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Design and characterization of a Golden Gate-compatible modular toolkit for promoter circuit assembly

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List of Abbreviations

CDS DNA Coding Sequence

CPEC Circular Polymerase Extension Cloning

DNA Deoxyribonucleic Acid

E. coli *Escherichia coli*

GFP Green Fluorescent Protein

GGA Golden Gate Assembly

IPTG Isopropyl β-d-1-thiogalactopyranoside

MEFL Molecules of Equivalent Fluorescein

mRNA Messenger Ribonucleic Acid

MoClo Modular Cloning

OD Optical Density

ORI Origin of Replication

oRibo Orthogonal Ribosome

PR Right Promoter

PL Left Promoter

PCR Polymerase Chain Reaction

P_X Promoter named X

RNA Ribonucleic Acid

RBS Ribosome Binding Site

SBOL Synthetic Biology Open Language

TU Transcriptional Unit

tRNA Transfer RNA

TF Transcription Factor

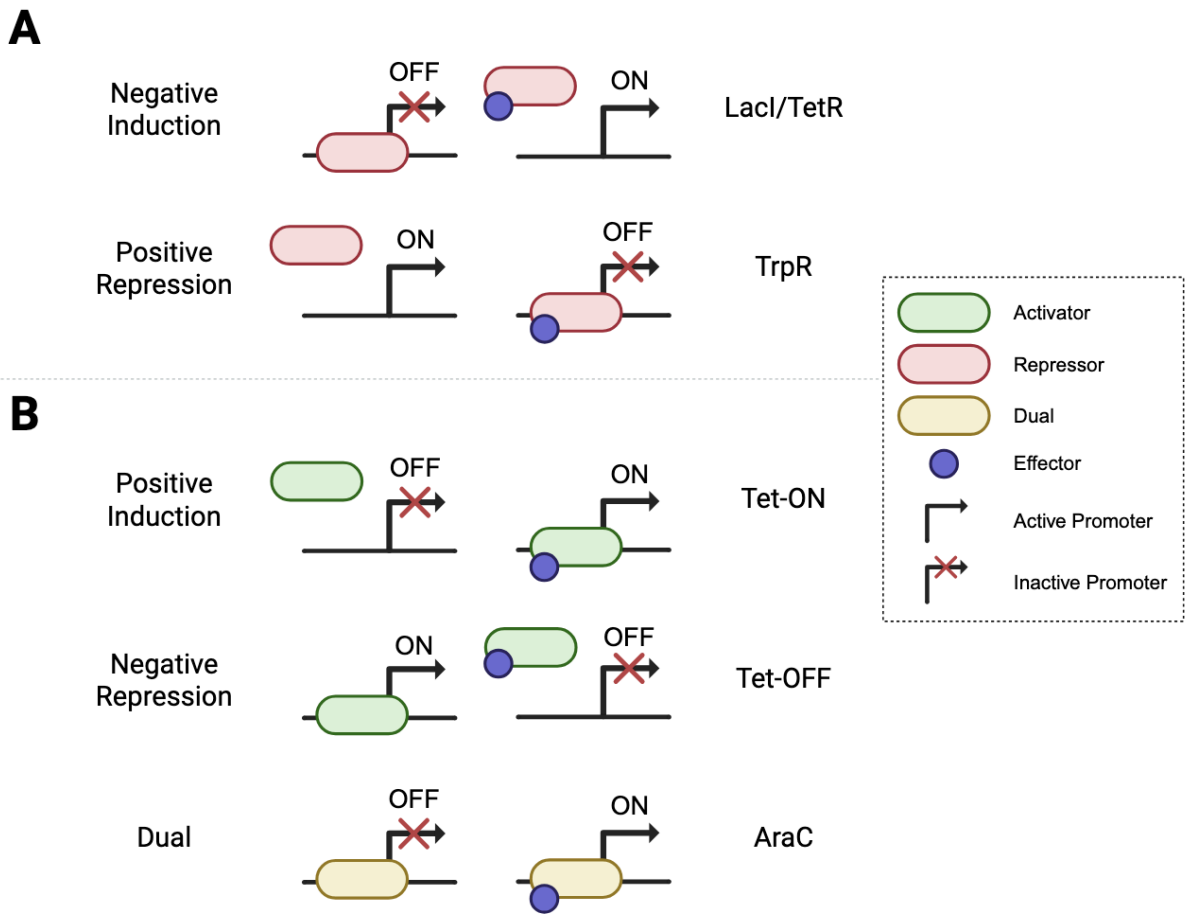

Figure 1. Regulatory roles of prokaryotic transcription factors. This scheme generally describes mechanisms of regulation seen at the level of transcription for prokaryotic operons. Green and red rounded rectangles represent transcription factors with activation (green) or repression (red) mechanisms. In the presence of their ligand, positive TFs bind to DNA, while negative TFs are released from it. Purple effectors represent activating molecules. At the right of each mechanism is a protein which exhibits this behaviour. **A**: Mechanisms exhibited by repressor TFs. Negative induction is also known as derepression. Binding of the TF to DNA represses transcription. **B**: Mechanisms exhibited by activator and dual TFs. Binding of the TF to DNA activates transcription. Dual TFs may either repress and activate while bound to DNA, depending on ligand availability. *Created in BioRender.*

I – Introduction to the general scientific context

Transcriptional regulation in synthetic biology

The regulation of gene expression is a fundamental aspect of cellular function, governing the precise control of protein production in response to environmental and intracellular signals. There are many different biological mechanisms for gene regulation at different stages of the central dogma. Notable examples are: genetic regulation through recombinase-mediated inversion, transcriptional regulation through DNA-binding transcription factor proteins (“TFs”), and translational regulation through RNA secondary structures (hairpins, riboswitches) or RNA interference. Transcriptional regulation is a prominent area of research, and many mechanisms relating the interaction between TFs, DNA binding sites, and RNA polymerase complexes have been revealed. Together, these components exhibit finely-tuned control over gene expression.

Prokaryotic transcription factors activate or repress gene expression

Prokaryotic transcription factors may exert control of gene expression through two possible mechanisms. These are (i) activation, where TF binding to an operator site helps form the RNA polymerase complex at the promoter, and (ii) repression, where TF binding physically prevents polymerase recruitment or blocks elongation (Fig. 1). Often, this activity is modulated by small molecular ligands that induce conformational changes in a TF, altering its affinity for its operator binding site. This behaviour gives rise to positive and negative systems, where transcription is switched either ON (1) or OFF (0) in response to a signal (Wang et al., 2011). For example, the LacI repressor loses affinity for its cognate operator upon binding allolactose, which derepresses the lac promoter (Lewis, 2005); the TetR/tTA/rtTA transcription factors are controlled by tetracycline, enabling the widely used Tet-ON and Tet-OFF systems (Zhou et al., 2006); LuxR acts as a transcriptional activator only upon binding acyl homoserine lactone (Yang et al., 2009); and TrpR represses transcription when bound to tryptophan (Merino et al., 2008). In addition, not all TFs are able to bind to a ligand. Some repressors act constitutively, so their regulatory effect is determined solely by their intracellular concentration and half-life.

Promoter abstraction enables construction of synthetic circuits

The predictable nature of TF-operator interactions has provided synthetic biologists with a growing repertoire of genetic parts with which they can engineer novel cellular behaviors.

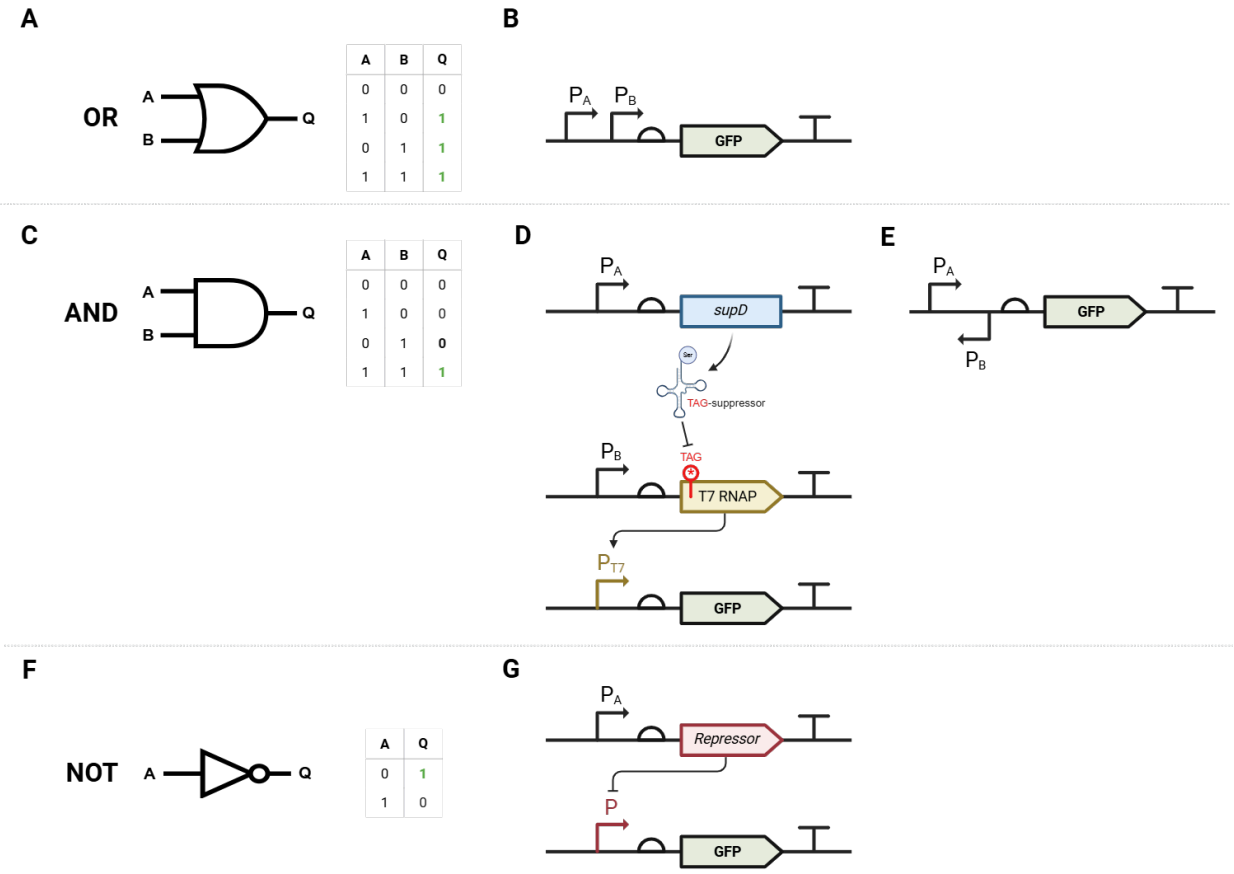

Figure 2. Primitive logic gates achievable using transcription factors. **A**: OR gate symbol and truth table. **B**: general promoter arrangement of an OR gate within a gene. **C**: AND gate symbol and truth table. **D**: Amber suppression AND gate which utilizes a anti-TAG $tRNA^{Ser}$ under P_A and a T7 RNA Polymerase gene with early amber stop codon (TAG). **E**: general promoter arrangement of an AND gate with "subtractive" topology. P_A is a positive activator, and P_B is a positive repressor or negative inducer. **F**: NOT gate symbol and truth table. **G**: NOT gate implementation within a genetic circuit. Red "repressor" gene is a constitutively active repressor. *A and B: inputs; Q: output. Truth table: 0 = absent/inactive; 1 = present/active. P_A and P_B represent promoters responsive to inputs A and B. Synthetic Biology Open Language (SBOL) symbols: half-circle: ribosome binding site; bent arrow: promoter; T: terminator; pointed rectangle: protein coding sequence (Quinn et al., 2015). Created in BioRender.*

Early works have demonstrated that regulatory components could be abstracted from their native context and recombined to produce predictable, non-natural functions (Müller et al., 2025). Moreover, many efforts have been made to evaluate the functional characteristics of transcription factor binding sites, not only allowing creation of chimeric promoters such as P_{trc10} but also their *de novo* prediction through computational approaches. Conventionally, circuits in computer science are described by a topology of connected logic gates, inputs, and controls which give rise to complex function. In synthetic biology, this concept was adapted to describe the use of different promoters to produce a desired outcome in response to various input stimuli; in essence, logical and reproducible expression of genes. At a higher level, tools such as CELLO enable the automated *in silico* design of complex logic circuits from characterized genetic parts (Nielsen et al., 2016). Synthetic toggle switches, repressilators, and logic gates all contribute to a rich and growing library of circuits for programming cellular decision-making.

Topologies and mechanisms of logic gates

The most straightforward logic gate is the OR gate, which is active when one or both of the two compared inputs are present (Fig. 2A). Normally, this is exhibited by the placement of two promoters that are each able to induce expression of a gene (Fig. 2B). The construction of OR gates using this topology is contingent upon the upstream promoter's ability to "pass through" the second downstream promoter. For example, as P_{BAD} is both a transcription activator and a repressor, occupation of the P_{BAD} operator in the absence of arabinose will block activity of any upstream promoter (Tamsir et al., 2011).

An AND gate produces an output only in the presence of both input signals (Fig. 2C). The commonly described topologies of this circuit include use of two transcriptional components that are codependent. Subtractive AND gates utilize two opposed promoters which compete for transcription activation. A viral T7 RNA polymerase gene with early amber stop codons that only produces functional polymerase in the presence of an amber-suppressor transfer RNA that is charged with an amino acid to continue translation can control the formation of the RNA polymerase complex on a gene with the T7 promoter (Fig. 2D). By having the sense promoter positively activated and the antisense promoter negatively repressed (such as *via* Tet-OFF), transcription only occurs when there is activation of P_A and repression of P_B (Fig. 2E). While the former topology is demonstrated and successful in the literature, it leaves very little room for more complex designs involving different gates interacting with each other.

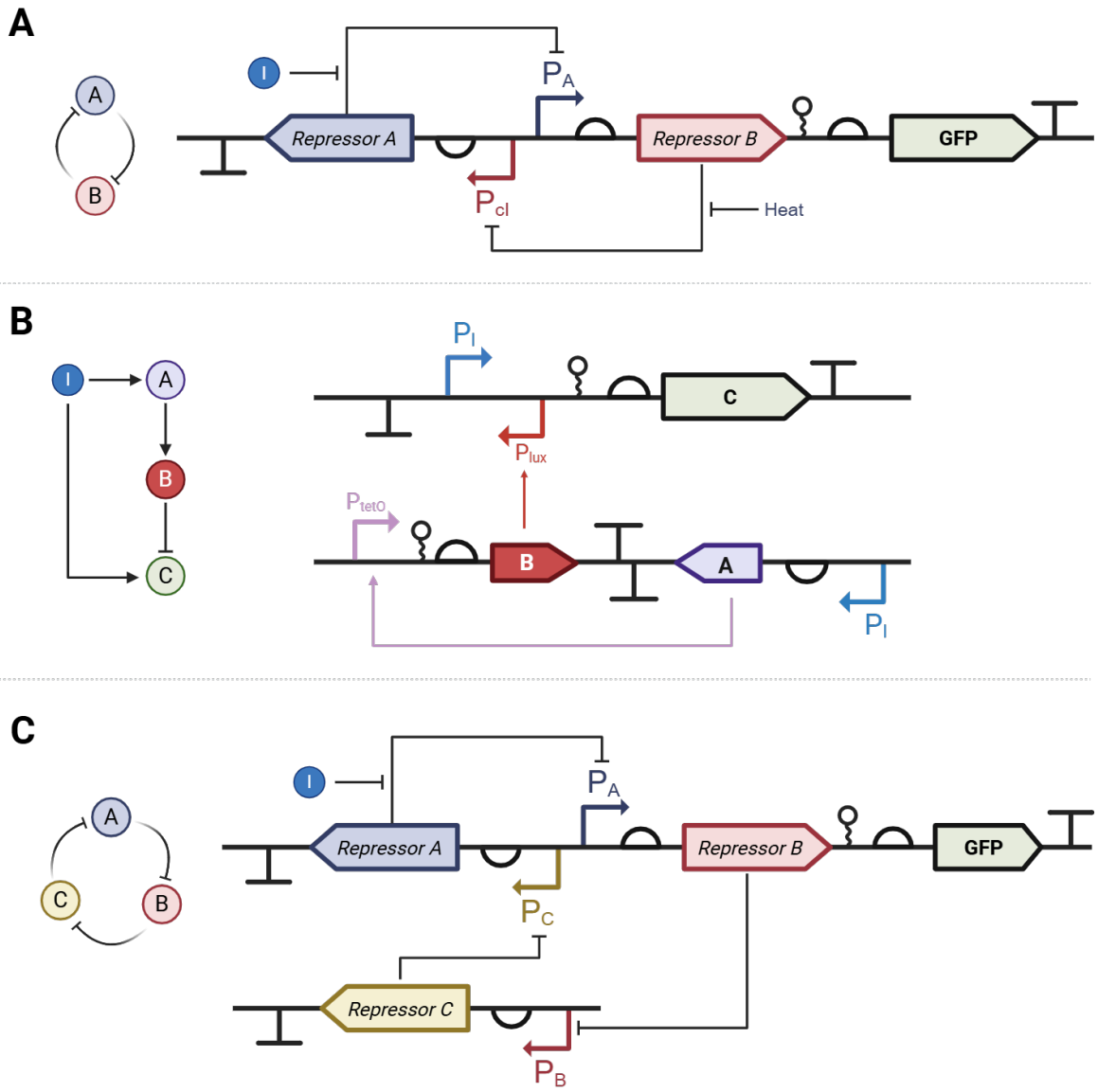

Figure 3. Topologies and genetic construction of representative dynamic genetic circuits. **A**: Bistable switch composed of repressors A and B. Specifically, P_{cI} is repressed by Repressor B, and inducible by heat shock. P_A is repressed by Repressor A, and inducible by an effector "I" (blue circle). P_A transcribes an mRNA which contains a self-cleaving ribozyme insulator, thus co-expressing Repressor B and GFP reporter genes. **B**: Incoherent feedforward loop that is responsive to effector "I" (blue circle) via promoter P_A. Transcription factor A activates expression of factor B which is positioned antisense to P_A. This has a repression effect on the expression of C, the final product. **C**: Repressilator composed of three repressors which repress in series. When P_A is active, the reporter is also expressed, exhibiting an oscillatory behaviour. *SBOL symbols as in Fig. 2; pin with wavy line: mRNA insulator (self-cleaving ribozyme). Created in BioRender.*

NAND and NOR gates are functionally complete

Both AND and OR gates can be negated using a NOT gate (Fig. 2F), forming NAND and NOR gates. This is achieved by placing the next component under a normally-ON repressible promoter. NAND and NOR gates are unique in their ability to be “functionally complete”; in short, able to replicate the functionality of any other logic gate. Negation is a simple task in biological circuits, effected by the production of a constitutive repressor TF which inhibits expression of the output gene. Thus, achieving this in practice not only depends on the ability to negate any gate, but on ensuring that the components used can be freely combined without cross-talk.

Dynamic genetic circuits

Beyond replicating Boolean logic gates, synthetic gene circuits can be designed to exhibit dynamic temporal behaviors using feedback and feedforward loops. Bistable switches exploit mutual repression between two transcriptional units to produce a system with two stable steady states (Fig. 3A) (Huan and Wang, 2024). Oscillators such as the repressilator extend this principle to three or more (in odd numbers) mutually repressing components, exhibiting periodic gene expression whose periods are determined by the degradation rates and expression levels of their constituent TFs (Elowitz and Leibler, 2000). Incoherent feedforward loops, where an input signal simultaneously activates an output and induces a delayed repression of that same output, produce an ephemeral pulse response to a sustained input; useful for input rising edge detection and filtering out transient signals (Fig. 3B) (Litovco et al., 2021). Repressilators are oscillating circuits which contain an odd number of mutually repressed genes (Fig. 3C). These are able to create periodic intervals of gene expression, applicable as an internal clock.

Cloning methods and assembly standards

The construction of synthetic gene circuits requires the precise assembly of multiple genetic parts in a specific order, a task that becomes increasingly complex as circuit size and complexity grows. Early cloning methods relying on conventional Type II restriction enzymes and ligation were limited by the scarcity of unique restriction sites and the incompatibility of fragments generated by different enzymes, making the assembly of multi-part constructs laborious and stochastic. Moreover, as these enzymes recognize palindromic sequences, cut sites or scars typically remain in the final construct. The usage of Type IIS (shifted) restriction enzymes that cleave DNA asymmetrically at a fixed distance from their recognition site proposes a solution to this problem. They enable the

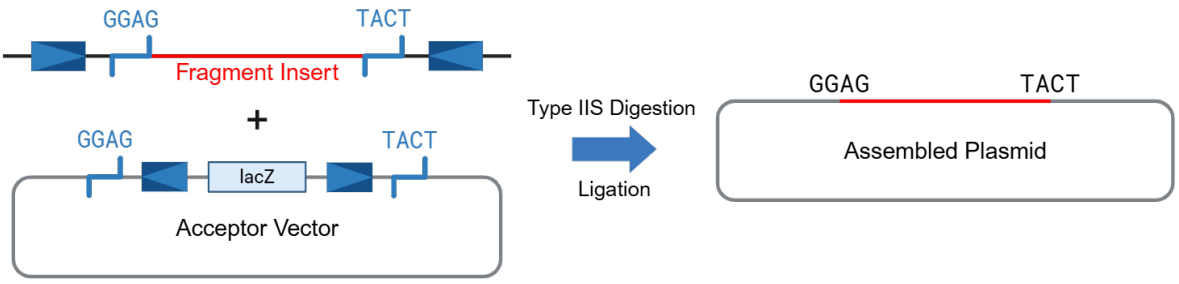

Figure 4. Golden Gate Assembly of a single insert into an acceptor vector. Blue boxes correspond to directional cut sites of a Type IIS restriction enzyme such as BsaI, BsmBI, and BbsI. The 5′ and 3′ overhangs match, and are able to assemble together in a restriction-ligation reaction. Restriction binding sites are removed, and a scarless ligation is achieved. This method can be extended to many fragments in one construction. *Created in BioRender.*

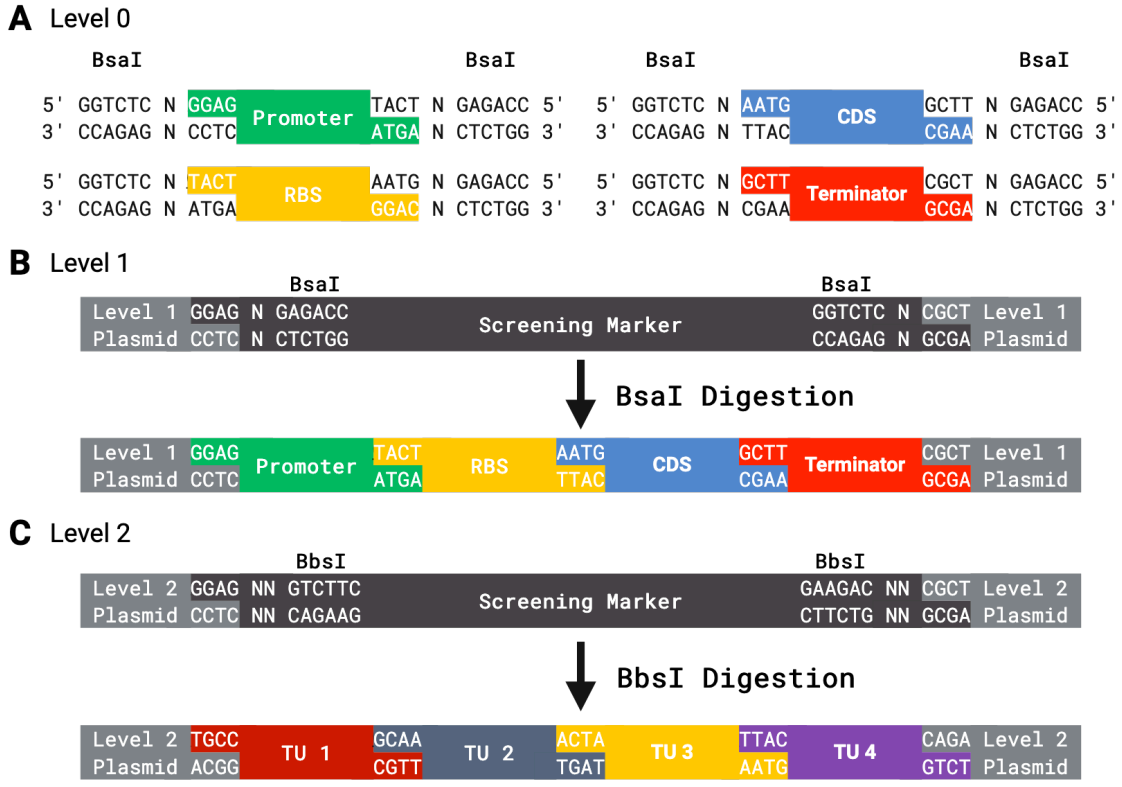

Figure 5. Illustration of the MoClo hierarchical cloning scheme. This scheme shows the MoClo cloning workflow which uses the BsaI and BbsI Type IIS restriction enzymes. **A**: generalized overhang choice for individual Level 0 parts. **B**: Level 1 transcriptional unit (TU) assembly using Level 0 parts and BsaI. At Level 0 construction, Loop and MoClo share overhang design and assembly restriction enzyme. **C**: MoClo Level 2 assembly using Level 1 TUs and BbsI. *Adapted from https://parts.igem.org/Help:Standards/Assembly/Type_IIS. Created in BioRender.*

generation of rational, defined DNA overhangs outside the recognition sequence. This property is exploited by Golden Gate Assembly (hereafter GGA), during which multiple fragments ligate in a reproducible order with no scar sequences at the junctions (Fig. 4). This method also enables one-pot reactions using only one restriction enzyme with ligase and DNA, reducing the likelihood that sequences require domestication (removal of illegal internal cut sites).

Hierarchical DNA assembly

Building upon GGA, the Modular Cloning (MoClo) standard comprises a framework for hierarchical assembly. First, linear DNA sequences are adapted using PCR or DNA synthesis and subcloned into "Level −1" vectors. These basic genetic sequences are then inserted into a Level 0 plasmid by GGA and formalized as "Level 0" parts; namely, promoters, ribosome binding sites (RBSs), coding sequences (CDSs), and terminators (Fig. 5A). Within the framework, Level 0 parts are given specific DNA overhangs. These are then combined into "Level 1" transcriptional units (Fig. 5B), which may stand alone or finally be assembled into multi-gene constructs as "Level 2" (Fig. 5C) (Weber et al., 2011). The Loop cloning standard enables further iterative cycles of assembly allowing for constructs unlimited in size (Pollak et al., 2019).

Generally, assembly frameworks contain formalized overhang sequences which flank different functional regions of a gene. These standards have been widely adopted in the synthetic biology community, enabling the reuse and exchange of parts between laboratories and organizations. Moreover, it allows for abstracting functions from their primary sequences, meaning that laboratories can use the same parts to assemble the same device and expect consistent results (Weber et al., 2011). The use of GGA-based assembly standards are supported by the iGEM[1] Foundation's RFC[1000], which provides guidelines for interoperability.

Control over the promoter sequence within current frameworks is limited

While MoClo and related hierarchical systems have accelerated the construction of multi-gene devices, granularity at the promoter level is rather limited. Their treatment of the promoter as an indivisible unit limits the ability to fine-tune transcriptional inputs or rapidly swap regulatory elements across circuit architectures. Increasingly complex synthetic gene circuits demand an approach with finer modularity. In practice, the promoter region is a composite of interacting elements whose combinatorial arrangement determines the logical properties of

[1] iGEM: International Genetically Engineered Machine

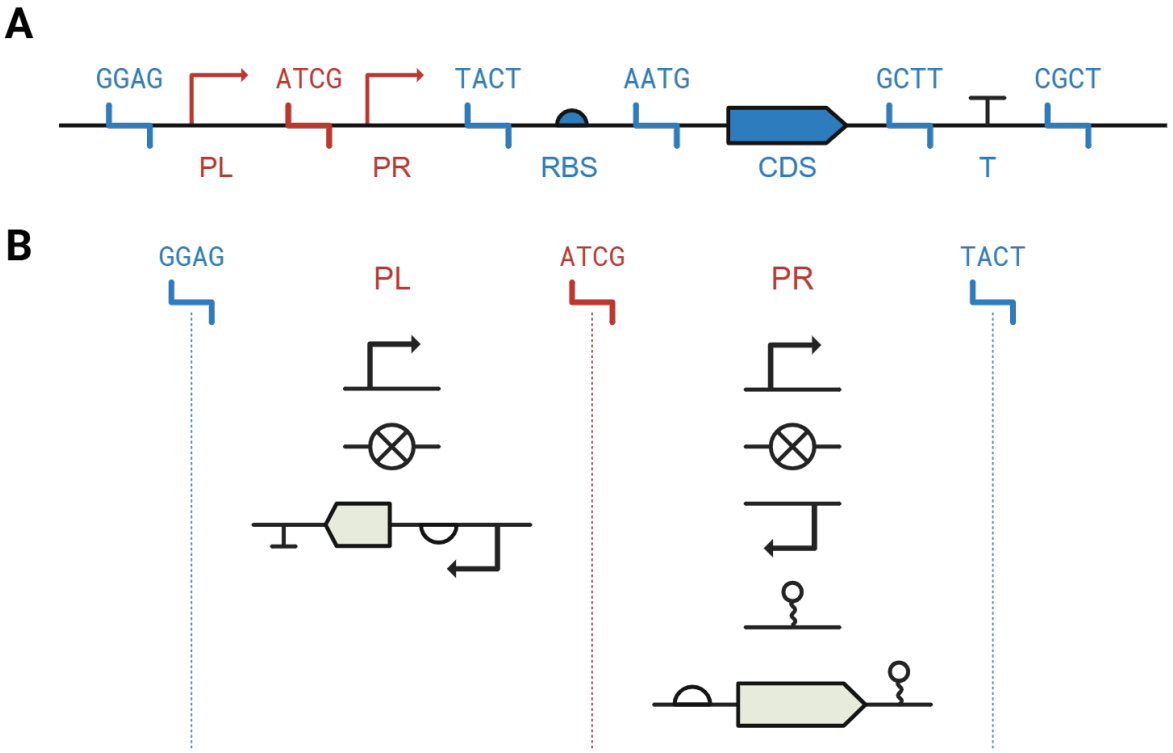

Figure 6. Scheme depicting the expanded MoClo standard with PL/PR architecture. **A**: MoClo standard overhangs (blue) with PL and PR parts and new ATCG overhang (red). Depicted parts, when assembled, would constitute a complete TU. **B**: Variants of Level 0 parts which may be inserted at the PL or PR regions of a constructed TU. This is not exhaustive, but representative of possible constructs for this project. *SBOL symbols as in (Figs. 2,3) circle with cross: spacer or dummy (Quinn et al., 2015). Created in BioRender.*

gene expression. A cloning standard that treats these elements as independently exchangeable parts would enable circuit flexibility that is currently unavailable within the existing frameworks.

Design of the genetic circuit "promoter toolkit"

To address these limitations, the host lab conceptualized a genetic circuit "promoter toolkit." The central design philosophy of this toolkit proposes the subdivision of the promoter region into two independently exchangeable regions: a left promoter (PL) and a right promoter (PR), separated by a novel DNA overhang at their junction (Fig. 6A). This modularity enables three powerful applications. The first is the embedding of a TF expression cassette within PL, such that a device becomes self-sufficient with respect to the regulatory interaction it participates in (Fig. 6B). This is particularly useful for heterologous promoters whose TF is not natively present in the host organism. The second application is the placement of two independent operator sequences within the promoter region, enabling dual-input transcriptional control within a single Level 0 part (Fig. 6B). This provides an architectural basis for the logic gates previously described of various different topologies. The third is the embedding of a TF expression cassette within PR that is under control of PL, and insulated from the downstream RBS and coding sequence using a self-cleaving ribozyme called RiboJ (Clifton et al., 2018) which removes the cis-regulatory region from the final mRNA (Fig. 6B). This enables the construction of bistable switches and oscillators which function via mutual regulatory dependencies between devices. Together, these three applications make the PL/PR architecture a flexible and compact platform for encoding complex regulatory logic directly at the promoter level.

In practice, this subdivision will be implemented by splitting the MoClo promoter functional region (demarcated by the 5′ GGAG and 3′ TACT fusion sites) into two parts using a defined internal junction site (Fig. 6A). Based on an analysis of four-nucleotide overhang ligation fidelities (Potapov et al., 2018), the sequence ATCG was chosen for the junction overhang due to its low mismatch annealing frequency to other existing MoClo overhangs and its comparable ligation efficiency, minimizing the risk of misassembly while keeping efficiency high. The resulting PL and PR parts are cloned as Level 0 parts flanked by their respective fusion sites (5′-**GGAG**|PL|**ATCG**-3′ and 5′-**ATCG**|PR|**TACT**-3′) and are therefore fully compatible with the existing MoClo hierarchy without modification to downstream assembly levels.

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II – Scientific questions and objective of the project

The scientific question posed in this project is: "*Can a plasmid assembly standard with expanded functionality at the promoter region of a transcriptional unit enable the reliable construction and accurate dose-dependent response of synthetic gene circuits across diverse topologies and dynamic mechanisms?*"

Objective #1: Design a modular genetic circuit toolkit based on an expanded MoClo promoter assembly standard *in silico*

Both common and niche synthetic regulatory circuit topologies were selected from literature or designed *in silico* using inducible promoters; including P_{lac}, P_{tet}, P_{lux}, heavy metal-responsive, heat shock, and CELLO-described promoters (Jones et al., 2022; Nielsen et al., 2016). These are chosen for their ability to constitute logic gates, oscillators, and bistable switches. Can the breadth of these topologies be accommodated within a single unified modular framework, and does the PL/PR promoter split provide sufficient flexibility for combinatorial circuit design across all topologies?

Objective #2: Establish the reliability of the expanded MoClo standard through construction of Level −1, 0, and 1 genetic parts

The physical generation of the genetic toolkit will follow, utilizing *de novo* gene synthesis or PCR, and plasmid cloning to generate a library of Level −1 and Level 0 genetic parts, kept within various housekeeping plasmids. Moreover, the design of the PL/PR split promoter system will be subject to assembly of Level 1 transcriptional units and analyzed by PCR and next-generation sequencing. Does the `ATCG` junction perform with sufficient fidelity for reliable assembly, and can the expanded standard produce these constructs consistently?

Objective #3: Characterize the logical and dynamic behavior of assembled synthetic gene circuits *in vivo*

Level 1 and 2 test devices assembled from the toolkit will be characterized in *E. coli* NEB 10-beta using fluorescent protein reporters, measuring steady-state expression levels, dose-response curves, and kinetics across a range of effector concentrations. Do assembled circuits produce theoretically predicted outputs, and do circuits implementing the same logical function through different molecular topologies produce comparable dynamic behaviors?

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III – Methodological and experimental approaches used

Objective #1 – *In silico* design of the genetic toolkit

The sequences of all genetic parts will be designed and catalogued in SnapGene (Dotmatics, Boston, MA), which will also be used to simulate Golden Gate assemblies and verify overhang compatibility prior to ordering. The toolkit will comprise numerous Level 0 parts encoding cis-regulatory elements required for the construction of primitive AND, OR, NAND, and NOR gates, bistable switches, and incoherent feedforward loops (Figs. 2,3). Circuit topologies will be selected from literature or newly designed utilizing characterized inducible promoters. In addition to promoter parts, the toolkit will include reporter fluorescent proteins, standard terminators and ribosome binding sites, and ribozyme insulators.

Each selected genetic part will be flanked by the appropriate MoClo fusion site overhangs to direct their assembly during GGA into a transcriptional unit. For non-promoter Level 0 parts, the existing MoClo overhang set will be used without modification. For parts designed to occupy the PL and PR positions of the split promoter, the `GGAG|PL|ATCG` and `ATCG|PR|TACT` fusion site architecture will be applied. All part sequences will be analyzed *in silico* for internal illegal Type IIS restriction enzyme cut sites, which will be removed where required.

Theoretical design of genetic circuit assemblies to be put under test

To act as a positive control for each topology, a version of the circuit will be constructed identically to its literature source. This control will serve as a reference point against which toolkit-assembled variants can be compared. Then, equivalent circuits will be designed in which the promoter is substituted while all other elements are held constant. To do this, literature promoters will be substituted by others that act through similar regulatory mechanisms. For example, (i) P_{BAD} can be substituted by P_{xylS}, as they are both from the same TF superfamily but react to different effectors, and (ii) P_{tac} can be substituted by $P_{PsiTac1}$[2], as they share upstream sequence homology, but bind different transcription factors. Negative control constructs will also be constructed, which incorporate non-functional dummy sequences at one or both PL/PR sites, establishing that observed reporter output is specifically driven by the intended regulatory inputs rather than by leaky expression or read-through transcription events.

[2] iGEM Registry Link: BBa_K2448016.

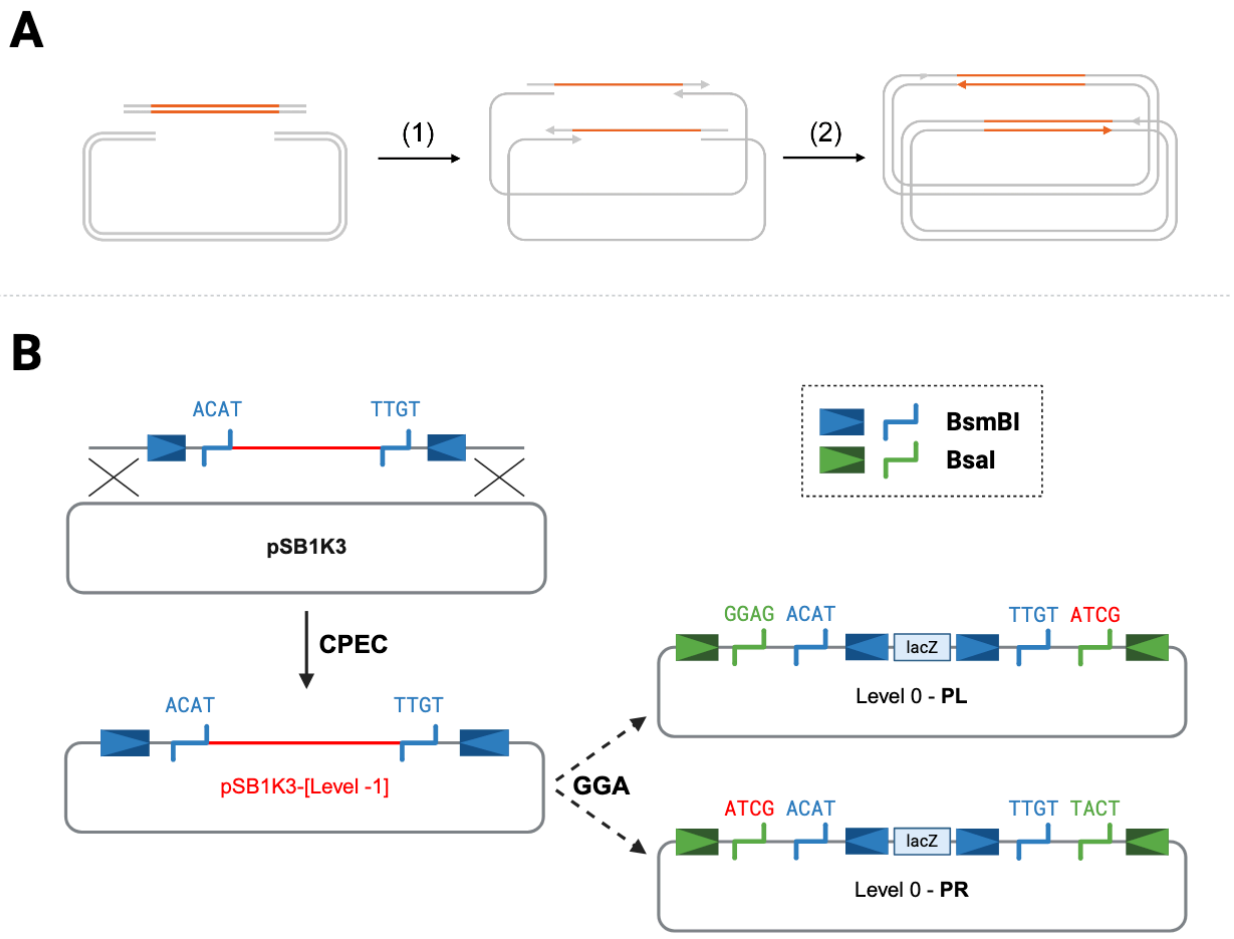

Figure 7. Cloning strategy for constructing Level −1 and Level 0 parts. **A**: Scheme describing circular polymerase extension cloning (CPEC). DNA is first denatured, then cooled such that homologous regions of DNA anneal (1). Then an extension step (2) performed by DNA polymerase completes the other strand of the plasmid, creating two nicked copies. Adapted from Quan and Tian (2009). **B**: Workflow from DNA fragments to Level 0 parts. After PCR or synthesis, a fragment is cloned into pAGM1311 using CPEC. Then, using Golden Gate Assembly, the part can enter a Level 0 vector corresponding to either PL or PR. Crossed lines represent homologous regions of DNA. *Created in BioRender.*

Objective #2 – Cloning assembly of DNA parts and devices

Assembly of Level −1 genetic parts

The physical construction of the toolkit parts will begin with the synthesis of preliminary Level −1 sequences, which encode basic, domesticated genetic elements that are destined to become Level 0 parts. Level −1 sequences may be assembled in tandem with other Level −1s, or alone to form Level 0s. The purpose of this level is to serve as a transition state between DNA fragments and standardized plasmids. The majority of Level −1 parts will either be amplified by PCR, or be ordered as synthetic DNA fragments from IDT (Coralville, IA) and Twist Bioscience (San Francisco, CA). These fragments will then be cloned into the pAGM1311 acceptor vector (which contains a kanamycin antibiotic resistance gene) via circular polymerase extension cloning (CPEC). CPEC is a method of DNA assembly which does not use restriction enzymes. Instead, it uses DNA polymerase to perform *ad hoc* homology-based ligation of two or more DNA molecules by subsequent melting-annealing-extension steps in a thermal cycler (Fig. 7A) (Quan and Tian, 2009; Quan and Tian, 2011).

The resulting Level −1 entry clones will be transformed into *Escherichia coli* NEB 10-beta[3], plated on selective medium containing kanamycin before being purified and sequence-verified by Nanopore sequencing (Oxford, UK) before passing through more assembly steps.

Assembly of Level 0 and 1 genetic parts using GGA

First, Level 0 acceptor vectors will be generated by swapping the insert region of the pSB1C5 plasmid with a region that can accept a Level −1 fragment upon GGA using BsmBI. Two of these vectors will be made in-house, one with built-in overhangs for PL, and another for PR (Fig. 7B). This allows any Level −1 part to be used in either the PL or PR position. This plasmid contains a chloramphenicol resistance cassette, and so it does not overlap with any plasmids used in this toolkit.

Then, Level −1 parts will be combined in a one-pot Golden Gate Assembly reaction to assemble Level 0 parts by their insertion into the appropriate acceptor vector (Fig. 7B). This first reaction is mediated by BsmBI and T4 DNA ligase over 15 cycles of digestion (37 °C) and ligation (16 °C) steps using a thermal cycler, followed by final digestion at 50 °C and enzyme inactivation

[3]NEB: New England Biolabs; Genotype: Δ(ara-leu) 7697 *ara*D139 *fhu*A Δ*lac*X74 *gal*K16 *gal*E15 e14$^-$ ϕ80dlacZΔM15 *rec*A1 *rel*A1 *end*A1 *nup*G *rps*L (Str^R) *rph spo*T1 Δ(mrr-hsdRMS-mcrBC)

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at 85 °C. These parts will also be transformed following assembly into *E. coli* NEB 10-beta and subsequently purified for PCR validation.

Next, using select Level 0 parts, Level 1 test GGA reactions will be performed using the BsaI restriction enzyme. These test assemblies will sample various possible combinations of PL and PR promoter parts using constant RBS, reporter, and terminator sequences in order to test the ligation efficiency of the novel promoter assembly system. In order to analyze these assemblies, they will be transformed into *E. coli* NEB 10-beta for clonal expansion, purified by mini-prep and then validated *via* Nanopore sequencing. From this data, we will observe the fidelity of the ATCG overhang resulting in correctly-ordered parts within each transcriptional unit. This will allow us to deduce whether this expanded assembly standard is suitable for generation of complex promoters through GGA, utilizing MoClo assembly standards.

Preparation of accessory vectors

Just prior to characterization, Level 2 accessory vectors will be constructed encoding the transcription factors required for circuit sensing function, including AraC, TetR, LacI, and XylR, among others. These will be assembled using the same GGA workflow described before and transformed into *E. coli* NEB 10-beta alongside the Level 1 test device plasmids. The level 2 accessory acceptor vector will be a high copy plasmid, either pSB1S3 or pJUMP44-2A, as it contains a spectinomycin resistance cassette and a pUC19-like origin of replication (ORI).

Objective #3 – Characterization of assembled genetic circuits

Dose-dependence and dynamics characterization

For characterization, *E. coli* cells harboring both a test device and an appropriate accessory vector will be grown overnight in 96-deep-well plates in LB medium supplemented with the appropriate antibiotics. Circuits will be induced within a normal 96-well plate during the exponential growth phase, ensuring that characterization captures gene expression dynamics under steady-state metabolism rather than under stationary phase conditions. Plates will be prepared using an OT-2 automated pipetting robot (Opentrons, New York), which will be programmed using Python to execute the dilution and dispensing steps in the correct order. Plates will be incubated within a fluorescence- and optical density-capable plate reader, with GFP fluorescence and optical density at λ = 600 nm (OD_{600}) measured every 15 minutes.

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All test transcription units built for characterization will utilize a superfolder green fluorescent protein (sfGFP) tagged with an LVA peptide. The LVA motif confers instability to the tagged protein, thus enabling GFP to be used in dynamic analyses by preventing toxic buildup that may interfere with cell function and also saturate fluorescence sensors (Andersen et al., 1998). Moreover, a fluorescence calibration assay will be conducted across all available plate readers to establish MEFL normalization curves using fluorescein standard solutions (Beal et al., 2020). This will ensure that fluorescence measurements are expressed in absolute units and are comparable across instruments, which is particularly important if multiple plate readers from different manufacturers are to be used in parallel to increase throughput. Fluorescence values will be normalized by OD_{600} to account for differences in cell density and converted to MEFL/particle.

Depending on the circuit being tested, I will employ one of two experimental strategies. For logic gates, dose-response assays will be performed by varying two effector concentrations independently across a 5x5 concentration matrix, yielding 25 conditions per circuit. With three biological replicates per condition, this design occupies 75 wells of a 96-well plate, with the remaining wells reserved for negative controls — including cells harboring no circuit plasmid for auto-fluorescence correction, and dummy PL/PR constructs as described in Objective 1. For dynamic circuits such as bistable switches and incoherent feedforward loops, kinetic assays will be performed over 24 hours to capture the full behavior of the circuit over time, with effector concentrations chosen based on the dose-response data obtained from the logic gate characterization and literature values from Litovco et al. (2021).

The characterization of these circuits will establish a quantitative behavioral baseline for their further application in the host laboratory. The PL/PR promoter architecture represents a generalized extension to the MoClo standard that is not limited to the circuit topologies described in this work. In principle, any regulatory element that can be encoded within a promoter region can be adapted to this framework, opening new possibilities for the combinatorial design of synthetic gene regulation.

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Abstract

Transcription factors are DNA-binding proteins that dynamically regulate gene expression. In prokaryotes, they regulate operons and genes logically, lending them to applications in synthetic biology as living logic gates. This project proposes a modular genetic circuit toolkit that extends the Modular Cloning Golden Gate Assembly standard. The modification allows Level 1 transcriptional units to accept two promoters; namely left (PL) and right (PR) promoters. This will be done by introducing a novel junction overhang, `ATCG`, between the canonical MoClo `GGAG`/`TACT` promoter overhangs. The resulting framework facilitates construction of AND, OR, and NOT gates, combinable at higher cloning levels into complex circuits. It also allows for building dynamic circuits such as bistable switches and incoherent feedforward loops for input control. Characterization will use fluorescent reporter test devices in dose-response and kinetics experiments. Altogether, the resulting toolkit will serve as a platform for the host lab and other researchers to create more complex circuits than possible using current logic gate assembly methods.

Les facteurs de transcription sont des protéines de liaison à l'ADN qui régulent dynamiquement l'expression des gènes. Chez les procaryotes, ils régulent les opérons et les gènes de manière logique, ce qui les rend utilisables en biologie synthétique comme portes logiques vivantes. Ce projet propose une boîte à outils de circuits génétiques modulaires qui étend la norme d'assemblage Golden Gate par Modular Cloning (MoClo). La modification permet aux unités de transcription de Niveau 1 d'accepter deux promoteurs : le promoteur gauche (PL) et le promoteur droit (PR). Ceci sera réalisé en introduisant une nouvelle séquence, `ATCG`, entre les séquences de jonction canoniques des promoteurs MoClo `GGAG`/`TACT`. Le cadre ainsi obtenu facilite la construction de portes AND, OR et NOT, combinables à des niveaux de clonage supérieurs en circuits complexes. Il permet également la construction de circuits dynamiques tels que des commutateurs bistables et des boucles de rétroaction incohérentes pour le contrôle des entrées. La caractérisation utilisera des dispositifs de test à rapporteur fluorescent dans des expériences de dose-réponse et de cinétique. Au final, la boîte à outils ainsi créée servira de plateforme au laboratoire hôte et à d'autres chercheurs pour créer des circuits plus complexes que ceux possibles avec les méthodes actuelles d'assemblage de portes logiques.

Keywords: *Gene regulation, genetic circuit, DNA toolkit, assembly standard, promoters.*