PANC-1 mini-Tn5 package

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User's manual

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NOTES

PANC-1 mini-Tn5 package

A colletion od Delivery System pUT mini-Tn5 plasmids carrying antibiotic selection markers and donor and helper strains.

For research use only

Contents

1. Product Description.

Mini-Tn*5* system is the generic name of a system derived of mini-Tn*5* transposon vectors, that can be used in the analysis, construction and manipulation of complex phenotypes in a wide range of Gram negative bacteria. This system includes cloning vectors (pUTmini-Tn*5*) and strains for mating experiments (donor and helper strains). The main feature of the cloning vectors of this system 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 the donor strain and the recipient strain. This system can be used for insertional mutagenesis, promoter probing and analysis of transcriptional terminators.

KIT COMPONENTS (CAT. NO. KT- 3260)

PRODUCT	QUANTITY	STORAGE	CAT. NO.
pUTmini-Tn5 Cm	8 µg	4ºC or -20ºC.	CV-3223
pUTmini-Tn5 Km	8 µg	4ºC or -20ºC.	CV-3224
pUTmini-Tn5 Sm/Sp	8 µg	4ºC or -20ºC.	CV-3225
pUTmini-Tn5 Tc	8 µg	4ºC or -20ºC.	CV-3226
Sequences CD	1 CD	-----	

! NOTE:

- All components of the Kit are shipped at room temperature. Upon arrival, store the kit components according to the directions in the table above.
- The plasmid is supplied dryed.
- CD-Rom supplied with the kit contains vectors sequences.

NOTES

11. Short protocol.

A. Cloning and transformation of donor strain.

1. Ligate the DNA fragment with pUTmini-Tn*5* vector, prior digestion with *Not* I.
2. Transform the ligation reactions into host strain λ*pir* cells and select the transformants by plating onto selective LB plates. Incubate overnight at 37°C.

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 10mM $MgSO_4$, vortex for a few seconds, transfer to a 5 ml disponsable 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), centrifugate 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 exconjugants.

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 5 ml 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.

2. Vectors and strains.

2.1. Vectors.

2.1.1. pUT mini-Tn5 vectors

All pUTmini-Tn*5* vectors included in this kit contain a mini-Tn*5* element, carrying an antibiotic resistance cassettes, flanked by I and O termini (terminal repeat sequences) of Tn*5*. The cassettes conferring resistance to tetracycline, streptomycin-spectinomycin and chloramphenicol are derived from Ω interposon and the cassette conferring resistance to kanamycin contains the kanamycin resistance interposon originally isolated from Tn*5* (1). These four mini-Tn*5* carry 156-nt inverted repeats consisting of the T4 phage gene 32 transcriptional terminators (strong transcriptional terminator) flanking the antibiotic resistance gene as well as synthetic translational stop codons in all three reading frames (1,2).

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

pUTmini-Tn5 Cm.

pUTmini-Tn*5* Cm is a 8686 basepair plasmid, that contains the minitransposon mini-Tn*5* Cm cloned in the pUT backbone. Mini-Tn*5* Cm carries as selection marker the *cat II* gene, that confers chloramphenicol resistance.

pUTmini-Tn5 Km.

pUTmini-Tn*5* Km is a 7575 basepair plasmid, that contains the minitransposon mini-Tn*5* Km cloned in the pUT backbone. Mini-Tn*5* Km carries as selection marker a kanamycin resistance gene. Mini-Tn*5* Km has one more I end at the left extreme of the kanamycin resistance gene, that itself originates from Tn*5*.

pUTmini-Tn5 Sm/Sp.

pUTmini-Tn5 Sm/Sp is a 7386 basepair plasmid, that contains the minitransposon mini-Tn5 Sm/Sp cloned in the pUT backbone. Mini-Tn5 Sm/Sp carries as selection marker the *add* gene, that confers streptomycin and spectinomycin resistante.

pUTmini-Tn5 Tc.

pUTmini-Tn5 Tc is a 7477 basepair plasmid, that contains the minitransposon mini-Tn5 Tc cloned in the pUT backbone. Mini-Tn5 Tc carries as selection marker the *tet* gene, that confers tetracycline resistance.

3. About mini-Tn5 System

Mini-transposons derivatives 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*, *Proteus*, *Vibrio*, *Bortedella*, *Brucella*, *Actinobacillus*, *Rhizobium*, *Acinetobacter*, *Rhodobacter*, *Agrobacterium*, *Alcaligenes* and several *Pseudomonads* (3). These vectors also simplify the generation of insertion mutants (insertion mutagenesis) and in vivo fusions with reporter genes (promoter probing). Mini-transposons cloning vectors have the advantages of natural transposons, but lack their disadvantages.

All pUTmini-Tn5 plasmids carry a backbone common (pUT backbone) and a mobile element variable (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 basepair 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. The modular nature of the mini-transposons facilitates the construction of mobile elements *à la carte*.

Hybrid transposons bearing the DNA segment of interest can be easily assembled with the help of the specialised cloning vector as pUC18*Not* or pUC18*Sfi* vector.

10. Related products.

PRODUCT		DESCRIPTION	Cat. No.
STRAINS	*E. coli* DH5α λ*pir*	Propagation of pUT plasmids	BS-3233
	E. coli CC118 λ*pir*	Propagation of pUT plasmids	BS-3235
	E. coli S17-1 λ*pir*	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/*lacZ*1	Mini-transposons derivatives vectors with a reporter gene	CV-3256
	pUT/*lacZ*2	Mini-transposons derivatives vectors with a reporter gene	CV-3257
	pUT/*phoA*	Mini-transposons derivatives vectors with a reporter gene	CV-3258
	pUT/*lux*AB	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

! NOTE:

• Alternatively, you can use exponential growing cells, obtained in the same day of the transformation directly from fresh colonies.

! IMPORTANT!: While the culture is growing place on ice or at 4°C LB medium that you will use in step 6.

4. When the culture reaches the desired OD_{600}, divide the cultures in microcentrifuge tubes (1 ml/tube) and place on ice for 5 minutes.
5. Centrifuge at 12000 r.p.m. for 30 seconds at room temperature.
6. Discard the medium and resuspend gently the pellets in 75 µl of cold LB medium. Place on ice for 5 minutes.
7. Add 75 µl of 2xTSS solution and mix gently. Replace on ice for 5 minutes.

IMPORTANT!: Invert the tube of TSS solution several times before use.

Now the cells are ready to perform the transformation!

TRANFORMATION PROCEDURE (STANDARD PROTOCOL)

8. Add 1-50 ng of DNA (supercoiled DNA) to the competent cells obtained in step 7. We recommend adding 5-10 µl of DNA; the DNA added do not must be more than 15 µl of volume (10% of the competent cell volume). Mix gently and place the transformation reaction on ice for 30 minutes.
9. Heat shock: Incubate the tubes by immersing in a 42°C water bath for exactly 45 seconds. Immediately replaces the tubes on ice.
10. Add 1 ml of LB medium to each tube. Incubate at 37°C for the time required to the resistance antibiotic expression (typical 60 min).
11. For each transformation reactions, we recommend diluting the cells 1:10 and plating out 100 µl of the 1:10 dilutions, 100 µl of the transformation (undiluted cells), and the rest of the cells concentrated for centrifugation, onto LB plates containing the appropriate selection antibiotics. Allow the plates to completely absorb any excess media.
12. Incubate the plates overnight at 37°C (12-14 hours).

The delivery system employed for all mini-Tn*5* transposons is the pUT plasmid. 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 $IS50_R$ (Tn*5* transposon), that lacks *Not* I sites and which encodes for the transposase needed for transposition of the mini-Tn*5* 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 favour DNA rearrangements or others forms of genetic inestability.

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. It also carry the origin of transfer oriT of plasmid RP4, which results in its efficient conjugal transfer to recipient strain from donor strains expressing RP4-conjugative functions (5). Delivery plasmids are thus mantained stably in λ*pir* lysogens or in *E. coli* strains with the *pir* gene recombined in their chromosome, and can be mobilised into target strains through RP4 transfer functions. However, once transferred it is unable to replicate in recipients that lack the π protein. The pUT plasmids are "suicide vectors" since the oriR6K is functional only in the presence of the *pir* gene and they cannot replicate in the recipient cells which have not the π protein. Delivery of the donor plasmids into selected host bacteria is accomplished through mating with the target strain.

One important feature of mini-Tn*5* 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.

ADVANTAGES of mini-Tn5 System vs OTHER DNA INTEGRATION INTO THE CHROMOSOME SYSTEMS

· 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 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 to clone from small fragments until large 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 (3). 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 (it's convenient that the vector has an array of unique cloning sites).

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

9. References.

1. **De Lorenzo V., Herrero M., Jakubzik U., Timmis K.** Mini-Tn5 Transposon Derivatives for Insertion Mutagenesis, Promoter Probing, and Chromosomal Insertion of Cloned DNA in Gram-Negative Eubacteria. Journal Bacteriology, 172, p. 6568-6572.

2. **Prentki P., Binda A., Epstein A.** Plasmid vectors for selecting IS*1*-promoted deletions in cloned DNA: sequence analysis of the omega interposon. Gene, 103, p 17-23.

3. **De Lorenzo V., Timmis K.N.** 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.** Transposon vectors Containing Non-Antibiotic Resistanse 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.** 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.** 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.

Chloramphenicol 25 mg/ml stock solution

Dissolve 125 mg chloramphenicol in 5 ml of ethanol.
Store the stock solution at 4°C.

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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 sterilizes the solution with a 0.22 µ filter.
Store the stock solution at –20°C.

Biomedal Cat. No. RS-3219

Streptomycin 10 mg/ml stock solution

Dissolve 0.1 g of streptomycin in 9 ml of deionised water.
Bring up to a 10 ml final volume with water deionised.
Filter sterilizes the solution with a 0.22 µ filter.
Store the stock solution at –20°C.

Biomedal Cat. No. RS-3221

Tetracycline 25 mg/ml stock solution

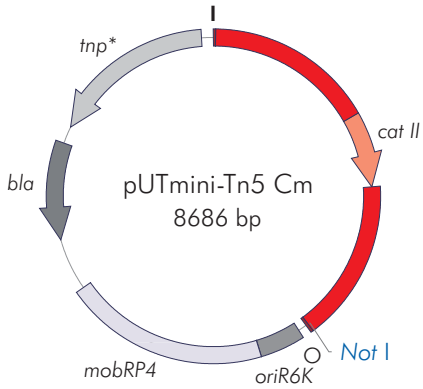
Dissolve 200 mg tetracycline in 5 ml water deionised/ 5 ml ethanol mix.
Store the stock solution at –20 °C.

Biomedal Cat. No. RS-3220

4. Vectors Maps.

pUTmini-Tn5 Cm

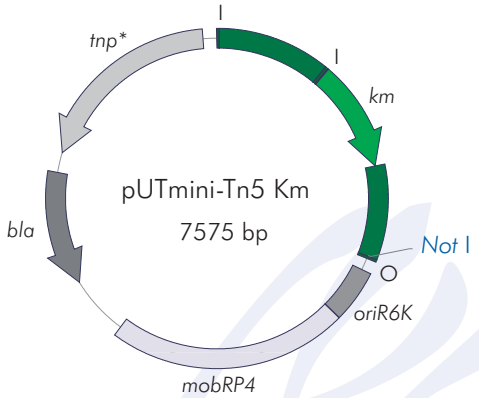
mini-Tn5 Cm: 1-3467
I termini: 1-19
O termini: 3449-3467
T4 gene transcription terminators (inverted repeats): 64-188/ 3259-3383
cat II coding region (Cm resistance): 1452-2090
R6K origin of replication: 3560-3940
*mob*RP4 origin of tranfer: 3944-5670
bla coding region (ampicillin resistance): 6130-6987
*tnp** coding region (transposase): 7139-8581



pUTmini-Tn5 Km

SEQUENCE REFERENCE POINT

miniTn5 Km: 1-2356
I termini: 1-19 / 831-849 (reverse complement)
O termini: 2338-2356
km coding region (kanamycin resistance): 857-1648
T4 gene 32 transcription terminators: 64-188/ 2148-2272 (inverted repeats)
R6K origin of replication: 2448-2829
*mob*RP4 origin of tranfer: 2833-4559
bla coding region (ampicillin resistance): 5018-5876
*tnp** coding region (transposase): 6031-7458



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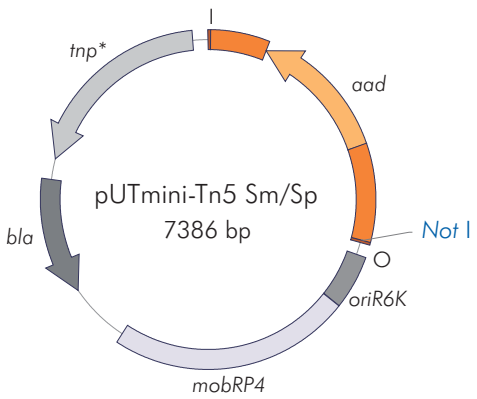
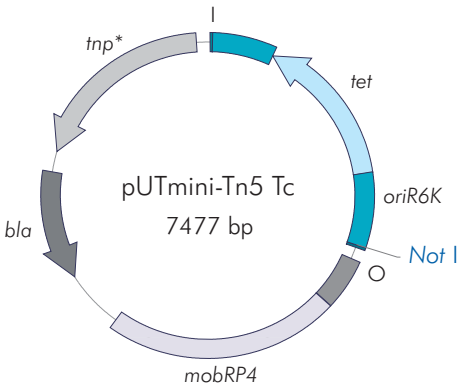
pUTmini-Tn5 Sm/Sp



SEQUENCE REFERENCE POINT

mini-Tn5 Sm/Sp: 1-2167
I termini: 1-19
O termini: 2149-2167
T4 gene transcription terminators (inverted repeats): 64-188/ 1959-2083
aad coding region (Sp/Sm resistance): 443-1450
R6K origin of replication: 2260-2640
*mob*RP4 origin of tranfer: 2644-4370
bla coding region (ampicilin resistance): 4830-5687
*tnp** coding region (transposase): 5842-7269

pUTmini-Tn*5* Tc



SEQUENCE REFERENCE POINT

mini-Tn5 Tc: 1-2258
I termini: 1-19
O termini: 2239-2258
T4 gene transcription terminators (inverted repeats): 64-188/ 2050-2174
tet coding region (Tc resistance): 489-1676
R6K origin of replication: 2351-2731
*mob*RP4 origin of tranfer: 2735-4461
bla coding region (ampicillin resistance): 4921-5768
*tnp** coding region (transposase): 5932-7372

8. Reagents and recipes.

A. Media and solutions.

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 adjusting the pH.

2. MATING SOLUTION.

Magnesium phosphate ($MgSO_4$ $7H_2O$) 1M solution

Dissolve 24.6 g of $MgSO_4 7H_2O$ in 100 ml of deionised water.
Autoclave sterilizes.

3. 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 sterilizes the solution with a 0.22 µ filter.
Store the stock solution at –20°C.

Biomedal Cat. No. RS-3217

7. Troubleshouting.

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 make and 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. Restriction analysis.

5.1. pUTmini-Tn5 Cm Restriction analysis.

RESTRICTION ENZYMES THAT DO NOT CUT pUTmini-Tn5 Cm

Aat II	*BbvC* I	*EcoICR* I	*Nru* I	*Sbf* I
Acc65 I	*BfrB* I	*EcoN* I	*Nsi* I	*Spe* I
Afl II	*Blp* I	*Fse* I	*Pme* I	*Srf* I
Ale I	*Bpl* I	*FspA* I	*PshA* I	*Swa* I
Apa I	*BsiW* I	*Kpn* I	*Psp0M* I	*Zra* I
Asc I	*BstB* I	*Mlu* I	*Psr* I	
AsiS I	*Bsu36* I	*Nco* I	*Rsr* II	
Bae I	*Cla* I	*Nde* I	*Sac* I	

RESTRICTION ENZYMES THAT CUT ONCE pUTmini-Tn5 Cm

Age I	*BssH* II	Dra III	*Pac* I	*Sph* I
Bcl I	*BssS* I	Drd I	*Pml* I	*Stu* I
Bmt I	*BstAP* I	EcoR V	*Pvu* I	*Xba* I
Bpu10 I	*BstE* II	Mfe I	*Sca* I	*Xcm* I
BsaX I	*BstX* I	Msc I	*SexA* I	*Xmn* I
BsrG I	*BstZ17* I	Nhe I	*SnaB* I	

5.2. pUTmini-Tn5 Km Restriction analysis

RESTRICTION ENZYMES THAT DO NOT CUT pUTmini-Tn5 Km

Aat II	*Bbs* I	*Bsi* WI	*Cla* I	*Fsp* AI	*Pac* I	*Psp* 0MI	*Spe* I
Afl II	*Bbv* CI	*Bsp* EI	*Ecl* 136II	*Mlu* I	*Pfl* MI	*Psr* I	*Srf* I
Apa I	*Bfr* BI	*Bsr* GI	*Eco* NI	*Nde* I	*Pme* I	*Sac* I	*Stu* I
Asc I	*Blp* I	*Bst* EII	*Eco* RV	*Nsi* I	*Ppu* 10I	*Sbf* I	*Swa* I
Bae I	*Bsa* XI	*Bst* XI	*Fse* I	*Oli* I	*Psh* AI	*Sgf* I	

RESTRICTION ENZYMES THAT CUT ONCE pUTmini-Tn5 Km

Acc 65I	*Bsg* I	*Dra* III	*Not* I	*Rsr* II	*Xba* I
Age I	*Bsm* I	*Kpn* I	*Nru* I	*Sca* I	*Xho* I
Alo I	*Bst* BI	*Mfe* I	*Pml* I	*Sex* AI	*Xmn* I
Bpl I	*Bst* Z17I	*Nco* I	*Psi* I	*Sna* BI	
Bpu 10I	*Bsu* 36I	*Nhe* I	*Pvu* I	*Sph* I	

5.3. pUTmini-Tn5 Sm/Sp Restriction analysis.

RESTRICTION ENZYMES THAT DO NOT CUT pUTmini-Tn5 Sm/Sp

Aat II	*Bae* I	*Bsi* WI	*Bsu* 36I	*Fse* I	*Nde* I	*Pfl* MI	*Psr* I	*Spe* I
Acc 65I	*Bbv* CI	*Bsp* EI	*Cla* I	*Fsp* AI	*Nru* I	*Pme* I	*Rsr* II	*Srf* I
Afl II	*Bfr* BI	*Bsr* GI	*Ecl* 136II	*Kpn* I	*Nsi* I	*Ppu* 10I	*Sac* I	*Stu* I
Apa I	*Bpl* I	*Bst* BI	*Eco* NI	*Mlu* I	*Oli* I	*Psh* AI	*Sbf* I	*Swa* I
Asc I	*Bsa* XI	*Bst* XI	*Eco* RV	*Nco* I	*Pac* I	*Psp* 0MI	*Sgf* I	

RESTRICTION ENZYMES THAT CUT ONCE pUTmini-Tn5 Sm/Sp

Age I	*Bsg* I	*Bst* Z17I	*Nhe* I	*Sca* I	*Xba* I
Alo I	*Bsm* I	*Drd* I	*Not* I	*Sex* AI	*Xho* I
Bbs I	*Bss* SI	*Hpa* I	*Pml* I	*Sna* BI	
Blp I	*Bst* API	*Mfe* I	*Psi* I	*Sph* I	
Bpu 10I	*Bst* EII	*Msc* I	*Pvu* II	*Tth* 111I	

5.4. pUTmini-Tn5 Tc Restriction analysis.

RESTRICTION ENZYMES THAT DO NOT CUT pUTmini-Tn5 Tc

Aat II	*Bae* I	*Bsa* XI	*Bst* EII	*Fse* I	*Nsi* I	*Psp* 0MI	*Sgf* I
Acc 65I	*Bbv* CI	*Bsi* WI	*Bst* XI	*Kpn* I	*Oli* I	*Psr* I	*Spe* I
Afl II	*Bfr* BI	*Bsp* EI	*Bsu* 36I	*Mlu* I	*Pac* I	*Rsr* II	*Srf* I
Apa I	*Blp* I	*Bsr* GI	*Cla* I	*Nco* I	*Pme* I	*Sac* I	*Stu* I
Asc I	*Bpl* I	*Bst* BI	*Ecl* 136II	*Nde* I	*Ppu* 10I	*Sbf* I	*Swa* I

2. Plate 100-500 µl of this suspension on selective LB agar medium with tetracycline. The tetracycline 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. Must be determined the minimal inhibitory concentration (MIC) in each case. Can be orientatives the concentrations which appears in the next table.

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. Other procedure consists to grow the exconjugants onto 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 Troubleshouting)

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:

- 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* like donor strains, to set up the biparental mating, you must follow the same protocol that when use triparental mating, but without helper strain. You must mix equal volumes (5-10 µl) of overnight cultures of the donor and recipient strain, in 5 ml of 10 mM $MgSO_4$, and proceed as described above.

6.3. 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 $MgSO_4$.

2. 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.

RESTRICTION ENZYMES THAT CUT ONCE pUTmini-Tn5 Tc

Age I	*Bsg* I	*Drd* I	*Mfe* I	*Psh* AI	*Sex* AI	*Xcm* I
Alo I	*Bss* HII	*Eco* NI	*Msc* I	*Psi* I	*Sna* BI	*Xho* I
Bbs I	*Bss* SI	*Eco* RV	*Not* I	*Pvu* I	*Sph* I	*Xmn* I
Bcl I	*Bst* Z17I	*Fsp* AI	*Nru* I	*Pvu* II	*Tth* 111I	
Bpu 10I	*Dra* III	*Hpa* I	*Pml* I	*Sca* I	*Xba* I	

6. Protocol.

6.1. Cloning and transformation of donor strain

To insert your DNA fragment in the chromosome of the recipient strain, first of all you must clone it into the pUTmini-Tn5 vector. We recommend that you clone your fragment in pUC18*Not* auxiliary plasmid, prior the cloning in pUT plasmid. The protocol should be as follows.

1. Excise the DNA fragment from pUC18*Not* plasmid by means of *Not* I digest.
2. Digest the pUTmini-Tn5 vector (transfer vector) with *Not* I. We recommend to treat the digested plasmid with alkaline phosphatase, to prevent re-ligation.
3. Ligate your DNA fragment and pUTmini-Tn5 backbone.
4. Transform the ligation reactions into λ*pir* cells. Select the transformants by plating onto LB /ampicillin (100 µg/ml)/ antibiotic that the pUTmini-Tn5 confers resistance (the minimal inhibitory concentration in each case) plates. Incubate at 37°C overnight.

! NOTE:

- You can use the TSS solution (BIOMEDAL, Cat.No. RS-3215, RS-3216) to perform the transformation.

6.2. Transfer of the DNA into the recipient strain.

To transfer the DNA cloned in pUTmini-Tn*5* plasmid 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 it is used the chromosomally integrated conjugal transfer functions of a specific host strain, such us *E. coli* SM10 λ*pir* or *E. coli* S17-1 λ*pir* (BIOMEDAL, Cat.No. BS-3234), into which the transposon vector DNA is transformed, the transfer is accomplished by means of 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 neccesary for the conjugal transfer of the miniTn*5* 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*. In this case, to mobilize the delivery plasmid directly from the donor strain into target cells, a triparental mating with a helper strain such as *E. coli* DH5α (pRK2013) (BIOMEDAL, Cat.No. BS-3236) or *E. coli* DH5α (pRK2073) (BIOMEDAL, Cat.No. BS-3263) is necessary.

! NOTE:

- To propagate pUTmini-Tn*5* in l*pir* strains, you can use the TSS solution to perform the transformation.

Triparental Mating

1. Grow overnight cultures in LB of each of the three strains: donor strain, recipient strain and helper strain:
   a. Inoculate a single colony of donor strain DH5α λ*pir* carrying the desired pUT derivative plasmid in LB medium containing 100 μg/ml ampicillin and the adequate concentration of the antibiotic of selection to ensure maintenance of the delivery plasmid. Incubate with shaking at 37°C overnight.
   b. Grow the recipient strain: Inoculate a single colony under the same conditions (at different temperature, if necessary), but preferably without selection (see Troubleshooting).
   c. Inoculate a single colony of *E. coli* DH5α (pRK2013) or *E. coli* DH5α (pRK2073) (if you use pUTmini-Tn*5* Km) 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 10 mM $MgSO_4$, vortex for a few seconds, transfer to a 5 ml disposable syringe, and filter throught a Millipore membrane, 13-25 mm diameter type HA (0.45 μm) or equivalent, placed on a reusable filter case.

! NOTE:

- To remove antibiotics from donor strain culture, an additional washing step of the culture is necessary.

! IMPORTANT!: Alternatively, mix the 10-50 μl of overnight cultures of helper strain, donor strain and recipient strain, centrifugate 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 dispense it. Let it dry. Go to step 4.

! NOTE:

- You can use alternative culture medium instead of LB, to favour recipient strain viability.
- 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).