Chapter 7
Production of Olfactory Receptors Using Commercial *E. coli* Cell-free Systems

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Abstract The first bottleneck in olfactory receptor (OR) structural and functional studies is to produce sufficient quantities of soluble, functional, and stable receptors. Other production systems have been used and summarized in other chapters of this book. We here show that commercial cell-free *in vitro* translation systems can be used to produce milligrams of soluble and functional olfactory receptors within several hours directly from plasmid DNA with select optimal detergents. These olfactory receptors can be purified using immunoaffinity 1D4 monoclonal antibody rhodopsin-tag and gel filtration, and can be analyzed using gel electrophoresis and with other standard techniques. The olfactory receptors and other scent-related receptors produced by the cell-free method fold properly and are able to bind their odorants.

7.1 Production of Olfactory Receptors is Required for Study and New Technology

The molecular basis of olfaction is poorly understood, primarily due to the extreme difficulty of producing sufficient quantities of soluble and functional olfactory receptors (ORs). Olfactory receptors belong to the G protein-coupled receptor family, which is characterized by seven transmembrane helical segments arranged in a barrel-like conformation. These transmembrane regions, which include the receptor-binding pocket, can cause problems with protein expression, and make it difficult to functionally stabilize the receptors outside of their native membrane environment. Receptor production in eukaryotic or bacterial cells frequently encounters problems such as low yields, cell toxicity, protein degradation, protein inhomogeneity and aggregation in internal compartments or inclusion bodies [1–5]. Cell-free *in vitro* translation is an alternative and enabling method allowing for rapid, cost-effective, high-yield protein expression [6–11]. The produced olfactory receptor proteins can

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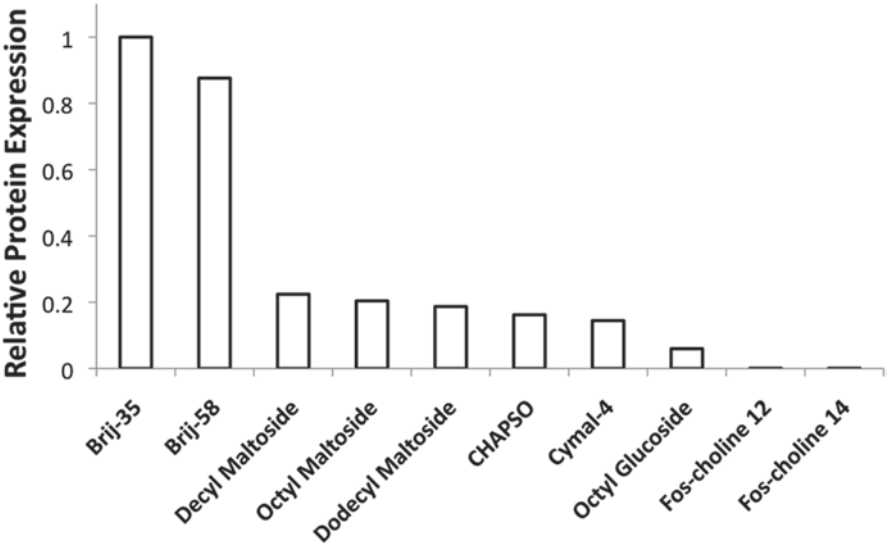

Fig. 7.1 Detergent screen for cell-free GPCR production. Detergent screen showing the relative expression of hOR17-210 in 10 different detergents. The Brij35 and Brij58 detergents yielded 4–5 times more protein than the next best detergent that was tested [8]

be purified using the affinity column chromatography of a monoclonal antibody 1D4 to very high purify [12–17], followed by a conventional gel filtration chromatography.

7.2 Cell-Free Olfactory Receptor Protein Production

Cell-free expression is an established technology for producing soluble proteins. This can be adapted for membrane proteins by including an appropriate detergent in the reaction mixture [7–11] (Fig. 7.1). Indeed, using the optimal detergent, it is possible to use cell-free systems to rapidly produce milligram quantities of receptors within several hours directly from plasmid DNA. Immunoaffinity chromatography and gel filtration chromatography can then be used to purify the expressed protein for structural and function studies (Fig. 7.2) [12–17].

Our study demonstrates that cell-free membrane protein production is a useful technology for expressing milligrams of olfactory receptors and other GPCRs (Table 7.1). The receptors could be purified to ~90% purity using immunoaffinity chromatography alone. CD measurements on a subset of purified olfactory receptors and other GPCRs showed that they had the predicted secondary structures, which suggests that they were properly folded (Fig. 7.3). Microscale thermophoresis indicated that the cell-free produced olfactory receptors and other GPCRs were functional by showing that the purified receptors could bind their

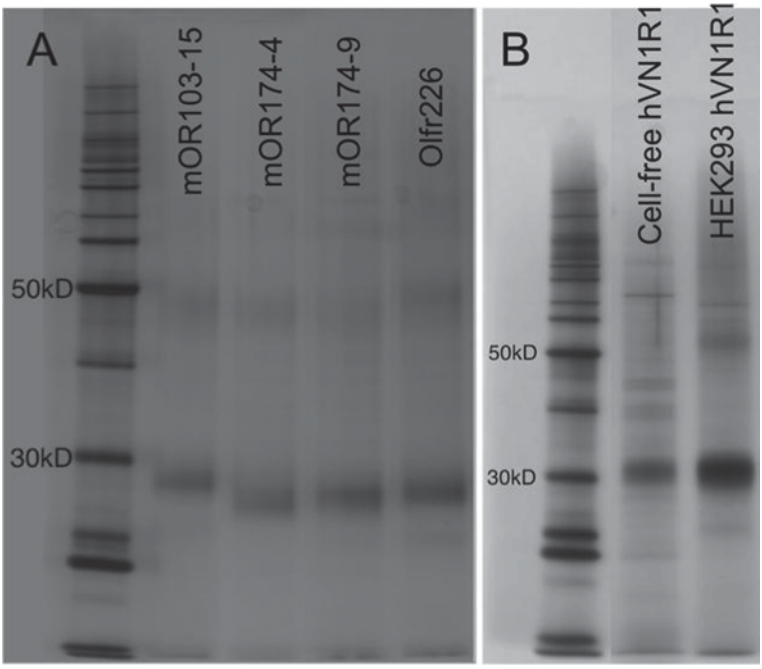

Fig. 7.2 Silver stains of purified olfactory receptors. **a**) Four cell-free expressed olfactory receptors. **b**) Comparison between cell-free and HEK293 expressed scent-related hVN1R1. Most olfactory receptors could be purified to >90% purity, and all showed two *bands* characteristic of a monomer and a dimer. The cell-free and HEK293 expressed receptors run at the same size, and have similar purities

reported small-molecule ligands (Fig. 7.4). Comparison of HEK293 and cell-free expressed protein (Figs. 7.2, 7.3 and 7.4) suggests that cell-free systems are a practical alternative to cell-based platforms for producing olfactory receptors and other GPCRs.

Although cell-free production is a mature technology for soluble proteins, very few membrane proteins have been produced, largely due to the lack of suitable detergents, requiring laborious detergent screens. We found that Brij-35 seemed to consistently be the optimal detergent for olfactory receptors and scent-related GPCRs. Brij-35 may not be optimal for all membrane proteins or GPCRs; the Brij family of detergents may function best with cell-free olfactory receptors and scent-related GPCRs and perhaps other membrane protein expressions. While the best detergent for protein production may not be the best detergent for downstream applications, we have shown that a single detergent exchange with FC14 is possible without compromising olfactory receptor's and other GPCR's structure and function. Since FC14 has been used to obtain protein structures, it should be possible to couple cell-free expression with crystal screens or NMR structural studies.

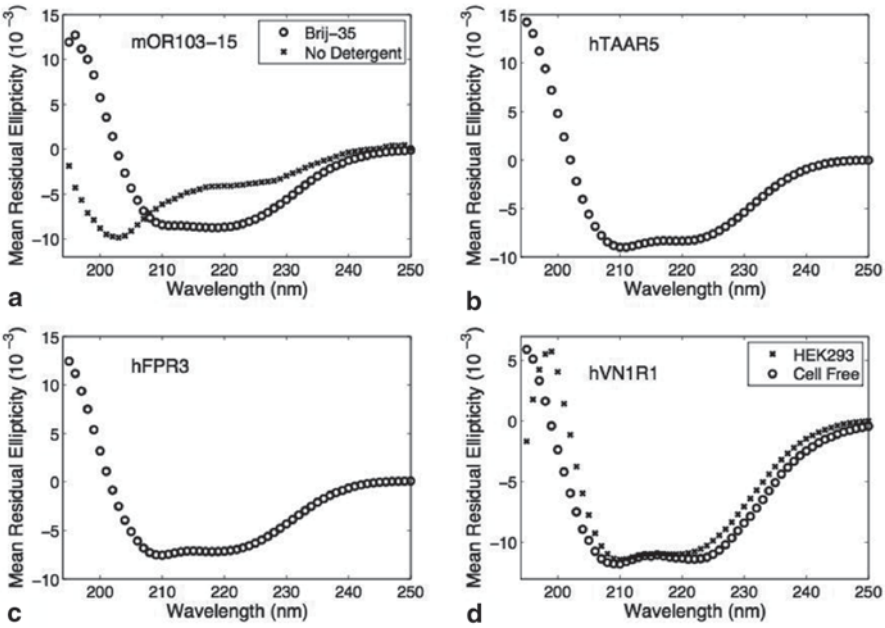

Fig. 7.3 Circular dichroism spectra of 5 purified scent-related GPCRs**. a**) Cell-free expressed mouse olfactory receptor mOR103-15 made with Brij-35 or no detergent, **b**) Cell-free expressed hTAAR5, **c**) Cell-free expressed hFPR3, and **d)** Cell-free and HEK293 expressed hVN1R1. All purified GPCRs have characteristic alpha-helical spectra, except mOR103-15 made without detergent. Since GPCRs have 7-transmembrane helices, and an overall α-helix content of ~50%, the CD spectra suggest that these receptors are properly folded. The near overlap of the spectra for cell-free and HEK293 expressed hVN1R1 suggests that both receptors are properly folded, and further indicates that cell-free produced scent-related receptors are comparable to those expressed in mammalian cells

In order to accelerate membrane protein structure and function studies, it is absolutely vital to develop simple, straightforward methods of producing sufficient quantities of membrane proteins. Commercial cell-free kits offer an attractive alternative to cell-based systems. Milligrams of protein can be produced within hours directly from plasmid DNA. The produced proteins can be purified quickly using conventional methods, and are amenable to detergent exchange for downstream applications. Using commercially available kits, the necessary reagents are easily and widely available, and results are reproducible. Although the 13 olfactory receptors and other scent-related GPCRs represent a small fraction of all receptors, it is the largest number presented in a single study with the same cell-free production method. Our ability to produce significant quantities of olfactory receptors and other GPCRs using commercial cell-free systems demonstrates the usefulness of this technology in the field. Indeed, the critical production bottleneck in membrane protein studies may potentially be overcome. Structure and function studies of olfactory receptors and other GPCRs may be stimulated and accelerated in the coming years.

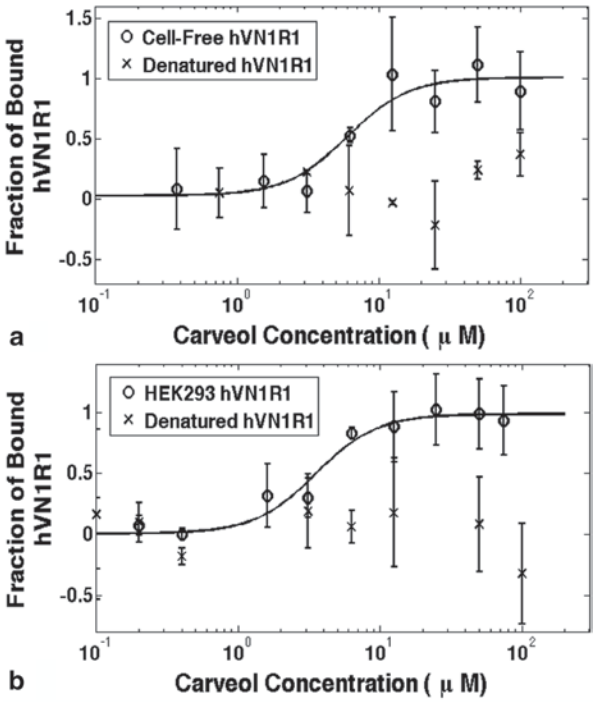

Fig. 7.4 Microscale thermophoresis measurements of purified GPCRs. **a**) Cell-free expressed hVN1R1 with and without heat-denaturation. **b**) HEK293 expressed hVN1R1 with and without heat-denaturation. The non-denatured receptors show typical sigmoidal binding curves, with plateaus at low and high concentrations. Cell-free expressed hVN1R1 has an EC_{50} of 6±2 μM, and HEK293 expressed hVN1R1 has an EC_{50} of 3.5±0.7 μM. The heat-denatured controls had flat responses or random amplitudes throughout the ligand titration range. These results show that hVN1R1 is binding carveol. Furthermore, the similar EC_{50} values and binding curves in **a**) and **b**) demonstrate that cell-free produced receptors function as well as HEK293 expressed receptors. The curves were normalized to the fraction of bound receptor. Each data point represents the mean of 3 independent experiments; error *bars* show the standard deviation. The binding curves were fit to the Hill equation. The binding results shown are representative of the data from other binding measurements

7.2.1 Reagents for Cell-Free Expression in Test Tubes

1. *E. coli* lysate (RiNA GmbH, http://rina-gmbh.eu/, Qiagen and Life Technologies). The lysate should be stored at −80 °C, thawed on ice, and used within 4 h of defrosting. It can be re-frozen and thawed once for optimal results. Repeated freezes and thaws of the lysate may not produce reproducible results. Small aliquots should be made from larger volumes of lysate for future uses.
2. Reaction buffer. The buffer should be stored at −80 °C, thawed on ice, and used within 4 h of defrosting. It can be re-frozen and thawed once for optimal results.

Table 7.1 Solubility and maximum yields of GPCRs produced using cell-free *in vitro* translation in the presence of Brij-35

GPCR	% solubility	Yield (mg)[a]	GPCR %	Solubility	Yield (mg)[a]
Olfr226	86±8	3.7	hOR17-209	88±4	2.5
mOR33-1	85±2	5.9	hOR17-210	91±2	4.5
mOR103-15	90±4	4.5	hFPR3	83±5	5.5
mOR106-13	86±13	2.4[b]	hTAAR5	90±1	4.5
mOR174-4	89±2	2	hVN1R1	88±0.1	0.4
mOR174-9	86±3	6	hVN1R5	85±2	1[b]
mOR175-1	81±8	2.5			

[a] Milligrams of receptor that could be produced in a 10 ml cell-free reaction. These yields were calculated from smaller batches of protein purified using immunoaffinity chromatography. Experiments showed that up to 1 mg/ml of protein could be produced, but that up to half could be lost during the purification process. The yields were determined by spectrophotometer readings

[b] These yields were calculated by comparing the intensities of the receptor samples against a sample with a known concentration

Aliquots can be made for larger volumes of buffer. The specific buffer varies with each kit, but contains all essential amino acids, RNA polymerases, ribosomes, elongation factors, etc.

3. Sterile, DNase-free and RNase-free water
4. Non-ionic Detergent Brij-35 [2% (10X) or 10% (50X) concentration]
5. Olfactory receptor gene ligated into the pIVex2.3d plasmid vector (Life Technologies)

7.2.2 Cell-Free Protein Production

1. Thaw the *E. coli* lysate, reaction buffer, and DNA on ice.
2. Add 175 µl of the *E. coli* lysate to a sterile, DNAse-free, RNAse-free Eppendorf tube Add the plasmid to the lysate so that the final DNA concentration is 1 µg/100 µl.
3. Add DNAase-free and RNAse-free water so that the final volume of the cell-free reaction will be 500 µl.
4. Add Brij-35 to a final concentration of 0.2% w/v.
5. Add 200 µl of the reaction buffer, and mix thoroughly with a pipette.
6. Briefly (~5 s) spin down the Eppendorf tube.
7. Place the cell-free reaction in an Eppendorf rack in a shaking incubator at 33 °C and 250 rpm for 1 h.
8. After the reaction is complete, spin it down in a microcentrifuge for 5 min at 10,000 rpm.
9. Carefully transfer the supernatant to a fresh tube without disturbing the pellet. The supernatant contains solubilized receptor.
10. The synthesized receptors can be purified or run on an SDS-PAGE gel immediately, or stored at −20 °C for longer periods of time.

7.3 Olfactory Receptor Purification

After production of olfactory receptors, they need to be purified from the cell-free system for subsequent studies and uses. We adopted an affinity purification system using a rhodopsin tag that was developed by Khorana and colleagues [18]. This monoclonal antibody-coupled bead has very high specificity and binding capacity. In most cases, a single step can purify olfactory receptor and other rho-tag proteins to near homogeneity [9–17]. Before affinity purification, the special antibody-coupled beads need to be prepared. It is described below.

7.3.1 Monoclonal Antibody Rho1D4 Bead Coupling

1. Rho1D4 monoclonal antibody (Cell Essentials, http://www.cell-essentials.com/) at 2–8 mg/ml (Cell Essentials, hybridoma 1B4-1) in coupling buffer (0.25 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, in milliQ water). If the antibody is not in the correct coupling buffer upon arrival, it must first be first dialyzed into the coupling buffer above. Otherwise, it will not couple very well and with low yield.
2. CNBr-activated Sepharose 4B (GE Healthcare)
3. Coupling buffer: 0.25 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, in milliQ water
4. HCl buffer: 1 mM HCl in milliQ water
5. Ethanolamine buffer: 1 M ethanolamine, pH 8.0 in milliQ water
6. Acetate buffer: 0.1 M NaOAc, 0.5 M NaCl, pH 4.0 in milliQ water
7. Sodium azide buffer: 0.05 % NaN_3 in PBS, pH 7.2

7.3.2 Method for Rho1D4 Monoclonal Antibody-Sepharose Bead Coupling

1. Suspend the sepharose beads in HCl buffer. The hydrated beads will swell: 1 g of bead powder will yield approximately 3.5 ml of bead slurry.
2. Wash the beads for 15 min with the HCl buffer in a sintered glass funnel and vacuum flask. The beads should be resuspended in the buffer as they fall out of solution. After ~1 min, a vacuum should be applied until the liquid is removed. Do not over dry the beads. Approximately 200 ml of HCl buffer should be used per gram of bead powder. Several aliquots may be necessary.
3. Add the washed beads to the antibody in coupling buffer. Add 20 ml of bead slurry to 130–200 mg of antibody. The ratio should be 5–10 mg of antibody per ml of bead slurry.
4. Rotate the slurry/antibody solution at 4 °C until the antibody is bound to the beads. The antibody concentration in the supernatant can be monitored by measuring its absorbance at 280 nm. When the concentration is below 5 % of the

original concentration, the binding reaction is complete. This procedure takes 4 h to overnight.
5. Remove the supernatant after the binding reaction is complete by spinning down the beads for 5 min at 2,000 rpm.
6. Remove excess antibody by washing the beads with 5 slurry volumes of coupling buffer.
7. Block remaining active groups with the ethanolamine buffer. Add a volume equal to the original supernatant volume, and rotate overnight at 4 °C or 2 h at room temperature.
8. Remove excess uncoupled antibody by washing the beads 4 times alternating between coupling buffer and acetate buffer. Use a sintered glass filter, and a wash volume at least 5 times the original slurry volume.
9. Suspend the beads in 1 slurry volume of sodium azide buffer, and store them at 4 °C.

7.3.3 Affinity Olfactory Receptor Purification Using Rho-Tag Monoclonal Antibody

1. Rho1D4 monoclonal antibody-coupled sepharose beads
2. DPBS (Life Technologies, 14190-250)
3. DNase1 (Life Technologies**,** 18047-019)
4. RNaseA (Life Technologies, 12091-039)
5. Sterile filtered water (0.22 μm) with a resistivity of at least 18 MΩ-cm.
6. Wash buffer: 0.2 % fos-choline-14 (FC14) (Anatrace/Affymatrix). This is made from a 10 % FC14 stock solution in DPBS.
7. Elution buffer: 800 μM elution peptide Ac-TETSQVAPA-NH_2 (with an acetylated N-terminus and amidated C-terminus) dissolved in wash buffer.
8. High pH buffer: 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5.
9. Low pH buffer: 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5.

7.3.4 Olfactory Receptor Purification Using 1D4 Rho-Tag Monoclonal Antibody

1. Pipette the necessary amount of antibody-coupled beads into a fresh tube. Mix the beads first by gently shaking them to ensure that they are homogeneously suspended. The binding capacity of fresh beads is ~0.7 mg/ml, and the capacity of regenerated beads is ~0.35 mg/ml.
2. Wash the beads with DPBS to remove excess sodium azide. Spin the beads down at 1,400 rpm for 1 min, and then let them sit for a minute to allow them to completely settle to the bottom of the tube. Using a pipette, slowly remove the supernatant without disturbing the bead pellet. Add one bead volume of DPBS

to re-suspend the pellet. Repeat this process three times. After the last repetition, do not add more DPBS.

3. Add the supernatant from the cell-free reaction to the washed beads.
4. Add 1 µl of DNAse and 1 µl of RNAse for each ml of cell-free reaction volume.
5. Rotate the supernatant with the beads overnight at 4 °C to capture the synthesized protein.
6. After the overnight rotation, spin the beads at 1,400 rpm for 1 min, and let them sit for 1 min to allow the bead pellet to settle. Remove the supernatant and transfer it to a tube labeled FT (Flow Thru). Save a small sample of the FT for analysis, and freeze the remainder at −80 °C in case the beads did not capture all of the synthesized receptors. Add one bead volume of wash buffer to the beads, and rotate at 4 °C for 10 min.
7. Wash the OR-bound beads to remove any impurities. For each wash, spin the tube at 1,400 rpm for 1 min, and allow it to sit for 1 min. Carefully remove the supernatant without disturbing the bead pellet, and transfer it to a fresh tube (labeled Wash 1, Wash 2, etc). Add 1 bead volume of wash buffer, and rotate at 4 °C for 10 min. Repeat this process until the absorbance at 280 nm of the removed supernatant is less than 0.01 mg/ml. Typically, 13–20 washes are required. The washes can be run overnight at 4 °C if necessary.
8. Elute the synthesized ORs from the beads. Add one bead volume of elution buffer to the beads, and rotate at room temperature for 1 h. Spin the beads at 1,400 rpm, and let them sit for 1 min. Carefully remove the supernatant without disturbing the bead pellet, and transfer it to a fresh, clean tube (labeled Elution 1, Elution 2, etc). Repeat this process until the absorbance of the removed supernatant at 280 nm is less than 0.01 mg/ml. The supernatant contains the synthesized receptors. Typically, 5–7 elutions are required. Any of the elutions can be run overnight at 4 °C if necessary.
9. The washes and elutions can be stored at 4 °C until they are ready for use.
10. The elutions can be pooled and concentrated in centrifugal units with 50 kDa molecular weight cut-off filters. If residual elution peptide must be removed (i.e. for circular dichroism), then the protein must be washed on the centrifugal units with excess wash buffer. 10 ml of wash buffer is usually sufficient to remove elution peptide from a concentrated protein sample with a total volume of 300 µl. If the receptors will be run on a size exclusion column, they must be concentrated to a volume that will fit in the loading loop. The receptors should be concentrated immediately prior to being loaded on the column to minimize aggregation and precipitation.
11. Used beads can be regenerated for re-use by washing them with 2–3 column volumes of alternating high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5) buffers. This cycle should be repeated 3 times followed by re-equilibration in binding buffer.

7.4 Gel Filtration for Further Purification

In order to separate the monomers from dimers, trimmers and multimers, it is important to further purify the olfactory receptors using conventional gel filtration method. This is to fractionate dimers, trimers and multimers for subsequent studies.

7.4.1 Materials and Reagents Required for Gel Filtration

1. Amicon Ultra Centrifugal Filter Units with Ultracel membranes (Millipore, 50 kDa MWCO membranes)
2. Sterile 96-well plates with V-shaped bottoms, ~500 µl/well capacity
3. Wash buffer: 0.2% FC14 in DPBS, sterile filtered through 0.22 µm filters

7.4.2 Gel Filtration Chromatography

1. Equilibrate the gel filtration column with at least 1–2 column volumes of wash buffer. We use a HiLoad 16/60 Superdex 200 column (GE Healthcare) on an ÄKTA Purifier FPLC system (GE Healthcare).
2. Load the freshly concentrated OR sample into the system.
3. Run the system at 0.3 ml/min, and monitor the UV absorbance at 215 nm and 280 nm. The monomeric form of our receptors typically exits the column at 60–65 ml. We collect the first 40 ml in a clean bottle. The remainder is collected in four 96-well V-bottom plates with 100 µl in each well.
4. Pool the appropriate eluted protein fractions together.
5. Concentrate the pooled fractions to the desired volume or concentration, and store them at 4 °C until they are ready for further analysis. Samples can be kept at −80 °C for long-term storage and should only be thawed once as repeated freeze-thaw cycles can induce protein aggregation.

7.5 Notes

1. Brij-35 has been the optimal detergent in our experiments. However, other groups have found other detergents to be optimal for their GPCRs, especially other polyoxyethylenes related to Brij-35 [7]. A preliminary detergent screen in which the cell-free reaction volumes are scaled down to a total volume of 25–50 µl may be necessary.
2. The olfactory receptor genes have a 5' NcoI site and a 3' XhoI site for ligation. They also have a C-terminal bovine rho1D4 epitope tag (TETSQVAPA) for puri-

fication followed by a double stop codon, and have potential glycosylation sites removed. The codons were optimized for *E. coli* expression.
3. The volumes listed here are for a total reaction volume of 500 µl. The volumes can be scaled up (to 5 ml) and down (50 µl) as necessary.
4. These are the optimal temperature, time, and rotation speed for the receptors we tested. We did not notice significant differences in olfactory receptor yield with longer incubation times (up to 6 h), rotation speeds up to 300 rpm, and at temperatures between 30 and 37 °C. However, the optimal conditions may vary with different receptors. In particular, lower temperatures can increase the yield of soluble receptor, while higher temperatures can increase the total receptor yield.

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References

1. Grisshammer R, Duckworth R, Henderson R (1993) Expression of a rat neurotensin receptor in *Escherichia coli*. Biochem J 295:571–576
2. Hampe W et al (2000) Engineering of a proteolytically stable human β2-adrenergic receptor/maltose-binding protein fusion and production of the chimeric protein in *Escherichia coli* and baculovirus infected insect cells. J Biotechnol 77:219–234
3. Sarramegna V, Talmont F, Demange P, Milon A (2003) Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification. Cell Mol Life Sci 60:1529–1546
4. McCusker EC, Bane SE, O'Malley MA, Robinson AS (2007) Heterologous GPCR expression: a bottleneck to obtaining crystal structures. Biotechnol Prog 23:540–547
5. Tate CG, Grisshammer R (1996) Heterologous expression of G-protein-coupled receptors. Trends Biotechnol 14:426–430
6. Ishihara G et al (2005) Expression of G protein coupled receptors in a cell-free translational system using detergents and thioredoxin-fusion vectors. Protein Expr Purif 41:27–37
7. Klammt C et al (2007) Cell-free production of G protein-coupled receptors for functional and structural studies. J Struct Biol 158:482–493
8. Kaiser L et al (2008) Efficient cell-free production of olfactory receptors: detergent optimization, structure, and ligand binding analyses. Proc Natl Acad Sci 105:15726–15731
9. Corin K et al (2011) A robust and rapid method of producing soluble, stable, and functional G-protein coupled receptors. PLoS ONE 6:e23036
10. Corin K et al (2011) Designer lipid-like peptides: a class of detergents for studying functional olfactory receptors using commercial cell-free systems. PLoS ONE 6:e25067
11. Wang X et al (2011) Peptide surfactants for cell-free production of functional G protein-coupled receptors. Proc Natl Acad Sci U S A 108:9049–9054
12. Takayama H et al (2008) High-level expression, single-step immunoaffinity purification and characterization of human tetraspanin membrane protein CD81. PLoS ONE 3:e2314
13. Cook BL et al (2008) Study of a synthetic human olfactory receptor 17-4: expression and purification from an inducible mammalian cell line. PLoS ONE 3:e2920
14. Cook BL et al (2009) Large-scale production and study of a synthetic GPCR: human olfactory receptor 17-4. Proc Natl Acad Sci U S A 106:11925–11930
15. Wang X et al (2011) Study of two G-protein coupled receptor variants of human trace amine-associated receptor 5. Sci Rep 1:e102

16. Corin et al (2011) Biochemical study of a bioengineered functional G-protein coupled receptor human vomeronasal type 1 receptor 1. Sci Rep 1:e172
17. Corin et al (2012) Insertion of T4-lysozyme (T4L) can be a useful tool for studying olfactory-related GPCRs. Mol Biosyst 8:1750–1759
18. Reeves PJ, Thurmond RL, Khorana HG (1996) Structure and function in rhodopsin: high-level expression of a synthetic bovine opsin gene and its mutants in stable mammalian cell lines. Proc Natl Acad Sci 93:11487–11492