yeast HSP104 gene (11), making it also available for the COOL procedure.

Although examples shown here are of plasmids used in yeast, the COOL procedure could be applied in any system. Notably, although BamHI, BgIII, and XhoI were used as examples here, any combination of restriction enzymes is valid for the procedure. We are using the procedure routinely for short sequences (up to 70 bp), but the procedure could be applicable to an insert of any length. It should be noted, however, that if long sequences are used, in particular in several repeats, verification by sequencing could be problematic. It is recommended for these cases to apply one unique insert (using the COOL procedure) that possesses a sequence that does not appear in other repeats and could serve as a recognition site for a sequencing primer.

REFERENCES


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Co-targeting a selectable marker to the Escherichia coli chromosome improves the recovery rate for mutations induced in BAC clones by homologous recombination

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Mice are a powerful genetic system for investigating human biology. The ability to express genes in specific tissues using transgenic mice and target mutations to any gene by homologous recombination in embryonic stem (ES) cells has greatly facilitated the functional genomic analysis of the mouse genome. However, it has become increasingly clear that the regulation of gene expression is complex and may require cis-acting elements that act at distances larger than most transgenic constructs. Bacterial artificial chromosome (BAC) clones are one possible solution to this problem. BAC clones are stably maintained in Escherichia coli and contain large inserts that are likely to contain all the cis-acting regulatory elements of a gene (1). Unfortunately, the manipulation of BAC clones by conventional molecular biology techniques is difficult. To address this problem, several groups have developed strategies that utilize homologous recombination in bacteria as a method to manipulate BAC clones in several ways, including targeting mutations into BAC DNA, inserting selectable marker and other sequences into BACs, and cloning specific sequences from BACs (2,3). This type of manipulation has been termed recombineering. Several mechanisms have been employed to initiate homologous re-

Figure 2. Validation of the controlled and ordered oligonucleotide ligation (COOL) procedure. An insert with XhoI and BamHI ends and a BglII site (similar to the one shown in Figure 1) was inserted in four cycles to the BamHI + XhoI digested –178(CYC1)-LacZ plasmid (10). PCR products were separated on 2% agarose gel and visualized on a UV table.
combination in BAC clones, and the two most frequently used are RecA-dependent recombination and ET recombination (RecE, RecT), which utilizes bacteriophage proteins (4–6). Both of these techniques can generate point mutations, deletions, and insertions into BAC sequences in a highly efficient manner. In practice, however, recombinining requires the construction of a targeting construct that contains a selectable marker, which is removed either by a second recombination event or, if the marker is flanked by specific sites, it can be removed by a site-specific recombination system such as cre-lox. In the former, multiple recombination steps are required and, in the latter, a single recombination site is left behind (3). Recent work using the λ phage recombination system and oligonucleotides showed that point mutations, deletions, and a 24-bp insertion could be targeted to a BAC containing the Brca2 gene without the need for a selectable marker (7). However, in this report, pools of potential recombinant clones were screened by PCR, and the highest frequency of recombination observed was 1:90 for a point mutation. Although this frequency is acceptable, the insertion of a larger sequence such as green fluorescent protein (GFP) is not feasible using oligonucleotides. Here we show that the recovery of targeted mutations in BAC clones using constructs lacking a selectable marker is significantly improved when a selectable marker is co-targeted to the E. coli chromosome.

The murine Mst1r locus encodes a receptor tyrosine kinase that is produced from two mRNAs, one that encodes a full-length receptor and a second that initiates from an internal promoter that encodes a truncated form of the receptor, called short form Stk or SF-Stk. This truncated form of the receptor is required for the pathogenesis of Friend erythroleukemia virus (8,9). In Friend virus-infected cells, the envelope glycoprotein of Friend virus, gp55, forms a complex with SF-Stk and the erythropoietin receptor (EpoR), which stimulates the proliferation of infected cells. In order to analyze the biochemistry of SF-Stk signaling during Friend virus infection, we wanted to generate a transgenic mouse line that expresses an epitope-tagged form of SF-Stk. Previous work had shown that an amino-terminal Myc-tag was compatible with the SF-Stk function (9). To ensure the proper expression of the transgene, we proposed to insert the Myc-tag into the Mst1r locus contained in a BAC clone by homologous recombination using the λ phage recombination system. We identified a BAC clone containing the Mst1r locus using the BAC tiling path generated by the mouse genome sequencing project and obtained this clone from the BACPAC Resource Center (Children’s Hospital Oakland Research Institute, Oakland, CA, USA) (10). This BAC clone was derived from C57BL/6 mice, which are resistant to Friend virus because of the 3-bp deletion in the SF-Stk promoter (8). Thus, two targeting events were required: first, using oligonucleotides, the 3-bp deletion was repaired, and second, using a 270-bp PCR fragment, we targeted the Myc-tag to the amino-terminus of SF-Stk.

The Mst1r-containing BAC clone was isolated using a NucleoBond® Plasmid Maxi Kit (BD Biosciences Clontech, Palo Alto, CA, USA). BAC DNA was electroporated into the DY380 strain, which contains a defective λ prophage (11). The expression of the recombination genes gex, bet, and gam is induced by shifting the culture temperature to 42°C for 15 min, and recombimogenic electroporation-competent cells were generated as previously described (5,11,12). To facilitate the recovery of correctly targeted BAC clones, we employed a new strategy whereby the Kanamycin resistance gene (KanR) was targeted to the E. coli galK gene in concert with the mutation in SF-Stk in the same electroporation reaction. The principle behind this strategy is to use an excess of SF-Stk targeting vector so that the probability that a KanR clone also contains the SF-Stk mutation is high (Figure 1A).

The galK targeting construct was generated by PCR using primers that contained 50 bp of galK homology, followed by 20 bp of homology to the KanR gene from pEGFPN3 (BD Biosciences Clontech), which resulted in a 1500-bp fragment that contained the KanR gene-flanked galK homology on either end. The sequences of the primers were galK-KanR forward 5’-GTTTGGCCGCGCATCGGATATC-CATTTCGCGAACGACTGTTGATA-GGCGAAACCCCTATTGTTTA-3’ and galK-KanR reverse 5’-TTTATAATTGTTCAGCGCACGTGCTGTCGAGCCGACCAGCTTCTCCCG-GAAATCTCCTGATGCGACGT-3’ (The underlined sequences indicate KanR homology.) The PCR conditions were 3 min at 95°C and 10 cycles of 1 min at 95°C, 1 min at 58°C, 2 min at 72°C, and then 20 cycles of 1 min at 95°C, 2 min at 72°C, followed by 1 cy-

**Figure 1. Co-targeting strategy.** (A) Outline of the general co-targeting strategy. (B) The SF-Stk repair oligonucleotide. The 3-bp insertion is indicated in bold. BAC, bacterial artificial chromosome.
clique of 7 min at 72°C. The 1.5-kb galK-KanR fragment was run on an agarose gel and purified.

Initial experiments showed that approximately 100 ng of the galK-KanR fragment would give rise to 50–60 KanR, CmR colonies. Kanamycin selection identifies potential galK-KanR recombinants, while chloramphenicol selects for BAC-containing colonies. Plating the colonies onto MacConkey galactose indicator agar (Sigma, St. Louis, MO, USA) showed that 70%–100% of the colonies contained insertions of KanR into galK (galK<KanR). To repair the 3-bp deletion in the SF-Stk promoter, we utilized two 43-mer oligonucleotides from complementary strands that could base pair through their 3’ 23 nucleotides. The additional 3 bp were contained in the middle of the double-stranded region (Figure 1B). Recent work has demonstrated that this arrangement of oligonucleotides is a very efficient recombination substrate in the λ-based recombination system (12). In the first experiment, 75% of galK oligonucleotide was used in conjunction with 100 ng of the galK-KanR fragment. This concentration represents approximately a 37-fold molar excess of oligonucleotide. Following electroporation, KanR, CmR colonies were selected, and a portion of these colonies were then tested on MacConkey galactose indicator agar. Seventy-one percent (25/35) of the KanR, CmR colonies had an insertion of KanR into galK. When 10 of those colonies were tested for repair of the 3-bp deletion by PCR, 20% were repaired. We repeated the experiment, but this time increased the amount of each oligonucleotide to 85 ng, which represented approximately a 42-fold molar excess of oligonucleotide. This time, 100% (35/35) of the KanR, CmR insertions were into galK, and 10 out of 10 galK<KanR colonies tested were repaired. These results demonstrate that by co-targeting a selectable marker to the E. coli chromosome, we can increase the efficiency of recovering targeted mutations in the BAC clones.

In the second step, we targeted a Myc epitope tag to the amino-terminus of SF-Stk. The repaired SF-Stk BAC clone was purified and electroporated into DY380 cells so that we could perform another round of the co-targeting strategy. The galK-KanR fragment was co-electroporated with a 270-bp PCR fragment that contained 50 bp of SF-Stk sequences flanking 6 repeats of the Myc epitope. In initial experiments, we utilized 140 ng of the Myc fragment and 115 ng of the galK-KanR fragment, which represents approximately an 8-fold molar excess of the Myc fragment over the galK-KanR fragment. One hundred percent (15/15) of the KanR, CmR colonies tested were insertions into galK, and 78% (7/9) of galK<KanR colonies tested had the Myc insertion. To confirm that the Myc epitope was correctly inserted in frame with the SF-Stk coding sequence, we PCR-amplified this region and sequenced multiple clones. In addition, the modified Stk BAC was fingerprinted by HindIII digest, which demonstrated that the two recombination events did not lead to unintended rearrangements of the BAC clone.

By co-targeting KanR to the galK locus on the E. coli chromosome, our strategy selects for bacteria that have undergone a recombination event. The use of an excess of BAC targeting construct ensures that a high frequency of BAC mutant clones will be identified in the subsequent screening of the galK<KanR colonies. Although we successfully targeted mutations with a 42-fold molar excess of mutant oligonucleotide and an 8-fold molar excess of the Myc tag construct, the optimal ratio of galK-KanR construct to mutant targeting construct will need to be determined for each mutation and BAC clone. This strategy also allows for the generation of mutations without the need to generate targeting constructs that contain selectable markers that will have to be removed later.

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REFERENCES


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