



Three Way Gateway Reactions for Modular Gene Targeting Vector Assembly

Final Gene Targeting Vectors are assembled from three component plasmids using a Gateway based system of modular replacement based on site specific recombination *in vitro*. More information on Gateway can be obtained at Invitrogen's website (www.invitrogen.com). Gateway sites employed in EUCOMM vectors are of two complimentary varieties, L site and R sites. L and R sites have specificities designated by a number. L sites react with R sites of identical specificity but not with other Gateway sites, eg L1 with R1 and L3 with R3 but **not** L1 with R3 or L1 with L2. The LR Gateway reaction leaves minimal 25bp Gateway B (specificity) sites in the final vector. The reaction is driven by a purified enzyme mix called LR Clonase (available from Invitrogen) and involves an *in vitro* incubation followed by a bacterial transformation step.

EUCOMM vector resources contain TWO Gateway based switching systems, each consisting of a set of two Gateway sites. Intermediate targeting vectors contain in total FOUR Gateway sites (R1/R2 and R3/4) which constitute these two independent switching systems.

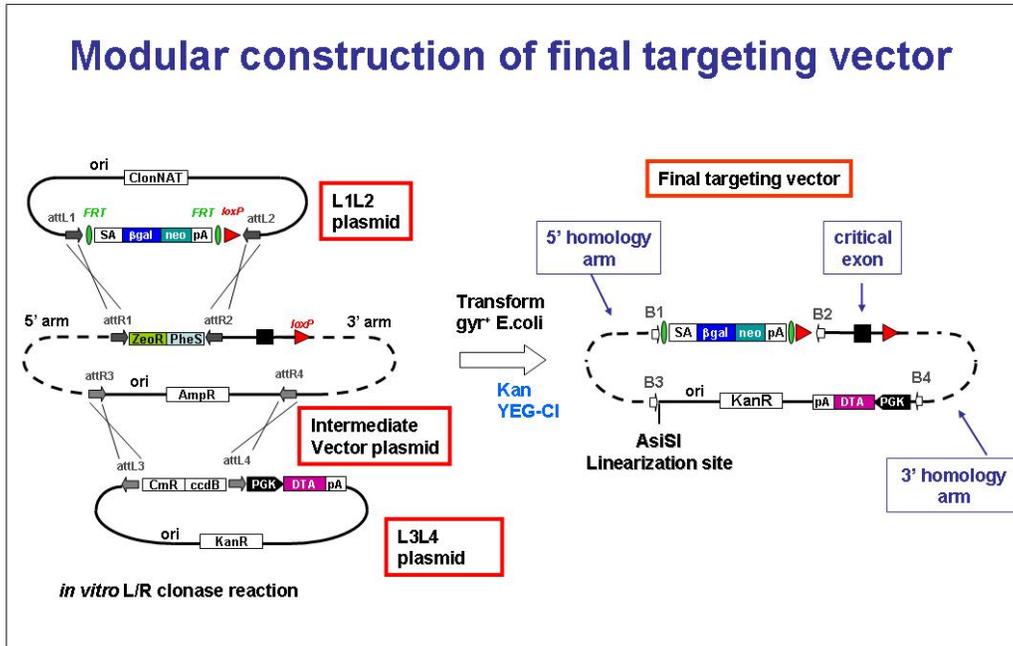
The first system is used to introduce a gene targeting element upstream of the critical exon and employs R1-L1 and R1-L2 Gateway sites. The R1 and R2 sites flank a negative selection marker (pheS, a conditional negative selection marker; see below) on the intermediate vector plasmid(ampR, ZeoR) and the L1 and L2 sites flank a gene targeting element(eg. -SA-T2-LacZ-T2-neo-pA) on a second targeting element plasmid (ClonNATR).

The second system is used to switch the gene targeting vector component (homology arms plus gene targeting element) to a new high copy number plasmid backbone containing a mammalian negative selection gene and employs R3-L3 and R4-L4 Gateway sites. The R3 and R4 sites are located at the ends of the homology arms of the gene targeting vector and the L3 and L4 sites flank a negative selection marker(ccdB) on the new plasmid backbone(KanR or SpecR) which also contains a mammalian negative selection cassette.

These steps can be efficiently combined into a single reaction containing three component plasmids:

1. "Intermediate targeting vector" plasmid with R1/R2 and R3/R4 Gateway sites. The intermediate targeting vector is gene specific and contains the homology arms, targeting cassette and AsiSI vector linearization site. It can be used to construct many different potential final targeting vectors in a modular fashion.
2. Gene targeting element plasmid with L1 and L2 Gateway sites. This will be referred to as "L1L2 plasmid". Various types of these plasmids are matched to characteristic of gene/exon reading frame, presence of a signal sequence transmembrane domain, necessity for a promoter in the targeting construct etc.
3. High copy number plasmid backbone containing DTA negative selection cassette with L4 and L4 Gateway sites. This will be referred to as "L3L4 plasmid".

A diagram of the reaction and the final targeting vector obtained is shown below.



Protocol

Plasmid DNA Preparation:

The three way Gateway reactions are highly dependent on DNA quality, probably due to the presence of contaminating proteins which block Gateway sites. Consequently, the use of standard alkaline mini-preps is not recommended for these reactions. It is highly recommended using plasmid prepared by ion exchange based systems such as Qiagen Midipreps. Silica membrane based mini-preps such as Qiaprep Minipreps(Qiagen) can also be employed, in this case add the optional wash with buffer PB to improve DNA quality.

In all cases plasmids have to be resuspended in TE Buffer (10mM Tris pH 8.0, 1mM EDTA) rather than in water or buffer alone. For Qiagen mini-preps, TE can be substituted for the elution buffer contained in the kit.

LR Clonase Enzyme Mix:

LR clonase contains a mixture of bacteriophage lambda(Int and Xis) and E. Coli(IHF) proteins in a 5X concentrated buffer solution.

The formulation which gives the greatest colony numbers is Gateway LR Clonase II Plus Enzyme Mix, Cat. No. 12538-120 (Invitrogen). Do not confuse LR Clonase with BP Clonase which is designed for use with different Gateway sites!



Three Way Gateway LR Clonase Reaction:

Total Volume 10ul; all plasmids in TE

1. Mix:

60 ng L1L2 Targeting element plasmid

60 ng L3L4 negative selection cassette/high copy number backbone plasmid

200 ng R1R2/R3R4 Intermediate Targeting Vector

2. Bring volume to 8ul with TE

3. Add 2ul of LR Clonase II PLUS enzyme mix (vortex Clonase before adding)

4. Mix well

5. Incubate at 25 °C about 18 hr(overnight, exact time not critical)

6. Add 1ul Proteinase K solution (included with LR Clonase);
incubate 37°C for 30 min.

7. Transform 2ul of reaction into 50ul chemically competent E.coli using standard procedures. For example, library efficiency DH0B cells (10^9 colonies per ug). Heat shock cells for 45 sec at 42 °C.

8. Cool on ice for 2 min, and add 400ul SOC medium.

9. Grow for one hour at 37 °C in a shaking incubator.

10. Plate on YEG (see instructions below) containing antibiotic in L3L4 plasmid (either Kan or Spec in EUCOMM resources). Grow plates in 37 °C incubator overnight

11. Yield of colonies should be in the order of 10's-100's per 100ul plated.

Confirming identity of clones:

1. First digest the putative final targeting vector with the linearization enzyme AsiSI (available from New England Biolabs, R0630). A single band of >20kb should be present after a successful 3 way gateway reaction.

2. Build a model of the predicted final targeting vector using the sequence of the intermediate vector, L1L2 targeting cassette and L3L4 backbone. Complete intermediate vector annotated sequences are available at (www.sanger.ac.uk/htgt). Digest with an enzyme which cuts the genomic arms several times and within the targeting cassette to confirm an intact clone.



3. Sequence junctions/critical regions of the vector. In our own high throughput quality control, these include:

- The ends of the genomic homology arms at the junctions of the Gateway B3 and B4 sites.
- The sequences surrounding the loxP site
- The junctions between the gene targeting elements and the genomic DNA in the targeting arms

Notes:

- The plasmids are present in approximately 1:1:1 molar ratio in the reaction. Do not add too much of the intermediate vector plasmid, as this can lower reaction efficiencies.
- Carrying out the reaction in TE (as opposed to water) really does make a difference.
- The LR Clonase II mix is quite rich in glycerol so proper mixing is essential.
- Colony size should be fairly uniform. If you obtain a mixture of colony sizes, pick the larger ones. Large numbers of tiny colonies indicate an incomplete reaction and the formation of vector co-integrates.
- Additional bands are occasionally observed in final vector preps. These are often due to the co-transformation of L1L2 plasmid (which has no negative selection) or potentially do to instability of the final vector. Our practical experience has been that if the >20kb AsiI linearized correcting targeting vector band constitutes >50% of the mass, the plasmid is suitable for gene targeting.
- **NEGATIVE SELECTION**

The Gateway reactions are driven by two separate negative selection systems.

The insertion of the L1L2 gene targeting element is driven by conditional negative marker PheS. PheS is a mutant version of phenylalanine t-RNA synthetase which will charge the toxic Phe analogue chl-Phe. Its toxic effect is dominant over WT PheS. WT E.Coli will grow normally on YEG-CI plates containing chl-Phe while PheS containing cells will not grow.

The switch to the L3L4 backbone is driven by ccdB negative selection. ccdB is toxic(it targets DNA gyrase) in wild type E. Coli but ccdB expressing constructs can be tolerated in special strains of E. Coli like DB3.1 or ccdB Survival(both available from Invitrogen).

Cells must be ccdB- and PheS – to grow in WT E. Coli on YEG selection agar.



A Modular System:

As the Gateway system is modular, the intermediate targeting vector can be used to assemble not only containing L1L2 targeting elements/ L3L4 plasmid backbones supplied directly by the EUCOMM program, but also any components developed by users themselves. We can supply a basic L1L2 ClonNAT R vector which can be used to construct novel gene targeting elements and a cassette which can convert other plasmids into L3L4 vectors. Please contact us directly if you wish to further exploit the modular aspects of the system.

Recipe for preparing YEG-CL agar for PheS selection:

LOW SALT YEG-CL AGAR

PER LITER:

5g Yeast Extract

5g NaCl

2g D, L-p-Cl-Phe (Aldrich 13,071-0)(store at RT)

15 g Agar

Mix thoroughly before autoclaving. The Cl-Phe does not go into solution until autoclaving of the mixture.

After melting the agar add Glucose to 0.4% final concentration (from 20% sterile stock) and appropriate antibiotics when agar is below 55 °C.