

Technical Protocol

BAC Subcloning Kit

By Red[®]/ET[®] Recombination

Version 2.4
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Please read

The products listed in this manual are for research purposes only. They are not designed for diagnostic or therapeutic use in humans, animals or plants. Success depends on following the protocols exactly as they are described. Do read the trouble-shooting guide before beginning your experiments. Red/ET Recombination is the intellectual property of Gene Bridges GmbH.

Safety

Some chemical reagents used with this system are dangerous if handled carelessly. Take care when using chemical reagents (such as isopropanol and ethidium bromide) and electrical apparatus (high-voltage power supplies, gel electrophoresis and electroporation apparatus). Follow the manufacturer's safety recommendations.

1. BAC Subcloning Kit

Introduction

The completion of large DNA-sequencing projects, including the Human Genome Project, has generated an extraordinary amount of primary sequence data. The next major challenge is to investigate the components that make up a genome, and is often called functional genomics. *Escherichia coli* vectors that can contain large inserts, such as bacterial artificial chromosomes (BACs), P1 vectors and P1 artificial chromosomes (PACs), offer several advantages for functional genomics. They can carry sufficient DNA to encompass most eukaryotic genes, including all *cis*-acting regulatory elements, as well as many eukaryotic gene clusters, prokaryotic regulons and many complete viral genomes, in a single molecule. However, conventional cloning methods rely on the use of restriction enzymes and *in vitro* purification steps, which preclude engineering of large molecules. Consequently, the usefulness of such molecules has been limited until recently.

Red/ET Recombination is the method that permits precise engineering of DNA molecules of any size, including very large ones such as BACs or the *E.coli* chromosome. It relies on homologous recombination *in vivo* in *E.coli* and allows a wide range of modifications with DNA molecules at any chosen position.

Homologous recombination is the exchange of genetic information between two DNA molecules in a precise, specific and faithful manner. These qualities are optimal for engineering a DNA molecule regardless of its size. Homologous recombination occurs through homology regions, which are stretches of DNA shared by the two molecules that recombine. Because the sequence of the homology regions can be chosen freely, any position on a target molecule can be specifically altered.

Red/ET Recombination utilizes homologous recombination and represents a revolutionary DNA engineering platform that addresses the limitations found in conventional methods.

BAC Subcloning kit

The BAC subcloning kit is designed to subclone DNA fragments of any size, including very large fragments (> 20 kb) from any type of bacterial artificial chromosomes (BACs, P1s, PACs) into a plasmid vector.

Contents of the kit (5 Eppendorf tubes + manual):

1. pSC101-BAD-gbaA^{tet}: The Red/ET recombination protein expression plasmid (20 ng/μl, 20 μl)
2. minimal vector: PCR-template for generating a linear vector carrying a ColE1 origin plus ampicillin resistant gene (50 ng/μl, 20 μl)
3. minimal vector PCR-product: A ColE1 origin plus ampicillin resistant gene already flanked by homology arms to be used in the control reaction for subcloning mouse Hoxa11 gene (15 kb) from a mouse BAC (100 ng/μl, 10 μl)
4. BAC-control+pSC101-BAD-gbaA^{tet}: Glycerol stock of *E.coli* DH10B strain already containing a control BAC (pBeloBAC11 backbone with a 100 kb insert of mouse genomic DNA) and the Red/ET plasmid pSC101-BAD-gbaA^{tet} to be used in the control reaction (500 μl, 25% glycerol)
5. pSub-Hoxa11: Glycerol stock of *E.coli* DH10B strain containing the plasmid which contains mouse Hoxa11 gene (15 kb) as positive control (500 μl, 25% glycerol)
6. This manual with protocols, maps and sequences.

Store tubes 1–3 and 5 at -20 °C, store tubes 4 at -80 °C

2. Overview of the strategy to subclone a gene from a BAC

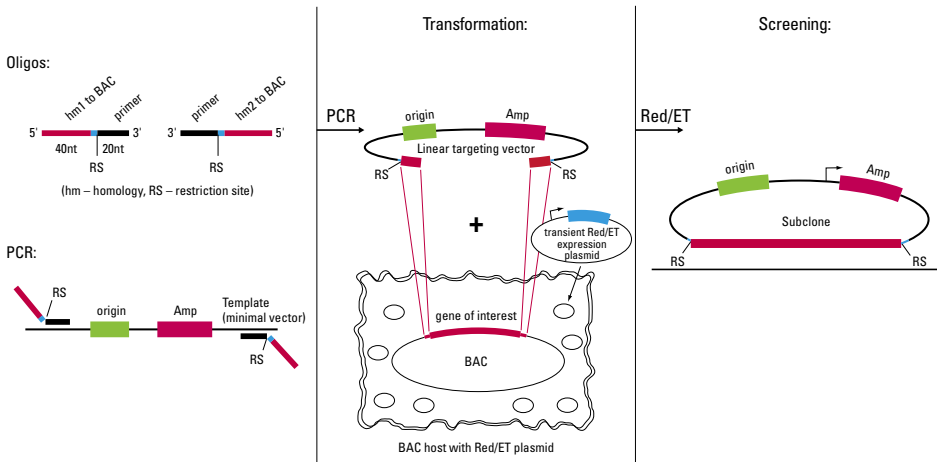


Figure 1: Oligos are designed containing stretches homologous (hm) to the fragment of the BAC which is to be subcloned. At their 3' ends, these oligos also contain primer sequences for amplification of the vector. Using these oligos a linear minimal vector with flanking homology arms is constructed in a PCR reaction. After transformation of the *E. coli* BAC host with the Red/ET expression plasmid, the expression of the genes necessary for recombination is induced. In the next step, the linear vector (PCR product with the added homology arms) is electroporated into the cells. Recombination will take place and the clones carrying the subcloned fragment are identified by selection for ampicillin resistance.

3. How Red/ET Recombination works

In Red/ET Recombination, also referred to as λ -mediated recombination, target DNA molecules are precisely altered by homologous recombination in *E. coli* which express the phage-derived protein pairs, either RecE/RecT from the λ prophage, or Red α /Red β from λ phage. These protein pairs are functionally and operationally equivalent. RecE and Red α are 5'-3' exonucleases, and RecT and Red β are DNA annealing proteins. A functional interaction between RecE and RecT, or between Red α and Red β is also required in order to catalyze the homologous recombination reaction. Recombination occurs through homology regions, which are stretches of DNA shared by the two molecules that recombine (Fig.2). The recombination is further assisted by λ -encoded Gam protein, which inhibits the RecBCD exonuclease activity of *E. coli*.

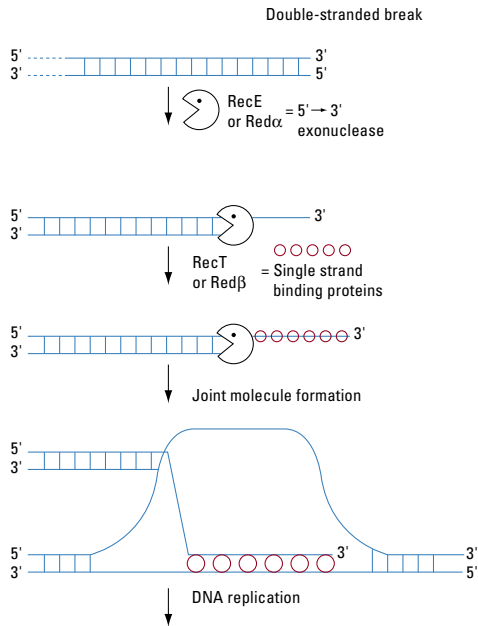


Figure 2: Mechanism of Red/ET recombination

A double-stranded break repair (DSBR) is initiated by the recombinase protein pairs, RecE/RecT or Red α /Red β .

First Red α (or RecE) digests one strand of the DNA from the DSB, leaving the other strand as a 3' ended, single-stranded DNA overhang. Then Red β (or RecT) binds and coats the single strand. The protein-nucleic acid filament aligns with homologous DNA. Once aligned, the 3' end becomes a primer for DNA replication.

The λ recombination functions can be expressed from a defective prophage integrated into the E.coli chromosome (e.g. Zhang et al. 2000) or from a plasmid (Fig. 5). In the latter case Red/ET recombination is transferable to the host strain in which the BAC resides, thereby avoiding the need to transform the BAC into a special strain.

Plasmid pSC101-BAD-gbaA-tet (Fig. 5) carries the λ phage *red $\gamma\beta\alpha$* operon expressed under the control of the arabinose-inducible pBAD promoter (Guzman et al. 1995) and confers tetracycline resistance.

The pBAD promoter is both positively and negatively regulated by the product of the *araC* gene (Schleif, 1992). AraC is a transcriptional regulator that forms a complex with L-arabinose. Arabinose binds to AraC and allows transcription to begin. In the presence of glucose or the absence of arabinose transcription is blocked by the AraC dimer.

The plasmid carries the $\text{red}\alpha$, β , γ genes of the λ phage together with the recA gene in a polycistronic operon under the control of an inducible promoter. The recombination window is therefore limited by transient expression of Red proteins. Thus, the risk of unwanted intra-molecular rearrangement is minimized.

While constitutive expression of the $\text{red}\gamma$ gene has a toxic effect in DH10B (recA^-) cells under some conditions, thus limiting the efficiency of recombination, tightly regulated expression of the γ gene together with simultaneous expression of the $\text{red}\alpha$ and β genes allows efficient homologous recombination between linear DNA fragments and BACs resident in such cells as DH10B.

The plasmid is a derivative of a thermo-sensitive pSC101 replicon which is a low copy number plasmid depending on the oriR101 . The RepA protein encoded by plasmid pSC101 is required for plasmid DNA replication and the partitioning of plasmids to daughter cells at division (Miller, Ingmer and Cohen 1995). Because the RepA protein is temperature-sensitive (Ts), cells have to be cultured at 30 °C to maintain the plasmid. pSC101 derivatives are easily curable at 37 °C to 43 °C.

Experiments have shown that after 2 h of cell growth at a temperature non-permissive for replication of this plasmid (i.e. 42 °C), the average plasmid copy number is sharply decreased; when these cells are returned to a permissive temperature, resumption of plasmid DNA replication restores copy number to normal. The copy number of the plasmid decreases by about 80% during four generations of bacterial cell growth at 42 °C. After return of the cultures to 30 °C, approximately the same number of generations of bacterial cell growth is required for the copy number of the plasmid to return to the level observed before (Miller, Ingmer and Cohen, 1995).

Since the plasmid is based on oriR101 it can be propagated in *E.coli* together with most ColE1-derived plasmids.

4. Oligonucleotide Design for Red/ET Recombination

To target your BAC at the site(s) you choose, you will need to attach short homology regions to a selectable marker. This is most conveniently done by ordering two oligonucleotides for use in PCR amplification (see Figure 3). Each oligonucleotide consists of two (or, if desired, three) parts:

1. Required Part A (A' for the other oligonucleotide) is the homology region, shared by the target molecule and the linear molecule. Choose the way you want to engineer your BAC. Often, you want to delete a section of your BAC. This is accomplished by replacing this section with the selectable marker. The homology regions are the 50bps directly adjacent to either side of the deleted section. You can delete from 0bp (i.e. make an insertion) to >100 kb. The exact sequences of the homology regions can be chosen freely, according to which position on the target molecule will be modified.
2. Optional Part B (B' for the other oligonucleotide): This part of the oligonucleotide allows useful sequences, such as HA-tags, Myc-tags, His-tags, or restriction sites, multiple cloning sites, site-specific recombination target sites, etc., to be incorporated into the recombinant product. By design, these will be incorporated into the recombinant product exactly where desired. If the introduction of such operational sequences is not needed, this piece can simply be omitted from the oligonucleotide design.

- Required Part C (C' for the other oligonucleotide): This piece, usually 18 to 24 nucleotides long, primes the PCR amplification of the selectable marker from the provided template (sequences are given on page 9).

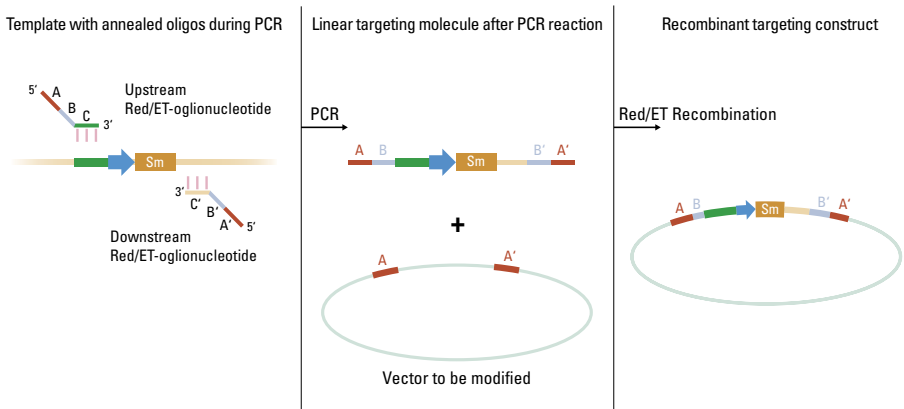


Figure 3: Practical steps involved in Red/ET. Fig. 3 illustrates the principle for modifying episomes such as bacterial artificial chromosomes (BACs). See text above for further details. *Sm*, selectable marker; the *small blue arrow* indicates a prokaryotic promoter. See Muyrers et al., 2001, for a recent overview of the possible modifications.

5. Media for antibiotic selection

All antibiotics are available from Sigma. Stock solutions should be stored at $-20\text{ }^{\circ}\text{C}$. For selective LB medium, the antibiotic is dissolved in LB medium to the indicated working concentration:

- Chloramphenicol stock solution $c = 30\text{ mg/ml}$ dissolved in ethanol. Working concentration $15\text{ }\mu\text{g/ml}$ for BACs and $50\text{ }\mu\text{g/ml}$ for high-copy plasmids.
- Ampicillin stock solution $c = 100\text{ mg/ml}$ dissolved in 50% ethanol. Working concentration $50\text{ }\mu\text{g/ml}$ for BACs and $100\text{ }\mu\text{g/ml}$ for high-copy plasmids.
- Tetracycline stock solution $c = 10\text{ mg/ml}$ dissolved in 75% ethanol. Working concentration for pSC101-BAD-gbaA $3\text{ }\mu\text{g/ml}$, for high copy plasmids $10\text{ }\mu\text{g/ml}$. Tetracycline is light sensitive.
- Kanamycin stock solution $c = 30\text{ mg/ml}$ dissolved in ddH₂O. Working concentration $15\text{ }\mu\text{g/ml}$ for BACs and $50\text{ }\mu\text{g/ml}$ for high-copy plasmids.
- Streptomycin stock solution $c = 50\text{ mg/ml}$ dissolved in ddH₂O. Working concentration $50\text{ }\mu\text{g/ml}$.

Selective LB plates are made by adding 15 g agar to 1 L LB medium. After boiling, cool to approx. $50\text{ }^{\circ}\text{C}$, add the required antibiotics to the working concentration and pour into petri dishes.

L-arabinose stock solution

Use 10% L-arabinose (Sigma A-3256) in ddH₂O, fresh or frozen in small aliquots at 20 °C.
Use 50 µl stock solution per 1.4 ml LB for induction of recombination protein expression from pSC101-BAD-gbaA.

6. Technical protocols

6.1 Protocols for generating linear vector by PCR reaction

Oligo design

Please follow the advice in Oligonucleotide Design (page 7) for Red/ET Recombination.
See the detailed sequence information of template in section 6.5.

i. Choose 50 nucleotides directly adjacent to the left of the site you want to change. Order an oligonucleotide with this sequence at the 5' end. At the 3' end of this oligo include the PCR primer sequence for amplification of the ColE1+amp template, given in *italics* below.

Upper oligonucleotide: 5'-(N)₅₀ TCACAGCTTGCTGTAAAGCGGATG -3'

ii. Choose 50 nucleotides directly adjacent to the right of the site you want to change and transfer them into the **reverse complement orientation**. Order an oligonucleotide with this sequence at the 5' end. At the 3' end of this oligo, include the 3' PCR primer sequence for the ColE1+amp template, given in *italics* below.

Lower oligonucleotide: 5'-(N)₅₀ GCTCTCCTGAGTAGGACAAATC -3'

If desired, include restriction sites or other short sequences in the ordered oligo(s) between the 5' homology regions and the 3' PCR primer sequences.

PCR

The oligonucleotides are suspended in ddH₂O at a final concentration of 25 pmol/µl. We present one standard PCR protocol, however any standard PCR protocol should yield satisfactory results.

PCR reaction (in 50 µl)

38.5 µl	ddH ₂ O
5.0 µl	10 x PCR reaction buffer
2.0 µl	5 mM dNTP
1.0 µl	upper oligonucleotide
1.0 µl	lower oligonucleotide
2.0 µl	Tn5-neo PCR-template (tube 2)
0.5 µl	Taq polymerase (5 U/µl)

- If necessary add 150 to 200 μ l mineral oil on top of the reaction to avoid evaporation.
 - An annealing temperature of 57–62 $^{\circ}$ C is optimal.
 - Thirty cycles; 1' 95 $^{\circ}$; 1' 57–62 $^{\circ}$ C; 2.5' 72 $^{\circ}$ C
1. Check 3 μ l PCR products on a gel to ensure the PCR was successful. The size of the PCR product is around 2.7 kb.
 2. Precipitate using 5 μ l 3 M NaAc, pH 7.0, and 150 μ l 100% ethanol. Mix well and precipitate for 5 min at -80° C or 30 min at -20° C. Spin down the DNA at maximal speed for 5 min.
 3. Carefully wash the pellet once with 500 μ l 70% ethanol. Be sure not to wash it away. You should see an obvious pellet at the bottom or along the walls of your tube.
 4. Dry the pellet at 37 $^{\circ}$ C using a heating block for 5 -10 min or vacuum dry for 2 min. Resuspend in 5 μ l 10 mM Tris-HCl, pH 8.0 (0.2 -0.5 μ g/ μ l).

6.2 Transformation of the Red/ET Plasmid pSC101- BAD-gbaA into the *E.coli* strain carrying the BAC with the gene of interest

Before starting

- Chill ddH₂O on ice for at least 2 hours.
 - Chill electroporation cuvettes (1 mm).
 - Cool an Eppendorf centrifuge to 2 $^{\circ}$ C.
1. Set up an overnight culture. Pick at least ten colonies carrying the BAC and inoculate them together in an Eppendorf tube containing 1.0 ml LB medium with appropriate antibiotics to select for your endogenous BAC or 15 μ g/ml chloramphenicol for the control experiment. Puncture a hole in the lid for air. Incubate at 37 $^{\circ}$ C over night with shaking.

Next day

2. Set up an Eppendorf tube containing fresh 1.4 ml LB medium conditioned with the same antibiotics as in step 1 and inoculate with 30 μ l of fresh overnight culture.
3. Culture for 2-3 hours at 37 $^{\circ}$ C, shaking at 1000 rpm.
4. Prepare the cells for electroporation
Centrifuge for 30 seconds at 11,000 rpm in a cooled Eppendorf benchtop centrifuge (at 2 $^{\circ}$ C). Discard the supernatant by quickly tipping out the supernatant twice, and place the tube on ice. Resuspend the pellet with 1 ml chilled ddH₂O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 μ l will be left in the tube with the pellet. Keep the tube on ice.
5. Take the Red/ET recombination protein expression plasmid pSC101-BAD-gbaA (tube 1). With a small pipette add 1 μ l to your cell pellet. Mix briefly. Keep the tube on ice. Transfer up to 30 μ l of cell suspension from the tube to the chilled electroporation cuvette.

6. Electroporate at 1350 V, 10 μ F, 600 Ohms. This setting applies to an Eppendorf® Electroporator 2510 using a 1 mm electroporation cuvette. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.
7. Resuspend the electroporated cells in 1 ml LB medium without antibiotics and return them to the Eppendorf tube.
8. Incubate at 30 °C for 70 min, shaking at 1000 rpm. (The Red/ET expression plasmid pSC101-BAD-gbaA will be lost at 37 °C).
9. Using a small loop, plate 100 μ l cells on LB agar plates containing tetracycline (3 μ g/ml) plus the appropriate antibiotics for the BAC [e.g. chloramphenicol (15 μ g/ml) for the control]. Incubate the plate at 30 °C overnight (or for at least 15 hours). Protect the plate from light by wrapping it up, because tetracycline is sensitive to light. Make sure the cells stay at 30 °C, or else the plasmid will be lost.
10. At the same time, use a loop to streak the control culture which already contains both a BAC and pSC101-BAD-gbaA^{tet} (tube 4) on a Cm + Tet (15 μ g/ml + 3 μ g/ml) plate and incubate at 30 °C over night. Protect the plate from light by wrapping it up.

6.3 Subcloning of a gene from a BAC by Red/ET

In the next step, the fragment which is to be subcloned will recombine into the linear vector leading to a circular molecule, which contains the ampicillin selection marker and a ColE1 origin of replication (minimal high copy vector).

Prepare electrocompetent cells from the BAC hosts that contain the Red/ET expression plasmid, shortly after inducing the expression of the recombination proteins.

In advance, prepare the linear vector DNA fragment with homology arms matching the fragment you would like to subclone from your BAC. Use tube 3 (linear vector PCR-product) and tube 4 (BAC-control+pSC101-BAD-gbaA^{tet}) to perform a control experiment in parallel.

Before starting

- Chill ddH₂O on ice for at least 2 hours
 - Chill electroporation cuvettes (1 mm)
 - Cool an Eppendorf centrifuge to 2 °C
1. To start overnight cultures, pick colonies from the plate you obtained in section 6.2, step 9 and from the control plate (step 10) and inoculate Eppendorf tubes containing 1.0 ml LB medium plus tetracycline (3 μ g/ml) and the appropriate antibiotics for the BAC [e.g. chloramphenicol (15 μ g/ml) for the control]. Pick at least 10 colonies of each. Puncture a hole in the lid for air. Incubate the cultures while shaking at 30 °C overnight.
 2. The next day, set up 4 lid-punctured Eppendorf tubes (2 for your own and 2 for control) containing 1.4 ml fresh LB medium conditioned with the same antibiotics as in step 1 and inoculate each with 30 μ l fresh overnight culture. Incubate the tubes at 30 °C for 2 hours, shaking at 1100 rpm until OD600 ~ 0.3.

3. Add 50 μl 10% L-arabinose to half of the tubes (1 for your own and 1 for control), giving a final concentration of 0.3%–0.4%. This will induce the expression of the Red/ET recombination proteins. Do not use D-arabinose. Leave the other tubes without induction as negative controls. Incubate at 37 °C, shaking for 45 min to 1 hour.
It is important that cells are incubated at 37 °C, the temperature at which all proteins necessary for the subsequent recombination are expressed. There are about 5 copies of this temperature-sensitive plasmid per cell, and during one hour there is approximately 1 doubling step, meaning any daughter cell will still have on average 2–3 copies left and will also go on expressing the recombination proteins. The plasmid is actually lost after electroporation and recombination, when cells are incubated at 37 °C over night.
4. Prepare the cells for electroporation
Centrifuge for 30 seconds at 11,000 rpm in a cooled Eppendorf benchtop centrifuge (at 2 °C). Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH₂O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 μl will be left in the tube with the pellet. Keep the tube on ice.
5. Add 1–2 μl (0.1–0.2 μg) of your prepared linear vector fragment with homology arms to the pellet in the Eppendorf tube, and pipette the mixture into the chilled electroporation cuvette. In parallel, pipette 2 μl from tube 3 into the control cells.
6. Electroporate at 1350 V, 10 μF , 600 Ohms. This setting applies to an Eppendorf® Electroporator 2510 using an electroporation cuvette with a slit of 1 mm. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.
7. Add 1 ml LB medium without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the Eppendorf tube. Incubate the cultures at 37 °C with shaking for 70 min. Recombination will now occur.
8. Streak the cultures with a loop (100 μl is sufficient) onto LB agar plates containing ampicillin (100 $\mu\text{g}/\text{ml}$). The plates should not contain tetracycline, otherwise the Red/ET Recombination protein expression plasmid (pSC101–BAD–gbaA) will either persist or the cells will die. Incubate the plates at 37 °C overnight. The Red/ET recombination protein expression plasmid (pSC101–BAD–gbaA) will disappear at 37 °C.

You should obtain >500 colonies and the ratio of correctly to incorrectly recombined clones should exceed 9:1. On the plates where the non-induced cultures were streaked only very few colonies should grow (background).

6.4 Verification of the obtained subclones

Colonies should be picked and cultured in 1 ml of LB medium with ampicillin over night to verify the successful recombination event. Plasmid DNA should be prepared and analyzed by restriction digestion.

For the control experiment, the restriction pattern of pSub-Hoxa11 is shown below (7759 bp, 3485 bp, 1959 bp, 1836 bp, 1730 bp, 692 bp, 422 bp; s. also figure 7).

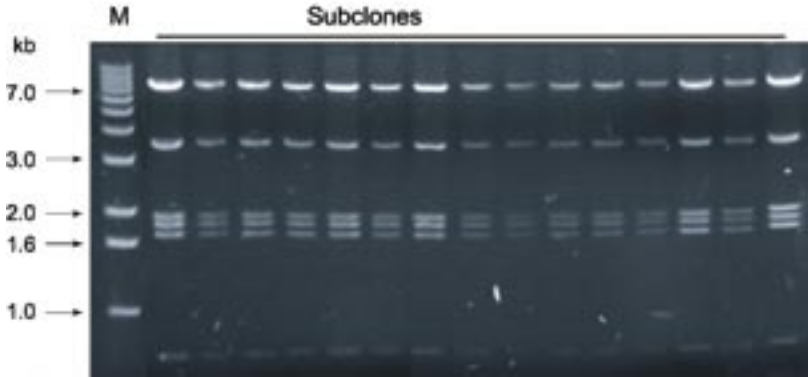


Figure 4: Restriction analysis of pSub-Hoxa11 subclones after *Bgl*I digestion.
M: 1 kb ladder from Gibco.
Lanes 1 to 15: different subclones containing the 15kb Hoxa11 gene.

6.5 Maps and Sequences

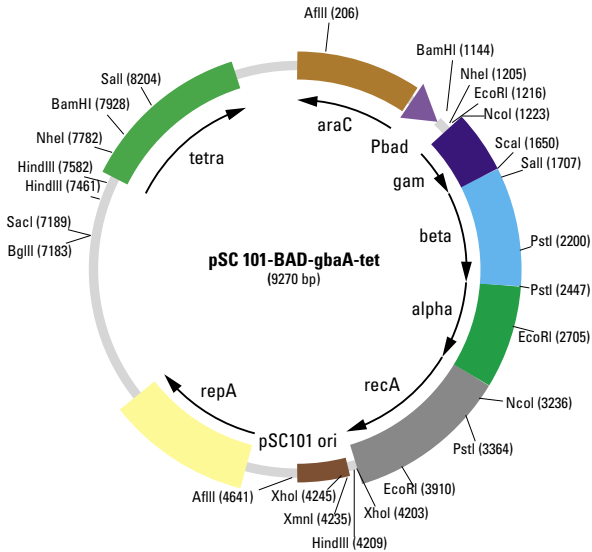


Figure 5: Map of the Red/ET expression plasmid pSC101-BAD-gbaA^{tet}. Transformation of *E.coli* hosts with this plasmid is selected for by acquisition of tetracycline resistance at 30 °C. Expression of the Red/ET recombination proteins is induced by L-arabinose activation of the BAD promoter at 37 °C.

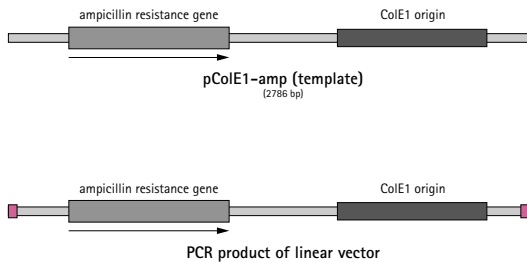


Figure 6: Map of the PCR template and PCR product of the control experiment. The pink colored regions at both ends of the PCR product resemble the introduced sequence, which is homologous to the *Hoxa11* gene (homology arms).

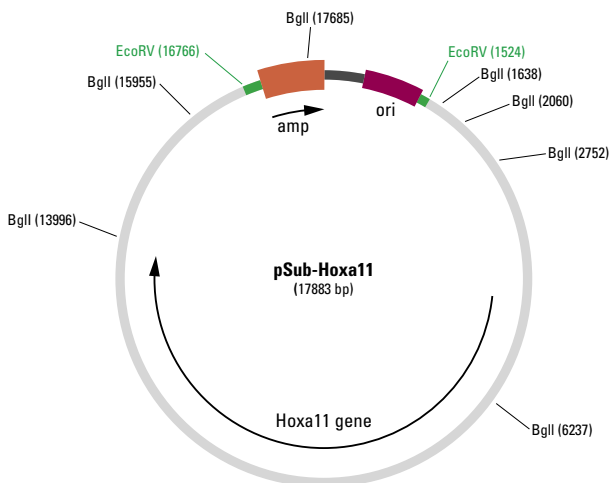


Figure 7: Map of the subclone (pSub-Hoxa11) obtained by the control reaction. A 15 kb fragment containing the mouse Hoxa11 gene of the original BAC is subcloned into a minimal vector. *BglI* restriction sites, which are used to control the successful recombination are indicated (s. also fig.4).

Oligonucleotides:

The oligonucleotides used to subclone a 15 kb fragment from the control BAC are given below. The homology arms are indicated in *italics*, the introduced EcoRV sites are indicated in bold and the sequence which primes the linear vector (PCR template) is underlined.

Upper:

5'-
*TGTCCACGTAGCACGAGCTGCTGATCACATCTCAGCGACCTCCGCCGATATC***ACAGCTTGCTGTAAGC**
GGATG-3'

Lower:

5'-
TCTCTCGGTGGAGAGAATGTGTATTACCAGGAAGAAAACCGACAATAGATATCGCTCTCCTGAGTAGG
ACAAATC-3'

EcoRV

TCTCTCGGTG GAGAGAATGT GTGTTATCAC CAGGAAGAAA ACCGACAATA GATATCGCTC
 TCCTGAGTAG GACAAATCCG CCGGGAGCGG ATTTGAACGT TGCGAAGCAA CGGCCCGGAG
 GGTGGCGGGC AGGACGCCCG CCATAAACTG CCAGGCATCA AATTAAGCAA AAGCCATCC
 TGACGGATGG CCTTTTGGC TTTCTACAAA CTCTTTTGT TATTTTTCTA AATACATTC
 AATATGTATC CGCTCATGAG ACAATAACCC TGATAAATGC TTCAATAATA TTGAAAAAGG
 AAGAGT ATG AGT ATT CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG
 GCA TTT TGC CTT CCT GTT TTT GCT CAC CCA GAA ACG CTG GTG AAA GTA AAA
 GAT GCT GAA GAT GAG TTG GGT GCA CGA GTG GGT TAC ATC GAA CTG GAT CTC
 AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG
 ATG AGC ACT TTT AAA GTT CTG CTA TGT GGC GCG GTA TTA TCC CGT GTT GAC
 GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG
 GTT GAG TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG GAT GGC ATG ACA GTA
 AGA GAA TTA TGC AGT GCT GCC ATA ACC ATG AGT GAT AAC ACT GCG GCC AAC
 TTA CTT CTG ACA ACG ATC GGA GGA CCG AAG GAG CTA ACC GCT TTT TTG CAC
 AAC ATG GGG GAT CAT GTA ACT CGC CTT GAT CGT TGG GAA CCG GAG CTG AAT
 GAA GCC ATA CCA AAC GAC GAG CGT GAC ACC ACG ATG CCT GTA GCA ATG GCA
 ACA ACG TTG CGC AAA CTA TTA ACT GGC GAA CTA CTT ACT CTA GCT TCC CGG
 CAA CAA TTA ATA GAC TGG ATG GAG GCG GAT AAA GTT GCA GGA CCA CTT CTG
 CGC TCG GCC CTT CCG GCT GGC TGG TTT ATT GCT GAT AAA TCT GGA GCC GGT
 GAG CGT GGG TCT CGC GGT ATC ATT GCA GCA CTG GGG CCA GAT GGT AAG CCC
 TCC CGT ATC GTA GTT ATC TAC ACG ACG GGG AGT CAG GCA ACT ATG GAT GAA
 CGA AAT AGA CAG ATC GCT GAG ATA GGT GCC TCA CTG ATT AAG CAT TGG TAA
 CTGTGACACC AAGTTTACTC ATATATACTT TAGATTGATT TACGCGCCT GTAGCGGCGC
 ATTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT
 AGCGCCCGCT CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCGC GCTTTCCTCG
 TCAAGCTTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC GGACATCGA
 CCCCAAAAAA CTTGATTTGG GTGATGGTTC ACGTAGTGG CCATCGCCTC GATAGACGGT
 TTTTCGCCCT TTGACGTTGG AGTCCACGTT CTTTAATAGT GGACTCTGT TCCAAACTTG
 AACAACTC AACCTATCT CGGGCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTT
 GGCCTATTGG TTAATAAATG AGCTGATTTA ACAAATTT AACGGAATT TTAACAAAAT
 ATTAACGTTT ACAATTTAAA AGGATCTAGG TGAAGATCCT TTTTGATAAT CTCATGACCA
 AAATCCCTTA AGTGTGATTT TCGTTCAC T GAGCGTCAGA CCCCCTAGAA AAGATCAAG
 GATCTTCTTG AGATCCTTTT TTTCTGCGCG TAATCTGCTG CTTGCAAACA AAAAAACCAC
 CGCTACCAGC GGTGGTTTGT TTGCCGGATC AAGAGCTACC AACTCTTTTT CCGAAGGTAA
 CTGGCTTCTAG CAGAGCGCAG ATACCAAATA CTGTCTTCT AGTGTAGCCG TAGTTAGGCC
 ACCACTCAA GAACCTCTGTA GCACCGCCTA CATACTCGC TCTGCTAATC CTGTTACCAG
 TGGCTGCTGC CAGTGGCGAT AAGTCGTGTC TTACCGGTT GGACTCAAGA CGATAGTTAC
 CGGATAAGGC CAGCGGCTCG GGCTGAACGG GGGGTTCTGT CACACAGCCC AGCTTGAGC
 GAACGACCTA CACCGAAGT AGATACCTAC AGCGTGAGCT ATGAGAAAGC GCCACGCTTC
 CGAAGGGAG AAAGGCGGAC AGGTATCCGG TAAGCGCAG GGTGCGAACA GGAGAGCGCA
 CGAGGGAGCT TCCAGGGGGA AACGCTGGT ATCTTTATAG TCTGTGCGG TTTGCGCCAC
 TCTGACTGA GCGTCGATTT TTGTGATGCT CGTCAGGGG GCGGAGCCTA TGGAAAAACG
 CCAGCAACGC GGCTTTTTTA CGTTCCTGCG CTTTTTGTG CCGTTTTGCT CACATGTTCT
 TTCCTGCGTT ATCCCCTGAT TCTGTGGATA ACCGTATTAC CGCCTTTGAG TGAGCTGATA
 CGCTCGCGC CAGCCGAACG ACCGAGCGCA GCGAGTCAGT GAGCGAGGAA GCGGAAGAGC
 GCCTGATGCG GTATTTTCTC CTTACGCATC TGTGCGGTAT TTCACACCGC ATAGGGTCA
 GGCTGCGCC CGACACCCGC CAACACCCGC TGACGCGCCC TGACGGGCTT GTCTGTCTCC
 GATCACCCT TACAGACAAG CTGTGATATC GCGCGGAGG TCGCTGAGAT GTGATCAGCA
 GCTCGTGCTA CGTGGACA *EcoRV*

Figure 8: Sequence of the PCR product, which is used in the control experiment. The red colored regions at both ends are the introduced homology arms to the *Hoxa11* gene. The sequence between the *EcoRV* sites (GATATC) reflects the minimal vector.

7. Troubleshooting

For homologous recombination the two DNA molecules must share two regions of perfect sequence identity. The most common problems are therefore 'wrong' nucleotides in one of the homology regions, which abolishes the recombination completely. Since oligos are used to add the homology regions they have to be synthesized properly and be of good quality. Take into account that long oligonucleotides (especially if they are longer than 80bp) require additional purification steps. Homologous recombination will also not take place if the sequence of the BAC is not determined 100% correctly for the homology region.

If you are trying to target a repeated sequence in your BAC, you may have problems because the homology region at the end of the linear selectable marker can go to more than one site. It is therefore best not to target repeats directly.

If after Red/ET there are no colonies or only a few visible, please check that the individual steps in the protocol were followed accurately. If the ratio of induced: uninduced antibiotic resistant bacterial colonies is not >100:1, check the following points.

1. Make sure the concentration of the appropriate antibiotics for your experimental design was accurate. Reduce the concentration of the antibiotic that you are using to select for the Red/ET event.
2. Incubation at 37 °C results in loss of the Red/ET expression plasmid. Perhaps the plasmid was lost before recombination took place.
3. Make sure that the cells were induced with L-arabinose and that the temperature was shifted from 30 °C to 37 °C. Without induction the recombination proteins will not be expressed.
4. Please check that you used **L-arabinose** not **D-arabinose**.
5. Did the electroporation cuvette remain intact during the pulse? Look at the cuvette. Has either electrode separated from the wall? If the cuvette is faulty, no DNA would have been transferred into the bacterial host.
6. The PCR-product should be concentrated and clean. Problematic PCR amplifications can mean that the recombination reaction fails. A DNA concentration of 0.3 µg/µl should be achieved before application.
7. Ensure that while preparing cells for electroporation, ddH₂O is sufficiently cooled and that the tubes are kept on ice.

If no bands are visible:

1. DNA should be directly extracted from bacteria. If you do not isolate DNA straight after cultivation, freeze the pelleted bacteria and prepare the DNA later.
2. The DNA from the mini preparation did not dissolve well in RNase water after precipitation. Make sure the DNA is sufficiently dried after precipitation.
3. Try the mini preparation with 6 ml cultures to obtain more starting material in case of large DNAs or low copy plasmids.

If the bands are not clearly visible:

1. The electrophoresis was run at over 4V/cm, resulting in poor resolution of various bands.
2. Make certain you added RNase during restriction digestion. RNA on the gel will make your DNA bands unclear or invisible.
3. Try the mini preparation with 6 ml cultures to obtain more starting material in case of large DNAs or low copy plasmids.

8. References and Patents

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9. Other products available from Gene Bridges

General information

- Red/ET Recombination is based on *in vivo* recombination. The intrinsic proofreading activity minimizes unwanted mutations in your construct.
- Besides all the necessary contents you need for your own experiment, each kit contains additional material to perform a control experiment intended to familiarize you with this technique.
- The Red/ET expression plasmid pSC101-BAD-gbaA-tet has to be propagated at 30 °C and will be lost at 37 °C.
- Kits are available for non-commercial research organizations. Commercial companies or for profit organizations require a sub-license to use Red/ET Recombination.

The products listed here can be ordered directly from our website: www.genebridges.com

BAC Modification Protocol – CD-ROM

Description:

- This 15-minute CD-ROM is a "Hands-On", audio-visual demonstration covering all the steps of the written protocol for the Quick and Easy BAC Modification Kit.

Quick and Easy BAC Modification Kit

Description:

- This kit is designed to modify any type of bacterial artificial chromosomes (BACs) within 1-2 weeks by using a kanamycin/neomycin cassette.
- This kit is optimized for basic modifications such as insertions or deletions of fragments in any type of bacterial artificial chromosomes (BACs) leaving a selectable marker gene.
- This kit can also be used to work on bacterial chromosome.
- High Red/ET efficiency plus convenient removal of the Red/ET recombination protein expression plasmid pSC101-BAD-gbaA after recombination.

Contents:

- Red/ET recombination protein expression plasmid pSC101-BAD-gbaA (amp or tet), an improved derivative of pSC101 ori (a temperature-sensitive origin). Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- BAC host *E. coli* strain DH10B already carrying the Red/ET plasmid pSC101-BAD-gbaA (tet).
- Tn5-neomycin resistance template to be used for your own experiments.
- Positive controls to introduce a Tn5-neo cassette into a 150 kb BAC.
- Detailed protocols, descriptions of plasmids, maps and sequences of oligos.

Counter-Selection BAC Modification Kit

Description:

- This kit is designed to modify any type of bacterial artificial chromosomes (BACs) within 2-3 weeks by using a counter selection cassette.
- The kit is designed for advanced BAC modification such as introducing short sequences (e.g. point mutations, loxP, restriction sites, etc.), insertion or deletion of non-selectable marker genes, fragment exchange without leaving a selection marker or any unwanted sequences.
- The included counter-selection cassette pRpsL-neo is based on Streptomycin selection which shows a much higher efficiency than pSacB-neo or comparable systems.
- This kit can also be used to work on bacterial chromosomes and common ColE1 origin plasmids.
- High Red/ET efficiency plus convenient removal of the Red/ET recombination protein expression plasmid pSC101-BAD-gbaA after recombination.

Contents:

- Red/ET Recombination protein expression plasmid pSC101-BAD-gbaA (tet), an improved derivative of pSC101 ori (a temperature-sensitive origin). Any *E. coli* strain can be made Red/ET proficient by transforming it with this plasmid.
- BAC host *E. coli* strain DH10B already carrying the Red/ET plasmid pSC101-BAD-gbaA (tet).
- pRpsL-neo template to be used for your own experiments.

- Positive controls for a point-mutation in a 150 kb BAC (BAC clone in the host carrying pSC101-BAD-gbaA, rpsL-neo PCR product flanked by homology arms for deletion in BAC backbone, BAC-rpsL-neo clone in the host as positive intermediate recombinant, oligo with homology arms and *XhoI* for replacing rpsL-neo cassette, BAC-*XhoI* clone as positive recombinant).
- Detailed protocols, descriptions of plasmids, maps and sequences of oligos.

Available soon:

Conditional Knock-out Kit

Description:

- This kit will be optimized to generate a conditional targeting construct based on any source of genomic DNA. The selection marker can be specifically removed from the targeted locus.
- No restriction sites necessary.
- No size limitations for the final targeting vector.

10. Purchaser Notification/Warranty

This product is the subject of European Patent No.1034260 (issued on 12.3.2003) (or PCT/EP98/07945) and United States Patent No. 6,355,412 (issued on 12th of March, 2002). The purchase of this product conveys to the purchaser the non-transferable right to use this product for research purposes only. The purchaser can not sell or otherwise transfer this product or its components to a third party. No rights are conveyed to the purchaser to use this product or its components for a commercial purpose. Commercial purposes shall include any activity a party receives consideration of any kind. These may include, but are not limited to use of the product or its components in manufacturing, to provide a service, information or data, use of the product for diagnostic purposes, or re-sale of the product or its components for any purpose, commercial or otherwise.

Products containing the *araB* promoter are sold under patent license for **research purposes only** and are non-transferable. Inquiries for any commercial use, including production of material to be sold commercially or used in production or in product development efforts which includes efforts toward regulatory approval, should be made directly to Xoma Corporation, Berkeley, California.

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Limited Warranty

Gene Bridges is committed to providing customers with high-quality goods and services. Gene Bridges assumes no responsibility or liability for any special, indirect, incidental or consequential loss or damage whatsoever. This warranty limits Gene Bridges GmbH's liability only to the cost of the product.

11. DNA Engineering Services available from Gene Bridges

Instead of performing your own DNA manipulations, let the Gene Bridges DNA Engineering Service do the work for you. We have worked with many commercial and research organizations across the world to provide DNA modifications in low- or high-copy plasmids, cosmids, PACs, BACs, the *E.coli* chromosome and other molecules.

The available DNA modifications are:

- Insertion of a selectable or non-selectable marker cassette
- Deletion of sequences of any size, ranging from 1 bp up to 200 Kb with or without leaving a selectable marker
- Point mutations
- Fusions
- Introduction of site specific targeting sites (loxP, FRT, etc.)
- Insertion of restriction enzyme recognition sites
- Subcloning of DNA pieces up to 150 Kb
- Transferring DNA fragments into multiple destination vectors
- BAC and cosmid stitching
- Substitutions

Contact our DNA Engineering Service through DNAService@genebridges.com, or see our website for details and prices.

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