1) Prepare 6 ml of 5X ISO Buffer in a 15 ml falcon tube as follows:

3 ml 1 M Tris-HCl pH 7.5

+ 150 ml 2 M MgCl2

+ 240 ml 100 mM dNTP mix (25 mM each: dGTP, dCTP, dATP, dTTP)

+ 300 ml 1 M DTT

+ 1.5 g PEG-8000

+ 300 ml 100 mM NAD

+  dH20 to

6 ml

Store at -20 C in 320 ml aliquots.

2) Prepare 1.2 ml of Gibson assembly master mix as follows:

320 ml 5X ISO Buffer

+ 0.64ml 10 U/ml T5 exonuclease\*

+ 20 ml 2 U/ml Phusion polymerase

+ 160 ml 40 U/ml Taq ligase

+  dH20 to

1.2 ml

Store at -20 C in 15 ml aliquots.

\*This is optimized for 20-150 bp sequence homology overlaps

3) Thaw a 15 ml aliquot of the Gibson assembly master mix, and keep on ice until use.

4) Measure the DNA concentration (ng/ml) of each assembly piece.

5) Add 100 ng of the linearized vector backbone and equimolar amounts of the other assembly pieces to the thawed 15 ml master mix in a 20 ml total volume assembly reaction mixture as follows:

linearized vector backbone (100 ng)

+ each additional assembly piece (to equimolar with backbone)

+ 15 ml Gibson assembly master mix

+  dH20 to

20 ml

6) Incubate the assembly reaction at 50 C for 60 minutes, and then place on ice.

7) Transform 5 ml of the assembly reaction into 100 ml of competent *E. coli* and/or run a diagnostic agarose gel to check for successful assembly.