Pooling and PCR as a method to combat low frequency gene targeting in mouse embryonic stem cells

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Abstract The introduction of germ line modifications by gene targeting in mouse embryonic stem (ES) cells has proven a fundamental technology to relate genes to mammalian biology. Critical aspects required for successful gene targeting have traditionally been experimental enhancements that increase the frequency or detection of homologous recombination within ES cells; however, the utilization of such methods may still result in the failed isolation of a positively targeted ES cell clone. In this study, we discuss the current enhancement methods and describe an ES cell pooling strategy that maximizes the ability to detect properly targeted ES cells regardless of an inherent low targeting efficiency. The sensitivity required to detect correctly targeted events out of a pool of ES cell clones is provided by polymerase chain reaction (PCR), and only those pools containing positives need to be expanded and screened to find individually targeted clones. This method made it possible to identify targeted clones from a screen of approximately 2,300 ES cell colonies by performing only 123 PCR reactions. This technically streamlined approach bypasses the need to troubleshoot and re-engineer an existing targeting

construct that is functionally suitable despite its low targeting frequency.

Keywords Homologous recombination · Gene targeting · Embryonic stem cells · Polymerase chain reaction

Introduction

Gene targeting in mouse embryonic stem (ES) cells has transformed the way in which scientists study gene function and in many cases the germ-line mutations derived by this technique can recapitulate important disease phenotypes present within the human population. Multiple factors such as sequence homology, positive and negative selection markers, DNA preparation, and ES cell culturing conditions need to be considered when designing a gene targeting experiment, such that sufficient targeting frequency is reached and positive recombinant ES cell clones are detected. Targeting frequencies in mouse ES cells are generally quite low and ranges between 1% and 10% are considered quite successful. In cases where positively targeted alleles are not detected after several ES cell electroporations, additional strategies must be employed to increase targeting frequency and/or detection.

Numerous methods have been adopted to increase gene-targeting frequency in ES cells. At a

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minimum, positive selection markers such as neomycin (Thomas and Capecchi, 1987) or hygromycin (Cruz et al. 1991; Mortensen et al. 1991) are inserted within the targeting construct and flanked by gene homology. In many cases these cassettes cause the desired disruption in the gene sequence by insertion or replacement, but as equally important, they reduce the background by allowing only those ES cell clones to grow which have integrated the targeting construct into the genome. Because random integrations appear much more prevalent than allele specific targeting events, negative selection markers such as TK (thymidine kinase) (Chauhan and Gottesman 1992) or DT (diphtheria toxin) (Yagi et al. 1990) have aided significantly in boosting the frequency of homologous recombination. By placing a negative selection gene at the end of either homology arm, constructs that do not lose the negative selection cassette (due to clipping during the homologous recombination event), render ES cells susceptible to enzymatic drug induced death. However, this system is not perfect, as a break in the negative selection marker during random integration may simulate a correctly targeted event. For this reason, a negative selection marker is often placed at both ends of a targeting construct and has been shown to enhance the selection of recombinants when using TK (Deng and Capecchi 1992).

Other methods such as increasing the length of homology of targeting arms can significantly enhance targeting frequency, however, length is only directly proportional to targeting frequency until a saturation point is reached (10–14 kb) (Deng and Capecchi 1992). A drawback to this method is that targeting vectors become increasingly difficult to manufacture as the plasmid size increases due to longer arms and additional selection marker cassettes. Increasing the length of homology also hampers ES cell screening for positive recombinants if PCR is to be used as the diagnostic, since amplification must span from the internal selection marker into flanking genomic sequence not present within the targeting vector.

Less traditional methods for enhancing targeting frequency have been tested at the ES cell level. Some researchers report increases in homologous recombination frequency by altering

the plating density of electroporated cells so as to limit significant death (Templeton et al. 1997), while others have shown an inverse linear correlation of targeting frequencies by changing the doubling time of ES cell growth through use of non-isogenic cell lines or manipulated growth medium (Udy et al. 1997). It is also known that the source strain of DNA used in construction of the targeting vector can have a significant affect. Isogenic DNA between a targeting vector and ES cell line can be 5–25 times more efficient for homologous recombination than nonisogenic DNA (Deng and Capecchi 1992; van Deursen and Wieringa 1992). Even the preparation of DNA used for electroporation can have a profound effect. In one instance it was shown that removal of vector sequence from the targeting DNA by restriction digestion increased the rate of homologous recombination by 10 fold (Sarig et al. 2000). For more information regarding gene targeting methods and protocols, the readers are referred to (Templeton et al. 1997; DeChiara 2001; Tessarollo 2001).

When considering the factors described above, great variability in the efficiency of homologous recombination still exists, suggesting that intrinsic properties of the DNA sequences themselves (low complexity, GC content) may also affect the process (Sarig et al. 2000). Thus, one may take many of the above parameters into consideration, and still observe an undetectable level of gene targeting for a well-designed targeting construct. Under these circumstances, rather than abandon or modify the original targeting construct, we have opted to increase the number of ES cells that can be screened by using a pooling strategy and PCR. This procedure allows easy screening of 10 fold or more ES cells for a rare event, which would have otherwise been missed during a conventional experiment.

Materials and methods

Construct preparation and ES cell electroporation

The targeting and PCR control constructs were designed as indicated in Fig. 1 using pBluescript

Fig. 1 ES cell targeting strategy. The targeting construct (a) is designed to remove exons 5, 6 and 7 of the H13 endogenous allele. (b) The targeting construct produces amplification (c–dashed line) upon homologous recombination into the locus of interest such that a neomycin

designed primer comes within proximity of a 5' flanking genomic primer. A control construct (d) simulates amplification of a positive targeting event (dashed line) even during random integration due to addition of adjacent 5' flanking genomic sequence (bold line)

II SK- (Stratagene) as the vector backbone. Both constructs were linearized with NdeI (New England Biolabs) and purified by phenol chloroform extraction followed by ethanol precipitation. For electroporation, R1 (129X1 × 129S1) ES cells were grown in 60 mm tissue culture plates on neo-resistant feeder cells. 1×10^6 ES cells were collected by centrifugation and resuspended in 400 μ l of cold PBS, combined with 5 μ g of linearized targeting vector in a BTX 2 mm cuvette for each of 10 electroporations, and electroporated using a BTX600 electroporator with the following settings: capacitance (C) = 425; volts (V) = 150; ohms (R) = setting 3. Electroporated cells were allowed to rest for 10 min at room temperature prior to distribution on 30–100 mm tissue culture plates containing feeder cells.

ES cell culture conditions

Feeder cells (G418 resistant primary fibroblasts derived from C57BL/6J mice) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-Brl) with 10% fetal bovine serum (FBS) (Hyclone). ES cells (provided by Dr. J. Rossant, The Hospital for Sick Children, Toronto/Canada) were cultured on feeders inactivated by irradiation (3,000 rads) in the presence of ES cell medium (DMEM with 16% FBS, 1% non essential amino acid (Gibco-BRL), 2 mM Glutamax (Gibco), 1 mM Sodium Pyruvate (Gibco), 1X PSN, 0.1 mM β -mecaptoethanol (Sigma), and 1000 U/ml leukemia inhibitory factor (LIF) (in

house)). ES cells were positively selected with G418 (Gibco) at a concentration of 200 μ g/ml. On day 2 negative selection was applied with 0.2 μ M FIAU (2'-fluoro-2'-deoxy-1-d-arabino-furanosyl-5-iodo-uracil) (with the exception of a G418 control plate used for calculation of TK-fold enrichment). On day 6, ES cells from each plate were disaggregated using 0.05% trypsin (Gibco) and split into two fractions (for PCR and archive). The fraction for archive was frozen back at -80°C in freeze media, 5:4:1 (FBS:culture medium:DMSO), while the other fraction was processed to isolate genomic DNA using the Wizard Genomic DNA Purification Kit (Promega, Inc.) following the manufacturers recommendations. Positive superpools identified by PCR were thawed and distributed into 4–100 mm plates containing G418. ES cells were allowed to develop for 6 days and individual colonies were distributed individually into a 96 well flat bottom feeder containing plate. After sufficient growth, this plate was duplicated, and PCR was performed on one replicate lysed with Lairds buffer (100 mM Tris pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl). Positively identified ES cell clones were expanded on 60 mm plates and archived in freeze media for future use.

PCR to detect gene targeting

To determine ES clones targeted for the H13 gene, an intronic H13 specific forward primer located outside of the targeting vector, but within

the control construct (H13flankF - CGGTGAT-CCTACTGAATTAACCCACCCTTG) and a neomycin specific reverse primer (NeoOutR - CCGTTTATCCTGCCACACCAGCAGGTC) were designed via Primer Express software v2.0 (Applied Biosystems, Inc.) with a melting temperature of 64°C to facilitate semi-long PCR. PCR was performed in standard 0.2 ml thin-walled tubes in a PTC-100 thermocycler (MJ Research, INC.). The PCR assay was performed in a 25 µl reaction volume containing 1X PC2 buffer (Ab Peptides), approximately 50 ng genomic DNA, 0.5 µM of each primer, 200 µM of each dNTP, 3 U of KlenTaqTM (Ab Peptides) and 0.04 U of PFU turbo[®] (Stratagene). Cycling conditions were as follows: an initial denaturation step at 96°C for 30 s, followed by 40 amplification cycles of 96°C for 20 s, 64°C for 30 s, 72°C for 3 min 30 s, and terminated by a final extension at 72°C for 7 min. PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Loss of homozygosity quantitative PCR

Wild type primers for exon 5 of the H13 gene (H13exon5F - TTGTGCTCTGGCTGCATACCTA, H13exon5R - GGATGTTCCAGTCAGG-TAAATCTCA) were designed using Primer Express software v2.0 (Applied Biosystems, Inc.) with a melting temperature of 60 degrees. ES cell DNA content was adjusted to achieve 25 ng per PCR reaction as determined using a Nanodrop DNA quantitation system. A standard curve (Fig. 5, black dots) was generated using four concentrations of wild type (non-targeted) genomic DNA on the ABI Prism 7700 using SYBR Green PCR Master Mix (Applied Biosystems, Inc.) under default cycling conditions. Twenty five nanograms of genomic DNA from either three non-targeted or targeted ES cell clones were amplified and DNA quantity was determined.

Results

Gene targeting strategy

To detect rare events in a large population, pooling of individual samples is often used to

minimize the amount of time and reagents required for effective screening. Thus far, a detailed method that describes the pooling of ES cells to screen for rare gene targeting events is lacking. To determine whether clones can be pooled and screened, such that positive ES clones are subsequently recovered, we tested our procedure using a targeting vector that has the potential to disrupt the mouse H13 minor histocompatibility gene (Mendoza et al. 1997), recently characterized as a signal peptide peptidase (Weihofen et al. 2002). The H13 knock out targeting strategy is illustrated in Fig. 1. A neomycin replacement construct was designed to remove exons 5 through 7 of the 12 exon gene. The resulting deletion would create a truncated protein by producing a stop codon at the junction of exon 4 and 8. The targeting vector contains 3.2 kb of 3' arm and a short 0.8 kb 5' arm, specifically designed to facilitate a robust PCR amplification from the positive selection marker into flanking genomic sequence not present within the construct. This PCR strategy negates the need to screen every ES cell colony by Southern blot. To enhance targeting frequency, a TK negative selection marker was included at the end of the 5' arm. In addition, we developed a construct identical to the targeting vector with the exception of an additional 400 bp of genomic flanking sequence on the 5' end of the 5' arm. This construct, when randomly integrated into ES cells, serves as a positive control for the PCR screening procedure by simulating a correctly targeted event. Identical primer sites for both the correctly targeted event and control construct are shown as arrows in Fig. 1.

ES cell pooling and colony screening

Under circumstances of unsuccessful gene targeting, additional ES cell electroporations can be performed to increase the number of neomycin positive ES colonies recovered and enhance the chance of obtaining a correctly targeted ES cell clone. However, a major limitation to this method is the laborious isolation and screening of thousands of ES cell colonies. To test whether neomycin positive ES cell colonies can be pooled to simplify the screening of ES cells from many

independent electroporations, we developed the strategy illustrated in Fig. 2. Ten electroporations are performed and subdivided amongst thirty 100 mm culture plates. Our initial plating estimate, based upon past experience, would be roughly 100 neo positive/TK negative clones per plate, or 3,000 clones total. The experimental design provided in Fig. 2 assumes that at least one plate will produce one positive clone. For simplicity, only one positive and one negative plate are illustrated. After ES cell growth, each plate is trypsinized and mixed to disaggregate all colonies into a homogenous single cell suspension. Each individual homogenous pool is then split into two aliquots. Since ES colonies are disaggregated into individual cell suspensions, a single positive colony per plate should result in positive cells within both split fractions (Fig. 2). For each plate, one aliquot is archived at -80°C , while the other is subjected to DNA extraction and PCR

Fig. 2 Multiple electroporations are plated (a), each plate is trypsinized (b), mixed to a single cell suspension (c) and split into two fractions (d). One aliquot is analyzed by PCR, while the other is frozen for potential recovery of the positive pool. A positive scenario is illustrated on the left, and a negative on the right. Positive clones are illustrated as filled dots

analysis. When a gene targeted positive plate is detected by PCR, that archived pool aliquot can then be thawed and replated to form both negative and positive ES cell colonies. Individual colonies can then be isolated and screened by PCR or Southern blot as in a traditional screening procedure.

To test whether PCR is sensitive enough to detect a single positive ES colony amongst a pool of negatives and feeder cells, we performed separate electroporations for both the targeting construct and the positive control construct. A single plate from the targeting construct electroporation containing approximately 100 neomycin resistant colonies was harvested, mixed into a single cell suspension and split into two aliquots as illustrated in Fig. 2 and described above. In addition, a single positive control ES colony was harvested, mixed in medium and split into two aliquots. One of these aliquots was then spiked into one of the targeting construct fractions. DNA was subsequently extracted from both the spiked and non-spiked aliquots and analyzed by PCR (Fig. 3). As anticipated, the positive control spiked ES cell fraction yielded a valid amplification product, whereas the non-spiked fraction was negative. Since no legitimate targeting events were present in the non-spiked pool, the positive PCR amplification was due exclusively to the single ES cell control clone. These results illustrate that PCR is sensitive enough to detect a single positive colony out of a milieu of at least 100 negative clones and feeder cells.

To test the overall procedure, we performed 10 electroporations with the targeting construct. Each electroporation was split into three separate aliquots and distributed to 100 mm feeder plates. We then sought to compare the effects of positive selection (only the G418 substrate) with both positive and negative selection (both G418 and the TK substrate, FIAU, respectively). Most plates were selected with G418 with FIAU added on day 2 for a total of 6 days of culture. For comparison, a separate single aliquot plate was selected on G418 only. Care was taken to prevent ES cell overgrowth, as faster growing clones could overwhelm the pool and/or differentiate. Total ES cell colonies were recorded, and each plate was then harvested and divided as illustrated in

Fig. 3 Testing the sensitivity of PCR to detect a single positive ES cell colony amongst a large pool of negatives and feeder cells. One half of one positive control ES cell colony is spiked into a pool of known negatives with feeder cells (**a**) and is detectable by PCR (**b**) expected band size indicated at the left

Fig. 2. As summarized in Table 1, the combined results indicate that the double selection procedure resulted in 6.6-fold enrichment in properly targeted clones.

Table 1 ES cell plating statistics

Average number of colonies per plate (Range)	75 (49–87) ^a
G418 only plate	498
TK enrichment (G418 + FIAU)	6.6 fold ^b
Total number of FIAU resistant colonies	2,311
Recombination frequency	≥ 5%

^a G418 positive selection + FIAU negative selection

^b Number of G418 colonies divided by the average number of doubly selected colonies

Determination of recombination frequency

The PCR screening (using the strategy illustrated in Fig. 1) revealed that each pool of ES cells contained at least one positively targeted ES cell colony (11 representative pool positives are shown in Fig. 4, top). To estimate recombination frequency, we established a lower limit on the total number of positive colonies required to insure at least one positive per plate using a Monte-Carlo statistical simulation. This method assumes that each colony could be placed on each plate with equal probability. The result of this calculation (10,000 iterations) gives a median value of 100 positive colonies, with a 95% confidence interval between 60 and 187. Given 2,021 total FIAU selected colonies, our recombination frequency estimate (100/2,021) is at least 5% or better (Table 1). To recover individual colonies, pool #1 was thawed and selected on G418 only. We recovered approximately 300 ES cell colonies, screened 96 for the targeted mutation, and detected six positives (three are shown in Fig. 4, bottom). Assuming ES cell colonies of approximate equal size, this value corroborates our recombination frequency estimate.

Loss of homozygosity assay

To further verify that our six positive clones had undergone a homologous recombination, we performed a quantitative “loss of homozygosity” PCR assay using primers that amplify the wild-type exon 5 (Fig. 5). This assay uses real-time PCR to quantitatively measure a 1:2 fold difference present within the genomic DNA starting material. A serial dilution with four concentrations of wild type ES cell DNA was used to generate a standard curve (Fig. 5, black dots). A defined quantity (25 ng) of either three non-targeted or three targeted ES cell clones (three technical replicates each) were amplified and cycle threshold (Ct) values calculated. As can be seen, all three targeted clones clustered to a population centered around 12.5 ng, half that of the three non-targeted ES cell clones (25 ng), thus illustrating that gene targeting had in fact removed one copy of exon 5. Two of the

Fig. 4 Identification of 11 positive superpools (top) and individual ES cell clones from thawed superpool #1 (bottom). Ten of 30 positive superpools are shown (top). Superpool #1 was thawed, plated and 96 colonies were screened by PCR. Three representative positives (of six total) are shown (bottom). Expected band size indicated to the left

ES cell clones that were verified as targeted by the loss of homozygosity assay were injected into blastocysts and multiple mice with high chimerism were obtained.

Discussion

Kim and Smithies originally suggested that an ES cell pooling strategy could be of value as a way to

Fig. 5 Quantitative loss of homozygosity assay. Black diamonds show the amplification efficiency (Y-axis, Ct-cycle threshold) of 3.125, 6.25, 12.5, 25 and 50 ng of wild type genomic DNA (three technical replicates each, error bars shown). Addition of 25 ng of three negative (triangles) and three positive ES cell lines (squares), illustrates that the positively targeted ES cell clones are missing one copy of exon 5. Three technical replicates each for each ES cell line are shown. X-axis is log base 10

combat low targeting frequency (Kim and Smithies 1988). Here, we demonstrate that ES pooling permits the facile screening of considerably more ES cells than a standard electroporation procedure. This is made possible because PCR is sensitive enough to detect a single positive out of a milieu of negatively targeted ES cell clones and feeder cells (Fig. 4, top). A positive pool fraction can then be thawed to screen and recover a clonal population of the positive ES cells (Fig. 4, bottom). Based upon the number of pools that are positive and the total number colonies present, one can use Monte-Carlo simulation to statistically estimate a lower level targeting frequency to determine how many ES cell clones should be picked from a positive pool fraction to ensure recovery. In our case, a 5% targeting frequency calculated at the pool level, informed us that approximately 100 colonies should yield on average five positives (we obtained six). At the pool level, an absence of any positives would certainly alert a researcher towards troubleshooting the targeting construct. In the case of this experiment, only 123 PCR reactions were needed to screen approximately 2,300 ES cell clones, due to the pooling strategy. In addition, the laborious task of isolating individual ES cell colonies was limited to only 96. This is much more efficient than hand picking hundreds or thousands of colonies and screening them by Southern blot. Screening individual ES cell clones by a quantitative loss of homozygosity assay can also limit the number of samples that need to be subjected to Southern-blot analysis to verify a targeted replacement or deletion.

A potential problem not addressed by the PCR assay is ES cell colonies seeded by both positive and negatively targeted ES cells. While this event is rare, even a few contaminating positive ES cells within a negative clone will appear as positive by non-quantitative PCR, and therefore, should not ultimately be used as a final replacement to verification by the Southern blot. However, the quantitative PCR loss of homozygosity assay should identify clones with substantial chimerism that have intermediate amplification efficiencies. In doing so, this method can bypass the Southern blot and verify that the majority of each clone carries predominantly targeted ES cells.

Although not performed in this experiment, PCR screening across both targeted arms can be used to show that the targeted construct has integrated correctly at both ends. In many cases, PCR of individual colonies is not possible due to the length of the targeting arms, or it is not feasible to design a control construct to verify that PCR is working. Therefore, some experimental design should be performed beforehand in order to use this pooling and screening method.

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