GENE BRIDGES
DNA ENGINEERING SPECIALISTS

Technical Protocol

Cat. No. K006

Quick & Easy *E. coli* Gene Deletion Kit

By Red®/ET® Recombination

Version 2.0 (January 2007)

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 Quick and Easy *E. coli* Gene Deletion Kit

Introduction

Targeted disruption of genes on the *E. coli* chromosome
Metabolic engineering to design and construct microorganisms suitable for the production of industrial products like ethanol or aromatic amino acids requires the disruption of specific genes on the bacterial chromosome. Regulatory circuits, the uptake of carbon and amino acids, the glycolytic and pentose phosphate pathway, as well as the common aromatic amino acid pathway have to be manipulated.
The complexity of the necessary modifications requires a tool allowing the precise knock-out or alteration of multiple genes without leaving antibiotic selection markers. Red/ET recombination first published under the name “ET cloning” (Zhang et al. 1998) and also known as “recombineering” is an easy to use modification system for prokaryotic functional genomics.

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 specifically be altered. Red/ET recombination allows you to choose **homology arms as short as 50 bp** for homologous recombination, which can easily be added to a functional cassette by long PCR primers.
Zhang and coworkers demonstrated in 1998 for the first time that a pair of phage coded proteins (RecE and RecT) only need 42bp 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). This method was used to disrupt the endogenous *lac*Z gene of *E. coli* strain JC9604 (Zhang et al 1998). One year later the system was extended by the same group in replacing *rec*E and *rec*T by their respective functional counterparts of phage lambda *red*Ŭ and *red*b (Muyrers et al. 1999).
Since the year 2000 the system, which is protected by several international patents from Gene Bridges, has been used by other academic groups to disrupt several chromosomal genes in *E. coli* (e.g. Datsenko and Wanner 2000, Yu et al. 2000).

The **Quick and Easy *E. coli* Gene Deletion Kit from Gene Bridges** provides an optimized system for the disruption of genes on the *E. coli* chromosome in a sequence precise manner, attainable within one week. The use of a FRT-flanked resistance cassette for the disruption or replacement of the targeted gene allows the subsequent removal of the selection marker by a FLP-recombinase step (optional), if required.
Two Red/ET expression plasmids are provided with the kit mediating either tetracycline or ampicillin resistance. The choice of the expression plasmid allows the user to perform Red/ET recombination even if one selection marker is already present in the *E. coli* strain.

The control experiment included in the kit demonstrates the easy and precise knock-out of a chromosomal gene in this case the major mannose transporter (man*XYZ*) of *E. coli* strain HS996, using the provided FRT flanked kanamycin-resistance cassette. The **recombination process is strictly controlled**, since the necessary genes are located on an expression plasmid carrying a temperature-sensitive origin of replication and can therefore only be propagated at 30°C. An increase of the temperature to 37°C results in a loss of the expression plasmid after recombination. In addition the expression of the proteins is tightly controlled by an inducible promoter opening just a short time window for the recombination process.

There is proven evidence that Red/ET recombination works not only in *E.coli,* but also in other microorganisms like *Salmonella* (e.g. Uzzau et al. 2001), *Shigella* (Taniya et al. 2003)*, Yersinia* (Derbise et al. 2003), *Serratia* (Sapriel, Wandersman and Delepelaire 2003) and *Citrobacter* (Mundy et al. 2004).

Remark: The efficiency of Red/ET recombination may vary between different *E.coli* strains.

Contents of the kit:

1. pRedET (tet): Red/ET expression plasmid (20 ng/µl, 20 µl)
2. pRedET (amp) : Red/ET expression plasmid (20 ng/µl, 20 µl)
3. FRT-PGK-gb2-neo-FRT template DNA: PCR-template (plasmid DNA) for generating a FRT-flanked PGK-gb2-neo cassette (50 ng/µl, 20 µl)
4. FRT-PGK-gb2-neo-FRT PCR-product: PGK-gb2-neo cassette flanked by FRT sites and 50 bp long homology arms for the control experiment (400 ng/µl, 10 µl)
5. *E. coli* cells + pRedET (tet): Glycerol stock of *E.coli* strain HS996 harboring the expression plasmid pRedET (tet) for the control experiment (500 µl, 25% glycerol)
6. *E. coli* cells Δ(man*XYZ*::kan): Glycerol stock of the modified *E.coli* strain HS996. The mannose transporter man*XYZ* is replaced by the FRT-flanked kanamycin resistance marker cassette (control experiment; 500 µl, 25% glycerol)
7. PCR primer 1: Amplification primer located upstream of the man*XYZ* deletion to confirm the correct insertion of the FRT-PGK-gb2-neo-FRT cassette in the *E .coli* chromosome. (10 µM, 20 µl)
8. PCR primer 2: Amplification primer located on the FRT-PGK-gb2-neo-FRT cassette to confirm its correct insertion in the *E .coli* chromosome. (10 µM, 20 µl)
9. PCR primer 3: Amplification primer located on the FRT-PGK-gb2-neo-FRT cassette to confirm its correct insertion in the *E .coli* chromosome. (10 µM, 20 µl)
10. PCR primer 4: Amplification primer located downstream of the man*XYZ* deletion to confirm the correct insertion of the FRT-PGK-gb2-neo-FRT cassette in the *E .coli* chromosome. (10 µM, 20 µl)
11. This manual with protocols, maps and sequences

Please store tubes 1-4 and 7-10 at -20°C, store tubes 5 and 6 at -80°C.

Please note: All materials necessary for the control experiment are provided with this kit. You must order your oligonucleotides (PCR primers) according to your experimental design before starting. High quality oligos yield highest recombination efficiencies.

2 Experimental Outline

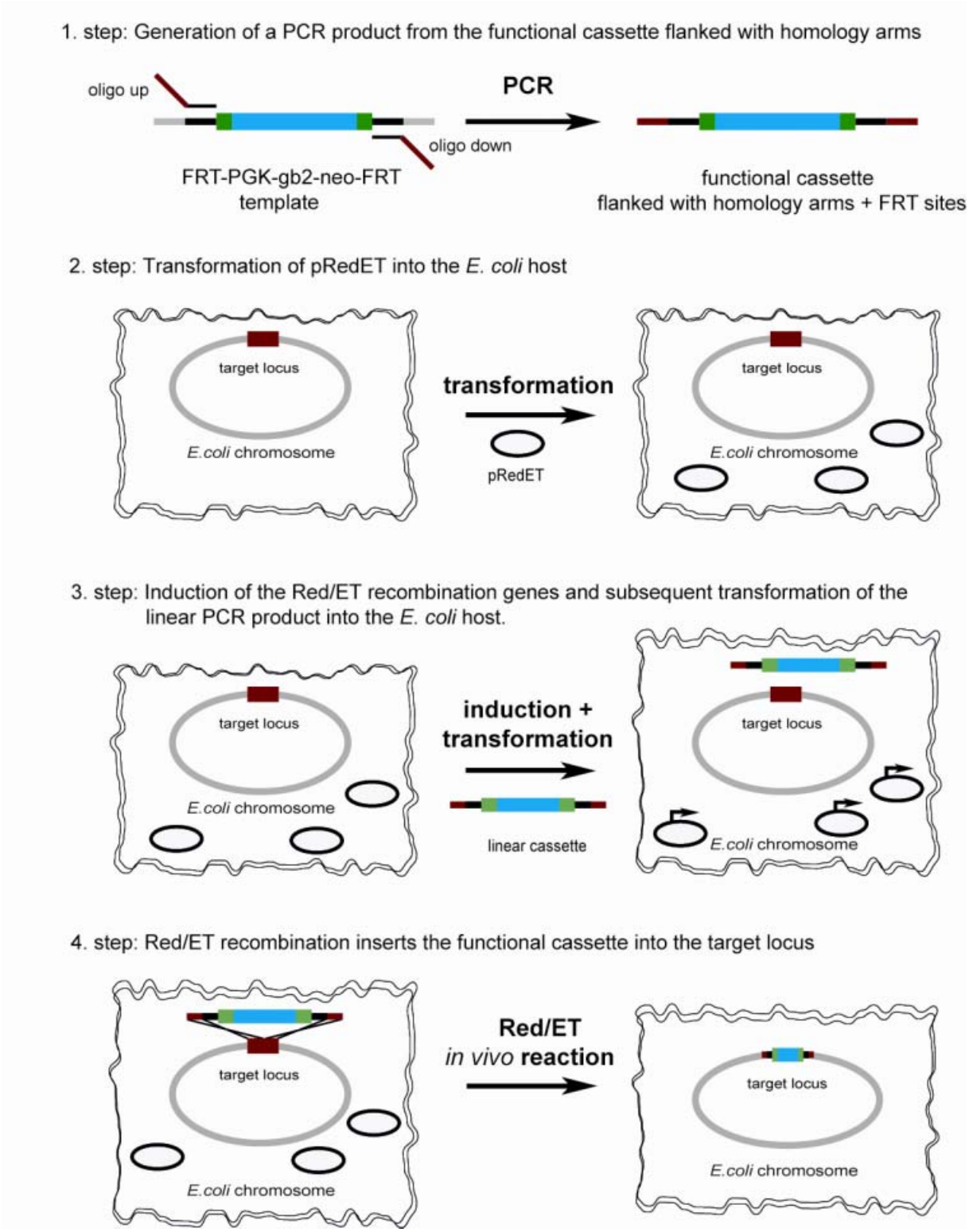

Figure 1: Flowchart of the experimental outline for the targeted disruption of genes on the *E. coli* chromosome.

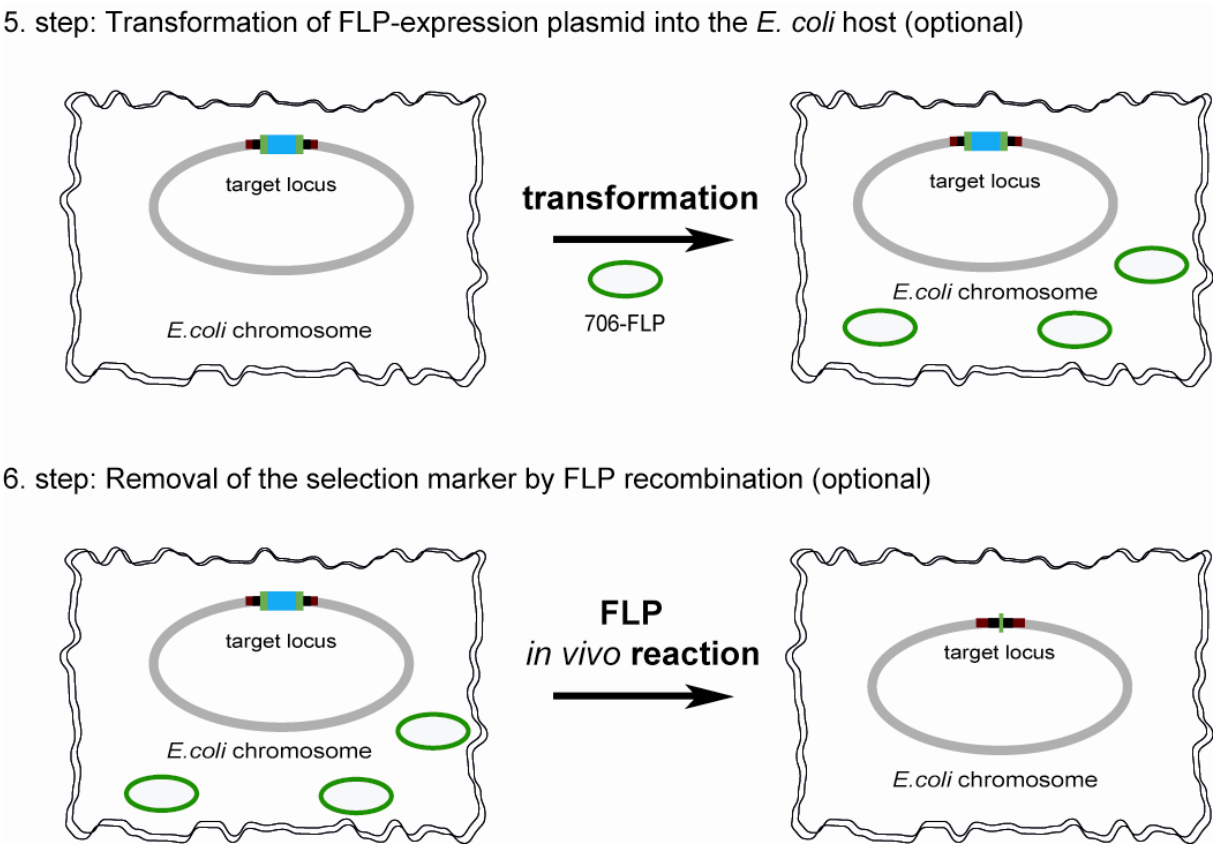

Figure 1 (continued): Flowchart of the experimental outline for the targeted disruption of genes on the *E. coli* chromosome.

In the first step 50bp long homology arms corresponding to the sequences flanking the insertion site on the chromosome are added to the functional cassette by PCR. In parallel the *E. coli* strain, which will 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. After induction, the cells are prepared for electroporation and the PCR product carrying the homology arms is electroporated (step 3). Red/ET recombination inserts the functional cassette into the target locus (step 4). Only colonies carrying the inserted modification (replacement of a gene by the FRT-PGK-gb2-neo-FRT cassette) will survive kanamycin selection on the agar plates.

Optionally, the kanamycin selection marker can be removed from the chromosome by transforming the cells with FLP expression plasmid (e.g. 706-FLP, Gene Bridges Cat. No. A103; step 5). Expression of the site specific FLP recombinase removes the selection marker from the target locus leaving a single FRT site as footprint behind (step 6).

3 How Red/ET Recombination works

In Red/ET Recombination, also referred to as ς -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 RedŬ/Redƀ from ς phage. These protein pairs are functionally and operationally equivalent. RecE and RedŬ are 5'- 3' exonucleases, and RecT and Redƀ are DNA annealing proteins. A functional interaction between RecE and RecT, or between RedŬ and Redƀ 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 ς-encoded Gam protein, which inhibits the RecBCD exonuclease activity of *E.coli*.

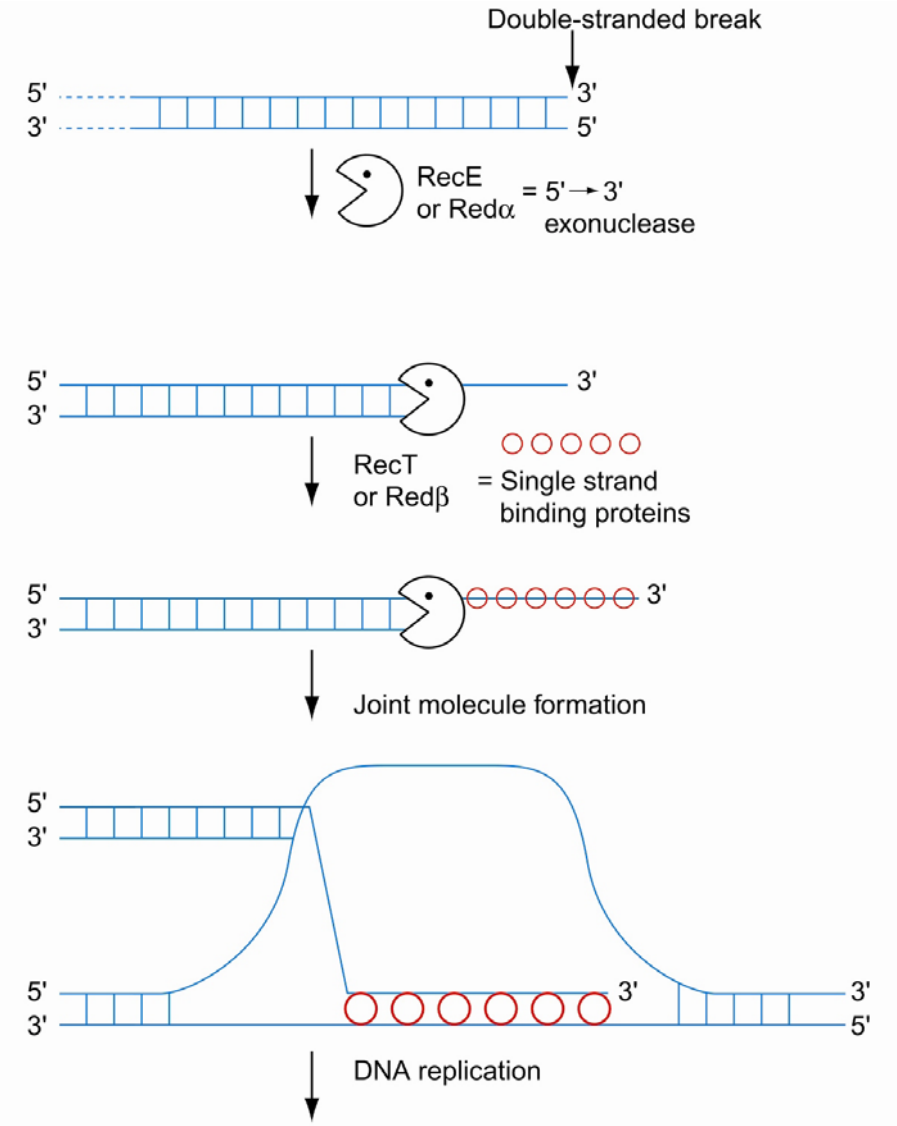

Figure 2: Mechanism of Red/ET Recombination.

Double-stranded break repair (DSBR) is initiated by the recombinase protein pairs, RecE/RecT or Redζ/Redη5

First Redζ (or RecE) digests one strand of the DNA from the DSB, leaving the other strand as a 3' ended, single-stranded DNA overhang. Then Redη (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 ς recombination proteins can be expressed from a plasmid (Figure 5) and are therefore transferable to any *E. coli* strain.

pRedET (Figure 5) carries the ɘ-phage *red*νηζ 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 *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 *redζ*, *η*, *ν* genes of the ς 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 intra-molecular rearrangement is minimized.

While constitutive expression of the *redɔ* 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 *ɔ* gene together with simultaneous expression of the *redŬ* and *b* 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 (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 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 the chromosome at the site(s) of choice, it is necessary to attach incorporate short homology regions into the functional cassette carrying the selectable marker “Sm”. This is most conveniently done by ordering two oligonucleotides for use in PCR amplification (Figure 3). Each oligonucleotide consists of two (or, if desired, three) parts:

1. Required Part A (A’ for the second oligonucleotide) is the homology region shared by the target molecule and the linear molecule. The homology regions are the 50 bp directly adjacent to either side of the insertion site. The exact sequences of the homology regions can be chosen freely, depending on the position on the target molecule to be modified.

2. Optional Part B (B´3 for the second oligonucleotide): This part of the oligonucleotide allows the incorporation of useful sequences, such as restriction sites. If the introduction of such operational sequences is not needed, this part can simply be omitted from the oligonucleotide design.

3. Required Part C (C’ for the second oligonucleotide): This piece, usually 18 to 24 nucleotides long, primes the PCR amplification of the selectable marker from the provided template.

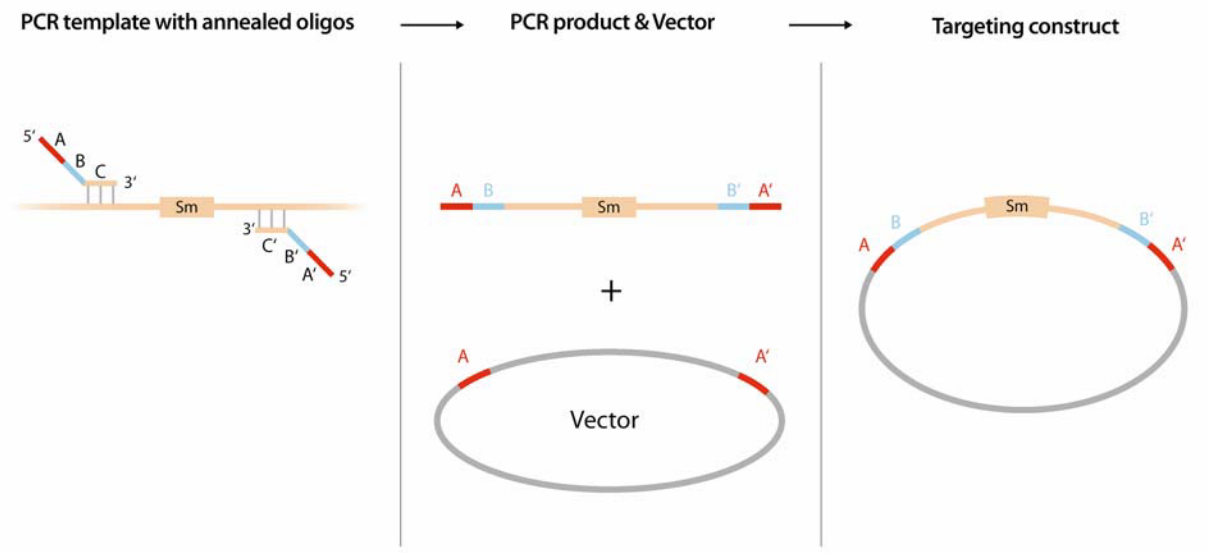

Figure 3: Practical steps involved in Red/ET.

The two oligos below were used to add the 50 bp homology regions for Red/ET Recombination to the FRT-PGK-gb2-neo-FRT cassette used in the control reaction (Figure 11). The fragments of the oligos which serve as homology arms are shown in plain text (Figure 3: Part A, respectively A'), the parts which serve as PCR primers for amplification of the cassette are *underlined* (Figure 3: Part C, respectively C'). These two oligos are not supplied with the kit.

Oligo 1 (upper oligo):
5'- GTTGATACATGGGGAGGCAGCCCGTTCAATGCTGCCAGCCGCATTGTCGT
AATTAACCCTCACTAAAGGGCGG-3'

Oligo 2 (lower oligo):
5'- CGAGCATTGGAATGTTAACGCCTGCAATGACTTCATAATGCTCTTTGTCGTAA
TACGACTCACTATAGGGCTCG-3'

Oligonucleotide Design for your target gene:

I) Choose 50 nucleotides $(N)_{50}$ directly adjacent upstream (5') to the intended insertion site. 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 FRT-PGK-gb2-neo-FRT cassette, given in *italics* below.

Upper oligonucleotide: 5'-$(N)_{50}$ *AATTAACCCTCACTAAAGGGCG* -3'

II) Choose 50 nucleotides $(N)_{50}$ directly adjacent downstream (3') to the intended insertion site 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 FRT-PGK-gb2-neo-FRT cassette, given in *italics* below.

Lower oligonucleotide: 5'-$(N)_{50}$ *TAATACGACTCACTATAGGGCTC* -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.

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 in LB medium to the indicated working concentration:

1. Chloramphenicol stock solution c = 30 mg/ml dissolved in ethanol. Working concentration 50 σg/ml.

2. Ampicillin stock solution c = 100 mg/ml dissolved in 50% ethanol. Working concentration 50 σg/ml5

3. Tetracycline stock solution c = 10 mg/ml dissolved in 75% ethanol. Working concentration for pRedET is 3 σg/ml. Tetracycline is light sensitive.

4. Kanamycin stock solution c = 30 mg/ml dissolved in dd$H_2$0. Working concentration 15 σg/ml.

Selective LB plates are made by adding 15 g agar to 1 L LB medium. After boiling, cool to approx. 50°C, add the required antibiotics to yield the appropriate working concentrations and pour into petri dishes.

L-arabinose stock solution

Use 10% **L-arabinose** (Sigma A-3256) in ddH_2O, fresh or frozen in small aliquots at -20°C. Use 50 µl stock solution per 1.4 ml LB for induction of recombination protein expression from pRedET. Frozen aliquots should not undergo more than three freeze-thaw cycles.

6 Technical protocol

6.1 Generating a functional cassette flanked by homology arms

PCR

The oligonucleotides are suspended in ddH_2O at a final concentration of 10 µM. We present as an example a standard PCR protocol for the use of Phusion DNA Polymerase (Finnzyme). However, any other DNA Polymerase together with the corresponding PCR protocol according to the instructions of the manufacturer should yield satisfactory results.

PCR reaction (in 50 µl)

34.5 µl	dH_2O
10.0 µl	5 x HF PCR reaction buffer
2.0 µl	5 mM dNTP
1.0 µl	upper oligonucleotide
1.0 µl	lower oligonucleotide
1.0 µl	FRT-PGK-gb2-neo-FRT PCR-template (tube 3)
0.5 µl	Phusion polymerase (5 U/µl)

- An annealing temperature of 57°- 62°C is optimal.
- PCR Profile: Initial denaturation step 30 sec 98°C; thirty cycles: 10 sec 98°C, 30 sec 55°C, 90 sec 72°C; final elongation step 10 min 72 °C.
- Check a 5 µl aliquot of the PCR product on a gel to ensure the PCR was successful. The size of the PCR product for the FRT-PGK-gb2-neo-FRT cassette is 1737 bp.
- Purify the PCR product either by running the whole PCR sample on an agarose gel and subsequent gel extraction or directly by Spin Column (e.g. „ “Min Elute Gel Extraction Kit“; Qiagen). Adjust the DNA concentration to 100-400 ng/µl.

6.2 Transformation with Red/ET expression plasmid pRedET

Before starting with the experiment, please streak out your *E. coli* culture on LB plates to obtain singly colonies.

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. Puncture a hole in the lid for air. Incubate at 37°C overnight with shaking.

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.

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

2. Culture for 2-3 h at 37°C, shaking at 1000 rpm.

3. Prepare the cells for electroporation
   Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge 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 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, 10σF, 600 Ohms. This setting applies to an Eppendorf® Electroporator 2510 using a 1 mm electroporation cuvette. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.

6. Resuspend the electroporated cells in 1 ml LB medium without antibiotics and return them to the microfuge tube.

7. Incubate at 30°C for 70 min, shaking at 1000 rpm.
   (The Red/ET expression plasmid pRedET will be lost at 37°C).

8. Using a small loop, plate 100 µl cells on LB agar plates containing tetracycline (3 µg/ml) or ampicillin (50 µg/ml) depending on which pRedET plasmid you used in step 4. Use a loop to streak the control culture (tube 5: *E. coli* cells + pRedET) on an LB agar plate with tetracycline (3 σg/ml). Incubate the plates at

30°C overnight (or for at least 15 h). Protect the plates from light by wrapping them up, because tetracycline is sensitive to light. Make sure the cells stay at 30°C, otherwise the plasmid will be lost.

6.3 Disruption of a chromosomal DNA fragment by the FRT-flanked PGK-gb2-neo cassette

In the next step, prepare electro-competent cells that contain the Red/ET expression plasmid, shortly after inducing the expression of the recombination proteins.
In advance, prepare the linear DNA fragment (the FRT-PGK-gb2-neo-FRT cassette) with homology arms that you will use to replace the DNA fragment on the chromosome. Use tube 4 (FRT-PGK-gb2-neo-FRT PCR-product) to perform the 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 either tetracycline (3 µg/ml) or ampicillin (50 µg/ml) depending on which pRedET plasmid you used in your experiment. Also pick one colony from the control plate and inoculate one microfuge tube containing 1.0 ml LB medium plus tetracycline (3 µg/ml) each. Puncture a hole in the lid of the tubes for air. Incubate the cultures while shaking at 30°C over night 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 approximately 2 h, shaking at 1100 rpm until OD600 ~ 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 microfuge benchtop centrifuge (at 2°C). 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 (400-800 ng) of your prepared linear FRT-PGK-gb2-neo-FRT 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 2 µl from tube 4 into each of the two tubes of the control.

6. Electroporate at 1350 V, 10 σF, 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 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 3 hours. Recombination will now occur.

8. Spin down the whole culture for 30 seconds and remove 900µl of the supernatant. Resuspend the cells in the remaining medium and streak out the cultures with a loop onto LB agar plates containing kanamycin (15 µg/ml). The plates should not contain tetracycline or ampicillin; otherwise the Red/ET Recombination protein expression plasmid (pPRedET) will either persist or the cells will die. Incubate the plates at 37°C overnight. The Red/ET Recombination protein expression plasmid (pRedET) will disappear at 37°C. You should obtain >100 colonies and the ration of induced:uninduced bacterial colonies should exceed 10:1.

   Note: Since a thin layer of unmodified *E. coli* cells is generally present all over the plate and also beneath the obtained colonies, the clones should be purified from these traces by streaking them out on a fresh LB agar plate containing kanamycin (15 µg/ml). Single colonies from this plate should then be analyzed by PCR to confirm the correct insertion of the cassette into the *E. coli* chromosome.

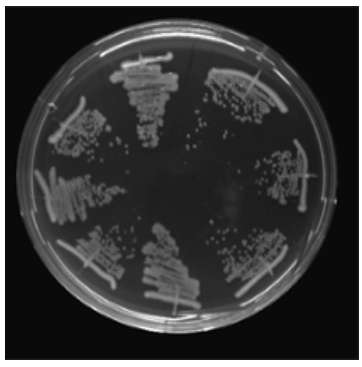

Figure 4: Eight colonies were streaked out on a fresh plate to obtain single colonies, which were then analyzed by PCR.

6.4 Verification of successfully modified genome by PCR analysis

Analyze the obtained colonies by colony PCR (e.g. pick a single colony and resuspend it in 30 µl of sterile water. Boil the sample at 98°C for 5 minutes and take an aliquot of 2 µl of the suspension as template for your PCR reaction).
For the control experiment, two pairs of primers are included (tube 7 to 10) amplifying a 503bp fragment for the left border (tube 7 and 8) and a 425bp fragment for the right border of the inserted fragment (tube 9 and 10). Since for each pair one primer is located on the chromosome and the second one on the cassette only a correctly recombined fragment will yield the expected PCR product confirming the correct insertion of the FRT-flanked kanamycin/neomycin resistance cassette.

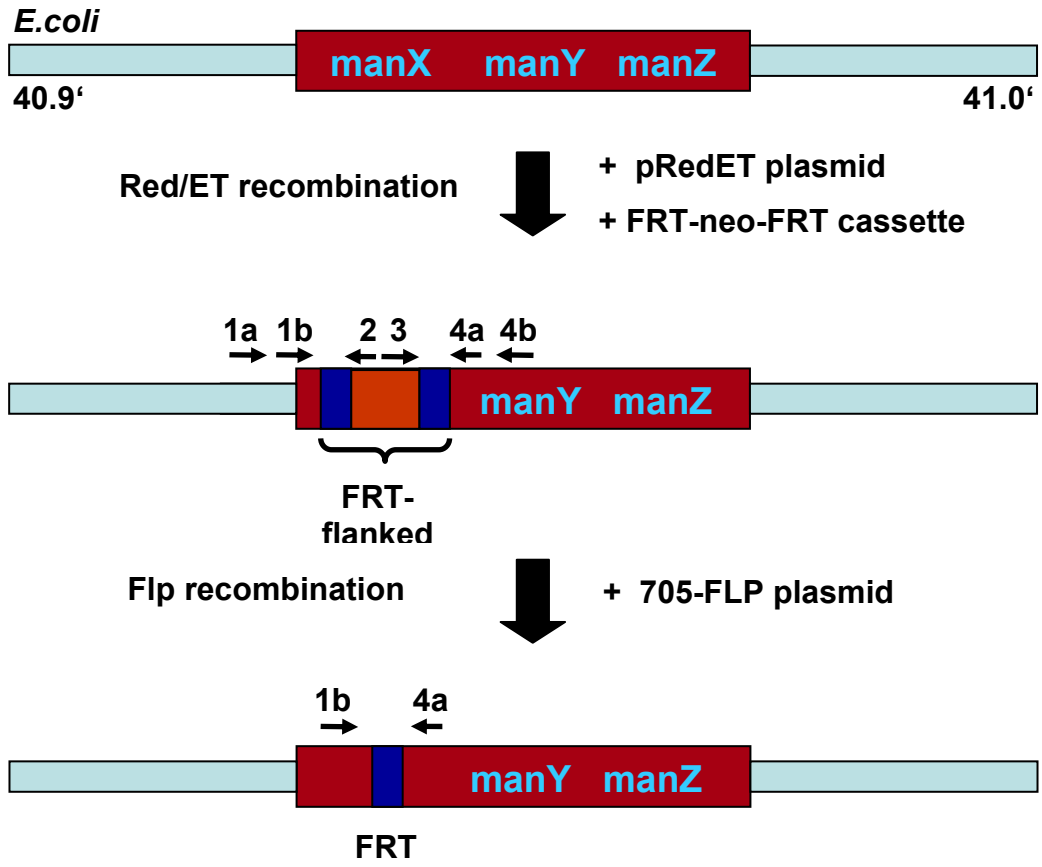

Figure 5: Confirmation of the correctly integrated FRT-flanked kanamycin/neomycin selection cassette. Primers 1b, 2, 3 and 4a are supplied with the kit.

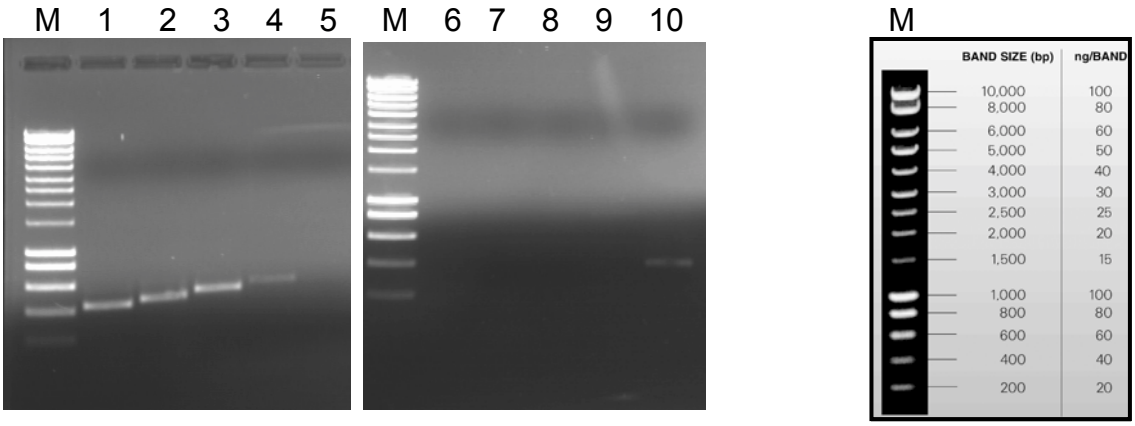

Figure 6: PCR primer combinations 3/4a (lane 1), 3/4b (lane 2), 1b/2 (lane 3) and 1a/2 (lane 4) confirm the correct insertion of the cassette by Red/ET recombination. After FLP recombination primer combination 1b/4a (lane 5) amplifies the DNA fragment without the cassette. M: Hyperladder I (Bioline).

6.5 Removal of the selection marker by FLP/FLPe expression (optional)

Since the kanamycin/neomycin selection marker is flanked by FRT sites, the cassette can be removed by transformation of a FLP expression plasmid (e.g. 706-FLP, Gene Bridges Cat. No. A103) into the cells and subsequent expression of FLP site specific recombinase.

Prepare electro-competent cells from a clone harboring the FRT-flanked kanamycin/ neomycin cassette on the chromosome and electroporate the FLP expression plasmid into the cells. 706-FLP carries a pSC101 origin of replication. The plasmids will maintain low copy and replicate at 30°C. They will not propagate and will get lost when incubated at 37°C. The expression of the FLP-recombinase is driven by the thermo-sensitive promoter cI578 (ς_{PR} promoter). Therefore, the expression is repressed at 30°C and induced at 37°C. The plasmid carries a tetracycline resistance gene.

Day 5:

1. Set up an overnight culture. Pick one or two colonies carrying the FRT-flanked kanamycin/neomycin cassette and inoculate them in microfuge tubes containing 1.0 ml LB medium and 50µg/ml kanamycin. Puncture a hole in the lid for air. Incubate at 37°C overnight with shaking.

Day 6:

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. Set up a microfuge tube containing fresh 1.4 ml LB medium conditioned with 50µg/ml kanamycin and inoculate with 30 µl of fresh overnight culture.

3. Culture for 2-3 h at 37 ºC, shaking at 1000 rpm.

4. Prepare the cells for electroporation
   Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge 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 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 the cells and keep the tube on ice.

5. Add 1 µl of the expression plasmid, e.g. 706-FLP, to your cell suspension. Mix briefly and keep the tube on ice. Transfer the cell suspension from the tube to the chilled electroporation cuvette.

6. Electroporate at 1350 V, 10 σF, 600 Ohms. This setting applies to an Eppendorf® Electroporator 2510 using a 1 mm electroporation cuvette. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.

7. Resuspend the electroporated cells in 1 ml LB medium without antibiotics and return them to the microfuge tube.

8. Incubate at 30°C for 70 min, shaking at 1000 rpm. (The expression plasmid 706-FLP 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 kanamycin (15 µg/ml). Incubate the plates at 30°C overnight (or for at least 15 h). Protect the plate from light by wrapping it up, as tetracycline is sensitive to light. Make sure the cells stay at 30°C, otherwise the expression plasmid will be lost.

Day 7:

10. Pick a single colony and grow the cells in 1 ml of LB medium without antibiotics at 30°C for 2-3 h.

11. Change the temperature to 37°C and incubate overnight. FLP/FLPe protein will be expressed at this temperature and the FRT sites will be recombined. At the same time, the expression plasmid cannot replicate any more and will get lost.

Day 8:

12. Take a loop and streak a sample of the culture on a fresh LB agar plate. To obtain single colonies one drop is enough. Incubate overnight at 37°C. The next day, analyze twelve single colonies by PCR for the successful removal of the selection marker. Alternatively you can streak the colonies on two different LB agar plates in parallel, one conditioned with 15µg/ml kanamycin, the other one without addition of antibiotics. Clones growing only on LB agar plates without kanamycin but no longer on kanamycin conditioned LB agar plates have successfully removed the selection marker by FLP recombination.

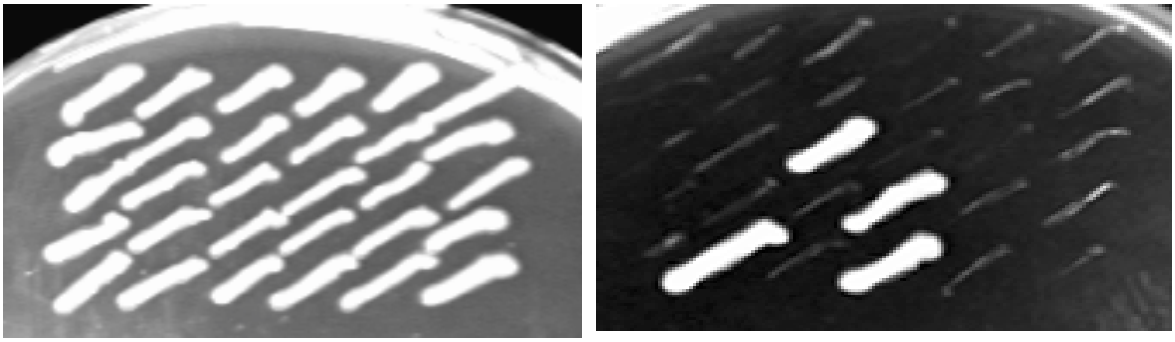

Figure 7: Marker test: Single Colonies were streaked out on LB plates (left) and LB+kanamycin plates (right) in parallel. The four clones growing on the kanamycin plate still contain the selection marker cassette, while all other clones lost the selection maker by FLP recombination.

6.6 Maps and Sequences

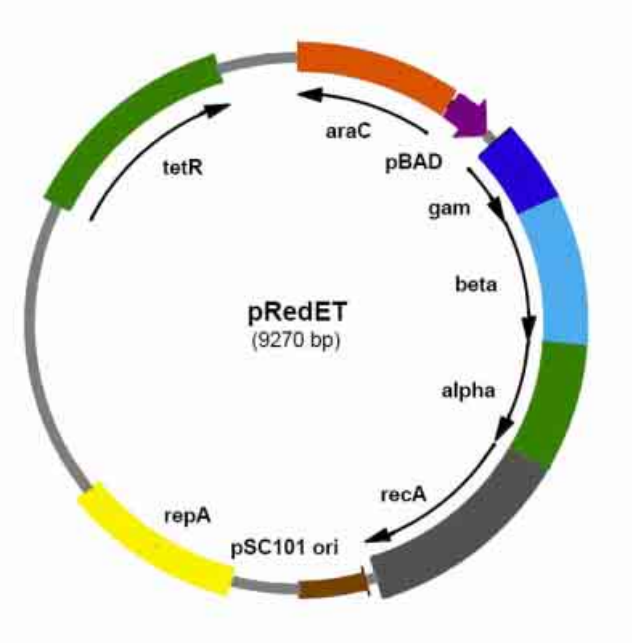

Figure 8: Map of the Red/ET expression plasmid pRedET (tet). Transformation of *E.coli* hosts with this plasmid is selected for by acquisition of tetracycline resistance at 30°C. Expression of the Red/ET Recombination proteins is induced by L-arabinose activation of the BAD promoter at 37°C. The second version of the expression plasmid supplied with the kit [pRedET (amp)] is identical but carries an ampicillin resistance marker instead of the tetracycline resistance gene.

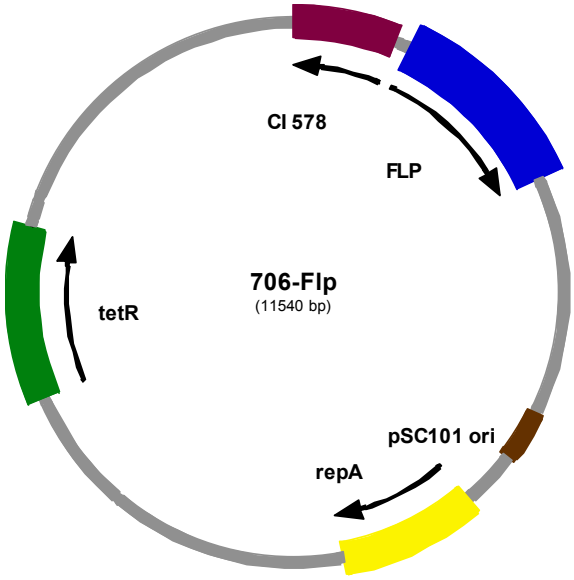

Figure 9: Map of the plasmid 706-FLP. Transformation of *E.coli* hosts with this plasmid is selected for by acquisition of tetracycline resistance at 30°C. Expression of the FLPe recombination proteins is induced by a temperature shift to 37°C.

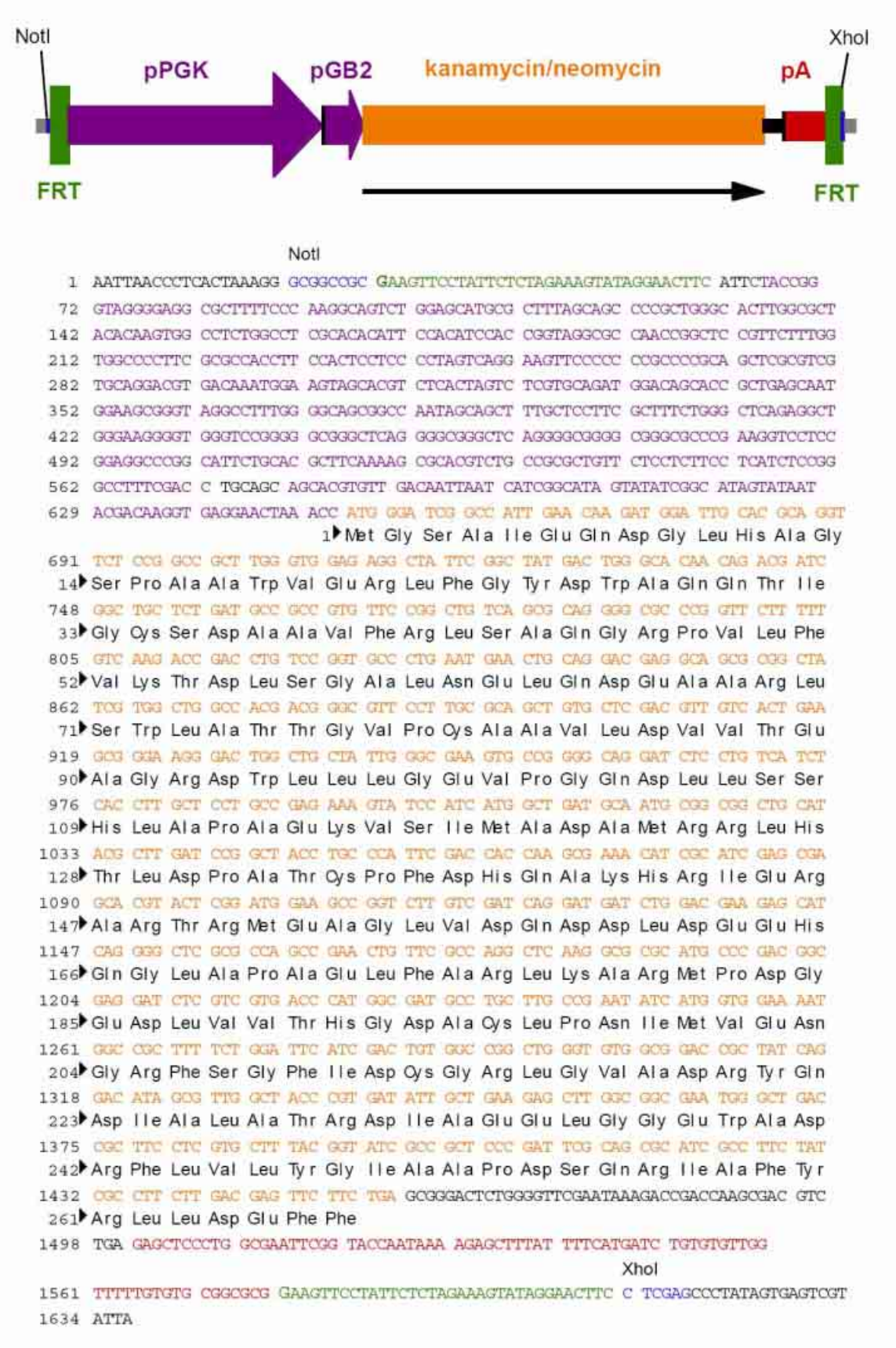

Figure 10: Map of the FRT-PGK-gb2-neo-FRT cassette.

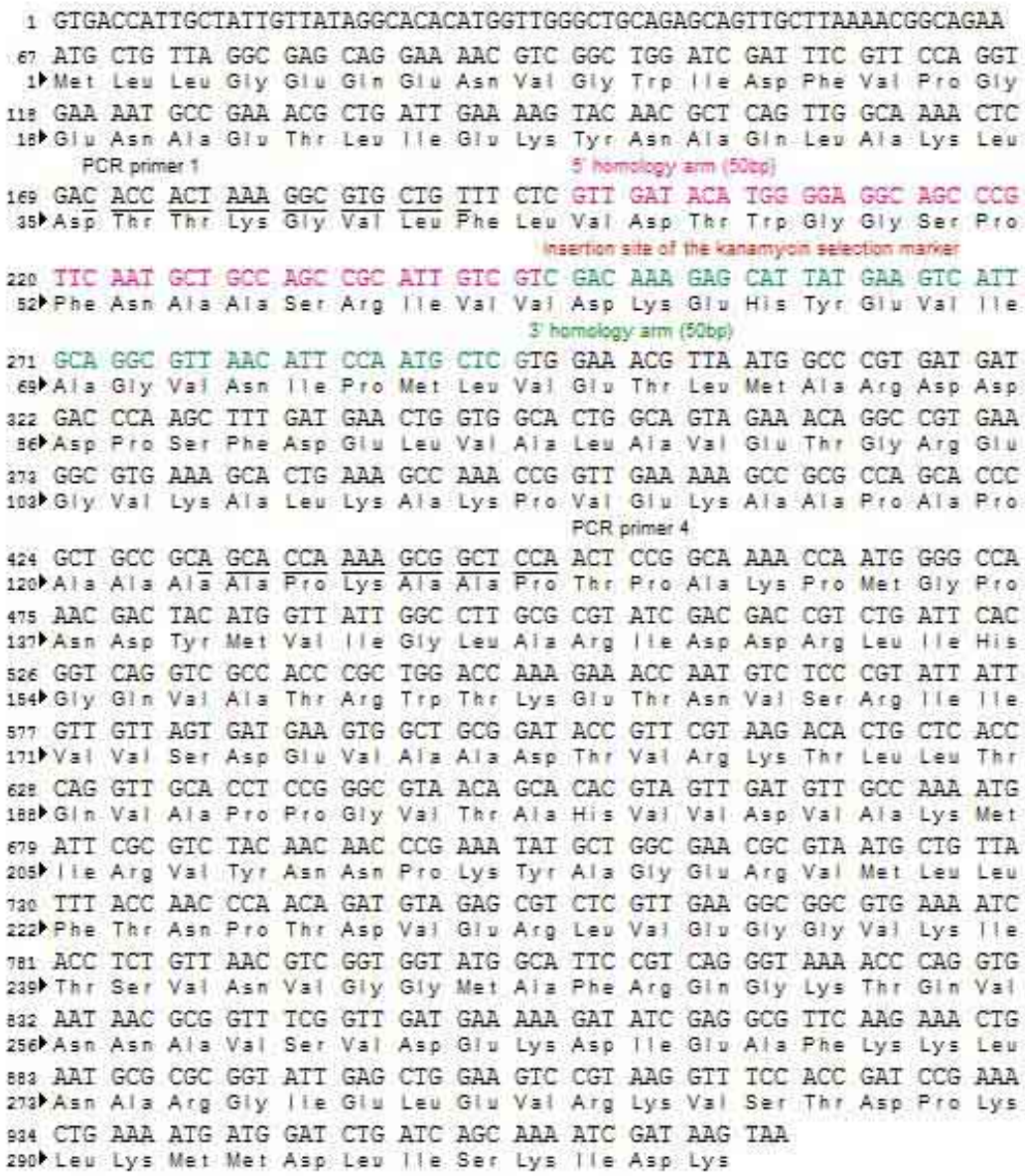

Figure 11: *man*X gene locus on the *E. coli* chromosome.

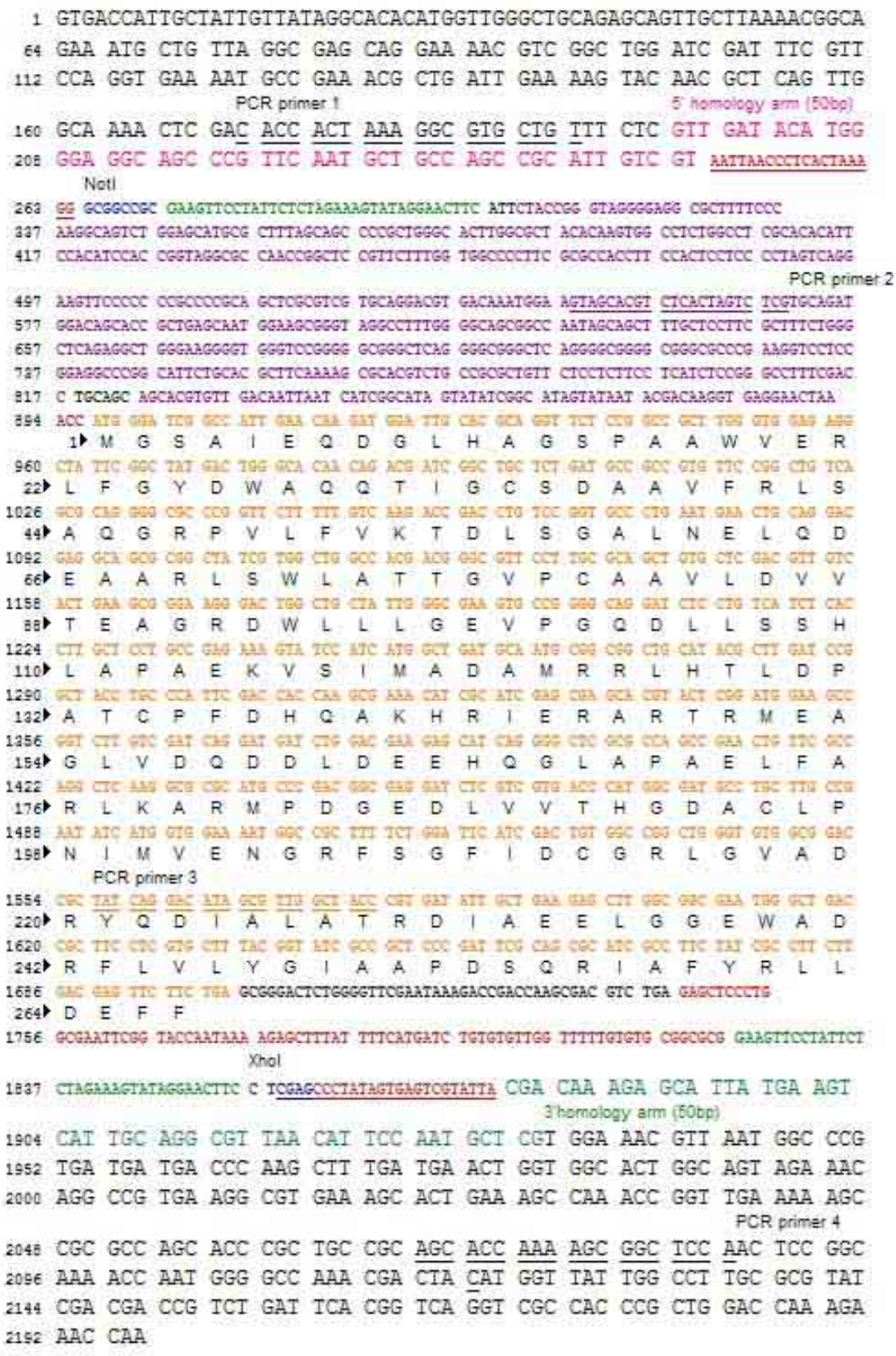

Figure 12: *man*X gene locus after insertion of the FRT-flanked kanamycin/neomycin selection marker cassette.

Oligonucleotides:

The four oligonucleotides labeled “PCR primer 1” to “PCR primer 4” are designed to verify the correct insertion of the FRT-flanked kanamycin/neomycin resistance marker cassette. They are supplied with the kit (tubes 7 to 10).

“PCR primer 1”: 5’-CACCACTAAAGGCGTGCTGT-3’

“PCR primer 2”: 5’- CGAGACTAGTGAGACGTGCTAC-3’

“PCR primer 3”: 5’- TATCAGGACATAGCGTTGGCTACC-3’

“PCR primer 4”: 5’- TGGAGCCGCTTTTGGTGCT-3’

7 Troubleshooting

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

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

For homologous recombination the two DNA molecules must share two regions of perfect sequence identity. Several wrong nucleotides in the homology region can completely abolish recombination. Since oligonucleotides are used to add the homology regions they have to be synthesized properly and be of excellent quality. Take into account that long oligonucleotides (especially if they are longer than 80bp) require additional purification steps, such as HPLC. Please ask you local oligonucleotide supplier for the recommended purification. Homologous recombination will also not take place if the targeted sequence on the chromosome is not determined 100% correctly for the homology region.

If you are trying to target a repeated sequence, 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 region on the chromosome

- the sequence which should be recognized by the homology arm is different or absent (check for the presence by amplifying the region by PCR and sequence the PCR product if necessary)

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

- there was no expression plasmid present in the cells; e.g. the cells were grown at 37°C instead of 30°C (check for tet resistance),
- 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 .

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

If you obtain a **high number** of colonies on both plates, it indicates that there are still traces of the circular (or supercoiled) plasmid used to prepare the linear fragment left in the sample. Since the transformation efficiency of linear fragments is 10^4-fold less than of circular DNA molecules you may obtain a background even if no traces were visible on an agarose gel.
If the linear DNA fragment was obtained by restriction digestion, use less DNA and gel purify the fragment. If the linear cassette was obtained by PCR, set up a *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 check all parameters mentioned in the section entitled: “no colonies after Red/ET Recombination”.

High number of “false positive” colonies on the induced plates:

If you obtain a **high number** of colonies on the induced plates for which you cannot confirm the correct location of the insertion, please double check the sequences you used as homology arms for the recombination by BLAST analyses against the *E. coli* genome. Avoid highly conserved regions and repetitive sequence stretches since otherwise the primer may guide the cassette to a wrong location.

Modification of *E. coli* strains/ Modification of the *E. coli* genome:

We observed that the efficiency of Red/ET recombination varies between different *E. coli* strains. Although the overall number of colonies was less for strain W3110 in all experiments performed, the percentage of clones for which the correct insertion was detected was even higher than for strain MG1655. Besides a higher background of unspecific insertion events for MG1655, we also observed that the two expression plasmids (pRedET and 706-FLP) persisted much longer at 37°C in MG1655 cells in comparison to W3110. For MG1655 and derived strains, a marker test (LB/LB+tet) similar to the one described for 706-FLP (page 20) is highly recommended to confirm the loss of the pRedET plasmid.

8 References and Patents

8.1 References

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8.2 Patents

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

- Stewart, A.F., Zhang, Y., and Buchholz, F. 1998. Novel DNA cloning method. *European Patent No.1034260 (issued on 12th of March, 2003), United States Patent No 6,509,156.*

- Stewart, A.F., Zhang, Y., and Muyrers, J.P.P. 1999. Methods and compositions for directed cloning and subcloning using homologous recombination. *United States Patent No. 6,355,412 (issued on 12th of March, 2002).*

- Youming Zhang, A. Francis Stewart, and Joep P.P. Muijrers. 2001. Improved RecT or RecET cloning and subcloning method. *European Patent Application No. 01 117 529.6*

- Stewart, A.F., Zhang, Y., and Muyrers, J.P.P. 2001. Recombination method. European Patent Application No. 0103276.2

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

9 Purchaser Notification/Warranty

This product is the subject of European Patent No.1034260 (issued on 12th of March 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
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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.

10 Other products available from Gene Bridges

General information

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

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

K001: Quick and Easy BAC Modification Kit

Description:

- This kit is designed to modify any type of bacterial artificial chromosomes (BACs) within 1-2 weeks by using a Kanamycin/Neomycin cassette
- This kit is optimized for basic modifications such as insertions or deletions of fragments in any type of bacterial artificial chromosomes (BACs) leaving a selectable marker gene.
- This kit can also be used to work on bacterial chromosomes and common ColE1 origin plasmids.
- High Red/ET efficiency plus convenient removal of the Red/ET Recombination protein expression plasmid pRedET after recombination.

Contents:

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

K002: Counter-Selection BAC Modification Kit

Description:

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

Contents:

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

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 a 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.

K007: Quick and Easy RNAi Rescue Kit

Description:

- Rescue the RNAi phenotype with a gene resistant to RNAi degradation (ex. mouse BACs for human cells and vice versa).
- BACs include promoters, introns, exons, regulatory regions and therefore express the gene at physiological levels and with different splice variants.
- This kit is designed as a quick and easy solution for the rescue of an RNAi phenotype using BACs (for transient and stable transfections).
- All components you need for the generation of the RNAi rescue construct included (except for the BAC).
- Red/ET recombination allows the Chloramphenicol resistance gene found on all BAC backbones (pBeloBAC, pBAC3.6, pTARBAC) to be replaced by the SNAP26M-kanamycin/neomycin cassette within 1 week.
- The SNAP cassette is easily detected by TMR-Star dye. Cells carrying the gene variant resistant to RNAi degradation can be identified within 30min.

Contents:

- Red/ET Recombination protein expression plasmid pRedET. Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- Ready-to-go SNAP26M-kanamycin/neomycin cassette flanked by homology arms
- *E.coli* strain HS996 already carrying the pRed/ET plasmid and an unmodified BAC clone for the control experiment.
- Positive control BAC containing the SNAP26M-cassette.
- Primers necessary to check for correct replacement of Chloramphenicol resistance gene with SNAP26M-cassette.
- Reagents needed for SNAP-detection including the SNAP-tag substrate TMR-Star
- 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)

Additional strains and plasmids:

- A101: FLP Expression Strain: 294-Flp
- A102: FLP Expression Plasmid: 705-Flp (cm resistance marker)
- A103: FLP Expression Plasmid: 706-Flp (tet resistance marker)
- A111: Cre Expression Strain: 294-Cre
- 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

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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