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Expression and Purification of Functionally Active Serotonin 5-HT$_{2A}$ Receptor in Insect Cells Using Low-titer Viral Stock

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[Abstract] The serotonin 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) is a member of the GPCR family that is important for various neurological functions and whose dysregulation causes many mental health disorders. Structural investigations of 5-HT$_{2A}$R require the production of functionally active receptors expressed from eukaryotic cell cultures. In this protocol, we describe a step-by-step method to express and purify serotonin 5-HT$_{2A}$R using a baculoviral expression vector system in Sf9 cell cultures, derived from our work with the rat (matching Uniprot ID P14842) and human (matching Uniprot ID P28223) 5-HT$_{2A}$Rs. A unique feature of this method is the utilization of cell culture additives to infect cells at low multiplicity of infection, thereby using several fold less quantity of viral titer compared to prior methods without the additive. This protocol can be tweaked to selectively over-express glycosylated or non-glycosylated forms of the receptor by varying the post-infection harvest times.

Keywords: Serotonin 5-HT$_{2A}$ receptor, GPCR production, Low MOI expression, Glycosylation, baculoviral expression, Sf9 cell culture

[Background] Over the last decade structural investigations on GPCRs have surged due to development of methods for high yield expression of functionally active receptors (Granier and Kobilka, 2012). Among those, the serotonin GPCRs are a small but diverse set of receptors that play important roles in neuromodulation and their dysfunction is linked to a number of mental health disorders (Berger *et al.*, 2009). Of the many methods available employing eukaryotic hosts for protein expression, including yeast, insect and mammalian cells, the use of Sf9 cells to express GPCRs in conjunction with baculoviral expression system has emerged as a prominent one due to its high yield along with reliability (Saarenpaa *et al.*, 2015; Wiseman *et al.*, 2020). However, various methods continue to be developed to overcome the various drawbacks of existing methods. There is no uniform, single method to express all types of GPCRs with sufficiently high yield to enable structural studies. This is partly due to the diversity of these receptors as well as modifications needed in their sequences to enable crystallographic studies. The advent of high-resolution single particle cryo-electron microscopy (CryoEM) circumvents some of the stringent requirements for crystallization and is more amenable towards studies on native receptors. One critical requirement for existing methods using baculoviral expression in insect cells is to obtain a high titer viral stock, which is non-trivial in many cases. Moreover for protein production, infecting cells typically at multiplicities of infection ~1-5 requires the

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use of significant volumes of viral titer, which gets depleted over time. Using multiple batches of viral titers can increase batch-to-batch variability. Hence, methods that consume much smaller quantities of viral titer are strongly desired. In addition, the native forms of many GPCRs, including serotonin receptors, have extensive glycosylation which is not desirable in all situations. Thus, any method that can provide either predominantly glycosylated or non-glycosylated form of receptor, preferably by a simple change of protocol, is also desirable. Here, we describe a method to express and purify 5-HT_{2A}R using baculoviral expression system in Sf9 cells that requires minimal quantities of viral titers compared to existing methods and can select the extent of glycosylation by varying the post-infection harvest times. This protocol, based on prior work (Wacker *et al.*, 2013; Mahesh *et al.*, 2018; Mozumder *et al.*, 2020), has been standardized to prepare samples for high-resolution CryoEM structure determination and it is applicable to other GPCRs, membrane proteins and other soluble proteins employing a similar expression and purification strategy.

Materials and Reagents

1. 6-well plate (BD Falcon, catalog number: 353046)
2. 24-well plate (BD Falcon, catalog number: 353047)
3. Whatman glass microfiber filters, Grade GF/B (Merck Millipore, catalog number: WHA1821025)
4. Electroporation cuvettes (Bio-Rad, catalog number: 1652086)
5. Sf9 cells, adapted to serum free medium (Life Technologies, catalog number: 11496-015)
6. *E. coli* DH10Bac cells (Life Technologies, catalog number: 10361-012)
7. pFastBac HT cloning vector kit, containing control plasmid (Life Technologies, catalog number: 10584-027) or pFastBac1 cloning vector kit, containing control plasmid (Life Technologies, catalog number: 10360014)
8. Baculoviral Titer kit, containing anti-gp64-PE conjugated antibody and control virus (Expression Systems, catalog number: 97-101)
9. Purelink HiPure Plasmid Miniprep Kit (Life Technologies, catalog number: K210002)
10. ESF 921 Insect cell culture medium (Expression Systems, catalog number: 96-001-01)
11. Grace's Insect cell culture medium (Life Technologies, catalog number: 11595030)
12. Production Boost Additive (Expression Systems, catalog number: 95-006-100)
13. Cellfectin II (Life Technologies, catalog number: 10362100)
14. Alexa Fluor 488 goat anti-rabbit antibody (Life Technologies, catalog number: A11008)
15. Anti-5-HT_{2A}R rabbit antibody (Neuromics, catalog number: RA24288)
16. Anti-5-HT_{2A}R rabbit polyclonal antibody (Abcam, catalog number: ab66049)
17. Anti-FLAG(R), antibody produced in rabbit (Sigma-Aldrich, catalog number: F7425)
18. HRP-conjugated goat anti-rabbit antibody (Merck Millipore, catalog number: AP187P)
19. Cholesteryl hemisuccinate (Avanti Polar Lipids, catalog number: 850524)
20. n-dodecyl-β-D-maltopyranoside (DDM) (Anatrace, catalog number: D310S)

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21. α-iodoacetamide (Merck Millipore, catalog number: 407710)
22. [^{3}H]-Ketanserin (Perkin-Elmer, catalog number: NET791250UC)
23. Ketanserin tartrate (Alfa Aesar, catalog number: J62798)
24. Serotonin Hydrochloride (Acros Organics, catalog number: AC215021000)
25. SIGMAFAST Protease Inhibitor Cocktail tablet (Sigma-Aldrich, catalog number: S8830)
26. Polyethylenimine (Sigma-Aldrich, catalog number: P3143)
27. Ni-NTA Agarose (Qiagen, catalog number: 30210)
28. Liquid nitrogen
29. Penicillin Streptomycin (Invitrogen, catalog number: 15070063)
30. Kanamycin sulfate (Calbiochem, catalog number: 420411)
31. Gentamicin sulphate (Himedia, catalog number: TC026)
32. Tetracycline (Himedia, catalog number: TC036)
33. Bovine Serum Albumin (Himedia, catalog number: MB083)
34. Bluo Gal (Invitrogen, catalog number: 15519028)
35. Trypan Blue (Life Technologies, catalog number: 15250)
36. Scintillation fluid Cocktail ‘T’ (Spectrochem, catalog number: 030399)
37. Isopropyl β-d-1-thiogalactopyranoside (IPTG) (Himedia, catalog number: MB072)
38. YT HiVeg Broth (Himedia, catalog number: MV1251)
39. LB medium (Himedia, catalog number: MV1245)
40. Agar Powder (Himedia, catalog number: RM301)
41. Na_2HPO_4 (Himedia, catalog number: MB024)
42. KH_2PO_4 (Himedia, catalog number: TC011)
43. HEPES (Calbiochem, catalog number: 5320)
44. $MgCl_2$ (SRL, catalog number: 69396)
45. KCl (Himedia, catalog number: MB043)
46. NaCl (Himedia, catalog number: TC046)
47. EDTA (Sigma-Aldrich, catalog number: E5134)
48. SDS (SRL, catalog number: 54468)
49. Methanol (Finar, catalog number: 67-56-1)
50. Glacial Acetic Acid (SRL, catalog number: 90868)
51. Glycine (Himedia, catalog number: MB013)
52. Tween 20 (Himedia, catalog number: MB067)
53. Imidazole (Himedia, catalog number: MB019)
54. Glycerol (Himedia, catalog number: MB060)
55. BCA Protein Assay Kit (Pierce, catalog number: PI23227)
56. LB agar plate for blue-white colony selection (see Recipes)
57. Sf9 cell culture medium (see Recipes)
58. Phosphate Buffered Saline (PBS) pH 7.4 (see Recipes)
59. Hypotonic buffer (see Recipes)

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60. Hypertonic buffer (see Recipes)
61. Resuspension buffer (see Recipes)
62. Solubilization buffer (see Recipes)
63. Equilibration buffer (see Recipes)
64. Wash buffer I (see Recipes)
65. Wash buffer II (see Recipes)
66. Elution buffer (see Recipes)
67. Radioligand binding buffer (see Recipes)
68. SDS-PAGE Running buffer (see Recipes)
69. Coomassie Brilliant Blue staining solution (see Recipes)
70. SDS-PAGE destaining solution (see Recipes)
71. Transfer buffer for Western blotting (see Recipes)
72. TBST buffer (see Recipes)
73. Blocking buffer (see Recipes)

Equipment

1. -80 °C freezer (ESCO, model: UUS-439-A-1)
2. Bio-safety Cabinet Class II A2 type (ESCO, model: AC2-4S8)
3. Electroporator (Bio-Rad, model: Micropulser electroporator, catalog number: 165-2100)
4. Flow cytometer (BD Biosciences, BD LSRFortessa, model: LSR Fortessa)
5. Liquid scintillation counter (Perkin Elmer, model: Tri-Carb 2810 TR)
6. Ultracentrifuge (Thermo, model: 46901-WX90) with T865 rotor for 30 ml tubes
7. Incubator Shaker (Sartorius, Brand, model: Certomat BS-T), used for large volume cultures
8. Incubator Shaker (Jeiotech, Brand, model: ISS-4075R), used for maintenance and small volume cultures
9. Bacterial incubator (BIS-1824)
10. Fluorescence inverted microscope (Leica, model: DMIL LED FluoTrinoculer inverted microscope, serial number: 362094)
11. Refrigerated centrifuge (Hermle, model: Z446K), used for larger volume (> 2 ml) or plate centrifugation
12. Refrigerated centrifuge (Eppendorf, model: 5417R), used for smaller volume (≤ 2 ml) centrifugation
13. Probe Sonicator (Takashi, model: U150), equipped with a 6 mm diameter probe
14. Gel electrophoresis set for SDS PAGE (GE Lifesciences, model: SE250)
15. Mini PAGE Blotting System (GE Lifesciences, Brand, catalog number: SBE2010-PB)
16. Manifold Vacuum Filtration (Millipore, catalog number: M2536-1EA)
17. Visible Fluorescent Western Blot Imaging System (Azure, model: Azure Biosystems c400)

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Software

1. FACSDiva v6.2 (BD Biosciences) for flow cytometric analysis
2. Prism (GraphPad, version 5.01), or free alternatives (*e.g.*, R Bioconductor packages), for fitting of radioligand binding assay data
3. Leica Microscope Imaging software (Leica), for estimation of cell density, viability and monitoring

Procedure

This protocol is based on our prior work with rat (Mahesh *et al.*, 2018) and human (Mozumder *et al.*, 2020) 5-HT_{2A}Rs. The rat receptor (matching Uniprot ID P14842), sub-cloned from an earlier plasmid (Bhattacharyya *et al.*, 2006), was over-expressed with an N-terminal histidine tag. The human receptor (matching Uniprot ID P28223) was sub-cloned from a codon-optimized sequence, having a stabilizing mutation $M182^{3.41}W$ and expressed with FLAG and histidine tags in the N- and C-terminal ends, respectively. The procedure to express and purify functionally active 5-HT_{2A}R consists of the following steps: (A) Bacmid preparation, (B) Preparation of high-titer viral stock, (C) Expression of 5-HT_{2A}R in Sf9 cell culture, (D) Purification of 5-HT_{2A}R, and, (E) Radioligand binding assay.

A. Bacmid preparation
   1. Add 2 µl of plasmid (50 ng/µl), containing the 5-HT_{2A}R cloned into a pFastBac1 or pFastHT vector (containing a FLAG or histidine tag at the 5′ or 3′ end of the receptor) to 100 µl of *E. coli* DH10Bac cells on ice.
   2. Mix gently, incubate for 30 min on ice and transfer to chilled electroporation cuvettes. Transform by electroporation using program “Ec2” if using cuvettes of 2 mm electrode gap.
   3. Immediately add 1 ml of ice-cold 2YT medium, without antibiotics, and transfer to culture tubes for incubation at 37 °C, shaking at 225 rpm for 4 h.
   4. Prepare 10-fold serial dilutions of the cells (*e.g.*, 1, 10^{-1}, 10^{-2}, 10^{-3}) and plate 100 µl of each dilutions on LB agar plates for blue-white colony selection (Recipe 1) and incubate plates for 48 h.
   5. Pick white colonies from any plate with sufficiently isolated colonies, separately, into 15 ml of LB medium containing 50 µg/ml kanamycin, 10 µg/ml tetracycline, 7 µg/ml gentamicin and incubate overnight at 37 °C, 180 rpm. Centrifuge at 6,000 *x g* for 5 min at 4 °C to harvest cells and purify bacmids from the pellet using the “Purelink HiPure Plasmid MiniprepKit” according to manufacturer’s protocol.
   6. Store the bacmids at -20 °C.
   7. **Recommended Step:** Verify the integrity of recombinant 5-HT_{2A}R sequence by running PCR using manufacturer specified pUC/M13 forward and reverse primers and following

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manufacturer's protocol. These primers are specific for the bacmid sequence and would bind to upstream and downstream sites flanking mini-*att*Tn7 site sandwiching the 5-HT_{2A}R gene.

B. Preparation of high-titer viral stock

1. Incubate healthy Sf9 cells from suspension culture (see Note 2) in 6-well plate (8 x 10^5 cells per well) at 27 °C. Wait for ~1 h to allow cells to adhere to the surface of the well. Wash cells twice with antibiotics free 2 ml of Grace's insect cell medium to remove residual non-adherent cells and adjust the plated cells to antibiotics free conditions.
2. Mix 1.5 µg freshly prepared bacmid and 8 µl of Cellfectin II reagent, separately, into 100 µl of antibiotics-free Grace's insect cell medium. Mix both the solutions and incubate for 30 min at room temperature. Then add 800 µl of antibiotics-free Grace's insect cell medium into the 200 µl transfection mixture and add the mixture drop-wise evenly into each well. Incubate for 4 h at 27 °C. For untransfected (control) cells, prepare and add the Cellfectin II reagent without any bacmid to the well.
3. Wash cells in each well with 2 ml of regular Sf9 cell culture media (Recipe 2) and incubate for 5-6 days till the completion of infection.
4. Compare the transfected cells with untransfected ones (see Note 2) and at the end of infection, harvest viral stock by mild centrifugation at 500 *x g* at 4 °C. Collect the supernatant (*i.e.*, the culture medium) and discard the pellet. The supernatant is the P0 baculoviral stock.
5. Dilute the P0 stock at a multiplicity of infection (MOI) of ~0.01-0.1 into a small volume of ~10 ml Sf9 cell culture in shake flask and incubate at 27 °C, 130 rpm for 48 h. Harvest the supernatant containing the culture medium by centrifugation at 500 *x g* at 4 °C and store this as P1 stock. (see Notes 4 and 5 for details on selecting the MOI and the time of harvest)
6. Repeat above step by diluting P1 stock into larger volumes (~250 ml) of Sf9 culture, at MOI ~0.01–0.1 and harvest the supernatant containing the culture medium by centrifugation and store this as P2 stock. The P2 stock is to be used for protein production.
7. Immediately filter all harvested baculovirual stocks using a 0.2 µm low protein binding syringe filter.
8. Measure the baculoviral titer for P1 and P2 stocks using the Baculoviral Titer kit by following manufacturer's protocol using flow cytometry (Figure 1). Briefly,
    a. Seed approximately 200 µl of Sf9 cells (2 x 10^6 cells/ml) from a healthy batch of suspension culture (see Note 2) into each well of a 24-well plate.
    b. Serially dilute the baculoviral stock whose titer is to be measured in 10-fold dilution steps using culture medium as the diluent (*e.g.*, 1:5, 1:50, 1:500 and so on). Subsequently, add 200 µl of the diluted baculovirus into each well of the 24-well plate containing 200 µl of plated cells, leading to an additional 2-fold dilution (and net dilution of 1:10, 1:100, 1:1,000 and so on).
    c. Incubate cells as suspension culture for 18 h at 27 °C, 170 rpm. Harvest cells by mild centrifugation for 3 min to maintain cells suitable for cytometry. Wash cells twice with

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ice-cold PBS buffer (Recipe 3) and stain with anti-gp64-PE conjugated antibody for 20 min at 4 °C in the dark.

d. Wash cells twice with ice-cold PBS buffer and resuspend in PBS buffer containing 1% BSA.

e. Analyze cell count by flow cytometer and obtain viral titer as per manufacturer's protocol for "Baculoviral Titer kit" (Figures 1A-1D).

9. Store working stocks (*e.g.*, part of the P2 stock) at 4 °C for up to 6 months and the rest, including all P0 and P1 stocks, at -80 °C. For freezing, aliquot baculoviral stocks to minimize free-thaw cycles, add 3% BSA and store at -80 °C. Store all aliquots (at 4 °C and -80 °C) in light protection tubes or wrap tubes by Aluminum foil to protect from sunlight.

C. Expression of 5-HT_{2A}R in Sf9 cell culture

1. Incubate 20-50 ml of healthy Sf9 cells at a density of 2-3 x 10^6 cells/ml in shake flasks at 27 °C, 130 rpm and scale up to the desired volume maintaining cell viability in rapid growth phase (see Note 2).
2. Infect cells at MOI of 0.1 and incubate at 27 °C for 30 h at 130 rpm. Add 1% (v/v) Production Boost Additive (PBA) to the cell culture and incubate for 66 h. Alternately in absence of PBA, infect cells at MOI of 5 and incubate for 48 h (see Note 2 for factors affecting the protein expression).
3. Harvest cells by centrifugation at 3,000 *x g*, for 10 min at 4 °C.
4. Wash the pellet with ice cold PBS buffer and store at -80 °C after flash freezing in liquid nitrogen.
5. For the expression of low- or high-glycosylated forms of the receptor, harvest the culture after 48 h or 120 h, respectively, after the time of addition of PBA. Run a Western blot analysis of the cell lysate (Figure 1E) and select the most appropriate harvest time for optimal extent of glycosylation (see Note 6).

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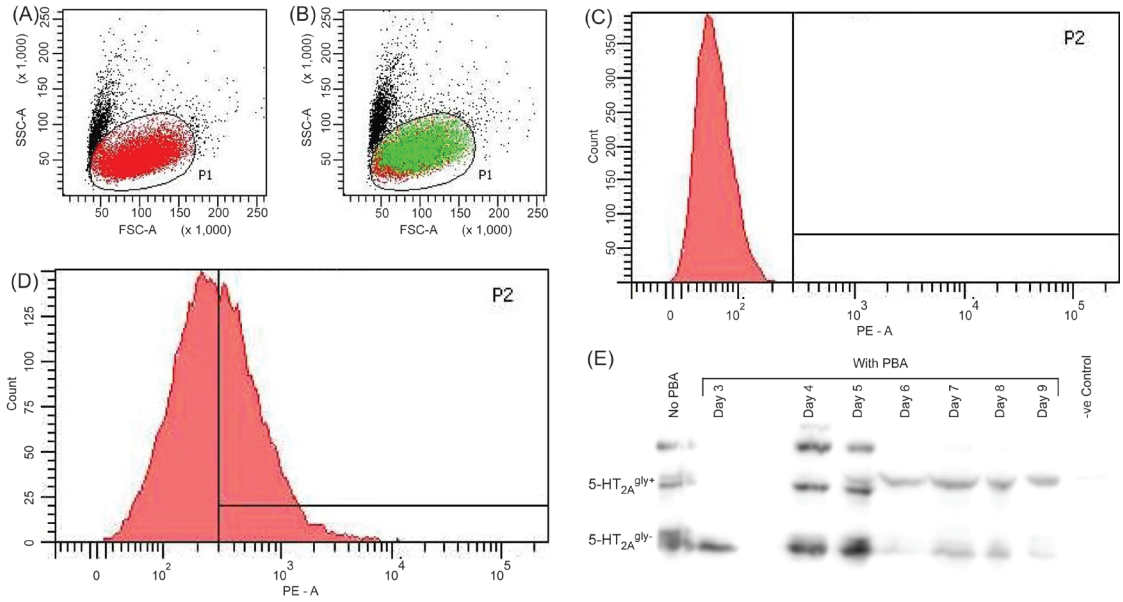

Figure 1. Estimation of baculoviral titer using flow cytometry. Representative dot plot analysis of flow cytometry data for (A) uninfected Sf9 cells (red dots) and, (B) Sf9 cells infected with P2 baculovirus (green), and stained by anti-gp64 PE-conjugated antibody. Histogram plots of cell count as a function of PE signal, indicate distinct populations for (C) uninfected Sf9 cells, gated (black oval) in (A), as well as (D) PE-positive infected Sf9 cells, gated (black oval) in (B), providing an estimate of baculoviral infection due to expression of gp64 in Sf9 cells. The signal in P2 quadrant increases (or decreases) proportionally with higher (or lower) viral loads in serially diluted baculoviral stocks as noted in Step B8b. (E) Western blot analysis of the variation of glycosylated and non-glycosylated 5-HT_{2A}R in absence of PBA (Lane 1, from left) and presence of PBA (Lanes 2-8), with post-infection harvest times shown above respective lanes. The negative control corresponds to cells that were not transfected by bacmid. 5-HT_{2A}^{gly+} and 5-HT_{2A}^{gly-} indicate the 5-HT_{2A}R with and without glycosylation, respectively.

D. Purification of 5-HT_{2A}R

1. Resuspend cell pellet from approximately 1 L culture in 30 ml of ice-cold hypotonic buffer (Recipe 4), followed by mild sonication on ice (pulse on for 10 s, off for 30 s, 10% power, for 3 cycles). In this and all subsequent steps of purification, cell pellets are resuspended by pipetting.
2. Ultracentrifuge the cell suspension at 150,000 *x g*, 4 °C for 30 min. Discard the supernatant and repeat the washing step with hypotonic buffer two more times.
3. Wash the residual pellet with 30 ml of ice-cold hypertonic buffer (Recipe 5), ultracentrifuge at 150,000 *x g*, 4 °C for 30 min and discard the supernatant.
4. Wash the pellet with ice-cold PBS (Recipe 3) to remove residual media and store the pellet at -80 °C until further use.
5. Resuspend the membrane pellet in 4 ml of resuspension buffer (Recipe 6) and incubate for 1 h at room temperature.

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6. Add α-iodoacetamide to a final concentration of 2 mg/ml and incubate further for 30 min at 4 °C (see Note 7).
7. Solubilize the ~4 ml solution after α-iodoacetamide treatment in 4 ml of solubilization buffer (Recipe 7) to achieve a 1:1 dilution and incubate for 2 h at 4 °C by gentle tumbling.
8. Ultracentrifuge the solubilized membranes at 150,000 *x g*, 4 °C for 30 min, collect the supernatant and discard the pellet.
9. Add concentrated solutions of buffered NaCl and imidazole to the solubilized protein in supernatant to match the equilibration buffer (Recipe 8).
10. Add the solubilized protein solution to (approximately) 2 ml of pre-equilibrated Ni-NTA resin and incubate overnight at 4 °C by gentle tumbling.
11. Collect the resin by centrifugation at 500 x *g* at 4 °C for 10 min. Wash the resin four times with 3 column volumes (CVs) of wash buffer I (Recipe 9), followed by four washes of 2 CV wash buffer II (Recipe 10).
12. Elute the protein by five washes of 1 CV elution buffer (Recipe 11) and collect the supernatant separately for each wash, since the 1st wash contains the maximum protein with decreasing concentrations in subsequent washes, and enables easier detection during electrophoresis (Figure 2A). Pool the purified fractions and exchange into an appropriate imidazole free buffer (*e.g.*, Elution buffer without imidazole) and concentrate by ultrafiltration.

E. Radioligand binding assay

1. Resuspend membrane pellet from approximately 50 ml of Sf9 cell culture in approximately 5 ml of radioligand binding buffer (Recipe 12). Adjust volumes of membrane pellet or resuspension buffer in order to have approximately 50 µg total protein (see Note 8) in 500 µl reaction volume for each reaction to be performed in triplicates. Estimate membrane protein concentration using BCA assay.
2. For competition binding assay (Figure 2B):
   Incubate the resuspended membrane pellet in triplicates with 3 nM [^{3}H]-ketanserin (hot ligand) and increasing concentrations of serotonin (cold ligand), ranging from 1 pM to 100µM (corresponding approximately to 10^{-3} to 10^5 times the dissociation constant, K_d), for 1 h at room temperature in the dark.
3. For saturation binding assay (Figure 2C):
   a. Incubate the resuspended membrane pellet in triplicates with increasing concentrations of [^{3}H]-ketanserin (hot ligand), ranging from 0.25 nM to 10 nM (corresponding approximately to 0.25 to 10 times the dissociation constant, K_d), for 1 h at room temperature in the dark.
   b. For determination of non-specific binding, incubate the reaction with 10 µM serotonin (cold ligand).
4. At the end of reaction, harvest each reaction using a 12-well cell harvester by filtering through 25 mm GF/B filters pre-soaked in 0.3% polyethylenimine.
5. Wash each filter thrice with 2 ml of ice cold 50 mM Tris buffer (pH 7.4) and dry overnight in air.

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6. Add 5 ml scintillation fluid into each vial containing dried filters followed by measurement in scintillation counter (Figures 2B and 2C).

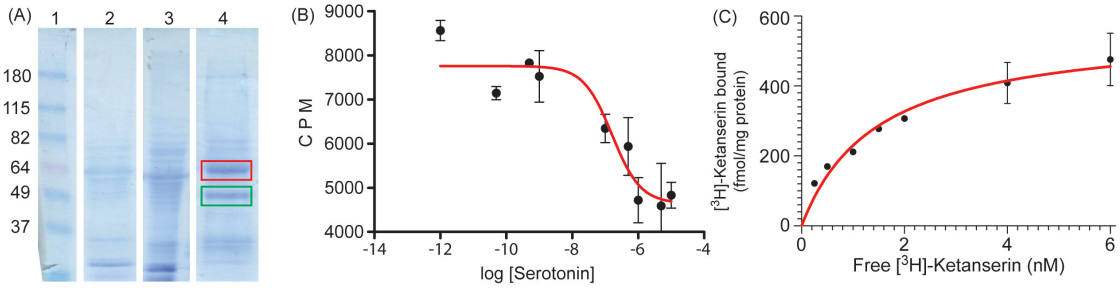

Figure 2. Results of purification and radioligand binding assay for 5-HT$_{2A}$R. (A) 10% SDS PAGE analysis of various fractions of purification steps, after staining with Coomassie. Lanes 1-4 indicate, Molecular weight marker (Ln 1), crude cell lysate (Ln 2), flow through after loading the solubilised protein on to the resin (Ln 3), eluted protein after dialysis in imidazole free buffer and concentration (Ln 4). The glycosylated and non-glycosylated 5-HT$_{2A}$R are shown in red and green rectangles, respectively. (B) Competition binding assay plot of 5-HT$_{2A}$R using 3 nM [^{3}H]-ketanserin in presence of increasing concentrations of serotonin, reveal a Ki of 39 nM for membrane bound receptors (logKi = -7.41 ± 0.29). (C) Saturation binding assay plot of [^{3}H]-ketanserin specific binding to 5-HT$_{2A}$R reveals a dissociation constant (K_d) of 1.47 ± 0.39 nM with maximum number of binding sites (B_{max}) of 567 ± 57 fmol/mg of protein. For (B) and (C), curves shown are from representative experiments performed in triplicates and values for K_d, logKi and B_{max} represent the mean (± SEM) obtained from triplicate sets of measurements.

Data analysis

1. Verify the presence of 5-HT$_{2A}$R in all desired fractions using Western blotting using anti-His and/or anti-FLAG antibodies (see Recipes 13-18 for appropriate buffers).
2. Purity of the eluted fractions should be verified by SDS PAGE, if desired by concentrating the necessary fractions and stained by Coomassie dye or Silver staining methods (Figure 2A).
3. Determine the baculoviral titer from an equation obtained from a standard curve obtained using a control virus of known titer, followed by viral stocks of unknown titers. Manufacturer of the baculoviral titer kit (Expression Systems Inc) provides the control virus as well as an excel sheet for calculating the titers.
4. For radioligand binding assay, normalized data points should be fitted to one-site Specific binding model or one-site–Fit K_i for saturation binding or competition binding assays, respectively using GraphPad Prism, v5.01.

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Notes

1. The use of a control plasmid, expressing a different protein but using the same baculoviral vector expression system, is recommended as a positive control to detect expression of the target protein on a comparative basis. Cells without infection serve as negative control. Plasmids, expressing proteins GUS and CAT, are provided along with pFastBac1 and pFastBac-HT cloning vectors kits, respectively, and can be used in exactly the same way as the plasmid containing the 5-HT_{2A}R.
2. Use of healthy Sf9 cells is critically important to the yield and reproducibility of the work. Typically, fast growing Sf9 cells with densities of 2-3 x 10^6 cells/ml, doubling times of ~24 h and viability > 95% were maintained strictly at 27 ± 0.5 °C and shaking at 130 rpm. 50 U/ml penicillin and 50 µg/ml of streptomycin in ESF921 medium were used for all cultures, unless otherwise mentioned. For suspension cultures, infection can be verified by observing cells on a hemocytometer at regular intervals under an inverted microscope. Cells show enlargement during the initial 24 h, followed by lowering of growth (*i.e.*, increase of doubling time) and increased granularity in the 24-72 h time followed by progression to cell lysis and reduced cell viability after 72 h. Cell viability can be measured by Trypan blue assay. For plate cultures, cell detachment from plate surface would be increasingly observed in 24-72 h.
3. Even though smaller volume of baculovirus is needed with PBA additive, the use of high-titer (> 1 x 10^8 pfu/ml) is recommended in absence of PBA. The transfection efficiency may be optimized, by varying the ratio of bacmid:cellfectin, to obtain higher titers of initial stocks, which may enable increasing the titer concentration upon subsequent passages.
4. Preparation of high-titer viral stock is usually an iterative process, and may require passaging the P0 and P1 stocks at various multiplicities-of-infections (MOI), ranging from 0.01 to 0.1 to increase viral titers, as well as selecting the optimal harvest times. The MOI is the ratio of plaque forming units (pfu) of virus particles/number of cells in culture. Typically, P2 stock will provide sufficient volume for protein production; however, if necessary, another passage may be done to obtain P3 stock. Higher passages are not recommended. Longer harvest times may lead to lowering of viral titer, possibly due to cell lysis; nonetheless, the harvest time may be optimized to select for the highest baculoviral titer.
5. For baculoviral titer estimation, the process has to be first calibrated using a control virus of *known* titer, provided in the Baculoviral titer kit. Volumes of P0 stock may be insufficient for titer estimation, hence titers of P1 and P2 should be measured, although an estimate of P0 stock's titer is necessary to start the process (which can be taken as 10^6 pfu/ml). In addition to titer estimation using Baculoviral titer kit and flow cytometric method, it is also feasible to obtain viral titers using plaque assays, quantitative real-time PCR and observations of infected viable cell size (Janakiraman *et al.*, 2006).
6. The yield of 5-HT_{2A}R is interdependent on the volume of PBA (up to ~10%), time of addition of PBA (18-30 h), the MOI of virus addition (0.001-20) and the post-infection harvest time (up to 2

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weeks), in addition to various other factors like the culture volume and quality of Sf9 cells. Hence, it may be prudent to optimize these factors, especially if it is more economical to expend baculoviral stock and save PBA, or vice versa. Moreover, as noted previously (Mozumder *et al.*, 2020), the extent of glycosylation in cultures grown with PBA can be modulated by varying the post-infection harvest time. Hence the time of harvest may also be optimized to select the desirable amount of glycosylation, by running Western blot analysis of cell lysate from cultures grown over a range of post-infection harvest times (see Recipes 13-18 for suitable buffers for Western blotting).

7. α-iodoacetamide is added to block any free thiol groups with the goal to prevent protein aggregation by non-specific disulfide bonds (Milic and Veprintsev, 2015). The effect of α-iodoacetamide treatment may be optimized by varying the amount and duration of treatment, and may be avoided (or substituted with a suitable alternative reducing agent) if desired, especially for other proteins.
8. The amount of membrane protein for saturation binding assay is to be chosen to avoid ligand depletion effects, *i.e.*, the drop in ligand concentration after binding should be minimal (less than ~10% of total ligand concentration). Theoretical estimates of B_{max}, which provide the maximum number of binding sites (fmol) per mg of total protein (obtained by BCA assay), may be used to estimate the bound ligand, and hence ligand depleted, per reaction. Increase the reaction volume or reduce the protein used per reaction if ligand depletion is observed.

Recipes

1. LB agar plate (for blue-white colony selection)
   25 g/L Luria Bertani Broth powder
   15 g/L Agar
   50 μg/ml kanamycin
   10 μg/ml tetracycline
   7 μg/ml gentamycin
   100 μg/ml of Bluo-gal
   40 μg/ml of IPTG
2. Sf9 cell culture medium
   ESF921 medium
   50 U/ml penicillin
   50 μg/ml of streptomycin
3. Phosphate Buffered Saline (PBS) pH 7.4
   10 mM Na_2HPO_4
   1.8 mM KH_2PO_4
   137 mM NaCl
   2.7 mM KCl

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4. Hypotonic buffer
   10 mM HEPES (pH 7.5)
   10 mM $MgCl_2$
   20 mM KCl
   SIGMAFAST Protease Inhibitor Cocktail tablet–add immediately before use
5. Hypertonic buffer
   10 mM HEPES (pH 7.5)
   10 mM $MgCl_2$
   20 mM KCl
   1 M NaCl
   SIGMAFAST Protease Inhibitor Cocktail tablet–add immediately before use
6. Resuspension buffer
   10 mM HEPES (pH 7.5)
   10 mM $MgCl_2$
   20 mM KCl
   150 mM NaCl
   SIGMAFAST Protease Inhibitor Cocktail tablet–add immediately before use
7. Solubilization buffer (to be used for 1:1 dilution)
   Note: For other dilutions, adjust concentrations of n-dodecyl-β-D-maltopyranoside and cholesteryl hemisuccinate to achieve final concentrations of 0.5% (w/v) and 0.2% (w/v), respectively.
   50 mM HEPES (pH 7.5)
   150 mM NaCl
   1% (w/v) n-dodecyl-β-D-maltopyranoside
   0.4% (w/v) cholesteryl hemisuccinate
   SIGMAFAST Protease Inhibitor Cocktail tablet
8. Equilibration buffer (or binding buffer, matching the loading of protein into resin)
   30 mM HEPES (pH 7.5)
   10 mM $MgCl_2$
   20 mM KCl
   400 mM NaCl
   0.5% (w/v) n-dodecyl-β-D-maltopyranoside
   0.2% (w/v) cholesteryl hemisuccinate
9. Wash buffer I
   50 mM HEPES (pH 7.5)
   800 mM NaCl
   0.1% (w/v) n-dodecyl-β-D-maltopyranoside
   0.02% (w/v) cholesteryl hemisuccinate
   20 mM imidazole

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10% (v/v) glycerol

10. Wash buffer II
    50 mM HEPES (pH 7.5)
    150 mM NaCl
    0.05% (w/v) n-dodecyl-β-D-maltopyranoside
    0.01% (w/v) cholesteryl hemisuccinate
    50 mM imidazole
    10% (v/v) glycerol
11. Elution buffer
    50 mM HEPES (pH 7.5)
    150 mM NaCl
    0.05% (w/v) n-dodecyl-β-D-maltopyranoside
    0.01% (w/v) cholesteryl hemisuccinate
    250 mM imidazole
    10% (v/v) glycerol
12. Radioligand binding buffer
    50 mM Tris-HCl (pH 7.4)
    10 mM $MgCl_2$
    0.1 mM EDTA
13. SDS-PAGE Running buffer
    25 mM Tris
    192 mM glycine
    0.1% SDS
14. Coomassie Brilliant Blue staining solution
    40% (v/v) Methanol
    10% (v/v) Glacial Acetic Acid
    1 g of Comassie Brilliant Blue
15. SDS-PAGE destaining solution
    40% (v/v) Methanol
    10% (v/v) Glacial Acetic Acid
16. Transfer buffer for Western blotting
    25 mM Tris
    192 mM glycine
    20% methanol
17. TBST buffer (Tris-buffered saline with Tween 20)
    20 mM Tris, pH 7.5
    150 mM NaCl
    0.1% Tween 20
18. Blocking buffer

5% (w/v) skimmed milk powder in TBST buffer

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Competing interests

The authors declare no conflict of interest.

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