Notes:

- pGwyRNAi contains the ccdB gene, so must maintain it in DB3.1 or other compatible strain.
- Use LB + Amp for growth.
- Use original RNAi vector for a transformation positive control. It is not good to use pGwyRNAi as your positive control since it contains so much junk between the R1 and R2 sites.
- pGwyRNAi is about 4kb larger than the original RNAi vector.
- Pdk is an arabidopsis intron that is supposedly spliced out during processing. Less than 50nt of intervening sequence between your inverted repeat will remain if this is spliced out. I have not verified experimentally that this occurs in my system, but I was able to achieve silencing of PNPase.
- There are two versions of pGwyCrRNAi: pdk fwd (#5) or pdk rev (#2), with the pdk intron in opposite orientations in each. Since the vector undergoes a double recombination when putting your sequence into it, each parent vector will produce both pdk in the correct and the incorrect orientation in the plasmid resulting from the recombination (see the note in a bullet below). It shouldn't matter which one you start with, but I've found that certain recombination reactions seem to work better with one or the other, so it pays to try the LR reaction with both parent vectors.
- The fragment can exist in either orientation (either fwd-pdk-rev or rev-pdk-fwd) and work just fine.
- I used fragments of 250-350bp. The shorter they are, the less chance that you will silence something unintended. If you fool around with the BLAST conditions in JGI chlamy3.0, you can get it to find all the other short regions in the genome that your possible RNAi fragment has matches to. However, I have found that ligation into pENTR works best if I PEG purify my fragments after PCR and gel extraction, and fragments much shorter than 250 may not purify well.
- I use half volumes in the LR recombination reaction, and we use the original LR recombinase. I do not linearize anything prior to recombination. You need very little of the plasmids for the recombination.
- I find it necessary to incubate overnight at RT my recombination reaction to get the best yields.
- I also transform commercially prepared cells, like chem. Competent Top10 from Invitrogen to get better yield after the recombination.
- It is safest to screen about 20 transformed colonies from the recombination reaction, because the recombination is not that efficient.
- Recombination can result in the pdk intron either remaining in its proper orientation of flipping around. You need to check this.
- I screen my final plasmids with
    - EcoRI digest: Should give you one backbone band and one band of size 2Xyour gene fragment + pdk intron.
    - Cla1/SacI digest: one backbone band and one band (1800 bp + 1X insert) = correct orientation, one band (1000bp + 1X insert) = incorrect pdk orientation.

- Regarding Cla1, when I screened my colonies I didn't realize there was a ClaI site near the Sac1 site. Doesn't change much, except that you probably don't need Sac1 in the above digest to get the same result. There is also another ClaI site around EcoRI that is crossed out in the map because it has on overlapping Dam methylase site and will not cut when propagated in most bacterial lines. I never see the band resulting from the DNA between the 3rd and 4th ClaI site, it is basically just the size of your RNAi insert and too small to pick up in the digest.
- I cut my vector with SacI prior to transformation and use 1ug per 2 x 10^7 cells
- We transform by electroporation.
- Use a range of concentrations of 5FI for selection and always have your melted agar the same temperature before adding the 5-FI. We've found it to be remarkable sensitive to temperature. It is easy to add too much and get no colonies as well! We've always had to do a "no DNA" negative control transformation to be sure the selection was working.