

Generation of Tetracycline-Inducible Mammalian Cell Lines by Flow Cytometry for Improved Overproduction of Membrane Proteins

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Abstract

Overexpression of mammalian membrane proteins in mammalian cells is an effective strategy to produce sufficient protein for biophysical analyses and structural studies, because the cells generally express proteins in a correctly folded state. However, obtaining high levels of expression suitable for protein purification on a milligram scale can be challenging. As membrane protein overexpression often has a negative impact on cell viability, it is usual to make stable cell lines where the protein of interest is expressed from an inducible promoter. Here we describe a methodology for optimizing the inducible production of any membrane protein fused to GFP through the isolation of clonal cell lines. Flow cytometry is used to sort uninduced cells and the most fluorescent 5% of the cell population are used to make clonal cell lines.

Key words Membrane protein, Overexpression, GFP, GPCR, HEK293 cell line, Flow cytometry, Transporter

1 Introduction

Expressing eukaryotic membrane proteins in mammalian cells has the advantage of providing a near-native environment that supports the production of fully functional protein [1]. For many integral membrane protein targets, such as G protein-coupled receptors, ion channels, and transporters, a mammalian expression system is the most effective system for producing properly folded and functional protein [2–4]. We have found that creating stable cell lines in HEK293 cells using an inducible TetR expression system [5], such as the T-Rex™ system, produces the best results [2, 3]. However, crystallization of eukaryotic membrane proteins for structural studies by X-ray crystallography requires milligram quantities of protein. Although some membrane proteins have been crystallized after overexpression in mammalian cells [6–9],

the purification of milligram amounts of protein can be challenging if expression levels are below one million copies/cell. In this protocol we describe a method to obtain cell lines producing milligram quantities of target protein by expressing membrane protein-GFP fusion proteins from a tetracycline-inducible promoter and using flow cytometry to create overexpressing clonal cell lines. These cell lines can be passaged many times with no reduction in expression levels of the target protein, which is not the case when flow cytometry has been used to overexpress membrane proteins using constitutive expression from a strong promoter [10].

To make a stable cell line using the T-Rex™ system, a plasmid expressing the protein-GFP fusion of interest from a tetracycline-inducible CMV promoter is transfected into HEK293(TetR) cells where it integrates randomly into the host cell genome. The expression level of the transgene depends to a large extent on where it has integrated. This positional effect on expression levels is then exploited by selecting for those cells where the random integration results in high levels of expression. Normally in a polyclonal cell line there is a wide range of expression levels both before and after induction. We have found that sorting the top 5 % of fluorescent cells of the uninduced population of a polyclonal cell line will generate clonal cell lines with the highest likelihood of high levels of induced expression (*Andréll et al. unpublished*). Usually we start the process by performing a transfection of the plasmid DNA expressing the cDNA of interest and setting up six polyclonal cell lines from this transfection. Sorting by flow cytometry will then be performed only on the polyclonal cell line that shows the widest distribution of fluorescence in the uninduced state. Sorting at low cell numbers in a 96-well plate will give rise to 40–60 different cell lines, which ensures that an overexpressing cell line will be found. Initial analysis of these cell lines is accomplished by flow cytometry and fluorescence microscopy, which together assess both the quantity and quality of the expressed fusion protein. As the cell lines are passaged, their viability and growth potential are also selected for, which allows easy identification of cell lines suitable for large-scale culture and downstream purification of milligram quantities of target protein.

The methodology we have developed is suitable for any integral membrane protein that is usually found at the plasma membrane and can be fused to GFP without affecting its functionality. Although the methodology will also work for membrane proteins localized to intracellular organelles, fluorescence microscopy may not be able to differentiate between correctly folded protein and misfolded protein localized in the endoplasmic reticulum, so it may be necessary to use a different assay to assess if misfolded protein is present, e.g., a differential detergent solubility assay [3].

2 Materials

Prepare all stocks and buffers at room temperature using ultrapure water, MilliQ grade (18 Ω M cm at 25 °C), and filter using a 0.22 μ m filter. All tissue culture work must be performed inside a microbiological safety cabinet. Use sterile equipment and buffers if in contact with cells. Wear nitrile gloves and a lab coat when working with mammalian cells.

2.1 Equipment (See Note 1)

1. Microbiological safety cabinet.
2. Temperature and CO₂ regulated incubator set at 37 °C and 5% CO₂.
3. Personal protection equipment: lab coat, nitrile gloves, safety glasses.
4. Pipette filler, such as S1 Pipette filler (Thermo Scientific).
5. Pipettes: 5, 10, 25, and 50 ml sterile.
6. Benchtop centrifuge suitable for 50 ml conical-bottom tubes.
7. Aspirator, such as VACUSAFE (Integra) used with sterile aspirator pastettes (Alpha laboratories LW4811).
8. Cell counting equipment, such as Countess™ automated cell counter (Life technologies) with Countess™ cell counting chamber slides (Life technologies C10228).
9. Fluorescent microscope, such as a Leica DMI LED Fluo (Leica Microsystems) coupled to a Lumen 200 fluorescence illumination system (Prior Scientific) and a QI click camera (QI imaging) and computer.
10. Flow cytometry analyzer, such as FACSCalibur™ (BD Biosciences).
11. Flow cytometry cell sorter, such as MoFlo™ Legacy (Beckman Coulter).
12. Flow cytometry data analysis program, such as FlowJo vX.0.7.
13. Liquid nitrogen and liquid nitrogen cell line storage dewar.

2.2 Tissue Culture Consumables (See Note 2)

1. T175 flask, such as 175 cm² tissue culture flask with vented cap. 10 cm plate, such as 100×20 mm tissue culture dish.
2. 96-well, 24-well, and 6-well tissue culture treated plates.
3. Polystyrene round bottom tube, Falcon® 5 ml, 12×75 mm, sterile (*see Note 3*).
4. 1.5 ml Eppendorf microcentrifuge tubes, sterile.
5. Cell strainer, such as CellTrics® 50 μ m filters, sterile or nonsterile (*see Note 4*).
6. Cryogenic vial.

7. 15 ml and 50 ml conical-bottom tubes, sterile.
8. Steriflip-GP, 50 ml disposable vacuum filter system, 0.22 μm , sterile.

2.3 Mammalian Expression System (See Note 5)

1. T-REx™-293 cell line (Life technologies) (*see Note 6*).
2. Construct DNA: pcDNA™4/TO mammalian expression vector (Life technologies) containing the gene of interest C-terminally tagged with eGFP.

2.4 Reagents, Stock Solutions, and Buffers

1. Blasticidin stock solution (5 mg/ml): Prepare inside microbiological safety cabinet. Dissolve 50 mg Blasticidin S HCl powder in 10 ml water. Filter sterilize using a Steriflip-GP 50 ml. Aliquot into 20 \times 0.5 ml and store at $-20\text{ }^{\circ}\text{C}$.
2. Doxycycline stock solution (1 mg/ml): Per 1 ml water, add 1 mg of doxycycline hyclate for a 1 mg/ml stock. Filter sterilize using Steriflip-GFP 50 ml. Make up fresh for each use.
3. Phosphate buffered saline (PBS) solution.
4. Cell buffer: 20 mM Tris pH 7.4, 100 mM NaCl with added Complete EDTA-free protease inhibitors according to manufacturer's instruction.
5. GeneJuice™ transfection reagent.

2.5 Media

1. Complete media: Prepare inside microbiological safety cabinet. Take one 500 ml bottle of Dulbecco's Modified Eagle Medium high glucose GLUTAMAX™ supplement pyruvate (DMEM), and add one aliquot of blasticidin stock solution for a final 5 $\mu\text{g}/\text{ml}$ concentration and add 50 ml of certified Fetal Bovine Serum (FBS) (*see Notes 7 and 8*).
2. Serum-free media: DMEM only.
3. Antibiotic selection media: Prepare inside microbiological safety cabinet. Add 1 ml Zeocin™ (100 mg/ml) to one 500 ml bottle of complete media for a final 200 $\mu\text{g}/\text{ml}$ concentration.
4. Cell freezing media: Add 47.5 ml complete media to 2.5 ml sterile Dimethyl sulfoxide Hybri-Max™ (DMSO) in a 50 ml conical-bottom tube for a final concentration of 5% DMSO.
5. Induction media: Per 1 ml selection media add 1 μl doxycycline stock solution for a 1 $\mu\text{g}/\text{ml}$ final concentration of doxycycline. Make up fresh for each use.

3 Methods

A protocol overview is presented in Fig. 1.

3.1 Polyclonal Cell Line Generation

1. Grow T-REx™-293 cells observing proper mammalian tissue cell culture technique. Use good tissue cell culture practice

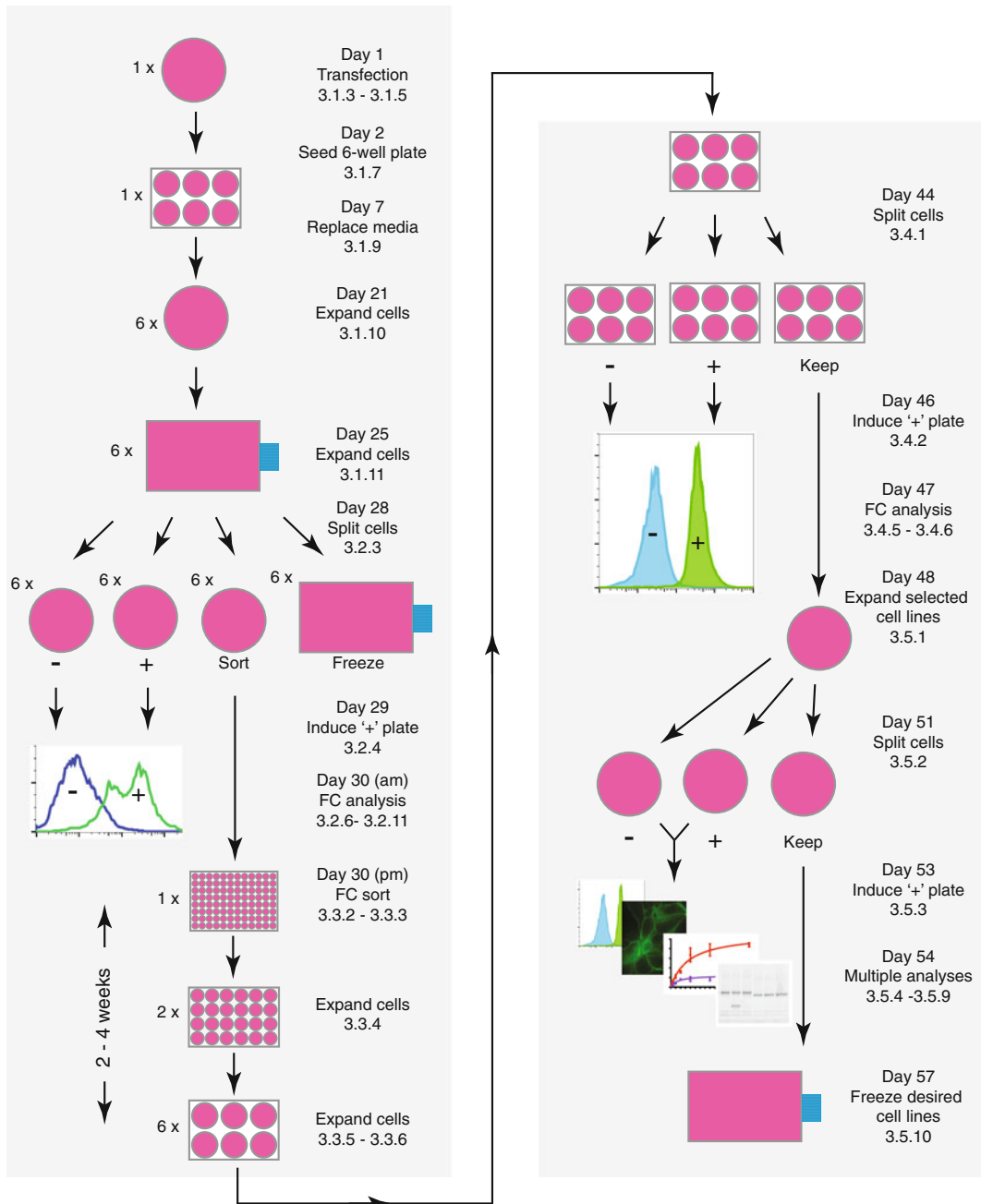


Fig. 1 Protocol overview. For each step we have given an approximate time and a reference to the section in the main text where it is discussed. The timings will vary depending on the rate of growth of individual cell lines and the protein/construct being expressed. The number of plates typically used for a single target is indicated above the cartoon of the tissue culture plate used (either a 10 cm plate, 6-well plate, 24-well plate, 96-well plate or a T175 flask). Abbreviations: *FC* flow cytometry; ‘-’, no added tetracycline; ‘+’, induced with tetracycline

throughout to keep cells healthy at high viability for good results. All tissue culture work must be performed inside a microbiological safety cabinet. Use sterile solutions if in contact with cells. All media must be warmed to 37 °C prior to use. All cells are grown by incubating them in an incubator set at 37 °C and 5% CO₂. Scale up cells into T175 tissue culture flasks.

2. To set up plates for transfection, harvest a confluent T175 tissue culture flask in 12 ml complete media. Unless otherwise stated cells are harvested by removing the media with an aspirator, washing the cells gently without dislodging them by swirling PBS over them (*see Note 9*), removing the PBS by aspiration, and finally dislodging the cells by pipetting the media over them repeatedly and pipetting the cell solution up and down to homogenize it. When harvesting flasks, the flask can be tapped by hand to dislodge the cells prior to pipetting them. Seed 1/6th of cells (2 ml) into a 10 cm tissue culture plate containing 8 ml complete media. Set up one 10 cm tissue culture plate per construct and one additional 10 cm plate as a negative control. After 24 h the 10 cm plates should be 70–80% confluent prior to transfection (*see Note 10*).
3. For each plate, pipette 800 µl serum-free media into a sterile microcentrifuge tube and add 18 µl GeneJuice™ transfection reagent. Vortex to mix and incubate at room temperature for 5 min.
4. Add 6 µg of construct DNA. Mix gently by pipetting and incubate at room temperature for 15 min. During this incubation replace the media of the 70–80% confluent 10 cm plate with 10 ml complete DMEM media.
5. Add the DNA/GeneJuice™ mixture dropwise to the plate. Tilt plate side-to-side and backwards and forwards for a gentle mix (*see Note 11*), put it in an incubator and leave for 24 h. For the negative control plate, repeat all the same steps but do not add any construct DNA to the transfection reagent.
6. For each transfected plate and the negative control: prepare a 6-well tissue culture plate by pipetting 4 ml antibiotic selection media into each well. Harvest the transfected plate in 5 ml of selection media. Count the cells using, for instance, a Countess™ automated cell counter.
7. Seed each well in the 6-well plate with ~400,000 cells to allow six polyclonal cell line selections to be done in parallel (*see Note 12*).
8. Set up a transfection efficiency control plate by adding 4 ml of the harvested cells to a 10 cm tissue culture plate. Add 6 ml complete media containing 10 µg doxycycline for a final concentration of 1 µg/ml and induce for 24 h.

9. Estimate the transfection efficiency by looking at the positive control plate in a fluorescence microscope. Compare the white light image with the green fluorescence image to estimate percentage transfection efficiency (*see Note 13*). After 5–7 days no live cells should be visible under the microscope in negative control plates (*see Note 14*). Depending on the transfection efficiency the transfected cells will either (a) die massively in the 6-well plates or (b) become confluent rapidly without any cell death. In the case of (a) the media should be replaced once colonies have started to grow (*see Note 15*). In the case of (b) proceed directly to **step 10**.
10. Once the media in each 6-well has started to become more yellow in color, harvest the cells in fresh 4 ml selection media and add them to a 10 cm tissue culture plate containing 6 ml selection media. If the cells did not die during the 6-well plate stage (9b), then this first passage will trigger cell death. The surviving cells will grow as colonies. Once grown to when they are visible to the eye, resuspend the colonies in 10 ml fresh selection media in order to get uniform confluency.
11. When confluent harvest the cells from each 10 cm plate in 10 ml selection media and add to a T175 flask containing 15 ml selection media.
12. Defrost a vial of T-REx™-293 cells to be used as a nonfluorescent control during flow cytometry analysis and cell sorting.

3.2 Choosing a Polyclonal Cell Line for Sorting

1. To set up plates for flow cytometry analysis, when the T175 flasks are confluent harvest the cells in 9 ml selection media. For each T175 flask containing a polyclonal cell line label three 10 cm plates as “–” (uninduced), “+” (induced), and “sort” and a T175 flask as “freeze.”
2. Add 9 ml selection media to the “–”, “+”, and “sort” plates and 20 ml selection media to the T175 “freeze” flask.
3. Harvest each T175 flask in 9 ml selection media. Add 1.5 ml harvested cells to the “–” plate, add 2 ml of the harvested cells to the “+” plate, add 1.5 ml of harvested cells to the “sort” plate, and add the remaining cells to the “freeze” flask.
4. When the “+” plates are 70–90% confluent, induce them by replacing the media with 10 ml induction media.
5. After 24 h induction image the “+” and “–” plates in a fluorescence microscope (Fig. 2a–c) (*see Note 16*).
6. Harvest the cells from the “+” plate in 10 ml PBS. Make sure the cells are well dispersed and have a cell density of $\sim 1 \times 10^6$ cells/ml (*see Note 17*). Harvest parental T-REx™-293 cells to use as a negative control in a similar manner.
7. Transfer 0.5 ml of each sample into a polystyrene round bottom tube. These are the samples for the flow cytometry analyzer.

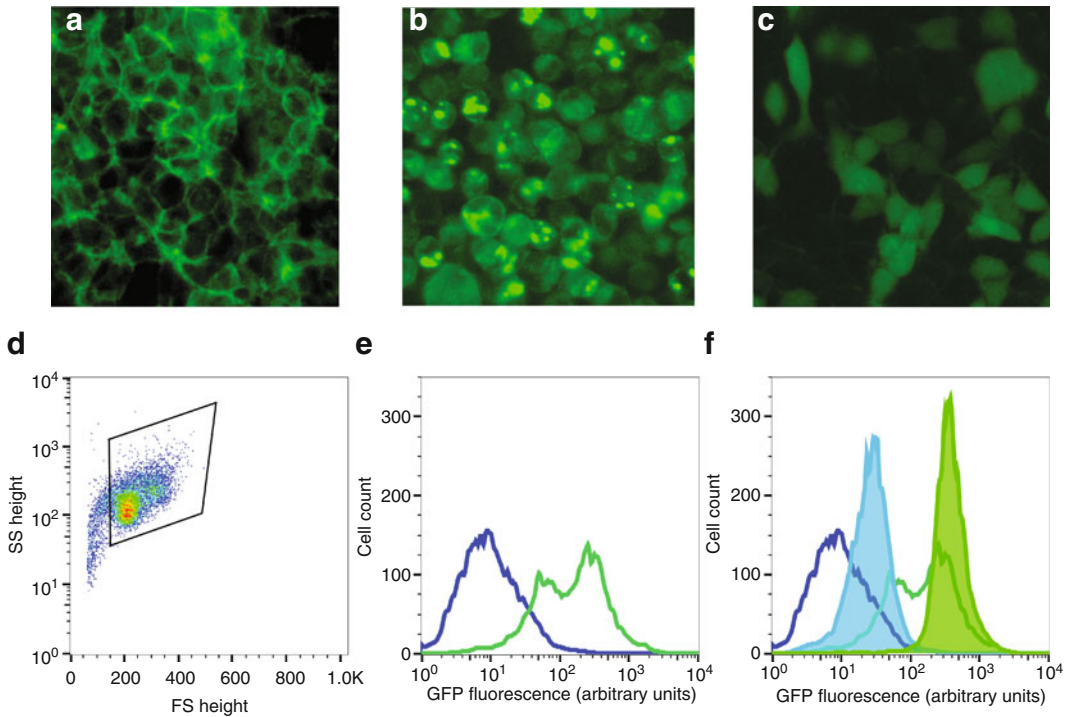


Fig. 2 (a–c) Fluorescence microscope images of cell lines expressing target membrane proteins showing different cellular localizations; (a) correctly localized at the plasma membrane with no obvious intracellular aggregates, (b) undesirable aggregates of the membrane protein-GFP fusion are clearly visible intracellularly as intense areas of fluorescence, (c) GFP fluorescence is decoupled from target membrane protein expression and low levels of soluble GFP are observed throughout the cell. Fluorescence brightness has been adjusted separately for each image for clarity. (d) During flow cytometry, intact cells are selected using a gate defined by the rhomboid in the Forward Scatter (FS) versus Side Scatter (SC) plot. (e) Histograms of an uninduced polyclonal cell line (*blue line*) and an induced polyclonal cell line (*green line*). (f) Histograms of the final clonal cell line, uninduced (*filled blue area*) and induced (*filled green area*), overlaid on the original polyclonal cell line histogram

8. Put the remaining cells in a 50 ml conical-bottom tube and keep on ice during the analysis **steps 9–11**, Subheading **3.2**.
9. Set up the flow cytometry analyzer according to manufacturer's instruction with the parental T-REx™-293 cell sample as an untransfected control. Adjust the voltage on the photomultiplier tube (PMT) detecting the GFP fluorescence so that the fluorescence value falls between 0 and 10 on the fluorescence log scale (arbitrary units).
10. Collect flow cytometry data from each test sample using the same PMT voltage set for the untransfected control sample. Use a Forward Scatter (FS) versus Side Scatter (SS) plot to select a region to gate the intact cells, avoiding the smaller cell debris and larger cell aggregates (Fig. 2d). Collect 10,000 events.

11. Analyze the data with a suitable analyzer program, *e.g.*, FlowJo. Use the FS versus SS plot to select intact cells (Fig. 2d) and represent the selected cells as a histogram with GFP fluorescence on the x-axis (Fig. 2e). Select polyclonal cell lines to sort from based on broadness of fluorescent peak when induced, inducibility, and presence of a highly fluorescent subpopulation (*see Note 18*). Discard all cell lines not meeting the selection criteria.
12. Count the “+” and “-” cell samples in the 50 ml conical-bottom tubes on ice corresponding to the selected cell lines.
13. Spin down the cells for 5 min at 4000 rpm, aspirate or decant the supernatant.
14. Resuspend the cell pellet in 50 ml PBS, spin down the cells for 5 min at 4000 rpm, aspirate or decant the supernatant.
15. Resuspend the cell pellet in cell buffer at 10 million cells/ml and transfer to a microcentrifuge tube. Label and freeze samples in liquid nitrogen and store at -80°C (*see Note 19*).
16. Once the T175 “freeze” flask is confluent, harvest the cells in 10 ml PBS, spin them down for 5 min at 1500 rpm, aspirate or decant the supernatant.
17. Resuspend the cell pellet gently in 4 ml of cell freezing media. Gently aliquot 4×1 ml into cryogenic vials, freeze cells in -80°C freezer, and transfer the next day to liquid nitrogen cell line storage dewar (*see Note 20*).

3.3 Clonal Cell Line Generation by Flow Cytometry Sorting

1. Prepare 1×96 -well plate per selected polyclonal cell line with 200 μl selection media/well. Keep plates in an incubator until ready for use.
2. Harvest the uninduced cells from one “sort” plate at a time in 10 ml selection media. Filter cells through a sterile cell filter and transfer 1 ml of $\sim 1 \times 10^6$ cells/ml to a 12×75 mm polystyrene round bottom tube.
3. Set up the sorter following the manufacturer’s instructions. Use a FS versus SS plot to select a region to gate the intact cells avoiding the smaller cell debris and larger cell aggregates. Use a second plot of Pulse Width versus Forward Scatter to set a gate to exclude cells of a greater width, *i.e.*, to exclude doublets. Single intact cells from the top 5% of the uninduced cell population were selected using the sort logic. 10 cells/well were sorted into the prepared 96-well plate. Repeat the procedure for each selected polyclonal cell line (*see Note 21*) and place plates into an incubator for growth.
4. The first confluent wells will appear 10–14 days post-sorting (*see Note 22*).

5. As the wells become confluent, harvest the cells from each well in the media they have grown in and transfer them to a 24-well plate containing 1 ml selection media per well.
6. As these become confluent harvest the cells from each well in the media they have grown in and transfer them to a 6-well plate containing 3 ml selection media per well (*see Note 23*).

3.4 Selection of Sorted Cell Lines by Flow Cytometry Analysis

1. To set up plates for flow cytometry analysis of sorted cell lines, when each 6-well becomes confluent, harvest the cells from each well in 6 ml selection media. Label three new 6-well plates “+”, “-” and “keep.” Add 2 ml selection media into each well. Transfer 2.5 ml/2 ml/1.5 ml of the harvested cells into the “+”, “-”, and “keep” plates respectively (*see Note 24*).
2. Induce the “+” wells when 70–90% confluent (~48 h after passaging) by replacing the media with 4 ml induction media.
3. After 24 h induction image the “+” and “-” plates in a fluorescence microscope (*see Note 25*).
4. After a 24 h induction harvest each well in the “+” and “-” plates in 2 ml PBS. Transfer 0.5 ml into a polystyrene round bottom tube for flow cytometry analysis.
5. Use the set up from Subheading 3.2, **step 9**. Check the settings by running a test sample of parental T-REx™-293 cells. Collect the flow cytometry data as in Subheading 3.2, **step 10** on all induced (“+”) and uninduced (“-”) samples.
6. Analyze the flow cytometry data similar to Subheading 3.2, **step 11**. Select which cell lines to keep based on the inducibility and median value (*see Note 26*). Discard the other cell lines.

3.5 Further Analysis of Selected Sorted Cell Lines

1. To expand selected sorted cell lines, go back to the “keep” plates and, when confluent, harvest each selected cell line in 3 ml selection media and add them to a 10 cm plate containing 7 ml selection media.
2. To set up plates for further analysis, when confluent, harvest each 10 cm plate in 10 ml selection media, split the cells into three 10 cm plates containing 8 ml selection media each by adding 4 ml of cells to the plate labeled “+” and 3 ml each into the plates labeled “-” and “keep.”
3. Induce the “+” plate when 70–90% confluent by replacing the media with 10 ml of induction media.
4. After 24 h induction, image the “+” and “-” plates in a fluorescence microscope to verify the correct localization of the fusion protein (Fig. 2a–c) (*see Note 27*).
5. Harvest the “+” and “-” plates in 10 ml PBS. Make sure the cells are well dispersed and of $\sim 1 \times 10^6$ cells/ml density. Harvest parental T-REx™-293 cells to use as a negative control

in a similar manner. Transfer 0.5 ml for flow cytometry analysis in a polystyrene round bottom tube. Put the remaining cells in a 50 ml tube and keep on ice during analysis **step 6**, Subheading **3.5**.

6. For flow cytometry analysis, use the set up in Subheading **3.2**, **step 9**. Check the calibration by running a sample of parental T-REx™-293 cells. Collect flow cytometry data as in Subheading **3.2**, **step 10** on all induced (“+”) and uninduced (“-”) samples. Analyze the flow cytometry data similar to Subheading **3.2**, **step 11** (*see Note 28*).
7. Count the “+” and “-” cell samples in the 50 ml conical-bottom tubes on ice corresponding to the selected cell lines. Spin down the cells for 5 min at 4000 rpm, aspirate or decant the supernatant.
8. Resuspend the cells in 50 ml PBS, spin down the cells for 5 min at 4000 rpm, aspirate or decant the supernatant.
9. Resuspend the cells in cell buffer at 10 million cells/ml and transfer to a microcentrifuge tube. Label and freeze samples in liquid nitrogen and store at -80°C (*see Note 29*).
10. To freeze selected cell lines, expand the 10 cm “keep” plate by harvesting the cells when confluent in 10 ml selection media and adding them to a T175 flask containing 15 ml selection media. When confluent, harvest the cells in 10 ml PBS, spin down the cells at 1500 rpm 5 min, aspirate away the supernatant carefully, and resuspend the cells in 4 ml freezing media. Aliquot the cells in the freezing media into 4×1 ml cryogenic vials, freeze at -80°C and transfer the next day to a liquid nitrogen cell storage dewar (*see Note 30*).

4 Notes

1. The brands we use are suggested, but a different brand with the same function may be substituted.
2. It is important that plate and flasks are tissue culture treated and sterile. We recommend vented caps on the tissue culture flasks to reduce the risk of contamination.
3. These tubes are compatible with the BD FACSCalibur and MoFlow machines. If other flow cytometry machines are used, use the sample holders recommended for them.
4. Use sterile cell filters if preparing samples for flow cytometry sorting. Nonsterile filters are fine for preparing samples for flow cytometry analysis.
5. It is important to use an inducible mammalian expression system that allows for the random integration of the transgene, such as the T-REx™ system used here.

6. Alternatively, an equivalent cell line expressing the TetR repressor protein can be used, such as the HEK293S-TetR [4] or HEK293S-GnTI- [11].
7. The certified FBS is guaranteed not to contain tetracycline, which is essential to retain the cells in an uninduced state.
8. Blasticidin is used to select for the presence of the TetR gene in the T-REx™-293 parental cell line.
9. Washing the cells with PBS removes debris and provides cleaner flow cytometry data.
10. The amount of cells split into the 10 cm tissue culture plate may have to be adjusted to reach 70–80% confluency the next day.
11. Do not swirl the plate as this will concentrate the transfection mix at the center of the plate and prevent uniform spread.
12. Each of the six polyclonal cell lines will be slightly different since they originate from different batches of cells.
13. If there is no fluorescence upon induction of the transfected control plates then the transfection has failed. Make sure the fluorescent microscope is working. When repeating the transfection make sure that the cells are healthy with no contamination, that the 10 cm tissue culture plate is 70–80% confluent prior to transfection, that the GeneJuice™ batch is fresh, and that the doxycycline stock is fresh and used at the correct concentration. Importantly, double-check that the construct is compatible with expression in the T-REx™ system and that the purified plasmid DNA is of sufficient quality.
14. In general dying cells will be round and floating in the media and live cells will be growing adherently to the bottom of the plate in an extended morphology. If there are live cells present in the negative control the antibiotic selection has failed. Repeat the transfection and cell seeding steps with a freshly prepared antibiotic selection media using fresh certified FBS and Zeocin™. If the negative control cells fail to die in the antibiotic selection media again, defrost a fresh vial of T-REx™-293 cells and repeat all steps.
15. When changing the media be careful to not disturb the cells, particularly if the colonies are small, as this may lead them to be washed away.
16. If the GFP fluorescence localizes to the plasma membrane it indicates that the fusion protein is intact, that the membrane protein has folded properly, and that it is likely to be functional (Fig. 2a). In this instance, the fluorescence intensity is proportional to the fusion protein expression levels. However, misleading fluorescence values could come from high levels of free GFP in the cell or from intensely fluorescent retained material of aggregated fusion protein. When looking at the cells in a

fluorescence microscope these two false positives can easily be discriminated between. Any retained aggregated fusion protein will be seen as a clump of very bright material in the cytosol (Fig. 2b). If there are high levels of free GFP, the entire cytoplasm will be uniformly fluorescent (Fig. 2c). Discard any polyclonal cell line containing these false positives.

17. If the harvested cells contain clumps or are very dense they may clog up the flow cell of the flow cytometry analyzer. Avoid this by filtering cells through a cell filter to remove any clumps and/or by diluting the cell sample with PBS to ~0.5 million cells/ml density.
18. Polyclonal cell line selection criteria: (1) A suitable polyclonal cell line to sort from should have a broad fluorescent peak once it is induced, indicating expression variability. (2) It is preferable if the cell line is fully inducible. The inducibility of a cell line is measured by how many cells are positive for GFP fluorescence upon induction, *i.e.*, they have a fluorescence value higher than the highest fluorescence measured from negative control cells. The percentage of positive cells in a cell line is indicative of how many cells carry the transgene, which ideally should be more than 90%. (3) The presence of a highly fluorescent subpopulation compared to the other polyclonal cell lines. Important: the decision of which polyclonal cell line to sort from is based on the flow cytometry data from the induced cells, but the flow cytometry sorting should be done using the uninduced cells.
19. These cell samples are useful to compare with the final sorted cell lines when verifying expression levels.
20. Polyclonal cell lines are particularly prone to detrimental changes to expression levels with increasing number of passaging. Hence it is important to freeze several aliquots of all cell lines that meet the selection criteria.
21. During sorting, live cells are chosen according to FS and SC profile (not propidium iodide).
22. As a rule ~50% of the wells will grow and become confluent. Even though the plate was seeded with 10 cells/well most of the cell lines that grow will originate from only 1–2 cells and occasionally from 3 to 4 cells (determined under the microscope by checking the colony formation in each well). If only one cell per well is used, over 95% of the cells will die. While the cell lines are not necessarily monoclonal, the resulting clonal cell lines behave as such, with relatively narrow fluorescent peaks that keep constant for 20+ passages (Fig. 2f).
23. The expansion process is an important indirect cell line selection process. The speed of growth will vary depending on how many cells per well are growing, but also on the individual

cells in the well and how well they cope with the uninduced constitutive expression of the transgene. Those cell lines that have constitutive expression levels compatible with healthy growth will expand in a timely manner. Cell lines unable to expand at this stage can automatically be culled, since they would not be useful for extended culture or large-scale growth. Because the expansion time will vary, this part of the protocol can be very labor intensive and requires careful planning. The steps from here until the end of the protocol will happen on different days or on overlapping days depending on the batch of cell lines. At this stage it is therefore very important to keep on top of the process and passage cells when confluent but not overgrown. High viability of the cells is extremely important at all times in order to ensure good expression levels and comparable cell lines during the cell line selection steps.

24. The varying amounts of cells in the three different plates will ensure their confluency at staggered time points. From this point it is important to label each cell line individually.
25. The imaging of potentially hundreds of cell lines in a day is very time-consuming. If there is a time constraint this step can be skipped. If doing so, be aware that, when making the cell line selection based on fluorescence in Subheading 3.4, **step 6**, the top fluorescent cell lines may contain free GFP and/or retained aggregated fusion protein giving rise to misleadingly high fluorescent values. Therefore select a surplus of cell lines to carry forward for further analysis.
26. Evaluating the clonal cell lines for GFP fluorescence using flow cytometry can be extremely quick and data from hundreds of cell lines can easily be collected in a couple of hours. Analyzing the data in a suitable program such as FlowJo allows for quick and easy compilation of statistics and histograms from which a first cell line selection can be made. There are three selection criteria for a suitable clonal cell line to be carried forward from this point. (1) The cell line should be inducible with 99% of cells positive for GFP fluorescence after induction. If the cell line is not fully inducible, it suggests the presence of a small subpopulation not carrying the transgene. During extended time in culture such a small subpopulation can take over the cell line since they grow better as they experience less metabolic demand, toxicity, and stress than those cells carrying the transgene. These cells could be re-sorted if other better cell lines are not available. (2) Populations of clonal cells before and after induction should exhibit narrow fluorescence profiles compared to the polyclonal cell line (Fig. 2f). The narrowness of the peaks suggests that the cell line is clonal. Broad peaks or multiple peaks are indicative of a polyclonal cell line and should

be re-sorted or discarded at this stage. (3) The cell line should have a high median fluorescence value, which is the value where half of the cells have higher fluorescence values and half of the cells have lower fluorescence values. There is no absolute median value that can be used as a cut-off, since fluorescence is in arbitrary units and depends on the flow cytometry analyzer used, the calibration of the machine, and the protein expressed. Once a high expressing cell line has been verified its fluorescence median (on a particular flow cytometry analyzer) can become the fluorescence selection criteria. The first time this protocol is carried out, keep the 10–20 cell lines with the highest median values that also fulfill the other two selection criteria. Note that the induced cells with the highest fluorescent median are also likely to have a high fluorescent median when uninduced (Fig. 2f) (*Andréll et al. unpublished*).

27. If the GFP fluorescence localizes to the plasma membrane, it suggests that the fusion protein is intact and that the membrane protein has folded properly and is likely to be functional (*see Note 16* and Fig. 2a). The selected sorted cell lines are likely to have a relatively high basal expression level when uninduced and these will show very low fluorescence under a fluorescent microscope.
28. It is not unusual for the median of each cell line to differ at this stage compared to the initial data from Subheading 3.4, **step 5**. The data from the scaled up 10 cm plate is more reliable since the cell line has stabilized at this point. Commonly, the cell lines with a very high basal expression level struggle to expand in a timely manner, which will be apparent at this point. These can be discarded as they are unsuitable for extended culturing and large-scale growth.
29. Use these cell samples for further verification of functional expression levels: such as (1) In-gel fluorescence and/or western blot. The in-gel fluorescence will verify the presence of the full-length fluorescent fusion protein, the absence/presence of free GFP, and the level of degradation of the fusion protein (containing the GFP moiety). (2) Radioligand binding assay. Provided there is a radiolabeled ligand of sufficient affinity to the target protein, this can provide quantification of functional copies of target membrane protein per cell. (3) Fluorescence size exclusion chromatography (FSEC). This will allow the evaluation of the quality of fusion protein when solubilized with different detergents. This is useful to evaluate which cell line to carry forward into large-scale production for protein purification purposes.
30. It is very important to have frozen aliquots stored of each selected sorted cell line.

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References

1. Andréll J, Tate CG (2013) Overexpression of membrane proteins in mammalian cells for structural studies. *Mol Membr Biol* 30:52–63
2. Tate CG, Haase J, Baker C, Boorsma M, Magnani F, Vallis Y, Williams DC (2003) Comparison of seven different heterologous protein expression systems for the production of the serotonin transporter. *Biochim Biophys Acta* 1610:141–153
3. Thomas J, Tate CG (2014) Quality control in eukaryotic membrane protein overproduction. *J Mol Biol* 426:4139–4154
4. Reeves PJ, Kim JM, Khorana HG (2002) Structure and function in rhodopsin: a tetracycline-inducible system in stable mammalian cell lines for high-level expression of opsin mutants. *Proc Natl Acad Sci U S A* 99:13413–13418
5. Yao F, Svensjo T, Winkler T, Lu M, Eriksson C, Eriksson E (1998) Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum Gene Ther* 9:1939–1950
6. Standfuss J, Edwards PC, D'Antona A, Fransen M, Xie G, Oprian DD, Schertler GF (2011) The structural basis of agonist-induced activation in constitutively active rhodopsin. *Nature* 471:656–660
7. Standfuss J, Xie G, Edwards PC, Burghammer M, Oprian DD, Schertler GF (2007) Crystal structure of a thermally stable rhodopsin mutant. *J Mol Biol* 372:1179–1188
8. Gruswitz F, Chaudhary S, Ho JD, Schlessinger A, Pezeshki B, Ho CM, Sali A, Westhoff CM, Stroud RM (2010) Function of human Rh based on structure of RhCG at 2.1 Å. *Proc Natl Acad Sci U S A* 107:9638–9643
9. Deupi X, Edwards P, Singhal A, Nickle B, Oprian D, Schertler G, Standfuss J (2012) Stabilized G protein binding site in the structure of constitutively active metarhodopsin-II. *Proc Natl Acad Sci U S A* 109:119–124
10. Mancia F, Patel SD, Rajala MW, Scherer PE, Nemes A, Schieren I, Hendrickson WA, Shapiro L (2004) Optimization of protein production in mammalian cells with a coexpressed fluorescent marker. *Structure* 12:1355–1360
11. Reeves PJ, Callewaert N, Contreras R, Khorana HG (2002) Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. *Proc Natl Acad Sci U S A* 99:13419–13424