This vector can be a little more difficult than most to work with because 90% of the plasmid prep is ssDNA and therefore unclonable. I have included a high efficiency cloning protocol but you should be able to successfully clone with much less DNA and smaller enzymes that are more typically used.

**Day 1**

Start 10mL pSL2680 in LB Km50.  Grow overnight at 37C.

**Day 2**

Perform miniprep on all 10mL pJU2680

In the end elute from the column with 50uL of water.

Digest all 50ul of miniprep with aarI (Thermo fisher) using the following reaction mix:

10ul aarI buffer

50ul plasmid prep

2ul of 50X aarI oligo

4ul aarI enzyme

34ul water

Incubate at 37C for 4 hours

Gel extract the digest

Excise largest band (~12kb). There is usually a brighter smaller band that is ssDNA that is generated during the denaturation step of the plasmid prep due to the perpetual nicked nature of the plasmid in the cells.

Elute gel extraction with 20uL water.

**Day 3**

Anneal oligos with the following reaction mixture

10ul 100uM stock of gRNAL primer

10ul 100uM stock of gRNAR primer

5ul ligase buffer

25ul water

1ul PNK enzyme

Place in thermocycler with the following program:

30 min 37C

5 min 95C

Cool to 4C at 0.1C/sec ramp rate

The most important part is to cool the reaction as SLOWLY as possible to allow proper annealing

Dilute annealed oligos 1:50.

Ligate diluted oligos into pSL2680 digest from the previous day with the following reaction mix:

8.0 ul pSL2680 gel extraction

0.5ul of annealed oligos that have been diluted 1:50

1ul ligase buffer

0.5ul ligase

Incubate overnight at 16C.

**Day 4**

Transform all 10ul ligation reaction into XL1-blue as follows

Transformations are plated on L-Km50 + IPTG/X-gal for blue/white screening of inserts.

**Day 5**

Pick white colonies from the transformation, patch them on Km50 and verify with colony PCR.

Start 10mL cultures of the correct colonies in L-Km50

**Day 6**

Miniprep the cultures that you started the previous day.

Sequence gRNA insert to verify that it is correct

Synthesize the homologous repair template using highfidelity PCR.

Typically you will make left and right halves with any changes you want to make. The middle primers contain the changes. The two halves should overlap by 30 bp for Gibson assembly

Gel extract PCR reactions and elute in 25uL water.

**Day 7**

Digest correctly identified plasmid from sequencing results using kpnI, make sure to dephosphorylate very well to prevent recircularization of backbone. Use the following reaction:

15ul FD buffer (green)

75ul plasmid prep

3ul kpnI

4ul FastAP (dephosphorylates vector)

53ul water

Incubate for 4 hours at 37C

Gel extract digest and elute in 25ul water. I usually gel extract the digest and PCR reactions from above on the same gel.

**Day 8**

Concentrate gel extraction of digest and perform Gibson assembly.

Concentrate 25ul of cut plasmid DNA into 6uL using a speedvac.

Measure concentration of the three samples (digested plasmid and left and right halves of repair template PCR). I typically nanodrop 1uL of each.

Assemble final vector with Gibson assembly:

Gibson assembly requires exactly 5uL of correctly mixed DNA. All fragments should be in a 1:1:1 stoichiometry by mols of DNA fragments rather than ng DNA. I typically try to use 200ng of vector and a 1X molar ratio of each fragment. Total volume must be 5uL.

Add 15uL of Gibson master to the 5ul of DNA. Mix.

Incubate for 1 hour at 50C.

Transform Gibson reaction into e.coli. Plate on L-Km50.

**Day 9**

Pick colonies and check with colony PCR

Select 2 positive colonies. Start 10mL of each in L-Km50 for plasmid preps.

**Day 10**

Sequence final homologous repair template. If it is correct you are done with cloning.