Chapter 34

Measuring Recombination Proficiency in Mouse Embryonic Stem Cells

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Abstract

A method is presented to measure homologous recombination in mouse embryonic stem cells by both gene targeting and short-tract gene conversion of a double-strand break (DSB). A fluorescence-based reporter is first gene targeted to the *Hprt* locus in a quantifiable way. A homing endonuclease expression vector is then introduced to generate a DSB, the repair of which is also quantifiable.

Key words Recombination, Double-strand break (DSB), *Hprt*, Mouse embryonic stem (ES) cells, Green fluorescent protein (GFP), Flow cytometry, Gene targeting, Gene conversion, I-*Sce*I, Homing endonuclease

1 Introduction

Homologous recombination (HR) is an important process in mitotically dividing mammalian cells [1]. Although poorly defined mechanistically, two processes involving HR are gene conversion and gene targeting. In these related processes, a particular chromosomal locus (the "recipient") is altered such that it becomes modified to that of a different locus (the "donor"). In both cases, there is a necessity that the recipient and donor sequences possess significant lengths of sequence homology, which is thought to "guide" transfer of information from the donor locus into the recipient locus through some as yet undetermined base-pairing mechanism. In gene conversion, the donor is located in the genome, whereas in gene targeting the donor is exogenously supplied.

Gene conversion is an important DNA repair mechanism for maintaining genomic integrity in mammalian cells, and, reflecting its role in DNA repair, it is strongly stimulated by a double-strand break (DSB) in the recipient locus [2]. Gene targeting is a valuable

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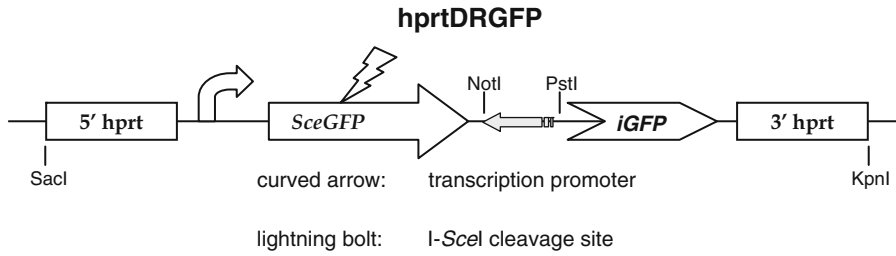

Fig. 1 Schematic of the targeted conversion of the mouse *Hprt* gene. *Curved arrow*, transcription promoter; *lightning bolt*, I-*Sce*I cleavage site; *gray arrow*, puromycin resistance gene. See text for details

molecular biology tool for the generation of mutant cell lines and potentially for gene therapy, since in a gene targeting reaction the exogenously supplied DNA is used to alter a specific chromosomal sequence in a defined way (for review, *see* ref. 3).

This chapter describes the use of the plasmid phprtDRGFP [4] (Fig. 1) in a system for assaying DSB-mediated gene conversion in a mouse *Hprt* gene targeting context. In this system, the DR-GFP reporter is the first gene targeted to the mouse *Hprt* locus in a quantifiable way, and then the cells successfully targeted with the reporter are transfected with a separate plasmid (pCβASce) [5], which encodes the I-*Sce*I homing endonuclease that will generate the gene-conversion-triggering DSB. The efficiency of this gene conversion is also quantifiable.

When cells are supplied with an exogenous gene targeting construct, the construct can integrate into either random loci (nontargeted) or into the homologous locus (targeted). Gene targeting efficiency is usually expressed as the proportion of targeted to total integrations (nontargeted plus targeted). In an organism like yeast, this efficiency approaches 100 %. In mammalian cells, the gene targeting efficiency seems to be strongly influenced by genomic context, but is usually on the order of a few percent. When the hprtDRGFP fragment is introduced into mouse embryonic stem (ES) cells, the *Hprt* targeting arms can direct integration of the construct to the native *Hprt* locus [6]. When this occurs, exon 2 of the endogenous *Hprt* gene is replaced by the DR-GFP reporter. This deletion of *Hprt* exon 2 inactivates the gene, rendering cells resistant to the nucleotide analogue 6-thioguanine (6-TG). Incorporation of the DR-GFP reporter carries with it a gene conferring resistance to puromycin. Hence, the efficiency of gene targeting can be determined from the fraction of transfected cells that are resistant to both puromycin and 6-TG (i.e., have undergone targeted integration at *Hprt*) vs the fraction of cells that are resistant to puromycin in the absence of 6-TG selection (i.e., have undergone nontargeted integration anywhere in the genome).

After identification of cells that have successfully integrated the DR-GFP reporter at the *Hprt* locus, DSB-induced gene conversion can be quantitated by assaying green fluorescent protein (GFP) after transfection of these cells with an expression vector for the I-*Sce*I endonuclease (pCβASce) [7]. The upstream GFP repeat (*SceGFP*) is nonfunctional owing to insertion of a recognition sequence for I-*Sce*I; hence, I-*Sce*I expression will generate a DSB in this repeat. The break can then be repaired by several mechanisms including nonhomologous end joining, single-strand annealing, and gene conversion. Gene conversion can be further mechanistically subdivided into processes involving short or long DNA tracts, with or without crossing-over. Short-tract gene conversion without crossing-over represents the majority of these events [8] and results in repair of the DSB using the downstream internal fragment GFP repeat (*iGFP*) as a template. The result is that *SceGFP* becomes a constitutively expressed functional *GFP*$^+$ gene, and the host cells acquire green fluorescence. The fraction of I-S*ce*I-transfected cells that repair the break by short-tract gene conversion without crossing-over then becomes easily quantifiable by flow cytometry. In wild-type mouse ES cells, this fraction is on the order of several percent.

Thus, using the hprtDRGFP/pCβASce system, it is possible to quantify both gene targeting and recombinagenic repair of DSBs in cells of differing genotypes, especially with variants of DNA repair genes. This approach also works effectively using wild-type cell lines transfected to express dominant-negative constructs of DNA repair genes, and can also be adapted for the use of small inhibitory double-stranded RNA molecules (siRNA) [9]. Please note that the following protocol is simply an update and revision of that previously published [10].

2 Materials

2.1 Embryonic Stem Cell Culture

1. A well-characterized line of mouse ES cells (e.g., J1, E14, available from Dr. Jasin, m-jason@ski.mskcc.org).
2. Tissue culture incubator.
3. Laminar flow tissue culture hood.
4. 10-cm Tissue culture plates.
5. 70 % ethanol.
6. Ca^{+2}/Mg^{+2}-free phosphate buffered saline (PBS): 200 mg/l KCl, 200 mg/l KH_2PO_4, 8 g/l NaCl, 2.16 g/l $Na_2HPO_4{\cdot}7H_2O$. Filter-sterilize and store at room temperature indefinitely (also available commercially).
7. ES cell medium: mix 500 ml high glucose Dulbecco's modified Eagle's medium (DMEM), 75 ml ES cell-qualified fetal bovine serum (FBS; *see* **Note 1**), 6 ml 100× penicillin/streptomycin (10,000 U/ml each, stock), 6 ml 100× nonessential

amino acids (10 mM each, stock), 6 ml 100× l-glutamine (200 mM stock), 6 ml dilute 2-mercaptoethanol (dilution is 21.6 µl of stock 2-mercaptoethanol in 30 ml of PBS), and 60 µl LIF (stock 10^7 U/ml; available as ESGRO from Chemicon, Temecula, CA). Store at 4 °C for up to several weeks. Store all stock solutions at 4 °C for routine use, or freeze at –20 °C for long-term storage.

8. Trypsin–EDTA solution: 0.2 % trypsin, 1 mM EDTA in PBS. Store at 4 °C for routine use. For long-term storage freeze at –20 °C.
9. Clinical centrifuge (e.g., Marathon Model 8K, Fisher, Pittsburgh, PA).
10. 4 mg/ml Puromycin (Sigma, St. Louis, MO) in PBS.
11. 10 mg/ml 6-TG (Sigma) in 1 N NaOH.
12. Dimethyl sulfoxide (DMSO).
13. Cryovials.
14. 100 % Methanol.
15. Giemsa stain.

2.2 Preparation and Analysis of Targeting Plasmid

1. Plasmid phprtDRGFP (available from Dr. Jasin).
2. Restriction enzymes: *Sac*I, *Kpn*I, *Eco*RV (e.g., New England Biolabs, Beverly, MA).
3. Agarose (molecular biology grade, e.g., Invitrogen, Carlsbad, CA) and agarose gel apparatus, including power supply (e.g., Owl Scientific, Portsmouth, NH).
4. Gel loading buffer: mix 600 µl 50 % glycerol, 50 µl 1 % bromophenol blue in 100 % ethanol, 50 µl 1 % xylene cyanol in ethanol, 60 µl Tris–HCl buffer, pH 8.0, 60 µl 500 mM EDTA, pH 8.0, and 180 µl water. Store at room temperature.
5. DNA size markers (e.g., λDNA digested with *Bst*EII, Invitrogen).
6. 8 M LiCl.
7. 100 and 75 % Ethanol (cold).
8. Tabletop microfuge (e.g., Eppendorf 5415D, Fisher).
9. 1/10× TE: 1 mM Tris–HCl, 0.1 mM EDTA, pH 8.0. Filter-sterilize and store at room temperature.

2.3 Transfecting ES Cells with the Targeting Plasmid

1. Electroporator (e.g., GenePulser II, Bio-Rad, Hercules, CA).
2. Electroporation cuvettes (0.8 ml with a gap width of 0.4 cm, Fisher).
3. 96-, 24-, and 6-Well tissue culture plates.

2.4 Preparing Genomic DNA from Transfectants

1. SALT-X genomic DNA extraction solution: 400 mM NaCl, 10 mM Tris–HCl, 2 mM EDTA, pH 8.0, 2 % sodium dodecyl sulfate (SDS), 0.4 mg/ml proteinase K. Freeze 10-ml aliquots at –20°C.

2. Hybridization oven.
3. Saturated NaCl solution.
4. Isopropanol.
5. 75 % Ethanol (room temperature).
6. Spectrophotometer.

2.5 Southern Hybridization

1. Restriction enzymes: *Hind*III, *Pst*I, *Sac*I, *Not*I (New England Biolabs).
2. Gel purification kit (e.g., GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences, Piscataway, NJ).
3. Blotting membrane (e.g., GeneScreen Plus charged nylon membrane [NEN, Boston, MA] works well when following the alkaline transfer instructions provided by the manufacturer).
4. Radiolabeling kit (e.g., Prime-It II Random Primer Labeling Kit, Stratagene, La Jolla, CA).
5. ProbeQuant G-50 Micro Column (size exclusion; Amersham).
6. Southern blot hybridization solution: mix equal amounts of 1 M Na_2HPO_4 and 2 mM EDTA, pH 8.0, 2 % bovine serum albumin (BSA), 10 % SDS. Stock solutions can be stored at room temperature indefinitely. The SDS in the mixed stock solutions will tend to precipitate at room temperature. It will go back into solution when heated to 65 °C.
7. 20× SSC (3 M NaCl, 0.3 M Na citrate, pH 7.0): mix 175.3 g NaCl and 88.2 g Na citrate in 800 ml H_2O. Adjust to pH 7.0 with HCl, if necessary, and adjust volume to 1 l. Store indefinitely at room temperature.

2.6 Measuring Homologous Recombination at a Defined Double-Strand Break

1. Plasmid pCβAsce (available from Dr. Jasin).
2. High-capacitance electroporator (e.g., Gene Pulser II with Capacitance Extender Plus, Bio-Rad).
3. Flow cytometer (e.g., FACScan [488 nm argon laser], BD Biosciences, San Jose, CA).

3 Methods

Mouse ES cells grow very well in culture. Log-phase growth has a doubling time on the order of 18 h. It is necessary to culture ES cells in the presence of LIF to prevent their spontaneous differentiation and loss of pluripotency. ES cells preferentially grow in clumps piled on top of one another. A healthy, nondifferentiated culture of ES cells will show discrete large “patches” of cells with individual cells not distinguishable within the patch. Additionally,

the patches should show sharp, bright borders under a phase contrast microscope on low power, indicative of their 3D piled-up nature. In contrast, unhealthy and/or differentiated ES cells grow as flat monolayers of individually distinguishable cells that appear dull under phase contrast.

Caution: All manipulations must be carried out in a laminar flow tissue culture hood.

3.1 Preparing a Tissue Culture Plate for ES Cells

1. Completely coat the bottom of a tissue culture plate with a 0.1 % gelatin solution, e.g., use 3 ml for a 10-cm diameter plate. Make sure that the bottom of the plate is completely covered by tilting the plate back and forth a few times. Let the gelatin sit on the plate for a minute or two. Store sterile gelatin solution at room temperature.
2. Completely aspirate off the gelatin solution but do not allow the plate to dry out. Leaving too much gelatin on the plate will "drown" the ES cells.
3. Add an appropriate volume of ES cell media to the plate. 8–10 ml is appropriate for a 10-cm plate (*see* **Note 2**).

3.2 Thawing Frozen ES Cells

1. Remove the vial of cells from frozen storage, and wipe it down with 70 % ethanol.
2. Open and then reclose the vial briefly to allow air pressure to equilibrate. (Skip this step if opening a sealed glass vial.)
3. Wearing gloves, hold the vial of cells in your hand until partially thawed.
4. Mix the partially thawed cells by inverting the vial a few times.
5. Pour the partially thawed cells into the ES cell medium on a prepared tissue culture plate.
6. Thaw the cells completely by swirling in the medium in the plate.
7. Place immediately in a 37 °C humidified tissue culture incubator with 5 % CO_2.

3.3 Subculturing ES Cells

1. Transfer ES cell plate(s) from the incubator to a laminar flow tissue culture hood.
2. Aspirate the medium.
3. Add an appropriate volume of trypsin–EDTA solution to the cells and tilt the plate back and forth several times to ensure even treatment; 2 ml for a 10-cm plate works well.
4. After the majority of the cells detach from the plate (usually requires a minute or two of gentle rocking), add at least 2 vol of complete tissue culture medium to the trypsinized cells and pipette up and down several times to disperse the cell clumps

and generate a single cell suspension. Do not allow cells to sit in the trypsin–EDTA solution longer than necessary as they will lyse. Cells will be stable after dilution into medium as the serum in the medium stops the action of the trypsin. After addition of the medium, the cell density of the cellular suspension can be measured with a hemocytometer, if desired.

5. Add an appropriate volume of the dispersed ES cells to the fresh ES cell medium in a prepared tissue culture plate and replace in the incubator. Split ratios of 10:1 work well. 20:1 splits are possible, if necessary. Splits of greater than 20:1 are not recommended.

3.4 Freezing ES Cells

1. Trypsinize cells from a 50 % confluent 10-cm plate as in Subheading 3.3, **step 3**.
2. Centrifuge the single-cell suspension of ES cells in medium for 5 min at 500 × *g* in a clinical centrifuge.
3. Aspirate the trypsin–EDTA–medium from the cell pellet.
4. Resuspend the cell pellet completely in 1 ml of 90 % ES cell medium/10 % sterile DMSO.
5. Add to a labeled freezer vial.
6. Freeze slowly by either using a freezing container at –80 °C or by placing the cell-containing vial directly in the *vapor* phase of a liquid nitrogen freezer. Do *not* place cells directly in the liquid phase of a liquid nitrogen freezer for the actual freezing process.
7. Short-term storage (several days) at –80 °C is acceptable. For long-term storage (more than 1 week), store in a liquid nitrogen-cooled freezer. Both liquid and vapor phase storage are acceptable. The cells stored in liquid nitrogen remain viable for several years.

3.5 Determining ES Cell Drug Sensitivity

It is necessary to determine for each ES line what level of drug selection will kill nonresistant cells. For hprtDRGFP, the selective drugs are puromycin and 6-TG. In general, we find that a final concentration of 10 μg/ml 6-TG is appropriate for all cell lines, but that the concentration of puromycin must be determined empirically.

1. Prepare ten 10-cm tissue culture plates, each with 9 ml ES cell medium.
2. Trypsinize a 50 % confluent 10-cm plate of ES cells, add medium and centrifuge for 5 min at 500 × *g* in a clinical centrifuge.
3. Aspirate the medium from the pellet, resuspend the pellet in 10 ml medium and add 1 ml of the cell suspension to each of the prepared 10-cm plates, for a total volume of medium and cells of 10 ml per plate.

4. Add puromycin to each plate to give final concentrations of 0, 0.1, 0.18, 0.32, 0.56, 1.0, 1.8, 3.2, 5.6, and 10.0 μg/ml puromycin.
5. Incubate cells at 37 °C in a humidified incubator with 5 % CO_2 for 5 days.
6. Note minimal concentration of puromycin that was necessary to kill *all* of the cells, i.e., no viable attached cells remain on the plate. For most ES cell lines, this concentration is typically in the range of 1–2 μg/ml puromycin.

3.6 Staining Colonies on a Tissue Culture Plate

1. Aspirate medium from the plate.
2. Treat with 100 % methanol for 30 s.
3. Rinse briefly with water.
4. Stain with dilute Giemsa solution (typically a 10:1–20:1 dilution of stain in water—consult instructions from the supplier) until colonies are stained dark blue.
5. Rinse away the stain completely with water and let the plate air-dry.

3.7 Preparation of the Targeting Plasmid

The vast majority of mouse ES lines in current use are derived from male mice. The goal is to target the hemizygous, X chromosome-linked, *Hprt* locus in male ES cells with the hprtDRGFP targeting construct to (1) determine the targeting efficiency at this locus, and (2) derive stable integrants harboring the DR-GFP reporter gene at a defined locus in order to perform the gene conversion assay.

1. Linearize the plasmid phprtDRGFP at the ends of the targeting arms (Fig. 1): Digest 70 μg of plasmid for each cell line to be transfected in a total restriction digest volume of 400 μl with 100 U of *Sac*I and 100 U of *Kpn*I overnight at 37 °C.
2. Verify that the plasmid has been correctly linearized: Digest 1 μl of the *Sac*I/*Kpn*I-digested DNA with *Eco*RV (4 U) in a total digestion volume of 15 μl at 37 °C for 1 h. As a control, add 1 μl of the *Sac*I/*Kpn*I-digested DNA to 14 μl of water.
3. Add 3 μl of gel loading buffer to the *Eco*RV digest and the control. Load and run on a 0.8 % agarose gel with suitable size markers.
4. If the *Sac*I/*Kpn*I digest was complete, the control lane should have two bands of 9,611 and 2,856 bp. The *Eco*RV-digested DNA should give three bands of 4,982; 4,629; and 2,856 bp.
5. If the *Sac*I digest was incomplete, the *Eco*RV digest will show a higher band at 7,485 bp and under-representation of the 4,629-bp band. If the *Kpn*I digest was incomplete, the *Eco*RV digest will show a higher band at 7,838 bp and

underrepresentation of the 4,982-bp band. In either case, the control DNA will show a higher band at 12,467 bp. In the event of an incomplete digest, add another 20 U of the appropriate enzyme, and digest again overnight. Then repeat *Eco*RV treatment and gel analysis.

6. Ethanol-precipitate the complete *Sac*I/*Kpn*I digest by adding 40 µl of 8 M LiCl and 800 µl of 100 % ethanol. Vortex briefly and incubate at room temperature for 3 min, and then microfuge at 12,000 × *g* for 3 min.
7. A white DNA pellet should be clearly visible. Decant the supernatant and add 500 µl 75 % ethanol. Invert the tube several times to mix and then centrifuge briefly to get the pellet back to the bottom of the tube.
8. Pipet off the 75 % ethanol and allow the pellet to air-dry (do *not* vacuum-dry the pellet). Dissolve the pellet completely in 70 µl 1/10× TE overnight at room temperature.

3.8 Transfecting ES Cells with the Targeting Plasmid

1. Two days before electroporation, seed 2×10^6 ES cells onto a 10-cm tissue culture plate. Incubate at 37 °C in a humidified incubator with 5 % CO_2.
2. Warm bottles of ES cell medium and trypsin–EDTA solution to 37 °C.
3. Aspirate the medium from the cells to be transfected and add prewarmed medium. Incubate cells for 4 h at 37 °C after this change to fresh medium.
4. Prepare ten 10-cm tissue culture plates with 9 ml medium each.
5. Place 70 µl of linearized targeting plasmid in a 1.0 × 0.4-cm electroporation cuvette.
6. Add 10 ml prewarmed medium to a sterile 15-ml tube.
7. Aspirate the medium from the ES cells, and add 2 ml warmed trypsin–EDTA.
8. When cells start to detach, add 4 ml warmed ES medium. Resuspend the cells well, then centrifuge for 5 min at 500 × *g* in a clinical centrifuge.
9. Resuspend the cell pellet in 650 µl room temperature PBS by pipeting up and down. Add the cell suspension in PBS to the electroporation cuvette with the added plasmid DNA and mix by pipeting up and down (*see* **Note 3**).
10. Electroporate the plasmid into the cells with an electroporator set to 0.8 kV, 3 µF (*see* **Note 4**). Immediately add 1,300 µl (2 × 650 µl) of medium from the 15 ml tube to the electroporation cuvette. Pipet up and down to mix and add the entire contents back to the 15-ml tube.

11. Add 1 ml of the electroporated cell suspension to each of the ten prepared 10-cm plates, for a final vol of medium and cells of 10 ml, and incubate overnight at 37 °C in a humidified incubator with 5 % CO_2.
12. Add an appropriate amount of puromycin (*see* Subheading 3.5) to each of the transfected plates and replace in the incubator for an additional 3 days.
13. There should be significant cell killing after 3 days of puromycin selection. Replace the medium on all plates with fresh puromycin-containing medium and continue to incubate until colonies are barely visible to the naked eye (another 3 days typically).
14. After colonies start to become visible (approximately 6 days post-transfection), add 6-TG stock solution to a final concentration of 10 μg/ml to nine of the ten transfected plates, leaving one plate with puromycin without 6-TG.
15. After colonies on the plate with puromycin alone are easily visible to the naked eye (an additional 2–3 days typically, for a total of 8–10 days post transfection) stain the plate and count the colonies. This count represents the total of both random and targeted integration. At this time, also change medium on the remaining nine plates to fresh medium with puromycin and 6-TG.
16. When the puromycin-/6-TG-resistant (i.e., doubly resistant) colonies are 2–3 mm in diameter, they are ready to pick. First, count how many of the doubly resistant colonies there were on the nine doubly selected plates in total. The gene targeting efficiency is the ratio of the number of these targeted colonies to the total number of stable integrants (from the plate with puromycin alone) normalized to the total number of cells transfected under each drug selection condition. For wild-type cells, this value is on the order of a few percent.
17. Replace the medium on the nine targeted (doubly selected) plates with PBS. With a sterile pipet tip, remove 18 of the doubly resistant colonies to individual wells in a 96-well plate, each well containing 20 μl trypsin–EDTA solution.
18. Incubate at 37 °C for 5 min, then add 180 μl fresh medium and disperse the colonies by pipeting up and down. Transfer the cell suspensions to individual wells of a gelatin-precoated 96-well plate. Place in the incubator for several days until cells are well established in the wells.
19. Expand these individual colonies progressively through growth on 24-well plates, 6-well plates, and finally to individual 10-cm plates.
20. After expansion, freeze stocks of the clones and prepare genomic DNA for verification of targeting by Southern blot.

3.9 Preparing Genomic DNA from Transfectants

There are many procedures for preparing genomic DNA from tissue culture cells, but this one is included because it is particularly simple and inexpensive [11]. Adequate DNA is isolated from mouse ES cells from either a semi-confluent well of a 6-well plate, or from about one-fourth of a 10-cm plate.

1. Trypsinize and suspend cells in medium as in Subheading 3.1, **step 3**.
2. Pellet an appropriate volume of cells and remove supernatant.
3. Add 400 µl SALT-X solution. Resuspend by agitation and incubate at 55–65 °C in hybridization oven until the solution clears completely (ranges from overnight to several days; *see* **Note 5**).
4. Transfer digested cells to a 1.5-ml microcentrifuge tube. Add 300 µl NaCl-saturated water and shake the tube vigorously. Do *not* vortex. A white precipitate should form immediately.
5. Centrifuge for 3 min to pellet proteins. If the pellet is not solid, shake vigorously again and repeat this step.
6. Transfer all of the supernatant to a new 1.5-ml microcentrifuge tube and recentrifuge. (This step is optional, but recommended.)
7. Transfer 600 µl of the supernatant to a new microcentrifuge tube. Avoid any pellet and/or cloudiness.
8. Add 420 µl room temperature isopropanol and mix by repeated gentle inversion. Precipitated DNA should be evident. Let sit for 3 min at room temperature.
9. Pellet genomic DNA in a microcentrifuge at 12,000 × *g* for 3 min. Rinse the pellet with room temperature 75 % ethanol, carefully aspirate the ethanol, let air-dry, and resuspend in 100 µl 1/10× TE overnight at room temperature.
10. Ensure that the genomic DNA is well dissolved and measure the DNA concentration by taking an OD_{260} reading in a spectrophotometer. Adjust the concentration of genomic DNA to 1 µg/µl with water and gentle agitation. The DNA will be stable at room temperature for several weeks or can be frozen for long-term storage.

3.10 Verifying Targeted Integration of phprtDRGFP by Southern Blot

Individual transfected clones are screened by Southern blot to verify that the reporter construct has integrated in an intact manner into the *Hprt* locus. A radiolabeled probe consisting of the *GFP* coding sequence is used, and genomic DNA is digested with enzymes that cut between the *GFP* repeats in phprtDRGFP (e.g., *Pst*I, *see* Fig. 1) and in the genome outside the construct. If the reporter has integrated correctly, two bands (and only two bands) of well-defined length should be observed. For a *Pst*I digest, for example, the bands should be 8,177 and 3,755 bp, corresponding

to targeted integration on the 5′ and 3′ sides, respectively. For a *Sac*I/*Not*I digest, the 3′- and 5′-specifc bands should be 7,488 and 5,126 bp, respectively. Colonies resistant to both puromycin and 6-TG typically show greater than 95 % correct targeted integration in wild-type cells.

1. Isolate *GFP* coding sequence for use as a probe. Digestion of plasmid phprtDRGFP with *Hind*III yields three fragments of 9,363; 2,298; and 806 bp. Gel-purify the 806 bp fragment using a suitable kit according to manufacturer's instructions.
2. Digest 8 μg of genomic DNA from each isolated 6-TG-resistant clone with *Pst*I or with a combination of *Sac*I and *Not*I. Run the digestion products on a 0.8 % agarose gel with suitable size markers. Take a picture of the gel to locate the size markers.
3. Blot the gel onto a suitable membrane.
4. Radiolabel 15 ng of the *GFP* coding sequence probe with α[^{32}P]dCTP or α[^{32}P]dATP. 10 pg of whatever size marker used above in **step 2** can be included in the reaction to radiolabel the marker bands.
5. Purify the radiolabeled probe from the unincorporated radionucleotides and primers using a ProbeQuant G-50 Micro Column.
6. Hybridize the probe with the membrane in hybridization solution overnight at 65 °C.
7. Rinse the membrane with successive 30-min rinses with 2× SSC/0.1 % SDS (twice), 1× SSC/0.1 % SDS (twice) and finally 0.5× SSC/0.1 % SDS (once) all at 65 °C. Dry the membrane and expose to film for several days.

3.11 Measuring Homologous Recombination at a Defined DSB

Transfection of phprtDRGFP-targeted cells with the pCβASce expression vector for the I-*Sce*I homing endonuclease will specifically generate a DSB in the *SceGFP* gene (*see* Fig. 1). Homologous recombination via short-tract gene conversion without crossing-over involving the downstream *iGFP* repeat will generate a functional *GFP*$^{+}$ gene, giving rise to cells which constitutively express GFP protein. The proportion of cells expressing functional GFP can then be easily measured by flow cytometry. A flow cytometry core facility can perform this analysis if you do not have direct access to a flow cytometer. The practical limit of detection with this procedure is on the order of 0.01 % fluorescent cells. Wild-type cells generally show homologous repair of a few percent.

1. Two days before electroporation, seed 2×10^6 phprtDRGFP-targeted ES cells onto a 10-cm tissue culture plate. Incubate at 37 °C in a humidified incubator with 5 % CO_2.

2. Warm a bottle each of ES cell medium, trypsin–EDTA solution and PBS to 37 °C.
3. Aspirate the medium from the cells to be transfected and add prewarmed medium. Incubate cells for 4 h at 37 °C after this change to fresh medium.
4. Add 50 μg pCβASce in a volume less than 80 μl to a 1.0×0.4-cm electroporation cuvette.
5. Prepare two 10-cm tissue culture plates with 10 ml medium each.
6. Aspirate the medium from the cells and add 2 ml warmed trypsin–EDTA solution. When cells have substantially detached from the plate, add 4 ml warmed medium and resuspend cells thoroughly.
7. Add 0.5 ml of the cell suspension to one of the prepared 10-cm plates. This will serve as the untransfected control. Place this plate back in the incubator.
8. Centrifuge the remaining cell suspension at 500×*g* for 5 min in a clinical centrifuge.
9. Aspirate the medium from the pellet. Add 650 μl of warmed PBS to the pellet and resuspend by pipeting up and down. Add the cells suspended in the PBS to the electroporation cuvette with the pCβASce plasmid DNA and thoroughly mix by pipeting up and down.
10. Immediately electroporate in a high-capacitance electroporator at 1,000 μF, 0.25 kV (*see* **Note 6**).
11. Immediately add 2×650 μl of medium from the prepared 10-cm plate to the electroporation cuvette. Pipet vigorously up and down to resuspend the electroporated cells. Pour back onto the 10-cm plate, swirl, and immediately place in the 37 °C humidified incubator with 5 % CO_2.
12. The following day, rinse the electroporated plate with warmed PBS, removing as much cellular debris as possible, and add fresh medium.
13. Split the unelectroporated control plate, if necessary, while the cells on the electroporated plate grow to a semi-confluent state (usually 2–3 days).
14. Trypsinize cells from the untransfected and each transfected plate into cellular suspensions. Replate 1/10 vol of cells suspended in medium from each plate onto a freshly prepared 10-cm plate.
15. Analyze 1/10 vol of the cells suspended in medium by flow cytometry for the presence of green fluorescence (from expression of GFP). This is the preliminary analysis. We use a Becton Dickinson FACScan (488-nm argon laser) with the following settings given in Table 1.

Table 1
Argon laser settings

Parameter	Voltage	Amplification	Scale
FSC (forward scatter)	10^{-1}	4.8×	Linear
SSC (side scatter)	380 V	1.0×	Linear
FL1 (green fluorescence)	460 V	1.0×	Log
FL2 (orange fluorescence)	525 V	1.0×	Log

We set the threshold to FSC 52, and use 25 % FL2–FL1 compensation. Your settings will depend upon your particular instrument

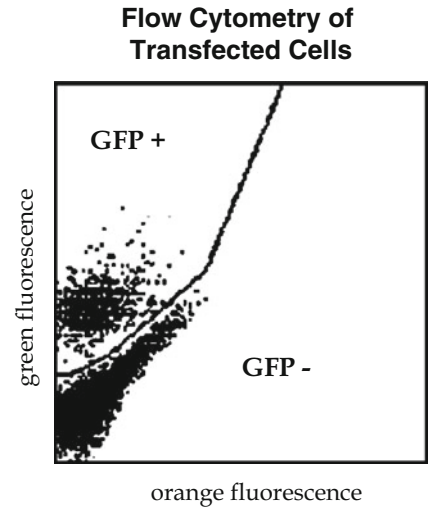

Fig. 2 Flow cytometric analysis of transfected cells. *GFP* green fluorescent protein. See text for details

16. Set up a gate on SSC vs FSC to select for cells with a well-defined size and shape, taking care to eliminate debris and clumps. We typically collect fluorescent information from 10,000 cells within the gated SSC vs FSC population.
17. From this gated population, plot FL1 (green fluorescence) vs FL2 (orange fluorescence). The nonfluorescent cells will fall on the FL1/FL2 diagonal. The cells that underwent homologous recombination to restore a functional *GFP* gene will form an obvious discrete population shifted "greenward" on the FL1 axis, away from the FL1/FL2 diagonal. Set a gate to quantify these cells (Fig. 2).
18. When the split cells have grown to a semi-confluent state, trypsinize, resuspend in medium, and reanalyze by flow cytometry (**steps 15–17**) to get the final values for green fluorescence.

4 Notes

1. This serum has been specifically tested for the ability to support undifferentiated ES cell growth (e.g., Invitrogen).
2. Recommended depth of medium is 3 mm. Less medium tends to have nutrients consumed and pH altered too rapidly, whereas greater depths lead to poor gas exchange.
3. The PBS is actually slightly hypotonic to the cells. Extended suspension in PBS will render the cells more fragile and lead to greater cell killing and lower transfection efficiencies.
4. These electroporation conditions are very mild. There should be almost no cell killing. These conditions are suitable for electroporation of linearized plasmid DNA only—circular or supercoiled plasmid will not successfully transfect under these conditions.
5. The digestion process can be enhanced by periodically agitating the mixture. If the digestion process is incomplete, the proteins will not pellet cleanly in subsequent steps and genomic DNA will be difficult to recover.
6. These conditions suitable for efficient electroporation of circular and supercoiled plasmid are quite harsh and should kill approx 50 % of the cells. If excessive cell killing is noted, reduce the electroporation voltage, typically in 20-V increments. If little cell killing is noted, the electroporation voltage can be increased to give greater transfection efficiency.

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