

Technical Protocol

Cat. No. K003

BAC Subcloning Kit

By **Red®/ET® Recombination**

Version 2.8 (January 2012)

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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 and specific 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 sequences 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:

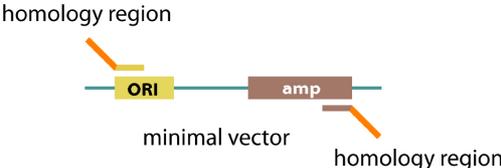
1. pRedET (*tc^R*): Red/ET expression plasmid (20 ng/μl, 20 μl)
2. minimal vector: PCR-template for generating a linear vector carrying a ColE1 origin plus ampicillin resistant (*amp^R*) gene (50ng/μl, 20 μl)
3. minimal vector PCR-product: A ColE1 origin plus ampicillin resistant gene (*amp^R*) already flanked by homology arms to be used in the control reaction for subcloning the mouse Hoxa11 gene (15kb) from a mouse BAC (100 ng/μl, 10 μl)
4. *E. coli* cells + control BAC + pRedET (*tc^R*): Glycerol stock of *E.coli* strain DH10B harboring the expression plasmid pRedET (*tc^R*) as well as a pBeloBAC11 derivative for the control experiment (500 μl, 25% glycerol)
5. pSub-Hoxa11: Glycerol stock of *E.coli* strain DH10B harboring the plasmid which contains the mouse Hoxa11 gene (15kb) as positive control (500 μl, 25% glycerol)

Please store tubes 1-3 at -20°C, and tubes 4 and 5 at -80°C.

Kit manual with protocols, maps and sequences.

2 Experimental Outline

Step 1:

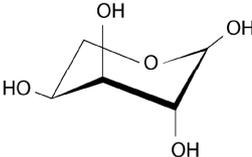


Step 2:

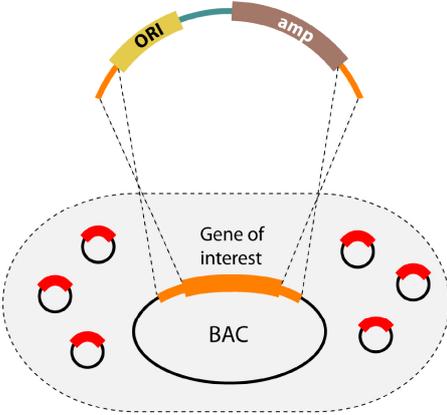
+ pRed/ET



+ L-Arabinose
30° -> 37°



Step 3:



Subclone:

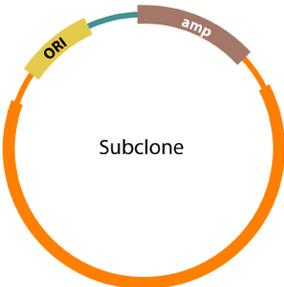


Figure 1: Flowchart of the experimental outline for subcloning a gene or part of a gene from a BAC into a plasmid.

In the first step oligonucleotides are designed containing stretches homologous (hm) to the fragment of the BAC which is to be subcloned. At their 3' ends, these oligonucleotides also contain primer sequences for amplification of the vector. Using these oligonucleotides a linear minimal vector with flanking homology arms is constructed in a PCR reaction. In the second step the *E. coli* strain carrying the BAC, which is to be modified, is transformed with the expression plasmid pRedET. The expression of genes mediating Red/ET is induced by the addition of L-arabinose. In the third 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. Only colonies with a circularized (recombines) vector will survive ampicillin selection on the agar plates.

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 Rac 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 (Figure 2). The recombination is further assisted by λ -encoded Gam protein, which inhibits the RecBCD exonuclease activity of *E. coli*.

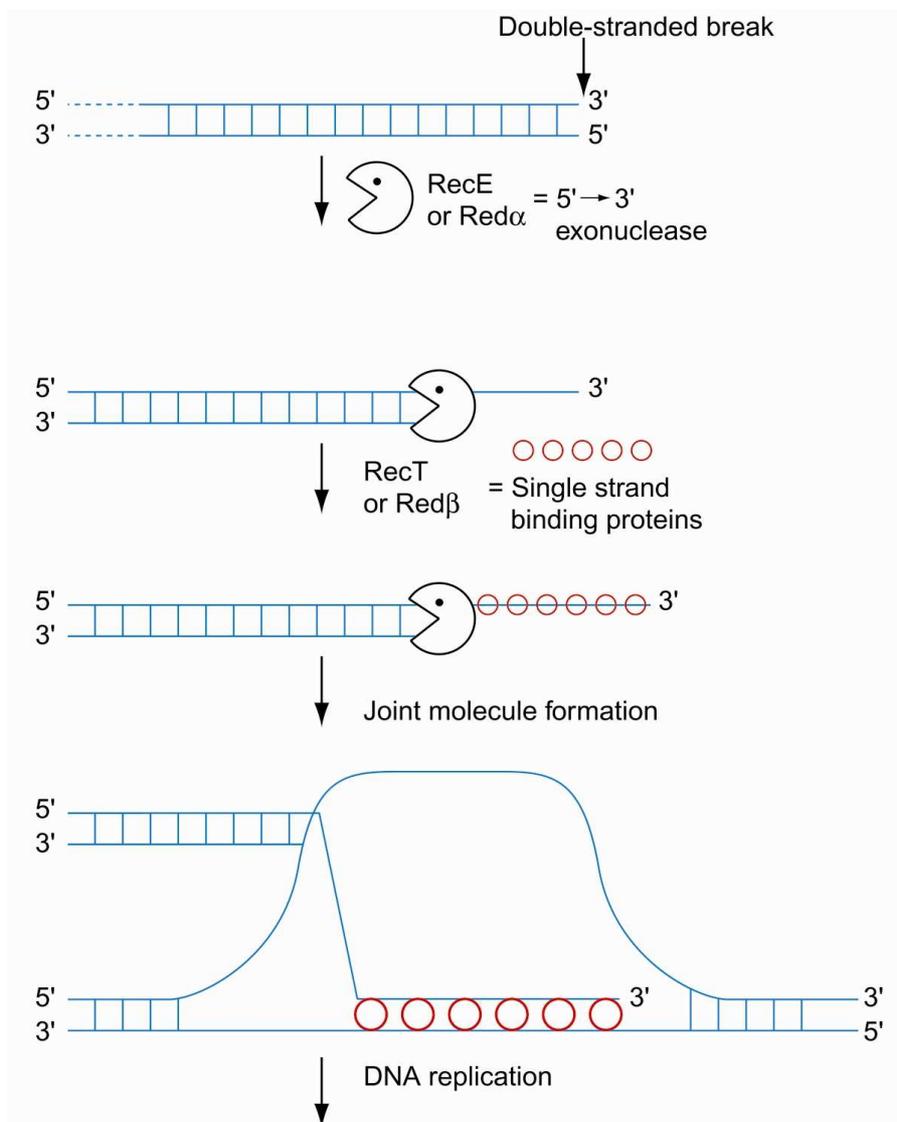


Figure 2: Mechanism of Red/ET Recombination.

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 genes can be expressed from a plasmid (Figure 5) and are therefore transferable to any *E. coli* strain.

pRedET (Figure 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 (tc^R).

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 *red* α , 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 the transient expression of Red proteins. Thus, the risk of unwanted intramolecular rearrangement is minimized.

While constitutive expression of the *red* γ gene has a toxic effect in (*recA*-) cells like DH10B or HS996 under some conditions, thus limiting the efficiency of recombination, tightly regulated expression of the γ gene together with simultaneous expression of the *red* α and β genes allows efficient homologous recombination between linear DNA fragments and plasmids resident in cells such as DH10B.

pRedET is a derivative of a thermo-sensitive pSC101 replicon, which is a low copy number plasmid dependent on 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 (T^S), 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 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 minimal vector containing 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. For the homology regions, choose the last 50bp at either end of the part you want to subclone from the BAC.
2. Optional Part B (B' for the other oligonucleotide): This part of the oligonucleotide allows useful sequences, such as restriction sites, multiple cloning sites, etc. to be incorporated into the recombinant product B and/or B'. 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.
3. Required Part C (C' for the other oligonucleotide): This sequence, usually 18 to 24 nucleotides long, primes the PCR amplification of the minimal vector from the provided template (sequences are given on page 10).

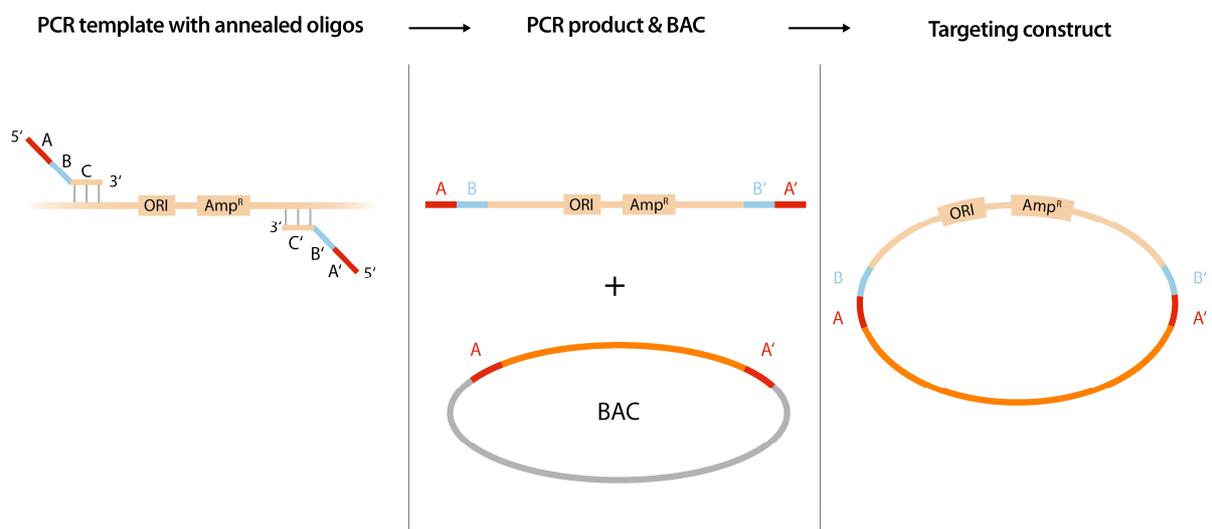


Figure 3: Practical steps involved in Subcloning by Red/ET recombination. Fig. 3 illustrates the principle for designing oligonucleotides to generate a linear vector with homology arms. See text above for further details.

5 Media for antibiotic selection

All antibiotics are available from Sigma. Stock solutions should be stored at -20°C. For selective LB medium, the antibiotic is dissolved in LB medium to the indicated working concentration:

1. Chloramphenicol (Cm) stock solution $c = 30$ mg/ml dissolved in ethanol. Working concentration 15 μ g/ml for BACs and 50 μ g/ml for high-copy plasmids.
2. Ampicillin (Amp) stock solution $c = 100$ mg/ml dissolved in 50% ethanol. Working concentration 50 μ g/ml for BACs and 100 μ g/ml for high-copy plasmids.
3. Tetracycline (Tc) stock solution $c = 10$ mg/ml dissolved in 75% ethanol. Working concentration for pSC101-BAD-gbaA 3 μ g/ml, for high copy plasmids 10 μ g/ml. Tetracycline is light sensitive.
4. Kanamycin (Km) stock solution $c = 30$ mg/ml dissolved in ddH₂O. Working concentration 15 μ g/ml for BACs and 50 μ g/ml for high-copy plasmids.
5. Streptomycin (Str) stock solution $c = 50$ mg/ml dissolved in ddH₂O. Working concentration 50 μ g/ml.

Selective LB plates are made by adding 15 g agar to 1 L LB medium. After boiling, cool to approx. 50°C, add the required antibiotics to yield the appropriate working concentrations 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 μ l stock solution per 1.4 ml LB for induction of recombination protein expression from pRedET. Frozen aliquots should not undergo more than three freeze-thaw cycles.

6 Technical protocol

6.1 Protocols for generating a linear vector by PCR reaction

Oligonucleotide design

Please follow the advice in Oligonucleotide Design (page 8) for Red/ET Recombination. See the detailed sequence information of template in section 6.5.

i. Choose 50 nucleotides at the 3' end of your gene, which you want to subclone. Order an oligonucleotide with these 50 nucleotides at the 5' end. At the 3' end of this sequence include the PCR primer sequence for amplification of the ColE1 + *amp^R*-template, given in *italics* below.

** Forward oligonucleotide: 5'-(N)₅₀ *GCTCTCCTGAGTAGGACAAATC* -3'

ii. Choose 50 nucleotides at the 5' end of your gene, which you want to subclone and transfer them into the **reverse complement orientation**. Order an oligonucleotide with this sequence at the 5' end. At the 3' end of this sequence include the PCR primer sequence for the ColE1 + *amp^R*-template, given in *italics* below.

** Reverse oligonucleotide: 5'-(N)₅₀ *TCACAGCTTGTCTGTAAGCGGATG* -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 dH₂O 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	dH 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	Minimal vector PCR-template (tube 2)
0.5 μl	Taq polymerase (5 U/μl)

- An annealing temperature of 57°- 62°C is optimal.
- Thirty cycles; 1' 95°; 1' 57°-62° C; 2.5' 72° C

1. Check 3 μ l of PCR products on a gel to ensure the PCR was successful. The size of the PCR product is around 2.7kb (s. page 17)
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°C using a heating block for 5 -10 min or vacuum dry for 2 min. Resuspend in 5 μ l 10mM Tris-HCl, pH 8.0 (0.2 -0.5 $\mu\text{g}/\mu\text{l}$).

6.2 Transformation with Red/ET expression plasmid pRedET

Before starting with the experiment, please streak out the glycerol stock of the BAC clone you obtained from the stock center on LB plates conditioned with Cm.

Day 1:

1. Set up an overnight culture. Pick one or two colonies and inoculate them in microfuge tubes containing 1.0 ml LB medium plus Cm (15 $\mu\text{g}/\text{ml}$). Puncture a hole in the lid for air. Incubate at 37°C overnight with shaking.

Day 2:

Before starting:

- Chill ddH₂O (or 10% glycerol) on ice for at least 2 h.
 - Chill electroporation cuvettes (1 mm gap).
 - Cool benchtop centrifuge to 2°C .
1. Set up one or two microfuge tubes containing fresh 1.4 ml LB medium plus Cm (15 $\mu\text{g}/\text{ml}$) and inoculate with 30 μ l of fresh overnight culture.
 2. Culture for 2-3 h at 37°C , shaking at 1,000 rpm.
 3. Prepare the cells for electroporation
Centrifuge for 30 sec at 11,000 rpm in a cooled (2°C) microfuge benchtop centrifuge. 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 μ l will be left in the tube with the pellet. Resuspend cells and keep the tube on ice.
 4. Take the Red/ET Recombination protein expression plasmid pRedET (tube 1). Add 1 μ l to your cell pellet. Mix briefly. Keep the tube on ice. Transfer the cell suspension from the tube to the chilled electroporation cuvette.

5. 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.
6. Resuspend the electroporated cells in 1 ml LB medium without antibiotics and return them to the microfuge tube.
7. Incubate at 30°C for 70 min, shaking at 1,000 rpm.
(The Red/ET expression plasmid pRedET will be lost at 37°C).
8. Using a small loop, plate 100 μ l cells on LB agar plates containing Tc (3 μ g/ml) plus Cm (15 μ g/ml) for the BAC. Incubate the plates at 30°C overnight (or for at least 15 h). Protect the plates from light by wrapping them up, because Tc is sensitive to light. Make sure the cells stay at 30°C, otherwise the Red/ET plasmid will be lost.
9. At the same time, use a loop to streak the control culture which already contains both a BAC and pRedET (tube 4) on a Cm + Tc (15 μ g/ml + 3 μ g/ml) plate and incubate at 30°C overnight. 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 *ampR* selection marker and a ColE1 origin of replication (minimal high copy vector).

Prepare electro-competent cells from the BAC hosts that contain the pRed/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 (minimal vector PCR-product) and tube 4 (*E. coli* cells + control BAC + pRedET (*tc^R*)) to perform a control experiment in parallel.

Day 3:

1. To start overnight cultures, pick one colony from the plate you obtained in 6.2, step 8 and inoculate one microfuge tube containing 1.0 ml LB medium plus Tc (3 μ g/ml) and Cm (15 μ g/ml) for the BAC. Also pick one colony from the control plate. Puncture a hole in the lid of the tubes for air. Incubate the cultures while shaking at 30°C overnight.

Day 4:

Before starting:

- Chill ddH₂O (or 10% glycerol) on ice for at least 2 h.
- Chill electroporation cuvettes (1 mm gap).
- Cool benchtop centrifuge to 2°C.

2. The next day, set up 4 lid-punctured microfuge tubes (2 for your own experiment and 2 for control experiment) containing 1.4 ml fresh LB medium conditioned with the same antibiotics as in step 1. Inoculate two of them with 30 μ l fresh overnight culture for your experiment, the other two with 30 μ l of the overnight culture from the control. Incubate the tubes at 30°C for approximately 2 h, shaking at 1,100 rpm until OD₆₀₀ ~ 0.3.
3. Add 50 μ l 10% L-arabinose to one of the tubes for your own experiment and to one of the control tubes, 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 all at 37°C, shaking for 45 min to 1 h.

Note: 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 overnight.

4. Prepare the cells for electroporation

Centrifuge for 30 sec at 11,000 rpm in a cooled (2°C) microfuge benchtop centrifuge. Discard the supernatant by quickly tipping it out 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 μ l will be left in the tube with the pellet. Resuspend cells and keep the tubes on ice.

5. Add 1-2 μ l (100-200 ng) of your prepared linear vector PCR product to the pellet to each of the two microfuge tubes (induced and uninduced), and pipette the mixture into the chilled electroporation cuvettes. In parallel, pipette 1 μ l from tube 3 into each of the two tubes of the control.
6. Electroporate at 1350 V, 10 μ F, 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 microfuge tube. Incubate the cultures at 37°C with shaking for 70 minutes. Recombination will now occur.
8. Streak the cultures with a loop (100 μ l is sufficient, if necessary plate all) onto LB agar plates containing ampicillin (100 μ g/ml). The plates should not contain Tc, otherwise the Red/ET Recombination protein expression plasmid (pRedET) will either persist or the cells will die. Incubate the plates at 37°C overnight. The Red/ET recombination protein expression plasmid (pRedET) will disappear at 37°C. You should obtain >100 colonies and the ratio of induced:uninduced bacterial colonies should exceed 10:1.

6.4 Verification of the obtained subclones

Colonies should be picked and cultured in 1 ml of LB medium with Amp overnight to verify the successful recombination event. Plasmid DNA should be prepared and analyzed by restriction digest.

For the control experiment, the restriction pattern of pSub-Hoxa11 after *Bgl*I digest is shown below (7759bp, 3485bp, 1959bp, 1836bp, 1730bp, 692bp, 422bp; s. also figure 8).

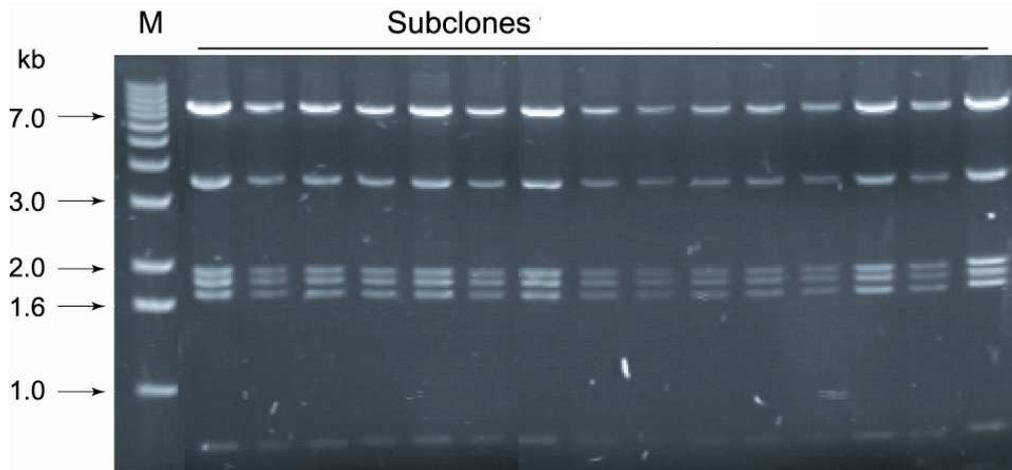


Figure 4: Restriction analysis of pSub-Hoxa11 subclones after *Bgl*I digest. *M*: 1 kb ladder from Gibco. *Lanes 1 to 15*: different subclones containing the 15kb Hoxa11 gene.

6.5 Maps and Sequences

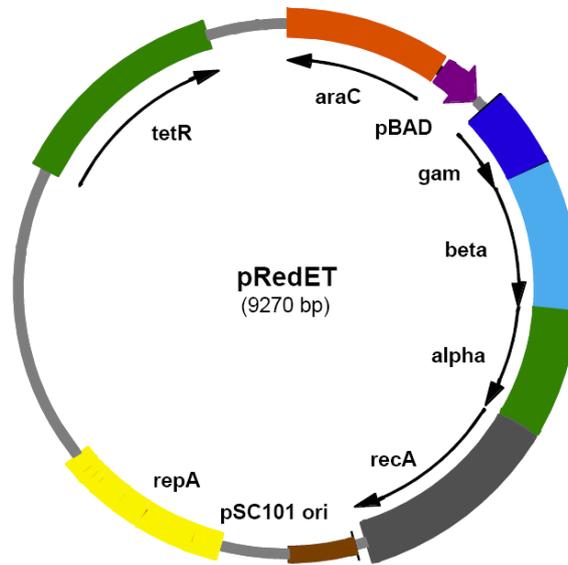


Figure 5: Map of the Red/ET expression plasmid pRedET (tc^R). Transformation of *E.coli* hosts with this plasmid is selected for by acquisition of tetracycline resistance at 30°C. Expression of the Red/ET Recombination proteins is induced by L-arabinose activation of the BAD promoter at 37°C.

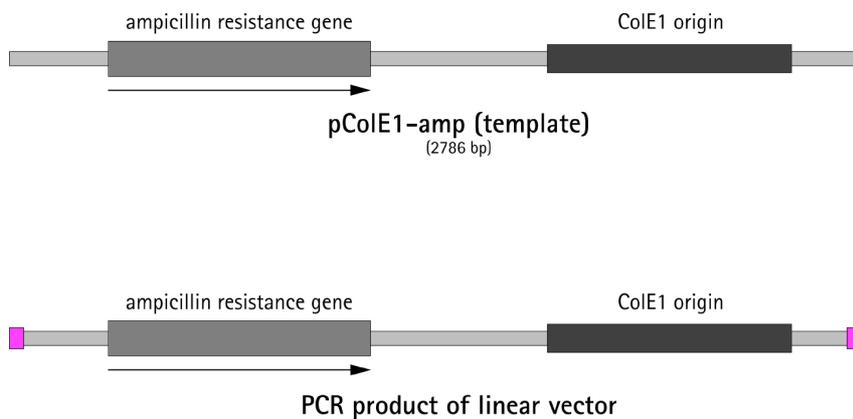


Figure 6: Map of the PCR template and PCR product of the control experiment. The pink colored regions at both ends of the PCR product represent the introduced sequence, which is homologous to the Hoxa11 gene (homology arms).

EcoRV

TCTCTCGGTG GAGAGAATGT GTGTTATCAC CAGGAAGAAA ACCGACAATA GATATCGCTC
 TCCTGAGTAG GACAAATCCG CCGGGAGCGG ATTTGAACGT TGCGAAGCAA CGGCCCGGAG
 GGTGGCGGGC AGGACGCCCC CCATAAACTG CCAGGCATCA AATTAAGCAG AAGGCCATCC
 TGACGGATGG CTTTTTGGC TTTCTACAAA CTCTTTTGTT TATTTTTCTA AATACATTCA
 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 CAG 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
 CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT TACGCGCCCT GTAGCGGCGC
 ATTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT
 AGCGCCCGCT CTTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG
 TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC GGCACCTCGA
 CCCCCAAAAA CTTGATTTGG GTGATGGTTC ACGTAGTGGG CCATCGCCCT GATAGACGGT
 TTTTTCGCCCT TTGACGTTGG AGTCCACGTT CTTTAATAGT GGACTCTTGT TCCAAACTTG
 AACAACACTC AACCCATCTC CGGGCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTT
 GGCCTATTGG TTAATAAATG AGCTGATTTA AAAAAAATTT AACGCGAATT TTAACAAAAT
 ATTAACGTTT ACAATTTAAA AGGATCTAGG TGAAGATCCT TTTTGATAAT CTCATGACCA
 AAATCCCTTA ACGTGAGTTT TCGTTCCACT GAGCGTCAGA CCCCCTAGAA AAGATCAAAG
 GATCTTCTTG AGATCCTTTT TTTCTGCGCG TAATCTGCTG CTTGCAAACA AAAAAACCAC
 CGCTACCAGC GGTGGTTTGT TTGCCGGATC AAGAGCTACC AACTCTTTTTT CCGAAGGTAA
 CTGGCTTCAG CAGAGCGCAG ATACCAAATA CTGTCCTTCT AGTGTAGCCG TAGTTAGGCC
 ACCACTTCAA GAACTCTGTA GCACCGCCTA CATACTCGC TCTGCTAATC CTGTTACCAG
 TGGCTGCTGC CAGTGGCGAT AAGTCGTGTC TTACCGGGTT GGACTIONA CGATAGTTAC
 CGGATAAGGC GCAGCGGTTC GGCTGAACGG GGGGTTCTGT CACACAGCCC AGCTTGAGC
 GAACGACCTA CACCGAAGT AGATACCTAC AGCGTGAGCT ATGAGAAAGC GCCACGCTTC
 CCGAAGGGAG AAAGGCGGAC AGGTATCCGG TAAGCGGCAG GGTGCGAACA GGAGAGCGCA
 CGAGGGAGCT TCCAGGGGGA AACGCCTGGT ATCTTTATAG TCCGTGTCGGG TTTGCGCCACC
 TCTGACTTGA GCGTCGATTT TTGTGATGCT CGTCAGGGGG GCGGAGCCTA TGGAAAAACG
 CCAGCAACGC GGCCTTTTTA CGGTTCCCTGG CCTTTTGCTG GCCTTTTGCT CACATGTTCT
 TTCTGCGT ATCCCCTGAT TCTGTGGATA ACCGTATTAC CGCCTTTGAG TGAGCTGATA
 CCGCTCGCCG CAGCCGAACG ACCGAGCGCA GCGAGTCAGT GAGCGAGGAA GCGGAAGAGC
 GCCTGATGCG GTATTTTCTC CTTACGCATC TGTGCGGTAT TTCACACCGC ATAGGGTCAT
 GGCTGCGCCC CGACACCCGC CAACACCCGC TGACGCGCCC TGACGGGCTT GTCTGCTCCC
 GGCATCCGCT TACAGACAAG CTGTGATATC GCGGCGGAGG TCGCTGAGAT GTGATCAGCA
 GCTCGTGCTA CGTGGACA *EcoRV*

Figure 7: Sequence of the PCR product used in the control experiment. The red colored regions at both ends are the introduced homology arms, which are homologous to the *Hoxa11* gene. The sequence between the *EcoRV* sites (GATATC) reflects the minimal vector.

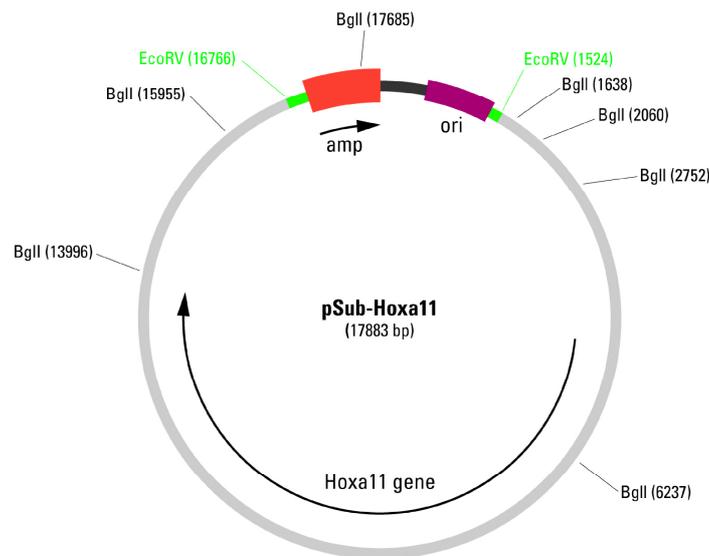


Figure 8: Map of the subclone (pSub-Hoxa11) obtained in the control reaction. A 15 kb fragment containing the mouse Hoxa11 gene of the original BAC is subcloned into a minimal vector. *Bgl*I restriction sites, which are used to check the successful recombination, are indicated (s. also fig.4)

6.6 Oligonucleotides

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

Upper:

5'-
TGTCCACGTAGCACGAGCTGCTGATCACATCTCAGCGACCTCCGCCGCGATAT
CACAGCTTGTCTGTAAGCGGATG-3'

Lower:

5'-
TCTCTCGGTGGAGAGAATGTGTGTTATCACCAGGAAGAAAACCGACAATAGATA
TCGCTCTCCTGAGTAGGACAAATC-3'

7 Troubleshooting

Problems with the recombination reaction can be caused by a number of different factors. Please review the information below to troubleshoot your experiments.

We highly recommend performing a positive control experiment using the components provided in the kit.

For homologous recombination the two DNA molecules must share two regions of perfect sequence identity. Several wrong nucleotides in the homology region can completely abolish recombination. Since oligonucleotides are used to add the homology regions they have to be synthesized properly and be of excellent quality. Take into account that long oligonucleotides (especially if they are longer than 80 bp) require additional purification steps, such as HPLC. Also note that the electronic sequences provided for BACs may not be 100% correct.

Observation:

No colonies on your plate after Red/ET Recombination:

If you do not obtain any colonies after recombination, the following parameters should be checked:

1) The PCR product

- could be wrong (check it by restriction digest or sequencing)
- could be degraded (check an aliquot on an agarose gel)
- could have incorrect homology arms. Please double-check the oligonucleotides used to generate the homology arms for quality and correctness. If necessary verify the sequence by sequencing of the PCR product.
- may not be enough; increase the amount of PCR product from approximately 200 ng up to 500 ng. Please take into consideration that you may also increase non-specific background.

2) The BAC

- may be instable and may have rearranged. Digest the BAC and run on a gel (preferably PFGE) to confirm the approximate size.
- may contain some repeats in the region you are targeting. Re-check sequence.
- could be wrong; make sure that you have the right BAC by isolating DNA and checking the region of the homology arms by PCR. If necessary sequence the PCR product to verify the region of homology. Some BACs are wrongly annotated, inherently instable or a mixture of more than one BAC.

3) The Red/ET reaction did not take place because

- there was no expression plasmid present in the cells; e.g. the cells were grown at 37°C instead of 30°C (check for tc^R),
- no or the wrong type of arabinose was used for induction (please make sure you use L-arabinose!)
- some strains (e.g. JM109, DH5alpha) are less efficient in Red/ET Recombination than others. DH10B, HS996, GeneHogs or TOP10 are our preferred strains.
- in very rare cases an elongation of the reaction time for the recombination from 70 min (incubation of electroporation) to up to four hours is necessary for successful recombination.

4) Problems with and after the electroporation:

- cells are not competent enough to take up the linear DNA fragment. Please make sure that the cells were kept on ice and that the water (respectively 10% glycerol) is sufficiently cold. Linear DNA has been shown to be about 10^4 -fold less active than DNA transformed in circular form (Eppendorf Operation Manual Electroporator 2510 version 1.0). Make sure that the time constant is around 5 ms.
- please make sure that there is no arching during the electroporation process.
- please make sure that after electroporation the cells are plated on the appropriate concentration of antibiotics depending on the copy number of the plasmid or BAC (see page 10).

Similar number of colonies on both plates, the induced and the uninduced one:

If you obtain a **high number** of colonies on both plates, it indicates that there are still traces of the circular (or supercoiled) plasmid used to prepare the linear fragment left in the sample. Since the transformation efficiency of linear fragments is 10^4 -fold less than of circular DNA molecules you may obtain a background even if no traces were visible on an agarose gel.

If the linear DNA fragment was obtained by restriction digestion, use less DNA and gel purify the fragment. If the linear cassette was obtained by PCR, set up a *DpnI* digest for your PCR product and gel purify it at the end.

If you obtain a very **low number** of colonies on both plates, it indicates that the overall efficiency of Red/ET Recombination is very low. In this case please check all parameters mentioned in the section entitled: “no colonies after Red/ET Recombination”.

You cannot separate the recombined plasmid from the non-recombined one after recombination even after re-transformation (high copy plasmid):

In very rare cases we have observed that after recombination it is difficult to separate the original plasmid from the recombined one. If you cannot separate them by dilution of the plasmid and re-transformation, you can choose a single cutting restriction enzyme and digest the plasmid for a few minutes. After re-ligation and re-transformation the two plasmids should be separated even when they were tangled (intertwined?) before.

8 References and Patents

8.1 References

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8.2 Patents

Red/ET recombination is covered by one or several of the following patents and patent applications:

- Stewart, A.F., Zhang, Y., and Buchholz, F. 1998. Novel DNA cloning method. *European Patent No.1034260 (issued on 12th of March, 2003), United States Patent No 6,509,156.*
- Stewart, A.F., Zhang, Y., and Muyrers, J.P.P. 1999. Methods and compositions for directed cloning and subcloning using homologous recombination. *United States Patent No. 6,355,412 (issued on 12th of March, 2002).*
- Youming Zhang, A. Francis Stewart, and Joep P.P. Muijrrers. 2001. Improved RecT or RecET cloning and subcloning method. *European Patent Application No. 01 117 529.6*
- Stewart, A.F., Zhang, Y., and Muyrers, J.P.P. 2001. Recombination method. *European Patent Application No. 0103276.2*

These patents and patent applications are owned by Gene Bridges, or owned by the EMBL and exclusively licensed to Gene Bridges.

9 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 for which 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.

The use of homologous recombination for commercial purposes may infringe the intellectual property covered by the EP 419,621 patent family.

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.

Xoma Corporation
2910 Seventh Street
Berkeley, CA 94710

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.

10 Other products available from Gene Bridges

General information

- Kits are available for non-commercial research organizations. Commercial companies or for-profit organizations require a sub-license to use Red/ET Recombination.

The complete product list as well as the all information how to order the kits in your country is given on our website: www.genebridges.com

K001: 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 chromosomes and common ColE1 origin plasmids.
- High Red/ET efficiency plus convenient removal of the Red/ET Recombination protein expression plasmid pRedET after recombination.

Contents:

- Red/ET Recombination protein expression plasmid pRedET. Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- BAC host *E.coli* strain HS996 already carrying the Red/ET plasmid.
- Tn5-neomycin resistance template to be used for your own experiments.
- Positive controls to introduce a Tn5-neo cassette in a 150 kb BAC.
- Detailed protocols, descriptions of plasmids, maps and sequences.

K002: 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 sites, 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 pRedET after recombination.

Contents:

- Red/ET Recombination protein expression plasmid pRedET. Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- BAC host *E.coli* strain HS996 already carrying the Red/ET plasmid.
- pRpsL-neomycin template to be used for your own experiments.
- Positive controls to introduce a point-mutation in a 150 kb BAC.
- Detailed protocols, descriptions of plasmids, maps and sequences.

**K004: Quick and Easy Conditional Knockout Kit (FRT/FLPe) and
K005: Quick and Easy Conditional Knockout Kit (loxP/Cre)**

Description:

- This kit is designed to integrate FRT or loxP sites into large vectors at any position within 2 weeks.
- Single FRT or loxP sites are inserted by Red/ET recombination of FRT or loxP flanked functional cassettes into any designated locus with subsequent removal of the selection marker by FLPe or Cre recombinases.
- Conditional targeting constructs can be generated either by a repetitive insertion of the functional cassette supplied with the kit or by combination with other functional cassettes offered by Gene Bridges.
- The functional cassette supplied with the kit (FRT-PGK-gb2-neo-FRT or loxP-PGK-gb2-neo-loxP) combines a prokaryotic promoter (gb2) for expression of kanamycin resistance in *E. coli* with an eukaryotic promoter (PGK) for expression of neomycin resistance in mammalian cells.
- High Red/ET efficiency plus convenient removal of the Red/ET Recombination protein expression plasmid pRedET after recombination.

Contents:

- Red/ET Recombination protein expression plasmid pRedET. Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- FRT or loxP flanked kanamycin/neomycin resistance template (FRT-PGK-gb2-neo-FRT or loxP-PGK-gb2-neo-loxP) to be used for your own experiments.
- Expression plasmid for FLPe or Cre site specific recombinase in *E. coli* cells
- Positive controls to introduce a single FRT site into a 15 kb high copy plasmid.
- Detailed protocols, descriptions of plasmids, maps and sequences.

K006: Quick and Easy *E.coli* Gene Deletion Kit

Description:

- This kit is designed to knock-out or alter genes on the *E. coli* chromosome in less than one week.
- Red/ET recombination allows the exchange of genetic information in a base pair precise, specific, and faithful manner.
- An FRT-flanked kanamycin resistance marker cassette is supplied with the kit which can be used to replace a gene on the *E. coli* chromosome.
- Red/ET recombination can replace fragments as large as 30kb from the chromosome.
- The use of a FRT-flanked resistance cassette for the replacement of the targeted gene allows the subsequent removal of the selection marker by a FLP-recombinase step, if required. (FLP expression plasmids can be purchased from Gene Bridges).
- Multiple knock-outs can be generated either by a repetitive insertion of the functional cassette supplied with the kit or by combination with other functional cassettes offered by Gene Bridges.
- Strictly controlled recombination process due to an optimized design of the pRedET expression plasmid. The genes for the Recombination proteins are under the control of an inducible promoter and the plasmid carries a temperature sensitive origin of replication for a convenient removal of the plasmid after recombination.

Contents:

- Two Red/ET Recombination protein expression plasmids pRedET (*tc^R*) and pRedET (*amp^R*). Any *E. coli* strain can be made Red/ET proficient by transformation with these plasmids.
- FRT flanked kanamycin resistance template (FRT-PGK-gb2-neo-FRT) to be used for your own experiments.
- Positive controls to replace the gene for mannose transporter (*manX*) on the *E. coli* chromosome.
- Detailed protocols, descriptions of plasmids, maps and sequences.

Additional functional cassettes:

- A001: Pro- and Eukaryotic Neomycin Selection Cassette (PGK-gb2-neo)
- A002: FRT flanked, Pro- and Eukaryotic Neomycin Selection Cassette (FRT-PGK-gb2-neo-FRT)
- A003: loxP flanked, Pro- and Eukaryotic Neomycin Selection Cassette (loxP-PGK-gb2-neo-loxP)
- A004: FRT flanked, Pro- and Eukaryotic Neomycin Selection Cassette plus loxP site (FRT-PGK-gb2-neo-FRT-loxP)
- A005: FRT flanked, Pro- and Eukaryotic Neomycin Selection Cassette plus loxP site 2nd version (loxP-FRT-PGK-gb2-neo-FRT)
- A006: FRT flanked Chloramphenicol Selection Cassette (FRT-cm-FRT)
- A007: loxP flanked Chloramphenicol Selection Cassette (loxP-cm-loxP)
- A008: FRT flanked Ampicillin Selection Cassette (FRT-amp-FRT)
- A009: loxP flanked Ampicillin Selection Cassette (loxP-amp-loxP)
- A010: FRT flanked, Pro- and Eukaryotic Hygromycin Selection Cassette (FRT-PGK-gb2-hygro-FRT)
- A011: loxP flanked, Pro- and Eukaryotic Hygromycin Selection Cassette (loxP-PGK-gb2-hygro-loxP)

Additional strains and plasmids:

- A104: Enhanced FLP Expression Plasmid 707-FLPe with tetracycline resistance marker for use in *E. coli* only
- A105: Enhanced FLP Expression Plasmid 708-FLPe with chloramphenicol resistance marker for use in *E. coli* only
- A112: Cre Expression Plasmid: 705-Cre (cm resistance marker)
- A113: Cre Expression Plasmid: 706-Cre (tet resistance marker)
- A201: Enhanced Eukaryotic FLP Expression Plasmid: pCAGGS-FLPe

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 work for many commercial and research organisations across the world to provide DNA modifications in low- or high-copy plasmids, cosmids, BACs and the *E.coli* chromosome.

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 more than 100 kb with or without leaving a selectable marker
- Replacement of genes on the *E.coli* chromosome
- Point mutations
- Fusions
- Introduction of site specific targeting sites (loxP, FRT, etc.)
- Insertion of restriction enzyme recognition sites
- Subcloning of DNA pieces up to 60 kb
- Transferring DNA fragments into multiple destination vectors
- BAC and cosmid stitching
- Substitutions

Contact our DNA Engineering Service by email to contact@genebridges.com, or go to www.genebridges.com for details and prices.

Gene Bridges GmbH

Commercial Centre

Im Neuenheimer Feld 584

69120 Heidelberg

Tel. +49 (0)6221-13 70 811

Fax +49 (0)6221-13 70 829

contact@genebridges.com

www.genebridges.com

