6th lanes show the amount of protein before and after elution from beads.

When comparing beads pre-elution and post elution one can see that trace amounts of precipitated protein present on the beads. Analysis of the 1st lane and 2nd lanes show that a significant quantity of 6His-hODF recombinant protein was purified under these conditions. Yet other proteins such as Hsc-70 chaperone protein and other bacterial proteins have also been co-purified; this lowers purity.

We purified the 6His tag fusion protein using Ni-NTA bead slurry. The beads have an affinity to histidine residues. Imidazole a derivative of histidine has a higher affinity to the bead slurry therefore it binds to the beads eluting the 6His fusion protein.

Protein quantification can be done using Bradford assay. Yet, there are multiple bands on both lanes which make it impossible to quantify protein using Bradford assay. Therefore, we conducted a BSA assay to compare different amounts of BSA to the band thickness. The SDS gel indicated that the purified hODF band is similar to 500ng-1ug BSA which makes the concentration 125ng/ul-250ng/ul (Figure 26).

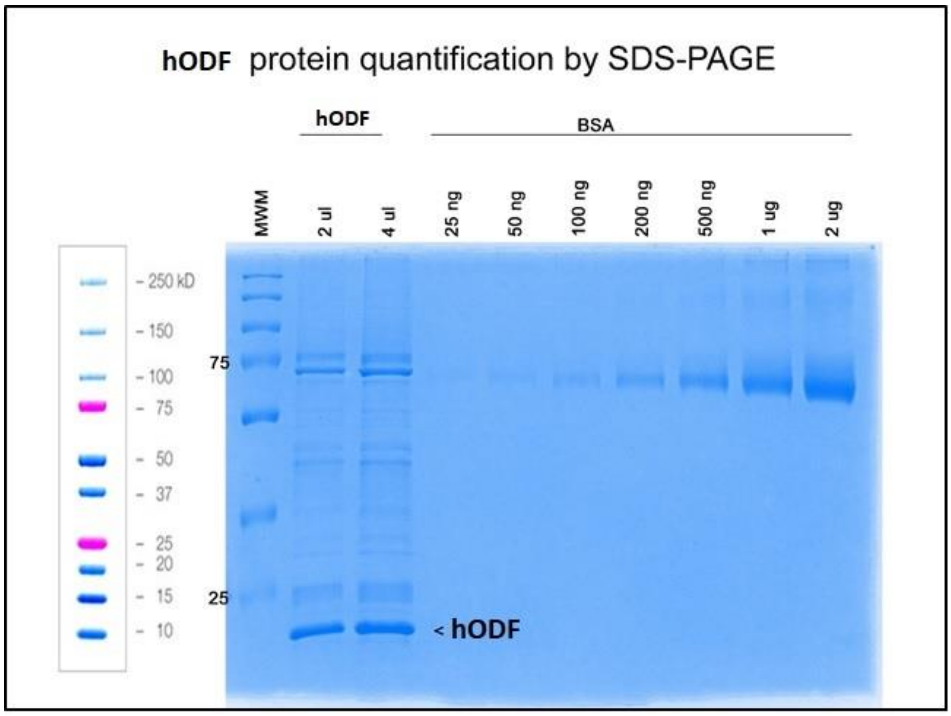


Figure 26. Protein quantification by SDS PAGE. A gradient of BSA is loaded on the gel to compare it with the samples. The 2uL and 4uL samples show almost the same amount on the 1ug band indicating that the eluted amount of protein is about 1ug.

The amount of hODF could not be determined with Bradford assay due to the extra bands present. Therefore, we used a BSA assay and approximated hODF protein amounts at 500-1000ng per 4 uL, which makes the concentration 125-250 ng/uL.

4.8 GST-hODF Cleavage and Purification

Apart from histidine tag purification, instead of eluting the protein, we cleaved off hODF with a protease known as PreScission. GST is a 26kDa protein. This brings up the possibility that GST could affect hODF's conformation. Therefore, cleavage is preferred instead of eluting a fusion protein (Figure 27).

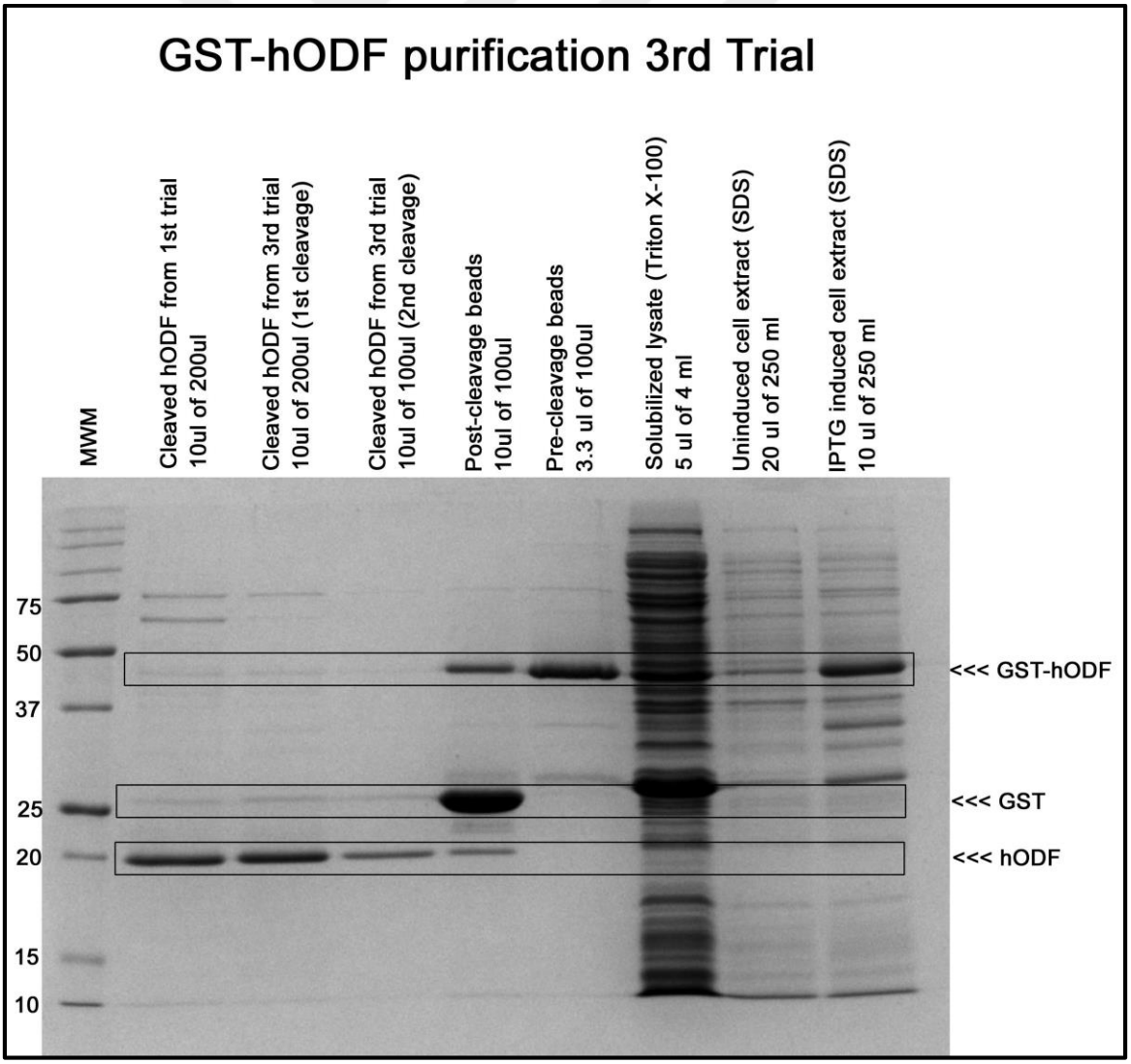


Figure 27. GST-hODF purification SDS PAGE Analysis. 15% Coomassie blue stained gel lanes have been annotated in the image.

The last two lanes show that the induction was successful due to the thick band which is at 46kDa (26kDA GST and 20 kDa hODF). We also checked for protein on beads before cleavage and after cleavage. As expected before cleavage the beads have a 46kDa band which is our fusion protein. On the beads after cleavage, the 46kDa band suggests that there is some fusion protein left on the beads. After cleavage we obtained a 20kDa band which is the size of hODF protein. We have also done a second cleavage to see if more hODF can be eluted. The band on the second cleavage shows that only an additional small quantity of hODF can be obtained after the second cleavage reaction. We repeated the purification and cleavage protocol several times to optimize the expression level and purity of the product. Our last trial, where we sonicated the bacterial cells longer to increase cell lysis, yielded a higher amount of purified protein (Figure 28).

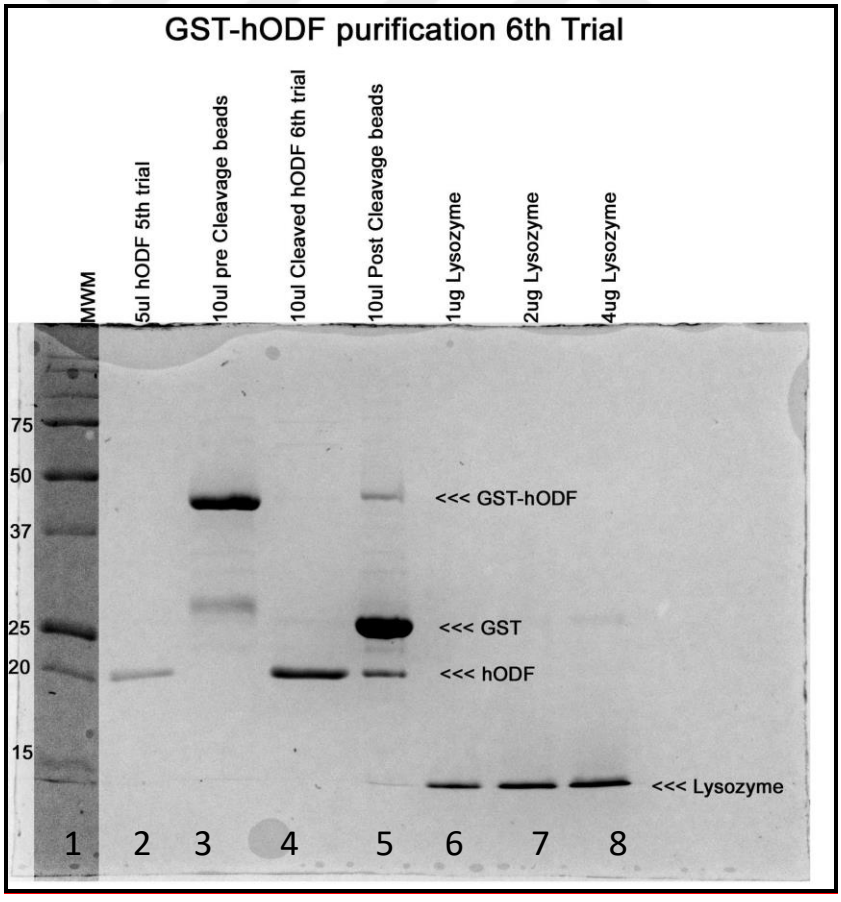


Figure 28. GST-hODF Purification 6th trial. Lane 6-8 show different amounts of lysozyme to visually compare with hODF and estimate the amount purified protein. The various protein products have been demarcated by arrows.

The 3rd lane shows the GST-hODF bound to the beads. On the 4th lane, the prominent band shows the cleavage to be successful, whereas the 5th lane shows that minute amounts of the fusion protein and cleaved hODF is still left but the prominent band shows the GST protein left on the beads after cleavage. Variable amounts of lysozyme proteins were used to visually compare the various protein products.

5. DISCUSSION

In this study, to be able to study osteoclastogenesis, we aimed to produce hODF recombinant protein and use it in downstream applications such as RAW264.7 osteoclastogenesis assay (Nguyen & Nohe, 2017) and in vitro binding assays.

We initially amplified the relevant DNA fragments via PCR using genomic DNA obtained from HEK293 cells and used them in molecular cloning experiments. Yet, Sanger sequencing of various cloned vectors showed that HEK293 cells carry a missense mutation in their genome that results in an amino acid substitution (these data were not shown). As it was not possible to know whether the mutated hODF will be fully active once it was produced recombinantly and purified by affinity chromatography techniques, we decided to repeat the cloning experiments using genomic DNA derived from another human cell line. We used genomic DNA obtained from HCT116 (*Homo sapiens* colorectal carcinoma) cells and repeated the cloning experiment. All of the insert DNA derived from HCT116 cells turned out to be 100% correct and therefore they were used in subsequent experiments.

We amplified a region containing 7 nucleotides from exon 3, full sequences of exon 4, and exon 5 which constitute the extracellular domain of hODF. We fused these fragments and cloned them into three separate vectors pET28a, pGEX6P1, and pcDNA3.1 each using different expression or purification strategies. Since hODF contains two glycosylation sites, at amino acid position 171 and 198, a mammalian expression system needs to be used. Therefore, we used a mammalian expression vector, pcDNA3.1, and cloned a leader peptide and 6His-hODF into it. Although, this was the ideal option for recombinant hODF production, due to time and reagent constraints, within the context of this thesis, we decided to discontinue using pcDNA3.1/mammalian expression system. Current research shows that biologically active hODF could be successfully produced using a bacterial expression system (Lu et al., 2012). Therefore, we decided to go on with pET28a and pGEX6P1 and discontinued using the pCDNA3-6His-hODF.

Simultaneously, we cloned hODF into these two vectors. We first checked whether the recombinant protein could be produced by the bacterial cells by conducting an induction assay. This was done to see how efficient our fusion protein could be induced. Both GST and 6His tagged hODF expression was detectable in bacterial lysates, however the former was in much higher quantities. We continued with the two different affinity purification strategies utilizing Ni-NTA and glutathione conjugated beads. Our results showed that a high amount of hODF protein could be purified using the pET28a-hODF vector/Ni-NTA affinity chromatography. Yet, other proteins beside our fusion protein were also co-purified. This reduces the overall purity therefore, we discontinued using this vector.

Afterwards, we decided to continue with the pGEX6P1-hODF vector/glutathione affinity chromatography. GST is a 26kDa moiety which could affect the conformation and activity of hODF protein. To avoid this, instead of eluting the whole fusion protein we decided to cleave off hODF using an HRV3C (same as PreScission Protease) proteolytic cleavage site, This, eventually leaves GST on the beads and pulls down hODF. Our results showed that our 20kDa hODF protein was successfully cleaved and eluted. The purified hODF protein was in higher purity compared to our trials using the pET28a vector system. We could obtain up to 100ug purified recombinant hODF per liter of bacterial culture.

Within the scope of this thesis study, we were not able to perform any in vivo assays such as RAW264 osteoclastogenesis assay or in vitro binding assays such as ELISA or SPR. The follow up work will involve testing the activity of the purified hODF via such assays. If the activity levels are not found satisfactory, the pcDNA3.1-6His-hODF will be used for purification in mammalian expression system. For this purpose, the vector needs to be transfected into the suspension culture adapted CHO-DG44 cells and transfected cells need to be selected in culture medium containing G418. pcDNA3.1 vector includes a neomycin gene that confers resistance to the G418. The transfected cells will be gaining resistance to G418 and they will secrete 6His-hODF fragment to the extracellular medium as they have a leader peptide sequence prior to 6His-hODF amino acid sequence. The recombinant protein will be glycosylated in the secretory pathway of the CHO cells and can be purified using Ni-NTA beads from the media.

Another widely used expression vector in *E. coli*, as an alternative to pET28a and pGEX6P, utilizes a maltose binding domain tag system (pMAL) . Using this method, upon IPTG induction a

soluble maltose binding protein (MBP)-fusion protein is produced, which can be subsequently purified with MBP Trap affinity chromatography and treated with Tobacco Etch Virus nuclear inclusion endopeptidase (TEV protease) to remove the MBP fusion protein. Previous studies have shown that hODF can be efficiently purified using the combination of MBP system and TEV protease cleavage (Park et al., 2015). This method could have been selected for this study to produce recombinant hODF. However, we did not have the reagents for the above mentioned expression/purification system and therefore we sufficed with the available expression systems.

6. CONCLUSION AND RECOMMENDATIONS

Osteoclast differentiation factor enables macrophage lineage cells to differentiate to osteoclasts. Here in this study we set out to produce recombinant hODF consisting of only the extracellular domain using a bacterial expression system.

Also, tartrate-resistant acid phosphatase (TRAP) (Walsh & Choi, 2014) is a critical gene of osteoclast development and therefore, it could be used as biochemical marker of osteoclast differentiation. Yet, research also shows that macrophages and osteoclasts under a light microscope could be undistinguishable and that immunofluorescence staining for cathepsin K could be used to distinguish osteoclast cells from macrophage polykaryons (Nguyen & Nohe, 2017). The purified protein could also be characterized using ELISA and protein activity could be measured via Surface Plasma Resonance (SPR) (Zhang et al., 2009). Also other protein characterization methods could be used such as peptide mapping, x-ray crystallography to check if the protein is in its correct conformation.

Our results showed that although pET28a-hODF expression plasmid in conjunction with Ni-NTA affinity chromatography yielded more of our fusion protein, yet, the purity was low. As for pcDNA3.1-6His-hODF, it was an alternative mammalian expression system. Due to time constraints we were not able to pursue expression using this system in mammalian cells. The third expression system was pGEX6P1-hODF which contains a GST tag, a tag protein that has an affinity to glutathione. We were successful in expressing the GST-hODF fusion protein and also eluting by protease cleavage. From this study we learned that we are able to introduce hODF into BL21 cells and express hODF using a bacterial expression system with >70% purity.

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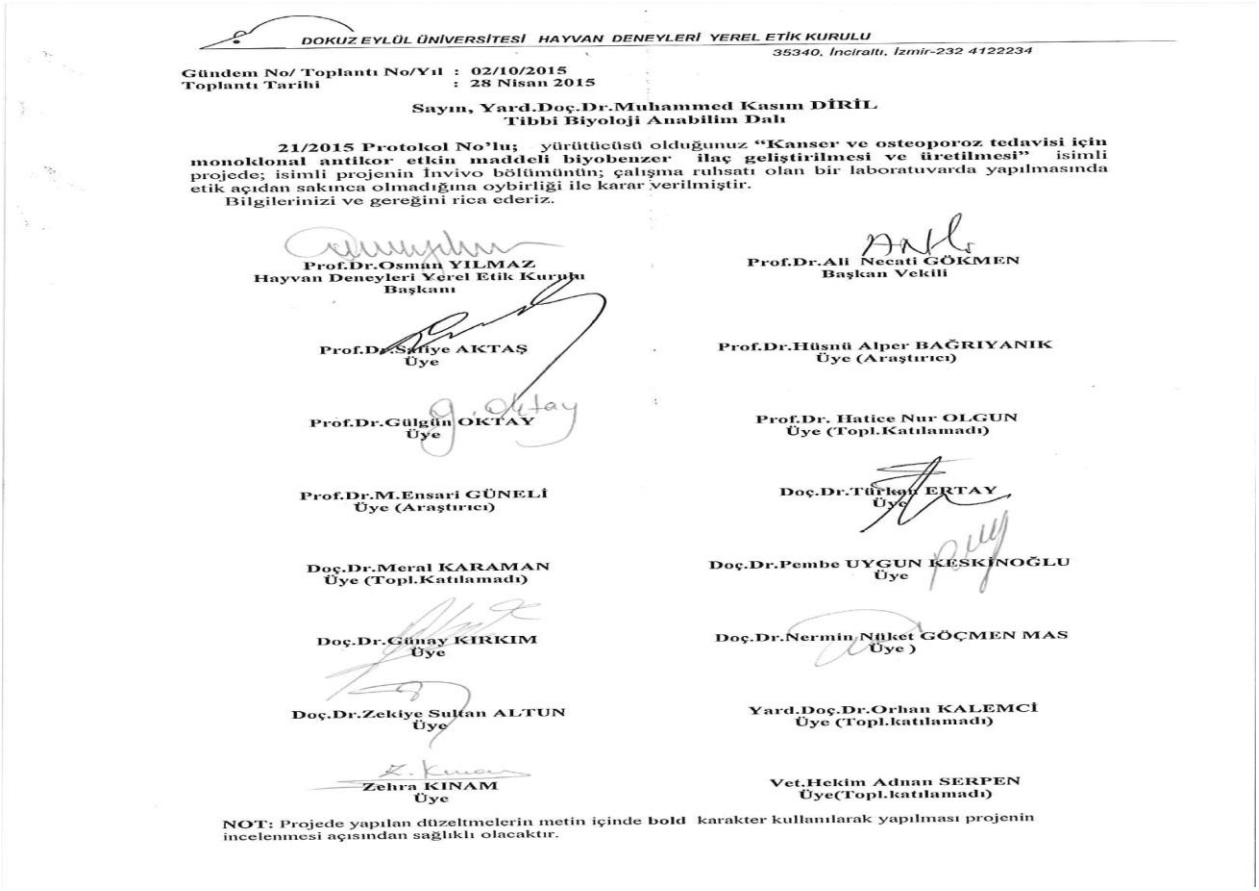
8. APPENDIX

8.1 Research Ethics Committee Approval



DOKUZ EYLÜL ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU

35340, İnciraltı, İzmir-232 4122234

Gündem No/ Toplantı No/Yıl : 02/10/2015
Toplantı Tarihi : 28 Nisan 2015

Sayın, Yard.Doç.Dr.Muhammed Kasım DİRİL
Tıbbi Biyoloji Anabilim Dalı

21/2015 Protokol No'lu; yürütücüsü olduğunuz **"Kanser ve osteoporoz tedavisi için monoklonal antikor etkin maddeli biyobenzer ilaç geliştirilmesi ve üretilmesi"** isimli projede; isimli projenin İnvivo bölümünün; çalışma ruhsatı olan bir laboratuvarda yapılmasında etik açıdan sakınca olmadığına oybirliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Prof.Dr.Osmun YILMAZ
Hayvan Deneyleri Yerel Etik Kurulu
Başkanı

Prof.Dr.Ali Necati GÖKMEN
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Zehra KINAM
Üye

Vet.Hekim Adnan SERPEN
Üye(Topl.katılamadı)

NOT: Projede yapılan düzeltmelerin metin içinde **bold** karakter kullanılarak yapılması projenin incelenmesi açısından sağlıklı olacaktır.

8.2 Curriculum Vitae

Muhammet Memon

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EDUCATION

Country Code	Unviersity	Faculty/Institute	Field	Degree	Year of Graduation
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TR	Ege University	Ege University	Biochemistry	BA	2009

RESEARCH FIELDS

Molecular Biology and Genetics, Recombinant Protein Production

ACADEMIC EXPERIENCES

Internship at CeBiTec in the fields of Proteomics and Metabolomics, Department of Proteome and Metabolome Research, Faculty of Biology, Bielefeld University, Bielefeld, Germany (2008)
Attended a 30-day practical course at the Genetic Engineering and Biotechnology Institute, Marmara Research Center, Gebze, Kocaeli, Turkey (2007)