Screening and large-scale expression of membrane proteins in mammalian cells for structural studies

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Structural, biochemical and biophysical studies of eukaryotic membrane proteins are often hampered by difficulties in overexpression of the candidate molecule. Baculovirus transduction of mammalian cells (BacMam), although a powerful method to heterologously express membrane proteins, can be cumbersome for screening and expression of multiple constructs. We therefore developed plasmid Eric Gouaux (pEG) BacMam, a vector optimized for use in screening assays, as well as for efficient production of baculovirus and robust expression of the target protein. In this protocol, we show how to use small-scale transient transfection and fluorescence-detection size-exclusion chromatography (FSEC) experiments using a GFP-His_8–tagged candidate protein to screen for monodispersity and expression level. Once promising candidates are identified, we describe how to generate baculovirus, transduce HEK293S GnTI$^-$ (*N*-acetylglucosaminyltransferase I-negative) cells in suspension culture and overexpress the candidate protein. We have used these methods to prepare pure samples of chicken acid-sensing ion channel 1a (cASIC1) and *Caenorhabditis elegans* glutamate-gated chloride channel (GluCl) for X-ray crystallography, demonstrating how to rapidly and efficiently screen hundreds of constructs and accomplish large-scale expression in 4–6 weeks.

INTRODUCTION

Since the initial observation that insertion of a human cytomegalovirus (CMV) promoter or a Rous sarcoma virus (RSV) promoter into an *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV; from here on referred to as baculovirus) transfer vector allowed for the expression of foreign genes in hepatocytes and other mammalian cell lines[1,2], BacMam has been used for a growing number of applications. These applications include drug discovery (identification and development of new therapeutic agents) through recombinant protein expression for cell-based functional assays using G protein–coupled receptors (GPCRs)[3,4], nuclear receptors[5], ion channels[6,7] and ATP-binding cassette drug transporters[8]. More recently, BacMam has been used for large-scale protein production for crystallography[9–20]. The success of these applications, however, depends in part on the efficient production and amplification of baculovirus and on subsequent large-scale transduction and heterologous protein expression. In addition to these challenges, obtaining sufficient quantities of membrane protein for crystallography is frequently compounded by low levels of expression and instability of the candidate membrane protein, thus requiring screening of many constructs. Furthermore, some mammalian membrane proteins require specific post-translational modifications and a near-native lipid environment, thus rendering expression in insect cells or in yeast untenable. Taken together, these complexities can result in a high cost for heterologous membrane protein expression in mammalian cells, and thus improving the efficiency of the process is important.

Here we describe methods for screening constructs and optimizing heterologous expression of membrane proteins from BacMam-transduced HEK293S GnTI$^-$ (*N*-acetylglucosaminyltransferase I–negative) cells for purification and crystallization (**Fig. 1**). We have constructed a vector (pEG BacMam) for high-level expression in mammalian cells using elements derived from a previously described vector, pVLAD[10]. Once genes of candidate membrane proteins are fused in frame with a GFP tag and cloned into pEG BacMam, they can be rapidly screened for expression and monodispersity using transient transfection in adherent cells coupled with FSEC[21,22]. We also optimized virus amplification and protein expression protocols such that the cost and time for expressing most of the membrane proteins in HEK293 GnTI$^-$ cells are similar to or better than those of expression in Sf9 cells.

This protocol is exemplified using two proteins expressed in mammalian cells: *Gallus gallus* cASIC1 (refs. 16,23) and *C. elegans* GluCl[24,25]. This protocol is now in standard use in our laboratory for mammalian-expressed membrane proteins[15–17,20].

Development of the protocol

To increase the heterologous expression of challenging membrane proteins, we first constructed pEG BacMam for

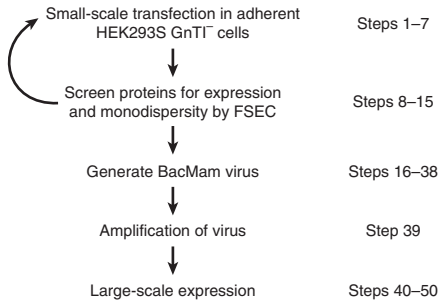

Figure 1 | Flowchart of the overexpression of membrane proteins in HEK293S GnTI cells. After one or more rounds of screening, a few potential candidates are chosen for large-scale protein expression.

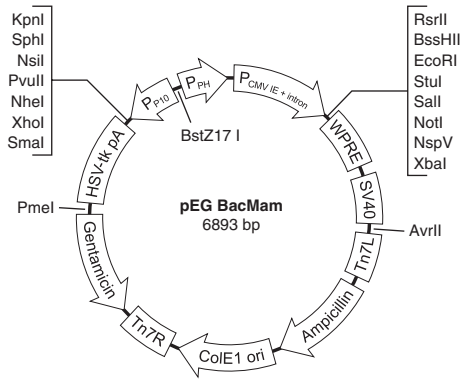

Figure 2 | Map of BacMam expression vector. For expression in mammalian cells, genes of interest are cloned into the multiple cloning site behind the CMV promoter using unique restriction sites. Elements that are important for high-level expression are shown, including those that are important for transcription initiation (CMV promoter), transcription termination (SV40 poly-A late signal) and mRNA processing (intron and WPRE motif). Also indicated are the elements that are important for insect cell expression and baculovirus amplification, including promoters (polh and p10), terminators (SV40 and HSV-tk), transposon elements (Tn7L and Tn7R) and resistance markers (ampicillin and gentamicin). IE, immediate/early.

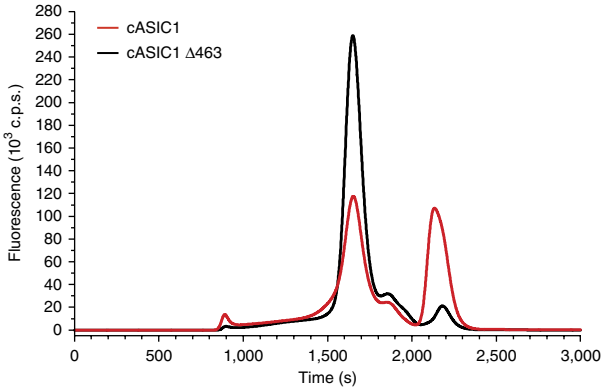

Figure 3 | Screening constructs by small-scale transient transfection in HEK293S GnTI⁻ cells and FSEC; 1 µg of N-terminal EGFP-tagged full-length cASIC1 or cASIC1 Δ463 (cASIC1, truncated 64 residues from the carboxy termini) subcloned into pEG BacMam was transfected separately into HEK293S GnTI⁻ cells, as described in the text. Cells were collected 48 h after transfection, and solubilized extracts were analyzed by FSEC to determine the behavior of the fusion proteins. c.p.s., counts per second.

high-level protein expression in mammalian cells with the ability to express multiprotein complexes from a single vector (**Fig. 2**). To do this, we chemically synthesized genetic elements derived from the previously described BacMam vector pVLAD[10], which include a strong CMV promoter for robust transcription, a synthetic intron for efficient RNA splicing and mRNA processing, and a WPRE motif for efficient mRNA processing, stability and export. These chemically synthesized elements were combined with the pFBDM (ref. 26), a bicistronic vector with a restriction enzyme module that allows the assembly of multiple expression cassettes, to generate pEG BacMam. After the gene of interest is cloned into pEG BacMam, we screen constructs by small-scale transfection/FSEC before moving to the time-consuming process of virus amplification[21].

In our research to optimize protein expression, we compared the expression of cASIC1 and GluCl in mammalian cells and insect cells. We show that fivefold more GluCl pentamer can be obtained in mammalian cells. In the case of cASIC1, not only can twofold more trimer be obtained in mammalian cells but also the protein is more monodisperse and experiences less spontaneous cleavage of the GFP-His_8 tag.

Although other HEK cell lines can be used, for screening and expression we typically use HEK293S GnTI⁻ cells, a mammalian cell line that expresses proteins that are more mannose-rich and are thus easily removed using endoglycosidases such as EndoH (ref. 27). Although the use of these cells and EndoH can reduce the heterogeneity caused by complex glycans that can create problems in crystallographic studies, it may not be beneficial for every protein. Therefore, it is advantageous to test protein expression in other mammalian cell lines, as well as to determine whether the use of EndoH affects the solubility and the heterogeneity of the glycoprotein.

To determine the expression level and monodispersity of the candidate membrane protein, HEK293S GnTI⁻ cells are transfected with the pEG BacMam plasmid containing the gene of interest; collected after 48 h; and then solubilized in a buffer containing *n*-dodecyl-β-D-maltoside ($C_{12}M$), maltose-neopentyl glycol[28] (MNG-3), or other detergent. The resulting supernatant is analyzed by FSEC (**Fig. 3**). As shown in **Figure 3**, removing the 64 residues from the C terminus of cASIC1 (cASIC1 Δ463) increases monodispersity and reduces cleavage of the GFP-His_8 tag. In addition to removing flexible termini, there are many methods that can be used to optimize the expression and stability of proteins, including codon optimization, surface entropy reduction and thermostability mutations[29–31]. Small-scale transfection followed by whole-cell solubilization and FSEC allows the screening of hundreds of candidates in ≤1 month.

Once a promising candidate is identified, the plasmid is transformed into the DH10Bac *Escherichia coli* strain to generate the recombinant bacmid DNA, which is then used to transfect insect cells to generate BacMam virus. We have detailed our methods for isolation of bacmid DNA, transfection of Sf9 cells and baculovirus amplification, which we use to reduce costs and ensure good-quality BacMam virus. We have found for some constructs that the multiplicity of infection (MOI) during virus amplification is 10–100-fold below the range recommended by the Bac-to-Bac system (Invitrogen) protocol (http://www.lifetechnologies.com/us/en/home/life-science/protein-expression-and-analysis/protein-expression/insect-expression/bac-to-bac-baculovirus-expression-system.html). We have also found that a low MOI (MOI of 2 or less) is sufficient for mammalian cell transduction and that too much virus results in low cell numbers, possibly owing to too much Sf9 medium or virus added to the culture. Therefore, before virus amplification or transduction of mammalian cells for protein expression, virus titer should be determined using the endpoint dilution assay[32], flow cytometric assay[33,34] or the viral plaque assay[35].

In addition to MOI, we also explored different growth and expression conditions for BacMam-transduced HEK293S GnTI⁻ cells to boost protein expression. After testing several types of media for the growth of suspension cells, we found that the use of Gibco FreeStyle 293 expression medium (Invitrogen) allowed

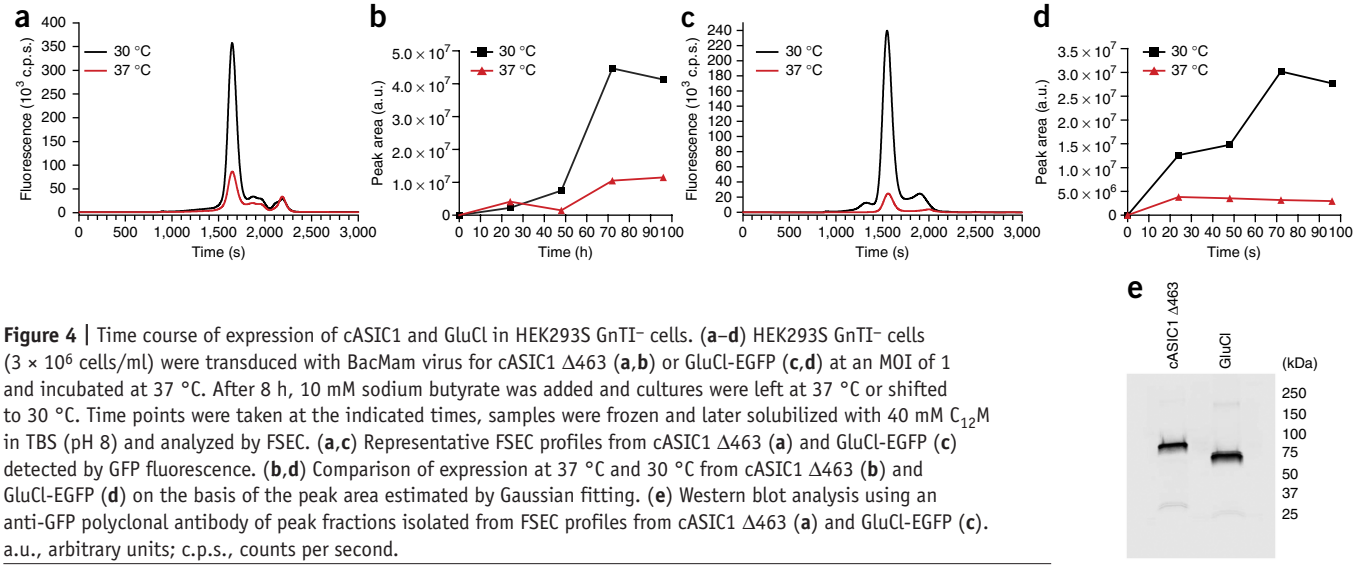

Figure 4 | Time course of expression of cASIC1 and GluCl in HEK293S GnTI⁻ cells. (**a**–**d**) HEK293S GnTI⁻ cells (3×10^6 cells/ml) were transduced with BacMam virus for cASIC1 Δ463 (**a**,**b**) or GluCl-EGFP (**c**,**d**) at an MOI of 1 and incubated at 37 °C. After 8 h, 10 mM sodium butyrate was added and cultures were left at 37 °C or shifted to 30 °C. Time points were taken at the indicated times, samples were frozen and later solubilized with 40 mM $C_{12}M$ in TBS (pH 8) and analyzed by FSEC. (**a**,**c**) Representative FSEC profiles from cASIC1 Δ463 (**a**) and GluCl-EGFP (**c**) detected by GFP fluorescence. (**b**,**d**) Comparison of expression at 37 °C and 30 °C from cASIC1 Δ463 (**b**) and GluCl-EGFP (**d**) on the basis of the peak area estimated by Gaussian fitting. (**e**) Western blot analysis using an anti-GFP polyclonal antibody of peak fractions isolated from FSEC profiles from cASIC1 Δ463 (**a**) and GluCl-EGFP (**c**). a.u., arbitrary units; c.p.s., counts per second.

for increased growth rates and reduced cell clumping of HEK293S GnTI⁻ cells in suspension. To further minimize cell clumping, we also assessed the growth of suspension cells in different vessels, including square bottles, flat-bottom flasks and baffled Erlenmeyer flasks. We found that baffled flasks minimized cell clumping and promoted cell growth. To reduce costs, the polycarbonate Erlenmeyer flasks can be washed, autoclaved and used again up to 20 times. If after autoclaving the filter in the cap deteriorates, a replacement cap can be purchased.

Previously, it has been shown that lowering the temperature after transduction or transfection enhances protein expression in mammalian cells[36–43]. In a time-course experiment for cASIC1, we found that at the optimal collection time of 72 h after transduction, there was a 4.3-fold increase in expression at 30 °C compared with that at 37 °C (**Fig. 4a**). Gaussian fitting, as described previously[21,22], was performed on the FSEC profiles to determine the peak area of cASIC1 trimer. A graph of the trimer peak area shows that the expression of cASIC1 is higher at 30 °C than at 37 °C for most time points (**Fig. 4b**). To monitor the expression of GluCl in HEK293S GnTI⁻ cells, an EGFP GluCl gene fusion[25,44] was cloned into pEG BacMam and used to generate BacMam virus. At the optimal collection time of 72 h after transduction for cells expressing GluCl, there was a 9.5-fold increase in expression at 30 °C with higher expression of GluCl at all time points at 30 °C (**Fig. 4c**,**d**). To show that the proteins present in the major peaks for cASIC1 and GluCl are of the expected molecular weight, peak fractions were collected and analyzed by SDS-PAGE, followed by western blot analysis using an antibody against GFP (**Fig. 4e**). In fact, for the expression of most proteins in HEK293S GnTI⁻ cells, we have found that lowering the temperature during expression increases protein yields at least twofold (A.G., C.-H.L., K.H.W., J.C.M., D.P.C. and E.G., unpublished data). In some cases, lowering the temperature is essential in order to obtain monodisperse, well-folded protein.

Finally, the use of histone deacetylase inhibitors has been shown to enhance protein expression in HEK293S cells (refs. 10,45). We found that for most membrane proteins histone deacetylase inhibitors boost expression. For cASIC1, there is a sevenfold increase in expression at 72 h after transduction, as well as higher expression of cASIC1 at all time points at which HEK293S GnTI⁻ cells are treated with 10 mM sodium butyrate (**Fig. 5a**,**b**). Previously published data suggest that valproic acid is more efficient than sodium butyrate at enhancing recombinant protein production from mammalian cells[45]. However, we find that

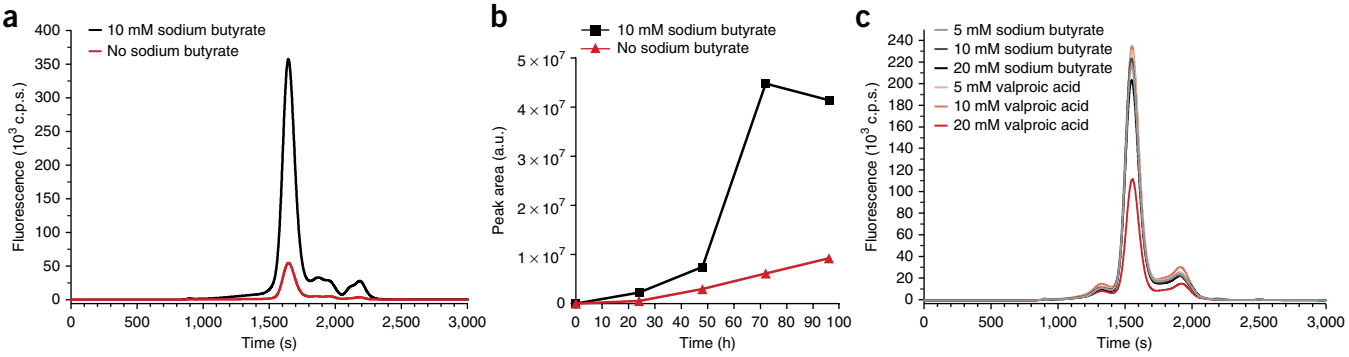

Figure 5 | Effect of histone deacetylase inhibitors on the expression of cASIC1 and GluCl in HEK293S GnTI⁻ cells. (**a**,**b**) Comparison of cASIC1 expression with and without sodium butyrate; representative FSEC profiles from cASIC1 Δ463 detected by GFP fluorescence (**a**) and comparison of expression from cASIC1 Δ463 on the basis of the peak area estimated by Gaussian fitting (**b**) in the presence or absence of sodium butyrate. (**c**) Representative FSEC profiles from GluCl-EGFP in the presence of sodium butyrate or valproic acid. c.p.s., counts per second.

both sodium butyrate and valproic acid enhance protein expression from BacMam-transduced HEK293S GnTI⁻ cells (**Fig. 5c**). Typically, sodium butyrate is added to cultures between 8 and 24 h after transduction; however, the amount and time of sodium butyrate addition should be optimized for each protein. Overall optimization of conditions (construct, MOI, cell density, temperature and collection time of BacMam-transduced HEK293S GnTI⁻ cells) could either increase expression or decrease aggregation, leading to more properly folded protein, and therefore the most favorable conditions for each protein should be determined before attempting a large-scale expression.

Other applications of the method

The protocol described here could also be used to optimize the expression of heteromers or protein complexes in mammalian cells. One option to simultaneously express multiple proteins is to co-infect with multiple BacMam viruses (with an optimized MOI for each virus). Alternatively, pEG BacMam could be used to express multiprotein complexes by combining two pEG BacMam plasmids using unique restriction enzyme sites. As a result, multiple genes could be transduced by a single BacMam virus, allowing for the simultaneous expression of two or more genes.

Some variants of pEG BacMam also contain vesicular stomatitis Indiana virus glycoprotein (VSIV-G) under control of the P10 promoter (a baculovirus-specific promoter). VSIV-G is a viral capsule protein important for mediating viral entry, and it has been shown to increase the transduction efficiency of baculovirus for some mammalian cells[46]. The P10 promoter could be used to drive the expression of VSIV-G in insect cells, allowing the incorporation of VSIV-G into the baculovirus to enhance transduction from other mammalian cell lines, such as the human lung carcinoma line A549 and the human hepatoma lines HepG2 and HuH7 (ref. 46).

Comparison with other methods

Many methods have been used for the overexpression of mammalian proteins, including plasmid transfection, stable cell lines and viral expression systems such as Sindbis virus, vaccinia virus and Semliki Forest virus (SFV), as well as AcMNPV[47]. Compared with BacMam, each of these methods has advantages and disadvantages in terms of cost, time, efficiency, safety and reproducibility.

One such transient expression method involves transfection of plasmid DNA into adherent cells or cultures, and overexpression is either immediate or induced, depending on the promoter. Plasmid transfection is fast, safe and easy to use for high-throughput screening[48–50]. However, if commercial transfection reagents and plasmid isolation kits are used, plasmid transfection can be expensive for large-scale expression. Furthermore, the level of expression using plasmid transfection can be limited by the plasmid size, the number of plasmids transfected and cytotoxic effects that have been observed with many transfection reagents. The use of BacMam can be cheaper than plasmid transfection for large-scale expression and multiple rounds of expression. In addition, BacMam is not limited by the gene number or size. In our hands, the level of protein expression, especially multisubunit proteins, is higher with BacMam than with plasmid transfection.

Stable expression in mammalian cells requires the integration of a transfected transgene into the cell's genome using Geneticin or other selection methods. Once clonal cell lines are generated and sorted for high-level producers, long-term overexpression from stably transfected cells can be robust, easy and consistent[51,52]. Furthermore, stable cell lines can be generated using regulated expression, such as the tetracycline-inducible expression system, thus allowing for large-scale expression of proteins that are cytotoxic to the cell[51,53]. Although stable expression enables the production of large quantities of protein, when compared to BacMam generating a stable cell line is time consuming.

Although other viral expression systems for protein expression such as lentiviruses[54], adenoviruses[55], Sindbis virus[56,57], vaccinia virus[58] and SFV[59,60] exist, SFV has been used to express a large number of membrane proteins consisting of mostly GPCRs[47]. To make virus, candidate genes are cloned into SFV plasmid and used as template for RNA synthesis. The RNA is then co-transfected (either using electroporation or liposome reagents) with helper RNA and packaged into SFV particles that can then be used to infect cells in culture. Although SFV can be easily used for

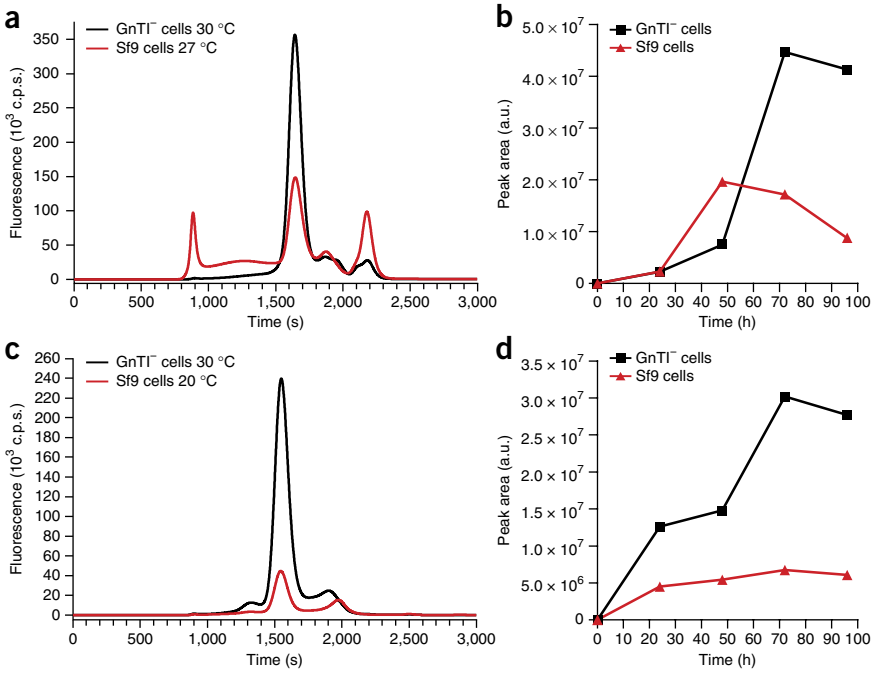

Figure 6 | Comparison of expression time course in HEK293S GnTI⁻ cells and Sf9 cells. (**a**–**d**) HEK293S GnTI⁻ cells were transduced with BacMam virus for cASIC1 Δ463 (**a**,**b**) or GluCl-EGFP (**c**,**d**), as described above. Sf9 cells (3×10^6 cells per ml) were infected with baculovirus cASIC1 Δ463 (**a**,**b**) or GluCl-EGFP (**c**,**d**) and placed at 27 °C. After 18 h, Sf9 cultures were left at 27 °C or shifted to 20 °C. Time points were taken, frozen and solubilized as described above. (**a**,**c**) Representative FSEC profiles from cASIC1 Δ463 (**a**) and GluCl-EGFP (**c**) detected by GFP fluorescence. (**b**,**d**) Comparison of expression from cASIC1 Δ463 (**b**) and GluCl-EGFP (**d**) on the basis of the peak area estimated by Gaussian fitting. a.u., arbitrary units; c.p.s., counts per second.

small-scale studies by transfecting synthesized RNA into cells and by analyzing expression, using SFV for large cultures is more challenging than BacMam owing to the amount of RNA that is needed to make virus for large-scale studies.

An effective method using BacMam was recently described to produce milligram quantities of proteins sufficient for crystallization[10]. However, the methods outlined therein to produce recombinant BacMam virus using the BD BaculoGold system are not as cost-efficient as the Bac-to-Bac method if multiple constructs are expected to be generated. In addition, we optimized growth and expression conditions using BacMam in order to express sufficient amounts of our desired membrane proteins.

Another commonly used method for the overexpression of proteins is AcMNPV baculovirus infection of insect cells. Although insect cells such as Sf9 cells have been used in our laboratory previously to provide sufficient protein for crystallization studies[25,61–64], some membrane proteins require a near-native environment to help ensure functional expression. The advantages for the expression of eukaryotic membrane proteins in mammalian cells over Sf9 include improved post-translational modifications such as *N*-linked glycosylation[65,66] and a different lipid environment that contains higher amounts of cholesterol. We performed a side-by-side comparison of protein expression from insect cells and mammalian cells. We found a twofold increase in cASIC1 trimer and an increase in homogeneity (**Fig. 6a**,**b**), and we found that fivefold more GluCl pentamer can be obtained in mammalian cells (**Fig. 6c**,**d**).

MATERIALS

REAGENTS

- pEG BacMam plasmid containing the gene of interest in frame with a GFP tag and a tag appropriate for affinity chromatography (such as a His_8 tag) cloned downstream of the CMV promoter (**Fig. 2**)
- HEK293S GnTI$^-$ cells (ATCC, cat. no. CRL-3022)
- Gibco FreeStyle 293 expression medium (Life Technologies, cat. no. 12338-018)
- US certified γ-irradiated FBS (Life Technologies, cat. no. 0984018DJ)
- DMEM (with 4.5 g per liter glucose, L-glutamine and sodium pyruvate; Corning/Cellgro, cat. no. 10-013)
- Opti-MEM I reduced serum medium (Life Technologies, cat. no. 31985-088)
- Lipofectamine 2000 (Life Technologies, cat. no. 11668-027)
- PBS (without calcium and magnesium; Corning/Cellgro, cat. no. 21-040-CM)
- Trypsin-EDTA (Corning/Cellgro, cat. no. 25-052-CV)
- Tris base (Fisher Scientific, cat. no. BP152)
- NaCl (Sigma-Aldrich, cat. no. 59888)
- $C_{12}M$ (Affymetrix, cat. no. D310)
- PMSF (Sigma-Aldrich, cat. no. 78830)
- Leupeptin (Sigma-Aldrich, cat. no. L0649)
- Aprotinin (Sigma-Aldrich, cat. no. A1153)
- Pepstatin (Sigma-Aldrich, cat. no. P4265)
- Sodium butyrate (Sigma-Aldrich, cat. no. 303410)
- Valproic acid (Sigma-Aldrich, cat. no. P4543)
- DH10Bac competent cells (Life Technologies, cat. no. 10361-012)
- Sf9 Easy Titer cell line[32]
- Cellfectin II reagent (Life Technologies, cat. no. 10362-100)
- Sf9 cells (Life Technologies, cat. no. 12659017)
- Sf-900 III serum-free medium (SFM) (Life Technologies, cat. no. 12658-027)
- QIAprep spin miniprep kit (Qiagen, cat. no. 27104)
- HyClone SFX-Insect medium (GE/Hyclone, cat. no. SH30278.02)
- G 418 disulfate salt (Sigma-Aldrich, cat. no. A1720-1g)
- Trypan blue solution (Corning/Cellgro, cat. no. 25-900-CL)

EQUIPMENT

- Incubator (Thermo Scientific, cat. no. 3950)
- Tissue culture plate (100 mm; BD Falcon, cat. no. 35300)
- Tissue culture plate (six well; BD Falcon, cat. no. 353046)
- Erlenmeyer baffled flasks (2,000 ml; BioExpress, cat. no. F-5909-2000B)
- Nunc EasYFlask (75 cm^2, Filter Cap; Thermo Scientific, cat. no. 156499)
- Syringe filters (polyethersulfone (PES) 13 mm diameter, 0.22 µm, polypropylene (PP) housing; Argos, cat. no. FE12S)
- Filter systems (250 ml, 0.22 µm; Corning, cat. no. 430767)
- Virus Counter 2100 (ViroCyt)
- Optima TL ultracentrifuge (Beckman)
- Fluorescence-detection size-exclusion chromatography system (FSEC; Equipment Setup)[21]
- Tissue culture plate (Costar 96-well black clear-bottom plates; Costar/Corning, cat. no. 3603)
- Sterile disposable reagent reservoirs (50 ml white; Costar/Corning, cat. no. 4870)
- Cluster tube system eight-tube strip (Costar/Corning product, cat. no. 4413)
- Cell scrapers (handle 18 cm blade 1.8 cm; Corning, cat. no. 353085)
- Hemocytometer (Hausser Scientific, cat. no. 1492)
- Shimadzu fluorometer (RF-20A)

REAGENT SETUP

DMEM medium To 500 ml of DMEM medium, add 50 ml of FBS. Store the medium at 4 °C for 1 month.

Suspension medium To 1 liter of FreeStyle 293 Expression medium, add 20 ml of FBS. Store the medium at 4 °C for 1 month.

Box 1 | Growth and maintenance of suspension HEK293S GnTI$^-$ cells

● **TIMING 15 min**

1. Slough off HEK293S GnTI$^-$ cells from a T-75 flask at 80% confluency using 25 ml of FreeStyle293 expression medium (Invitrogen) supplemented with 2% (vol/vol) FBS.
2. Transfer the cell suspension to a 125-ml baffled flask and place it on an orbital shaker within a 37 °C incubator in the presence of 8% CO_2.

▲ **CRITICAL STEP** The cell suspension should not exceed >40% of the baffled flask size.

3. After 24 h, dilute the cells to 0.5 × 10^6 cells per ml and maintain the suspension-adapted cells between 0.2 and 3 × 10^6 cells per ml at 40% of the vessel size for baffled flasks.

? TROUBLESHOOTING

Box 2 | Endpoint dilution assay ● TIMING 1 h

The following procedure is for one 96-well black plate that can be used to titer four viruses, and it is based on ref. 32. Adjust the volumes as necessary.

Growth and maintenance of adherent Sf9 Easy Titer cell line

1. When Sf9 Easy Titer cell line reach 90% confluency, aspirate off the medium and wash the cells with 5 ml of PBS.
2. Aspirate off the PBS and add 2 ml of trypsin for 30 s.
3. Add 10 ml of HyClone SFX-Insect medium supplemented with 5% (vol/vol) FBS and 150 µg/ml G418.
4. Scrape the cells gently with a sterile cell scraper.
5. Dilute the cells 1:4 in a 100-mm tissue culture dish and incubate them at 27 °C.

End-point dilution assay

6. By using a 100-mm tissue culture dish of Sf9 Easy Titer cells that is 90% confluent, follow steps 1–4 above.
7. Make a 10-ml stock of Sf9 Easy Titer cells at a density of 0.75×10^6 cells per ml in a sterile culture reservoir.
8. By using the multichannel pipette, seed 100 µl per well of a 96-well black plate (75,000 cells per well) and allow the cells to attach for ~15 min at 25 °C.
9. By using the sterile, deep-well eight-strip clusters, make stocks of virus from 10^{-1} to 10^{-8} in HyClone SFX-Insect medium supplemented with 5% (vol/vol) FBS and 150 µg/ml G418 (360 µl of medium + 40 µl of virus).

(i) 360 µl of medium + 40 µl of P1 virus = 10^{-1}
(ii) 360 µl of medium + 40 µl of 10^{-1} dilution = 10^{-2}
(iii) 360 µl of medium + 40 µl of 10^{-2} dilution = 10^{-3}
(iv) 360 µl of medium + 40 µl of 10^{-3} dilution = 10^{-4}
(v) 360 µl of medium + 40 µl of 10^{-4} dilution = 10^{-5}
(vi) 360 µl of medium + 40 µl of 10^{-5} dilution = 10^{-6}
(vii) 360 µl of medium + 40 µl of 10^{-6} dilution = 10^{-7}
(viii) 360 µl of medium + 40 µl of 10^{-7} dilution = 10^{-8}

10. Once the cells have attached to the 96-well plate (~20 min incubation at 27 °C), remove the medium and replace it with the 100 µl of diluted virus using a multichannel pipette.
11. Infect each virus in triplicate.
12. Incubate the 96-well plate at 27 °C.
13. At 72 h after infection, count the number of green foci in the dilution that gives <10 foci per well. To calculate viral titer, use the following equation:

Average number of foci × dilution factor × 10 = plaque-forming units (p.f.u.)/ml

? TROUBLESHOOTING

Suspension medium To 1 liter of HyClone SFX-Insect medium, add 50 ml of FBS and 150 µg/ml G418. Store the medium at 4 °C for 1 month.
Sodium butyrate (2 M) Dissolve 11 g of sodium butyrate with water to a final volume of 50 ml, and filter-sterilize using a 0.2-µm filter inside a biological safety cabinet. Store it at −20 °C for at least 1 month.
Tris-buffered saline (TBS) Mix 20 mM Tris-HCl (pH 8) and 150 mM NaCl. Store TBS at room temperature (RT; 25 °C) for at least 1 month.
Solubilization buffer Mix 20 mM Tris-HCl (pH 8), 150 mM NaCl and 40 mM C_{12}M. Chill the buffer to 4 °C. Immediately before use, add 1 mM PMSF, 200 µM aprotinin, 2 µg/ml leupeptin and 2 µM pepstatin A. Discard any unused buffer.
FSEC buffer Mix 20 mM Tris HCl (pH 8), 150 mM NaCl and 1 mM C_{12}M. Filter the buffer using a 0.2-µm filter. Store the buffer at 4 °C for up to 1 week.
Purification of plasmid DNA Purify plasmid DNA using the QIAprep spin miniprep kit (Qiagen) or another suitable method.
Growth and maintenance of adherent HEK293S GnTI$^-$ cells Cells are cultured as previously described[51].
Growth and maintenance of suspension HEK293S GnTI$^-$ cells HEK293S GnTI$^-$ cells are maintained as described in **Box 1**.
Growth and maintenance of Sf9 cells Sf9 cells are maintained as suspension cultures at 27 °C in Sf-900 III SFM medium. Isolation of bacmid DNA, transfection of Sf9 cells and amplification of virus are methods modified from the Bac-to-Bac system (Invitrogen), which we use to reduce costs and ensure production of good-quality BacMam virus.
Growth and maintenance of Sf9 Easy Titer cell line Sf9 Easy Titer cell lines are maintained as adherent cells at 27 °C in HyClone SFX-Insect medium, as described in **Box 2**.
BacMam virus titer determination Determine the titer of the BacMam virus using one of the several methods for virus titer determination. We prefer using the Sf9 Easy Titer cell line and the endpoint dilution assay[32] (**Box 2**) or the flow cytometric assay[33,34].

EQUIPMENT SETUP

FSEC In our laboratory, this is performed as described by Kawate and Gouaux[21]. The analyte is loaded onto a Superose 6 column (10/30, Amersham Biosciences) that has been pre-equilibrated with FSEC buffer. Separation is performed at a flow rate of 0.5 ml/min. The eluent from the SEC column is passed through a Shimadzu fluorometer (RF-20A) fitted with a flow cell, as described in the manufacturer's instructions. The fluorometer settings are as follows: band-pass, 3 nm/3 nm; excitation, 488 nm; emission, 507 nm; time increment, 1 s; integration time, 1 s; and recording time, 3,000–3,600 s. Calibration with known quantities of GFP have demonstrated that 1–10 ng of GFP can readily be detected.

PROCEDURE

Cell seeding (day 1) ● TIMING 15 min

1| Add 1 × 10^6 HEK293S GnTI$^-$ cells in 2 ml of DMEM supplemented with FBS to each well of a six-well culture plate. Incubate the cells at 37 °C with 5% CO_2 for 16–24 h.

! CAUTION Cell cultures are a potential biohazard. Work in an approved laminar flow hood using aseptic techniques, and check institutional and governmental guidelines for recommended protective clothing and proper disposal of waste before performing experiments.

? TROUBLESHOOTING

Small-scale transient transfection to screen constructs (day 2) ● TIMING 45 min

2| For each well, prepare an autoclaved 1.5-ml centrifuge tube. By using a pipette, add 4 µl of Lipofectamine 2000 into 50 µl of Opti-MEM I.

3| Add 1 µg of Qiagen miniprep–purified DNA into 50 µl of Opti-MEM I in a separate 1.5-ml centrifuge tube.

4| Add DNA/Opti-MEM I mixture to the Opti-MEM/Lipofectamine mixture; gently mix and incubate it for 20 min at RT.

5| Pipette the Opti-MEM I–DNA mixture dropwise onto 70–80% confluent HEK293S GnTI$^-$ cells. Ensure even dispersal.

6| After 8–24 h, replace the medium with DMEM plus 10 mM sodium butyrate.

7| Incubate the cells at 37 °C with 5% CO_2 for 2 d.

Screening the constructs by FSEC for monodispersity and expression level (day 4) ● TIMING 3 h

8| Aspirate off the medium and wash the transfected adherent cells carefully with 2 ml of TBS.

9| Add 1 ml of TBS to each well, collect the cells and transfer them to a 1.5-ml centrifuge tube.

10| Centrifuge the cells at 1,500*g* for 5 min at 4 °C.

11| Remove the supernatant and resuspend the cell pellet in 200 µl of solubilization buffer.

12| Nutate the samples for 1 h at 4 °C.

13| Centrifuge the solubilized sample at 70,000*g* in a TL100 ultracentrifuge for 40 min at 4 °C.

14| Collect the supernatant and analyze 50 µl by FSEC[21]. Allow 1 h for each sample to be analyzed by FSEC. Samples should be stored at 4 °C until analysis.

15| Identify the best-expressed and monodisperse candidate via FSEC (**Fig. 3** and Kawate and Gouaux[21]).

Transformation of DH10Bac *E. coli* (day 5) ● TIMING 1 h

16| Transform purified plasmid DNA into DH10Bac *E. coli* for transposition into the bacmid, as described in the Bac-to-Bac system protocol (Invitrogen; http://www.lifetechnologies.com/us/en/home/life-science/protein-expression-and-analysis/protein-expression/insect-expression/bac-to-bac-baculovirus-expression-system.html).

Inoculation of bacmid-containing cultures (day 7) ● TIMING 15 min

17| Inoculate 5 ml of LB medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline with a white colony, and allow the cells to grow overnight at 37 °C.

Isolation of bacmid (day 8) ● TIMING 1 h

18| Centrifuge the cells for 10 min at 1,500*g* at RT.

19| (Optional) Make a glycerol stock of the DH10Bac *E. coli* containing the bacmid DNA for future bacmid DNA isolation. In an autoclaved 1.5-ml centrifuge tube, take 250 µl of cell suspension (from Step 17) and add 250 µl of sterile 50% (vol/vol) glycerol; mix it and store it for years at –80 °C.

20| Discard the supernatant and resuspend the pellet in 200 µl of P1 (Qiagen kits). Transfer the suspension into a 1.5-ml centrifuge tube.

21| Add 200 µl of P2 (included in Qiagen kits) and mix by inverting the centrifuge tube a few times.
! CAUTION Do not vortex the samples, as this could shear the bacmid DNA.

22| Add 200 µl of N3 (included in Qiagen kits) and mix by inverting the centrifuge tube a few times; centrifuge the tube for 10 min at 1,500*g* at RT.

23| Transfer the supernatant to a 2-ml centrifuge tube, add 1 ml of isopropanol and gently invert the tube.

24| Place the tube for 10 min in a –20 °C freezer.

25| Centrifuge the tube at 1,500*g* for 15 min at RT.

26| Remove the supernatant, and preserve the pellet. Add 1 ml of 70% (vol/vol) ethanol and wash the pellet by gently inverting the centrifuge tube.

27| Centrifuge the tube at 1,500*g* for 15 min at RT.

28| Remove the supernatant and dry the pellet for 5 min.

29| Resuspend the pellet in 50 µl of autoclaved Milli-Q water. Determine the concentration of the bacmid DNA.
! CAUTION Do not pipette samples more than one or two times, as this could shear the bacmid DNA.
■ **PAUSE POINT** Store the bacmid DNA at 4 °C until you are ready to proceed with Step 30 (up to 3 d).

Transfection of Sf9 cells with bacmid ● TIMING 2 h
30| Seed 9 × 10^5 of Sf9 cells in 2 ml of Sf-900 medium per well of a six-well plate.
? TROUBLESHOOTING

31| Incubate the cells at 27 °C until they attach (~20 min).

32| While waiting for the cells to attach, add 8 µl of Cellfectin II to 100 µl of Sf-900 III SFM medium in centrifuge tubes for each transfection.

33| In a different centrifuge tube, add 1 µg of bacmid DNA to 100 µl of Sf-900 III SFM.

34| Mix the Cellfectin II/Sf-900 III SFM media mixture and the bacmid DNA/Sf-900 III SFM mixture, and incubate for 30 min at RT.

35| Change the medium in each well with 2 ml of Sf-900 III SFM medium, and add the Cellfectin II/DNA mixture dropwise onto the Sf9 cells. Ensure even dispersal.

36| Incubate the cells for 72 h in a 27 °C incubator (make sure to have water inside the incubator to prevent strong evaporation of the medium).

37| Collect the supernatant containing P1 virus (~2 ml from each well), and filter the medium containing P1 virus into a 2-ml centrifuge tube using a 3-ml syringe fitted with a small 0.2-µm filter. This is a stock of P1 virus that should be stored at 4 °C light protected for up to a month. Add 2% (vol/vol) FBS to the stabilized virus stock. It might also be helpful to use the titerless infected cells preservation and scale-up (TIPS) method[67] to preserve Sf9 cells infected with P1 virus.

38| Determine the titer of the P1 BacMam virus using the Sf9 easy titer cell line and the end-point dilution assay or by using the Virus Counter 2100.
? TROUBLESHOOTING

Infection of Sf9 cells with P1 virus to produce P2 virus ● TIMING 2 h

39| On the basis of the desired volume of P2 virus, add P1 virus to an MOI of 0.1 to 0.0001 to Sf9 cells that are 1.0–1.5 × 10^6 cells per ml in an Erlenmeyer flask of the corresponding size.

▲ **CRITICAL STEP** For the amplification of some viruses, we have found that it is essential to infect at a lower MOI than recommended by the Bac-to-Bac system (Invitrogen). Therefore, it may be important to determine the optimal MOI for the virus amplification before making a large amount of P2 virus.

? TROUBLESHOOTING

40| Incubate the Sf9 cells infected with the P1 virus for 96 h in a 27 °C orbital shaker at 115 r.p.m.

▲ **CRITICAL STEP** For the amplification of some viruses, the collection time of the P2 BacMam virus may need to be optimized. We advise initially trying 72 and 96 h.

41| Centrifuge the cells at 8,000*g* for 15 min at 4 °C, and collect the supernatant containing P2 virus.

42| Filter the supernatant using disposable 0.2-μm filters (50-ml Steriflip filters from Millipore for small amounts or 250-ml, 0.5 l or 1-liter Corning filter systems for large amounts). Add 2% (vol/vol) FBS to the stabilized virus stock. This is a stock of P2 virus that should be stored at 4 °C, protected from light (we use aluminum foil) for up to 1 month.

43| Determine the titer of the P2 BacMam virus using the Sf9 Easy Titer cell line and the endpoint dilution assay or by using the Virus Counter 2100.

? TROUBLESHOOTING

Transduction of suspension HEK293S GnTI⁻ cells with BacMam virus ● TIMING 2 h

44| Expansion of HEK293S GnTI⁻ cells should be prepared in advance (~10 d in advance) so that a sufficient amount of cells are available on day 15. To expand HEK293S GnTI⁻ cells, determine the total number of cells and percent viability using a hemocytometer and trypan blue exclusion, and make sure that the density of the cells are 2.5–3 × 10^6 cells per ml (from **Box 1**).

45| When a 25-ml culture of HEK293S GnTI⁻ cells are 2.5–3 × 10^6 cells per ml, dilute the culture to 0.2 × 10^6 cells per ml in 200 ml and incubate the cells on an orbital shaker within a 37 °C incubator in the presence of 8% CO_2 for ~5 d until the density is 3 × 10^6 cells per ml.

? TROUBLESHOOTING

46| On the basis of the volume of cells needed (2.4–6.4 liters), calculate the volume of medium that you need to add to dilute the culture to a seeding density of 0.2 × 10^6 cells per ml. We prefer to have a starting density of 0.2 × 10^6 cells per ml. For 2.4 liters, one will need 4.8 × 10^8 cells, ~2.2 liters of medium and three 2-liter flasks.

47| Aseptically add the appropriate volume of prewarmed growth medium into the culture flask (the total volume should be 800 ml in a 2-liter flask). Split the culture to multiple flasks as needed, and incubate the cells on an orbital shaker within a 37 °C incubator in the presence of 8% CO_2 for ~5–6 d until the cells reach a density of 2–3.5 × 10^6 cells per ml.

48| Add BacMam P2 virus at an MOI of 1 to infect 2.4 liters of HEK293S GnTI⁻ cells at a density of 2–3.5 × 10^6 cells per ml, and incubate the cells on an orbital shaker within a 37 °C incubator in the presence of 8% CO_2.

▲ **CRITICAL STEP** The amount of virus added should not exceed >10% of the culture volume.

49| After 8–24 h at 37 °C, add 10 mM sodium butyrate and incubate the cells on an orbital shaker within a 30 °C incubator in the presence of 8% CO_2.

▲ **CRITICAL STEP** The incubation temperature for the BacMam-transduced HEK293S GnTI⁻ cells should be determined before attempting a large-scale expression (**Fig. 4**). In addition, the amount and time of sodium butyrate addition should be optimized for each protein (**Fig. 5**).

50| Collect the cells 60–90 h after transduction by centrifugation for 20 min at 6,200*g* at 4 °C.

▲ **CRITICAL STEP** The collection time of BacMam-transduced HEK293S GnTI⁻ cells should be determined before attempting a large-scale expression (**Fig. 4**).

? TROUBLESHOOTING

■ **PAUSE POINT** The cell pellets can be stored at –80 °C for weeks until you are ready to proceed with protein purification[15–17,20].

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1, 30, 39, 43, **Boxes 1** and **2**	Change in medium color and/or the presence of a foul smell	Contamination of cell culture owing to improper sterile technique	Ensure that proper sterile technique is used. Clean and disinfect hood work surfaces before and after every manipulation. Before working in the hood, disinfect gloves with 70% (vol/vol) ethanol. Spray all items to be placed in the hood (medium bottles, pipetting devices, outside of the plastic wrap of the sterile containers and so on) with 70% (vol/vol) ethanol and wipe them with paper towels
		Contaminated virus	Ensure that the virus has been filter sterilized
		Contaminated reagent	Filter-sterilize histone deacetylase inhibitors (i.e., sodium butyrate) in the hood
38	Low virus titer	Sf9 cells infected at wrong MOI	Generate P2 virus using lower MOI (0.05–0.0001)
		Poor quality bacmid	Remake the bacmid ensuring that the bacmid culture is inoculated from a white colony and that the bacmid DNA is not sheared during purification
45,50	Low cell harvest density	Poor-quality cells	It is important not to let cells overgrow (i.e., to subculture them on a regular basis) and to avoid using cells that have undergone more than 30 continuous passages since being raised from liquid nitrogen. Check the health and viability of cells before each experiment
	Low expression	Infection at the wrong MOI, nonoptimal expression conditions, toxicity of membrane protein when overexpressed	Always determine the titer of the BacMam virus to calculate a MOI. Screen for optimal temperature, harvest time and concentration of histone deacetylase inhibitors
		Virus is not stable when stored for extended periods	Remake the P2 virus starting from either the P1 virus or a glycerol stock of DH10Bac *E. coli* containing bacmid DNA Use TIPS method to preserve Sf9 cells infected with P1 virus

● TIMING

The entire protocol, starting from transfection (Step 1) to the collection of BacMam virus–transduced suspension HEK293S GnTI$^-$ cells (Step 50), takes ~3 weeks to complete if a promising candidate is identified (Step 14). The hands-on timing for each stage of the PROCEDURE is summarized below.

Step 1, cell seeding: 15 min

Steps 2–7, small-scale transient transfection to screen constructs: 45 min

Steps 8–15, screening the constructs by FSEC for monodispersity and expression level: 3 h

Step 16, transformation of DH10Bac *E. coli*: 1 h

Step 17, inoculation of bacmid-containing cultures: 15 min

Steps 18–29, isolation of bacmid: 1 h

Steps 30–38, transfection of Sf9 cells with bacmid (producing P1 virus): 2 h (45 min for transfection + 15 min for P1 virus collection + 1 h for virus titer determination using the Virus Counter 2100)

Steps 39–43, infection of Sf9 cells with P1 virus to produce P2 virus: 2 h (15 min for infection of Sf9 cells with P1 virus + 45 min for collection of P2 virus + 1 h for virus titer determination using the Virus Counter 2100)

Steps 44–50, expansion of HEK293S GnTI$^-$ cells and transduction of suspension HEK293S GnTI$^-$ cells with BacMam virus: 2 h

Box 1, growth and maintenance of suspension HEK293S GnTI$^-$ cells: 15 min

Box 2, endpoint dilution assay: 1 h

ANTICIPATED RESULTS

This protocol (as outlined in **Fig. 1**) has been used in our laboratory to successfully express cASIC1, *Drosophila melanogaster* dopamine transporter (DAT), *N*-methyl-D-aspartate (NMDA) receptors and many other membrane proteins in HEK293S GnTI$^-$ cells[15,16,20,61]. The time it takes to identify a promising candidate (**Fig. 3**) is likely to vary substantially depending on (for example) the number of flexible regions to be removed, surface entropy reduction mutations and thermostability mutations. Although few changes are made for cASIC1, several construct changes were needed for DAT and NMDA to obtain the best-expressed and monodisperse candidate via FSEC[15–17,20]. Once a promising candidate is identified, the most favorable conditions for MOI, cell density, expression time, temperature (**Fig. 4**) and the presence of histone deacetylase inhibitors (i.e., sodium butyrate; **Fig. 5**) should be determined for each protein before attempting a large-scale expression.

The protocol for optimized expression can be completed in ~3 weeks. Purification of membrane proteins from transduced HEK293S GnTI$^-$ cell pellets (which may include affinity chromatography, tag cleavage, removal of *N*-linked glycosylation and size-exclusion chromatography), depending on the candidate protein, can produce 0.25–1.5 mg of protein per liter of medium sufficient for crystallization. The protein expression and yield can vary depending on factors such as the titer of the virus, the toxicity of the protein when expressed and the stability of the protein.

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AUTHOR CONTRIBUTIONS A.G. screened and optimized expression conditions for cASIC1 and GluCl. A.G., C.-H.L., K.H.W., J.C.M. and D.P.C. optimized the cell growth and virus amplification conditions. C.-H.L. designed the BacMam construct and performed initial characterization of the BacMam construct. I.B. and T.A. cloned and optimized the cASIC1 and GluCl pEG BacMam constructs, respectively. K.C.G. and S.F. provided the pVLAD construct, incubator configuration and consultations to optimize cell growth during the early stages of the project. All authors wrote and edited the manuscript.

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