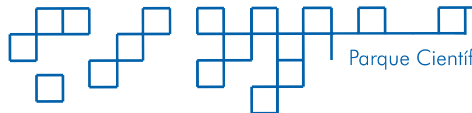
pUT *lacZ1* vector



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User's manual

Biomedal

NOTES

pUT *lac*Z1 vector

Delivery System pUTmini-Tn5 vector carrying a minitransposon containing a promoterless *trp*'-`*lacZ* to generate translational fusions.

For research use only

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1. Product Description

1.1. pUT *lacZ1* vector

pUT *lacZ1* is a 10.5 Kb plasmid, that is included in the collection of pUTmini-Tn*5* derivative vectors. This vector contains the minitransposon mini-Tn*5lacZ1* cloned in the pUT backbone. Mini-Tn*5lacZ1* has a promoterless trp'-'lacZ fusion with its Shine-Dalgarno sequence downstream of the Tn5 I end of the transposon (1). Since neither termini have transcriptional terminators in any of the possible frames, this vector is designed for generation of type I (transcriptional) *lacZ* fusions (2). The minitransposon also carries a gene upstream of the Tn5 O end that confers kanamycin resistance as selection marker.

Selection for resistance to	Kanamycin
Replication Origin	R6K
Transfer Origin	RP4oriT
Antibiotic Resistance	Ampicillin, Kanamycin
Host strain	*E. coli* λ*pir* strains
Copy number	Low

PRODUCT	QUANTITY	STORAGE	CAT. NO
pUT *lacZ1*	8 µg	4°C or -20°C	CV-3256

! NOTE: The plasmid is supplied dried.

10. Short Protocol.

A. Transformation of donor strain.

1. Transform S17-1 λ*pir* or DH5α λ*pir* cells with pUT*lacZ2*.
2. Select the transformants by plating onto LB /ampicillin (100 µg/ml)/ kanamycin (25 µg/ml) plates. Incubate at 37°C overnight.

B. Transfer of the DNA into the recipient strain (triparental mating).

Filter system

1. Mix the 10-50 µl of overnight cultures of the donor, recipient and helper strains (1:1:1 mixtures of cultures overnight), in 5 ml of 10 mM $MgSO_4$, vortex for a few seconds, transfer to a 5 ml disposable syringe, and filter through a Millipore membrane (or equivalent).
2. Remove carefully the drained membrane on the agar surface of an LB plate (cell side up), avoiding bubbles formation.
3. Incubate the plates at 30-37 °C for 8-18 hours.

Drop system

1. Mix the 10-50 µl of overnight cultures of the donor, recipient and helper strains (1:1:1 mixtures of cultures overnight), centrifuge the mixture and discard the supernatant. Resuspend the pellet in 20 µl of LB medium and place on a LB plate. Let to dry the drop on the plate.
2. Incubate the plates at 30-37 °C for 8-18 hours.

C. Selection of exconjungants.

1. To filter system: Resuspend the filter with the mating mix in 5 ml of 10 mM $MgSO_4$.
   To drop system: Recover the mating mix on the plate and resuspend in 5ml of 10 mM $MgSO_4$.
2. Plate 100-500 µl of this suspension on selective LB agar medium containing tetracycline.
3. Incubate the plates at the optimal growth temperature of the recipient strain until colonies become visible.
4. Confirm that the acquisition of the selected phenotype is due to an authentic transposition event and not to integration of the delivery plasmid in a recipient replicon or its illegitimate replication in the new host, by Southern blot, PCR analysis or growing the exconjugants onto medium containing β-lactam antibiotic.

9. Related Products.

PRODUCT		DESCRIPTION	Cat. No.
STRAINS	*E. coli* DH5α *λpir*	Propagation of pUT plasmids	BS-3233
	E. coli CC118 *λpir*	Propagation of pUT plasmids	BS-3235
	E. coli S17-1 *λpir*	Propagation of pUT plasmids	BS-3234
	E. coli DH5α (pRK2013)	Mating helper strain	BS-3236
	E. coli DH5α (pRK2073)	Mating helper strain	BS-3263
PRIMERS	pUToriR6K	Amplification by PCR (selection of positive clones) and sequencing of cloned fragments	PR-3280
VECTORS	pUT mini-Tn5 Cm	Mini-transposons derivates vectors (repeat insertion events)	CV-3223
	pUT mini-Tn5 Km	Mini-transposons derivatives vectors (repeat insertion events)	CV-3224
	pUT mini-Tn5 Sm/Sp	Mini-transposons derivatives vectors (repeat insertion events)	CV-3225
	pUT mini-Tn5 Tc	Mini-transposons derivatives vectors (repeat insertion events)	CV-3226
	pUT mini-Tn5 Tel	Mini-transposons derivatives vectors (repeat insertion events)	CV-3227
	pUT/*lacZ1*	Mini-transposons derivatives vectors with a reporter gene	CV-3256
	pUT/*lacZ2*	Mini-transposons derivatives vectors with a reporter gene	CV-3257
	pUT/*phoA*	Mini-transposons derivatives vectors with a reporter gene	CV-3258
	pUT/*luxAB*	Mini-transposons derivatives vectors with a reporter gene	CV-3259
	pUC18*Not*	Auxiliary plasmid for cloning	CV-3282
	pUC18*NotSfi*	Auxiliary plasmid for cloning	CV-3248
ANTIBIOTICS	Apicillin	Test culture	RS-3217
	Chloramphenicol	Test culture	RS-3218
	Kanamycin	Test culture	RS-3219
	Streptomycin	Test culture	RS-3221
	Tetracycline	Test culture	RS-3220
REAGENTS and SOLUTIONS	Potassium Tellurite	Selection of transformants	RS-3222
	TSS	Competent cells preparation	RS-3215/ RS-3216

1.2. pUT mini-Tn5 vectors features.

All pUTmini-Tn5 plasmids carry a common backbone (pUT backbone) and a variable mobile element (mini-Tn5). The mobile element (mini-Tn5 elements) consists of an *Sfi* I cassette containing a selection marker and a single *Not* I site outside of the cassette that can be used for cloning foreign DNA fragments. Flanking these two features are the 19 base pair I and O termini of Tn5 transposon. In this vectors any heterologous DNA segment can be cloned within the boundaries of a mini-Tn5 element and finally inserted into the chromosome of Gram negative bacteria target (3).

The backbone pUT includes the R6K origin of replication sequence, the RP4oriT origin of transfer sequence, the *bla* gene sequence (ampicillin resistance) and the *tnp** gene sequence.

The *tnp** is a mutant *tnp* gene of $IS50_R$ (Tn5 transposon), and encodes for the transposase needed for transposition of the mini-Tn5 elements. *tnp** carries a single mismatch that changed the GCG codon specifying Ala-168 of the *tnp* gene to the Ala codon GCC. This change eliminate the *Not* I site without changing the structure of the *tnp* product (4).The *tnp** gene is presented in cis but external to the mobile element. Due to the loss of the *tnp** gene after insertion, minitransposons are stably inherited and do not cause DNA rearrangements or others forms of genetic instability.

Plasmids having the R6K origin of replication require the R6K-specified replication protein π and can be maintained only in host strains producing this protein (*λpir* strain). It also carry the origin of transfer oriT of plasmid RP4, which results in efficient conjugal transfer to recipient strain from donor strains expressing RP4-conjugative functions (5). Delivery plasmids are thus mantained stably in *λpir* lysogens or in *E. coli* strains with the *pir* gene recombined in their chromosome, and can be mobilised into target strain cells through RP4 transfer functions. Delivery of the donor plasmids into selected host bacteria is accomplished through mating with the target strain.

2. General usages. Applications of pUT/*lac*Z1.

Mini-Tn5 vectors are used in the analysis, construction and manipulation of complex phenotypes in a wide range of Gram negative bacteria. The main virtue of these cloning vectors is their ease for insertion of one or more segments of heterologous DNA in the chromosome of the strain of interest, by means of a mating between a donor strain and a recipient strain. As cloning vectors, are useful to stably maintain a recombinant phenotype in the recipient strain.

Mini-transposons derivative vectors provide a straightforward tool to clone and insert foreign genes stably into the chromosomes of a variety of Gram negative bacteria such *E.coli*, *Klebsiella*, *Salmonella*, *Brucella*, *Proteus*, *Vibrio*, *Bortedella*, *Actinobacillus*, *Rhizobium*, *Acinetobacter*, *Rhodobacter*, *Agrobacterium*, *Alcaligenes* and several *Pseudomonads* (1). This vector also simplifies the generation of insertion mutants (insertion mutagenesis), *in vivo* fusions with reporter genes (promoter probing) and analysis of transcriptional terminators.

One important feature of mini-Tn5 elements is that, as a result of the loss of the transposase-cognate inhibitor along the pUT system after transposition (transposase function is not maintained in target cells), a single recipient strain can be used for repeated insertion events with differentially marked minitransposons. Multiple insertions in the same strain are therefore only limited by the availability of distinct selection markers.

pUT *lac*Z1 is included into mini-Tn5 derivatives vectors that have been adapted for promoter probing, by means of generation of type I gene fusions (transcriptional) with target genes. These vectors do not have transcriptional terminators within the I and O terminal sequences of Tn5, and the insertion of the minitransposon in the appropriate orientation downstream of chromosomal promoters may activae the expression of a promoterless gene placed within the mobile unit (1). pUT *lac*Z1 is a vector designed to generate random gene fusions, with *lacZ* as reporter gene.LacZ is the most convenient reporter available, but may be troublesome. The differential permeability to X-Gal among different strains may cause misleading estimations of promoter activity. In most cases, the fluorescent substrate of β-galactosidase, methylumbelliferyl-β-D-galactopiranoside (MUG), is preferable to X-Gal for measure promoter activity.

4. ANTIBIOTICS AND ADDITIVES

Ampicillin 100 mg/ml stock solution
Dissolve 1 g ampicillin in 9 ml of deionised water.
Add deionised water to a 10 ml volume final.
Filter sterilize the solution with a 0.22 µ filter.
Store the stock solution at –20°C.

Biomedal Cat. No. RS-3217

Kanamycin 25 mg/ml stock solution
Dissolve 0.25 g of kanamycin in 9 ml of deionized water.
Bring up to a 10 ml final volume with water deionized.
Filter sterilize the solution with a 0.22 µ filter.
Store the stock solution at 4°C.

Biomedal Cat. No. RS-3219

8. References.

1. De Lorenzo, V. Timmis K.N. 1994. Analysis and Construction of Stables Phenotypes in Gram-Negative Bacteria with Tn5- and Tn10-Derived Minitransposons. *Methods in Enzimology*, 235, p. 386-405.
2. De Lorenzo V., Herrero M., Jakubzik U., Timmis K.N. 1990. Mini-Tn5 transposon Derivatives for Insertion Mutagenesis, Promoter probing and Chromosomal Insertion of Cloned DNA in Gram-Negative Eubacteria. *Journal of Bacteriology*, 172, p. 6568-6572.
3. De Lorenzo V., Timmis K.N. 1994. Analysis and Construction of Stables Phenotypes in Gram-Negative Bacteria with Tn5- and Tn10-Derived Minitransposons. *Methods in Enzimology*, 235, p. 386-405.
4. Herrero M., De Lorenzo V., Timmis K.N. 1990. Transposon vectors Containing Non-Antibiotic Resistance Selection Markers for Cloning and Stable Chromosomal Insertion of Foreign Genes in Gram-Negative Bacteria. *Journal Bacteriology*, 172, p.6557-6567.
5. De Lorenzo V., Herrero M., Sánchez J., Timmis K.N. 1998. Mini-transposons in microbial ecology and environmental biotechnology. *FEMS Microbiology Ecology*, 27, p. 211-224.
6. Boyd D., Weiss D.S., Chen J.C., Beckwith J. 2000. Towards single-Copy gene expression systems making gene cloning physiologically relevant: Lamda Inch, a simple Escherichia coli plasmid-chromosome shuttle system. *Journal Bacteriology*, 182, p. 842-847.

7. Reagent and Receipes.

1. MEDIA

LB (per liter) (liquid)

· 10 g tryptone
· 5 g yeast extract
· 10 g NaCl

Dissolve in 950 ml of deionised water.
Adjust pH to 7.0 with 5 N NaOH.
Add deionised water to 1 l final volume.
Autoclave and let cool to below 55°C.

LB agar (plates)
Exactly like liquid LB but adding 15 g agar after adjust the pH.

2. MATING SOLUTION

Magnesium phosphate ($MgSO_4$ $7H_2O$) 1M solution
Dissolve 24.6 g of $MgSO_47H_2O$ in 100 ml of deionised water.
Autoclave sterilize.

3. β-GALACTOSIDASE ASSAY REAGENTS

Z-Buffer

· 16.1 g $Na_2HPO_47H_2O$
· 5.5 g $NaH_2PO_4H_2O$
· 0.75 g KCl
· 0.246 g $MgSO_47H_2O$

Dissolve in 950 ml of deionised water.
Adjust pH to 7.0.
Add deionised water to 1 l final volume.
Do not autoclave.
Store at –20°C in 50 ml aliquots.
Add 135 μl of β-mercaptoethanol/50 ml Z-buffer, before use it.

ONPG
Dissolve 0.8 g ONPG in 200 ml of Z-buffer.
Store at –20°C in 10 ml aliquots.

3. Advantages of mini-Tn5 Vectors vs other chromosome integration system.

- Ease for insertion of one or more segments of the heterologous DNA in the chromosome of the strain of interest (any heterologous DNA segments can be inserted into the chromosome of target cells after a few simple genetic manipulations).

- Conditional replication to allow selection for integration into the chromosome ("suicide plasmids"). The mini-Tn5 would be able to transpose from the plasmid to the chromosome but when succesive cell divisions occur, the plasmid vector would be lost to the population.

- Insert foreign DNA stably into the chromosomes.

- Wide range of inserted fragments size. pUTmini-Tn5 plasmids allow the cloning of variable size fragments. Heterologous DNA of 12 Kb cloned in mini-Tn5 derivatives are transposed at frequencies in the range of those observed with insert-lacking transposon (1). In other system the range of sizes is more restricted. For example, lambda Inch vectors system allow to insert fragments a total of about 7 Kb (6).

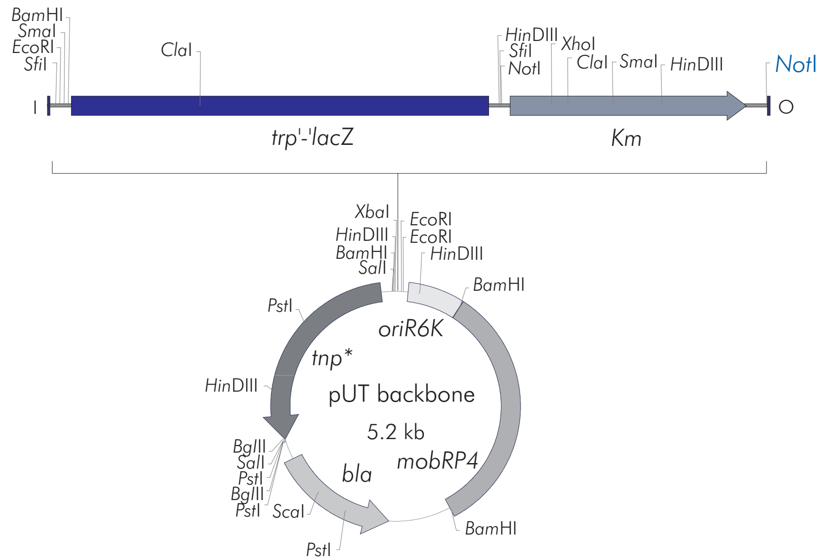
- Inserted DNA segments remain stably inherited and produce no burden to the carrier strain. Posibility of multiple insertions in the same strains (only limited by the available selection markers), as a result of the loss of the transposase function, which is not maintained in target cells.

- Easy modification of the cloning vector, because of the modular nature of the constructions.

- Use of cleavage sites of the rare cutters *Not* I and *Sfi* I (for cloning convenience the vector has an array of unique cloning sites).

- The pUTmini-Tn5 plasmids are transferable to a variety of bacteria.

4. pUT *lac*Z1 map.



5. Protocol.

5.1. Transformation of donor strain

To insert mini-Tn*5* *lac*Z1 into the chromosome of a recipient strain, first of all you must transform the donor strain with pUT*lac*Z1 vector. The protocol should be as follows.

1. Transform S17-1 λ*pir* or DH5α λ*pir* cells with pUT*lacZ1*.
2. Select the transformants by plating onto LB /ampicillin (100 µg/ml)/ kanamycin (25 µg/ml) plates. Incubate at 37°C overnight.

! NOTE:, You can use the TSS solution to perform the transformation (Biomedal, Cat. No. RS-3215, RS-3216).

6. Troubleshooting

PROBLEM	WHY?	SOLUTIONS
Loss of pUT plasmids.	Plasmid derivatives cannot be maintained into delivery λ*pir* strains	Using of alternative donor strains.
Low or zero yield of exconjugants.	Selection conditions are inappropriate.	It is advisable to perform assay with the target cells before the matting/plating is carried out, because the minimal inhibitory concentration of the toxic compound used as marker can vary considerably among genera and strains.
	Zero or low frequency of RP4-mediated transfer.	Properties of target cells such as restriction system, surface exclusion phenomena may lead to low frequency or zero transfer. If the target strain is unable to act as a recipient for RP4, the pUT plasmid cannot be introduced at high frecuency. Also, some changes in the mating protocol can be introduced. For example, avoid the pregrowth of the recipient strains on antibiotic medium prior to mating, since traces of the antibiotic present in the suspension or accumulated by the recipient cells can kill donor cells.
All exconjugants are resistant to ampicillin or others β-lactam antibiotics	The exconjugant phenotype is not due to an authentic transposition events	Shorter times. Lower temperatures (30°C). To *E. coli* recipient strains use LB medium with 0.1 M citrate to avoid the recipient strain infection by λ*pir* phage.

5.4. β-galactosidase assay.

pUT/lacZ2 carries *lacZ* gene as reporter. You can monitor expression levels by β-gal activity.

1. Take 1 ml pellet from induced and uninduced expression culturers, with a known OD_{600}. Resuspend the pellet in 1 ml of Z buffer.

2. Mix 100 µl of sample, 900 µl of Z buffer, 2 drops of chloroform and 1 drop of 0.1% SDS.

3. Vortex the tubes for 10 seconds.

4. Equilibrate samples to room temperature for 15 minutes to evaporate the chloroform.

5. Add 200 µl of ONPG (in Z buffer) to each tube.

6. After a yellow color develops, stop the reaction by adding 500 µl of 1 M of Na_2CO_3 1 M solution to each tube, following the same timed addition order. Record, in minutes, the incubation time.

7. Read the absorbance of the samples at OD_{420}.

8. Determine the Miller units of expression by means of the formula:

$$\text{Miller units} = \frac{(OD_{420}) \times (1000)}{(t_{min}) \times (OD_{600}) \times (\text{dilution})} \times 1000$$

5.2. Transfer of the DNA into the recipient strain

To transfer the minitransposon into the recipient strain, the genes that code for mobilisation and chromosomal transfer (*tra* and *mob* genes), are necessary. These genes can be present in a host strain or can be provided by way of a helper plasmid. When employing host strains such as *E. coli* SM10 λ*pir* or *E. coli* S17-1 λ*pir*, the transformed transposon DNA is transfered via biparental mating. The transfer can also be achieved by triparental mating mediated by a helper plasmid.

In the triparental mating the conjugatable suicide minitransposon donor is introduced into the recipient strain by a helper plasmid, which self mobilises from its own host into the donor strain and provides the genes necessary for the conjugal transfer of the miniTn5 from the donor host strain to the recipient, provided the vector plasmids contain the specific recognition site for mobilisation.

Since some λ*pir* RP4 strains like *E. coli* SM10 λ*pir* or *E. coli* S17-1 λ*pir*, cannot stably maintain the pUT plasmids, we recommend other strains to propagate this vectors without problems, such as DH5α λ*pir*. To mobilize the delivery plasmid directly from the donor strain into target cells in this case, a triparental mating with a helper strain such as *E. coli* DH5α (pRK2013) or *E. coli* DH5α (pRK2073) is necessary.

! NOTE: To propagate pUTmini-Tn5 in λ*pir* strains, you can use the TSS solution to perform the transformation (Biomedal, Cat. No. RS-3215, RS-3216).

Tripartental mating

1. Grow overnight cultures in LB of each of the three strains: donor strain, recipient strain and helper strain:

- Inoculate a single colony of donor strain DH5α λ*pir* carrying the desired pUT vector or derivative plasmid in LB medium containing 100 µg/ml ampicillin and 25 µg/ml of kanamicyn (the adequate concentration to ensure maintenance of the delivery plasmid). Incubate with shaking at 37°C overnight.
- Grow the recipient strain: Inoculate a single colony under the same conditions (at different temperature, if necessary), but preferably without selection (see Troubleshooting).
- Inoculate a single colony of *E. coli* DH5α (pRK2073) in LB medium, preferably without selection.

2. Mix the 10-50 µl (~10^8 cells) of overnight cultures of each of the three strains (1:1:1 mixtures of cultures overnight), in 5 ml of 10mM $MgSO_4$, vortex for a few seconds, transfer to a 5 ml disponsable syringe, and filter through a Millipore membrane, 13-25 mm diameter type HA (0.45 µm) or equivalent, placed on a reusable filter case.

! NOTE:
- pRK2073 plasmid confers streptomycin/spectinomycin resistance.
- To remove antibiotics from donor strain culture, an additional washing step is necessary.

IMPORTANT ! : Alternatively, mix the 10-50 µl of overnight cultures of helper strain, donor strain and recipient strain, centrifuge the mix and discard the supernatant. Resuspend the pellet in 20 µl of LB medium or 10 mM $MgSO_4$ using vortex and place it on LB plate, without dispersing it. Let it dry. Go to step 4.

! NOTE:
- When you use the filter system, if too many donor, recipient or helper cells are used and the membrane clogs, it is better to refilter a more diluted mix.
- If you need only few insertions, 10 µl drops of cultures of donor, recipient and helper strains can be mixed and spotted on an LB plate, which is then dried and incubated for several hours before the mating mixture is streaked out on selective medium (go to step 4).

3. After the filtering, remove carefully the drained membrane on the agar surface of an LB plate (cell side up). You can help yourself with sterile tweezers, preferably with curved tips.

! NOTE:
- You can use alternative culture medium instead of LB, to favour recipient strain viability.
- Air bubbles should be avoided between the filter and agar surface.

4. Incubate the plates at 30-37 °C for 8-18 hours.

! NOTE:
- Several alternatives to mating protocol are possible, according to individuals needs. Strains ratios (donor/recipient/helper), temperatures, time of mating and culture medium can be changed to suit specific requeriments of the recipients.

Biparental mating

If you use *E. coli* SM10 λ*pir* or *E. coli* SM17-1 λ*pir* as donor strains, to set up the biparental mating, you must follow the same protocol as when using triparental mating, but without helper strain. You must mix equal volumes (10-50 µl) of overnight cultures of the donor and recipient strain, in 5 ml of 10 mM $MgSO_4$, and proceed as described above.

5.3. Selection of exconjugants

The selection of clones with the minitransposon inserted in the chromosome is a critical step. The procedure is as follows.

1. If you use filter system:
   Resuspend the filter with the mating mix in 5 ml of 10 mM $MgSO_4$.
   If you use drop system:
   Recover the matting mix on the plate using a sterile inoculating loop and resuspend in 5 ml of 10 mM $MgSO_4$.

! NOTE: This suspension can be kept at 4°C for several weeks.

2. Plate 100-500 µl of this suspension on selective LB agar medium with kanamycin. The kanamycin concentration must be adequate to ensure maintenance of the delivery plasmid, but you must determine the minimal inhibitory concentration of antibiotic.

! NOTE: When the marker carried by the minitransposon is an antibiotic resistance determinant, optimal concentrations of antibiotics to the selection can vary among strains. The minimal inhibitory concentration (MIC) must be determined in each case. The concentrations in the table below can be used as a guideline.

ANTIBIOTIC	ORIENTATIVE MIC
Chloramphenicol	5-50 µg/ml
Kanamycin	25-75 µg/ml
Streptomycin	50-100 µg/ml
Spectinomycin	50-100 µg/ml
Tetracycline	2.5-15 µg/ml

3. Incubate the plates at the optimal growth temperature of the recipient strain until colonies become visible.

4. Confirm that the acquisition of the selected phenotype is due to an authentic transposition event and not to integration of the delivery plasmid in a recipient replicon or its illegitimate replication in the new host. The insertion can be confirmed by Southern blot or PCR analysis. Another procedure consist of growing the exconjugants on medium containing β-lactam antibiotic, because authentic transposition results in the loss of the portion of the delivery plasmid containing *bla* gene and the exconjugants will be sensitive to ampicillin and other β-lactams (see Troubleshooting).