Internal Epitope Tagging of Proteins Using Transposon Tn7

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Abstract

This is a protocol for *in vitro* transposon mutagenesis for introduction of internal epitope tags. It is from:
Zordan RE, Beliveau BJ, Trow JA, Craig NL, and Cormack BP (2015) Avoiding the Ends: Internal Epitope Tagging of Proteins Using Transposon Tn7. *Genetics* 200:47-58; doi:10.1534/genetics.114.169482

Please see the full manuscript for additional details.

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.

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Guidelines

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)
ddH2O = doubly distilled (MilliQ) H2O

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
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 MgCl2
10% glycerol

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

1. Brachmann, C.B. et al. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115-132 (1998).
2. Winzeler, E.A. et al. Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285, 901-906 (1999).
3. Gamas, P. & Craig, N.L. Purification and characterization of TnsC, a Tn7 transposition protein that binds ATP and DNA. Nucleic acids research 20, 2525-2532 (1992).
4. Choi, K.Y., Li, Y., Sarnovsky, R. & Craig, N.L. Direct interaction between the TnsA and TnsB subunits controls the heteromeric Tn7 transposase. Proceedings of the National Academy of Sciences of the United States of America 110, E2038-2045 (2013).

Before start

We highly recommend performing diagnostic restriction digests on plasmid pools from each phase of this protocol. See the guidelines for details.

Materials

phenol:chloroform:IAA 0883-100ml by Amresco
FseI - 100 units R0588S by New England Biolabs
PmeI - 500 units R0560S by New England Biolabs
ApaLI - 2,500 units R0507S by New England Biolabs
T4 DNA Ligase - 20,000 units M0202S by New England Biolabs
Gateway LR clonase II 11791-100 by Life Technologies

Qiagen Hi-Speed MidiPrep kit 12643 by Qiagen

MegaX DH10B T1R Electrocomptent Cells C6400-03 by Life Technologies

Protocol

In vitro transposition protocol

Step 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	ddH2O

NOTES

Tracey DePellegrin 22 Sep 2015

This protocol delineates the steps to perform a small (20 µl) "- enzyme" negative control reaction and a large (80 µl) "+ 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.

In vitro transposition protocol

Step 2.

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

In vitro transposition protocol

Step 3.

Make the enzyme mixture. Combine:

7.49 µl	TnsA
3.31 µl	Storage buffer A
2.00 µl	TnsB
6 µl	Storage buffer B
8 µl	$TnsC^{A255V}$
8 µl	Storage buffer C
5.2 µl	50% glycerol

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

NOTES

Tracey DePellegrin 06 Sep 2015

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.

In vitro transposition protocol

Step 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

In vitro transposition protocol

Step 5.

Add 4 µl enzyme mix to the “+ enzyme” tube. Flick tube to mix

In vitro transposition protocol

Step 6.

Add 1 µl of the buffer mixture to the “- enzyme” tube. Flick tube to mix.

In vitro transposition protocol

Step 7.

Incubate both tubes at 37°C for 10 minutes on a PCR heat block.

DURATION

00:10:00

In vitro transposition protocol

Step 8.

Add 300mM MgOAc to the tubes:

For “+ enzyme” reactions, add 4.2 µl 300mM MgOAc
For “- enzyme” reaction, add 1.05 µl 300mM MgOac

In vitro transposition protocol

Step 9.

Incubate at 37°C for 1 hour on a PCR block

DURATION

01:00:00

In vitro transposition protocol

Step 10.

Incubate at 75°C for 5 minutes on a PCR block to heat-kill the enzymes.

DURATION

00:05:00

Clean-up of transposition reactions

Step 11.

Transfer “+ enzyme” and “- enzyme” reactions to 15ml microfuge tubes Bring the volume of each up to 100 µl.

Clean-up of transposition reactions

Step 12.

Add 100 µl phenol:chloroform:IAA. Vortex to mix.

AMOUNT

100 µl Additional info:

REAGENTS

phenol:chloroform:IAA [0883-100ml](#) by [Amresco](#)

Clean-up of transposition reactions

Step 13.

Spin 5 minutes, 4°C, 13500 rpm in a microfuge.

DURATION

00:05:00

Clean-up of transposition reactions

Step 14.

Remove and discard organic (bottom) layer.

Clean-up of transposition reactions

Step 15.

Add 100 µl chloroform. Vortex to mix.

Clean-up of transposition reactions

Step 16.

Spin 5 minutes, 4°C, 13500rpm in microfuge.

DURATION

00:05:00

Clean-up of transposition reactions

Step 17.

Transfer aqueous (top) later to a new 1.5ml microfuge tube.

Clean-up of transposition reactions

Step 18.

Add 10 µl 3M NaOAc.

Clean-up of transposition reactions

Step 19.

Add 220 µl ice-cold 100% EtOH and chill at -20°C for 15 minutes.

DURATION

00:15:00

Clean-up of transposition reactions

Step 20.

Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.

DURATION

00:15:00

Clean-up of transposition reactions

Step 21.

Wash DNA pellet with 500 µl ice-cold 70% EtOH.

Clean-up of transposition reactions

Step 22.

Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.

DURATION

00:05:00

Clean-up of transposition reactions

Step 23.

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

Clean-up of transposition reactions

Step 24.
Optional: Cleaned transposition reactions may be stored at -20°C prior to transformation.

Transformation of transposition reactions: For “- enzyme” reactions

Step 25.
Combine 2 µl DNA with 20 µl MegaX cells in a chilled electroporation cuvette.

AMOUNT
20 µl Additional info:

REAGENTS
MegaX DH10B T1R Electrocomptent Cells C6400-03 by Life Technologies

NOTES
Tracey DePellegrin 07 Sep 2015
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.

Transformation of transposition reactions: For “- enzyme” reactions

Step 26.
Electroporate at 2.0 kV, 200Ω, 25µF, according to protocol.

Transformation of transposition reactions: For “- enzyme” reactions

Step 27.
Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.

Transformation of transposition reactions: For “- enzyme” reactions

Step 28.
Transfer 900 µl cells to a 1.5ml microfuge tube.

Transformation of transposition reactions: For “- enzyme” reactions

Step 29.
Recover at 37°C for 1 hour.

DURATION
01:00:00

Transformation of transposition reactions: For “- enzyme” reactions

Step 30.
Plate 9 µl (1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan .

Transformation of transposition reactions: For “- enzyme” reactions

Step 31.
Grow plates overnight at 37°C.

DURATION
18:00:00

Transformation of transposition reactions: For “+ enzyme” reactions

Step 32.
Combine 4 µl DNA with 40 µl MegaX cells in one chilled electroporation cuvette.

AMOUNT

40 µl Additional info:

REAGENTS

MegaX DH10B T1R Electrocomptent Cells C6400-03 by Life Technologies

Transformation of transposition reactions: For “+ enzyme” reactions

Step 33.

Electroporate at 2.0 kV, 200Ω, 25µF, according to protocol.

Transformation of transposition reactions: For “+ enzyme” reactions

Step 34.

Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.

Transformation of transposition reactions: For “+ enzyme” reactions

Step 35.

Transfer 900 µl cells to a 500ml flask containing 150 mls Isosensistest media.

Transformation of transposition reactions: For “+ enzyme” reactions

Step 36.

Recover at 37°C for 1 hour.

DURATION

01:00:00

Transformation of transposition reactions: For “+ enzyme” reactions

Step 37.

Plate 150 µl(0.1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan

Transformation of transposition reactions: For “+ enzyme” reactions

Step 38.

Add 150 µl of Kan (30mg/ml stock) and 300µl Tmp (5mg/ml stock) to the flask.

Transformation of transposition reactions: For “+ enzyme” reactions

Step 39.

Grow plates and culture overnight at 37°C.

DURATION

18:00:00

Transformation of transposition reactions

Step 40.

The following morning, count the colonies growing on each plate. Calculate the number of independent transformants in your “+ enzyme” pools.

Transformation of transposition reactions

Step 41.

Make a glycerol frozen stock of the “+ enzyme” overnight culture. Store at -80°C

Transformation of transposition reactions

Step 42.

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 43 directly.

DURATION

00:15:00

Transformation of transposition reactions

Step 43.

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).

REAGENTS

Qiagen Hi-Speed MidiPrep kit 12643 by Qiagen

NOTES

Tracey DePellegrin 07 Sep 2015

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: Linearize mutagenized DNA pool

Step 44.

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.

REAGENTS

ApaLI - 2,500 units R0507S by New England Biolabs

NOTES

Tracey DePellegrin 07 Sep 2015

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.

Gateway LR mobilization to create expression pools: Linearize mutagenized DNA pool

Step 45.

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.

Gateway LR mobilization to create expression pools: Linearize mutagenized DNA pool

Step 46.

Digest 37°C for 1 hour.

DURATION

01:00:00

Gateway LR mobilization to create expression pools: Linearize mutagenized DNA pool

Step 47.

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.

AMOUNT

2 µl Additional info:

REAGENTS

ApaLI - 2,500 units R0507S by New England Biolabs

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 48.

Add 82µl ddH2O to the remaining ApaLI-digested DNA, to bring it to a final volume of 100µl.

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 49.

Add 100µl phenol:chloroform:IAA. Vortex to mix.

AMOUNT

100 µl Additional info:

REAGENTS

phenol:chloroform:IAA 0883-100ml by Amresco

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 50.

Spin 5 minutes, 4ºC, 13500 rpm in microfuge. Remove and discard organic (bottom) layer.

DURATION

00:05:00

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 51.

Add 100 µl chloroform. Vortex to mix.

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 52.

Spin 5 minutes, 4°C, 13500 rpm in microfuge. Transfer aqueous (top) later to a new 1.5ml microfuge tube.

DURATION

00:05:00

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 53.

Add 10 µl 3M NaOAc.

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 54.

Add 220µl ice-cold 100% EtOH and chill at -20°C for 15 minutes.

DURATION

00:15:00

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 55.

Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.

DURATION

00:15:00

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 56.

Wash DNA pellet with 500µl ice-cold 70% EtOH.

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 57.

Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.

DURATION

00:05:00

Gateway LR mobilization to create expression pools: Clean the linearized DNA

Step 58.

Resuspend DNA in 20µl 10mM Tris. DNA will be 45ng/µl.

Gateway LR mobilization to create expression pools: Gateway LR Reaction – Move ORF to destination vector

Step 59.

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

NOTES

Tracey DePellegrin 07 Sep 2015

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

Gateway LR mobilization to create expression pools: Gateway LR Reaction – Move ORF to destination vector

Step 60.

For the “+LR” experimental reaction, combine 16µl of the DNA mixture from step 59 and 4µl Invitrogen LR Clonase II in a PCR tube. For the “-LR” control reaction, combine 4µl DNA mixture from step 59 and 1µl TE in a PCR tube. Incubate both reactions overnight at 25°C on the PCR block.

DURATION

18:00:00

Gateway LR mobilization to create expression pools: Gateway LR Reaction – Move ORF to destination vector

Step 61.

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.

Gateway LR mobilization to create expression pools: Gateway LR Reaction – Move ORF to destination vector

Step 62.

Incubate at 37°C for 10 minutes on the PCR block.

DURATION

00:10:00

Gateway LR mobilization to create expression pools: Gateway LR Reaction – Move ORF to destination vector

Step 63.

Clean reactions: Transfer reactions to 1.5ml microfuge tubes. Perform phenol/chloroform extraction and EtOH precipitation as described before (“Gateway LR mobilization to create expression pools: Clean linearized DNA”)

Gateway LR mobilization to create expression pools: Gateway LR Reaction – Move ORF to destination vector

Step 64.

Resuspend “+LR” experimental DNA in 6µl TE (final [DNA] = 50ng/ul).

Gateway LR mobilization to create expression pools: Gateway LR Reaction – Move ORF to destination vector

Step 65.

Resuspend “-LR” negative control DNA in 4µl TE (final [DNA] = 18.75ng/ul).

Transform Gateway LR reactions: For “- enzyme” reactions

Step 66.

Combine 2µl DNA with 20µl MegaX cells in a chilled electroporation cuvette.

AMOUNT

20 µl Additional info:

REAGENTS

MegaX DH10B T1R Electrocomptent Cells C6400-03 by Life Technologies

NOTES

Tracey DePellegrin 07 Sep 2015

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.

Transform Gateway LR reactions: For “- enzyme” reactions

Step 67.

Electroporate at 2.0 kV, 200Ω, 25µF, according to protocol.

Transform Gateway LR reactions: For “- enzyme” reactions

Step 68.

Add 1ml Recovery media (provided with MegaX cells) to the cuvette and pipet up and down to resuspend.

Transform Gateway LR reactions: For “- enzyme” reactions

Step 69.

Transfer 900µl cells to a 1.5ml microfuge tube.

Transform Gateway LR reactions: For “- enzyme” reactions

Step 70.

Recover at 37°C for 1 hour.

DURATION

01:00:00

Transform Gateway LR reactions: For “- enzyme” reactions

Step 71.

Plate 9µl (1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan .

Transform Gateway LR reactions: For “- enzyme” reactions

Step 72.

Grow plates overnight at 37°C.

DURATION

18:00:00

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 73.

Combine 2µl DNA with 20µl MegaX cells in one chilled electroporation cuvette.

AMOUNT

20 µl Additional info:

REAGENTS

MegaX DH10B T1R Electrocomptent Cells C6400-03 by Life Technologies

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 74.

Electroporate at 2.0 kV, 200Ω, 25µF, according to protocol.

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 75.

Add 1ml Recovery media (provided with MegaX cells) to the cuvette and pipet up and down to

resuspend.

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 76.

Transfer 900µl cells to a 1.5ml microfuge tube.

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 77.

Recover at 37°C for 1 hour

DURATION

01:00:00

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 78.

Transfer contents of microfuge tube to a 500ml flask containing 150mls LB. Swirl to mix.

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 79.

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

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 80.

Add 150µl of Car (100mg/ml stock) to the flask.

Transform Gateway LR reactions: For “+ enzyme” reactions

Step 81.

Grow plates and culture overnight at 37°C.

DURATION

18:00:00

Transform Gateway LR reactions

Step 82.

The following morning, count the colonies growing on each plate. Calculate the number of independent transformants in the “+ enzyme” pools.

Transform Gateway LR reactions

Step 83.

Make a glycerol frozen stock of the “+ enzyme” overnight culture. Store at -80°C.

Transform Gateway LR reactions

Step 84.

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 85 directly.

DURATION

00:15:00

Transform Gateway LR reactions

Step 85.

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”).

REAGENTS

Qiagen Hi-Speed MidiPrep kit 12643 by Qiagen

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

Step 86.

Combine 1µl “xpC” expression pool DNA (50ng/µl) with 20µl MegaX cells in one chilled cuvette.

AMOUNT

20 µl Additional info:

REAGENTS

MegaX DH10B T1R Electrocomptent Cells C6400-03 by Life Technologies

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

Step 87.

Electroporate at 2.0 kV, 200Ω, 25µF, according to protocol.

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

Step 88.

Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.

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

Step 89.

Transfer 900µl cells to 150mls Isosensitest media (in a 500ml flask).

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

Step 90.

Recover at 37°C for 1 hour

DURATION

01:00:00

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

Step 91.

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

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

Step 92.

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

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

Step 93.

Add 300µl of Tmp (5mg/ml stock) to the flask.

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

Step 94.

Grow plates and culture overnight at 37°C.

DURATION

18:00:00

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

Step 95.

The following morning count the colonies growing on each plate. Calculate the number of independent transformants in your pools.

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

Step 96.

Make a glycerol frozen stock of the overnight culture. Store at -80°C.

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

Step 97.

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 98 directly.

DURATION

00:15:00

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

Step 98.

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").

Remove Tn7L end with FseI digest

Step 99.

Digest 500ng of xpT pool with *FseI*; use 100ng of xpT pool for a "no enzyme" control.

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

REAGENTS

FseI - 100 units R0588S by New England Biolabs

NOTES

Tracey DePellegrin 23 Sep 2015

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 FseI digest

Step 100.

Incubate restriction digests and control reactions at 37°C for 1hr.

DURATION

01:00:00

Remove Tn7L end with FseI digest

Step 101.

Heat inactivate FseI by incubating reactions at 65°C for 20 minutes.

REAGENTS

FseI - 100 units R0588S by New England Biolabs

DURATION

00:20:00

Remove Tn7L end with FseI digest

Step 102.

Run all of the "no enzyme" control and 2µl (100ng of DNA) from FseI digest on an agarose gel to confirm restriction digest was successful. The uncut control should contain supercoiled plasmid, and the FseI 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.

REAGENTS

FseI - 100 units R0588S by New England Biolabs

Ligate to recircularize plasmid after FseI digestion

Step 103.

Add 12 µl of dH2O to remaining FseI-digested xpT material to bring final volume to 20 µl.

Ligate to recircularize plasmid after FseI digestion

Step 104.

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

REAGENTS

T4 DNA Ligase - 20,000 units M0202S by New England Biolabs

Ligate to recircularize plasmid after FseI digestion

Step 105.

Ligate at room temperature, 30 minutes.

DURATION

00:30:00

Ligate to recircularize plasmid after FseI digestion

Step 106.

Heat inactivate ligase enzyme by incubating at 65°C, 20 minutes

DURATION

00:20:00

Clean the ligated DNA after removal of Tn7L

Step 107.

Add 50µl ddH2O to the ligated DNA, to bring it to a final volume of 100µl.

Clean the ligated DNA after removal of Tn7L

Step 108.

Add 10 µl 3M NaOAc. Add 220µl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.

DURATION

00:15:00

Clean the ligated DNA after removal of Tn7L

Step 109.

Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.

DURATION

00:15:00

Clean the ligated DNA after removal of Tn7L

Step 110.

Wash DNA pellet with 500µl ice-cold 70% EtOH.

Clean the ligated DNA after removal of Tn7L

Step 111.

Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.

DURATION

00:05:00

Clean the ligated DNA after removal of Tn7L

Step 112.

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

Step 113.

Combine 1µl of DNA (at 50ng/µl) with 20µl MegaX cells in a chilled electroporation cuvette.

AMOUNT

20 µl Additional info:

REAGENTS

MegaX DH10B T1R Electrocomptent Cells C6400-03 by Life Technologies

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 114.

Electroporate at 2.0 kV, 200Ω, 25µF, according to protocol.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 115.

Add 1ml Isosensitest to the cuvette and pipet up and down to resuspend.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 116.

Transfer 900µl cells to a 1.5ml microfuge tube.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 117.

Recover at 37°C for 1 hour.

DURATION

01:00:00

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 118.

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

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 119.

For “-ligase” control reactions:

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.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 120.

For “+ligase” reactions:

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.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 121.

Transfer remainder of the “+ligase” reaction to a flask containing 150ml Isosensitest. Add 300µl 500x

Tmp stock.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 122.

Grow plates and culture at 37°C overnight.

DURATION

18:00:00

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 123.

The following morning, pull plates and count colonies. Calculate the number of independent transformants in your “+ ligase” pools.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 124.

Make a glycerol frozen stock of the “+ ligase” overnight culture. Store at -80°C.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 125.

Pellet the remainder of the “+ ligase” overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm.

DURATION

00:15:00

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 126.

Pellet can be stored at -20°C, or continue to step 127 directly.

Transform ligated DNA after removal of Tn7L: For both “- ligase controls” and “+ ligase” reactions

Step 127.

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 PmeI digest

Step 128.

Digest 1 µg of xpT-L pool with PmeI; use 100ng of xpT-L pool for a “no enzyme” control.

No enzyme	**PmeI**	
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	PmeI
To 10µl total	To 10µl total	dH2O

REAGENTS

PmeI - 500 units R0560S by New England Biolabs

Remove Tn7R end with PmeI digest

Step 129.

Incubate restriction digests and control reactions at 37°C for 1hr.

DURATION

01:00:00

Remove Tn7R end with PmeI digest

Step 130.
Heat inactivate PmeI by incubating reactions at 65°C for 20 minutes.

DURATION
00:20:00

Remove Tn7R end with PmeI digest

Step 131.
Run all of the “no enzyme” control and 1µl (100ng of DNA) from PmeI digest on an agarose gel to confirm restriction digest was successful. The uncut control should contain supercoiled plasmid. The PmeI 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

Step 132.
Add ddH2O to remaining PmeI-digested xpT-L material to bring final volume to 100µl.

NOTES
Tracey DePellegrin 07 Sep 2015
We found that residual PmeI may interfere with the success of the ligation reaction.

Clean digest prior to ligation

Step 133.
Add 100µl phenol:chloroform:IAA. Vortex to mix.

AMOUNT
100 µl Additional info:

REAGENTS
phenol:chloroform:IAA 0883-100ml by Amresco

Clean digest prior to ligation

Step 134.
Spin 5 minutes, 4°C, 13500 rpm in microfuge. Remove and discard organic (bottom) layer.

DURATION
00:05:00

Clean digest prior to ligation

Step 135.
Add 100 µl chloroform. Vortex to mix.

Clean digest prior to ligation

Step 136.
Spin 5 minutes, 4°C, 13500 rpm in microfuge.

DURATION
00:05:00

Clean digest prior to ligation

Step 137.
Transfer aqueous (top) later to a new 1.5ml microfuge tube.

Clean digest prior to ligation

Step 138.
Add 10 µl 3M NaOAc.

Clean digest prior to ligation

Step 139.
Add 220µl ice-cold 100% EtOH and chill at -20°C for 15 minutes.

DURATION
00:15:00

Clean digest prior to ligation

Step 140.
Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.

DURATION
00:15:00

Clean digest prior to ligation

Step 141.
Wash DNA pellet with 500µl ice-cold 70% EtOH.

Clean digest prior to ligation

Step 142.
Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.

DURATION
00:05:00

Clean digest prior to ligation

Step 143.
Resuspend DNA pellet (may not be visible) in 20µl of 10mM Tris pH 7.5.

Ligate to recircularize plasmid after PmeI digestion

Step 144.
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

REAGENTS
T4 DNA Ligase - 20,000 units M0202S by New England Biolabs

NOTES
Tracey DePellegrin 07 Sep 2015
Longer overnight reaction is performed to encourage ligation of blunt ends from PmeI digest.

Ligate to recircularize plasmid after PmeI digestion

Step 145.
Ligate at 16°C, 16 hours.

DURATION
16:00:00

Ligate to recircularize plasmid after PmeI digestion

Step 146.
Heat inactivate ligase enzyme by incubating at 65°C, 20 minutes.

DURATION
20:00:00

Clean the ligated DNA after removal of Tn7R

Step 147.
Add 50µl ddH2O to the ligated DNA, to bring it to a final volume of 100µl

Clean the ligated DNA after removal of Tn7R

Step 148.
Add 10 µl 3M NaOAc. Add 220µl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.

DURATION
00:15:00

Clean the ligated DNA after removal of Tn7R

Step 149.
Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.

DURATION
00:15:00

Clean the ligated DNA after removal of Tn7R

Step 150.
Wash DNA pellet with 500µl ice-cold 70% EtOH.

Clean the ligated DNA after removal of Tn7R

Step 151.
Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.

DURATION
00:05:00

Clean the ligated DNA after removal of Tn7R

Step 152.
Resuspend DNA in 4µl 10mM Tris. DNA will be roughly 50ng/µl.

Transform ligated DNA after removal of Tn7R

Step 153.
Combine 1µl of DNA (112ng/µl) with 20µl MegaX cells in a chilled electroporation cuvette.

AMOUNT
20 µl Additional info:

REAGENTS
MegaX DH10B T1R Electrocomptent Cells C6400-03 by Life Technologies

NOTES
Tracey DePellegrin 23 Sep 2015
Follow steps 153-156 **for both** "- control" and "+ ligase" reactions:

Transform ligated DNA after removal of Tn7R

Step 154.
Electroporate at 2.0 kV, 200Ω, 25µF, according to protocol.

Transform ligated DNA after removal of Tn7R

Step 155.
Add 1ml Isosensitest to the cuvette and pipet up and down to resuspend.

Transform ligated DNA after removal of Tn7R

Step 156.
Transfer 900µl cells to a 1.5ml microfuge tube.

Transform ligated DNA after removal of Tn7R

Step 157.
For "-ligase" reactions:

Recover at 37°C for 1 hour in microfuge tube.

DURATION

01:00:00

Transform ligated DNA after removal of Tn7R

Step 158.

For “-ligase” reactions:

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.

Transform ligated DNA after removal of Tn7R

Step 159.

For “-ligase” control reactions:

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

Transform ligated DNA after removal of Tn7R

Step 160.

For “-ligase” reactions:

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.

Transform ligated DNA after removal of Tn7R

Step 161.

For “-ligase” reactions:

Grow plates and culture at 37°C overnight.

DURATION

18:00:00

Transform ligated DNA after removal of Tn7R

Step 162.

For “+ligase” control reactions:

Transfer the cells to a 500ml flask containing 150mls LB. Recover at 37°C for 1 hour.

DURATION

01:00:00

Transform ligated DNA after removal of Tn7R

Step 163.

For “+ligase” control reactions:

Plate 150µl of recovered cells (0.1% of total cells) onto prewarmed LB+Kan and Iso+Tmp+Kan plates.

Transform ligated DNA after removal of Tn7R

Step 164.

For “+ligase” control reactions:

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

Transform ligated DNA after removal of Tn7R

Step 165.

For “+ligase” control reactions:

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.

Transform ligated DNA after removal of Tn7R

Step 166.

For “+ligase” control reactions:

Add 150µl 100x Car stock to culture flask. Grow plates and flask at 37°C overnight.

DURATION

18:00:00

Transform ligated DNA after removal of Tn7R

Step 167.

The following morning, pull plates and count colonies. Calculate the number of independent transformants in your “+ ligase” pools.

Transform ligated DNA after removal of Tn7R

Step 168.

Make a glycerol frozen stock of the “+ ligase” overnight culture. Store at -80°C.

Transform ligated DNA after removal of Tn7R

Step 169.

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 170 directly.

Transform ligated DNA after removal of Tn7R

Step 170.

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”).

REAGENTS

Qiagen Hi-Speed MidiPrep kit 12643 by Qiagen