Chapter 7

A Robust, Rapid, and Simple Method of Producing Olfactory Receptors Using Commercial *E. coli* Cell-Free Systems

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Abstract

The first bottleneck in olfactory receptor (OR) studies is producing sufficient quantities of soluble, functional, and stable receptors. Commercial cell-free in vitro translation systems can be used to produce milligrams of soluble and functional receptors within several hours directly from plasmid DNA. The receptors can be purified using immunoaffinity chromatography and gel filtration, and can be analyzed using gel electrophoresis and with other standard techniques.

Key words In vitro translation, Olfactory receptors, Odorant ligand-binding, Microscale thermophoresis

1 Introduction

The molecular basis of olfaction is poorly understood, primarily due to the difficulty of expressing sufficient quantities of soluble and functional olfactory receptors (ORs). ORs 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 productions in eukaryotic or bacterial cells frequently encounter 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 allowing for rapid, cost-effective, high-yield protein expression (6–11).

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. 1). Indeed, by 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.

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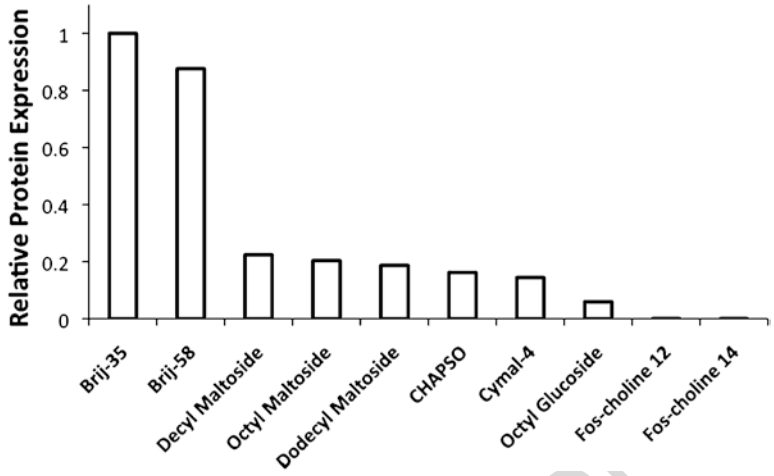

Fig. 1 Detergent screen showing the relative expression of hOR17-210 in ten different detergents. The Brij35 and Brij58 detergents yielded four to five times more protein than the next best detergent that was tested (8)

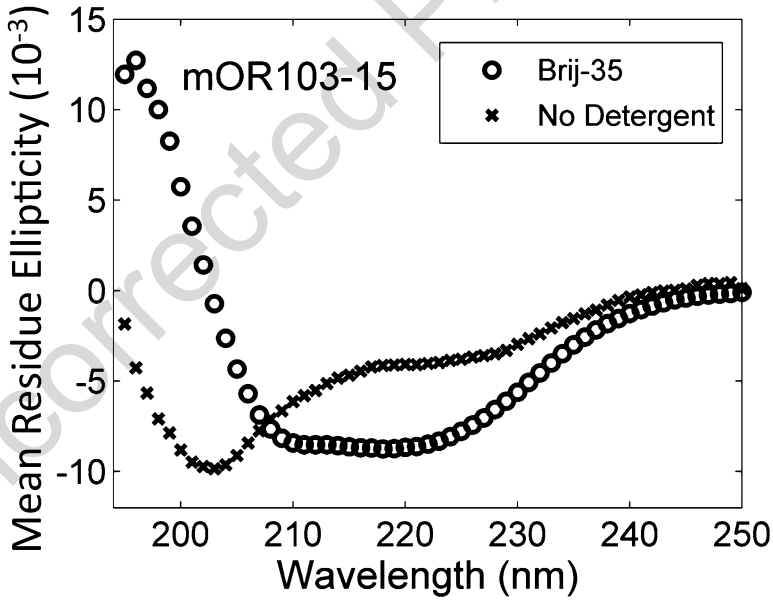

Fig. 2 Circular dichroism scan of mOR103-15 expressed in a cell-free reaction with and without detergent. The receptors expressed in a cell-free system could be purified and analyzed using circular dichroism. The scan showed a detergent is necessary for proper folding. Without a detergent, the purified protein has a random coil secondary structure. With a detergent (Brij-35), the secondary structure is helical, as expected for a GPCR (8)

Immunoaffinity chromatography and gel filtration chromatography can then be used to purify the expressed protein for structural and functional studies (Fig. 2).

2 Materials

2.1 Cell-Free Expression

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 refrozen and thawed once for optimal results. Aliquots can be made for larger volumes of lysate.
2. Reaction buffer. The buffer should be stored at −80 °C, thawed on ice, and used within 4 h of defrosting. It can be refrozen and thawed once for optimal results. 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. Brij-35 (see Note 1).
5. Olfactory receptor gene ligated into the pIVex2.3 vector (Life Technologies) (see Note 2).

2.2 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 coupling buffer upon arrival, it must first be dialyzed into the coupling buffer.
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.

2.3 Receptor Purification

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/Affymetrix). 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.

2.4 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.

3 Methods

Commercial cell-free expression kits can be used to produce milligrams of soluble, function, and stable olfactory receptors. The most significant factor affecting expression levels and solubility is the choice of detergent. For example, the Brij family of detergents has repeatedly yielded high levels of receptors, while the fos-cholines, which are optimal for purification from HEK293 cells, result in almost no receptor expression (7, 8). This is likely due to their non-ionic nature, as ionic detergents can interfere with transcriptional and translational machinery. Factors like incubation temperature and time typically had negligible effects on expression. Although the optimal expression conditions must be validated for each protein of interest, and the solubility and functionality of the expressed receptors analyzed, the following protocol has proven to be optimal for most of the receptors that we have tried to express.

3.1 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 (see Note 3).
3. Add the plasmid to the lysate so that the final DNA concentration is 1 µg/100 µl.
4. Add DNAase-free and RNAse-free water so that the final volume of the cell-free reaction will be 500 µl.
5. Add Brij-35 to a final concentration of 0.2 % w/v (see Note 1).
6. Add 200 µl of the reaction buffer and mix thoroughly with a pipette.
7. Briefly spin down the eppendorf tube.
8. Place the cell-free reaction in an eppendorf rack in a shaking incubator at 33 °C and 250 rpm for 1 h (see Note 4).

9. After the reaction is complete, spin it down in a microcentrifuge for 5 min at 10,000 rpm. [AU1]
10. Carefully transfer the supernatant to a fresh tube without disturbing the pellet. The supernatant contains solubilized receptor.
11. The synthesized receptors can be purified or run on an SDS-PAGE gel immediately, or stored at −20 °C for longer periods of time.

3.2 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 overdry 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. [AU2]
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 four times, alternating between coupling buffer and acetate buffer. Use a sintered glass filter and a wash volume at least five times the original slurry volume.
9. Suspend the beads in 1 slurry volume of sodium azide buffer and store them at 4 °C.

3.3 Receptor Purification

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 1 bead volume of DPBS to pellet to resuspend 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 1 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 1 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 cutoff 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 reuse 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 three times followed by re-equilibration in binding buffer.

3.4 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 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.

4 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 OR genes have a 5′ *Nco*I site and a 3′ *Xho*I site for ligation. They also have a C-terminal rho1D4 epitope tag (TETSQVAPA) for purification 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 and down as necessary.
4. These are the optimal temperature, time, and rotation speed for the receptors we tested. We did not notice significant differences in OR 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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AU2	Please provide the g-force value for 2,000 rpm.	
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