

GATEWAY TOL2 PROTOCOLS

Protocol 1: Generate appropriate entry clone (BP reaction)

1. Generate primers with attB sites on the ends: see instructions on p. 4.
2. PCR-amplify sequence using Phusion polymerase (from Finnzymes, distributed by NEB) – note that the reaction conditions for Phusion differ from those for other PCR enzymes. Depending on the template I'm using, I make up 1 or 2 x 50µl rxns.
 - 2.b. If starting from a plasmid template that is kanamycin resistant: Perform DpnI digest:
 - To 50 µl PCR rxn, add 5 µl of 10x buffer and >5 U DpnI
 - Incubate at 37° for 15 minutes
 - Heat-inactivate DpnI at 65° for 15 minutes
3. Gel-purify PCR product using your method of choice, elute in 30µl, determine concentration by spectrophotometer, and move directly to the next step!

Note: The attB sequences at the ends of the PCR product are fragile, and without them intact the BP reaction cannot take place. If you absolutely cannot go from PCR reaction to BP reaction in one day, it is preferable to save the completed PCR reaction at 4°C rather than proceeding with the purification.

Note 2: If your purified PCR product is not concentrated enough to get 50 fmoles in 7 µl, don't proceed. Try reamplifying from this PCR reaction to get more product.
4. Calculate the amount (in ng) of purified PCR product to add to the BP reaction:
$$\text{ng} = (x \text{ fmoles}) (N \text{ bp}) (660 \text{ fg/fmoles}) (1\text{ng}/10^6 \text{ fg})$$

x – number of fmoles of insert to include, usually 50 for BP rxn (but see note below)
N – length of insert
5. Set up BP reaction
 - Obtain appropriate donor vector (pDONR P4-P1R for p5E; pDONR221 or pENTR kit for pME; pDONR P2R-P3 for p3E) diluted to 150 ng/µl
 - Combine:

attB PCR product (20-50 fmoles)	1-7 µl
pDONR vector (150 ng/µl)	1 µl
TE buffer pH 8.0	to 8 µl

Note: Combine insert and donor vector at equal molarity, but do not add more than 150 ng of insert (so, use 50 fmol of each unless the insert is quite large; then scale down amounts of both insert and donor vector accordingly)
 - Remove BP Clonase II enzyme mix from -20° or -80°C (we distribute the enzyme into 10µl aliquots and store them at -80°, leaving one aliquot at -20°)
 - Add 2 µl BP Clonase II enzyme mix. Mix well.
 - Incubate BP reactions at 25°C for 1-18 hours (if insert is over 4kb, incubate reaction overnight)
6. Add 1 µl of Proteinase K solution, incubate 10 minutes at 37°C
7. Transform 1 µl of BP reaction into competent cells (homemade DH5a cells are sufficient) and plate on LB + 50 µg/ml kanamycin plates
8. Select colonies for overnight growth in LB + kan
9. Miniprep and perform diagnostic digests; sequence if desired using M13 Forward and Reverse primers.

Protocol 2: Generate Destination clone (LR reaction)

1. Obtain destination vector (eg, pDestTol2pA2, Tol2kit clone #394; or pDestTol2CG2, Tol2kit clone #395 which contains cmlc2:egfp transgenesis marker) diluted to 20 fmol/ μ l (103 ng/ μ l for clone #395)
2. Combine:

p5E vector (20 fmol)	\	
pME vector (20 fmol)	→	1-7 μ l (total for all 3 components)
p3E vector (20 fmol)	/	
pDest vector (20 fmol)		1 μ l
TE buffer pH 8.0		to 8 μ l

Note: Calculate the quantity of each entry vector to add using the formula listed in Step 4 of the BP reaction protocol; use the entire length of each entry vector (not just the segment you're interested in) for the calculation.
3. Remove LR Clonase II Plus enzyme mix from -80° , thaw on ice (LR Clonase is extremely labile, so we distribute the enzyme into 10 μ l aliquots and store them all at -80°)
4. Add 2 μ l LR Clonase II Plus enzyme mix. Mix well.
5. Incubate LR reactions at 25°C for 16 hours
6. Add 1 μ l of Proteinase K solution, incubate 10 minutes at 37°C .
7. Transform:
 - Dilute 50 μ l of One Shot Top10 Electrocompetent Cells with 150 μ l ice-cold water
 - Transfer 50 μ l to ice-cold electroporation cuvette
 - Transform cells with 2-5 μ l of LR reaction
 - Add 150 μ l SOC media
 - recover for 2 hours at 37°C
 - plate on LB + 50-100 $\mu\text{g/ml}$ ampicilin plates (two for plates each LR reaction, warmed to 37°C for 30 minutes before plating, plated with 50 μ l and 150 μ l)
8. Select colonies for overnight growth in LB + amp
9. Miniprep and perform diagnostic digests. (Note that there is no easy way to sequence destination clones; we rely on diagnostic digests alone.)
10. If using a homemade miniprep to isolate DNA, purify it by running through a PCR column to remove all phenol/chloroform before injecting.
11. Dilute DNA to 50 ng/ μ l for injections. Combine 1:1 with 50 ng/ μ l RNA before injecting (make DNA/RNA mix fresh).

Protocol 3: Generate Transposase RNA for coinjection with destination clone

1. Linearize pCS2FA plasmid (Tol2kit clone #396):

- Combine:

DNA (25 µg)	_____ µl
10x buffer (NEB #3)	20 µl
100x BSA	2 µl
NotI	4 µl
H ₂ O	to 200 µl

- Digest 2 hours at 37°C

2. Precipitate

- Add:

3 M NaOAc pH 5.2	22 µl
100% EtOH	555 µl

- mix

- Precipitate for 15 minutes at -80°C

- Spin 15 minutes at 4°C, discard supernatant

- Wash pellet with 1 ml 70% EtOH, spin 5 minutes, discard supernatant, dry pellet

- Resuspend pellet in 100 µl H₂O

- Determine concentration using a spectrophotometer.

3. Transcribe (using Ambion mMessage mMachine SP6 kit)

- Combine:

linearized pCS2FA DNA (1 µg)	_____ µl
2xNTP/CAP	10 µl
10x rxn buffer	2 µl
enzyme mix (SP6)	2 µl
nuclease-free H ₂ O	to 20 µl

- Transcribe 2.5 hours at 37°C

- Add 1 µl Turbo DNase

- Incubate 30 minutes at 37°C

4. Purify RNA (using Roche mini QuickSpin Columns)

- Resuspend matrix

- Remove cap, then tip

- Spin 1 minute at 3000 rpm to pack matrix, transfer column to new tube

- Apply Transcription reaction to column

- Spin 4 minutes at 3000 rpm

5. Determine concentration using a spectrophotometer. Dilute RNA to 50 ng/µl, run on an RNA gel to confirm quality, distribute 10µl aliquots and freeze at -80°C. We treat these aliquots as one-use so as to ensure high quality of RNA for each injection.

Note: This protocol should make enough RNA to last a long, long time. I did two Transcription reactions side-by-side recently, and generated nearly 1ml of diluted RNA!

Notes and Related Information

Links:

Chi-Bin Chien Lab (Utah): http://chien.neuro.utah.edu/tol2kitwiki/index.php/Main_Page
Tol2kit blog: <http://tol2kit.blogspot.com/>
Lawson Lab (UMass): <http://lawsonlab.umassmed.edu/gateway.html>

Manual:

We, like the Chien lab, use Invitrogen's Multisite Gateway manual Version D. An archived copy of this is available through the Chien lab website.

Designing primers for Gateway: standard purity (desalted) is sufficient for most applications; for efficient cloning of large PCR products (greater than 5kb), HPLC or PAGE-purified primers are recommended.

1. For p5E vector

- Forward primer (attB4): GGGG ACAACTTTGTATAGAAAAGTTG [18-25 bp template-specific sequence]
- Reverse primer (attB1R): GGGG ACTGCTTTTTTGTACAAACTTG [18-25 bp template-specific sequence]

2. For pME vector

- Forward primer (attB1): GGGG ACAAGTTTGTACAAAAAGCAGGCT [18-25 bp template-specific sequence]

Note: Be sure the fragment you're amplifying includes a Kozak sequence and a start site.

- Reverse primer (attB2R): GGGG ACCACTTTGTACAAGAAAGCTGGGT [18-25 bp template-specific sequence; see note below on stop codon use]

Note:

If you want to make a fusion construct between the product of this gene and the product of the p3E vector, do not include the stop codon in the reverse primer, and include one extra nucleotide after the attB2R site to maintain the proper reading frame.

If you want the product of this gene expressed or localized separately from the product of the p3E vector, include the stop codon in the reverse primer.

3. For p3E vector

- Forward primer (attB2): GGGG ACAGCTTCTTGTACAAAGTGG [18-25 bp template-specific sequence]

Note: If you want to make a fusion construct between the product of this gene and the product of the pME vector, add two extra nucleotides before the start codon to maintain the proper reading frame.

- Reverse primer (attB3R): GGGG ACAACTTTGTATAATAAAGTTG [18-25 bp template-specific sequence]

Growing Gateway vectors:

- Donor vectors (pDonr) and Destination vectors (pDest) prior to recombination contain ccdB suicide genes and must be grown in ccdB-tolerant cells.
- pDonrP4P1R is prone to self-recombination and therefore requires a special isolation strategy; see Chi-Bin Chien's Tol2Kit website for details and a protocol.
- Entry clones are kan-resistant.
- Destination clones are amp-resistant.

Terminology:

pDonr – “donor vector,” the vector into which a fragment of interest is recombined in a BP reaction; contains a ccdB suicide gene and therefore must be grown in ccdB-tolerant cells. Vectors contain attP sites specific to the entry clone they are intended to produce

- pEntr – a series of kits available from Invitrogen that allow the user to TOPO-clone a fragment of interest into a vector containing attR1 and attR2 sites (creating a pME vector without needing to go through a BP reaction). Use of these kits does not require primers containing attB sites!
- p5E, pME, p3E – “entry clones” for each of the three sections of the final multisite gateway construct, the products of BP reactions; fragments of interest are flanked by specific attL or attR sites to ensure ordered directional assembly in the LR reaction (attL4 and attR1 for p5E; attL1 and attL2 for pME; attR2 and attL3 for p3E)
- pDest – “destination vector,” the vector into which entry vectors are recombined to generate a final vector in an LR reaction; contains a ccdB suicide gene and therefore must be grown in ccdB-tolerant cells. Also used in the name of the completed clone generated in an LR reaction.

Nomenclature:

- Entry clones are referred to by their multisite component identity followed by the insert identity

p5E-hsp70	hsp70 promoter
p5E-βactin2	beta-actin promoter (ubiquitous)
pME-EGFPCAAX	membrane-localized (prenylated) EGFP
pME-palmmCherry	membrane-localized (palmitylated) mCherry
p3E-IRES-nlsEGFPpA	IRES driving nuclear EGFP plus SV40 late polyA
p3E-polyA	SV40 late polyA signal
- Destination clones are referred to by pDest- followed by all three of their component inserts
 - pDest-mitfa:palmmCherry-pA
 - pDest-hsp70:csf1rDN-EGFP
 - pDest-βactin2:nlsmCherry-IRES-EGFPCAAX