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TECHNOLOGY REPORT

Building a Zoo of Mice for Genetic Analyses: A Comprehensive Protocol for the Rapid Generation of BAC Transgenic Mice

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Summary: Transgenic mice are highly valuable tools for biological research as they allow cell type–specific expression of functionally instrumental genes. In this protocol, the generation of bacterial artificial chromosome (BAC) transgenic constructs is described. We give an overview of different transgenic inserts, such as fluorescent proteins (alone or in combination with Cre variants), diphtheria toxin receptor, lacZ, and light-activated ion channels. The most reliable and versatile approach to express these genes is by using BACs, which allow "highjacking" of the expression pattern of a gene without characterizing its transcriptional control elements. Here, we describe the necessary cloning techniques compared with conventional transgenesis. With the provided "toolbox" of already available transgene constructs, the generation of the BAC transgenes is made easy and rapid. We provide a comprehensive outline how to insert the different transgenes into a chosen BAC by either ET cloning or recombineering. We also describe in detail the methods to identify the correct insertion and the integrity of the final BAC construct, and finally, the preparation of the BAC DNA for oocyte injection is described. genesis 48:264–280, 2010. **© 2010 Wiley-Liss, Inc.**

Key words: transgenic mouse; Cre; CreER^{T2}; tdTomato; channelrhodopsin; lacZ; EGFP; bacterial artificial chromosome; recombineering; ET recombination

Transgenesis and gene targeting have revolutionized the way research in the mouse is performed. Some of the most striking examples (see Fig. 1) of how transgenesis has changed life science are the various applications of the Cre-loxP system (Abremski and Hoess, 1984; Sauer and Henderson, 1988) and of the different fluorescent proteins (Giepmans *et al.*, 2006). With the introduction of cell-specific (Gu *et al.*, 1994; Lakso *et al.*, 1992; Orban *et al.*, 1992) and inducible Cre-mediated recombination (Feil *et al.*, 1997; Schwenk *et al.*, 1998), spatially and temporally restricted gene deletion has become possible, thus avoiding embryonic lethality or confounding effects through compensation and pathway adaptations. Reporter proteins allow identification of subsets of cells (Kleinfeld and Griesbeck, 2005) and tracing of cell types throughout development and in vivo (Charite *et al.*, 1995). A specific application of these two systems lies in fate mapping by marking a cell lineage with a marker through Cre-mediated removal of a transcriptional stop unit within a precursor cell type, thus resulting in constitutive marker expression in the progeny (Zinyk *et al.*, 1998). Another class of frequently used proteins expressed from transgenes leads to or enables the ablation of specific cell lineages. Although transgenic expression of diphtheria toxin A (Breitman *et al.*, 1987) results in immediate death of the respective cell type, use of herpes simplex thymidine kinase (Bush *et al.*, 1998; Heppner *et al.*, 2005) or diphtheria toxin receptor (Buch *et al.*, 2005; Jung *et al.*, 2002; Saito *et al.*, 2001) allows induced cell ablation by injection of ganciclovir or diphtheria

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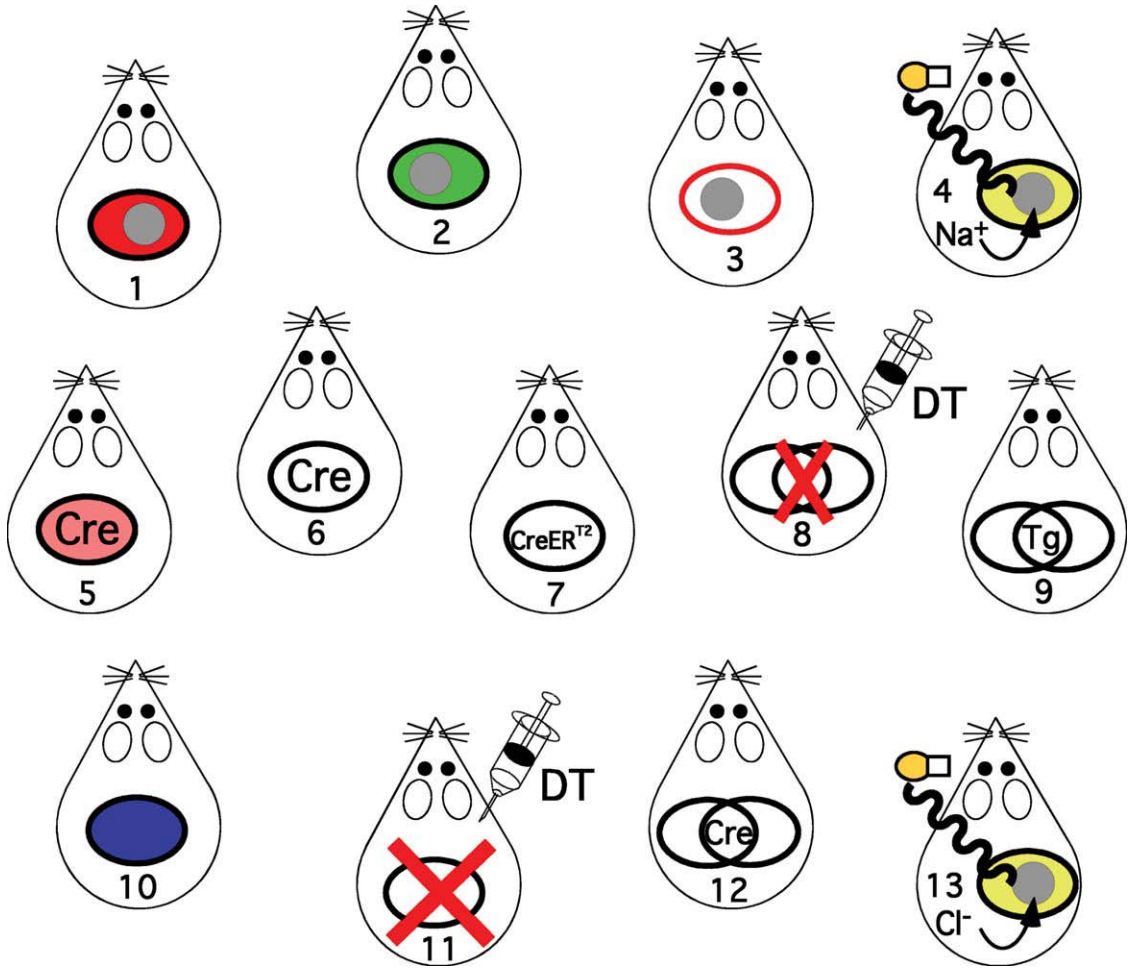

FIG. 1. A zoo of BAC transgenic mice. This scheme depicts the use of BAC transgenic mice for functional and genetic analysis of cell and gene function. One BAC can be used to generate various transgenic mouse strains that allow such diverse analyses as in situ identification of the cell, lineage tracing, recombinase expression, or even expression of light-gated ion channels. Further information about the used constructs are found in Table 1, where the numbers indicated within the different mouse strains of this figure are assigned to the respective function and constructs.

toxin, respectively. Furthermore, the combination of a fluorescent protein with light-gated ion channels and pumps, such as channelrhodopsin (Arenkiel *et al.*, 2007; Wang *et al.*, 2007) or halorhodopsin (Zhao *et al.*, 2008), allows light-inducible electrophysiological manipulation of single cells or of specific subtypes of cells in clusters of neurons.

Independently from the chosen application, the generation of a transgenic mouse strain is the result of random integration of the transgene (Tg) into the mouse genome via pronucleus injection. In conventional transgenesis, the transgenic construct consists of transcriptional control elements (promoter, enhancer, and silencer), an open reading frame (ORF),

FIG. 2. Setting up the Sepharose™ column. The picture shows how the Sepharose™ column is filled by use of a syringe and infusion tube. As described in the protocol, the cotton plug of the 5-ml pipette is sucked to the tip of the pipette. The Sepharose™ solution is slowly brought into the pipette by placing the infusion tube at the bottom (through the top). It is critical to avoid generating bubbles.

and a polyadenylation (pA) signal. As the characterization of transcriptional control elements is time consuming and position effects tend to make the outcome of conventional transgenesis difficult to predict, researchers have also used gene targeting to generate Tg-expressing mouse lines (so-called knock-in lines, Rickert *et al.*, 1997). Although these lines correctly represent the transcriptional expression pattern of the gene whose promoter was hijacked, such knock-in transgenes in most cases also constitute a null allele of the respective gene resulting in a gene deficiency when homozygous.

An alternative and a lot more reliable approach to achieve precise Tg expression is to express the Tg from a bacterial artificial chromosome (BAC) construct. This approach enables an expression that mimics the natural expression of the gene which transcriptional control is being used (Gong *et al.*, 2003; Yang *et al.*, 1997). One major advantage of this method is that by placing the transgene into 150–200 kb of genomic sequence, presumably containing the necessary promoter (enhancer and silencers), the detailed characterization of such transcriptional control elements becomes obsolete. In addition, this method is in most cases not as susceptible to position effects as conventional transgenesis. Furthermore, BAC transgenesis has the advantage of being fast with a construction time for the transgenic construct of less than 4 weeks once the system is properly setup. Moreover, transgene insertion into the mouse genome is carried out by oocyte injection and does not include time-consuming work with embryonic stem cells. Because most BAC transgenes show less variability in expression pattern through position effects, fewer transgenic founders have to be generated and screened to find lines which display the desired pattern. Nevertheless, in contrast to targeted insertions, BAC transgenesis frequently yields lines that carry multiple insertions in the genome, which may compensate for low transcriptional activity of a specific but weak promoter.

The two methods presented here that are used to introduce transgenes into BACs, the ET cloning (ET-mediated recombination, Zhang *et al.*, 1998), and the recombineering system (Red-mediated recombination, Liu *et al.*, 2003), rely on the use of phage-derived recombinases, which facilitate the introduction of a transgene flanked by short homology arms into the BAC by homologous recombination in *E. coli*. As the sequence of the homology regions can be chosen freely, almost any position on a target DNA can be specifically altered. Consequently, this technique bypasses the problem of finding suitable restriction sites in the genomic region to be modified. Therefore, recombineering/ET cloning is the tool of choice for generation of BAC transgene constructs to produce transgenic animals.

In this protocol, we describe in detail how to use either of the recombination systems to generate a BAC transgene construct and how to prepare the BAC DNA for microinjection into mouse oocytes. We show how to find, order, and confirm the appropriate BAC.

MATERIALS

Reagents

SOC medium (Invitrogen, 1554-34); Luria Broth Base (Invitrogen, 12795-027); Luria Broth Agar (Invitrogen, 2700-025); Phusion™ High-Fidelity PCR Kit (Finnzymes, F-553S); Glycerol (Fluka, 49770); Chloramphenicol (Sigma/Supelco, 442513); Ampicillin (Sigma, A2804); Kanamycin (Sigma, K 1637); L-(+)-Arabinose (Sigma, A3256); Tetracycline (BioChemika, 87128-25G); Spermine (Sigma, tetrahydrochloride, #S-1141); Spermidine (Sigma, trihydrocholoride, #S-2501); GeneJET™ Fast PCR Master Mix (Fermentas, K0211); PhasePrep™ BAC DNA Kit (Sigma, NA0100-1KT); Ladderman™ Labeling Kit (Takara, 6046); EasyTides™ Deoxycytidine 5′-triphosphate, [(α-^{32}P] (PerkinElmer, NEG513H250UC); Illustra™ MicroSpin™ S-200 HR Columns (GE Healthcare, 27-5120-01); Sepharose™ CL-4B (Amersham Pharmacia, 17-0150-01); Sonicated fish sperm DNA (Roche, 114 671 400 01); GeneRuler™ 1 kb Plus (Fermentas, SM1333); MidRange PFG Marker II (New England Biolabs, N3552 S).

Equipment

Molecular biology equipment (fridge, freezers, incubator, centrifuges, pipettes, plastic ware, water bath, tips, agarose gel electrophoresis setup, and glass ware); Thermal cycler; Centrifugation flask (250 ml); 50-ml conical tubes; Electroporator (Eppendorf electroporator 2510); Gene Pulser™ electroporation cuvette 0.1 cm (BioRad, 151 582); 10-ml sterile syringe; Infusion tube; Syringe filter Acrodisc 0.1 μm (Pall, 4651); 3MM Chr Paper (Whatman, 303 091 7); Paper towels; Isotope lab equipped for ^{32}P32 including: hybridization oven (with bottle roller), bag sealer, heat plate, thermoblock, and 1.5-ml tube centrifuge; Pulse-field apparatus (e.g., Biorad CHEF-DR II System™); Southern blot membrane: Biodyne® B 0.45 μm (PALL, 60 208); Degassing setup: vacuum flask (Büchner flask, Kitasato flask), plug, tubing, and pump; BioMax™ film (Kodak, 829 498 5).

Plasmids and Bacteria

Plasmids. pSC101-BAD-gbaA-tetra (GeneBridges, Dresden, Germany) and 706-FLP (GeneBridges, Dresden, Germany). A list of further constructs is given in Table 1.

Bacteria. SW105 (Recombineering website:http://recombineering.ncifcrf.gov/default.asp#bacterial_strains, National Cancer Institute); Top 10 (Invitrogen, C66411) or DH5α (Invitrogen, 11319019).

Reagent Setup

Injection buffer

1,000x Polyamine stock. 30 mM spermine and 70 mM spermidine. Dissolve the spermine and spermidine together in autoclaved distilled water, filter sterilize (0.2-μm filters), and store at −20°C.

Note: As the polyamines are very hygroscopic, only small quantities (1 g) should be ordered and used at once.

Table 1
Toolbox of constructs useful for the generation of BAC transgenic mice

Construct	Function	Constructed by	# In Figure 1
Cre frt Kan(R) frt	Cre expression	Buch	6
iCre frt Kan(R) frt	Cre expression	Zeidler[a]	6
$CreER^{t2}$ frt Kan(R) frt	Tamoxifen-inducible Cre	Buch	7
tdTomato frt Kan(R) frt	Strong red fluorescent color	Buch and Kräutler	1
dsRED-Mst frt Kan(R) frt	Red fluorescent color at plasma membrane	Zeidler (Zeidler, 2009)	3
Mst-tdTomato frt Kan(R) frt	Strong red fluorescent color at the plasma membrane	Halin Winter[b] and Buch (Halin Winter; Trichas *et al*., 2008)	3
lacZ frt Kan(R) frt	β-Gal expression, histological staining of cells with X-Gal	Zeidler (Zeidler, 2009)	10
tdTomato-pest 2A Cre frt Kan(R) frt	Short half life red color + Cre	Buch and Haak	5
CreDo and CreAc frt Kan(R) frt	Cre expression in dual marker populations	Tertilt[c] and Buch (Tertilt)	12
loxP STOP Kan(R) loxP-DTR	Cre inducible DTR expression (dual marker)	Barchet[d] and Buch (Barchet)	8, 11
loxP STOP Kan(R) loxP	Inducible gene expression	Barchet and Buch (Barchet)	9
ChR2(H134R)-EYFP frt Kan(R) frt	Light-inducible cation channel	Johansson	4
eNpHR-EYFP frt Kan(R) frt	Light-inducible anion channel	Johansson	13
TAP frt Hyg(R) frt	Placing a TAP tag at the N-terminus of the target protein	Testa (Testa *et al*., 2003)	
loxP IRES bGeo/Kan(R) pA loxP	Generating polycistronic mRNA for lacZ expression	Testa (Testa *et al*., 2003)	
EGFP exon 1 loxP Kan(R) loxP EGFP exon 2 S-peptide	N-terminal tagging	Poser (Poser *et al*., 2008)	2
S-peptide EGFP IRES Kan(R)	C-terminal tagging	Poser (Poser *et al*., 2008)	2
GFP loxP Kan(R) loxP	N-terminal tagging, GFP expression	Lin (Lin *et al*., 2007)	2
GFP FRT Kan(R) FRT	N-terminal tagging, GFP expression	Tallini (Tallini *et al*., 2006)	2
Luc loxP Neo(R) Sp(R)	Luciferase expression	Magin-Lachmann (Magin-Lachmann *et al*., 2003)	

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Microinjection buffer

Concentrations	Amount	Stock solution
10 mM Tris-HCl, pH 7.5	0.5 ml	1 M Tris-HCl, pH 7.5 (autoclaved)
0.1 mM EDTA, pH 8.0	10 μl	0.5 M EDTA, pH 8.0 (autoclaved)
100 mM NaCl	1 ml	5 M NaCl (autoclaved)
1× Polyamines	50 μl	1,000× Polyamines mix
	Fill up to 50 ml with H_2O	

Note: Prepare fresh and discard unused microinjection buffer.

Filter sterilize (0.1 μm), degas the buffer using the degassing device.

Note: Depending on the injection facility, the injection buffer may vary, check with your service provider.

Antibiotics. Ampicillin 100 mg/ml dissolved in ethanol (50%), chloramphenicol 30 mg/ml dissolved in ethanol (100%), kanamycin 100 mg/ml dissolved in H_2O, and tetracycline 3 mg/ml dissolved in methanol (100%).

Hybridization buffer

Concentration	Amounts	Stock Solution
1 M NaCl	11.8 g NaCl	
50 mM Tris-HCl pH 7.5	10 ml	1 M Tris-HCl pH 7.5
10% Dextran sulfate (Fluka BioChemika 31 403)	20 g Dextran sulfate	
1% SDS	20 ml	10% SDS solution
250 μg/ml fish sperm DNA	10 ml	5 mg/ml fish sperm DNA
	fill up to 180 ml, dissolve at 65°C	
	fill to 200 ml, freeze 30 ml aliquots	

10 M NaOH: Dissolve 200 g NaOH in water to a final volume of 500 ml; 5 M NaCl: Dissolve 292 g NaCl in water to a final volume of 1 l; 0.4 M NaOH: 40 ml 10 M NaOH in 960 ml water.

Transfer buffer. 0.4 M NaOH, 0.6 M NaCl (40 ml 10 M NaOH and 120 ml 5 M NaCl, fill up to 1 l with water).

Neutralization buffer. 0.5 M Tris-HCl, 0.6 M NaCl, pH 7.0 (35.1 g NaCl, 60.1 g Tris for 1 l, equilibrate to pH: 7.0 with HCl).

Equipment Setup

Separation column

I. Take a 5-ml serological pipette and pull out the cotton plug using forceps.
II. Twist two of the cotton filaments around each other and place them bent as before into the pipette. Seal the top of the pipette with your finger, apply vacuum to the tip, and swiftly remove the seal to shoot the cotton down to the tip, creating a liquid-permeable plug.
III. Clamp the pipette vertically (tip down) in a holder and place a 50-ml collection tube under the tip.
IV. Homogenize the Sepharose™ by careful inversion, degas about 10 ml Sepharose™ and 5 ml injection buffer per column, and store on ice.

Note: Always prepare fresh solutions for the day of separation.

Note: By degassing the Sepharose™/injection buffer, the formation of bubbles later during the procedure is avoided.

V. Connect a 10-ml syringe to a sterile perfusion tube (luer lock) and cut the tube to a similar length as the pipette. Mix the Sepharose™ with the injection buffer and fill the syringe by sucking the mixture through the tube.
VI. Insert the tube into the pipette until it almost reaches the cotton plug (see Fig. 2). Slowly press the mixture into the pipette while simultaneously pulling the tube out of the pipette leaving the opening 1–2 cm below the liquid surface. Liquid will start to drop out of the pipette while the Sepharose™ stays in the column. Repeat until the Sepharose™ surface is about 3–4 cm below the top opening of the pipette.

Note: Avoid introducing bubbles into the matrix of the column.

VII. To add injection buffer, take out the plunger of a 50-ml syringe and insert the syringe with a bit of force into the top of the pipette. Seal the connection between syringe and pipette with Parafilm® and fill 30 ml of degassed injection buffer into the syringe. Wait until the injection buffer passed through the column, which afterward is ready for use.

PROCEDURE

Identification and Preparation of the Appropriate BAC

1. Go to www.ensembl.org, choose “mouse”.
2. Search for your gene (preferably using the gene name nomenclature, e.g., *slc6A5*). In the result page, expand “Gene” and click on “*Mus musculus*.” On the next page, click on the appropriate search result. Under “location” click on “chromosome 7: 57,166,139-57,219,226” (for *slc6A5*).
3. This transfers you to the “region in detail” overview. In the left-side menu, click on “configure this page.” A pop-up window appears. In this window, click on “external data” and mark BAC map “normal.” In the upper right corner, click “SAVE and close,” which closes the pop-up window.
4. Choose one or more BACs in which your gene is located (if possible in the middle of the genomic sequence). You may want to zoom out to get a better overview of the available BACs (see Fig. 3).
5. To export a BAC sequence, click on the graphical bar representing your chosen BAC. This reveals its location on the chromosome under “start and end.” Open a new window in your web browser (go to the same URL by copy-paste). Under “location” type in the locations of left and right end of your BAC and click “go.” Choose “export data” in the left side menu. In the new window choose “Genebank” as your output format. Scroll down and choose the features you want to have included in the genebank output file and click “next.” On the next screen, choose “text.” Ensemble extracts all the bases between your defined start and end points as text and includes the gene information into the file. Save page as txt document.
6. Import the BAC sequence into your favorite DNA analysis/cloning program. If you do not have such a program one option is ApE, which is freeware and available under http://www.biology.utah.edu/jorgensen/wayned/ape/. Alternatives are VectorNTI (Invitrogen) or CLC main workbench (CLC bio), which need to be licensed.
7. Order BAC from a resource such as www.chori.org (BACPAC Resource Center).
8. To add the Vector backbone sequence to your BAC go to “http://bacpac.chori.org/,” choose in the menu “products” the button “vectors,” click on “detailed map and sequence information about our vectors,” and download the right sequence as genebank file. The information of the used vector is provided at the ordering information.

Note: It is important to create two construct files of the BAC in which the backbone is placed in both possible orientations. Later, these construct files are used to determine the actual direction of the insert in the vector backbone.

FIG. 3. Identification of an appropriate BAC. The Contig view of the gene IL17RD in the Ensembl database is shown (http://www.ensembl.org). An optimal BAC would not include the neighboring genes (as indicated by red lines), thus avoiding unexpected phenotypes from overexpression of these. However, in many cases (as is also the case in this example), this may not be possible and parts of or even full neighboring genes may be included in the chosen BAC. In these cases, precautions may have to be taken to exclude experimental artifacts from the resulting gene duplications. A possible BAC is indicated by red arrow. This BAC includes only parts of a neighboring gene.

Amplification and Verification of the BAC

Throughout the procedures described in this protocol, the presence and integrity of the BAC has to be confirmed and eventually the right modification has to be verified. We observed that occasionally incorrect BACs are provided by the supplier, BACs are lost during the culturing of the bacteria, or BACs undergo large-scale recombinations during handling. To monitor the BAC recombination procedure, we therefore recommend a combination of PCR analysis, restriction fragment length analysis, and Southern blot analysis. For this purpose, design PCR primers for at least three PCR reactions of 300–500 bp product length, to allow a quick confirmation of the identity and integrity of the BAC (see Fig. 4). Place the PCR primers in such a way that you will get products from the very 5′ and 3′ ends of the genomic sequence within the BAC. A third primer pair should amplify the region into which you want to place the ORF of the Tg. Additionally, you may also want to sequence from the BAC backbone into the insert. The second method is a simple restriction digest of the BAC. This generates usually a rather complex pattern which should be compared with the in silico prediction. In the end, the modification of the BAC is confirmed by Southern blot analysis using probes outside of the integrated restriction cassette and its homology arms (so-called external probes). These probes should be tested before starting the recombination and thus yield a final proof of the presence of the gene of interest in the BAC.

Amplification of the BAC (Maxiprep)

9. The BAC will arrive as stab culture. Inoculate some of the stab into 3 ml LB medium (with suggested amount of the respective antibiotic; usually 15 μg/ml chloramphenicol) and place in shaking incubator at 37°C (250 rpm) over night.
10. On the following day, streak out on LB plate (15 μg/ml chloramphenicol) and place in incubator at 37°C over night.
11. Next day, pick one colony and grow in 3 ml LB (15 μg/ml chloramphenicol) over the day at 37°C (250 rpm).
12. Prepare glycerol stock by mixing 300 μl 50% glycerol (autoclaved) with 700 μl culture; store at −80°C.
13. Transfer rest of culture into 1-l LB (15 μg/ml chloramphenicol) and grow over night, shake at 37°C (250 rpm).
14. Perform DNA extraction with PhasePrep™ BAC DNA Kit according to the manufacturer's recommendations.

Note: To prevent shearing of the BAC DNA, pipette with large orifice tips and store at 4°C (do not freeze!).

Generation of the Targeting Cassette

The targeting cassette contains the ORFs of the Tg and a kanamycin resistance (Kan(R)) gene. The selection cassette is flanked by two FRT sites to allow excision by the Flp recombinase. The homology arms required for the recombination processes are added during the PCR amplification of the Tg-Kan(R) cassette. The primers are designed in such a way that they contain 50-bp homology arms (see Fig. 4). To secure best primer quality, it is advisable to order PAGE-purified oligonucleotides. As your PCR primers are different for each target gene, PCR conditions may vary. Nevertheless, you may start with the conditions outlined by us. Do always use a polymerase with proofreading activity! It may take considerable time to generate the PCR product. As a second approach, you may perform a two-step PCR, because in many instances the PCR involving 70–90 bp primers does not function efficiently enough to yield sufficient product for recombination. In this approach, in a first PCR reaction, the homology arms are added by use of the respective oligonucleotides introduced above. In a second PCR reaction, a sample of the first PCR product is subsequently used together with "conventional" primers (ca. 21 bp) placed at the end of each homology arm. It is sometimes worthwhile to add a rare restriction enzyme between the annealing section and the homology section of the 3′ primer to facilitate better fingerprinting of the BAC after recombination. You may also clone your PCR product into a PCR cloning vector. However, make sure that you can recover the insert with restriction enzymes cutting adjacent to the homology arms. For recombination the insert is cut out and gel purified. This method results in higher yield of the targeting cassette, especially in cases of difficult PCRs, but any mutation you have in your clone will be propagated in the following steps.

As an example, we describe here the PCR reactions which you can use for the Cre and CreER^{t2} plasmids described in this protocol (Cre frt Kan(R) frt and CreER^{t2} frt Kan(R)frt):

1	Placing the Cre exactly in place of the endogenous ATG	Forward	Cre	**ATG**CCCAAGAAGA AGAGGAAGGTGTC
		Forward	CreER^{T2}	**ATG**GGCGCCACGA GTGATGAGGTTC
		Reverse	Cre/ CreER^{t2}	CGCGCCGCGACA TTTTGAAG
2	Taking the partial Kozak sequence from the plasmid	Forward	Cre	CGACCATGCCCA AGAAG
		Forward	CreER^{t2}	TTAATTAACCACC **ATG**GGC
		Reverse	Cre/ CreER^{t2}	CTTAAGCTTGG AAAAGCTGG

Note: Add the homology arms to the oligonucleotides

Note: Order the primers as gel purified (PAGE-purified).

Recommended protocol to amplify the targeting cassette:

	Amount
10× Taq buffer -$MgCl_2$	5 μl
Flanked primer for	5 μl of 5 μM
Flanked primer rev	5 μl of 5 μM
dNTP	2 μl 10 mM (each)
$MgCl_2$ (50 mM)	2.5 μl (titrate 2.0, 2.2, 2.5)
DMSO	3 μl (titrate 0, 1.5, 3, 4.5 μl)
Pfu polymerase	0.1 U
Taq polymerase	2 U
Template	50 ng
H_2O	fill up to a total volume of 50 μl

The cycling conditions are as follows: 95°C—5′; 95°C—30″; 58°C—1′; 72°C—4′; go to 2 35 times; 72°C—10′; 10°C—hold.

Generation of Electrocompetent Bacteria

Note: For good electrocompetence, it is important that the bacteria are kept cold during the process and that mechanical stress is reduced to a minimum.

15. Puncture lid of 1.5-ml Eppendorf vial for ventilation. Add 1 ml of LB (including the required antibiotics), inoculate with a bacterial clone, and grow at 37°C (1,000 rpm; 32°C for SW105 bacteria) overnight.
16. Add 30 μl of overnight culture to 1.4-ml LB (including the required antibiotics) in new punctured Eppendorf.
17. Grow at 37°C (1,000 rpm; 32°C, 4 hrs for SW105 bacteria) for 3 h.
18. Centrifuge at 4°C, 11,000 rpm, 30 s.
19. Remove supernatant.

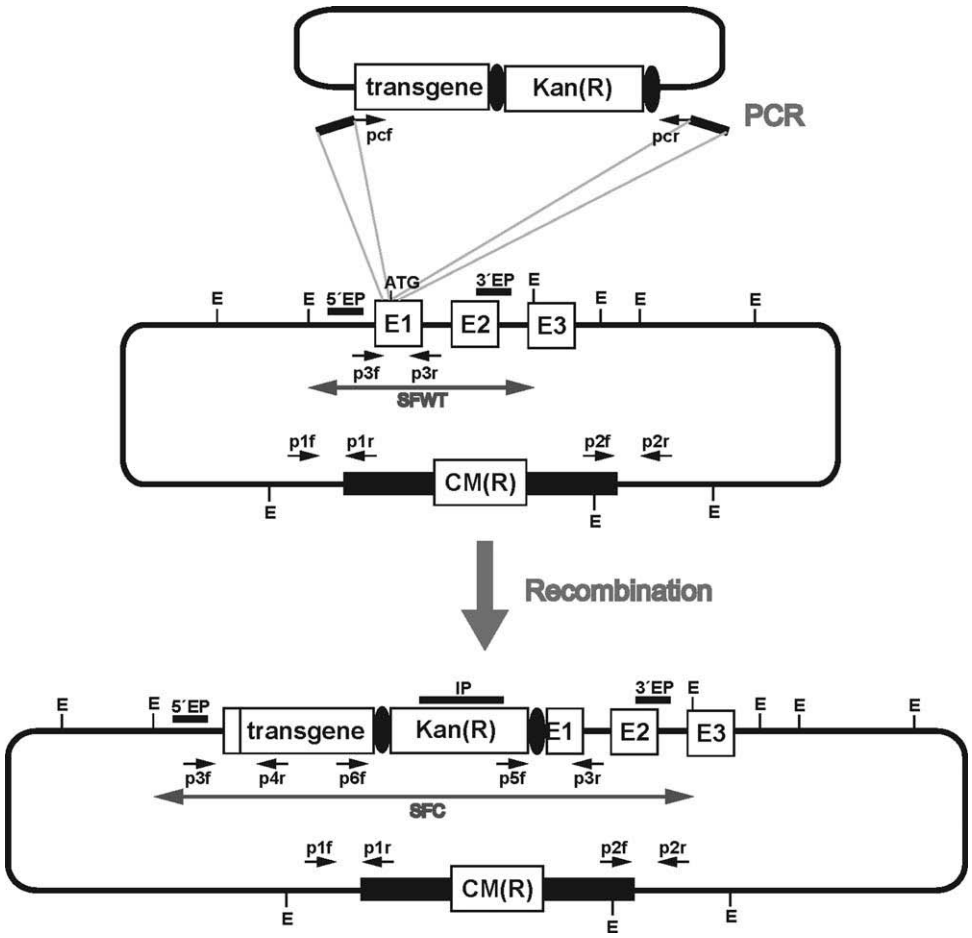

FIG. 4. Scheme of the genotyping of the original and modified BAC. Scheme of the recombination of the PCR product carrying Tg and Kan(R) into a BAC. Shown is how a PCR product of the ORF of Tg and Kan(R) is generated that carries 50-bp long homology arms. These homology arms direct the recombination process into the BAC at the chosen location, usually the ATG of your target gene. Before recombination is performed the integrity of the BAC is confirmed by PCR using a primer pair around the ATG (p3f and p3r, product should be sequenced) and primer pairs at the backbone insert boundaries (p1f and p1r, p2f and p2r). Restriction fragment length analysis is performed with a restriction enzyme (E). This or another enzyme is used for Southern blot analysis using the external probes 5′EP and 3′EP. The size of the Southern blot band is indicated as SFWT. After recombination, the integration at the appropriate location is confirmed by PCR using primers p3f with p4r and p5f with p3r. The overall integrity is confirmed by restriction fragment length analysis and appropriate integration shown by Southern blot analysis using external probes. The new size of the Southern blot fragment is indicated with SFC. In addition, a hybridization with an internal Kan(R) probe is performed to exclude other additional integration sites or presence of the plasmid coding for the Tg-Kan(R) cassette. E1–E3, exon 1 to exon 3; black ovals, FRT sites; small arrows, PCR primers; E, restriction enzyme cutting site; EP, external probe; IP, internal probe; two-sided arrow, size of Southern blot fragment; Kan(R), kanamycin resistance; CM(R), chloramphenicol resistance.

20. Resuspend in 1-ml ice-cold dH_2O.
21. Repeat once from step 18 on.
22. Add 20–30 μl dH_2O.
23. Directly perform the electroporation (electrocompetent bacteria can also be frozen in 10% glycerol).

Recombination of Mini Targeting Cassette into the BAC

Different systems are available for modification of BACs using recombination-based techniques (see Fig. 5). The first system taking advantage of homologous recombination in bacteria was published by the group of Nathaniel Heintz (Yang *et al*., 1997). This system was a breakthrough for BAC manipulation and laid the foundation for future systems. In our laboratories, we now use two later developed methods, ET-cloning and recombineering:

Method A was introduced by the laboratory of Francis Stewart (Zhang *et al*., 1998) under the name ET cloning (ET-mediated recombination) and makes use of the RecET system derived from the prophage Rac. In ET cloning, the BAC remains in the same bacterial clone

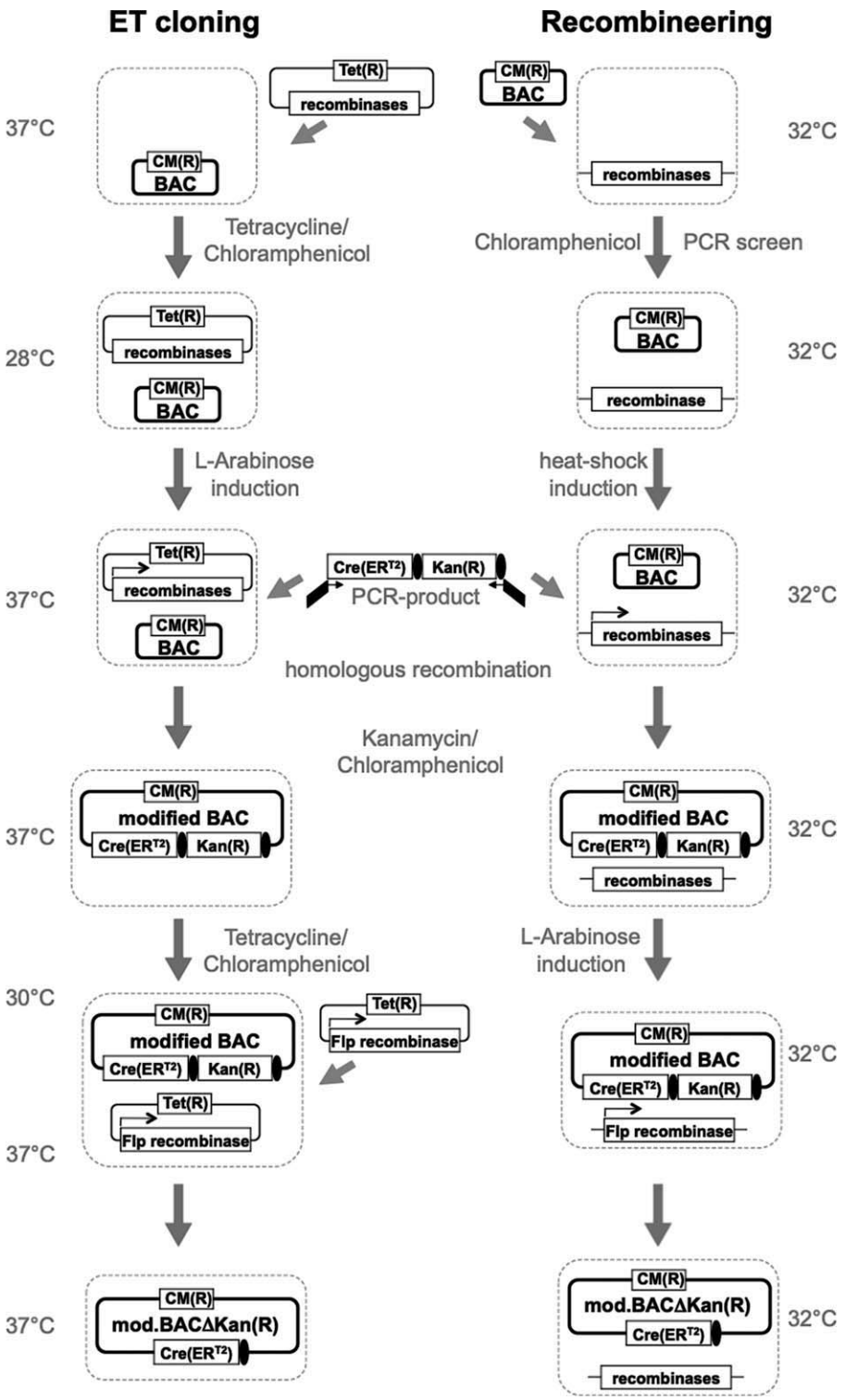

FIG. 5. Scheme of the bacterial recombination systems for BAC engineering. In ET cloning (left flow chart) a tetracycline-resistant (Tet(R)) plasmid carrying the recombinases is transformed into the bacteria carrying the BAC, which confers chloramphenicol resistance (CM(R)). The BAC and recombinase carrying bacteria have to be cultured at 28°. In the presence of L-arabinose, the recombinases are expressed. The genes for transgene and for kanamycin resistance (Kan(R)) are PCR-amplified with primers including 50 bp of homology region for the target region in the BAC. The PCR product is transformed into the recombinase-expressing bacteria that subsequently integrate the PCR product into the BAC. The plasmid coding for the recombinases is lost by culture at 37°C. To remove Kan(R), a FLP-expressing plasmid is transformed into the modified bacteria and expression of the recombinase is induced by heat shock. In recombineering (right flow chart), the recombinases are integrated into the genome of the bacteria. Therefore, the BAC has to be transformed into these bacteria, and the presence of the BAC confirmed by chloramphenicol resistance and PCR. The recombinases are induced by heat shock. After transformation with the PCR product carrying Tg and Kan(R), these are integrated into the BAC generating the final transgenic construct. For removal of Kan(R), the FLP recombinase gene that is integrated in the genome of SW105 bacteria is induced by L-arabinose.

and the recombinases as well as the targeting construct are introduced via electroporation. The recombinase-carrying and tetracycline-resistant plasmid is propagated at 28°C, the recombinases induced by L-arabinose, and finally the recombinase-carrying plasmid lost by culture at 37°C. The advantage of this method lies in the fact that the BAC, which can be difficult to transform, stays in the bacteria throughout the whole protocol.

Method B was developed in the laboratory of Neal Copeland (Liu *et al*., 2003) and uses Red recombinase derived from bacteriophage λ integrated into the bacterial genome. In this system, the BAC is electroporated into the recombinase-carrying host strain. The recombination system is induced by heat shock and recombination takes place after the targeting cassette is transformed into the bacteria.

We currently cannot recommend one of these methods over the other. We think that the researcher should start with the method for which it is easier to obtain the required items and switch to the other system if encountering problems. A possible guide for the decision is (Copeland *et al*., 2001), which shows possible advantages and disadvantages of both methods.

(A) Using ET cloning (see Fig. 5). The plasmid pSC101-BAD-gbaA-tetra (available from GeneBridges, Dresden) carries the λ phage redγβα operon expressed under the control of the L-arabinose-inducible pBAD promoter (Guzman *et al*., 1995) and confers tetracycline resistance. The plasmid is temperature sensitive and low copy number. It is propagated at 28°C and rapidly lost at 37°C.

24a. Ten colonies of bacteria carrying the BAC are picked and made electrocompetent (see steps 14–23).
25a. 1 μl of pSC101-BAD-gbaA is added to the cell slurry and the mixture transferred into a chilled electroporation cuvette (BioRad, 0.1 cm). Electroporation is performed according to the specifications of your electroporator (e.g., 1,350 V, 10 μF, 600 Ω with the Eppendorf electroporator 2510).
26a. The electroporated cells are resuspended in 1 ml LB medium and incubated at 30°C (1,000 rpm) for 60–80 min.
27a. 100 μl of these cells are plated on LB agar plates containing tetracycline (3 μg/ml) and chloramphenicol (15 μg/ml). The plates are incubated at room temperature (<28°C) for at least 24 h.
28a. Ten colonies of these recombinase-carrying bacteria are picked and inoculated together in a ventilated tube containing LB medium conditioned with tetracycline and chloramphenicol. The tube is incubated at 30°C (250 rpm) overnight.
29a. The next day, two new tubes containing 1.4 ml fresh LB (with 15 μg/ml chloramphenicol) are inoculated with 30 μl of overnight culture and incubated at 30°C (1,000 rpm) for about 2 h, until an OD_{600} of 0.2 is reached.
30a. 20 μl of 10% (w/v) L-arabinose is added to one of the tubes to induce the expression of the recombinases, and the bacteria are incubated at 37°C (1,000 rpm) for 40 min (cells should not grow further than OD600 ∼ 0.4).
31a. Make bacteria electrocompetent (steps 18–23).
32a. Electroporate 1 μl (200 ng) of the amplified Tg-Kan(R) cassette into the bacteria (see step II), resuspend in 1 ml of LB medium, and incubate at 37°C for 70 min. During this period recombination occurs. Plate bacteria on LB agar plates (15 μg/ml chloramphenicol, 15 μg/ml kanamycin) and incubate at 37°C overnight.

(B) Using recombineering (see Fig. 5). The SW 105 bacteria strain expresses the recombinases upon heat shocking because of a heat-sensitive promoter. Therefore, the bacteria need to be cultured at 32°C. This strain also contains the gene for FLP-recombinase under the control of an L-arabinose-inducible promoter.

24b. Generate electrocompetent SW105 (see steps 14–23).
25b. Electroporate the BAC (30–100 ng) into electrocompetent SW105. Electroporation is performed according to the specifications of your electroporator (e.g., 1,350 V, 10 μF, 600 Ω with the Eppendorf electroporator 2510). Culture bacteria in 500 μl LB media.
26b. 100 μl of these cells are plated (15 μg/ml chloramphenicol) and incubated at 32°C overnight.
27b. Pick 10 colonies and grow overnight in 3-ml LB media (15 μg/ml chloramphenicol).
28b. Use 1 μl of overnight culture to PCR screen for the presence of the BAC (see Methods Section), make glycerol stocks from the positive ones.

Note: Do not skip this step because you get many false-positive clones.

29b. Make electrocompetent bacteria from 1 to 3 positive clones (see steps 14–22).

Note: Heat shock bacteria to express the recombinases by incubating at 42°C (water bath, occasional stirring) for 15 min between steps 17 and 18.

30b. Electroporate 200 ng of the Tg-Kan(R) cassette (including homology arms) into the BAC-containing electrocompetent bacteria (perform at least two electroporations in parallel). Resuspend bacteria in 1 ml LB medium and incubate at 32°C (250 rpm) for 1 h.
31b. Plate onto two LB plates (15 μg/ml kanamycin and 15 μg/ml chloramphenicol).
32b. Grow at 32°C for about 18 h.

Analysis of (Putatively) Recombined Clones

33. Pick all clones (usually 1–10 clones) that appear after 18–24 h and grow overnight in 3–5 ml LB (15 μg/ml chloramphenicol and 15 μg/ml kanamycin).

Note: Do not pick clones that appear later.

34. Use 1 μl of overnight culture to screen for targeted BAC (PCR III) and nontargeted BAC (see Methods Section PCRs II and III, the PCRs can also be combined to form a competitive-Multiplex-PCR (see Fig. 4) primer sets p3f, p3r, p4r and p3f, p3r, p5f). Continue with the clones that have been positive for only the band(s) detecting the homologous inserted targeting cassette (PCR III) and analyze these further. Do not continue with clones that are positive for PCR 1, thus still carry the wild-type BAC.
35. Prepare Maxiprep of 2–4 positive clones.
36. Perform PCRs I and III for reconfirmation.
37. Perform restriction fragment length analysis (see Methods Section). Run wild-type BAC next to your recombinants. Take pictures at different time points and analyze your band pattern thoroughly according to your in silico prediction.
38. Perform Southern blot using 5′ and 3′ external and internal probes (see Methods Section). Include the wild-type BAC as a control. The modified BACs should give you the predicted band shifts. The internal probe (kanamycin probe) should give you only one band. In some cases, it is possible to combine steps 37 and 38 by blotting the gel of the restriction fragment length analysis.

The correct sequence of the Tg should be confirmed in the targeted BAC by sequencing. It is also advisable to sequence the FRT sites to assure that the resistance cassette can be removed at any time later. Some sequencing facilities allow sequencing on the BAC. However, in most cases, the inserted cassette should be amplified by PCR and sequenced.

Excision of Kan(R)

The reason for the generation of BAC transgenic mice is the presence of most if not all endogenous transcriptional control elements without the need for their characterization. This results in an expression pattern that is identical to the one of the gene being used. As there are reports in which the presence of the Kan(R) has resulted in unexpected phenotypes (Fiering *et al.*, 1995; Scacheri *et al.*, 2001), likely because of altered transgene expression as a result of the strong PGK promoter, it is recommendable to remove the Kan(R) from the BAC construct. However, it is noteworthy that in some instances mice carrying the resistance cassette showed higher specific Cre activity than the same strain after removal of the Kan(R) cassette (Schmidt-Supprian *et al.*, 2007, and own observation). If you choose to remove the resistance gene, you either have the choice of removing it in bacteria or later in the mouse. For removal in the mouse you cross to a *FLP*-deleter mouse strain (Rodriguez *et al.*, 2000) that recombines the FLP sites in the germ line. This approach is expensive and time consuming and requires the presence of the respective *FLP*-deleter strain in your animal facility. It also may lead to the resolution of concatamers. Alternatively, you can also remove the Kan(R) in bacteria. In that case you would, however, usually settle for only injecting the modified BAC that lacks Kan(R). Again, you have two options for removing the resistance gene: (a) You can introduce by transformation a plasmid allowing heat-shock inducible expression of the FLP recombinase or (b) you can use L-arabinose-inducible expression of the FLP recombinase when your modified BAC is in the SW105 bacteria.

(a) Excision of Kan(R) by transformation with the FLP-expressing plasmid 706-FLP. The plasmid 706-FLP (GeneBridges, Germany) is a low copy number plasmid that can be propagated below 30°C and is lost at 37°C. It carries a Tet(R) and the gene for the FLP recombinase under a heat-inducible promoter. It is the method of choice for removing the Kan(R) gene if you do not already have your BAC in SW105 bacteria.

39a. Make the bacteria carrying your modified BAC electrocompetent (steps 14–24), culture during the procedure in LB medium (15 μg/ml chloramphenicol and 15 μg/ml kanamycin).

Note: Test for remaining ET-recombinase and Tet(R) containing clones by plating 50 μl of culture onto LB plates (3 μg/ml tetracycline) and incubating overnight at 37°C. If clones appear, plate bacteria containing your modified BAC onto LB plates (15 μg/ml chloramphenicol, 15 μg/ml kanamycin, primary plate), culture overnight at 37°C, transfer individual clones onto duplicate LB plate (3 μg/ml tetracycline), and incubate overnight. Keep primary plate. Continue work only with clones from primary plate that were not resistant to tetracycline

40a. Add 1 μl of 706-FLP (10–100 ng) to the bacteria slurry, carefully mix, and transfer into a chilled electroporation cuvette (BioRad, 0.1 cm). Electroporation is performed according to the specifications of your electroporator (e.g., 1,350 V, 10 μF, 600 Ω with the Eppendorf electroporator 2510).
41a. The electroporated cells are resuspended in 1-ml LB medium and incubated at 30°C (1,000 rpm) for 60–80 min.
42a. 100 μl of these cells is plated on LB agar plates containing tetracycline (3 μg/ml) and chloramphenicol (15 μg/ml). The plates are incubated at room temperature (<28°C) for at least 24 h.
43a. Ten colonies of these FLP recombinase-carrying bacteria are picked and individually cultured in

a ventilated tube containing 3-ml LB medium (15 μg/ml chloramphenicol). The tube is incubated at 30°C (250 rpm) for 2–3 h and then overnight at 37°C (250 rpm).

(b) Excision of Kan(R) using the SW105 bacteria. SW105 bacteria carry the gene for the FLP recombinase inserted into their genome under the control of an L-arabinose-inducible promoter. Thus, if your BAC is already in these bacteria, you can remove Kan(R) by culture in the presence of L-arabinose. You should keep in mind, however, that this promoter is somewhat leaky and already without induction some FLP-mediated deletion of the Kan(R) is performed.

39b. Inoculate the clone in 3-ml LB medium (15 μg/ml chloramphenicol) and grow to light turbidity of OD_{600} 0.2 (at 32°C (250 rpm) for about 5 h).
40b. Add 33 μl L-arabinose (10% in H_2O) resulting in a final concentration of 0.1%. Grow for another hour.
41b. Plate 10 μl onto agar plates: one containing 15 μg/ml chloramphenicol and another containing 30 μg/ml kanamycin, grow in incubator at 32°C overnight.
42b. Compare number of clones on both plates. If the excision was successful the plate containing kanamycin should have only few colonies compared with the chloramphenicol plate.
43b. Pick clones from the chloramphenicol plate for further analysis and grow in 3-ml LB medium (15 μg/ml chloramphenicol) at 32°C (250 rpm) overnight.
44. PCR screen for absence of Kan(R) on 1 μl overnight culture, PCR IV and V should give you the expected smaller bands, PCR III with primers p3f and p4r should still give you the same band, and PCR III with primers p5f and p3r should give you no band (see Fig. 4).
45. Prepare glycerol stocks of confirmed clones and prepare a MaxiPrep.
46. Perform restriction fragment length analysis (see Methods Section). Run wild-type BAC, modified BAC, and modified BAC lacking Kan(R). Take pictures at different time points and analyze your band pattern thoroughly according to your in silico prediction.
47. Perform Southern blot using 5′ and 3′ external and internal Kan(R) probes (see Methods Section and Fig. 4). Include the wild-type and modified BAC as controls. The modified Kan(R)-deleted BACs should give you the predicted band shifts. However, the Kan(R) probe should show no band. In some cases, it is possible to combine steps 46 and 47 by blotting the gel of the restriction fragment length analysis.

Note: If all your Kan(R)-deleted clones show the presence of some Kan(R) in the Southern blot or PCR analysis, you may want to subclone them. Plate the bacteria onto LB plates (15 μg/ml chloramphenicol, primary plate), culture overnight at 37°C, transfer individual clones onto duplicate LB plate (15 μg/ml kanamycin), and incubate overnight at 37°C. Keep primary plate. Repeat your tests for absence of Kan(R) and presence of the modified BAC with clones from primary plate that were not resistant to kanamycin.

Preparation of the Modified BAC for Oocyte Injection

48. Digest 50 μg of BAC DNA with 100 U restriction enzyme in 300–400 μl overnight. It is desirable to cut away the vector backbone with a rare cutter such as *Not I*. If you cannot use *Not I* it is in many cases also possible to just linearize the BAC construct using *Asc I*.
49. Prepare injection buffer.
50. Heat-inactivate the enzyme.
51. Add 200 μl injection buffer and 10 μl 6× loading dye.
52. Prepare a Sepharose™ separation column (see Materials and Fig. 2).
53. After the injection buffer has left the syringe, carefully remove the syringe, wait until liquid level reaches the Sepharose™ and add digested BAC.
54. When the liquid level reaches the surface of the Sepharose™ add about 1-ml injection buffer and start to collect 500 μl fractions.
55. Repeat adding injection buffer until 10 ml has been added. Collect a total of 20 fractions.

Separation through the column functions in this setup similar to gel electrophoresis. Small fragments of DNA pass through the matrix easier than large fragments. As a result, the genomic DNA and the cut-out vector backbone can be separated from the BAC construct without a gel extraction step. Broken-up genomic DNA and the vector backbone elute in the earlier phases, whereas the BAC construct elutes later.

Note: Never let the Sepharose™ run dry.

56. Run a pulse-field gel with 20 μl from each of the first 20 fractions. Include MidRange PFG Marker II (120 V, 20 h, switch rate optimized to your construct size; see manual of power supply).
57. Choose the fraction that shows the smallest contamination of genomic DNA (smears) compared to BAC construct (to be expected in fraction 6–10, Fig. 6).
58. Quantitate the DNA content in the three cleanest fractions by spectrophotometry.
59. Have your DNA injected.

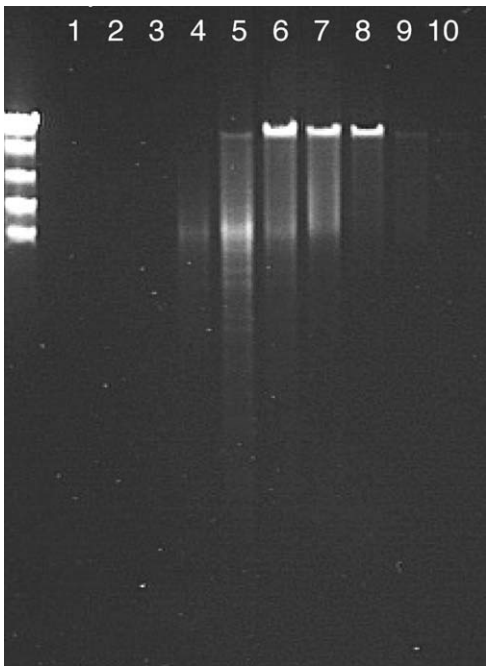

FIG. 6. Isolation of finished BAC construct. Agarose gel of collected DNA fractions separated by pulse-field electrophoresis. Fraction 4 contains only bacterial genomic DNA, seen in the lower range around 25 kB. Fractions 5–9 carry the BAC construct (upper band) and decreasing amount of bacterial genomic DNA (lower band). Fraction 8 was used for oocyte injections.

Note: Although it is preferable to have the BAC construct injected into the pronuclei of oocytes from the mouse strain selected for subsequent experiments, it must be noted that most common laboratory inbred strains such as C57BL/6 and BALB/c are also the most challenging when it comes to generating transgenics by pronuclear injection. In reality, therefore, the transgenic founders are frequently generated in strains more conducive to transgenesis such as CBF1 or BDF1 hybrids or FVB/N inbred strains, which yield good numbers of robust oocytes with well defined pronuclei.

METHODS SECTION: ANALYSIS OF BAC INTEGRITY AND MODIFICATION

(A) PCR Tests

PCR is used to verify the identity of the BAC during the procedure but also to identify correct modification and to provide material for sequencing. It is recommended to plan all PCR reactions at the very beginning and order the respective primers. All the following PCR reactions can be performed on a 1-μl sample of overnight culture or on a colony.

I. PCR screening for the 3′ and 5′ end of the BAC insert (300–500 bp product) by placing one primer in the backbone and the other primer in the BAC (see Fig. 4).

Note: In the initial PCR analysis combine the primers in all four possible ways to determine the orientation of the BAC insert in relation to the backbone.

II. PCR verification of the putative insertion site. Amplify a region of around 600–800 bp around the ATG that you plan to target. Sequence the product to verify that you will order the correct homology arms for the recombination step. Small changes would decrease recombination frequency significantly (see Fig. 4, primer pair p3f and p3r).

III. PCR-based confirmation of the insertion of the targeting cassette Tg-Kan(R) into the BAC on the 5′ and 3′ end of the insertion. For both reactions, place one primer inside the cassette and one primer outside (see Fig. 4, primer pairs p3f and p4r; p5f and p3r).

Note: Do not place any primers inside a homology arm.

IV. PCR amplification of the inserted cassette. Amplify the targeting cassette (Fig. 4 primers p3f and p3r) and sequence the ORF of the Tg as well as the FRT sites.

V. PCR identification of modified BAC from which Kan(R) has been removed by FLP-mediated recombination (Fig. 4, primer pair p6f and p3r).

(B) Restriction Fragment Length Analysis (see Fig. 7)

A good method to assess the integrity of the BAC after recombination is restriction fragment length analysis. By carefully picking restriction cutters and comparing obtained digestion patterns with the correct band pattern predicted in silico, often the presence of almost the entire BAC can be verified. At a later point in the protocol, restriction analysis can be used to verify the correct point of integration by doing a side-by-side analysis comparing the wild-type BAC with the modified BAC, confirming loss/gain of band(s) within the site of integration in the wild-type BAC (see Fig. 7). It is necessary to obtain a good distribution of the digestion band product sizes for reliable identification of the bands. If this is hard to achieve, a pulse-field gel may be run to provide a better separation in the higher band range (see point 56).

I. Identify restriction enzymes that have about 20–30 sites in your BAC.

II. Digest 1–5 μg BAC DNA with your restriction enzyme.

III. Run digested DNA and DNA ladder at 30 V for 15–24 h on a 0.7% agarose gel of >20 cm length and take pictures at different time points.

IV. Compare result with in silico prediction.

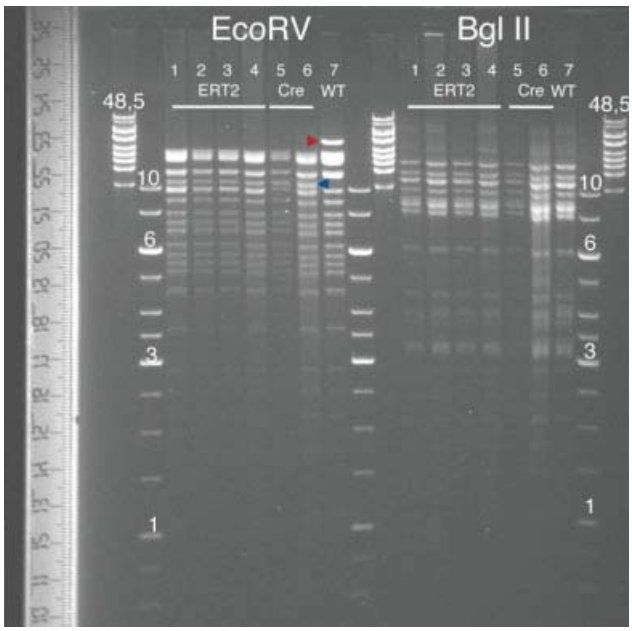

FIG. 7. Restriction fragment length analysis. Agarose gel showing separated DNA digestion (*EcoRV* and *Bgl II*) of the finished BAC constructs compared to the wild-type nontargeted BAC. The correct site of integration of the Tg open reading frame is confirmed by the loss of a high-range band (red arrow) and the gain of low-range band (blue arrow). In addition, the digests show the correct integrity of the modified BACs, by comparing the digest pattern between wild-type BAC and transgenic BAC (ladder sizes in kilobases). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

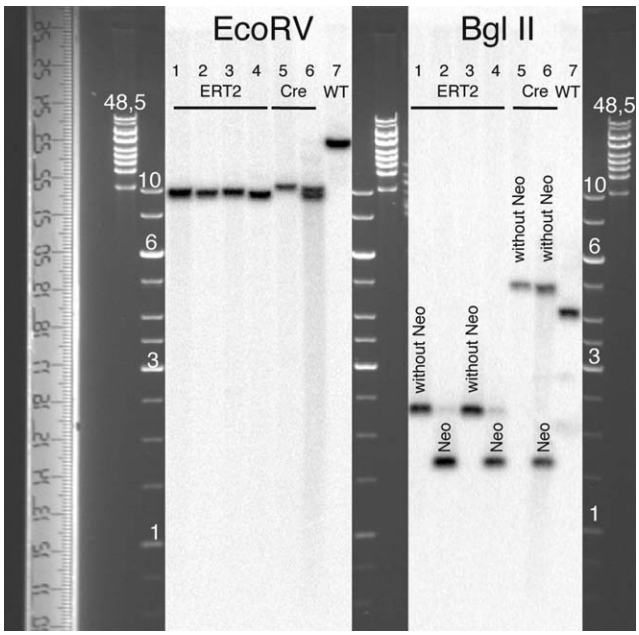

FIG. 8. Southern blot analysis of modified BAC. (Southern blot overlaid on top of the original agarose gel, Fig. 7). An external probe at the 3′ end was used to identify the correct integration site for the targeting cassette, and loss of the Kan(R) cassette was verified. *EcoRV* digest: BAC clones 1–4 are positive for the correct integration and show the expected band size compared with wild-type BAC. Clones 5 and 6 also show the expected shift, but in addition, clone 6 displays a band pattern indicating a mixed clone containing BACs with and without Kan(R). The digests of the transgenic clones 1–4 with *BglII* confirm the results of the EcoRV digest but in addition convincingly show that clones 2 and 4 still carry Kan(R). In this digest, the mixed composition of clone 6 is obvious, while it also demonstrates the lack of Kan(R) in clone 5 (ladder sizes in kb).

(C) Southern Blot Analysis (see Fig. 8)

The final test to confirm the correct integration point into the BAC is done by Southern blot analysis using probes outside the homology arms of the integrated Tg cassette (so-called external probes). In addition, an internal probe (the kanamycin-resistance probe) is used to control for the presence of only a single integration and to control for unnoticed contamination of a nonlinearized targeting cassette.

I. PCR-amplify and gel-purify your external probes and the Kan(R) ORF probe. Dilute purified DNA to 30 ng/μl.
II. Digest 1 μg of your BAC DNA with the appropriate restriction enzyme(s).
III. Run a 0.7% agarose gel of the digested BAC DNA at 30 V over night, load also 1 kb Plus marker.
IV. Take a picture including a ruler that is visible in UV light.
V. Shake gel in 0.4 M NaOH for 15 min.

Attention: NaOH is corrosive.

VI. Prepare transfer solution (0.4 M NaOH and 0.6 M NaCl).
VII. Place two 5-cm stacks of paper towels next to each other.

Note: Throughout steps IX to XIII, it is critical to enable an efficient transfer by creating a compact stack. By rolling over each new layer with a 25-ml serological pipette air bubbles are squeezed out generating optimal contact within the sandwich.

VIII. On top of the paper place three, in transfer solution soaked, 3MM Chr papers that are slightly larger than the gel.
IX. Wet the membrane in transfer buffer and place it on the 3MM Chr papers.
X. Place the gel on the membrane and place three wet 3MM Chr papers on top of the gel.
XI. Build a bridge of 3MM Chr paper (two layers) to a reservoir with transfer solution that is placed at least 5 cm higher than the top of the stack.
XII. Transfer for at least 4 h or overnight, then disassemble the blot, and neutralize membrane in neutralization buffer for 10 min.
XIII. Bake membrane between 3MM Chr paper at 65–70°C in the hybridization oven for 1 h (blot can be stored at RT for a long time when dry).

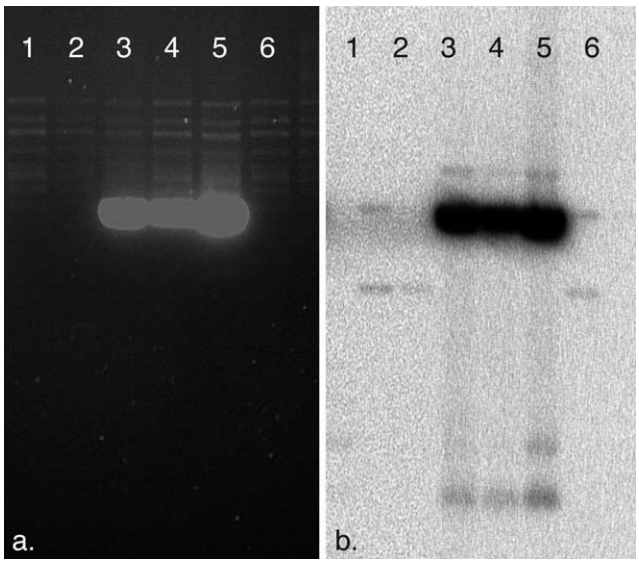

FIG. 9. Contamination of targeted BAC with targeting cassette vector. Agarose gel of modified BAC clones after digest with *Bgl II*. The presence of a strong band indicates contamination by circular and replicating vector from which the targeting cassette was amplified (**a**. lanes 3–5). Southern blot analysis using a Kan(R) probe was used to verify the correct 3′ integration of the targeting cassette into the BAC (5.2 kb), also revealing the contamination with the nonlinearized targeting cassette vector (**b**. lanes 3–5, 7.4 kb).

XIV. Prewet membrane with 2× SSC and then incubate while rotating with 15–20 ml of prehyb./hybridization solution at 65°C over night (at least 4 h) in hybridization oven.

XV. Label your probe according to the Ladderman® labeling kit manual.

Attention: ^{32}P is a β-radiation source. Shield with Plexiglas. Follow the guidelines of your country and institution.

XVI. Prepare S-200 HR columns by first vortexing, snap off the bottom seal, and then centrifuge of the liquid into a tube (1 min at 3,000 rpm). Discard liquid and place column back into the provided screw cap tube. Add 100 μl water to the probe, transfer the probe to the column, and centrifuge (1 min at 3,000 rpm). Compare the counts in column to the counts in probe (should be around 1/2 each). Discard column.

XVII. Tighten the screw cap thoroughly and place tube containing the flow-through (probe) in boiling water for 5 min.

Note: Make sure to seal the tubes properly to avoid leakage of radioactivity during boiling.

XVIII. Place the tube on ice for 5 min.

XIX. Add the probe to the hybridization tube by pipetting it into the prehybridization solution and hybridize at 65°C over night.

Note: Avoid pipetting directly onto the membrane.

XX. Next day, wash twice at 65°C with 2× SSC (preheated to 65°C) for 5 min, measure counts (should be around 200). If necessary, wash with increasing stringency by using 2× SSC 0.1% SDS, 1× SSC 0.1% SDS, 0.5× SSC 0.1% SDS 10 min each, measure counts between each wash.

XXI. Place membrane in plastic bag and seal. Place the membrane in a cassette with BioMax™ film for 2 h and develop. Repeat overnight if necessary.

TIMING

Step 1	Order and delivery of BAC and primers	10 days
Step 2	Preparation of targeting cassette	1 week
Step 3	BAC-targeting and screening	1 week
Step 4	Confirmation of the modified BAC	2–4 weeks
Step 5	BAC-microinjection	Depends on your microinjection service

TROUBLESHOOTING

Step	Problem	Possible reason	Solutions and suggestions
PCR amplification of targeting cassette	The targeting cassette including the homology arms cannot be amplified	Difficult sequence	Clone homology arms into the vector containing Tg-Kan(R), cut out insert and use for targeting
Recombineering	No positive clone after five electroporations	Cells are not electrocompetent	Test electrocompetence by transforming a common plasmid (e.g., pBluescript)
		Homology arms are too short or contain wrong bases	Reorder the primers and generate a new PCR product of the Tg-Kan(R) cassette. Sequence also the homology region on the BAC

(Continued)

TROUBLESHOOTING (Continued)

Step	Problem	Possible reason	Solutions and suggestions
	Unintentional loss of Kan(R)	Leakage of FLP expression in the SW 105 strain	When you decide to keep Kan(R) you should preferably use another bacterial strain than SW 105 (e.g., SW 106) for recombineering. Alternatively, retransform modified BAC into other bacteria.
BAC screen PCR	No result in screening PCR	Suboptimal conditions	Using 1 μl from a overnight culture as template has proven to be more reliable than a colony PCR
BAC screen restriction digest	In restriction digest the band patterns differ from your expectation	No bands or only high-molecular-weight bands are visible	Increase amount of used BAC DNA (digest: 1 μg for expected fragments up to 2 kb size; 5 μg for smaller fragments)
		Some or many bands are missing	Part of your BAC got lost due to internal recombination, redo targeting
		High amount of genomic bacterial DNA	Use the BAC DNA-Kit for DNA-Preparation (PhasePrep™ BAC DNA-Kit, Sigma)
		Incomplete digest	Your enzyme may be methylation-sensitive, change enzyme; too short incubation; enzyme not effective; not enough enzyme
		Additional weaker bands	Your enzyme may have star activity. Reduce digestion time or change enzyme
BAC screen Southern blot	Appearance of two bands for 5′ or 3′ probe but no or only a weak band with Kan(R) probe	The kanamycin resistance was lost during culture in the SW 105 strain	See above under: Recombineering; "Unintentional loss of Kan(R)"
	Bands have not expected sizes	Wrong in silico map or wrong insertion into the BAC	Check your in silico maps and predictions, redo targeting
	Too strong and un-expected bands in agarose gel and with Kan(R) probe (Fig. 9)	Possible contamination of replicating targeting vector	Redo targeting after isolating targeting cassette on gel

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