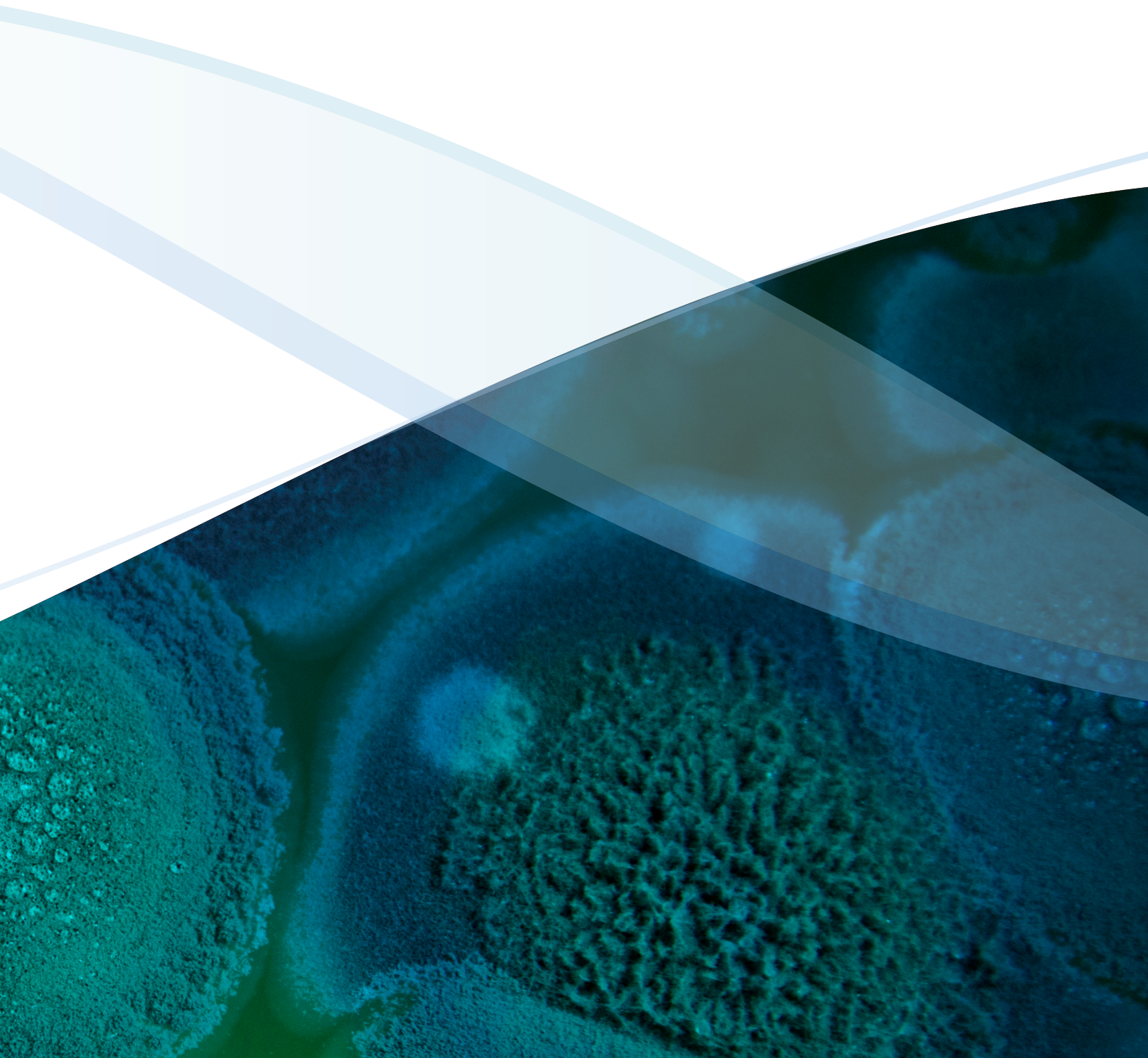
ATCC®

Synthetic Biology Solutions User Guide

Biological Systems Design and Development



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PRODUCT OVERVIEW

The ATCC Synthetic Biology Yeast Tool Kit, which was developed by the Weiss laboratory at MIT, is based on a framework[1] designed to assemble multiple transcriptional units (TUs) for the flexible delivery of genetic circuits in yeast. This approach combines advances in Gateway® recombination-based cloning strategies,[2,3] Gibson Assembly®,[4,6] and unique nucleotide sequences for determining the position of every TU to permit the development of genetic circuits comprising multiple promoter-gene pairs. The system operates in two stages (Figure 1): First, a single TU comprising a promoter-gene pair is generated via Gateway recombinase technology; and second, multiple coded TUs are positionally assembled together into a vector backbone using Gibson Assembly.

The initial design to full circuit construction takes approximately 4-7 days and does not require gel extractions or polymerase chain reactions, thus reducing potential mutation rates. Additionally, multiple TUs can be built in parallel, rather than sequentially or in a hierarchical reaction, allowing individual elements to be swapped with ease (e.g., changing a promoter to enable higher levels of expression or using a fluorescent protein color). As part of the tool kit, ATCC offers constitutive promoters, inducible promoters, transcriptional regulators, a selection marker, and two empty donor vectors for investigators to build additional promoters and genes for assembly. Moreover, the necessary backbones to create up to 7 single TUs and transform yeast with site-specific integration, low-copy, or high-copy vectors are available (Table 2).

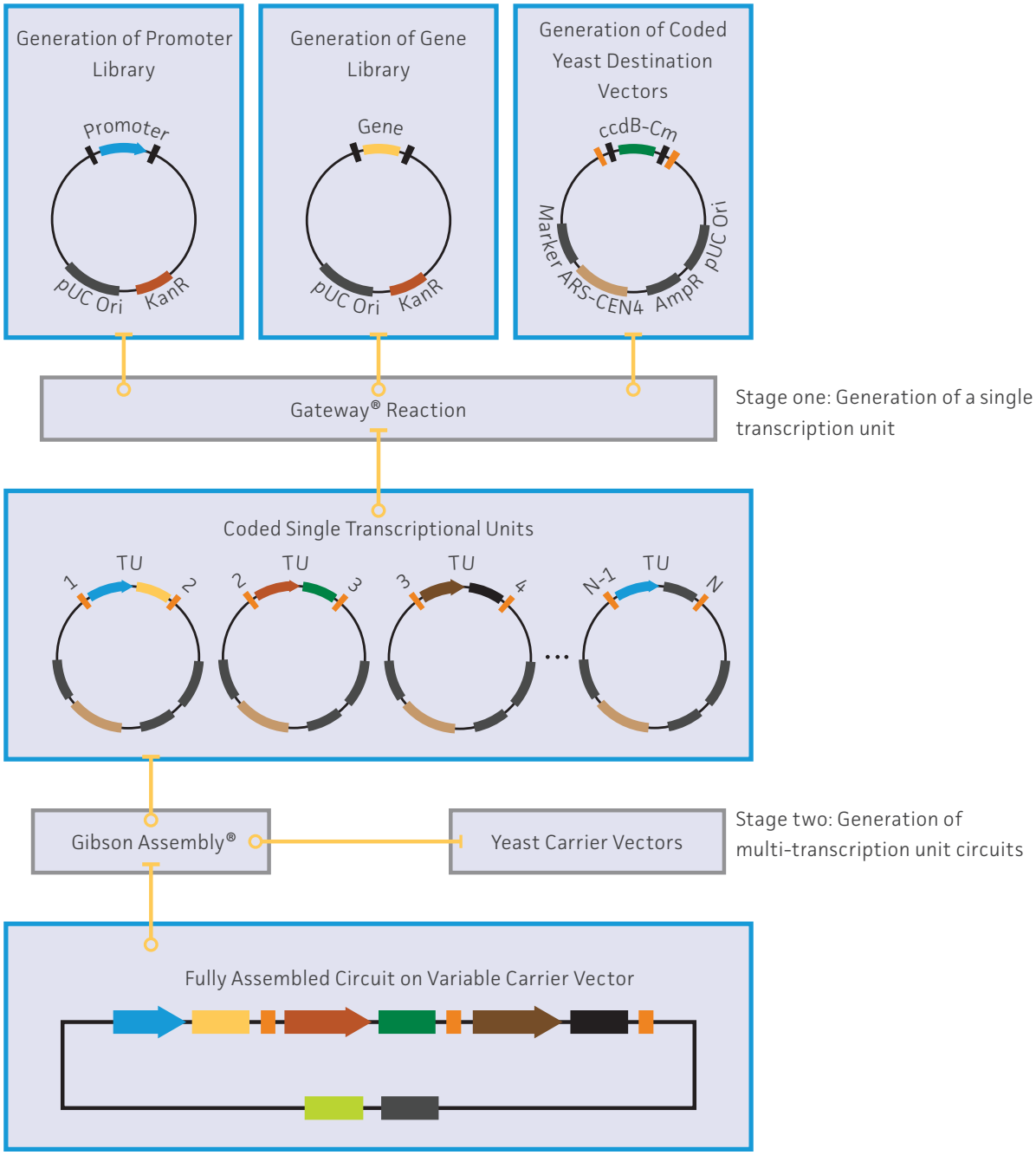

Figure 1: **Schematic of the two-stage Gateway/Gibson Assembly strategy using ATCC Synthetic Biology Yeast Tool Kit components**

APPLICATION EXAMPLES

Example 1: How to build a construct that enables constant ectopic expression of a single gene of interest

This construction demonstrates how to express a green fluorescent protein (GFP) under the control of a strong yeast promoter (YPD1). It involves two Gateway reactions and one Gibson Assembly reaction (Figure 2). Before performing the Gateway and Gibson Assembly reactions, the GFP gene should first be cloned into the entry vector pENTR_L1L2_DONR1 (ATCC® SB-1013™) to generate the construct pENTR_L1L2_GFP. Additional detailed information on how to build a pENTR_L1L2_Gene construct can be viewed in the Appendix.

STAGE 1: GATEWAY REACTION

1 Each TU is assembled into a destination vector using the Gateway LR reaction, according to manufacturer's instructions5.
   a In the first TU assembly, pDEST-12y (ATCC® SB-1000™), pENTR_L4R1_YPD1 (ATCC® SB-1041™), and the pre-built pENTR_L1L2_GFP construct are required.
   b In the second TU assembly, pDEST-23y (ATCC® SB-1001™), pENTR_L4R1_TEF2 (ATCC® SB-1044™), and pENTR_L1L2_KanMX (ATCC® SB-1006™) are required.

2 After performing the Gateway reaction, a portion of the reaction mixture is transformed into ccdB-sensitive, competent *E. coli* cells under antibiotic selection using ampicillin.

3 Assembly clones containing the TU of interest can be verified via restriction mapping or sequencing.

STAGE 2: GIBSON ASSEMBLY

1 Assembly requires four genetic parts: Two TUs, a PacI-linearized low-copy carrier, and a PCR-amplified adapter containing the kanamycin resistance gene (Kan). Detailed information on the Gibson Assembly reaction can be viewed in the manufacturer's instruction manual6.
   a Each TU built using the Gateway reaction, together with the two unique 45 bp adjacent sequences, can be released from the vector using the restriction enzyme I-SceI.
   b The low-copy carrier pCarrier 3 (416) (ATCC® SB-1004™) is digested via the restriction enzyme PacI to linearize the vector, resulting in two unique 45 bp sequences (1 and X) present at the ends of the linearized vector.
   c The Kan adapter is PCR-amplified using the primer pair Seq3-KanF and KanR-SeqX (Table 1); any of the ATCC pENTR entry vectors (e.g., ATCC® SB-1041™) can be used as a DNA template. The PCR product will contain unique 45 bp sequences (3 and X) at the ends of the DNA fragment.

2 The assembly order is determined by the unique 45 bp sequences (1, 2, 3, and X) located at the ends of each fragment.

3 After complete assembly of the construct in *E. coli*, the plasmid is then isolated and transformed into yeast under antibiotic selection using G418.

4 Following successful assembly and yeast transformation, the expression of GFP can be analyzed by cytometry.

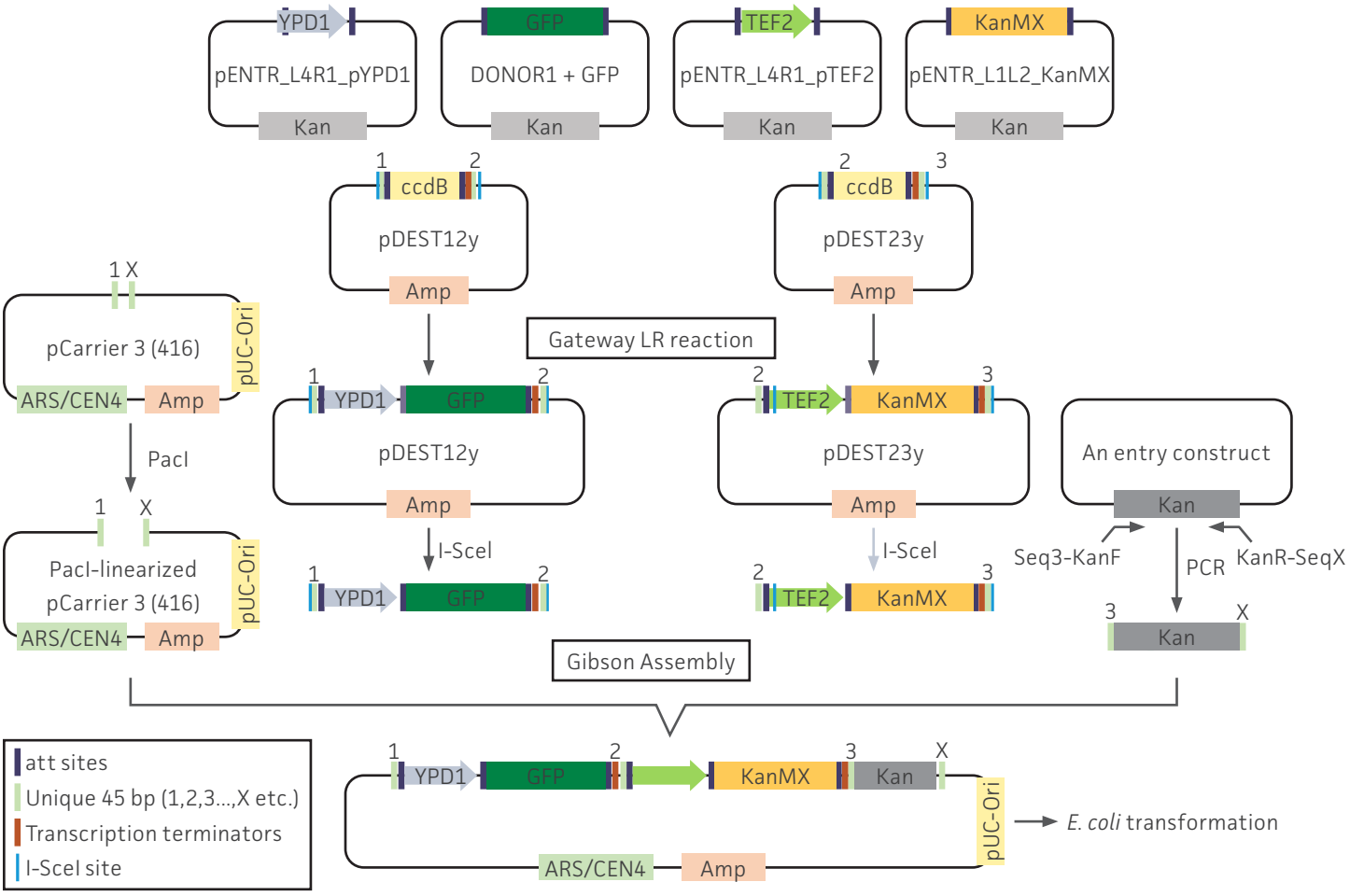

Figure 2: **Schematic of how to build a construct that enables constant ectopic expression of a single gene of interest**

Table 1: **PCR primers for generating SeqN – SeqX adapters**

Name	Sequence*
KanR-SeqX	**GGTTAGGCGACTGTTATAACTTACCTCTGTAATACTAGTGATACC**ATGAGCTTGCGCCGT
Seq1-KanF	**GGTTTACCGAGCTCTTATTGGTTTTCAAACTTCATTGACTGTGCC**TGGCCCGTGTCTCAA
Seq2-KanF	**GGTGCGTTTTTATGCTTGTAGTATTGTATAATGTTTTTAAGATCC**TGGCCCGTGTCTCAA
Seq3-KanF	**GGTCTAATACCCAATCTCTCGTCTTATCCAGATGTTTTATACGCC**TGGCCCGTGTCTCAA
Seq4-KanF	**GGTGAATTCCCTTATGTGAGTGTAAAAGGCAGGCGAGTTTGTCCC**TGGCCCGTGTCTCAA
Seq5-KanF	**GGTTGCTTGCAAAAGCAGTAATTGGAAAGCACTCTCAAAGAATCC**TGGCCCGTGTCTCAA
Seq6-KanF	**GGTAGATAAGTTGATTTAGCCATAAAATATTGTTTCCGTGACCCC**TGGCCCGTGTCTCAA
Seq7-KanF	**GGTTCTGAGTCACGGCTTCATTGGCATTCCGTACAACGAACGTCC**TGGCCCGTGTCTCAA
Seq8-KanF	**GGTCCTCAGAGAGCCTATAGCGGTAAAACAACACCATGCATCCCC**TGGCCCGTGTCTCAA

*The unique 45 bp codes are highlighted in bold

Example 2: **How to build an inducible expression system that is integrated into the genome**

This construction demonstrates how to build an inducible expression system integrated into the genome that comprises a single gene (e.g., a yellow fluorescent protein [YFP]) mediated by the antibiotic 2,4-diacetylphloroglucinol (DAPG) and the transcriptional repressor PhlF (Figure 3A). It involves three Gateway reactions and one Gibson Assembly reaction. Before performing the Gateway and Gibson Assembly reactions, a reporter gene (e.g., YFP) should first be cloned into the entry vector pENTR_L1L2_DONR1 (ATCC® SB-1013™) to generate the construct pENTR_L1L2_YFP. Additional detailed information on how to build a pENTR_L1L2_Gene construct can be viewed in the Appendix.

STAGE 1: GATEWAY REACTION

1. Three TUs are built in destination vectors using the Gateway reaction, according to manufacturer's instructions5.
   a. In the first TU assembly, pDEST-12y (ATCC® SB-1000™), pENTR_L4R1_YPD1 (ATCC® SB-1041™), and pENTR_L1L2_PhlF (SB-1032™) are required.
   b. In the second TU assembly, pDEST-23y (ATCC® SB-1001™), pENTR_L4R1_ GPD-PhlF (ATCC® SB-1070™), and pENTR_L1L2_YFP are required.
   c. In the third TU assembly, pDEST-34y (ATCC® SB-1002™), pENTR_L4R1_TEF2 (ATCC® SB-1044™), and pENTR_L1L2_KanMX (ATCC® SB-1006™) are required.
2. After performing the Gateway reaction, a portion of the reaction mixture is transformed into ccdB-sensitive, competent *E. coli* cells under antibiotic selection using ampicillin.
3. Assembly clones containing the TU of interest can be verified via restriction mapping or sequencing.

STAGE 2: GIBSON ASSEMBLY

1. Assembly requires five genetic parts: Three TUs, a PCR-amplified Kan-resistant adapter, and a PacI-linearized integrating carrier. Detailed information on the Gibson Assembly reaction can be viewed in the manufacturer's instruction manual6.
   a. Each TU built in a destination vector, together with two unique 45 bp adjacent sequences, can be released from the vector using the restriction enzyme I-SceI.
   b. The integrating carrier pCarrier 8 (integ) (ATCC® SB-1005™) is digested via the restriction enzyme PacI to linearize the vector, resulting in two unique 45 bp sequences (1 and X) present at the ends of the linearized vector.
   c. The Kan-resistant adapter is PCR-amplified using the primer pair Seq4-KanF and KanR-SeqX (Table 1); any of the ATCC pENTR entry vectors (e.g., ATCC® SB-1041™) can be used as a DNA template. The PCR product will contain unique 45 bp sequences (4 and X) at the ends of the DNA fragment.
2. The assembly order is determined by the unique 45 bp sequences (1, 2, 3, 4, and X) located at the ends of each fragments.
3. After complete assembly of the construct in *E. coli*, the plasmid is then isolated and digested with I-SceI to release the construct together with the two adjacent homothallic (HO) fragments (~480 bp) for yeast transformation. Two HO sequences flanking the construct mediate HO locus integration via homologous recombination (Figure 3B).

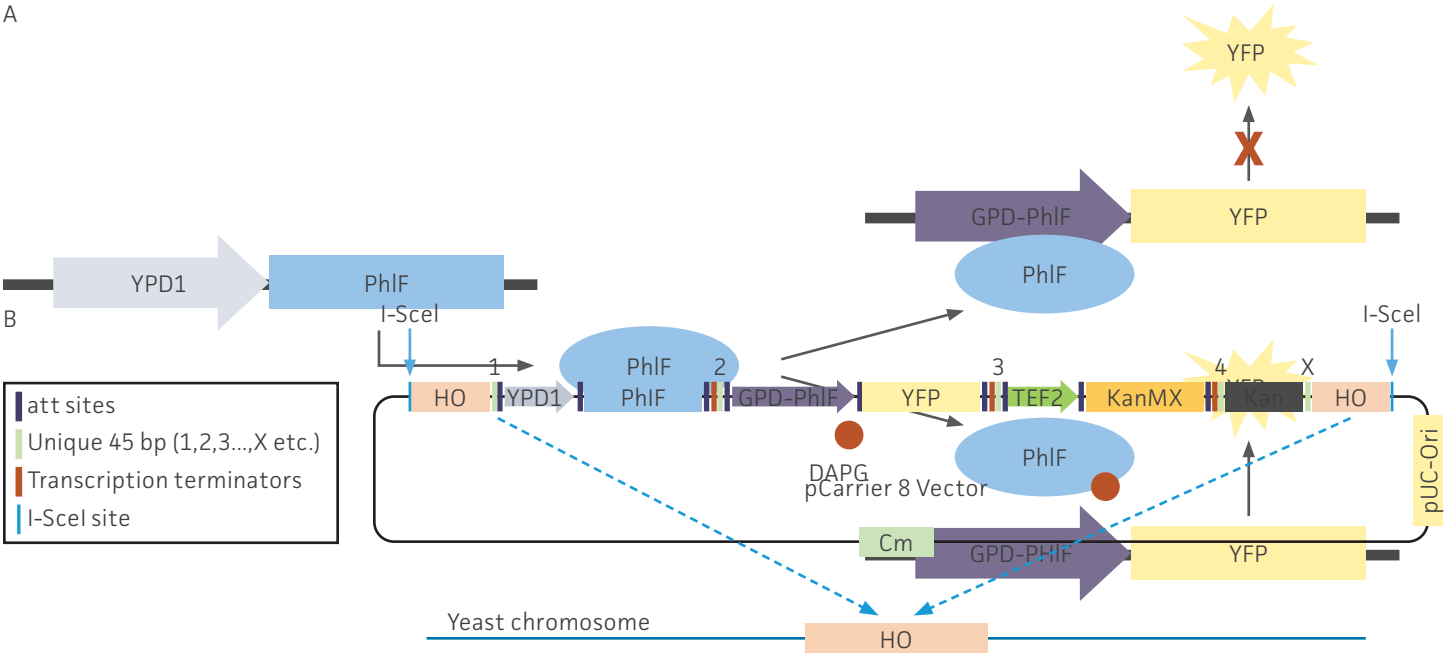

Figure 3: Schematic of how to build a DAPG-inducible circuit that is integrated into the genome. A) Diagram of the DAPG/PhlF system using YFP as a reporter. The transcriptional regulator PhlF binds to the PhlF operator region of the synthetic promoter GPD-PhlF in the absence of DAPG; this binding inhibits YFG expression. The addition of DAPG, which binds to the PhlF regulator, results in the dissociation of PhlF from the promoter; this leads to the steady expression of YFP. B) Diagram of a gene circuit assembled in an integration vector. Following the complete assembly of three TUs along with the Kan adapter into the integration vector, the gene circuit flanked by two HO sequences is released from the vector via the I-SceI restriction enzyme. The gene circuit then integrates into the HO locus in the yeast chromosome via homologous recombination.

FREQUENTLY ASKED QUESTIONS

1 **What if I don't get any colonies after the transformation of TUs built using the Gateway reaction?**

Ensure the competency of the host cells and that plates are supplemented with the correct antibiotic by using a pUC19-Amp control with each transformation. For each Gateway reaction, verify that all necessary genetic parts were added; because the reaction results in recombination, missing any one of these components will result in the lack of a successful product.

2 **What if I don't get any colonies after the transformation of Gibson Assembly products?**

Ensure cell competency of the host cells and that plates are supplemented with the correct antibiotics (CM + Kan) by using a control with each Gibson Assembly reaction. For Gibson Assembly reactions, verify that all necessary genetic parts were added in equimolar ratios and that the Gibson mix is fresh (most commercial mixes include a positive control).

3 **What is the purpose of the adapter sequence?**

Because each carrier vector has a defined Sequence 1 and Sequence X flanking 45 bp regions, but individual networks may range in size from 1 to 7 parts, the adapter bridges the varying sizes to a single carrier. Additionally, by including a kanamycin resistance gene, the dual selection of carrier and adapter improve the final success rate of finding a correct Gibson clone.

4 **How do I generate my own ENTR promoter or gene plasmids for use?**

As part of the tool kit, we have included two DONR plasmids that have blue-white screening capabilities and Golden Gate cloning compatibility. Simply PCR or synthesize the promoter or gene of interest as described in the Appendix and follow a single-step cloning reaction.

PRODUCT LISTING

Table 2: ATCC® Synthetic Biology Yeast Tool Kit

ATCC® No.	Product Name	Product Description
SB-1000™	pDEST-12y	Gateway destination vector-12
SB-1001™	pDEST-23y	Gateway destination vector-23
SB-1002™	pDEST-34y	Gateway destination vector-34
SB-1003™	pDEST-45y	Gateway destination vector-45
SB-1015™	pDEST-56y	Gateway destination vector-56
SB-1016™	pDEST-67y	Gateway destination vector-67
SB-1017™	pDEST-78y	Gateway destination vector-78
SB-1004™	pCarrier 3 (416)	Yeast low copy vector
SB-1024™	pCarrier 7 (426)	Yeast high copy vector
SB-1005™	pCarrier 8 (integ)	Yeast integration vector
SB-1006™	pENTR_L1L2_KanMX	Entry vector carrying a yeast selection marker
SB-1013™	pENTR_L1L2_DONR1	Gateway empty entry vector-1
SB-1014™	pENTR_L4R1_DONR2	Gateway empty entry vector-2
SB-1008™	pENTR_L4R1_pHOG1	Entry vector carrying yeast constitutive promoter, pHOG1
SB-1035™	pENTR_L4R1_pHOT1	Entry vector carrying yeast constitutive promoter, pHOT1
SB-1036™	pENTR_L4R1_pSSRE	Entry vector carrying yeast constitutive promoter, pSSRE
SB-1038™	pENTR_L4R1_pTR-SSRE	Entry vector carrying yeast constitutive promoter, pTR-SSRE
SB-1041™	pENTR_L4R1_pYPD1	Entry vector carrying yeast constitutive promoter, pYPD1
SB-1044™	pENTR_L4R1_pTEF2	Entry vector carrying yeast constitutive promoter, pTEF2
SB-1104™	pENTR_L4R1_pCYC1MIN	Entry vector carrying yeast constitutive promoter, pCYC1MIN
SB-1105™	pENTR_L4R1_pSKN7	Entry vector carrying yeast constitutive promoter, pSKN7
SB-1046™	pENTR_L4R1_pGAL1	Entry vector carrying yeast inducible promoter, pGAL1
SB-1050™	pENTR_L4R1_pTETO7	Entry vector carrying a tetracycline response element (TRE)
SB-1055™	pENTR_L4R1_BetI-pCYC1MIN	Entry vector carrying a synthetic inducible promoter, BetI-pCYC1MIN
SB-1056™	pENTR_L4R1_BM3R1-pCYC1MIN	Entry vector carrying a synthetic inducible promoter, BM3R1-pCYC1MIN
SB-1059™	pENTR_L4R1_LitR-pCYC1MIN	Entry vector carrying a synthetic inducible promoter, LitR-pCYC1MIN
SB-1060™	pENTR_L4R1_PhlF-pCYC1MIN	Entry vector carrying a synthetic inducible promoter, PhlF-pCYC1MIN
SB-1061™	pENTR_L4R1_QacR-pCYC1MIN	Entry vector carrying a synthetic inducible promoter, QacR-pCYC1MIN
SB-1066™	pENTR_L4R1_pGPD-BM3R1	Entry vector carrying a synthetic repressible promoter, pGPD-BM3R1
SB-1069™	pENTR_L4R1_pGPD-LitR	Entry vector carrying a synthetic repressible promoter, pGPD-LitR
SB-1070™	pENTR_L4R1_pGPD-PhlF	Entry vector carrying a synthetic repressible promoter, pGPD-PhlF
SB-1071™	pENTR_L4R1_pGPD-QacR	Entry vector carrying a synthetic repressible promoter, pGPD-QacR
SB-1031™	pENTR_L1L2_rtTA	Entry vector carrying a regulator, rtTA (reverse tetracycline-controlled transactivator)
SB-1032™	pENTR_L1L2_PhlF	Entry vector carrying a TetR homolog-based repressor, PhlF
SB-1086™	pENTR_L1L2_BM3R1	Entry vector carrying a TetR homolog-based repressor, BM3R1
SB-1089™	pENTR_L1L2_LitR	Entry vector carrying a TetR homolog-based repressor, LitR
SB-1090™	pENTR_L1L2_QacR	Entry vector carrying a TetR homolog-based repressor, QacR
SB-1094™	pENTR_L1L2_BetI-VP64	Entry vector carrying a TetR homolog-based repressor, BetI-VP64
SB-1095™	pENTR_L1L2_BM3R1-VP64	Entry vector carrying a TetR homolog-based repressor, BM3R1-VP64
SB-1098™	pENTR_L1L2_LitR-VP64	Entry vector carrying a TetR homolog-based repressor, LitR-VP64
SB-1099™	pENTR_L1L2_PhlF-VP64	Entry vector carrying a TetR homolog-based repressor, PhlF-VP64
SB-1100™	pENTR_L1L2_QacR-VP64	Entry vector carrying a TetR homolog-based repressor, QacR-VP64
SB-2000™	Basic Yeast Synthetic Biology Tool Kit	The basic tool kit consists of 4 destination vectors (SB-1000™ , SB-1001™ , SB-1002™ , and SB-1003™), 3 carrier vectors (SB-1004™, SB-1005™, and SB-1024™), 2 empty entry vectors of the Gateway system (SB-1013™ and SB-1014™), a Kanamycin/G418-resistant gene (SB-1006™), 4 yeast constitutive promoters (SB-1008™, SB-1035™, SB-1041™, SB-1105™), and an inducible promoter (SB-1046™)
SB-2001™	rtTA/TETO7 activation pair	TRE promoter (ATCC® SB-1050™) and its regulator rtTA (ATCC® SB-1031™)

ATCC® No.	Product Name	Product Description
SB-2002™	BetI activation pair (TetR Homolog)	Synthetic promoter BetI-pCYC1MIN (ATCC® SB-1055™) and its regulator BetI-VP64 (ATCC® SB-1094™)
SB-2003™	BM3R1 activation pair (TetR Homolog)	Synthetic promoter BM3R1-pCYC1MIN (ATCC® SB-1056™) and its regulator BM3R1-VP64 (ATCC® SB-1095™)
SB-2004™	LitR activation pair (TetR Homolog)	Synthetic promoter LitR-pCYC1MIN (ATCC® SB-1059™) and its regulator LitR-VP64 (ATCC® SB-1098™)
SB-2005™	PhlF activation pair (TetR Homolog)	Synthetic promoter PhlF-pCYC1MIN (ATCC® SB-1060™) and its regulator PhlF-VP64 (ATCC® SB-1099™)
SB-2006™	QacR activation pair (TetR Homolog)	Synthetic promoter QacR-pCYC1MIN (ATCC® SB-1061™) and its regulator QacR-VP64 (ATCC® SB-1100™)
SB-2008™	BM3R1 repression pair (TetR Homolog)	Synthetic promoter pGPD-BM3R1 (ATCC® SB-1066™) and its regulator BM3R1 (ATCC® SB-1086™)
SB-2009™	LitR repression pair (TetR Homolog)	Synthetic promoter pGPD-LitR (ATCC® SB-1069™) and its regulator LitR (ATCC® SB-1089™)
SB-2010™	PhlF repression pair (TetR Homolog)	Synthetic promoter pGPD-PhlF (ATCC® SB-1070™) and its regulator PhlF (ATCC® SB-1032™)
SB-2011™	QacR repression pair (TetR Homolog)	Synthetic promoter pGPD-QacR (ATCC® SB-1071™) and its regulator QacR (ATCC® SB-1090™)

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5 Invitrogen. Gateway Technology: A universal technology to clone DNA sequences for functional analysis and expression in multple systems. Revision 1.0, 2003.

6 New England BioLabs, Inc. Gibson Assembly® Master Mix: Instruction Manual. Version 3.3, 2017.

7 New England BioLabs, Inc. NEB® Golden Gate Assembly Mix: Instruction Manal. Version 2.0, 2017.

APPENDIX

Generation of custom L1L2 gene ENTR or L4R1 promoter vectors using Golden Gate Assembly

We recommend using the Golden Gate cloning method to insert a gene or a promoter of interest into the pENTR_L1L2_DONR1 (ATCC® SB-1013™) or pENTR_L4R1_DONR2 (ATCC® SB-1014™) vectors, respectively (Figure 4). The vectors contain two BsaI sites flanking the LacZ alpha gene, enabling the selection of positive clones containing the insertion via screening on plates supplemented with X-Gal and IPTG. The gene or promoter can be generated by either gene synthesis or PCR. The ends of the DNA fragment should include a BsaI site and 4 bp overhang (Figure 4A). For the detailed Golden Gate assembly protocol, please follow the manufacturer's instruction7.

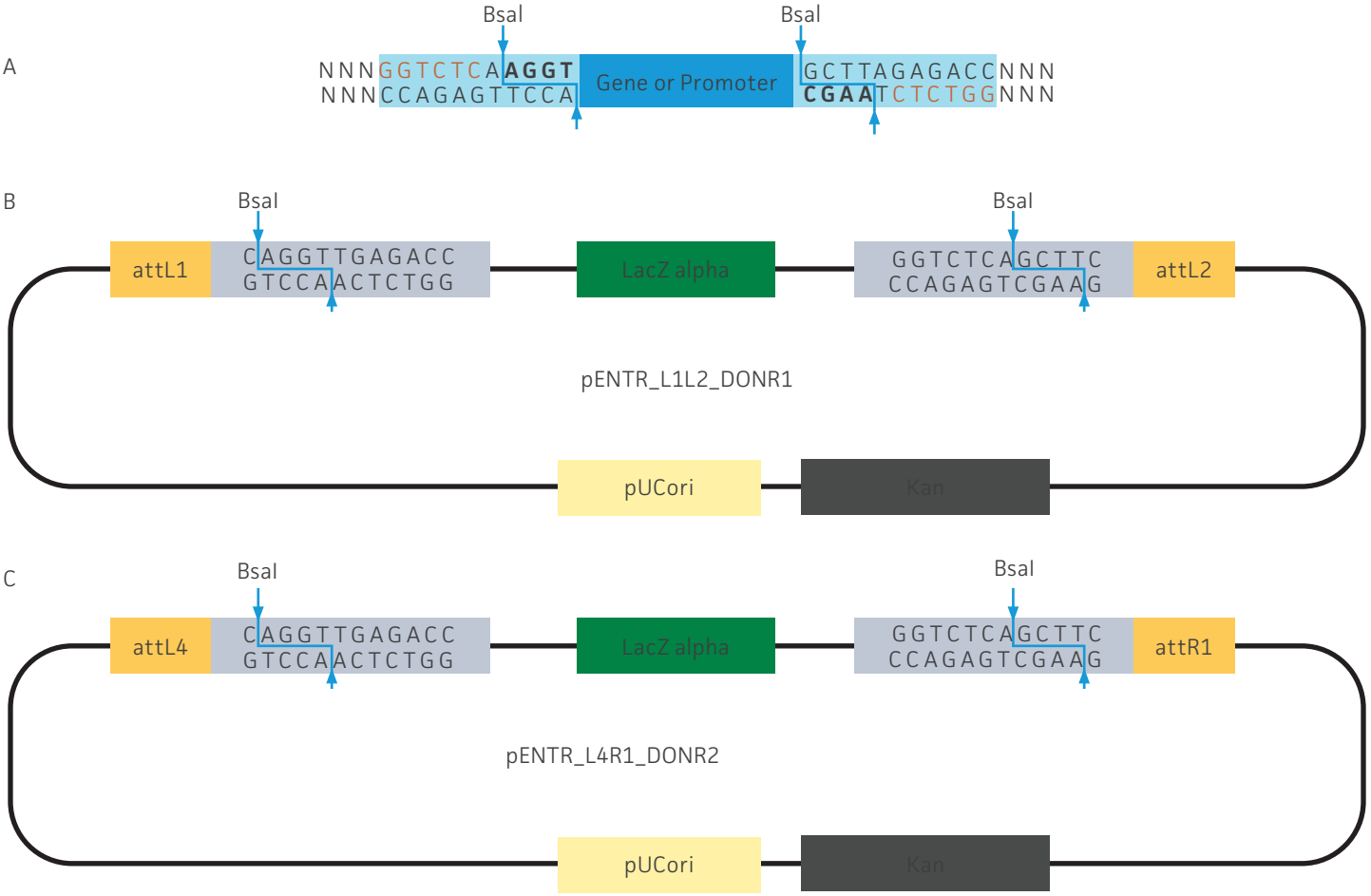

Figure 4: A) Sequence information for PCR-amplified or synthesized gene or promoter of interest. The ends of the DNA fragment should include a BsaI site (red) and a 4 bp overhang (bold). B) Genetic map for pENTR_L1L2_DONR1. C) Genetic map for pENTR_L4R1_DONR2.

Disclaimers

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