Generation of Flp-intm-ready DG44 and Lec 3.2.8.1 CHO cell lines for quick and easy constitutive protein expression

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The well-characterized cell line Chinese hamster ovary (CHO) has been used to produce numerous biopharmaceuticals and is an important tool for basic research. However, introducing foreign DNA into specially modified CHO cells such as DG44 and Lec 3.2.8.1 can sometimes be an arduous process. Here we show that the Flp-intm plasmid can be modified to produce a fluorescent tracer protein tag (mCherrytm) as a fusion reporter, to allow for the rapid selection of single-cell sorted, isogenic Flp-intm-ready DG44 and Lec 3.2.8.1 cell lines. These two cell lines are stable and viable and may be useful for applications such as antibody production and crystallographic studies. Here we provide key details on how the modified pFRT/CherryZeo plasmid may be used to incorporate Flp-intm technology into virtually any desired target cell line in a fast, safe and reliable manner.

Rapid generation of desired cell lines expressing proteins of interest is a major hurdle when working with mammalian systems. Although well-characterized mammalian cells such as HEK293 and Chinese hamster ovary (CHO) have been used for the accurate production of bioproducts that closely mimic their natural counterparts, mammalian cell lines are notoriously expensive to culture and their slow growth relative to other systems can restrict their usage [1]. Lipofectamine or electroporation are widely used as brute-force methods of introducing foreign DNA into mammalian cells, but they carry limitations such as integration in transcriptionally silent regions as a result of random integration and low transfection efficiency [2]. As an alternative method, lentiviral systems have been designed to incorporate foreign DNA material with high efficiency into dividing cells [3], although their use

requires complex handling authorizations and inherent intrinsic hazards. A relatively new alternative to these transfection hurdles was introduced by Schlake and Bode [4], who first described the Flp-intm transfection and expression system. This technology was then adopted to integrate genes in mammalian systems for quick and reliable constitutive expression of proteins [5]. As originally designed, the Flp-intm system is a two-step approach. First, one needs to integrate the Flp-intm cassette into the desired target cell line using Lipofectamine or electroporation methods [6]. Once the Flp-intm cassette is stably incorporated into the genome of the target cell line for expression, a second step is performed wherein a plasmid (e.g., pcDNA5/ Flp recombination target [FRT]) carrying the cDNA for the gene of interest (GOI) is used to incorporate the GOI into the host genome with co-transfection of the Flp-recombinase-

encoding pOG44 plasmid [6]. Although the recombinase transfection steps are relatively straightforward, the stable incorporation of the Flp-intm construct to generate the host cell line is the rate-limiting step as it relies on brute-force DNA integration into the genome. Although several ready-made host Flp-intm cell lines are commercially available (CHO, HEK-293, CV-1, Jurkat, BHK and 3T3), these cell lines often do not have optimal features for expression of particular proteins of interest.

Lec 3.2.8.1 CHO cells were initially developed by Stanley [7]. These cells lack N-type glycosylation as a result of a mutation destroying several N-glycosylation enzymes [7]. Thus these cells lack the ability to attach complex N-type glycans to proteins, but still retain the less complex O-glycosylation pathways; the end result is that cells homogeneously glycosylate proteins. This property is highly desired

METHOD SUMMARY

An new version of the Flp-intm pFRT/LacZeo plasmid was develop using fluorescence instead of luminescence in order to accurately select cells that integrated the Flp-intm cassette in their genome. The new plasmid pFRT/CherryZeo has the same characteristics than the old but is 3k bp shorter and allows for automatic single-cell sorting using FACS.

was substituted with a monomeric cherry sequence, (mCherry, Tsien version [13]), fused to the Zeocin resistance gene. The size of the resultant plasmid was reduced from 8106 bp to 5030 bp. (Figure 1, map and mCherry sequence substituted).

In order to test the concept of the fluorescently modified Flp-in[™] plasmid for a gene of interest, we designed and cloned a human aquaporin 3 (AQP3, Accession: NM_004925.3) cDNA construct (designed by DCS) expressing turbo green fluorescent protein (tGFP [Evrogen, Moscow, Russia], into the pcDNA5/FRT plasmid). A peptide linker (IDAVLLE) was placed at the C terminus of AQP3 in order to further separate the GFP protein from the AQP3 tetramers. The full amino acid sequence of this construct is shown in Figure 4D. The 1655 bp construct was then custom-synthesized by Biomatik (DE, USA) and subcloned into a pBluescript SK II (+) plasmid. The cDNA construct was then removed using Not1 flanking restriction sites and subcloned into a Not1-digested pcDNA5/FRT plasmid. Hygromycin-resistant colonies were sequenced until plasmids that contained the construct in the correct orientation were identified.

Transfection

DG44-CHO cells (4×10^5) were transfected with 10–11 μg of Sca1-digested pFRT/CherryZeo plasmid using Lipofectamine 2000 (Life technologies, CA, USA). Lec 3.2.8.1 cells (1×10^6) were electroporated with 2–3 μg of Apa1-digested pFRT/CherryZeo plasmid using an Amaxa Nucleofactor II electroporator using program U24 (Lonza, Germany) following the manufacturer's instructions.

In order to test the Flp-in[™]-ready cells, Lipofectamine 2000 was used to transfect 5×10^4 Lec 3.2.8.1 or 4×10^5 DG44 CHO cells with our GOI, in this case 100 ng of circular AQP3-GFP_pcDNA5/FRT plasmid and 900 ng of the helper pOG44 plasmid (Flp recombinase) in 96-well plates in the presence of 1 mM sodium butyrate using a modified manufacturer's protocol (Life Technologies).

Mass spectrometry analysis of constitutively expressed AQP3-GFP

In order to determine if our transfected cells were expressing the AQP3 protein,

three aliquots of cell lysates, each of 5 μL ($= 5 \mu\text{g} = \sim 90 \text{ pmol}$), were reduced with TCEP, alkylated with IAM and digested with trypsin, chymotrypsin or proteinase K. After digestion, the trypsin digest was split and half was further digested with Glu-C (double digest). Digests in progress using chymotrypsin and proteinase K were sampled at different time points for the optimal conditions and multiple runs carried out on the Orbitrap. The digests were then run by nanoLC-MS/MS on an Orbitrap Velos Pro (ThermoFisher Scientific, MA, USA) using a 1 h gradient through a C18 reverse-phase column. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1).

Confocal imaging

pFRT/mCherryZeo plasmid expression and incorporation was visualized using a Leica DMI 6000 B Inverted microscope under a 10 $\times$ objective. Scale bar = 30 μm . Images

were captured using Metamorph software (MDS Analytical Technologies, CA, USA).

Rapid identification of genetically altered cell lines constitutively expressing genes of interest can be a major hurdle. Here we describe the modification of a commercially available recombinase plasmid system (Flp-in[™]) to generate Flp-in[™]-ready cell lines that can be selected and subcloned using FACS. Using our newly modified pFRT/mCherryZeo plasmid, we were able to rapidly transfect and identify Lec 3.2.8.1 and DG44 cells that were isogenic Flp-in[™]-competent cell lines.

The pFRT/CherryZeo plasmid is 3 kb shorter than its predecessor pFRT/LacZeo

Using our approach, we swapped the 3051bp β -galactosidase protein with fluorescent 708 bp mCherry gene, thus shortening the length of the plasmid by 3 kb. The resulting plasmid retained the

Figure 2. Preparation of Flp-in[™]-ready Lec 3.2.8.1 and DG44 CHO cell lines. CHO-Lec 3.2.8.1 (A) or CHO-DG44 (B) cell lines were resistant to zeocin and expressed red fluorescence as a result of incorporating the fusion mCherry- and zeocin-resistant proteins. Scale bar = 30 μm .

Table 1. Comparison of old versus new method.

	Old method	New method
Plasmid size	pFRT/LacZeo 8106 kp	pFRT/CherryZeo 5030 kb
Timeline	Very variable	<1 Month
Cell clones selection	Manual	Automated (FACS sorting)
Selection	Luminescent (requires substrate for detection)	Fluorescent (no substrate requirement for detection)
Advantages	1. Long timeline 2. No fluorescence interference with future projects	1. Reduced timeline 2. Reduced plasmid size increase cassette integration 3. Fluorescence is used for cell selection; single cell selection is easy
Disadvantages	1. Time consuming 2. Manual selection of clones; single-cell selection difficult 3. Additional testing is needed for selection of high-expressing clones (western blot, etc)	1. Presence of mCherry precludes usage with future protein of interest

Flp-intm machinery as well as all endogenous restriction sites needed for linearization (Figure 1). The utility of the modified plasmid was demonstrated by incorporation into the genome of Lec 3.2.8.1 and

DG44 CHO cell lines (Figure 2A & B). Table 1 summarizes the advantages and disadvantages of using the modified pFRT/mCherryZeo plasmid.

The pFRT/CherryZeo plasmid can be incorporated into Lec 3.2.8.1 & DG44 CHO cell lines & identified by fluorescence

pFRT/mCherryZeo was linearized using the Apa-1 restriction enzyme. 10 µg of plasmid was then introduced into 1×10^6 Lec 3.2.8.1 cells by electroporation using the Amaxa Nucleofector II cell electroporation system (Lonza, MD, USA) or 4×10^5 DG44 CHO cells using Lipofectamine 2000. Both cell lines were maintained under zeocin selection for approximately 15 days. As seen in Figure 2, transfected Lec 3.2.8.1 and DG44 cells developed positive red foci (Figure 2A & B). Figure 3 shows the selection scheme for identifying mCherry-transfected cells. Representative nontransfected Lec 3.2.8.1 and DG44 cells are shown in panels A & E (Figure 3). Zeocin-resistant, positive (red) transfectants were then trypsinized and single-cell sorted directly into 96-well plates using a FACS Aria Sorter (Becton Dickinson, MD, USA; Figure 3B & F) and allowed to expand for approximately 10 days. After expansion, wells expressing the highest amount of mCherry fluorescence were further expanded into 6-well plates in order to increase cell number. Expanded cells continued to show mCherry expression after 20 days in culture (total of 30 days post-sorting; Figure 3C & G). For comparison, nontransfected Lec 3.2.8.1 and DG44 cells expanded in a similar manner show no positive cells (Figure 3D & H).

Figure 3. Sorting and generation of isogenic Lec 3.2.8.1 and DG44 CHO cell lines. A & E show wild-type Lec 3.2.8.1 and DG44 CHO cells. B & F show the sorting strategy for Lec 3.2.8.1 and DG44 CHO selected on mCherry positive cells. C & G show pFRT/CherryZeo-transfected Lec 3.2.8.1 or DG44 CHO cells approximately 30 days after sorting; D & H represent negative controls with only lipofectamine, expanded in a similar manner to Lec 3.2.8.1 cells shown in C & G. FSC: Forward scatter; SCC: Side scatter.

Figure 4. Flp-In™ ready Lec 3.2.8.1 and DG44 CHO- cell lines are easily transfected with a gene of interest. CHO-Lec 3.2.8.1 (A) or CHO-DG44 (B) were transfected with a circular pcDNA5/FRT plasmid carrying the AQP3-GFP gene. After 10 days under hygromycin selection, both cell lines expressed the protein of interest. (C) Commercially available Flp-In™ CHO cells were used as a positive control comparator following transfection with pcDNA5/FRT plasmid carrying the AQP3-GFP gene. Scale bar = 30 μ m. (D) The full AQP3-tGFP construct used to test the Flp-in cell lines for constitutive gene expression. (E) AQP3-tGFP sequence: The entire sequence is reproduced here for ease of accessing. In blue is shown AQP3, yellow is the linker and green is the tGFP: MGRQKELVSRGEMLHRYRLLRQALAECLGLTLLVMFGCGSVAQVVLRSRTHGGFLTINLAFGFAVTLGILI-AGQVSGAHLNPAVTFAMCFLAREPWIKLPIYTLAQTLAGLAGIVFGLYYDAIWHFADNQLFVSGPNGTA-GIFATYPSGHLDMINGFFDQFIGTASLIVCVLAIVDPYNNPVRGLEAFVGLVVLVIGTSMGFNSGYAVN-PARDFGRLFTALAGWGSVFTTGQHWVWVPIVSPLLGSIAGVFVYQLMIGCHLEQPPSNEEENVKLAH-VKHKEQIIAVLLEMESDESLPAMEIECRITGTNLGVFEVLVGGEGTPEQGRMTNKMSTKGALTFSPY-LLSHVMGYGFYHFGTYPGYENPFLHAINNGGTYNTRIEKYEDGGVLHVSFYSRYEAGRVIGDFKVMGTG-FPEDSVIFTDKIRSNATVEHLHPMGDNDLDGSFTRTFLSLRDGGYSSVVDSHMHFKSAIHPILQNGGPM-FAFRVEEDHSNTELGIVEYQHAFTKTPDADAGEERV (F) Mass spectrometry analysis of transfected cell lysates identified 67% of the AQP3-GFP protein extracted from representative constitutively expressing AQP3 cell lines.

Flp-In™ Lec 3.2.8.1 and DG44 readily incorporate genes of interest

Using the AQP3-GFP construct as a positive control, we transfected 5×10^4 Flp-In™-ready Lec 3.2.8.1 and DG44 CHO cell lines using Lipofectamine 2000. After 10 days of selection pressure under hygromycin, both Lec 3.2.8.1 and DG44 cell lines developed AQP3-GFP-expressing foci (Figure 4, A & B). The commercial Flp-In™ Life technologies CHO cell line is shown as positive control in Figure 4C. The full amino acid sequence of this construct is shown in Figure 4D. Mass spectrometry analysis was performed on SDS-PAGE bands obtained from extracted AQP3-GFP protein, and 67% protein coverage was obtained (Figure 4F).

We report the generation of two CHO cell lines (Lec 3.2.8.1 and DG44) using

a modified Flp-In™ expression system. The pFRT/mCherryZeo-modified plasmid allowed for selection of transfection-ready host cells in a relatively short time period. Taking advantage of flow cytometry selection by fluorescence, we generated both cell lines in less than 3 months, as compared with other traditional methods which can take substantially longer (Table 1). The use of the pFRT/CherryZeo plasmid allowed for accurate single-cell sorting in order to select the highest expressing cell clones allowing for a positive ratio of 100% while minimizing false-positive ratio to almost zero. Conventional non-FACS-based methods require single-cell dilution assays using a micro pipette to capture the cell colonies in the bottom of the wells in order to obtain isogenic cell lines. This process is intrinsically inaccurate and can lead to cell mosaicism that is hard to detect or correct.

Using a FACS-based method increases selection ease as the Flp-In™-ready clones are isogenic and the Flp-In™ pcDNA5/FRT expressing the gene of interest can be readily identified. This modification allows for more rapid selection of the stably transfected host clones, but can still be used with the conventional (and commercially available) pcDNA5/FRT plasmid and helper plasmid (DG044) recombinase system for expression of genes of interest. We acknowledge that in this current manuscript we do not assess how many copies of the FRT cassette was incorporated into each cell line. This is important because of the known problem of gene silencing over time if multiple copies are incorporated. However, assessment of copy number was beyond the scope of this paper, whose intent was to demonstrate the modification of the original pFRT/LacZeo plasmid in order to increase its ease of use for multiple purposes is possible and that pFRT/mCherryZeo could be used as an alternative with distinct advantages compared with traditional LacZ methodology [8].

Author contributions

Drs Soler and McCormick had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy. Study concept and design: Soler and McCormick. Acquisition of data: Soler, Young and Vahedi-Faridi. Analysis and interpretation of data: Soler and McCormick. Drafting of the manuscript: Soler, Vahedi-Faridi and McCormick.

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