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Point Mutations of the Putative Follitropin Receptor Caveolin Interaction Motif Do Not Disrupt Receptor Function

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ABSTRACT

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Human follicle stimulating hormone (hFSH) is a hormone found in humans that is synthesized and secreted by the anterior pituitary and targets the ovaries and testes. This specificity of targeting is accomplished by the interaction of hFSH with its receptor (hFSHR) on target cells. hFSHR is a G protein-coupled receptor that localizes to domains in the cell membrane known as lipid rafts. The mechanism of translocation of the hFSHR into lipid rafts is unknown. Our hypothesis is that translocation occurs through interaction of hFSHR with the protein caveolin via a specific sequence in the hFSHR; a putative caveolin interaction motif (CIM). The canonical caveolin interaction motif, FXFXXXXFXXF, where F is any aromatic amino acid such as phenylalanine (F), tyrosine (Y) or tryptophan (W) is found in the fourth transmembrane domain of hFSHR in amino acids 479-489 (FAFAAALFPIF). Here, the 4 critical phenylalanine residues match the motif. The goal of the current research has been to produce stable cell lines expressing isoforms of hFSHR with one of each of the four phenylalanines mutated to leucine. The mutant receptors were expressed in the cells and qualitatively demonstrated normal signaling when stimulated by hFSH. Future studies will focus on analyzing quantitative measurement of signaling and the location of the mutant receptor on the cell surface. Understanding the caveolin interaction motif of hFSHR could give us better understanding of the mechanism of hFSHR localization to lipid rafts and may give insight into a novel way to regulate FSH signaling and function.

Acknowledgements

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Introduction

Plasma Membrane

Every cell is constructed with a plasma membrane separating the interior of the cell from its surroundings. As shown in Figure 1, this membrane is a combination of lipids, proteins, and in the case of eukaryotes, sterols. [1] The most common lipids in the

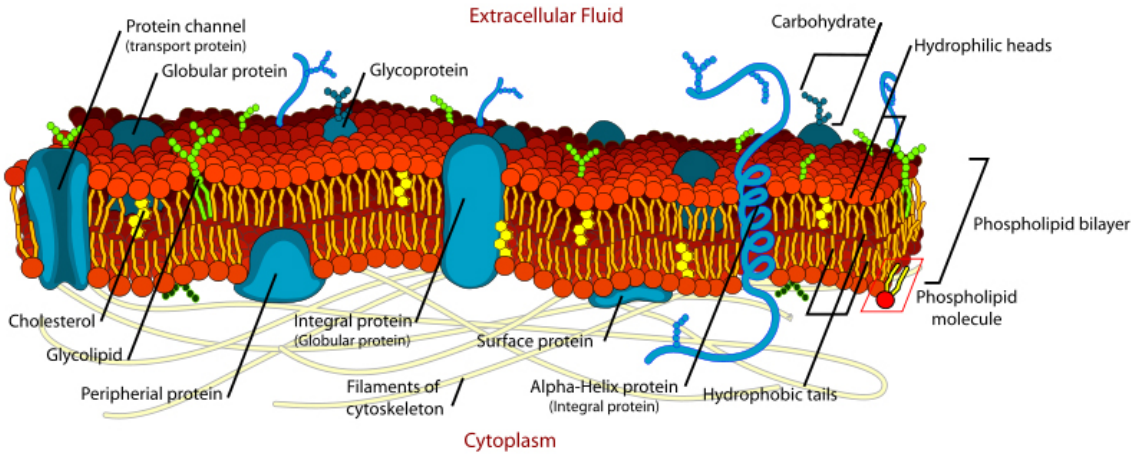


Figure 1. Singer-Nicolson Augmented model of the plasma membrane. [25]

membrane are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin. [2] These lipids all have cylindrical Van der Waals envelopes (Figure 2); when the lipids are in aqueous environments, the hydrophobic tails are pushed together forming a bilayer. This is known as the hydrophobic effect. [3] Because the middle of the plasma membrane is hydrophobic, the cell is impermeable to water-soluble molecules.

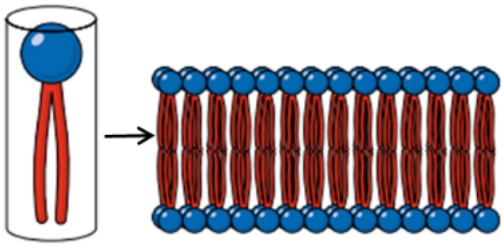


Figure 2. Cylindrical, bilayer lipid and the bilayer it forms in aqueous solutions. . [26]

Although not all of the lipids have hydrocarbon tails with double bonds, when double bonds are present, they are usually cis, introducing kinks into the tails. [2] This makes them difficult to pack together and for this reason, the tails move freely within the membrane; there is also uncatalyzed lateral diffusion of the lipids themselves. [2] In animal cells, cholesterol is present in the membrane to increase or decrease fluidity, as necessary; it inserts itself into the bilayer, between lipids, with its polar hydroxyl group. [2] The presence of cholesterol in between the lipids keeps the fatty acid tails from coming together and freezing at low temperatures. At high temperatures, cholesterol does the opposite. Its presence in between lipids interferes with the rotation of the fatty acid tails, limiting their movement. [2]

G-Protein Coupled Receptors

Within the membrane are many different families of receptors. One of the largest superfamilies of membrane-bound receptors is the g protein coupled receptors (GPCRs). [4] GPCRs make up about 3% of all human genes representing between 750 and 800 different GPCRs. Individual cells express less than 100 different GPCRs, a number of different G protein subunits, and various G protein-linked effectors. [4] Transmission of a signal by GPCRs requires physical interaction between the receptor, a guanosine nucleotide-binding protein (G protein) subunit, and an effector molecule. [4] Signal transduction occurs when the receptor

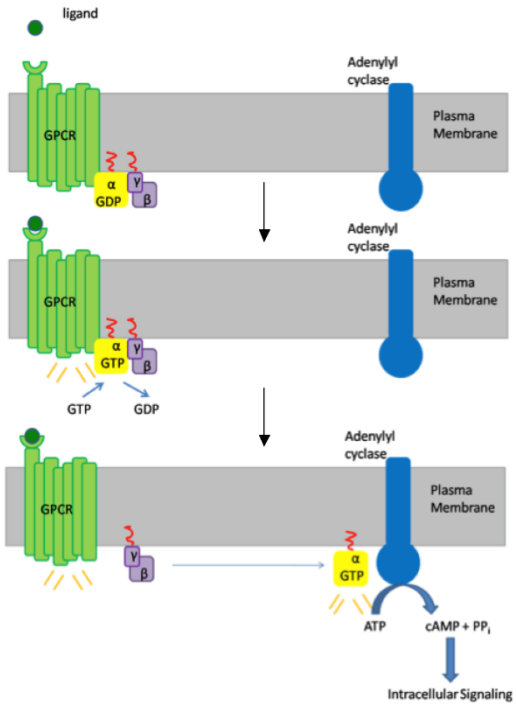


Figure 3. The activation of adenylyl cyclase as a result of ligand binding to a GPCR. Binding activates the GPCR which then promotes the exchange of GDP for GTP in the alpha subunit. The alpha unit can then activate effector proteins, such as adenylyl cyclase.

is activated by its ligand. This interaction causes a conformational change in the receptor that allows it to function as a guanine exchange factor (GEF). [5] As a GEF, the receptor acts on its coupled, inactive G protein on the cytosolic side of the membrane. It facilitates the release of bound GDP from the G protein and binding of free GTP from the cytosol, activating the G protein. Once activated, the G protein dissociates from the receptor and binds to a nearby effector protein, usually an enzyme. This binding alters the activity of the enzyme. [5]

These components required for signaling, however, are expressed on the cell surface at relatively low levels, less than 10,000/cell. [6] At these concentrations, a uniform distribution of the components would be extremely unlikely to allow for interaction and sufficient signal transduction, but somehow signal transduction still occurs rapidly and efficiently. One mechanism that may explain how signal transduction is facilitated is that cells concentrate signaling molecules within membrane microdomains. [4]

Lipid Rafts

The multitude of saturated hydrocarbon chains in cell membrane sphingolipids allows for cholesterol to be tightly intercalated. [7] This leads to membrane rigidity and the formation of microdomains known as lipid rafts. [8] The tight packing of the cholesterol and sphingolipids within the membrane causes the high melting temperature of these microdomains. [9] Rafts can be characterized as caveolar or non-caveolar; caveolar lipid rafts are flask shaped invaginations in the membrane and non-caveolar rafts are planar microdomains. [10]

Studies have concluded that lipid rafts provide both a spatial and temporal meeting point for signaling molecules. It is believed that lipid rafts can coordinate specific signaling events by influencing the inclusion or exclusion of receptors and their

associated effector molecules. [10] Lipid rafts also influence g protein-coupled-receptor signaling (GPCR). [10] Lipid rafts can localize or move any of the components required for transducing the signal as a way of modifying receptor signaling. Studies show, however, that GPCR components are more likely to be confined within lipid rafts. [4] This may explain how stable complexes are able to be formed between the separate components of the GPCR pathway. Disrupting lipid rafts has resulted in reductions in G protein coupling and binding of agonists to GPCRs, providing support to the theory that lipid rafts facilitate coupling of the G protein and receptor. [4] Many of these couplings have been found to occur in the previously mentioned lipid raft subsets called caveolae.

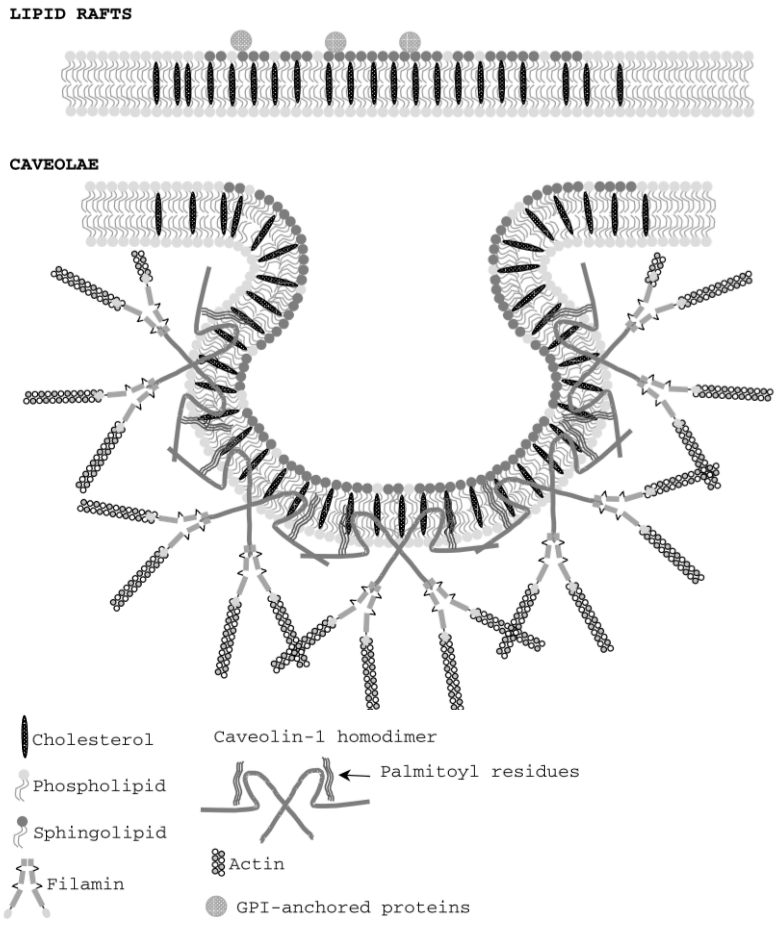


Figure 4. Linear and Caveolar Lipid Rafts [27]

Caveolae

Caveolae are thought to be a subset of lipid rafts that comprise about 1% of the plasma membrane surface area of the cells that express them. They are approximately 50-100 nm in diameter and were first identified during the 1950s by their "flask-shape" on the cell surface. In addition to the usual characteristics of lipid rafts, caveolae characteristically contain caveolin proteins localized on the inner leaflet of the membrane bilayer. [11] Caveolae are present in cells from most tissues but are particularly abundant in adipocytes, endothelial cells, type I pneumocytes, fibroblasts, and smooth and striated muscle cells. Alternatively, cells such as neurons and lymphocytes normally lack caveolae. [12] Within caveolin expressing cells, caveolae function in transcytosis, endocytosis, exocytosis, cholesterol homeostasis, and receptor and ion-channel expression, activation, and desensitization. [12]

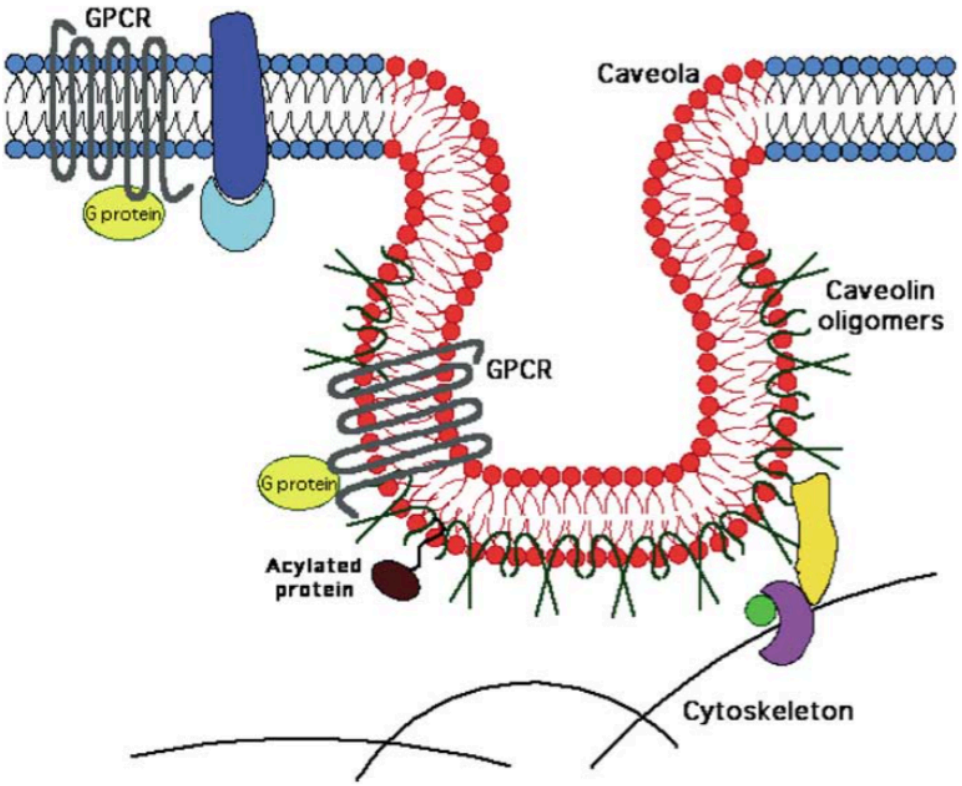


Figure 5. Schematic representation of the lipid and protein organization of caveolae [4]

Caveolin

Caveolin proteins are located solely on the cytoplasmic side of the plasma membrane and are considered the molecular markers for caveolae. Three different isoforms of the protein exist: caveolin-1, caveolin-2, and caveolin-3. Caveolae generally form in cells expressing caveolin-1, the predominant isoform, or caveolin-3, the striated muscle-specific isoform. [13] Caveolin-2 is found in hetero-oligomers with caveolin-1 and caveolin-3, but it is not clear if caveolin-2 can induce caveolae biosynthesis on its own. [14] The three isoforms have a similar structure consisting of a hairpin loop with both the C and N termini on the cytoplasmic side of the membrane. Knock out experiments performed in mice show that the isoforms have different patterns of regulation of enzymatic and functional activities. [12]

Within caveolae, caveolin has three major functions. First, it helps maintain the structural integrity of the caveolae. The curvature of the membrane creates physical stress on the membrane that caveolin helps to alleviate by "filling in" the stretched side of the membrane (Figure 4). [15] Secondly, caveolin acts as a scaffolding protein. In this role, caveolin recruits various molecules into the raft region. Lastly, caveolin acts as a protein chaperone and functions in cell membrane trafficking. [15]

Multiple domains have been identified within the structure of the caveolin monomer. The scaffolding domain that exists likely serves as an anchor for signaling components. Caveolin also contains and oligomerization domain. This is the site of interaction where the high-weight oligomers seen in the caveolae are formed. [15]

Caveolin Interaction Motif

Previous research has suggested that signaling proteins form direct protein-protein interactions with the scaffolding domain of caveolin (CSD) through a conserved peptide

sequence, the caveolin binding motif (CBM). [16] These consensus CBMs are hydrophobic and rich in aromatic residues. The typical sequences are ϕxϕxxxxϕ or ϕxxxxϕxxϕ or the combined sequence ϕxϕxxxxϕxxϕ, where ϕ is a phenylalanine, tyrosine or tryptophan residue and x can be any amino acid (figure 6). [16]

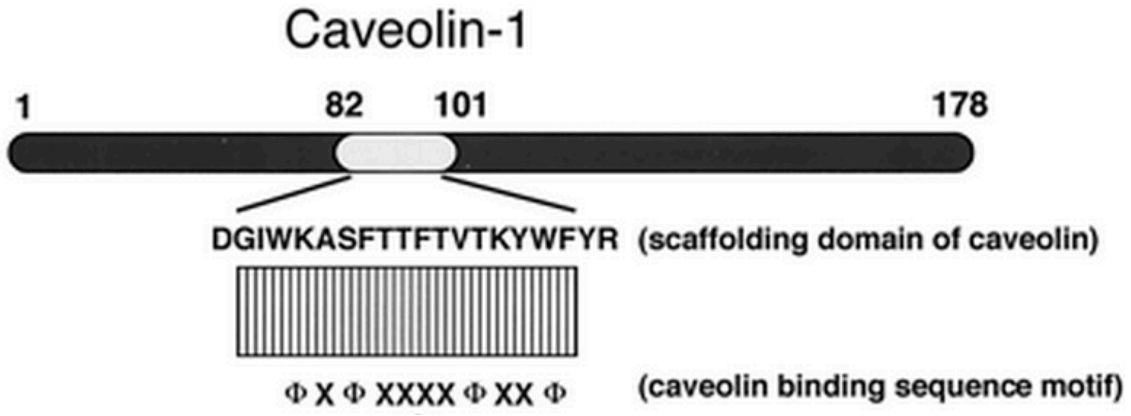


Figure 6. Caveolin interaction motif and conjugate amino acid sequence on scaffolding domain of caveolin [14]

Human Follicle Stimulating Hormone Receptor

Follicle stimulating hormone, or follitropin, is a gonadotropin hormone secreted by the gonadotroph cells in the anterior pituitary. [17] In order to transduce a signal, FSH binds to its receptor, follicle stimulating hormone receptor (FSHR). FSHR is found on the surface of granulosa cells surrounding the immature follicle and oocyte in ovaries of women and on Sertoli cells in the testes of men. [17] Activation of FSHR by FSH directly promotes follicle maturation and estrogen production in females. [18] In males, FSH plays a role in establishing the population of Sertoli cells in the testicular seminiferous tubules and in the maintenance of sperm quality and number. [17]

These aforementioned effects are the result of FSHR signaling through what is known as its canonical pathway. When FSH binds to FSHR, the receptor transduces a signal through two different pathways, the canonical pathway and what is believed to be a desensitization and internalization pathway.

FSHR is a GPCR (Figure 7). The canonical pathway is dependent on FSHR's action through it coupled G protein. FSHR is primarily coupled to the G protein $G_S\alpha$. [19]

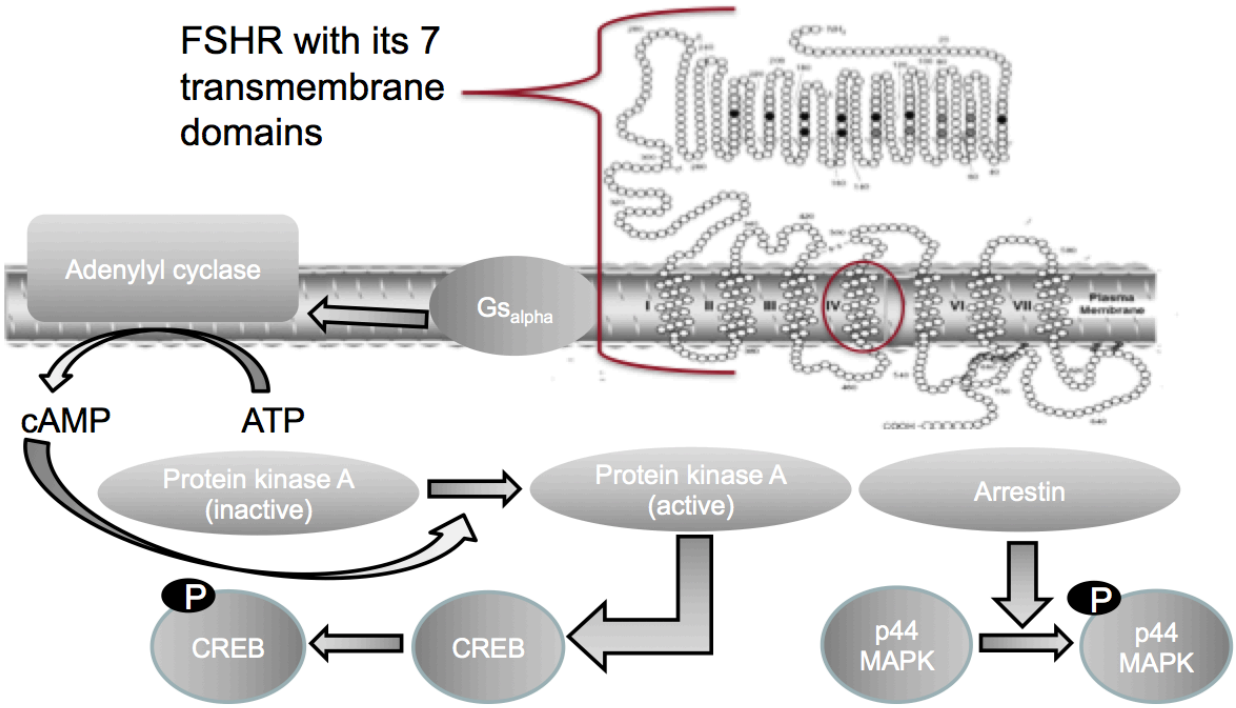


Figure 7. hFSHR and its signaling pathways

When activated, $G_S\alpha$ alters the activity of the enzyme adenylyl cyclase, stimulating the production of cyclic AMP (cAMP). cAMP then activates Protein kinase A (PKA), which can activate the cAMP Response Element Binding Protein (CREB) pathway (Figure 3). [19] This results in the previously discussed endocrine effects of FSH.

Prolonged exposure to FSH, however, leads to desensitization. This is decreased sensitivity and response to the hormone. [20] Desensitization is a result of receptor uncoupling, which decreases receptor function and occurs relatively rapidly after receptor stimulation. [20] FSHR is uncoupled from the $G_S\alpha$ subunit due to phosphorylation of serine and threonine residues on the C terminal end of the receptor. This event results in the recruitment of inhibitor proteins known as arrestins. [21] Arrestins then recruit other

molecules that play a role in receptor down-regulation. [20] This is a slower desensitization process. The number of FSHRs on the cell surface decrease due to internalization through clathrin coated pit or caveolae endocytotic pathways. [22] Receptor numbers also begin to decrease as a result of decreased transcription, translation, or mRNA half-life.

Human FSHR (hFSHR), like other GPCRs, has been shown to preferentially localize within microdomains in the cell membrane known as lipid rafts during signaling. [23] More specifically, hFSHR has been observed to associate with caveolae (Cohen Lab). Research has shown that lipid rafts function as spatial and temporal meeting places for the molecules required to transduce a signal, like GPCRs. [24]

FSHR And Lipid Rafts

The hFSHR sequence contains a canonical combined motif between amino acids 479-489 (Figure 8) with each of the ϕ's of the CBD being a phenylalanine.[10] In Figure 8, these residues are colored in with 479 as green and 489 as blue. This provides a possible mechanism for the interaction of hFSHR with caveolin, although it does not help to understand the functional significance of either the sequence or the interaction.

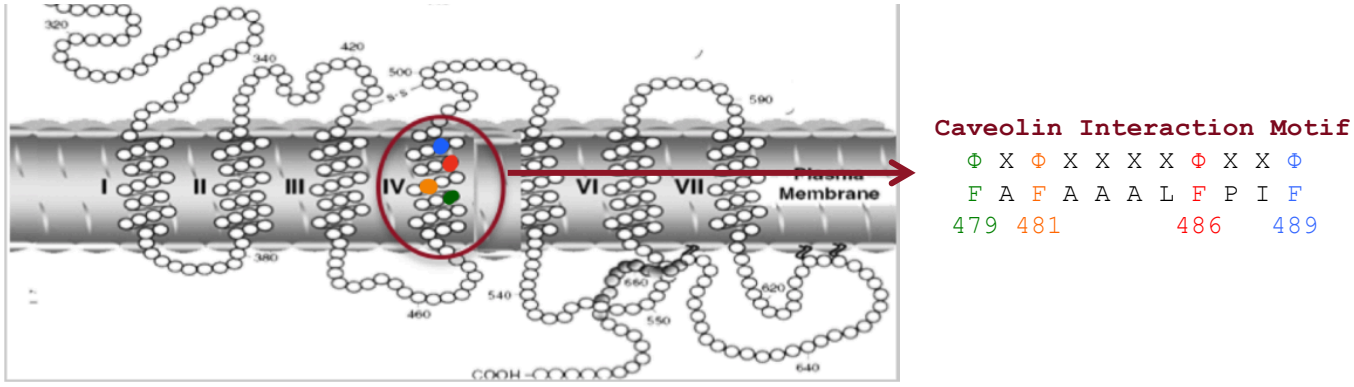


Figure 8. hFSHR with combined caveolin interaction motif circled [17]

To study the interactions of hFSHR with caveolin, mutated receptors have been synthesized. Members of the Cohen lab individually mutated each of the phenylalanine residues of the CBD to a leucine residue using Splicing by Overlap Extension (Table 1).

Table 1. FSH receptor Mutagenesis Oligonucleotides for Splicing by Overlap Extension

FSH Receptor Mutagenesis Oligonucleotides	
Normal Sequence	5'GTCATGGTGATGGGCTGGATTTTTGCTTTTGCAGCTGCCCTCTTTCCCATCTTTGGCATCAGCAGCTACATGAAG 3' 3'CAGTACCACTACCCGACCTAAAAACGAAAACGTCGACGGGAGAAAGGGTAGAAACCGTAGTCGTCGATGTACTTC 5'
F479L	5'GGCTGGATCCTTGCTTTTGCAGCTGCCCTC3' C primer BamHI 3'CAGTACCACTACCCGACCTAGGAACGAAAACG5' B primer GCAAAAGCAAGGATCCAGCCCATCACCATGAC
F481L	5'GATTTTTGCGCTAGCAGCTGCCCTCTTTCCC3' C primer NheI 3'CACTACCCGACCTAAAAACGCGATCGTCGACGG5' B primer GGCAGCTGCTAGCGCAAAAATCCAGCCCATCAC
F486L	EarI 5'GCAGCTGCTCTTCTTCCCATCTTTGGCATCAGC3' C primer 3'CTAAAAACGAAAACGTCGACGAGAAGAAGGGTAG5' B primer GATGGGAAGAAGAGCAGCTGCAAAAGCAAAAATC
F489L	AvrII 5'TTTCCCATCCTAGGCATCAGCAGCTACATGAAG3'C primer 3'CGACGGGAGAAAGGGTAGGATCCGTAGTCG5' B primer GCTGATGCCTAGGATGGGAAAGAGGGCAGC

The next step of this research is to transfect the mutated receptors into HEK293 cells. These transfected cells will be examined for proper expression of the receptor. If the receptors are expressed properly, they will be tested for co-immunoprecipitation with caveolin and proper signal transduction.

Once the interaction between hFSHR and the lipid rafts is better understood, research can be focused on possible pharmaceutical interventions for infertility and novel birth control. Specifically, research will be focused on synthesis of two different drugs, one that would enhance interaction between the receptor and caveolin and one that would interfere with the interactions.

Methods

Cell Culture and DNA Transfection

HEK293 cells were grown and maintained as monolayer cultures at 37°C in DMEM medium supplemented with 10% fetal bovine serum and penicillin:streptomycin. The cells were grown to 50-70% confluence before being transfected using X-tremeGENE HP DNA Transfection Reagent Roche) and the respective DNA. The HEK293 cells were transfected with wild-type or mutant FSHR plasmids for expression, localization, and signaling assays.

SDS-PAGE and Western Blotting

HEK293 cells were grown to a monolayer and transfected. The cell's proteins were then extracted by detergent lysis. An Igepal/DOC lysis buffer (1% Igepal, 0.4% deoxycholate, 10mM Tris-Cl pH 7, 6.6mM EDTA) was used with a 1X protease inhibitor cocktail. After BCA Assay analysis, the protein samples were linearized by treatment with SDS and separated on 10% poly-acrylamide gels by molecular mass. The proteins were then transferred to a PVDF membrane and blocked with 5% milk in TBST. The membranes were then probed with various antibodies. Anti-hFSHR mAb 106.105 (105) was used to probe for hFSHR, P-p44/42 MAPK Rabbit Ab for phosphorylated p44, P-CREB Rabbit Ab for phosphorylated CREB, and p38 MAPK Rabbit Ab for total p38. Finally, the membranes were probed with anti-mouse HRP antibody when probing with 105 and with anti-rabbit HRP when probing with phospho-p44, phospho-CREB, or p38.

Immunohistochemistry

HEK293 cells were grown on glass cover slips and then transfected with wild-type or mutant FSHR plasmids. These cells were fixed using 4% paraformaldehyde in phosphate buffered saline (PBS). Primary antibody for the detection of FSHR was monoclonal antibody mAb106.105, the kind gift of Dr. James Dias (Wadsworth Center, Albany, NY). Goat anti-mouse with an Alexa-Red fluorophore was used as the secondary antibody.

Stable Selection

HEK293 cells were grown and transfected as described in *Cell Culture and DNA Transfection.* The medium was then changed to one including G418 antibiotic in order to select for only the cells that were successfully transfected. After several days in the G418 medium, single colonies of the cells were transferred to their own wells of a 24-well dish for their amplification. Once they reached confluence, they were moved to 12-well dishes, 6-well dishes, t-25 flasks, and finally t-75 flasks.

Results

Single point mutations of the caveolin interaction motif do not disrupt receptor expression.

SDS-PAGE and western blotting assays were performed to determine whether mutations of single aromatic residues of the caveolin interaction motif disrupted the expression of the mutant receptors by cells. Results show that all four of the mutant receptors, and the wild type receptor, were successfully transfected into HEK293 cells and the cells were able to express them (Figure 9).

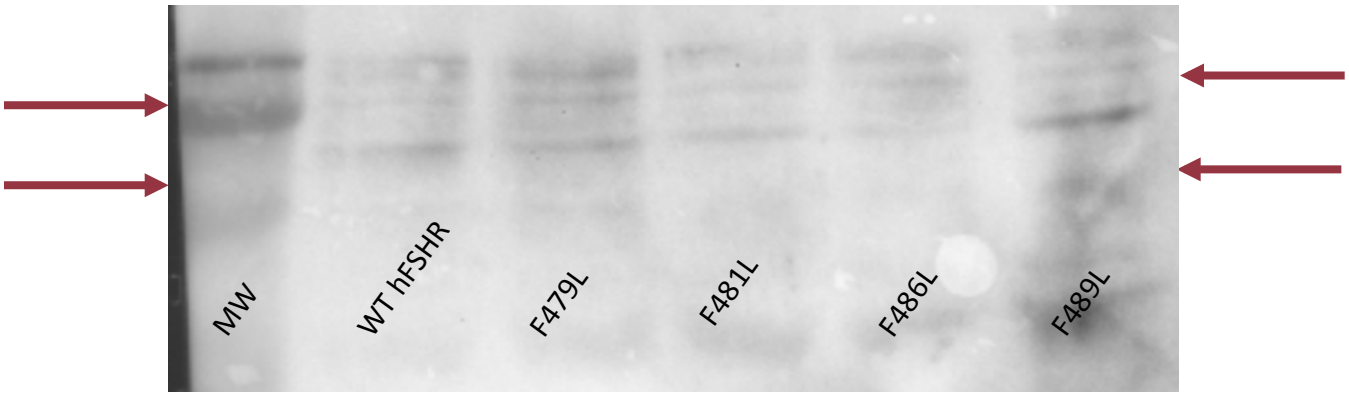


Figure 9. Western blot of lysates transfected with wild-type and mutant hFSHR plasmids. Probed with anti-hFSHR mAb 106.105 for the detection of FSHR.

Expression of the mutant receptors was also analyzed by fluorescence microscopy. Results support that the cells were successfully transfected with the wild type and mutant receptors and the cells express each of the receptors (Figure 10).

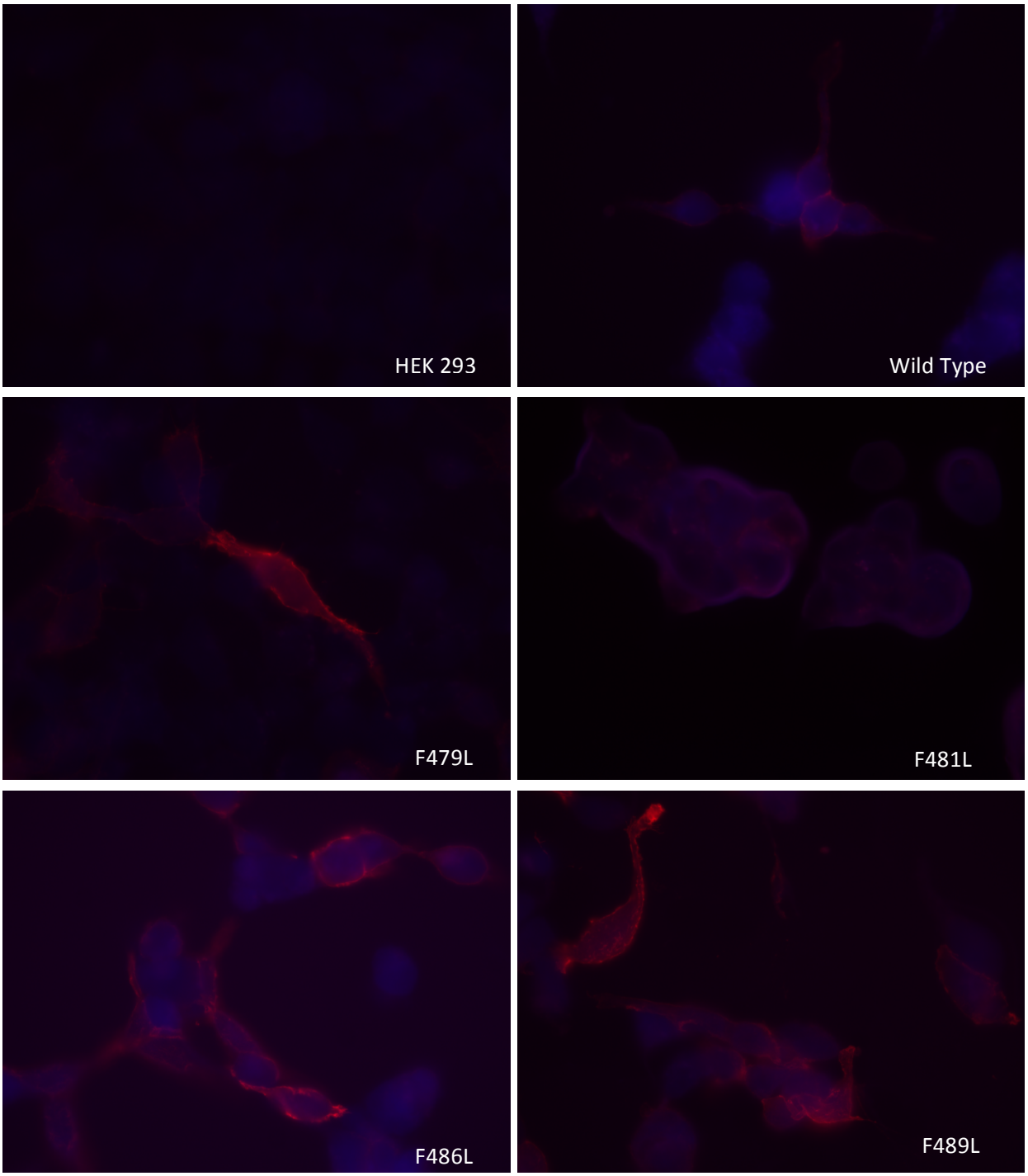


Figure 10. Fluorescence imaging of HEK293 cells transfected with nothing, wild type hFSHR, and the three hFSHR single mutant plasmids. Cells were stained with Alexa 594 Goat anti-Mouse Ab and DAPI.

Single point mutations of the caveolin interaction motif do not disrupt receptor signaling

SDS-PAGE and western blotting assays were performed to determine whether mutations of single aromatic residues of the caveolin interaction motif disrupted the ability of the mutant receptors to signal through the CREB and MAPK pathways when exposed to hormone for 0, 5, and 15 minutes before protein extraction. Results show that all four of the mutant receptors, and the wild type receptor, successfully signaled through both pathways in response to hormone stimulation (Figures 11 and 12).

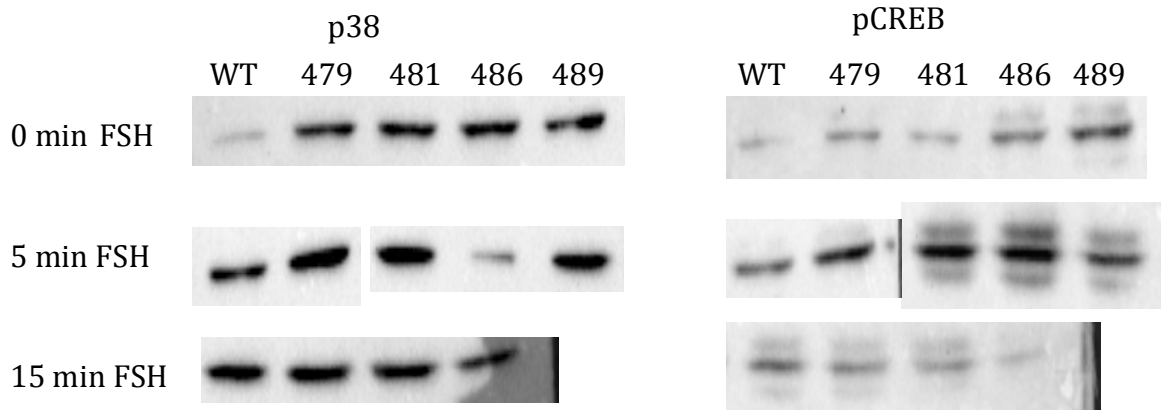
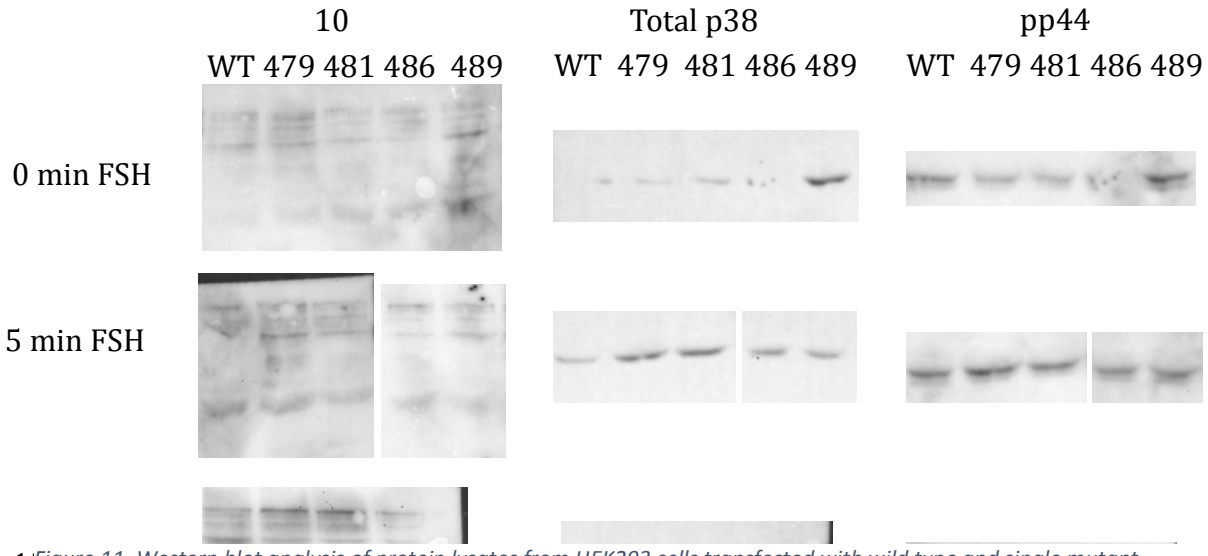


Figure 12. Western blot analysis of protein lysates from HEK293 cells transfected with wild type and single mutant hFSHR plasmids probed with p38 MAPK Rabbit Ab as a loading control and P-CREB Rabbit Ab to determine signaling by the canonical pathway.



1. *Figure 11. Western blot analysis of protein lysates from HEK293 cells transfected with wild type and single mutant hFSHR plasmids probed with anti-hFSHR mAb 106.105 to show hFSH expression, p38 MAPK Rabbit Ab as a loading control and P-p44/42 MAPK Rabbit Ab to determine signaling by the internalization pathway.*

Discussion

Four variant hFSHR plasmids with mutations in the caveolin interaction motif were previously created using splicing by overlap extension. The phenylalanine residues of the caveolin interaction motif were mutated to leucine residues at amino acids 479, 481, 486, and 489 of the hFSHR. These plasmids were transfected into human embryonic kidney (HEK293) cells for various assays to determine the effect of the mutations on properties of the receptor.

Western blot analysis using anti-hFSHR mAb 106.105, an hFSHR-detecting antibody, demonstrated that the wild type and mutant receptors were present in the transfected cells. Therefore, the single residue mutations did not affect the ability of the cells to transcribe and translate the plasmids into receptor proteins.

In order to determine if the mutant receptors could also be localized to the cell membrane by HEK293 cells, fluorescence microscopy assays using fluorescently tagged mAb 106.105 were performed. Results show that the wild type and mutants receptors were indeed localized to the cell membrane in the HEK293 cells. The transfected cells were not permeablized prior to incubation with mAb 106.105 and the fluorescently tagged secondary antibody so any fluorescence seen should correspond to antibody bound to receptors on the cell surface.

Receptor localization to the cell surface was also confirmed by western blot analysis using anti-Phospho p44 and anti-Phospho-CREB. These antibodies detect phosphorylated p44 and CREB molecules. p44 and CREB are phosphorylated downstream of two FSHR signaling pathways. Levels of phosphorylated p44 and CREB were measured at 0, 5, and 15 minutes of exposure to FSH in order to determine if the

mutant receptors signaled like the wild type receptor. Results show that the wild type and mutant receptors each signal in response to hormone stimulation. Variability in gel loading was observed (based on the total p38 staining in Figures 11 and 12) which prevents quantitative analysis of signaling activation by the mutant receptors. However, any signaling is demonstrative of the presence of hFSHR CIM mutants on the cell membrane.

Taken together, these results indicate that single mutations of a phenylalanine residue of the caveolin interaction motif do not disrupt completely disrupt expression, localization, and signaling of the hFSHR. The qualitative assays performed did not rule out the possibility that the mutations impacted the level of expression, localization to the cell surface, or signaling efficiency of the hFSHR mutants as compared to the wild type.

Future experiments should include selection of stable cell lines expressing the mutant hFSHRs. With stably selected cell lines, the quantitative expression assays can be performed and once expression is determined for the different mutants, the surface localization and signaling can be normalized to the number of receptors. This will allow the researchers to compare the mutant and wild type receptors quantitatively on a per-receptor basis and the effect of the mutations on receptor properties will be better understood.

Once the effect of the single mutations is better understood, the focus can be turned to creating multiple mutants and performing different assays to see if additional mutations of the phenylalanine residues of the caveolin interaction motif will result in disruption of proper hFSHR function.

References

1. Singleton P. *Bacteria in biology, biotechnology and medicine.* 6th ed. USA: Wiley; 2004.

2. Cooper G. *The cell: A molecular appraoch.* 2nd ed. Sunderland, MA: Sinauer Associates; 2000.

3. Tanford C. *The hydrophobic effect: Formation of micelles and biological membranes.* 2nd ed. J. Wiley; 1980.

4. Ostrom RS, Insel PA. The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: Implications for molecular pharmacology. *Br J Pharmacol*. 2004;143(2):235-245.

5. Lehninger A, Nelson DL, Cox MM. *Lehninger's principles of biochemistry.* 4th ed. New York, NY: W. H Freeman; 2005.

6. Alousi AA, Jasper JR, Insel PA, Motulsky HJ. Stoichiometry of receptor-gs-adenylate cyclase interactions. *FASEB J*. 1991;5(9):2300-2303.

7. Simons K, Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol*. 2000;1(1):31-39.

8. London E. Insights into lipid raft structure and formation from experiments in model membranes. *Curr Opin Struct Biol*. 2002;12(4):480-486.

9. Brown DA, London E. Structure and function of sphingolipid-and cholesterol-rich membrane rafts. *Journal of Biological Chemistry*. 2000;275(23):17221-17224.

10. Allen JA, Halverson-Tamboli RA, Rasenick MM. Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci*. 2007;8(2):128-140.

11. Anderson RG. The caveolae membrane system. *Annu Rev Biochem*. 1998;67:199-225.

12. Cohen AW, Hnasko R, Schubert W, Lisanti MP. Role of caveolae and caveolins in health and disease. *Physiol Rev*. 2004;84(4):1341-1379.

13. Song KS, Scherer PE, Tang Z, et al. Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J Biol Chem*. 1996;271(25):15160-15165.

14. Razani B, Woodman SE, Lisanti MP. Caveolae: From cell biology to animal physiology. *Pharmacol Rev*. 2002;54(3):431-467.

15. Parton RG, Hanzal-Bayer M, Hancock JF. Biogenesis of caveolae: A structural model for caveolin-induced domain formation. *J Cell Sci*. 2006;119(Pt 5):787-796.

16. Collins BM, Davis MJ, Hancock JF, Parton RG. Structure-based reassessment of the caveolin signaling model: Do caveolae regulate signaling through caveolin-protein interactions? *Dev Cell*. 2012;23(1):11-20.

17. Dias JA, Cohen BD, Lindau-Shepard B, Nechamen CA, Peterson AJ, Schmidt A. Molecular, structural, and cellular biology of follitropin and follitropin receptor. *Vitam Horm*. 2002;64:249-322.

18. Huhtaniemi I. The parkes lecture. mutations of gonadotrophin and gonadotrophin receptor genes: What do they teach us about reproductive physiology? *J Reprod Fertil*. 2000;119(2):173-186.

19. Dierich A, Sairam MR, Monaco L, et al. Impairing follicle-stimulating hormone (FSH) signaling in vivo: Targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci U S A*. 1998;95(23):13612-13617.

20. Irannejad R, von Zastrow M. GPCR signaling along the endocytic pathway. *Curr Opin Cell Biol*. 2014;27:109-116.

21. Shukla, Arun K., Kunhong Xiao, and Robert J. Lefkowitz. Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends in biochemical sciences*. 2011;36(9):457-469.

22. Gagnon AW, Kallal L, Benovic JL. Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the beta2-adrenergic receptor. *J Biol Chem*. 1998;273(12):6976-6981.

23. Chini B, Parenti M. G-protein coupled receptors in lipid rafts and caveolae: How, when and why do they go there? *J Mol Endocrinol*. 2004;32(2):325-338.

24. Allen JA, Halverson-Tamboli RA, Rasenick MM. Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci*. 2007;8(2):128-140.

25. McCarthy EM. The fluid mosaic model. http://www.macroevolution.net/fluid-mosaic-model.html. Updated 2015. Accessed 12/15/15, 2015.

26. Johnson M. Detergents: Triton X-100, tween-20, and more. http://www.labome.com/method/Detergents-Triton-X-100-Tween-20-and-More.html. Updated 2013. Accessed 12/15/15, 2015.

27. Quest AF, Leyton L, Parraga M. Caveolins, caveolae, and lipid rafts in cellular transport, signaling, and disease. *Biochem Cell Biol*. 2004;82(1):129-144.

For life science research only.
Not for use in diagnostic procedures.

Roche

X-tremeGENE HP DNA Transfection Reagent

For transient and stable transfection of eukaryotic cells

Version 08
Content version: January 2014

Cat. No. 06 365 752 001	Trial-pack
Cat. No. 06 366 244 001	0.4 ml
Cat. No. 06 366 236 001	1 ml
Cat. No. 06 366 546 001	5 × 1 ml

Store at –15 to –25°C

1. What this Product Does

Number of Tests

Using the standard procedure, 1 ml of X-tremeGENE HP DNA Transfection Reagent can be used to perform up to 10,000 transfections in 96-well plates.

Formulation

X-tremeGENE HP DNA Transfection Reagent is a proprietary blend of lipids and other components supplied in 80% ethanol, filtered through 0.2 µm pore size membrane, and packaged in glass vials. It does not contain any ingredients of human or animal origin.

Storage and Stability

Store X-tremeGENE HP DNA Transfection Reagent at –15 to –25°C, with the lid tightly closed. The reagent is stable until the expiration date printed on the label when stored under these conditions.

- X-tremeGENE HP DNA Transfection Reagent remains fully functional even after repeated opening of the vial (at least five times over a two-month period), as long as the vial is tightly recapped and stored at -15 to -25°C.
- Note that the shipping temperature of this product is different from the storage temperature. These different temperatures will not affect product performance or product stability.

Special Handling

- ⚠ After removing the amount required, tightly close the vial with the lid immediately after use.
- ⚠ Always bring the vial to +15 to +25°C and mix X-tremeGENE HP DNA Transfection Reagent prior to removing the amount required vortexing for one second.
- ⚠ Do not aliquot X-tremeGENE HP DNA Transfection Reagent; store in the original glass vials.
- ⚠ Minimize the contact of undiluted X-tremeGENE HP DNA Transfection Reagent with plastic surfaces.
- ⚠ For use, the minimum amount of X-tremeGENE HP DNA Transfection Reagent: DNA complex is 100 µl. Complex formation at lower volumes can significantly decrease transfection efficiency.
- ⚠ Do not use tubes or microplates made of polystyrene for X-tremeGENE HP Transfection Reagent : DNA complex preparation. When not able to avoid polystyrene materials, make certain to pipet the transfection reagent directly into the serum-free medium (*e.g.,* Opti-Mem).
- ⚠ Do not use siliconized pipette tips or tubes.

Additional Equipment and Reagents Required

Additional reagents and equipment required to perform transfection assays using X-tremeGENE HP DNA Transfection Reagent include:

➤ **Standard Laboratory Equipment**.
- Standard cell culture equipment (*e.g.,* biohazard hoods, incubators)
- Standard pipettes and micropipettes
- Vortex mixer

➤ **For Plasmid Preparation**
- Purified plasmid stock (0.1 – 2.0 µg/µl) in sterile TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer or sterile water
- Genopure Plasmid Midi Kit* or Genopure Plasmid Maxi Kit* to prepare plasmid

➤ **For Verification of Vector Function**
- Assay appropriately for transfected gene
- G-418 Solution* or Hygromycin B* (optional for stable transfection experiments)

➤ **For Transfection-Complex Formation**
- Opti-MEM I Reduced Serum Medium or serum-free medium
- Sterile polypropylene tubes or round-bottom 96-well plates

➤ **Growing Cells**
- Select subconfluent cultures in log phase for preparation of cell cultures
- Quantify cell number to reproducibly plate the same number of cells

Application

X-tremeGENE HP DNA Transfection Reagent is a high performance transfection reagent, free of animal-derived components. Benefits of X-tremeGENE HP DNA Transfection Reagent include:

- Designed to transfect a broad range of eukaryotic cells, including insect cells, many cell lines not transfected well by other reagents, and hard-to-transfect cell lines (*e.g.,* HT-1080, K-562, HepG2).
- Can be successfully used in a variety of applications, such as gene expression analysis and protein production using transiently transfected cells, generation of stable cell lines, expression of shRNA for gene knockdown studies, drug discovery programs, and target evaluation. Samples and detailed transfection protocols are available at ***http://www.powerful-transfection.com.***
- Produces minimal cytotoxicity or changes in morphology when adequate numbers of cells are transfected, eliminating the requirement to change media after adding the transfection complex.
- Suitable for transient and stable transfection.
- Functions very well in the presence or absence of serum.

2. How to Use this Product

2.1 Before You Begin

Required Amount of X-tremeGENE HP DNA Transfection Reagent

To optimize, first transfect a monolayer of cells that is 70 – 90% confluent, using 1:1, 2:1, 3:1 and 4:1 ratios of microliter (µl) X-tremeGENE HP DNA Transfection Reagent to microgram (µg) DNA. A ratio of 3:1 of microliter (µl) X-tremeGENE HP DNA Transfection Reagent to microgram (µg) DNA has been shown to be optimal for many cell types.

Lower cell confluencies have also been tested successfully.

The recommended starting concentration is a 3:1. For most cell types, these X-tremeGENE HP DNA Transfection Reagent to DNA ratios provide excellent transfection efficiency.

Further optimization may increase transfection efficiency in your particular application. In addition to varying the ratio, other parameters may also be evaluated, such as the amount of transfection complex added. For additional optimization guidelines, see Section 3, Troubleshooting and visit ***http://www.powerful-transfection.com.***

Plasmid DNA

- For best results, accurately determine the plasmid DNA concentration using 260-nm absorption; estimates of DNA by measuring gel band density are not recommended. Determine DNA purity using a 260 nm/280 nm ratio (the optimal ratio is 1.8).
- Prepare the plasmid DNA solution using sterile TE (Tris/EDTA) buffer or sterile water at a concentration of 0.1 to 2.0 µg/µl.
- Use high quality DNA preparation kits to obtain endotoxin-free DNA.

Cell Culture Conditions

- Minimize intra- and inter-experimental variance in transfection efficiency using cells that are regularly passaged, proliferating well in a log-growth phase, and plated at a consistent density.
- For best results, accurately quantify cell concentration using a hematocytometer or automated system.
- Cells must be healthy and free of Mycoplasma.
- Cells should have a low passage number to achieve best results.

Other Media Additives

In some cell types, antimicrobial agents (*e.g.,* antibiotics and fungicides) commonly included in cell-culture media may adversely affect the transfection efficiency of X-tremeGENE HP DNA Transfection Reagent. If possible, exclude additives in initial experiments. Once high-efficiency conditions have been established, these components can be added back while monitoring transfection results. Cell growth and/or transfection efficiency may be affected by variations in serum quality and medium formulations.

Verification of Vector Function

Optimize transfection conditions using a known positive-control reporter gene construct before transfecting cells with a new vector construct:

- Determine transfection efficiency using a reporter gene assay, such as β-Gal*, Luciferase*, or SEAP*.
- Sequence flanking vector insert regions to verify the integrity of your new construct.

2.2 Preparation of Cells for Transfection

Adherent Cells: Plate cells approximately 24 hours before transfection making sure cells are at the optimal concentration in the appropriate cell culture vessel.

Suspension Cells: Plate freshly passaged cells at optimal concentration.

2.3 Transfection Procedure

❶ Allow X-tremeGENE HP DNA Transfection Reagent, DNA and diluent to equilibrate to +15 to +25°C. Briefly vortex the X-tremeGENE HP DNA Transfection Reagent vial.

❷ Dilute DNA with appropriate diluent (*e.g.,* serum-free medium) to a final concentration of 1 µg plasmid DNA /100 µl medium (0.01 µg/µl). Mix gently.

❸ Place 100 µl of diluent, containing 1 µg DNA into each of four sterile tubes labeled 1:1, 2:1, 3:1, and 4:1.

⚠ Use a minimum of 100 µl of diluent. Lower volumes may significantly decrease transfection efficiency.

Use sterile tubes or tissue culture treated round-bottom, 96-well plates to produce the complex.

❹ Pipet the X-tremeGENE HP DNA Transfection Reagent (1, 2, 3, or 4 µl) directly into the medium containing the diluted DNA without coming into contact with the walls of the plastic tubes. Mix gently.

⚠ To avoid adversely affecting transfection efficiency, do not allow undiluted X-tremeGENE HP DNA Transfection Reagent to come into contact with plastic surfaces. Do not use siliconized pipette tips or tubes.

❺ Incubate the transfection reagent:DNA complex for 15 minutes at +15 to +25°C.

Some ratios and cell types may required longer incubation (up to 30 min). Determine this for your particular cell line and the ratio used.

❻ Remove the culture vessel from the incubator. Removal of growth medium is not necessary. Add the transfection complex to the cells in a dropwise manner.

See Table 1 to determine component amounts corresponding to the surface area of the cell culture vessel used.

Gently shake or swirl the wells or flasks to ensure even distribution over the entire plate surface. If available, use a rotating platform shaker for 30 seconds at low speed for mixing 96-well plates.

Once the transfection reagent: DNA complex has been added to the cells, there is no need to replace with fresh medium (as may be necessary with other transfection reagents)

❼ Following transfection, incubate cells for 18 – 72 hours before measuring protein expression. The duration of incubation will depend on many factors, including the transfected vector construct, the cell type being transfected, the cell medium, cell density, and the type of protein being expressed. After the incubation period, measure protein expression using an assay appropriate for your system.

Notes:

As with any experiment, include appropriate controls. Prepare culture wells with cells that remain untransfected, cells with transfection reagent alone, and cells with DNA alone.

For stable transfection experiments, the complex-containing medium should be left unchanged until the cells are passaged. At that time, include appropriate selection antibiotics (*e.g.,* G 418 Solution or Hygromycin B).

To prepare transfection complexes for different-sized containers or parallel experiments, adjust component amounts corresponding to the surface area of the cell culture vessel used (see Table 1).

For ease-of-use when transfecting small volumes into 96-well plates containing 0.1 ml culture medium per well, prepare 100 µl of transfection complex, and then add 10 µl to each well (depending on cell type).

The optimal ratio of transfection reagent to DNA, and the optimal total amount of complex, will depend on the cell line, cell density, day of assay, and gene expressed.

After performing the optimization experiment in which several different ratios are tested, select a ratio in the middle of the plateau optimum for future experiments.

Tab. 1: Guidelines for Preparing X-tremeGENE HP DNA Transfection Reagent: DNA Complex for Various Culture Vessel Sizes

Culture vessel	Surface Area (cm^2)	Total volume of medium (ml)	Suggested amount of 100 µl transfection complex to add to each well (µl)	DNA (µg) using 1:1 or 4:1 Ratio	Final amount of X-tremeGENE HP DNA Transfection Reagent (µl) using 1:1 Ratio	Final amount of X-tremeGENE HP DNA Transfection Reagent (µl) using 4:1 Ratio
96-well plate (1 well)	0.3	0.1	10	0.1	0.1	0.4
48-well plate (1 well)	1.0	0.3	30	0.3	0.3	1.2
24-well plate (1 well)	1.9	0.5	50	0.5	0.5	2
12-well plate (1 well)	3.8	1	100	1	1	4
35-mm dish	8	2	200	2	2	8
6-well plate (1 well)	9.4	2	200	2	2	8
60-mm dish	21	5	500	5	5	20
10-cm dish	55	10	1000	10	10	40
T-25 flask	25	6	600	6	6	24
T-75 flask	75	20	2000	20	20	80

2.4 Troubleshooting

Observation	Possible Cause	Recommendation
Low Transfection Efficiency	Suboptimal X-tremeGENE HP DNA Transfection Reagent : DNA ratio	Titrate optimal X-tremeGENE HP DNA Transfection Reagent : DNA ratio. Refer to the text in Section 2.1 “Before you begin”.
	Insufficient number of cells	Determine optimal cell density for each cell type. For most cell types, 70 – 90% confluence at transfection is optimal.
	X-tremeGENE HP DNA Transfection Reagent : DNA complexes did not form well	Prepare complexes in serum-free medium (*e.g.,* Opti-MEM). Do not use siliconized pipet tips or tubes. Do not aliquot the X-tremeGENE HP DNA Transfection Reagent.
	Incubation time of transfection	Determine the optimal incubation time (18 - 72 h). Optimal for most cell types and plasmids is 24 – 48h.
	Inhibition by media components	Some media components (*e.g.,* polyanions) may influence the transfection.
	Low volume of X-tremeGENE HP DNA Transfection Reagent : DNA complex	The minimum amount of X-tremeGENE HP DNA Transfection Reagent to DNA complex is 100 µl. Complex formation at lower volumes may significantly decrease the transfection efficiency; refer to the text in Section 1, “Special Handling”.
High Cytotoxicity	Cell density not optimal	For each cell type, the optimal density should be determined. For most cell types, 70 - 90 % confluence at transfection is recommended, but other confluencies may increase cell viability.
	Cells are cultured in serum-free medium	Transfection using X-tremeGENE HP DNA Transfection Reagent in cells cultured in serum-free medium is possible, however, toxicity may be higher when serum is absent.
	X-tremeGENE HP DNA Transfection Reagent : DNA complexes and cells not mixed well	Add X-tremeGENE HP DNA Transfection Reagent dropwise to the cells. Gently rock the dish/plate back and forth and from side to side to evenly distribute the complexes.
	Plasmid preparation contaminated with endotoxin	Use highly purified, contaminant-free DNA for transfection.
	Transfected protein is cytotoxic or is produced at high levels	Reduced viability or slow growth rates may be due to high levels of protein expression, with cellular metabolism directed toward production of the heterologous protein. Note that the expressed protein may also be cytotoxic at the expressed levels.
	Too much transfection complex for number of cells	Increase the number of plated cells, and/or decrease the total amount of complex added to the cells.

Protein Extraction Protocol

1) Do all steps on ice or at 4^{o}.

2) Rinse cells with 1X PBS (1 ml, ice cold)

3) Add 0.5 ml lysis buffer/well

4) Incubate on ice for 20 min.

5) Scrape into microfuge tube

6) Transfer extract to Dounce homogenizer and homogenize with 10 strokes of Dounce homogenizer (tight pestle)

7) Transfer extract back to microfuge tube

8) Spin in microfuge in cold room for 10 min at max speed

9) Transfer supernatant to new tube. Save pellet.

Igepal-DOC lysis buffer
1% Igepal
0.4% deoxycholate
10mM Tris pH7.
6.6mM EDTA

-add 1X protease inhibitor cocktail before using lysis buffers

SDS polyacrylamide gel electrophoresis and Western Blot

Materials
BCA protein assay (Pierce Biotech, cat. #23225)
Mini-PROTEAN II gel apparatus (Biorad)
Costar gel-loading tips (Krackler Scientific, cat. #MN520R-LRS)
Prestained SDS-Page broadrange molecular weight standard (NEB, cat. #P7708S)
Trans-blot Semi-Dry Transfer cell (Biorad)
Immobilon-P membrane (Millipore, cat. #IPV00010)
heat-sealable bags (Kapak, cat. #TRS-95250)
Kodak Biomax light autoradiagraphy film, 13 x 18 cm (Perkin Elmer Life Sciences, cat. #868-9358)
Radtape (Diversified Biotech, cat. #RAD-10)

Reagents
TEMED (Sigma)
Tween-20 (Sigma)
Price Chopper non-fat dry milk
Isopropanol (Sigma)

Gel Buffers
Acrylamide solution (30% acrylamide/0.8% bis-acrylamide)
-dissolve 30g acrylamide (FW=71.08), 0.8g bis-acrylamide (FW=154.17) in a total volume of 100ml water. Filter through 0.2um filter.

4X Running Gel Buffer (1.5M Tris, pH 8.8)
-dissolve 36.3g Tris Base (FW=121.1) in 150ml water. Adjust to pH 8.8. Add water to total volume of 200ml. Filter through 0.2um filter.

4X Stacking Gel Buffer (0.5M Tris, pH 6.8)
-dissolve 12.1g Tris Base (FW=121.1) in 150ml water. Adjust to pH 6.8. Add water to total volume of 200ml. Filter through 0.2um filter.

10% SDS
-dissolve 10g SDS (FW=288.38) in a total volume of 100ml water.

10% Ammonium Persulfate
-dissolve 1.0g APS (FW=228.2) in a total volume of 10ml water. Store at -20˚C in 50-100ul aliquots.

1M Tris, pH 6.8
-dissolve 12.1g Tris Base (FW=121.1) in 80ml water. Adjust to pH 6.8. Add water to total volume of 100ml. Filter through 0.2um filter.

6X SDS Sample Buffer (0.375M Tris pH 6.8, 12% SDS, 60% glycerol, 0.6M DTT, 0.06% bromophenol blue)

-combine 3.75ml 1M Tris-Cl, pH 6.8, 6ml glycerol, 1.2g SDS (FW=288.38), 0.93g DTT (FW=154.2), 6mg bromophenol blue. Add water to total volume of 10ml. Store at -20˚C in 0.5ml aliquots.

2X SDS Sample Buffer (0.125M Tris pH 6.8, 4% SDS, 20% glycerol, 0.2M DTT, 0.02% bromophenol blue)

-combine 2.5ml 4X Tris pH 6.8 (stacking gel buffer), 2ml glycerol, 4ml 10% SDS (FW=288.38), 0.31g DTT (FW=154.2), 2mg bromophenol blue. Add water to total volume of 10ml. Store at -20˚C in 0.5ml aliquots.

1X SDS Sample Buffer (0.0625M Tris pH 6.8, 2% SDS, 10% glycerol, 0.1M DTT, 0.01% bromophenol blue)

5X Electrophoresis Buffer (0.125M Tris, 0.96M glycine, 0.5% SDS)

-dissolve 30.3g Tris Base (FW=121.1), 144.1g glycine (FW=75.07), and 10g SDS (FW=288.38) in a total volume of 2L water.

1X Electrophoresis Buffer (0.025M Tris, 0.192M glycine, 0.1% SDS)

-add 200ml 5X electrophoresis buffer to 800ml water.

Western Blot Buffers

Transfer Buffer (0.048M Tris, 0.039M glycine, 20% methanol, 0.00375% SDS)

-dissolve 11.64g Tris Base (FW=121.1), 5.86g glycine (FW=75.07) in ~1500ml water. Add 0.750ml 10% SDS (FW=288.38). Add 400ml methanol. Add water to total volume of 2L. No pH adjustment necessary.

10X TBST Wash Buffer (0.1M Tris HCl, 1.5M NaCl, 0.5% Tween-20)

-dissolve 31.52g Tris HCl (FW=157.6), 175.32g NaCl (FW=58.44) in ~1900ml water. Add 10ml Tween-20. Adjust to pH 7.2. Add water to total volume of 2L.

1X TBST Wash Buffer (0.01M Tris HCl, 0.15M NaCl, 0.05% Tween-20)

-add 100ml 10X TBST to 900ml water.

5% milk Blocking Buffer

-Dissolve 5g milk in a total volume of 100ml 1X TBST.

Method

1. Determine cell number or protein concentration to be loaded onto gels and what volume to load per well. Note max volumes for different comb configurations.

2. Usually add sample to Tris or PBS and then add sample buffer (2 ul sample + 18 ul PBS + 3.3 ul 6X sample buffer).

3. Sonicate cells briefly on ice to homogenize. Purified protein samples do not need to be sonicated.

4. Preparing resolving and stacking gels (for BioRad Mini-PROTEAN II): Make sure glass plates are clean. Use Sparkleen or Alconox powder to clean plates. Rinse with distilled water and then 95% EtOH. Wipe dry with KimWipes. Select a comb and spacer (ex. 0.75 mm). Place spacers in between the inner and outer glass plates. Make sure that the spacers are flush with the plates. Insert plates into the clamping assembly and tighten screws. Vacuum grease can be used on the casting stand gaskets to prevent buffer leakage. Snap assembly into the casting stand. Prepare resolving gel. Pour gel and overlay with 1X Electrophoresis Buffer or isopropanol (use 3.5ml resolving gel when using 0.75mm spacers and 7.0ml gel for 1.5mm spacers, this will produce a gel approx. 3.25" x 2"). Look for a division between the overlay and resolving gel, this means the gel is polymerized. Pour off overlay, use thin strips of Whatman paper to remove any excess. Pour stacking gel and insert comb. Remove comb when stacking gel is polymerized. Use a syringe and a 12 gauge needle to wash stacking gel wells. Snap clamp assembly into electrode assembly. Vacuum grease can be used on electrode assembly gaskets to prevent buffer leakage.

5. Boil samples 5 minutes at 100°C, and then spin tubes at 13,000rpm for ~1min. in RT micro centrifuge. Load samples using Costar Gel-Loading Tips.

6. Place electrode assembly with loaded samples into an electrophoresis chamber. Fill the electrode assembly carefully with 1X Electrophoresis Buffer. Fill the electrophoresis chamber with 1X Electrophoresis Buffer. Run gels at 100V for stacking gel, 100-150V for resolving gel (can increase to 200V for resolving gel if running on ice).

7. To transfer mini- gels to Immobilon-P membrane using the BioRad Semi-Dry Transfer Cell: Remove gels from glass plates. Cut stacking gel away and discard. Incubate gels in Transfer Buffer for 20 - 40 min. Cut out 6 pieces of Whatman paper and 1 piece of Immobilon-P membrane for each gel that is being transferred. They should be 3.25" x 2". Incubate Immobilon-P membrane in Methanol for ~10 - 20 seconds. Wet Immobilon-P membrane and Whatman paper in Transfer Buffer for 5 - 10 minutes before assembling the gel sandwich according to the BioRad Reference Guide. Transfer 1 mini-gel for 15 min. at 15 V, max current. Transfer 2 mini-gels for 30 min at 15 V, max current. Make sure that pre-stained MW markers transfer to Immobilon-P membrane.

8. Western Blot:

-Block membrane in 20ml blocking buffer overnight at 4°C or 1h at room temperature on rocker in a seal-a-meal bag.
-Dilute Ab in blocking buffer (1:1000 for serum or 5ug / 10ml for purified Ab). Incubate blots with Ab for 1h at rom temperature or overnight at 4°C on rocker in a seal-a-meal bag.
-Wash blots with 50ml 1X TBST on orbital shaker 3 times, 5 minutes each.
-Dilute HRP conjugated Ab 1:10,000. Incubate blots with secondary Ab for 1h at room temperature or overnight at 4°C on rocker in a seal-a-meal bag.
-Wash blots with 50ml 1X TBST on orbital shaker 3 times, 5 minutes each.
-Add 5ml chemiluminescent substrate to each blot, incubate for 5 minutes. Place blots in between transparency film. Push out air bubbles. Develop Western blot.

Do a 1 minute exposure to check signal intensity. Continue with longer or shorter exposures as needed.
(Max. exposure length = 60 min. Min exposure length = 1 sec.)
-For high backgrounds, wash in TBST then re-develop.

Resolving Gel

	7.5% gel (15ml)	10% gel (15ml)	15% gel (15ml)
Water	7.5ml	6.25ml	3.75ml
30% acrylamide/ 0.8% bis-acrylamide	3.75ml	5ml	7.5ml
4X Running Buffer	3.75ml	3.75ml	3.75ml
10% SDS	150ul	150ul	150ul
10% APS	50ul	100ul	50ul
TEMED	10ul	20ul	10ul

Running Gel: 7.5, 10, or 15% acrylamide, 0.375M Tris-Cl pH 8.8, 0.1% SDS, 0.33% APS, 0.66% TEMED

Stacking Gel

	4% gel (10ml)
Water	6.1ml
30% acrylamide/ 0.8% bis-acrylamide	1.3ml
4X Stacking Buffer	2.5ml
10% SDS	100ul
10% APS	100ul
TEMED	40ul

Stacking Gel: 4% acrylamide, 0.125M Tris-Cl pH 6.8, 0.1% SDS, 0.05%APS, 0.2% TEMED

Protocol for non-permeabilizing IHC.

1. Wash cells with 0.5ml 1X PBS
2. Fix cells for 10 minutes at RT with 0.5ml 4% paraformaldehyde in 1X PBS
3. Aspirate PFA and add 0.5ml 10mg/ml Glycine in 1X PBS for 10 minutes
4. Block cells for 20 minutes with 0.5ml 5% BSA in 1X PBS
5. Wash with 0.5ml 1X PBS.
6. Incubate with primary antibody for 60 minutes. Antibody concentration is 1.0 µg/ml in 0.5ml PBS with 5% BSA.
7. Wash with three 0.5ml changes of PBS for 5 minutes each.
8. Incubate for 45 minutes with secondary antibody diluted to 1µg/ml in 0.5ml PBS with 5% BSA.
9. Wash with three 0.5ml changes of PBS.
10. Mount coverslip with Vectashield or 90% glycerol in PBS with n-propyl galate (about 10ml).
11. Store slides in a dark location at 4° C