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Introduction of the negative selection marker into replacement vectors by a single ligation step

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ABSTRACT

Gene targeting is a powerful method for introducing mutations into the genome of embryonic stem cells. The most widely used approach is the positive–negative selection method in which a gene encoding a negative selection marker is cloned into the replacement vector to obtain an enrichment of properly targeted clones. Here, we present an alternative means to introduce any given negative selection marker at the ends of a replacement vector using a single ligation step, thereby avoiding laborious cloning procedures. Our results demonstrate that this fast and simple method consistently provides a high level of enrichment of appropriately targeted clones.

Gene targeting by homologous recombination is a powerful method widely used to mutate genes in embryonic stem (ES) cells (1). In mammalian cells, DNA integration into specific chromosomal loci is very rare compared with random integration events. Different approaches have been used to identify the small fraction of cells in which homologous recombination took place. The 'positive–negative' protocol developed by Mansour *et al*. (2) is based upon the additional cloning of the herpes simplex virus-thymidine kinase (HSV-*tk*) gene in one or both ends of the replacement vector which contains the *neo* gene. This strategy relies on the principal that those cells in which random integration has occurred will retain the HSV-*tk* gene and thus, will be specifically eliminated by the antiviral agent gancyclovir (GANC). By this means an enrichment in properly targeted clones is obtained. Although this approach is being widely used, the cloning of additional sequences in an already large plasmid carrying long regions of homologous sequences (7–10 kb) to improve targeting efficiency (3,4) is a laborious and time-consuming procedure, often accompanied by additional difficulties, such as plasmid instabilities and limited choices of unique restriction sites. Furthermore, the handling and the purification of plasmids >15 kb is not easy. Moreover, when the goal is not simply to disrupt the gene of interest but rather to replace it by introduction of a reporter gene such as *lacZ* in addition to the *neo* gene, the cloning of additional sequences to achieve enrichment can be a particularly difficult task.

In this report, we present an alternative procedure to introduce any negative selection marker at the ends of a replacement vector using a simple ligation reaction, thereby avoiding laborious cloning procedures and plasmid size limitations. Briefly, the plasmids carrying the targeting sequences and the negative marker are first linearized by two enzymes (in our case *Spe*I and *Hin*dIII, respectively) that become compatible only after a 2-base partial fill-in reaction. If necessary, appropriate restriction sites could be easily created by the use of adaptor and linker methodology. The partial fill-in of the ends of the two plasmid molecules prevents their circularization during the ligation reaction, and additionally ensures the absence of intermolecular products in the reaction (e.g. replacement vector:replacement vector or negative marker:negative marker). Furthermore, a 1.5–1.8× molar excess of the plasmid containing the negative marker in the ligation reaction will favour the formation of products that will carry the latter at least at one end of the replacement vector. It is important to note that the employment of conventional cloning procedures to introduce the negative selection marker into the targeting vector requires at least 10 days of laborious work, while using this alternative method, the final linear form of the replacement vector can be obtained ready to use within 1 day. Additionally, potential problems concerning the structural integrity of the replacement vector due to plasmid instabilities or purification procedures are avoided.

We have tested the above procedure with three different replacement vectors: FGF-5-neo (10.5 kb), FGF-5-LacZ (14.4 kb) (G.T., unpublished results) and Bcl-2-LacZ (15.6 kb) (5). As a negative selection marker we used the HSV-*tk* gene under the control of the phosphoglycerate kinase gene (PGK) promoter (6). The ligation reaction (5 µg, 20 µl) was carried out for 4 h in the presence of 600 U T4 DNA ligase (New England BioLabs). The end products of the ligation reaction, including appropriate controls, were monitored by agarose gel electrophoresis. In each case, 0.5×10^6 D3 ES cells were transfected by electroporation with 5 µg of ligated linear DNA (7). Prior to transfection, the ends of the DNA molecules used in either co-ligation or co-transfection experiments were modified by a dideoxynucleotide addition reaction (8). The results of these experiments are shown in Table 1. A high level of enrichment (2.4–3.6-fold) of the efficiency of negative selection was observed for each of the three co-ligated vectors. Co-transfection of the PGK-*tk* plasmid and the replacement vector were made using the same molar ratio as in the corresponding co-ligation experiments (1.5–1.8×) provided only a 1.5-fold enrichment. In all cases, the identification of properly targeted clones was done using suitable probes to characterize in

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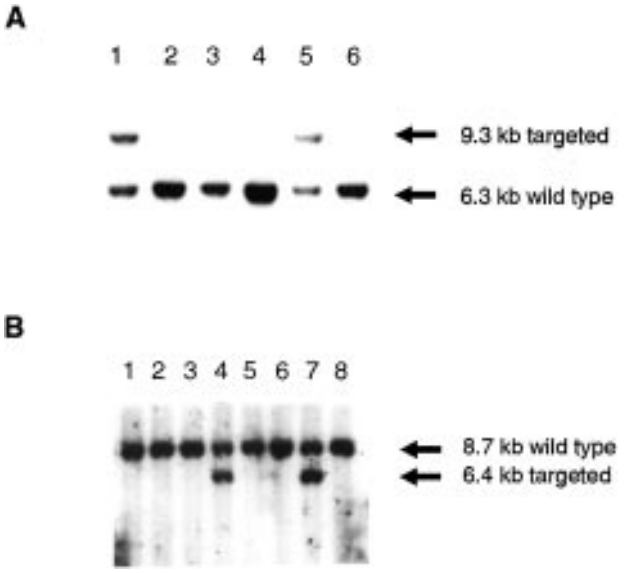

Figure 1. Representative Southern blot analysis of selected ES clones. Genomic DNA derived from individual double-drug resistant ES clones, obtained from the co-ligation experiments, was digested with the restriction enzymes *Eco*RIx*Kpn*I (*bcl-2*) and *Eco*RI (*fgf-5*) and subjected to Southern blot analysis. In both cases the DNA fragments used as probes, contained sequences outside of the homologous genomic region included in the targeting vectors. (**A**) *bcl-2* disruption: ES clones carrying a properly targeted allele are represented in lanes 1 and 5; (**B**) *fgf-5* inactivation: properly targeted recombination ES clones are shown in lanes 4 and 7.

detail the ends of the homologous recombination events. Real homologous recombination events were obtained with FGF-5-LacZ and Bcl-2-LacZ co-ligated vectors (Fig. 1). Moreover, in the case of the Bcl-2-LacZ co-ligation experiment, proper homologous recombination events were observed in 2 out of 25 clones analysed, whereas in the corresponding co-transfection experiment no homologous recombination was detected in 48 clones tested (5). Targeted clones obtained from these co-ligation experiments were used to generate Bcl-2 (5) and FGF-5 (9) deficient mice.

It is important to note that using the same PGK-*tk* cassette cloned into two different replacement vectors the enrichment was only 1.5–1.8-fold. Although these vectors were designed to target other loci than those employed in our study, the higher efficiency of the negative selection observed in our procedure as compared with the conventional cloning can be explained by the fact that the HSV-*tk* cassette is not readily accessible to exonucleolytic degradation either due to the presence of the negative selection marker to some extent at both ends of the targeting vector and/or to the presence of plasmid sequences flanking the PGK-*tk* cassette (10). It is well known that the inefficiency of the GANC selection is mostly caused by exonucleolytic degradation progressing from the ends of the targeting vectors (11).

Table 1. Comparison of the effect of co-transfection and co-ligation on the efficiency of the negative selection

Vector		Number of colonies		Enrichment
		$G418^r$	$G418^r/GANC^r$	(in fold)
FGF-5-neo	co-transfection	204	136	1.5
	co-ligation	158	65	2.4
FGF-5-LacZ	co-transfection	120	78	1.5
	co-ligation	112	42	2.7
Bcl-2-LacZ	co-transfection	138	102	1.4
	co-ligation	138	38	3.6

In conclusion, this fast and simple method to introduce a negative selection marker into a targeting replacement vector provides a high efficiency of negative selection, thus significantly reducing the amount and time of work needed to screen and identify clones that have undergone homologous recombination.

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