

GATEWAY CLONING Seydoux Lab

For more information on Gateway cloning please consult

<http://www.invitrogen.com/Content/Online%20Seminars/gateway/gatewayhome.html>

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Step 1: Clone your favorite ORF into a Gateway entry vector.

The logic here is to PCR your ORF in such a way that it becomes flanked by attB recombination sites (attB1 and attB2) and then use these sites to recombine your ORF into pDONR201 to create an entry clone.

1. Design 5' and 3' GATEWAY primers to amplify ORF of interest.

1. 5' primer (5' to 3'): GATEWAY forward sequence (attB1), 2 bases*, 18 bases from the 5' end of your ORF.

*These two bases should NOT be AA, AG or GA otherwise you will create a STOP when adding the GATEWAY forward sequence. These extra bases are needed to put your ORF in the proper frame. Double check that your ORF is in the proper reading frame.

2. 3' primer (5' to 3'): GATEWAY reverse sequence (attB2), 18 bases from the 3' end of your ORF including STOP (**antiparallel**).

*Be sure to include a STOP codon at the end of your ORF to avoid translating the downstream recombination sequence.

2. PCR your favorite gene with these primers, and perform a BP reaction between the PCR product and Invitrogen's pDONR201 (see protocol below). The resulting clone will be your ORF's entry clone. It is a good idea to sequence the ORF in the entry clone before proceeding further to make sure no errors were introduced during the PCR. The primers we typically use to verify the sequence in the entry clone are:

5' - TCGCGTTAACGCTAGCATGGATCTC

3' - GTAACATCAGAGATTTTGAGACAC

Step 2: Transfer your ORF from the entry clone into the destination vector.

Once your ORF is in an entry clone, it is ready to be transferred by recombination to any GATEWAY destination vectors. The *pie-1* destination vectors are designed to create an in frame fusion between GFP and your ORF (pID3.01, pKR2.40) or between the *pie-1* ATG and your ORF (pID2.02). Always double check (at least on paper) that your ORF is in the correct frame and has a STOP codon at its 3' end *before* the GATEWAY recombination sequence.

1. Perform a LR reaction between your entry clone and one of the *pie-1* destination vectors (see protocol below). The resulting clone is ready for transformation into worms (see Seydoux lab Bombardment protocol if you are using the *unc-119* vectors).

Gateway Cloning BP Reaction

(ORF + pDONR201 = Kan^R Entry clone)

In a 1.5 ml microcentrifuge tube on ice, combine:

- 2 µl BP reaction buffer
- 1 µl Empty entry vector pDONR201 (see note below)
- 2 µl BP Clonase Enzyme Mix
- 1.25 µl PCR product
- 3.75 µl TE pH 8.0

Vortex to mix, briefly microcentrifuge, incubate at 25° for 1 hour

Add 1 µl Proteinase K, briefly vortex, briefly microcentrifuge, incubate at 37° for 10 minutes. Transform 1-5 µl of BP reaction into 50 µl aliquot of subcloning efficiency DH5 α competent cells. (The unrecombined pDONR201 will not grow in DH5 α cells since it contains a “death gene” which requires growth in DB3.1 cells).

Plate transformation on LB plates containing 50 µg/ml kanamycin. The Kan^R colonies are your entry clones.

Gateway Cloning LR Reaction

(Entry clone + Destination vector = Amp^R expression clone ready for transformation into worms)

In a 1.5 ml microcentrifuge tube on ice, combine:

- 4 µl LR reaction buffer
- 2 µl Empty destination vector (see note below)
- 4 µl LR Clonase Enzyme Mix
- 2.5 µl Entry clone (1mg/ml)
- 7.5 µl TE pH 8.0

Vortex to mix, briefly microcentrifuge, incubate at 25° for 1 hour

Add 1-2 µl Proteinase K, briefly vortex, briefly microcentrifuge, incubate at 37° for 10 minutes. Transform 1-5 µl of LR reaction into 50 µl aliquot of subcloning efficiency DH5 α competent cells. (The unrecombined destination vector will not grow in DH5 α cells since it contains a “death gene” which requires growth in DB3.1 cells).

Plate transformation on LB plates containing 50 µg/ml ampicillin. The Amp^R colonies are your final expression clone ready for transformation. (The original entry clone will not grow since it is kanamycin resistant, NOT Amp resistant.)

Notes

- CAUTION: Unrecombined pDONR201 and destination vectors must be propagated in DB3.1 bacteria and plated on plates supplemented with antibiotic (kanamycin for pDONR201, and ampicillin for destination vectors).

-Both BP and LR enzyme mixes are stored at -80°. Thaw on ice for 2min, gently tap the tube to mix, *quickly* return to -80° after use.

-BP buffer, LR buffer, and Proteinase K are stored at -20°.

-Both BP and LR reactions can be stored at -20° before transformation.