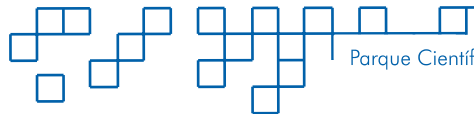




pCNB4 vector



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User's manual
Biomedal

pCNB4vector

Delivery System pUTmini-Tn5 vector carrying mini-Tn5 *nahR/Psal* minitransposon, that contains *Psal* promoter along with its regulator gene *nahR*.

For research use only

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pCNB4 vector

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1. Product Description.

1.1. pCNB4 vector.

pCNB4 (pUTmini-Tn5 *nahR/Psal*) is a 8.5 Kb plasmid, that is included in the collection of pUTmini-Tn5 derivative vectors. This vector contains the minitransposon mini-Tn5 *nahR/Psal* cloned in the pUT backbone. This minitransposon contains outward- facing *Psal* promoter from the catabolic plasmid NAH (naphthalene degradation) of *Pseudomonas putida*, along with its cognate regulatory *nahR* sequence (1). The *nahR* gene, encodes for a positive regulator of the *Psal* promoter, which is activated by salicylate. pCNB4 plasmid contains the salicylate-responsive promoter (*Psal*) of catabolic plasmid NAH7(1). Mini-Tn5 *nahR/Psal* carries, an Ω -Km^R element (selection marker) that confers kanamycin resistance, between I termini and *nahR* structural sequence. The *Psal* promoter is oriented toward a unique Not I site within the minitransposon, to clone heterologous genes downstream from catabolic promoter.

C. Selection of exconjugants.

1. To filter system: Resuspend the filter with the mating mix in 5 ml of 10 mM MgSO₄.

To drop system: Recover the mating mix on the plate and resuspend in 5ml of 10 mM MgSO₄.

2. Plate 100-500 μ l of this suspension on selective LB agar medium containing kanamycin.

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.

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| | |
|-----------------------------|--------------------------------------|
| Selection for resistance to | Kanamycin |
| Replication Origin | R6K |
| Transfer Origin | RP4oriT |
| Antibiotic Resistance | Ampicillin, Kanamycin |
| Host strain | <i>E. coli</i> λ pir strains |
| Copy number | low |

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| PRODUCT | QUANTITY | STORAGE | CAT. NO |
|---------|-----------|--------------|---------|
| pCNB4 | 8 μ g | 4°C or -20°C | CV-3254 |

! NOTE: The plasmid is supplied dried.

10. Short protocol.

A. Cloning and transformation of donor strain.

1. Ligate the DNA fragment with pUTmini-Tn5 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 μ l of overnight cultures of the donor, recipient and helper strains (1:1:1 mixtures of cultures overnight), in 5 ml of 10 mM MgSO₄, 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 μ l 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 μ l 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.

1.2. pUTmini-Tn5 vectors features.

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 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 (2).

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 (3). 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 (λ pir strain). 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 (4). Delivery plasmids are thus maintained stably in λ pir 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 pCNB1.

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 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*, *Brucella*, *Proteus*, *Vibrio*, *Bordetella*, *Actinobacillus*, *Rhizobium*, *Acinetobacter*, *Rhodobacter*, *Agrobacterium*, *Alcaligenes* and several *Pseudomonas* (5). This vectors 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.

pCNB4 is a plasmid with a property that can be exploited in different applications, including generation of conditional phenotypes dependent on the presence of specific effectors and generation of non polar mutations (there are not transcriptional terminators in the whole minitransposon).

9. Related products.

| PRODUCT | | DESCRIPTION | Cat. No. |
|------------------------|-------------------------------|--|---------------------|
| STRAINS | <i>E. coli</i> DH5α λpir | Propagation of pUT plasmids | BS-3233 |
| | <i>E. coli</i> CC118 λpir | Propagation of pUT plasmids | BS-3235 |
| | <i>E. coli</i> S17-1 λpir | Propagation of pUT plasmids | BS-3234 |
| | <i>E. coli</i> DH5α (pRK2013) | Mating helper strain | BS-3236 |
| | <i>E. coli</i> 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 derivatives 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 |
| | pUC18Not | Auxiliary plasmid for cloning | CV-3282 |
| | pUC18NotSfi | 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 |
| | Salicylic acid | Expression inducer | RS-3285 |

8. References.

1. **De Lorenzo V., Fernández S., Herrero M., Jakubzik U., Timmis K.N.** Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. *Gene*, 130, p. 41-46.
2. **De Lorenzo V., Timmis K.N.** Analysis and Construction of Stable Phenotypes in Gram Negative Bacteria with Tn5- and Tn10-Derived Minitransposons. *Methods in Enzymology*, 235, p. 386-405.
3. **Herrero M., De Lorenzo V., Timmis K.N.** 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.
4. **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.
5. **De Lorenzo V., Timmis K.N.** Analysis and Construction of Stable Phenotypes in Gram Negative Bacteria with Tn5- and Tn10-Derived Minitransposons. *Methods in Enzymology*, 235, p. 386-405.
6. **Boyd D., Weiss D.S., Chen J.C., Beckwith J.** Towards single-Copy gene expression systems making gene cloning physiologically relevant: Lambda Inch, a simple *Escherichia coli* plasmid-chromosome shuttle system. *Journal Bacteriology*, 182, p. 842-847.

pCNB5 is a plasmid with a property that can be exploited in a number of applications, including generation of non polar mutations and analysis of transcriptional terminators. In the absence of insertions at *Not I* site, random integration of the minitransposon into the chromosome of a target bacterium can generate phenotypes whose expression is dependent on the addition of IPTG to medium (1). Three types of applications of the mini-Tn5 *lacI^q/Ptrc* contained into pCNB5 are:

- 1) Generation of IPTG-dependent conditional phenotypes and non polar mutations. Since there are no transcriptional terminators within the *O* end of mini-Tn5, transcription from *Ptrc* goes through the *Not I* site and continues beyond the site of insertion of the transposon into downstream chromosomal genes, thereby generating in certain cases IPTG-conditional phenotypes (2).
- 2) Stably regulated expression of recombinant genes. Cloning of heterologous genes into the *Not I* site produces hybrid transposons in which the expression of the phenotype encoded by the cloned genes can be triggered by addition of *lac* inducers.
- 3) Genetic analysis of transcriptional termination activity, by means of insertions of terminators or operator sequences between *Ptrc* and a downstream reporter gene (2).

It should be noted that the *Ptrc* promoter has a significant activity in the absence of induction. Optimal concentrations of IPTG and other conditions such as temperature, time of induction and composition medium have to be determined.

3. Advantages of mini-Tn5 Vectors 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 successive 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 big 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. Possibility 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 (is convenient that the vector has an array of unique cloning sites).
- The pUTmini-Tn5 plasmids are transferable to a variety of bacteria.

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.
Filters 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 deionised water.
Bring up to a 10 ml final volume with water deionised.
Filters sterilize the solution with a 0.22 μ filter.
Store the stock solution at 4°C .

Biomedal Cat. No. RS-3219

Salicylate 1 M (sodium salt)

Dissolve 3.202 g of salicylic acid (sodium salt) in 18 ml of deionised water.
Bring up to a 20 ml final volume with deionised water.
Filters sterilize the solution with a 0.22 μ filter.
Store the stock solution at 4°C .

Biomedal Cat. No. RS-3285

7. Reagents and recipes.

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)

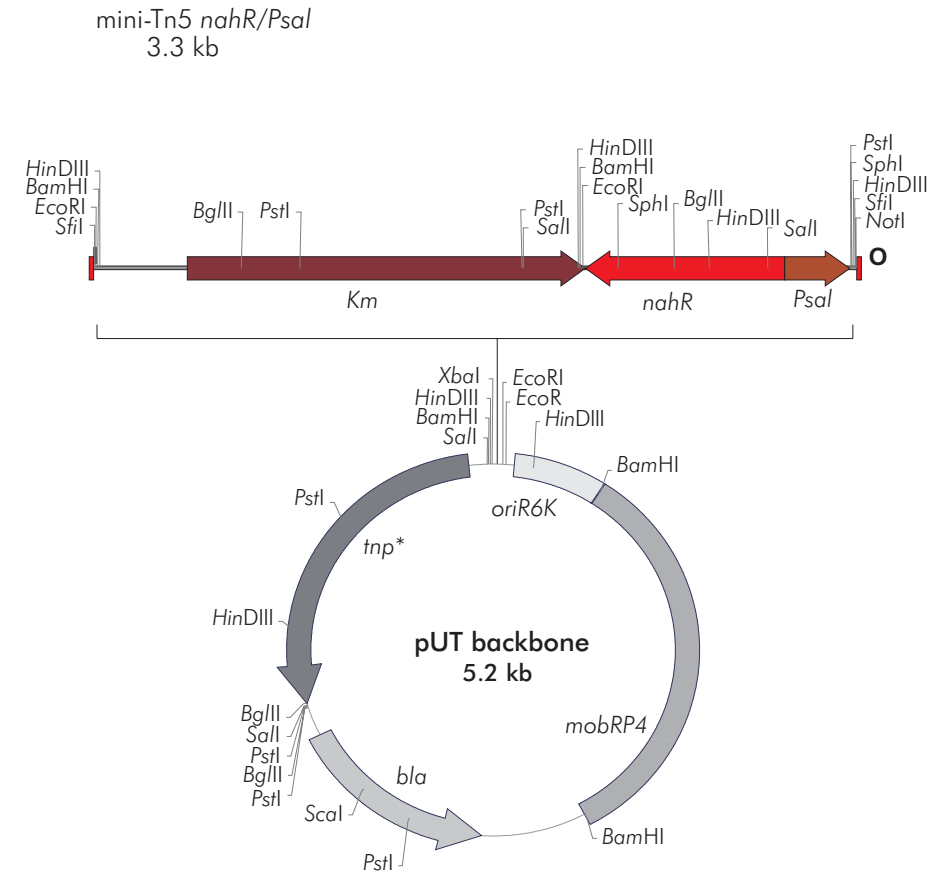
Exactly like liquid LB but adding 15 g agar after adjusting the pH.

2. Mating solution

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

Dissolve 24.6 g of $MgSO_4 \cdot 7H_2O$ in 100 ml of deionised water.
Autoclave sterilize.

4. pCNB5 map.



5. Protocol.

5.1. Cloning and transformation of donor strain (heterologous DNA under control of *P_{trc}*).

To insert a DNA fragment into the chromosome of a recipient strain, first of all you must clone it into the pCNB5. We recommend cloning your fragment into pUC18*Not* auxiliary plasmid (Biomedal, Cat. No. CV-3282), prior the cloning in pCNB5 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 pCNB5 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 pCNB5 backbone.
4. Transform the ligation reactions into S17-1*λpir* or DH5α *λpir* cells. Select the transformants by plating onto LB /ampicillin (100 µg/ml)/ kanamycin (25 µg/ml) plates µg/ml. Incubate at 37°C overnight.

! NOTE:

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

5.2. Transfer of the DNA into the recipient strain.

To transfer the DNA cloned in pCNB4 or 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 it is used the chromosomally integrated conjugal transfer functions of a specific host strain, such as *E. coli* SM10 *λpir* or *E. coli* S17-1 *λpir*, 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.

6. Troubleshooting.

| PROBLEM | WHY? | SOLUTIONS |
|---|--|---|
| Loss of pUT plasmids. | Plasmid derivatives cannot be maintained into delivery <i>λpir</i> strains | Using of alternative donor strains. |
| Low or zero yield of exconjugants. | Selection conditions are inappropriate. Zero or low frequency of RP4-mediated transfer. | It is advisable to make and assay with the target cells before the mating/plating is carried out, because the minimal inhibitory concentration of the toxic compound used as marker can vary considerably among genera and strains. 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 frequency. 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 <i>E. coli</i> recipient strains use LB medium with 0.1 M citrate to avoid the recipient strain infection by <i>λpir</i> phage. |

| 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 Troubleshooting).

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5.4. Induction of expression.

The transcription from *Psal* promoter is activated when the host bacteria encounters certain aromatic compounds, such as salicylates. To induce the expression from *Psal* promoter in the exconjugants, we recommend growing the cells with aeration until $OD_{600}=0.4$ and after adding the expression inducer, to a final concentration of 2 mM salicylate (1:500 dilution).

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 *λpir* RP4 strains like *E. coli* SM10 *λpir* or *E. coli* S17-1 *λpir*, cannot stably maintain the pUT plasmids, we recommend other strains to propagate this vectors without problems, such as DH5 α *λpir*. 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* DH5 α (pRK2013) or *E. coli* DH5 α (pRK2073) is necessary.

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

Triparental mating

- 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 DH5 α *λpir* carrying the desired pUT derivative plasmid in LB medium containing 100 µg/ml ampicillin and 25 µg/ml of kanamycin (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* DH5 α (pRK2073) in LB medium, preferably without selection.
- 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 MgSO₄, vortex for a few seconds, transfer to a 5 ml disposable 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 μ l of overnight cultures of helper strain, donor strain and recipient strain, centrifuge the mix and discard the supernatant. Resuspend the pellet in 20 μ l of LB medium or 10 mM MgSO₄ using vortex and place it on LB plate, without dispense 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 μ l 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).

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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 requirements 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 that when use triparental mating, but without helper strain. You must mix equal volumes (10-50 μ l) of overnight cultures of the donor and recipient strain, in 5 ml of 10 mM MgSO₄, 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 MgSO₄.

If you use drop system:

Recover the mating mix on the plate using a sterile inoculating loop and resuspend in 5 ml of 10 mM MgSO₄.

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! NOTE:

- This suspension can be kept at 4°C for several weeks.

2. Plate 100-500 μ l 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. Must be determined the minimal inhibitory concentration (MIC) in each case. Can be orientatives the concentrations which appear in the next table.