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	μl	2 M $MgCl_2$
+	240	μl	100 mM dNTP mix (25 mM each: dGTP, dCTP, dATP, dTTP)
+	300	μl	1 M DTT
+	1.5	g	PEG-8000
+	300	μl	100 mM NAD
+	_____		dH_20 to
	6	ml	

Store at -20 C in 320 μl aliquots.

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

	320	μl	5X ISO Buffer
+	0.64	μl	10 U/μl T5 exonuclease*
+	20	μl	2 U/μl Phusion polymerase
+	160	μl	40 U/μl Taq ligase
+	_____		dH_20 to
	1.2	ml	

Store at -20 C in 15 μl aliquots.

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

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

4) Measure the DNA concentration (ng/μl) 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 μl master mix in a 20 μl total volume assembly reaction mixture as follows:

			linearized vector backbone (100 ng)
+			each additional assembly piece (to equimolar with backbone)
+	15	μl	Gibson assembly master mix
+	_____		dH_20 to
	20	μl	

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

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