Text S1. Success rates with representative BioBrick and primer combinations.

The following description is based on surveying over 300 independent reactions, typically with an annealing temperature of either 57C or 59C, 28-32 cycles, with a starting template of 5-500pg, and an extension time appropriate for the target. The template was sometimes a diluted, purified plasmid, and in other times it was the product of a previous PCR reaction. We found there to be no significant difference between these two template sources in terms of success in a PCR reaction, although low template concentration appeared to be a problematic in many cases.

BioBricks amplified, across all primer combinations, included but were not limited to the following (BBa_ designation omitted, % successful reactions listed in parentheses. 'Recalcitrant' BioBricks, or ones that were difficult to amplify, are underlined): I13521 (100%), I732005 (28%), J04450 (65%), J04500 (58%), K081012 (80%), K081014 (67%), K0133906 (60%), K123002 (85%), K123002 (14%), K515005 (84%), K592006 (16%), K592009 (36%), K592012 (38%), R0010 (77%), R0040 (100%), with an average of 20 different reactions for each BioBrick.

Organized by primer combination, the same set featured the following success rates: V1-F&R (72%), V2-F&R (25%), V3-F&R (75%), V6-F&R (51%). Using a reverse control primer (VR), the following success was obtained: V1 (80%), V2 (54%), V3 (100%), V6 (79%). Using a forward control primer, the following success was obtained: V1 (92%), V6 (80%). Overall, this set of reactions featured a success rate of 68%. When pairing both control primers, a success rate of 87% was achieved.

It should be noted that some of the reactions may have failed due to improper annealing temperature, degraded DNA or low template concentration, human error or other technical issues (as reflected by the less than perfect number for the control primers). Furthermore, the V7 primer set, used on BioBricks such as (BBa prefix omitted) K081014, K515005, R0010, K1033906, K592012, K322921, K132000, I732018, featured a success rate of 100%.

Tables

Table S1. Primers used in the study. Oligonucleotide primers were synthesized by Integrated DNA technologies (IDT). The underlined portion of the sequence represents the expected overhang resulting from BsaI cleavage. All primers above were designed for this study except for VF2 and VR, which are described elsewhere and listed as BioBricks G00100 and G00101, respectively, in the Registry of Standard Biological Parts.

Name	Sequence (5'-3')	Purpose
Backbone_R1	CggatccGGTCTCc**tgcag**tccggcaaaaaagggc	Amplification of plasmid backbone for GoldenGate assembly (TGCA & AATT)
Backbone_F1	gcggatccGGTCTCg**aatt**ccagaaatcatccttagcg	
Backbone_F_actc	gcggatccGGTCTCg<u>actc</u>ccagaaatcatccttagcg	
Backbone_R_actc	CggatccGGTCTCc<u>actc</u>gtccggcaaaaaagggc	
Backbone_F_aggt	gcggatccGGTCTCg<u>aggt</u>ccagaaatcatccttagcg	
Backbone_R_aggt	CggatccGGTCTCc<u>aggt</u>gtccggcaaaaaagggc	
Backbone_F_tact	gcggatccGGTCTCg<u>tact</u>ccagaaatcatccttagcg	
Backbone_R_tact	CggatccGGTCTCc<u>tact</u>gtccggcaaaaaagggc	
Backbone_R_tcat	CggatccGGTCTCc<u>tcat</u>gtccggcaaaaaagggc	Amplification of plasmid backbone for GoldenGate assembly (Indicated overhangs, compatible with other fragments)
Backbone_F_ggag	gcggatccGGTCTCg<u>ggag</u>ccagaaatcatccttagcg	
Backbone_R_ggag	CggatccGGTCTCc<u>ggag</u>gtccggcaaaaaagggc	
Backbone_F_gagt	gcggatccGGTCTCg<u>gagt</u>ccagaaatcatccttagcg	
Backbone_R_gagt	CggatccGGTCTCc<u>gagt</u>gtccggcaaaaaagggc	
Backbone_F_acct	gcggatccGGTCTCg<u>acct</u>ccagaaatcatccttagcg	
Backbone_R_acct	CggatccGGTCTCc<u>acct</u>gtccggcaaaaaagggc	
VF_E2	gccggact<u>gcag</u>GAGACCggatccGtatataaacgcagaaaggccc	Mutagenesis of plasmid backbone for creation of dropout vector
VR_R2	ctgg<u>aatt</u>cGAGACCggatccgcagtttacagctagctcagtcctagg	
AAGG_F1	TGGAATTCGCGGCCGCTTCTAGAGGTCTCC<u>AAGG</u>CTAGATG	Introduction of indicated overhang upstream of biobrick (coding regions only)
ACTC_F1	TGGAATTCGCGGCCGCTTCTAGAGGTCTCC<u>ACTC</u>CTAGATG	
AGGT_F1	TGGAATTCGCGGCCGCTTCTAGAGGTCTCC<u>AGGT</u>CTAGATG	
AATT_F1	TGGAATTCGCGGCCGCTTCTAGAGGTCTCC<u>AATT</u>CTAGATG	
AAGG_F2	TGGAATTCGCGGCCGCTTCTAGAGGTCTCC<u>AAGG</u>CTAGA	Introduction of indicated overhang upstream of biobrick (minimal scar)
ACTC_F2	TGGAATTCGCGGCCGCTTCTAGAGGTCTCC<u>ACTC</u>CTAGA	
AGGT_F2	TGGAATTCGCGGCCGCTTCTAGAGGTCTCC<u>AGGT</u>CTAGA	
AATT_F2	TGGAATTCGCGGCCGCTTCTAGAGGTCTCC<u>AATT</u>CTAGA	
AAGG_F3	GATTTCTGGAATTCCTAGAGGTCTCC<u>AAGG</u>GCGGCCGCTCCTAGA	Introduction of indicated overhang upstream of biobrick (extended scar)
ACTC_F3	GATTTCTGGAATTCCTAGAGGTCTCC<u>ACTC</u>GCGGCCGCTCCTAGA	
AGGT_F3	GATTTCTGGAATTCCTAGAGGTCTCC<u>AGGT</u>GCGGCCGCTCCTAGA	
AATT_F3	GATTTCTGGAATTCCTAGAGGTCTCC<u>AATT</u>GCGGCCGCTCCTAGA	
AAGG_F6	cgctaaGGATGATTTCTGGAATTGGTCTCC<u>AAGG</u>CTAGA	Introduction of indicated overhang upstream of biobrick (mismatch)
ACTC_F6	cgctaaGGATGATTTCTGGAATTGGTCTCC<u>ACTC</u>CTAGA	
AGGT_F6	cgctaaGGATGATTTCTGGAATTGGTCTCC<u>AGGT</u>CTAGA	
AATT_F6	cgctaaGGATGATTTCTGGAATTGGTCTCC<u>AATT</u>CTAGA	
AAGG_R1	CTGCAGCGGCCGCTACTAGTAGGTCTCC<u>CCTT</u>TAGTA	Introduction of indicated overhang downstream of biobrick (mismatch)
ACTC_R1	CTGCAGCGGCCGCTACTAGTAGGTCTCC<u>GAGT</u>TAGTA	
AGGT_R1	CTGCAGCGGCCGCTACTAGTAGGTCTCC<u>ACCT</u>TAGTA	
TGCA_R1	CTGCAGCGGCCGCTACTAGTAGGTCTCC<u>TGCA</u>TAGTA	
AAGG_R6c	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>CCTT</u>TAGTA	Introduction of indicated overhang downstream of biobrick (mismatch)
ACTC_R6c	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>GAGT</u>TAGTA	
AGGT_R6c	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>ACCT</u>TAGTA	
TACT_R6c	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>AGTA</u>TAGTA	
GGAG_R6c	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>CTCC</u>TAGTA	
AATG_R6c	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>CATT</u>TAGTA	
GCTT_R6c	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>AAGC</u>TAGTA	
TGCA_R6c	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>TGCA</u>TAGTA	
CCTT_R6	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>AAGG</u>TAGTA	Use in combination with above primer sets for introduction in reverse orientation
GAGT_R6	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>ACTC</u>TAGTA	
ACCT_R6	CCTTGCCCTTTTTTGCCGGACTGCAGGTCTCC<u>AGGT</u>TAGTA	

TGCA_F6	cgctaaGGATGATTTCTGGAATTGGTCTCC<u>TGCA</u>CTAGA	
ACCT_F6	cgctaaGGATGATTTCTGGAATTGGTCTCC<u>ACCT</u>CTAGA	
CCTT_F6	cgctaaGGATGATTTCTGGAATTGGTCTCC<u>CCTT</u>CTAGA	
GAGT_F6	cgctaaGGATGATTTCTGGAATTGGTCTCC<u>GAGT</u>CTAGA	
GAGT_F7	CGCTAAGGATGATTTCTGGAggTCtCGgagtCTTCTAGA	
ACTC_F7	CGCTAAGGATGATTTCTGGAggTCtCGactcCTTCTAGA	
AAGG_F7	CGCTAAGGATGATTTCTGGAggTCtCGaaggCTTCTAGA	
CCTT_F7	CGCTAAGGATGATTTCTGGAggTCtCGccttCTTCTAGA	Introduction of indicated overhang downstream of biobrick (mismatch)
AGGT_F7	CGCTAAGGATGATTTCTGGAggTCtCGaggtCTTCTAGA	
ACCT_F7	CGCTAAGGATGATTTCTGGAggTCtCGacctCTTCTAGA	
TACT_F7	CGCTAAGGATGATTTCTGGAggTCtCGtactCTTCTAGA	
ATGA_F7	CGCTAAGGATGATTTCTGGAggTCtCGatgaCTTCTAGA	
GAGT_R7	CCTTGCCCTTTTTTGCCGGACTGgtctcGgagtTACTAGTA	
ACTC_R7	CCTTGCCCTTTTTTGCCGGACTGgtctcGactcTACTAGTA	
AAGG_R7	CCTTGCCCTTTTTTGCCGGACTGgtctcGaaggTACTAGTA	
CCTT_R7	CCTTGCCCTTTTTTGCCGGACTGgtctcGccttTACTAGTA	Introduction of indicated overhang downstream of biobrick (mismatch)
AGGT_R7	CCTTGCCCTTTTTTGCCGGACTGgtctcGaggtTACTAGTA	
ACCT_R7	CCTTGCCCTTTTTTGCCGGACTGgtctcGacctTACTAGTA	
TACT_R7	CCTTGCCCTTTTTTGCCGGACTGgtctcGtactTACTAGTA	
ATGA_R7	CCTTGCCCTTTTTTGCCGGACTGgtctcGatgaTACTAGTA	
2nd_F2	TGGAATTCGCGGCCGCCTAGAGGTC	
2nd_R2	CTGCAGCGGCCGCTACTAGTAGGTC	Use in combination with the above to create assembly in a two-reaction or phased approach
2nd_F3	GATTTCTGGAATTCCTAGAGGTC	
2nd_F6	cgctaaGGATGATTTCTGGAATTGGTC	
2nd_R6	CCTTGCCCTTTTTTGCCGGACTGCAGGTC	
VF2	TGCCACCTGACGTCTAAGAA	Control primers for amplication (standard sequence used by the iGEM community)
VR	ATTACCGCCTTTGAGTGAGC	
RFP_seqF	GCAAGACGGTGAGTTC	
RFP_seqR	GAACTCACCGTCTGGC	
GFP_seqF	CTTTGCGAGATACCCAGATCATA	
GFP_seqR	GTCTTGTAGTTCCCGTCATCTT	
R0010_seqF	gttggccgattcattaatgcagctgg	Primers for Sanger sequencing. Creating using the PrimerQuest tool from Integrated DNA technologies against sequences for BioBricks BBa_: K081014 (RFP), K081012 (GFP), R0010 (Promoter), I732018 (LacZ-alpha), K132000 (KanR), and K322921 (SacB).
R0010_seqR	gtgtaaagcctggggtgcctaatgag	
LacZ_seqF	caacgtctgactgggaaa	
LacZ_seqR	cggggcctcttcgctattac	
KanR_seqF	gtttggttgatgcgagtgattt	
KanR_seqR	gactgaatccggtgagaatgg	
SacB_seqF	gaacatcaacggtgtagaggatta	
SacB_seqR	ctgagctgtagttgccttcat	

Table S2. Primer hybridization motif occurrence in iGEM distribution kit sequences. Different primers from the V6 set are named according to Table S1, and the 3' end which includes the target hybridization sequence and the BsaI overhang are shown. BioBricks in which this 3' sequence is present in either the forward or reverse direction are indicated in the 3rd and 4th columns, respectively (the BBa_ prefix is omitted from the BioBrick name or ID). * There were over 50 reverse matches for primer 'GAGT_R6', not all of them are indicated for the sake of brevity.

Name	3' Sequence	Off-Target Forward Matches	Off-Target Reverse Matches
AAGG_F6	AAGGCTAGA	P10302	None
ACTC_F6	ACTCCTAGA	K823015	K314202, K1680009, K530005
AGGT_F6	AGGTCTAGA	K863204	None
TACT_F6	TACTCTAGA	K838000	None
GGAG_F6	GGAGCTAGA	None	None
GCTT_F6	GCTTCTAGA	None	None
AATG_F6	AATGCTAGA	None	K1483001, I746101
AATT_F6	AATTCTAGA	P10001	None
AAGG_R6c	CCTTTAGTA	R0053, I6040, I7108, I6084, I6070, Q04530, E0615, K1472601, K654058	None
ACTC_R6c	GAGTTAGTA	None	K112806, K777109, K133016, K133010, K847101
AGGT_R6c	ACCTTAGTA	None	K1172303, K763005
TACT_R6c	AGTATAGTA	K395602, K496003	None
GGAG_R6c	CTCCTAGTA	K813002	K1185000
AATG_R6c	CATTTAGTA	None	K1114100, K1114101, K1114102, K1114103
GCTT_R6c	AAGCTAGTA	P10201	K517000
TGCA_R6c	TGCATAGTA	None	K1789000, K515100
CCTT_R6	AAGGTAGTA	None	
GAGT_R6	ACTCTAGTA	K137058, K880002, K228009, B0025	I13013, K624001, K581012, K316003, K523013, K1031410, K349003…...*
ACCT_R6	AGGTTAGTA	None	K133046, K823042
TGCA_F6	TGCACTAGA	K581012, K568004, K568001	None
ACCT_F6	ACCTCTAGA	None	None
CCTT_F6	CCTTCTAGA	P10300	None
GAGT_F6	GAGTCTAGA	None	None

Table S3. Primer annealing to select BioBricks as predicted by Benchling. For each category of BioBrick, at least two representatives were chosen and the sequence was screened for primer binding using the suite of tools provided in the Benchling website (https://benchling.com/, last accessed February 19th, 2023). Several selected Biobricks are shown here. The position on the + or – strand of the BioBrick sequence for primer binding is indicated, as well as the predicted melting temperature in Celsius. In some cases, a high melting temperature and matching nucleotide number did not suggest amplification, since the mismatches were near the 3'. Reactions that had reasonable matches near the 3' end that were subsequently tested (see Figure S5) are highlighted. The one tested primer that led to apparent off-target amplification when tested is emphasized in bold font.

BioBrick	Type	Primers	Position	Tm (C)	Matches	Gaps
K608351	Coding	2nd_F6	172	50.9	14	5
		AATG_R6c	-213	54.6	14	5
		2nd_F3	-114	50.6	13	5
		TACT_R6c	-24	45	11	5
		TGCA_R6c	-24	48.9	11	4
K808003	Composite	2nd_R6	45	59	16	4
		2nd_F3	54	49	12	4
		2nd_R6	-48	55.9	10	4
		2nd_R2	-151	52.7	11	6
		2nd_F6	58	48.4	12	5
K542004	Device	2nd_R2	1506	49.8	12	5
		2nd_R2	-217	45.8	11	3
		TACT_R6c	-824	53.6	15	5
		2nd_R2	700	59.7	14	4

		TACT_F6	209	48.1	13	4
K863201	DNA	AAGG_F6	-525	51.4	13	3
		2nd_R6	134	57	12	3
		AAGG_R6c	-23	57.1	15	6
		2nd_F3	62	52.2	15	5
K819010	Generator	2nd_F6	-299	58	19	5
		Backbone_F	325	53	16	4
		AGGT_F6	635	50.4	13	5
		2nd_R2	-52	52.7	12	3
		GGAG_F6	171	50.3	10	4
K539627	**Intermediate**	GGAG_F6	-1358	46.9	11	3
		GGAG_F6	**-757**	**60.8**	**20**	**6**
		2nd_F6	968	48.4	14	4
		GCTT_F6	-757	59.4	19	6
		GGAG_R6c	-678	56	14	5
K880002	Measurement	AAGG_R6c	-277	46.1	12	3
		AATG_F6	-5	48.9	12	5
		AATG_R6c	-813	51.9	13	3
		2nd_F6	-1065	52.2	14	7
		2nd_F3	993	49	12	5
K1758375	Other	AATG_F6	86	50.4	14	4
		GGAG_R6c	435	49.7	12	3
		AGGT_R6c	435	48.3	12	4
		TGCA_R6c	435	48.9	12	4
		AATG_F6	462	51.7	14	6

Table S4. Ligation frequency matrix. Primer generated overhangs were tested for their predicted ligation fidelity using the NEB Beta tool 'Ligase Fidelity Viewer (v2)', accessed at https://ggtools.neb.com/viewset/run.cgi (accessed on April 28th, 2022) [2,3] with the BsaI-HFv2 37 static setting. Numbers represent a normalized ligation count, with colors representing a good Watson-crick pair (dark blue), trace mismatch ligations (light tan), or high-count mismatch ligations (orange). Primers used in the assembly proposed in Figure S5 are bound by the purple rectangle.

	GAGT	ACTC	AGGT	ACCT	AAGG	CCTT	TACT	AGTA	AATT	AATT	TGCA	TGCA	GGAG	CTCC	AATG	CATT	GCTT	AAGC
GAGT	0	306	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACTC	306	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AGGT	0	0	0	375	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACCT	0	0	375	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AAGG	0	0	0	0	0	402	0	0	0	0	0	0	0	0	0	1	0	0
CCTT	0	0	0	0	402	0	0	0	0	0	0	0	0	0	4	0	0	0
TACT	0	0	0	0	0	0	0	361	0	0	0	0	0	0	0	0	0	0
AGTA	0	0	0	0	0	0	361	0	0	0	0	0	0	0	0	0	0	0
AATT	0	0	0	0	0	0	0	0	419	419	0	0	0	0	0	0	0	0
AATT	0	0	0	0	0	0	0	0	419	419	0	0	0	0	0	0	0	0
TGCA	0	0	0	0	0	0	0	0	0	0	380	380	0	0	0	0	0	0
TGCA	0	0	0	0	0	0	0	0	0	0	380	380	0	0	0	0	0	0
GGAG	0	0	0	0	0	0	0	0	0	0	0	0	0	255	0	0	0	0
CTCC	0	0	0	0	0	0	0	0	0	0	0	0	255	0	0	0	0	0
AATG	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	391	0	0
CATT	0	0	0	0	1	0	0	0	0	0	0	0	0	0	391	0	0	0
GCTT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	363
AAGC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	363	0

Figures

Figure S1. Sequence verification of BsaI site incorporation into PCR product with V6 primers. The BioBrick BBa_J04450 was amplified using primers ACTC_F6 and VR. Successful amplification was verified by gel electrophoresis and the resulting PCR product was purified and sent for sequencing using the RFP_seqR primer, which starts in the middle of the BioBrick and reads in the reverse direction towards the 5' end. The resulting chromatogram is shown, together with an inset that highlights the BsaI site (the recognition site is shaded blue) and the BioBrick specific sequence (shaded pink). When compared to the sequence of BBa_J04450 present in the Registry of Standard Biological Parts (http://parts.igem.org/cgi/sequencing/one_blast.cgi?id=34138, last accessed February 3rd, 2023), taking into account the reverse orientation, the addition of ACTC site from the primer can be seen. The BBa_J04450 specific sequence is shown in pink. Note how this removes the XbaI site from the finished product, preserving the ability to carry out 3A assembly later and maintaining compatibility. For reference, the binding of primer 2nd_F6, used in the two-stage approach, is shown in green.
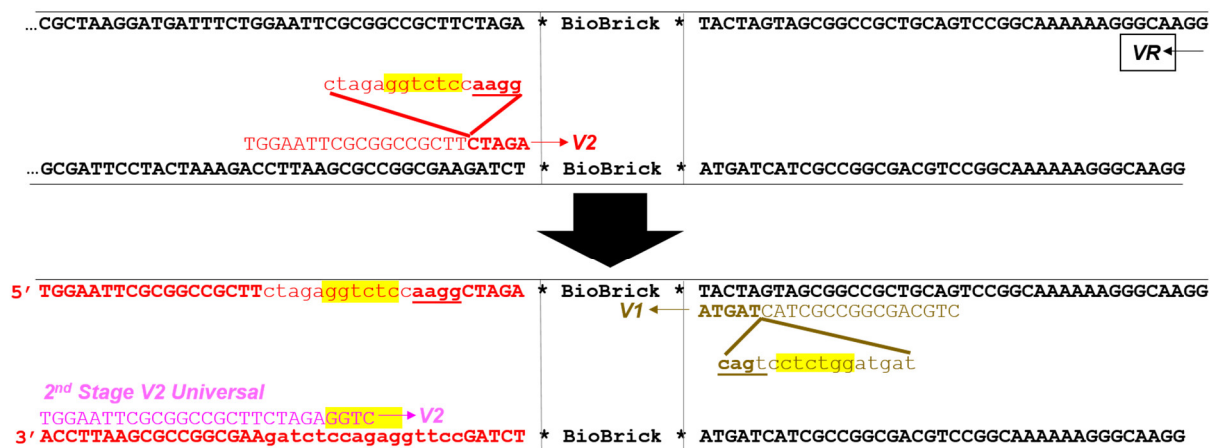
Figure S2. Two-Step strategy for GEM-Gate amplification. Some of the GEM-gate primers, such as the one shown here (V2, red), feature limited homology on the 3' end and may be sensitive to PCR conditions. In this amplification strategy, these primers are first paired with a control primer (VR) located further downstream of this region. This more robust reaction (top line) creates a product (bottom line) that contains the BsaI site derived from the primer (red sequence). In the 2nd reaction, a universal primer designed for this type of product, but independent of the overhang (pink) is paired with another universal reverse primer (brown).
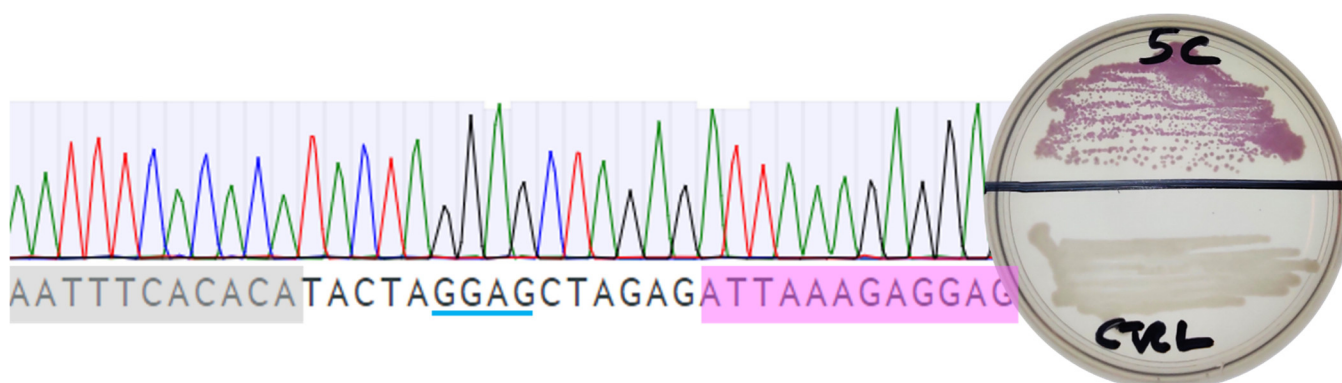
Figure S3. Sequence and functional verification of two-fragment assembled product. BioBrick BBa_R0010 (a promoter) was amplified with AATT_F6 and GGAG_R6c, whileBBa_ K081014 (RFP) was amplified with GGAG_F6 and TGCA_R6c. For the backbone, pSB1C3 was amplified with Backbone_F and Backbone_R (for a representative example, see Figure S9) Following successful amplification, these Bsa-I site containing PCR products were included in a Golden Gate assembly reaction and transformed into bacteria. Subsequent colonies were selected, plasmid DNA was mini-prepped, and sequenced using the VF2 primer. A portion of the chromatogram from a representative clone is shown. The region that matches the 3' end of the BBa_R0010 sequence is shown in grey, while the region matching the 5' region of BBa_K081014 is shown in pink. The scar sequence contains the expected GGAG overhang, underlined. This is shown alongside a plate demonstrating the expression of RFP in the clone, as compared to an empty vector control, after overnight growth at 37^0C.
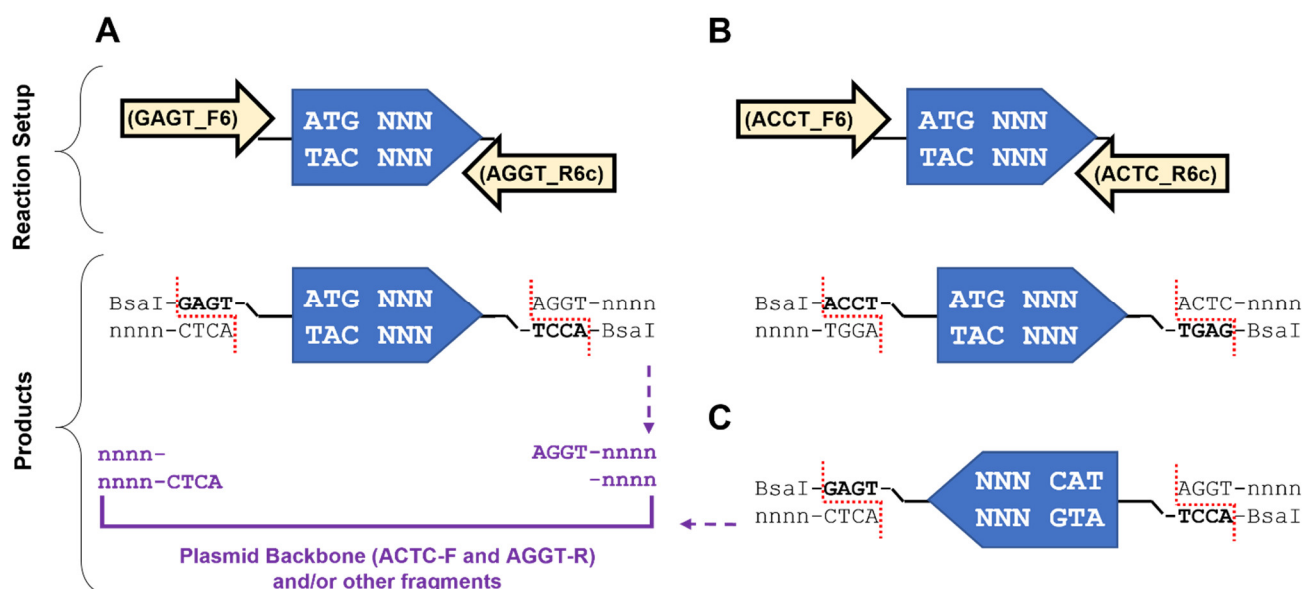
Figure S4. Assembly of BioBrick in forward and reverse orientation. A) Amplification of a directional BioBrick, such as a protein coding sequence starting with ATG, with the indicated primer pair leads to a fragment which will have a 5' GAGT overhang and a 3' AGGT overhang, which can be ligated with a cognate set of overhangs on a plasmid or other fragments (shown in purple). B) A similar amplification, but with a different primer pair, leads to a different product. C) This product, when flipped, can be used in the same assembly as before (as indicated by the purple dashed arrows). The direction of the blue arrow indicates the direction of the reading frame.
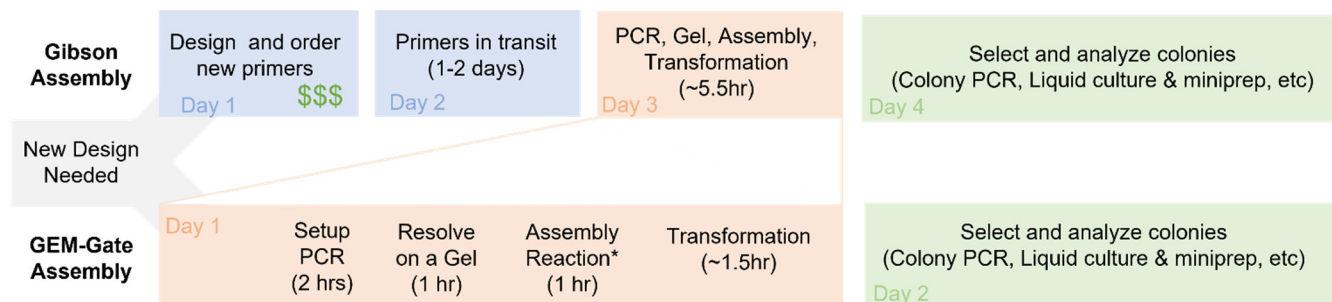
Figure S5. Comparison of workflow for GEM-Gate and Gibson assembly. Upon deciding that a new combination of BioBricks is needed for an experiment, researchers may proceed with Gibson Assembly (top row) or GEM-Gate Assembly (bottom row). Gibson assembly has the added requirement that new primers must be purchased, which adds an expense and a delay, which can vary depending on the speed of synthesis and shipping (blue squares). Since GEM-Gate does not require purchase of new primers for this specific design, but instead relies on a small set that could have been ordered in advance (and used for other projects), most of the tasks can be completed in a single day (orange). This includes the amplification using GEM-Gate primers, verification by gel electrophoresis, an assembly reaction (which can be faster or slower than Gibson assembly, according to the kit or enzyme used), and a transformation. All of these tasks must be completed in Gibson assembly as well, as will all subsequent tasks to confirm the presence of the correct assembled product. GEM-Gate (and Golden Gate assembly) has the added advantage that a successful assembly can be tested for directly before transformation, using flanking primers on the vector backbone (i.e. VF2 and VR for pSB1C3).
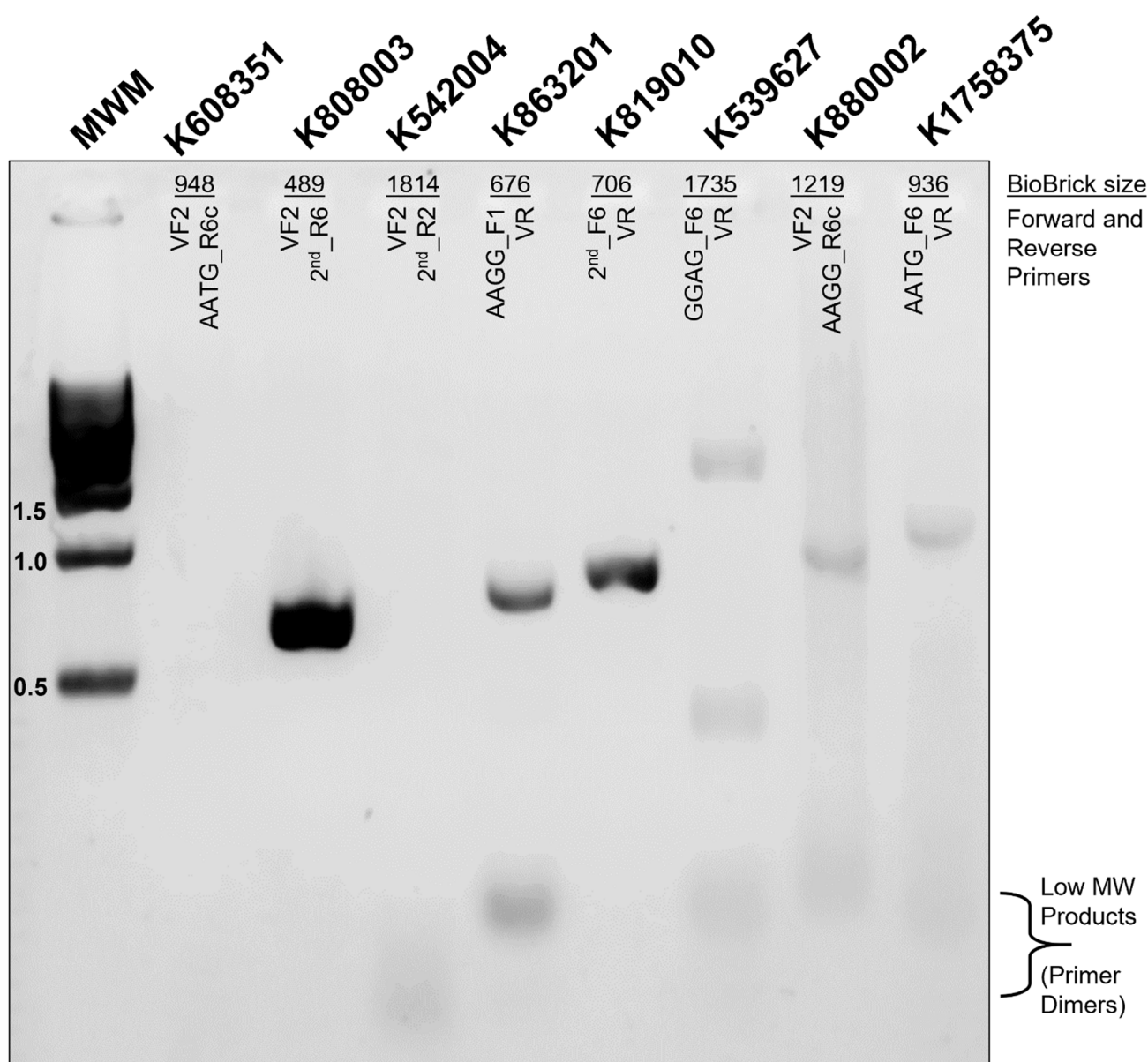
Figure S6. Amplification of selected BioBricks to test for internal or off-target amplification. Prescreened BioBricks, representing potentially problematic sequences from different categories (Coding, Composite, Device, DNA, Generator, Intermediate, Measurement, and Other, respectively) were amplified by the indicated primers via PCR. The BioBricks are indicated above the gel, with the BBa prefix omitted. The size of each BioBrick is listed and compared against the molecular weight marker (MWM, 1Kbp+ Ladder from New England Biolabs), although amplification with control primers adds ~150bp to the size. In this preliminary experiment, 6/8 of the reactions produced an amplicon, and 1 of these reactions (K539627) produced an apparent off-target product. It is worth noting that one of these reactions (K542004) failed to produce an amplicon, but no product is expected since a 2nd stage primer is used without first amplification with the GEM-gate primers.

Figure S7. Proof-reading polymerase corrects primer mismatches. RFP (BBa_K081014) was amplified using the AAGG_F6 and TACT_R6 primers and the Q5 DNA Polymerase from New England Biolabs. The sequence of the primer is shown in blue, with homology to the target sequence (the pSB1C3 prefix sequence, shown in black) indicated by uppercase type and the blue shaded box. Portions of the BsaI and the overhang represent mismatches to the target or template, and are shown in bold type, while the corresponding sequence in the template is orange. The resulting PCR product was sequenced, and the chromatogram trace is shown. Note that this trace gives the reverse complement to the sequence indicated at the top; for clarity, the BioBrick specific sequence is shown in green in both places and the reference sequences are reversed (note the indicated 5' and 3' termini). Nucleotides corresponding to the primer:template mismatch generate polymorphisms and low sequencing read quality. While the primer should be incorporated into the product without changes (which would give a sequence trace of CCTT), this result indicates that the mismatches are actually repaired by the proof-reading Q5 polymerase (with the predominate product AATC, with only the third nucleotide correct, as indicated by the blue shading). Two mismatch induced repairs, changing the intended primer sequence into that of the template, are indicated by solid or dashed gray lines. This result is representative of several other templates and PCR products, particularly with this forward primer.
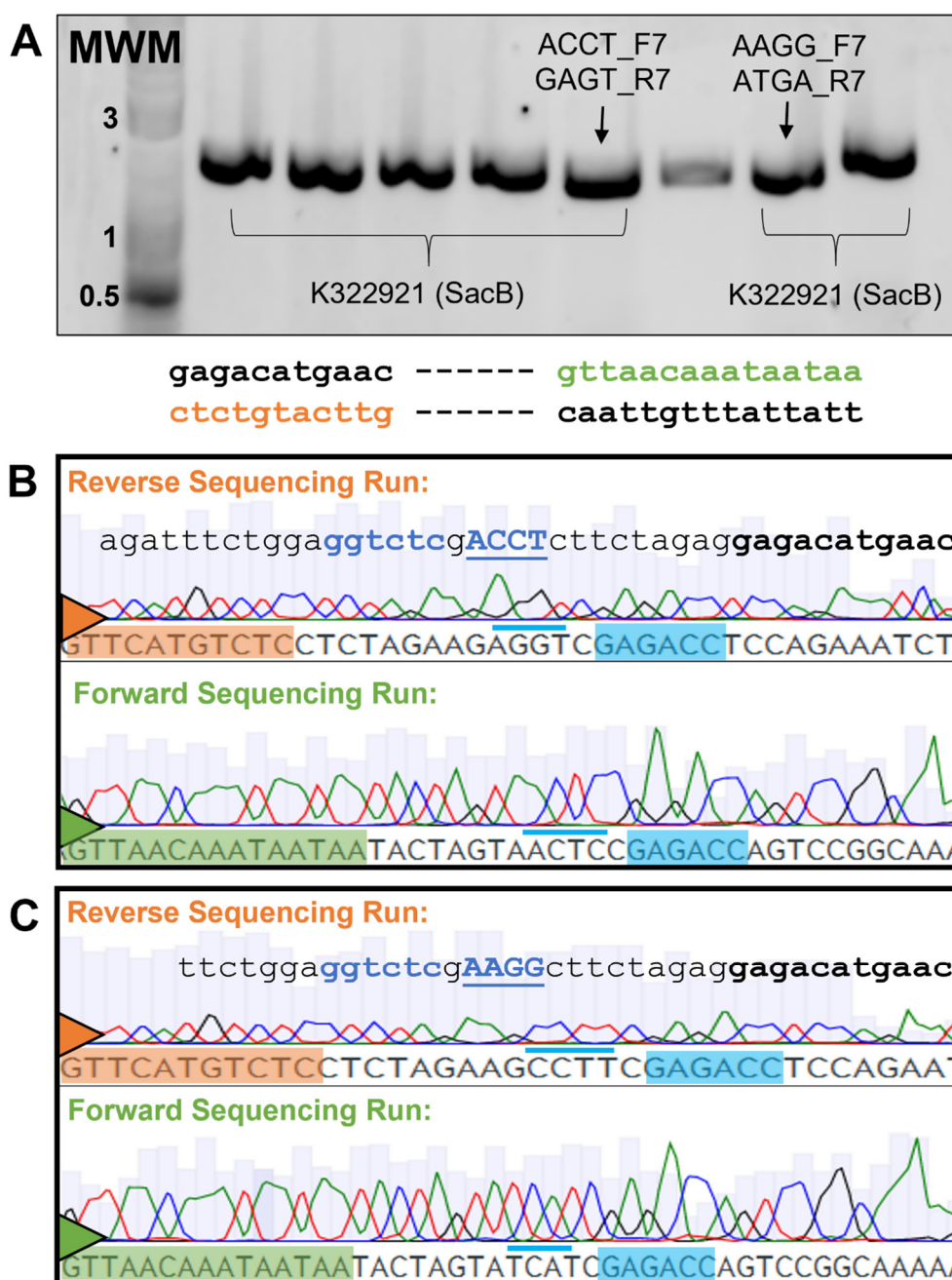
Figure S8. Amplification and Sequence Verification using V7 Primers. A) The BioBrick BBa_K322921, which contains the coding region for the gene SacB, was recovered from the iGEM distribution kit in the context of plasmid pSB1C3. Approximately 20pg of recovered DNA was immediately amplified using a variety of primers and the resulting fragments were resolved on an Agarose gel. The dsDNA sequence of the 5′ and 3′ ends of the BioBrick sequence was retrieved from the registry and is shown below the gel. B) The PCR product from using primers ACCT_F7 and GAGT_R7 was sequenced using the internal SacB_seqF and SacB_seqR primers. The chromatogram trace for the sequencing runs are shown, with the region of the sequence matching either the downstream region (green) or complement of the upstream region (orange) highlighted. The overhang sequence is indicated by a blue line, and the BsaI site (or its complement) is highlighted in blue. For the reverse sequencing run, the reverse complement of the chromatogram is rewritten above the trace, with the BsaI recognition and overhang highlighted in blue. C) The same as B, but for the PCR product using primers AAGG_F7 and ATGA_R7. Compare this sequencing result to that in Figure S7; this set of primers does not lead to correction of primer:template mismatches. These results are representative of other V7 primer and template combinations, including LacZ-alpha, RFP, GFP, and KanR, which were subsequently assembled successfully (See Figures S10, S11 for more details).
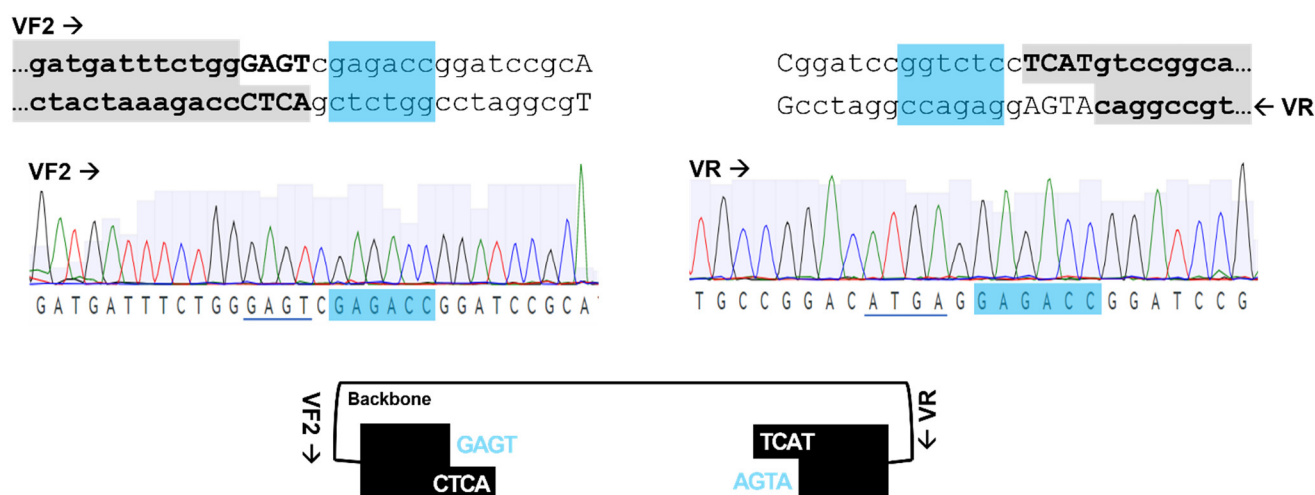
Figure S9. Sequence verification of amplified Backbone. The plasmid pSB1C3 was used as a template with primers Backbone_F_ACTC and Backbone_R_TCAT to generate the type of DNA fragment shown in Figure 3 (reproduced here, bottom). The expected sequence and chromatogram trace for the termini of this fragment are shown (top), with the BsaI recognition sequence shaded in blue and the overhang underlined in blue. Upon BsaI digestion, sequence shaded in gray remains as part of the fragment, while the sequence in white is removed. This DNA fragment was assembled with others (Figure S8) and produced functional plasmids (Figures S10, S11).
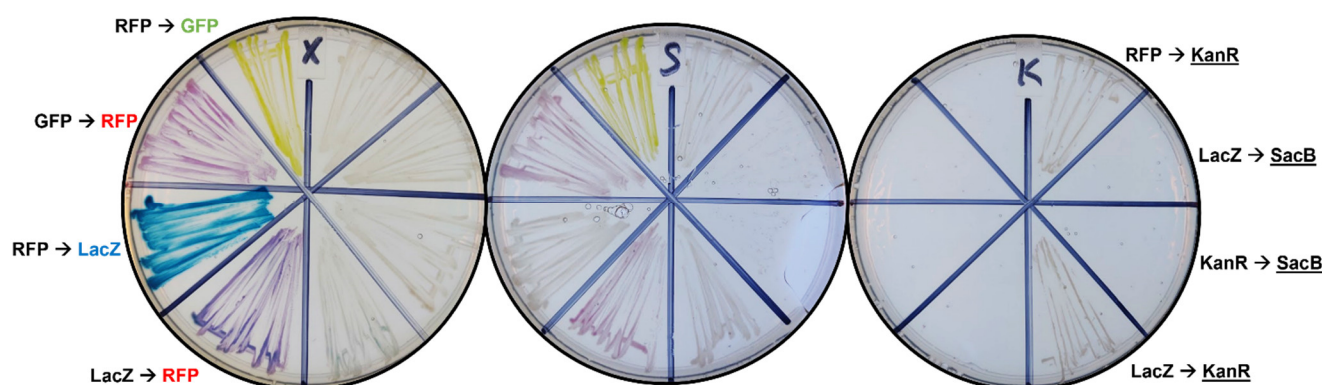

Figure S10. Functional verification of three-fragment assembled products. A variety of coding Bio-Bricks that lack a promoter, including SacB (BBa_K322921), RFP (BBa_K081014), GFP (BBa_K515005), KanR (BBa_132000), and LacZ-alpha (BBa_I732018), were amplified with V7 primers and assembled together with a promoter (BBa_R0010) and backbone in the fashion shown in Figure 3A (SacB termini sequence shown in Figure S8, Backbone termini sequence shown in Figure S9). Several representative strains resulting from these reactions are shown here on LB plates with chloramphenicol, 1mM IPTG, and either 0.02% Xgal (X), 0.6% sucrose (S), or 50ug/mL Kanamycin (K). In these assemblies, the only gene downstream of the promoter should be expressed. These plates where inoculated with strains resulting from the following assemblies, listed counter-clockwise: RFP-Promoter-GFP, GFP-Promoter-RFP, RFP-Promoter-LacZ, LacZ-Promoter-RFP, LacZ-Promoter-KanR, KanR-Promoter-SacB, LacZ-Promoter-SacB, RFP-Promoter-KanR. Strain color or growth on sucrose (toxic for cells expressing SacB), or kanamycin (toxic unless cells express KanR) are consistent with predictions about expression. However, unexpected and leaky expression of LacZ from the LacZ-Promoter-RFP is sufficient to give a mixed color when grown on X-gal plates. A similar faint blue can be seen in the LacZ-Promoter-SacB strain. Serendipitously, this result helps to demonstrate that these gene were indeed assembled into a single product.
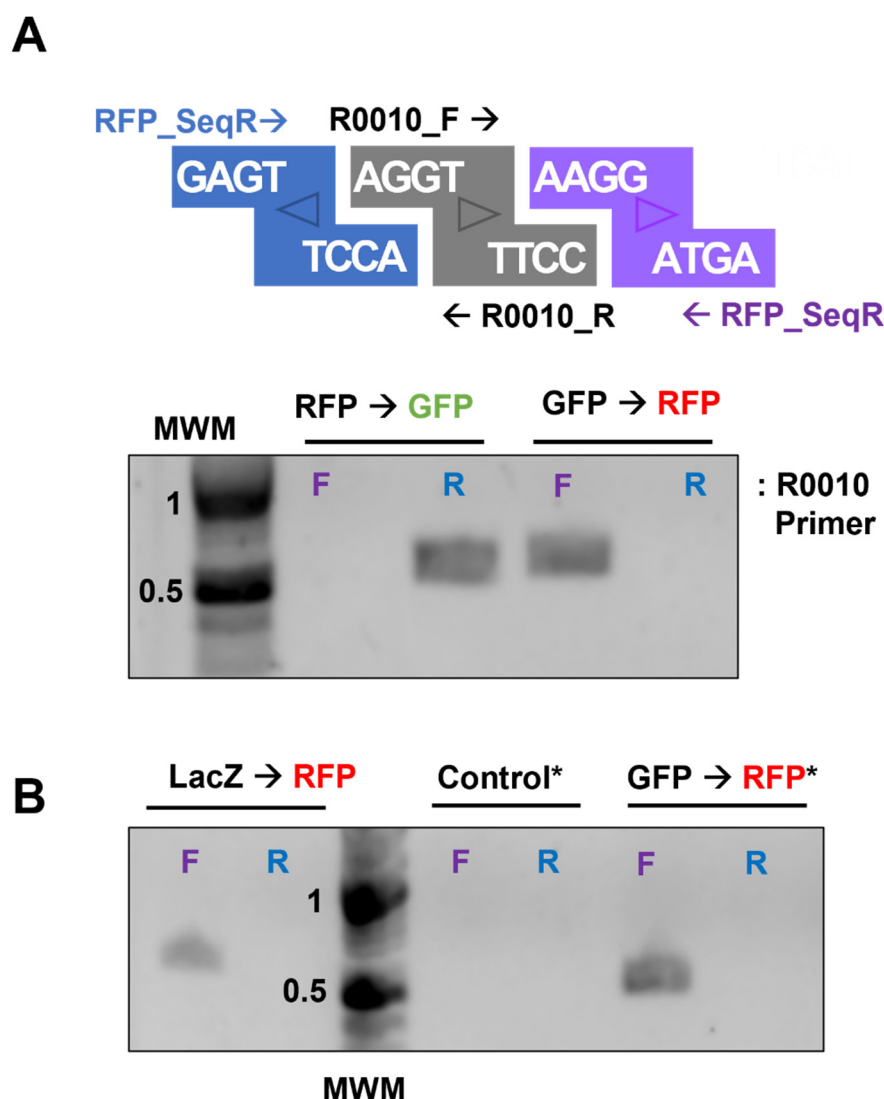
Figure S11. PCR verification of RFP orientation in three-fragment assembled products. Purified plasmid DNA from strains in Figure S10 (namely, the RFP->GFP and GFP->RFP strains, which produce green and red fluorescence, respectively, and the LacZ->RFP strain, which is also red) was obtained via a QIAgen QuickLyse miniprep and diluted $1:10^3$ in water. A) This was then used as a template in a diagnostic PCR with Taq polymerase, the RFP_SeqR primer, and either the R0010_SeqF primer or the R0010_SeqR primer. In two of these strains, the order of the genes is flipped. In the RFP->GFP strain, the RFP gene is upstream of the promoter and in the reverse orientation (in the 'blue' position, consistent with the assembly strategy from Figure 3A). Thus, amplification should occur with RFP_SeqR and R0010_SeqR only. In the GFP->RFP strain, the RFP gene is downstream of the promoter (in the 'purple' position) and should only be amplified by other primer combination. This predicted result was confirmed by gel electrophoresis. Note that the RFP->GFP strain produces green fluorescence, but the PCR result shown here demonstrates that the RFP gene is also present in that construct. B) A similar result is observed for other representative strains, such as those obtained from the lacZ->RFP assembly. This diagnostic PCR can also be used to test for the success of an assembly reaction prior to transformation (these reactions are indicated by an asterisk). A control reaction, which contained 10-fold excess DNA but no BsaI or T4 DNA Ligase, was diluted $1:10^3$ and used as a template for this PCR. As expected, this produces no result, since no assembly between R0010 and K081014 has occurred. In contrast, the assembly reaction that contained the enzyme produced the expected bands when tested in the same way. It is worth noting that this is a viable method to check for successful Golden Gate assembly prior to transformation, but would likely produce false positives for checking Gibson assembly reactions. In the gel images, size of select bands in the molecular weight marker (MWM) are shown in kilobases.

Supplemental References

1. Davies, K. From the Bench to Benchling. *GEN Edge* **2020,** *2.1*: 303–309. Available online at https://benchling.com (last accessed February 19th, 2023)