MAKING GATEWAY COMPATIBLE VECTORS
WITH DAVID DANKORT

Reagents:

Plasmid to be converted (1-5 µg)
Gateway conversion manual
Calf Intestinal Alkaline Phosphatase (CIAP)
Appropriate Gateway® reading frame cassette (5 ng/µl)
T4 DNA Ligase
ccdB Survival™ T1 Phage-Resistant Cells
SOC media
Chloramphenicol (15ug/ml) + Ampicillin (100ug/ml) LB agar plates

Helpful hints

When converting a plasmid to be a destination vector try to do a few things. (1) Remove as much of the "multicloning region" as possible. (2) Keep the frame in mind when converting fusion vectors (e.g epitope tagged vectors).

Protocol
(day1)

1. Cut 1-5ug of plasmid DNA. Preferably this will be with an enzyme that leaves blunt ends. If not, one must blunt end the overhangs (5' overhangs with Klenow, 3' with T4 DNA polymerase).

(optional) One at this point can gel purify the fragment or run it through a gel purification column to remove the restriction enzyme and change the buffers.

2. Dephosphorylate blunt ends with Calf Intestinal Alkaline Phosphatase (CAIP) as per manufacturers instruction. I often dephosphorylate as the plasmid is digested.

This is absolutely necessary to prevent the vector from 'collapsing' on itself.

3. Remove CIAP.

This can be done through gel purification or through heat-inactivation for 15 minutes at 65°C.

4. Quantify DNA and adjust to 20-50ng/ul

It is always good to run out a bit on a gel to be sure everything has worked as expected.

Note: at no point do you <u>have to</u> gel purify the vector. In fact it might work better if you don't because you will lose DNA at each purification step.

5. Set up a ligation as follows:

2 µl	Dephosphorylated vector (20-50 ng)
1 µl	5X T4 DNA ligase buffer
1 µl	Gateway® reading frame cassette (10 ng)
1 µl	T4 DNA ligase 1 unit

This is exactly half of what the manual calls for. The reason for this is simple – 1ul of the ligation is actually used. In fact it is 1ul of a 1 in 5 dilution that is used. The ligation above is sufficient for 15 transformations.

Normally I am a fan of NEB ligase however Invitrogen's ligase buffer has PEG in it, which works better for blunt end ligations. I have never tested this rigourously.

6. Ligate at room temperature for 1 hour

At this point prewarm your plates to 37°C.

After the ligation the manual states that one should dilute the ligation reaction 5 fold in TE (i.e add 20ul TE) prior to transformation. Presumably this is because at these concentrations there may be a chemical present that would be a problem for the bacteria. I suggest that one remove 2ul to a separate tube and add 12ul to the remaining 3ul of ligation product.

The bacteria used HAVE to be DB3.1, which are now called ccdB Survival™ T1 Phage-Resistant Cells. Most other strains of bacteria should be killed by the ccdB gene resident in the Gateway cassette.

7a. Transform ccdB resistant bacteria. If using those that come with the kit (one-shots), do the following and then go to step 8.

Add 1 µl of the diluted ligation reaction into a vial of One Shot® cells and mix gently. **Do not mix by pipetting up and down**.

Incubate the vials on ice for 30 minutes.

Heat-shock the cells for 30 seconds at 42°C without shaking.

Remove the vials from the 42°C water bath and place them on ice for 2 minutes.

7b. Transform ccdB resistant bacteria. If using home-made bacteria (from Z-competents), do the following and then go to step 8.

Set up 2 transformations

- Add 1 µl of the diluted ligation reaction to 40ul of competent bacteria.
- To a separate tube add 1ul of the undiluted ligation reaction to 40ul of competent bacteria

Mix gently - **do not mix by pipetting up and down**.

Incubate the vials on ice for 10-30 minutes.

8. Add 250 µl of room temperature S.O.C. Medium to each tube and incubate for 1 hour at 37°C horizontally while shaking no more than 225rpm.

9. Spin down in microcentrifuge at 1000rpm for 2 minutes or long enough to pellet the bacteria. Remove 150ul of media.

10. Gently resuspend and plate the entirety on to LB-Chlorenphenico/Ampicillin plates.

11. Incubate at 37°C overnight.

Analyzing Transformants
(day2-5)

Reagents:

Reagents to isolate miniprep DNA and carry out restriction analysis

ccdB Survival T1 Phage-Resistant competent bacteria

ccdB Sensitive competent bacteria (Mach1, XL1, DH5a... not NEB Turbo's or any bacteria that carries the F1 resistance plasmid)

Ampicillin (100ug/ml) LB agar plates

Do not be surprised if there are few colonies on the plates. Often I get fewer than 20. Remember there is little that can survive under double selection (this is why you don't need to purify the DNA prior to ligation). While it is obvious that you must screen for orientation of the Gateway cassette what is less obvious is the importance of screening for functionality of the cassette. There are several hurdles that must be surmounted before you use such a plasmid you make

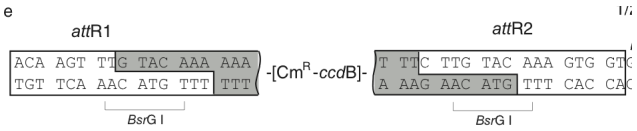
Testing for insert/orientation

1. Pick colonies simultaneously inoculating bacteria to a LB-Chloramphenicol – Ampicillin plate (to keep the bacteria for a few days.) and to 3ml LB-Chlor/Amp media.

> *Having a "hard copy", i.e. the plate is very useful as you will need to streak these out again to get a single colony before maxi-prepping verified positives.*

2. Grow bacteria overnight while shaking and isolate miniprep DNA as per usual

3. Screen the plasmids for the Gateway cassette. This can be done with *BsrGI* digestion although I generally use the fact that there are *BamHI* sites within the cassette about 700bp apart.



4. Screen for the proper orientation of the Gateway cassette. This is easily done as there a *NotI* site at the 5'end and a *SalI* site at the 3' end of the cassette.

Now you should have a couple of test plasmids in either orientation – this is hurdle #1. The question, 'is are they functional?' This is tested by retransforming these plasmids back into ccdB resistant bacteria and into bacteria that <u>should</u> be sensitive to the actions of the ccdB gene.

Testing the ccdB function

5. Take 1ul of each "positive" mini-prepped DNA and dilute it with 19ul of TE.

6. Add 2ul of diluted DNA to 20ul of DB3.1 and separately to 20ul normal (Mach1, XL1) bacteria, mix gently and incubate for 5-10 minutes on ice.

> *I have successfully use 1ul into 10ul. Use the transformation protocol one normally would use for the bacteria on hand. Here I use Z-comps. Note you do not need super great bacteria, nor do you need to incubate for a long time because the amount of input DNA is huge here.*

There are two controls you may wish to carry along side the first couple times you do this (or every time): (i) Transform 10ng of any non-gateway Amp-resistant plasmid into the senstive strain and for good form into the DB3.1s as well. (ii) Transform 10 ng of a gateway destination vector into the two bacterial strains.

7. Add 50 ul of LB and directly plate on LB-Amp plates and incubate overnight at 37°C

There is no need to allow the bacteria to recover because Ampicillin does not affect DNA or protein synthesis.

At this point you should have two plates for each plasmid, plus two sets of control plates. The DB3.1 plates are simply a control for your plasmid and transformation protocol. The other plate, the "sensitive" bacteria (Mach1, DH5a) are the important ones This is a hypothetical and expected results

	DB3.1	**Sensitive bacteria (mach1)**
non gateway vector	many colonies	many colonies
destination vector	many colonies	*few or no colonies
test plasmid #1	many colonies	many colonies
test plasmid #3	many colonies	many colonies
test plasmid #4	Few colonies	Few or No colonies
test plasmid #8	**many colonies**	**Few or No colonies**

In the above example test plasmids #1 and #3 are garbage – for whatever reason they can be efficiently propagated in sensitive cells suggesting something is wrong with the ccdB gene. Test plasmid #4 probably is at a really low concentration or has some impurity affecting transformation. It would not be a "lead plasmid". Test Plasmid #8 is the one you want. Notice that it produces many colonies in the DB3.1 bacteria but very few in the sensitive bacteria. This is exactly what you would predict.

*Note you will often get a couple of colonies with this much DNA. They key thing here is that the number of colonies should be significantly fewer in the sensitive bacteria with a destination vector relative to that of a non-gateway vector (best to use the original non-converted plasmid). The manual suggests a 10,000 fold difference in bacterial numbers is expected.

At this point you now have a Gateway destination vector that appears to work to kill sensitive bacteria dead (hurdle #2).

8. Go back to the original bacteria "hard-copy" from day 1. Streak this out so that you get single colonies and use one to inoculate a large liquid culture grown in LB containing Chlor&Amp.

Testing the plasmid for recombination

9. Set up an LR reaction

1 µl Destination vector (150 ng)

3 µl Entry clone (bGal) (50-150 ng/ul) (must have a different selection marker)
1 µl LR Clonase II reaction mixture

Given the expense of the LR Clonase I tend to set up a negative control (ie. a reaction lacking the Entry clone using TE instead). After doing this once for a vector, it is documented and not done again.

10. Incubate at room temperature (25°C) for at least 1 hour. For larger plasmids/insert (>10kb) incubate up to 18hours.

11. Add 0.5 µl of the Proteinase K solution to each reaction and incubate for 10 minutes at 37°C.

12. Transform 1 µl of the LR recombination reaction into sensitive bacteria as above and select for expression clones (in this case on LB-Amp plates). Screen the clones as appropriate.

Note: The remaining LR reaction may be stored at -20°C for up to 1 week before transformation.