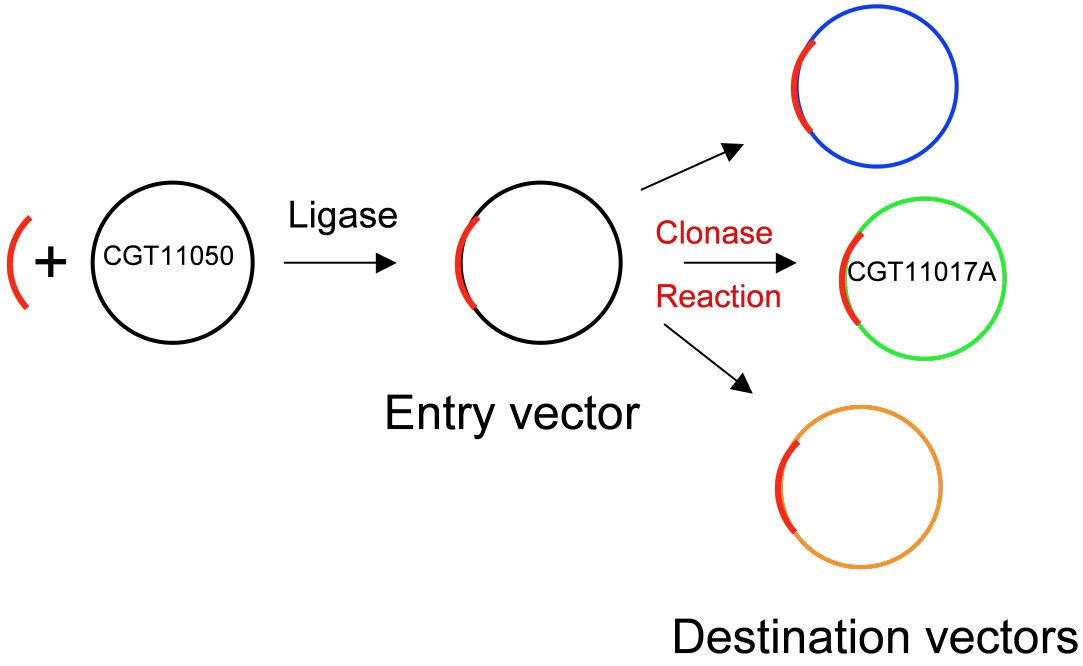
Gateway Cloning Protocol

(Clough Lab Edition)

This document is a modification of the Gateway cloning protocol developed by Manju in Chris Taylor's lab

With the Gateway cloning system, a PCR fragment is first cloned into an Entry Vector using standard cloning techniqes (i.e. DNA ligase). The resulting clone may then be transferred to one or more functional Destination Vectors using the LR clonase enzyme. The LR clonase cloning step is very easy and efficient, requiring little time or effort.



Overview of Gateway cloning process

1. Perform PCR of gene of interest.

2. Clone PCR product into entry vector (CGT11050) using traditional methods (ligase).

3. Verify PCR insert by restriction digest, PCR, and/or DNA sequencing.

4. Clone insert into destination vector(s) using LR Clonase ezyme.

5. Verify destination vector clone by PCR.

6. Transform destination vector into agrobacterium (e.g. *A. rhizogenes*).

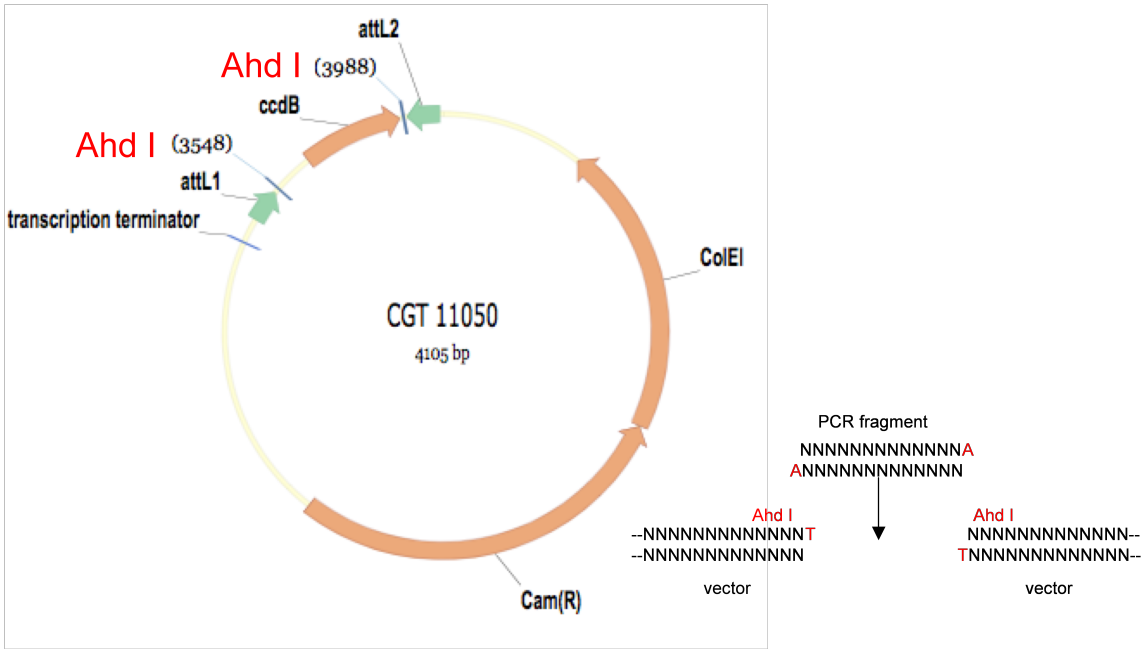
7. Verify agro clone by PCR.

8. Transform soybean tissue (e.g. roots) with agro clone.

9. Assay for gene silencing or transgene expression.

1. Entry Vector Preparation

Entry vector CGT11050 is cut with **Ahd I*** to yield cloning site for PCR fragment.



- Cutting with Ahd I results in a 3' T overhang
- Taq Polymerase (PCR) results in a 5' A overhang

* Note that Ahd I cuts CGT11050 twice, resulting in <u>two fragments</u>: 440 bp and 3665 bp. We do not worry about purifying the 3665 vector fragment from the 440 bp fragment.

<u>Minipreps to obtain CGT11050 vector</u>

CGT11050 plasmid prep yield is low, so you need to compensate.
Performing several minipreps seems to give a better yield than one midi-prep.
Grow *four* 5 ml cultures of CGT11050 in LB media + Chloramphenicol.
Use 5 ml for each miniprep.
Using a 2ml tube, add 1.5 ml culture, spin 2 min @12,000 rpm,
discard supernatant; add 1.5 ml to same tube, spin, discard, etc.
until all 5 ml of culture are spun down into one tube.
Use Qiaprep Spin Miniprep Kit to purify the plasmid.
Warm elution buffer EB to 70^{o} C to maximize elution efficiency.
You should obtain appx. 100ng/ul of plasmid when eluted in 50 ul.

Ahd I digest of CGT11050 vector

DNA:	30ul (appx. 3 ug; use more or less accordingly)
10x Buffer:	10 ul
100x BSA:	1 ul
Ahd I:	3 ul
H_2O:	57 ul
Total:	100 ul

Incubate at 37° C for 2 hours.
Heat-kill enzyme at 65° C for 20 minutes.
Add 100 ul P:C:I (25:24:1)
Vortex for 1 minute
Spin @13,000 rpm for 10 minutes
Transfer the aqueous layer to a new 1.5ml tube
Add 50 ul C:I (24:1)
Vortex for 1 minute
Spin @13,000 rpm for 5 minutes
Transfer the aqueous layer to a new 1.5ml tube
Check to see if any of the chloroform transferred; if it did, remove it from the bottom of the tube with a pipet.
Add 1/10th volume of 3M Sodium Acetate (usually you will recover 80-90 ul of the aqueous layer after the extractions; bring the total volume to 90 ul and then add 10 ul of Sodium Acetate)
Add 3 volumes of Etanol (usually 300 ul)
Incubate at -20° C for 1 hour to precipitate DNA
Spin @13,000 rpm for 10 minutes, then discard supernatant
Wash with 200 ul of 70% Et-OH
Spin @13,000 rpm for 5 minutes, then discard supernatant
Air dry the pellet (will be difficult to see, or possibly invisible)
Resuspend pellet in 20 ul sterile H_2O

Verifying CGT11050/Ahd I digest

Determine DNA concentration using Nanodrop
Run appx 800-1000ng on a 1% agarose gel (this should be about 1/4 or 1/5 of your entire sample)
Ahd I digest cuts CGT11050 twice and should yield two bands:

1) **440 bp**
2) **3665 bp**

Total size = 4105 bp

Only the upper band is used for cloning, but we do not worry about separating out the lower band. Excess PCR product is used to out compete it.

2. PCR of gene of interest

Fresh, clean PCR product is critical for successful cloning into the Ahd I site, as PCR products tend to lose their A-overhangs with time. The PCR, cleanup, and ligation should all be performed on the same day.

Optimal fragment size is 150-300 bp, located in the 3' UTR (or 5' UTR) of the gene. Taq polymerase should be used in order to obtain the A-overhangs needed for cloning into the Ahd I sites. Standard lab PCR protocols should be used to obtain a single clean band. Once a satisfactory band is produced, run 2 to 4 reps of a 40-50 ul PCR reaction to obtain PCR product for cloning. Combine the reactions, check 5-10 ul on an agarose gel, and then use a Quiaquick PCR Cleanup Kit on the remaining amount. Measure concentration with the Nanodrop.

3. Ligation of PCR product into CGT11050 entry vector

Ligation reaction should contain a 3:1 ratio of insert:vector. Keep in mind that this is ratio refers to the number of ends of the two pieces of DNA being ligated and not the mass (ng) of the samples. Perform calculations accordingly.

Ligation Reactions

The NEB Quick Ligation Kit is used (this kit uses concentrated DNA ligase, which is essential for the inefficient T/A cloning of PCR products). Ligation reaction volumes should be kept as small as possible (10-15ul is best). Adjust vector and insert volumes below according to your sample concentrations.

CGT11050 vector:	1 ul (30-50 ng total)
Insert:	5 ul (500-1000 ng total)
H_2O	0.5
2x Ligase Buffer:	7.5 ul
Ligase:	1 ul
Total:	15 ul

Perform in a **0.5ml** microfuge tube
Combine vector with insert, and adjust volume to 7.5 ul with H_2O.
Add 7.5 ul Quick Ligation Buffer and mix.
Add 0.5 ul Quick T4 DNA Ligase and mix thoroughly.
Incubate @ room temp for 5 minutes, then 15^{o} C overnight in thermalcycler.

You should also do a vector-only ligation as a control.

E. coli Transformations

We are using heat-shockable supercompetent cells.
Check the manufacturer's protocol for transformation instructions.
Use 5-10 ul of the above ligation reaction for each transformation.
Ligation efficiency will be low, so after the 1 ml shake stage you need to concentrate the cells by centrifugation before plating.
Transfer the 1 ml shake to a new 1.5ml tube.
Spin @5000 rpm for 2 minutes.
Decant supernatant and resuspend cells in 100-200 ul of SOC.
Plate out onto 2 LB/Chlor plates; you may want to plate a low volume and a high volume plate (e.g. 50 ul and 150 ul, if total volume is 200 ul); avoid plating volumes less than 50 ul because spreading will be uneven.
Incubate overnight @37^{o} C (may need to incubate 24 hours for colonies to fully form).

Verify entry vector clones by PCR and/or sequencing

Grow minipreps of clones (3 ml culture for each) @37^{o} C overnight.
Make a glycerol stock for each clone (700 ul 50% glycerol + 300 ul culture).
Use 2 ml for each miniprep.
Perform PCR on miniprep DNA to check for insert (include controls).
It takes very little plasmid DNA for PCR (1-5 ng is enough for 1 reaction)
Clones may also be sequenced with original primers to verify insert.

4. Cloning into destination vector CGT11017

Transfer of the insert from vector CGT11050 to CGT11017 is mediated by the LR Clonase enzyme, so no restriction enzyme digest or conventional ligation reaction is needed. You simply mix the two vectors, add LR Clonase, and incubate.

Minipreps to obtain CGT11017 vector

Same protocol as for 11050, but 11017 is kanamycin resistant

CGT11017 plasmid prep yield is low, so you need to compensate.
Grow *four* 5 ml cultures of CGT11017 in LB media + kanamycin.
Use 5 ml for each miniprep.
Using a 2ml tube, add 1.5 ml culture, spin 2 min @12,000 rpm, discard supernatant; add 1.5 ml to same tube, spin, discard, etc. until all 5 ml of culture are spun down into one tube.
Use Qiaprep Spin Miniprep Kit to purify plasmid.
Warm elution buffer EB to 70^{o} C to maximize elution efficiency.
You should obtain appx. 100ng/ul of plasmid when eluted in 50 ul.

LR Clonase reactions

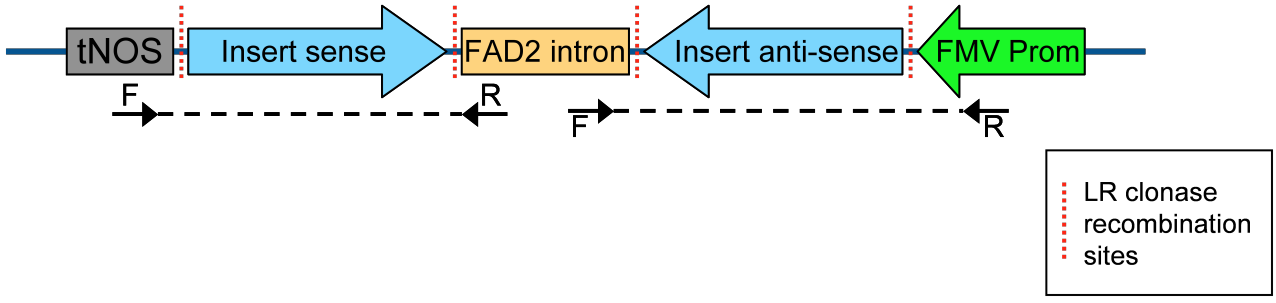
Determine concentrations for both entry (11050) and destination (11017) vectors. This is a critical step; you must have correct concentrations of entry and destination vector for the LR clonase reaction. Greater concentrations will not work in the reaction.
Add the following to a 0.5ml PCR tube at room temp:

1 µL Entry Vector (50-150 ng)
1 µL Destination Vector (150 ng)
2 µL sterile water (TE can also be used)

LR Clonase should be thawed on ice for 2 min then vortexed twice for 2 sec each time. Flash spin.
Add 1µL LR clonase mix (buffer is included) to the reaction.
Vortex to mix, then flash spin.
Incubate for at least 1 hour at 25°C in a thermalcyler.
(If binary vectors are used as destination vectors, this reaction should be ran at 25°C overnight.)
Add 0.5 µL Proteinase K solution to stop the reactions
Vortex briefly and flash spin.
Incubate samples at 37°C for 10 min.
Transform into DH5α cells by heat shock.
Plate onto an LB plate with the correct antibiotic (kanamycin).

Verify entry vector clones by PCR

Due to the sense-antisense incorporation of your insert into the vector, you cannot use your cloning primers for sequencing. Special primers, which anneal to vector sequences flanking the inserts, are used to identify clones containing inserts.



tNOS-F	attgccaaatgtttgaacga
FAD2-F	caccaacccaccagaaaataa
FAD2-R	atttgtggcaatccctttca
FMV-R	gcagtgacgaccacaaaaga

Primers are designed to amplify regions shown above. If you obtain PCR fragments using these primers, then the assumption is that the LR clonase reaction was successful. The main thing that can go wrong is for the FAD2 intron to be flipped (reversed). If that happens, then the FAD2 primers will not be in the proper orientation for amplification. Thus, a negative PCR result is interpreted as an incorrect clone.
PCR fragment sizes for empty vector are as follows:

tNOS-F + FAD2-R: 1980 bp
FAD2F + FMV-R: 2191 bp

It should be noted that LR clonase recombination removes some DNA from the vector, so determining PCR fragment insert size is not a simple matter of adding your fragment length to the above molecular weights.

A secondary verification of your insert (sense and antisense) may be performed by nested PCR. PCR is first performed using the primers above. Bands are cut out of the gel, then bands are subjected to the freeze/thaw method to extrude the DNA. This DNA serves as a template for PCR using the original cloning primers.

You may also verify your inserts by sequencing using the above primers. In this manner, sense and antisense orientation of your insert may be verified.