PCR targeting system in *Streptomyces coelicolor* A3(2)

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Introduction

Many bacteria are not readily transformable with linear DNA because of the presence of the intracellular *recBCD* exonuclease that degrades linear DNA. However, the λ RED (*gam, bet, exo*) functions promote a greatly enhanced rate of recombination when using linear DNA. By exploiting this, Datsenko and Wanner (2000) made 40 different disruptions on the *E. coli* chromosome by replacing the wild-type sequences with a selectable marker generated by PCR using primers with 36 nt homology extensions.

The strategy for PCR-targeting for mutagenesis of *Streptomyces coelicolor* is to replace a chromosomal sequence within a *S. coelicolor* cosmid (Redenbach *et al*., 1996) by a selectable marker that has been generated by PCR using primers with 39 nt homology extensions. The inclusion of *oriT* (RK2) in the disruption cassette allows conjugation to be used to introduce the PCR targeted cosmid DNA into *S. coelicolor*. Conjugation is much more efficient than transformation of protoplasts and it is readily applicable to many actinomycetes (Matsushima *et al*., 1994). The potent methyl-specific restriction system of *S. coelicolor* is circumvented by passaging DNA through a methylation-deficient *E. coli* host such as ET12567 (MacNeil *et al*., 1992).

Vectors containing *oriT* (RK2; Pansegrau *et al*., 1994) are mobilisable *in trans* in *E. coli* by the self-transmissible pUB307 (Bennett *et al*., 1977, Flett *et al*., 1997) or the non-transmissible pUZ8002, which lacks a *cis*-acting function for its own transfer (Kieser *et al*., 2000).

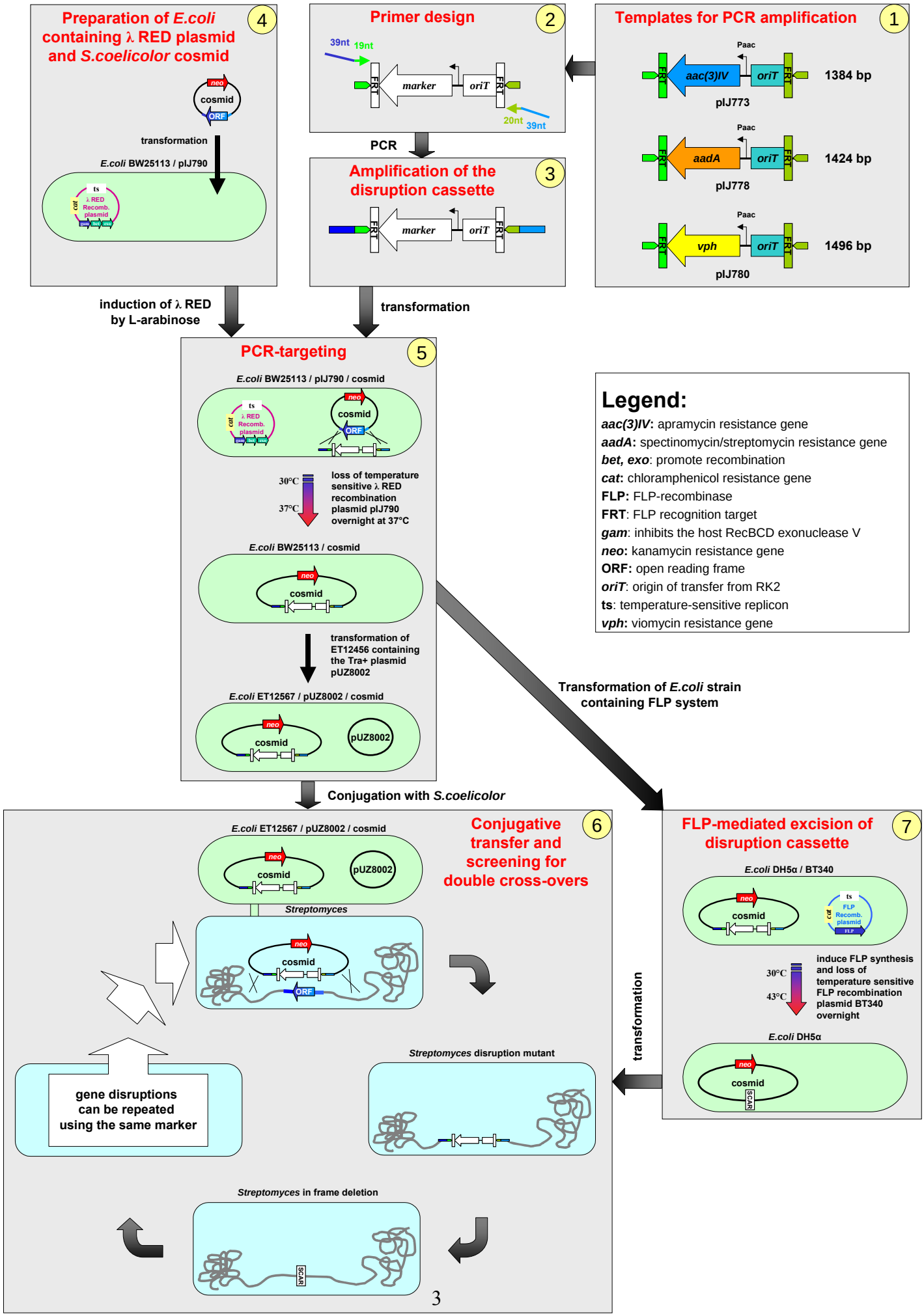
To adapt the procedure of λ RED mediated recombination for *Streptomyces*, cassettes for gene disruptions were constructed that can be selected both in *E. coli* and in *Streptomyces* (Table 1). After a single disruption with an *oriT*-containing cassette, further disruptions can be performed on the same cosmid using *oriT*-free cassettes containing alternative selective markers. The λ RED recombination plasmid pKD20

(*E. coli* Genetic Stock Center CGSC Strain # 7637) was modified by replacing the ampicillin resistance gene *bla* with the chloramphenicol resistance gene *cat*, generating pIJ790, to permit selection in the presence of Supercos1-derived cosmids (ampicillin and kanamycin resistance).

Name of plasmid	Resistance-marker	Resistance	Concentration for *E. coli*	*oriT*	Size of template
pIJ773 Fig. 5	*aac(3)IV*	apramycin	50 μg/ml LB	+	1382 bp
pIJ778 Fig. 6	*aadA*	spectinomycin streptomycin	50 μg/ml LB 50 μg/m LB	+	1425 bp
pIJ779.	*aadA*	spectinomycin- streptomycin	50 μg/ml LB 50 μg/ml LB	-	1057 bp
pIJ780 Fig.7	*vph*	viomycin	30 μg/ml DNA	+	1497 bp
pIJ781	*vph*	viomycin	30 μg/ml DNA	-	1622 bp

Table 1: Disruption cassettes containing different resistance markers with and without *oriT*: All disruption cassettes were cloned into the *Eco*RV site of pBluescript SK II (+) allowing the isolation of a *Eco*RI/*Hind*III fragment for use as template for the PCR reaction. The size of the cassettes includes the 19 bp and 20 bp primer site (see section 2: "primer design") which are identical in all disruption cassettes. The resistance genes with or without *oriT* are flanked by FRT sites (FLP recognition targets) which allows FLP-mediated excision of the cassette (see section 7: "FLP-mediated excision of the disruption cassette").

Fig. 1: Flowchart of gene disruption by PCR-targeting



Protocol (see Flowchart Fig. 1)

1 Purification of the PCR template (resistance (-*oriT*) cassette)

Using whole plasmids as templates for the PCR can result in a high proportion of antibiotic-resistant transformants without gene disruption. This is caused by traces of CCC DNA that compete with the linear PCR fragment and result in the occurrence of false positive transformants. Using gel-purified disruption cassettes as templates prevents the occurrence of false positives.

1. Digest ~ 10 µg plasmid DNA (see Table 1) with 50 U *Eco*RI (Roche) and 50 U *Hin*dIII (Roche) in 1 X buffer B (Roche) in a 100 µl reaction.
   - **A 2938 bp vector fragment and a fragment 14 bp larger than the size of the cassette given in Table 1 should be generated.**
2. Run the digest on a 20 x 20 x 0.25 cm (100 ml) 1% TAE (1x) agarose gel at 5V/cm for 2 - 3 h in 1x TAE buffer.
   - **Longer runs exhaust the buffer capacity and destroy the gel unless the buffer is recycled.**
3. Cut out the cassette band from the gel and purify using the Qiagen gel extraction kit. The purified fragment is stored in 10 mM Tris.HCl (pH 8) at a concentration of 100 ng / µl at –20°C.
4. Absence of plasmid DNA is tested by using 1µl (100 ng) of purified cassette DNA to transform highly competent *E. coli* DH5α cells (10^8/µg). Plate on LB agar containing 100 µg/ml carbenicillin. If any transformants appear, repeat steps 2-4.

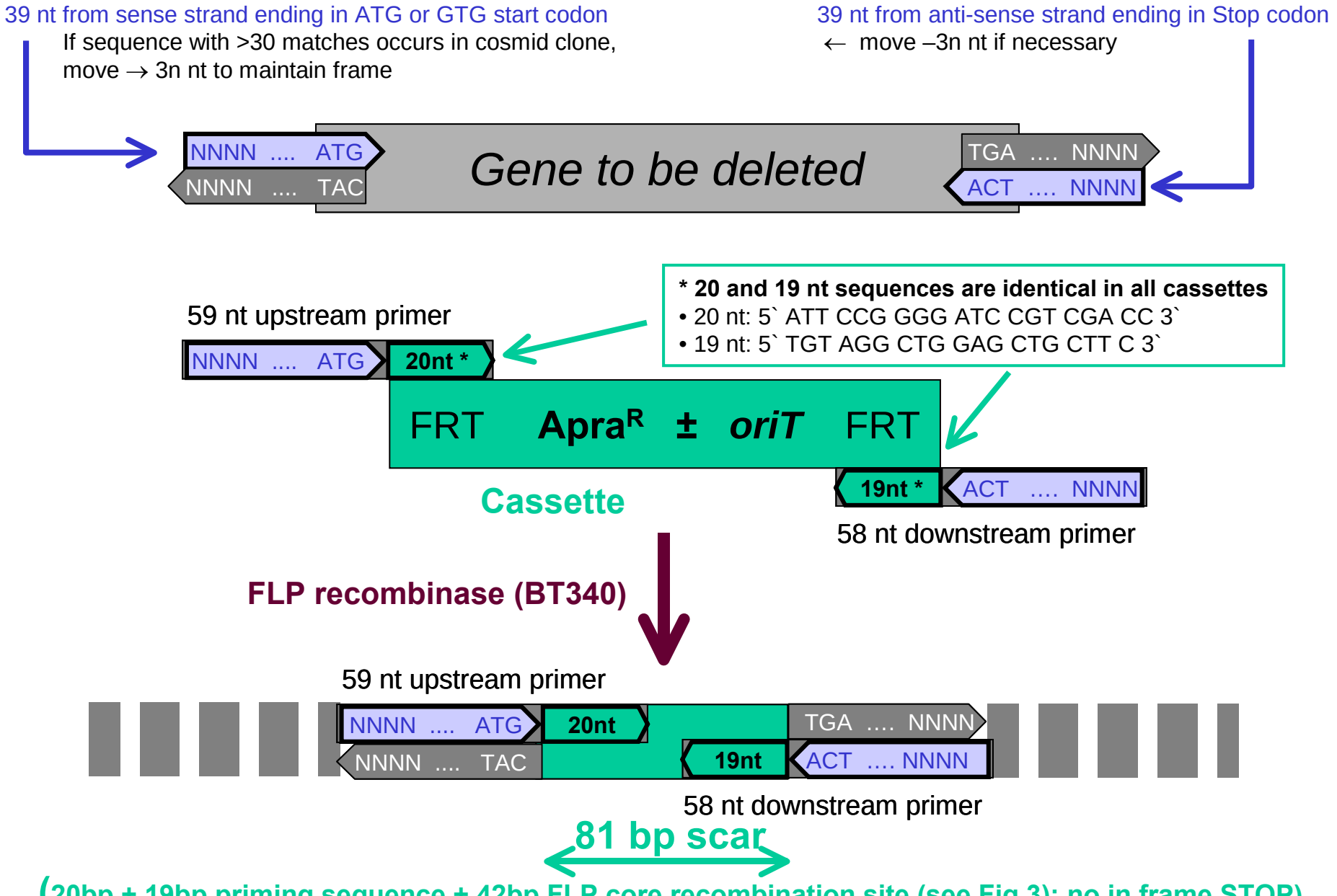
2 Design of long PCR primers

For each gene disruption, two long PCR primers (58 nt and 59 nt) are required. Each has at the 5´end 39 nt matching the *S. coelicolor* sequence adjacent to the gene to be inactivated, and a 3´sequence (19 nt or 20 nt) matching the right or left end of the disruption cassette (all cassettes have the same "right" and "left" ends). The precise positioning of the 39 nt sequence as indicated in Fig. 2 is important for creating in-frame deletions by FLP recombinase-induced excision of the resistance marker (see section 7).

- **The 5´- 39 nt sequence of the forward primer (upstream primer; Fig. 2) must be from the coding strand of the gene of interest and its 3' end must be in the correct reading frame with respect to the replaced gene. The 5´- 39 nt sequence of the reverse primer (downstream primer; Fig. 2) must be from the *complementary* strand.**
- **To prevent unwanted recombination, a BlastN search is performed comparing each 39 nt sequence with the "real cosmid" (sequences at the Sanger Centre Homepage in the folder ftp.sanger.ac.uk/pub/S_coelicolor/cosmid_inserts and on the CD in the folder /S_coelicolor/cosmid inserts). The perfect match should be found but no other matches >30 bp. If necessary, the 39 nt sequence is shifted in 3 nt steps until the above criteria are met.**

Fig.2: Designing PCR primers for making an *in-frame* deletion

(the example illustrates a complete deletion)



3 PCR amplification of the extended resistance cassette

All PCR amplifications are performed using the Expand high fidelity PCR system according to the manufacturer's instructions (Roche). Reaction conditions:

• Primers (100 pmoles/µl)	0.5 µl each	50 pmoles each
• Template DNA (100 ng/µl)	0.5 µl	50 ng ≈ 0.06 pmoles
• Buffer (10x)	5 µl	1 x
• dNTPs (10 mM)	1 µl each	50 µM each
• DMSO (100 %)	2.5 µl	5%
• DNA polymerase (2.5 U/µl)	1 µl	2.5 Units
• Water	36 µl	
• Total volume	50 µl	

Cycle conditions:

1. Denaturation:	94°C, 2 min	
2. Denaturation:	94°C, 45 sec	} 10 cycles
3. Primer annealing:	**50°C**, 45 sec	
4. Extension:	72°C, 90 sec	
5. Denaturation:	94°C, 45 sec	} 15 cycles
6. Primer annealing:	**55°C**, 45 sec	
7. Extension:	72°C, 90 sec	
8. Final extension:	72°C, 5 min	

5 µl of the PCR product is used for analysis by gel electrophoresis. The expected sizes are 78 bp larger than the sizes of the disruption cassettes listed in Table **1** (because of the 2 x 39 bp 5´-primer extensions). The remaining 45 µl of the PCR product is purified using the Qiagen PCR purification kit according to the manufacturer's instructions. The PCR product is finally eluted from the columns with 12 µl of water (~200 ng/µl).

4 Introduction of *S. coelicolor* cosmid clone into *E. coli* BW25113/pIJ790 (λ RED recombination plasmid) by electroporation

pIJ790 contains the resistance marker *cat* (chloramphenicol resistance) and a temperature sensitive origin of replication (requires 30°C for replication).

1. Grow *E. coli* BW25113/pIJ790 overnight at 30°C in 10 ml LB (Luria-Bertani medium; Sambrook *et al*., 1998) containing chloramphenicol (25 μg/ml).
2. Inoculate 100 μl *E. coli* BW25113/pIJ790 from overnight culture in 10 ml SOB (Hanahan, 1983) containing 20 mM $MgSO_4$ (add 200 μl of 1M stock to 10 ml SOB) and chloramphenicol (25 μg/ml).
3. Grow for 3-4 h at 30°C shaking at 200 rpm to an OD_{600} of ~ 0.4.
4. Recover the cells by centrifugation at 4000 rpm for 5 min at 4°C in a Sorvall GS3 rotor (or equivalent).
5. Decant medium and resuspend the pellet by gentle mixing in 10 ml ice-cold 10 % glycerol.
6. Centrifuge as above and resuspend pellet in 5 ml ice-cold 10 % glycerol, centrifuge and decant. Resuspend the cell pellet in the remaining ~ 100 μl 10 % glycerol.
7. Mix 50 μl cell suspension with ~ 100 ng (1-2 μl) of cosmid DNA. Carry out electroporation in a 0.2 cm ice-cold electroporation cuvette using a BioRad GenePulser II set to: 200 Ω, 25 μF and 2,5 kV. The expected time constant is 4.5 – 4.9 ms.
8. Immediately add 1 ml ice cold LB to shocked cells and incubate shaking for 1h at 30°C.
9. Spread onto LB agar containing carbenicillin (100 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml).
10. Incubate overnight at 30°C.
11. Transfer one isolated colony into 5 ml LB containing antibiotics as in (9) above.
12. Incubate overnight at 30°C. This culture will be used as a pre-culture for generating competent cells to be transformed with the extended resistance cassette.

5 PCR targeting of the *S. coelicolor* cosmid

E. coli BW25113/pIJ790 containing a *S. coelicolor* cosmid is electro-transformed with the extended resistance cassette. **The example described uses the apramycin – *oriT* disruption cassette from pIJ773.** Table **1** lists alternative cassettes and their resistance determinants.

1. Inoculate a 10 ml SOB (*without* $MgSO_4$) culture containing carbenicillin (100 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml) with 1% of the overnight culture of *E. coli* BW25113/pIJ790 and the *S. coelicolor* cosmid. Add 100 μl 1M L-arabinose stock solution (final concentration is 10 mM, induces *red* genes).
2. Grow for 3-4 h at 30°C shaking at 200 rpm to an OD_{600} of ~ 0.4.
3. Recover the cells by centrifugation at 4000 rpm for 5 min at 4°C in a Sorvall GS3 rotor (or equivalent).
4. Decant medium and resuspend the pellet by gentle mixing in 10 ml ice-cold 10% glycerol.
5. Centrifuge as above and resuspend pellet in 5 ml ice-cold 10 % glycerol, centrifuge and decant. Resuspend the cell pellet in remaining ~ 100 μl 10 % glycerol.
6. Mix 50 μl cell suspension with ~ 100 ng (1-2 μl) of PCR product. Carry out electroporation in a 0.2 cm ice-cold electroporation cuvette using a BioRad GenePulser II set to: 200 Ω, 25 μF and 2,5 kV. The expected time constant is 4.5 – 4.9 ms.
7. Immediately add 1 ml ice cold LB to shocked cells and incubated shaking 1 h at 37°C (or 30°C if further gene disruptions will be made on the same cosmid; see below).
8. Spread onto LB agar containing carbenicillin (100 μg/ml), kanamycin (50 μg/ml) and apramycin (50 μg/ml). If no further gene disruptions will be made on this cosmid, incubate overnight at 37°C to promote the loss of pIJ790. (If further disruptions are planned propagate overnight at 30°C and include chloramphenicol (25 μg/ml) so that pIJ790 is retained).

- **If no colonies are obtained after 16 h growth at 37°C, repeat the experiment starting with a 50 ml SOB culture instead of 10 ml culture for generating electrocompetent cells. Try to concentrate the cells as much as possible by removing all of the remaining 10% glycerol. Resuspend the cell pellet in 50 µl 10% glycerol and use for electroporation.**
- **After 12 – 16 h growth at 37°C different colony-sizes are observed. Cultivating for longer time results in an increased background of small colonies, which are false positives. It is important to note that at this stage wild-type and mutant cosmids exist within one cell. The transformation with a PCR product and its integration in the cosmid DNA by homologous recombination will not occur in all copies of the cosmid molecules in one cell. One copy of a cosmid containing the incoming resistance marker is sufficient for resistance to this antibiotic. Normally, the larger the size of a colony, the more copies of mutagenised cosmids are present. Inoculating a large colony in 5 ml LB liquid cultures containing carbenicillin (100 µg/ml), kanamycin (50 µg/ml) and apramycin (50 µg/ml) result in a growth at 37°C to a cell density (OD_{600} ~ 0.1 – 0.3) within 3-4 h (*E. coli* BW25113 without pIJ790 grows very fast). After 6 h plasmid DNA can be isolated and tested by restriction analysis and/or PCR using the primers described below.**
- **PCR analysis with a primer pair (test primers) priming just ~ 100 bp outside the region affected by homologous recombination will generate the expected fragment after gene disruption, but will usually also generate the wild-type fragment, caused by remaining wild-type copies within the same transformant. These will be lost during the subsequent transformation step into the methylation-deficient *E. coli* host ET12567 containing the non-transmissible plasmid pUZ8002 (this is not a problem anyway because wild-type copies lack the *oriT*).**
- **Notes on viomycin selection: selecting for viomycin^R depends critically on the amount of salt in the medium; more viomycin is required at higher salt concentrations. For a clean selection of *E. coli* clones, use DNA agar or 2xYT agar containing 30 µg/ml viomycin (see Kieser *et al.*, 2000).**

For multiple gene replacements, choose an *oriT*-containing disruption cassette for the first knock-out, and a cassette without *oriT* and different resistance markers for further gene disruptions.

The gene disruption is confirmed by restriction analysis and/or PCR. Cosmid DNA of transformants is isolated from a 6 h, 37°C, 5 ml LB culture containing carbenicillin (100 µg/ml), kanamycin (50 µg/ml) and apramycin (50 µg/ml). Alkaline lysis followed by phenol/chloroform extraction produces cosmid DNA suitable for restriction analysis.

Cosmid CCC DNA isolation

1. Resuspend the cell pellet from 1 ml culture by vortexing in 100 µl solution I (50 mM Tris/HCl, pH 8; 10 mM EDTA).
2. Immediately add 200 µl solution II (200 mM NaOH; 1% SDS) and mix by inverting the tubes 10x.
3. Immediately add 150 µl solution III (3 M potassium acetate, pH 5.5) and mix by inverting the tubes 5x.
4. Spin at full speed in a microcentrifuge for 5 min at room temperature.
5. Immediately extract supernatant with 400 µl phenol/chloroform, vortex 2 min and spin at full speed in a micro centrifuge for 5 min.
6. Transfer the upper phase and add 600 µl 2-propanol. Leave the tubes on ice for 10 min.
7. Spin as above and wash the pellet with 200 µl 70% ethanol.
8. Spin as above and leave the tube open for 5 min at room temperature to dry the pellet. Resuspend the pellet in 50 µl 10mM Tris/HCl (pH 8) and use 10 µl for restriction digest.
    - **Omitting the phenol/chloroform extraction step results in degradation of the cosmid DNA. Use of miniprep-columns without including a phenol/chloroform extraction is not recommended.**

Verification of positive transformants by PCR requires an additional pair of 18 – 20 nt test primers which anneal 100 – 200 bp upstream and downstream of the 39 bp recombination region. (These primers can also be used later to verify the FLP-mediated excision of the resistance cassette.)

• Primers (100 pmoles/µl)	0.2 µl each	20 pmoles each
• Template DNA (~50 ng/µl)	1 µl	50 ng
• Buffer (10x)	5 µl	1 x
• dNTPs (10 mM)	1 µl each	50 µM each
• DMSO (100 %)	2.5 µl	5%
• DNA polymerase (2.5 U/µl)	1 µl	2.5 Units
• Water	36.1 µl	
• Total volume	50 µl	

Cycle conditions:

1. Denaturation:	94°C, 2 min	
2. Denaturation:	94°C, 45 sec	} 30 cycles
3. Primer annealing:	55°C, 45 sec	
4. Extension:	72°C, 90 sec	
5. Final extension:	72°C, 5 min	

5 µl of the PCR product is used for gel electrophoresis.

6 Transfer of the mutant cosmids into *Streptomyces*

If the target *Streptomyces* for mutagenesis carries a methyl-sensing restriction system (as is the case for *S. coelicolor* and *S. avermitilis*), it is necessary to passage the cosmid containing an apramycin resistance-*oriT* cassette through a non-methylating *E. coli* host. To achieve this, it is introduced by transformation into the non-methylating *E. coli* ET12567 containing the RP4 derivative pUZ8002. The cosmid is then transferred to *Streptomyces* by intergeneric conjugation (see Table 2 for resistance markers). If the target *Streptomyces* for mutagenesis does **not** carry a methyl-sensing restriction system (as is the case for *S. lividans*), common *E. coli* strains such as DH5α containing pUZ8002 can be used instead.

Description	**Name**	**Replication**	**Carb^R**	**Cml^R**	**Kan^R**	**Tet^R**
S. coelicolor cosmid clones	Supercos 1		Carb^R		Kan^R	
λ Red plasmid	pIJ790	t^s		Cml^R		
FLP recombinase plasmid	BT340	t^s	Carb^R	Cml^R		
OriT$^-$ RP4 derivative	pUZ8002				Kan^R	
OriT$^+$ RP4 derivative	pUB307				Kan^R	
Non-methylating *E. coli*	ET12567			Cml^R		Tet^R

Table 2. Resistance markers of vectors, helper plasmids and strains (carbenicillin resistance (Carb^R), chloramphenicol resistance (Cml^R), kanamycin resistance (Kan^R), tetracycline resistance (Tet^R), temperature sensitive replicon (t^S)). See Table **1** for replacement cassettes.

1. Prepare competent cells of *E. coli* ET12567/pUZ8002 grown at 37ºC in LB containing kanamycin (25 μg/ml) and chloramphenicol (25 μg/ml) to maintain selection for pUZ8002 and the *dam* mutation, respectively. (ET12567 has a doubling time > 30 min.)
   - **High competence is required when Dam-methylated plasmids are introduced into a dam$^-$ strain.**
2. Transform competent cells with the *oriT*-containing cosmid clone, and select for the incoming plasmid only using apramycin (50 μg/ml) and carbenicillin (100 μg/ml) .

3. Inoculate a colony into 10 ml LB containing apramycin (50 μg/ml), chloramphenicol (25 μg/ml) and kanamycin (50 μg/ml). Grow overnight at 37ºC.
    - **Chloramphenicol^S or Kanamycin^S segregants arise frequently among transformants, so set up more than one culture. The kanamycin selection is probably ineffective because both the cosmid and pUZ8002 confer resistance (Table 2).**
4. Inoculate 100 μl overnight culture into 10 ml fresh LB plus antibiotics as above and grow for ~ 4 h at 37°C to an OD_{600} of 0.4.
5. Wash the cells twice with 10 ml of LB to remove antibiotics that might inhibit *Streptomyces*, and resuspend in 1 ml of LB.
6. While washing the *E. coli* cells, for each conjugation add 10 μl (10^8) *Streptomyces* spores to 500 μl 2 × YT broth. Heat shock at 50°C for 10 min, then allow to cool.
7. Mix 0.5 ml *E. coli* cell suspension and 0.5 ml heat-shocked spores and spin briefly. Pour off most of the supernatant, then resuspend the pellet in the c. 50 μl residual liquid.
8. Make a dilution series from 10^{-1} to 10^{-4} each step in a total of 100 μl of water.
9. Plate out 100 μl of each dilution on MS agar + 10mM $MgCl_2$ (without antibiotics) and incubate at 30°C for 16-20 h.
10. Overlay the plate with 1 ml water containing 0.5 mg nalidixic acid (20 μl of 25 mg/ml stock; selectively kills *E. coli*) and 1.25 mg apramycin (25 μl of 50 mg/ml stock). Use a spreader to lightly distribute the antibiotic solution evenly. Continue incubation at 30°C.
11. Replica-plate each MS agar plate with single colonies onto DNA plates containing nalidixic acid (25 μg/ml) and apramycin (50 μg/ml) with and without kanamycin (50 μg/ml). Double cross-over exconjugants are kanamycin^S and apramycin^R. (DNA gives fast, non-sporulating growth.)
12. Kanamycin^S clones are picked from the DNA plates and streaked for single colonies on MS agar (promotes sporulation) containing nalidixic acid (25 μg/ml) and apramycin (50 μg/ml).
13. Confirm kanamycin sensitivity by replica-plating onto DNA plates containing nalidixic acid (25 μg/ml) with and without kanamycin (50 μg/ml).
14. Purified kanamycin sensitive strains are then verified by PCR and Southern blot analysis.

- **Typically, ~ 10 % of the exconjugants are double cross-over recombinants. The frequency of double cross-overs depends on the length of the flanking regions of homologous DNA on the cosmid. If < 1 kb is left on one side of the disrupted gene, obtaining kanamycin^S double cross-over types directly on the conjugation plates may be difficult. It may be necessary to streak out several exconjugants for single colonies on MS agar without antibiotics. After 3-5 days growth replica-plate onto DNA with and without kanamycin.**

Antibiotic	Stock mg/ml	µl for 1 ml overlay	Final conc. after flooding µg/ml	Concentration in	
				MS, DNA µg/ml	R2YE µg/ml
Apramycin	**50**	**25**	**50**	**50**	**50**
Kanamycin	**50**	**100**	**200**	**50**	**200**
Spectinomycin	**200**	**25**	**200**	**400**	**400**
Streptomcyin	**10**	**25**	**10**	**10**	**10**
Viomycin	**30**	**25**	**30**	**30**	**NA**
Nalidixic acid	**25 in 0.3 M NaOH**	**20**	**20**	**25**	**25**

Table 3: Antibiotic concentrations for selection on *S. coelicolor* MS conjugation plates, DNA replica plates or R2YE protoplast regeneration plates (Note some small differences from Kieser *et al*., 2000).

7 FLP-mediated excision of the disruption cassette

The disruption cassettes are flanked by FRT sites (FLP recognition targets). Expression of the FLP-recombinase in *E. coli* removes the central part of the disruption cassette, leaving behind a 81 bp "scar" sequence which, in the preferred reading frame (bold in Fig. 3), lacks stop codons.

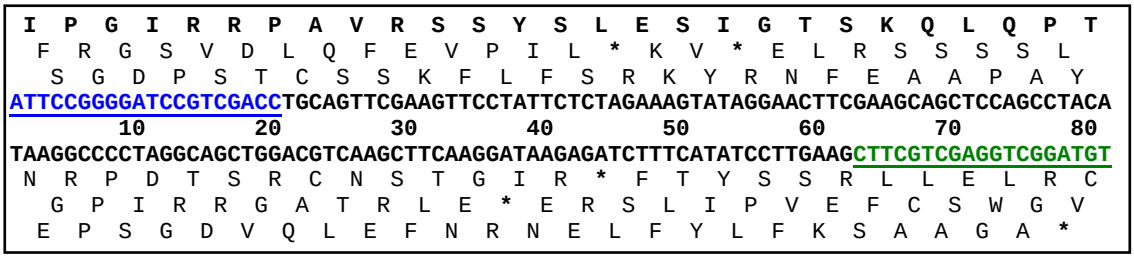


Fig. 3: Sequence of the 81 bp "scar" sequence remaining after FLP-mediated excision of the disruption cassette. The translation of the preferred reading frame is printed bold. The 20 and 19 nt priming sites are underlined and printed in colour. (Fig. **2** explains the determination of the reading frame.)

★ indicate stop codons,

priming site (20 nt) priming site (19 nt)

This allows the generation of (hopefully) non-polar, unmarked in-frame deletions and repeated use of the same resistance marker for making multiple knock-outs in the same cosmid or in the same strain. *E. coli* DH5α cells containing the temperature sensitive FLP recombination plasmid BT340 (Datsenko and Wanner, 2000; can be obtained from the *E. coli* Genetic Stock Center: CGSC Strain# 7629) are transformed with the mutagenised cosmid DNA (obtained in section 5). BT340 contains ampicillin and chloramphenicol resistance determinants and is temperature sensitive for replication (replicates at 30°C). FLP synthesis and loss of the plasmid are induced at 42°C (Cherepanov and Wackernagel, 1995).

1. Grow *E. coli* DH5α/BT340 overnight at 30°C in 10 ml LB containing chloramphenicol (25 μg/ml).
    - **Transforming *E. coli* BW25113/cosmid::apramycin (mutagenised cosmid) with the plasmid BT340 is not recommended because the isolates after PCR targeting may still contain copies of undisrupted cosmid DNA (see page 10, second paragraph).**
2. Inoculate 100 μl *E. coli* DH5α/BT340 from overnight culture into 10 ml LB containing chloramphenicol (25 μg/ml).
3. Grow for 3-4 h at 30°C shaking at 200 rpm to an OD_{600} of ~ 0.4.
4. Recover the cells by centrifugation at 4000 rpm for 5 min at 4°C in a Sorvall GS3 rotor (or equivalent).
5. Decant medium and resuspend the pellet by gentle mixing in 10 ml ice-cold 10 % glycerol.
6. Centrifuge as above and resuspend pellet in 5 ml ice-cold 10 % glycerol, centrifuge and decant. Resuspend the cell pellet in remaining ~ 100 μl 10% glycerol.
7. Mix 50 μl cell suspension with ~ 100 ng (1-2 μl) of mutagenised cosmid DNA. Carry out electroporation in a 0.2 cm ice-cold electroporation cuvette using a BioRad GenePulser II set to: 200 Ω, 25 μF and 2,5 kV. The expected time constant is 4.5 – 4.9 ms.
8. Immediately add 1 ml ice cold LB to shocked cells and incubate shaking for 1 h at 30°C.
9. Spread onto LB agar containing apramycin (50 μg/ml) and chloramphenicol (25 μg/ml).
10. Incubate for 2 d at 30°C (*E. coli* DH5α/BT340 grows slowly at 30°C).
11. A single colony is streaked on an LB agar plate without antibiotics for single colonies and grown overnight at 42°C to induce expression of the FLP recombinase followed by the loss of plasmid BT340.
12. Make two masterplates by streaking 20 – 30 single colonies with a toothpick first on a LB agar plate containing apramycin (50 μg/ml) and then on a LB agar plate containing kanamycin (50 μg/ml).

13. Grow the masterplates overnight at 37°C. Apramycin^S kanamycin^R clones indicate the successful loss of the resistance cassette and are further verified by restriction and PCR analysis.
    - **Typically, ~ 10 % of the single colonies after non-selective growth lose the incoming resistance marker and the plasmid BT340 simultaneously.**
    - **Using the same test primers as in section 5 (annealing ~ 100 bp upstream and downstream of the 39 nt primer sequence) should produce a PCR product of ~ 300 bp (200 bp + 81 bp "scar"). PCR fragments can be sequenced using the amplification primers for verification.**

Replacing resistance cassette inserts in *S. coelicolor* with the unmarked "scar" sequence

The chromosomal apramycin resistance cassette insert in *S. coelicolor* is replaced by the "scar" sequence. This is achieved by homologous recombination between the chromosome and the corresponding "scar cosmid" prepared in 7. The procedure differs from section 6 because the cosmid lacks *oriT*, and the desired product is antibiotic sensitive. Therefore, it is necessary to introduce the scar cosmid into *Streptomyces* by protoplast transformation, and then select for kanamycin resistant *Streptomyces* containing the entire scar cosmid integrated by a single crossover. Restreaking to kanamycin-free medium, followed by screening for concomitant loss of kanamycin resistance and apramycin resistance, then identifies the desired *Streptomyces* clones.

Preparation of *Streptomyces coelicolor* protoplasts

1. Add 25 ml YEME medium to a baffled flask. Add ~ 0.1 ml spore suspension and required growth factors. Incubate 36-40 h at 30°C in an orbital incubator shaker.
    - **Cultures of *S. lividans* and *S. coelicolor* are ready for harvesting when they start to produce red pigment**
2. Pour culture broth into a 20 ml screw cap bottle and spin in the bench centrifuge (~ 1000 x g, 10 min).
    - **Before centrifugation, examine the culture for contamination by unicellular bacteria, usually indicated by turbidity: the *Streptomyces* mycelium sediments**

quickly while unicellular contaminants remain suspended. In case of doubt, use the microscope.

3. Discard the supernatant carefully; the pellet is easily disturbed.
   - **If the mycelium does not pellet add 5 ml sterile water to reduce the density of the medium and centrifuge again.**
4. Resuspend pellet in 15 ml 10.3% sucrose and spin in bench centrifuge as above. Discard supernatant.
5. Repeat step 4.
   - **The mycelial pellet, without added liquid, can be stored frozen at –20°C**
6. Resuspend mycelium in 4 ml lysozyme solution (1 mg/ml P buffer, filter sterilised); incubate at 30°C, 15-60 min.
7. Draw in and out of a 5 ml pipette three times and incubate for a further 15 min.
   - **This helps to free protoplasts from the mycelium so that they will pass through the cotton wool filter used in step 9. At least with *S. lividans,* it is possible to obtain transformants with unfiltered material, but the washing (steps 9-10) is still needed to remove lysozyme.**
8. Add 5 m1 P buffer. Repeat step 7.
9. Filter protoplasts through cotton wool (using a filter tube) and transfer to a plastic tube.
10. Sediment protoplasts gently by spinning in a bench centrifuge (~ 1000 x g, 7 min).
11. Discard supernatant and suspend protoplasts in 1 ml P buffer.
    - **At this and any other steps when pelleted protoplasts are to be resuspended, resuspend in the remaining drop of liquid by tapping the side of the tube repeatedly with a finger until the protoplasts are dispersed to form a creamy suspension, then add the suspending P buffer (otherwise the protoplast pellet is difficult to disperse). Avoid vortexing, which induces foaming and consequent lysis. To freeze the protoplasts for storage, place samples of the protoplast suspension in small plastic tubes, close them and place them in ice in a plastic beaker. Place the beaker at –70°C overnight. Free the frozen protoplasts in their tubes from the ice and store at –70°C. To thaw, shake the frozen tube under running warm water (i.e. freeze slowly, thaw quickly). To assess the proportion of non-protoplasted units in the suspension, samples can be diluted in parallel in P buffer and in dilute detergent (~ 0.01% SDS) and plated on regeneration plates. Any colonies arising after dilution in detergent are likely to have arisen from non-protoplasted units.**

Rapid small-scale transformation of *Streptomyces coelicolor*

1. Dispense 50 µl samples of protoplasts (~ 10^{10}/ml) into as many tubes as there are transformations.
   - **We usually spin the protoplasts down immediately before the transformation experiment. This eliminates substances that may have leaked out of the protoplasts during storage and the contents of protoplasts which have lysed spontaneously (which may include nucleases).**
2. Complete steps 2a-c for each transformation individually.
   a. Add up to 5 µl DNA solution to protoplasts and mix immediately by tapping tube.
   b. Add 200 µl 25% PEG 1000 in P buffer and mix by pipetting up and down four times (be careful not to contaminate the barrel of the pipette).
   c. Spread protoplast suspension (100-200 µl) on two dried R2YE plates. Use P buffer to make dilutions if required.
      - **1 ml glass pipettes can be used instead of spreaders. The solution will spread to some extent by itself if the plates are left on a horizontal surface.**
3. Incubate plates at 30°C. After 14-20 h, flood for kanamycin selection. Score for resistant colonies after 3 d.
4. Select single colony and streak non-selectively for single colonies on MS agar plates and grow 3-4 d at 30°C.
5. Replica-plate to DNA agar plates with apramycin (50 µg/ml) or kanamycin (50 µg/ml) to screen for apramycin^S and kanamycin^S transformants.

The λ RED recombination plasmid pIJ790

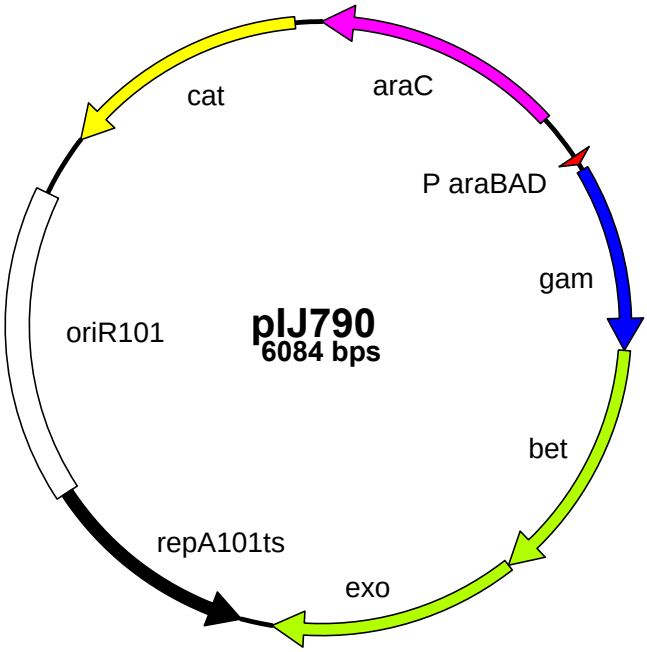


Fig. 4: The λ RED recombination plasmid pKD20 (*E. coli* Genetic Sock Center CGSC Strain# 7637; Datsenko and Wanner, 2000) was modified by replacing the ampicillin resistance gene *bla* by the chloramphenicol resistance gene *cat*, generating pIJ790. Unlike pKD20, pIJ790 can be selected in presence of Supercos1.

Legend:

araC: encodes arabinose activator

bet: encodes single strand DNA binding protein

cat: chloramphenicol resistance gene

exo: exonuclease gene, promotes recombination together with *bet*

gam: gene product inhibits the host exonuclease V

oriR101: origin of replication

P araBAD: L-arabinose inducible promotor

repA101ts: temperature-sensitive replication

The following digestions can be used to verify this plasmid.

*Bam*HI: 6084 bp; *Eco*RI: 2872 bp, 1703bp, 1509 bp;

*Nco*I: 3927 bp, 2157 bp; *Pst*I: 5873 bp, 247 bp

Template plasmids

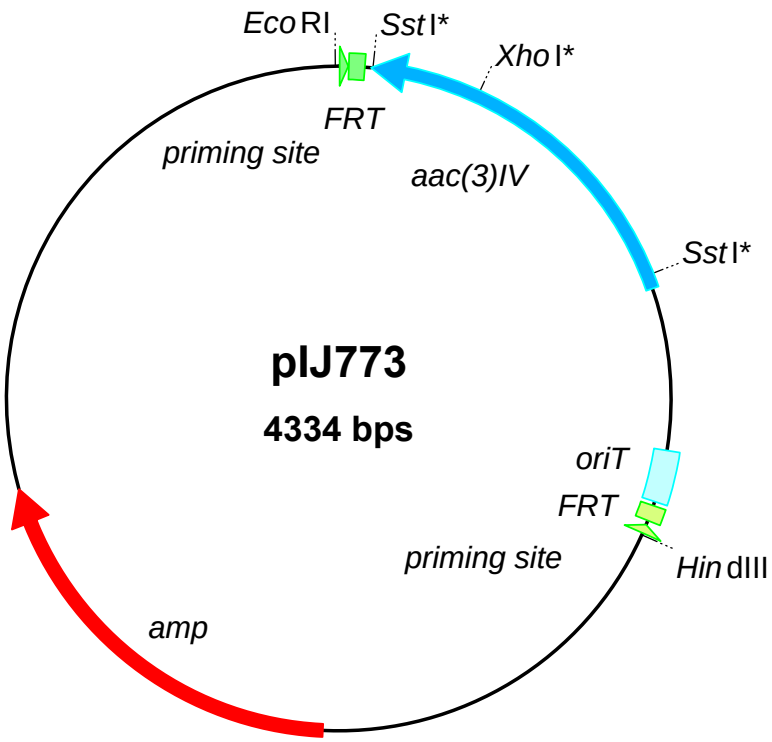


Fig. 5: Template plasmid pIJ773 containing the apramycin resistance gene *aac(3)IV* (AC=X99313) and the *oriT* of plasmid RP4 (=RK2) (AC=L27758), flanked by FRT sites (FLP recognition targets, see Datsenko and Wanner, 2000). The disruption cassette was cloned into the *Eco*RV site of pBluescript KS (+) allowing its isolation as a 1382 bp *Eco*RI/*Hin*dIII fragment.

* indicates suitable restriction sites for verification of mutagenised cosmid DNA by restriction analysis (for example: *Sst*I generates a 751 bp internal fragment within the disruption cassette).

```
LOCUS       pIJ773       4334 bp    DNA   CIRCULAR SYN        02-AUG-2002
DEFINITION  Ligation of Apra-oriT disruption cassette into the EcoRV site of
pBluescript SK(+)
ACCESSION   pIJ773
KEYWORDS    .
SOURCE      Unknown.
  ORGANISM  Unknown
            Unclassified.
REFERENCE   1  (bases 1 to 4334)
  AUTHORS   Gust et al., 2003,
  JOURNAL   Proc. Natl. Acad. Sci. USA 100(4), 1541-1546
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     CDS             21..54
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     CDS             complement (66..851)
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                     /product="apramycin resistance gene"
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     CDS             complement (1352..1371)
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 421  cgaggagaag tacctgccca tcgagttcat ggacacgggc gaccgggctt gcaggcgagt
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3301  gtttgccgga tcaagagcta ccaactcttt ttccgaaggt aactggcttc agcagagcgc
3361  agataccaaa tactgtcctt ctagtgtagc cgtagttagg ccaccacttc aagaactctg
3421  tagcaccgcc tacatacctc gctctgctaa tcctgttacc agtggctgct gccagtggcg
3481  ataagtcgtg tcttaccggg ttggactcaa gacgatagtt accggataag gcgcagcggt
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3601  tgagatacct acagcgtgag ctatgagaaa gcgccacgct tcccgaaggg agaaaggcgg
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3721  gaaacgcctg gtatctttat agtcctgtcg ggtttcgcca cctctgactt gagcgtcgat
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3841  tacggttcct ggccttttgc tggccttttg ctcacatgtt ctttcctgcg ttatcccctg
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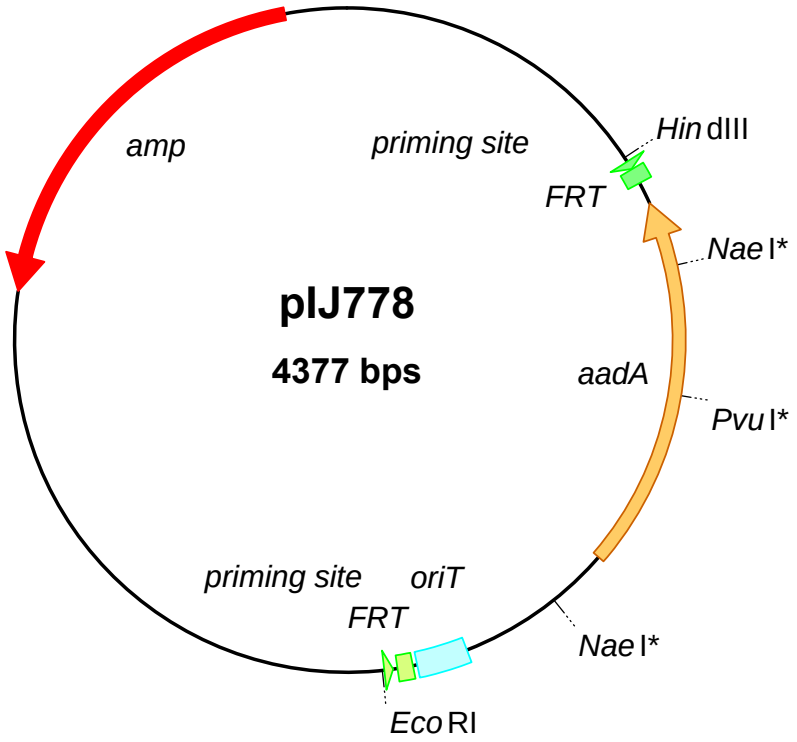

Fig. 6: Template plasmid pIJ778 containing the streptomycin and spectinomycin resistance gene *aadA* (AC=M60473) and the *oriT* of plasmid RP4 (=RK2) (AC=L27758), flanked by FRT sites (FLP recognition targets, see Datsenko and Wanner, 2000). The disruption cassette was cloned into the *Eco*RV site of pBluescript KS (+) allowing its isolation as a 1425 bp *Eco*RI/*Hin*dIII fragment.

* indicates suitable restriction sites for verification of mutagenised cosmid DNA by restriction analysis (for example: *Nae*I generates a 782 bp internal fragment within the disruption cassette).

```
LOCUS       pIJ778       4377 bp    DNA   CIRCULAR SYN        02-AUG-2002
DEFINITION  Ligation of Spec,Strep-oriT disruption cassette into the EcoRV site of
pBluescript SK(+)
ACCESSION   pIJ778
KEYWORDS    .
SOURCE      Unknown.
  ORGANISM  Unknown
            Unclassified.
REFERENCE   1  (bases 1 to 4377)
  AUTHORS   Gust et al., 2003
  JOURNAL   Proc. Natl. Acad. Sci. USA 100(4), 1541-1546
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     CDS             1931..2040
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                     /region="FRT"
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     CDS             complement
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      121 GATAGGGTTG AGTGTTGTTC CAGTTTGGAA CAAGAGTCCA CTATTAAAGA ACGTGGACTC
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      301 CCCCCGATTT AGAGCTTGAC GGGGAAAGCC GGCGAACGTG GCGAGAAAGG AAGGGAAGAA
      361 AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG GTCACGCTGC GCGTAACCAC
      421 CACACCCGCC GCGCTTAATG CGCCGCTACA GGGCGCGTCG CGCCATTCGC CATTCAGGCT
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      541 AGGGGGATGT GCTGCAAGGC GATTAAGTTG GGTAACGCCA GGGTTTTCCC AGTCACGACG
      601 TTGTAAAACG ACGGCCAGTG AATTGTAATA CGACTCACTA TAGGGCGAAT TGGGTACCGG
      661 GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGATGTG TAGGCTGGAG CTGCTTCGAA
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     2161 CGCGGTGGAG CTCCAGCTTT TGTTCCCTTT AGTGAGGGTT AATTCCGAGC TTGGCGTAAT
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     2281 GAGCCGGAAG CATAAAGTGT AAAGCCTGGG GTGCCTAATG AGTGAGCTAA CTCACATTAA
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2581 GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC
2641 GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG
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3721 GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG CGAGTTACAT
3781 GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA
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4141 CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG
4201 CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC
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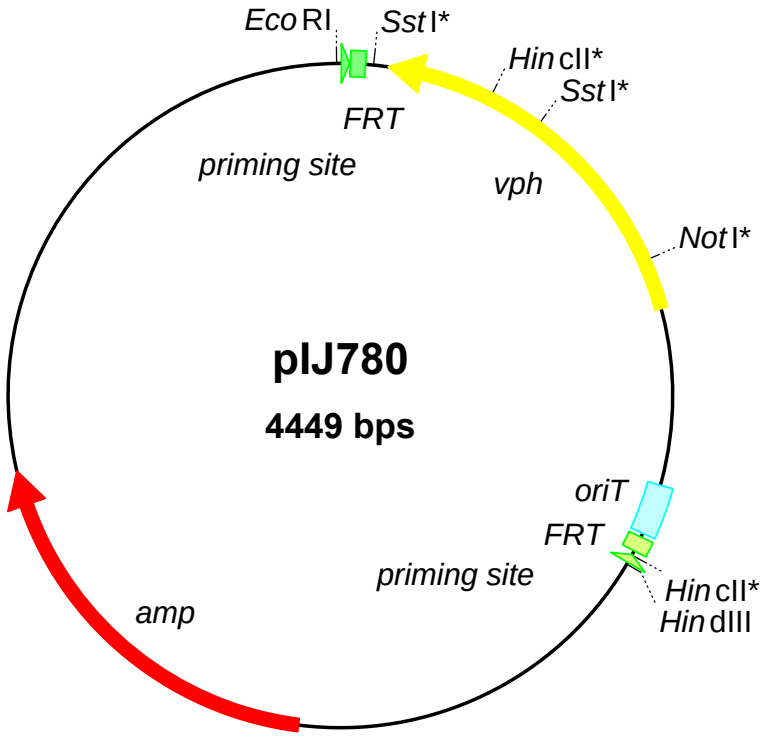

Fig. 7: Template plasmid pIJ780 containing the viomycin resistance gene *vph* (AC=X99314) and the *oriT* of plasmid RP4 (=RK2) (AC=L27758), flanked by FRT sites (FLP recognition targets, see Datsenko and Wanner, 2000). The disruption cassette was cloned into the *Eco*RV site of pBluescript KS (+) allowing its isolation as a 1497 bp *Eco*RI/*Hin*dIII fragment.

* indicates suitable restriction sites for verification of mutagenised cosmid DNA by restriction analysis (for example: *Hinc*II generates a 1137 bp internal fragment within the disruption cassette).

```
LOCUS       pIJ780       4449 bp    DNA   CIRCULAR SYN        02-AUG-2002
DEFINITION  Ligation of Vio-oriT disruption cassette into the EcoRV site of
pBluescript SK(+)
KEYWORDS    .
SOURCE      Unknown.
  ORGANISM  Unknown
            Unclassified.
REFERENCE   1  (bases 1 to 4449)
  AUTHORS   Gust et al., 2003
  JOURNAL   Proc. Natl. Acad. Sci. USA 100(4), 1541-1546
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     CDS             complement (102..925)
                     /gene="vph"
                     /product="viomycin resistance gene"
     CDS             1306..1415
                     /region="oriT"
                     /product="origin of transfer (RK2)"
     CDS             1424..1457
                     /region="FRT"
                     /product="natural FRT site"
     CDS             complement (1466..1485)
                     /region="priming site 20 bp"
                     /product="ATT CCG GGG ATC CGT CGA CC"
     CDS             2314..3174
                     /gene="amp"
                     /product="b-lactamase"
BASE COUNT      977 a   1241 c   1194 g   1037 t
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        1 GTGTAGGCTG GAGCTGCTTC GAAGTTCCTA TACTTTCTAG AGAATAGGAA CTTCGGAATA
       61 GGAACTTCAT GAGCTCAGCC AATCGACTGG CGAGCGGCAT CCTACCGGTA GCCGCTGAGG
      121 CCGTCGGCGA GTTCCTCCTC GTCGCCGTCG CGCTGCGCGT AGAGGGCCTG CTGGAGTGCG
      181 AAGGTGCCTC GGATCGCCGA GATCCGCTCG GCCGTTCCGT TGTCGGCCCA GCCGCCGAGC
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      301 GCCGGGTCGC CGATGCCGAC CTCGTCCCAG TCGACGACGC CGCTCATGCG CGGCACTCCG
      361 TCCACCGTCT CCCACAGGAC GTTCTCGCCG CCGAGGTCAC CGTGGACCAC CGCGGAGGTG
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     1021 TGTTGCAAAT AGTCGGTGGT GATAAACTTA TCATCCCCTT TTGCTGATGG AGCTGCACAT
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4321 GGAAACAGCT ATGACCATGA TTACGCCAAG CTCGGAATTA ACCCTCACTA AAGGGAACAA
4381 AAGCTGGAGC TCCACCGCGG TGGCGGCCGC TCTAGAACTA GTGGATCCCC CGGGCTGCAG
4441 GAATTCGAT
//
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Strains:

Strain designation: BW25113/pIJ790

Plasmid: pIJ790 *[oriR101], [repA101*(ts)*]*, *araBp-gam-be-exo*

Chromosome: (Δ(*araD-araB*)*567*, Δ*lacZ4787*(::*rrnB-4*), *lacIp-4000*($lacI^Q$), λ^-, *rpoS369*(Am), *rph-1*, Δ(*rhaD-rhaB*)568, *hsdR514;* Datsenko and Wanner, 2000);

this strain should be grown on rich medium containing chloramphenicol (25 μg/ml) at 30°C.

Strain designation: DH5α/pIJ773

Plasmid: pBluescript KS (+), *aac(3)IV*, *oriT* (RK2), FRT sites

chromosomal marker : see Stratagene,

this strain should be grown on rich medium containing carbenicillin (100 μg/ml) and apramycin (50 μg/ml) at 37°C.

Strain designation: DH5α/pIJ778

Plasmid: pBluescript KS (+), *aadA*, *oriT* (RK2), FRT sites

chromosomal marker : see Stratagene,

this strain should be grown on rich medium containing carbenicillin (100 μg/ml), streptomycin (50 μg/ml) and spectinomycin (50 μg/ml) at 37°C.

Strain designation: DH5α/pIJ780

Plasmid: pBluescript KS (+), *vph*, *oriT* (RK2), FRT sites

chromosomal marker : see Stratagene,

this strain should be grown on DNA (Difco Nutrient Agar), 2XYT broth or DNB (Difco Nutrient broth) containing carbenicillin (100 μg/ml) and viomycin (30 μg/ml) at 37°C.

Strain: DH5α/BT340 (Datsenko and Wanner, 2000) **can be ordered from:**

E. coli Genetic Stock Center

830 Kline Biology Tower

MCD Biology Department

266 Whitney Ave. Box 208103

Yale University

New Haven, CT 06520-81033

Tel. (0044) (203) 432-9997 Fax –6161

email: berlyn@cgsc.biology.yale.edu

webpage: http://cgsc.biology.yale.edu

Strains DH5α/pIJ779 and DH5α/pIJ781 will be send separately on request, please contact:

Plant Bioscience Limited, Dr Karin Schofield

Technology Acquisition & Technology ManagerNorwich Research Park, Colney, Norwich, NR4 7UH, UKEmail: karin@plantbioscience.com

Tel: +44 (0) 1603 456500; Fax: +44 (0) 1603 456552

www.plantbioscience.com

Media: for more detailed information see:

Kieser T, Bibb MJ, Buttner MJ, Chater KF and Hopwood DA (2000)

Practical Streptomyces Genetics, John Innes Foundation, Norwich Research Park, Colney, Norwich NR4 7UH, England

Agar Media

Note: MS medium (Mannitol Soya flour Medium) is also known as SFM medium (Kieser *et al*., 2000).

R2 Medium (Okanishi *et al.,* 1974; Hopwood and Wright, 1978)
Make up the following solution:

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.6H_2O$	10.12 g
Glucose	10 g
Difco Casaminoacids	0.1 g
Distilled water	800 ml

Pour 80 ml of the solution into 250 ml Erlenmeyer flasks each containing 2.2 g Difco Bacto agar. Close the flasks and autoclave. At time of use, remelt the medium and add to each flask the following autoclaved solutions in the order listed:

KH_2PO_4 (0.5%)	1 ml
$CaCl_2.2H_2O$ (3.68%)	8 ml
L-proline (20%)	1.5 ml
TES buffer (5.73%, adjusted to pH7.2)	10 ml
[1]Trace element solution	0.2 ml
NaOH (1N) (unsterilised is OK)	0.5 ml
Required growth factors for auxotrophs	0.75 ml each
(For stock solutions, see Kieser *et al.,* 2000)	

[1]Trace element solution (l^{-1}):

$ZnCl_2$	40 mg
$FeCl_3.6H_2O$	200 mg
$CuCl_2.2H_2O$	10 mg
$MnCl_2.4H_2O$	10 mg
$Na_2B_4O_7.10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	10 mg

R2YE Medium

Make up in the same way as R2, but add 5 ml of Difco yeast extract (10%) to each 100 ml flask at time of use

R5 Medium

This is an alternative way of making R2YE. It obviously saves time at the bench. For most purposes it seems satisfactory, but there is some folklore suggesting that R2YE made by the traditional process gives more reproducible results.

Make up the following solution:

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.6H_20$	10.12 g
Glucose	10 g
Difco Casaminoacids	0.1 g
[1]Trace element solution	2 ml
Difco yeast extract	5 g
TES buffer	5.73 g
Distilled water to	1000 ml

[1] The same as in R2 and R2YE.

Pour 100 ml of the solution into 250 ml Erlenmeyer flasks each containing 2.2 g Difco Bacto agar. Close the flasks and autoclave. At time of use, re-melt the medium and add to each flask in the order listed:

KH_2PO_4 (0.5%)	1 ml
$CaCl_2.2H_2O$ (5M)	0.4 ml
L-proline (20%)	1.5 ml
NaOH (1N)	0.7 ml
Any required growth factors for auxotrophs (see Kieser *et al.*, 2000)	0.75 ml each

Mannitol Soya flour Medium (MS) Hobbs *et al.* (1989). Sometimes referred to as "SFM".

Agar	20 g
Mannitol	20 g
[1]Soya flour	20 g
Tap water	1000 ml

[1]Use soya flour from a health food shop or supermarket, not the expensive material from a laboratory supplier.

Dissolve the mannitol in the water and pour 200 ml into 250 ml Erlenmeyer flasks each containing 2 g agar and 2 g soya flour. Close the flasks and autoclave *twice* (115 ºC , 15 min), with gentle shaking between the two runs.

Difco nutrient agar (DNA)

Place 4.6 g Difco Nutrient Agar in each 250 ml Erlenmeyer flask and add 200 ml distilled water. Close the flasks and autoclave.

L agar

Agar	10 g
Difco Bacto tryptone	10 g
NaCl	5 g
Glucose	1 g
Distilled water	up to 1000 ml

Dissolve the ingredients, except agar, in the distilled water and pour 200 ml into 250 ml Erlenmeyer flasks each containing 2 g agar. Close the flasks and autoclave.

Liquid media

Yeast extract-malt extract medium (YEME)

Difco yeast extract	3 g
Difco Bacto-peptone	5 g
Oxoid malt extract	3 g
Glucose	10 g
Sucrose	340 g (34% final)
Distilled water	up to 1000 ml

After autoclaving, add:

$MgCl_2.6H_2O$ (2.5M)	2 ml/litre (5mM final)

For preparing protoplasts, also add:

[1]Glycine (20%)	25 ml/litre (0.5% final)

[1]Different *Streptomyces* strains may need different concentrations of glycine; 0.5% is best for *S. lividans* and *S. coelicolor*.

Tryptone soya broth (TSB)

Oxoid Tryptone Soya Broth powder (CM129)	30 g
Distilled water	1000 ml

Difco nutrient broth (DNB)

Difco Nutrient Broth powder	8 g
Distilled water	1000 ml

L (Lennox) broth (LB)

Difco Bacto tryptone	10 g
Difco yeast extract	5 g
NaCl	5 g
Glucose	1 g
Distilled water	1000 ml

2 X YT medium

Difco Bacto tryptone	16 g
Difco Bacto yeast extract	10 g
NaCl	5 g
Water	1000 ml

Buffers

P (protoplast) buffer

Make up the following basal solution:

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.6H_20$	2.02 g
[1]Trace element solution	2 ml
Distilled water to	800 ml

[1] The same as in R2 and R2YE.

Dispense in 80 ml aliquots and autoclave. Before use, add to each flask in order:

KH_2PO_4 (0.5%)	1 ml
$CaCl_2.2H_20$ (3.68%)	10 ml
TES buffer (5.73%, adjusted to pH7.2)	10 ml

T (transformation) buffer

Mix the following sterile solutions:

Sucrose (10.3%)	25 ml
Distilled water	75 ml
[1]Trace element solution	0.2 ml
K_2SO_4 (2.5%)	1 ml

[1] The same as in R2 and R2YE.

To 9.3 ml of the above solution add:

$CaCl_2$ (5M)	0.2 ml
[1]Tris-maleic acid buffer	0.5 ml

[1]Make up a 1M solution of Tris and adjust to pH8.0 by adding maleic acid. For use, add 3 parts by volume of the above solution to 1 part by weight of PEG 1000, previously sterilised by autoclaving.

L (lysis) buffer (Thompson *et al*., 1982)

Mix the following sterile solutions:

Sucrose (10.3%)	100 ml
TES buffer (5.73%, adjusted to pH7.2)	10 ml
K_2SO_4 (2.5%)	1 ml
[1]Trace element solution	0.2 ml
KH_2PO_4 (0.5%)	1 ml
$MgCl_2 \cdot 6H_2O$ (2.5M)	0.1 ml
$CaCl_2$ (0.25M)	1 ml

[1] The same as in R2 and R2YE.

This stock solution keeps indefinitely. Just before use dissolve lysozyme in a sample of the solution at a concentration of 1 mg/ml and sterilise by filtration.

The CD-ROM includes:

1. Manual as Microsoft Word document and Adobe Acrobat document (X:/Protocol/)
2. Sequences of template plasmids as plain sequence files and as EMBL files (X:/Template plasmids/)
3. Sequences of the *S. coelicolor* real cosmids (X:/S_coelicolor/cosmid inserts)

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