File S1

Protocol

In vitro Transposon Mutagenesis for Introduction of Internal Epitope Tags

REQUIRED REAGENTS

Purified TnsA, TnsB, and TnsC^{A255V} enzymes [3, 4]

TnsA stock = 150ng/μl in Storage Buffer A

TnsB stock = 200ng/μl in Storage Buffer B

TnsC ^{A255V} stock = 500ng/μl in Storage buffer C

Tn7 donor vector: pRZ101 (Tn7-FLAG donor vector). Dilute to 25ng/μl in 10mM Tris pH8.0

Entry vector of target gene: *DCW1* entry vector. Dilute to 50ng/μl in 10mM Tris pH8.0

Invitrogen MegaX DH10B T1R electrocompetent *E. coli* cells

300mM Magnesium Acetate (MgOAc)

phenol:chloroform:IAA (25:24:1) (Amresco 0883-100ml)

Chloroform

3M Sodium Acetate (NaOAc)

100% ethanol (EtOH)

70% EtOH

FseI (New England Biolabs R0588S)

PmeI (New England Biolabs R0560S)

ApaLI (New England Biolabs R0507S)

T4 DNA ligase and buffer (New England Biolabs M0202S)

Gateway LR clonase II (Life Techonologies 11791-100)

Qiagen Hi-Speed MidiPrep kit (Qiagen 12643)

MegaX DH10B T1R Electrocomptent Cells (Life Technologies C6400-03)

LB media

Oxoid Isosensitest media (Iso) (agar: OXCM0471B, liquid broth: OXCM0473B)

Carbenicillin (100mg/ml = 1000x stock)

Kanamycin (30mg/ml = 1000x stock)

Trimethoprim (5mg/ml in DMSO = 500x stock)

ddH_2O = doubly distilled (MilliQ) H_2O

BUFFERS

Use sterile-filtered MilliQ water to make all buffers. If possible, make all buffers in plastic containers; residual detergent on glassware may inhibit the transposition reaction.

Standard buffers

20mM ATP in 125mM Tris pH 7.5 (store at -20°C for at most 1 month)
20mM DTT in 125mM Tris pH 7.5 (store at -20°C for at most 1 month)
100mM ATP in 250mM HEPES (pH8.0) – for making TnsC storage buffer
1M DTT in 150mM HEPES (pH8.0) – for making TnsA and TnsC storage buffers
1M DTT in 1M Tris pH 7.5 – for making TnsB storage buffer
10mM Tris - for elution of mutagenized plasmid pools from midiprep kit
50% glycerol

Tns storage buffers

Store at -20°C for at most 6 months. We do not recommend refreezing and rethawing of the storage buffers, so store them in small aliquots and discard after use.

Storage buffer A
25mM HEPES pH8.0
150mM NaCl
1mM EDTA
1mM DTT (in HEPES)
10% glycerol

Storage buffer B
25mM TrisHCl pH8.0
500mM NaCl
1mM EDTA
1mM DTT (in Tris)
25% glycerol

Storage buffer C
25mM HEPES pH8.0
1M NaCl
0.1mM EDTA
2.5mM DTT (in HEPES)
1mM ATP
10mM $MgCl_2$
10% glycerol

***In vitro* transposition protocol**

This protocol delineates the steps to perform a small (20μl) "- enzyme" negative control reaction and a large (80μl) "+ enzyme" experimental mutagenesis reaction in parallel. The enzymes and buffers are mixed together in a master mix (5.5-reaction sized, to account for pipetting error) and later split into appropriate reaction sizes.

1) Make reaction mix. Combine:
   - 17.6 μl target DNA (880 ng) (*DCW1* entry vector)
   - 8.8 μl Tn7 donor DNA (220 ng) (pRZ101)
   - 11 μl 20mM ATP
   - 11 μl 20mM DTT
   - 56.1 μl ddH_2O

2) Aliquot reaction mix into two PCR tubes. Dispense 76μl into the "+ enzyme" reaction tube, and 19μl into the "- enzyme" reaction tube.

3) Make the enzyme mixture. Combine:
   - 7.49 μl TnsA
   - 3.31 μl Storage buffer A
   - 2.00 μl TnsB
   - 6μl Storage buffer B
   - 8μl $TnsC^{A255V}$
   - 8μl Storage buffer C
   - 5.2μl 50% glycerol

   Mix by flicking tube gently. Keep on ice while setting up transposition reaction.

   Note 1: The ratio of TnsA, TnsB, and $TnsC^{A255V}$ in this mixture was determined empirically. You may need to optimize the relative amount of each enzyme using your purified enzyme stocks.

   Note 2: You may refreeze the enzyme mixture at -80°C and rethaw twice, but will have decreased transposition efficiency with each thaw. We do not recommend refreezeing the stock solution of the individual Tns enzymes, so take care to store these in small aliquots so as to avoid wasting purified enzyme.

4) Make the buffer mixture for the "- enzyme" control. Combine:
   - 10.8 μl Storage buffer A
   - 8.0 μl Storage buffer B
   - 16.0 μl Storage buffer C
   - 5.2 μl 50% glycerol

5) Add 4μl enzyme mix to the "+ enzyme" tube. Flick tube to mix.
6) Add 1μl of the buffer mixture to the "- enzyme" tube. Flick tube to mix.
7) Incubate both tubes at 37°C for 10 minutes on a PCR heat block.
8) Add 300mM MgOAc to the tubes:
   For "+ enzyme" reactions, add 4.2μl 300mM MgOAc
   For "- enzyme" reaction, add 1.05μl 300mM MgOac
9) Incubate at 37°C for 1 hour on a PCR block
10) Incubate at 75°C for 5 minutes on a PCR block to heat-kill the enzymes.

Clean-up of transposition reactions

11) Transfer "+ enzyme" and "- enzyme" reactions to 1.5ml microfuge tubes. Bring the volume of each up to 100µl.
12) Add 100µl phenol:chloroform:IAA. Vortex to mix.
13) Spin 5 minutes, 4°C, 13500 rpm in a microfuge. Remove and discard organic (bottom) layer.
14) Add 100µl chloroform. Vortex to mix.
15) Spin 5 minutes, 4°C, 13500rpm in microfuge. Transfer aqueous (top) later to a new 1.5ml microfuge tube.
16) Add 10µl 3M NaOAc. Add 220µl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
17) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
18) Wash DNA pellet with 500µl ice-cold 70% EtOH.
19) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
20) Resuspend DNA pellets in desired volume of 10mM Tris pH 7.5.
    For "+ enzyme" reactions, use 8 µl Tris pH 7.5
    For "- enzyme" reaction, use 4 µl Tris pH 7.5
21) Optional: Cleaned transposition reactions may be stored at -20°C prior to transformation.

Transformation of transposition reactions

Transform cleaned transposition reaction DNA into Invitrogen MegaX DH10B T1R electrocompetent *E. coli* cells. One set of steps is described for the "+ enzyme" reactions, another for "- enzyme" reactions. They can be performed in parallel, but are separated here for clarity.

For "- enzyme" reactions:

22A) Combine 2μl DNA with 20μl MegaX cells in a chilled electroporation cuvette.
23A) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
24A) Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.
25A) Transfer 900μl cells to a 1.5ml microfuge tube.
26A) Recover at 37°C for 1 hour.
27A) Plate 9μl (1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan .
28A) Grow plates overnight at 37°C.

For "+ enzyme" reactions:

22B) Combine 4μl DNA with 40μl MegaX cells in one chilled electroporation cuvette.
23B) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
24B) Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.
25B) Transfer 900μl cells to a 500ml flask containing 150 mls Isosensistest media.
26B) Recover at 37°C for 1 hour
27B) Plate 150μl (0.1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan
28B) Add 150μl of Kan (30mg/ml stock) and 300μl Tmp (5mg/ml stock) to the flask.
29B) Grow plates and culture overnight at 37°C.

30) The following morning, count the colonies growing on each plate. Calculate the number of independent transformants in your "+ enzyme" pools.
31) Make a glycerol frozen stock of the "+ enzyme" overnight culture. Store at -80°C.
32) Pellet the remainder of the "+ enzyme" overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 33 directly.
33) Purify the DNA from the "+ enzyme" culture cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the mutagenized *DCW1**FLAG entry vector pool (ep).

Note: You can monitor the plasmid population content by restriction enzyme digestion of the DNA pool. We have observed about 50% of the plasmid pool will be unmutagenized target vector. We suspect that the MegaX may take up multiple plasmids during transformation and do not segregate them properly.

Gateway LR mobilization to create expression pools

Linearizing the mutagenized entry pool increases the efficiency of the Gateway LR reaction. Additionally, it minimizes the amount of Kan-marked plasmid that is present in the subsequent pools.

Linearize mutagenized DNA pool

1) Identify a restriction enzyme that cuts only in the backbone of the mutagenized *DCW1**FLAG pool. We chose ApaLI, which cuts in the origin of replication.
2) Digest 1μg of DNA from the *DCW1**FLAG mutagenized entry pool in a 20μl reaction. Include a negative control – use 100ng of DNA in a 20μl reaction.
3) Digest 37°C for 1 hour.
4) Run the entire negative control reaction, and 2μl of the ApaLI digestion, on a gel to verify the backbone is fully digested. If digestion was successful, proceed.

Clean the linearized DNA

5) Add 82μl ddH2O to the remaining ApaLI-digested DNA, to bring it to a final volume of 100μl.
6) Add 100μl phenol:chloroform:IAA. Vortex to mix.
7) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Remove and discard organic (bottom) layer.
8) Add 100 μl chloroform. Vortex to mix.
9) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Transfer aqueous (top) later to a new 1.5ml microfuge tube.
10) Add 10 μl 3M NaOAc. Add 220μl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
11) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
12) Wash DNA pellet with 500μl ice-cold 70% EtOH.
13) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
14) Resuspend DNA in 20μl 10mM Tris. DNA will be 45ng/μl.

Gateway LR Reaction – Move ORF to destination vector

1 reaction volume uses 150ng destination vector and 150ng (mutagenized, linearized) entry vector

15) Make a 2.5x reaction mixture DNA, which will be split into +LR and –LR reactions. Combine: 371.25ng (8.25μl) mutagenized, linearized entry vector
    371.25ng pRZ159 (*DCW1* destination vector)
    Bring to 20μl total volume with TE
16) For the "+LR" experimental reaction, combine 16μl of the DNA mixture from step 15 and 4μl Invitrogen LR Clonase II in a PCR tube. For the "-LR" control reaction, combine 4μl DNA mixture from step 15 and 1μl TE in a PCR tube. Incubate both reactions overnight at 25°C on the PCR block.
17) Stop the LR reaction by adding proteinase K to the reactions: Add 2μl proteinase K to the "+LR" experimental reaction; Add 0.5μl proteinase K to the "-LR" negative control.
18) Incubate at 37°C for 10 minutes on the PCR block.
19) Clean reactions: Transfer reactions to 1.5ml microfuge tubes. Perform phenol/chloroform extraction and EtOH precipitation as described before (steps 5-13 under "Clean linearized DNA")
20) Resuspend "+LR" experimental DNA in 6μl TE (final [DNA] = 50ng/ul).
21) Resuspend "-LR" negative control DNA in 4μl TE (final [DNA] = 18.75ng/ul).

Transform Gateway LR reactions

Transform cleaned LR reaction DNA into Invitrogen MegaX DH10B T1R electrocompetent *E. coli* cells. One set of steps is described for the "+ enzyme" reactions, another for "- enzyme" reactions. They can be performed in parallel, but are separated here for clarity.

Note that the Car and Tmp selections (intended to select only those expression plasmids with a DCW1 orf and a Tn insertion) are performed in sequential rounds of transformation. We found simultaneous Car and Tmp drug selection seemed to pressure cells into maintaining both an unmutagenized DCW1 expression vector and a mutagenized entry vector.

For "- enzyme" reactions:

22A) Combine 2μl DNA with 20μl MegaX cells in a chilled electroporation cuvette.
23A) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
24A) Add 1ml Recovery media (provided with MegaX cells) to the cuvette and pipet up and down to resuspend.
25A) Transfer 900μl cells to a 1.5ml microfuge tube.
26A) Recover at 37°C for 1 hour.
27A) Plate 9μl (1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan .
28A) Grow plates overnight at 37°C.

For "+ enzyme" reactions:

22B) Combine 2μl DNA with 20μl MegaX cells in one chilled electroporation cuvette.
23B) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
24B) Add 1ml Recovery media (provided with MegaX cells) to the cuvette and pipet up and down to resuspend.
25B) Transfer 900μl cells to a 1.5ml microfuge tube.
26B) Recover at 37°C for 1 hour
27B) Transfer contents of microfuge tube to a 500ml flask containing 150mls LB. Swirl to mix.
28B) Plate 150μl (0.1% of total cells) onto five types of selective media – LB+Car, LB+Kan, Iso + Tmp, and Iso+Tmp+Kan , Iso+Tmp+Car
29B) Add 150μl of Car (100mg/ml stock) to the flask.
30B) Grow plates and culture overnight at 37°C.

30) The following morning, count the colonies growing on each plate. Calculate the number of independent transformants in the "+ enzyme" pools.
31) Make a glycerol frozen stock of the "+ enzyme" overnight culture. Store at -80°C.
32) Pellet the remainder of the "+ enzyme" overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 33 directly.
33) Purify the DNA from the "+ enzyme" culture cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the expression pool, selected for Car^R ("xpC").

Retransform for 2nd round drug selection: Select expression pool for plasmids with Tn insertions

34) Combine 1μl "xpC" expression pool DNA (50ng/μl) with 20μl MegaX cells in one chilled cuvette.
35) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
36) Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.
37) Transfer 900μl cells to 150mls Isosensitest media (in a 500ml flask).
38) Recover at 37°C for 1 hour
39) Plate 150μl (0.1% of total cells) onto five types of selective media – LB+Car, LB+Kan, Iso + Tmp, and Iso+Tmp+Kan , Iso+Tmp+Car
40) Make a 1:1000 dilution of recovered cells (2μl cells into 2ml Isosensitest media). Plate 150μl of a 1:1000 dilution of cells onto Iso+Tmp, LB+Car, and Iso+Tmp+Car plates
41) Add 300μl of Tmp (5mg/ml stock) to the flask.
42) Grow plates and culture overnight at 37°C.
43) The following morning count the colonies growing on each plate. Calculate the number of independent transformants in your pools.
44) Make a glycerol frozen stock of the overnight culture. Store at -80°C.
45) Pellet the remainder of the overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 46 directly.
46) Purify the DNA from the pelleted cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the expression pool, now selected for Tmp^R ("xpT").

Restriction digestions to remove Tn7 DNA from plasmids

At this point, the pools contain expression plasmids with mutagenized target ORF. This is based on the ability to grown in MegaX cells – confirming the expression backbone instead of the entry or destination vectors, and the selection for both Car^R and Tmp^R - confirming the expression backbone (Car^R) and the presence of Tn7 (Tmp^R). At this time, the Tn7 ends and Tmp^R gene are no longer needed, and are removed from the plasmids using a series of restriction digests.

Remove Tn7L end with *Fse*I digest

1) Digest 500ng of xpT pool with *Fse*I; use 100ng of xpT pool for a "no enzyme" control.

No enzyme	***Fse*I**	
100 ng	500 ng	xpT pool DNA
1 μl	1 μl	10x NEB buffer #4
1 μl	1 μl	10x BSA
0 μl	0.5 μl	*Fse*I
To 10μl total	To 10μl total	dH2O

2) Incubate restriction digests and control reactions at 37°C for 1hr.
3) Heat inactivate *Fse*I by incubating reactions at 65°C for 20 minutes.
4) Run all of the "no enzyme" control and 2μl (100ng of DNA) from *Fse*I digest on an agarose gel to confirm restriction digest was successful. The uncut control should contain supercoiled plasmid, and the *Fse*I digest should have a high MW band of linearized plasmid DNA; the Tn7L fragment is 211bp long, though this fragment is sometimes not visible. If successful, continue with protocol.

Ligate to recircularize plasmid after *Fse*I digestion

5) Add 12μl of dH2O to remaining *Fse*I-digested xpT material to bring final volume to 20μl.
6) Set up ligation reaction, and a negative control, as shown:

- control	**+ ligase**	
10 μl	10 μl	DNA (200ng)
5 μl	5 μl	10x T4 ligase buffer
5 μl	5 μl	10mM ATP
0 μl	2.5 μl	T4 DNA ligase
30 μl	27.5 μl	dH2O
50 μl	50 μl	TOTAL

7) Ligate at room temperature, 30 minutes.
8) Heat inactivate ligase enzyme by incubating at 65°C, 20 minutes

Clean the ligated DNA after removal of Tn7L

9) Add 50μl ddH2O to the ligated DNA, to bring it to a final volume of 100μl.
10) Add 10 μl 3M NaOAc. Add 220μl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
11) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
12) Wash DNA pellet with 500μl ice-cold 70% EtOH.
13) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
14) Resuspend DNA in 4μl 10mM Tris. DNA will be roughly 50ng/μl

Transform ligated DNA after removal of Tn7L

For both "- ligase controls" and "+ ligase" reactions:

15) Combine 1μl of DNA (at 50ng/μl) with 20μl MegaX cells in a chilled electroporation cuvette.
16) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
17) Add 1ml Isosensitest to the cuvette and pipet up and down to resuspend.
18) Transfer 900μl cells to a 1.5ml microfuge tube.
19) Recover at 37°C for 1 hour.
20) Make a 1:10 dilution of recovered cells (22μl cells + 200μl Isosensitest). Plate 90μl (1% of total cells) onto prewarmed LB+Kan and Iso+Kan+Tmp plates

For "-ligase" control reactions:

21A) Make a 1:1000 dilution of recovered cells (3μl of the 1:10 diluted cells + 297μl Isosensitest). Plate 90μl (0.01% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates.

For "+ligase" reactions:

21B) Make a 1:10,000 dilution of recovered cells (1μl of the 1:10 diluted cells + 999μl Isosensitest). Plate 90μl (0.001% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates.

22) Transfer remainder of the "+ligase" reaction to a flask containing 150ml Isosensitest. Add 300μl 500x Tmp stock.
23) Grow plates and culture at 37°C overnight.
24) The following morning, pull plates and count colonies. Calculate the number of independent transformants in your "+ ligase" pools.
25) Make a glycerol frozen stock of the "+ ligase" overnight culture. Store at -80°C.
26) Pellet the remainder of the "+ ligase" overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 37 directly.
27) Purify the DNA from the "+ ligase" culture cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the expression pool after removal of Tn7L ("xpT-L").

Remove Tn7R end with *Pme*I digest

21) Digest 1 μg of xpT-L pool with *Pme*I; use 100ng of xpT-L pool for a "no enzyme" control.

No enzyme	***Pme*I**	
100 ng	1000 ng	xpT pool DNA
1 μl	1 μl	10x NEB buffer #4
1 μl	1 μl	10x BSA
0 μl	0.5 μl	*Pme*I
To 10μl total	To 10μl total	dH2O

22) Incubate restriction digests and control reactions at 37°C for 1hr.
23) Heat inactivate *Pme*I by incubating reactions at 65°C for 20 minutes.
24) Run all of the "no enzyme" control and 1μl (100ng of DNA) from *Pme*I digest on an agarose gel to confirm restriction digest was successful. The uncut control should contain supercoiled plasmid. The *Pme*I digest should have a high MW band of plasmid backbone DNA, and the released Tn7R end should be visible at 863bp. If successful, continue with protocol.

Clean digest prior to ligation

We found that residual PmeI may interfere with the success of the ligation reaction.

25) Add ddH2O to remaining *Pme*I-digested xpT-L material to bring final volume to 100μl.
26) Add 100μl phenol:chloroform:IAA. Vortex to mix.
27) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Remove and discard organic (bottom) layer.
28) Add 100 μl chloroform. Vortex to mix.
29) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Transfer aqueous (top) later to a new 1.5ml microfuge tube.
30) Add 10 μl 3M NaOAc. Add 220μl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
31) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
32) Wash DNA pellet with 500μl ice-cold 70% EtOH.
33) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
34) Resuspend DNA pellet (may not be visible) in 20μl of 10mM Tris pH 7.5.

Ligate to recircularize plasmid after *Pme*I digestion

Longer overnight reaction is performed to encourage ligation of blunt ends from PmeI digest.

35) Set up ligation reaction (and a negative control) as shown:

- control	**+ ligase**	
10 μl	10 μl	DNA (200ng)
5 μl	5 μl	10x T4 ligase buffer
5 μl	5 μl	10mM ATP
0 μl	2.5 μl	T4 DNA ligase
30 μl	27.5 μl	dH2O
50 μl	50 μl	TOTAL

36) Ligate at 16°C, 16 hours.
37) Heat inactivate ligase enzyme by incubating at 65°C, 20 minutes

Clean the ligated DNA after removal of Tn7R

38) Add 50μl ddH2O to the ligated DNA, to bring it to a final volume of 100μl.
39) Add 10 μl 3M NaOAc. Add 220μl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
40) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
41) Wash DNA pellet with 500μl ice-cold 70% EtOH.
42) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
43) Resuspend DNA in 4μl 10mM Tris. DNA will be roughly 50ng/μl

Transform ligated DNA after removal of Tn7R

For both "- control" and "+ ligase" reactions:

44) Combine 1μl of DNA (~ 112ng/μl) with 20μl MegaX cells in a chilled electroporation cuvette.
45) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
46) Add 1ml Isosensitest to the cuvette and pipet up and down to resuspend.
47) Transfer 900μl cells to a 1.5ml microfuge tube.

For "-ligase" control reactions:

48A) Recover at 37°C for 1 hour in microfuge tube.
49A) Make 1:10 dilution of recovered cells (30μl cells + 270μl LB). Plate 90μl (1% of total cells) onto prewarmed LB+Kan and Iso+Tmp+Kan plates.
50A) Make a 1:100 dilution of recovered cells (40μl of 1:10 dilution of cells, 360μl LB). Plate 90μl (0.1% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates
51A) Make a 1:1000 dilution of recovered cells (40μl of 1:100 dilution of cells, 360μl LB). Plate 90μl (0.01% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates.
52A) Grow plates at 37°C overnight

For "+ligase" control reactions:

48B) Transfer the cells to a 500ml flask containing 150mls LB. Recover at 37°C for 1 hour.
49B) Plate 150μl of recovered cells (0.1% of total cells) onto prewarmed LB+Kan and Iso+Tmp+Kan plates.
50B) Make a 1:10 dilution of recovered cells (60μl of cells, 540μl LB). Plate 150μl (0.01% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates
51B) Make a 1:100 dilution of recovered cells (50μl of 1:10 dilution of cells, 450μl LB). Plate 150μl (0.001% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates.
52B) Add 150μl 100x Car stock to culture flask. Grow plates and flask at 37°C overnight

53) The following morning, pull plates and count colonies. Calculate the number of independent transformants in your "+ ligase" pools.
54) Make a glycerol frozen stock of the "+ ligase" overnight culture. Store at -80°C.
55) Pellet the remainder of the "+ ligase" overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 37 directly.
56) Purify the DNA from the "+ ligase" culture cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the expression pool after removal of both Tn7L and Tn7R("xpT-L-R").

Diagnostic digests

We highly recommend performing diagnostic restriction digests on plasmid pools from each phase of this protocol. This helps to characterize the proportion of each pool that is mutagenized, identify any lingering donor plasmid that persists in the expression pools, and helps determine the complexity of the pool (ie, is the Tn inserted at many locations throughout the target ORF?).

We suggest performing many restriction digests in parallel. Use an enzyme that cuts only in the donor backbone, one that cuts only in the expression backbone, one that cuts in the Tn7L, and one that cuts in Tn7R. Perform "no enzyme" controls, as well. Include one double digestion which cuts in the epitope tag and one in the backbone – this will determine the complexity of the mutagenized pool. Perform restriction digest on the following DNA samples:

1) Unmutagenized target donor vector
2) wt expression vector
3) mutagenized donor pool
4) xpC pool
5) xpT pool
6) xpT-L pool
7) xpT-L-R pool

References

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