

BASIC: A New Biopart Assembly Standard for Idempotent Cloning Provides Accurate, Single-Tier DNA Assembly for Synthetic Biology

Marko Storch,^{†,||} Arturo Casini,^{†,||} Ben Mackrow,[†] Toni Fleming,[‡] Harry Trehwitt,[†] Tom Ellis,[§] and Geoff S. Baldwin^{*,†}

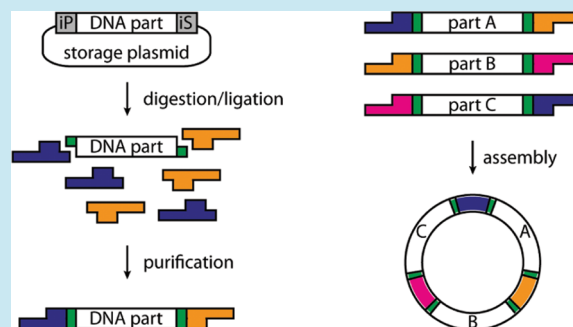
[†]Department of Life Sciences & Centre for Synthetic Biology & Innovation, Imperial College, South Kensington Campus, London, SW7 2AZ, U.K.

[‡]Dr. Reddy's Chirotech Centre, Milton Road, Cambridge, CB4 0PE, U.K.

[§]Department of Bioengineering & Centre for Synthetic Biology & Innovation, Imperial College, South Kensington Campus, London, SW7 2AZ, U.K.

S Supporting Information

ABSTRACT: The ability to quickly and reliably assemble DNA constructs is one of the key enabling technologies for synthetic biology. Here we define a new Biopart Assembly Standard for Idempotent Cloning (BASIC), which exploits the principle of orthogonal linker based DNA assembly to define a new physical standard for DNA parts. Further, we demonstrate a new robust method for assembly, based on type II restriction enzyme cleavage and ligation of oligonucleotides with single stranded overhangs that determine the assembly order. It allows for efficient, parallel assembly with great accuracy: 4 part assemblies achieved 93% accuracy with single antibiotic selection and 99.7% accuracy with double antibiotic selection, while 7 part assemblies achieved 90% accuracy with double antibiotic selection. The linkers themselves may also be used as composable parts for RBS tuning or the creation of fusion proteins. The standard has one forbidden restriction site and provides for an idempotent, single tier organization, allowing all parts and composite constructs to be maintained in the same format. This makes the BASIC standard conceptually simple at both the design and experimental levels.



The ability to build newly designed DNA constructs easily, quickly and with high accuracy is one of the key enabling technologies of synthetic biology,^{1,2} and the adoption of a standard format for the assembly of genetic components is part of this vision.^{1,3} The BioBrick standard⁴ is a restriction-ligation-based format, and its usefulness stems from the principle of idempotency, where assembled parts retain the prefix and suffix of the original, enabling successive rounds of hierarchical cloning. However, a recent survey⁵ has highlighted that most synthetic biology researchers now use Gibson's isothermal method for their DNA assembly.⁶ This suggests that the advantages of being able to assemble five or more fragments of DNA in parallel and having no forbidden sequences or scars outweighs the usefulness of a widely adopted standard in the eyes of many researchers.

Although the Gibson method can be adapted to a physical standard framework using synthetic sequences to guide assembly,^{3,7,8} it is mostly used *ad hoc*, with customized parts that are generally prepared *via* PCR amplifications. This has led to a return to bespoke assembly, where each reaction requires design, optimization and verification. Furthermore, reliance on PCR can compromise fidelity through errors in amplification and is inefficient for very long sequences or those containing high GC content and repeat sequences. PCR is also difficult to

implement in an automated workflow because reactions for individual parts have to be optimized and verified. A recent approach excludes PCR⁷ but requires upstream cloning to define downstream assembly order, thus extending the workflow.

Aside from BioBricks, alternative restriction-based standards have been developed, including GoldenBraid^{9,10} and MoClo,¹¹ which are based on the Golden-Gate¹² protocol that employs type II restriction enzymes. A common feature of these approaches is that the entry vector of a part defines its position in the final destination vector, so that changing the order of the parts requires an additional round of cloning. Both MoClo and GoldenBraid adopt a tiered approach, which takes advantage of the consistent layout of transcription units: in the first tier of assembly, where elementary parts such as promoters, ORFs and terminators are assembled into transcription units, a fixed and predefined part order is adopted. In this way, the first round of assembly never requires changing entry vectors. The same strategy cannot be used in the second tier of assembly, where transcription units are assembled into multigene constructs,

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since it is usually necessary to retain complete freedom of design. Here MoClo adopts a parallel approach, which requires cloning in a different vector for each possible position, while GoldenBraid adopts a sequential approach that minimizes the number of vectors necessary but only allows pairwise assembly. It was previously suggested that the relative advantages of MoClo and GoldenBraid were mutually exclusive.⁹

To address the limitations of current assembly technologies, we have developed Biopart Assembly Standard for Idempotent Cloning (BASIC) to bring together six key concepts: standard reusable parts; single-tier format (all parts are in the same format and are assembled using the same process); idempotent cloning; parallel (multipart) DNA assembly; size independence; automatability. Our previous assembly strategy was based on Modular Overlap Directed Assembly with Linkers (MODAL),³ which introduced the concept of computationally derived orthogonal linkers.¹³ To address these key concepts we have developed a new method based on robust restriction/ligation reactions to ligate orthogonal oligonucleotide linkers with single stranded overhangs that define the assembly order. To further address many of the requirements of assembling DNA parts and biological pathways,¹ we have enabled hierarchical cloning within a single-tier format and demonstrated that the linkers themselves can be used as composable parts encoding RBS sequences or peptide linkers for fusion proteins. This has been achieved within a standard format that facilitates reuse of both linkers and parts.

The core of the BASIC physical DNA standard is constituted by the integrated prefix and suffix sequences (*iP/iS*), which were designed to be back compatible with our previous MODAL strategy³ where they can act as PCR priming sites; alternatively, we here define the BASIC assembly method, based on simple robust reactions. The *iP* and *iS* sequences were also designed to ensure compatibility with the creation of fusion proteins, either by BASIC or MODAL, by optimizing the amino acid coding of both the short BASIC scars and the full *iP/iS* sequences (Figure 1a).

To avoid PCR, yet retain the advantages of overlap directed DNA assembly, we have revisited pre-PCR methods, where oligonucleotides were routinely ligated onto DNA ends to either provide restriction enzyme sites, or compatible sticky ends to direct molecular cloning.¹⁴ The BASIC standard defines two inward-facing *BsaI* recognition sites to release the parts from a storage vector, leaving a 4 bp scar on the prefix end and a 6 bp scar on the suffix (Figure 1b). Digestion yields different 4 bp overhangs at the prefix and suffix, enabling end-specific ligation. Ligation of partially double-stranded oligonucleotide DNA linkers is performed simultaneously with *BsaI* digestion (Figure 1c). Nonligated oligonucleotide linkers are then removed by a purification step to yield linker-adapted parts. Final assembly is achieved by annealing the linker-adapted parts in an ionic buffer at elevated temperature. No ligase is required in the final step and the nicked plasmid generated is readily repaired *in vivo* following transformation. Full details of the protocol and optimization of the method are provided in the Supporting Information.

Linker sequences to guide assembly were an expanded set of 7 linkers based on our previously used 40% GC content linkers designed by R2oDNA Designer^{3,13} (Tables S3 and S4). We split each 45 bp linker sequence across 2 parts, with each containing a 12 bp double stranded region on the outer side and sharing the central 21 base single stranded overlap region as top and bottom strands (Figure 1b). Double-stranded

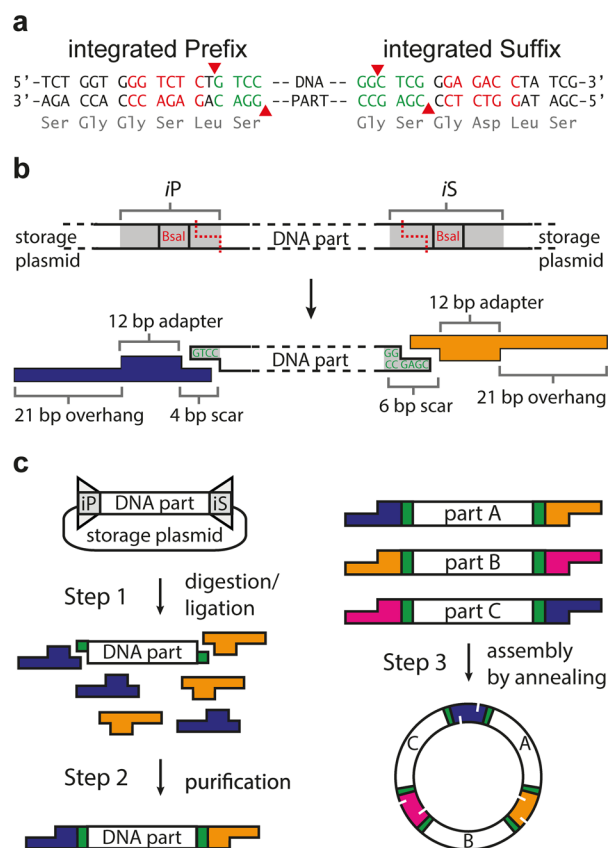


Figure 1. BASIC standard and method. (a) Sequence of the integrated prefix (*iP*) and suffix (*iS*); the *BsaI* recognition sequence is shown in red with the cut sites marked (red triangles); the amino acid translation for each codon in the *iP/iS* are shown. (b) During the assembly process *iP* and *iS* are cut to produce different sticky ends that enable differential ligation of linkers onto each end. (c) The BASIC assembly workflow. Step 1: linkers are attached by simultaneous digestion and ligation. Step 2: unligated excess linkers are removed *via* magnetic bead purification. Step 3: purified linker-adapted parts are mixed and annealed in an ionic buffer to generate the final construct. [Protocol provided in the Supporting Information.]

regions at the sites of linker ligation are necessary for efficient activity of T4 DNA ligase.¹⁵ The use of 21 bp overlaps enables elevated temperatures during final assembly by complementary annealing, facilitating the kinetics, thermodynamics and specificity of the homology-search process.

To evaluate the efficiency and accuracy of our assembly method, and benchmark against our previous Gibson-based work, we generated a number of parts in BASIC format with *iP* and *iS* sequences flanking each part of interest (Table S2). This formatting step only ever has to be performed once for any part since the storage plasmid carries no positional information for the DNA assembly process, which is directed through the subsequent choice of linkers. Parts prepared include those that are essential for cell survival (origin of replication (MB1), kanamycin resistance (Kan) chloramphenicol resistance (Cm), combined origin and kanamycin (Kan-MB1), and others that produce fluorescent proteins (GFP and RFP, both as expression cassettes and as separate open reading frame parts).

Benchmarking DNA assembly reactions were performed by creating plasmids in a modular format from this parts library. Constructs comprising 2–6 component parts with a single antibiotic marker, and 2–7 parts with double antibiotic

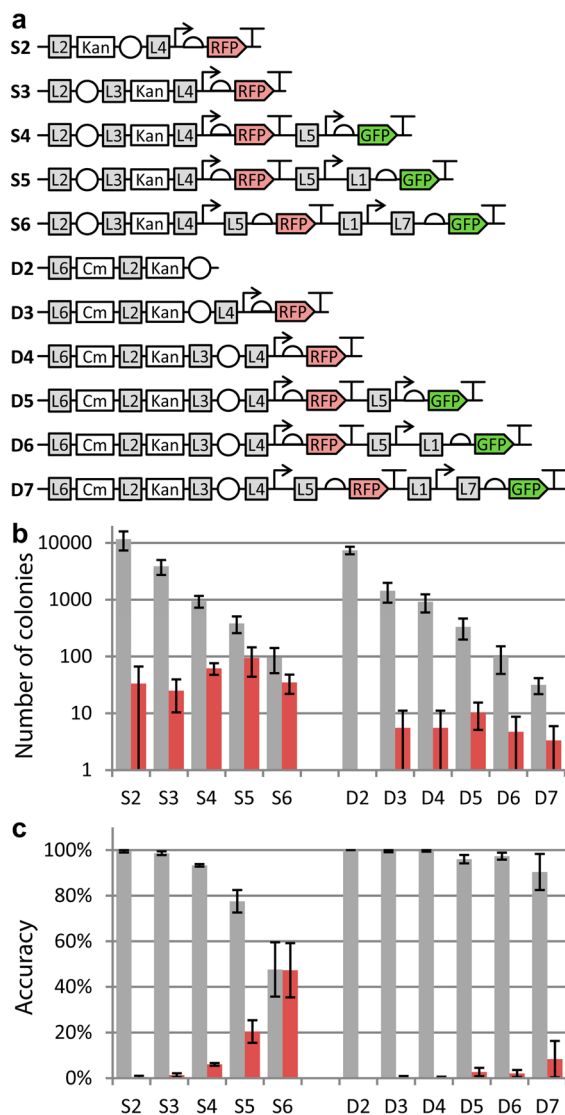


Figure 2. BASIC allows for highly efficient multi part assembly. (a) Benchmarking DNA assembly reactions were performed creating constructs with 2–6 parts using single antibiotic selection (S2–S6) and 2–7 parts with double antibiotic selection (D2–D7) [Table S1]. (b) The number of colonies returned from each assembly is shown as the average of 4 repeat reactions with standard error of the mean (SEM; gray bars); the total number of incorrect assemblies that either had no fluorescence or incorrect fluorescence profiles are also shown (red bars). (c) The accuracy of each assembly reaction was assessed as the percentage of colonies with the correct fluorescence profile for the designed assembly (gray bars); percentage of incorrect assemblies are also shown (red bars). All data is shown as the average of 4 repeat reactions with SEM.

selection were chosen for evaluation (Figure 2a). All final constructs (apart from D2) contain a fluorescent reporter, and accuracy of assembly was thus evaluated by observing the correct expression of reporters while assessing each construct's ability to replicate and confer the appropriate antibiotic resistance.

BASIC assembly reactions were performed four times for each of the 11 designated test constructs following an optimized protocol (Supporting Information). Assembly efficiency was determined from the number of colonies and accuracy as the percentage of colonies with the correct

antibiotic resistance expressing the correct fluorescent reporters (Figure 2).

The results of the DNA assembly benchmarking reveal that the efficiency of assembly decreases exponentially with the number of parts involved (Figure 2b). However, even with 6 or 7 parts, reactions routinely returned between 40 and 150 colonies, while 3–4 part assembly routinely returned more than 1000 colonies, demonstrating the overall efficiency of the process.

The more critical measure for DNA assembly is accuracy. With single antibiotic selection there is the possibility that the storage plasmid that carries either the Kan-MB1 composite part or the Kan cassette can return a viable nonfluorescent colony if it is not completely digested in the first step of the protocol. The assembly efficiency decreased exponentially with increasing number of parts, but the number of incorrect assemblies (which includes both white background colonies and colonies with the wrong fluorescent reporters) remained relatively constant. The incorrect assemblies thus became a larger proportion of the colony count, decreasing accuracy (Figure 2c). To address this we included a second antibiotic resistance cassette, chosen so that the final construct could be selected using double antibiotic selection without any of the starting constructs conferring resistance. This significantly reduces the proportion of incorrect assemblies, indicating that these arise largely through carryover of storage plasmids when only a single antibiotic marker is used (Figure 2b and 2c). The double antibiotic selection strategy thus provides a significant improvement in the accuracy of BASIC assembly and was therefore adopted as the standard method in subsequent assemblies.

Since the orthogonal linker sequences provide positional watermarks in the final assembly, they may be used to validate assemblies since they provide ideal PCR primer sites. This strategy was used to evaluate the 5-part assembly, demonstrating the flexibility in reordering parts simply by changing the linker combinations ligated to each part. We assessed the assembly order of these reactions as well as the seven part construct (D7) by performing PCR reactions with a forward primer for the first linker and reverse primers for each of the other 4 or 6 linkers. The PCR products exhibit the anticipated ladder of increasing size demonstrating the correct order and presence of each part in the assembly (Figure S1). This provides a useful screening method for DNA assembly verification and because the linkers are standardized, the PCR verification primers are also standardized (Table S13). Since the DNA assembly workflow starts with plasmid DNA and does not involve PCR amplification, there is less need to sequence the final construct.

In many cases it is advantageous to assemble a limited number of parts together in a module and then combine different modules to create more complex systems or to reuse modules in different assemblies. The single-tier approach of BASIC therefore requires an idempotent method by which the *iP* and *iS* sequences can be recapitulated during DNA assembly. The objective therefore was to encode *iP* and *iS* on linkers attached during DNA assembly, while avoiding any modification to the protocol. To achieve this we investigated DNA methylation as a strategy to protect the *BsaI* site from digestion during the assembly process.

The cognate DNA methyltransferase of the *BsaI* restriction modification system is a C-5 methyltransferase, but its target within the *BsaI* recognition sequence is not known.¹⁶ We have therefore determined the pattern of methylation protection

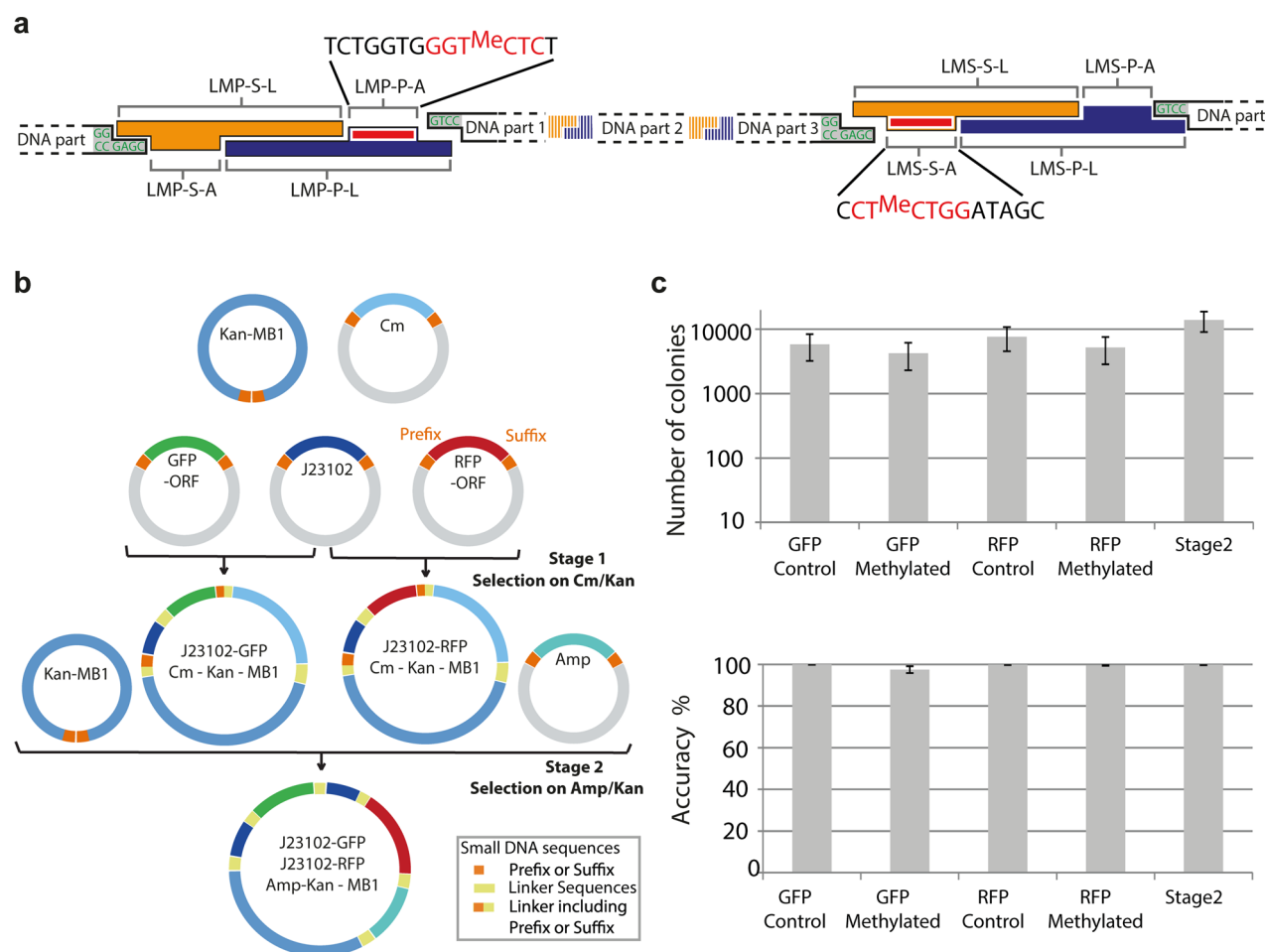


Figure 3. Hierarchical assembly using methylated linkers. (a) Linker design to recapitulate *iP* and *iS* adjacent to the parts being assembled. The methylated cytosine is located on the adapter oligonucleotide, which prevents digestion of the linker during the assembly process. (b) Workflow to test idempotent DNA assembly using methylated linkers: in stage 1 GFP and RFP expressing cassettes are assembled flanked by *iP* and *iS*, backbone Kan-MB1 and Cm parts are located outside of *iP* and *iS* and so are not carried through in subsequent assembly rounds. In stage 2 the previously assembled expression cassettes are used to assemble a double fluorescence reporter. (c) Data from assembly reactions is shown for reactions with methylated linkers, control reactions with nonmethylated linkers and stage 2 reactions. Data shown is the average of 4 repeat reactions with SEM for the number of colonies returned and the accuracy, determined as the percentage of colonies with the correct fluorescence profile.

through *in vitro* digestion of fluorescently labeled oligonucleotides, with each of the 4 cytosine residues within the recognition site methylated in turn. The restriction digests clearly reveal that methylation of the bottom strand only partially protects the DNA from digestion, while methylation of either cytosine in the top strand effectively protects the DNA from digestion by BsaI (Figure S2). We therefore propose a general single-tier workflow for BASIC, where *iP* and *iS* are recapitulated around the constructed cargo during assembly by methylation of specified linker oligonucleotides to avoid cleavage during the combined digestion/ligation step (Figure 3a).

To demonstrate this approach we separately constructed GFP and RFP expression cassettes from individual parts encoding a constitutive promoter (J23102) and RBS-ORFs for GFP and RFP: these cassettes were then used in a second round of assembly to construct a dual fluorescence plasmid (Figure 3b). Parallel reactions were also performed with nonmethylated linkers to benchmark the efficiency of the idempotent assembly compared to standard linkers [for a detailed list of assembly order see Table S7]. The 4-part first round of assembly proceeded with 99% accuracy and an

efficiency that was only 10% lower than that with standard linkers (Figure 3c). The expression cassette constructs were then successfully used for construction of the dual reporter plasmid. This demonstrates that methylation of a single cytosine in the BsaI recognition sequence provides sufficient protection against BsaI digestion to enable an idempotent strategy without modification of the protocol. Maintaining the same protocol for all stages of assembly and for all parts ensures an easy workflow for both bench-scale work and automation.

One feature of synthetic biology is the ability to rationally compose parts to provide either tunable or predictable behavior. Using custom RBS sequences to regulate protein translation has become increasingly common.^{17–20} The use of synthetic linker sequences provides the opportunity to encode small parts within the linker, such as RBS sequences. In line with our modular standardized approach to DNA assembly, we chose to tune the output of fluorescent reporters by encoding known RBS sequences of different strengths with the expectation that local sequence context would provide additional variability.¹⁹ Four RBS sequences were selected from the iGEM Parts Registry, and encoded onto the double stranded portion of the prefix linker (Figure 4a; Table S8). Two

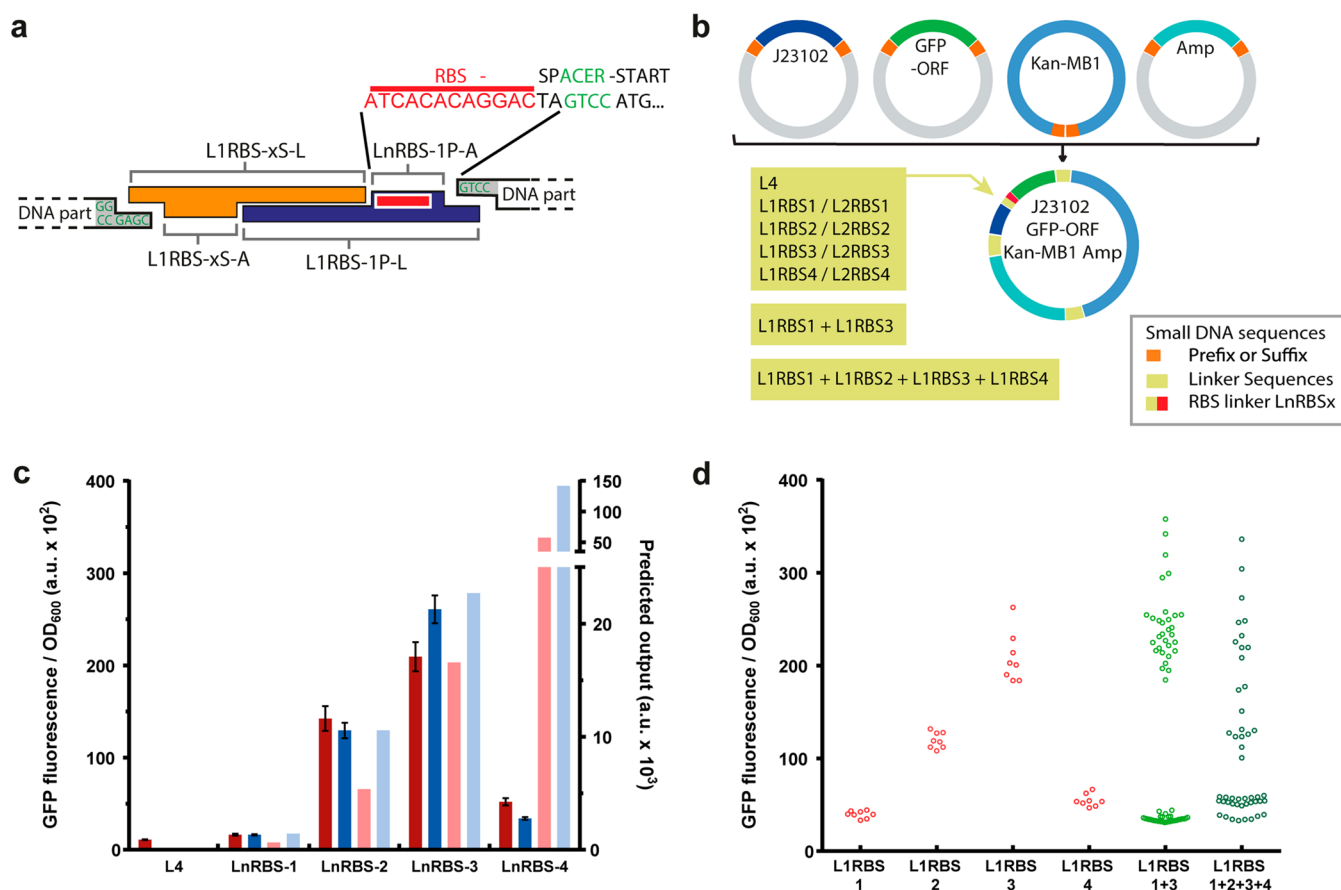


Figure 4. Tuning translation with RBS linkers. (a) RBS sequences were encoded on the double stranded portion of the ligated linker oligonucleotide with a spacing region to ensure efficient translation [Table S8]; the single stranded overlap of the linker does not encode the RBS and multiple RBS sequences were encoded with the same linker homology (L_nRBS_x , where n denotes the homology type within a series of x different RBS sequences). (b) Assembly strategy for constructs to test 4 RBS sequences within two different linker contexts and a control linker that does not encode an RBS (L4). (c) GFP expression was evaluated after 6 h growth and is shown normalized to OD₆₀₀ for no RBS control (L4) and RBS 1 to RBS 4 with linker 1 (dark red bars) and linker 2 (dark blue bars). Predicted expression levels were calculated for all 4 RBS sequences in both linker contexts using the RBS calculator¹⁸ and these are plotted for linker 1 (light red bars) and linker 2 (light blue bars). (d) Assembly reactions were performed with single RBS linkers and also combinations of both two (RBS 1,3) and four (RBS 1–4) linkers to create a library of expression variants. Expression levels for randomly selected colonies of these assemblies are shown as a dot plot.

linker overhang sequences were designed using R2oDNA Designer software¹³ that are suitable for assembly with the 4 RBS sequences used. These two linker overhangs are orthogonal to the other linkers used in this paper and thus it was possible to generate a library of RBS sequences that can be incorporated in two different locations within a single assembly.

To evaluate the tunability of protein expression using RBS-linkers, 4-part assemblies were performed with the four different strength RBS linkers to join a constitutive promoter to a GFP ORF part without an RBS, but with a start codon adjacent to its *iP* (Figure 4b). Two sets of assemblies were performed to evaluate the degree of variation caused by the minor context change produced by changing the overhang sequence in the two sets of RBS linkers (Table S8). The four RBS sequences clearly give distinct levels of GFP expression, while there is no significant difference due to the overhang sequence context of linker 1 vs linker 2 (Figure 4c).

Additionally, we evaluated the potential to perform combinatorial library assembly by including multiple RBS linkers for a single part using a combination of RBS 1 and RBS 3 in one instance and of all four RBS linkers in the other. To evaluate the combinatorial RBS assemblies, a number of individual colonies were randomly selected from a quadrant

of the plate and grown out in culture. Comparison of expression levels for assemblies with a single RBS linker demonstrate that each colony tested exhibited a fluorescence expression within the expected range for the RBS sequences used. An even distribution of all RBS sequences included was also observed, demonstrating that there was no obvious bias between the RBS sequences chosen. All possibilities of RBS variants in the library construction were found within a relatively small number of colonies analyzed. Furthermore, none of the randomly selected colonies for either the specific or library constructions were incorrect, again demonstrating the overall accuracy of the assembly process.

The constructed sequences were computationally evaluated for predicted expression strength using the reverse mode of the RBS Calculator¹⁸ (Figure 4c; Table S15). It is interesting to note that the expected levels of expression follow the anticipated order for RBS 1–3, while RBS 4 gives significantly lower than expected output. For the different linker contexts, the computational analysis predicted a significant difference in protein output when the RBS sequences were combined with the different linker sequences. However, experimental results demonstrate that there was minimal variation in the protein output when the RBS sequences were placed in different linker

In common with other standards, adoption of BASIC can facilitate the sharing and reuse of parts and this is enhanced here by the single-tier format of our approach. It would also be possible to reuse parts designed for other type II methods like Golden Gate simply by changing the linker ligation overhang sequence. While a significant number of oligonucleotides are required for this method, their standardization and long-term viability means that economies of scale rapidly accumulate as more people within a single laboratory or group of laboratories adopt the methodology (an evaluation of cost is provided in Table S16). Furthermore, the robustness and reliability of all the steps in the BASIC protocol will facilitate translation of the workflow to an automated liquid handling platform.

METHODS

BASIC Assembly Protocol. A full protocol for laboratory use is provided as online Supporting Information.

Methods. Full details of all other materials and methods are provided in online Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Supporting figures and tables are provided to give further details on protocol development and methods. In addition a stand-alone lab protocol is also provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*E-mail: g.baldwin@imperial.ac.uk.

Author Contributions

[†]M.S. and A.C. contributed equally to this work

Notes

The authors declare no competing financial interest.

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