pCNB1 vector

User's manual

Biomedal

Avda. Américo Vespucio, 5 E • Planta 1ª - Módulo 12
Parque Científico y Tecnológico Cartuja 93 • 41092 SEVILLA España/Spain
Tel. (+34) 954.081.276 • Tel/Fax: (+34) 954.081.279
Web: www.biomedal.com • Email: info@biomedal.com

NOTES

pCNB1vector

Delivery System pUTmini-Tn5 vector carrying mini-Tn5 *xylS/Pm* minitransposon, that contains outward-facing catabolic promoter *Pm* along with its regulator gene *xylS*.

For research use only

Contents

1. Product Description.

1.1. pCNB1 *vector*.

pCNB1 (pUTmini-Tn*5 xylS/Pm)* is a 9 Kb plasmid, that is included in the collection of pUTmini-Tn*5* derivative vectors. This vector contains the minitransposon mini-Tn*5 xylS/Pm* cloned in the pUT backbone. This minitransposon contains an outward- facing *Pm* promoter from the catabolic plasmid TOL of *Pseudomonas putida,* along with its regulatory *xylS* sequence (1). Pm is the promoter of the meta-cleavage operon of the TOL plasmid. Mini-Tn*5 xylS/Pm* carries as selection marker a gene that confers kanamycin resistance, between I termini and *xylS* gene sequence, which is transcribed from the Km^R gene promoter. *Pm* promoter activity can be triggered by addition of certain aromatic compounds, such as alkyl- and halobenzoates. Transcription is initiated from *Pm* when its cognate regulatory protein XylS is activated by substrates of the meta-cleavage pathway (benzoate, toluate) and halo- and alkyl-substitutes structural analogues (1). The *Pm* promoter is oriented toward a unique *Not* I site within the minitransposon, to clone heterologous genes downstream from catabolic promoter.

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
pCNB1	8 µg	4°C or -20°C	CV-3253

! **NOTE:** The plasmid is supplied dried.

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 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.

10. 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 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.

1.2. pUTmini-Tn5 vectors features.

All pUTmini-Tn*5* plasmids carry a backbone common (pUT backbone) and a mobile element variable (mini-Tn*5*). The mobile element (mini-Tn*5* elements) consists of a *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 Tn*5* transposon. In these vectors any heterologous DNA segment can be cloned within the boundaries of a mini-Tn*5* 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 $IS50_R$ (Tn*5* transposon), and 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 eliminates 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 inestability.

Plasmids having the R6K origin of replication require the R6K-specified replication protein p 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 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 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*, *Bortedella*, *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.

pCNB1 is a plasmid with a property that can be exploited in different applications, including generation of conditional phenotypes dependent on the presence of specifics effectors and generation of non polar mutations (there are not transcriptional terminators in the whole minitransposon).

9. 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/*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	Ampicillin	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
	Sodium benzoate	Expressium inducer	RS-3284

8. References.

1. De Lorenzo V., Fern{andez S., Herrero M., Jakubzik U.,Timmis K.N. Enginnering 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 Stables Phenotypes in Gram-Negative Bacteria with Tn5- and Tn10-Derived Minitransposons. Methods in Enzimology, 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 Stables Phenotypes in Gram Negative Bacteria with Tn5- and Tn10-Derived Minitransposons. Methods in Enzimology, 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: Lamda Inch, a simple Escherichia coli plasmid-chromosome shuttle system. Journal Bacteriology, 182, p. 842-847.

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 alkyl- and halobenzoates to medium (1). Mini-Tn*5 xylS/Pm* enable the engineering of strains expressing heterologous genes that are regulated by aromatic compounds, including several xenobiotic compounds (environmental pollulants).Three types of applications of the mini-Tn*5 xylS/Pm* contained into pCNB1 are:

1) Generation of benzoate/alkylbenzoates/halobenzoates-dependent conditional phenotypes and non polar mutations. Since there are no transcriptional terminators within the O end of mini-Tn*5*, transcription from *Pm* goes through the *Not* I site and continuous beyond the site of insertion of the transposon into downstream chromosomal genes, thereby generating in certain cases benzoate/alkylbenzoates/halobenzoates - conditional phenotypes. The construction of stable strain exhibiting halo/alkyl aromatic regulated conditional phenotypes in the absence of antibiotic selection can be required for bioremediation applications.

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 *Pm* inducers.

3) Genetic analysis of transcriptional termination activity, by means of insertions of terminators or operator sequences between *Pm* and a downstream reporter gene.

It should be noted that the regulatory module *xylS* benzoate responsive/*Pm* target, exhibit a tighter regulation than other systems as *lacI*^{q}*Ptrc* system (100 fold induction compared to 10-20 fold induction ratio), and this system uses cheap inducers.

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-Tn*5* 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-Tn*5* plasmids allow the cloning of variable size fragments. Heterologous DNA of 12 Kb cloned in mini-Tn*5* derivatives are transposed at frequencies in the range of those observed with insert-lacking transposon (2). 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-Tn*5* 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

Benzoate 1 M (sodium salt)

Dissolve 14.41 g of benzoate (sodium salt) in 90 ml of deionised water.
Bring up to a 100 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-3284

7. Reagents and recipes.

1. Media.

LB (per liter) (liquid)
· 10 g tryptone
· 5 g yeast extract
· 10 g NaCl

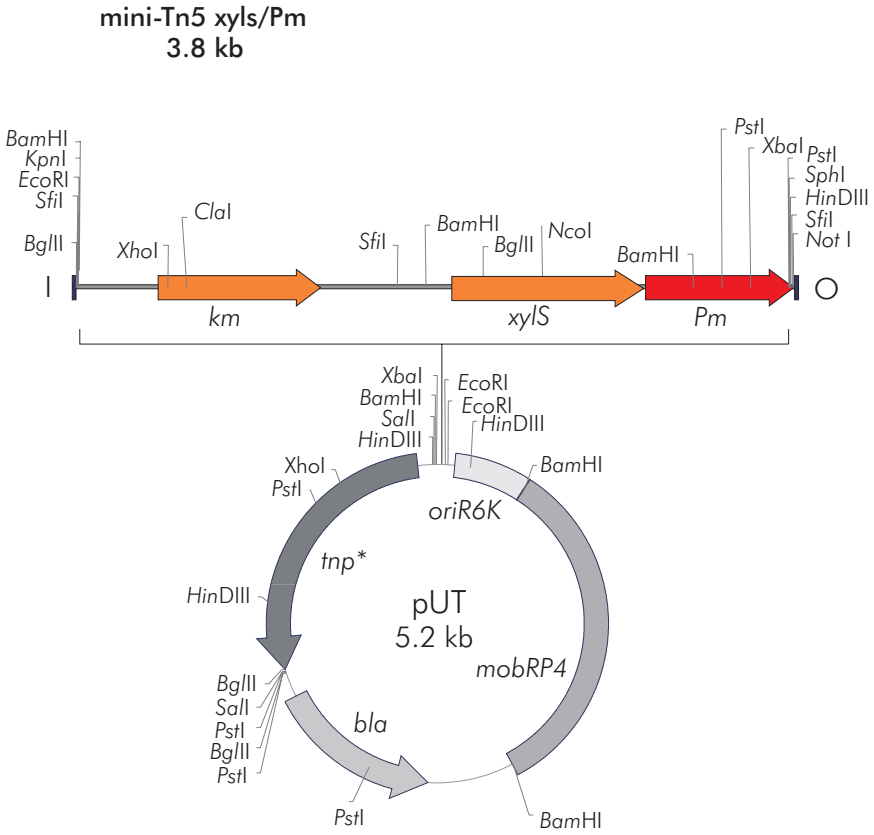
Dissolve in 950 ml of deionised water.
Adjust pH to 7.0 with 5 N NaOH.
Add deionised water to 1 l final volume.
Autoclave and let cool to below 55°C.

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

2. Mating solution.

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

4. pCNB1 map



5. Protocol.

5.1. Cloning and transformation of donor strain (heterologous DNA under control of Pm promoter)

To insert a DNA fragment into the chromosome of a recipient strain, first of all you must clone it into the pCNB1. We recommend that you clone your fragment into pUC18*Not* auxiliary plasmid (Biomedal, Cat. No. CV-3282), prior the cloning into pCNB1 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 pCNB1 vector (transfer vector) with *Not* I. We recommend treating the digested plasmid with alkaline phosphatase, to prevent re-ligation.
3. Ligate your DNA fragment and pCNB1 backbone.
4. Transform the ligation reactions into S17-1 λ*pir* or DH5α λ*pir* 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 transformatio (Biomedal, Cat. No. RS-3215, RS-3216).

5.2. Transfer of the DNA into the recipient strain

To transfer the DNA cloned in pCNB1 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 λ*pir* or *E. coli* S17-1 λ*pir*, 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 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.

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 b-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).

5.4. Induction of expression

The transcription from *Pm* promoter is actived when the host bacteria encounters certain aromatic compounds, such as salicylates. To induce the expression from *Pm* 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 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*. 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-Tn*5* in λ*pir* strains, you can use the TSS solution to perform the transformation. (Biomedal, Cat. No. RS-3215, RS-3216).

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 25 μg/ml of kanamycin (the adequate concentration 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 (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 10 mM $MgSO_4$, 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 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 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 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 that when use 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. Must be determined the minimal inhibitory concentration (MIC) in each case. Can be orientatives the concentrations which appear in the next table.