Robust Cell Line Development Using Meganucleases

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Summary

Cell line development for protein production or for the screening of drug targets requires the reproducible and stable expression of transgenes. Such cell lines can be engineered with meganucleases, sequence-specific endonucleases that recognize large DNA target sites. These proteins are powerful tools for genome engineering because they can increase homologous gene targeting by several orders of magnitude in the vicinity of their cleavage site. Here, we describe in details the use of meganucleases for gene targeting in Chinese hamster ovary-K1 cells, with a special emphasis on a gene insertion procedure using a promoter-less marker gene for selection. We have also monitored the expression of genes inserted by meganucleases-induced recombination, and show that expression is reproducible among different targeted clones, and stable over a 4 mo period. These experiments were conducted with the natural yeast *I-SceI* meganuclease, but the general design and process can also be applied to engineered meganucleases.

Key Words: Cell line development; double-strand break; gene targeting; homologous recombination; *I-SceI*; meganucleases; protein production.

1. Introduction

Homologous recombination is a powerful tool for genome engineering. Since the first gene targeting experiments in yeast more than 25 yr ago (*1,2*), homologous recombination (HR) has been used to insert, replace, or delete genomic sequences in a variety of cells (*3–5*). However, targeted events occur at a very low frequency in mammalian cells. The frequency of HR can be significantly increased by a specific DNA double-strand break (DSB) in the targeted locus (*6,7*). Such DSBs can be delivered with meganucleases, sequence-specific endonucleases that recognize large DNA target sites (>12 bp). Because of their exquisite specificity, these proteins can cleave a unique chromosomal sequence without affecting global genome integrity. Natural meganucleases are essentially represented by homing endonucleases, a widespread class of proteins found in eukaryotes, bacteria, and archae (*8*). Early studies of the *I-SceI* and homothallic switching endonuclease

From: *Methods in Molecular Biology*, vol. 435: *Chromosomal Mutagenesis*
Edited by: G. Davis and K. J. Kayser © Humana Press Inc., Totowa, NJ

have illustrated how the cleavage activity of these proteins initiates HR events in living cells and demonstrated the recombinogenic properties of chromosomal DNA DSBs (9,10). Since then, meganucleases-induced recombination has been successfully used for genome engineering purposes in bacteria (11), mammalian cells (6,7,12–14), mice (15), and plants (16,17).

However, the use of meganucleases-induced recombination has long been limited by the repertoire of natural meganucleases; although several hundreds of other meganucleases had been identified in between (8), the diversity of cleavable sequences was too limited to address the complexity of genomes. In fact, only artificial loci, wherein an I-SceI cleavage site had been introduced, could be engineered. Recently, the making of artificial meganucleases, based on homing endonucleases (18,19) or zinc-finger proteins (20,21), has considerably enlarged the number of sequences that can be targeted. The production of such new proteins opens the door for the engineering of natural chromosomal sequences.

Here we present a general overview on how to use meganucleases to induce gene correction, gene insertion, or gene replacement. We provide precise protocols for the creation and characterization of gene targeting events, and give a brief survey of the impact of insertion or deletion size on the efficiency of the process, with a special emphasis on gene insertion. Gene insertion can be used, for example, to insert genes of interest in specific loci, for heterologous protein production. Recombinant therapeutic proteins are today mostly produced in mammalian cells such as Chinese hamster ovary (CHO), mouse SP2/0 and NS0 cells, or the human PerC.6 cell line, stably transfected with the gene of interest (22). In the process of selecting highly expressing clones, the level and stability of protein expression are two major criteria. Furthermore, obtaining reproducible results from one clone to another would be an advantage in terms of screening efforts. These principles also apply to the generation of cells for screening of specific drug targets such as G protein-coupled receptors. In order, to illustrate the various advantages of meganucleases-induced gene targeting for this specific kind of application, we provide protocols to monitor the expression of inserted genes and data of expression stability following gene targeting.

In all the experiments described in **Subheading 3.**, we use the I-SceI meganuclease, which remains today the “gold standard” for DSB-induced recombination, in terms of efficacy and specificity. However, the same protocols can be used to induce similar levels of gene correction or insertion with engineered meganucleases derived from the I-CreI homing endonuclease, which were described in recent reports (18,19).

2. Materials

2.1. Cell Culture and Transfection

1. Phosphate-buffered saline (PBS) (Invitrogen-Life Science, Carlsbad, CA).
2. Kaighn's modified F-12 medium (F12-K) (Invitrogen-Life Science) is supplemented with 2 mM L-glutamine, penicillin (100 UI/mL), streptomycin (100 µg/mL), amphotericin B

(Fongizone) (0.25 $\mu\text{g/mL}$) (Invitrogen-Life Science), and 10% fetal bovine serum (Sigma-Aldrich Chimie, St. Louis, MO).

3. Freezing medium: complete F12-K medium supplemented with 10% Dimethylsulfoxide.
4. Puromycin dihydrochloride (Sigma-Aldrich Chimie). For CHO-K1, the concentration of 10 $\mu\text{g/mL}$ is used.
5. Hygromycin B solution (Sigma-Aldrich Chimie). For CHO-K1, the concentration of 0.6 mg/mL is used.
6. Trypsin-EDTA solution (Invitrogen-Life Science).
7. Versene solution (Invitrogen-Life Science).

2.2. Molecular Characterization of Targeted Events by Southern Blot Using Nonradioactive Probes

2.2.1. Genomic DNA (gDNA) Preparation and Digestion

1. gDNA lysis buffer: 0.5% sodium dodecyl sulfate (SDS), 40 mM Tris-HCl, 40 mM EDTA, 200 mM NaCl, pH 7.5. Store at 4°C.
2. Proteinase K (20 mg/mL) (Eurobio, Les Ulis, France).
3. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

2.2.2. DNA Electrophoresis and Transfer

1. Running buffer: 0.5X Tris-acetate EDTA: 20 mM Tris-acetate, 1 mM EDTA pH 8.0. Prepare a 50X stock solution.
2. Denaturation buffer: 0.5 M NaOH, 1.5 M NaCl. Store at room temperature.
3. Neutralization buffer: 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl. Store at room temperature.
4. Transfer buffer: 10X SSC (1.5 M NaCl, 0.15 M Na-citrate). Make 20X stock solution. Store at room temperature.
5. Hybond N+ membrane (Amersham, Little Chalfont, England).
6. 3 MM Chr Whatmann paper (Schleicher and Schuell, Maldstone, England).

2.2.3. Probe Labeling

1. Digoxigenin (DIG) DNA-labeling kit (Roche Diagnostics, Mannheim, Germany).
2. Nucleospin column (Macherey-Nagel, Düren, Germany).

2.2.4. Hybridization

1. Hybridization buffer: 0.5 M phosphate buffer pH 7.2, 7% SDS, 1 mM EDTA. Store at room temperature.

2.2.5. Wash and Anti-DIG Probing

1. Wash buffer: 40 mM phosphate buffer pH 7.2, 1% SDS. Store at room temperature.
2. Buffer I: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5. Prepare a 10X stock solution. Adjust pH with NaOH. Store at room temperature. Add 0.3% Tween-20 before use.
3. Buffer II: buffer I supplemented with 10% (v/v) blocking reagent.
4. Buffer III: 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl_2 . Store at room temperature.

5. Alkaline phosphatase-conjugated anti-DIG Fab (Roche Diagnostics, Mannheim, Germany). Store at 4°C.
6. Blocking reagent 10X (Roche Diagnostics, Mannheim, Germany). Store at 4°C.
7. CDP star—chemiluminescence substrate for alkaline phosphatase—(Roche Diagnostics, Mannheim, Germany). Store at 4°C.

2.3. Protein Production

2.3.1. Measure of β -Galactosidase Activity

1. Lysis buffer: 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100. Store at 4°C.
2. 100X Mg buffer: 100 mM MgCl₂, 35% β -mercaptoethanol. Store at room temperature.
3. Orthonitrophenyl- β -D-galactopyranoside (ONPG): prepare a 8 mg/mL solution in water. Store at -20°C.
4. 0.1 M Na₂HPO₄/NaH₂PO₄ pH 7.5.

2.3.2. Antibodies and FACS Reagents

1. Biotin-conjugated mouse antihuman CD4 monoclonal antibody (Becton Dickinson, San Jose, CA).
2. Biotin-conjugated mouse isotype control monoclonal antibody (Becton Dickinson).
3. Streptavidin-conjugated PhycoErythrin (Streptavidin-PE) (Becton Dickinson).
4. FACS buffer: PBS supplemented with 2% fetal bovine serum.

3. Methods

Meganucleases (and more generally gene targeting) can be used to trigger gene correction as well as gene insertion, deletion, or replacement. Obtaining the desired kind of events does not simply depend on a standard protocol, but also on the design of the targeting vector, also referred to as “repair matrix,” because they actually provide a matrix or template for the DSB-repair process initiated by I-SceI and other meganucleases. In this chapter, we will provide a few examples for the design of the repair matrix for gene correction, gene insertion, and gene replacement.

We take, as an example, experiments that were performed on a model cell line with a *puromycin-resistance* marker as target gene. This gene is under the transcriptional control of the human EF1 α promoter, and about 1 kb of additional EF1 α sequences, corresponding to the two first untranslated exons and first intron, are present between the promoter and the *puromycin-resistance* gene. Furthermore, a cleavage site for the natural endonuclease I-SceI was inserted 132 bp downstream of the ATG of *puromycin-resistance* gene, thus inactivating this gene. The construct has been stably integrated in CHO-K1 cell genome in single copy. Then, different repair matrix were constructed, to modify this locus by gene targeting.

The first repair matrix was designed for the correction of the *puromycin-resistance* gene (**Fig. 1A**). Gene correction should result in the removal of 22 bp including the I-SceI cleavage site, and the consequent restoration of a functional

Fig. 1. Design of reporter system and repair matrix for gene correction (A), gene insertion (B), and gene replacement (C). The puromycin-resistance gene is interrupted by an I-SceI cleavage site (CS) and under the control of the EF1 α promoter. For gene correction, the repair matrix includes 1.8 kb of homologous sequence corresponding to the two untranslated exons (E1, E2) and first intron and a full length puromycin gene. For gene insertion, the repair matrix corresponds to a promoter-less hygromycin-resistance gene alone or with a complete expression cassette that is flanked by two stretches of homologous sequences (1.1 kb and 2.3 kb). The repair matrix for gene replacement is very similar to the insertion matrix except that (i) the 3' homology sequence starts after the puromycin polyA signal (ii) all sequences from the puromycin-resistance gene have been removed from the 5' homologous sequence. Homology lengths are depicted by hatched boxes. GOI stands for gene of interest (β -galactosidase or CD4 in this study).

puromycin-resistance cassette. The gene correction matrix includes a noninterrupted *puro*^R gene, with EF1 α sequences in 5' (these sequences include exon 1 and 2 and intron 1, but not the promoter). It shares a total of 1.8 kb of homology with the targeted locus, with 1.1 kb in 5' of the I-SceI site, and 0.7 kb in 3'.

Second, a series of three insertion vectors were designed by cloning inserts of various sizes in the *puromycin-resistance* gene (Fig. 1B). These repair matrix are intended to insert novel sequences within the targeted locus. The heterologous inserts are surrounded by 1.1 kb of homologous sequences in 5' (the EF1 α exons and intron), and 2.3 kb in 3' (longer than in the gene correction vector). The simplest

insertion vector includes a promoter-less *hygromycin-resistance* cassette of 1.3 kb, whereas the two others contain an additional expression cassette, coding for the *Escherichia coli* β -galactosidase enzyme or the human CD4 transmembrane protein, and resulting in total inserts length of 5.5 and 4.4 kb, respectively. Note that in these insertion vectors, the *hygromycin-resistance* gene is modified by addition in its 5' end of the first 132 bp (44 first amino acids) of the *puromycin-resistance* gene (132 bp upstream of the I-SceI site), resulting in the production of a fusion protein that confers resistance to hygromycin B.

Third, one can envision, in several applications, the total replacement of the *puromycin-resistance* gene by another selection marker. To meet this purpose, a gene replacement vector was produced by modifying the 3' homologous sequence of the first gene insertion vector (**Fig. 1C**). In this construct, the 3' homologous sequence (about 2 kb in length) started 2 kb after the end of the *puromycin-resistance* ORF, and all sequences from the *puromycin-resistance* gene (132 bp) have been removed from the 5' homologous sequence (for a total deletion of 3 kb). The 5' homologous sequence is also modified by removing all sequences from the *puromycin-resistance* gene.

In the experiments described in **Subheading 3.**, the repair matrix and the I-SceI expression vector (**Fig. 1**) are cotransfected in our model cell line. For maximal efficiency, we use a vector wherein I-SceI is placed under the control of the cytomegalovirus promoter and described in a former report (7). On targeted insertion, the functional *puromycin*- or *hygromycin-resistance* gene is placed downstream of the EF1 α sequences, and transcribed from the EF1 α promoter. In contrast, random insertion will not create an expressed *puromycin* or *hygromycin-resistance* cassette unless the cassette is fortuitously inserted just downstream of a functional promoter. As such “promoter trap” events are supposed to be very rare, our system allows for a considerable enrichment for targeted events among transformed puro^R or hygro^R cell lines. This kind of “refinement” is not compulsory, but can greatly simplify the task of the researcher.

3.1. Cell Transfection and Selection

1. All the transfection experiments were done with the Amaxa Electroporation system (Amaxa GmbH, Koeln, Germany), which ensures more than 50% transfection efficiency.
2. The adherent cells are washed once with PBS, then incubated with trypsin-EDTA solution for 5' at 37°C and collected in a 15-mL conical tube (**Note 1**). After centrifugation at 300g for 5 min, the supernatant is discarded and the cell pellet is resuspended in 10 mL of complete F12K medium. Cells are numerated, centrifuged again, and adjusted at the concentration of 2×10^7 cells/mL in Amaxa solution T.
3. 2–15 μ g of plasmid DNA (**Note 2**) is added to 100 μ L of cells (2×10^6 cells) in an Amaxa electroporation cuvet. The cells and DNA are gently mixed before insertion in the electroporation chamber of the Amaxa electroporation apparatus (**Note 3**). For CHO-K1 cells, the manufacturer recommends the use of program U-23.

4. After electroporation, 0.5 mL of prewarmed complete F12K medium is added to the cuvet and cells are gently transferred into a 10-cm culture dish containing 10 mL of prewarmed complete F12K medium. Dishes are incubated in a 37°C, 5% CO₂ humidified incubator for 24 h.
5. After a 24 h recovery period, transfected cells are washed once with PBS, then incubated with trypsin-EDTA solution for 5' at 37°C and collected in a 15-mL conical tube. Cells are counted and cloned in 96-well plates at the density of 500 cells/well in complete F12K medium supplemented with the selecting reagent. Examples of selecting reagent: puromycin solution is added at the final concentration of 10 µg/mL. Hygromycin B solution is added at the final concentration of 0.6 mg/mL.
6. About 10 d later, puromycin (puro^R)- or hygromycin (hygro^R)-resistant clones can be counted. Positive clones are amplified by sequential passage onto 12-well plates, 6-well plates, and finally 10-cm dishes. The selecting agent is maintained through all these steps. At this point, clones are cryo-conserved in freezing medium.

The frequencies of puro^R and hygro^R cells are summarized in **Table 1**. These frequencies are established by dividing the number of wells containing puro^R and hygro^R cells by the total number of plated cells (For puro^R cells, this number is an underestimation: if puro^R cells occur at a frequency of 3×10^{-3} , and if 500 cells were plated per well, there might be two puro^R clones in several). However, such phenotypes can result either from gene targeting, or from random integration of the repair matrix downstream of an active promoter. Indeed, hygro^R-resistant clones (but for unknown reasons, no puro^R clone) were also obtained when the repair matrix were transfected without the I-SceI expressing vector (**Table 1**). Therefore, the frequencies of targeted events can only be obtained after molecular characterization.

3.2. Molecular Characterization of Targeted Events by Southern Blot Using Nonradioactive Probes

To identify targeted events, the gDNA of hygro^R and puro^R clones can be analyzed by Southern blotting, using restriction enzymes that will discriminate targeted loci from nonrecombined ones. An example is shown on **Fig. 2**.

3.2.1. gDNA Preparation and Digestion

1. Confluent cells from 10-cm dish are collected in a 15-mL conical tube. Cells are washed once with PBS. After centrifugation (300g) supernatants are discarded and cell pellets are thoroughly vortexed.
2. 500 µL of gDNA lysis buffer is added to cell pellets and incubated at room temperature for 30 min. 15 µL of proteinase K solution (20 mg/mL) is added to the lysates. Incubate at 56°C overnight.
3. The next day, 10 mL of ethanol 85% is added. Mix gently then incubate for 10 min at room temperature. The gDNA pellet will sediment at the bottom of the tube.
4. DNA pellet is transferred in a 1.5-mL microfuge tube and centrifuged at 16,000g for 30 min at 4°C. Supernatants are discarded. DNA pellets are rinsed with 0.5 mL ethanol 70%, centrifuged again for 10 min at 16,000g. A maximum of ethanol is

Table 1
Summary of Gene Targeting Experiments

Nature of repair matrix	Gene correction (Fig. 1A)			Gene insertion (Fig. 1B)		Gene replacement (Fig. 1C)
Genes inserted		NA		<i>hygro^R</i>	<i>hygro^R + LacZ</i>	<i>hygro^R</i>
Size of insertion cassette (kb)		0		1.3		1.3
I-SceI expressing vector	-	+	-	+	+	+
Frequency of <i>hygro^R</i> or <i>puro^R</i> clones	0	3×10^{-3}	10^{-4}	5×10^{-4}	1.7×10^{-4}	5.5×10^{-4}
Total targeted events among <i>hygro^R</i> or <i>puro^R</i> clones	NA	30/30	0/27	29/37	4/12	9/36
Targeted events associated with random insertions	NA	0/30	NA	5/29	2/4	3/9
Frequency of targeted event among transfected cells	0	3×10^{-3}	0	3.9×10^{-4}	1.6×10^{-4}	1.4×10^{-4}

NA: not applicable.

Fig. 2. Southern blot analysis of hyg^R clones using nonradioactive probes. Here, we present the analysis of clones obtained with the repair matrix containing the hyg^R - $LacZ$ 5.5 kb insert. **(A)** Principle of analysis. gDNA is digested with restriction enzymes *Bgl*II and *Eco*RV. After electrophoresis and DNA transfer, the blot is hybridized with a probe corresponding to the first intron. In the parental clone, a 10 kb band corresponding to the nontargeted $puro^R$ locus should be detected. On targeted insertion of the hyg^R :: $LacZ$ cassette, the 10 kb band disappears and should be replaced by a 5.2 kb band. In nontargeted clones, additional bands of various sizes correspond to random insertion of the repair matrix. **(B)** Southern blotting. **Lane 1**; control parental cell line, **lane 2**; CHO-K1 cell line before transformation with reporter cassette, **lane 3**; linearized repair matrix plasmid, **lanes 4–20**; hyg^R clones selected after transfection. Random insertion is observed in **lanes 4–7**. **Lane 8** likely corresponds to integration of the hyg^R ORF without the intron. Targeted insertion is observed in **lanes 9–11** and **13–20**. Targeted insertion can also be associated with random insertion, revealed by an additional band (**lane 12**).

removed. DNA pellets are dried for 5 min at 37°C (**Note 4**) before the addition of 100–200 μ L of TE buffer. Resuspended DNA is incubated at 65°C until complete dissolution (**Note 5**). DNA concentration is measured at 260 nm with a spectrophotometer.

5. Usually, 10 μ g of gDNA is digested overnight with a two- to fivefold excess of enzymes.

3.2.2. DNA Electrophoresis and Transfer

1. Digested gDNA are loaded on a 0.8% agarose gel in 0.5X Tris-acetate EDTA buffer and run at 5 V/cm for 1 h then 8.3 V/cm for 4–5 h for a total migration length of 12–14 cm (**Note 6**). Take a photograph.
2. The gel is soaked first in denaturation buffer for 30 min at room temperature under gentle agitation, then in neutralization buffer for 30 min and again in new neutralization buffer for 15°C. Finally, the gel is soaked in 10X SSC for 5°C.

3. The separated DNA is blotted onto Hybond N+ nylon membrane by capillarity in 10X SSC overnight. Briefly, the gel is positioned on a piece of Whatmann 3 MM paper that has been previously soaked in transfer buffer. The extremities of the paper are lying in a tank containing the transfer buffer. The piece of nylon membrane (previously soaked in ddH₂O) is placed on the top of the gel and air bubbles are removed by rolling a clean plastic pipet. Three sheets of Whatmann 3 MM paper are placed onto the membrane. The upward flow of buffer will be ensured by a stack of paper towels positioned on the top. Finally, a glass plate with a weight is applied to maintain a tight connection between the layers.
4. The next morning, the membrane is removed from the transfer system and incubated a few minutes in 40 mM Na₂HPO₄. The membrane is then dried on a piece of Whatmann 3 MM paper and DNA is cross-linked to the membrane using a Ultraviolet Stratalinker (Stratagene) (**Note 7**).

3.2.3. Probe Labeling

1. 1 µg of DNA probe (between 500 bp and 1 Kb long) is denatured for 10 min at 100°C in a water bath.
2. The denatured DNA is labeled with Klenow enzyme (12 IU) in the presence of random hexamer oligonucleotides, deoxynucleotides, and DIG-conjugated dUTP at 37°C overnight in a final reaction volume of 100 µL.
3. The next day, the probe is purified on a Nucleospin column (Macherey—Nagel, Düren, Germany) and aliquoted. The probe aliquots are stored at -20°C until use.

3.2.4. Hybridization

1. The membrane is soaked in ddH₂O for 5 min then placed into a hybridization bottle with 20 mL of hybridization buffer. The bottle is incubated for 1 h in a hybridization oven at 68°C.
2. The DIG-labeled probe (use about 15 ng/mL hybridization buffer) is denatured for 10°C in a water bath at 100°C, then is added to 20 mL of hybridization buffer. The hybridization is performed at 68°C overnight.

3.2.5. Wash and Anti-DIG Probing

1. The probe is recovered and stored at -20°C (**Note 8**). The blot is washed two times in washing buffer (equilibrated at 68°C) at 68°C for 5 min then once in 100 mL of buffer I at room temperature under agitation on a retro orbital shaker (Certomat® RM, B. Braun Biotech International GmbH, Melsungen, Germany) (30 rpm).
2. The membrane is wrapped in a plastic bag and 20 mL of buffer II is added. This step corresponds to the blocking step. The membrane is shaken for 30 min at 250 rpm. Remove the buffer and add 20 mL of buffer II with 0,0375 U/mL of anti-DIG antibody. Incubate membrane for another 30 min (250 rpm).
3. The membrane is removed from the plastic bag and placed immediately in buffer I. The membrane is washed two times for 30 min under gentle agitation (30 rpm).
4. Finally, the membrane is incubated for 3 min in buffer III (**Note 9**).
5. The membrane is placed on a plastic sheet. 3 mL of buffer III plus 10 µL of CDP star is carefully layered over the membrane and a second sheet of plastic is placed on top of it. After 5 min of incubation, excess of liquid is wiped out and the plastic sheets are sealed.

6. The membrane is exposed in a cassette to an X-ray film at room temperature. Time of exposure will depend on the probe efficiency. Usually 4 h exposure is enough but can be longer (overnight for example) (**Note 10**).

Results are summarized in **Table 1**. For gene correction, the frequency of *puro*^R clones reflects the frequency of targeted events, as only corrected puromycin gene can be selected. With the other cassettes, the frequency of gene targeting seems to decrease when the size of the insert increases. As shown on **Fig. 2** (Lane 12), targeted insertion can be associated with random insertion in the same clone, but these double events remain a minority, except with the 4.4 kb cassette (two out of four targeted clones). In addition, the replacement of the whole *puro*^R gene with additional 3' sequences (3 kb) seems to be more demanding than the simple insertion of the *hygro*^R cassette. However, there is only a two-fold decrease in efficiency. Altogether, these results argue that large insertions (5.5 kb) and deletions (3 kb) can be obtained by meganucleases-induced gene targeting. Note that when the repair matrix were transfected without the I-SceI expressing plasmid, we never could obtain any targeted clone (*see Table 1*).

3.3. Protein Production

Insertion of different heterologous sequences at the same locus can be interesting for protein production or drug screening purpose. The level of protein expression and its stability are both major criteria during cellular clone selection. Furthermore, reproducibility of expression would decrease the screening effort and allow for better planification. An insertion in the same locus would ensure reproducible levels of expression independently of the inserted sequences. As an example, we monitor the expression level of two different proteins (β -galactosidase and human CD4) over a 4 mo period after I-SceI-induced gene insertion. In these experiments, only clones with targeted insertions and without additional random inserts were considered.

3.3.1. Measure of β -Galactosidase Activity

1. Cells are washed twice in PBS then incubated with 5 mL of trypsin-EDTA solution. After 5 min incubation at 37°C, cells are collected in a 15-mL conical tube and counted.
2. 2×10^6 cells are incubated with 200 μ L of lysis buffer on ice for 30 min.
3. Cell lysates are centrifuged for 2 min at 10,000g at 4°C. Supernatants are transferred in a clean 1.5-mL microfuge tube.
4. 20 μ L of 1/10th dilution of cell lysates are mixed with: 2 μ L of Mg 100X buffer, 22 μ L of ONPG (8 mg/mL) solution and 156 μ L of 0.1 M Na₂HPO₄ pH 7.5 in a 96-well plate.
5. Plates are incubated at 37°C for 45 min. Optical density is read at 415 nm using a microplate reader (Model 550, BioRad, Hercules, CA).

The production level of β -galactosidase has been measured in this way for 4 different targeted clones during 16 wk. The graph shown on **Fig. 3A** displayed the

Fig. 3. (Continued)

mean level of expression for these 4 clones as compared with nonproducing cell line, showing a very small dispersion of the measurements.

3.3.2. FACS Detection of CD4-Expressing Cells

1. Cells are washed twice in PBS and incubated with 2 mL of Versene solution (**Note 11**). After 5 min incubation at 37°C, cells are collected in a 15-mL conical tube. The cells are counted.
2. 10⁶ cells are transferred in 5-mL tube (Falcon, 2058) and centrifuged at 300g for 5 min at 4°C. Cells are washed once with FACS buffer. Cell pellets are resuspended in 20 μ L of biotin-conjugated anti-CD4 or biotin-conjugated isotype control antibody. After 30 min of incubation on ice, cells are washed once in FACS buffer. Cell pellets are then incubated with 20 μ L of streptavidin-conjugated PE for 30 min on ice and protected from light. The cells are washed once in FACS buffer and finally resuspended in 0.5 mL of FACS buffer.
3. The cells sample are analyzed on a FACS vantage II (BD Bioscience, San Jose, CA) using a 488 nm Ion-Argon laser. The emitted fluorescence (emission wavelength at ~580 nm) is collected in the fluorescence 2 channel.

The level of CD4 expression of a targeted clone has been analyzed by FACS for two different targeted clones during 16 wk. The pattern of CD4 expression for one of these clones is shown on **Fig. 3B**. For the other one the pattern is very similar (Data not shown). Thus, as for β -galactosidase, we demonstrated that the level of expression is very similar from clones to clones and that the expression is very stable over a long period of time.

Fig. 3. Stability of protein expression in targeted clones. **(A)** β -galactosidase expression was assayed by the ONPG test for 4 independent targeted clones ($\blacklozenge$) and for the parental cell line ($\blacksquare$) over a period of 16 wk. The mean and standard deviation of expression levels for these 4 clones is plotted against time. **(B)** CD4-expression of a targeted clone was measured by FACS over a period of 16 wk. FACS displays for week 2 and 16 are shown. The CD4 expression of the targeted clone (bold line) is compared with the CD4⁻, parental cells (light line) and to endogenous CD4 expressing cells (dotted line).

4. Notes

1. For optimal transfection efficiency, the cells should not be more than 80% confluent. For the same reason, we recommend to use cells with low passage number.
2. For optimal transfection efficiency, the plasmid DNA must be produced with an endotoxin-free preparation technique.
3. The cells should not stay more than 20 min in solution T.
4. gDNA pellet is quite difficult to resuspend in TE, unless if not dried completely.
5. The resuspension time may vary from sample to sample. It can take several hours. Do not hesitate to gently mix the DNA from time to time.

6. Trays, combs, and electrophoresis apparatus is carefully cleaned with soap and rinsed with distilled water. This step is crucial because dirty materials can impair the final result.
7. At this step, the membrane can be stored between two pieces of Whatmann paper for a long period of time.
8. The same probe can be used three to four times. When using a new aliquot, we recommend performing a first hybridization with a useless membrane. We noticed that the background is diminished when a probe has been used one or two times.
9. When performing the anti-DIG antibody revelation, it is important not to let the membrane dry up during the steps. Otherwise, a very high background will occur.
10. Hybridized membranes can be stored at -20°C in their plastic bag. Alternatively, they can be stripped by a short incubation in denaturation buffer (10 min) followed by two quick bath in distilled water first and then in 200 mM Na_2HPO_4 , pH 7.2. The membrane is dried on Whatmann paper and stored at room temperature. This membrane can be reprobed but usually the reprobing does not give satisfactory results.
11. Because the CD4 protein is expressed at the cell surface, the Versene solution is used to collect the cells. This solution is preferred to the trypsin-EDTA solution because the trypsin can degrade surface molecules. As the Versene solution is less efficient than the trypsin-EDTA solution, it is recommended to gently scrap the cells after the 5 min incubation before the addition of medium. Furthermore, multiple samples are treated one at a time.

Acknowledgment

We thank Luc Mathis for critical reading of the manuscript.

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