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Confirmation of Recombination Site Functionality in Gene Targeting Vectors using Recombinase-Expressing Bacteria

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Abstract

Recognition sequences for the site-specific DNA recombinases Cre and FLP are commonly incorporated into gene targeting vectors for the purposes of removing selection markers or generating conditional alleles. Gene targeting vectors typically contain a positive selection marker, such as the neomycin resistance gene, flanked by loxP sites. Thus, the selection marker can be removed by breeding to a mouse strain which expresses Cre recombinase in its germ line. Conditional knockout vectors typically have one or more exons flanked by loxP sites and the positive selection marker flanked by FRT sites. Thus, the selection marker is removed with FLP recombinase and the knockout allele is generated in tissues expressing Cre recombinase. Because the generation of mice by gene targeting in embryonic stem (ES) cells is an expensive and time-consuming process, it is important to confirm that the recombination sites in your targeting vector are functional prior to electroporation of ES cells. This chapter describes a simple method for testing the functionality of loxP and FRT sites *in vivo* using Cre- or FLP-expressing bacteria.

1. Introduction

Technology allowing mutagenesis of the mouse genome via homologous recombination in embryonic stem (ES) cells has yielded thousands of mouse mutants. Phenotypic analysis of these mutant strains has provided important information regarding the physiological functions of individual genes.

Gene targeting in ES cells is accomplished by first generating a targeting vector consisting of a positive selection marker, usually the neomycin resistance gene (neo) whose expression is driven by a regulatory sequence such as that of the phosphoglycerate kinase (PGK) gene that is active in mouse ES cells. The PGK–neo cassette is flanked by 5′ and 3′ arms of homology to the gene of interest. Typically for knockout experiments the arms of homology exclude one or more exons important for gene function. Thus, following homologous recombination, the mutant allele lacks these exons and hopefully produces a nonfunctional protein product or no protein product at all. The targeting vector may also contain a negative selection marker outside the homologous arms to select against random integration. Because the inclusion of PGK–neo in the targeted allele may interfere with gene function, it is common to design the targeting vector such that the PGK–neo cassette is flanked by recognition sequences for either Cre or FLP recombinase (Fig. 9.1A). In this manner the PGK–neo cassette may be removed after identification of correctly targeted ES cell clones.

Creation of conditional knockout alleles is also a very common gene targeting strategy. These alleles are designed such that critical exons are flanked by recognition sites for Cre recombinase. Breeding to an appropriate tissue-specific Cre-expressing mouse strain will yield tissue-specific excision of the critical exons and thus a deletion of gene function in that particular tissue. Targeting vectors created for this purpose usually contain both loxP and

FRT sites. The loxP sites flank the critical exons and the FRT sites flank the positive selection marker (PGK-neo; Fig. 9.1B). These mice can be bred to germ line FLP-expressing mice to permanently remove the PGK-neo cassette and a tissue-specific Cre-expressing line to generate conditional knockout mice for phenotypic analysis.

Generating mutant mice through gene targeting in ES cells is a costly and time-consuming process. Thus, it is advised to take all precautions necessary to ensure that the targeting vector is correctly designed. The method described in this chapter was developed to ensure the recombination sites of your targeting vector are functional *in vivo*. Nonfunctional recombination sites may occur due to molecular cloning errors while making the targeting vector or uncontrollable errors during homologous recombination. While we absolutely advise sequencing the targeting vector to confirm everything is correct, including the recombination sites, we also recommend using the following simple method to functionally test the recombination sites *in vivo* prior to electroporation into ES cells.

2. Materials

Cre-expressing *Escherichia coli* strains: BNN132 (ATCC, cat # 47059) (Elledge *et al.*, 1991), EKA133 (ATCC, cat # 47041) (Ayres *et al.*, 1993), BS1365 (Sauer and Henderson, 1988), or 294-CRE (Buchholz *et al.*, 1996).

FLP-expressing *E. coli* strains: PS393 (Babineau *et al.*, 1985) and 294-FLP (Buchholz *et al.*, 1996).

Your gene targeting vector.

3. Method

Figure 9.2 shows a flow diagram of the basic method described below.

Dilute the targeting vector to a concentration of ~3 ng/ μ l.

Thaw a 100 μ l aliquot of recombinase-expressing competent bacteria on ice (~5 min).

Add 1 μ l of targeting vector (3 ng) and mix by gently flicking the tube with your finger.

Incubate on ice for 30 min.

Heat shock at 42 °C for 45 s. Place immediately back on ice. Keep on ice for 2 min.

Add 500 μ l of LB or SOC and shake for 1 h at 37 °C.

Spread 20 and 50 μ l of cell suspension on LB agar plates containing the appropriate antibiotic for your targeting vector. Incubate overnight at 37 °C.

Pick 20 colonies and inoculate 2–3 ml LB cultures with appropriate antibiotic. Incubate overnight in a 37 °C shaking incubator.

Prepare plasmid DNA from the minicultures using standard procedures.

Analyze the DNA for recombination by either restriction enzyme digestion or PCR (see example below).

Expect mosaicism, that is, there will be a mixture of intact and recombined plasmid DNA, because the Cre and FLP recombinases expressed in the bacteria exhibit a varying degree of

activity. In normal practice, they will not recombine and “pop-out” the intervening sequence 100% of the time. The presence of a smaller band of the correct size in a clone indicates that the recombination sites are functional *in vivo* and it is safe to proceed with ES cell electroporation.

4. Example of Results

Figure 9.3A and B illustrates the results of restriction enzyme analysis of a gene targeting vector introduced into Cre-expressing bacteria. In this example, the targeting vector yields fragments of 8.3 and 5.2 kb when digested with *Bam*HI. Following removal of PGK–neo by Cre recombinase, the 8.3 kb fragment is reduced to 6.5 kb. This difference can be easily distinguished on an agarose gel. As explained above, there is mosaicism; the results indicate both the presence and absence of Cre-mediated recombination. In this example, Clone 1 shows complete recombination; whereas Clones 2–4 exhibit varying degrees of recombination.

Figure 9.3C and D illustrates the results of analysis by PCR. In this experiment, two reactions were performed for each clone. Primer set F1/R1 yields no band in the presence of PGK–neo because the extension time is too short to produce 2.1 kb products. However, in the absence of PGK–neo this primer set yields a product of 360 bp. Primer set F2/R1 yields a 550 bp product in the presence of PGK–neo and no product in the absence. In this experiment, Clone 1 shows complete recombination (presence of 360 bp product, but no 550 bp product); whereas Clones 2–4 exhibit varying degrees of recombination.

5. Summary

Gene targeting in ES cells and the subsequent generation of correctly targeted strains of mice is a time-consuming and expensive process. For this reason, it is advisable to take all precautions necessary such that the mice generated will have the precise mutation that you designed. In this chapter we have described a simple method to test the functionality of loxP and FRT recombination sites *in vivo*. A positive result from this simple procedure will give an investigator confidence that the gene targeting plasmid that is introduced into ES cells has functional recombination sites. Likewise, a negative result saves an enormous amount of time, energy, and money that would have been expended in the screening of ES cells and generation of mice.

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Figure 9.1.

Gene targeting strategies using loxP and FRT sites. (A) Traditional targeting vector with PGK-neo flanked by loxP sites. (B) Conditional knockout vector with an exon flanked by loxP sites and PGK-neo flanked by FRT sites.

Figure 9.2.
Outline of experimental procedure.

Figure 9.3.

Analysis of recombination in bacteria. (A) For this targeting vector *Bam*HI digestion yields fragments of 8.3 and 5.2 kb prior to recombination and 6.5 and 5.2 kb following removal of the PGK-neo cassette by Cre recombinase. (B) Example of a restriction enzyme digestion. Clone 1 = complete excision; Clones 2–4 = varying degrees of excision. (C) Testing the functionality of recombinase recognition sequences by PCR. In this example two PCR reactions were performed for each clone. Primer set F1/R1 yields no band if PGK-neo is present (2.1 kb is too long for the extension time) and a 360 bp band if PGK-neo is removed by Cre recombinase. Primer set F2/R1 yields a 550 bp band if PGK-neo is present and no band if it is removed. (D) Clone 1 = complete excision; Clones 2–4 = partial excision.