

Technical Protocol

Quick and Easy BAC Modification Kit

By Red[®]/ET[®] Recombination

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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. Quick and Easy 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), P1 vectors and P1 artificial chromosomes (PACs), offer several advantages for functional genomics. They can carry sufficient DNA to encompass most eukaryotic genes, including all cis-acting regulatory elements, as well as many eukaryotic gene clusters, prokaryotic regulons and many complete viral genomes, in a single molecule. However, conventional cloning methods rely on the use of restriction enzymes and *in vitro* purification steps, which preclude engineering of large molecules. Consequently, the usefulness of such molecules has been limited until recently.

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

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

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

Quick and Easy BAC Modification kit

The Quick and Easy BAC Modification kit is designed to introduce basic modifications such as insertions and deletions in any type of bacterial artificial chromosomes (BACs) within 1-2 weeks.

Contents of the kit (7 Eppendorf tubes + manual):

1. **pSC101-BAD-gbaA^{tet}**: The Red/ET recombination protein expression plasmid (20 ng/μl, 20 μl)
2. **Tn5-neo**: PCR-template for generating a Tn5-neomycin cassette (50 ng/μl, 20 μl)
3. **Tn5-neo PCR-product**: A Tn5-neomycin resistance cassette already flanked by homology arms to be used in the control reaction (100 ng/μl, 10 μl)
4. **BAC-control+pSC101-BAD-gbaA^{tet}**: Glycerol stock of *E.coli* DH10B strain already containing a control BAC (pBeloBAC11 backbone with a 150kb insert of mouse genomic DNA) and the Red/ET plasmid pSC101-BAD-gbaA^{tet} to be used in the control reaction (500 μl, 25% glycerol)
5. **BAC-control-neo**: Glycerol stock of *E.coli* DH10B strain containing the modified control BAC. A Tn5-neomycin resistance cassette was introduced by Red/ET recombination into the backbone of the control BAC (500 μl, 25% glycerol).
6. **Oligo check-up**: Amplification primer to check the modified control BAC (25 μM, 20 μl)
7. **Oligo check-down**: Amplification primer to check the modified control BAC (25 μM, 20 μl).
8. This manual with protocols, maps and sequences

Store tubes 1-3 and 6-7 at -20° C, store tubes 4 and 5 at -80° C

2. Overview of one-step insertion of a selectable marker gene

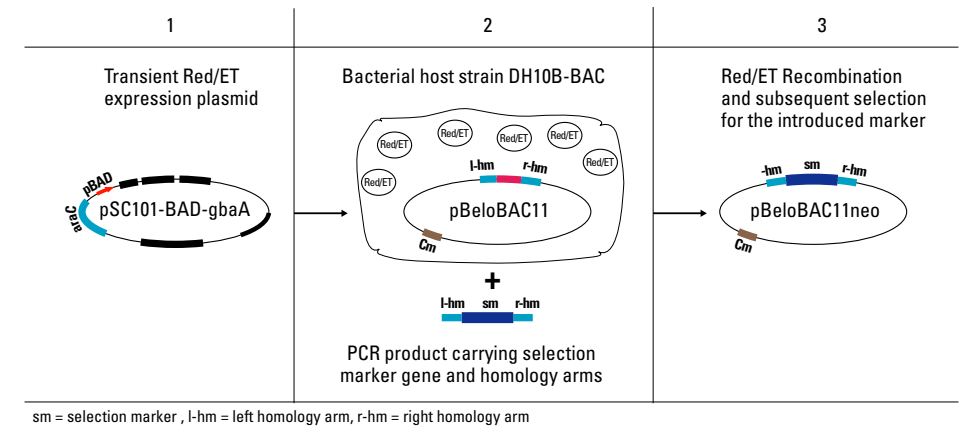


Figure 1: Mutagenesis strategy for inserting a selectable marker gene into a BAC. All nucleic acids needed for a control experiment are provided with this kit.

1. **Transform *E. coli* BAC host with the Red/ET plasmid pSC101- BAD-gbaA.** In a first step pSC101-BAD-gbaA is transferred into the *E. coli* host that contains the BAC.
2. **Modify a BAC by Red/ET.** The expression of genes mediating Red/ET is induced by adding L-arabinose and a temperature shift from 30 °C to 37 °C. After induction the cells are prepared for electroporation and the PCR product with the added homology arms is electro-porated.
3. **Extract the modified BAC DNA.** Select for the antibiotic resistance conveyed by the selectable marker in the PCR product to identify colonies carrying successfully modified BACs. A subsequent DNA mini preparation is used to confirm the desired BAC modification.

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

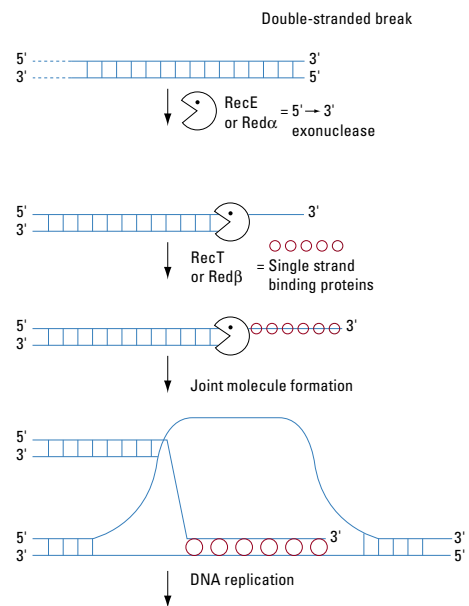


Figure 2: Mechanism of Red/ET recombination

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

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

The λ recombination functions can be expressed from a defective prophage integrated into the *E. coli* chromosome (e.g. Zhang et al. 2000) or from a plasmid (Fig. 6). In the latter case Red/ET recombination is transferable to the host strain in which the BAC resides, thereby avoiding the need to transform the BAC into a special strain.

Plasmid pSC101-BAD-gbaA-tet (Fig. 6) carries the λ phage *red $\gamma\beta\alpha$* operon expressed under the control of the arabinose-inducible pBAD promoter (Guzman et al. 1995) and confers tetracycline resistance.

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

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

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

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

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

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

4. Oligonucleotide Design for Red/ET Recombination

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

1. Required Part A (A' for the other oligonucleotide) is the homology region, shared by the target molecule and the linear molecule. Choose the way you want to engineer your BAC. Often, you want to delete a section of your BAC. This is accomplished by replacing this section with the selectable marker. The homology regions are the 50 bps directly adjacent to either side of the deleted section. You can delete from 0bp (i.e. make an insertion) to >100 kb. The exact sequences of the homology regions can be chosen freely, according to which position on the target molecule will be modified.
2. Optional Part B (B' for the other oligonucleotide): This part of the oligonucleotide allows useful sequences, such as HA-tags, Myc-tags, His-tags, or restriction sites, multiple cloning sites, site-specific recombination target sites, etc., to be incorporated into the recombinant product.

By design, these will be incorporated into the recombinant product exactly where desired. If the introduction of such operational sequences is not needed, this piece can simply be omitted from the oligonucleotide design.

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 9).

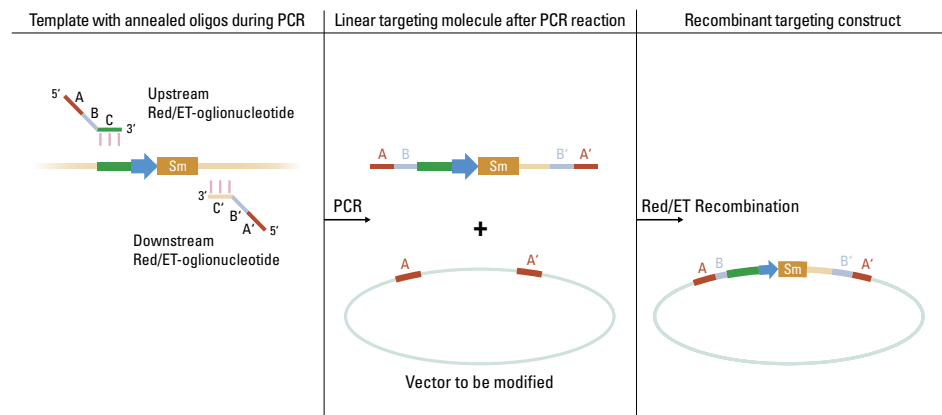


Figure 3: Practical steps involved in Red/ET. Fig. 3 illustrates the principle for modifying episomes such as bacterial artificial chromosomes (BACs). See text above for further details. *Sm*, selectable marker; the *small blue arrow* indicates a prokaryotic promoter. See Muyrers et al., 2001, for a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

5. Media for antibiotic selection

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

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

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

L-arabinose stock solution

Use 10% L-arabinose (Sigma A-3256) in ddH₂O, fresh or frozen in small aliquots at $-20\text{ }^{\circ}\text{C}$.

Use $50\text{ }\mu\text{l}$ stock solution per 1.4 ml LB for induction of recombination protein expression from pSC101-BAD-gbaA.

6. Technical protocols

6.1 Generation of a Tn5-neo PCR product flanked by homology arms

Oligo design

Please follow the advice in Oligonucleotide Design (page 7) 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 neomycin selection cassette, given in italics below.

Upper oligonucleotide: $5'-(N)_{50}\text{ TGGACAGCAAGCGAACCGGAATTGC}-3'$

- 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 neomycin selection cassette, given in italics below.

Lower oligonucleotide: $5'-(N)_{50}\text{ TCAGAAGAACTCGTCAAGAAGGCG}-3'$

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 ddH₂O at a final concentration of $25\text{ pmol}/\mu\text{l}$. We present a standard PCR protocol, however any standard PCR protocol should yield satisfactory results.

PCR reaction (in $50\text{ }\mu\text{l}$)

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

- If necessary add 150 to 200 µl mineral oil on top of the reaction to avoid evaporation.
- An annealing temperature of 57–62 °C is optimal.
- Thirty cycles; 1' 95°; 1' 57–62 °C; 1' or longer 72 °C

1. Check 3 µl PCR product on a gel to ensure the PCR was successful.
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 °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 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 °C using a heating block for 5–10 min or vacuum dry for 2 min. Resuspend in 5 µl 10 mM Tris-HCl, pH 8.0 (0.2–0.5 µg/µl).

6.2 Transformation with the Red/ET Plasmid pSC101- BAD-gbaA

Before starting

- Chill ddH₂O on ice for at least 2 hours.
- Chill electroporation cuvettes (1 mm).
- Cool an Eppendorf centrifuge to 2 °C.

STAGE 1

1. Set up an overnight culture. Pick at least ten colonies carrying the BAC and inoculate them together in an Eppendorf tube containing 1.0 ml LB medium with appropriate antibiotics to select for your endogenous BAC. Puncture a hole in the lid for air. Incubate at 37 °C over night with shaking.

Next day

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

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

6.3 Modify a BAC by Red/ET

In the second stage, (Stage 2) prepare electro-competent cells from the BAC hosts that contain the Red/ET expression plasmid, shortly after inducing the expression of the recombination proteins (gbaA).

In advance, prepare the linear DNA fragment with homology arms that you will insert into your BAC. Use tube 3 (Tn5-neomycin PCR-product) and tube 4 (BAC-control+pSC101- BAD-gbaA^{ect}) to perform a control experiment in parallel.

Before you start

- Chill ddH₂O on ice for at least 2 hours.
- Chill electroporation cuvettes (1 mm).
- Cool down an Eppendorf centrifuge to 2 °C.

STAGE 2

1. To start overnight cultures, pick colonies from the plate you obtained in section 6.2, step 9 and from the control plate (step 10) and inoculate Eppendorf tubes containing 1.0 ml LB medium plus tetracycline (3 µg/ml) and the appropriate antibiotics for the BAC [e.g. chloramphenicol (15 µg/ml) for the control]. Pick at least 10 colonies of each. Puncture a hole in the lid for air. Incubate the cultures while shaking at 30 °C over night.
2. The next day, set up 4 lid-punctured Eppendorf 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 and inoculate each with 30 µl fresh overnight culture. Incubate the tubes at 30 °C for 2 hours, shaking at 1100 rpm until OD₆₀₀ ~ 0.3.

3. Add 50 μ l 10% L-arabinose to half of the tubes (1 for your own and 1 for control), giving a final concentration of 0.3%–0.4%. This will induce the expression of the Red/ET recombination proteins. Do not use D-arabinose. Leave the other tubes without induction as negative controls. Incubate at 37 °C, shaking for 45 min to 1 hour.

It is important that cells are incubated at 37 °C, the temperature at which all proteins necessary for the subsequent recombination are expressed. There are about 5 copies of this temperature-sensitive plasmid per cell, and during one hour there is approximately 1 doubling step, meaning any daughter cell will still have on average 2–3 copies left and will also go on expressing the recombination proteins. The plasmid is actually lost after electroporation and recombination, when cells are incubated at 37 °C over night.

4. Prepare the cells for electroporation

Centrifuge for 30 seconds at 11,000 rpm in a cooled Eppendorf benchtop centrifuge (at 2 °C). Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH₂O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 μ l will be left in the tube with the pellet. Keep the tube on ice.

5. Add 1–2 μ l (0.1–0.2 μ g) of your prepared linear fragment with homology arms to the pellet in the Eppendorf tube, and pipette the mixture into the chilled electroporation cuvette. In parallel, pipette 2 μ l from tube 3 into the control cells.
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 Eppendorf tube. Incubate the cultures at 37 °C with shaking for 70 min. Recombination will now occur.
8. Streak the cultures with a loop (100 μ l is sufficient) onto LB agar plates containing the appropriate antibiotics for the BAC [e.g. chloramphenicol (15 μ g/ml) for the control] and kanamycin (15 μ g/ml). The plates should not contain tetracycline; otherwise the Red/ET Recombination protein expression plasmid (pSC101–BAD–gbaA) will either persist or the cells will die. Incubate the plates at 37 °C overnight. The Red/ET recombination protein expression plasmid (pSC101–BAD–gbaA) will disappear at 37 °C. You should obtain >500 colonies and the ratio of correctly to incorrectly recombined BACs should exceed 9:1.

Nearly all colonies growing on the agar plates conditioned with the appropriate antibiotics will have successfully recombined by Red/ET. Notably, although most kanamycin-resistant 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 secondary recombination events varies greatly from one BAC to another.

To find out which clones have been modified without rearrangement, isolate the BAC DNA. Pick 10–20 colonies from your experiment and 2 from the control. Also pick colonies from the original

unmodified BAC plates for DNA preparation and comparison. Check 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 either b) DNA sequencing, using external primers to sequence across the recombination site(s) and/or c) PCR amplification of the insertion site using externally located primers. As a further control, tube 5 contains *E.coli* harboring the correctly modified product of the control reaction. You can streak out these cells, pick colonies, culture and prepare a BAC mini-prep DNA for analysis by a), b), c) above.

6.4 Verification of modified BACs by PCR and restriction analysis

Oligos 'Check-up' (tube 6) and 'Check-down' (tube 7) are supplied for checking the successfully modified control BAC in a polymerase chain reaction (PCR). Use the bacterial colonies as a template for a colony-PCR. The primers amplify a 1066 bp fragment from the unmodified control BAC and a 1133 bp fragment after insertion of the neomycin cassette (fig. 4). As a further control, restriction digestion of mini-prep DNA of the modified BAC can be performed (fig. 5; protocol s. page 14).

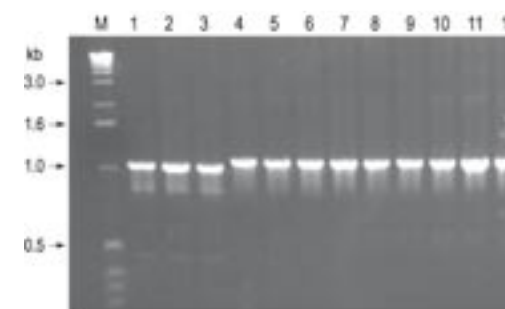


Figure 4: PCR results verifying the successful Red/ET Recombination. *M*: 1 kb ladder from Gibco. *Lanes 1 to 3*: unmodified control BAC resulting in a 1066 bp band. *Lanes 4 to 12*: successfully modified BACs containing the inserted Tn5-Neo cassette resulting in a 1133 bp band

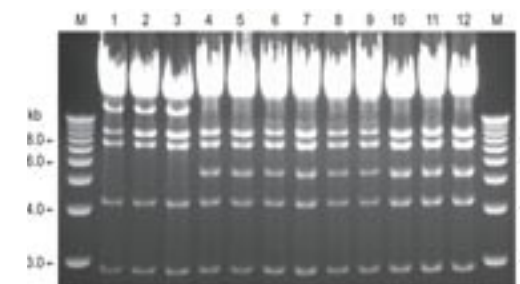


Figure 5: Restriction analysis of original and modified BACs after *Xho*I digestion. *M*: 1 kb ladder from Gibco. *Lanes 1 to 3*: unmodified BACs. *Lanes 4 to 12*: successfully modified BACs with the inserted Tn5-neo cassette.

Protocol: Preparation of BAC DNA for analytical purposes

1. Pick colonies and inoculate 2 ml of LB culture containing the appropriate antibiotics. Incubate at 37 °C over night with shaking at 1100 rpm.

Next day

2. Spin down the 2 ml overnight cultures for 1 min at 11,000 rpm.
3. Discard the supernatant and resuspend the cell pellet in 200 µl buffer P1 with RNase (from QIAGEN DNA Maxi-preparation Kit).
4. Add 200 µl of buffer P2 (Qiagen) and mix by inverting the tube several times.
5. Add 200 µl of buffer P3 (Qiagen) and mix by inverting the tube several times.
6. Spin down the white lysate at highest speed for 4 min.
7. Transfer the clear supernatant into a new 1.5 ml-Eppendorf tube and add 0.50 ml of 2-propanol.
8. Mix by inverting the tube and spin down the DNA at highest speed for 5 min.
9. Discard the supernatant and add 1 ml of 70% ethanol to rinse the pellet (be careful not to loose the small white pellet).
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. While the DNA is drying prepare a master mix for the subsequent restriction digest (e.g *Xho*I for the control experiment):

1.2 µl	10x restriction buffer
1.0 µl	RNase water 10 µg/ml
0.3 µl	restriction enzyme
9.5 µl	ddH ₂ O
<hr/>	
12.0 µl	
13. Mix well and carefully resuspend the dry DNA pellet in 12 µl digestion mix. Incubate at 37 °C for 2 to 3 hours. Subsequently, run the samples on an agarose gel for analysis.

6.5 Maps and Sequences

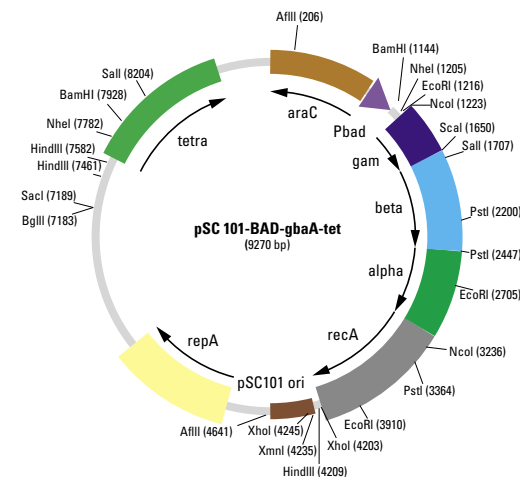


Figure 6: Map of the Red/ET expression plasmid pSC101-BAD-gbaA^{tet}. Transformation of *E.coli* hosts with this plasmid is selected for by acquisition of tetracycline resistance at 30 °C. Expression of the Red/ET recombination proteins is induced by L-arabinose activation of the BAD promoter at 37 °C.

Oligonucleotides used for the control reaction included in this kit:

The two oligonucleotides labeled 'check-up' and 'check-down' are designed for verification of the correctly recombined BAC clones. They are supplied with the kit (tubes 6 and 7).

'check-up': 5'-GTCGATCAGACTATCAGCGTGAG-3'

'check-down': 5'-TACCGAGCTCGAATTCGCCCTATAG-3'

The two oligos below were used to add the 50 bp homology regions (*italics*) for Red/ET recombination to the neomycin selection cassette used in the control reaction. The parts of the oligos which serve as PCR primers for amplification of the Tn5-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'-

TAGAACGGAGTAACCTCGGTGTGCGTTGTATGCCTGCTGTGGATTGCTGCTGGACAGCAAGCGAA
CCGGAATTGC-3'

Lower:

*Xho*I

5'-

TACCGAGCTCGAATTCGCCCTATAGTGAGTCGTATTACAATTCAGTGGCCCTCGAGTCAGAAGAA
CTCGTCAAGAAGGCG-3'

Sequence of the PCR product:

TAGAACGGAGTAACCTCGGTGTGCGGTTGTATGCCTGCTGTGGATTGCTGCTGGACAGCAAGCGCC
GGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAACTGGATGGCT
TTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGAT
CGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTA
TTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTGAGC
GCAGGGGCGCCCGTTCTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGAC
GAGGCAGCGCGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGT
CACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCT
CACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGTTGAT
CCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTAATCGGATGG
AAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGA
GTTCCGCCAGGCTCAAGGCGCGCATGCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCC
TGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGT
GTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGA
ATGGGCTGACCGCTTCTCGTGTTCAGGATCGCCGCTCCCGATTGCGCAGCGCATCGCCTTCTAT
CGCCTTCTTGACGAGTCTTCTGACTCGAGGGCCAGTGAATTGTAATACGACTCACTATAGGGCGA
ATTCGAGCTCGGTA

7. Troubleshooting

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

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

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

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

If no bands are visible:

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

If the bands are not clearly visible:

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

8. References and Patents

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- 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).*
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9. Other products available from Gene Bridges

General information

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

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

BAC Modification Protocol – CD-ROM

Description:

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

BAC Subcloning Kit

Description:

- This kit is optimized for subcloning of DNA fragments from BACs, PACs, P1 origin based vectors and cosmids.
- No restriction sites necessary.
- No size limitations for the fragment to be subcloned.
- High Red/ET Recombination efficiency combined with the convenient removal of the Red/ET plasmid pSC101-BAD-gbaA after recombination.

Contents:

- Red/ET recombination protein expression plasmid pSC101-BAD-gbaA (amp or tet), an improved derivative of pSC101 ori (a temperature-sensitive origin). Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- Linear vector carrying a ColE1 origin plus ampicillin resistance gene to be used for the subcloning experiment.
- Positive control experiment for subcloning a 15 kb fragment from a control BAC into the vector delivered with the kit.
- Protocols, descriptions of plasmids, maps and sequence of oligos.

Counter-Selection BAC Modification Kit

Description:

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

Contents:

- Red/ET Recombination protein expression plasmid pSC101-BAD-gbaA (tet), an improved derivative of pSC101 ori (a temperature-sensitive origin). Any *E. coli* strain can be made Red/ET proficient by transforming it with this plasmid.
- BAC host *E. coli* strain DH10B already carrying the Red/ET plasmid pSC101-BAD-gbaA (tet).
- pRpsL-neo template to be used for your own experiments.
- Positive controls for a point-mutation in a 150 kb BAC (BAC clone in the host carrying pSC101-BAD-gbaA, rpsL-neo PCR product flanked by homology arms for deletion in BAC backbone, BAC-rpsL-neo clone in the host as positive intermediate recombinant, oligo with homology arms and *XhoI* for replacing rpsL-neo cassette, BAC-*XhoI* clone as positive recombinant).
- Detailed protocols, descriptions of plasmids, maps and sequences of oligos.

Available soon:

Conditional Knock-out Kit

Description:

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

10. Purchaser Notification/Warranty

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

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

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Limited Warranty

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

11. DNA Engineering Services available from Gene Bridges

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

The available DNA modifications are:

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

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

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