

Chapter 2

Using the Flp-In™ T-Rex™ System to Regulate GPCR Expression

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Abstract

The development of a cell-based system that allows the integration of a gene of interest (GOI), such as a G protein-coupled receptor (GPCR), into a specific site on the genome, has made the generation of mammalian cell lines able to express such proteins easy and efficient. Flp-In™ stable cell lines are isogenic and hence protein expression is constant across a population of cells. A useful addition to the Flp-In™ system (Flp-In™ T-Rex™) allows this expression to be controlled by the addition of a small molecule inducer to the cell culture medium. Stable cell lines generated as described here can be used to great advantage in the study of receptor pharmacology signalling and oligomerisation.

Key words: Flp-In™ T-Rex™, GPCR expression, Inducible locus, Stable expression, Doxycycline induction, Tet repressor

1. Introduction

Expression of G protein-coupled receptors (GPCRs) in a heterologous system using a variety of mainly mammalian cells has become the norm when studying the pharmacology and function of these crucial signal transduction system components. Stable expression of GPCRs, that is when a gene or cDNA is integrated into the host cell genome, is even more useful to furthering the understanding of the mechanisms involved in cell signalling. For this reason, cell lines that express stably and reliably the proteins of interest, in this case GPCRs are routinely created in many laboratories (1, 2). Transiently transfected cells are commonly used as they are quick to produce but they have many disadvantages, notably large differences in expression level among the population

of cells and between experiments. The FLP-In™ T-Rex™ system (Invitrogen Life Technologies; www.invitrogen.com) is designed to create cell lines which stably express proteins of interest in an isogenic and inducible manner. This removes possible variation in expression levels or patterns due to different sites of integration into the chromosome of the gene of interest (GOI) and allows cells to be grown without expression of the protein of interest until such time as this is required. The ability to induce expression by addition to the culture medium of a small molecule has several advantages over either transient transfection, or the generation of constitutively expressing “conventional” stable cell lines. (1) There is no need for repeated, highly variable and expensive transient transfections. (2) All the FLP-In™ T-Rex™ stables derived from the same parental cells are integrated at the same site and hence have the same genetic background allowing comparisons to be made. (3) The FLP-In™ T-Rex™ stable cell lines require only polyclonal selection rather than the isolation of individual colonies. (4) Stable cell lines can be grown to the required density before induction of the GOI, thus avoiding potentially detrimental effects upon cell growth (see Fig. 1). (5) The level of expression can be regulated by varying the concentration of the inducing agent (tetracycline/doxycycline). (6) Since expression of the GOI is only induced when required, there is less likelihood of this expression being lost due to non-expressing cells outgrowing those which are still expressing the GOI.

The generation of a FLP-In™ T-Rex™ cell line (that is, a parental cell line which can receive and express a GOI) requires the integration of two plasmids, one containing a *Flp* Recombination

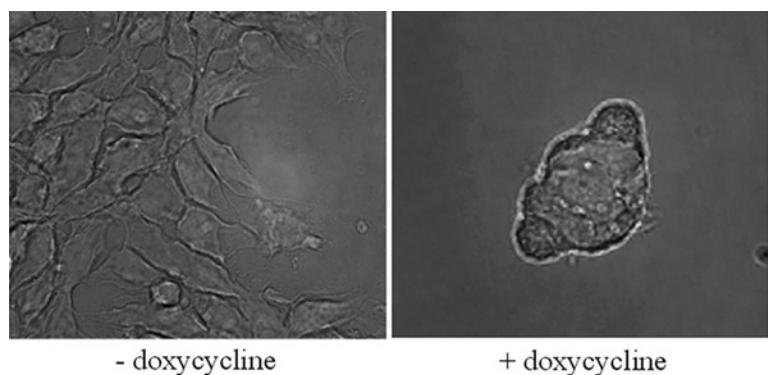


Fig. 1. The effect of expression of the human ghrelin receptor upon cell growth. The *left hand panel* shows a FLP-In™ T-Rex™-293 cell line harbouring the human ghrelin receptor prior to induction. The *right hand panel* shows cells of the same line after induction with doxycycline. The rounding and poor viability of the cells after induction of the ghrelin receptor is related to the high constitutive activity of this receptor through G_q/G_{11} family G proteins as it is prevented by both a ghrelin receptor inverse agonist and an inhibitor of G_q/G_{11} G proteins (K. A. Bennett and G. Milligan, unpublished observations).

Target (FRT) site (3) and the other, a gene expressing the tetracycline repressor (tet repressor) (4). These are maintained within the genome by conferring resistance to the antibiotics zeocin and blasticidin, respectively. It is possible to purchase various cell lines which have been modified in this way from a commercial source (Invitrogen Life Technologies). A list of the available cell lines can be found in Table 1. It can be seen from this that while there are several Flp-In™ cell lines (which have the FRT integration site) and T-Rex™ cell lines (which have the tet repressor system), there is to date only the Flp-In™ T-Rex™-293 (based upon HEK293 cells) which has both. Therefore, in order to work in a background other than HEK293 cells, the plasmids must be obtained and integrated by the user into the desired cell line, for instance, Madin-Darby Canine Kidney (MDCK) cells, which may be used as a polarised cell model (J.H. Robben and G. Milligan, unpublished results). This procedure involves the transfection and integration of the FRT site plasmid (pFRT/*lacZeo*), the selection of a number of colonies by zeocin resistance and the verification of the β -galactosidase activity that this plasmid confers (Fig. 2). One limitation of the Flp-In™ system is that assays based on β -galactosidase expression and/or complementation are limited in these cells by the high level of β -galactosidase activity derived from the pFRT/*lacZeo* plasmid. Southern blotting analysis is then used to distinguish a clone with only one copy of the FRT site. The plasmid pcDNA6/TR, which expresses the tet repressor protein, is then transfected into this clone and integrants (independent of the FRT site) selected by blasticidin resistance and checked for β -galactosidase activity (Fig. 2). While this procedure is within the capabilities of many laboratories, the use of the established Flp-In™ T-Rex™-293 cell line clearly makes sense if appropriate to the proposed study.

The expression of GPCRs using this system has been found to be an efficient and reliable means of generating cell lines that

Table 1
Commercially available Flp-In™ TRex™ cell lines

Flp-In™ cell lines	T-REx™ cell lines	Flp-In™ T-REx™ cell lines
Flp-In™-293	T-REx™-293	Flp-In™ T-REx™-293
Flp-In™-CV-1	T-REx™-HeLa	
Flp-In™-CHO	T-REx™-CHO	
Flp-In™-BHK	T-REx™-Jurkat	
Flp-In™-3T3		
Flp-In™-Jurkat		




- Flp-In™ T-REx™ parental 293 cells, resistant to zeocin and blasticidin

- co-transfect pcDNA5/FRT/TO/GPCR and pOG44

- pcDNA5/FRT/TO/GPCR integrates at FRT site catalysed by Flp recombinase from pOG44. Resistant to blasticidin and hygromycin. TetR protein binds tetOp and represses GPCR expression

- add tetracycline / doxycycline to culture medium

- tetracycline / doxycycline binds tetR protein which is released by tetOp allowing GPCR expression

Tet repressor (tetR) = 
 Tetracycline / doxycycline = 
 GPCR = 

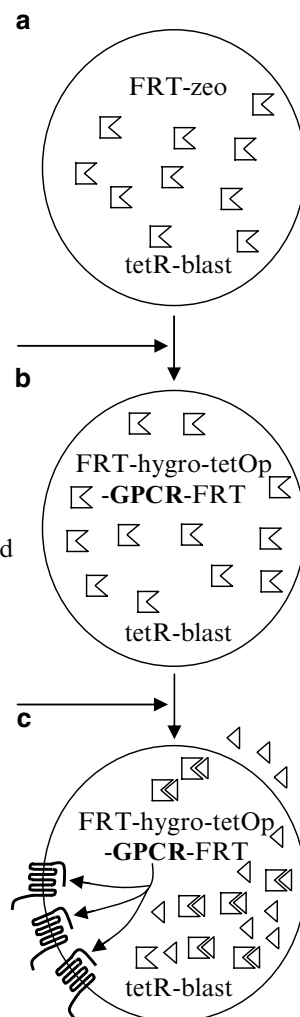


Fig. 2. Schematic representation of the process of making Flp-In™ T-Rex™ cell lines which express a GPCR in a stable and inducible manner. (a) Shows parental Flp-In™ T-Rex™ HEK293 cells, containing the FRT site and expressing the tet repressor. After transfection, (b), the pcDNA5/FRT/TO/GPCR integrates at the FRT site and the tet repressor protein binds to the tetOp region of the integrated pcDNA5/FRT/TO/GPCR which controls the GPCR expression, repressing it. (c) Addition of tetracycline or doxycycline binds the tet repressor, releasing the tet operator and enabling GPCR expression.

are simple to maintain, but which can express GPCRs within a few hours at a level determined by the degree of induction. The system is compatible with the use of epitope tags and fluorescent tags at the N- or C- termini of the GPCR (Figs. 3 and 4). Novel tagging technologies, such as the SNAP/CLIP tagging system (Covalys, www.covalys.com/New England Biolabs Inc; www.neb.com), can also be used.

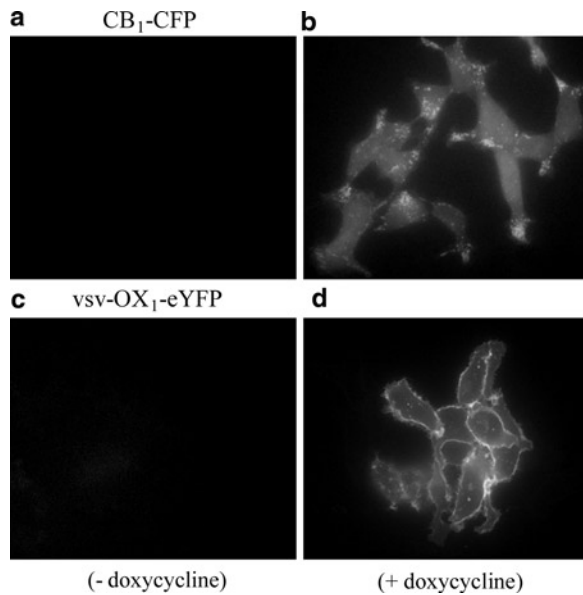


Fig. 3. Induction and expression of GPCRs using the Flp-In™ T-Rex™ system. Panels (a) and (b) show cells of a stable line which harbour the human cannabinoid CB₁ receptor fused at its carboxy-terminal to cyan fluorescent protein (CFP). (a) Non-induced cells (no doxycycline), (b) cells which have been induced with 0.5 µg/mL doxycycline. Panels (c) and (d) are equivalent, but show cells harbouring the human orexin 1 receptor (OX₁) fused to enhanced yellow fluorescent protein (eYFP).

2. Materials

2.1. Equipment

1. Tissue culture plastics.
2. “Mr. Frosty”-type Cryo Freezer container (Nalgene).
3. 22 mm thickness 0 glass cover slips.

2.2. Reagents

1. pcDNA5/FRT/TO (Invitrogen Life Technologies).
2. pOG44 (Invitrogen Life Technologies).
3. Flp-In™ T-Rex™ -293 host cells (Invitrogen Life Technologies).
4. Dulbecco’s Modified Eagle Medium 1× (DMEM): + 4.5 g/L glucose, + L-glutamine, – pyruvate.
5. Foetal bovine serum (FBS) tetracycline negative, European Union approved: Added to DMEM to 10% (v/v) of final volume.
6. Penicillin/streptomycin solution: 10,000 units/mL penicillin and 10 mg/mL streptomycin in 0.9% (w/v) NaCl diluted

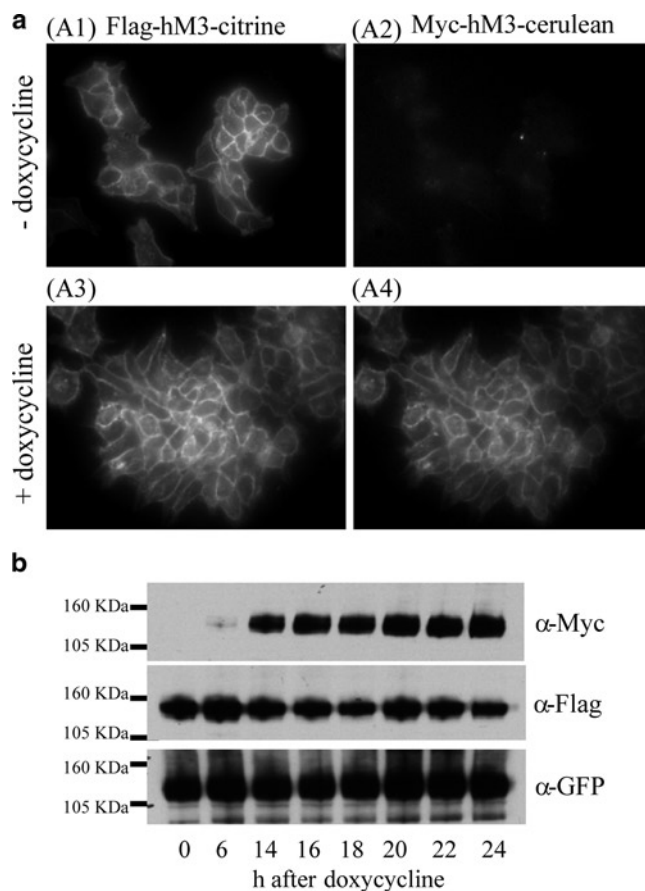


Fig. 4. Induction of a double stable cell line expressing c-Myc-tagged human muscarinic M_3 receptor fused to cerulean fluorescent protein (c-Myc-hM₃-cerulean), inducibly and flag-tagged human muscarinic M_3 receptor fused to citrine fluorescent protein (flag-hM₃-citrine), constitutively. Flag-hM₃-citrine is present in the presence and absence of doxycycline (panels **a1** and **a3**, respectively), while c-Myc-hM₃-cerulean can only be observed when doxycycline is present (panels **a4** and **a2**, respectively). Western blots of lysates of these cells treated with doxycycline for the times indicated are shown in panel (**b**). Positive signals are seen in all lanes with the anti-GFP and anti-Flag antisera as these are able to detect the constitutively expressed component (flag-hM₃-citrine), but are only present after 6–14 h doxycycline treatment with the anti-c-Myc antiserum as this is specific for the inducible component, c-Myc-hM₃-cerulean.

1:100 into DMEM to give 100 $\mu\text{g}/\text{mL}$ of streptomycin and 100 units/ mL of penicillin.

7. Complete DMEM: DMEM with the addition of 10% (v/v) FBS and penicillin/streptomycin (100 units/ mL /100 $\mu\text{g}/\text{mL}$, respectively).
8. Zeocin (Invitrogen Life Technologies): 1 g at 100 mg/ mL in water.
9. Complete DMEM + zeocin: As complete DMEM (see item 7), but with the addition of 50–500 $\mu\text{g}/\text{mL}$ zeocin.

10. Blasticidin S HCl (Invitrogen Life Technologies): 50 mg dissolved in 10 mL water (5 mg/mL), 0.22 µm filtered and stored at -20°C as appropriate aliquots.
11. Complete DMEM + zeocin + blasticidin: As complete DMEM + zeocin (see item 9) but with the addition of blasticidin to a final concentration of 5 µg/mL.
12. Ultrapure sterile water.
13. Complete DMEM + blasticidin: As complete DMEM + zeocin + blasticidin (see item 11), but with the zeocin omitted.
14. Hygromycin B (Roche Diagnostics): 1 g in 20 mL water (50 mg/mL).
15. Complete DMEM + blasticidin + hygromycin: As complete DMEM + blasticidin (see item 13), but with the addition of hygromycin B to a final concentration of 0.5–2 µg/mL.
16. Tetracycline hydrochloride (Sigma-Aldrich Company Ltd): Dissolved in water to 1 mg/mL and passed through a 0.22 µm filter. Stored at -20°C as 0.5 mL aliquots.
17. Doxycycline hyclate (Sigma-Aldrich Company Ltd.): Dissolved in water to 1 mg/mL and passed through a 0.22 µm filter. Stored at -20°C as 0.5 mL aliquots.
18. 2× RIPA buffer: 100 mM HEPES pH 7.5, 300 mM NaCl, 2% (v/v) Triton TX-100, 1% (w/v) sodium deoxycholate and 0.2% (w/v) SDS, stored at 4°C. 1× RIPA consists of 25 mL 2× RIPA with the addition of 10 mM NaF, 5 mM EDTA, 10 mM sodium phosphate buffer pH 7.5, and 5% (v/v) ethylene glycol made up to a final volume of 50 mL with water. Protease inhibitors (see item 21) should be added according to the manufacturer's instructions. This solution is stable for up to 1 week at 4°C.
19. Poly-D-Lysine hydrobromide: Add 50 mL of sterile water to 5 mg in the bottle and store at 4°C. This solution only has to come into contact with cover slips or tissue culture plastic to form an adequate coating and, if kept sterile may be reused repeatedly.
20. HEPES buffer pH 7.4: For 1 L, add 7.6 g NaCl (final 130 mM), 0.37 g KCl (final 5 mM), 4.77 g HEPES (final 20 mM) and 1.8 g glucose (final 9 mM) to 900 mL water. When dissolved, add 1 mL 1 M MgCl₂ and pH to 7.2. Correct volume to 1 L with water and add 1 mL 1 M CaCl₂. This solution is stable for up to 1 month at 4°C, but should be discarded if it shows signs of microbial growth.
21. Complete protease inhibitors (Roche Diagnostics).
22. Geneticin/G418.
23. Complete DMEM + blasticidin + hygromycin + G418: As complete DMEM + blasticidin + hygromycin (see item 15),

but with the addition of G418 to a final concentration of 1 mg/mL dissolved in DMEM and added back to the bottle via a 0.22 μ m filter.

24. pcDNA3 (Invitrogen Life Technologies, no longer available, replaced with pcDNA 3.1).
25. pcDNA5/FRT/TO and TOPO vectors (Invitrogen Life Technologies).
26. Plasmid Maxi kit (Qiagen).
27. Endofree Plasmid Maxi kit (Qiagen).
28. pFRT/lacZeo (Invitrogen Life Technologies).
29. pcDNA6/TR (Invitrogen Life Technologies).
30. HEK-293 cells: Human embryonic kidney 293 cells (Health Protection Agency Culture Collections).
31. Dimethyl sulphoxide (DMSO).
32. 1 \times PBS: 120 mM NaCl, 25 mM KCl, 10 mM Na₂HPO₄ and 3 mM KH₂PO₄, pH 7.4. This solution can be made up at 10 \times concentrated, but pH must be checked on dilution.
33. 0.25% Trypsin-EDTA solution.
34. Lipofectamine Reagent (Invitrogen Life Technologies): Used according to manufacturer's instructions.
35. Lipofectamine 2000 (Invitrogen Life Technologies): Used according to manufacturer's instructions.
36. Polyethyleneimine (PEI) 25 kDa linear (Polysciences): 1 mg/mL in sterile 150 mM NaCl. Stored as aliquots at -20°C .
37. Non-essential amino acids (NEAA) (Invitrogen Life Technologies).

3. Methods

In order to express a GOI, it must first be subcloned into the multiple cloning site of the plasmid pcDNA5/FRT/TO. This is then co-transfected into the Flp-InTM T-RexTM cell line with a plasmid (pOG44) expressing the Flp recombinase (Fig. 2). Upon integration of the pcDNA5/FRT/TO containing the GOI into the FRT site, the cells are rendered hygromycin resistant and zeocin sensitive, allowing selection of the required integrants. Hygromycin resistance is conferred from the pcDNA5/FRT/TO and zeocin sensitivity is due to inactivation of the *lacZeo* fusion gene by the integration process. Expression of the GOI is repressed by the tet repressor protein which binds to the tet operator O₂ sequence upstream of the GOI and prevents

transcription. In order to induce expression tetracycline or doxycycline must be added to the culture media (Figs. 2 and 3). This then binds to the tet repressor protein, releasing it from the tet operator sequence, which in turn allows transcription and translation of the GOI.

3.1. Generation of Expression Construct

1. Design a cloning strategy for the GOI by assessing the multiple cloning site of pcDNA5/FRT/TO. This expression vector contains the FRT site needed for recombinase-mediated integration of the GOI into the genome (see Note 1).
2. Ligation of insert to vector, transformation into *Escherichia coli* (DH5 α or XL-1 Blue strains) and the selection of transformants are carried out by standard molecular biological techniques (see ref. 5 for general techniques).
3. Analyse positive transformants by restriction digest of miniprep DNA and confirm reading frame and orientation by DNA sequencing (see Note 2).
4. Purify plasmid DNA containing the GOI-pcDNA5/FRT/TO construct. Purify also the Flp recombinase vector (pOG44) ready for co-transfection (see Note 3).

3.2. Growth and Maintenance of Flp-In™ T-Rex™ -293 Host Cells

1. Flp-In™ T-Rex™ -293 host cells (derived from HEK293 parental cells) are available as frozen stocks from Invitrogen Life Technologies, but if desired they (or cell lines of alternative lineage) can be generated in the laboratory (see Note 4). This cell line stably expresses the tetracycline repressor protein (tet repressor) and contains a single integration target site or FRT site (see Note 5).
2. To thaw cells from frozen stock, remove vial from liquid nitrogen and thaw as quickly as possible at 37°C in a water bath.
3. Just before completely thawed, transfer the cell suspension to a sterile 15 mL centrifuge tube containing 10 mL of complete DMEM medium. Centrifuge at 300 $\times g$ for 5 min at room temperature. After spinning, remove media, resuspend the cell pellet in 1 mL complete DMEM and transfer to a T-75 flask containing 9 mL of fresh complete DMEM medium (without blasticidin or zeocin). Incubate cells in a humidified incubator at 37°C and 5% CO₂ for 16–24 h.
4. Refresh medium using complete DMEM containing 5 μ g/mL of blasticidin HCl and 100 μ g/mL zeocin (complete DMEM + zeocin + blasticidin) (see Note 6).
5. Incubate and check daily until cells reach about 80% confluency when they are ready to split for further expansion.
6. We recommend freezing cells as soon as possible to keep stocks of the lowest possible passage number (see Note 7).

**3.3. Transfection
of Host Cell Lines
to Generate Stable,
Inducible Flp-InTM
T-RexTM -293
Expression Cell Lines**

1. Seed host Flp-InTM T-RexTM -293 cells into 10 cm dishes and incubate with complete DMEM + zeocin + blasticidin medium.
2. Integration of the construct containing the GOI into the Flp-InTM T-RexTM -293 genome is dependent on the Flp recombinase from the pOG44 plasmid. Therefore, an appropriate ratio of pOG44 and GOI-pcDNA5/FRT/TO plasmids may have to be determined for each experimental system. However, in our experience working with Flp-InTM T-RexTM -293 cells, a ratio of at least 9:1 (w/w) pOG44/GOI-pcDNA5/FRT/TO gives good results. For instance, 7.2 µg pOG44 and 0.8 µg GOI-pcDNA5/FRT/TO is a good starting point.
3. Prepare DNA mixture in ultrapure sterile water. Prepare also a negative control with empty pcDNA5/FRT/TO but pOG44.
4. Remove medium from cells and replace with complete DMEM supplemented with blasticidin, but *without* zeocin (complete DMEM + blasticidin).
5. Transfect DNA using the preferred method (see Note 8).
6. 24 h after transfection remove the media and replace with fresh complete DMEM + blasticidin (see Note 9).
7. 48 h after transfection split the cells into several fresh T-75 flasks to a density of less than 25% confluent. Keep cells in complete DMEM with blasticidin but without zeocin for at least another 24 h or until cells attach to flask.
8. Begin the selection of integrants by removing the medium and replacing it with complete DMEM supplemented with the optimal concentration of hygromycin B required for selection of your cell line (complete DMEM + blasticidin + hygromycin) (see Note 10). For most applications involving Flp-InTM T-RexTM -293 cells, we find that 0.5–2 µg/mL works well.
9. Refresh medium with complete DMEM + blasticidin + hygromycin every 2–3 days until visible foci appear (see Note 11).
10. 2–20 hygromycin B resistant foci should be evident between 10 and 15 days after transfection (see Note 12). The whole polyclonal population of cells should be pooled, expanded and screened for tetracycline-regulated expression (see Note 13). Alternatively, independent clones may be isolated if desired (see Note 14).
11. Freeze a stock of cells of the lowest passage possible as soon as the cell line is established.
12. For subsequent use of these cells, the growing medium should be complete DMEM + blasticidin + hygromycin.

3.4. Methods for Screening GPCR Expression in Flp-In™ T-Rex™- 293 Cell Lines

3.4.1. Screening for GPCR Function Using Pharmacological Assays

1. Seed Flp-In™ T-Rex™-293 expressing the GPCR of interest at the desired density with complete DMEM + blasticidin + hygromycin and incubate until attached to plastic.
2. Refresh medium with that containing the appropriate concentration of tetracycline or doxycycline to induce expression (see Note 15).
3. Incubate cells for 24 h (see Note 16).
4. Harvest cells and prepare membranes (as required, see refs. 1, 2 for method), use appropriate assay for the GPCR of interest.

3.4.2. Screening for Specific Doxycycline- Induced Expression by Western Blotting

This method can be used with cell lines expressing GPCRs fused to an appropriate epitope tag and those for which there is a specific antiserum/antibody against the GPCR.

1. Induce cells with optimal concentration of doxycycline and for the pre-determined length of time. Incubate also a plate or flask of non-induced cells to be used as negative control (see Note 17).
2. Prepare cell lysates using 1× RIPA buffer supplemented with a protease inhibitor cocktail tablet or any other equivalent method.
3. Perform SDS-PAGE gel electrophoresis and transfer of samples onto nitrocellulose membranes according to manufacturer's instructions.
4. Blot using specific antiserum/antibody and analyse results (6).

3.4.3. Screening by Microscopy

This method may be used when the GPCR has been tagged with a fluorescent protein and you are interested in seeing *in vivo* localisation of the receptor (for example, membrane localisation, see Fig. 3).

1. Seed cells onto poly-D-lysine coated glass cover slips and incubate until attached.
2. Induce GPCR expression with the required concentration of doxycycline for 24 h. Leave one coverslip without doxycycline to use as negative control.
3. Remove cover slips off medium and wash twice in warm HEPES buffer pH 7.4.
4. Visualise with microscope. All cells present in the induced sample should show receptor expression and no fluorescence should be detected in those which are non-induced (7).

3.5. Generation of Double Stable Cell Lines in a Flp-In™ T-Rex™-293 Background

This is a very useful approach to study protein–protein interactions and particularly receptor homo- and hetero-dimerisation. It involves the introduction of a second cDNA that expresses constitutively, into a cell line already harbouring one cDNA at the inducible locus (see Fig. 4).

1. Seed Flp-In™ T-Rex™-293 cells harbouring the GPCR at the inducible locus into 10 cm dishes and incubate with complete DMEM + blasticidin + hygromycin medium until 60–70% confluent.
2. Prepare DNA construct for the second GPCR, in ultrapure sterile water.
3. Transfect inducible stable cells with 5–10 µg DNA using the chosen method for transfection (see Note 18).
4. 24 h after transfection remove media and replace with fresh complete DMEM + blasticidin + hygromycin.
5. 48 h after transfection, split cells into several 10 cm dishes to various cell densities, always lower than 20% confluent, using complete DMEM + blasticidin + hygromycin (see Note 19).
6. Refresh medium with complete DMEM + blasticidin + hygromycin supplemented with G418 (complete DMEM + blasticidin + hygromycin + G418) every 2–3 days until visible foci appear (see Note 20). Foci comprising at least 20–50 cells should be evident between 10 and 15 days after transfection.
7. Pick individual clones using “cloning rings” and withdraw the cells with 100–500 µL with complete DMEM + blasticidin + hygromycin + G418 medium into 24 well plates (see Note 21).
8. Feed clones and expand until there are sufficient cells for screening (see Note 22).
9. Clones should be checked for constitutive expression of the second cDNA and inducible expression of the gene under the tet-inducible promoter control (see Fig. 4).
10. Freeze a stock of cells of the lowest passage as soon as the cell line is established.

4. Notes

1. We recommend that the GOI insert contains a Kozak sequence (such as *GGATCC*), immediately prior to the start codon and an appropriate stop codon in order to facilitate correct translation. We routinely include epitope amino or carboxy terminal tags, such as Flag (DYKDDDDKC), c-Myc (EQKLISEEDL), vsv-G (YTDIEMNRLGK), or HA (YPYDVPDYA), to which well characterised commercial antisera/antibodies are available (see Chapter 4). In addition, fluorescent tags consisting of proteins based upon the many variants of GFP from *Aequorea victoria* may be added which can be very useful during the characterisation of receptor expression and subsequent use of the newly created cell line.

The addition of such tags during the cloning of the receptor can be done easily using standard PCR-based techniques (see Chapter 4). The pcDNA5/FRT/TO/TOPO vector can be used as alternative system for cloning PCR fragments into pcDNA5/FRT/TO. This system uses the well-established TOPO cloning technology and does not require ligase and many other post-PCR steps making this a good option when rapid and highly efficient cloning is required. It is worth mentioning at this point that it might be helpful to design a cloning strategy that allows simple swapping of the GOI fragment from pcDNA5/FRT/TO to and from the vector pcDNA3, for example, to facilitate transient transfection or generation of constitutive double stables (see Subheading 3.5).

2. Inserts cloned into pcDNA5/FRT/TO should be sequenced using primers annealing to the human cytomegalovirus promoter region (CMV forward: 5'-CGCAAATGGGCGGTA GGCGTG-3') and to the bovine growth hormone region (BGH reverse 5'-TAGAAGGCACAGTCGAGG-3') present in the backbone plasmid. It is also recommended that specific internal sequencing primers for the GOI are designed to confirm the sequence at the gene-plasmid and gene-tag ligation points.
3. Plasmid DNA must be of highest purity, with no contaminating salts, organic solvents or bacterial debris, for instance, lipopolysaccharide or endotoxin. We routinely purify DNA using Qiagen Plasmid Maxi kit or Endofree Plasmid Maxi kit, but any other equivalent method of obtaining high quality DNA is acceptable.
4. Generation of the Flp-In™ T-Rex™ -293 cell line is achieved by transfecting the pFRT/lacZeo and pcDNA6/TR plasmids into HEK-293 cells. Transfection and subsequent selection of the cells expressing the pFRT/lacZeo plasmid is performed first and the resulting cell line is used as host for pcDNA6/TR transfection. The final resulting stable cell line is resistant to zeocin, resistant to blasticidin and exhibits β -galactosidase activity as conferred by the pFRT/lacZeo plasmid. For further details on how to create such cell lines, see the Invitrogen Web site.
5. The FRT site is achieved by the integration of the pFRT/lacZeo plasmid into a transcriptionally active region of the genome followed by zeocin selection. The tet repressor is stably expressed after integration of the original pcDNA6/TR plasmid in the genome and expression is maintained under blasticidin selection.
6. The addition of zeocin to the medium selects cells with the FRT site. Concentrations of zeocin between 50 and 1,000 $\mu\text{g/mL}$ should be tested to find the minimum selective concentration

when generating a new Flp-In™ T-Rex™ host cell line. It is important to note that the effects of zeocin on cells are considerably different from that of other antibiotics, such as hygromycin or geneticin. Cells do not round up and detach as it would be expected, but they suffer dramatic morphological changes. They augment in size and develop an abnormal cell shape, increasing the number of empty cytoplasmatic vesicles. Finally, sensitive cells break down completely only leaving “string-like” cellular debris behind. Cells that are resistant to zeocin do not present any of these characteristics and should look indistinguishable from any other cells.

7. Cells should be in their growing phase and at least 70% confluent at the time of freezing. The cells may be frozen in complete media supplemented with 10% DMSO or in FBS supplemented with 10% DMSO. Cells are washed in 1× PBS, trypsinised and pelleted by centrifugation. The cells are then resuspended in freezing medium and aliquoted into 1 mL cryo-vials that should be rapidly transferred to a -80°C freezer. For ideal cryopreservation, freezing should progress at a rate of 1°C per minute so using a “Mr. Frosty”-type Cryo Freezer container or a homemade equivalent, such as a beaker insulated with a thick layer of cotton wool, is recommended. It is advisable to check cell viability after 24 h of freezing.
8. Transfection can be carried out with standard reagents available for mammalian transfection, such as Lipofectamine or Lipofectamine 2000. A more economical alternative method uses PEI (polyethyleneimine 25 kDa linear (8)). This method does not require serum-free medium, such as Optimem (which is required for the use of Lipofectamine) and the presence of antibiotics does not interfere with the transfection.
9. Integration of the GOI into the FRT site disrupts the *lacZeo* gene and therefore zeocin resistance is abolished. For this reason after co-transfection of GOI-pcDNA5/FRT/TO and pOG44, the zeocin must be removed from the medium. The integration also places the hygromycin resistance gene of pcDNA5/FRT/TO into frame with the integration site ATG codon and under the control of the SV₄₀ promoter. Expression of this gene then confers hygromycin resistance.
10. It is advisable to generate a hygromycin B kill curve of the host cells, Flp-In™ T-Rex™-293 in this case, to determine the optimal antibiotic concentration that kills any untransfected cells. We recommend testing concentrations ranging from 0.5 to 200 $\mu\text{g}/\text{mL}$.
11. Once hygromycin B selection starts, massive cell death is apparent with only a small number of cells remaining attached to the

- plastic. During this period, refresh medium very carefully to avoid losing these cells.
12. In addition to the inherent variation in the efficiency of transfection, it must be taken into account that the recombinase-mediated integration of the GOI is a rare event; therefore, you should not expect a large number of integrants. However, anything between 2 and 20 foci should be expected within 10 days of transfection. If no apparent foci are visible after 15–20 days, it is likely that there is a problem with some aspect of the process. Media, reagents, plasmids, and cells should all be rechecked.
 13. While maintaining Flp-In™ T-Rex™-293 inducible stable cell lines in culture, special attention must be paid to the type of FBS used to supplement the medium. The serum must be free of any traces of tetracycline as this induces gene expression upon binding to the tet repressor. Only FBS that is either European Union approved or tested as tetracycline negative must be used.
 14. Given that the host cell line is a single integrant of the FRT site, the resulting stable cell line after GOI integration is an isogenic population with each cell carrying only one copy of the GOI at that particular integration site. Therefore, it should show homogeneous levels of expression throughout and is possible to use the pooled population. However, it is still possible to isolate and screen independent clones derived from independent foci, if required.
 15. We have replaced the use of tetracycline with doxycycline as it has a longer half-life than tetracycline (at least 24 h) and seems to have the same mechanism of action in this system (see the Invitrogen product literature for more information). The doxycycline concentration used as standard starting point is 1 µg/mL, but we recommend optimising this concentration for your particular system. A good experiment to choose the optimal concentration would be to titrate the amount of doxycycline required to modulate gene expression with a dose curve using concentrations ranging from 1 ng/mL to 1 mg/mL.
 16. To find the optimal doxycycline concentration, it is important to find out how long it takes for gene induction to be optimal/maximal. Therefore, we routinely carry out a time course of doxycycline induction in which we use the concentration that gives the best level of expression.
 17. As a negative control, it is a good idea to have a cell lysate of host parental Flp-In™ T-Rex™-293 cells to use as further control for the specificity of the antibody/antiserum.

18. The second DNA construct can be in pcDNA3 or any other expression vector that is compatible with those already present. It is important to pay attention to the antibiotics used for selection in this case.
19. We recommend using a 1 mg/mL G418 concentration as a starting point. As in the cases of the other antibiotics, it is a good idea to perform a kill curve for G418 with the parental cell line to find the best drug concentration.
20. We advise that cells should be split into 10 cm dishes as this facilitates the process of picking the individual clones. Cells should be seeded at low density to ensure the generation of well separated, individual colonies. We routinely split the transfected cells 1:50 or even 1:100 at this stage. Splitting into several plates for each dilution increases the chances of obtaining positive clones.
21. To pick individual foci, we use “cloning rings” made with either 0.5 cm cut from the wide end of a 1,000 μ L pipette tip or the top half of a 0.5 mL tube, with the lid and conical bottom removed. These rings are then autoclaved. Mark the position of the foci on the bottom of the plate and remove the media. In order to assist the detachment of the cells from the plate with trypsin, it should be washed carefully with warmed 1 \times PBS. Spread a very thin layer of vacuum grease on the ring and place over the foci, surrounding it. Make sure that the ring is tightly stuck to the plate and that is not overlapping with any neighbouring foci. Add 100 μ L of medium into the ring and carefully pipette up and down to separate the cells from the plate. Transfer medium with cells into a well containing fresh medium in a 12 or 24 well plate. If foci are already quite confluent, 50 μ L of trypsin can be used to encourage detachment of the cells.
22. Double stabiles can be slow to grow, particularly just after selection or while recovering from frozen storage and so it is often beneficial to add NEAA to the medium, until the cells are growing normally.

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