High-Efficiency Expression of Yeast-Derived G-Protein Coupled Receptors and 19F Labeling for Dynamical Studies

Chapter *in* Methods in molecular biology (Clifton, N.J.) · January 2018

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Chapter 19

High-Efficiency Expression of Yeast-Derived G-Protein Coupled Receptors and ^{19}F Labeling for Dynamical Studies

Libin Ye, Alexander P. Orazietti, Aditya Pandey, and R. Scott Prosser

Abstract

We describe a detailed protocol for heterologous expression of the human adenosine A_{2A} G-protein coupled receptor (GPCR), using *Pichia pastoris*. Details are also provided for the reconstitution and functional purification steps. Yields of 2–6 mg/g membrane were obtained prior to functional purification (ligand column purification). Typically, functional purification reduced overall yields by a factor of 2–4, resulting in final functional production of 0.5–3 mg/L membrane. Yeast is an excellent protein expression system for NMR given its high tolerance for isotope-enriched solvents and its ability to grow in minimal media.

Key words *P. pastoris*, GPCR, Membrane protein, Functional purification, $A_{2A}R$

1 Introduction

In eukaryotes, signal transduction associated with vision, sensory-, autonomic-, and neurotransmitter-mediated response, inflammation, and cell homeostasis are regulated by a diverse class of membrane receptors, known as G-protein coupled receptors (GPCRs) G-protein coupled receptors (GPCRs). There are over 800 human GPCRs, all of which possess a common 7-transmembrane motif [1]. Signal transduction is generally regulated by an extracellular ligand that when bound to the orthosteric binding pocket of the receptor establishes an allosteric path between the GPCR and intracellular signaling proteins such as G proteins or β-arrestins. One third of currently marketed drugs [2] target GPCRs and many drug discovery efforts are currently focused on their application to treat cancer, obesity, cardiac disease, and neurodegenerative diseases. Considering their location in the plasma membrane and their ubiquitous role in the regulation of signal transduction, there is an immense interest in understanding receptor and drug interactions.

Ranajeet Ghose (ed.), *Protein NMR: Methods and Protocols*, Methods in Molecular Biology, vol. 1688,
DOI 10.1007/978-1-4939-7386-6_19,

Despite their widespread interest to the medical community, progress in understanding the relationship between GPCR structure and function has been mired by challenges associated with obtaining high yield of functionally reconstituted receptor. The past decade has witnessed a renaissance for structural biology in terms of capacity to determine high-resolution crystal structures of GPCRs in complex with specific ligands or, most recently, binding partners such as G proteins and β-arrestins.

Methodological breakthroughs in X-ray crystallography have recently provided high-resolution structures of dozens of GPCRs since 2007, spanning representative functional classes [3]. In contrast to more than 50 unique GPCR X-ray crystal structures currently available, a single structure of a chemokine receptor, CXCR1, has been obtained by solid-state NMR [4]. Current NMR initiatives have thus far focused on the identification of mutations that improve fold stability (and spectroscopic resolution) at higher temperatures while addressing issues associated with heterologous expression that allow for the facile incorporation of ^{13}C,^{15}N, and ^{2}H isotopes [5–8]. Studies of related 7-transmembrane receptors suggest that the requisite resolution is at hand to allow for triple-resonance (^{1}H, ^{13}C, ^{15}N) experiments and subsequent structure studies by NMR [9–13]. However, the greatest challenge with each receptor is to identify a robust heterologous expression system that generates functional isotopically enriched receptors. Insect cells remain promising systems for the expression of GPCRs and many NMR initiatives are focused on the use of insect cells containing deuterated solvent and ^{15}N, ^{13}C, and ^{2}H-enriched amino acids derived from algal cells or yeast extract [14]. Insect cells nevertheless have a reputation for being prohibitively costly and less versatile when factoring in the cost of isotope-enriched media. The approach in our lab and others has been to make use of yeast (e.g., *Saccharomyces cerevisiae, Pichia pastoris, Kluyveromyces lactis,* and *Yarrowia lipolytica*) expression systems, in part owing to their high tolerance for D_2O, amenability to the introduction of inexpensive ^{13}C and ^{15}N sources [15–19], and high cell densities. Yeast expression systems are also relatively inexpensive, can accomplish post-translational modifications, and have been shown to provide very high yields of mammalian proteins, including GPCRs [20, 21].

Despite the technical challenges, there are several key advantages that NMR brings to the study of GPCRs. First, fusion constructs that are often employed in crystallography to obtain crystals, are generally not a part of the NMR regimen, and in many cases, it is possible to make use of wild-type sequences, assuming expression is not unduly compromised. GPCRs are also reputed to undergo both slow and fast exchange between multiple conformations, associated with distinct functional states [22–25]. While this complicates NMR structure studies and backbone assignments, the

spectroscopic characterization of specific functional states and their exchange rates greatly enriches our understanding of the conformational landscape and mechanisms associated with receptor activation. In particular, ^{19}F NMR is sensitive to subtle changes in electrostatic and van der Waals environment and thus to the activation process, allowing the characterization of the conformational landscape as a function of ligand and/or interacting species [22, 23, 26]. While ^{19}F-enriched amino acids (in particular, fluoro-aromatic residues) can in principle be incorporated into GPCRs [27], the majority of ^{19}F NMR spectroscopic studies of GPCRs have been accomplished via thiol-specific tagging of optimized fluorinated derivatives [28]. Most recently, in our lab, we succeeded in high-yield expression, ^{19}F-labeling, and reconstitution of two class A GPCRs using *Pichia pastoris* [29, 30]. Herein, using the adenosine A_2 receptor ($A_{2A}R$) as an example, we provide details of our *in-house* protocol associated with screening of transformants, expression, reconstitution in detergents, and purification of functional receptor for NMR sample preparation.

2 Materials

2.1 Materials and Apparatus Commonly Used for Molecular Biology

1. pPIC9K vector (*see* **Note 1**).
2. *Pichia pastoris* SMD1163 strain (*see* **Note 2**).
3. Glycerol.
4. 1 M ice-cold sterile sorbitol.
5. Geneticin® 418.
6. XL 10-Gold® ultracompetent *E. coli* cells.
7. YPD plate: 1% yeast extract, 2% peptone, 2% glucose, and 1.5% agar.
8. YPD broth: 1% yeast extract, 2% peptone, 2% glucose.
9. YNBD plate: 1.34% yeast nitrogen base without amino acid, 1% glucose, 4×10^{-5}% D-Biotin, and 1.5% agar.
10. *BamHI*-HF, *NotI*-HF, and *PmeI*-HF restriction enzymes.
11. T4 DNA ligase.
12. GenElute™ plasmid miniprep kit (Sigma-Aldrich).
13. 100% ethanol.
14. 1.5 mL Eppendorf tubes.
15. 2 mm electroporation cuvette.
16. 14 mL round-bottom Falcon tubes.
17. UV-vis spectrometer.
18. Spectrophotometer (NanoDrop™ 2000).
19. Electroporation system (Gene Pulser II from Bio-Rad).

2.2 Materials for Screening of Transformants

1. YPD plates with different concentrations of Geneticin® 418.
2. All materials for electroporation as listed in Subheading 2.1.
3. BMGY medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB without amino acids, 4×10^{-5}% (w/v) D-Biotin, 1% (w/v) glycerol, 0.1 M phosphate buffer at pH 6.5.
4. BMMY medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base without amino acids, 4×10^{-5}% (w/v) D-Biotin, 0.5% (w/v) methanol, 0.1 M phosphate buffer at pH 6.5, 0.04% (w/v) histidine and 3% (v/v) DMSO, 10 mM theophylline.
5. Methanol.
6. P1 buffer: 50 mM HEPES, pH 7.4.
7. Lysis buffer P2: 50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 100 U Zymolyase.
8. Anti-His6 peroxidase kit.
9. Nitrocellulose membranes.
10. Blocking buffer: 125 mM NaCl, 25 mM Tris base, pH 7.5, 0.3% Tween-20, and 3% nonfat milk.
11. Incubation buffer: Anti-His antibody diluted to 1:2000 with blocking buffer.
12. Washing buffer: 125 mM NaCl, 25 mM Tris base, pH 7.5, 0.3% Tween-20.

2.3 Materials for Functional Ligand Column Preparation

1. Xanthine amine congener ≥96% (Sigma-Aldrich).
2. DMSO.
3. Affi-Gel® 10 (BioRad).
4. Isopropanol.
5. Trisaminomethane (Tris).
6. Acetic acid.
7. NaN_3.

2.4 Materials for Cell Culture and Induced Expression

1. YPD plates.
2. YPD broth.
3. Buffered glycerol complex (BMGY) medium.
4. Buffered methanol complex (BMMY) medium.
5. Methanol.
6. Anti-foam A.

2.5 Materials for Membrane Preparation and Functional Purification

1. Buffer P1: 50 mM HEPES, pH 7.4
2. Lysis buffer P2: 50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 100 U Zymolyase, EDTA-free-proteinase inhibitor (Sigma-Aldrich).
3. Ultracentrifuge.
4. Buffer P3: 50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM theophylline, 10% glycerol, EDTA-free-protease inhibitor, solution of 1% (Lauryl Maltose Neopentyl Glycol) MNG (Anatrace), 0.2% cholesterol hemisuccinate (CHS), and 20 mM imidazole.
5. TALON® Metal Affinity Resin (Clontech Laboratories, Inc.).
6. Binding Buffer P4: 50 mM HEPES, pH 7.4, 100 mM NaCl, 2mM theophylline, 10% glycerol, EDTA-free protease inhibitor, solution of 0.1% MNG and 0.02% CHS, 20 mM imidazole.
7. Buffer P4+1: 50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM theophylline, 10% glycerol, EDTA-free protease inhibitor, solution of 0.1% MNG, and 0.02% CHS.
8. Buffer P5: 50 mM HEPES, pH 7.4, 0.1% MNG, 0.02% CHS, 100 mM NaCl.
9. Buffer P6: 50 mM HEPES, pH 7.4, 100 mM NaCl, solution of 0.1% MNG and 0.02% CHS, 250 mM imidazole.
10. Buffer P7: 50 mM HEPES, pH 7.4, 0.1% MNG, 0.02% CHS.
11. Buffer P8: 50 mM HEPES, pH 7.4, 0.1% MNG,0.02% CHS, 100 mM NaCl, 20 mM theophylline.

3 Methods

3.1 Gene Manipulation and Transformant Optimization

3.1.1 Preparation of Competent Cells

1. SMD1163 cells were streaked from a −80 °C stored glycerol stock onto YPD plates and incubated for 3–5 days at 30 °C before making competent cells (*see* **Note 3**).
2. A single colony from streaked YPD plates was inoculated into 10 mL YPD broth, and incubated with shaking at 275 rpm at 30 °C until an OD_{600} of ~3.0 was reached.
3. The 10 mL cell broth was then re-inoculated into 500 mL YPD broth and the OD_{600} was continuously monitored until such time that an OD_{600} of 1.0–1.3 was reached (typically requiring 20–24 h).
4. The cell pellets were collected into pre-autoclaved centrifugation tubes, upon centrifugation at 1500 × *g* for 5 min at 4 °C.
5. The cell pellets were washed twice with 10× autoclaved ice-cold distilled H_2O (*see* **Note 4**).

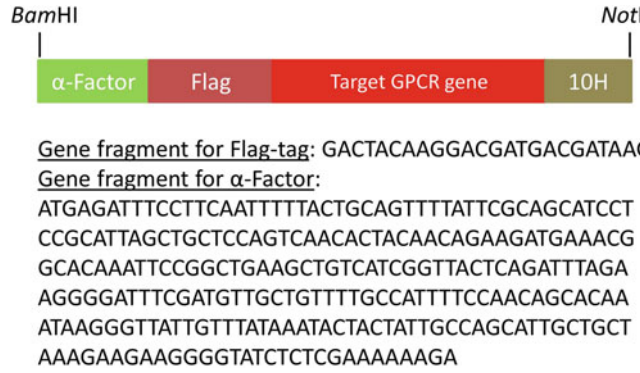

Fig. 1 Gene fragment inserted into the pPIC9K vector, containing the target gene, surrounded by α-Factor signal peptide, FLAG-tag, and His_6-tag

6. The final cell pellets were resuspended in 1 mL 1 M sterile ice-cold sorbitol (*see* **Note 5**).
7. 80 μL of resuspended cells were aliquoted into 1.5 mL pre-chilled sterile Eppendorf™ tubes, for electro-transformation (*see* **Note 6**).

3.1.2 Preparation of Linearized Plasmid Containing Target Gene

Prior to electroporation, the plasmid should be ready in accordance with the following procedure.

1. The target gene fragment (Fig. 1) including the target adenosine A_{2A} (2-317) receptor [23] gene, a fragment of *Saccharomyces cerevisiae* derived from N-terminal α-Factor signal peptide (α-Factor), an N-terminal FLAG-tag, and a C-terminal His_{10}-tag gene fragment were ligated by multi-PCR reactions.
2. Both the products from the PCR reaction and pPIC9K plasmid were digested for 2 h at 37 °C by restriction enzymes *BamHI*-HF and *NotI*-HF.
3. DNA gel electrophoresis was applied to both digested PCR products and the pPIC9K plasmid and the resulting products were purified via a QIAquik® Gel Extraction Kit.
4. The extracted PCR products and empty pPIC9K were then ligated by T4 DNA ligase.
5. The plasmid thus obtained was amplified in XL 10-Gold® ultracompetent cells and extracted by GenElute™ plasmid miniprep kit.
6. The concentration of the extracted plasmid was next measured using a NanoDrop™ 2000 spectrophotometer.
7. In accordance with the concentration of the extracted plasmid, *PmeI*-HF was added and incubated for 2 h to fully linearize the plasmid.

8. *PmeI*-HF enzyme was denatured at 80 °C for 30 min (Note: This step is OPTIONAL).
9. The linearized plasmid was then precipitated by 70% ethanol (*see* **Note 7**).
10. The precipitated DNA was then micro-centrifuged for 1 min at 13,000 × *g*.
11. The supernatant was discarded and the precipitated linearized DNA was dried under a fume hood or vacuum for 20–30 min.
12. The linearized DNA was dissolved in distilled water to a final concentration of 500–1000 ng/μL.

3.1.3 Electroporation

The electroporation method was used to achieve multi-copy insertion of the plasmid.

1. 5–10 μg (about 10 μL) linearized plasmid was gently mixed with 80 μL freshly prepared *P. pastoris* competent cells and kept on ice for 5 min before electroporation.
2. The mixture of plasmids and competent cells was transferred to a prechilled 2 mm electroporation cuvette.
3. Electroporation was performed on a Gene Pulser II.
4. The electroporation conditions were 1500–2000 V charging voltage, 25 μF capacitance, and 400 Ω resistance (*see* **Note 8**).
5. 1 mL ice-cold 1 M sorbitol was immediately added to the transformation cuvette.
6. The sample was then transferred to a sterile 14 mL round-bottom tube and incubated for 2–3 h at 30 °C without shaking.
7. 200 μL of medium containing transformed plasmids was spread on YNBD plates, and incubated for 3–5 days until colonies appeared.

3.2 High-Yield Transformant Screening

The screening to obtain a desirable transformant was carried out in two stages, that is, high-copy transformants screening and high-yield expression transformants screening. The main procedure for screening high-copy transformants was in accordance with the previous publication [31] in addition to a secondary screening cycle to increase the probability of high-copy transformants.

3.2.1 Two-Stage Screening Approach for High-Copy Transformants

1. After 3–5 days incubation at 30 °C, the transformants typically appeared on histidine-deficient YNBD plates with the number of colony-forming units (CFU) varying from hundreds to thousands.
2. The transformants were then inoculated individually onto YPD plates containing 0.1 mg/mL G418 for an additional 3–5 days incubation at 30 °C.

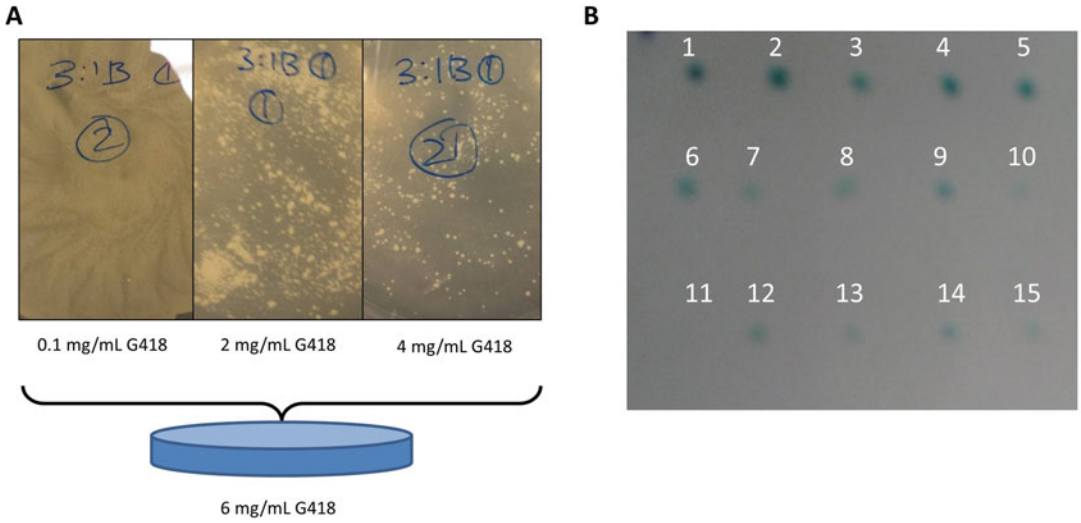

Fig. 2 The secondary screening plates containing different concentrations of G418 (**a**) and immunoblot images for crude expression screening of colonies chosen from 6 mg/mL G418 plates (**b**)

3. The colonies were subsequently transferred to YPD plates with 2 mg/mL G418 for an additional incubation period of 5–7 days; transformants were then transferred onto YPD plates containing 4 mg/mL G418 and incubated for an additional 5–7 days (*see* **Note 9**).
4. The colonies with high-copy target-gene integration can be easily judged from colony size/morphology.
5. The colonies resistant to high concentrations of G418 were selected to make competent cells for second-cycle screening (*see* **Note 10**).
6. The secondary electro-transformation was performed using a high-copy transformant from the first cycle screening to make competent cells.
7. After transformation, the secondary transformants were directly spread onto YPD plates with different G418 concentrations (2 mg/mL and 4 mg/mL), rather than YNBD plates (*see* **Note 11**).
8. The colonies that grew on 4 mg/mL G418 YPD plates were further transferred onto 6 mg/mL G418 YPD plates for an additional 5–7 day incubation period.
9. 10–15 high-copy colonies were picked from YPD plates containing 6 mg/mL G418 for further expression to screen high-yield transformants (Fig. 2).

3.2.2 High-Yield Transformants Screening via Immunoblotting

1. 10–15 different single colonies from YPD plates containing 6 mg/mL G418 were inoculated into 5 mL BMGY medium in 14 mL falcon tubes, and were cultured at 30 °C for at least 24 h with shaking (275 rpm) until an OD_{600} between 2 and 6 was obtained.
2. The medium was then transferred into 250 mL BMMY media in Erlenmeyer flasks and was further cultured at 22 °C for 60 h (275 rpm) (*see* **Note 12**).
3. During cell growth, 0.5% methanol was added every 18 h to induce receptor expression.
4. At the end of induction, the cell pellets were collected after centrifugation at 4000 × *g* for 15 min.
5. The cell pellets were washed once using buffer P1.
6. The cell pellets were resuspended into lysis buffer P2 and left at room temperature for 1 h (*see* **Note 13**).
7. The cell pellet suspensions were then vortexed at 2000 rpm for 2 h at 4 °C in the presence of a slurry of 5 mm glass beads.
8. The disrupted cell pellets were centrifuged at 8000 × *g* for 30 min at 4 °C to discard unbroken cells and cellular debris.
9. The supernatant containing cell membrane was collected and directly applied to immunoblotting membrane.
10. Immunoblotting was performed by anti-His_6-peroxidase.
11. 1 μL of the supernatant was blotted on nitrocellulose membrane and allowed to dry (*see* **Note 14**).
12. The membrane was placed in blocking buffer for 1 h at room temperature.
13. The membrane was then transferred to incubation buffer containing anti-His_6-peroxidase antibody (1:2000) at room temperature for 2 h.
14. The membrane was then washed three times with washing buffer, followed by distilled water.
15. The membrane was visualized by reacting with BM Blue POD substrate (*see* **Note 15**).

3.3 Ligand Column Synthesis

Obtaining functional receptor is critical for NMR studies. For this purpose, affinity purification using a column with immobilized ligand is the key to removing misfolded receptor. The synthesis of an XAC ligand (xanthine amine congener, a functionalized antagonist) column for the adenosine A_{2A} receptor was closely followed from the protocols proposed by Nakata et al. and Grisshammer et al. [32, 33] with slight modification, as described below:

1. 0.01 mmol XAC (4.285 mg) was dissolved in 10 mL of DMSO (*see* **Note 16**).

2. Prior to XAC addition, Affi-Gel® 10 was washed extensively (at least 20×) with ice-cold isopropanol and then briefly with DMSO (*see* **Note 17**).
3. The moist gel cake was immediately resuspended in 120 mL DMSO containing 51 mg XAC.
4. The gel suspension was incubated at room temperature overnight with slow continuous rotation/shaking.
5. The reaction was stopped by washing the gel with DMSO extensively.
6. The gel was further washed sequentially with distilled H_2O, 1 M Tris, and distilled H_2O.
7. The washed gel was incubated with 200 mM Tris acetate buffer, pH 8.0, for 24 h at 4 °C.
8. The gel was then washed with distilled water extensively.
9. Finally, the XAC-agarose was stored at 4 °C in 0.02% NaN_3.
10. The amount of covalently bound XAC was estimated by monitoring the absorbance at 310 nm in 0.01 N HCl in comparison to that of the starting XAC solution.

3.4 Cell Culture and Induced Expression

1. The high yield transformant was streaked from a glycerol stock onto YPD agar plates and was incubated for 3–5 days until clear single colonies appeared on the plates.
2. A single colony was inoculated in 4 mL YPD broth at 30 °C with shaking at 275 rpm overnight (usually 12–14 h).
3. The cells were then transferred into the 200 mL BMGY medium and cultured at 30 °C for about 20–24 h until the OD_{600} reached 2–6.
4. The cell pellets were spun down at 4000 × *g* for 15 min, and were resuspended in the 1 L BMMY medium to begin expression under induction with methanol at 20–22 °C.
5. 0.5% methanol was added every 18 h to continue induction of receptor expression.
6. After 60 h methanol induction, cell pellets were harvested for receptor purification.

3.5 Membrane Preparation, ^{19}F Labeling, and Functional Purification

1. The cell pellets were washed one time with buffer P1.
2. Harvested cells were suspended in 4 mL of Lysis buffer P2 at a ratio of 1:4 (*see* **Note 18**) and left at room temperature (20 °C) for 1 h.
3. Yeast cell walls were further disrupted by the addition of 5 mm glass beads to the resuspended cells and vortexing at 4 °C for 2 h.

4. Intact cells and cell debris were separated from the membrane suspension by low-speed centrifugation (8,000 × *g*, 30 min, 4 °C).
5. The supernatant was collected and centrifuged at 100,000 × *g* for 1 h.
6. The cell pellet was dissolved in 25 mL buffer P3 under continuous shaking for about 1–2 h, at 4 °C, until complete resuspension was achieved.
7. TALON® Metal Affinity Resin was washed with distilled water twice and with binding buffer P4 once, then combined with the resuspended membrane under gentle shaking for at least 2 h at 4 °C.
8. The suspension was then centrifuged at 4 °C to collect $A_{2A}R$ conjugated TALON® Metal Affinity Resin at 500 × *g* for 2 min (*see* **Note 19**).
9. The $A_{2A}R$-conjugated resin was washed extensively (at least 5×) with buffer P4+1 to remove nonconjugated receptor and impurities in solution.
10. The mixture of TALON® resin and supernatant was incubated at 4 °C on a shaker for 30 min with 100 μM Tris (2-carboxyethyl)phosphine hydrochloride (TCEP).
11. The TCEP was washed out immediately with buffer P4+1 twice, and the receptor was resuspended in P4+1 buffer.
12. 200 μM ^{19}F tag (BTFMA) was added into the solution, and incubated at 4 °C on a shaker for 6 h under nitrogen.
13. The solution was washed once with buffer P4+1 and another aliquot of ^{19}F tag was added. The solution was then incubated for an additional 6 h.
14. $A_{2A}R$-conjugated Talon resin was loaded on an empty column, washed with 10× bed volumes of buffer P5, and the apo $A_{2A}R$ was eluted with at least 5× bed volume of buffer P6.
15. The eluate containing apo-state $A_{2A}R$ was concentrated to 5 mL.
16. The NaCl and imidazole concentrations in the solution were decreased by the addition of 15 mL buffer P7.
17. The XAC-agarose gel was washed with distilled water twice, and equilibrated with buffer P7.
18. The receptor was then incubated with XAC-agarose gel for at least 1 h with gentle shaking at 4 °C to allow for $A_{2A}R$ binding to XAC agarose.
19. $A_{2A}R$-conjugated XAC agarose was packed on a disposable column.
20. The column was washed with buffer P7 for 2× column volumes to remove non-bound receptor.

21. $A_{2A}R$ was then eluted with 10× bed volumes of buffer P8.
22. The eluted samples were concentrated into 30–40 mL.
23. *See* **Note 20** for alternatives to the steps described below.
24. The sample was combined with TALON® resin which was then incubated for 2 h with the functional purified $A_{2A}R$.
25. The functional $A_{2A}R$-conjugated resin was packed on a column and washed extensively with buffer P5 (at least 10× column volumes) to remove all theophylline (*see* **Note 21**).
26. Functional apo $A_{2A}R$ was then eluted out by buffer P6.
27. Functional apo $A_{2A}R$ was concentrated into 1–2 mL and dialyzed twice against Buffer P5 with dilution factor of 1×10^6.
28. Functional apo-$A_{2A}R$ can now be prepared for NMR samples or other biophysical measurements.

4 Notes

1. The modified vector has a *BamHI* restriction site in front of the α-Factor, and a *NotI* restrict site following his-tag; refer to Fig. 1.
2. A methylotrophic yeast *P. pastoris* protease-deficient strain—SMD1163 (*Δhis4, Δpep4, Δprb1)*, which is deficient in both proteinases A (encoded by *pep4*) and B (encoded by *prb1*) genes, was used as an expression host system.
3. The strain was kept in 15% glycerol stock. The normal cultured cells should emit a fragrant smell.
4. Complete cell dispersal can be achieved by gently pipetting up and down during wash. Cell pellets should always be kept on ice.
5. At this point, the cell solution is very viscous and quite difficult to pipette by a micropipettor. However, this viscous condition is needed. Cells should not be diluted by adding more ice-cold sorbitol.
6. To reach high efficiency, always make fresh *P. pastoris* competent cells for electro-transformation.
7. At this point, linearized DNA can usually be seen by visual inspection after ethanol addition.
8. During electroporation, quickly wipe off moisture from the cuvette and insert into the electroporation device.
9. In this process, the same colonies were re-transferred onto grid plates with different concentrations of G418. For instance, the same colony will be simultaneously transferred onto plates containing 2 mg/mL and 4 mg/mL G418, respectively. As

different colonies will have unique copies of the gene of interest, the different survival probabilities for distinct colonies will be easily used for judging the copy number of colonies. It should be noted the procedures of making the secondary competent cells and electroporation were the same as in the first electroporation step.

10. Note the procedures for making the secondary competent cells and electroporation are unchanged from the first electroporation step.
11. Usually, hundreds of CFUs will appear on plates containing 4 mg/mL G418 at this stage, in contrast to the first-cycle screening where directly spreading the medium onto plates with 4 mL/mL G418 produces no visible colony.
12. Keep the temperature at 20–22 °C to increase the yield of the functional receptor.
13. Ensure the sample is left at room temperature to optimize Zymolyase activity and ensure cell walls are disrupted. Alternatively, you can use lyticase instead of Zymolyase.
14. Nitrocellulose membranes are essential as PVDF membranes are not suitable for immunoblotting with supernatant applied directly onto the membrane.
15. Different transformants will give rise to different intensities for the immunoblots and the transformant showing strongest intensity should be used for further expression.
16. MW of XAC is 428.5. The XAC concentration is 1 mM when dissolved in 10 mL of DMSO. For 20 mL of Affi-Gel® 10, 51 mg XAC must be dissolved in 120 mL DMSO. Note 12 mL XAC in DMSO for 1 mL of packed gel.
17. Keep the temperature at 10 °C; otherwise, DMSO and Affi-Gel® 10 will be freeze below this temperature.
18. 1 g cell pellets were resuspended in 4 mL Lysis buffer.
19. Do not exceed 1000 × *g*; otherwise the resin will be crushed. Refer to the manual for different resins from different suppliers.
20. The following steps can be alternatively replaced by extensive dialysis against buffer P6.
21. This and subsequent steps are required to return the receptor to an apo state.

References

1. Lv X, Liu J, Shi Q, Tan Q, Wu D, Skinner JJ, Walker AL, Zhao L, Gu X, Chen N, Xue L, Si P, Zhang L, Wang Z, Katritch V, Liu ZJ, Stevens RC (2016) In vitro expression and analysis of the 826 human G protein-coupled receptors. Protein Cell 7(5):325–337. doi:10.1007/s13238-016-0263-8

2. Lundstrom K (2016) New winds in GPCR-based drug discovery. Future Med Chem 8 (6):605–608. doi:10.4155/fmc-2016-0008
3. Katritch V, Cherezov V, Stevens RC (2013) Structure-function of the G protein–coupled receptor superfamily. Annu Rev Pharmacol Toxicol 53(1):531–556. doi:10.1146/annurev-pharmtox-032112-135923
4. Park SH, Das BB, Casagrande F, Tian Y, Nothnagel HJ, Chu M, Kiefer H, Maier K, De Angelis AA, Marassi FM, Opella SJ (2012) Structure of the chemokine receptor CXCR1 in phospholipid bilayers. Nature 491 (7426):779–783. doi:10.1038/nature11580
5. Scott DJ, Plückthun A (2013) Direct molecular evolution of detergent-stable G protein-coupled receptors using polymer encapsulated cells. J Mol Biol 425(3):662–677. doi:10.1016/j.jmb.2012.11.015
6. Klenk C, Ehrenmann J, Schütz M, Plückthun A (2016) A generic selection system for improved expression and thermostability of G protein- coupled receptors by directed evolution. Sci Rep 6:21294. doi:10.1038/srep21294
7. Schütz M, Schöppe J, Sedlák E, Hillenbrand M, Nagy-Davidescu G, Ehrenmann J, Klenk C, Egloff P, Kummer L, Plückthun A (2016) Directed evolution of G protein- coupled receptors in yeast for higher functional production in eukaryotic expression hosts. Sci Rep 6:21508. doi:10.1038/srep21508
8. Isogai S, Deupi X, Opitz C, Heydenreich FM, Tsai C-J, Brueckner F, Schertler GFX, Veprintsev DB, Grzesiek S (2016) Backbone NMR reveals allosteric signal transduction networks in the β_1-adrenergic receptor. Nature 530 (7589):237–241. doi:10.1038/nature16577
9. Etzkorn M, Raschle T, Hagn F, Gelev V, Rice AJ, Walz T, Wagner G (2013) Cell-free expressed bacteriorhodopsin in different soluble membrane mimetics: biophysical properties and NMR accessibility. Structure 21(3): 394–401. doi:10.1016/j.str.2013.01.005
10. Gautier A, Kirkpatrick JP, Nietlispach D (2008) Solution-state NMR spectroscopy of a seven-helix transmembrane protein receptor: backbone assignment, secondary structure, and dynamics. Angew Chem Int Ed Engl 47 (38):7297–7300. doi:10.1002/anie.200802783
11. Gautier A, Mott HR, Bostock MJ, Kirkpatrick JP, Nietlispach D (2010) Structure determination of the seven-helix transmembrane receptor sensory rhodopsin II by solution NMR spectroscopy. Nat Struct Mol Biol 17(6):768–774. doi:10.1038/nsmb.1807
12. Nietlispach D, Gautier A (2011) Solution NMR studies of polytopic alpha-helical membrane proteins. Curr Opin Struct Biol 21 (4):497–508. doi:10.1016/j.sbi.2011.06.009
13. Nisius L, Rogowski M, Vangelista L, Grzesiek S (2008) Large-scale expression and purification of the major HIV-1 coreceptor CCR5 and characterization of its interaction with RANTES. Protein Expr Purif 61(2):155–162. doi:10.1016/j.pep.2008.06.001
14. Opitz C, Isogai S, Grzesiek S (2015) An economic approach to efficient isotope labeling in insect cells using homemade ^{15}N-, ^{13}C- and ^{2}H-labeled yeast extracts. J Biomol NMR 62 (3):373–385. doi:10.1007/s10858-015-9954-3
15. Fan Y, Emami S, Munro R, Ladizhansky V, Brown LS (2015) Isotope labeling of eukaryotic membrane proteins in yeast for solid-state NMR. Methods Enzymol 565:193–212. doi:10.1016/bs.mie.2015.05.010
16. Morgan WD, Kragt A, Feeney J (2000) Expression of deuterium-isotope-labelled protein in the yeast pichia pastoris for NMR studies. J Biomol NMR 17(4):337–347
17. Fan Y, Shi L, Ladizhansky V, Brown LS (2011) Uniform isotope labeling of a eukaryotic seven-transmembrane helical protein in yeast enables high-resolution solid-state NMR studies in the lipid environment. J Biomol NMR 49(2): 151–161. doi:10.1007/s10858-011-9473-9
18. Sugiki T, Shimada I, Takahashi H (2008) Stable isotope labeling of protein by *Kluyveromyces lactis* for NMR study. J Biomol NMR 42 (3):159–162. doi:10.1007/s10858-008-9276-9
19. Wood MJ, Komives EA (1999) Production of large quantities of isotopically labeled protein in *Pichia pastoris* by fermentation. J Biomol NMR 13(2):149–159
20. Niebauer RT, Robinson AS (2006) Exceptional total and functional yields of the human adenosine A2a receptor expressed in the yeast *Saccharomyces cerevisiae*. Protein Expr Purif 46 (2):204–211. doi:10.1016/j.pep.2005.09.020
21. O'Malley MA, Lazarova T, Britton ZT, Robinson AS (2007) High-level expression in *Saccharomyces cerevisiae* enables isolation and spectroscopic characterization of functional human adenosine A_{2a} receptor. J Struct Biol 159(2):166–178. doi:10.1016/j.jsb.2007.05.001
22. Liu JJ, Horst R, Katritch V, Stevens RC, Wuthrich K (2012) Biased signaling pathways in β_2-adrenergic receptor characterized by ^{19}F-NMR. Science 335(6072):1106–1110. doi:10.1126/science.1215802

23. Ye L, Van Eps N, Zimmer M, Ernst OP, Prosser RS (2016) Activation of the A_{2A} adenosine G-protein-coupled receptor by conformational selection. Nature 533(7602):265–268. doi:10.1038/nature17668
24. Horst R, Stanczak P, Stevens RC, Wüthrich K (2012) β_2-adrenergic receptor solutions for structural biology analyzed with microscale NMR diffusion measurements. Angew Chem Int Ed 52(1):331–335. doi:10.1002/anie.201205474
25. Horst R, Liu JJ, Stevens RC, Wüthrich K (2013) β2-adrenergic receptor activation by agonists studied with ^{19}F NMR spectroscopy. Angew Chem Int Ed 52(41):10762–10765. doi:10.1002/anie.201305286
26. Manglik A, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, Lerch MT, Kobilka TS, Thian FS, Hubbell WL, Prosser RS, Kobilka BK (2015) Structural insights into the dynamic process of β_2-adrenergic receptor signaling. Cell 161(5):1101–1111. doi:10.1016/j.cell.2015.04.043
27. Kitevski-LeBlanc JL, Prosser RS (2011) Current applications of ^{19}F NMR to studies of protein structure and dynamics. Prog Nucl Magn Reson Spectrosc 62:1–33. doi:10.1016/j.pnmrs.2011.06.003
28. Ye L, Larda ST, Li YFF, Manglik A, Prosser RS (2015) A comparison of chemical shift sensitivity of trifluoromethyl tags: optimizing resolution in ^{19}F NMR studies of proteins. J Biomol NMR 62(1):97–103. doi:10.1007/s10858-015-9922-y
29. Asada H, Uemura T, Yurugi-Kobayashi T, Shiroishi M, Shimamura T, Tsujimoto H, Ito K, Sugawara T, Nakane T, Nomura N, Murata T, Haga T, Iwata S, Kobayashi T (2011) Evaluation of the *Pichia pastoris* expression system for the production of GPCRs for structural analysis. Microb Cell Fact 10:24. doi:10.1186/1475-2859-10-24
30. Yurugi-Kobayashi T, Asada H, Shiroishi M, Shimamura T, Funamoto S, Katsuta N, Ito K, Sugawara T, Tokuda N, Tsujimoto H, Murata T, Nomura N, Haga K, Haga T, Iwata S, Kobayashi T (2009) Comparison of functional non-glycosylated GPCRs expression in *Pichia pastoris*. Biochem Biophys Res Commun 380 (2):271–276. doi:10.1016/j.bbrc.2009.01.053
31. Scorer CA, Clare JJ, McCombie WR, Romanos MA, Sreekrishna K (1994) Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. Nat Biotechnol 12:181–184
32. Weiss HM, Grisshammer R (2002) Purification and characterization of the human adenosine A_{2a} receptor functionally expressed in *Escherichia coli*. Eur J Biochem 269(1):82–92
33. Nakata H (1989) Purification of A_1 adenosine receptor from rat brain membranes. J Biol Chem 264(28):16545–16551