GENE BRIDGES
DNA ENGINEERING SPECIALISTS

Technical Protocol

Cat. No. K002

Counter-Selection BAC Modification Kit

(Advanced BAC Modification Kit)

By Red®/ET® Recombination

Version 3.2 (January 2012)

CONTENTS

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 Counter-Selection BAC Modification 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) 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 relies on homologous recombination *in vivo* in *E. coli* and allows a wide range of modifications of DNA molecules of any size and at any chosen position. Homologous recombination is the exchange of genetic material between two DNA molecules in a precise, specific and accurate 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.

Zhang and coworkers demonstrated in 1998 for the first time that a pair of phage coded proteins (RecE and RecT) only need 42 bp long homology arms to mediate the homologous recombination between a linear DNA molecule (e.g. a PCR product) and circular DNA (plasmid, BAC or *E. coli* chromosome). One year later the system was extended by the same group in replacing *recE* and *recT* by their respective functional counterparts of phage lambda *redα* and *redβ* (Muyrers *et al*.; 1999).

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

rpsL-neo counter-selection system

Besides the well established sucrose based counter-selection system (*sacB-neo*), Gene Bridges has developed a new selection and counter-selection system based on the *rpsL* gene (*rpsL-neo*) and Streptomycin selection.
The Streptomycin sensitivity system takes advantage of the fact that the S12 ribosomal protein is the target of Streptomycin, a widely used antibiotic. Mutations in the *rpsL* gene encoding this protein are responsible for resistance to high concentrations of Streptomycin. However, resistance is recessive in a merodiploid strain. When both wild-type and mutant alleles of *rpsL* are expressed in the same strain, the strain is sensitive to Streptomycin, possibly because of a general inhibition of translation by the wild-type ribosome.
Most of the commonly used *E. coli* strains (e.g. DH10B, HS996, DH12S, TOP10) carry a mutation in the *rpsL* gene resulting in Streptomycin resistance, which is a prerequisite for this technology.
If the wild-type *rpsL* gene is introduced via a plasmid into such an *E. coli* host, the strain will become Streptomycin sensitive again. Using this system, we developed a new counter-selection system based on an *rpsL-neo* cassette. Selection using the antibiotics Streptomycin and Kanamycin is very efficient. Therefore, overnight incubation is sufficient to achieve recombined clones. In addition, the entire cassette is just 1.3 kb in size in comparison to around 3 kb of the *sacB-neo* cassette.

REMINDER: Please make sure that the *E. coli* strain you are working with is Streptomycin resistant, since the counter-selection only works in strains carrying a mutated *rpsL* gene.

Introduction of a non-selectable marker by Red/ET Recombination using the Counter-Selection Modification kit

This kit is designed for BAC (bacterial artificial chromosome) modifications like insertion or deletion of non-selectable marker genes, fragment exchange without leaving a selection marker or introducing short non-selectable sequences like point mutations, loxP-sites or restriction sites. In a two-step approach a counter-selection cassette is first introduced at the location to be modified and in the second step replaced by non-selectable DNA.

Contents of the kit:

1. pRedET (*tc*^{R}): Red/ET expression plasmid (20 ng/µl, 20 µl)
2. *rpsL-neo* template DNA: PCR-template for generating a *rpsL*-neomycin (kanamycin) counter-selection/selection cassette (10 ng/µl, 20 µl)
3. *rpsL-neo* PCR-product: *rpsL*-neomycin cassette flanked by homology arms at the 5' and 3' end for the control experiment (100 ng/µl, 10 µl)
4. BAC-repair: Oligonucleotide to generate a point mutation resulting in an additional *Xho*I restriction site (25µM, 10µl)
5. control BAC + pRedET (*tc*^{R}): Glycerol stock of *E.coli* strain HS996 harboring the expression plasmid pRedET (*tc*^{R}) as well as a pBeloBAC11 (*cm*^{R}) derivate for the control experiment (500 µl, 25% glycerol)
6. BAC-*rpsL-neo* + pRedET (*tc*^{R}): Glycerol stock of *E.coli* strain HS996 harboring the expression plasmid pRedET (*tc*^{R}) as well as the modified control BAC (*cm*^{R}) with the inserted *rpsL-neo* cassette (500 µl, 25% glycerol)
7. BAC-repaired: Glycerol stock of *E.coli* strain HS996 harboring the modified pBeloBAC11 derivate (*cm*^{R}) after replacement of the counter-selection cassette by the BAC-repair oligonucleotide (500 µl, 25% glycerol).
8. PCR primer "oligo check-up": Amplification primer to confirm the correct recombination in the control experiment (10 µM, 20 µl)
9. PCR primer "oligo check-down": Amplification primer to confirm the correct recombination in the control experiment (10 µM, 20 µl)

Please store tubes 1-4 and 8-9 at -20℃, store tube s 5-7 at -80℃.

Kit manual with protocols, maps and sequences.

2 Experimental Outline

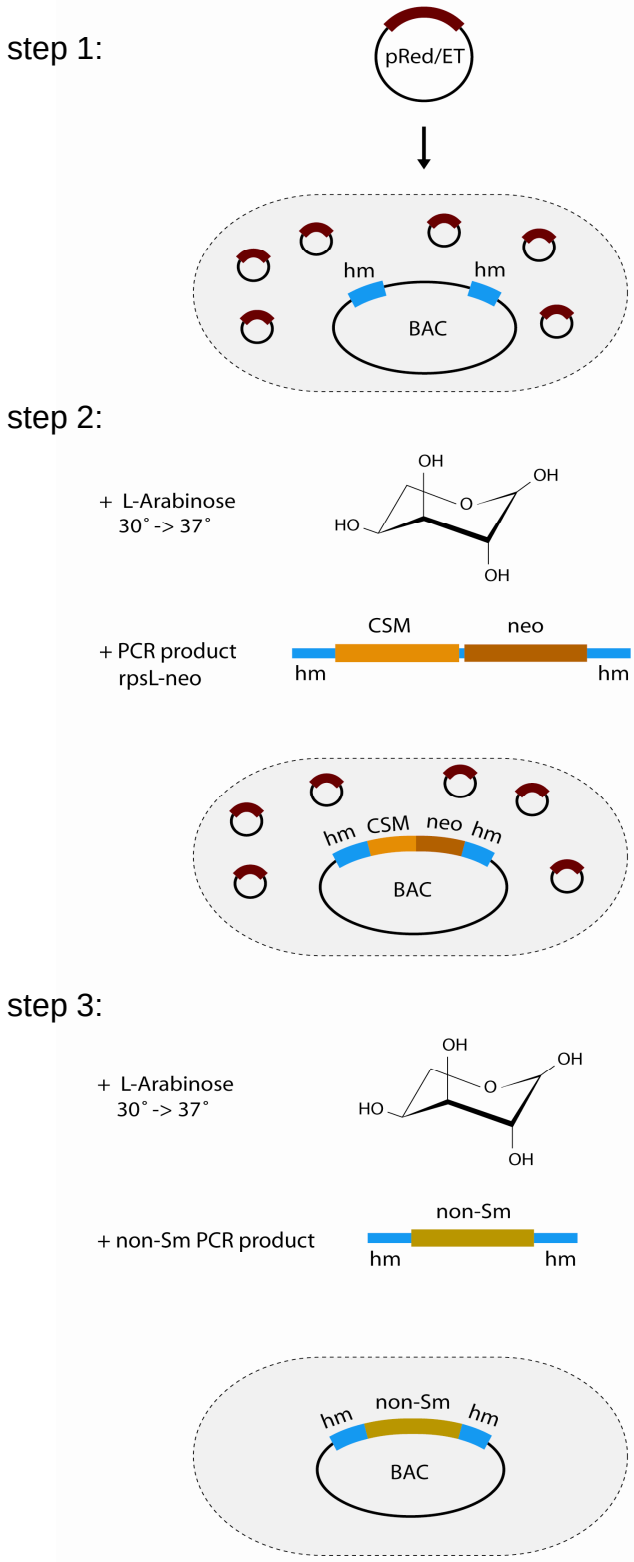

Figure 1: Flowchart of the experimental outline for the insertion of a non-selectable marker gene (e.g. a point mutation) into a BAC.

Step 1: The *E. coli* strain carrying the BAC, which is to be modified, is transformed with the expression plasmid pRedET.

Step 2: The expression of genes mediating Red/ET is induced by the addition of L-arabinose and a temperature shift from 30℃ to 37℃ . After induction the cells are prepared for electroporation and the linear *rpsL-neo* counter-selection/selection cassette (PCR product) flanked by homology arms “hm” is electroporated. Red/ET recombination inserts the functional cassette into the target locus. Only colonies carrying the modified BAC will survive Kanamycin selection on the agar plates. Grow **at 30℃** to allow the pRedET plasmid to persist in the cells.
The successful integration of the counter-selection/selection cassette will be monitored by PCR or DNA mini preparation. Due to the insertion of the *rpsL-neo* cassette cells will become **Streptomycin sensitive**. Single colonies must be analyzed to confirm the necessary Streptomycin sensitive phenotype before performing step 3.

Step 3: The expression of genes mediating Red/ET is induced by the addition of L-arabinose and a temperature shift from 30℃ to 37℃ . After induction the cells are prepared for electroporation. The non-selectable DNA, which can be either just an oligonucleotide harboring the right and the left homology arms of the selection cassette and a point mutation (control reaction) or a gene flanked by homology arms, will be electroporated. Red/ET recombination will replace the *rpsL-neo* counter-selection/selection cassette by the non-selectable DNA.
Only colonies which lost the selection/counter-selection cassette will grow on Streptomycin containing plates.
The successful integration of the non-selectable DNA will be monitored by PCR or DNA mini preparation.

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 Redα/Redβ from λ phage. These protein pairs are functionally and operationally equivalent. RecE and Redα are 5‘- 3‘ exonucleases, and RecT and Redβ are DNA annealing proteins. A functional interaction between RecE and RecT, or between Redα and Redβ 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*.

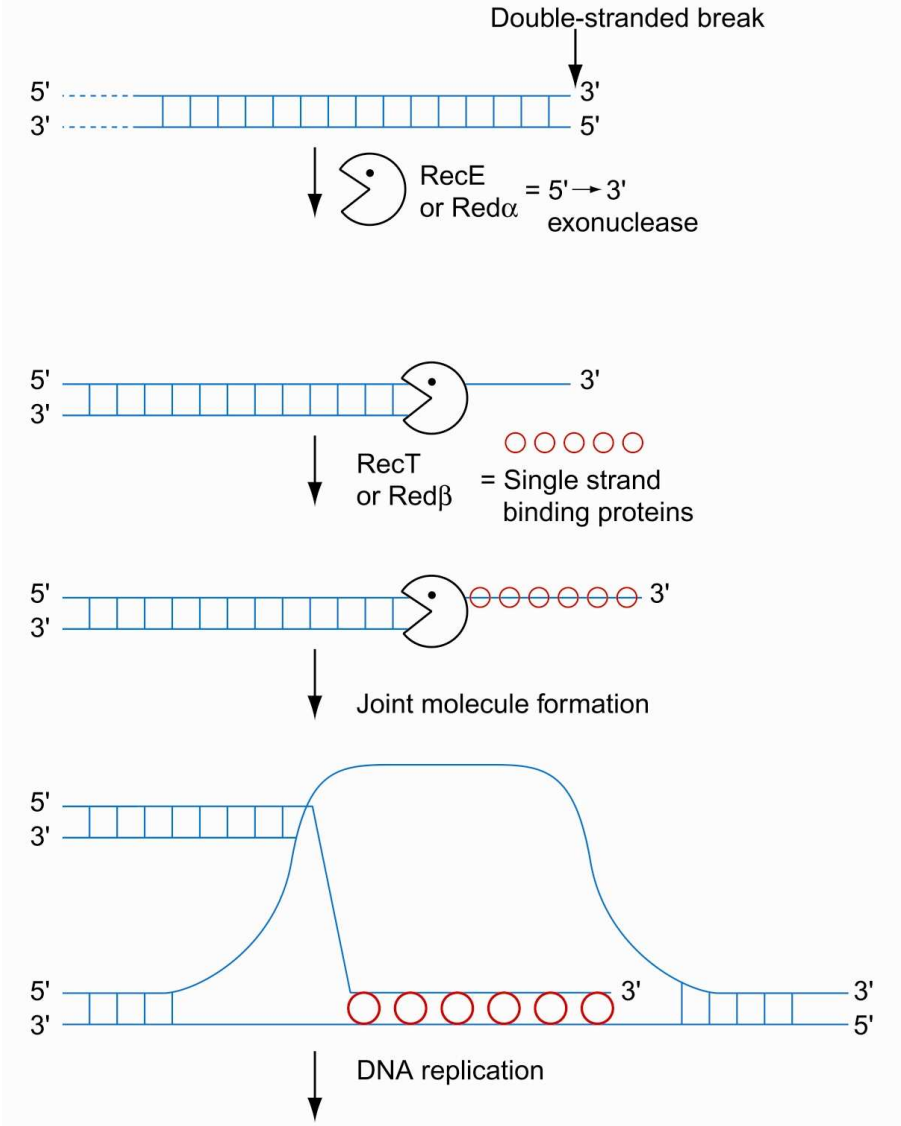

Figure 2: Mechanism of Red/ET Recombination.

Double-stranded break repair (DSBR) is initiated by the recombinase protein pairs, RecE/RecT or Redα/Redβ.

First Redα (or RecE) digests one strand of the DNA from the DSB, leaving the other strand as a 3' ended, single-stranded DNA overhang. Then Redβ (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 proteins can be expressed from a plasmid (Figure 6) and are therefore transferable to any *E. coli* strain.

pRedET (Figure 9) carries the λ phage *red* γβα 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 *ara*C 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* γβα 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 th e 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 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 50 bp directly adjacent to either side of the deleted section. You can delete from 0 bp (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.

3. 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 24).

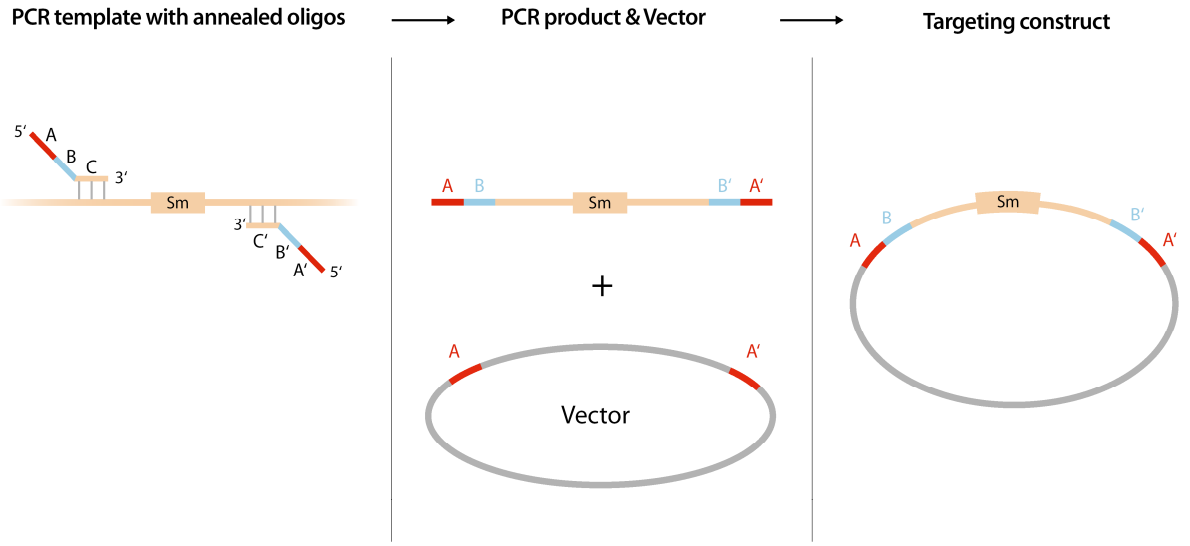

Figure 3: Practical steps involved in Red/ET. Fig. 3 illustrates the principle for modifying episomes such as bacterial artificial chromosomes (BACs). See text above for further details. Sm, selectable marker.

5 Media for Antibiotic Selection

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

1. Chloramphenicol (Cm) stock solution c = 30 mg/ml dissolved in ethanol. Working concentration 15 µg/ml for BACs, 50 µg/ml for high-copy plasmids.

2. Tetracycline (Tc) stock solution c = 10 mg/ml dissolved in 75% ethanol. Working concentration for pRedET is 3 µg/ml. Tetracycline is light sensitive.

3. Kanamycin (Km) stock solution c = 30 mg/ml dissolved in dd$H_2$0. Working concentration 15 µg/ml for BACs, 50 µg/ml for high-copy plasmids.

4. Streptomycin (Str) stock solution c = 50 mg/ml dissolved in dd$H_2$0. Working concentration 50 µg/ml.

Selective LB plates are made by adding 15 g agar to 1 L LB medium. After boiling, cool to approx. 50℃, 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 ddH_2O, fresh or frozen in small aliquots at -20℃. Use 50 µl 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 Generation of a rpsL-neo PCR product flanked by homology arms

Oligonucleotide design

Please follow the advice in Oligonucleotide Design (page 9) for Red/ET Recombination. The example used for the positive control reaction included in this kit is presented.

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 *rpsL-neo* counter selection cassette, given in *italics* below.

Upper oligonucleotide: 5'-$(N)_{50}$ *GGCCTGGTGATGATGGCGGGATCG* -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 *rpsL-neo* counter selection cassette, given in *italics* below.

Lower oligonucleotide: 5'-$(N)_{50}$ *TCAGAAGAACTCGTCAAGAAGGCG* -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 dH_2O at a final concentration of 10 pmol/µl. We present one standard PCR protocol, however any standard PCR protocol should yield satisfactory results.

<u>PCR reaction (in 50 µl)</u>

39.5 µl	dH_2O
5.0 µl	10 x PCR reaction buffer
2.0 µl	5 mM dNTP
1.0 µl	upper oligonucleotide
1.0 µl	lower oligonucleotide
1.0 µl	rpsL-neo 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 µl PCR products on a gel to ensure the PCR was successful. The size of the PCR product is for the *rpsL-neo* cassette is 1320 bp (plus homology arms).
2. Precipitate using 5 µl 3 M NaAc, pH 7.0, and 150 µl 100% ethanol. Mix well and precipitate for 5 min at -80℃ or 30 min at -20 ℃. Spin down the DNA at maximal speed for 5 min.
3. Carefully wash the pellet once with 500 µl 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℃ using a heating block for 5 -10 min or vacuum dry for 2 min. Resuspend in 5 µl 10mM Tris-HCl, pH 8.0 (0.2 -0.5 µg/µl).

As an alternative use one of the commercial available PCR purification kits!

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 with appropriate antibiotics to select for your endogenous BAC. Puncture a hole in the lid for air. Incubate at 37℃ overnight with shaking.

2. For testing of the Streptomycin resistance (*str*^{R}), streak some colonies carrying the BAC on agar plates containing 50µg/ml Str in addition to the appropriate antibiotics for the BAC. The colonies should grow on Str plates (***rpsL-neo* counter selection only works in *E. coli* strains carrying a mutated *rpsL* gene conferring a *str*^{R} phenotype**).

Day 2:

Before starting:

- Chill ddH_2O (or 10% glycerol) on ice for at least 2 h.
- Chill electroporation cuvettes (1 mm gap).
- Cool benchtop centrifuge to 2℃.

1. Set up one or two microfuge tubes containing fresh 1.4 ml LB medium with appropriate antibiotics and inoculate with 30 µl of fresh overnight culture.

2. Culture for 2-3 h at 37℃, 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 ddH_2O, 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. Resuspend cells and keep the tube on ice.

4. Take the Red/ET Recombination protein expression plasmid pRedET (tube 1). Add 1 µl 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, 10µF, 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℃ for 70 min, shaking at 1,000 rpm.
   (The Red/ET expression plasmid pRedET will be lost at 37℃).

8. Using a small loop, plate 100 µl cells on LB agar plates containing Tc (3 µg/ml) plus Cm (15 µg/ml) for the BAC. Incubate the plates at 30℃ 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℃, otherwise the Red/ET plasmid will be lost.

6.3 Inserting the rpsL-neo cassette into a BAC

In the next step, prepare electro-competent 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 DNA fragment (the *rpsL-neo* counter-selection cassette) with homology arms that you will insert into your BAC. Use tube 3 (*rpsL-neo* PCR-product) and tube 5 (control BAC + pRedET) 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 µg/ml) and Cm (15 µg/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℃ overnight.

Day 4:

Before starting:

- Chill ddH_2O (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 µl fresh overnight culture for your experiment, the other two with 30 µl of the overnight culture from the control. Incubate the tubes at 30°C for 2 h, shaking at 1100 rpm until OD_{600} ~ 0.3.

3. Add 50 µl 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 ddH_2O, 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. Resuspend cells and keep the tubes on ice.

5. Add 1-2 µl (100-200 ng) of the linear *rpsL-neo* cassette 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 µl from tube 3 into each of the two tubes of the control.

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 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 µl is sufficient, if necessary plate all) onto LB agar plates containing Cm (15 µg/ml), Km (15 µg/ml) and Tc (3 µg/ml). Incubate the plates at **30℃** overnight to keep pRed/ET in the host strain. The Red/ET recombination protein expression plasmid (pRed/ET) would get lost at 37℃. The plates should be incubated longer than 20 hours to obtain large colonies.

The ratio of induced : uninduced bacterial colonies should exceed 100 : 1. An example is shown on Figure 4.

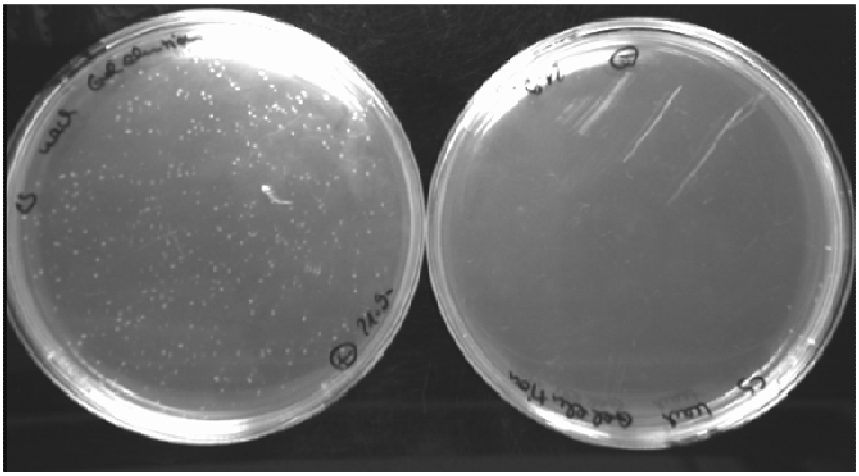

Figure 4: Typical result of a Red/ET recombination experiment. On the left plate the arabinose induced sample was streaked on a LB plated conditioned with Cm (15 µg/ml), Km (15 µg/ml) and Tc (3 µg/ml). The right plate shows the result of the uninduced control plate.

9. Pick 10 single colonies from the “induced” plates and inoculate each of them in 100 µl of LB medium with Cm+Km+Tc (15+15+3 µg/ml).

10. In parallel pick 1 colony from the original BAC plate as control and inoculate 100 µl of LB medium with Cm (15 µg/ml).

11. Incubate the tubes from steps 9 and 10 at 30℃ with shaking at 1100 rpm for 1-2 hours and use these cultures for step 12, 13, and 14.

12. After the incubation time, use a loop to streak a small sample of the culture on LB plates conditioned with Str (50 µg/ml) plus Km (15 µg/ml) plus Cm (15 µg/ml) as well as on LB plates conditioned with Km (15 µg/ml) plus Cm (15 µg/ml). Incubate the plates at 37℃ over night to test the function of the *rpsL-neo* cassette (see Figure 5).

13. Transfer 30 µl of culture from step 11 into 2 ml of fresh LB culture with the appropriate antibiotics (Cm+Km or Cm). Incubate at 37℃ over night with shaking at 1,100 rpm. These cultures will be used for preparing BAC DNA and/or for PCR verification (page 20).

14. Add 300 µl of fresh LB medium conditioned with Cm+Km+Tet (15+15+3 µg/ml) to the tubes from step 11 and incubate them at 30℃ over night. These cultures will be used for the second round of Red/ET recombination to replace the *rpsL-neo* cassette by a non-selectable gene or an oligonucleotide.

Nearly all colonies growing on the agar plates conditioned with the appropriate antibiotics [Cm (15 µg/ml), Km (15 µg/ml) and Tc (3 µg/ml)] will have successfully undergone Red/ET Recombination. Nevertheless, since the introduced *rpsL* gene was amplified by a PCR reaction, some molecules may carry a mutation leading to a Str^R phenotype although the *rpsL-neo* cassette is still present.
Such a mutation in the *rpsL* part of the cassette would result in a high background in the second Red/ET recombination step, when correctly recombined clones will be selected by Str. The BAC-*rpsL-neo* clones should therefore be confirmed by functional test and restriction digestion analysis or PCR before starting the second round of Red/ET recombination (see Figure 5). In general one or two out of eight clones analyzed show some growth on the LB plate conditioned with Str.

15. Check the Str plate from step 12 and identify the clones which didn't grow on this plate.

16. Place the streptomycin sensitive clones from step 14 at 4℃ till the BAC-*rpsL-neo* clones are confirmed by digestion or PCR.

After successful confirming that the clones contain the *rpsL-neo* insertion in the BAC (restriction pattern or PCR product) and that the *rpsL* gene is not mutated (functional test: str^S), you can go on with the next steps.

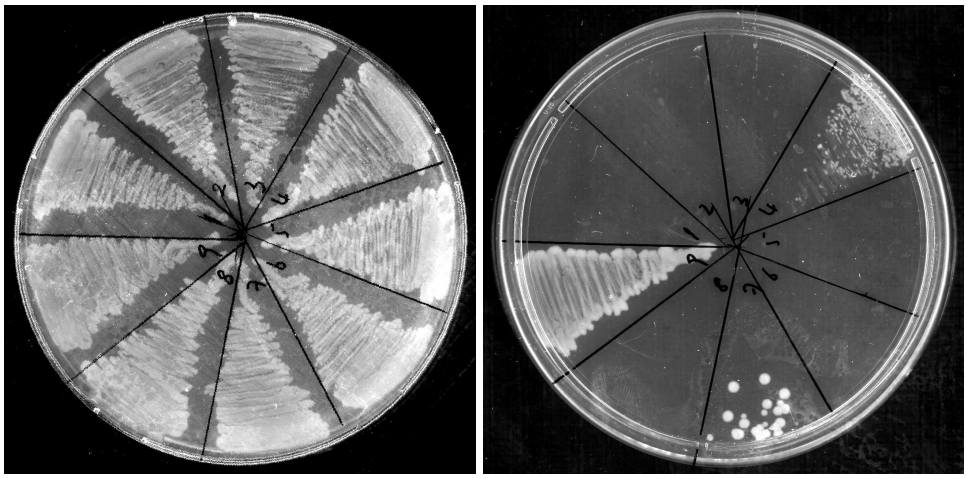

Figure 5: Typical result of a Streptomycin growth test (left plate: LB + Km + Cm; right plate: LB + Km + Cm + Str) after insertion of the *rpsL-neo* cassette by Red/ET recombination. Eight colonies carrying the *rpsL-neo* cassette were analyzed (no.1 to 8), clone number 9 is a km^R control clone.

Protocol: Preparation of BAC DNA for analytical purposes

(Next day)

1. Spin down the 2 ml overnight cultures for 1 min at 13,200 rpm.

2. Discard the supernatant and resuspend the cell pellet in 200 µl buffer P1 with RNase (from QIAGEN DNA Maxi-preparation Kit).

3. Add 200 µl of buffer P2 (Qiagen) and mix by inverting the tube several times.

4. Add 200 µl of buffer P3 (Qiagen) and mix by inverting the tube several times.

5. Spin down the white lysate at highest speed for 15 min.

6. Transfer the clear supernatant into a new 1.5 ml-Eppendorf tube and add 0.50 ml of 2-propanol.

7. Mix by inverting the tube and spin down the DNA at highest speed for 15 min.

8. Discard the supernatant and add 0.5 ml of 70% ethanol to rinse the pellet (be careful not to loose the small white pellet).

9. Spin down the DNA at highest speed for 10 min.

10. Clean the inner wall of the tube with a piece of tissue or cotton stick.

11. Dry the pellet under the speed vacuum for 2 min or leave the tube open on the bench for 5 to 10 min until the DNA pellet is completely dry. Do not overdry the pellet otherwise the DNA will become difficult to re-dissolve.

12. Resuspend the dry DNA pellet in 30 µl ddH_2O.

6.4 Replacing the rpsL-neo cassette by a non-selectable DNA

In the next step, the *rpsL-neo* cassette will be replaced by a single-stranded oligonucleotide or any non-selectable DNA flanked by homology arms.

Prepare electro-competent cells from the BAC hosts that contain the correctly inserted counter-selection cassette as well as the Red/ET expression plasmid, shortly after inducing the expression of the recombination proteins.
In advance, prepare the linear DNA fragment with homology arms that you will insert into your BAC. Use the linear 'BAC-repair' oligonucleotide from the kit (tube 4) to perform a control experiment in parallel.

Day 1:

1. Set up overnight cultures (LB medium conditioned with Cm+Km+Tet (15+15+3 µg/ml)) from a single colony of your experiment and of the control and incubate them at 30°C over night. Puncture a hole in the lid of the tubes for air. Both colonies must show a verified *str*^{S} phenotype (see section 6.3 step 14).

Day 2:

Before starting:

- Chill ddH_2O (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 Cm (15 µg/ml), Km (15 µg/ml) and Tc (3 µg/ml). Inoculate two of them with 30 µl fresh overnight culture for your experiment, the other two with 30 µl of the overnight culture from the control. Incubate the tubes at 30°C for 2 h, shaking at 1100 rpm until OD_{600} ~ 0.3.

3. Add 50 µl 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 ddH_2O, 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. Resuspend cells and keep the tubes on ice.

5. Add 1-2 µl (100-200 ng) of the linear non-selectable DNA fragment with homology arms 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 µl from tube 4 into each of the two tubes of the control.

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 microfuge tube. Incubate the cultures at 37℃ with shaking for 70 minutes. Recombination will now occur.

8. Streak the cultures with a loop (between 20 µl and 100 µl is in general sufficient, if necessary plate all) onto LB agar plates containing the appropriate antibiotics for the BAC [e.g. Cm (15 µg/ml) for the control] and Str (50 µg/ml). The plates should not contain Tc, otherwise the Red/ET expression plasmid will either persist or the cells will die.

9. Incubate the plates at 37 ℃ over night. The Red/ET recombination protein expression plasmid (pRedET) will disappear at 37℃.

You should obtain >50 colonies and the ratio of induced to uninduced bacterial colonies should exceed 10:1. Depending on the amount of cells plated there might be some very limited growth or a kind of smear visible as a background on both plates which can lead to misinterpretations. Nevertheless, single colonies should be clearly visible at least on the arabinose induced plates (see Figure 6).

The recombination efficiency of the last step normally exceeds 90%. Although most *str*^{R} colonies will contain the correct BAC recombinant, it is possible that secondary recombination, usually deletions between internal repeats in the BAC, can also occur. The frequency of such secondary recombination events varies greatly from one BAC to another. If the targeted BAC vector is inherently unstable due to direct repeats, larger numbers of clones have to be screened to confirm correctly recombined ones.

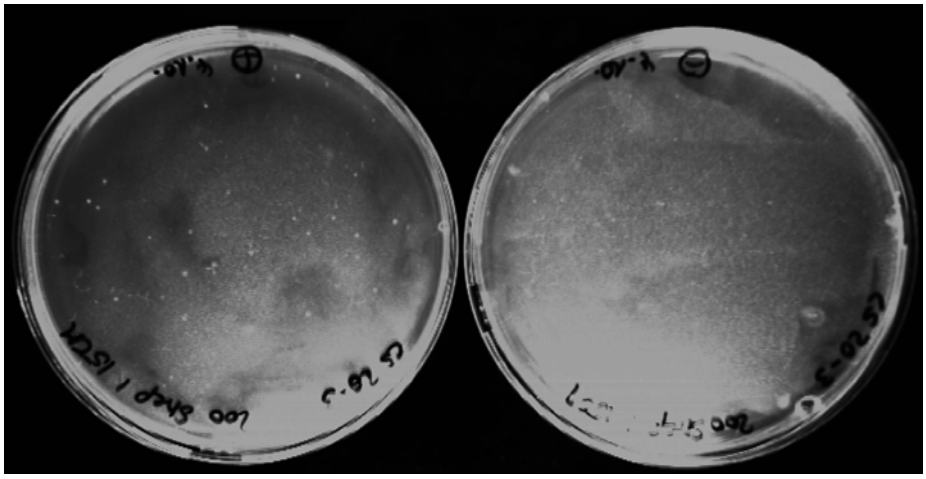

Figure 6: Typical result of a counter-selection experiment. On the left plate 50 µl of the arabinose induced sample was streaked on a LB plated conditioned with Cm (15 µg/ml) and Str (50 µg/ml). The right plate shows the result of the uninduced control plate.

To find out which clones have been modified without rearrangement, isolate the BAC DNA. Pick 10-20 colonies from the experiment and 2 from the control. Also pick colonies from the original unmodified BAC plates for DNA preparation and comparison. Analyze these DNA preparations using a) Restriction digestion of mini-prep DNA followed by electrophoresis (this is our preferred method because secondary recombination events can be detected) and/or b) PCR amplification of the insertion site using externally located primers with subsequent sequencing across the recombination site(s).

As a further control, tube 7 contains *E. coli* harboring the correctly modified product of the control reaction.

6.5 Verification of successfully modified BAC by PCR analysis

Analyze several colonies by colony PCR (e.g. pick a single colony and resuspend it in 30 µl of sterile water. Boil the sample at 98℃ for 5 minutes and take an aliquot of 2 µl of the suspension as template for your PCR reaction).
Two pairs of control primers are included in the kit (tube 8 and 9). The primers bind to the pBeloBAC11 backbone and amplify a 1066 bp fragment from the unmodified control BAC, a 1797 bp fragment after insertion of the *rpsL-neo* cassette, and a 476 bp fragment after replacement of the *rpsL-neo* cassette by the 'BAC repair' oligo (see Figure 7).
As a further control, restriction digestion of mini-prep DNA can be performed (Figure 8).

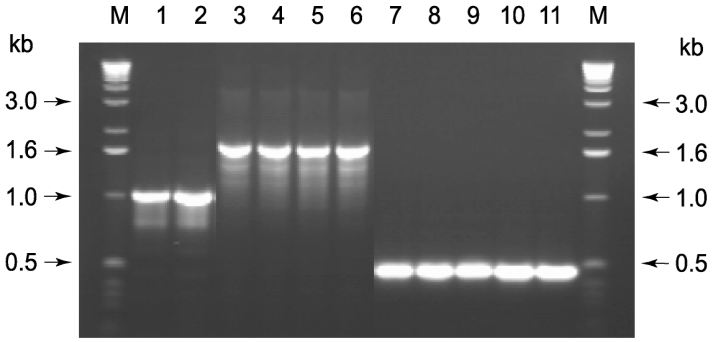

Figure 7: PCR results verifying the successful Red/ET Recombination of the control BAC. *M:* 1 kb ladder from Gibco. *Lanes 1 and 2*: unmodified control BAC resulting in a 1066 bp band. *Lanes 3 to 6*: successfully modified BACs containing the inserted *rpsL-neo* cassette showing a 1797 bp band. *Lanes 7 to 11*: BACs with introduced point mutation resulting in a 476 bp band.

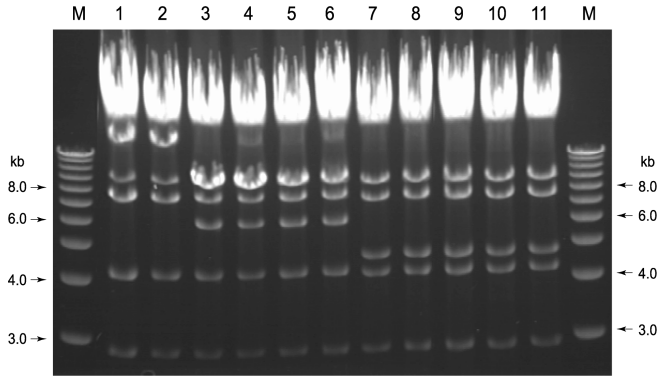

Figure 8: Restriction analysis of the original and the modified control BAC after *Xho*I digestion. *M*: 1 kb ladder from Gibco. *Lanes 1 and 2*: unmodified BACs. *Lanes 4 to 6*: successfully modified BACs with the inserted *rpsL-neo* cassette. *Lanes 7 to 11*: BACs with introduced point mutation.

6.6 Maps and sequences

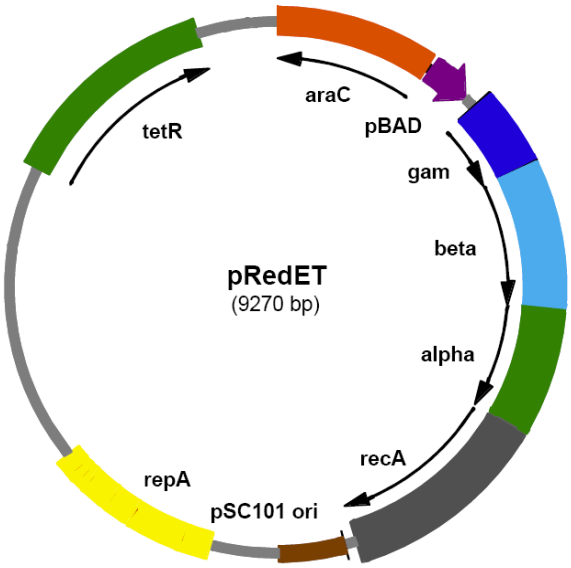

Figure 9: Map of the Red/ET expression plasmid pRedET (tc^R). Transformation of *E. coli* hosts with this plasmid is selected for by acquisition of tc^R at 30℃. Expression of the Red/ET Recombination proteins is induced by L-arabinose activation of the BAD promoter at 37℃.


```
1     GGCCTGGTGA TGATGGCGGG ATCGTTGTAT ATTTCTTGAC ACCTTTTCGG CATCGCCCTA
61    AAATTCGGCG TCCTCATATT GTGTGAGGAC GTTTTATTAC GTGTTTACGA AGCAAAAGCT
121   AAAACCAGGA GCTATTTA ATG GCA ACA GTT AAC CAG CTG GTA CGC AAA CCA CGT
175   GCT CGC AAA GTT GCG AAA AGC AAC GTG CCT GCG CTG GAA GCA TGC CCG CAA
226   AAA CGT GGC GTA TGT ACT CGT GTA TAT ACT ACC ACT CCT AAA AAA CCG AAC
277   TCC GCG CTG CGT AAA GTA TGC CGT GTT CGT CTG ACT AAC GGT TTC GAA GTG
328   ACT TCC TAC ATC GGT GGT GAA GGT CAC AAC CTG CAG GAG CAC TCC GTG ATC
379   CTG ATC CGT GGC GGT CGT GTT AAA GAC CTC CCG GGT GTT CGT TAC CAC ACC
430   GTA CGT GGT GCG CTT GAC TGC TCC GGC GTT AAA GAC CGT AAG CAG GCT CGT
481   TCC AAG TAT GGC GTG AAG CGT CCT AAG GCT TAA GGAGGACAATC ATG ATT GAA
534   CAA GAT GGA TTG CAC GCA GGT TCT CCG GCC GCT TGG GTG GAG AGG CTA TTC
585   GGC TAT GAC TGG GCA CAA CAG ACA ATC GGC TGC TCT GAT GCC GCC GTG TTC
636   CGG CTG TCA GCG CAG GGG CGC CCG GTT CTT TTT GTC AAG ACC GAC CTG TCC
687   GGT GCC CTG AAT GAA CTG CAG GAC GAG GCA GCG CGG CTA TCG TGG CTG GCC
738   ACG ACG GGC GTT CCT TGC GCA GCT GTG CTC GAC GTT GTC ACT GAA GCG GGA
789   AGG GAC TGG CTG CTA TTG GGC GAA GTG CCG GGG CAG GAT CTC CTG TCA TCT
840   CAC CTT GCT CCT GCC GAG AAA GTA TCC ATC ATG GCT GAT GCA ATG CGG CGG
891   CTG CAT ACG CTT GAT CCG GCT ACC TGC CCA TTC GAC CAC CAA GCG AAA CAT
942   CGC ATC GAG CGA GCA CGT ACT CGG ATG GAA GCC GGT CTT GTC GAT CAG GAT
993   GAT CTG GAC GAA GAG CAT CAG GGG CTC GCG CCA GCC GAA CTG TTC GCC AGG
1044  CTC AAG GCG CGC ATG CCC GAC GGC GAG GAT CTC GTC GTG ACC CAT GGC GAT
1095  GCC TGC TTG CCG AAT ATC ATG GTG GAA AAT GGC CGC TTT TCT GGA TTC ATC
1146  GAC TGT GGC CGG CTG GGT GTG GCG GAC CGC TAT CAG GAC ATA GCG TTG GCT
1197  ACC CGT GAT ATT GCT GAA GAG CTT GGC GGC GAA TGG GCT GAC CGC TTC CTC
1248  GTG CTT TAC GGT ATC GCC GCT CCC GAT TCG CAG CGC ATC GCC TTC TAT CGC
1299  CTT CTT GAC GAG TTC TTC TGA
```

Figure 10: Sequence of the *rpsL-neo* selection/counter-selection cassette.

Oligonucleotides:

The two oligonucleotides labeled ‘check-up’ and ‘check-down’ are designed for verification of the correctly recombined BAC clones by PCR. They are supplied with the kit (tubes 8 and 9).

‘check-up’: 5’-GTCGATCAGACTATCAGCGTGAG-3’

‘check-down’: 5’-TACCGAGCTCGAATTCGCCCTATAG-3’

The underlined sequence of the ‘BAC-repair’ oligonucleotide (tube 4) constitutes the left homology arm. The sequence shown in *italics* constitutes the right homology arm. The additional *Xho*I restriction site is marked in bold.

BAC-repair:

5´- TGGCCTCCACGCACGTTGTGATATGTAGATGATAA**CTCGAG***GGCCAGTG AATTGTAATACGACTCACTATAGGGCG* -3´

The oligos below were used to add the 50 bp homology regions (*italics*) for Red/ET recombination to the *rpsL-neo* selection cassette used in the control reaction. The parts of the oligos which serve as PCR primers for amplification of the *rpsL-neo* cassette are underlined. An additional *Xho*I site (bold) was introduced between the homology region and the PCR primer of the ‘lower’ oligonucleotide. These two oligos are not supplied with the kit.

Upper:

5’-*TGACGTGGTTTGATGGCCTCCACGCACGTTGTGATATGTAGATGATAATC*GG CCTGGTGATGATGGCGGGATCG-3’

Lower:

5’-*TACCGAGCTCGAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCC***CTC GAG**TCAGAAGAACTCGTCAAGAAGG-3’

7 Troubleshooting

7.1 Problems with the detection of Streptomycin sensitive clones

The detection of Streptomycin resistant (*str*^{R}) clones after the second Red/ET recombination step is sometimes difficult. When a larger amount of cells is plated a background smear may lead to the misinterpretation that the Streptomycin selection did not work well. The *str*^{R} colonies are nevertheless clearly visible in such a situation (see Figure below).

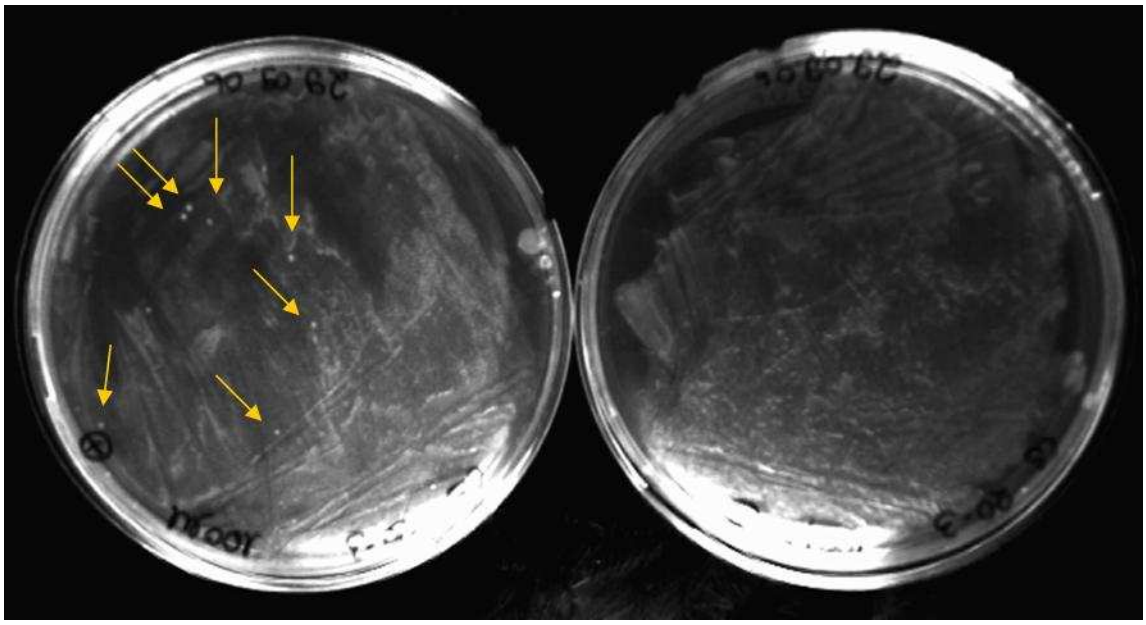

Figure 11: Counter-selection experiment with background smear. On the left plate 100 µl of an arabinose induced sample were streaked on a LB plated conditioned with Cm (15 µg/ml) and Str (50µg/ml). The right plate shows the result of the uninduced control plate. Arrows indicate *str*^{R} colonies.

7.2 Problems with the Red/ET recombination

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.

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

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℃ instead of 30℃ (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 un-induced 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 *Dpn*I 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 control all parameters mentioned in the section for “no colonies after Red/ET Recombination”.

8 References and Patents

8.1 References

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- Zhang Y., Buchholz F., Muyrers J.P.P. and Stewart A.F. 1998. A new logic for DNA engineering using recombination in Escherichia coli. *Nature Genetics* 20, 123-128.

- Zhang Y., Muyrers J.P.P., Testa G. and Stewart A.F. 2000. DNA cloning by homologous recombination in *Escherichia coli. Nature Biotech.* 18, 1314-1317.

- Zhang Y., Muyrers P.P.J., Rientjes J. and Stewart A.F. 2003. Phage annealing proteins promote oligonucleotide-directed mutagenesis in *Escherichia coli* and mouse ES cells. *BMC Molecular Biology.* 4, 1-14.

8.2 Patents

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

- PCT/EP98/07945, Novel DNA Cloning Method (ET) Priority date December 5, 1997;
- European Patent no. 1034260 by Stewart *et al.* and related patents and applications;
- U.S. Patent Application no. 09/350,830 filed July 9, 1999, Directed Cloning and Subcloning;
- US Patent nos. 6,355,412 and 6,509,156B by Stewart *et al.* and related patents and applications;

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.

K003: BAC Subcloning Kit

Description:

- This kit is optimized for subcloning of DNA fragments from BACs and cosmids.
- No restriction sites necessary.
- Fragments up to 20 kb can be subcloned.
- 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.
- Linear vector carrying a ColE1 origin of replication plus Ampicillin resistance gene to be used for the subcloning experiment.
- Positive controls to subclone a 15 kb fragment from a control BAC into the vector delivered with the kit.
- 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