

Overexpressing human membrane proteins in stably transfected and clonal human embryonic kidney 293S cells

Sarika Chaudhary^{1-3,5}, John E Pak^{1-3,5}, Franz Gruswitz¹⁻³, Vinay Sharma⁴ & Robert M Stroud¹⁻³

¹Department of Biochemistry and Biophysics, University of California, San Francisco, California, USA. ²Center for the Structure of Membrane Proteins, University of California, San Francisco, California, USA. ³Membrane Protein Expression Center, University of California, San Francisco, California, USA. ⁴Department of Bioscience and Biotechnology, Banasthali University, Rajasthan, India. ⁵These authors contributed equally to this work. Correspondence should be addressed to R.M.S. (stroud@msg.ucsf.edu).

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X-ray crystal structures of human membrane proteins, although potentially of extremely great impact, are highly underrepresented relative to those of prokaryotic membrane proteins. One key reason for this is that human membrane proteins can be difficult to express at a level, and at a quality, suitable for structural studies. This protocol describes the methods that we use to overexpress human membrane proteins from clonal human embryonic kidney 293 (HEK293S) cells lacking *N*-acetylglucosaminyltransferase I (GnTI⁻), and was recently used in our 2.1-Å X-ray crystal structure determination of human RhCG. Upon identification of highly expressing cell lines, suspension cell cultures are scaled up in a facile manner either using spinner flasks or cellbag bioreactors, resulting in a final purified yield of ~0.5 mg of membrane protein per liter of medium. The protocol described here is reliable and cost effective, can be used to express proteins that would otherwise be toxic to mammalian cells and can be completed in 8–10 weeks.

INTRODUCTION

The overexpression of human membrane proteins, in both quantities and qualities amenable to structural studies, is often an immense challenge for numerous reasons. At a fundamental level, the biosynthesis of both prokaryotic and eukaryotic membrane proteins requires two events to occur^{1,2}. First, the newly synthesized polypeptide chain must be inserted into the membrane via recognition by the evolutionarily conserved Sec translocon^{3,4}. This prerequisite for Sec translocon engagement can be seen as placing a lower ceiling on membrane protein biosynthesis relative to soluble protein biosynthesis, given that the Sec translocon can become saturated when membrane proteins are overexpressed^{5,6}. The manner in which the Sec translocon can recognize heterologous sequences can lower this ceiling even further⁷, especially in those cases of expressing human membrane proteins in the most commonly used system for protein expression, *Escherichia coli*⁸. Second, the inserted polypeptide must laterally traverse into the lipid bilayer where folding into the correct three-dimensional structure predominately occurs^{1,2,9}. Given that the composition of lipid bilayers differ substantially between humans and other species^{10,11}, and given the pronounced effects that lipids and sterols have on membrane protein structure and function^{12–14}, it is apparent that lipid composition is a key variable in determining the yields of functional membrane proteins that are produced.

In addition to these two fundamental principles of membrane protein biosynthesis, eukaryotic membrane proteins often require additional post-translational modifications not found in prokaryotes. *N*-linked glycosylation of eukaryotic membrane proteins, for instance, can be of crucial importance for the folding of membrane proteins possessing large extracellular domains, given that these glycans serve as ligands for the ER-resident molecular chaperones, calnexin and calreticulin¹⁵. Nevertheless, *N*-linked glycosylation is often seen as an impediment toward structure determination via X-ray crystallography, given that these glycans are often very large, heterogeneous and conformationally flexible¹⁶. Indeed, considerable

effort has been placed into restricting the heterogeneity of these *N*-linked glycans via mutagenesis, glycosidases and/or inhibitors, for the purpose of structural studies^{17–20}.

The use of mammalian cell expression systems is becoming increasingly popular for the overexpression of mammalian membrane proteins for structural studies^{21,22}, likely owing to their near-native translocation machinery, lipid milieu and post-translational modifications. A suspension-adapted HEK293S cell line lacking GnTI⁻ has been successfully used to overexpress a wide variety of mammalian membrane proteins^{23–25}, including that of human RhCG, whose X-ray crystal structure was recently reported in our laboratory²¹. Developed by Gobind Khorana and colleagues to overexpress large quantities of functional rhodopsin²⁰, the HEK293S GnTI system possesses features that make it amenable for structural studies. First, the lack of GnTI restricts *N*-linked glycans to a homogeneous Man₅-GlcNac₂ structure, which greatly facilitates their enzymatic removal via endo- and exoglycosidases. Second, the system uses a tetracycline-inducible promoter, allowing for protein expression to be induced once high-density cell cultures are established (discussed further in Induction of membrane protein expression). As compared with other lower eukaryotic expression systems that have been used to express mammalian membrane proteins for structural studies, such as baculovirus-infected SF9 insect cells²⁶, expression from HEK293S GnTI⁻ cells is typically more labor intensive and less cost effective. Nevertheless, given that the folding and proper trafficking of mammalian membrane proteins can depend crucially on the translocation machinery, lipid milieu and post-translational modification present in the expression system¹⁴, the use of mammalian cell expression systems is likely to ensure the greatest probability of producing properly folded and functional human membrane proteins.

In this protocol, we describe the methods that we used to overexpress quantities of pure, homogeneous and stable human RhCG from stably transfected and clonal HEK293S GnTI⁻ cells,

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for use in crystallization. Expression trials of human RhCG were performed using *E. coli*, *Saccharomyces cerevisiae* and HEK293S GnTI⁻ cells; however, only in the case of HEK293S GnTI⁻ cells was RhCG expressed at high levels sufficient for structural studies. In addition, three human Rh proteins were transiently expressed in HEK293S GnTI⁻ cells, with RhCG having the highest level of solubilized membrane protein (Fig. 1). The protocol described here is of use to those that require long-term overexpression of a human membrane protein (e.g., in antigen production, drug discovery, biochemical characterization or structural studies), and it assumes that suitable expression constructs have already been generated and screened (see Experimental design). A summary of the various steps of the protocol are discussed in detail below.

Stable versus transient expression in HEK293S cells

There are two ways by which a transfected transgene can be overexpressed in HEK293S cells, either transiently or stably. In transient expression, a sufficient quantity of plasmid DNA is transfected into cell cultures of varying sizes (milliliter to liter scales), after which overexpression is either immediate or induced, depending on the promoter type of the plasmid. Continued passage of transiently transfected cells results in a dilution of cells that are transfected; therefore, each overexpression trial must be preceded by transfection. Large-scale transient expression, typically involving cell cultures in the 1- to 10-liter range, is becoming increasingly popular given the flexibility of the system and its potential for high-throughput overexpression trials^{17,19,27}.

Stable expression, on the other hand, occurs from a transgene that has been stably integrated into the transfected cell's genome. Stably transfected cell lines are generated by introducing Geneticin to the cell culture over a period of 2–3 weeks, which selects for cells that have stably integrated plasmid DNA (possessing the transgene and Geneticin resistance). Clones of stably transfected cell lines can then be generated and scaled up for transgene overexpression. The generation of clonal, stably transfected HEK293S cell lines is initially slower and more technically challenging relative to large-scale transient expression. Nevertheless, once a clonal cell line is generated, long-term overexpression from stably transfected cells can be much more facile and consistent compared with transient expression, given that the purification of large quantities of plasmid DNA followed by separate large-scale transfections is not required.

We have explored overexpression of human RhCG from both transiently and stably transfected HEK293S cell lines, using a pACMV-tetO expression vector in both cases, and we have found that expression levels are significantly higher in clonal, stably

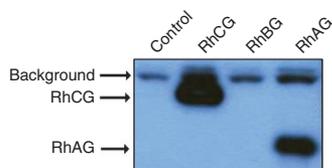


Figure 1 | Expression testing of full-length human Rh membrane proteins in transiently transfected HEK293S GnTI⁻ cells. A measure of 10 µg of RhAG, RhBG and RhCG, subcloned into pACMV-tetO, was separately transfected into HEK293S GnTI⁻ cells grown in a 10-cm² tissue culture plate. Cells were induced with doxycycline (24 h after transfection), collected (24 h post induction) and analyzed via anti-histidine western blotting, as described in the text. On the basis of these results, RhAG and RhCG were chosen as candidates for stable cell line generation.

transfected HEK293S cells. Nevertheless, in those cases in which shorter timescales and/or higher throughput are favored over longer-term and consistent expression levels, cell lines and expression vectors designed for transient expression can be explored^{17,28}.

Stable cell line generation

Stably transfected HEK293S cells are generated by selecting for successful integration of the *neo* gene using the antibiotic Geneticin, following a workflow as shown in Figure 2. Selection occurs in a 10-cm² tissue culture plate, where it takes approximately 2–3 weeks for foci of Geneticin-resistant colonies to appear, after which the resistant foci are clonally expanded over a period of an additional 2–3 weeks (approximately). Although we used Geneticin for selection, other drugs and resistance gene combinations can be used, such as puromycin and puromycin acetyltransferase, respectively.

The level of transgene expression for any given stably transfected and clonal HEK293S cell line can vary substantially. Integration of plasmid DNA into the HEK293S genome is random; therefore, the level of transgene expression will be determined in large part by position effects. In addition, given that the site of recombination within the plasmid sequence is random, no transgene expression will be observed if the site of recombination disrupts either the coding sequence or the promoter of the transgene. We have found that expanding 24 clonal cell lines and screening these clones for expression via western blotting is most efficient to find the highest-expressing clones. Nevertheless, in those cases in which screening 24 clonal cell lines is insufficient to identify highly expressing clones, or in those cases in which a large number of clonal cell lines are to be generated in parallel, higher-throughput cloning and screening strategies could be explored. For example, higher-throughput cloning into 96-well plates, via FACS or limited dilution plating followed by fluorescence or ELISA quantification of expression levels, can be used to facilitate cloning and screening for highly expressing mammalian cell lines²⁹.

Assessment of expression levels from clonal cell lines

Small-scale detergent solubilizations, using both octyl-β-D-glucopyranoside (OG) and *N*-dodecyl-β-D-maltopyranoside (DDM), are performed on all clonally expanded cell lines. The use of two or more detergents at this stage is optional, as detergent solubilization screening can be performed further downstream upon identification of the most highly expressing clone. DDM should be used for small-scale solubilizations if a single detergent is to be assessed, given its greater ability to solubilize membrane proteins compared with OG³⁰.

The solubilized material from each clonal cell line is assessed for membrane protein expression via anti-fusion tag western blotting. Comparison of the 'before spin' (BS) sample, which reveals total membrane protein expression levels, with the 'after spin' (AS) sample, which reveals successfully solubilized protein expression levels, indicates the degree of solubilization. Caution should be exercised in those cases where solubilization only occurs in fos-choline-type detergents, given their potential to denature membrane proteins³¹. Although the western blotting protocol used for assessing membrane protein expression levels is not quantitative, relative expression levels between clones can be assessed, allowing for the identification of clones that express high levels of detergent-soluble membrane proteins. It is the amount of detergent-soluble membrane protein (i.e., AS) produced, and not the total amount

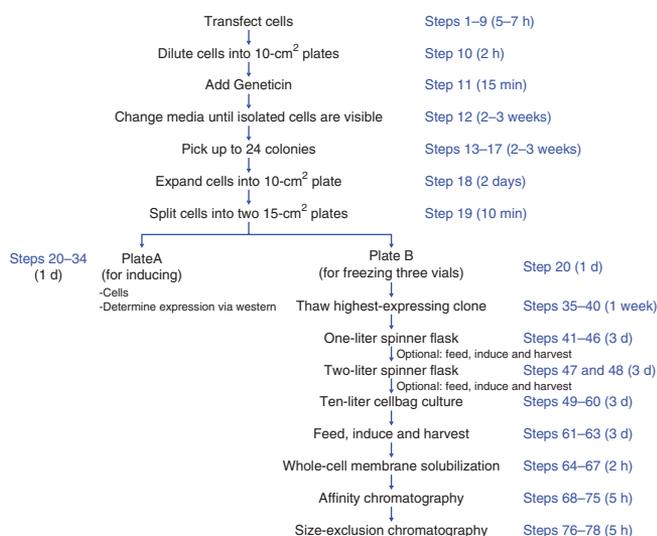


Figure 2 | Workflow for the overexpression of mammalian membrane proteins in stably transfected and clonal HEK293S GnTI⁻ cells.

of membrane protein (i.e., BS) produced, that defines the usable expression level of a particular cell line.

Medium- and large-scale suspension cultures

Once a highly expressing, clonal cell line has been identified, medium (approximately 1- to 3-liter spinner flasks) and/or large (~10-liter cellbag bioreactor) suspension cultures are established for overexpression trials. The HEK293S GnTI⁻ cells used during the course of this work are adaptable for suspension growth²⁰, allowing for the establishment of higher densities of cell cultures relative to adherent cultures. This adaptation from an adherent monolayer to suspension cultures is brought about solely by changing the medium; the suspension medium lacks the calcium required for forming an adherent monolayer. In practice, adherent HEK293S GnTI⁻ cells do not take long to be adapted to grow as a suspension culture in DMEM supplemented with serum^{20,32}. Nevertheless, the growth of HEK293 cells in suspension is not always facile and, depending on the type of cells and media used, can require that the cells be adapted for suspension growth over a period of several weeks²⁸. The suspension medium used in this manuscript is a cost-effective alternative to commercially available, serum-free formulations designed for suspension cell cultures and, in addition to a lack of calcium, it includes iron-supplemented bovine calf serum (BCS), and the nonionic detergent Pluronic F-68, with Primatone RL/UF added later as a supplement. Iron-supplemented BCS is a cost-effective alternative to FBS, whereas Primatone RL/UF increases the viability and density of mammalian cells grown in suspension³³. Pluronic F-68 has been shown to both reduce cell adherence and protect cells against high levels of shear stress introduced by sparging and stirring in the suspension culture³⁴, in a mechanism thought to involve the nonionic detergent coating the plasma membranes of the suspension cultures³⁴. This suspension medium permits us to grow HEK293S GnTI⁻ cells as a suspension culture, without requiring a lengthy adaptation period from an adherent culture.

The scale of cell culture that is established depends largely on what is known of the membrane protein being expressed. At the initial stages of the structure determination of human RhCG, cells were grown, induced and collected on a periodic basis in 1- to 2-liter

spinner flasks. These 1- to 2-liter spinner flask cultures were used to determine the optimal parameters for isolating pure, homogeneous and stable RhCG—parameters that included detergent type, pH, glycerol, and protease concentrations and incubation periods (for fusion tag removal). Once optimal purification conditions were determined, suspension cultures were established in larger 10-liter cellbags, or occasionally in three 3-liter spinner flasks. Although cellbag bioreactors are expected to establish higher densities of suspension cultures relative to spinner flasks, owing to more efficient oxygen transfer into the medium, the observed densities of RhCG HEK293S cells in cellbag and spinner flask cultures were comparable (approximately 1–1.5 × 10⁶ cells per ml). We have not, however, explored whether increasing or decreasing the rate of air/oxygen sparging into the cellbag can increase cell densities. Given the comparable cell densities observed for RhCG HEK293S in cellbags and spinner flasks, we have also established large-scale (three 3 liter) spinner-flask cultures of RhCG HEK293S, which are more facile and more cost-effective compared with cellbag cell cultures.

Induction of membrane protein expression

In the HEK293S GnTI⁻ system, all membrane proteins are expressed from a tetracycline-inducible promoter. This is an advantage in the overexpression of membrane proteins in particular, as the intrinsic function of certain membrane proteins (e.g., channels, G protein-coupled receptors and so on) can be cytotoxic to the cell, thereby preventing high-density cell cultures from being established^{14,32,35,36}. For example, it has been shown that stable cell lines constitutively expressing the serotonin transporter (SERT) can only be generated in the presence of SERT inhibitors, possibly owing to the fact that the intrinsic functions of SERT (serotonin transport and channel-like activity) severely stress the cell in the absence of SERT inhibitors¹⁴. The use of a tetracycline-inducible promoter, therefore, delays the expression of potentially toxic membrane proteins until high-density cell cultures are established.

In the protocol described here, membrane protein expression is induced by adding an appropriate amount of doxycycline (a tetracycline antibiotic) to the suspension culture, either grown in spinner flasks or cellbags. For RhCG, we have found that induction with doxycycline at a cell density of ~1.0 × 10⁶ cells per ml, followed by collection of the cells 36 h later, is optimal for RhCG expression; nevertheless, these parameters should be empirically determined for each particular membrane protein to be overexpressed. Sodium butyrate is also added to the suspension cultures at the time of induction, given its ability to increase protein expression levels from mammalian cells³⁷; however, it alone does not induce expression.

Whole-cell solubilization and membrane protein purification

Solubilization of RhCG from whole HEK293S cells was performed subsequently to harvesting the medium- and large-scale suspension cultures. We have found that whole-cell solubilizations are faster to perform and can result in higher yields of extracted RhCG compared with solubilization from a membrane preparation. Solubilization of membrane proteins from a membrane preparation, however, should be explored if the final purity of membrane protein after whole-cell solubilization is insufficient.

A two-step purification scheme was used for the purification of human RhCG. First, FLAG purification was performed, with either OG or DDM, from whole-cell-solubilized RhCG. This step will

depend on the type of transgene that is integrated into the clonal cell; in the case of RhCG, the expression vector (pACMV-tetO; ref. 20) was modified to possess an N-terminal FLAG tag and a C-terminal histidine tag, potentially allowing for two types of affinity purification to be performed. The FLAG-purified RhCG was purer and of better yield as compared with the immobilized metal affinity chromatography (IMAC)-purified RhCG; therefore, we did not explore IMAC purification in detail. Second, size-exclusion chromatography (SEC) was performed, which resulted in the purification of RhCG to near homogeneity. SEC is an excellent metric for both the purity of a membrane protein (pure proteins will have discrete and near-monodisperse major peaks) and its stability (stable proteins will possess few changes in chromatographic profile over multiple days). Purified membrane proteins that are both near-monodisperse and stable, such as was found with human RhCG, are excellent candidates for crystallization trials.

Experimental design

Before undertaking the experiments described in this protocol, it is important to screen potential constructs for both expression quality and quantity. The use of fluorescence SEC (FSEC) at this stage to assess protein quality and quantity from transiently transfected HEK293 cells is therefore suggested, in that this will help to ensure that only suitable expression constructs are selected for stable cell line generation³⁸. Constructs suitable for structural studies will possess single, monodisperse FSEC profiles of high magnitude³⁸. An alternative approach is to perform anti-fusion tag western blotting of transiently transfected HEK293S GnTI⁻ cells, in a similar manner as described in this protocol (see Steps 26–34). Constructs that

possess a weak signal on a western blot could then be redesigned, or possibly codon optimized, depending on the importance of the particular membrane protein to the laboratory. The screening of constructs via FSEC or western blotting is also important in that, in our experience, it is not possible to predict a priori whether a particular membrane protein class will express well in HEK293S GnTI⁻ cells, as the most highly expressing clonal cell lines produced in our laboratory, to date, are an ammonia transporter (RhCG), a cation transporter, a class B GPCR and a protease.

Although the entire procedure described here takes approximately 8–10 weeks to perform, the procedure can be paused at week 6 by freezing the clonal cell line. It is important to ensure that personnel are available to maintain the HEK293S GnTI⁻ cells up to week 6, as clonal cell line generation can fail if the procedure is not followed and the cells are unnecessarily stressed (e.g., by failing to change the medium promptly, allowing the cells to become overconfluent and so on.). This requires approximately 0.5–3 h of cell culture work per day, 1–4 d per week, depending on the stage of the procedure. Similarly, once HEK293S GnTI⁻ cells are collected from spinner flask or WAVE cellbag suspension cultures, the whole-cell membrane solubilization and purification steps should be performed immediately, given that the membrane protein of interest may be unstable; this most closely follows the procedure performed for the solubilization, purification and crystallization of human RhCG. Nevertheless, the procedure can be modified following the cell harvesting stage to create an additional pause point by preparing a membrane fraction and freezing these membranes at –80 °C (ref. 39) for future solubilization and purification.

MATERIALS

REAGENTS

- HEK293S GnTI cells (ATCC, cat. no. CRL-3022)
- pACMV-tetO (see **Supplementary Fig. 1** for full sequence)
- Opti-MEM I (UCSF Cell Culture Facility, cat. no. CCFAC008 or Invitrogen, cat. no. 31985070) **▲ CRITICAL** All reagents to be used for cell culture work (media, antibiotics, chemicals and so on) should be of tissue culture grade, if available.
- DMEM with high glucose (UCSF Cell Culture Facility, cat. no. CCFAA005 or Invitrogen, cat. no. 32430027)
- DMEM with high glucose without Ca salts (UCSF Cell Culture Facility, cat. no. CCFDA003 or Invitrogen, cat. no. 21068028)
- D-PBS (without calcium and magnesium; UCSF Cell Culture Facility, cat. no. CCFAL003 or Invitrogen, cat. no. A1285601)
- Penicillin-streptomycin (100×; UCSF Cell Culture Facility, cat. no. CCFGK004 or Invitrogen, cat. no. P4333-100ML)
- Sodium bicarbonate (Sigma, cat. no. S5761)
- Trypsin (0.05% (wt/vol) trypsin) with EDTA in saline A (UCSF Cell Culture Facility, cat. no. CCFGP002 or Invitrogen, cat. no. 25300-054)
- Geneticin (50 mg ml⁻¹; Gibco, cat. no. 10131-027)
- Blastidicin S HCl (50 mg; Invitrogen, cat. no. R210-01)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-019)
- Pluronic (Sigma, cat. no. P1300)
- Sodium butyrate (Sigma, cat. no. 303410)
- Doxycycline hyclate (Sigma, cat. no. D9891)
- Primatone RL/UF (Kerry Bio Science, cat. no. 5X00130)
- FBS (JRSscientific Inc., cat. no. 43652)
- Cell preservation medium (UCSF Cell Culture Facility, cat. no. CCFHH002)
- Iron-supplemented BCS (Hyclone, cat. no. SH30072.03)
- Tris base (Fisher Scientific, cat. no. BP154-1)
- NaCl (Fisher Scientific, cat. no. 7647-14-5)
- Glycerol (Fisher Scientific, cat. no. BP229-4)
- Anti-FLAG M2 affinity gel (Sigma, cat. no. A2220)

- FLAG peptide (3×; Sigma, cat. no. F4799)
- OG (Anatrace, cat. no. O 311)
- DDM (Anatrace, cat. no. D 310)
- PMSF (Sigma, cat. no. P7626)
- Complete EDTA-free protease inhibitor cocktail tablets (Roche, cat. no. 11 873 580 001)
- Western blotting reagents (Thermo, cat. nos. 1856135 and 1856136)
- Benzonase (Sigma, cat. no. E1014)
- DTT (Sigma, cat. no. D0632)
- 3C protease (homemade)
- Thrombin (Novagen, cat. no. 69671-3)
- Mouse IgG-agarose (Sigma, cat. no. A0919)
- Ethanol
- Glacial acetic acid
- Distilled water
- Liquid nitrogen
- 293 SFM II (optional; Invitrogen, cat. no. 11686-029)
- Coomassie blue stain

EQUIPMENT

- CryoTube vials (Nunc, cat. no. 377267)
- Cloning cylinder (Bel-Art products, cat. nos. 378470100, 378470200 and 378470300)
- Tissue culture plate (six well; BD Falcon, cat. no. 353046)
- Tissue culture plate (24 well; BD Falcon, cat. no. 353047)
- Tissue culture dish (100 mm; BD Falcon, 353003)
- Tissue culture dish (150 mm; BD Falcon, 353025)
- Sterile alcohol prep pads (Fisher HealthCare, 06-669-62)
- Precast (4–20% wt/vol) SDS-PAGE (Bio-Rad, cat. no. 456-1096)
- Dounce homogenizers (7, 15 and 40 ml; Kontes Glass Co.)
- Bottle (1,000 ml; Beckman)
- Bottle (250 ml; Nalgene)
- Inverted light microscope (Olympus CK2 or equivalent)



- Hemocytometer (Hausser Scientific or equivalent)
- Class II biological safety cabinet (Baker)
- CO₂ incubator (Sanyo)
- Superdex 10/300GL (GE Healthcare)
- Fast-performance liquid chromatography station (Akta, Shimadzu or equivalent)
- AHB spin flask comp (1 and 3 liter (Bellco)) ▲ **CRITICAL** Ensure that each flask has been cleaned and autoclaved before use (see EQUIPMENT SETUP).
- WAVE Bioreactor (GE Healthcare)
- Cellbag (GE Healthcare or Flex Concepts)
- Nutator (BD)
- Ultrasonic water bath (Branson)
- Mosquito (TTP LabTech)
- Optima L-90K Ultracentrifuge (Beckman)
- Type 45 Ti rotor (Beckman)
- Glass Econo-column (Bio-Rad, cat. no. 737-1517)
- Luer syringe (5 ml; BD)
- Syringe filters (0.22 and 0.45 μm; Pall)
- Centrifuge tubes (15 and 50 ml; Corning)
- Amicon Ultra-15 (50 kDa or 100 kDa molecular weight cutoff) centrifugal filters (Millipore, cat. nos. UFC905024 and UFC910024)
- Felt-tip pen
- Sterile forceps
- Dewar flask
- Nitrocellulose membrane
- Nutator

REAGENT SETUP

DMEM medium To 1 liter of DMEM–high glucose, add 10 ml of penicillin-streptomycin (100×) and 100 ml of iron-supplemented BCS. Filter-sterilize inside a biological safety cabinet by using a 0.2-μm syringe filter. Store at 4 °C for up to 1 year. ▲ **CRITICAL** The addition of serum to DMEM is suggested for all cell culture work as, in our experience, HEK293S GnT[−] cells do not passage well in DMEM alone. Iron-supplemented BCS is added to both DMEM and suspension medium, as it is a cost-effective alternative to FBS, suitable for most cell culture work (see DMEM (10% (vol/vol) FBS) for exception).

DMEM (10% (vol/vol) FBS) To 1 liter of DMEM–high glucose, add 10 ml of penicillin-streptomycin (100×) and 100 ml of FBS. Filter-sterilize inside a biological safety cabinet by using a 0.2-μm syringe filter. Store at 4 °C for up to 1 month. ▲ **CRITICAL** The medium that is used during the drug selection process and foci expansion contains FBS, in place of iron-supplemented BCS, in order to better ensure cell viability.

Selection medium To 1 liter of DMEM–high glucose, add 10 ml of penicillin-streptomycin (100×), 100 ml of FBS, 40 ml of Geneticin and 1 ml of Blastidicin S HCl. Filter-sterilize inside a biological safety cabinet by using a 0.2-μm syringe filter. Store at 4 °C for up to 1 month. ▲ **CRITICAL** The medium that is used during the drug selection process and foci expansion contains FBS, in place of iron-supplemented BCS, in order to ensure better cell viability.

Suspension medium To 1 liter of DMEM–high glucose without calcium salts, add 10 ml of penicillin-streptomycin (100×), 10 ml of Pluronic, 0.3 g of Primatone RL/UF, 100 ml of BCS and 3.7 g of sodium bicarbonate. Filter-sterilize inside a biological safety cabinet with a 0.2-μm syringe filter. Store at 4 °C for up to 1 month. ▲ **CRITICAL** A serum-free medium formulated for the growth of HEK293S in suspension, such as 293 SFM II, may also be used; however, serum-free medium is typically fivefold more expensive than suspension medium.

Suspension medium (for cellbag bioreactor suspension cultures) To 7 liters of DMEM–high glucose without calcium salts, add 70 ml of penicillin-streptomycin (100×), 700 ml of BCS, 70 ml of Pluronic, 2.1 g Primatone RL/UF and 25.9 g sodium bicarbonate. Prepare before use.

Blasticidin solution (5 mg ml^{−1}) Dissolve 50 mg of Blastidicin S HCl in 10 ml of autoclaved water. Filter-sterilize inside a biological safety cabinet with a 0.2-μm syringe filter and store 1-ml aliquots at −20 °C. ▲ **CAUTION** Blastidicin is toxic. Always wear gloves, a mask, a laboratory coat and safety glasses. Prepare the solution inside a biological safety cabinet. ▲ **CRITICAL** Aliquot in small volumes suitable for one-time use and store them at −20 °C for 6–8 weeks.

Doxycycline hyclate (20 mg ml^{−1}) To prepare 20 mg ml^{−1} of doxycycline solution, dissolve 100 mg of doxycycline hyclate in 5 ml of autoclaved water. Filter-sterilize inside a biological safety cabinet with a 0.2-μm syringe filter and store 1-ml aliquots at −20 °C for 6–8 weeks. ▲ **CRITICAL** Doxycycline should not be exposed to direct sunlight.

Sodium butyrate (500 mM) Dissolve 27.5 g of sodium butyrate with autoclaved water to a final volume of 500 ml and filter-sterilize by using a 0.2-μm syringe filter inside the biological safety cabinet. Store at room temperature (20–25 °C) for 6–8 weeks.

Primatone RL/UF (10% wt/vol) Dissolve 3 g of Primatone RL/UF in 30 ml of autoclaved water and filter-sterilize by using a 0.2-μm syringe filter inside the biological safety cabinet. Prepare immediately before use.

Glucose (20% wt/vol) Dissolve 100 ml of glucose in 500 ml of autoclaved water and filter-sterilize by using a 0.2-μm syringe filter inside the biological safety cabinet. Store at 4 °C for 2–3 months.

Pluronic (10% wt/vol) Dissolve 50 ml of Pluronic in 500 ml of autoclaved water and filter-sterilize with a 0.2-μm filter inside the biological safety cabinet. Store at 4 °C for 6–8 weeks.

Western solubilization buffer (2×) Mix 40 mM Tris base (pH 7.4), 200 mM NaCl and 20% (vol/vol) glycerol. Immediately before use, add 1 mM PMSF and one complete EDTA-free protease inhibitor cocktail tablet. Chill to 4 °C and discard any unused buffer.

Lysis buffer Mix 20 mM Tris base (pH 7.4), 100 mM NaCl and 10% (vol/vol) glycerol. Immediately before use, add 1 mM PMSF and one complete EDTA-free protease inhibitor cocktail tablet. Chill to 4 °C and discard any unused buffer.

SEC buffer Mix 20 mM Tris base (pH 7.4), 100 mM NaCl, 10% (vol/vol) glycerol and 40 mM OG (or 0.5 mM DDM). Store at 4 °C for up to 1 week.

Culture of HEK293S GnT[−] cells Periodically split into 10-cm² tissue culture plates using DMEM. In a biosafety cabinet, gently wash the cells with 5 ml of D-PBS and aspirate off the medium. Add 1 ml of trypsin, and incubate at room temperature for 1 min. Gently shake the tissue culture plate to facilitate dislodging of the cells from the plate. Add 5 ml of DMEM, resuspend the cells and pellet at low speed for 5 min. Discard the DMEM, add 1 ml of DMEM to resuspend the cells and add half of the cells to a new 10-cm² tissue culture plate containing 10 ml of DMEM. For all cell culture work, HEK293S cells are maintained in a humidified incubator set to 5% CO₂/95% air and 37 °C. ▲ **CRITICAL** HEK293 cells, as with mammalian cells in general, are very easily contaminated. Therefore, it is of utmost importance that sterile tissue culture techniques be followed at all times. All surfaces (i.e., biosafety cabinet, microscopes and so on) should be cleaned with 70% (vol/vol) ethanol before use (see TROUBLESHOOTING table). Gloves (cleaned with 70% (vol/vol) ethanol) and lab coats must be worn at all times.

EQUIPMENT SETUP

Spinner flask Clean thoroughly by separating the different parts of the flask. Add 10% (vol/vol) glacial acetic acid and stir overnight at room temperature. The next day, remove the 10% (vol/vol) glacial acetic acid and rinse the spinner flask very well to remove any trace of acid. Fill the spinner flask with distilled water and perform two liquid autoclaves for 30 min each, and then a final dry autoclave for 30 min. Allow the flasks to cool to room temperature before use.

WAVE bioreactor Assemble the WAVE bioreactor in a tissue culture room according to the manufacturer's protocols.

PROCEDURE

Cell seeding (day 1) ● **TIMING** ~15 min

1| Split HEK293S cells to ~40% confluency into each well of a six-well tissue culture dish, using DMEM, so that the next day the cells are approximately 70–80% confluent for transfection. In practice, this can be performed by resuspending the

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cells from one confluent 10-cm² tissue culture plate with 6 ml of DMEM, and then seeding ~400 μ l of this into a well containing 2 ml of DMEM.

? TROUBLESHOOTING

Transfection (day 2) ● TIMING ~5–7 h (45 min transfection + 4–6 h incubation + 15 min medium exchange)

2| On the day of transfection, ensure that the cells are approximately 70–80% confluent.

? TROUBLESHOOTING

3| In an autoclaved 1.5-ml centrifuge tube, dilute 1.5 μ g of plasmid DNA (in up to 10 μ l of autoclaved H₂O) with 240 μ l of Opti-MEM I.

4| Repeat Step 3 with positive control DNA (i.e., typically a pACMV-tetO construct containing a well-expressed transgene) and 10 μ l of autoclaved H₂O as negative control.

5| For each transfection to be performed, dilute 30 μ l of Lipofectamine 2000 with 720 μ l of Opti-MEM I in a separate 1.5-ml centrifuge tube and incubate for 5 min at room temperature.

6| Gently mix the diluted DNA samples (Steps 3 and 4) with the diluted Lipofectamine reagent (Step 5; total volume = 500 μ l), and incubate for 30 min at room temperature to allow for the formation of DNA-liposome complexes.

7| During the 30-min incubation of Step 6, replace the medium of the cells with 1.5 ml of Opti-MEM I.

8| To one well of the six-well cell culture plate, add 500 μ l of DNA-liposome complex dropwise. A six-well tissue culture plate can thus be used to transfect four separate constructs, in addition to the positive and negative controls. Mix gently by tilting the plate back and forth and incubate for 4–6 h at 37 °C in a CO₂ incubator.

9| Replace the medium with DMEM and incubate overnight at 37 °C.

Expansion (day 3) ● TIMING ~2 h

10| Split the cells from one well of the six-well plate into five 10-cm² tissue culture plates, at a dilution ratio ranging from 1:20 to 1:400, as shown in **Figure 3**, and then incubate overnight at 37 °C.

? TROUBLESHOOTING

Geneticin selection (day 4) ● TIMING ~15 min

11| Exchange the DMEM in each of the 10-cm² plates with 10 ml of selection medium and incubate overnight at 37 °C.

Generation of stably transfected cell colonies ● TIMING 2–3 weeks

12| Exchange the selection medium in each of the 10-cm² plates with 10 ml of fresh selection medium. During the first week of selection, the medium should be replaced every other day, whereas after 1 week the medium can be replaced every 3–4 d. The whole process takes approximately 15–20 d, after which single colonies of adequate size for clonal expansion (~1 cm in diameter) are formed.

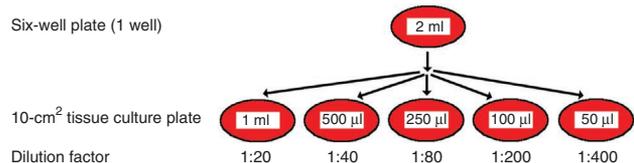
? TROUBLESHOOTING

Generation of clonal cell lines ● TIMING 2–3 weeks

13| By using a felt-tip pen, draw a circle on the back of the tissue culture plate around up to 24 single, well-isolated cell colonies. Hold the plate toward a light source for better colony visualization.

▲ **CRITICAL STEP** Colonies must be single and well isolated from neighboring colonies in order to ensure that clonal cell lines are generated. Of the five cell dilutions prepared in Step 10, only a few well-isolated colonies are typically observed for the most-diluted plate (1:400), whereas less well-isolated colonies are observed for the least-diluted (1:20) plate. Choose a total of 24 colonies from all of the five plates.

Figure 3 | Dilution of transfected HEK293S GnTI⁻ cells before drug selection. Cells from one well of a six-well tissue culture plate (2 ml total) are diluted into five 10-cm² tissue culture plates (indicated in the boxes) by plating the appropriate amount of cells. Each 10-cm² plate contains 10 ml of DMEM. The final dilution factor is indicated for each plate.



14| On the basis of the size of the colony, choose either a small-, medium- or large-sized cloning cylinder. Use sterile forceps to pick up the sterile cylinder, and then apply autoclaved grease to the bottom of the cylinder to ensure that the cylinder will seal to the plate.

15| Aspirate off the medium from the plate and wash the cells with 5 ml of D-PBS. Carefully, without disturbing the colony or touching any other colony, place a cylinder over the colony such that the colony is centered in the middle of the cylinder. Gently press the cylinder down to adhere it to the surface.

▲ **CRITICAL STEP** Make sure to completely aspirate off the D-PBS, as any remaining D-PBS will prevent the cylinder from adhering well to the plate.

16| On the basis of the size of the cloning cylinder, add between 50 and 200 μ l of trypsin. Dislodge the cells by gently pipetting up and down without disturbing the cylinder. For each colony, transfer the trypsinized cells to a single well of a 24-well tissue culture plate containing 1 ml of DMEM (10% (vol/vol) FBS). Incubate at 37 °C in a CO₂ incubator for 22–24 h. DMEM (10% (vol/vol) FBS), as opposed to selection medium, is used at this stage in order to ensure viability of the newly picked colony.

17| On the next day, exchange the DMEM in each well with 1 ml of selection medium. Keep exchanging with fresh selection medium every 3–4 d until the cells are confluent.

18| At confluency, aspirate off the medium and add 100 μ l of trypsin. Transfer the resuspended cells to one well of a six-well tissue culture plate containing 2 ml of selection medium. Transfer any residual cells left over in the 24-well plate using this 2 ml of selection medium. Once the cells reach confluency, aspirate off the medium and add 200 μ l of trypsin. Transfer the resuspended cells to a 10-cm² plate containing 10 ml of the selection medium.

19| Once the cells reach ~90% confluency, split the cells into two 15-cm² tissue culture plates. Incubate at 37 °C until the plates reach confluency.

20| Use one of the 15-cm² tissue culture plates to make frozen cell stocks as described in **Box 1**. Use the other 15-cm² tissue culture plate for assessing membrane protein expression levels (Steps 21–34).

21| Aspirate off the medium, wash the cells with 10 ml of D-PBS and add 25 ml of DMEM, being careful not to dislodge the cells.

22| To induce the cells, add doxycycline (final concentration = 2 μ g ml⁻¹) and sodium butyrate (final concentration = 5 mM), and incubate at 37 °C.

23| After 24 h, aspirate off the medium, wash the cells with 10 ml of D-PBS and add 2 ml of trypsin.

24| Resuspend the trypsinized cells thoroughly in 10 ml of DMEM and pellet the cells at low speed for 4–5 min.

25| Aspirate off all of medium and flash-freeze the cell pellet in liquid nitrogen. Store the frozen cells at –20 °C until at least 12 clones have been produced before proceeding to Step 26 for small-scale solubilization and western blotting analysis.

■ **PAUSE POINT** For future use, cell pellets can be stored in a –80 °C freezer for weeks, or in a liquid N₂ storage Dewar for years.

Box 1 | Making frozen cell stocks ● TIMING ~30 min

1. Aspirate off the medium, wash the cells with 10 ml of D-PBS and add 2 ml of trypsin.
2. Resuspend the trypsinized cells with 10 ml of DMEM, transfer to a 15-ml centrifuge tube and pellet the cells at low speed for 4–5 min.
3. Aspirate off the medium and gently resuspend the cells in 3 ml of cell preservation medium, ensuring that there are no large clumps of cells.
4. Make three frozen cell stocks by transferring 1 ml of resuspended cells to each of three 1.5-ml cryovials. Transfer the vials to Cryo container and store at –80 °C.
5. After 24 h, transfer the cryovials to a liquid nitrogen storage Dewar. Use these frozen stocks to expand highly expressing clones (Step 35).

PROTOCOL

Assessment of membrane protein solubilization and expression levels from clonal cell lines ● TIMING 1 d

26| Remove the frozen cell pellets from $-20\text{ }^{\circ}\text{C}$ and thaw on ice. Measure the weight of the pellet. Add 0.5 ml of 2× western solubilization buffer (no detergent) per 0.1 g of cells to each tube.

27| Resuspend the pellet with a pipette. Aliquot the resuspended cells equally into two glass tubes containing an equal volume of either 40 mM DDM or 400 mM OG, in H_2O .

28| Place the samples in a water bath sonicator and sonicate for 1 min. Incubate the samples on ice for 1 min. Repeat the sonication and incubation-on-ice cycle five times.

29| Transfer 400 μl of sonicated sample into a 1.5-ml ultracentrifuge tube containing a small magnetic stir bar. Add 0.2 μl of Benzonase and stir at $4\text{ }^{\circ}\text{C}$ for 30 min at moderate speed.

30| Take a 50- μl aliquot as a BS sample.

31| Spin the remaining sample at 200,000g for 20 min at $4\text{ }^{\circ}\text{C}$ with a Type 45 Ti ultracentrifuge rotor. Transfer to a clean 1.5-ml centrifuge tube labeled as AS.

32| For each clone, resolve 10 μl of BS and AS samples by reducing SDS-PAGE.

33| Transfer to a nitrocellulose membrane according to the manufacturer's protocols.

34| Perform a western blot analysis using either anti-His or anti-FLAG HRP-conjugated antibodies, according to the manufacturer's protocols. The two clones that possess the highest level of solubilized membrane protein (i.e., the strongest band in the AS lane) are saved for further cell culture scale-up (Step 35). The remaining clones can either be discarded or saved; we typically save frozen stocks of the eight most highly expressing clonal cell lines, until we establish that our best clone expresses from a 1-liter spinner flask. The levels of membrane protein expression from each clone can be expected to be highly variable (Fig. 4).

▲ **CRITICAL STEP** It is important to immunoblot both the BS and AS samples for each clone, given that the BS sample reveals the amount of membrane protein produced, whereas the AS sample reveals the amount that is solubilized. A low amount of solubilized AS signal, relative to BS, can indicate that either the detergent is not sufficient for solubilization (as is often the case with OG) or that the membrane protein is not well folded. In addition, in cases of low solubilization, additional variables such as different detergents, pH and salt concentration should be assessed for their effect on solubilization.

Expansion of cells from liquid nitrogen–frozen stocks ● TIMING 1 week

35| Once the best-expressed clone has been determined via western blotting, transfer one vial of cells from the liquid nitrogen storage Dewar (Box 1) to dry ice. In the tissue culture room, incubate the vial in a $37\text{ }^{\circ}\text{C}$ water bath for 1–2 min until the cells are almost completely thawed.

36| Remove the vial from the $37\text{ }^{\circ}\text{C}$ water bath. Thoroughly clean the outside of the vial with 70% (vol/vol) ethanol before opening it in the biosafety cabinet.

37| Add 0.5–1 ml of DMEM to the vial, resuspend gently and add the resuspended cells with 10 ml of DMEM to a 15-ml centrifuge tube.

38| Centrifuge at low speed for 5 min, aspirate off the medium and resuspend the cells in 10 ml of DMEM medium. Transfer cells to a 10- cm^2 tissue culture plate and incubate overnight at $37\text{ }^{\circ}\text{C}$.

▲ **CRITICAL STEP** Inadequate removal of the cell preservation medium before seeding can result in a complete loss of cell viability.

39| Aspirate off the medium, which should contain many floating (i.e., dead) cells. Add 10 ml of DMEM and incubate at $37\text{ }^{\circ}\text{C}$ until the plate reaches confluency.

40| Split cells into two 15- cm^2 tissue culture plates (see Step 19), and continue splitting until ten confluent 15- cm^2 tissue culture plates are obtained.

? TROUBLESHOOTING

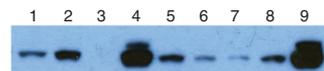


Figure 4 | Expression levels from stably transfected and clonal HEK293S GnTI⁻ cell lines as determined by western blotting. Each lane corresponds to the AS sample from a separate clonal cell line (clones 1–9), visualized via anti-FLAG western blotting. All clones were solubilized using DDM.

Cell cultures (1 liter) in spinner flasks ● **TIMING** ~3 d

41 Resuspend the cells from ten 15-cm² tissue culture plates, following the protocol as described in **Box 1** with the exception that suspension medium is substituted for DMEM. This is typically performed five plates at a time.

42 Transfer the cells to a 1-liter spinner flask containing 500 ml of suspension medium.

43 Add the remaining suspension medium (~400 ml) to the spinner flask, and ensure that the lids of the two spouts of the flask are tightly closed before removing the spinner flask from the biosafety cabinet.

44 Place the flask on top of a magnetic stir plate in the 37 °C incubator and stir it at ~65 r.p.m. To ensure gas exchange between the incubator and the flask, loosen both caps of the spinner flask, ensuring that enough thread is still held to prevent the caps from being removed from the flask via a straight pull.

▲ **CRITICAL STEP** Ensure that 2 ml of resuspended cells (from a total volume of 12 ml) from one 15-cm² tissue culture plate is used to seed a new 15-cm² tissue culture plate. This ‘maintenance plate’ can be used in the future to seed further 1-liter spinner flasks.

45 The next day, measure the cell density using a hemocytometer. The cell density should be ~0.5 × 10⁶ cells per ml. Continue to incubate at 37 °C at ~65 r.p.m.

▲ **CRITICAL STEP** To avoid contamination, ensure that the lids of the spinner flask are closed before removal of the flask from the incubator.

? **TROUBLESHOOTING**

46 The next day, again measure the cell density, which should now be approximately 0.7–1.0 × 10⁶ cells per ml. At this point, proceed to either generate two 1-liter spinner flasks (Step 47) or feed and induce the cell culture (Step 61), depending on the final scale of cell culture that is desired.

Cell cultures (2 liters) in spinner flasks ● **TIMING** ~3 d

47 Warm 1 liter of suspension medium to 37 °C in a water bath. Transfer half of the cells from a 1-liter spinner flask (density = approximately 0.7–1.0 × 10⁶ cells per ml) into a second sterilized 1-liter spinner flask. Add 500 ml of suspension medium to each spinner flask. Ensure that the lids of both flasks are partially loosened (see Step 44) after placing the flasks in the incubator. Incubate overnight at 37 °C at ~65 r.p.m.

48 Measure the cell density, which should be approximately 0.7–1.0 × 10⁶ cells per ml. At this point, proceed to either inoculating a 10-liter WAVE bioreactor (Step 49) or to feeding and inducing the cell cultures (Step 61). Optionally, additional 1- or 3-liter spinner flasks can be generated and maintained in an incubator (**Fig. 5a**).

Cell cultures (10 liters) using WAVE cellbag bioreactors ● **TIMING** ~3 d

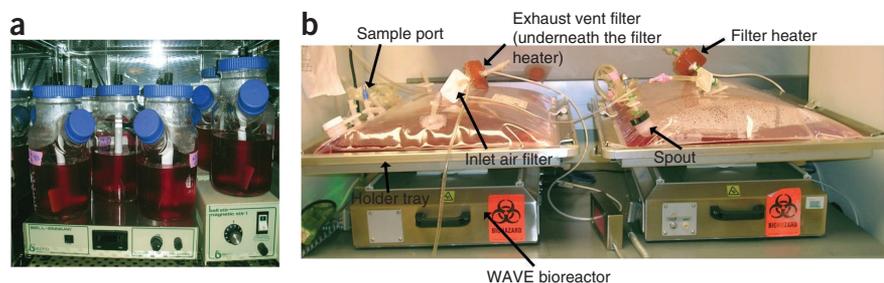
49 Warm up 7 liters of suspension medium in a 37 °C water bath.

50 In the biosafety cabinet, remove the outer packaging from a 10-liter cellbag. Clamp the inlet air and exhaust vent filters. Refer to **Figure 5b** for cellbag and WAVE bioreactor features.

51 Pour all 7 liters of suspension medium into the cellbag. Wipe the opening of the cellbag with sterile 70% (vol/vol) ethanol wipes before closing the bag.

52 Secure the cellbag to the holder tray of the rocking unit according to the manufacturer’s protocols. Connect the air line (mixed with 5% CO₂) from the MIX OUT port of the pump to the inlet air filter on the cellbag and unclamp the inlet air filter.

Figure 5 | Medium- and large-scale HEK293S GnTI⁻ cell cultures. **(a)** Up to seven 1-liter spinner flasks can be maintained in a single CO₂ incubator. Alternatively, up to three 3-liter spinner flasks can be maintained (not shown). **(b)** Two 10-liter WAVE bioreactors, using cellbags manufactured by GE Healthcare (left) and Flex Concepts Inc. (right), are shown. Relevant features of the cellbag and WAVE bioreactor, as discussed in the text, are highlighted.



PROTOCOL

53| Attach the filter heater to the exhaust vent filter to reduce condensation, and plug it into the FILTER HEATER port on the back panel of the base of rocking unit.

▲ **CRITICAL STEP** Condensation will nevertheless build up on the exhaust vent filter over time. To prevent blockage of the vent, the vent filter should be periodically flicked to remove excess condensation.

54| Set the airflow on the pump to 0.25 LPM (liters per min) in order to inflate the cellbag. Set the temperature to 37 °C.

55| Turn on the rocking unit and set the rocking speed to 15 r.p.m. Verify that the rocking unit is functional.

56| After approximately 15–30 min, verify that the cellbag bioreactor is fully inflated (i.e., taut with no creases observed) and secured to the tray and rocking unit. Unclamp the exhaust vent filter and verify that air is being released through the pressure control valve by pressing gently on the cellbag.

57| After ~1.5 h, clamp the inlet air and exhaust vent filters, remove the filter heater, turn off the air pump and remove the air line from the inlet air filter. Remove the cellbag from the rocking unit and transfer it to the biosafety cabinet.

58| Inoculate the cellbag by slowly pouring the entire contents of the two 1-liter spinner flasks (see Step 47) into the cellbag. Wipe the opening of the cellbag with sterile 70% (vol/vol) ethanol wipes.

59| Repeat Steps 52–56 to inflate the cellbag. Once it is inflated, set the rocking speed to 22 r.p.m., and rock overnight.

60| To measure the cell density and viability, first stop the rocker and then sterilize the sample port with 70% (vol/vol) ethanol wipes. Attach a 5-ml Luer syringe to the sterilized sample port, unclamp the sample port tubing and withdraw approximately 3–5 ml of cells. Reclamp the sample port tubing, remove the syringe and sterilize the sample port with 70% (vol/vol) ethanol wipes. When the cell density reaches $\sim 1.0 \times 10^6$ cells per ml (typically 2–3 d), proceed to feeding and inducing the cellbag (Step 61).

▲ **CRITICAL STEP** Removing the syringe before reclamping the sample port tubing can result in contamination of the cellbag.

Induction and collection of cell cultures ● **TIMING** ~3 d

61| Feed the cells with 20% (wt/vol) glucose (100 ml per 10-liter WAVE bag, 10 ml per 1-liter spinner flask) and 10% (wt/vol) Primatone RL/UF (300 ml per 10-liter WAVE bag, 30 ml per 1-liter spinner flask). Incubate for 24 h at 37 °C.

? **TROUBLESHOOTING**

62| Induce the cells (typical cell density of $\sim 1 \times 10^6$) with doxycycline (final concentration = 2 µg per ml) and sodium butyrate (final concentration = 5 mM). Incubate for 24–36 h at 37 °C.

▲ **CRITICAL STEP** For RhCG, an incubation period of 36 h after induction resulted in the highest amount of protein expression. For novel membrane protein targets, the optimal incubation period should be empirically determined.

63| Take an aliquot of cells and measure the cell density and viability. Transfer the entire contents of the spinner flask to 1 liter and 250 ml centrifuge bottles. Pellet the cells by spinning at 5,000g for 10 min. Decant the supernatant, wash the pellets with 20 ml of D-PBS per liter of cell culture and transfer the pellets to 50-ml centrifuge tubes. Pellet again at 5,000g for 10 min. Decant the supernatant and keep the pellet on ice until you are ready to solubilize it.

? **TROUBLESHOOTING**

Whole-cell membrane solubilization ● **TIMING** 2 h

64| Weigh the cell pellet. Resuspend the cells with 0.5 ml of lysis buffer per 0.1 g of cells. Add an equal volume of lysis buffer containing 40× critical micelle concentration of an appropriate detergent, as determined in Step 34. Transfer the sample to a Dounce homogenizer and semisolubilize the sample with 30 strokes. Perform the Dounce homogenization in multiple steps if the total volume is too large for a single homogenizer.

▲ **CRITICAL STEP** Although RhCG was well solubilized in this manner using OG, we have found that other human membrane proteins are very poorly solubilized in OG and only well solubilized in DDM. Therefore, the optimal [detergent], pH and [NaCl], as determined in Step 34 should be used for solubilization.

65| Stir at moderate speed for 1 h at 4 °C. Optionally, aliquot 30 µl as a BS sample for western blotting analysis (Step 34).

66 | Transfer the entire contents of the beaker to an ultracentrifuge tube, and spin the cells at 75,000*g* in a Type 45 Ti ultracentrifuge rotor for 45 min at 4 °C.

67 | Filter the supernatant (possessing solubilized membrane proteins) through a 0.45- μ m filter. Optionally, aliquot 30 μ l as an AS sample for western blotting analysis (see Step 34).

FLAG affinity and SEC ● **TIMING** ~1 d

68 | For every 2 liters of suspension culture, wash 1 ml of FLAG resin (i.e., 2 ml of slurry) with SEC buffer (no DTT) according to the manufacturer's protocols.

69 | Add the supernatant from Step 67 to the washed FLAG resin. Transfer the mixture to 50-ml centrifuge tubes and gently mix on a Nutator for 2 h at 4 °C.

70 | Load the entire contents onto a glass Econo-column at 4 °C. Collect the flow-through containing the unbound material.

71 | Wash the FLAG resin with 10–20 column volumes of SEC buffer (no DTT). Collect the washes.

72 | Elute the protein from the FLAG resin by adding 1 ml of 100 μ g ml⁻¹ FLAG peptide in SEC buffer (no DTT). Repeat five times, eluting the sample in a separate 1.5-ml centrifuge tube each time. Add DTT to a final concentration of 2 mM to each elution fraction.

73 | Resolve the flow-through, wash and elute 1–5 samples by reducing SDS-PAGE. Stain the gel with Coomassie blue according to standard protocols. Pool the elution fractions that possess the overexpressed membrane protein.

? TROUBLESHOOTING

74 | Optionally, remove the fusion tag(s) by adding the appropriate proteases, following the manufacturer's recommended concentrations. In the case of RhCG, 10 μ l of 3C and 10 μ l of thrombin were initially used to test for enzymatic removal of the FLAG and histidine tags, respectively. Incubate the reaction overnight on a Nutator at 4 °C.

75 | Regenerate and store the FLAG resin for future use by following the manufacturer's protocols.

76 | Equilibrate a Superdex 10/300 GL SEC column with two column volumes of SEC buffer at 0.1 ml min⁻¹.

77 | Concentrate the appropriate elutions (from Step 73) to a final volume of approximately 200–400 μ l by using an ultracentrifugal filtration device with a 50-kDa cutoff at 4 °C. Inject the entire volume into the equilibrated Superdex 10/300 GL size-exclusion column.

▲ CRITICAL STEP It is important, if possible, to minimize the number of injections on the size-exclusion column. A large number of dilute injections (i.e., as performed with an autosampler) will result in a larger peak fraction volume. Subsequent concentration of such a large volume of membrane protein can increase the amount of detergent in the concentrated sample, especially in the case of DDM, which can impede crystallization. In the case of RhCG, however, free detergent micelles were easily separated from RhCG by SEC and did not concentrate readily using the 50-kDa cutoff filtration device.

78 | Pool fractions of the desired peak, concentrate to ~5 mg ml⁻¹ by using a centrifugal filtration device (e.g., Amicon) with a 50- or 100-kDa cutoff at 4 °C and proceed to crystallization trials with commercial membrane protein screens. Yields of human membrane proteins from HEK293S cells are typically modest; therefore, crystallization screening using nanoliter liquid handlers (such as TTP LabTech's mosquito) is of critical importance. We have used the mosquito to screen a variety of detergent-solubilized membrane proteins, and have found that the presence of the detergent typically has no adverse impact on liquid handling.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

PROTOCOL

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1, 10–12, 40, 45, 61	Contamination	Improper sterile technique	Ensure that proper tissue culture technique is observed. Ensure that all surfaces are cleaned with 70% (vol/vol) ethanol before use
2	Cells do not adhere to the plate	Incorrect medium or tissue culture plate	Check that the medium contains calcium. Ensure that tissue culture-treated plates are being used. Check for contamination
12	No cell death upon drug selection	Inadequate drug concentration or cell contamination	Use fresh Geneticin (as it is light sensitive) and repeat selection. If cells are still viable, induce the cells and check for protein expression; the cell line may be contaminated with another stably transfected cell line
45	Low cell density	Low viability, low inoculum density, and/or contamination	Wait an additional 24 h and check the cell density. Measure the cell viability. Feed cells with glucose and Primatone. Check for possible contamination
63	Low cell viability after induction	Toxicity of the membrane protein when overexpressed	Harvest the cells early (i.e., at ~24 h). Increase the serum percentage to 15% (vol/vol)
73	Loss of membrane protein expression	Inactivation of the transgene	Thaw a fresh vial of cells. Make new frozen cell stocks from these thawed cells
	Low purity	Suboptimal binding and washing protocol	Increase [NaCl] and [glycerol]. Decrease binding time. Increase wash volumes. Preclear the lysate with mouse IgG-agarose to remove proteins with nonspecific binding

● TIMING

The entire protocol, starting from transfection (Step 1) to SEC purification (Step 78), takes approximately 8–10 weeks to complete. Once a stable cell line is generated and characterized, however, 1- to 2-liter spinner flask cultures can be generated in ~2 weeks' time, whereas 10-liter cellbag cell cultures can be generated in ~3 weeks. Multiple spinner flask and/or cellbag cell cultures can be grown in parallel, given adequate access to the appropriate equipment (e.g., incubators, WAVE bioreactors and so on). The timing for each stage of the PROCEDURE is summarized below and in **Figure 2**.

Step 1, cell seeding: ~15 min

Steps 2–9, transfection: approximately 5–7 h (45 min transfection + 4–6 h incubation + 15 min media exchange)

Step 10, expansion: ~2 h

Step 11, Geneticin selection: ~15 min

Step 12, generation of stably transfected cell colonies: 2–3 weeks

Steps 13–25, generation of clonal cell lines: 2–3 weeks

Steps 26–34, assessment of membrane protein solubilization and expression levels from clonal cell lines: 1 d

Steps 35–40, expansion of cells from liquid nitrogen–frozen stocks: 1 week

Steps 41–46, cell cultures (1 liter) in spinner flasks: ~3 d

Steps 47 and 48, cell cultures (2 liter) in spinner flasks: ~3 d

Steps 49–60, cell cultures (10 liter) using WAVE cellbag bioreactors: ~3 d

Steps 61–63, induction and collection of cell cultures: ~3 d

Steps 64–67, whole-cell membrane solubilization: ~2 h

Steps 68–78, FLAG affinity and SEC: ~1 d

Box 1, making frozen cell stocks: ~30 min

ANTICIPATED RESULTS

We have overexpressed human RhCG, in addition to other human membrane proteins, in HEK293S GnTI⁻ cells using the process described here. For each membrane protein that was expressed, the progression through the protocol was very similar (**Fig. 2**). Approximately 2–3 weeks after drug selection, single foci of cells are typically observed for at least one plating dilution (**Fig. 3**), and clonal cell lines can be generated 2–3 weeks after this. The number of clonal cell lines generated can vary, however, as occasionally only <10–12 individual colonies in total are observed. The amount of membrane protein produced from each clone is likely to vary substantially, thereby necessitating the use of western blotting to assess expression

levels (Fig. 4). Although human RhCG was well solubilized with OG, in our experience, DDM is a preferred detergent given its greater ability to solubilize membrane proteins from HEK293S cells. For those human membrane proteins that cannot be successfully solubilized with DDM (Table 1), solubilization with harsher detergents such as FC-14 can be attempted; however, caution should be exercised in these cases given the potential for solubilizing an inactive form of the membrane protein³¹.

A clonal cell line can typically be used indefinitely; human RhCG was expressed from a single, clonal cell line over the course of ~1 year with no appreciable loss of protein expression. In addition, cell lines can be frozen at any point and thawed at a later time, again with no appreciable loss of protein expression. In rare cases, membrane protein expression levels from clonal cell lines have been found to decrease upon continued passage; in these cases, care must be exercised to thaw fresh cells and create new frozen stocks before each cell culture scale-up.

The use of both spinner flasks and cellbag WAVE bioreactors (Fig. 5) for cell culture scale-up is recommended, given that their respective scales complement one another. It is to be expected that cellbag cultures should reach a higher density, given its more optimal oxygen transfer; however, we did not observe this for the RhCG HEK293S cells. Rarely, for certain clonal cell lines, viability has been observed to decrease markedly after induction with doxycycline (Table 1). In these cases, the cells should be collected earlier than normal (Step 63), which will ultimately result in less membrane protein purified per liter of cell culture medium. The optimal conditions for membrane protein stability (i.e., pH, [salt], [glycerol] and so on) can typically be determined using protein purified from a 1-liter spinner flask. For human membrane proteins expressed in HEK293S cells, FLAG affinity purification typically results in higher yields and better purity compared with IMAC purification. Final yields of purified membrane proteins from stably transfected and clonal HEK293S cells are typically up to ~0.5 mg of protein per 1 liter of medium.

Note: Supplementary information is available via the HTML version of this article.

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