MULTISITE GATEWAY OVERVIEW

Creating entry clone:
Amplify 2 DNA fragments using primers with attB sites on them (see next page).

Recombination of attB and attP produces attL site. However, recombination of attBr and attPr produces attR site. These reactions are accomplished by BP Clonase enzyme.

Recombination of both fragments into a 2-component destination vector:
**STORAGE**

Vectors: -20
BP Clonase: -80 (6 months if -20)
LR Clonase: -80 (6 months if -20)

**GATEWAY PRIMERS**

<table>
<thead>
<tr>
<th>att sites flanking insert</th>
<th>Primer sequence</th>
<th>Entry vector</th>
</tr>
</thead>
</table>
| B4 and B1r                | F: GGGG ACA ACT TTG TAT AGA AAA GTT G  
                          | R: GGG AC TGC TTT TTT GTA CAA ACT TG | pDONR P4-P1r |
| B1 and B2                 | F: GGGG ACA AGT TTG TAC AAA AAA GCA GGC T |
                          | B: GGGG AC CAC TTT GTA CAA GAA AGC TGG GT | pDONR 221/207 |

**SEQUENCING ENTRY CLONES**

**pDONR 221 and pDONR P4-P1R:**

To check for mistakes use the following primers to check entry clones:
F: GTAAAAACGACGGCCAG  
R: CAGGAAACAGCTATGAC

**pDONR 207:**

F: TCGCGTTAAGCGCTAGCATGGATCTC  
R: GTAACATCAGAGATTTTGGAGACAC
PROTOCOLS

BP Recombination reaction:
1. Mix:
   a. AttB PCR product (20-50fmol) – 0.5-3.5ul
   b. PDONR vector (supercoiled, 150 ng/ul) – 0.5ul
   c. 1x TE Buffer, pH 8.0 – 4 ul
2. Vortex BP Clonase II briefly and add 1ul to the mixture.
3. Incubate at 25C for 1 hour.
4. Add 1ul of 2ug/ul Proteinase K and incubate at 37C for 10 min. (Important – improves efficiency by 100x)
5. Transform 2-3ul of the reaction into competent E. coli.

LR Recombination reaction
1. Mix:
   a. Entry clones (10 fmoles each) – 0.5-3.5 ul
   b. PDESTINATION (supercoiled, 20 fmol) – 0.5 ul
   c. 1x TE, pH 8.0 – 4 ul
2. Vortex LR Clonase briefly and add 1ul to the mixture.
3. Incubate at 25C for 16 hours (overnight)
4. Add 1ul of 2ug/ul Proteinase K and incubate at 37C for 10 min. (Important – improves efficiency by 100x)
5. Transform the whole reaction into competent E. coli.

Transformation of Library Efficient DH5 competent cells
1. Mix 50ul of cells with the reaction mixture
2. Leave on ice for 30 min.
3. Heat shock for 45 sec at 42C (DO NOT leave cells in the water bath for longer than 45 sec).
4. Add 500ul of SOC and incubate at 37C for 1 hour
5. Spread 150ul on LB plate with appropriate antibiotic.
### 6. SUMMARY OF AVAILABLE DESTINATION VECTORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Purpose</th>
<th>pDONR required</th>
<th>Selectable marker</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBGWFS7</td>
<td>Promoter analysis</td>
<td>pDONR 221</td>
<td>Bar, Sm/Spr</td>
<td>attR1/attR2, GFP and GUS</td>
</tr>
<tr>
<td>pKGWFS7</td>
<td>Promoter analysis</td>
<td>pDONR 221</td>
<td>Kan, Sm/Spr</td>
<td>attR1/attR2, GFP and GUS</td>
</tr>
<tr>
<td>pB7GWIWG2(II)</td>
<td>RNAi construct</td>
<td>pDONR 221</td>
<td>Kan, Sm/Spr</td>
<td>attR1/attR2</td>
</tr>
<tr>
<td>pK7GWIWG2D(II)</td>
<td>RNAi construct</td>
<td>pDONR 221</td>
<td>Kan, Sm/Spr</td>
<td>attR1/attR2</td>
</tr>
<tr>
<td>pK7GWIWG2(II)</td>
<td>RNAi</td>
<td>pDONR 221</td>
<td>Kan, Sm/Spr</td>
<td>attR1/attR2</td>
</tr>
<tr>
<td>pK7m24GW,3</td>
<td>2 fragment multisite</td>
<td>pDONR 221 / pDONR P4-P1r</td>
<td>Kan, Sm/Spr</td>
<td>attR2/attR4 Terminator</td>
</tr>
<tr>
<td>pH7m24GW,3</td>
<td>2 fragment multisite</td>
<td>pDONR 221 / pDONR P4-P1r</td>
<td>Hyg, Sm/Spr</td>
<td>attR2/attR4 Terminator</td>
</tr>
<tr>
<td>pBm42GW,3</td>
<td>2 fragment multisite</td>
<td>pDONR 221 / pDONR P4-P1r</td>
<td>Bar, Sm/Spr</td>
<td>attR2/attR4 No terminator</td>
</tr>
<tr>
<td>pB7m24GW,3</td>
<td>2 fragment multisite</td>
<td>pDONR 221 / pDONR P4-P1r</td>
<td>Bar, Sm/Spr</td>
<td>attR2/attR4 Terminator</td>
</tr>
<tr>
<td>pK2GW7</td>
<td>OX or antisense</td>
<td>pDONR 221/207</td>
<td>Kan, Sm/Spr</td>
<td>35S:: attR1-attR2</td>
</tr>
<tr>
<td>pK7GWIWG2(I)</td>
<td>RNAi</td>
<td>pDONR 221/207</td>
<td>Kan, Sm/Spr</td>
<td>35S:: attR1-attR2-intron-attR2-attR1</td>
</tr>
<tr>
<td>pB2WG7</td>
<td>OX or antisense</td>
<td>pDONR 221/207</td>
<td>Bar, Sm/Spr</td>
<td>35S::attR2-attR1</td>
</tr>
<tr>
<td>pB7WG2D</td>
<td>OX with visible marker</td>
<td>pDONR 221/207</td>
<td>Bar, Sm/Spr</td>
<td>35S::attR1-attR2</td>
</tr>
<tr>
<td>pMK7S*NFm14GW</td>
<td>Promoter analysis</td>
<td>pDONR P4-P1R</td>
<td>Kan, Sm/Spr</td>
<td>attR4-attL1::GFP-GUS</td>
</tr>
<tr>
<td>pK8WGIm24GW</td>
<td>Tissue specific RNAi</td>
<td>pDONR P4-P1R and pDONR 221</td>
<td>Kan, Sm/Spr</td>
<td>attR4-attR2-cat(intron)-attR2-attR1</td>
</tr>
</tbody>
</table>
Notes: 35S, KNAT1, UFO, STM, WUS, ML1, SUC2, CmGAS1, rolC and TobRB7 promoters are cloned into this plasmid and are flanked by L1 and L2 sites (refer to diagram on page 1)
pDONR 221

Notes: Preferred plasmid for multisite gateway reactions (is used to clone the gene of interest). See diagram on page 1.
Notes: This plasmid is used to clone 5’ (upstream) DNA fragment (e.g., promoter). See diagram on page 1.
UFO in pDONR 207

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L1   UFO   L2
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```
T2  T1
pUC ori

pDONR™207

Gentamicin
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ML1 in pDONR 207
KNAT1 in pDONR 207

L1  KNAT1  L2

T2  T1

pUC ori  Gentamicin

pDONR™ 207
SUC2 in pDONR 207
TobRB7 in pDONR 207
WUS in pDONR 207

pDONR™ 207

pUC ori

Gentamicin

T1, T2
35S in pDONR 207

pDONR™ 207

Gentamicin

pUC ori

T2 → T1
pBGWFS7

Notes: Promoter analysis. Drives expression of GFP and GUS.

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Notes: 2-fragment multisite cloning vector. Use pDONR P4-P1r to clone 5' (upstream) DNA fragment (eg. Promoter) and pDONR 221 to clone 3' (downstream) DNA fragment (eg. gene). See page 1 for diagram.

pH7m24GW,3

Notes: 2-fragment multisite cloning vector. Use pDONR P4-P1r to clone 5' (upstream) DNA fragment (eg. Promoter) and pDONR 221 to clone 3' (downstream) DNA fragment (eg. gene). See page 1 for diagram.

Notes: 2-fragment multisite cloning vector. Use pDONR P4-P1r to clone 5’ (upstream) DNA fragment (eg. Promoter) and pDONR 221 to clone 3’ (downstream) DNA fragment (eg. gene). See page 1 for diagram.

Important – this plasmid does NOT have a terminator.

Notes: 2-fragment multisite cloning vector. Use pDONR P4-P1r to clone 5' (upstream) DNA fragment (eg. Promoter) and pDONR 221 to clone 3' (downstream) DNA fragment (eg. gene). See page 1 for diagram.

The website where the plasmid was ordered lists it as having NO marker, however, the map shows Bar.

Overexpression or antisense

Hairpin RNAi expression

Overexpression or antisense

Overexpression together with a visible marker

pMK7S*NFm14GW

Promoter analysis plasmid for sequences cloned into pDONR P4-P1R

2-fragment destination vector for tissue specific expression of hairpin RNAi