

A recombineering based approach for high-throughput conditional knockout targeting vector construction

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ABSTRACT

Functional analysis of mammalian genes *in vivo* is primarily achieved through analysing knockout mice. Now that the sequencing of several mammalian genomes has been completed, understanding functions of all the genes represents the next major challenge in the post-genome era. Generation of knockout mutant mice has currently been achieved by many research groups but only by making individual knockouts, one by one. New technological advances and the refinements of existing technologies are critical for genome-wide targeted mutagenesis in the mouse. We describe here new recombineering reagents and protocols that enable recombineering to be carried out in a 96-well format. Consequently, we are able to construct 96 conditional knockout targeting vectors simultaneously. Our new recombineering system makes it a reality to generate large numbers of precisely engineered DNA constructs for functional genomics studies.

INTRODUCTION

Sequencing and analysis of the mouse and human genomes have led to the identification of ~25 000 genes as well as hundreds of conserved non-coding regions (1–3). Functional characterization of the genes and the potential regulatory elements genome-wide represents a major challenge in the post-genome sequencing era. While there are a range of experimental and informatics tools that address gene function, analysing knockout (KO) mice produced through gene targeting in mouse embryonic stem (ES) cells is still the most widely used approach to understand mammalian gene

function (4). KO mutant lines have been generated for hundreds of mouse genes (5). Analyses of these mutant mice have provided fundamental insights into mammalian gene functions. In general, there are several phenotypic outcomes in the KO mice. The mutant mice may have the exact phenotypes anticipated based on a gene's expression pattern, *in vitro* assays, bioinformatics predictions or symptoms in human patients. Yet, in some cases, the mutant mice may not have any obvious phenotype, which might be due to genetic redundancy, nature of the KO alleles, genetic background effects, investigator's knowledge or incomplete phenotyping spectrums. For other genes, the mutants die *in utero* owing to the critical roles of these genes in embryonic development, thereby precluding studying their functions in late development or in specific tissues of adult mice.

To circumvent the embryonic lethality problem and to investigate gene function temporally and spatially, conditional knockout (cko) approaches have been developed (6,7). The current cko strategy takes advantage of the Cre-*loxP* site-specific recombination system that functions well in mouse cells (8). In a typical cko allele, the critical exon(s) of a gene is flanked by two *loxP* sites so that it can be deleted by spatial and temporal Cre expression. With the advances of DNA manipulation technologies, it is now possible to generate a multi-purpose allele of a gene that can serve as a conventional KO, a conditional KO and a reporter allele (9).

The first step in generating a cko allele is to construct a targeting vector in *E. coli* that is subsequently transfected into mouse ES cells for homologous recombination. We and others have previously described methods for constructing targeting vectors using recombineering, which is based on highly efficient homologous recombination systems from bacteriophages (10–15). In the system using homologous recombination encoded by λ phage that we developed, a temperature-dependent repressor

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(cI857) tightly controls λ prophage expression, and, thus, the recombination functions of λ phage can be transiently supplied by shifting cultures to 42°C for 15 min (14,16). Three proteins encoded by λ are required for the homologous recombination function in recombinering (17–19): Exo, Beta and Gam. Exo is a 5′–3′ exonuclease that acts on linear double-strand DNA (dsDNA) to generate 3′ single-strand DNA (ssDNA) overhangs for recombination. Beta binds to the ssDNA overhangs that are longer than 35 nt and stimulates complementary strand annealing. The recombination is further enhanced by Gam, which inhibits RecBCD-dependent, linear DNA degradation activities of the host cell. The λ prophage-based recombinering systems have proven to be very efficient due to the fact that the three genes are expressed in single copy from their natural operon and from the strong promoter, p_L , and that the three proteins are in appropriate molar ratio as they form a complex *in vivo*. The tightly regulated expression also ensures that the Beta and Exo proteins are present only after induction. This, together with the short induction time (15 min) for the expression of the recombination activities, help to minimize the potential genomic rearrangements caused by prolonged presence of the phage Red recombination proteins. To make a cko vector from a BAC, the first step is usually to transform the BAC DNA into recombinering-competent *E. coli* cells. Subsequently, selection cassettes with *loxP* sites are inserted via recombinering into genomic regions that flank the exon to be deleted (10).

We describe here a high-throughput recombinering system, which can generate conditional targeting vectors in a rapid efficient manner. A key reagent for this new system includes a complete λ phage that is replication defective in BAC-harboring DH10B *E. coli* cells but still retains its full heat-inducible homologous recombination functions. A second reagent useful for high-throughput recombinering is a set of low-copy plasmids (pSim) that contain *exo*, *bet* and *gam* in their native phage operon, p_L , under λ CI repressor control (20). By using cells carrying either of these two new reagents, thousands of BAC clones can be easily made recombinering competent by either simple λ infection or plasmid transformation. Further improvement of recombinering in these systems allowed all the steps for making targeting vectors to be performed in 96-well plates, making the whole process suitable for high-throughput operations. Our new recombinering system described here has solved a key technical bottleneck for the genome-wide targeted mutagenesis programmes in the mouse (5,21).

MATERIALS AND METHODS

Construction of the replication-defective phage

The replication-defective λ phage, λ cI₈₅₇ *ind1* Cro_{TYR26amber} P_{GLN59amber} *rex*< >*tetRA*, was created by combining several different mutations to mimic the defective prophage strain that we have used for most of our recombinering studies. The cI857 mutation allows temperature induction by inactivating the CI repressor.

The Cro mutation enhances p_L operon and recombination gene expression analogous to the Cro deletion of the defective prophages. Wild-type λ lysogens die within 7 min after a normal prophage induction due to *in situ* λ DNA replication in the bacterial chromosome carried out by O and P functions of λ (22). The P mutation causes a defect in λ replication allowing induction of the prophage for at least 15 min. Replication-competent lysogens kill the host with less than a 10-min induction. The *tetRA* genes are added to select for the presence of the lysogenic prophage in the strain using tetracycline resistance.

The construction of λ cI₈₅₇ Cro_{TYR26amber} was described previously (23). The amber mutation in Cro is at codon 26 changing it from a tyrosine TAT codon to the amber TAG codon. A high-titre lysate was prepared from this mutant phage on strain N720, which contains the *supF* amber suppressor for tyrosine tRNA allowing Cro expression by the phage. Note that this phage mutant does not form plaques on a non-suppressor strain like W3110 or DH10B or even on a *supE* glutamine suppressor tRNA strain; presumably because glutamine cannot be substituted for tyrosine at this position in Cro.

The mutation P_{GLN59amber} was first created by Campbell (24) as an amber (*sus*) mutation in the λ replication gene P at glutamine codon 59. This phage mutant called λ P80 forms plaques on the C600 *supE* suppressor strain but not on DH10B or the N720 *supF* strain, i.e. just the reverse of the Cro mutation above. This mutation is a C>T change in position 39759 of the λ DNA sequence. A high-titre lysate was prepared from this mutant phage on strain C600. From the pure phage preparation, we amplified the entire P gene carrying the amber mutation by PCR using Invitrogen Platinum HiF polymerase and cleaned the product with a Qiagen kit. We next did recombinering with the phage λ cI₈₅₇ Cro_{TYR26amber} by crossing it with the linear DNA PCR product carrying the P80 mutation using the protocol outlined in the Oppenheim's article above (23).

We detected the recombinant phage containing both the Cro and P amber mutations on a tryptone agar Petri plate overlaid with two bacterial top agar layers of 2.5 ml each. In the first agar layer was the N720 bacterial strain on which the Cro amber mutant parent phage could form plaques but on which the double Cro P amber mutant would not form plaques because of the P80 mutation. In the top layer was the LE392 strain, which allows both amber mutations to be functionally suppressed and which is used for infection by phage from the recombinering cross described above. The desired double mutant, recombinant λ cI₈₅₇ *ind1* Cro_{TYR26amber} P_{GLN59amber} is distinguished from the parental λ cI₈₅₇ Cro_{TYR26amber} because the P mutant forms cloudy plaques due to its inability to grow on the bottom layer, and the P⁺ parental phage forms clear plaques by growing on both layers. These cloudy recombinant plaques were purified on LE392 and tested for plaquing on LE392, N720 and C600. As expected, they formed plaques efficiently only on the LE392 double suppressor strain. We amplified the DNA of the P gene from the recombinant and demonstrated that it contained the mutation P80 creating a BfaI site.

We next modified the phage by replacing the *rexArexB* genes with *tetRA* encoding tetracycline resistance. This was done following the same procedure as outlined previously (23) using recombinering with the λ *cI₈₅₇ ind1 Cro_{TYR26amber}P_{GLN59amber}* recombinant phage and a PCR product of the *tetRA* genes with flanking homologies designed to delete the *rexArexB* genes. The recombinering was done in the LE392 derivatives. Following the recombinering cross, the resultant phage lysate was used to infect LE392, and tetracycline-resistant lysogens were selected on LB-tet plates at 32°C. Lysogens were purified and tested for sensitivity to phages λ , *λimm21*, T4, and T4 *rII*. The strain was resistant (immune) to λ but not *λimm21* or T4 as expected for a λ lysogen. This λ lysogen was sensitive to T4 *rII* which is expected if the T4 *rII* exclusion functions RexAB are absent. The lysogen was induced to produce a phage lysate by shifting an exponentially growing culture in LB to 42°C for 15 min, and then shifting to 39°C until cell lysis (60–90 min). For long term storage, the λ lysate was prepared by centrifuging the lysed culture with a couple drops of added chloroform. We routinely obtained λ lysates with titres greater than 10⁹ p.f.u./ml.

Construction of pSim plasmids

Plasmid pSim6 was constructed by recombination the λ defective prophage into pSC101 plasmid backbone (20). The Amp^R coding sequence was present as a substitute for the *rexAB* genes. We made pSim17 and pSim18 by replacing *amp* in pSim6 with either Blastidicin-or Hygromycin-resistant coding sequences using the recombinering functions expressed from the plasmid.

PCR products for recombinering

All DNA oligos in this study were purchased from Sigma. Linear templates to be used for PCR amplification were prepared as follows: The retrieval vector (PL611) was digested with EcoRI and BamHI, the *loxP-F3-PGK-EM7-Neo-F3* selection cassette was cut with NotI and SalI, and the *I-SceI-Bsd-I-CeuI* cassette was digested with EcoRI and BamHI. The digestion mixtures were run on a gel and bands corresponding to *Bsd* (526 bp), *Neo* (2 kb) and PL611 (3 kb) were excised and purified using Qiagen purification kit. To avoid the background from uncut plasmids carrying these cassettes, we tested the gel-purified PL611 and the two selection cassettes by transforming 1.0 ng of them into DH10B cells. If there were any background drug-resistant colonies, the cassettes were re-purified and tested again until they were clean, thereby ensuring that all the Bsd^R or Kan^R cells selected out in liquid media after recombinering should be the recombinant ones. The restriction maps of the selection cassettes and PL611 are depicted in Supplementary Figures 2–6.

To generate products for targeting or retrieving, 1.0 ng of each of the three linear purified products above was used as a template for PCR reaction. PCR amplification was carried out using Extensor Hi-Fidelity PCR Master Mix 2 (2X, ABgene). Twenty-five microlitres of the master

mix was added to 1 μ l of template (1.0 ng), 2 μ l of each primer (10 μ M) and 20 μ l of PCR-grade water. PCR was performed using PTC-225 PCR machine (Peltier Thermal Cycler) with the following settings: 94°C for 4 min, this was followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 1 min (*Bsd*) or 2–3 min (*Neo* and retrieval backbone). This was then followed by 68°C for 5 min. After PCR reactions, 0.5 μ l of exonuclease I (10 U, from either New England Biolabs or Epicentre) was added per 50 μ l of PCR products and incubated at 37°C for 1 h followed by heat inactivation at 80°C for 20 min. The PCR products were then purified using Qiagen mini-preparation columns and eluted in 50 μ l of PCR-grade water.

For 96-well operations, all primers were synthesised in 96-well plates so that they could be used directly in a 96-well PCR reaction plate. After Exo I treatment and heat inactivation, PCR products were precipitated with ethanol in 96-well PCR plates. Here, 100 μ l ethanol with 2 μ l 5 N NaCl were added to each well of 50 μ l PCR reaction. DNA was precipitated by centrifuging the plate at 3700 r.p.m. (2250 g) for 30 min and washed once with 70% ethanol. Air-dried PCR products were dissolved in 100 mM CaCl₂ for chemical transformation. Alternatively, for electroporation, DNA was dissolved in 50 μ l water. Three micrograms of amplified DNA was usually obtained from one PCR reaction.

Making one or a few targeting vectors using the mobile recombinering reagents

Targeting selection cassettes to BACs.

(1) Using replication-defective λ phage

Day 1. Inoculate BAC cells into 1 ml LB with Chloramphenicol (12.5 μ g/ml), 1% maltose, for overnight growth at 37°C with shaking.

Day 2. Cells were collected and washed once with 1 ml 10 mM MgSO₄ and resuspended in 100 μ l 10 mM MgSO₄. One microlitre containing greater than 1 million lambda phage was added to 100 μ l of the BAC cells and the mixture was incubated at 32°C for 20 min. One millilitre LB was added to the tube that was incubated for another hour before the cells are plated to a Tet-LB plate. The plate was incubated at 32°C for overnight. The lysogenization frequency was ~1%, as expected, and infection yielded ~10 000 Tet^R colonies.

(2) Using pSim plasmids

Day 1. Inoculate BAC cells into 1 ml LB with chloramphenicol (12.5 μ g/ml), for overnight growth at 37°C with shaking.

Day 2. Cells were collected and washed with cold water three times and were electroporated with pSim18 (1.0 ng) in 50 μ l water. Electroporation condition: 200 Ohms (capacitance, 25 microFD, 0.1 cm gap, 1.8 kV). The transformation mixture was added with 1 ml LB and was incubated for 1 h at 32°C. The cells were plated onto a LB-Hygro (75 μ g/ml) plate and incubated at 32°C for overnight.

If chemical transformation was used, cells were collected and were washed with 100 mM MgCl₂, 100 mM CaCl₂ and then with 100 mM CaCl₂. Finally, cells were resuspended in 50 µl 100 mM CaCl₂ with 1.0 ng pSim18. The cell-DNA mixture was heat shocked at 42°C for 2 min. The transformation mixture was added with 1 ml LB and was incubated for 1 h at 32°C before plated onto a LB-Hygro plate. The plate was incubated at 32°C for overnight.

Day 3. Pick up one Tet^R or Hygro^R colony and inoculate into 1 ml LB with chloramphenical and tetracycline (12.5 µg/ml each) or hygromycin (75 µg/ml), for overnight growth at 32°C with shaking.

Day 4. Inoculate 25, 35, 45 and 55 µl of the overnight culture into four 15-ml tubes (or into four wells of a 96-well deep plate) with 1 ml fresh LB in each tube. Shake at 32°C for 2 h. Without measuring OD, transfer the cells from the four tubes into wells of a 42°C heat block (Grant Instrument, Cambridge, UK). Incubate for 15 min without shaking. Transfer the heat block to an ice bucket. The temperature of heat block dropped to 0°C within 2 min. The metal heat block was chilled on ice for 5 min. Cells were transferred to four 1.5-ml eppendorf tubes and centrifuged at maximum speed for 25 s and combined at the washing steps. For using electroporation, cells were washed three times with cold water, electroporated with Bsd PCR product (3 µg) in 50 µl water. For using chemical transformation, cells were collected and were washed once with 100 mM MgCl₂, 100 mM CaCl₂ and then with 100 mM CaCl₂. Finally, cells were resuspended in 50 µl 100 mM CaCl₂ with 3 µg Bsd PCR product. Here, 1 ml LB was added to the transformation mixture, which was incubated for 1 h at 32°C before plated onto a LB-Bsd (75 µg/ml) plate. Alternatively, the transformation mixture was added to one well of a 96-well plate that had 1 ml Bsd-LB media (75 µg/ml).

Day 5. Pick up 10 Bsd colonies to test whether they still retained the replication-defective λ phage or pSim18 by growing them either in LB-Tet or LB-Hygro. The positive cells were used for the next round of recombineering-targeting *Neo* cassette to the BACs. If Bsd^R cells were growing in a 96-well plate, 150 µl of the cells were transferred to another well in the same 96-well plate that had 1 ml of Bsd-Tet-LB or Bsd-Hygro-LB media to select for retaining the prophage or pSim plasmid.

Recombineering *Neo* cassette was performed exactly as described above except recombinants are selected on LB-Kan plates (20 µg/ml).

Retrieving. Day 1. Bsd^R-Kan^R-Tet^R (λ lysogen) or Bsd^R-Kan^R-Hygro^R (if using pSim18) BAC cells were inoculated into 1 ml LB with the antibiotics for overnight growth at 32°C.

Day 2. Recombineering was performed as described above using either electroporation or chemical transformation with PCR-amplified PL611 (~3 µg). Transformants were plated on an Amp plate (50 µg/ml) and grew at 32°C for overnight. Alternatively, transformant mixture was inoculated into 1 ml LB with 50 µg/ml ampicillin and grew overnight at 32°C.

Day 3. Individual Amp^R colonies were inoculated into LB-Amp media. Alternatively, if transformation mixture was inoculated into LB, plasmid was prepared from the Amp^R liquid culture and 1.0 ng plasmid was transformed into DH10B or recombineering-competent *E. coli* cells (EL350 and DY380). The transformants were selected in 1 ml Kan-LB media.

Day 4. Plasmid mini-preparation.

Recombineering for making 96 targeting vectors

The procedure for manipulating 96 BACs is essentially the same as described above for making the *Meox1* targeting vector. All BACs in this study except Bcl11a BAC (C3) were from the end-sequenced and indexed mouse 129 BAC library (25) and were ordered from the Sanger Institute Archive group (http://www.sanger.ac.uk/cgi-bin/software/archives/new_clone_login.cgi). The flow chart in Figure 4 illustrated the recombineering steps for vector construction in 96-well plates. We used a 12-channel pipette (Matrix) that has 850 µl capacity in the experiments. Therefore, all recombineering steps in 96-well plates used 850 µl LB for the 2 h growth of diluted overnight cultures.

Day 1. Individual BACs were inoculated into 850 µl LB medium with 12.5 µg/ml Chloramphenical, 1% maltose in a 96-well deep well plate and grew at 37°C overnight.

Day 2. Cells were collected by centrifugation at 3700 r.p.m. (2250 g) at 4°C for 5 min and were resuspended in 50 µl 10 mM MgSO₄ solution. Here, 1–10 µl phage lysate was added to the cell suspension. The mixture was incubated at 32°C for 20 min. This was followed by the addition of 500 µl LB and 1 h incubation at 32°C. Lysogens were selected in Tet-LB media (by adding 500 µl Tet-LB at 25 µg/ml) at 32°C.

Day 3. 25, 35, 45 and 55 µl of the Tet^R overnight culture (OD₆₀₀ ~1.2–1.4) was transferred to 850 µl fresh LB in 96-well plates so that cultures from one original 96-deep-well plate were eventually transferred to four 96-well plates (Figure 5).

After 2-h growth at 32°C, cells were transferred to four 96-well metal heat-blocks at 42°C. Cells were kept at 42°C for 15 min for induction of recombination functions and then the heat block was immediately put into wet ice so that the temperature of the block was lowered to 0°C within 2 min. The cooled induced cell cultures were transferred back to regular 96-well plates that were centrifuged at 3700 r.p.m. for 5 min at 0°C.

In the chemical transformation protocol, the cells from four individual 96-well plates were combined and suspended in 850 µl 100 mM MgCl₂, 100 mM CaCl₂, pelleted again and washed with 100 mM CaCl₂. These cells were finally suspended in 50 µl ice-cold 100 mM CaCl₂ containing the PCR product. The DNA-cell mixture was heat shocked at 42°C for 2 min in a PCR machine and transferred to a 96-well plate with 500 µl fresh LB. The plate was incubated at 32°C for at least 2 h before 500 µl Bsd (150 µg/ml)-LB media was added to each well.

Day 4. 150 µl overnight culture was transferred into fresh Bsd (75–100 µg/ml)-Tet (12.5 µg/ml)-LB media and

cultured for another 24–48 h for cell growth to reach saturation.

Day 5. cultures were ready for targeting the *Neo* cassette to the BACs. The protocol of targeting the *Neo* cassette to BACs in a 96-well plate was performed using the same procedure for targeting the *Bsd* cassette as described above, except Kan was used in the selection.

Once the Bsd^R/Kan^R/Tet^R BAC cells were obtained, the specific genomic DNA fragments were retrieved to PL611.

Day 1. Overnight cultures of Bsd^R/Kan^R/Tet^R BAC cells at 32°C were inoculated into fresh LB for 96-well recombineering using PCR-amplified PL611. Recombinant cells carrying the retrieved plasmids were selected in Ampicillin LB media. Because retrieving a genomic fragment from a BAC was generally less efficient than targeting a small selection cassette to a BAC, we obtained fewer colonies after retrieving. As a result, it may take up to 2 days to grow enough Amp^R cells for plasmid preparation from 850 µl culture in each well.

Day 3. 96-Well plasmid preparation. We prepared plasmids using a simple isopropanol/ethanol precipitation protocol for the crude DNA preparations that were sufficient for re-transformation to remove the background plasmids. Briefly, Amp^R cells in each well of a 96-well plate were pelleted and suspended in 250 µl P1 solution (from Qiagen). This was followed by the addition of 250 µl P2 solution to each well. Finally, 350 µl N3 solution was added to each well and mixed. The plate was centrifuged at 3700 r.p.m. for 20 min at 4°C. The supernatant from each well was transferred to a new 96-well plate with 750 µl isopropanol in each well. DNA was pelleted by centrifuging the plate at 3700 r.p.m. for 30 min at 4°C. Plasmid DNA was washed with 70% ethanol, air dried and finally dissolved in 50 µl water. One microlitre of the plasmid DNA was transformed into either customarily or commercially made preparations of chemical competent DH10B cells. Transformants were selected in 850 µl Kan-LB media (20 µg/ml).

Day 4–5. Plasmid DNA was isolated using a commercial 96-well plasmid kit. To confirm the identities of the retrieved plasmids, they were usually either sequenced at the plasmid backbone-genome insert junctions or digested for restriction patterns.

Replacement of the *Bsd* cassette with the *lacZ* reporter

The *lacZ* reporter cassette was isolated from PL613 respectively with I-SceI and I-CeuI double digestion (purchased from New England Biolabs). The purified fragment was used for ligating to the retrieved plasmid digested with I-SceI and I-CeuI.

In a typical ligation, 10 µl of purified digested retrieved plasmids, 12 µl of digested PL613, 2.5 µl of T4 DNA ligase buffer and 1 µl of T4 DNA ligase (NEB). This reaction was incubated at room temperature for 2 h. Here, 5 µl of the ligation products was transformed into commercially purchased chemical competent cells, or to DH10B-λ competent cells for the next recombineering step to introduce the *MCITK* cassette to the plasmid backbone. The transformed cells were selected on Puro/Kanamycin plates or in Hygromycin/Kanamycin LB medium,

depending on which reporter was chosen. Puromycin media (powder) was purchased from InvivoGen.

Recombineering of the *MCITK* cassette

To add *MCITK* to the targeting vectors for negative selection in ES cells, we constructed a cassette where *MCITK-Cm* cassette that is flanked by ~600 bp sequences identical to *pBR322*. This enabled us to target *MCITK* to the retrieval plasmid backbone by simple recombineering. *MCITK-Cm* plasmid DNA was digested with NotI and SalI. The 4.2-kb fragment was purified as described above for recombineering.

The targeting vectors were transformed into DH10B-λ cells that were selected in Kan/Amp-LB media in 96-well plates. Overnight cultures were diluted into fresh LB and incubated for a further 2 h. These cells were then heat shocked at 42°C for 15 min to induce recombination activities and were processed for chemical transformation. We used 100 ng purified *MCITK-Cm* cassette for each recombineering reaction. The recombinant cells were selected out in Kan/Cm-LB.

Cre or Flpe recombinase expression

To confirm the functionality of *loxP*, *FRT* and *F3* sites in the targeting vectors, the targeting vector plasmid DNA was transformed into EL350 and EL250 *E. coli* cells that express Cre and Flpase respectively upon L-arabinose induction. The procedure was performed as described previously (10,14).

Genotyping ES cells by long-range PCR

AB2.2 ES cells transfected with the linearized targeting vectors were selected in 150 µg/ml G418 (active component) and 2 µM ganciclovir (Ganc). Genotyping targeted ES cell clones by long-range PCR was performed as following. ES cell colonies were picked into 96-well feeder plates. After 3 days, cells were trypsinized in 50 µl trypsin for 10–15 min with subsequent addition of 50 µl ES cell media. Here, 30 µl was disposed into two 96-well cell culture plates that were gelatinized for DNA preparation. For the remaining cells (~30 µl), 30 µl 2× freezing media (DMEM, 20% FCS, 20% DMSO) was added and mixed. Cells in the plate were kept in a sealed styrofoam box at –80°C. Cells in the DNA plates were allowed to grow for further 2 days and DNA were prepared as previously published (26). DNA was dissolved in 30 µl water. Here, 2 µl of DNA was diluted in 18 µl water in a 96-well PCR plate and heated at 99°C for 10 min using a PCR machine to denature DNA. The plate was centrifuged at 3700 r.p.m. for 5 min. Here, 1 µl of the denatured DNA was used for the long-range PCR. The remaining DNA in the original DNA plate was used for restriction digestion and Southern blotting.

PCR amplification was carried out using Extensor Hi-Fidelity PCR Master Mix 2 (2×, ABgene). Here, 12.5 µl of the master mix was added to 1 µl of template, 1 µl of each primer (10 µM) and 9.5 µl of PCR-grade water. PCR was performed using PTC-225 PCR machine (Peltier Thermal Cycler) with the following settings: 94°C for 4 min, this was followed by 35 cycles of 94°C for 30 s,

60°C for 30 s and 68°C for 6 min (6–7 kb) or 3 min (2–3 kb). This was then followed by 68°C for 10 min.

Primers for genotyping *Meox1* targeted ES cell clones:
For 5' side diagnosis:

Meox1-5'-F: 5'-CTGATGCTCCACCTCTGTTGCTA
GCACACT

LacZ reverse: 5'-CAAGGAAACCCTGGACTACTGC
GCCCTA

For 3' side diagnosis:

BpA F: GAAAGAACCAGCTGGGGCTCGACTA
GAG

Meox1-3'-R: 5'-CAGGTGCCTGTGTTCTTCTTGAA
GAGATAC

RESULTS

The λ phage based recombineering is currently the most commonly used method for engineering DNA in *E. coli*. This is achieved, in many cases, by first transforming BAC DNA into special *E. coli* strains that harbour a defective λ prophage (14,16). For obscure reasons, some BACs are more difficult to transform, or the initial transformation leads to unwanted DNA rearrangements. Furthermore, re-transformation of BAC DNA from the original library isolates is not suitable for high-throughput operations. To address these problems with the current λ prophage recombineering systems, we made new mobile recombineering reagents and used them in a high-throughput recombineering protocol for making mouse KO targeting vectors.

Construction of a replication-defective λ phage and pSim plasmids

To create a mobile system that is suitable to efficiently deliver recombineering function to a large number of indexed BAC clones or to convert entire libraries, we constructed a replication-defective λ phage λ *cI₈₅₇ ind1* Cro^{TYR26amber} P^{Gln59amber} *rex*<>*tetRA*, which has two amber mutations in genes Cro and P at codons 26 and 59, respectively, and a tetracycline-resistance cassette in *rex* (Figure 1A).

High-titre lysates of this multiply mutant phage can be made on strain LE392 carrying tRNA suppressor alleles for the amber mutants. The resultant mutant λ phage lysate was infectious but did not lyse non-suppressive strains such as DH10B where most BAC libraries are constructed and maintained. The lysogens can be easily selected with tetracycline at 32°C.

Besides the new λ phage, we have developed and tested an alternative plasmid-based recombineering system, pSim, which is a temperature-sensitive, low-copy plasmid (pSC101) that carries the *exo*, *bet* and *gam* genes under the control of their native *p_L* operon in a mini-prophage (20) (Figure 1B). Three versions of this pSim plasmid, pSim-6 (Amp), pSim-17 (Blasticidin) and pSim-18 (Hygromycin), were constructed and were used for high-throughput studies. The only difference among these three plasmids is that they carry different antibiotic-resistant gene-coding sequences driven by the *p_{RM}* promoter that also directs the CI repressor gene transcription. pSim plasmids are easier

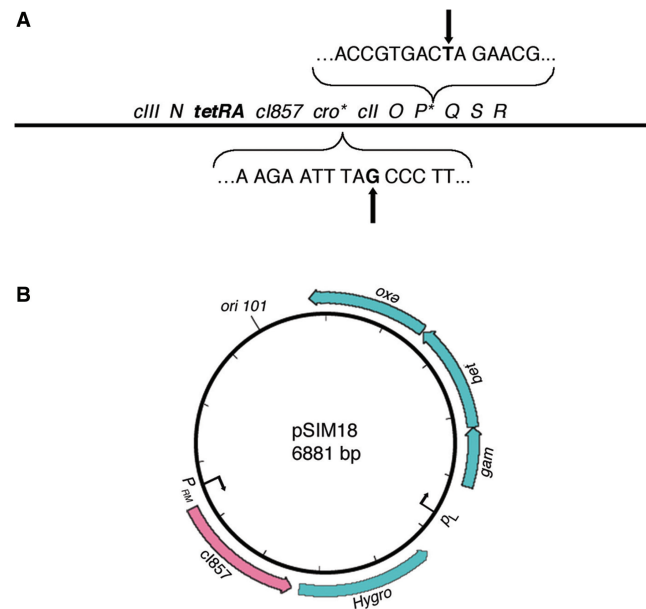


Figure 1. The new mobile recombineering reagents. (A) A schematic diagram of the replication-defective λ phage with the two amber mutations in Cro and P genes. Arrows point to the nucleotide changes in the amber mutations. (B) pSim plasmids provide an alternative convenient route for recombineering. The Amp coding sequence in pSim6 plasmid is engineered to either Blasticidin- (pSim17) or Hygromycin (pSim18)-resistant gene-coding sequences, which are driven by *P_{RM}* promoter. All the pSim plasmids have the *pSC101* temperature sensitive, low-copy replication origin.

to use than the mini-lambda we made previously (27) since integration of mini-lambda into the *E. coli* genome is relatively inefficient. BAC cells harbouring pSim plasmids showed efficient recombineering in each type of experiment described in this study (data not shown). Details of using pSim plasmids in recombineering can be found in the Materials and methods section. Since it is easier to introduce recombineering functions into 96 BACs in a 96-well plate, or into an entire BAC library, by simple λ phage infection rather than plasmid transformation, we describe here the new high-throughput recombineering procedures, focussing on using the replication-defective λ phage.

Recombineering with short homology using chemical transformation in 96-well plates

To test recombineering using the replication-defective λ , we infected BAC-C3 (containing mouse *Bcl11a* gene) with the phage and used the resultant Tet^R lysogen in the experiments. The goal was to insert a *Neo/Kan* cassette into this BAC by using a 2.8-kb DNA fragment (from plasmid PL440) in which the *Neo/Kan* cassette is flanked by two 400-bp genomic DNA homology arms. The heat-induced lysogenic cells were electroporated with the DNA fragment using a conventional *E. coli* transformation condition (16). We obtained thousands of Kanamycin-resistant (Kan^R) recombinant colonies, demonstrating that the new λ was efficient in recombineering. We also noticed more cells lysed during electroporation compared to using either regular BAC cells or heat-induced

recombineering competent cells with a defective λ prophage (EL350), suggesting that upon heat induction the slight leakiness of lysis genes *S* and *R* might contribute to the sensitivities of these cells to high voltages (28). Plating post-electroporation cells on LB plates demonstrated that 30% of the DH10B- λ lysogens survived electroporation compared to about half of the cells that survive when regular DH10B cells are used. Importantly, no noticeable DNA rearrangements were detected in the BACs after heat induction (data not shown).

To find out whether chemical transformation was sufficient for recombineering in the heat-induced λ lysogens, we compared the recombineering efficiencies in BAC-C3 BAC lysogen cells using either chemical transformation (see Materials and methods section) or conventional electroporation. With 50 ng of the gel-purified 2.8-kb fragment from PL440, similar numbers of Kanamycin-resistant (Kan^R) colonies were obtained from either chemical transformation (2004 Kan^R/3 \times 10⁹ surviving cells) or electroporation (3400 Kan^R/6 \times 10⁸ surviving cells), although the absolute recombineering efficiency of recombinants (Kan^R) versus total survival cells was eight times lower in chemical transformation. Nevertheless, chemical transformation is simpler, does not require any special equipment, and can easily be adapted into a 96-well format.

In general, 50-bp homology is routinely used for targeting a selection cassette to the *E. coli* genome by recombineering. This is usually achieved by using two 70-mer PCR primers that have 50 nt for homology and 20 nt for PCR amplification. To examine whether the new λ prophage system is sufficient to recombine with short homologies, we replaced the *Neo* coding sequence in PL451 (*PGK-EM7-Neo-bpA*) plasmid (10) with the coding sequence of a Puromycin resistance gene (*Puro*), indicating that the new λ phage recombineering system had provided high enough recombineering efficiencies with relatively short homology arms.

Next, we investigated whether it is possible to obtain enough recombinants using cells cultured in a small volume. We repeated the above experiment of replacing the *Neo* coding sequence with *Puro*. We inoculated 50 μ l overnight culture into 1 ml LB in a 96-well plate for the 2-h incubation. After chemical transformation, we obtained \sim 200 Puro^R colonies, demonstrating that it was indeed feasible to scale down the culture volume to perform recombineering in a 96-well plate.

Construction of a conditional targeting vector for the *Meox1* gene in 96-well plates

We next made a cko targeting vector using the new recombineering reagents and the 96-well recombineering protocol. The vector design was a cko strategy, where a reporter is integrated into the targeted allele to allow the detection of endogenous gene expression (9), and combined with our past experience in gene targeting (Figure 2).

To construct a targeting vector, we first incorporated a 526-bp Blasticidin selection marker (*Bsd*) flanked by two rare cutter sites (I-SceI/I-CeuI) to the 5' of the region to be

deleted (Figure 2A). Empirically, we find that smaller selection cassettes generally give rise to better recombineering efficiencies. After the *Bsd* is integrated to the BAC, the *loxP-F3-Neo-F3* cassette is subsequently targeted to the 3' side of the region to be deleted (Figure 2A). *F3* is a mutant variant of the wild-type *FRT* site. It can recombine with another *F3* site to excise *Neo* but not with wild-type *FRT* sites (29). The *Neo* cassette has a *PGK* promoter and an *EM7* promoter so it is functional in both mammalian cells and *E. coli*. Next, the modified genomic DNA fragment with the two selection markers (*Bsd* and *Neo*) was retrieved from the BAC to a modified *pBR322* plasmid (PL611) (Figure 2B). We used *pBR322* rather than a *pUC19* backbone to reduce the instability problems associated with cloning mouse genomic DNA into the very high-copy pUC-type plasmid. Finally, the *Bsd* cassette was removed *in vitro* with I-SceI/I-CeuI double digestion and replaced with a reporter (*lacZ*) as illustrated in Figure 2C. The *lacZ* reporter, with its splicing acceptor site (*SA*) and polyadenylation site, serves to detect transcription expression of the targeted gene and at the same time may disrupt the gene and possibly create a loss of function allele. To produce a cko allele, the *lacZ* and *Neo* cassettes can be excised by expressing Flp either in ES cells, or preferably in the mouse germline (Figure 3A). After Cre-*loxP*-mediated deletion of the genomic region, *FRT* and *F3* sites remain at the targeted locus, providing an anchor point for *FRT-F3*-mediated cassette exchange in cultured cells (29).

Based on this design and using the 96-well recombineering reagents and protocol, we made a cko vector of the mouse *Meox1* gene. The KO strategy for the *Meox1* locus is to flank the last two exons with *loxP* sites to create a deletion of \sim 3.5 kb (Figure 3A). The six primers for introducing the two selection markers and for retrieving the genomic DNA fragment were designed by a software for genome-wide targeting strategies (D.M. and P.L., unpublished data). These primers have 70–80 nt of mouse genomic sequences for recombineering homology and 20 nt complementary to the selection cassettes (Supplementary Table 2).

Cells of BAC bMQ-434G24 (containing *Meox1*), ordered from an end-sequenced and indexed 129S5 BAC library (25), were infected by the replication-defective phage and λ lysogenic cells were selected either on Tet-LB plates or in liquid Tet-LB in a 96-well plate. In the latter case, because of the high titre of the λ lysate, thousands of cells became λ lysogens and the Tet^R lysogenic liquid culture in the 96-well plate was used for recombineering the next day. After *Meox1* BAC lysogens were heat induced to express *red* genes and chemically transformed with the PCR-amplified *Bsd* cassette, recombineering mixture was selected with 1 ml of Bsd-LB media at 32°C in a 96-well plate.

To introduce the 3'*loxP* site, the Bsd^R/Tet^R cells in a 96-well plate were heat induced and transformed with PCR-amplified *loxP-F3-PGK-EM7-Neo-F3* cassette. The transformed cells were selected directly in LB-Kan media in a 96-well plate.

To retrieve the genomic DNA fragment, PCR-amplified retrieval vector (PL611) with 70-bp homology on each side

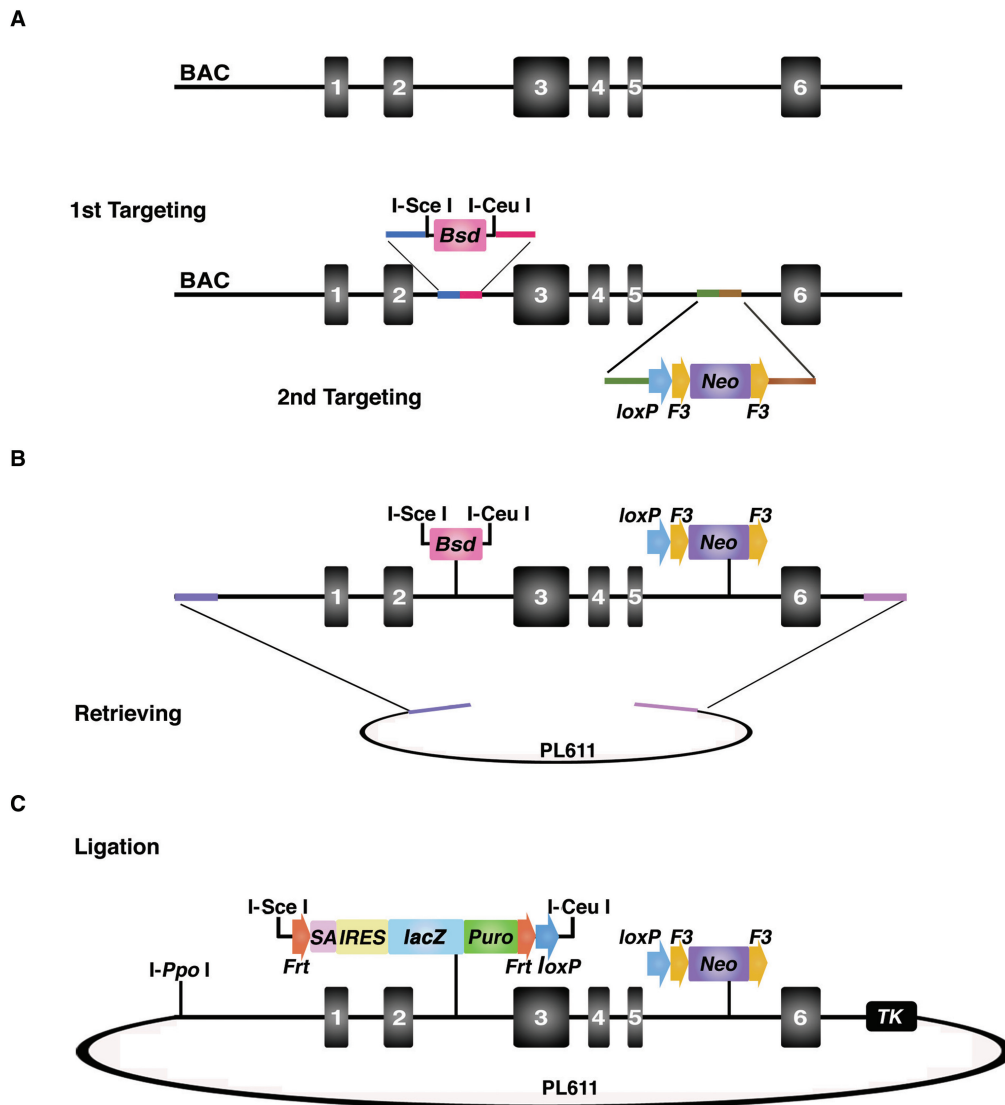


Figure 2. Construction of conditional knockout targeting vectors using the new recombinering reagents. (A) The genomic structure of a locus with exons 3–5 to be deleted in the cko allele. The *Bsd* cassette flanked by two rare cutter sites, I-SceI and I-CeuI, is targeted to the 5' side of the intended deletion region. Subsequently, the *loxP-F3-PGK-EM7-Neo-F3* (*Neo*) cassette is targeted to the 3' side of the deletion region. The point mutation present in the *Neo* coding sequence of PL452 and PL451 plasmids (10,36) was corrected in this *Neo* cassette which resulted in higher resistance to Kanamycin in *E. coli* and a 2-fold increase in the number of G418-resistant ES colonies. Coloured lines represent the short homology arms in recombinering. (B) The genomic DNA fragment is retrieved from the BAC to PL611, which has the Amp^R gene. In a typical cko vector, we choose 4–5-kb genomic DNA as the left homology arm (5'), and 2–3 kb as the right homology arm (3'). The genomic DNA region to be deleted is generally between 1 and 7 kb. (C) The *Bsd* cassette can conveniently be replaced by a reporter, i.e. *lacZ*, in a simple ligation reaction. The final targeting vector has the reporter flanked by two *FRT* sites followed by a *loxP* site at the 5' side of the intended deletion region, and a *F3* flanked *Neo* cassette providing positive selection in ES cells. The negative selection marker *TK* is added to the vector backbone by recombinering. The vector is linearized with the rare-cutter I-PpoI.

was transformed into heat-induced Bsd^R/Kan^R/Tet^R cells. Up to 100 colonies were usually obtained for each retrieval experiment in chemical transformation. Analysis of these colonies showed that many of them were simple rearrangement or end-joining of the original retrieval vector and did not contain the retrieved fragment, an observation also made by other laboratories using recombinering to retrieve or to perform gap repair (Figure 3B, lane 1). To eliminate this problematic background, we selected the retrieval transformants directly in Amp-LB media in a 96-well plate and made a mini-preparation from Amp^R

cells that represented a mixture of true recombinants as well as the background (Figure 3B, lane 2). Because the correctly recombined plasmid should retain the *Bsd* and *Neo* selection markers targeted to the genomic DNA region, the background plasmids could easily be eliminated by transforming the plasmid DNA mixture into regular DH10B or its recombinering-ready sub-strains (DH10B- λ , EL350 or DY380) and selecting transformants in Kan-LB (Figure 3B, lane 3).

Once the *Bsd* cassette was replaced by the *lacZ* reporter, and the functionality of *loxP*, and the *FRT/F3* sites was

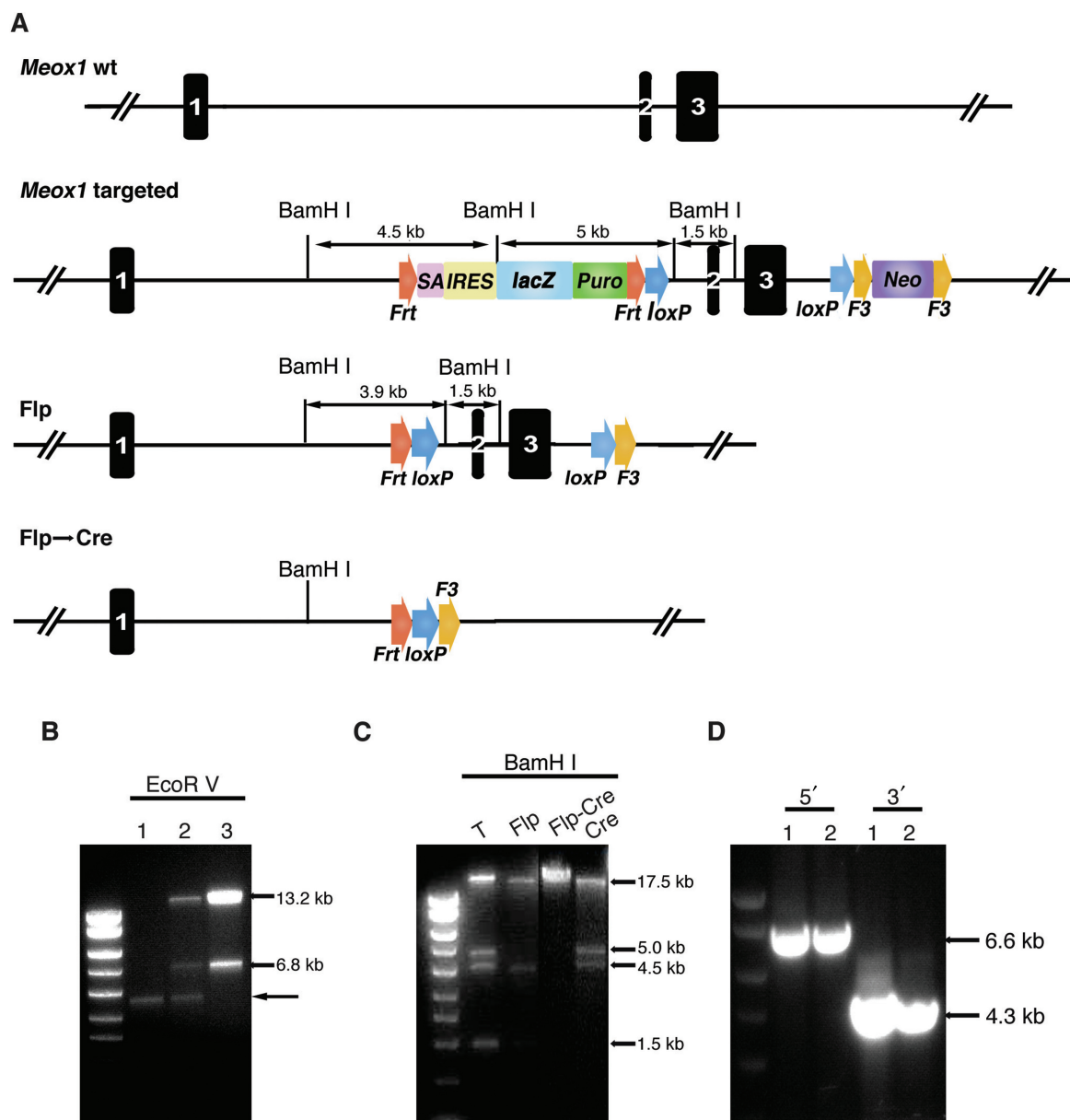


Figure 3. Targeting at the mouse *Meox1* locus. (A) Construction of the *Meox1* conditional knockout (cko) allele using the design depicted in Figure 2. The *lacZ* reporter is targeted to the intron 1 and the *Neo* cassette is in intron 3. Flp excises both *lacZ* reporter and the *Neo* cassette, leaving behind a cko allele. Cre recombinase deletes the region between the two *loxP* sites. One *FRT*, one *loxP* and one *F3* site still remain after Cre-*loxP*-mediated deletion. (B) Elimination of retrieving background by re-transformation. After the *Bsd* and *Neo* cassettes were targeted to the BAC, the genomic DNA was retrieved to PL611 (*Amp^R*). Many of the *Amp^R* colonies were the rearranged PL611 backbone (lane 1). When the plasmid preparation from pooled *Amp^R* colonies was examined, there were some true retrieved fragments besides the PL611 rearranged band (lane 2). Once the plasmid mixture was re-transformed into DH10B cells that were selected in Kan-LB, only the correctly recombined plasmid survived and background plasmids were eliminated (lane 3). The correctly retrieved plasmid had the *Bsd* cassette and produced the 13.2-kb and 6.8-kb fragments after EcoRV digestion. Arrow points to the rearranged retrieval vector backbone (PL611). (C) Test of the functionality of *FRT*, *F3* and *loxP* sites. The final targeting vector was digested BamHI which generated four fragments: 17.5, 5.0, 4.5 and 1.5 kb. The digestion pattern changed after Flp or Cre excision of the selection cassettes as anticipated. T: targeting vector. (D) Long-range PCR identification of targeted ES clones. The internal primers were from the *lacZ* or the *Neo* cassette, and the external ones from genomic DNA immediately outside the homology arms. The gel image shows PCR amplification of two targeted clones. For the 5' diagnosis, a 6.6-kb fragment was amplified (lanes 1 and 2) and for the 3' side, the 4.3-kb junction fragment was detected in the targeted clones.

confirmed (Figure 3C), the *Meox1* targeting vector was linearized with I-PpoI prior to being transfected into AB2.2 mouse ES cells.

Correct recombinant ES clones were identified by PCR using external and internal (*lacZ* or *Neo*) primers

(Figure 3D), and were confirmed on Southern blots using external probes (data not shown). Twenty-five percent of the G418^rGanc^r clones were correctly targeted at both the 5' and the 3' side, and had the desired *lacZ* reporter cko of the *Meox1* locus.

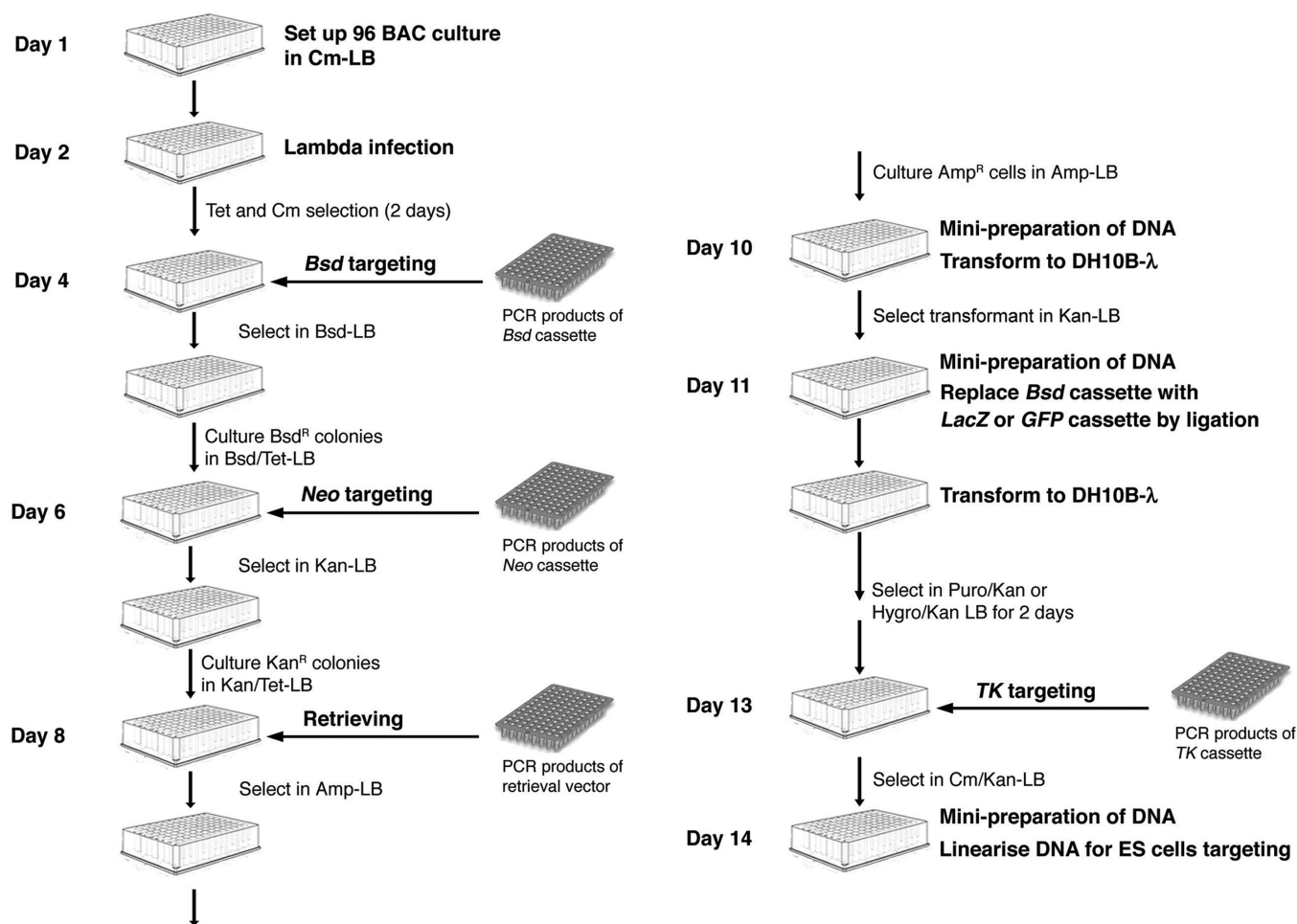


Figure 4. Schematic diagram of recombinering steps in 96-well plates. Ninety-six BACs corresponding to the 96 genes on the mouse chromosome 11 were seeded into a 96-well plate. The BAC cells were infected by the replication-defective λ phage and lysogens were selected in Tet-LB media. The *Bsd* and *Neo* cassettes were consecutively targeted to the BACs in recombinering reactions performed in 96-well plates. Retrieved plasmid preparations were transformed back into DH10B cells to eliminate rearranged retrieval plasmid backbone. *MCITK*, with a Chloramphenicol (Cm) cassette, was targeted to PL611 to provide a negative selection in ES cells.

Construction of multiple cko targeting vectors in 96-well plates

We selected 96 genes on the mouse chromosome 11 to make KO vectors simultaneously because it is one of the chromosomes sequenced and annotated at the Sanger Institute. The primers for the 96 KO vectors were generated by the vector design software (Supplementary Table 2), and were ordered through the Sanger's high-throughput oligo ordering pipeline. The intended deletion regions in these genes are generally in the range of 3–5 kb. BACs from the 129S5 BAC library corresponding to these genes were infected with λ phage and selected with tetracycline directly in a 96-well plate in liquid media. The procedure for recombinering in 96-well format is depicted in Figure 4.

Escherichia coli DH10B carrying BACs harbouring different genomic inserts grow at different rates. To ensure that at least some cells for any given BAC in the 96-well plate would achieve optimal growth density for recombinering and transformation, we inoculated various amounts of the overnight culture of Tet^R lysogens into

0.85 ml fresh LB for the 2 h incubation at 32°C prior to heat induction. For example, we usually inoculated 25, 35, 45, 55 μ l of the overnight cell culture into 850 μ l fresh LB in four wells of four 96-well plates respectively (Figure 5). This added step should increase the likelihood that some cells in at least one well for any BAC would reach optimal growth density (log phase) for heat induction and recombinering after 2 h incubation at 32°C.

In our first attempt to manipulate 96 BACs simultaneously, we obtained 94 retrieved targeting vectors that were subsequently confirmed by sequencing the junctions between the plasmid backbone and the genomic DNA inserts, and by restriction digestion. Figure 6 shows the restriction digestion patterns of these KO vectors.

DISCUSSION

To develop a robust high-throughput recombinering protocol, we developed two mobile recombinering-enabling systems. The λ phage provides a very efficient

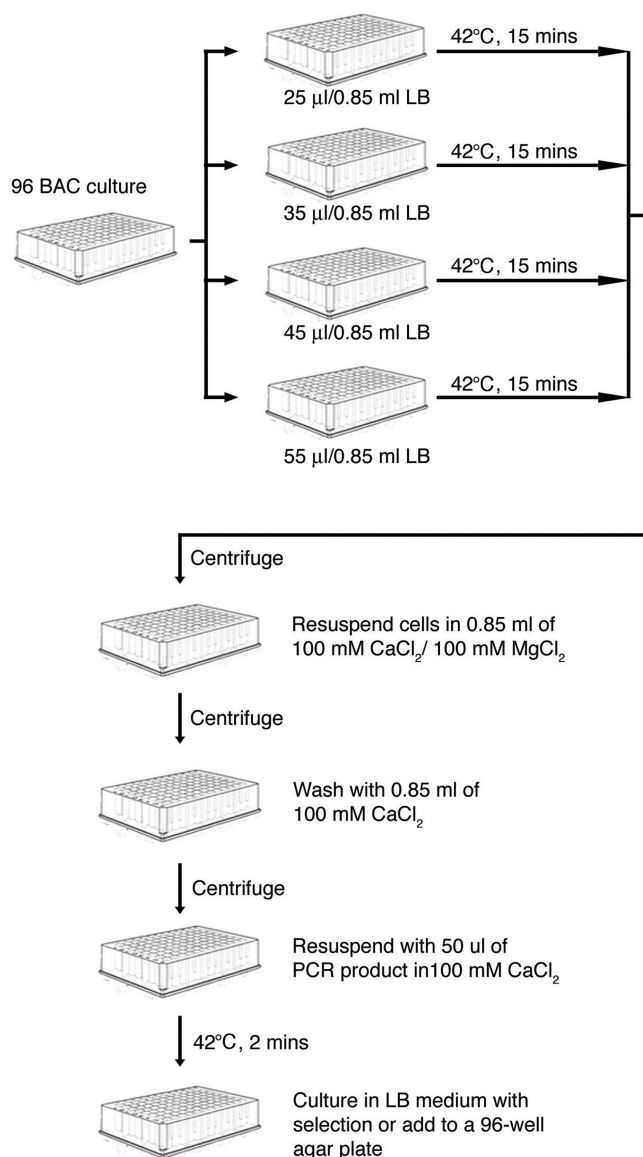


Figure 5. Increasing the recombineering efficiency by inoculating different amounts of cells in 96-well plates. *Escherichia coli* cells harbouring BACs are growing differently due to the nature and the sizes of genomic DNA inserts. Efficient recombineering and transformation require cells growing in log phase. To increase the likelihood that cells of a particular BAC reach the optimal growth condition prior to heat induction and recombineering, four 96-well plates were used for the 2 h culture. Each plate had different amounts of the overnight culture. Cells from the four plates were combined after heat induction and were transformed with PCR products.

and convenient way to deliver recombineering functions to many BAC clones or an entire BAC library by simple phage infection. On the other hand, pSim plasmids serve as an effective alternative vehicle for the same purpose. Both of these recombination systems are robust. In this article, we primarily described the replication-defective λ system, but pSim plasmids delivered similar results and might be more convenient to use if one needs to manipulate only one or a few BACs using the standard recombineering protocols because most molecular biology

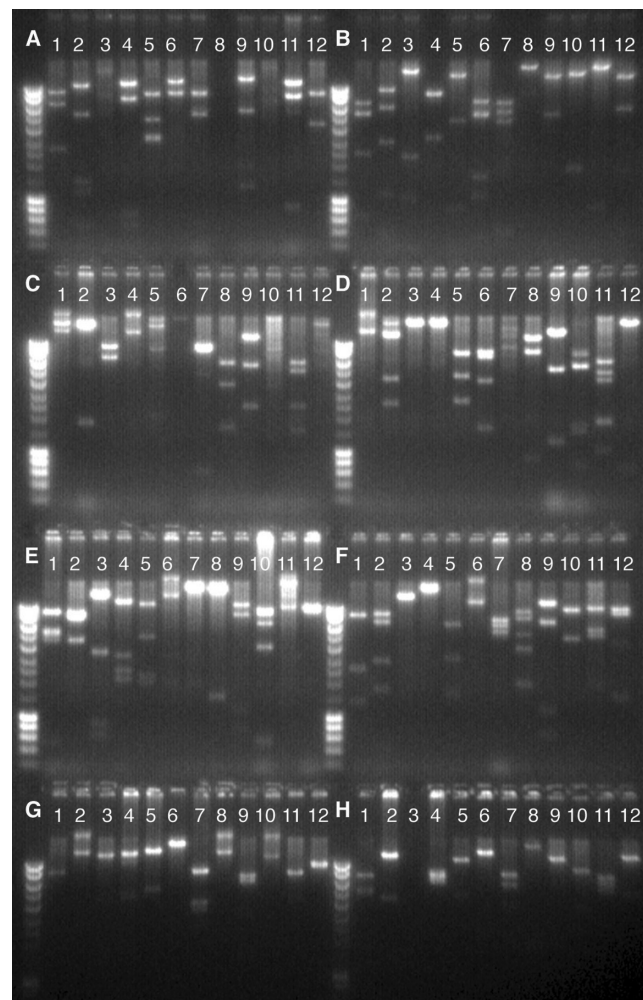


Figure 6. Targeting vectors constructed in 96-well plates. We started with 96 BACs in a 96-well plate and, after three rounds of recombineering, obtained 94 vectors. Recombineering did not work for the two failed BACs (lanes A8 and H3) at the *Bsd* targeting step because these two wells were empty or clear after *Bsd* selection. The gel image shows the BamHI restriction digestion patterns of these plasmids. The vectors still carried the *Bsd* cassette, which could be replaced by various reporters.

laboratories are more familiar with using plasmid transformation.

We also improved the previous recombineering protocols at several steps. For example, we used a relatively large amount of PCR products (3 μ g) in transformation in order to compensate for using fewer *E. coli* cells growing in 96-well plates and for the less efficient chemical transformation. To obtain clean PCR-amplified selection cassettes, we used *E. coli* Exo nuclease I to digest the excessive PCR primers and ssDNA so that any potential competition between the single-strand primers and the double-strand PCR products for the same target DNA in recombineering would be eliminated (see Materials and methods section). When a selection marker is targeted to single-copy BACs, the genomic region can have significant effects on expression of this marker in *E. coli* and thus on detection of recombineering events. Therefore, we used

strong, clean selection markers *Bsd* and a codon-corrected *PGK-EM7-Neo-bpA* (Figure 2).

To increase the chance that for any given BAC at least some cells would be in the optimal growth phase for recombineering or transformation, we inoculated different amounts of overnight cultures from one 96-well plate to four 96-well plates for the 32°C–2 h incubation prior to heat induction (see Figure 5). In practice, this did not substantially increase the workload because the recombineering procedure itself requires a minimal amount of physical work. We found that many of the Amp^R colonies in retrieval experiments were end-joined products of the linear PCR-amplified retrieval plasmid backbone. This background was completely eliminated by transforming mini-prepared DNA of the retrieved plasmid mixture to *E. coli* and selecting cells in Kan-LB, thus avoiding picking and characterizing individual colonies.

It is known that increased homology length improves recombineering efficiencies (12,16,30,31) and using PCR-amplified long homology arms (200–500 bp) further substantially improved recombineering reliability and efficiency (10,31). However, using PCR-amplified long homology arms is time consuming and is not suitable for a 96-well-plate-based recombineering protocol. Therefore, we chose 70–80 bp homology in this study even though 50 bp might be sufficient for a majority of the recombineering reactions. The relatively long homology (70–80 bp) should reduce the impact of SNPs among mouse strains and short stretches of identical sequences in the homology arms on recombineering efficiencies (32).

Using the new λ phage system, we demonstrated that chemical transformation yielded sufficient recombinants. This has enabled us to easily perform recombineering in 96-well plates. Not surprisingly, BAC cells containing the λ lysogen or harbouring the pSim plasmids were very efficient in recombineering using conventional electroporation protocols (16) (data not shown). A recent publication has also demonstrated that recombineering using λ *red* genes, together with a transiently supplied *RecA* function, worked well in manipulating *Caenorhabditis elegans* BACs using *E. coli* cells cultured in eppendorf tubes (33). The system we described here takes advantage of growing cells in 96-well plates, uses chemical transformation and is based on the very efficient λ recombination system where all three *red* genes are in their native operon and are controlled by the strong p_L promoter. Importantly, our system does not require *RecA* for efficient recombineering, the function of which can lead to rearrangements of mammalian DNA in BACs. During the process of developing the new recombineering system, we made 103 targeting vectors out of the 105 mouse genes on chromosome 11 that were attempted (Supplementary Table 1). These vectors include the first vector (*Meox1*) made completely in 96-well plates, then the 8 vectors made simultaneously (data not shown) and finally 96 vectors constructed at once in a scaled-up operation.

The basic targeting vector design presented in this study comes from many years of gene targeting experience of the authors, even though the KO strategy, the selection markers and the use of *loxP*, *FRT* and *F3* sites described

in this article can all vary depending on individual research purposes. In general, we choose a long 5' homology arm so as to survive cellular nuclease attacks, and a shorter 3' homology arm to minimize the size of the vector and to facilitate PCR-mediated genotyping. In the plasmid vector, the negative selection marker (*MCITK*) is adjacent to the 3' arm and is protected by the rest of the plasmid backbone. Additionally, the use of *PGK-EM7-Neo-bpA* as the positive selection marker for homologous recombination in ES cells makes it possible to target genes that are both expressed and non-expressed in ES cells. For targeting in ES cells, the vectors are linearized at the 5' side to ensure that the basal transcription of a locus and the initiation of homologous recombination are coordinated (34,35), although we have not tested whether this coordination have an effect on the targeting efficiency. Nevertheless, the targeting vectors configured in this way, for inserting a selection cassette, have generally given rise to correct targeting frequencies that are in the range of 30–95% of the stable mouse ES cell transfectants, regardless of the expression status of a locus in ES cells. The genomic DNA regions between the two *loxP* sites in our conditional targeting vectors can be as large as 7.0 kb without reducing the targeting frequencies in ES cells. Our vectors therefore can be used to delete multiple exons after Cre-*loxP* recombination and generate null alleles. Using the recombineering system described in this study, we have recently generated the *lacZ*-tagged cko mouse lines for *Bcl11a* and *Bcl11b*, and obtained the true cko alleles after Flpe excised *lacZ* and *Neo* in the germline (S.C.L. and P.L., unpublished data). X-gal staining of the heterozygotes with the *lacZ* has demonstrated distinct expression patterns of the genes in embryonic development (Supplementary Figure 1).

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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Conflict of interest statement. None declared.

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