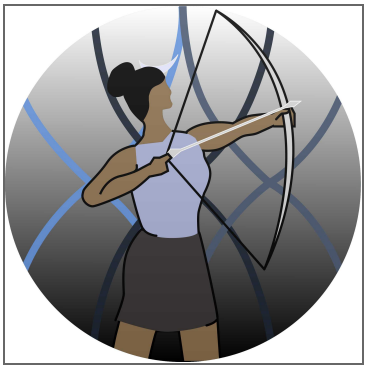

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Janelia Atalanta series plasmid cloning V.2

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ABSTRACT

The Janelia Atalanta plasmids allows simple cloning of a gRNA and homology arms for CRISPR/Cas9 mediated homology directed repair in Drosophila. Gateway-compatible arms are synthesized with appropriate attL recognition sequences and homology arms required for homology directed repair. One, or two, of the arms also encodes a sgRNA flanked by tRNA sequences, which is expressed at high levels from a U6 promoter. The sgRNA is spliced out of the transcript by endogenous tRNA maturation cellular machinery.

Protocol status: Working
We use this protocol and it's working

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Make plasmid DNA

1 Transform the pJAT into ccdB Survival cells following the standard protocol. Plate cells on LB _ Ampicillin (100ug/mL) and grow at 37°C at least 15 hours.

2 Pick one colony and grow in 3mL LB + antibiotics. I normally grow in Ampicillin, but the plasmid encodes three antibiotic resistance genes, and any single antibiotic—Ampicillin, Chloramphenicol, or Spectinomycin—is fine. Grow overnight shaking at 225RPM at 37°C. Perform standard mini-prep next day.
Antibiotic concentrations:
Ampicillin: 100 ug/mL
Chloramphenicol: 25 ug/mL
Spectinomycin: 50 ug/mL

Design and synthesize attL Gateway cloning arms

3 Using computational tools, such as https://www.flyrnai.org/crispr3/web/, identify a predicted high efficiency gRNA target site in the genome of interest. Identify homology arms that precisely, or nearly precisely flank the expected Cas9 cut site. It is optimal that the payload integrated during homology directed repair destroys the gRNA target site in the genome. I have not performed a comprehensive survey of effective homology arm lengths, but have not observed a difference in efficiency between arms containing 250 bp and 1000 bp.

4 Using sequence editing software cut and paste the 20 gRNA target recognition sequence and the left and right homology arms into the provided template sequences.
IMPORTANT: The gRNA sequence should be the 20 bp target sequence, excluding the NGG PAM sequence. The experiment will fail if you include the NGG sequence.
Double check to make sure you have inserted all the sequences in the correct orientation! For example, the gRNA should be in forward direction relative to target gRNA sequence (not necessarily in forward direction relative to the genomic target). In addition, the orientation of the homology arms in the synthesized attL fragments should match their orientation in the genome.

Some software "flips" sequences extracted from the genome, so double check that he orientation is as expected.

4.1

The attL pieces required for assembly are provided as attached files in Geneious and Genbank format. Ensure that the attL3 and attL4 (and, similarly, the attL5 and attL6) are oriented inwards, towards each other.
Replace the gRNA sequence indicated with your target 20bp gRNA. Then concatenate
attL3 + Homology Arm Left + attL4
and
attL5 + Homology Arm Right + attL6

attL_pieces.geneious attL_pieces.gb

4.2 Attached are examples of actual attL arms ordered from Twist for cloning into a pJAT plasmid and that were cloned successfully. The Genious file of the left homology arm includes annotations of the tRNAs and NNN placeholders for the gRNA sequence and homology arm.
The tRNA sequences can be replaced with other tRNAs, for example if working in different species and want to use species-specific tRNAs. We have observed high efficiency integration after replacing tRNAs. There are 20bp upstream of the second second tRNA (right after the gRNA scaffold) that were unlabelled in the original design. According to Fillip Port, these are part of a tRNA segment, and so it should be possible to remove them if changing out a tRNA gene. However, we have left these 20bp and changed the region marked as the second tRNA and observed high efficiency integration,

2R_5.gb 2R_5.geneious

4.3 Attached are example attL arms used for pBac-mediated scarless genome manipulation. The pBac arms are included in the synthesized arms, as illustrated in Figure 4 of Stern et al (2023), including a duplication of the target TTAA site. Everything between the TTAA sites, including the marker gene, can be removed by driving expression of pBac transposase. Genome modifications can be included in the homology arms flanking the pBac arms.

attL-pBac-attR.gb attL-pBac-attR.geneious

5 Synthesize the left and right homology arm pieces with the attL sites. I use Twist to synthesize arms and leave their adaptor sequences on. This is cheaper than removing the adaptors and the adaptors are removed during Gateway cloning. Also, Twist synthesis often fails if you specify adaptors off, probably because the repetitive attL sequences disrupt synthesis. Sometimes the Twist QC step will flag the sequence as too complex to be sequenced. This usually results from regions containing high variance in GC content. I have found that you can edit these AT or GC rich regions in the homology arms with one or a few mutations to balance the GC content. DO NOT edit the attL sites or the tRNA-gRNA-tRNA region!

Gateway cloning

6 Perform Gateway cloning reaction

1 uL 100 ng/uL double Gateway plasmid
0.5 uL 50 ng/uL attL3-tRNA-gRNA-LeftHomologyArm-attL4 synthesized dsDNA
0.5 uL 50 ng/uL attL5-RightHomologyArm-attL6 synthesized dsDNA
0.25 uL LR Clonase II enzyme mix (Thermo Fisher)

Incubate at least 2 hours at 25°C in thermocycler with heated lid.
Note 1: The published protocol specifies 1 hour incubation, but the double Gateway reaction is less efficient than single Gateway cloning and I have found that a longer incubation improves cloning efficiency. I often leave these reactions overnight.
Note 2: Thermo Fisher sells a Gateway Clonase that is advertised to be optimized for cloning multiple attL fragments into a single plasmid (as we are doing here). The enzyme is twice as expensive as the normal Clonase. So far, I have always had success with the original Clonase, so I haven't tried the optimized enzyme.

7 0.125 uL Proteinase-K (from Clonase kit)

Incubate 37°C 20 min in thermocycler with heated lid.
We have observed that this step does not appear to be critical for successful cloning.

8 Gently mix the entire ~2.25 uL reaction with 50 uL of DH5alpha (or equivalent) competent cells. I routinely use Zymo Mix & Go! competent cells which seem to give me higher cloning efficiency than other leading brands and are *considerably cheaper* than other brands. (For further cost savings, you can split the supplied 50uL aliquots into two 25 uL aliquots by gently pipetting 25uL into a new tube on ice.) I routinely see hundreds of colonies on plates. I very occasionally see only a few colonies, but they are usually correct.

9 Let mixture site on ice for 30 min

10 Gently, but rapidly move tube containing cells to 42°C block or water bath for 30 sec without shaking.

11 Move tube to ice for a few minutes.

12 Add 150 uL SOC

13 Tape tube on its side to base of a 37°C shaking incubator and shake at 300 RPM for 1 hour

14 Plate 100 uL on Agar plates containing Tryptone-Yeast extract + Spectinomycin + Sucrose and grow overnight at 37°C.

(Surprisingly, we have found, accidentally, that heat shock and outgrowth are not absolutely required for successful cloning.)

14.1 To make Tryptone-Yeast + Sucrose plates

Make the agar solution:

10 g tryptone
5 g yeast extract
15 g agar (or whatever amount you typically use per liter)
750 ml water
Autoclave

Separately make the sucrose solution:

100 g sucrose
250 ml water
Filter sterilize using a 0.2 micron filter.

After the autoclaved agar has cooled, but before it sets, mix the sucrose solution into the agar and add appropriate volume of antibiotics (in this case Spectinomycin to 100ug/mL) and pour plates.

15 Pick colonies into LB + Spectinomycin, grow overnight at 37°C with shaking and perform miniprep. QC plasmid either by whole plasmid Nanopre sequencing or by sequencing just the ends of the integrated arms with SP6 and T7 primers.