

The Drosophila Gateway™ Vector Collection

A resource developed by the Murphy lab for the Drosophila research community

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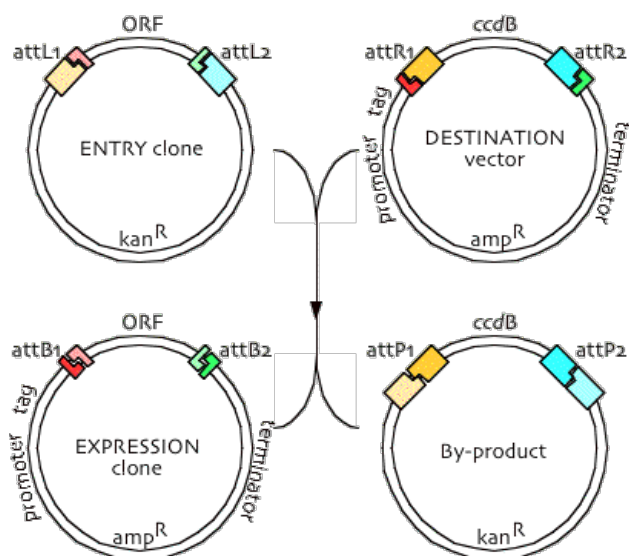


Figure 1 . The Gateway LR in vitro recombination reaction

Overview

The Drosophila Gateway™ Vector collection is a set of 68 Gateway-based vectors designed to express epitope-tagged proteins in Drosophila culture cells or flies. At its core is [Invitrogen's Gateway™](#) recombination cassette, which allows you to recombine an Open Reading Frame (ORF) of interest into any of the vectors using a simple and efficient in vitro reaction. The result is a fusion gene with your ORF placed in frame with one of 7 different epitope tags and expressed by one of 4 different promoters.

Gateway™ technology uses lambda integrase to recombine your ORF, flanked by attL1 and attL2 recombination sites, with the attR1 and attR2 recombination sites of a destination vector ([figure 1](#)). The result is a "swap" of your ORF with the cassette containing the *ccdB* gene in the destination vector. Successfully recombined expression clones can be selected based on their resistance to ampicillin and lack of toxicity to standard laboratory strains of *E. coli* (the *ccdB* gene product is toxic, which prevents the original destination vector from forming colonies).

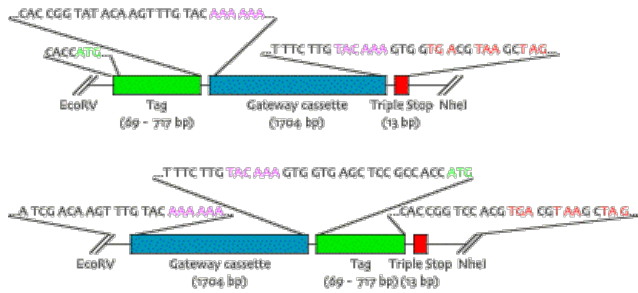


Figure 2 . N- and C-terminal Tag/Gateway fusion modules

The Drosophila Gateway Vector collection

We have constructed a set of 17 Gateway/Tag modules (Figure 2 and Table 1) for use in making different destination vectors. Each module contains one of seven epitope tags placed either 5' or 3' of the Gateway cassette, followed by stop codons in all three reading frames. These modules can be subcloned into any vector containing a promoter and terminator of interest as an EcoRV (blunt) - NheI (XbaI compatible) restriction fragment. This subcloning step is facilitated by the presence of a *chlR* gene within the Gateway cassette, allowing the desired clones to be selected based on their resistance to chloramphenicol.

| N-terminal modules | | C-terminal modules | | tag description (abs / em), source |
|--------------------|--------------|--------------------|--------|---|
| name | tag | name | tag | |
| GW | EGFP | WG | EGFP | GFP (489 / 509 nm), Clontech |
| CW | ECFP | WC | ECFP | CFP (435 / 475 nm), Clontech |
| VW | Venus | WV | Venus | improved YFP (515 / 528 nm), Nagai et al. 2002 |
| RW | mRFP | WR | MRFP | monomer RFP (584 / 607 nm), Campbell et al. 2002 |
| HW | 3xHA | WH | 3xHA | 3 HA epitopes |
| MW | 6xMyc | WM | 6xMyc | 6 Myc epitopes |
| FW | 3xFLAG | WF | 3xFLAG | 3 FLAG epitopes followed by enterokinase cleavage site, Sigma |
| FHW | 3xFLAG-3xHA | | | tandem FLAG / HA epitopes separated by enterokinase cleavage site |
| FMW | 3xFLAG-6xMyc | | | tandem FLAG / Myc epitopes separated by enterokinase cleavage site |
| W | none | | | |

Table 1. The 17 Gateway/Tag modules

The 17 modules have two or three letter designations, using combinations of a one-letter abbreviation for each epitope and "W" to stand for the Gateway

cassette. Thus, "GW" designates an EGFP tag placed 5' of the Gateway cassette, suitable for producing N-terminal fusions.

| name | abbr. | promoter | terminator | P-element? | antibiotic resistance | recommended use |
|------------------|-------|------------------|------------|------------|-----------------------|--|
| Actin5C | pA | Actin5C | SV40 | NO | <i>ampR</i> | tissue culture |
| Hsp70 | pH | Hsp70 | SV40 | NO | <i>ampR</i> | tissue culture |
| UAS _t | pT | UAS _t | SV40 | YES | <i>ampR</i> | GAL4-driven somatic expression in vivo |
| UAS _p | pP | UAS _p | K10 | YES | <i>ampR</i> | GAL4-driven somatic and female germline expression in vivo |

Table 2 . The four vectors currently available with the 17 Gateway/Tag modules

The 17 modules are currently available in four different vectors (Table 2). The Actin5C- and Hsp70-based vectors (Huynh and Zieler, 1999) are intended for high and moderate levels, respectively, of transient expression in tissue culture cells. The UAS_t vectors contain a GAL4-responsive promoter for expression in vivo in cells expressing GAL4 (Brand and Perrimon, 1993). The UAS_p vectors contain a GAL4-responsive promoter modified to allow expression in the female germline in addition to somatic cells (Rorth, 1998), although anecdotal evidence suggests that UAS_p vectors may have some basal expression in the absence of GAL4 and induce less strongly in response to GAL4 than UAS_t vectors.

How to get the vectors

Vectors are available from the [Drosophila Genomics Resource Center at Indiana University](#).

It has been a tedious and time-consuming process to arrange for permission to distribute the vectors from the various companies that hold applicable patents (Invitrogen, Sigma, Amersham, Aurora Biosciences, Riken Brain Science Institute, and UCSD/HHMI). Most of the arrangements have been finalized, with the exception of Amersham, which has reneged on their initial approval. It has also been impossible to make any progress with Amersham's lawyers due to the pending sale of Amersham to GE. Unfortunately, Amersham has legal control over distribution of GFP-based vectors, precluding us from distributing 24 of the 68 vectors at this time. We are continuing to work on ways around this legal roadblock and are hopeful that this barrier can be resolved, but we have no idea how long these negotiations will take. We also do NOT have blanket permission to distribute the mRFP-based vectors; however, interested labs can contact Roger Tsien and sign an individual MTA with HHMI/UCSD after which we can provide the 8 mRFP-based vectors.

As a further complication, we had planned on providing the entire collection of vectors as a microtiter plate of glycerol stocks, which were prepared LONG ago and currently sit in our freezer. Given that many people have been awaiting these vectors for 6 months or more, we can ship DNA of a small number of FLAG-, myc-, HA-, or mRFP-based vectors to not-for-profit institutions upon request. Information on obtaining a Material Transfer Agreement can be found [here](#).

Note that, under the terms of the MTA, you are not allowed to further distribute these vectors to parties that have not also signed an MTA with the Carnegie Institute. New labs and departing post-docs and students that want to take copies of these vectors should fill out their own MTA so that we don't get sued.

Organization of the vectors

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|--|------|------|------|------|------|------|-------|-----|---|----|------|----|
| | pAGW | pACW | pAVW | pAHW | pAMW | pAFW | pAFHW | pAW | | | pARW | |

| | | | | | | | | | | | |
|----------|------|------|------|------|------|------|-------|-----|--|--|------|
| A | | | | | | | | | | | |
| B | pAWG | pAWC | pAWV | pAWH | pAWM | pAWF | pAFMW | | | | pAWR |
| C | pHGW | pHCW | pHVW | pHHW | pHMM | pHFW | pHFHW | pHW | | | pHRW |
| D | pHWG | pHWC | pHVV | pHWH | pHWM | pHWF | pHFMW | | | | pHWR |
| E | pTGW | pTCW | pTVW | pTHW | pTMM | pTFW | pTFHW | pTW | | | pTRW |
| F | pTWG | pTWC | pTWV | pTWH | pTMM | pTWF | pTFMW | | | | pTWR |
| G | pPGW | pPCW | pPVW | pPHW | pPMW | pPFW | pPFHW | pPW | | | pPRW |
| H | pPWG | pPWC | pPWV | pPWH | pPMM | pPWF | pPFMW | | | | pPWR |

Table 3 . Organization of vectors in the microtiter plate

The vectors are organized in a microtiter plate ([table 3](#)). All vectors are resistant to ampicillin (in the vector backbone) and chloramphenicol (in the Gateway cassette). Plates that include the mRFP-based vectors will only be sent to labs that have signed a separate MTA with Roger Tsien; otherwise column 11 will be omitted. We have taken every precaution to ensure that the proper clones are in each well; however, **we strongly recommend that you verify the structure of the destination vectors you are using before proceeding with your experiments**. Suggestions for enzymes to cut vectors with specific epitopes are in [table 4](#), and the predicted restriction fragment sizes are available in TEXT and JPEG files listed with the sequence files ([table 7](#)).

| epitope | enzyme |
|---------|----------|
| GFP (1) | none (2) |
| CFP (1) | none (2) |
| Venus | PstI |
| 3xHA | NdeI |
| 6xMyc | NcoI |
| 3xFLAG | ClaI |
| mRFP | PstI |

(1) EGFP and ECFP cannot be distinguished from each other except by sequencing, and are also very similar to Venus with the exception of a PstI site in Venus.

(2) No useful cut sites exist in EGFP and ECFP, but can be distinguished based on size relative to the other epitopes.

Table 4 . Restriction enzymes with sites in the listed epitope for use in verifying destination vectors

FAQs

I strongly recommend thoroughly reading the [Gateway™ Technology manual](#) from Invitrogen. The information we've provided here is only intended to demonstrate how we routinely use the Gateway system in our lab.

How do I flank my ORF with attL1 and attL2 recombination sites?

Several options are available to produce an Entry clone with your ORF positioned in the proper reading frame. We routinely amplify the desired ORF and clone it into an Entry vector by a topoisomerase-catalyzed reaction (pENTR/D-TOPO Cloning Kit, Invitrogen catalog # K240020). Primers are designed with the sequence CACC on the 5' end of the 5' primer followed by the gene specific sequence in the first reading frame. The 5' end of the 3' primer should end with a complete codon ([Figure 3](#)). If you plan on making C-terminal fusions, your PCR product must include an ATG, typically immediately after the CACC tail, and must not contain a stop codon. Note that the CACC sequence is a consensus Kozak site and will be included in the final clone. The resulting construct

can also be used to make N-terminal fusions, but will contain a short tail at the C-terminus which can be detrimental to some proteins.

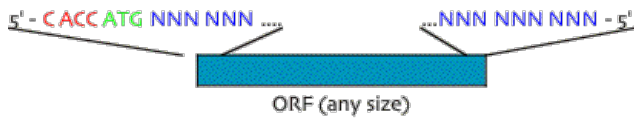


Figure 3 . PCR product design for TOPO cloning

The resulting Entry clone will have the sequence shown in [figure 4](#), where the AAA-AAA and TAC-AAA sequences represent the core of the attL1 and attL2 recombination sites.

For the PCR reaction, we routinely use a proofreading polymerase such as Pfx (Invitrogen catalog # 11708-021) to amplify from a cDNA, and have observed a PCR-induced error rate of ~1 / 15,000 bases after cloning from a 25-cycle amplification. In our hands, we need to use a much lower concentration of MgSO₄ in the PCR reaction (0.25 mM) than recommended by Invitrogen in order to get a PCR product.

We have found that the TOPO cloning reaction isn't as efficient or directional as claimed by Invitrogen. We recover a median of 42 colonies (range 17 - 356), of which a median of 25% (range 8 - 56%) contain the PCR product cloned in the correct orientation. However, the colonies can be rapidly screened by colony PCR using a vector specific and ORF specific primer. Afterwards we miniprep and sequence verify a single clone, which becomes our master Entry clone for recombination into the desired Destination vectors.

Entry clones can also be produced by including attB1 and attB2 sequences in the PCR primers and using a BP recombination reaction to recombine the attB sites with attP sites in a donor vector, resulting in an entry clone with your ORF flanked by attL1/attL2 recombination sites. This strategy can also be used to directly recombine your ORF into a Destination vector by sequentially carrying out a BP followed by a LR recombination reaction, as described in the Gateway manual. However, we prefer to use TOPO cloning to avoid ordering primers that include the 29-bp attB1/attB2 sequences. We also prefer sequence verifying the Entry clone which can then be used with many different Destination vectors.

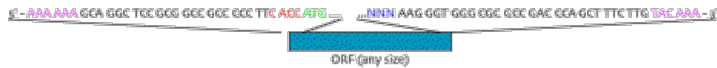


Figure 4 . Entry clone sequence after cloning into pENTR/D-TOPO

How do I ensure that my ORF will be in the proper reading frame after recombining into a destination vector?

As long as your ORF is in frame with the AAA-AAA sequence at the 5' end and the TAC-AAA sequence at the 3' end (e.g., [Figure 4](#)), the resulting clone will be in frame with either N-terminal or C-terminal epitope tags.

What materials do I need to use the Drosophila Gateway Vector Collection?

To make an Entry clone containing your ORF using pENTR/D-TOPO:

- pENTR/D-TOPO Cloning Kit (Invitrogen catalog # K240020, 20 reactions, \$20 / rxn), which includes TOP10 competent cells
- cDNA of your favorite gene (DGC clone or equivalent, \$20 per clone + shipping from [BACPAC](#))
- PCR primers to amplify the ORF (~\$12 / pair)
- Proofreading polymerase (e.g. Pfx, Invitrogen catalog # 11708-021 or equivalent, \$0.80 / rxn)
- LB-agar plates containing 50 ug/ml kanamycin
- Primers to sequence verify longer ORFs (>1000 bp) after cloning

We occasionally perform half-reactions of the TOPO cloning step to reduce costs, although this results in a 3 ul reaction volume which can be hard to manage.

To recombine an Entry clone with a Destination clone:

- miniprep DNA of an Entry clone containing your ORF
- miniprep DNA of the desired Destination vector
- Gateway LR Clonase Enzyme Mix (Invitrogen catalog # 11791-019, 20 reactions or 11791-043, 100 reactions; \$12.50 to \$14.75 / rxn)
- Library Efficiency DH5alpha competent cells (Invitrogen catalog # 18263-012 or equivalent; \$2.50 / 20 ul)
- LB-agar plates containing 100 ug/ml ampicillin

We routinely perform half-reactions (10 ul total volume) of the LR Clonase step to reduce costs, and recently had success with quarter-reactions (5 ul total volume).

Which destination vector should I use?

The beauty of this system is that you can easily make several constructs for different purposes. For example, we initially examine protein localization in

Drosophila KC cells using pHGW and pHWG (Hsp70 promoter, GFP fusions at either end). This way we can test which end of the protein better tolerates the epitope tag. Proteins that we want to further analyze in vivo are then swapped into pTGW or pTWG. The HA, Myc, and FLAG epitope tags are all useful for immunoprecipitations in culture or in vivo (we have only used the HA tag in tissue culture experiments).

We are just beginning to use the Venus and mRFP constructs. The Venus tag (EYFP) should be more versatile than EGFP -- it is brighter, works with standard GFP filter sets, and can be easily spectrally separated from ECFP for double labeling experiments. Consequently, we anticipate that it will become our main tag for use in transgenic flies. mRFP fluorescence is relatively weak, but it doesn't have the aggregation and toxicity problems of DsRed. We have successfully used it to look at protein localization in culture cells, but we expect that it will be more difficult to detect in flies, making it more practical to use Venus and ECFP for double-labeling experiments. We are currently experimenting with the tandem-dimer RFP and other red fluorescent proteins which may give useful increases in fluorescent intensity, but these are unlikely to be added to the vector collection until early 2004.

Invitrogen sells many other useful [destination vectors](#), although in some cases you may want to engineer your own. For example, they sell a N-terminal GST fusion vector for expression in *E. coli* (pDEST 15, Invitrogen catalog # 11802-014), but this vector lacks a cleavage site between the GST and your protein.

Invitrogen also sells yeast two-hybrid vectors (ProQuest, Invitrogen catalog # 10835-031), but they are low copy number vectors and we have had mixed success in obtaining sufficient expression levels to see an interaction.

I have an older Gateway manual that says I need to linearize the destination vector before in vitro recombination. Is this necessary?

No. Somewhere along the way Invitrogen changed their opinion on whether linearizing or relaxing your plasmids is necessary. The recombination reaction works fine with two supercoiled plasmids.

How can I sequence verify my expression clones?

In our hands the Gateway recombination reaction has been very reliable so we typically don't sequence-verify our expression clones. But before making transgenic flies you may want to sequence across the tag-gene junction to verify that the reading frame is intact. You may find the primers in [table 5](#) and [figure 5](#) useful.

| name | sequence | vector | purpose |
|-------|------------------------|--------------------|--|
| ACTf | GAGCATTGCGGCTGATAAGG | pA | from actin5C into 5' end |
| HSPf | TATAAATAGAGGCGCTTCGT | pH, pT | from hsp70 into 5' end |
| UASPf | GGCAAGGGTCGAGTCGATAG | pP | from UASp into 5' end |
| SVr | GGCATTCCACCACTGCTCCC | pA, pH, pT | from SV40 into 3' end |
| K10r | TGGTGCTATGTTTATGGCGC | pP | from K10 into 3' end |
| GW5f | ATCGAGGCCTGTCTAGAGAAGC | all | EcoRV site into 5' end (through tag) |
| GW3r | CTAGCTTACGTCACGTGGACC | C-terminal fusions | NheI site into 3' end (through tag) |
| GFPf | GGAGTACAACTACAACAGCC | G, C, V tags | middle towards 3' end, verify N-terminal G, C, V fusions |
| GFPPr | CTTCGGGCATGGCGGACTTG | G, C, V tags | middle towards 5' end, verify C-terminal G, C, V fusions |
| RFPf | GCTGCGCGGCACCAACTTCC | R tags | middle towards 3' end, verify N-terminal R fusions |
| RFPPr | GGACAGCTTCAAGTAGTCGG | R tags | middle towards 5' end, verify C-terminal R fusions |

Table 5 . Sequencing primers to verify recombinant clone reading frames

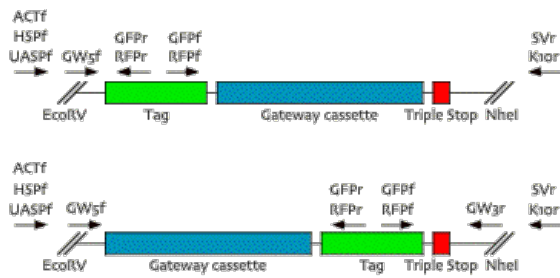


Figure 5 . Location of sequencing primers in N-terminal and C-terminal Tag/Gateway modules

What will be the amino acid sequence of my resulting protein?

Sequence files are available below for all the destination vectors ([table 7](#)). We typically construct a predicted sequence for each Entry clone (which is used for the sequence verification), and "subclone" the ORF into the desired destination vector by configuring Sequencher to treat the attL1/attL2 and attR1/attR2 recombination sites as large restriction sites. You can then easily extract the predicted amino acid sequence.

When using Entry clones made by topo-cloning into pENTR/D (as described above), the resulting amino acid sequence will be:

N terminal fusions: M - tag - HRYTSLYKKAGSAAAPFT - *your gene* - KGGRADPAFLYKVVVT*

C terminal fusions: *your gene* - KGGRADPAFLYKVVSSAT - tag - HRST*

The linkers are produced by flanking sequences in the pENTR/D vector and the attB1/attB2 recombination sites remaining in the final Expression clone. The extra C-terminal tail on N-terminal fusion proteins can be eliminated by including a stop codon at the end of your ORF prior to cloning into an Entry vector, but the resulting clone cannot be used to make C-terminal fusions.

What antibodies can I use to detect the different epitopes?

We have used the following antibodies ([Under Construction](#)):

| epitope | antibody | species | source | comments |
|---------|----------|---------|-------------------|--------------------------------|
| EGFP | | | | |
| ECFP | | | | |
| Venus | | | | |
| mRFP | | | | |
| Myc | 9E10 | mouse | Santa Cruz sc-40 | clean on Westerns, IPs, IF |
| Myc | A14 | rabbit | Santa Cruz sc-789 | lots of background on Westerns |
| HA | | | | |
| FLAG | M2 | mouse | Sigma F3165 | clean on Westerns, IPs, IF |

Table 6 . Antibodies for use with different epitope tags

If you have success (or failure) with other antibodies, please let me know and I'll update this list.

I have other questions. Where can I find answers?

The best place to start is the [Gateway™ Technology manual](#) from Invitrogen. If there are other questions and answers that would be helpful to include here, please let me know.

Vector sequence files

Sequence files are available for all vectors in several formats. These sequences were originally assembled using [Gene Construction Kit 2.5](#), a robust clone construction and annotation program available from [TextCo](#) for both Macintosh OS X and Windows. Consequently, the GCK files are more extensively annotated than the other file formats. The [Filemaker Pro 6](#) database contains all the vector sequences and locations of basic features such as the Gateway™ cassette, epitope tags, promoters, terminators, and antibiotic resistance genes. This database was used to make three sets of text files: 1) annotated files with the locations of basic sequence features followed by the vector sequence; 2) FASTA files with only the vector name and sequence; and 3) files with only the vector sequence (suitable for importing into Sequencher). All files are provided in both [Stuffit](#) and Zip compression formats.

| file type | .sit | .zip |
|-----------------------------------|--|--|
| Sequence Files | | |
| Filemaker Pro 6 database | FP6.sit (240 kb) | FP6.zip (308 kb) |
| Gene Construction Kit 2.5 | GCK.sit (380 kb) | GCK.zip (404 kb) |
| Annotated text files | Annotated.sit (192 kb) | Annotated.zip (216 kb) |
| FASTA text files | FASTA.sit (176 kb) | FASTA.zip (196 kb) |
| Sequence only text files | DNA.sit (176 kb) | DNA.zip (196 kb) |
| Restriction Fragment Sizes | | |
| Gene Construction Kit 2.5 | GCK.sit (12 kb) | GCK.zip (12 kb) |
| text files | text.sit (8 kb) | text.zip (8 kb) |
| JPEG files | jpeg.sit (216 kb) | jpeg.zip (164 kb) |

Table 7 . Downloadable versions of sequences and restriction maps for all Drosophila Gateway Vectors

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