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Research Article

GoldenBraid2.0 *E. coli*: a comprehensive and characterized toolkit for enterics

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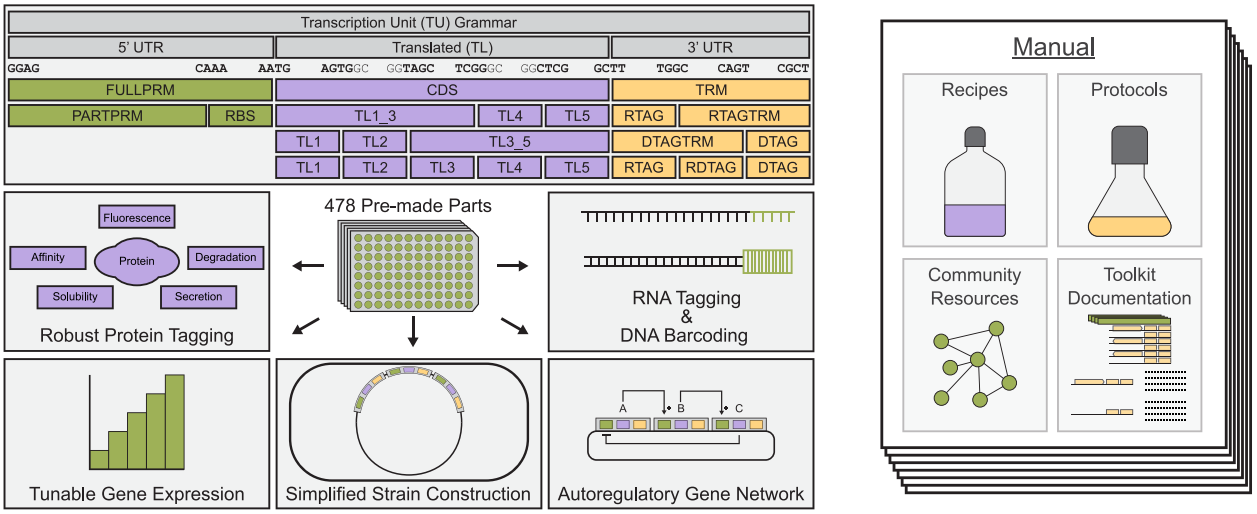
Abstract

Modular cloning systems streamline laboratory workflows by consolidating genetic 'parts' into reusable and modular collections, enabling researchers to fast-track strain construction. The GoldenBraid 2.0 modular cloning system utilizes the cutting property of type IIS restriction enzymes to create defined genetic 'grammars', which facilitate the reuse of standardized genetic parts and assembly of genetic parts in the right order. Here, we present a GoldenBraid 2.0 toolkit of genetic parts designed to accelerate cloning in the model bacterium *Escherichia coli*. This toolkit features 478 pre-made parts for gene expression and protein tagging as well as strains to expedite cloning and strain construction, enabling researchers to quickly generate functional plasmid-borne or chromosome-integrated expression constructs. In addition, we provide a complete laboratory manual with overviews of common reagent recipes, *E. coli* protocols, and community resources to promote toolkit utilization. By streamlining the assembly process, this resource will reduce the financial and temporal burdens of cloning and strain building in many laboratory settings.

Graphical Abstract



Keywords: cloning; molecular; genetic engineering; gram-negative bacteria

Introduction

Genetic cloning enables researchers to build custom expression constructs, reporter systems, genetic circuits and other genetic tools. While powerful, this process is frequently the bottleneck in experimental workflows, largely due to the time and effort required to design and generate genetic parts. *Escherichia coli* is widely regarded as the organism of choice for routine cloning, owing to its well-characterized genetics, rapid growth, and extensive catalogue of established protocols and vectors. Its robustness and ease of manipulation not only facilitate high-yield plasmid

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propagation but also provide a quick and reliable pipeline for the verification of newly assembled constructs. Meanwhile, modular cloning (MoClo) systems offer an effective solution to streamline cloning workflows and overcome bottlenecks [1]. These systems rely on fixed criteria, typically in the form of pre-determined restriction enzyme sites and standardized ligation overhangs [2], which make part design formulaic and allow previously designed components to be reused without additional modification. This approach has been embraced not only by established research labs but also by student teams competing in iGEM [3].

One form of modular cloning system, commonly referred to as Golden Gate [4] and its derivative GoldenBraid [5] cloning, uses type IIS restriction enzymes. These restriction enzymes cleave multiple bases away from their recognition sites (Fig. 1A). As such, a single enzyme can create any desired four-base pair overhang. Furthermore, as the enzyme binding site is distal to its cut site, the enzyme can cut again if the cut sequence is ligated back together. Conversely, if the newly cut sequence is ligated to another molecule that does not contain the enzyme binding site, the new ligation is stable and will not be cut. This means that type IIS enzymes allow for dual digestion–ligation reactions, where undesirable products—re-ligated input components—are repeatedly depleted and the desired ligation product is gradually enriched (Fig. 1B; Supplementary fig. S1).

As MoClo systems that use type IIS restriction enzymes are not limited to a single four-base overhang, any genetic construct can be designed to include the same restriction enzyme sites. Consequently, parts are grouped by function—e.g. promoters or coding sequences—and each group is assigned specific four-base overhangs that define the assembly 'grammar'. For instance, promoters may have the overhangs GGAG and AATG, while coding sequences may use AATG and GCTT, ensuring that, in a single digestion–ligation reaction, these parts will consistently assemble in the correct orientation. In the GoldenBraid 2.0 standard [6], each component of a transcription unit follows this grammar, making assembly of larger constructs both straightforward and scalable (Fig. 1C; Supplementary figs S2 and S3).

For storage and streamlined assembly of genetic parts, MoClo systems use a set of dedicated shuttle vectors. In GoldenBraid 2.0, a selected part is first 'domesticated' by polymerase chain reaction (PCR) amplification with grammar-specific primers, which also remove any internal type IIS restriction sites (Supplementary fig. S4). The resulting PCR product is then digested and ligated into a Universal Part Domestication plasmid (pUPD3) (Supplementary fig. S5). This plasmid both stores the genetic part and serves as the entry point for subsequent cloning reactions (Fig. 1D).

Once parts are domesticated, MoClo systems assemble the parts into defined assembly 'levels'. In the GoldenBraid 2.0 system, there are two levels: alpha (α) and omega (Ω). For an α assembly, domesticated parts comprising a single transcription unit are assembled onto a single α-level vector. For example, a domesticated promoter, coding sequence, and terminator can be assembled into a full transcription unit on an α-level vector. Alpha-level vectors are designed such that they contain grammar themselves, allowing the contents of an $\alpha 1$ vector to be combined with those of an $\alpha 2$ vector onto an Ω-level vector. Likewise, two Ω-level vectors can be combined into an α-level vector. As the GoldenBraid 2.0 vector grammar is designed to regenerate from α to Ω to α again, constructs of arbitrarily increasing size can be made (Fig. 1E).

Here we present a toolkit for *E. coli* strain building and genetic engineering that adheres to the GoldenBraid 2.0 assembly standard as instantiated by Matinyan *et al.* [7]. While MoClo toolkits [8–11] and strain-building resources [12–17] for *E. coli* already exist, our GoldenBraid 2.0 assembly standard provides a flexible and robust grammar system. In this toolkit, we aimed to create a comprehensive catalogue of pre-made genetic parts rather than relying predominantly on community-generated parts, and we further sought to integrate this MoClo system with existing strain-building tools and libraries available for plants [6], *Drosophila* [7, 18], and *Dictyostelium* [19].

Materials and methods

Bacterial culturing

All bacterial cloning was done in an *E. coli* DH5α derivative which expresses the pR6K Pir protein to allow replication of R6K plasmids. General culturing was done in Luria-Bertani (LB) media (Lennox formulation). Growth for plasmid isolation was done in LB supplemented with plasmid-specific antibiotics. When used, antibiotics were supplemented in growth media and plates at the following concentrations: carbenicillin (50 μg/ml), kanamycin A (30 μg/ml), chloramphenicol (12.5 μg/ml), tetracycline (12.5 μg/ml), and spectinomycin (50 μg/ml). When used, inducers/repressors were supplemented in growth media and plates at the following concentrations: glucose (0.2% w/v), arabinose (0.2% w/v), IPTG (100 μM), and anhydrotetracycline (200 ng/ml). For blue-white screening, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to antibiotic plates for a final concentration of 40 μg/ml. Unless otherwise indicated, all culturing was done at 37°C. Temperature-sensitive strains and constructs were cultured at 32°C as permissive temperature and 42°C for non-permissive temperature.

Bacterial strain building

Bacterial gene knockouts were sourced from the Keio collection [20]. Knockouts and chromosomally integrated genetic constructs were transferred across genetic backgrounds with standard P1*vir* transduction with selective antibiotic plates supplemented with 6 mM sodium citrate [21]. New genetic knockouts and chromosomally integrated constructs were generated using standard recombineering techniques [22]. For knockouts, the FRT-flanked kanamycin-resistance cassette of plasmid pKD13 was amplified as done for the generation of the Keio collection [20]. For new chromosomal constructs, 60 bp ssDNA oligomers were ordered with 20 bp regions that anneal to the desired insertion construct and 40 bp of homology to the desired insert locus. These primers were used to PCR amplify the insert of interest, forming the repair template. The resulting repair templates were integrated into the chromosome using λ Red recombineering in strain HME45 [23]. All knockouts were confirmed *via* PCR of the locus of interest. Allelic mutants were confirmed *via* Sanger sequencing and, when applicable, phenotypic screening for the allelic mutation.

Plasmids were introduced into strains through electroporation (1.40 kV voltage, 25 μF capacitance, 200 Ω resistance, and 0.1 cm cuvette gap length) or heat-shock-induced chemical competence.

For the construction of strains CH13363, CH13963, and CH13967 by Liu *et al.* [24], the protocol described in the supplemental methods section 'Coupling Recombineering with Cas9 Counter-Selection (Integrated λ Prophage Method)' was followed. Briefly, guide RNA were designed on plasmid ptetgRNA that target the edit locus (*fliP*, *flkB*, and *ytfH* 3' ssDNA break consensus sequences for CH13363, CH13963, and CH13967, respectively), confirmed to have lethal activity in wild-type strains, and then transformed into the recombineering strain tagged with I-41 for *fliP* or I-89 for *flkB* or *ytfH*. ssDNA recombineering

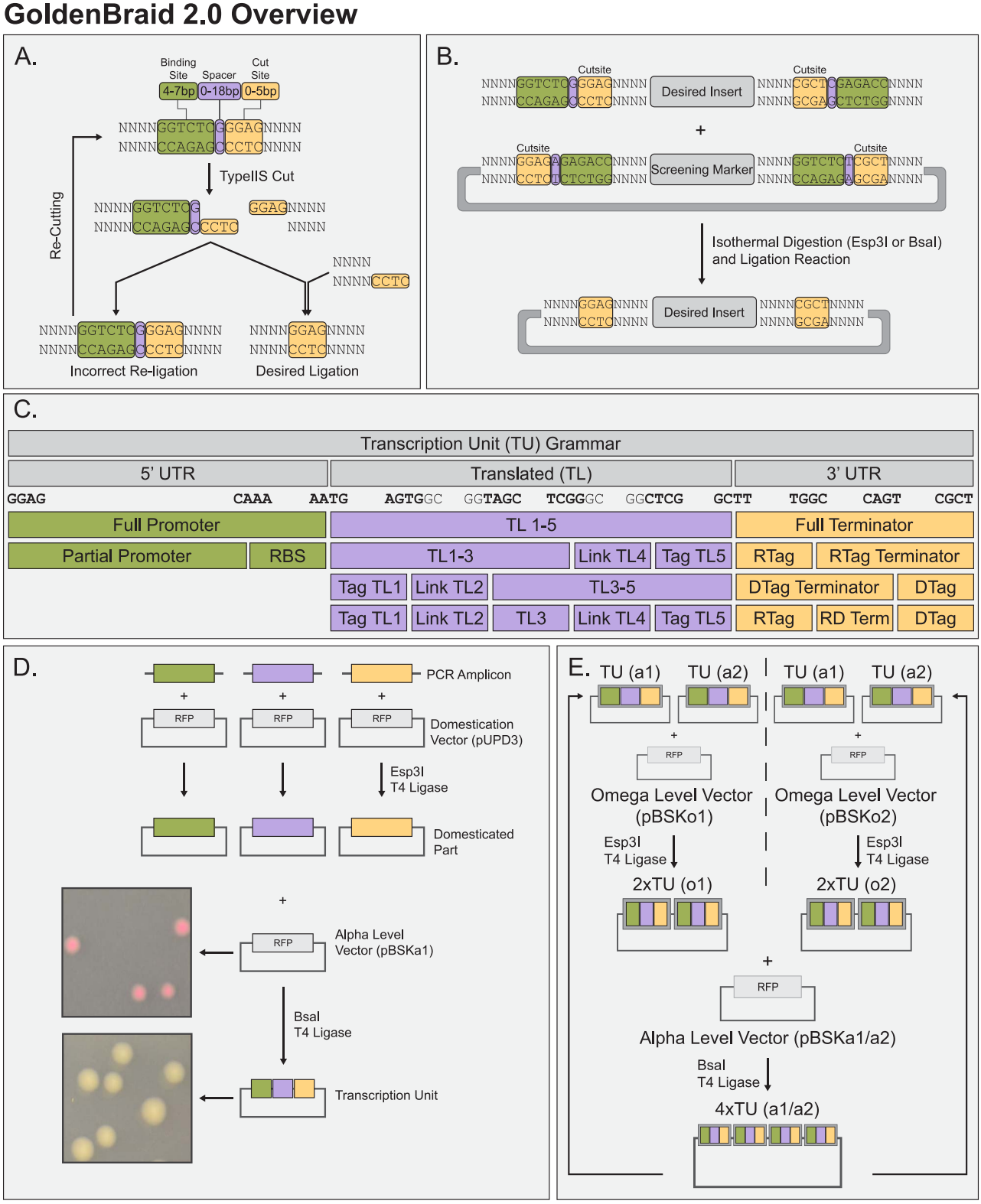

Figure 1. Overview of type IIS enzyme cloning and the GoldenBraid 2.0 cloning system. (A) General overview of type IIS enzyme cutting behaviour and its consequences. Type IIS enzymes cut (right; yellow) a distance from (middle; purple) their sequence binding site (left; green). If combined with DNA ligases, the product of this reaction can ligate to another piece of DNA, where it will no longer have a type IIS binding site and become stable. If the cut product re-ligates to itself, it will be cut by the type IIS enzyme again. (B) (Top) A linear PCR product with type IIS sites oriented 'inwards', such that the binding sites cut themselves out. (Middle) A plasmid vector with type IIS sites oriented 'outwards', such that binding sites cut themselves out along with a visually marked insert. (Bottom) The resulting ligation product of the digestion/ligation reaction if the cutsite 'grammar' (yellow highlight) matches between the linear PCR product (top) and plasmid (middle). (C) The GoldenBraid 2.0 'grammar' system applied to a bacterial transcription unit context. (D) The GoldenBraid 2.0 workflow for generating a single transcription unit. PCR amplicons for a promoter (left; green), coding sequence (middle; purple), and terminator (right, yellow) are 'domesticated' into a shuttle vector, pUPD3. These domesticated parts are then 'assembled' into another shuttle vector. (E) The GoldenBraid 2.0 system allows for sequential binary assembly of transcription units into increasingly larger and larger constructs.

templates were designed to ablate the guide RNA cut site and introduce the alternative DNA damage consensus motif. For the recombineering, the source strains containing λ RED functions, pCas9cr4, the ptetgRNA variant, and the I-Deconvoluter marker were induced for λ RED functions, transformed with the ssDNA repair template, recovered in LB media, and plated with Cas9 induction to counter-select against non-edited cells. The next day, colonies were isolated and the edits were confirmed by Sanger sequencing.

To generate the strain library needed to optimize the Arc-lexA-gfpmut2 layered gene circuit, the *Pant*-RBS-*lexA* library was transferred to the chromosome using CRISPR-mediated selection of successful recombinants as previously described by Marciano *et al.* [25] Briefly, the *PsulA-gfpmut2* reporter strain DCM153[pKD46] was induced for λ Red expression, made electrocompetent, and then co-transformed with pCas9-PlexA and a rescue template PCR product of the *Pant*-RBS-*lexA* plasmid DNA library amplified with primers dgkA-3 and TL5-LexA-bot. The *Pant* Golden Braid part includes ~150 bp of homology to the region upstream of the *PlexA* promoter and the *lexA* Golden Brain part provides ~600 bp of homology downstream of the *PlexA* promoter thereby enabling recombineering and replacement of the *PlexA* sequence targeted by pCas9-PlexA with the *Pant*-RBS library at the *lexA* locus. The resulting library is both chloramphenicol resistant (pCas9-PlexA) and kanamycin resistant (*lamB*:FRT-KanR-FRT).

GoldenBraid2.0 domestications and assemblies

GoldenBraid domestication and assembly reactions were assembled with Invitrogen T4 DNA Ligase Buffer, 1 unit Invitrogen T4 DNA Ligase, 20 units Esp3I (domestication or Ω assembly reactions) or 20 units BsaI-HFv2 (α assembly reactions), 40 ng of destination vector (pUPD3 for domestications; α1-, α2-, Ω1-, or Ω2-level vectors for assemblies), and 1:1 molar ratio of insert linear DNA (domestications) or input vectors (assemblies) in a final volume of 20 μl. This mixture was incubated at 37°C for 18 h, heat inactivated at 80°C for 20 min, and held at 4°C. Shorter 37°C incubations were done when desired. The resulting reaction mix was cleaned using 1X Ampure XP SPRI beads and transformed into DH5α *pir*$^+$ electrocompetent cells. Correct constructs were identified through blue-white screening for vectors containing inserts, PCR screening for inserts of the desired size, and sanger or whole plasmid sequencing to confirm parts/assemblies matched expected sequences.

For screening Arc-responsive *Pant*-RBS-*lexA* clones from the library, the pBSK-α1 cloning cassette was transferred into a derivative of the spectinomycin-resistant pMB1 ori vector pTargetF-FRT [26] to generate pDCM157-α1. The *arc* orf was domesticated into pUPD3 and then assembled into pDCM157-α1 with *Ptet* and t0 parts to generate pDCM157-α1-Arc.

As Esp3I appears to lose activity at −20°C, we stored aliquots of Esp3I at −80°C for long-term storage. Working stocks of Esp3I were stored at −20°C and generally used within 2 months.

Site-directed mutagenesis of GoldenBraid2.0 parts and plasmids

When small (<30 bp) changes were desired, plasmids were modified with site-directed mutagenesis. Briefly, primers were designed to divergently amplify plasmids at the site of interest. One or both primers contained 5′ overhangs that added to or edited the underlying DNA sequence at the primer site when amplified with Phusion High Fidelity DNA polymerase in HF buffer. The resulting amplicons were cleaned with 1X Ampure XP SPRI beads. In total, 2 μl of the cleaned amplicon was added to 2 μl 5X Invitrogen T4 DNA Ligase buffer, 1 μl Invitrogen T4 DNA Ligase, 1 μl Invitrogen T4 Polynucleotide Kinase, 1 μl Thermofisher DpnI, and 2 μl nuclease-free water for a 10 μl KLD reaction. This reaction mixture was left at room temperature overnight. The following day, this mixture was cleaned with 1X Ampure XP SPRI beads and transformed into DH5α *pir*$^+$ electrocompetent cells. Cells were then plated onto selective antibiotic and the correct underlying sequence was confirmed by Sanger sequencing.

GoldenBraid2.0 domestication of oligonucleotide dimers

For parts larger than that achievable with site-directed mutagenesis (>30 bp), but smaller than most PCR products (<120 bp), sets of ssDNA oligonucleotides were ordered that dimerized with 4 bp ssDNA overhangs corresponding to GoldenBraid2.0 vector insert overhangs. For parts larger than 60 bp, multiple sets of oligonucleotides were ordered that would 'stitch' together with their 4 bp overhangs. These ssDNA oligonucleotide pairs were dimerized by adding 1 μl of 100 μM forward oligonucleotide, 1 μl of 100 μM reverse oligonucleotide, 1 μl Invitrogen T4 Polynucleotide Kinase, 2 μl 5X T4 DNA Ligase buffer, and 5 μl nuclease-free water together to create an ssDNA phosphorylation mix. This phosphorylation mix was incubated at 37°C for 30 min, 95°C for 5 min, and ramped down to 25°C at 0.1°C/s to phosphorylate and dimerize the oligonucleotides. This mixture was diluted 1:25 in nuclease-free water and 1 μl of diluted mixture was used per oligonucleotide pair in relevant domestication or assembly reactions.

Quantification of construct expression levels

Transcriptional and translational fusions were attached to the fluorescent protein mScarlet-I through assembly onto p15a α1. The resulting assemblies were transformed into DH5α *pir*$^+$ and loaded into a Cytation 5 plate reader. The plate reader was set to grow cells in LB 50 μg/ml carbenicillin at 37°C. At 15-min intervals, the culture was agitated by 15 s of orbital mixing, followed by OD_{600} absorbance measurement and mScarlet-I fluorescence measurement (excitation: 569 ± 20 nm; emmission: 593 ± 20 nm). Expression levels were measured as mScarlet-I fluorescence at the 20-h growth curve timepoint for Fig. 3B–F and the 10-h timepoint for Fig. 5B, normalized to OD_{600} absorbance at that timepoint. Expression measurements for each construct were replicated three times.

For assaying GFP fluorescence levels of specific Pant-RBS-lexA clones, overnight biological replicates were diluted 1:100 into 1.3 ml of LB media containing spectinomycin and a range of anhydrotetracycline concentrations in a 24-well plate. The 24-well plates were incubated with shaking at 32°C for 20 h before reading OD_{600} absorbance and fluorescence (excitation: 475 ± 20 nm; emission: 518 ± 20 nm). Each of the ribosome binding sites (RBS) clones was assayed with four to six biological replicates.

Determining relative read counts from RBS libraries

The assembled pBSK-α1-Pant-LexA RBS library was transformed into NEBTurbo cells, plated onto kanamycin plates and the colonies were pooled prior to isolation of plasmid DNA. Pooled plasmid DNA was used in a PCR reaction using primers KP_A1_F1 and either SapI-pUC_ori (for the pUPD3-RBS library input) or TL5-LexA-bot (for the pBSK-α1-Pant-LexA RBS library). The PCR products were column purified and sequenced with Plasmidsaurus's Premium PCR sequencing service which uses

Oxford Nanopore's high-accuracy double reader-head PromethION Flow Cell (R10.4.1). Provided fastq files were then analysed using genBaRcode [27] to count RBS sequences as if they were barcodes. Invariant positions preceding the RBS sequences were used to generate the backbone sequence used by genBaRcode (GTCTCGCAAAGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNN for the pUDP3-RBS library and CGTCGACAAAGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNN for the pBSK-α1-Pant-LexA RBS library). One mismatch was allowed in the backbone sequence and reads with mismatches were manually added into the read count of the matching RBS.

Reagents

A complete list of reagents used in this study can be found in Supplementary tables S1–S3.

Biological resources

All toolkit plasmids and strains will be deposited to Addgene and available for purchase and are listed in Supplementary table S4. Plasmids used for toolkit part characterization are listed in Supplementary table S5. Oligonucleotide sequences used for plasmid domestication or assembly screening are listed in Supplementary table S6. Oligonucleotide sequences used to insert GoldenBraid assemblies into I-Deconvoluter loci, using the method shown in Fig. 7B, are listed in Supplementary table S7. Oligonucleotide sequences used to genotype insertions made into I-Deconvoluter loci, using the method shown in Fig. 7B, are listed in Supplementary table S8.

Statistical analysis

All error bars presented in this study represent 95% confidence intervals of the mean. Statistical significance as assessed in main text Figs 3B–F and 5B are from Student's two-sided *t*-tests.

Data availability/sequence data resources

Raw growth curve values are available upon request.

Data availability/novel programs, software, algorithms

No new computational resources were developed in the current study.

Web sites/data base referencing

No new websites were developed in the course of this work. All annotated sequence files relevant to the toolkit deposited to Addgene are present there. An additional public Benchling folder has been created which contains redundant annotated sequence files as well as additional files generated after completion of the toolkit itself (https://benchling.com/mattevolab/f_/xRGZHD3U-goldenbraid2-0-e-coli/).

Results

We have organized our toolkit into five modules, each addressing a different facet of *E. coli* genetic engineering. Module 1 provides the core cloning infrastructure—vector backbones and fundamental parts—allowing for high-yield plasmid preparation and reliable assembly (Fig. 2). Module 2 includes a comprehensive collection of promoters, RBS, and reporter elements, enabling fine-tuned control and easy measurement of gene expression (Fig. 3). Module 3 streamlines protein engineering through modular tags, linkers, and fusion components designed for purification, localization, or functional assays (Fig. 5). Module 4 covers transcription termination and nucleic acid tagging, incorporating versatile terminators, barcodes, and RNA-tagging sequences for efficient and easily tracked expression constructs (Fig. 6). Finally, Module 5 focuses on *E. coli* strain construction, offering antibiotic-resistance markers and integration tools for robust chromosomal modification (Fig. 7).

Module 1: core toolkit components

The original GoldenBraid 2.0 system relies on a set of pre-designed vectors [6]. These vectors contain an insert cassette with built-in restriction enzyme binding sites and a constitutively expressed *lacZ* fragment, which causes the vectors to metabolize X-Gal into a blue pigment. When used in a cloning reaction, blue colonies indicate an unmodified, or parental, vector and white colonies indicate candidates for correct assembly. As part of this toolkit, we built alternative vectors with RFP-based insert cassettes, to replace the standard blue/white *lacZ* screening. These RFP insert cassettes do not require the addition of X-Gal to plates, as undesired colonies will simply turn red (Fig. 1D; Supplementary fig. S6). The minimal system requires pUPD3, the domestication vector to clone a type IIS cleavage-site-free insert, and a set of α and Ω shuttle vectors (Fig. 2A) to assemble the genetic parts together. These core shuttle vectors are designed to allow for high plasmid yields to facilitate cloning (pBSK vectors) or to minimize vector carryover in downstream genetic manipulation (pR6K vectors cannot replicate without trans-complementation of the '*pir*' replication element present on the chromosome). We have also built *E. coli* expression vectors based on p15A, pMB1, and pSC101^{ts} origins of replication (Fig. 2B; Supplementary fig. S8). The low copy vector antibiotic-resistance markers are chosen such that no input vectors can cross-contaminate transformations of subsequent assemblies (Supplementary fig. S7). The original GoldenBraid2.0 standard provided a standardized vector insert grammar that permits transcription unit placement in both forward and reverse orientations. The provided vectors do not accommodate reverse orientation transcription units and are all provided in the forward orientation—however are compatible with the published reverse transcription unit parts.

The toolkit core also contains a strong, constitutive promoter, a set of fluorescent protein coding sequences, and a strong terminator (Fig. 2C). These core components allow one to easily verify that assemblies are working in their hands or to use it as a teaching module in the classroom. To maximize flexibility in construct design, we included a collection of 'stuffer' parts that allow users to intentionally skip specific elements of the genetic grammar (Fig. 2D). Each stuffer consists of a short, non-homologous DNA sequence (20 bp sequences that lack homology to any designed part or region of the *E. coli* genome [28]) flanked by the appropriate overhangs for its corresponding grammar position (e.g. promoter, RBS, or terminator). By inserting a stuffer in place of a functional part, users can maintain the required assembly structure without introducing active regulatory or coding elements. This feature is particularly useful for generating negative controls, assembling partial transcription units or larger operon structures, or designing modular constructs that can be easily modified at later stages. The use of stuffers thus preserves the integrity of the GoldenBraid 2.0 assembly process while offering enhanced flexibility for custom genetic designs.

Module 2: transcription control and reporters

While most published MoClo systems are submitted with a minimal set of pre-made parts, we aimed to deposit a comprehensive set of pre-made parts that can be quickly adopted for controlled

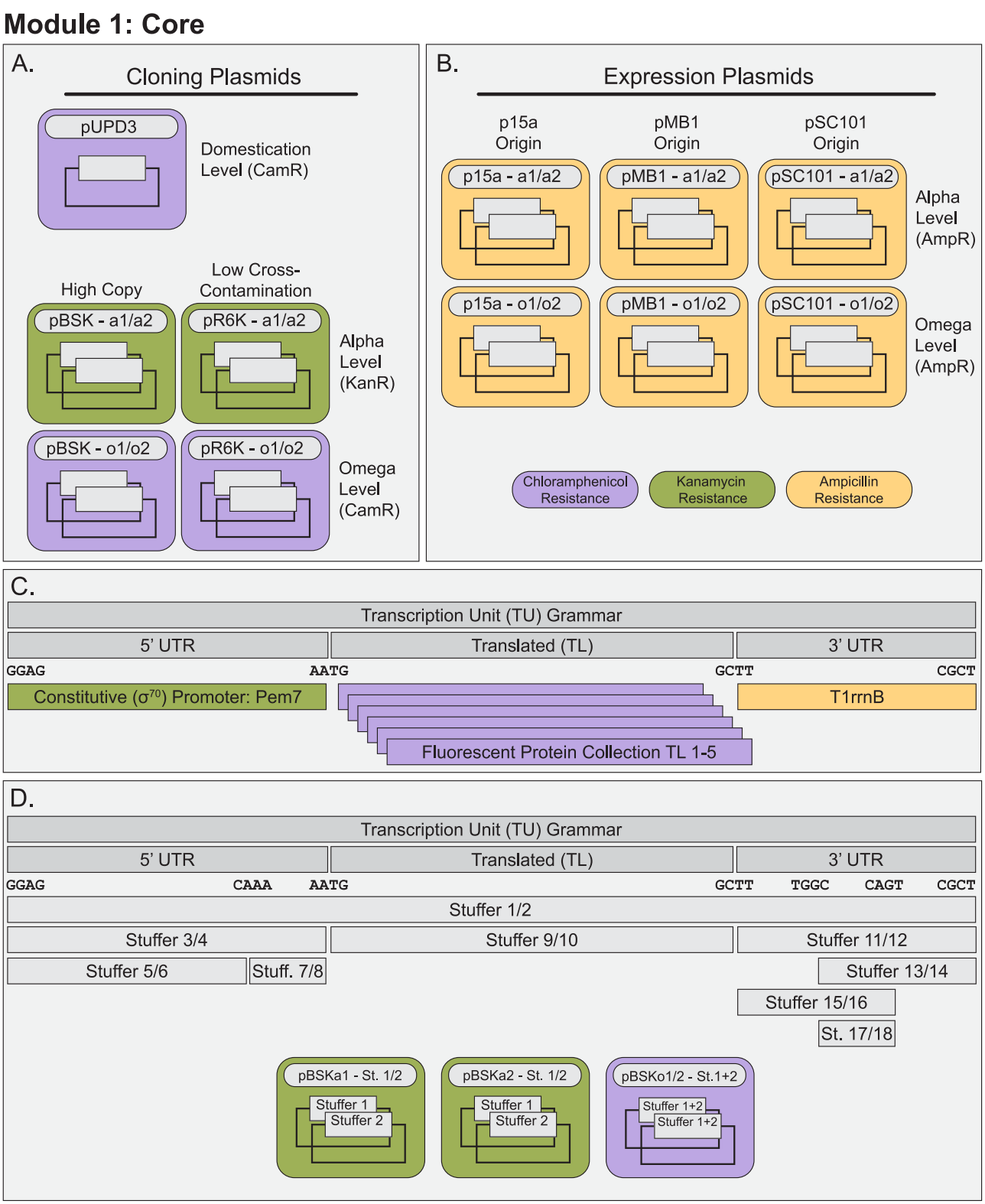

Figure 2. Module 1: Core toolkit components. (A) The minimum set of plasmids are shuttle plasmids for genetic part domestication (pUPD3) and sets of $\alpha 1$, $\alpha 2$, $\Omega 1$,and $\Omega 2$ shuttle vectors (pBSK set and pR6K set). The pUPD3 and the pBSK set are high copy number and thus give high plasmid yields. pR6K is divorced from its plasmid replication protein, Pir, meaning that recombineering amplicons derived from these plasmids are not at risk of introducing the template plasmid into strains during the recombineering process. (B) Additional sets of $\alpha 1$, $\alpha 2$, $\Omega 1$, and $\Omega 2$ expression plasmids have been constructed that are maintained readily in *E. coli* strains, but are of copy numbers suitable for experimental levels of gene expression. (C) A minimal set of parts necessary to create a working transcription unit are included in the core module. A strong, constitutive reporter, set of fluorescent proteins, and robust terminator are included. (D) A set of 20 bp stuffer parts were made to substitute for functional parts or entire transcription units. This allows one to create control constructs or non-standard assemblies.

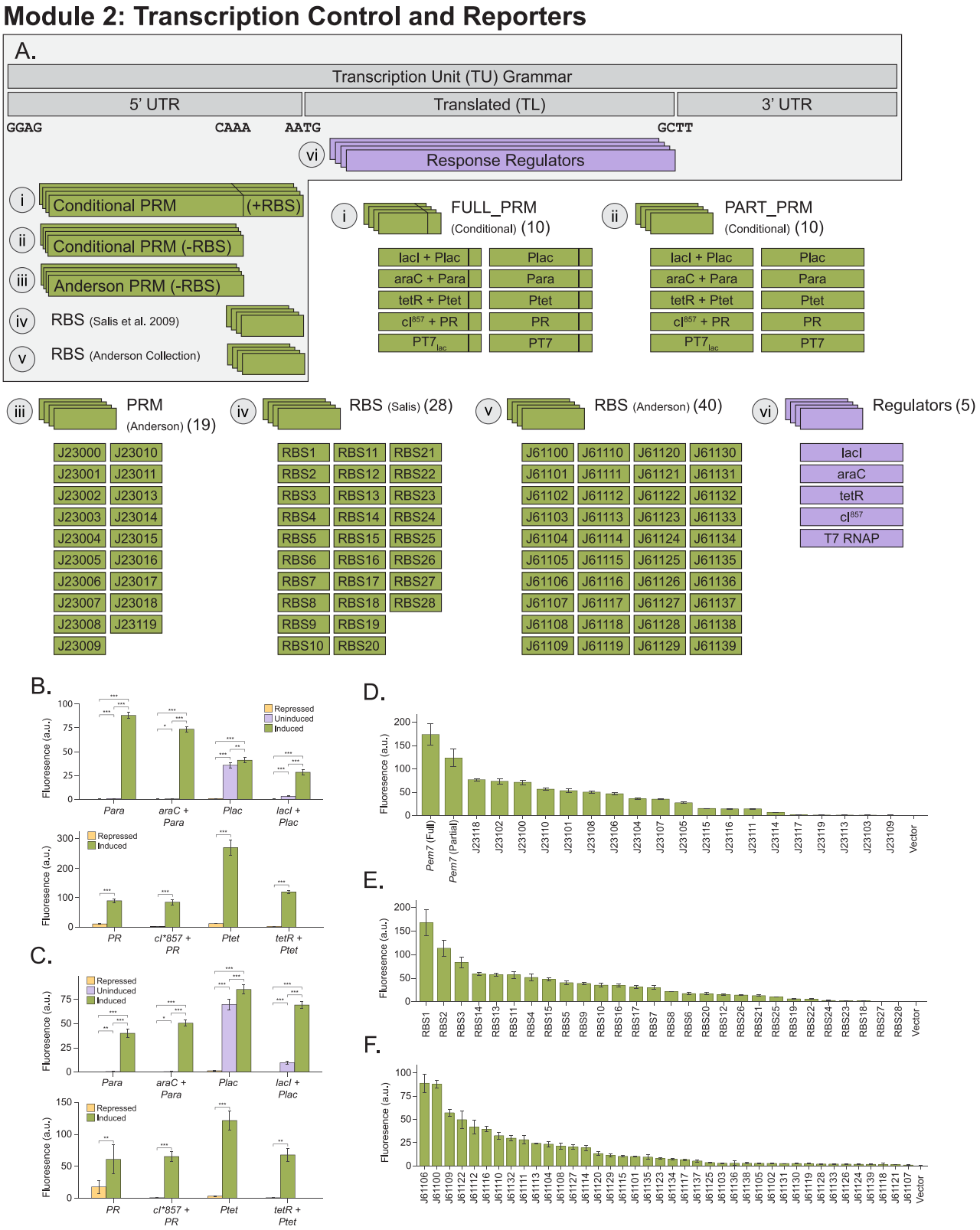

Figure 3. Module 2: Transcription control and reporters. (A) Upper vignette contains all relevant grammar configurations present in the module. Left side drop down contains catalogue of parts and part libraries present in the module. (B) Activity quantification (mScarlet-I fluorescence normalized to OD_{600} for panels B–F) of conditional FULL_PRM set with and without activation signal. (C) Activity quantification of conditional PART_PRM set, holding RBS constant (*RBS5*), with and without activation signal. (D) Activity quantification of Anderson promoter library, holding RBS constant (*RBS5*). (E) Activity quantification of Salis RBS library, holding promoter constant (*Para*). (F) Activity quantification of Anderson RBS library, holding promoter constant (*Para*).

gene expression. These part modules have been divided into groups based on their function within a transcription unit: transcriptional control (5' UTR), protein design (coding region), and transcript termination (3' UTR). Module 2 allows for control of transcription and translation levels for coding sequences in a transcription unit (Fig. 3A; Supplementary figs S9 and S10).

Parts in the transcription control module are deposited in one of four grammar types: full promoters (FULLPRM) which contain the promoter region and the native RBS, partial promoters (PARTPRM) which contain the promoter region up to the transcription start site but must be added to a RBS to cause protein expression, RBS which must be added to a partial promoter part to function, and finally, a set of coding sequence parts (CDS) that encode the response regulators for the supplied conditional promoters.

Firstly, we include a set of conditional promoters that are responsive to carbon sources (P_{lac} and P_{ara}), small molecule inducers (P_{tet}), temperature [$P_R(cI^{857})$], and the presence of the orthogonal T7 RNA polymerase (P_{T7} and P_{T7lac}) (Supplementary fig. S11). These promoters have been provided in the FULLPRM (Fig. 3A, i) and PARTPRM (Fig. 3A, ii) grammars and their conditional activity has been quantified (Fig. 3B and C).

Secondly, we include the popular Anderson constitutive, variable-strength promoter library from BioBrick [29] in the PARTPRM grammar (Fig. 3A, iii). Their activity has been quantified with a medium-strength Salis RBS library RBS (Fig. 3D).

Thirdly, we include two RBS libraries in the RBS grammar. The RBS grammar CAAA was chosen to minimize interference with the A-rich RBS sequences. Additional rationale for grammar choice and cross-compatibility between bacterial MoClo standards can be found in the supplement section 'Transcription Unit Grammar'. The first of which is the Anderson RBS library (Fig. 3A, v), which has been quantified using the P_{ara} promoter in presence of arabinose (Fig. 3F). The second library is the synthetic RBS library by Salis *et al.* [30] (Fig. 3A, iv) that consistently produces a range of expression levels in reporter constructs (Fig. 3E), even when taking into account the effects of coding sequence-dependent RNA folding.

Finally, we include the conditional reporter response regulators (i.e. *lacI* for P_{lac}) in the CDS grammar (Fig. 3A, vi). Alongside this, we include a set of FULLPRM (Fig. 3A, i) and PARTPRM (Fig. 3A, ii) conditional promoters that lack their response regulators. This allows for the convenient assembly of simple genetic circuits (such as feedback loops) out of the conditional promoters and their response regulators (Supplementary fig. S12).

To provide an example of the toolkit's usage, we applied it to the optimization of a layered gene circuit. The gene circuit was designed to allow for positive selection of repressor function of the DNA-binding Arc protein [31]. In the gene circuit, Arc represses expression of the LexA repressor, which in turn represses P_{sulA}-*gfpmut2* expression (Fig. 4A). By joining two repressor functions in series, an increase in GFP fluorescence is expected to correlate with Arc repressor function. We first assembled a P_{ant}-RBS_{1-28}-*lexA* library using an equimolar pool of the Salis RBS library, nanopore-sequenced the assembled library, and then nanopore-sequenced the library distribution after library transformation and outgrowth. Although sequence counts of the input Salis RBS library parts were evenly distributed, we found that the assembled and transformed P_{ant}-RBS_{1-28}-*lexA* library was enriched for weaker promoters (Fig. 4B). This suggests the previously observed fitness cost of *lexA* overexpression [32] may limit the contribution of strong RBS sequences to the library. Next, the P_{ant}-RBS_{1-28}-*lexA* library was recombineered into the native *lexA* locus in the chromosome of a P_{sulA}-*gfpmut2* reporter strain. We noticed some of the recombinant colonies fluoresced green even though Arc was not yet present in the cells. This suggests some RBS sequences in the library are too weak to supply enough LexA to repress the P_{sulA}-*gfpmut2* promoter. The chromosomally encoded library was pooled and then transformed with an assembled *tetR*-P_{tet}-*arc*-*t0* plasmid. The final strain pool containing: *tetR* + P_{tet}-*arc* (circuit layer 1), P_{ant}-RBS_{1-28}-*lexA* (circuit layer 2), and P_{sulA}-*gfpmut2* (circuit layer 3) was then induced for Arc with 200 ng/ml anhydrotetracycline and FACS was used to sort the top 0.03% most fluorescent cells. The sorted cells were then struck on agar plates to generate isolated colonies. Even though no anhydrotetracycline inducer was present in the plates, the majority of colonies now fluoresced green. We reasoned that high basal levels of fluorescence in the absence of inducer would decrease the dynamic range of our gene circuit. As such, to screen for dose-responsive gene circuits with a large dynamic range, non-fluorescent colonies were picked and further assayed over a range of anhydrotetracycline concentrations (Fig. 4C). Three unique RBS clones (P_{ant}-RBS_{20}, P_{ant}-RBS_{22}, and P_{ant}-RBS_{26}) were isolated that displayed the desired anhydrotetracycline-dependent dose response to GFP fluorescence induction (Fig. 4D). The ability to quickly assemble multiple layers of the gene circuit allowed us to isolate clones with the desired input–output behaviour.

Module 3: modular protein design

Module 3 permits modular recombinant fusion-protein design tagged either at the N- or C-terminus (Fig. 5A). Parts in this module are supplied based on a five-part coding sequences grammar. This grammar is denoted TransLated 1–5 (i.e. TL1–3 or TL5). N-terminal tags are TL1, C-terminal tags are TL5, Linkers are TL2, TL4, or TL2–4 to connect TL1 and TL3–5, TL1–3 and TL5, or TL1 and TL5, respectively. These configurations minimally allow for expression of a whole coding sequence, a TL1–5 part, and at most permit tagging both sides of a protein with custom linkers, TL1 tag + TL2 linker + TL3 insert + TL4 linker + TL5 tag.

We first include a set of TL1 and TL5 fluorescent tags that are monomeric (except Sirius), bright, quickly maturing, and span the visual spectrum [33–38] (Fig. 5A, i; Supplementary fig. S13). Secondly, we include a set of TL1 and TL5 affinity purification tags that permit protein immunofluorescence and affinity pulldowns (Fig. 5A, ii; Supplementary fig. S14). Thirdly, we included a set of commonly used TL1 and TL5 protein solubility tags to decrease the propensity of overexpressed proteins to aggregate (Fig. 5A, iii; Supplementary fig. S15). Fourthly, we include a set of TL1 protein secretion tags composed of entire secreted proteins or just Sec-pathway N-terminal signal peptides (Fig. 5A, iv; Supplementary fig. S16). Finally, we include TL5 tags corresponding to the amino acid sequence derived from the ssrA ncRNA that targets proteins for degradation (Fig. 5A, v), including degenerate versions of the ssrA tag with decreased degradation activity [39] (Fig. 5B).

To link expressed proteins and their provided tags, we included three sets of protein linkers [40] (Supplementary fig. S17). The first set consists of flexible linkers (Fig. 5A, vi). These flexible linkers include the commonly used G-G-G-S repeat tags of various lengths, as well as optimized flexible tags based on natural protein linkers. The second set consists of rigid linkers (Fig. 5A, vii). These rigid linkers are composed of various repeat lengths of the E-A-A-A-K alpha helix-forming amino acid sequence. The third set consists of sequence-specific protease-cleavable linkers (Fig. 5A, viii). These linkers can be cleaved by treating the linked protein with the corresponding protease *in vitro* or co-expressing the corresponding protease *in vivo*.

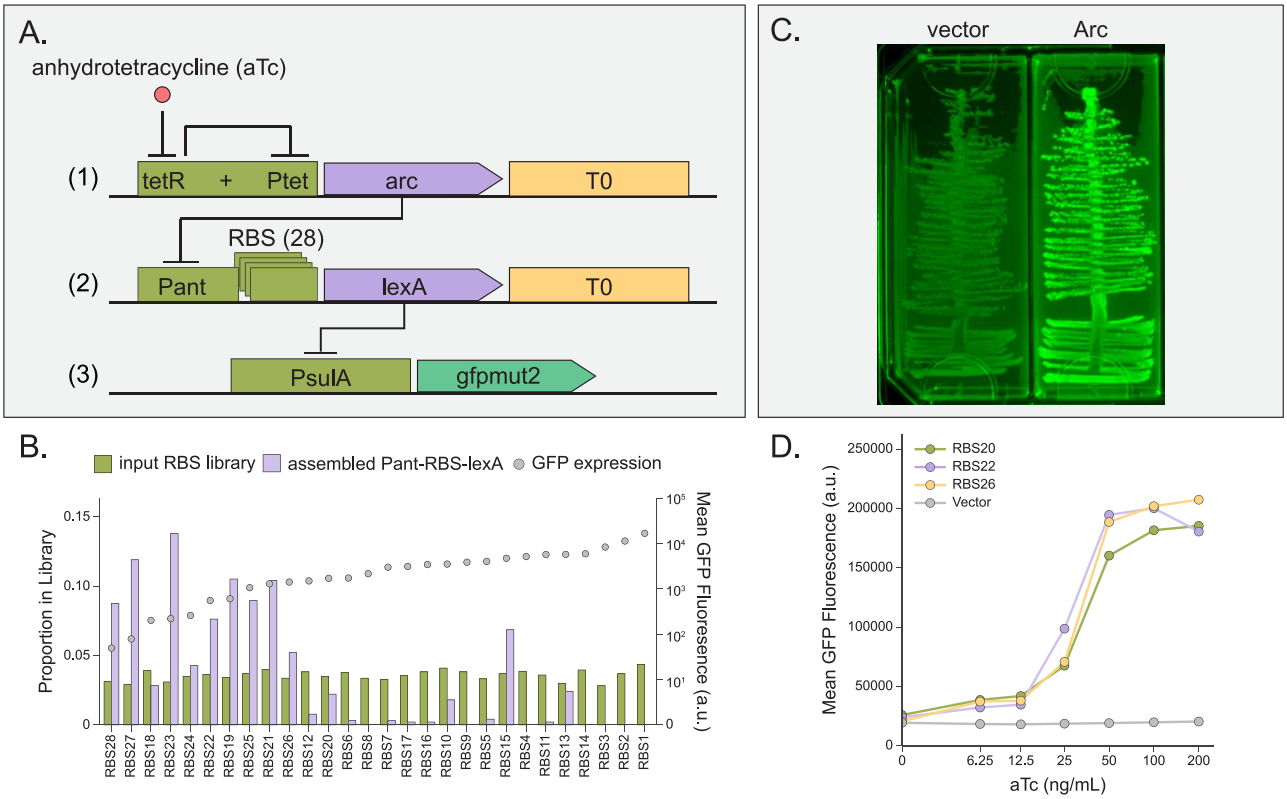

Figure 4. Construction and validation of a multi-layered gene circuit. (A) Overview of the anhydrotetracycline (aTc) inducible gene circuit. The *tetR-P*$_{tet}$*-arc-T0* (circuit layer 1) and *P*$_{ant}$*-RBS*$_{1–28}$ *library-lexA-T0* (circuit layer 2) were assembled separately and then either transformed (layer 1) or recombined (layer 2) into the chromosome of a strain with a *P*$_{sulA}$*-gfpmut2* reporter (layer 3). (B) Nanopore sequencing of RBS libraries before assembly (left; green bars) and after assembly (right; purple bars) shows a bias towards low expression RBS sequences (grey circles, right y-axis). (C) Agar plate of *P*$_{ant}$*-RBS*$_{26}$*-lexA*, *P*$_{sulA}$*-gfpmut2* reporter strain transformed with either an empty vector (left) or the assembled *P*$_{tet}$*-arc* plasmid (right). (D) Induction of *P*$_{sulA}$*-gfpmut2* in *P*$_{ant}$*-lexA* strains with RBS$_{20}$, RBS$_{22}$, or RBS$_{26}$ (used in panel C) transformed with *P*$_{tet}$*-arc* or an empty vector control ($N = 6$ [anhydrotetracycline 0, 12.5, 25, 50, and 100 ng/ml]; $N = 4$ [anhydrotetracycline 200 ng/ml]; $N = 2$ [anhydrotetracycline 6.25 ng/ml]).

In addition to the core of Module 3 described above, additional protein tagging parts have been provided for context-specific utility, including parts that enable post-translational protein fusion, proximity biotinylation, and two-hybrid assay constructs. Directions on how to assemble functional transcription units using these parts are elaborated on in the toolkit documentation (see Supplementary table S4; Supplementary fig. S18).

Module 4: transcription termination and tagging

The fourth set of pre-made parts permits nucleic acid tagging of transcription units and transcription termination (Fig. 6). Parts in this module are based on a three part 3' UTR grammar. This grammar allows for at most: a pre-terminator RNA tagging sequence (RTAG), a post terminator DNA tagging sequence (DTAG), and terminators compatible with RNA tagging (RTAGTRM), DNA tagging (DTAGTRM), both (RDTAGTRM), or no tagging at all (TRM) (Fig. 6, i–iv).

The RNA and DNA tagging parts were left at a minimal, as use of these tags is expected to be highly context-specific (i.e. to provide consistent adapter sequences for RT-qPCR and qPCR or to insert UMI libraries for high-throughput experiments). However, a fluorescent RNA adapter sequence, dBrocolli [41], was domesticated as an RTAG, to permit fluorescent quantification of RNA levels (Fig. 6, v). Additionally, 20 bp DNA barcodes [28] were domesticated as DTAGs, to permit rudimentary construct barcoding (Fig. 6, vi).

Strong sequence-dependent terminators were domesticated for all terminator grammars. All grammar includes strong terminators derived from Chen *et al.* [42] and the TRM grammar also includes the five strongest native *E. coli* terminators. The inclusion of this latter set of native terminators is to provide sequence diversity in the terminator portion of transcription units to discourage inter-terminator recombination events in large constructs.

Module 5: *E. coli* strain construction

Although our modules are assembled on plasmids, many experiments benefit from integrating these genetic constructs into the chromosome. Module 5 provides straightforward tools for chromosomal integration (Fig. 7A and B), offering either selectable, flippable antibiotic markers or a scarless CRISPR-based system to insert the desired module.

Chromosomal integration is achieved by subcloning constructs into plasmids containing FRT-flanked antibiotic-resistance markers—similar to those in pKD3 or pKD13 [22], which allows for the removal of the selection marker after insertion. These FRT-resistance-linked constructs can be PCR-amplified and then introduced into the chromosome *via* recombineering. To streamline this process, we transferred the FRT-flanked kanamycin-resistance marker from pKD13 [22] into α1- and α2-level pBSK vectors and expanded this cassette into a versatile marker set that confers resistance to ampicillin, kanamycin, chloramphenicol, tetracycline, and spectinomycin (Fig. 7A). This permits increased flexibility when working with antibiotic-resistant strains, such as strains containing Keio collection-derived knockouts (Kan^R), Tn10-marked alleles (Tet^R), or common plasmid vectors (Amp^R).

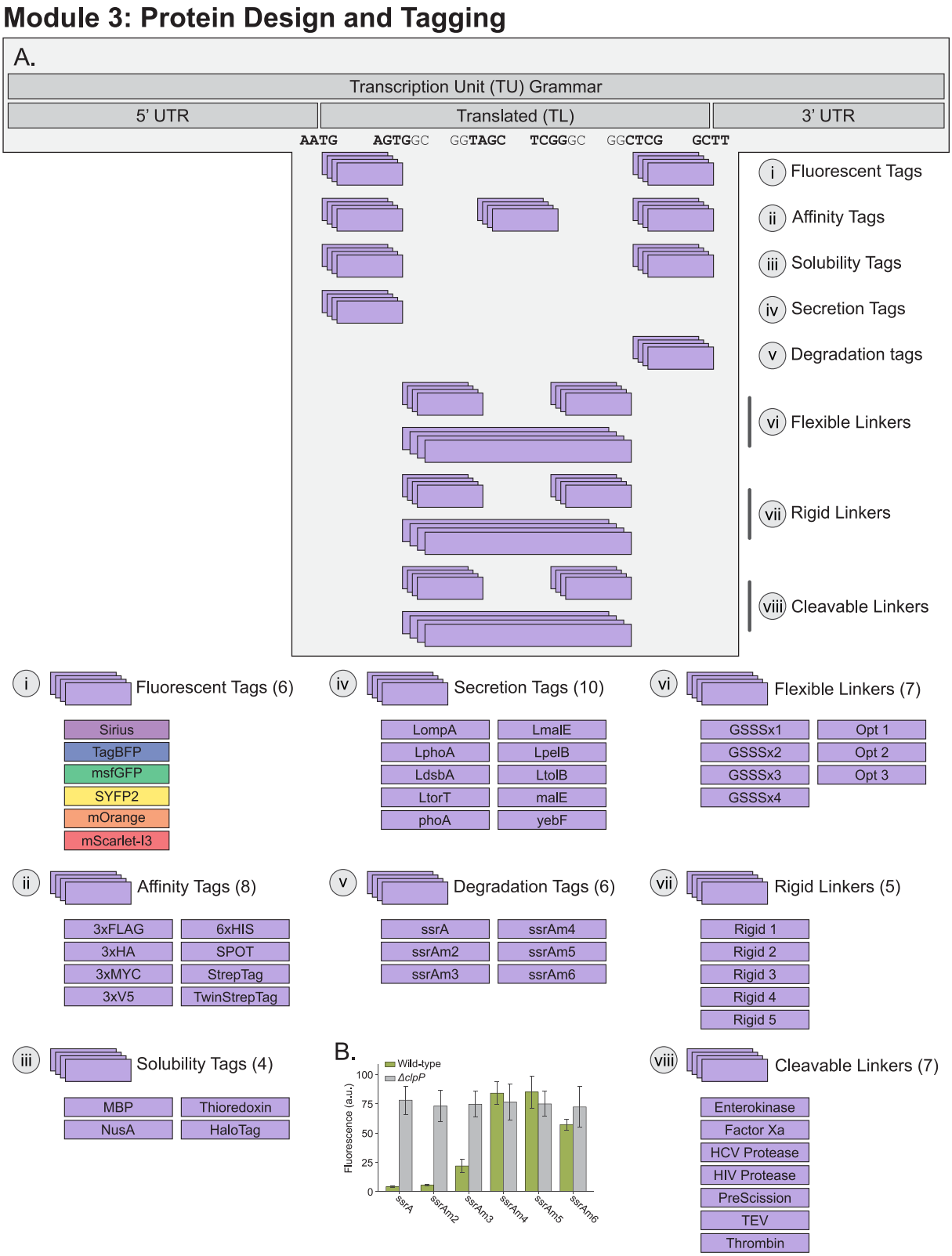

Figure 5. Module 3: Protein design and tagging. (A) Upper vignette contains all relevant grammar configurations present in the module. Left side drop down contains catalogue of parts and part libraries present in the module. (B) Quantification of ssrA-tag-dependent decrease in mScarlet-I expression levels.

The transcription units, including FRT-flanked antibiotic markers, are then co-assembled onto Ω-level pR6K shuttle vectors to create recombineering templates. Finally, once the complete genetic construct is assembled with the FRT-resistance cassette, a PCR template is generated for recombineering in a strain lacking the pR6K replication element (*pir*), which prevents pR6K replication and minimizes false-positive clones during selection (Fig. 7A). For the simple use case, where these excisable antibiotic cassettes

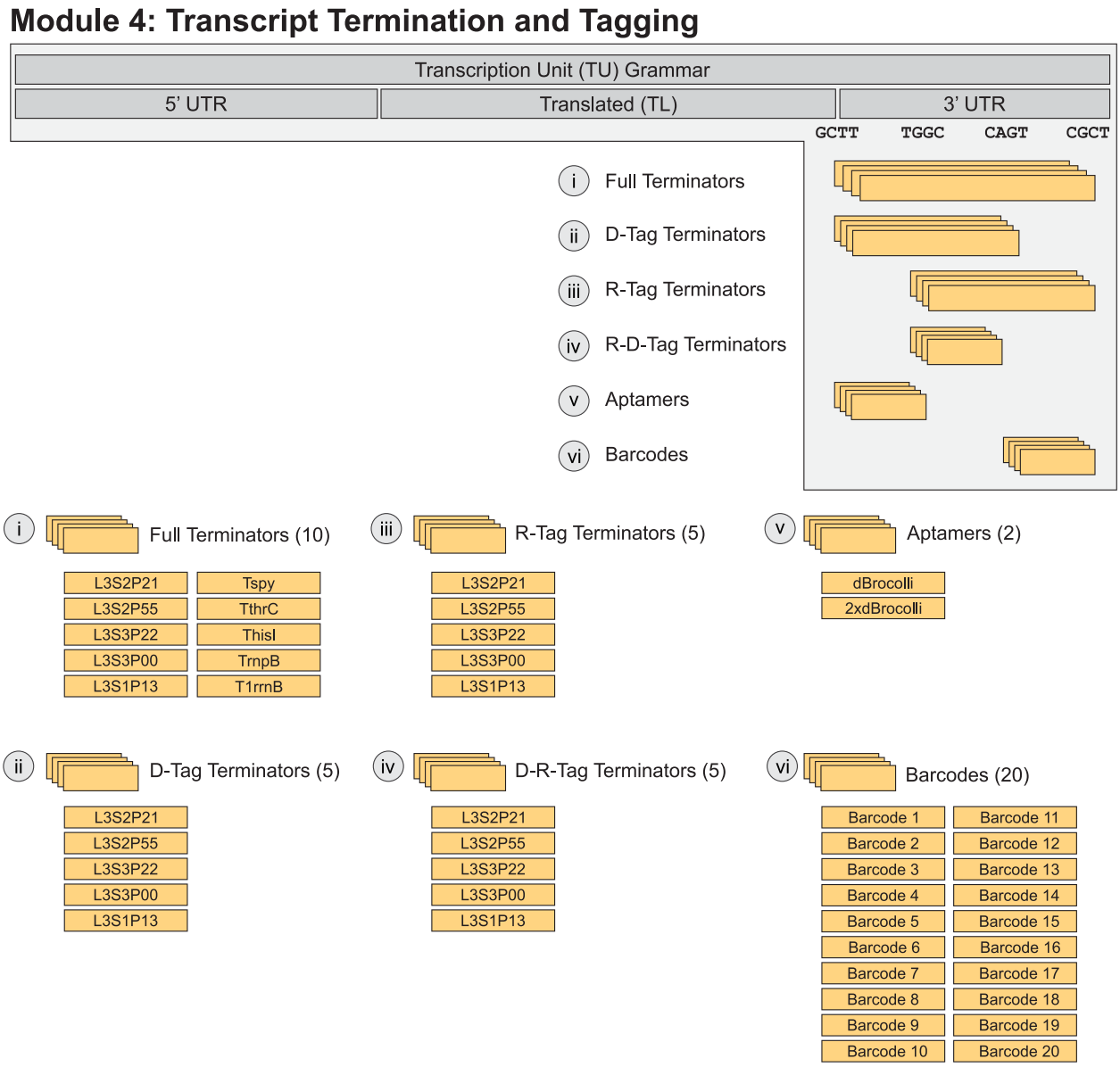

Figure 6. Module 4: Transcript termination and tagging. Upper vignette contains all relevant grammar configurations present in the module. Left side drop down contains catalogue of parts and part libraries present in the module.

are being used as PCR templates for gene knockouts, we have also provided each cassette pre-assembled onto an Ω-level pR6K vector.

Recent advances in bacterial strain engineering have replaced traditional antibiotic-based selection with CRISPR-Cas counter-selection, enabling marker-free gene editing [26, 43]. This approach is particularly advantageous when modifying complex gene operons or genetic contexts, as it permits precise edits without altering the surrounding chromosomal architecture; for instance, editing a gene within an operon without disrupting downstream gene expression *via* an antibiotic-resistance cassette. While marker-free editing preserves the native chromosomal context, it can complicate the transfer of edited alleles between different genetic backgrounds. To overcome this challenge, Nehring *et al.* [44] developed the I-Deconvoluter library, a collection of 94 intergenic FRT-flanked kanamycin-resistance cassettes that co-transduce with any genomic region at a high frequency, thereby facilitating the movement of alleles between strains. To facilitate scarless insertions that can later be transferred into a clean genetic background, we integrated the I-Deconvoluter marker library into a strain equipped with recombineering machinery and an anhydrotetracycline-inducible Cas9 (Fig. 7B). We built a compatible gRNA cloning plasmid with tight repression (ptetsgRNA), enabling Cas9 cutting only in the presence of anhydrotetracycline inducer during recombineering. This collection of 94 strains enables users to efficiently generate chromosomal, antibiotic marker-free engineered alleles or insert GoldenBraid constructs. In a simple use case, we amplified the mScarlet-I insert from pR6Kα1 containing homology arms detailed in Supplementary table S7. The resulting amplicon was recombineered in place of I-Deconvoluter I-1 in a single step by co-transforming the I-1, pCas9cr4 with the mScarlet-I amplicon and ptetsgRNA-FRT. Compared to the control where no mScarlet-I template DNA was electroporated, the recombineering step resulted in efficient gene replacement. In an applied use case, we used these recombineering strains to build marked variants

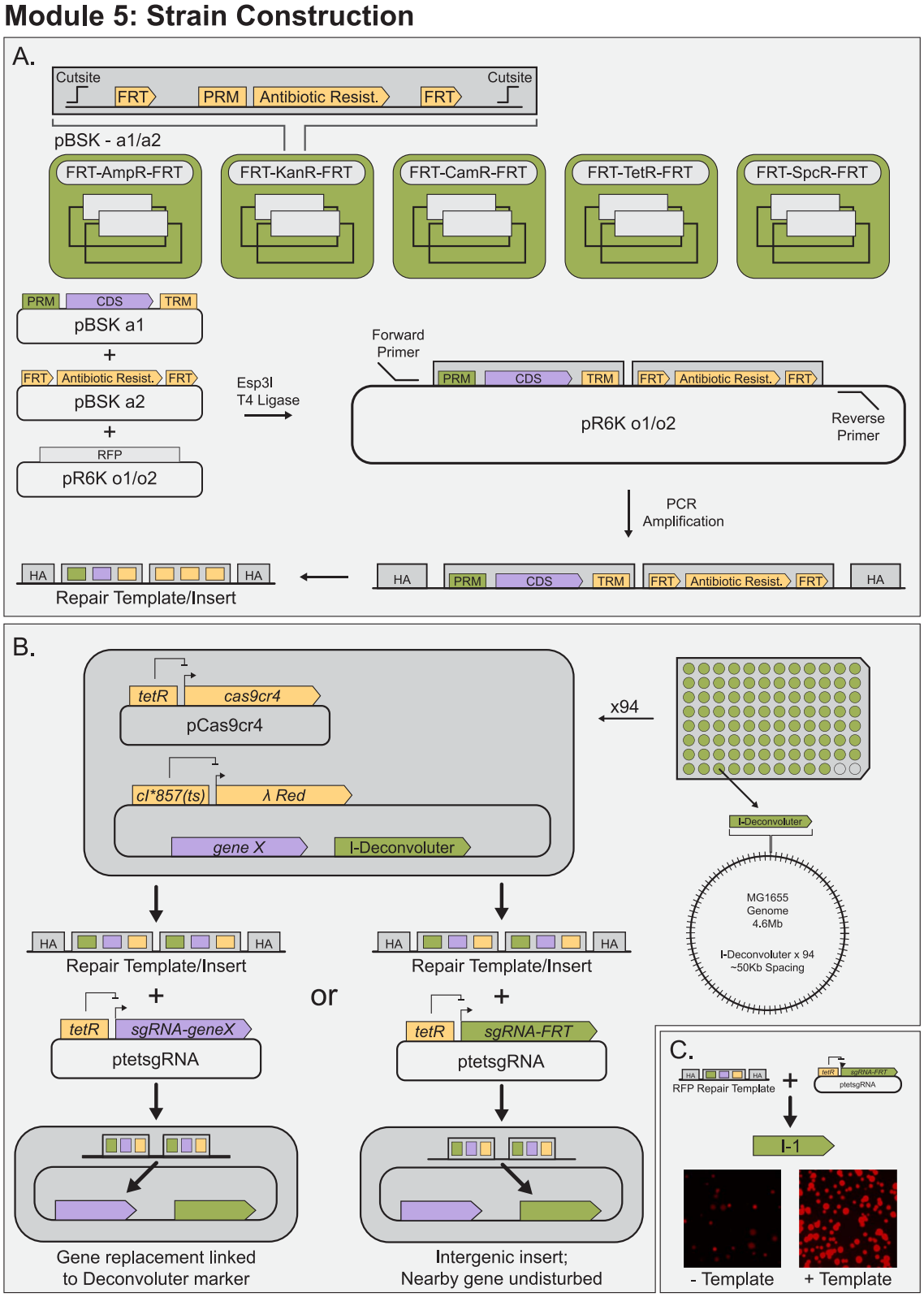

Figure 7. Module 5: Strain construction. (A) The standard FRT-flanked antibiotic-resistance cassette has been pre-assembled onto α1 and α2 vectors with ampicillin, kanamycin, chloramphenicol, tetracycline, and spectinomycin-resistance markers. These allow transcription units to be co-assembled onto Ω-level shuttles (pR6K set) to create FRT-flanked antibiotic marked recombineering templates. (B) To enable flexible CRISPR-Cas recombineering, a set of strains containing No-SCAR [43] recombineering machinery and the I-Deconvoluter library [44] were assembled. These strains allow for convenient marking of SCAR-free edits or flexibility inserting constructs across the genome. (Right) The notches on the chromosome depiction demonstrate the spacing of the I-Deconvoluter markers in the library. (C) The red fluorescent insert from red/white screening version of pR6Kα was amplified and used as a recombineering template for insertion into I-Deconvoluter site I-1. This repair template was co-transformed with ptetsgRNA-FRT and this resulted in red fluorescent chromosome integrants following Cas9 counter-selection against non-recombinants. Images are of red fluorescent signal acquired on an Azure 400 gel imager for recombineering electroporations containing ptetgRNA-FRT (– template) and ptetgRNA-FRT plus mScarlet-I recombineering template (+ template).

of DNA-damage-prone loci [24]. In these cases, the edits do not disrupt the local DNA context with an adjacent antibiotic-resistance marker, which could interfere with the DNA damage phenotype, yet can still be transduced *via* a distant I-Deconvoluter kanamycin-resistance cassette.

A supplementary lab manual

Finally, this toolkit was created with multiple objectives in mind. Firstly, to accelerate the research of established laboratories by streamlining their cloning workflows and saving time by pre-making general-use genetic parts. These laboratories will have little difficulty using these tools, as they have experience with bacteriology and have established recipes and protocols. Secondly, we would like to provide informative documentation on proper genetic cloning and toolkit parts that junior laboratory personnel can refer to. This is to assist undergraduate trainees and junior lab technicians who are frequently assigned the task of cloning constructs for more expansive projects, but themselves may not have the background to know how their constructs should behave or why the set of parts was chosen. Thirdly, undergraduate teams, such as those who participate in iGEM, are given Addgene toolkits, but may have limited access to protocol and recipe books. Thus, these teams must use their limited time searching for media/solution recipes or protocols that sometimes conflict.

For these reasons, we provide a working laboratory manual and genetic part documentation in the supplementary material. For established laboratories, the recipes and protocols will likely be redundant with established norms. For junior personnel, we hope that these resources prove useful for troubleshooting and general use.

Discussion

In this work, we present a comprehensive GoldenBraid 2.0 toolkit for *E. coli* that spans a broad range of genetic functions and applications. By building on a robust modular cloning framework, our toolkit simplifies an otherwise time-intensive and often repetitive process. A key goal was to provide ready-to-use, pre-characterized components that significantly reduce the burden of genetic assembly and strain construction, all while preserving a high degree of flexibility that allows users to interchangeably build, modify, and expand their constructs for various experimental designs. A particular strength of our approach lies in its uniform 'cloning grammar': each component—be it a promoter, RBS, coding sequence tag, or terminator—adheres to a consistent set of four-base overhangs. This grammar consistency removes the need for custom primer design or repeated cloning steps for each new element. Moreover, by employing a dual digestion–ligation strategy characteristic of type IIS restriction enzymes, we capitalize on a powerful iterative enrichment of desired ligation products. As a result, the GoldenBraid 2.0 system enables both reliable domestication of new parts and straightforward multi-part assemblies, even when those assemblies become progressively larger or more complex.

Our toolkit particularly benefits from its modularity and depth. In contrast to many published MoClo collections that feature minimal sets of pre-made parts [8, 9], here we tried to incorporate a broad catalogue of expression elements, antibiotic-resistance cassettes, integration markers, and specialized tags. This includes sets of constitutive and inducible promoters, characterized RBS libraries, multiple fluorescent reporter proteins, affinity tags, solubility tags, secretion tags, and customizable linkers. For researchers, this expanded range of parts accelerates experimentation: many 'off-the-shelf' combinations are immediately available for rapid prototyping. Users can, for example, screen several promoter and RBS strength combinations in parallel simply by swapping modules, or engineer protein fusions with a menu of solubility or affinity tags without creating new domestication constructs. An additional highlight is the integration of classical antibiotic-based selection (e.g. FRT-flanked antibiotic-resistance cassettes) with CRISPR-Cas-based counter-selection approaches. This dual strategy means that users can choose to generate marker-free modifications or incorporate antibiotic markers at targeted loci, depending on experimental needs. Incorporating a library of intergenic FRT-markers further adds flexibility in mapping or transferring edits between strains, especially valuable when multiple genetic backgrounds are in play.

Beyond utility in experienced research labs, we have also tailored this resource for less experienced users, including student teams (such as those competing in iGEM [3]) and junior personnel. The accompanying lab manual provides curated protocols and troubleshooting tips, streamlining the transition into modular cloning workflows. Given the emphasis on clarity and reproducibility, our documentation includes solution and media recipes, standard transformations, and recommended screening methods. We anticipate that such transparency will broaden the adoption of GoldenBraid-based cloning in teaching labs, core facilities, and collaborative projects where uniform and well-documented practices can greatly expedite progress. This toolkit and accompanying documentation create numerous opportunities for expansion. Specialized modules—such as those for post-translational modifications, CRISPR-based transcriptional regulation, or integration of synthetic circuit elements—could be readily integrated into our standardized format. Similarly, extension into high-throughput workflows, perhaps with automated DNA assembly platforms, stands to benefit substantially from the robust grammar and well-characterized parts described here. Finally, the capacity to combine antibiotic-free and antibiotic-linked methods in chromosomal engineering opens new doors for combinatorial and large-scale projects, particularly when systematically improving or repurposing *E. coli* strains for synthetic biology.

In conclusion, our GoldenBraid 2.0 *E. coli* toolkit provides a next-generation set of modular resources for cloning and strain construction, combining ease of use, versatility, and thorough documentation. By reducing the time and cost commonly associated with cloning and consolidating many fundamental genetic parts into a single resource, this toolkit offers an immediate and practical benefit to both established and emerging research programs. We anticipate that it will become a valuable community resource, fostering both rapid discovery and reproducible, open-source approaches to genetic engineering in *E. coli*.

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Author contributions

Cooke and Herman: Conceptualization. Cooke, Welch, Deus, Wen, and Marciano: Data curation. Cooke, Welch, and Marciano: Formal analysis. Marciano and Herman: Funding acquisition. Cooke, Welch, Deus, Wen, and Marciano: Investigation. Cooke and Welch: Methodology. Cooke: Project administration. Herman: Resources. Cooke and Welch: Software. Cooke and Herman: Supervision. Cooke, Welch, Deus, Wen, and Marciano: Validation. Cooke, Welch, and Marciano: Visualization. Cooke: Writing—original draft preparation. Cooke, Welch, Deus, Wen, Marciano, and Herman: Writing—review and editing.

Supplementary data

Supplementary data is available at *SYNBIO* online.

Conflict of interest: The authors declare no conflicts of interest.

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Data availability

All raw fluorescence measurements, growth curve data, and micrographs are available upon request to the corresponding author. All toolkit plasmid and strain resources will be deposited to Addgene prior to publication. All strains and plasmids used to validate the activity and behaviour of the toolkit parts (Supplementary table S5) are available upon request to the corresponding author. All toolkit part sequences can be found on Benchling (https://benchling.com/mattevolab/f_/xRGZHD3U-goldenbraid2-0-e-coli/).

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Synthetic Biology, 2025, **00**, 1–15
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