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Combinatorial metabolic pathway assembly approaches and toolkits for modular assembly

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ABSTRACT

Synthetic Biology is a rapidly growing interdisciplinary field that is primarily built upon foundational advances in molecular biology combined with engineering design principles such as modularity and interoperability. The field considers living systems as programmable at the genetic level and has been defined by the development of new platform technologies and methodological advances. A key concept driving the field is the Design-Build-Test-Learn cycle which provides a systematic framework for building new biological systems. One major application area for synthetic biology is biosynthetic pathway engineering that requires the modular assembly of different genetic regulatory elements and biosynthetic enzymes. In this review we provide an overview of modular DNA assembly and describe and compare the plethora of in vitro and in vivo assembly methods for combinatorial pathway engineering. Considerations for part design and methods for enzyme balancing are also presented, and we briefly discuss alternatives to intracellular pathway assembly including microbial consortia and cell-free systems for biosynthesis. Finally, we describe computational tools and automation for pathway design and assembly and argue that a deeper understanding of the many different variables of genetic design, pathway regulation and cellular metabolism will allow more predictive pathway design and engineering.

1. Introduction

Synthetic biology has the capacity to contribute towards the United Nations' Sustainable Development Goals, particularly those concerning health, nutrition, clean energy and responsible production. It allows not only improvements in the efficiency and sustainability of current production methods (Schmidt-Dannert and Lopez-Gallego, 2016) but also the development of fundamentally novel products that cannot be made with other technologies, such as advanced materials (Le Feuvre and Scrutton, 2018) and sensor-actuator therapeutic cells (Black et al., 2017).

The creation and regulation of genetic pathways for such purposes requires the assembly of DNA parts, for instance promoters, ribosome binding sites, coding sequences and terminators. In this review we focus on DNA assembly methods that can be used for combinatorial approaches, i.e. the parallel formation of many variants of a pathway to be optimised, usually where two or more elements are varied simultaneously (Jeschek et al., 2017). Combinatorial experiments facilitate the efficient optimisation of pathways in the absence of fully accurate predictive modelling in silico. For example, multiple candidate genes from diverse sources may be screened for each enzymatic function required in a metabolic pathway, and/or the expression level of each gene can be optimised by combinatorial testing of regulatory elements, including the option of dynamic optimisation by feedback control. Inappropriate expression levels can lead to the accumulation of toxic intermediates or overburdening the cell (Ghodasara and Voigt, 2017). For prokaryotes, the gene order and operon occupancy are additional variables that affect expression levels. Combinatorial DNA approaches (Section 3) have enabled the improved production of chemicals such as flavonoids (Carbonell et al., 2018), carotenoids (Naseri et al., 2019; Taylor et al., 2019) and acrylic acid (Ko et al., 2020), while information from combinatorial tuning experiments (Section 4) has aided the increased production of taxadiene (Ajikumar et al., 2010), squalene (Park et al., 2019), artemisinic acid (Fuentes et al., 2016) and 3-hydroxybutyrate (Karim et al., 2020) in various chassis.

DNA part combinations can be tested randomly by generating assemblies from large pooled libraries, or alternatively in a more controlled fashion using a Design of Experiments (DoE) approach to

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sample efficiently across the design space (see Section 6.1). The number of designs that can be characterised is likely to be limited by either the cost of DNA synthesis or the throughput of the chosen screening method; such constraints should be taken into consideration during library design. Importantly, the decreasing cost of gene synthesis is making it now more feasible to explore longer metabolic pathways or those that contain large enzymes.

In Section 5 we move to a broader definition of combinatorial pathway assembly to include cases where biosynthesis involves combinations of organisms or mixed cell extracts. These options expand the boundaries of what is possible using synthetic biology by separating incompatible or competing enzyme expression or functions and are currently areas of intensive research. As illustrated in Fig. 1, the process of metabolic pathway optimisation often involves multiple

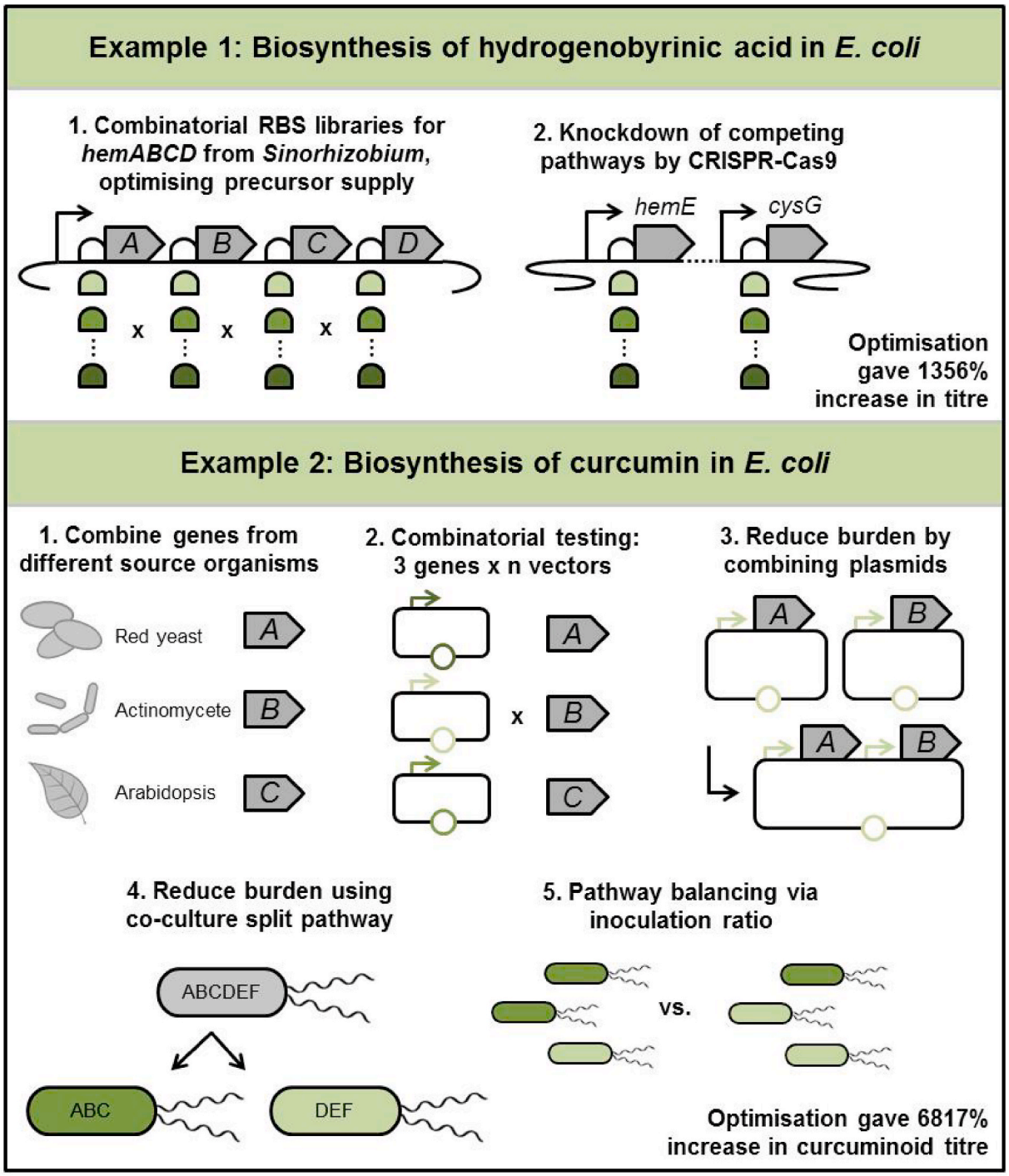

Fig. 1. Pathway assembly strategies for metabolic engineering, illustrated by the production of hydrogenobyrinic acid and curcumin in *Escherichia coli*. **Top panel**: in work by Jiang et al. (2020), classical combinatorial pathway assembly via multivariate optimisation (left) was augmented with debottlenecking approaches (right) to increase the titre of a vitamin B_{12} precursor. For step 1, different RBS libraries were used for each gene, each comprising 8 variants, and 288 clones were tested. Additional optimisation steps for *hemABCD* RBSs and promoter were also carried out (not shown). **Lower panel**: Strategies used by Rodrigues et al. (2020) to increase the titre of curcuminoids, including curcumin, in *E. coli*. A six-gene pathway using tyrosine as a substrate was assembled and optimised by several methods. Figures are presented using SBOL Visual glyphs. For simplicity, only the relevant elements are shown.

combinatorial techniques to ensure that the choice of genes, regulatory elements and chassis maximises product yield.

2. Considerations for DNA part design

2.1. Open reading frames

2.1.1. Choice of enzymes and codons

Candidate enzymes to be included in the pathway design can be identified through databases such as BRENDA (Jeske et al., 2019) or MetaCyc (Caspi et al., 2017), or through genome mining and homology searches. In some cases the best-performing combination of genes may originate from several diverse species, as demonstrated for long chain omega-3 fatty acid ('fish oil') biosynthesis in *Camelina sativa* seeds (Han et al., 2020) and tropane alkaloid biosynthesis in yeast (Srinivasan and Smolke, 2020). To accelerate pathway design, genes can be individually transcribed and translated in vitro and the resulting enzymes tested combinatorially, for example to explore natural product biosynthesis (Bogart et al., 2020) or investigate protein glycosylation pathways (Kightlinger et al., 2019).

For expression in a heterologous host, the original gene or cDNA can be cloned if donor genomic DNA is available; *E. coli* strains such as Rosetta™ enhance the translation of genes from eukaryotic sources by supplying rare tRNAs. Alternatively, a codon-optimised version can be synthesised. The codon choice influences the level of competition between protein elongation and mRNA degradation (Boël et al., 2016), in effect tuning the expression level. A simpler approach was demonstrated by Wu et al. (2019), who randomized only the initiation codons of three genes in a combinatorial fashion to improve levels of zeaxanthin production in *E. coli* ten-fold.

2.1.2. Variants and fusion proteins

Advances in both DNA synthesis and high-throughput screening (e.g. microbead Split and Mix assembly (Lindenburg et al., 2020)) enable many enzyme variants to be tested, either those with similar predicted functions from different organisms or mutant libraries that explore structural variations of a domain. The inclusion of de novo designed enzymes in pathways is also becoming a more feasible option thanks to increased understanding of protein structure-function relationships (Dawson et al., 2019). Fusion proteins with multiple or new functions can be encoded by combining open reading frames. The length and amino acid sequence of the linker affects the activity and interaction of domains, so optimisation may be needed; linker libraries and combinatorial approaches are available (Gräwe et al., 2020; G. Li et al., 2016). Individual domains of an enzyme can also be exchanged to generate novel products. For example, Bozhüyük et al. (2018) mixed and matched parts from multiple bacterial non-ribosomal peptide synthetase genes to make enzymes that produced new peptides.

2.1.3. Design features

Within an open reading frame, a plethora of design features are available to control the activity of the protein. For example, a light-oxygen-voltage (LOV) domain can be incorporated to allow precise activation using light, with steric unblocking or domain dissociation being the most common strategies (Seifert and Brakmann, 2018). In synthetic circuit design, protein degradation tags such as ssrA tags are often used to shorten response times, but care must be taken to avoid crosstalk between circuit components via competition for proteases (Butzin and Mather, 2018). An ssrA tag can also be paired with an upstream protease cleavage site in a clever scheme that uses conditional proteolysis to counteract leaky expression (Volke et al., 2020).

Spatial organisation within the cell is an important consideration for optimising the performance of metabolic pathways. Beyond the basic requirement for enzymes in eukaryotes to be directed to the appropriate compartments, often using localisation tags, the capacity to bring enzymes into close proximity increases intermediate flux and minimises crosstalk with other pathways. This can be achieved by, for example, co-localisation within an encapsulin compartment using a targeting peptide (Lau et al., 2018) or anchoring the enzymes to a spatial scaffold such as the endogenous functional membrane microdomains in bacteria, a technique used to increase GlcNAc titre in *Bacillus subtilis* as a proof of principle (Lv et al., 2020). Another option is to employ a pair of peptide tags with strong affinity to each other, such as RIDD and RIAD, to assemble key metabolic nodes (Kang et al., 2019).

Lastly, scientists in the twenty-first century are not restricted to designing enzyme pathways containing only the standard 20 amino acids. Over 200 different non-canonical amino acids have been incorporated into proteins through altering the genetic code machinery (Biava, 2020). Recent advances are reviewed elsewhere (Arranz-Gibert et al., 2019; Biava, 2020; Ros et al., 2020); key challenges include ensuring the orthogonality of the introduced aminoacyl tRNA synthetase/tRNA pair to the native system, the efficiency of incorporation, and providing a source of the amino acid either intracellularly through metabolic engineering or through uptake from the growth media. An alternative method, particularly suited to the incorporation of multiple different modifications, is to use orthogonal pairs of split inteins to insert synthetic peptides into proteins (Khoo et al., 2020). In all, these exciting advances allow new properties to be conferred on proteins to alter or enhance their function.

2.2. Non-coding regions

2.2.1. Transcriptional control

Promoter libraries allow the fine-tuning of multigene pathways in a combinatorial fashion and have been developed for many organisms commonly used for metabolic engineering. Notable recent examples include the screening of over 100 million synthetic promoters in *Saccharomyces cerevisiae*, many of which include transcription factor binding sites by chance (de Boer et al., 2020), and a large screen of promoters (randomised excluding the −35 and −10 regions) for cyanobacteria, of which a representative set of 37 were characterised further (Taylor and Heap, 2020). UP elements upstream of the −35 element in native *E. coli* promoters can also be modified to increase expression (Presnell et al., 2019). A new in vitro method employing bacterial cell lysates for transcription and RNA sequencing allows the profiling of thousands of regulatory elements in one reaction (rather than the separate wells required for fluorescence methods), showing good correlation with in vivo measurements regarding relative promoter strengths (Yim et al., 2019).

Bacterial promoters that indirectly sense electrical signals (Bhokisham et al., 2020) or small molecules (Meyer et al., 2019; Moser et al., 2018) have been developed to allow precise external control of pathways. Combinatorial logic gates can also be integrated into the design, using for example the large set of synthetic transcription factors and their cognate DNA operators developed for *E. coli* by Rondon et al. (2019); these allow robust and predictable dynamic regulation, although the more complex circuits (three or more transcription factors) were found to impact the cell growth rate (Rondon et al., 2019).

2.2.2. Translational control

For modulating prokaryotic enzyme expression at the translational level, the sequence of the ribosome binding site is critical. Predictive software such as RBS Calculator v2.1 (Espah Borujeni et al., 2014), NRP (non-repetitive parts) Calculator (Hossain et al., 2020), UTR Designer (Seo et al., 2013) or EMOPEC (Bonde et al., 2016) are used alongside screens to identify appropriate translational regulatory elements. For example, Liang et al. (2020) screened 256 RBS variants to tailor the translation rate of two transgenic enzymes for enhanced styrene production in *E. coli*. On a larger scale, Bonde et al. (2016) used Multiplex Automated Genome Engineering (MAGE) in *E. coli* to alter the Shine-Dalgarno sequence of a reporter gene to most possible combinations of six bases and assess its contribution to *E. coli* protein expression.

The resulting software, EMOPEC, allows expression levels to be tuned while minimising mRNA secondary structure changes. Accurate prediction of long-range and tertiary RNA structure, however, is still challenging (Pervouchine, 2018).

Cell-free transcription-translation reactions using crude cell lysates facilitate the rapid profiling of part collections for particular species; for example, 264 synthetic RBS variants were screened via GFP fluorescence in 2 μL reactions using a lysate of *Bacillus megaterium* (Moore et al., 2018). A similar high-throughput system has been tested for mammalian regulatory elements such as internal ribosome entry sites (IRES) although variation in performance between commercial cell lysate batches was observed (Kopniczky et al., 2020).

2.2.3. Flanking regions

A combinatorial testing approach can also include variation of the regions flanking the expression cassette(s); sequence optimisation upstream of the promoter was recently demonstrated in yeast using a randomised 30 bp library and overlap extension PCR (Lopez et al., 2020). The authors hypothesized that the effect was due to altered transcription factor binding and/or nucleosome occupancy. Regarding *E. coli*, Carr et al. (2017) carried out library screening to identify effective insulator-promoter pairs, whilst Zong et al. (2017) insulated core promoters and operators to prevent interactions, making the system more predictable and amenable to modelling.

2.3. DNA parts to aid screening and analysis

To test the function of regulatory elements such as promoters and RBSs, fluorescent reporter proteins are frequently used, often in conjunction with plate readers or flow cytometry. Issues with this method include protein stability, oxygen dependency and lack of standardisation (Decoene et al., 2018); fluorescence measurements should be quantified using an independent calibrant (Beal et al., 2018) to avoid collection of data in arbitrary units which precludes comparisons between datasets. The levels of specific mRNAs can be measured using fluorescence-enhancing RNA aptamers (summarised by Hennig and Neubacher, 2019) so that fluorescent proteins no longer have to be used as a proxy in transcriptional studies. Screening and analysis methods do not necessarily require the inclusion of special DNA sequences; for example, population or single-cell RNA sequencing can provide insights into regulatory network behaviour and help to check and debug genetic circuits (Appleton et al., 2017a; Gorochowski et al., 2017).

2.4. Vectors

2.4.1. Vector backbones

Many combinatorial pathway assembly methods rely on the use of *E. coli* or yeast cloning vectors as these invariably constitute the workhorses used by researchers; see Nora et al. (2019) for an overview. These vectors are available with various selection markers and origins of replication, with the latter determining plasmid copy number and host range. The choice of elements has a marked effect on the performance of bacterial expression vectors: SEVA Linkers (a nicking-enzyme based technology for backbone exchange) was used to test all combinations of six selection markers and five origins of replication, demonstrating a ten-fold improvement in beta-carotene yield from a four-gene pathway (Kim et al., 2016).

2.4.2. Rational rebuilding

Several groups have reported recent improvements to popular vectors. Shilling et al. (2020) modified the T7 promoter and translation initiation region of pET28a to increase protein expression; these changes are also applicable to some other pET vectors. Meanwhile, Staal et al. (2019) created a 1 kb minimal bacterial cloning plasmid (pICOz) by deleting regions of pUC18. This resulted in increased yields of the high copy plasmid from *E. coli* culture in terms of both moles and DNA mass. The small size would be advantageous for assembly techniques that involve PCR amplification of the backbone. These examples illustrate that a rational rebuilding of genetic element libraries, including traditional vector backbones, may enable a more rigorous approach to combinatorial assembly than relying on parts that are widely used for historic reasons.

2.5. Chassis choice and part registries

2.5.1. Host cells, toolkits and registries

The choice of host species for bioproduction depends on factors such as underlying metabolism, the ease and speed of culture and genetic engineering, secretion pathways, the need for post-translational modification, biosafety, and cost. These are reviewed elsewhere (Calero and Nikel, 2019; Kelwick et al., 2014). DNA assembly toolkits and part collections are available for an increasing number of metabolic engineering chassis organisms, as detailed in Table 1. Many of these have been made available to purchase from Addgene (www.addgene.org) or can be ordered from the researchers. Additional registries focus on parts developed following particular assembly standards. For example, the Registry of Standard Biological Parts (http://parts.igem.org) currently uses BioBrick RFC[10] and Type IIS as its de facto standards and catalogues over 20,000 parts, including collections for *E. coli*, *Bacillus subtilis* and plants. The database for Standard European Vector Architecture 3.0 (http://seva.cnb.csic.es/) lists prokaryotic plasmid vectors and cargo modules adhering to the SEVA assembly standards.

2.5.2. Photosynthetic hosts

Of particular interest from a sustainability perspective are photosynthetic chassis organisms, which use (sun)light and CO_2 as their energy and carbon sources. For example, cyanobacteria have been engineered to produce ethanol, isobutanol, isoprenoids, biohydrogen, sugars, glycerol and natural products (Knoot et al., 2018), although low yields currently hinder industrial production. The CyanoGate toolkit for modular cloning will assist further development in this field and includes 6 acceptor plasmids, 11 CDS parts including *dCas9*, 10 homology regions for insertion sites in *Synechocystis* or *Synechococcus*, 12 native promoters, 33 heterologous promoters and 21 terminators (Vasudevan et al., 2019). The development of this resource should also facilitate sharing and experimental standardization between laboratories. Since chloroplasts are of prokaryotic origin, the toolkit could be adapted for photosynthetic eukaryotes such as microalgae and plants.

2.5.3. New and fast-growing hosts

The bacterium *Vibrio natriegens* is another potential chassis for metabolic engineering, with its main appeals being extremely fast growth and a non-pathogenic nature. Tschirhart et al. (2019) found this organism to have a doubling time of 12 min in rich medium even with an increased metabolic load; the genetic basis of this rapid growth was recently analysed using CRISPRi (interference via Clustered Regularly Interspaced Short Palindromic Repeats) (Lee et al., 2019). Heterologous production of 2,3-butanediol, a precursor for synthetic rubber production, has been achieved as a demonstration of the metabolic potential and genetic tractability of this organism (Erian et al., 2020). It has been shown that *V. natriegens* is compatible with many commonly-used bacterial plasmids and other genetic elements (Table 1) and amenable to chemical and optogenetic induction pathways (Tschirhart et al., 2019). Although *E. coli* is still the most popular cloning host for plasmid assembly before transfer to other organisms, *V. natriegens* is a strong future competitor for this purpose due to its accelerated workflows.

2.6. Enhancing stability and minimising burden

2.6.1. Designing enhanced pathway stability

Maintaining the stability of optimised strains and pathways during

Table 1
Toolkits and part collections for a variety of chassis organisms.

Group	Organism	Resource name	Details	References
Eubacteria	*Acinetobacter baylyi*	n/a	Integrative vectors, promoters, RBSs. CRISPR/Cas9-based markerless insertion.	Biggs et al., 2020
	Bacillus (1)	Bacillus BioBrick Box 2.0	BioBrick parts: integrative and replicative vectors, selection markers, fluorescent proteins, promoters.	Popp et al., 2017 Parts at http://bgsc.org
	Bacillus (2)	n/a	Various part collections: native and synthetic promoters, RBSs, UP elements, proteolysis tags.	Reviewed by(Liu et al., 2019)
	Chromobacterium violaceum	n/a	Replicative vectors, promoters, selection markers, CRISPRi. Gibson Assembly used.	Liow et al., 2020
	E. coli (1)	CIDAR MoClo	MoClo parts (for CIDAR protocol): promoters, terminators, RBSs, fluorescent proteins.	Iverson et al., 2016. Available from Addgene
	E. coli (2)	CRIMoClo: Conditional-Replication, Integration and Modular MoClo	MoClo integrative vectors, targeting four chromosomal sites.	Vecchione and Fritz, 2019
	E. coli (3)	EcoFlex	MoClo parts: promoters, terminators, RBSs, purification tags, fluorescent proteins. 68-part plasmid assembled.	(Lai et al., 2018; Moore et al., 2016). Available from Addgene
	Halomonas spp.	n/a	Vectors, promoters, selection markers, GFP.	(X. Chen et al., 2018; Shen et al., 2018)
	Mycobacterium bovis BCG	n/a	BioBrick parts: vectors, promoters, selection markers, GFP.	Oliveira et al., 2019
	Pseudomonas putida	n/a	Integrative vectors, promoters, UP elements, RBSs.	Elmore et al., 2017
	Rhodococcus opacus	n/a	Replicative and integrative vectors, promoters, selection markers, CRISPRi.	DeLorenzo et al., 2018
	Shewanella oneidensis	n/a	BioBrick parts: conjugation vectors, promoters, selection markers, fluorescent proteins.	Cao et al., 2019
	Vibrio natriegens	n/a	Vectors, promoters, selection markers, terminators, RBSs, fluorescent proteins, degradation tags.	Tschirhart et al., 2019
	Cyanobacteria: *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus*	CyanoGate	96 MoClo parts: integrative and replicative vectors, promoters, selection markers, terminators, CRISPRi. Could be adapted for eukaryotic organelles e.g. chloroplasts.	(Vasudevan et al., 2019). Available from Addgene
	Gram negative (1)	BEVA: Bacterial Expression Vector Archive	Broad host range. Golden Gate/SEVA vectors for conjugation from *E. coli* to other bacteria.	Geddes et al., 2019
	Gram negative (2)	SEVA: Standard European Vector Architecture	Vectors with varied replication origin, selection marker and cargo in standard format.	(Martínez-García et al., 2020). Available at http://seva.cnb.csic.es/
Fungi	Filamentous fungi	FungalBraid	GoldenBraid parts: promoters, selection markers, fluorescent protein. Demonstrated in *Penicillium digitatum.*	Hernanz-Koers et al., 2018
	Issatchenkia orientalis	n/a	DNA Assembler used in this yeast. Characterised promoters and terminators.	Cao et al., 2020
	Kluyveromyces marxianus	n/a	MoClo parts: vectors, promoters, terminators, selection markers.	Rajkumar et al., 2019
	Pichia pastoris	Golden*Pi*CS	Golden Gate parts: integrative and replicative vectors, promoters, terminators, selection markers. Up to 8 transcription units per plasmid.	(Prielhofer et al., 2017). Available from Addgene
	Saccharomyces cerevisiae (1)	COMPASS: Combinatorial Pathway Assembly	High-throughput scheme for balancing expression of heterologous pathways in yeast. Employs homology arms for assembly, with 3 successive cloning levels. Uses a library of orthogonal, plant-derived artificial transcription factors.	(Naseri et al., 2019). Available from Addgene
	Saccharomyces cerevisiae (2)	COSPLAY: Combinatorial Swift Plasmid Assembly in Yeast	Golden Gate parts: integrative and replicative vectors, promoters, degrons, fluorescent proteins. Recapitulates features of pRSII vectors in Golden Gate format.	(Goulev et al., 2019). Available from Addgene
	Saccharomyces cerevisiae (3)	EasyClone-MarkerFree	BioBrick/USER based. Allows simultaneous insertion of 6 genes (2 genes into 3 genomic locations) using markerless CRISPR/Cas9.	(Jessop-Fabre et al., 2016). Available from Addgene
	Saccharomyces cerevisiae (4)	Yeast Toolkit	MoClo parts and assembly standard: integrative and replicative vectors, promoters, terminators, degradation tags. BioBrick-compatible.	(Lee et al., 2015; Rajakumar et al., 2019)
	Schizosaccharomyces pombe	n/a	Golden Gate parts: integrative vectors, promoters, terminators, selection markers, fluorescent proteins.	(Kakui et al., 2015)
	Yarrowia lipolytica (1)	YaliBricks	BioBrick parts: replicative vectors, promoters, introns.	(Wong et al., 2017). Available from Addgene
	Yarrowia lipolytica (2)	n/a	Golden Gate parts: replicative vectors, promoters, selection markers, fluorescent proteins.	(Celińska et al., 2017; Larroude et al., 2019). Available from Addgene
Microalgae	*Chlamydomonas reinhardtii*	Chlamydomonas MoClo Toolkit	119 MoClo parts for nuclear genome including riboswitch, 2A peptide and micro RNA backbones.	(Crozet et al., 2018) Available at AddGene or https://www.chlamycollection.org/products/moclo-toolkit/
	Nannochloropsis oceanica	n/a	Gateway vectors, bidirectional promoters, selection markers, GFP, luciferases.	(Poliner et al., 2020)
Plants	Chloroplasts	MoChlo	128 Golden Gate parts: integrative vectors for tobacco, maize and potato, promoters, UTRs, selection markers.	(Occhialini et al., 2019). Available at Addgene.
	Nucleus (1)	MoClo	A Golden Gate framework. Up to 6 transcription units in Level 2 assembly, which can be combined further. Originally for plants; standard now expanded to other organisms.	(Weber et al., 2011; Werner et al., 2012). Empty vector kit and plant parts kit available at Addgene.
	Nucleus (2)	GoldenBraid 4.0	A Golden Gate framework. Large parts collection with associated experimental data.	

(*continued on next page*)

Table 1 (*continued*)

Group	Organism	Resource name	Details	References
				(Sarrion-Perdigones et al., 2013; Vazquez-Vilar et al., 2017). Available at Addgene. Data-only at https://gbcloning.upv.es
	Nucleus (3)	n/a	Collection of 80 additional MoClo parts, mostly also compatible with GoldenBraid.	(Gantner et al., 2018). Available at Addgene.
	Nucleus (4)	n/a	Library of TF-binding cis-elements, minimal promoters and TF fusion proteins using elements from yeast regulatory systems. Used Golden Gate and jStack. Validated in *Nicotiana* and *Arabidopsis*.	(Belcher et al., 2020)
Mammals	Mammalian cells (1)	COMET: Composable Mammalian Elements of Transcription	Comprises 44 activating and 12 inhibitory zinc-finger TFs and 83 cognate promoters.	(Donahue et al., 2020)
	Mammalian cells (2)	EMMA: Extensible Mammalian Modular Assembly	A Golden Gate framework with 25 functional categories. Up to 3 transcription units but 5 proteins.	(Jones et al., 2019; Martella et al., 2017). Available at Addgene.
	Mammalian cells (3)	MoPET: Modular Protein Expression Toolbox	A Golden Gate framework with 53 defined DNA modules across 8 functional categories. Assemble up to 8 parts in one step (promoters, tags, linkers etc).	(Weber et al., 2017)
	Mammalian cells (4)	MTK: Mammalian ToolKit	Golden Gate. Over 300 parts including promoters, 3′UTRs, fluorescent proteins, insulator and P2A elements. Build up to 9 transcription units.	(Fonseca et al., 2019)
Cross-kingdom	n/a	MK: Multi Kingdom Golden Gate	A Golden Gate framework designed to allow part use across species, e.g. bacteria, fungi, plants, protista, frog oocytes, human cells.	(Chiasson et al., 2019). Available at Addgene.
	n/a	uLoop vector kits	Enables use of Universal Loop Assembly in many chassis including bacteria, yeast, plants and diatoms.	(Pollak et al., 2020). Available at Addgene.

large scale production can be a challenge, but the risk can be mitigated by certain design considerations at the outset. Using the example of mevalonic acid production in *E. coli*, Rugbjerg et al. (2018) used deep-sequencing to examine how a pathway mutates away from chemical production to regain fitness, finding that insertion sequences were the predominant mechanism. The authors demonstrated that this can be selected against by moving an essential gene to the end of the biosynthesis operon; in addition, homopolymeric tracts should be avoided within open reading frames as these can easily mutate and put the gene out of frame (Rugbjerg et al., 2018). Repeated elements, e.g. promoters, operators or terminators, can lead to deletion of the intervening sequence over time (Sleight et al., 2010) and in plants leads to gene silencing (Belcher et al., 2020), so access to a library of diverse parts is advantageous for combinatorial pathway assembly even if the genes require similar promoter strengths.

2.6.2. Improved chassis strains

Increasingly sophisticated genetic engineering tools are making it easier to modify host organisms to further enhance stability, generating strains that are more suitable as industrial chassis. In an *E. coli* BL21 (DE3) background, prophages were eliminated using genome shuffling and all remaining insertion sequences were inactivated by stop codon insertion with CRISPR/Cas-assisted MAGE (Umenhoffer et al., 2017). It was subsequently found that a similar outcome could be obtained in just one transformation step by employing a single CRISPRi plasmid to inhibit the transposition of insertion elements (Nyerges et al., 2019). This efficiently suppressed insertional mutagenesis in chromosomal and plasmid genes and could be adapted for other species in the future. Meanwhile, in the ambitious *S. cerevisiae* Sc2.0 project, every chromosome is gradually being replaced with a synthetic version in which (among other modifications) all mobile elements are designed out, potentially increasing genetic stability (Richardson et al., 2017).

2.6.3. Controlling host cell burden

Chromosomal integration of transgenes removes the burden of plasmid replication, allows more precise control over gene copy number and does not require constant antibiotic selection for stability (Jessop-Fabre et al., 2016; Vecchione and Fritz, 2019). For many host organisms both integrative and replicative plasmids are available (Table 1). Regardless of plasmid or chromosomal location or growth conditions, incoherent feedforward loops can be used to maintain constant gene expression in bacteria (Segall-Shapiro et al., 2018). The translational burden of a heterologous pathway should also be considered. The resource consumption of different designs can be modelled in silico using information from in vitro protein expression using cell-free lysates (Borkowski et al., 2018). In addition, as demonstrated using a GFP-based capacity monitor in *E. coli*, designs with similar expression outputs can inflict different levels of burden on the cell (Ceroni et al., 2015), endorsing the assessment of multiple pathway designs.

3. DNA assembly methods

Researchers have developed a huge variety of methods for assembling DNA fragments in a defined order (Table 2). These largely depend on enzymes such as restriction endonucleases, exonucleases, DNA polymerases, DNA ligase and/or recombinases (Fig. 2), with a varying degree of assistance in vivo from the cloning host organism. Aspects to consider when selecting an assembly method include the simplicity of design and laboratory steps, number of parts that can be assembled at once, cost of reagents, amount of DNA synthesis required, ratio of correct to incorrect clones, presence of scars, sequence limitations, scalability and suitability for automation.

The most popular techniques and standards for combinatorial assembly have been tabulated by Naseri et al. (2020) and described by Wiltschi et al. (2020), alongside schematic workflows of the processes required. However, all of the methods that we present in Table 2 are

Table 2
DNA assembly methods, with a focus on techniques enabling multi-insert assembly.

Category	Assembly method	Reference	Key features and capabilities
Overlapping oligonucleotides	RapGene	Zampini et al., 2015	Insert assembled from overlapping 60 nt oligos between 20-22 nt vector overhangs, displacing counter-selectable marker. Amplified in *E. coli*.
Type IIP RE based	Conventional restriction-ligation cloning	Cohen et al., 1973	No standardisation. Separate digestion and ligation steps required. Relies on presence of suitable RE sites or addition by PCR.
	3A: Three Antibiotic Assembly	Shetty et al., 2011	BioBrick improvement. Uses negative and positive selection to reduce the number of possible incorrect assembly products. No digest purification required. Vector background eliminated with *ccdB* insert. Two parts are assembled into vector.
	BglBricks	Anderson et al., 2010	Uses BioBrick principle but allows protein fusions through a glycine-serine 6 bp linker.
	BioBrick™	Knight, 2003	Two parts assembled into a destination vector using Type IIP REs, leaving 8 bp scar. Iterative process to add further parts.
	ePathOptimize	Jones et al., 2015	Uses isocaudomer REs (matching overhangs) to allow sequential assembly of 5-gene pathway with randomised 5-promoter library for combinatorial screening.
	iBrick	Liu et al., 2014	BioBrick principle but uses two homing endonucleases, I-SceI and PI-PspI, with rare >18 bp recognition sites. This avoids need for part domestication but creates 21 bp scar (7 amino acids if in CDS).
	IRDL: Improved Restriction Digestion-Ligation	Wang et al., 2014	Restriction and ligation in single 5–30 min 37 °C step; vector background eliminated with *ccdB* insert. RE inactivation is not necessary. 3-part assembly demonstrated.
	QGA: Quick Gene Assembly	Yamazaki et al., 2017	In vitro assembly of multiple Biobricks then ligation into vector. Uses magnetic beads conjugated to oligo-dT to purify DNA containing oligo-dA at each step. PCR and gel purification required; 6 bp scar between BioBricks. Tandem repeats and short DNA fragments are permissible. Requires only 4 universal primers for any assembly.
Type IIS RE based	3G Assembly: Golden Gate-Gibson	Halleran et al., 2018	Single-day assembly of multigene constructs. Transcription units flanked by unique nucleotide sequences (UNS) are constructed by Golden Gate then amplified and linked by Gibson Assembly. 6-gene assembly demonstrated; suitable for libraries.
	BASIC: Biopart Assembly Standard for Idempotent Cloning	(Storch et al, 2015, 2017)	Hierarchical cloning standard with single tier format enabled by methylase protection of RE sites. PCR-free; 1 forbidden RE site. BsaI-cut parts are ligated to oligo linkers, which determine assembly order. 21 bp overlaps, 6 bp scars facilitating fusion proteins. Allows same part to be used in multiple locations. 7-part assembly demonstrated.
	Body Double cloning	Tóth et al., 2014	Type IIS REs allow use of classic Type IIP RE cloning without prior part domestication.
	FASTR: Fully Automatic Single-Tube Recombination	Kotera and Nagai, 2008	Crude PCR products used directly in Type IIS RE/ligation one-pot reaction: aphidicolin inhibits polymerase and DpnI digests template DNA. *E. coli* transformation. Suitable for high throughput. Limitations: parts are not sequence-verified before assembly. Primer dimers may form unwanted parts.
	Golden Gate	(Engler et al., 2008; Engler and Marillonnet, 2014)	Popular method for hierarchical assembly with toolkits available for many organisms. Parts are stored in entry vectors then combined in hierarchical multipart assemblies, with order defined by short overhangs. Quick restriction –ligation method but extensive part domestication required.
	Loop Assembly	Pollak et al., 2019	Type IIS assembly alternating between 2 types of vector, reducing size of vector set required and allowing flexibility between levels. Use with LOOPDESIGNER software. Assembly of 16 genes (56 parts) and 38 kb demonstrated. Compatible with long-overlap assembly techniques.
	MASTER Ligation: Methylation-Assisted Tailorable Ends Rational	Chen et al., 2013	Similar to Golden Gate but avoids part domestication: MspJI cuts at methylated sites added to fragment ends but not internal (unmethylated) sites. MspJI can remove restriction scars. Methylated primers can be expensive.
	MetClo: Methylase-Assisted Hierarchical DNA Assembly	Lin et al., 2018	Golden Gate Assembly framework. Uses only one RE for hierarchical assembly, making use of methylase-protected and unprotected sites. Less part domestication required. Uses a methylase-expressing *E. coli* strain.
	MIDAS: Modular Idempotent DNA Assembly System	Van Dolleweerd et al., 2018	Allows nesting between existing parts. Allows genes in either orientation. Uses *pheS* negative marker in Level 2 vectors – unlike *ccdB*, this does not require special *E. coli* strain. Assembled 7 TUs one at a time into final vector. Pathway was then used in *Penicillium paxilli*.
	Mobius Assembly	Andreou and Nakayama, 2018	Golden Gate Assembly framework. Uses a low frequency cutter and chromogenic proteins for easy colony scoring. Validated with 16-gene construct.
	OLMA: Oligo-Linker Mediated Assembly	Zhang et al., 2015	Golden Gate reactions where short parts (promoters, RBS) are generated by staggered oligo annealing, with ends defining assembly order. 7-part assembly (3 genes) demonstrated for small combinatorial library.
	pHD: pHeaven's Door vectors	Schefer et al., 2014	For production of recombinant proteins in mammalian cells starting from the cDNA within 3 days. Golden Gate style one pot reaction; single insert demonstrated but more are possible; vector background eliminated with *ccdB* insert; bulk plasmid prep from transformation mix was reliable enough to use directly in transfection.
	PODAC: Protected Oligonucleotide Duplex Assisted Cloning	Van Hove et al., 2017	Uses single Type IIS RE (BsaI) for iterative assembly. New BsaI sites introduced for next round of assembly, protected by methylation. No vector

(*continued on next page*)

Table 2 (*continued*)

Category	Assembly method	Reference	Key features and capabilities
			levels required. 5-insert sequential assembly demonstrated but multiple inserts possible.
	PS-Brick	(S. Liu et al., 2019)	Seamless and iterative method using both Type IIP and IIS REs. BioBrick based. Uses T/A or blunt ends to avoid scars. Requires part domestication and separate restriction/ligation reactions.
	Scarless Stitching	Smanski et al., 2014	Scarless and modular. Bridging fragment is removed with MlyI digestion to give precise fusion between two parts only.
	Start-Stop Assembly	Taylor et al., 2019	Scarless and modular. 3 nt overhangs correspond to start and stop codons. Internal sites for 3 REs are forbidden. Only one destination vector needed. Demonstrated in *E. coli*.
	TNT: Three Nucleotides Cloning	De Paoli et al., 2016	Uses Type IIS REs with 3 nt overhang, useful for in-frame fusion. Requires *E. coli* cell line expressing M.TaqI methyltransferase. Part domestication can be avoided by a triplex oligo step, masking certain RE sites.
	Universal (u) Loop Assembly	Pollak et al. (2020)	Loop Assembly adapted for cross-kingdom use.
	VEGAS: Versatile Genetic Assembly System	(Chuang et al., 2018; Mitchell et al., 2015)	Transcription units are assembled by yeast Golden Gate, including adapters added for later [episomal] pathway assembly by homologous recombination in *S. cerevisiae*. 6-gene assembly and combinatorial use demonstrated.
Nicking endonuclease based	NE-LIC: Nicking Endonuclease Ligation-Independent Cloning	Wang et al., 2013	Uses nicking enzymes Nb.BbvCI, Nt.BspQI or Nb.BtsI for multiple fragment assembly. Produces controlled ssDNA overhangs containing scar and any overlap. Avoid internal sites in the parts. Recommended overlap 5–10 bp with T4 DNA ligase or 10–15 bp without.
Homology based	AFEAP: Assembly of Fragment Ends After PCR	Zeng et al., 2017	Two rounds of PCR generate 5′ overhangs. 5–8 nt is optimal. Ligase treatment then *E. coli* transformation. Shown for 200 kb assembly and up to 13 fragments.
	AQUA: Advanced Quick Assembly	Beyer et al., 2015	Enzyme-free after PCR step. Gel-purified fragments with >15 bp homology ends are mixed; *E. coli* transformation is sufficient to recombine them. Expression strains can be used directly. 6-part assembly demonstrated.
	Chew-back assembly	(Schmid-Burgk et al, 2012, 2014)	Steps: PCR with 45 nt primers, gel purification, chew-back, *E. coli* transformation. Precise chew-back by T4 DNA polymerase by inclusion of mononucleotide. 20 bp overlap designs are restricted to other 3 bases. 10 parts assembled in 2 steps.
	CLIVA: Cross-Lapping In Vitro Assembly	Zou et al., 2013	Enzyme-free after PCR step; scarless. PCR products with 38 bp overlaps are made using phosphorothioate primers, allowing single-stranded ends. Mixed and transformed into *E. coli*. 6-fragment 21 kb plasmid assembled.
	CPEC: Circular Polymerase Extension Cloning	(Quan and Tian, 2009, 2011)	Gel-purified parts with 15–25 bp homology ends are hybridised then extended with DNA polymerase. *E. coli* transformation. No sequence restrictions or scars. 5-part assembly demonstrated.
	DATEL: DNA Assembly with Thermostable Exonuclease and Ligase; sDATEL (simplified)	(Ding et al., 2017; Jin et al., 2016)	Assembled up to 10 (DATEL) or 4 (sDATEL) fragments. Parts with 30 bp overlap are hybridised, overhangs removed with Taq DNA polymerase and sealed with Taq DNA ligase.
	Gibson Assembly (Isothermal Assembly)	Gibson et al., 2009	Uses T5 exonuclease, DNA polymerase and Taq DNA ligase to assemble fragments with >15 bp homology ends in vitro in 15 min single step. 583 kb scarless assembly demonstrated. Uncontrolled exonuclease can be problematic for small parts; long primers can be costly.
	GTS: Guanine/Thymine Standard	Ma et al., 2019	Assembly standard with 1 bp scars (scarless around CDS). Parts with 1 nt overhangs (e.g. using phosphorothioate primers) are barcoded at both ends using stem-loop oligos, which create 15–20 bp overlaps. Allows combinatorial use of Gibson, CLIVA etc.
	Hot fusion	Fu et al., 2014	Similar to Gibson Assembly but no DNA ligase. Assembled up to 7 fragments. Parts with 17–30 bp overlaps are assembled using T5 exonuclease and Phusion polymerase. Nicks sealed by *E. coli* transformation. Blue-white screen allowed use of unpurified vector digest.
	LCR: Ligase Cycling Reaction	de Kok et al., 2014	Scarless method using bridging oligos and Ampligase thermostable DNA ligase to assemble up to 20 parts in one step. Parts can be reused easily between assemblies by utilizing different bridging oligos.
	MODAL: Modular Overlap-Directed Assembly With Linkers	Casini et al., 2014	Combines Gibson-style overlap method with part reusability for combinatorial design. Uses 15 bp adaptor sequences (75 bp synthetic DNA between parts), so best suited to combining whole gene cassettes. Use with R2oDNA Designer software. Compatible with *E. coli* and yeast assembly.
	M-PERL: Multigene Pathway Engineering with Regulatory Linkers	Liu et al., 2016	Uses OE-PCR to assemble transcription units, then in vivo recombination in yeast using overlapping linkers of 120–400 bp. To test modifications to TSS (transcriptional start sites) for yeast. Assembly of 8 genes demonstrated.
	Nimble Cloning	Yan et al., 2020	Standardisation of Gibson Assembly. Entry vector/PCR product is assembled directly with circular destination vector using SfiI (rare cutter) and T5 exonuclease. Assembly with 3 inserts demonstrated.
	One-Step SLIC: Sequence- and Ligation-Independent Cloning	Jeong et al., 2012	Fragments with 15 bp homology ends are treated with T4 DNA polymerase for 2.5 min to generate overhangs, annealed on ice and gap-repaired in *E.coli*. 4-part assembly demonstrated. Based on SLIC (Li and Elledge, 2012).
	Optimised USER: Uracil Excision Cloning	Cavaleiro et al., 2015	PCR with uracil-containing primers followed by uracil DNA glycosylase treatment creates overhangs for annealing. Overhangs and reaction conditions optimised for efficiency and accuracy. Scarless assembly of 6 parts demonstrated.
	PaperClip	(Trubitsyna et al, 2014, 2017)	

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Table 2 (*continued*)

Category	Assembly method	Reference	Key features and capabilities
			'Clips' (phosphorylated, annealed and ligated oligos) determine assembly order; a set of 4 oligos is required per part and allows assembly in any order from any type of part library. 8-part assembly demonstrated. GCC scars.
	PIPE: Polymerase Incomplete Primer Extension	(Klock et al., 2008; Klock and Lesley, 2009)	No enzymes required after PCR step. Uses any 15 bp overlap. Direct *E. coli* transformation of unpurified PCR mix, taking advantage of incomplete extension products. Best suited to parallel assembly of single-insert plasmids.
	PLICing: Phosphorothioate-based Ligase-Independent Cloning	Blanusa et al., 2010	Homology ends added by PCR using phosphorothioate primers. Products used directly in 10 min iodine cleavage/hybridisation. Scarless and fast but primers expensive. Demonstrated for single insert but multiple possible.
	PTO (Phosphorothioate) QuickStep	Jajesniak et al., 2019	Seamless cloning into any plasmid position; suitable for one insert or PCR mutagenesis library with no pre-cloning. Uses Q5 DNA polymerase, PTO primers, iodine and DpnI. Could be adapted for multiple inserts using overlapping PCR.
	SLiCE: Seamless ligation cloning extract	(Motohashi, 2015; Okegawa and Motohashi, 2015)	Scarless assembly of 2–3 parts with 15–19 bp homology ends. Uses the recombination activity of *E. coli* cell lysates in vitro. Lysate is cost-effective but long primers are needed.
	TEDA: T5 Exonuclease-Dependent Assembly	Xia et al., 2019	Similar to Gibson Assembly but uses T5 Exonuclease alone. Completed by *E. coli* transformation. Purified linear vector and inserts; assembled 2–4 fragments using 9–20 bp homology.
	TPA: Twin-Primer Non-Enzymatic DNA Assembly	Liang et al., 2017	Two PCRs per fragment, annealed to give overhangs. Parts with 20 bp overlap are hybridised. Completed by *E. coli* transformation. 10 fragments assembled.
	UNS-guided isothermal assembly (Unique Nucleotide Sequence)	Torella et al., 2014	Enables reuse of elements such as promoters and terminators. Demonstrated in *E. coli* and embryonic stem cells.
	Zebrα: Zero-Background Redα	Richter et al., 2019	Insert replaces counter-selectable marker in vector. Uses simplified SLiCE lysate for in vitro recombination.
CRISPR	C-Brick	(S. Y. Li et al., 2016)	BioBrick principle but uses Cpf1 & crRNAs (CRISPR) instead of REs: long recognition sites so part domestication not necessary. 6 bp (Gly-Ser) scar. 2 inserts per round. Gel purification required.
	CRISPR-CLONInG: CRISPR-Cutting and Ligation Of Nucleic acid In vitro via Gibson	Shola et al., 2020	CRISPR-Cas9 is used to precisely remove unwanted element from vector. Gibson Assembly then inserts new PCR-amplified element(s) with homology ends. Useful for vectors where convenient restriction sites are unavailable and PCR amplification would be problematic (e.g. large or repetitive sequence).

capable of assembling at least two parts into a vector backbone, which means that they can be used for at least basic combinatorial approaches if the two parts are libraries. To diversify parts for this library creation, the techniques available depend partly on the assembly method. Random mutagenesis can be carried out by error-prone PCR using $MnCl_2$ and/or imbalanced dNTPs, a method first developed several decades ago (Leung et al., 1989) but that continues to be useful for directed evolution experiments today (Jajesniak et al., 2019; Reed et al., 2019). Alternatively, degenerate oligonucleotides can be used in PCR or overlapping assemblies, or part libraries can be ordered. These methods apply to any part where variants are to be tested in a combinatorial fashion, including coding regions, regulatory elements or insulators.

In parallel to the introduction and tuning of heterologous genes, pathway engineering often involves manipulating host metabolism by deleting or altering the expression of endogenous genes. Current methodology for genome engineering is reviewed by Ren et al. (2020).

3.1. DNA synthesis and overlapping oligonucleotide assembly

When the part libraries to be assembled are not already available or easily generated using the methods above, de novo DNA synthesis is generally the first step. This is performed by combining phosphoramidite-synthesised overlapping oligonucleotides to create parts, either commercially or in-house (Hughes and Ellington, 2017). The accuracy and cost of DNA synthesis are key determinants of the amount of design space that can be explored. Recent advances in fidelity include a MutS error correction enzyme with improved stability (Zhang et al., 2020). Miniaturization and new technologies have reduced both the cost and environmental impact of DNA synthesis; 9600 genes can now be produced on a single silicon chip (www.twistbioscience.com). A new method for enzymatic rather than chemical synthesis of DNA is in development (Barthel et al., 2020; Palluk et al., 2018; Perkel, 2019) and is the basis of a benchtop DNA printer in development by DNA Script (www.dnascript.com).

3.1.1. In vitro assembly

DNA can be assembled from overlapping oligonucleotides in vitro, including with small-scale high-throughput methods such as microfluidics (Kong et al., 2007; Plesa et al., 2018) or microchips (Quan et al., 2011). Megacloning using error-free, microarray-derived oligonucleotides and a high tiling depth was used to produce 72 *cas9* genes from different species (Cho et al., 2018); this technique could be used to efficiently generate a library of gene variants to test in metabolic pathways after in silico genome mining. Generation by overlapping oligonucleotides can be problematic where the sequence contains repeats. Cooper and Hasty (2020) overcame this issue when making multiplex CRISPR arrays by a careful tiling design that avoided annealing and ligation within repeats and allowed single stranded gaps that were filled in by PCR.

3.1.2. In vivo assembly

It has been demonstrated that oligonucleotides can also be assembled into a vector in vivo using *S. cerevisiae* (Gibson, 2009) or *E. coli* (Zampini et al., 2015). The latter method, RapGene, requires a preannealing step in vitro but is faster overall due to the shorter doubling time of *E. coli*. In addition to the overlapping oligonucleotides, the *E. coli* method requires the use of type IIP restriction enzymes and the exonuclease activity of T4 DNA polymerase. These oligonucleotide-based assembly methods are most often used to generate single DNA sequence designs, but could be adapted for combinatorial assembly if the central (non-overlapping) portion of certain oligonucleotides was varied in a systematic way.

3.2. Restriction enzyme methods

Restriction-ligation techniques were established in the 1970s and have been the mainstay of cloning ever since. The first standardised assembly method was NOMAD (Rebatchouk et al., 1996), which used standard prefix and suffix restriction sites and allowed one insert per round of restriction and ligation. This was followed by the BioBricks

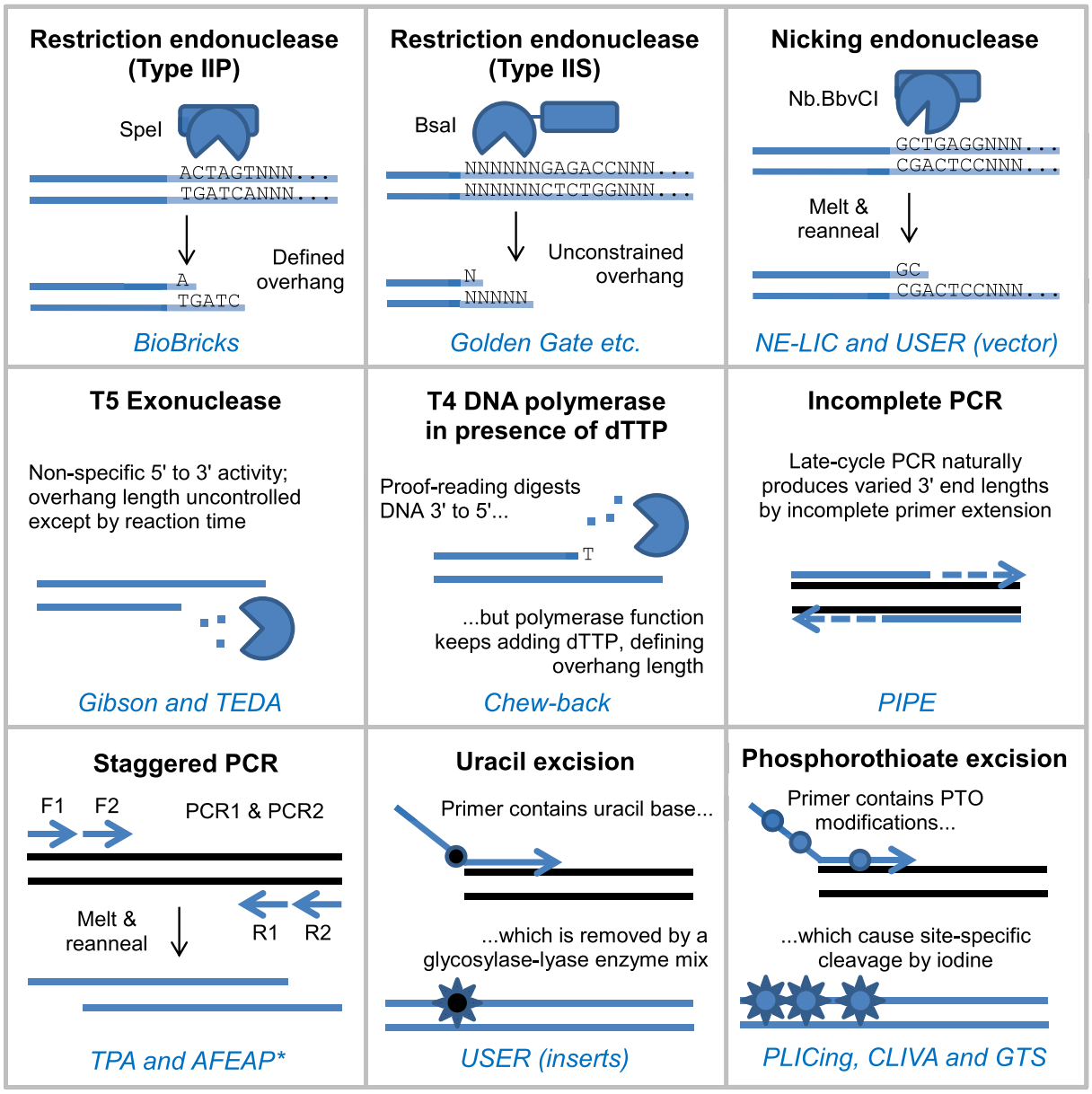

Fig. 2. Techniques for creating single strand DNA overhangs for part assembly. Nine techniques are shown, with the corresponding DNA assembly methods in blue (see Table 2 for references). Blue rectangles represent the recognition domain of an endonuclease, lined up against its cognate recognition sequence. Parallel horizontal lines represent double stranded DNA, with PCR templates in black and DNA to be assembled in blue. *AFEAP uses sequential PCRs.

standard (Knight, 2003) where two inserts are ligated into the vector backbone simultaneously, accelerating the cloning process. Both methods were designed to be idempotent, i.e. the output vector can be manipulated in the same way as the input vector for subsequent rounds of assembly. A modified protocol called Quick Gene Assembly allows five BioBricks to be assembled per day instead of two (Yamazaki et al., 2017); see Table 2.

3.2.1. *Golden Gate assembly*

Golden Gate assembly (Engler et al., 2008; Engler and Marillonnet, 2014) relies on Type IIS restriction enzymes; these cut outside their recognition domain, allowing multipart assembly through unconstrained sequence design for the single strand overhang. T4 DNA ligase is used to seal junctions. As the recognition sequence is not present in the desired ligation product, the reaction proceeds one-way in an efficient combined restriction-ligation step that is amenable to automation. Sequencing of the final assembly is not always necessary as the procedure uses pre-sequenced part libraries in entry vectors and no subsequent PCR amplification or gel purification. Golden Gate assembly can be used for combinatorial experiments; for example, 48 combinations of −35 and −10 promoter elements were tested in *E. coli* to identify those with high inducibility and low leakiness and to study the dynamic range (Y. Chen et al., 2018).

Three issues with the Golden Gate method are the need for part domestication (the removal of internal restriction sites), the variation in ligation efficiency between different overhang designs, and the presence of scars (typically 4 bp) at part junctions. These are all addressed by a new web application, iBioCAD GGA (HamediRad et al., 2019) which designs scarless assembly schemes and primers using existing areas around the junctions as overhangs, although at some expense to part reusability. These linker sets are carefully chosen to ligate efficiently while avoiding cross-reactions. Combinatorial assembly schemes can be designed by uploading multiple part sequences per location. The ligation fidelity and efficiency of different overhangs has also been studied by

others (Potapov et al, 2018a, 2018b).

3.2.2. Scarless assembly methods

Other techniques for the avoidance of scars are Scarless Stitching and Start-Stop Assembly. Scarless Stitching (Smanski et al., 2014) allows the assembly of two parts into a backbone per round of assembly. An initial Golden Gate reaction is performed to assemble three parts into a backbone using BsaI, with the central part being a bridging sequence encoding a lacZα peptide. In the same tube, the bridging sequence is then removed with MlyI digestion and blunt-end ligation to form a scarless junction between the two desired parts. This method is suitable to form transcription units that can be PCR amplified to add MoClo cohesive ends for subsequent higher order assembly using standard Golden Gate/MoClo procedures. Scarless Stitching was used in the first assembly step of a combinatorial library for the nitrogenase pathway in *Klebsiella* that comprised three T7 RNAP promoters, one terminator, twelve RBSs and seven spacers (Smanski et al., 2014). Although it was largely successful, some point mutations occasionally occurred (likely due to the PCR step), and in some experiments 1–2 bp deletions were found to be common at the junction site, so clones should be checked by sequencing.

Start-Stop Assembly (Taylor et al., 2019) is an alternative modular and scarless method in which the assembly of expression units (Level 1) uses a Type IIS restriction enzyme that leaves 3 bp overhangs, appropriate for junctions that are sensitive to scars such as those around the start and stop codons. As above, subsequent assemblies use classic Golden Gate 4 bp overhangs that have already proven to give high-fidelity assembly as scars are not so problematic at these levels. Up to five expression units can be combined at Level 2 and 15 at Level 3. The approach was validated using five libraries for carotenoid biosynthesis pathways, each produced combinatorially from up to eight genes, six promoters, six RBSs and four terminators to explore the design space. This included both monocistronic and operon configurations. The Start-Stop constructs developed by Taylor et al. (2019) are for prokaryotic expression, but the scheme is designed in such a way that only one destination vector is required (Level 2 or 3), facilitating the transfer of the approach to new shuttle vectors or organisms. One potential drawback of the scheme is that it cannot assemble fusion proteins, as a stop codon is used as the fusion point at the end of any coding region.

3.2.3. Type IIS modular assembly methods

To eliminate the requirement for construct-specific cloning strategies and facilitate sharing of parts between researchers, hierarchical Golden Gate syntax schemes such as MoClo (Weber et al., 2011) and GoldenBraid (Sarrion-Perdigones et al, 2011, 2013) have been developed. The MoClo standard requires many entry and destination vectors, while GoldenBraid uses fewer vectors but involves more cloning steps for large assemblies. These are reviewed elsewhere (Casini et al., 2015; Deb and Reshamwala, 2020; Vazquez-Vilar et al., 2020). A common syntax for MoClo and GoldenBraid was developed that uses 12 Type IIS overhangs defining different positions for eukaryotic genes (Patron et al., 2015).

Other Type IIS assembly systems are detailed in Table 2, including the recent developments of 3G Assembly (Halleran et al., 2018), BASIC (Storch et al, 2015, 2017), Loop Assembly (Pollak et al., 2019), MetClo, (Lin et al., 2018), PODAC (Van Hove et al., 2017), Universal Loop Assembly (Pollak et al., 2020), and VEGAS plus yeast Golden Gate (Chuang et al., 2018; Mitchell et al., 2015). These help to address some of the limitations of MoClo and GoldenBraid; for example, BASIC is an idempotent cloning framework for combinatorial assembly that only requires parts to be domesticated for one restriction enzyme, reducing the risk of altering part function. With this method the authors assembled constructs containing up to seven parts, with the possibility to include more by applying functionalised linkers (Storch et al., 2015). These advantages of idempotency and reduced part domestication are also shared by the MetClo and PODAC methods.

3.3. Non-restriction enzyme methods

To avoid issues associated with the use of restriction enzymes e.g. scars and part domestication, researchers have developed alternative DNA assembly methods.

3.3.1. Methods using in vivo recombination

Several of these methods utilise yeast homologous recombination (YHR). YHR has been used for routine cloning for more than 30 years (Kunes et al., 1985). However, it was not until relatively recently that this methodology was adapted for combinatorial pathway assembly with the implementation of the DNA assembler method (Shao et al., 2009). The authors first assembled individual expression cassettes using overlap-extension PCR. These were then assembled into larger biochemical pathways via YHR. Up to eight cassettes coding for D-xylose utilization and zeaxanthin biosynthesis pathways were assembled with efficiencies of 70–100%, depending on the number of fragments assembled. The authors suggested that between 30 and 50 genes encoding 100–200 kb DNA could be assembled through iterative cycles of transformation, selection and counter-selection, with the latter step freeing a *ura3* selectable marker for further cycles. A follow-up study used a variant of DNA assembler to engineer higher efficiency xylose and cellobiose utilizing pathways (Du et al., 2012), further demonstrating its utility. Several additional studies have also used YHR for combinatorial pathway assembly e.g. COMPASS (Naseri et al., 2019) and M-PERL (Liu et al., 2016). The latter M-PERL method used linker oligonucleotides to vary promoter sequences, reducing the number of expression cassettes that needed to be assembled prior YHR. A key limitation of these approaches is the amount of time required, roughly 1–2 weeks (Kouprina and Larionov, 2016; Shao et al., 2009). This derives from the slower doubling of *S. cerevisiae* compared to other alternative workhorses e.g. *E. coli*. As such, for smaller pathways and for those that do not require expression in yeast, alternative methods are generally preferred.

As an alternative to YHR, the AQUA cloning method uses the native ability of *E. coli* to assemble DNA fragments containing 15 bp overlapping regions (Beyer et al., 2015). Given the short doubling time of *E. coli*, cloning and recombinant protein expression can be completed within a single day. AQUA was subsequently used to assemble different variants of an auxin sensor and an A NIMPLY B logic gate, illustrating how it might be used for combinatorial assembly. However, depending on cell competency and strain, colony numbers can be low, limiting its application.

3.3.2. Methods using in vitro assembly steps

3.3.2.1. Gibson DNA assembly. To facilitate faster DNA assembly workflows and without the drawbacks associated with restriction enzymes, researchers have developed methods using alternative enzymes in vitro. One such method is Gibson DNA assembly (GDA) (Gibson et al., 2009). GDA utilises a combination of T5 exonuclease, DNA polymerase and *Taq* DNA ligase to assemble multiple DNA fragments in vitro. The method was originally demonstrated by assembling half the *Mycoplasma genitalium* genome (~300 kb) into a bacterial artificial chromosome, via a 3-fragment assembly, achieving 50% fidelity (Gibson et al., 2008). While the original method can be used to assemble up to 5 fragments in a one-pot reaction, a two-step variant was developed by Codex DNA where up to 15 fragments can be assembled simultaneously (https://codexdna.com/products/benchtop-reagents/gibson-assembly-kits). The two-step variant splits the exonuclease and annealing steps of the reaction from the polymerase and ligation steps, increasing fidelity. In addition to genome engineering, GDA has been used for combinatorial transcriptional rewiring of *Pichia pastoris*, improving the expression of recombinant proteins (Windram et al., 2017). In another example, a variation of GDA was used to improve the production of pristinamycin, a streptogramin antibiotic, via combinatorial metabolic engineering (Li

et al., 2015). Further variations on GDA have been proposed to reduce its cost (Fu et al., 2014; Xia et al., 2019) or improve its efficiency (Casini et al., 2014).

3.3.2.2. Ligase-dependent assembly. Other relevant DNA assembly methods using DNA ligases in vitro include ligase cycling reaction (LCR) (de Kok et al., 2014) and "DNA assembly method using thermal exonucleases and *Taq* DNA ligase" (DATEL) (Jin et al., 2016). LCR utilises bridging oligonucleotides with complementarity to DNA fragments in combination with a thermostable DNA ligase and thermocycling to join DNA fragments in a one-pot reaction. LCR in combination with a DoE approach was recently used to optimize the production of the flavonoid (2S)-pinocembrin in *E. coli*, improving titres 500-fold (Carbonell et al., 2018). Notably, LCR was shown to have a similar performance compared to YHR, achieving 60–100% fidelity for the assembly of 12 fragments. However, it has a higher cost compared to alternative methods given the requirement for phosphorylated primers and bridging oligonucleotides (de Kok et al., 2014). DATEL utilises *Taq* and *Pfu* DNA polymerases to degrade ssDNA flaps, formed when multiple dsDNA molecules sharing complementarity are annealed. The resulting nicked backbone is sealed by *Taq* DNA ligase. The method was able to ligate 2–10 fragments simultaneously with accuracies ranging between 74 and 100%. This facilitated the combinatorial assembly of the beta-carotene pathway (Jin et al., 2016).

3.3.2.3. Ligase-independent assembly. Except for GDA variants (Fu et al., 2014; Xia et al., 2019), the previously described methods all utilise DNA ligases to seal the backbone of constructs prior to transformation. An alternative strategy is to avoid the use of DNA ligases and rely on native, in vivo machinery to seal the nicks. A collection of these "ligase-independent" methods have been described and utilised for combinatorial assembly (Liang et al., 2017; Quan and Tian, 2009; Zou et al., 2013). These methods use a variety of strategies to anneal DNA fragments. For instance, Twin-Primer Assembly (TPA) utilises two, separate PCR reactions and subsequent re-annealing, followed by hybridisation to assemble constructs (Liang et al., 2017). TPA enabled the assembly of constructs from 5 to 10 fragments with fidelities ranging between 50 and 80%. Furthermore, pathways expressing glucoraphanin, a compound with potential neuroprotective and cardiovascular effects (Bai et al., 2015; Tarozzi et al., 2013), were synthesised using TPA (Yang et al., 2020).

Other ligase-independent methods use the enzymes present in bacterial lysates to recombine homologous DNA ends (Motohashi, 2015; Richter et al., 2019). Alternatively, bacteriophage lambda recombination proteins can be used for in vitro assembly; these recombine *attB* and *attP* sites that are positioned at the ends of fragments by PCR. MultiSite Gateway™ technology allows up to four fragments to be assembled in a vector in a predetermined order and has been used for the creation of complex plasmids for genomic integration in mammalian cells (Jäckel et al., 2016), among other applications. The in vitro version of Synthetic Chromosome Rearrangement and Modification by LoxPsym-mediated Evolution (SCRaMbLE) also relies on recombinases (Wu et al., 2018), in this case the Cre/loxP system from bacteriophage P1, and allows pathway flux optimisation by stochastically rearranging transcription units.

3.4. DNA sequence verification

Traditionally, the arrangement and DNA sequence of individual plasmid constructs was confirmed by techniques such as restriction digest and Sanger sequencing. However, high-throughput quality control methods suitable for combinatorial plasmid libraries are now available. In terms of short-read NGS sequencing, Shapland et al. (2015) used an automated workflow to sequence 4000 plasmids in one Illumina MiSeq run, while plasmids submitted to Addgene are sequenced using plexWell™ technology, which pools 96 plasmids per well. Long-read NGS sequencing is also employed: D'Amore et al. (2017) developed a high-throughput SMRT (Single Molecule, Real-Time) Gate method to validate plasmid inserts using Golden Gate and Pacific Biosciences technology. In addition, a new PCR barcoding system and analysis algorithm were recently described that facilitate fast high-throughput plasmid sequencing using Oxford Nanopore technology (Currin et al., 2019).

4. Methods and toolkits for enzyme balancing

Once the enzymes required for a metabolic pathway have been established, their expression must be balanced to achieve optimal product yields. The traditional screening approach is to vary the DNA cis-elements that regulate their expression at the transcriptional or translational levels, such as promoters and RBS, as discussed above. However, this laborious pathway-specific combinatorial DNA construction can be bypassed by gaining information on optimal expression in other ways, often followed by the informed selection of parts for the final hard-coded design. As illustrated in Fig. 3, pathway-blind toolkits developed for this purpose include those based on CRISPR, inducible transcription factors and sRNA translational downregulation.

CRISPRi and CRISPRa (CRISPR-mediated activation), which enable transcriptional regulation via a nuclease-deficient Cas9 protein, can be harnessed for pathway tuning by adjusting the presence or promoter strengths of a set of guide RNA sequences that each correspond to a different target gene's promoter region. For example, Liu et al. (2019) developed a reusable CRISPRa-based expression profile scanning platform for bacteria that takes advantage of the wide dynamic range permitted by eukaryotic-like σ^{54}-dependent promoters and by dxCas9. As a proof of principle, a five-gene violacein pathway in *E. coli* was tuned by upregulating three of the genes individually with a 27-member combinatorial guide RNA library prepared by Golden Gate assembly (three guide RNA genes each expressed using one of three promoters; Fig. 3a). CRISPRa systems that could facilitate pathway tuning are also available for mammalian cells (Li et al., 2017) and yeast (Cámara et al., 2020). In addition, a highly effective CRISPR/Cas9-based programmable transcriptional activator has recently been developed for plants, with a 10,000-fold increase in mRNA abundance demonstrated for an endogenous test gene in *Nicotiana* (Selma et al., 2019).

Meyer et al. (2019) recently developed *E. coli* 'Marionette' strains in which 12 inducer chemicals control 12 transcription factors in an orthogonal and tunable manner (Fig. 3b), allowing researchers to test different combinations of expression levels for many genes in highly automatable screens. Directed evolution was employed to ensure that each sensor, comprising a transcription factor and its target promoter, gave low background expression and a high dynamic range while minimising crosstalk. This sophisticated tuning system rests on decades of research into gene regulation in *E. coli* (Meyer et al., 2019) and similar tools are not yet available for the majority of industrial chassis organisms.

A third method to identify optimal enzyme balance is the combinatorial repression of target genes using sRNA; this binds to its cognate mRNA to inhibit translation initiation and stimulate RNA degradation (Fig. 3c). Binding occurs between the 15 nt sRNA tag region and its reverse complement sequence in the 5′UTR of the target mRNA. Six orthogonal sRNA:target pairs were created for pathway optimisation in bacteria, with each target sequence incorporated upstream of a test gene from a beta-carotene biosynthesis pathway (Ghodasara and Voigt, 2017). The rigorous development process included screening for optimal hammerhead ribozyme additions and strong (>120-fold) potential repression without off-target effects, then diversification of the chaperone-binding, ribozyme and terminator loop regions to prevent issues with genetic instability when using multiple sRNAs. The sRNAs, which can be used for any pathway, were each expressed from 16 different promoters of varying strengths. After identifying the most

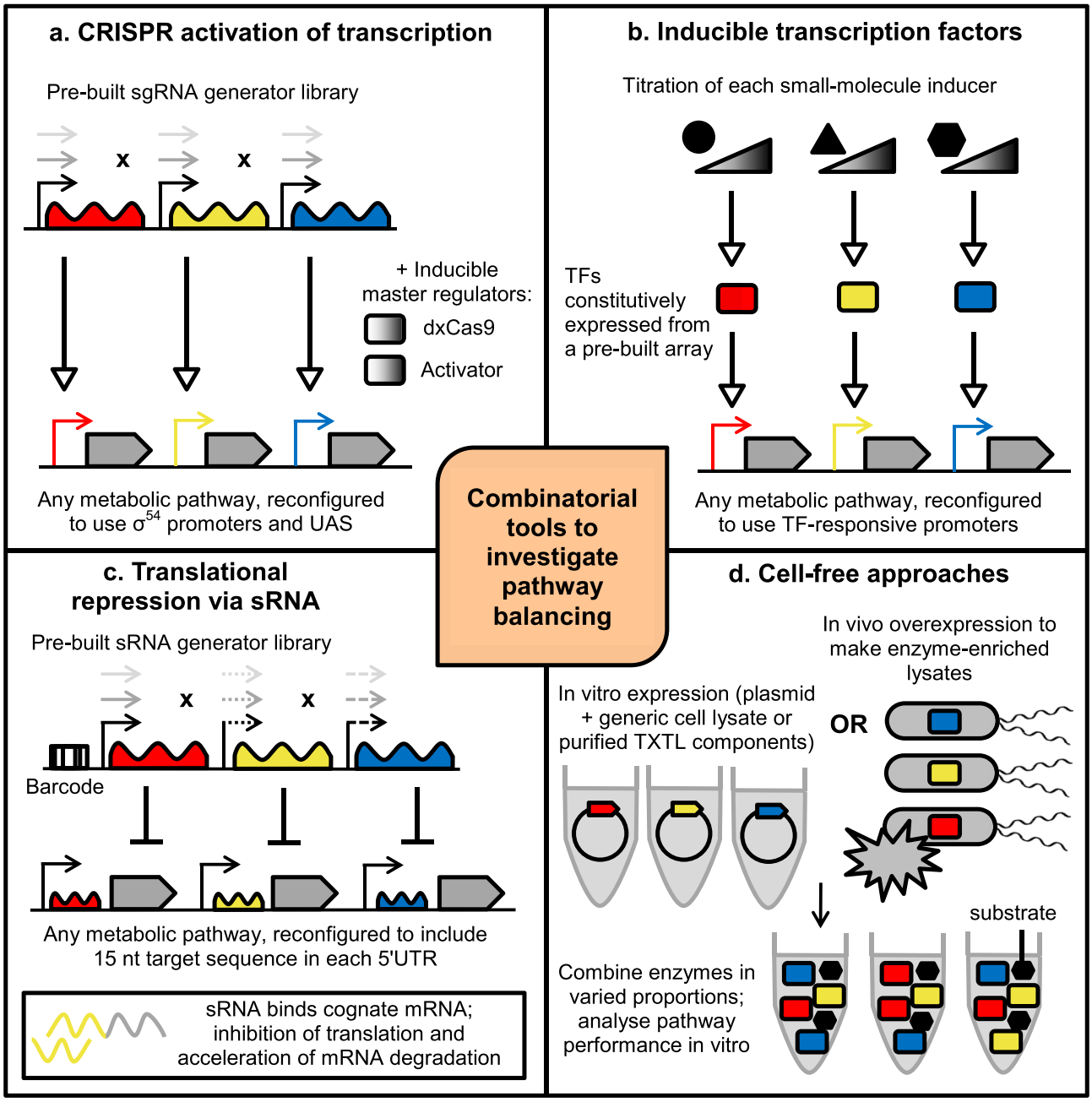

Fig. 3. Combinatorial tools to investigate pathway balancing without creating a pathway-specific combinatorial library, illustrating the approaches of Yang Liu et al. (2019), Meyer et al. (2019), Ghodasara and Voigt (2017) and Karim et al. (2020). See main text in Section 4 for more detail. RBS and terminator symbols are omitted for clarity. TF = transcription factor, TXTL = transcription and translation, UAS = upstream activating sequence.

productive strains, the authors recapitulated the balance of expression using a panel of T7-based promoters.

The most appropriate approach to pathway tuning depends in part on the chassis organism. The random integration of transgenes into the nuclear genomes of plant and mammalian cells by non-homologous end joining leads to variable expression and copy number; this heterogeneity can be exploited in combinatorial screens. In a proven approach for plant enzyme selection and balancing termed COSTREL (Fuentes et al., 2016), a core biosynthetic pathway is integrated into the chloroplast genome by homologous recombination for stable, high-level expression. The transplastomic line is then supertransformed with a collection of single-gene plasmids to allow the combinatorial integration of potential regulatory or accessory genes into the nuclear genome. This leads to variation in the identity, copy number and location (therefore expression level) of integrated genes across the transformant population. This method has been used to optimize the metabolic flux of a heterologous artemisinic acid biosynthesis pathway in tobacco (Fuentes et al., 2016) and improve plant drought tolerance (Schulz et al., 2020).

A pathway testing and tuning approach particularly suited to microbes that are slow-growing and/or difficult to engineer is the use of cell-free protein synthesis, exemplified by the iPROBE platform (Karim et al., 2020). In this method, genes are expressed individually from plasmids in vitro using crude cell lysates; the resulting enzymes are quantified and mixed in different combinations and ratios together with substrates and cofactors to enable pathway analysis and optimisation. The authors used iPROBE to improve 3-hydroxybutyrate production in *Clostridium autoethanogenum* (Karim et al., 2020). A number of cell-free approaches to pathway tuning, including iPROBE, are illustrated in

Table 3
Platforms and methods used for automated DNA assembly.

Platform	Assembly method using platform	References
Microfluidics	Golden Gate and cloning standards	(Linshiz et al., 2014; Shih et al., 2015)
	Gibson DNA assembly	(Linshiz et al, 2014, 2016; Shih et al., 2015)
Tip-based liquid handling robotics	Golden Gate and cloning standards	(Chao et al., 2017; Linshiz et al., 2014; Ortiz et al., 2017; Walsh et al., 2019)
	Gibson DNA assembly	https://codexdna.com/pages/bioxp-3200-system
	Ligase cycling reaction	(Carbonell et al., 2018)
	BASIC DNA assembly	(Exley et al., 2019; Storch et al., 2020)
Acoustic liquid handling robotics	Golden Gate and cloning standards	(Kanigowska et al., 2016; Mann et al., 2019; Ortiz et al., 2017; Rajakumar et al., 2019)
	Gibson DNA assembly	(Kanigowska et al., 2016)
	BASIC DNA assembly	(Exley et al., 2019)

Fig. 3d.

5. Alternatives to intracellular biomanufacturing

5.1. Microbial consortia

So far, we have considered techniques for combinatorial pathway assembly in which all elements are expressed in a single host cell line in the final production system. However, an intercellular approach combining natural or engineered organisms that each express parts of a metabolic pathway can be advantageous in increasing the yield or range of possible products.

There has been a surge of interest in the use of microbial consortia for bioproduction, with several recent reviews available (Arora et al., 2020; Lu et al., 2019; McCarty and Ledesma-Amaro, 2019; Sgobba and Wendisch, 2020). Novel bioproducts have been discovered by combinatorial screening of microbe pairs, such as an antibacterial borrelidin from the co-culture of an actinomycete and fungus (Yu et al., 2019), although many of these new compounds are the result of cryptic pathway induction rather than the sharing of intermediates. If the co-culture species are slow growing or product yields are low, genes can be transferred to more amenable and genetically tractable host organisms.

Where the aim of a consortium is simply to spread the metabolic burden of a well-characterised but resource-intensive pathway between cells, a single species can be used: for example, the naringenin biosynthetic pathway was split between two engineered *E. coli* strains to increase yield (Ganesan et al., 2017), as was the resveratrol pathway (Hong et al., 2020). Relative population densities can be tested and controlled through engineered quorum sensing (Stephens et al., 2019) or allocating different carbon substrates (Li et al., 2019). Pathways requiring multiple specialized environments or existing substrates can be distributed across two appropriate species, such as the biosynthesis of a paclitaxel precursor using an *E. coli* and *S. cerevisiae* co-culture (K. Zhou et al., 2015).

5.2. In vitro pathways

Commercial chemical production using combined purified enzymes, or using lysates from cell lines that express the relevant enzymes, is termed 'cell-free synthetic biochemistry' (reviewed by Bowie et al., 2020). Advantages include the ability to mix enzymes from different hosts, lack of competition for cellular resources, avoidance of product toxicity problems and the option to use non-physiological conditions, but technical and economic issues (e.g. enzyme stability and cofactor cost) will need to be overcome. One promising example of cell-free synthetic biochemistry is the production of hydrogen from starch and water using a pathway of 13 enzymes originating from plant, animal, yeast, bacterial and archaeal sources (Zhang et al., 2007).

Aside from biomanufacturing, this approach allows the efficient screening of enzyme combinations prior to in vivo work, such as for monoterpenoid pathways (Dudley et al., 2019), and the optimisation of enzyme ratios simply by combining different amounts of each extract (Section 4). It is also particularly suited to molecular diagnostics; for example, Silverman et al. (2020) combined four *E. coli* extracts, each overexpressing a different protein, as part of a biosensor for the water contaminant atrazine.

Cell-free protein synthesis shares many of the benefits of cell-free synthetic biochemistry and is starting to be considered as a chemical manufacturing platform using multi-enzyme combinations (e.g. Grubbe et al., 2020), augmenting its current roles in part characterisation (Section 2) and pathway optimisation (Section 4). These two biomanufacturing routes are compared in a recent review (Lim and Kim, 2019).

6. Computational tools and automation

6.1. DNA design software

Computational tools are available to assist with many stages of combinatorial pathway assembly, from selection of parts through to amplification of assembled DNA. The Synthetic Biology Open Language (SBOL) standard (Cox et al., 2018) for the representation of genetic data facilitates automated generation of computational models of genetic circuits from data stored in design repositories (Misirli et al., 2019). Circuits can be visualised using SBOL Visual glyphs (Madsen et al., 2019; Misirli et al., 2020) or the more flexible DNAplotlib (Der et al., 2017).

Biodesign automation tools were recently reviewed (Appleton et al., 2017b). These include BOOST, which takes into account determinants of DNA synthesis success such as repeats and secondary structure (Oberortner et al., 2017); and the algorithm of Blakes et al. (2014) which calculates the fewest concatenations required to assemble a whole library. The Edinburgh Genome Foundry provides a suite of over 20 open-source software applications for DNA design and assembly (https://edinburgh-genome-foundry.github.io/).

DoE can be used to explore the design space without making every single combination, selecting a subset of designs that will together provide maximum information. Tools such as Double Dutch (Roehner et al., 2016) are available to help design suitable DoE combinatorial libraries. For example, the yield of itaconic acid in *S. cerevisiae* was improved by testing different promoters and terminators for each of six genes in a novel pathway (Young et al., 2018), while Woodruff et al. (2017) adopted DoE to explore a huge design space for the optimisation of expression levels in a 16-gene nitrogen fixation pathway, using pools of retrievable composite parts. Ideally, different genetic designs and growth conditions (e.g. temperature, aeration and media composition) should be varied simultaneously with DoE. This approach was shown to increase the yield of 6-aminocaproic acid (6-ACA) in *E. coli* (H. Zhou et al., 2015).

6.2. Automation

High-throughput and complex combinatorial assembly projects can benefit from automation, saving time and reagents and reducing human error (Jessop-Fabre and Sonnenschein, 2019; Walsh et al., 2019). A range of DNA assembly methods have been automated using microfluidic platforms, tip-based liquid handling robotics or acoustic liquid

handlers (Table 3) either dedicated or in combination.

Each of these platforms offers specific strengths and challenges. For instance, digital microfluidic platforms can manipulate sub-microlitre assembly reaction volumes with high accuracy (Yehezkel et al., 2016). However, delivering material to and from the chip and accurately controlling temperatures is often challenging. To address this, 3D printing technology was recently applied to digital microfluidics, improving temperature stability and facilitating the delivery of larger volumes to reservoirs, while making this workflow more accessible (Moazami et al., 2019).

Tip-based liquid handling robotics are widely available and their ability to transfer liquids with high accuracy and precision (Bessemans et al., 2016) has led to a range of well-performing automated DNA assembly workflows (Table 3). Furthermore, the initial cost of these platforms can be low, making them accessible to most research groups. For instance, BASIC DNA assembly was recently automated using the Opentrons OT-2, which at a price of $8k will be available to most labs (Storch et al., 2020). When aiming at higher throughput, tip-based liquid handlers are limited by their lower transfer limits of 1 μL and relatively slow speed for cherry-picking operations (Walsh et al., 2019). These limitations restrict the ability to reduce cost by shrinking reaction volumes or scaling up throughput.

Acoustic-based liquid handlers utilise acoustic energy to transfer liquids in multiples of nanolitre volumes contactless between source and target plates. They are highly scalable and fast, dispensing liquids into microtiter plates with up to 1536 wells and assembling 96x, 5-part DNA assembly reactions in as little as 5 min (Ortiz et al., 2017). Their small volume operations can reduce costs dramatically, exemplified in 50 nL, 2-part Golden Gate reactions assembled at a cost of $0.01 per reaction (Kanigowska et al., 2016). It should however be recognised that later steps in the DNA assembly workflow, e.g. bacterial transformation, are typically conducted in larger volumes (Exley et al., 2019; Kanigowska et al., 2016; Mann et al., 2019; Ortiz et al., 2017; Rajakumar et al., 2019), meaning overall savings are not as significant given that reagents such as competent cells tend to constitute a larger percentage of total cost (Storch et al., 2020). Furthermore, these platforms typically have high initial and services costs, impeding their wide availability.

It is worth considering that the aforementioned platforms can work synergistically, drawing on individual strengths and facilitating the automation of more complex workflows. For instance, a complete DNA assembly workflow and validation consisting of Golden Gate assembly, *E. coli* transformation, plasmid extraction and digest validation has previously been automated using multiple robotic platforms including an acoustic dispenser, tip-based liquid handlers and a colony picker (Mann et al., 2019). Within this work, fast cherry-picking operations enabled by acoustic dispensers were paired with batch operations enabled by tip-based systems equipped with multi-channel pipetting heads, yielding a more effective setup. Cross-platform automation is further aided by versatile programming languages such as PR-PR (Linshiz et al, 2013, 2014).

7. Conclusion and outlook

Our ability to assemble optimal metabolic pathways has seen great progress in the last few years, with improved enzyme annotations allowing more sophisticated genome mining (Blin et al., 2019) and genetic tools and parts becoming available for many more potential chassis organisms (Table 1). As presented in this review, a great number of DNA assembly strategies have been developed to suit different requirements such as speed, cost effectiveness, or the ability to join parts without leaving scars. Furthermore, researchers investigating the optimal expression ratios of the enzymes within a pathway can now choose between directly swapping regulatory elements such as promoters or using one of the 'universal' toolkits for adjusting the balance of any pathway, if available for the chassis of interest.

Screening candidate pathways for the final product is no longer such a bottleneck now that various methods can bypass time-consuming sample preparation or extraction steps, at least for an initial evaluation. For example, rapid laser-enabled mass spectrometry directly from yeast colonies can semi-quantitatively detect a metabolite of interest at a rate of six colonies per minute (Gowers et al., 2019), and metabolite-specific biosensors that transduce detection into a signal are increasingly available (Carpenter et al., 2018). In addition, Syntrophic Co-culture Amplification of Production phenotype (SnoCAP) uses auxotrophic cross-feeding secretor and sensor strains for high-throughput metabolite screening of *E. coli* strain libraries in microplates or microdroplets (Saleski et al., 2019).

The development of automation platforms for DNA assembly has allowed more design space to be explored efficiently. Walsh et al. (2019) used 'Q-metrics' as a means to determine whether automation might lead to time or cost savings when compared to manual workflows; however, comparison between the various automation options for DNA assembly is still challenging and would require improved metrics such as ease of use, versatility, throughput and the costs of initial setup and consumables.

The issue of intellectual property (IP) can potentially affect several aspects of pathway assembly, particularly the use of parts, vector backbones and standards (Nielsen et al., 2018). An IP expert meeting (Minssen et al., 2015) made six recommendations for the synthetic biology community in this regard, such as encouraging scientists to employ tools that are unencumbered with IP rights when developing foundational technologies. The BioBrick™ Public Agreement facilitates the free sharing of DNA sequences across the synthetic biology community (https://biobricks.org/bpa) while the more recent Open Material Transfer Agreement (http://openmta.org/) provides a legal tool for sharing physical biomaterials in a simpler and less restrictive way than typical arrangements allow (Kahl et al., 2018).

As discussed by Szymanski and Scher (2019), DNA is not merely linear text comprising independent parts; inter- and intramolecular interactions, epigenetic modifications and environmental factors make the reality of these 3D polymers much more complex. Predictive software that incorporates these parameters is much more likely to result in a well-functioning design. In reality, combinatorial library screens will still be a necessity, but more accurate models should result in smarter library design, reducing the R&D resources needed to reach the same goal or increasing the attainable product yield.

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References

Ajikumar, P.K., Xiao, W.H., Tyo, K.E.J., Wang, Y., Simeon, F., Leonard, E., Mucha, O., Phon, T.H., Pfeifer, B., Stephanopoulos, G., 2010. Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. Science 330, 70–74. https://doi.org/10.1126/science.1191652.

Anderson, J.C., Dueber, J.E., Leguia, M., Wu, G.C., Arkin, A.P., Keasling, J.D., 2010. BglBricks: a flexible standard for biological part assembly. J. Biol. Eng. 4, 1. https://doi.org/10.1186/1754-1611-4-1.

Andreou, A.I., Nakayama, N., 2018. Mobius Assembly: a versatile Golden-Gate framework towards universal DNA assembly. PLoS One 13, e0189892. https://doi.org/10.1371/journal.pone.0189892.

Appleton, E., Densmore, D., Madsen, C., Roehner, N., 2017a. Needs and opportunities in bio-design automation: four areas for focus. Curr. Opin. Chem. Biol. 40, 111–118. https://doi.org/10.1016/j.cbpa.2017.08.005.

Appleton, E., Madsen, C., Roehner, N., Densmore, D., 2017b. Design automation in synthetic biology. Cold Spring Harb. Perspect. Biol. 9, a023978 https://doi.org/10.1101/cshperspect.a023978.

Arora, D., Gupta, P., Jaglan, S., Roullier, C., Grovel, O., Bertrand, S., 2020. Expanding the chemical diversity through microorganisms co-culture: current status and outlook. Biotechnol. Adv. 40, 107521 https://doi.org/10.1016/j.biotechadv.2020.107521.

Arranz-Gibert, P., Patel, J.R., Isaacs, F.J., 2019. The role of orthogonality in genetic code expansion. Life 9, 58. https://doi.org/10.3390/life9030058.

Bai, Y., Wang, X., Zhao, S., Ma, C., Cui, J., Zheng, Y., 2015. Sulforaphane protects against cardiovascular disease via Nrf2 activation. Oxid. Med. Cell. Longev, 407580. https://doi.org/10.1155/2015/407580.

Barthel, S., Palluk, S., Hillson, N.J., Keasling, J.D., Arlow, D.H., 2020. Enhancing terminal deoxynucleotidyl transferase activity on substrates with 3′ terminal structures for enzymatic de novo DNA synthesis. Genes 11, 102. https://doi.org/10.3390/genes11010102.

Beal, J., Haddock-Angelli, T., Baldwin, G., Gershater, M., Dwijayanti, A., Storch, M., de Mora, K., Lizarazo, M., Rettberg, R., 2018. Quantification of bacterial fluorescence using independent calibrants. PLoS One 13, e0199432. https://doi.org/10.1371/journal.pone.0199432.

Belcher, M.S., Vuu, K.M., Zhou, A., Mansoori, N., Agosto Ramos, A., Thompson, M.G., Scheller, H.V., Loqué, D., Shih, P.M., 2020. Design of orthogonal regulatory systems for modulating gene expression in plants. Nat. Chem. Biol. 1–9. https://doi.org/10.1038/s41589-020-0547-4.

Bessemans, L., Jully, V., de Raikem, C., Albanese, M., Moniotte, N., Silversmet, P., Lemoine, D., 2016. Automated gravimetric calibration to optimize the accuracy and precision of TECAN Freedom EVO liquid handler. J. Lab. Autom. 21, 693–705. https://doi.org/10.1177/2211068216632349.

Beyer, H.M., Gonschorek, P., Samodelov, S.L., Meier, M., Weber, W., Zurbriggen, M.D., 2015. AQUA Cloning: a versatile and simple enzyme-free cloning approach. PLoS One 10, e0137652. https://doi.org/10.1371/journal.pone.0137652.

Bhokisham, N., VanArsdale, E., Stephens, K.T., Hauk, P., Payne, G.F., Bentley, W.E., 2020. A redox-based electrogenetic CRISPR system to connect with and control biological information networks. Nat. Commun. 11, 2427. https://doi.org/10.1038/s41467-020-16249-x.

Biava, H.D., 2020. Tackling Achilles' Heel in synthetic biology: pairing intracellular synthesis of noncanonical amino acids with genetic-code expansion to foster biotechnological applications. Chembiochem 21, 1265–1273. https://doi.org/10.1002/cbic.201900756.

Biggs, B.W., Bedore, S.R., Arvay, E., Huang, S., Subramanian, H., Mcintyre, E.A., Duscent-Maitland, C.V., Neidle, E.L., Tyo, K.E.J., 2020. Development of a genetic toolset for the highly engineerable and metabolically versatile *Acinetobacter baylyi* ADP1. Nucleic Acids Res. 48, 5169–5182. https://doi.org/10.1093/nar/gkaa167.

Black, J.B., Perez-Pinera, P., Gersbach, C.A., 2017. Mammalian synthetic biology: engineering biological systems. Annu. Rev. Biomed. Eng. 19, 249–277. https://doi.org/10.1146/annurev-bioeng-071516-044649.

Blakes, J., Raz, O., Feige, U., Bacardit, J., Widera, P., Ben-Yehezkel, T., Shapiro, E., Krasnogor, N., 2014. Heuristic for maximizing DNA reuse in synthetic DNA library assembly. ACS Synth. Biol. 3, 529–542. https://doi.org/10.1021/sb400161v.

Blanusa, M., Schenk, A., Sadeghi, H., Marienhagen, J., Schwaneberg, U., 2010. Phosphorothioate-based ligase-independent gene cloning (PLICing): an enzyme-free and sequence-independent cloning method. Anal. Biochem. 406, 141–146. https://doi.org/10.1016/j.ab.2010.07.011.

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S.Y., Medema, M.H., Weber, T., 2019. antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. Nucleic Acids Res. 47, 81–87. https://doi.org/10.1093/nar/gkz310.

Boël, G., Letso, R., Neely, H., Price, W.N., Wong, K.H., Su, M., Luff, J.D., Valecha, M., Everett, J.K., Acton, T.B., Xiao, R., Montelione, G.T., Aalberts, D.P., Hunt, J.F., 2016. Codon influence on protein expression in *E. coli* correlates with mRNA levels. Nature 529, 358–363. https://doi.org/10.1038/nature16509.

Bogart, J.W., Cabezas, M.D., Vögeli, B., Wong, D.A., Karim, A.S., Jewett, M.C., 2020. Cell-free exploration of the natural product chemical space. ChemBioChem cbic, 202000452. https://doi.org/10.1002/cbic.202000452.

Bonde, M.T., Pedersen, M., Klausen, M.S., Jensen, S.I., Wulff, T., Harrison, S., Nielsen, A. T., Herrgård, M.J., Sommer, M.O.A., 2016. Predictable tuning of protein expression in bacteria. Nat. Methods 13, 233. https://doi.org/10.1038/nmeth.3727.

Borkowski, O., Bricio, C., Murgiano, M., Rothschild-Mancinelli, B., Stan, G.-B., Ellis, T., 2018. Cell-free prediction of protein expression costs for growing cells. Nat. Commun. 9, 1457. https://doi.org/10.1038/s41467-018-03970-x.

Bowie, J.U., Sherkhanov, S., Korman, T.P., Valliere, M.A., Opgenorth, P.H., Liu, H., 2020. Synthetic biochemistry: the bio-inspired cell-free approach to commodity chemical production. Trends Biotechnol. 38, 766–778. https://doi.org/10.1016/j.tibtech.2019.12.024.

Bozhüyük, K.A.J., Fleischhacker, F., Linck, A., Wesche, F., Tietze, A., Niesert, C.P., Bode, H.B., 2018. De novo design and engineering of non-ribosomal peptide synthetases. Nat. Chem. 10, 275–281. https://doi.org/10.1038/NCHEM.2890.

Butzin, N.C., Mather, W.H., 2018. Crosstalk between diverse synthetic protein degradation tags in *Escherichia coli*. ACS Synth. Biol. 7, 54–62. https://doi.org/10.1021/acssynbio.7b00122.

Calero, P., Nikel, P.I., 2019. Chasing bacterial chassis for metabolic engineering: a perspective review from classical to non-traditional microorganisms. Microb. Biotechnol. 12, 98–124. https://doi.org/10.1111/1751-7915.13292.

Cámara, E., Lenitz, I., Nygård, Y., 2020. A CRISPR activation and interference toolkit for industrial *Saccharomyces cerevisiae* strain KE6-12. Sci. Rep. 10, 1–13. https://doi.org/10.1038/s41598-020-71648-w.

Cao, M., Fatma, Z., Song, X., Hsieh, P.H., Tran, V.G., Lyon, W.L., Sayadi, M., Shao, Z., Yoshikuni, Y., Zhao, H., 2020. A genetic toolbox for metabolic engineering of *Issatchenkia orientalis*. Metab. Eng. 59, 87–97. https://doi.org/10.1016/j.ymben.2020.01.005.

Cao, Y., Song, M., Li, F., Li, C., Lin, X., Chen, Yaru, Chen, Yuanyuan, Xu, J., Ding, Q., Song, H., 2019. A synthetic plasmid toolkit for *Shewanella oneidensis* MR-1. Front. Microbiol. 10, 410. https://doi.org/10.3389/fmicb.2019.00410.

Carbonell, P., Jervis, A.J., Robinson, C.J., Yan, C., Dunstan, M., Swainston, N., Vinaixa, M., Hollywood, K.A., Currin, A., Rattray, N.J.W., Taylor, S., Spiess, R., Sung, R., Williams, A.R., Fellows, D., Stanford, N.J., Mulherin, P., Le Feuvre, R., Barran, P., Goodacre, R., Turner, N.J., Goble, C., Chen, G.G., Kell, D.B., Micklefield, J., Breitling, R., Takano, E., Faulon, J.-L., Scrutton, N.S., 2018. An automated Design-Build-Test-Learn pipeline for enhanced microbial production of fine chemicals. Commun. Biol. 1, 66. https://doi.org/10.1038/s42003-018-0076-9.

Carpenter, A.C., Paulsen, I.T., Williams, T.C., 2018. Blueprints for biosensors: design, limitations, and applications. Genes 9, 375. https://doi.org/10.3390/genes9080375.

Carr, S.B., Beal, J., Densmore, D.M., 2017. Reducing DNA context dependence in bacterial promoters. PLoS One 12, e0176013. https://doi.org/10.1371/journal.pone.0176013.

Casini, A., MacDonald, J.T., De Jonghe, J., Christodoulou, G., Freemont, P.S., Baldwin, G. S., Ellis, T., 2014. One-pot DNA construction for synthetic biology: the modular overlap-directed assembly with linkers (MODAL) strategy. Nucleic Acids Res. 42, e7.

Casini, A., Storch, M., Baldwin, G.S., Ellis, T., 2015. Bricks and blueprints: methods and standards for DNA assembly. Nat. Rev. Mol. Cell Biol. 16, 568–576. https://doi.org/10.1038/nrm4014.

Caspi, R., Billington, R., Fulcher, C.A., Keseler, I.M., Kothari, A., Krummenacker, M., Latendresse, M., Midford, P.E., Ong, Q., Ong, W.K., Paley, S., Subhraveti, P., Karp, P. D., 2017. The MetaCyc database of metabolic pathways and enzymes. Nucleic Acids Res. 46, 633–639. https://doi.org/10.1093/nar/gkx935.

Cavaleiro, A.M., Kim, S.H., Seppälä, S., Nielsen, M.T., Nørholm, M.H.H., 2015. Accurate DNA assembly and genome engineering with optimized uracil excision cloning. ACS Synth. Biol. 4, 1042–1046. https://doi.org/10.1021/acssynbio.5b00113.

Celińska, E., Ledesma-Amaro, R., Larroude, M., Rossignol, T., Pauthenier, C., Nicaud, J.-M., 2017. Golden Gate Assembly system dedicated to complex pathway manipulation in *Yarrowia lipolytica*. Microb. Biotechnol. 10, 450–455. https://doi.org/10.1111/1751-7915.12605.

Ceroni, F., Algar, R., Stan, G.B., Ellis, T., 2015. Quantifying cellular capacity identifies gene expression designs with reduced burden. Nat. Methods 12, 415–418. https://doi.org/10.1038/nmeth.3339.

Chao, R., Liang, J., Tasan, I., Si, T., Ju, L., Zhao, H., 2017. Fully automated one-step synthesis of single-transcript TALEN pairs using a biological foundry. ACS Synth. Biol. 6, 678–685. https://doi.org/10.1021/acssynbio.6b00293.

Chen, W.H., Qin, Z.J., Wang, J., Zhao, G.P., 2013. The MASTER (methylation-assisted tailorable ends rational) ligation method for seamless DNA assembly. Nucleic Acids Res. 41, e93. https://doi.org/10.1093/nar/gkt122.

Chen, X., Yu, L., Qiao, G., Chen, G.Q., 2018. Reprogramming Halomonas for industrial production of chemicals. J. Ind. Microbiol. Biotechnol. 45, 545–554. https://doi.org/10.1007/s10295-018-2055-z.

Chen, Y., Ho, J.M.L., Shis, D.L., Gupta, C., Long, J., Wagner, D.S., Ott, W., Josić, K., Bennett, M.R., 2018. Tuning the dynamic range of bacterial promoters regulated by ligand-inducible transcription factors. Nat. Commun. 8, 64. https://doi.org/10.1038/s41467-017-02473-5.

Chiasson, D., Giménez-Oya, V., Bircheneder, M., Bachmaier, S., Studtrucker, T., Ryan, J., Sollweck, K., Leonhardt, H., Boshart, M., Dietrich, P., Parniske, M., 2019. A unified multi-kingdom Golden Gate cloning platform. Sci. Rep. 9, 10131. https://doi.org/10.1038/s41598-019-46171-2.

Cho, N., Seo, H.N., Ryu, T., Kwon, E., Huh, S., Noh, J., Yeom, H., Hwang, B., Ha, H., Lee, J.H., Kwon, S., Bang, D., 2018. High-throughput construction of multiple cas9 gene variants via assembly of high-depth tiled and sequence-verified oligonucleotides. Nucleic Acids Res. 46, e55. https://doi.org/10.1093/nar/gky112.

Chuang, J., Boeke, J.D., Mitchell, L.A., 2018. Coupling yeast golden gate and VEGAS for efficient assembly of the violacein pathway in *Saccharomyces cerevisiae*. In: Methods in Molecular Biology. Humana Press Inc., pp. 211–225. https://doi.org/10.1007/978-1-4939-7295-1_14

Cohen, S.N., Chang, A.C.Y., Boyer, H.W., Helling, R.B., 1973. Construction of biologically functional bacterial plasmids in vitro. Proc. Natl. Acad. Sci. U.S.A. 70, 3240–3244. https://doi.org/10.1073/pnas.70.11.3240.

Cooper, R.M., Hasty, J., 2020. One-day construction of multiplex arrays to harness natural CRISPR-Cas systems. ACS Synth. Biol. 9, 1129–1137. https://doi.org/10.1021/acssynbio.9b00489.

Cox, R.S., Madsen, C., McLaughlin, J.A., Nguyen, T., Roehner, N., Bartley, B., Beal, J., Bissell, M., Choi, K., Clancy, K., Grünberg, R., Macklin, C., Misirli, G., Oberortner, E.,

Pocock, M., Samineni, M., Zhang, M., Zhang, Z., Zundel, Z., Gennari, J.H., Myers, C., Sauro, H., Wipat, A., 2018. Synthetic biology Open Language (SBOL) version 2.2.0. J. Integr. Bioinform. 15, 20180001 https://doi.org/10.1515/jib-2018-0001.

Crozet, P., Navarro, F.J., Willmund, F., Mehrshahi, P., Bakowski, K., Lauersen, K.J., Pérez-Pérez, M.E., Auroy, P., Gorchs Rovira, A., Sauret-Gueto, S., Niemeyer, J., Spaniol, B., Theis, J., Trösch, R., Westrich, L.D., Vavitsas, K., Baier, T., Hübner, W., De Carpentier, F., Cassarini, M., Danon, A., Henri, J., Marchand, C.H., De Mia, M., Sarkissian, K., Baulcombe, D.C., Peltier, G., Crespo, J.L., Kruse, O., Jensen, P.E., Schroda, M., Smith, A.G., Lemaire, S.D., 2018. Birth of a photosynthetic chassis: a MoClo toolkit enabling synthetic biology in the microalga *Chlamydomonas reinhardtii*. ACS Synth. Biol. 7, 2074–2086. https://doi.org/10.1021/acssynbio.8b00251.

Currin, A., Swainston, N., Dunstan, M.S., Jervis, A.J., Mulherin, P., Robinson, C.J., Taylor, S., Carbonell, P., Hollywood, K.A., Yan, C., Takano, E., Scrutton, N.S., Breitling, R., 2019. Highly multiplexed, fast and accurate nanopore sequencing for verification of synthetic DNA constructs and sequence libraries. Synth. Biol. 4, ysz025. https://doi.org/10.1093/SYNBIO/YSZ025.

D'Amore, R., Johnson, J., Haldenby, S., Hall, N., Hughes, M., Joynson, R., Kenny, J.G., Patron, N., Hertz-Fowler, C., Hall, A., 2017. SMRT gate: a method for validation of synthetic constructs on Pacific biosciences sequencing platforms. Biotechniques 63, 13–20. https://doi.org/10.2144/000114565.

Dawson, W.M., Rhys, G.G., Woolfson, D.N., 2019. Towards functional de novo designed proteins. Curr. Opin. Chem. Biol. 52, 102–111. https://doi.org/10.1016/j.cbpa.2019.06.011.

de Boer, C.G., Vaishnav, E.D., Sadeh, R., Abeyta, E.L., Friedman, N., Regev, A., 2020. Deciphering eukaryotic gene-regulatory logic with 100 million random promoters. Nat. Biotechnol. 38, 56–65. https://doi.org/10.1038/s41587-019-0315-8.

de Kok, S., Stanton, L.H., Slaby, T., Durot, M., Holmes, V.F., Patel, K.G., Platt, D., Shapland, E.B., Serber, Z., Dean, J., Newman, J.D., Chandran, S.S., 2014. Rapid and reliable DNA assembly via ligase cycling reaction. ACS Synth. Biol. 3, 97–106. https://doi.org/10.1021/sb4001992.

De Paoli, H.C., Tuskan, G.A., Yang, X., 2016. An innovative platform for quick and flexible joining of assorted DNA fragments. Sci. Rep. 6, 19278. https://doi.org/10.1038/srep19278.

Deb, S.S., Reshamwala, S.M.S., 2020. Recent progress in DNA parts standardization and characterization. In: Advances in Synthetic Biology. Springer Singapore, pp. 43–69. https://doi.org/10.1007/978-981-15-0081-7_4.

Decoene, T., De Paepe, B., Maertens, J., Coussement, P., Peters, G., De Maeseneire, S.L., De Mey, M., 2018. Standardization in synthetic biology: an engineering discipline coming of age. Crit. Rev. Biotechnol. 38, 647–656. https://doi.org/10.1080/07388551.2017.1380600.

DeLorenzo, D.M., Rottinghaus, A.G., Henson, W.R., Moon, T.S., 2018. Molecular toolkit for gene expression control and genome modification in *Rhodococcus opacus* PD630. ACS Synth. Biol. 7, 727–738. https://doi.org/10.1021/acssynbio.7b00416.

Der, B.S., Glassey, E., Bartley, B.A., Enghuus, C., Goodman, D.B., Gordon, D.B., Voigt, C. A., Gorochowski, T.E., 2017. DNAplotlib: programmable visualization of genetic designs and associated data. ACS Synth. Biol. 6, 1115–1119. https://doi.org/10.1021/acssynbio.6b00252.

Ding, W., Weng, H., Jin, P., Du, G., Chen, J., Kang, Z., 2017. Scarless assembly of unphosphorylated DNA fragments with a simplified DATEL method. Bioengineered 8, 296–301. https://doi.org/10.1080/21655979.2017.1308986.

Donahue, P.S., Draut, J.W., Muldoon, J.J., Edelstein, H.I., Bagheri, N., Leonard, J.N., 2020. The COMET toolkit for composing customizable genetic programs in mammalian cells. Nat. Commun. 11, 779. https://doi.org/10.1038/s41467-019-14147-5.

Du, J., Yuan, Y., Si, T., Lian, J., Zhao, H., 2012. Customized optimization of metabolic pathways by combinatorial transcriptional engineering. Nucleic Acids Res. 40, 142. https://doi.org/10.1093/nar/gks549.

Dudley, Q.M., Nash, C.J., Jewett, M.C., 2019. Cell-free biosynthesis of limonene using enzyme-enriched *Escherichia coli* lysates. Synth. Biol. 4, ysz003. https://doi.org/10.1093/synbio/ysz003.

Elmore, J.R., Furches, A., Wolff, G.N., Gorday, K., Guss, A.M., 2017. Development of a high efficiency integration system and promoter library for rapid modification of *Pseudomonas putida* KT2440. Metab. Eng. Commun. 5, 1–8. https://doi.org/10.1016/j.meteno.2017.04.001.

Engler, C., Kandzia, R., Marillonnet, S., 2008. A one pot, one step, precision cloning method with high throughput capability. PLoS One 3, e3647. https://doi.org/10.1371/journal.pone.0003647.

Engler, C., Marillonnet, S., 2014. Golden gate cloning. Methods Mol. Biol. 1116, 119–131. https://doi.org/10.1007/978-1-62703-764-8_9.

Erian, A.M., Freitag, P., Gibisch, M., Pflügl, S., 2020. High rate 2,3-butanediol production with *Vibrio natriegens*. Bioresour. Technol. Reports 10, 100408. https://doi.org/10.1016/j.biteb.2020.100408.

Espah Borujeni, A., Channarasappa, A.S., Salis, H.M., 2014. Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. Nucleic Acids Res. 42, 2646–2659. https://doi.org/10.1093/nar/gkt1139.

Exley, K., Reynolds, C.R., Suckling, L., Chee, S.M., Tsipa, A., Freemont, P.S., McClymont, D., Kitney, R.I., 2019. Utilising datasheets for the informed automated design and build of a synthetic metabolic pathway. J. Biol. Eng. 13, 8. https://doi.org/10.1186/s13036-019-0141-z.

Fonseca, J.P., Bonny, A.R., Kumar, G.R., Ng, A.H., Town, J., Wu, Q.C., Aslankoohi, E., Chen, S.Y., Dods, G., Harrigan, P., Osimiri, L.C., Kistler, A.L., El-Samad, H., 2019. A toolkit for rapid modular construction of biological circuits in mammalian cells. ACS Synth. Biol. 8, 2593–2606. https://doi.org/10.1021/acssynbio.9b00322.

Fu, C., Donovan, W.P., Shikapwashya-Hasser, O., Ye, X., Cole, R.H., 2014. Hot fusion: an efficient method to clone multiple DNA fragments as well as inverted repeats without ligase. PLoS One 9, e115318. https://doi.org/10.1371/journal.pone.0115318.

Fuentes, P., Zhou, F., Erban, A., Karcher, D., Kopka, J., Bock, R., 2016. A new synthetic biology approach allows transfer of an entire metabolic pathway from a medicinal plant to a biomass crop. Elife 5, e13664. https://doi.org/10.7554/eLife.13664.

Ganesan, V., Li, Z., Wang, X., Zhang, H., 2017. Heterologous biosynthesis of natural product naringenin by co-culture engineering. Synth. Syst. Biotechnol. 2, 236–242. https://doi.org/10.1016/j.synbio.2017.08.003.

Gantner, J., Ordon, J., Ilse, T., Kretschmer, C., Gruetzner, R., Löfke, C., Dagdas, Y., Bürstenbinder, K., Marillonnet, S., Stuttmann, J., 2018. Peripheral infrastructure vectors and an extended set of plant parts for the Modular Cloning system. PLoS One 13, e0197185. https://doi.org/10.1371/journal.pone.0197185.

Geddes, B.A., Mendoza-Suárez, M.A., Poole, P.S., 2019. A bacterial expression vector archive (BEVA) for flexible modular assembly of golden gate-compatible vectors. Front. Microbiol. 9, 3345. https://doi.org/10.3389/fmicb.2018.03345.

Ghodasara, A., Voigt, C.A., 2017. Balancing gene expression without library construction via a reusable sRNA pool. Nucleic Acids Res. 45, 8116–8127. https://doi.org/10.1093/nar/gkx530.

Gibson, D.G., 2009. Synthesis of DNA fragments in yeast by one-step assembly of overlapping oligonucleotides. Nucleic Acids Res. 37, 6984–6990. https://doi.org/10.1093/nar/gkp687.

Gibson, D.G., Benders, G.A., Andrews-Pfannkoch, C., Denisova, E.A., Baden-Tillson, H., Zaveri, J., Stockwell, T.B., Brownley, A., Thomas, D.W., Algire, M.A., Merryman, C., Young, L., Noskov, V.N., Glass, J.I., Venter, J.C., Hutchison, C.A., Smith, H.O., 2008. Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. Science (80-.) 319, 1215–1220. https://doi.org/10.1126/science.1151721.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–345. https://doi.org/10.1038/nmeth.1318.

Gorochowski, T.E., Espah Borujeni, A., Park, Y., Nielsen, A.A., Zhang, J., Der, B.S., Gordon, D.B., Voigt, C.A., 2017. Genetic circuit characterization and debugging using RNA-seq. Mol. Syst. Biol. 13, 952. https://doi.org/10.15252/msb.20167461.

Goulev, Y., Matifas, A., Heyer, V., Reina-San-Martin, B., Charvin, G., 2019. COSPLAY: an expandable toolbox for combinatorial and swift generation of expression plasmids in yeast. PLoS One 14, e0220694. https://doi.org/10.1371/journal.pone.0220694.

Gowers, G.O.F., Cameron, S.J.S., Perdones-Montero, A., Bell, D., Chee, S.M., Kern, M., Tew, D., Ellis, T., Takáts, Z., 2019. Off-colony screening of biosynthetic libraries by rapid laser-enabled mass spectrometry. ACS Synth. Biol. 8, 2566–2575. https://doi.org/10.1021/acssynbio.9b00243.

Gräwe, A., Ranglack, J., Weyrich, A., Stein, V., 2020. iFLinkC: an iterative functional linker cloning strategy for the combinatorial assembly and recombination of linker peptides with functional domains. Nucleic Acids Res. 48, e24. https://doi.org/10.1093/nar/gkz1210.

Grubbe, W.S., Rasor, B.J., Krüger, A., Jewett, M.C., Karim, A.S., 2020. Cell-free styrene biosynthesis at high titers. Metab. Eng. 61, 89–95. https://doi.org/10.1016/j.ymben.2020.05.009.

Halleran, A.D., Swaminathan, A., Murray, R.M., 2018. Single day construction of multigene circuits with 3G Assembly. ACS Synth. Biol. 7, 1477–1480. https://doi.org/10.1021/acssynbio.8b00060.

HamediRad, M., Weisberg, S., Chao, R., Lian, J., Zhao, H., 2019. Highly efficient single-pot scarless Golden Gate assembly. ACS Synth. Biol. 8, 1047–1054. https://doi.org/10.1021/acssynbio.8b00480.

Han, L., Usher, S., Sandgrind, S., Hassall, K., Sayanova, O., Michaelson, L.V., Haslam, R. P., Napier, J.A., 2020. High level accumulation of EPA and DHA in field-grown transgenic *Camelina* – a multi-territory evaluation of TAG accumulation and heterogeneity. Plant Biotechnol. J. 18, 2280–2291. https://doi.org/10.1111/pbi.13385.

Hennig, S., Neubacher, S., 2019. Fluorescent RNA tags: current and future applications. Future Med. Chem. 11, 2483–2485. https://doi.org/10.4155/fmc-2019-0207.

Hernanz-Koers, M., Gandía, M., Garrigues, S., Manzanares, P., Yenush, L., Orzaez, D., Marcos, J.F., 2018. FungalBraid: a GoldenBraid-based modular cloning platform for the assembly and exchange of DNA elements tailored to fungal synthetic biology. Fungal Genet. Biol. 116, 51–61. https://doi.org/10.1016/j.fgb.2018.04.010.

Hong, J., Im, D.K., Oh, M.K., 2020. Investigating *E. coli* coculture for resveratrol production with 13C metabolic flux analysis. J. Agric. Food Chem. 68, 3466–3473. https://doi.org/10.1021/acs.jafc.9b07628.

Hossain, A., Lopez, E., Halper, S.M., Cetnar, D.P., Reis, A.C., Strickland, D., Klavins, E., Salis, H.M., 2020. Automated design of thousands of nonrepetitive parts for engineering stable genetic systems. Nat. Biotechnol. https://doi.org/10.1038/s41587-020-0584-2.

Hughes, R.A., Ellington, A.D., 2017. Synthetic DNA synthesis and assembly: putting the synthetic in synthetic biology. Cold Spring Harb. Perspect. Biol. 9, 1–17. https://doi.org/10.1101/cshperspect.a023812.

Iverson, S.V., Haddock, T.L., Beal, J., Densmore, D.M., 2016. CIDAR MoClo: improved MoClo assembly standard and new *E. coli* part library enable rapid combinatorial design for synthetic and traditional biology. ACS Synth. Biol. 5, 99–103. https://doi.org/10.1021/acssynbio.5b00124.

Jäckel, C., Nogueira, M.S., Ehni, N., Kraus, C., Ranke, J., Dohmann, M., Noessner, E., Nelson, P.J., 2016. A vector platform for the rapid and efficient engineering of stable complex transgenes. Sci. Rep. 6, 34365. https://doi.org/10.1038/srep34365.

Jajesniak, P., Tee, K.L., Wong, T.S., 2019. PTO-quickstep: a fast and efficient method for cloning random mutagenesis libraries. Int. J. Mol. Sci. 20, 3908. https://doi.org/10.3390/ijms20163908.

Jeong, J.Y., Yim, H.S., Ryu, J.Y., Lee, H.S., Lee, J.H., Seen, D.S., Kang, S.G., 2012. One-step sequence-and ligation-independent cloning as a rapid and versatile cloning method for functional genomics studies. Appl. Environ. Microbiol. 78, 5440–5443. https://doi.org/10.1128/AEM.00844-12.

Jeschek, M., Gerngross, D., Panke, S., 2017. Combinatorial pathway optimization for streamlined metabolic engineering. Curr. Opin. Biotechnol. 47, 142–151. https://doi.org/10.1016/j.copbio.2017.06.014.

Jeske, L., Placzek, S., Schomburg, I., Chang, A., Schomburg, D., 2019. BRENDA in 2019: a European ELIXIR core data resource. Nucleic Acids Res. 47, D542–D549. https://doi.org/10.1093/nar/gky1048.

Jessop-Fabre, M.M., Jakočiūnas, T., Stovicek, V., Dai, Z., Jensen, M.K., Keasling, J.D., Borodina, I., 2016. EasyClone-MarkerFree: a vector toolkit for marker-less integration of genes into *Saccharomyces cerevisiae* via CRISPR-Cas9. Biotechnol. J. 11, 1110–1117. https://doi.org/10.1002/biot.201600147.

Jessop-Fabre, M.M., Sonnenschein, N., 2019. Improving reproducibility in synthetic biology. Front. Bioeng. Biotechnol. 7, 18. https://doi.org/10.3389/fbioe.2019.00018.

Jiang, P., Fang, H., Zhao, J., Dong, H., Jin, Z., Zhang, D., 2020. Optimization of hydrogenobyrinic acid biosynthesis in *Escherichia coli* using multi-level metabolic engineering strategies. Microb. Cell Fact. 19, 118. https://doi.org/10.1186/s12934-020-01377-2.

Jin, P., Ding, W., Du, G., Chen, J., Kang, Z., 2016. DATEL: a scarless and sequence-independent DNA assembly method using thermostable exonucleases and ligase. ACS Synth. Biol. 5, 1028–1032. https://doi.org/10.1021/acssynbio.6b00078.

Jones, J.A., Vernacchio, V.R., Lachance, D.M., Lebovich, M., Fu, L., Shirke, A.N., Schultz, V.L., Cress, B., Linhardt, R.J., Koffas, M.A.G., 2015. EPathOptimize: a combinatorial approach for transcriptional balancing of metabolic pathways. Sci. Rep. 5, 11301. https://doi.org/10.1038/srep11301.

Jones, S., Martella, A., Cai, Y., 2019. EMMA assembly explained: a step-by-step guide to assemble synthetic mammalian vectors. In: Methods in Enzymology. Academic Press Inc., pp. 463–493. https://doi.org/10.1016/bs.mie.2018.12.017

Kahl, L., Molloy, J., Patron, N., Matthewman, C., Haseloff, J., Grewal, D., Johnson, R., Endy, D., 2018. Opening options for material transfer. Nat. Biotechnol. 36, 923–927. https://doi.org/10.1038/nbt.4263.

Kakui, Y., Sunaga, T., Arai, K., Dodgson, J., Ji, L., Csikász-Nagy, A., Carazo-Salas, R., Sato, M., 2015. Module-based construction of plasmids for chromosomal integration of the fission yeast *Schizosaccharomyces pombe*. Open Biol 5, 150054. https://doi.org/10.1098/rsob.150054.

Kang, W., Ma, T., Liu, M., Qu, Jiale, Liu, Z., Zhang, H., Shi, B., Fu, S., Ma, J., Lai, L.T.F., He, S., Qu, Jianan, Wing-Ngor Au, S., Ho Kang, B., Yu Lau, W.C., Deng, Z., Xia, J., Liu, T., 2019. Modular enzyme assembly for enhanced cascade biocatalysis and metabolic flux. Nat. Commun. 10, 4248. https://doi.org/10.1038/s41467-019-12247-w.

Kanigowska, P., Shen, Y., Zheng, Y., Rosser, S., Cai, Y., 2016. Smart DNA fabrication using sound waves: applying acoustic dispensing technologies to synthetic biology. J. Lab. Autom. 21, 49–56. https://doi.org/10.1177/2211068215593754.

Karim, A.S., Dudley, Q.M., Juminaga, A., Yuan, Y., Crowe, S.A., Heggestad, J.T., Garg, S., Abdalla, T., Grubbe, W.S., Rasor, B.J., Coar, D.N., Torculas, M., Krein, M., Liew, F.M. (Eric), Quattlebaum, A., Jensen, R.O., Stuart, J.A., Simpson, S.D., Köpke, M., Jewett, M.C., 2020. In vitro prototyping and rapid optimization of biosynthetic enzymes for cell design. Nat. Chem. Biol. 16, 912–919. https://doi.org/10.1038/s41589-020-0559-0.

Kelwick, R., MacDonald, J.T., Webb, A.J., Freemont, P., 2014. Developments in the tools and methodologies of synthetic biology. Front. Bioeng. Biotechnol. 2, 60. https://doi.org/10.3389/fbioe.2014.00060.

Khoo, K.K., Galleano, I., Gasparri, F., Wieneke, R., Harms, H., Poulsen, M.H., Chua, H.C., Wulf, M., Tampé, R., Pless, S.A., 2020. Chemical modification of proteins by insertion of synthetic peptides using tandem protein trans-splicing. Nat. Commun. 11, 2284. https://doi.org/10.1038/s41467-020-16208-6.

Kightlinger, W., Duncker, K.E., Ramesh, A., Thames, A.H., Natarajan, A., Stark, J.C., Yang, A., Lin, L., Mrksich, M., DeLisa, M.P., Jewett, M.C., 2019. A cell-free biosynthesis platform for modular construction of protein glycosylation pathways. Nat. Commun. 10, 5404. https://doi.org/10.1038/s41467-019-12024-9.

Kim, S.H., Cavaleiro, A.M., Rennig, M., Nørholm, M.H.H., 2016. SEVA Linkers: a versatile and automatable DNA backbone exchange standard for synthetic biology. ACS Synth. Biol. 5, 1177–1181. https://doi.org/10.1021/acssynbio.5b00257.

Klock, H.E., Koesema, E.J., Knuth, M.W., Lesley, S.A., 2008. Combining the polymerase incomplete primer extension method for cloning and mutagenesis with microscreening to accelerate structural genomics efforts. Proteins Struct. Funct. Genet. 71, 982–994. https://doi.org/10.1002/prot.21786.

Klock, H.E., Lesley, S.A., 2009. The polymerase incomplete primer extension (PIPE) method applied to high-throughput cloning and site-directed mutagenesis. In: Methods in Molecular Biology: High Throughput Protein Expression and Purification. Humana Press, pp. 91–103. https://doi.org/10.1007/978-1-59745-196-3_6.

Knight, T., 2003. Idempotent Vector Design for Standard Assembly of Biobricks. MIT Libr. http://hdl.handle.net/1721.1/21168.

Knoot, C.J., Ungerer, J., Wangikar, P.P., Pakrasi, H.B., 2018. Cyanobacteria: promising biocatalysts for sustainable chemical production. J. Biol. Chem. 293, 5044–5052. https://doi.org/10.1074/jbc.R117.815886.

Ko, Y.S., Kim, J.W., Chae, T.U., Song, C.W., Lee, S.Y., 2020. A novel biosynthetic pathway for the production of acrylic acid through β-alanine route in *Escherichia coli*. ACS Synth. Biol. 9, 1150–1159. https://doi.org/10.1021/acssynbio.0c00019.

Kong, D.S., Carr, P.A., Chen, L., Zhang, S., Jacobson, J.M., 2007. Parallel gene synthesis in a microfluidic device. Nucleic Acids Res. 35, 61. https://doi.org/10.1093/nar/gkm121.

Kopniczky, M.B., Canavan, C., McClymont, D.W., Crone, M.A., Suckling, L., Goetzmann, B., Siciliano, V., MacDonald, J.T., Jensen, K., Freemont, P.S., 2020. Cell-free protein synthesis as a prototyping platform for mammalian synthetic biology. ACS Synth. Biol. 9, 144–156. https://doi.org/10.1021/acssynbio.9b00437.

Kotera, I., Nagai, T., 2008. A high-throughput and single-tube recombination of crude PCR products using a DNA polymerase inhibitor and type IIS restriction enzyme. J. Biotechnol. 137, 1–7. https://doi.org/10.1016/j.jbiotec.2008.07.1816.

Kouprina, N., Larionov, V., 2016. Transformation-associated recombination (TAR) cloning for genomics studies and synthetic biology. Chromosoma 125, 621–632. https://doi.org/10.1007/s00412-016-0588-3.

Kunes, S., Botstein, D., Fox, M.S., 1985. Transformation of yeast with linearized plasmid DNA. Formation of inverted dimers and recombinant plasmid products. J. Mol. Biol. 184, 375–387. https://doi.org/10.1016/0022-2836(85)90288-8.

Lai, H.E., Moore, S., Polizzi, K., Freemont, P., 2018. EcoFlex: a multifunctional MoClo kit for *E. coli* synthetic biology. In: Methods in Molecular Biology, pp. 429–444. https://doi.org/10.1007/978-1-4939-7795-6_25.

Larroude, M., Park, Y., Soudier, P., Kubiak, M., Nicaud, J., Rossignol, T., 2019. A modular Golden Gate toolkit for *Yarrowia lipolytica* synthetic biology. Microb. Biotechnol. 12, 1249–1259. https://doi.org/10.1111/1751-7915.13427.

Lau, Y.H., Giessen, T.W., Altenburg, W.J., Silver, P.A., 2018. Prokaryotic nanocompartments form synthetic organelles in a eukaryote. Nat. Commun. 9, 1311. https://doi.org/10.1038/s41467-018-03768-x.

Le Feuvre, R.A., Scrutton, N.S., 2018. A living foundry for synthetic biological materials: a synthetic biology roadmap to new advanced materials. Synth. Syst. Biotechnol. 3, 105–112. https://doi.org/10.1016/j.synbio.2018.04.002.

Lee, H.H., Ostrov, N., Wong, B.G., Gold, M.A., Khalil, A.S., Church, G.M., 2019. Functional genomics of the rapidly replicating bacterium *Vibrio natriegens* by CRISPRi. Nat. Microbiol. 4, 1105–1113. https://doi.org/10.1038/s41564-019-0423-8.

Lee, M.E., DeLoache, W.C., Cervantes, B., Dueber, J.E., 2015. A highly characterized yeast toolkit for modular, multipart assembly. ACS Synth. Biol. 4, 975–986. https://doi.org/10.1021/sb500366v.

Leung, D.W., Chen, E., Goeddel, D.V., 1989. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. Technique 1, 11–15.

Li, G., Huang, Z., Zhang, C., Dong, B.J., Guo, R.H., Yue, H.W., Yan, L.T., Xing, X.H., 2016. Construction of a linker library with widely controllable flexibility for fusion protein design. Appl. Microbiol. Biotechnol. 100, 215–225. https://doi.org/10.1007/s00253-015-6985-3.

Li, L., Zhao, Y., Ruan, L., Yang, S., Ge, M., Jiang, W., Lu, Y., 2015. A stepwise increase in pristinamycin II biosynthesis by *Streptomyces pristinaespiralis* through combinatorial metabolic engineering. Metab. Eng. 29, 12–25. https://doi.org/10.1016/j.ymben.2015.02.001.

Li, M.Z., Elledge, S.J., 2012. SLIC: a method for sequence- and ligation-independent cloning. Methods Mol. Biol. 852, 51–59. https://doi.org/10.1007/978-1-61779-564-0_5.

Li, S.Y., Zhao, G.P., Wang, J., 2016. C-Brick: a new standard for assembly of biological parts using Cpf1. ACS Synth. Biol. 5, 1383–1388. https://doi.org/10.1021/acssynbio.6b00114.

Li, Z., Wang, X., Zhang, H., 2019. Balancing the non-linear rosmarinic acid biosynthetic pathway by modular co-culture engineering. Metab. Eng. 54, 1–11. https://doi.org/10.1016/j.ymben.2019.03.002.

Li, Z., Zhang, D., Xiong, X., Yan, B., Xie, W., Sheen, J., Li, J.F., 2017. A potent Cas9-derived gene activator for plant and mammalian cells. Nat. Plants 3, 930–936. https://doi.org/10.1038/s41477-017-0046-0.

Liang, J., Liu, Z., Low, X.Z., Ang, E.L., Zhao, H., 2017. Twin-primer non-enzymatic DNA assembly: an efficient and accurate multi-part DNA assembly method. Nucleic Acids Res. 45, e94. https://doi.org/10.1093/nar/gkx132.

Liang, L., Liu, R., Foster, K.E.O., Choudhury, A., Cook, S., Cameron, J.C., Srubar, W.V., Gill, R.T., 2020. Genome engineering of *E. coli* for improved styrene production. Metab. Eng. 57, 74–84. https://doi.org/10.1016/j.ymben.2019.09.007.

Lim, H.J., Kim, D.-M., 2019. Cell-free metabolic engineering: recent developments and future prospects. Methods Protoc 2, 33. https://doi.org/10.3390/mps2020033.

Lin, D., O'Callaghan, C.A., O'Callaghan, C.A., 2018. MetClo: methylase-assisted hierarchical DNA assembly using a single type IIS restriction enzyme. Nucleic Acids Res. 46, e113. https://doi.org/10.1093/nar/gky596.

Lindenburg, L., Huovinen, T., van de Wiel, K., Herger, M., Snaith, M.R., Hollfelder, F., 2020. Split & Mix assembly of DNA libraries for ultrahigh throughput on-bead screening of functional proteins. Nucleic Acids Res. 48, e63. https://doi.org/10.1093/nar/gkaa270.

Linshiz, G., Jensen, E., Stawski, N., Bi, C., Elsbree, N., Jiao, H., Kim, J., Mathies, R., Keasling, J.D., Hillson, N.J., 2016. End-to-end automated microfluidic platform for synthetic biology: from design to functional analysis. J. Biol. Eng. 10, 1–15. https://doi.org/10.1186/s13036-016-0024-5.

Linshiz, G., Stawski, N., Goyal, G., Bi, C., Poust, S., Sharma, M., Mutalik, V., Keasling, J.D., Hillson, N.J., 2014. PR-PR: cross-platform laboratory automation system. ACS Synth. Biol. 3, 515–524. https://doi.org/10.1021/sb4001728.

Linshiz, G., Stawski, N., Poust, S., Bi, C., Keasling, J.D., Hillson, N.J., 2013. PaR-PaR laboratory automation platform. ACS Synth. Biol. 2, 216–222. https://doi.org/10.1021/sb300075t.

Liow, L.T., Go, M.K., Chang, M.W., Yew, W.S., 2020. Toolkit development for cyanogenic and gold biorecovery chassis *Chromobacterium violaceum*. ACS Synth. Biol. 9, 953–961. https://doi.org/10.1021/acssynbio.0c00064.

Liu, D., Liu, H., Li, B.Z., Qi, H., Jia, B., Zhou, X., Du, H.X., Zhang, W., Yuan, Y.J., 2016. Multigene pathway engineering with regulatory linkers (M-PERL). ACS Synth. Biol. 5, 1535–1545. https://doi.org/10.1021/acssynbio.6b00123.

Liu, J.K., Chen, W.H., Ren, S.X., Zhao, G.P., Wang, J., 2014. IBrick: a new standard for iterative assembly of biological parts with homing endonucleases. PLoS One 9, e110852. https://doi.org/10.1371/journal.pone.0110852.

Liu, S., Xiao, H., Zhang, F., Lu, Z., Zhang, Y., Deng, A., Li, Z., Yang, C., Wen, T., 2019. A seamless and iterative DNA assembly method named PS-Brick and its assisted metabolic engineering for threonine and 1-propanol production. Biotechnol. Biofuels 12, 180. https://doi.org/10.1186/s13068-019-1520-x.

Liu, Yanfeng, Liu, L., Li, J., Du, G., Chen, J., 2019. Synthetic biology toolbox and chassis development in *Bacillus subtilis*. Trends Biotechnol. 37, 548–562. https://doi.org/10.1016/j.tibtech.2018.10.005.

Liu, Yang, Wan, X., Wang, B., 2019. Engineered CRISPRa enables programmable eukaryote-like gene activation in bacteria. Nat. Commun. 10, 1–16. https://doi.org/10.1038/s41467-019-11479-0.

Lopez, C., Zhao, Y., Masonbrink, R., Shao, Z., 2020. Modulating pathway performance by perturbing local genetic context. ACS Synth. Biol. 9, 706–717. https://doi.org/10.1021/acssynbio.9b00445.

Lu, H., Villada, J.C., Lee, P.K.H., 2019. Modular metabolic engineering for biobased chemical production. Trends Biotechnol. 37, 152–166. https://doi.org/10.1016/j.tibtech.2018.07.003.

Lv, X., Wu, Y., Tian, R., Gu, Y., Liu, Y., Li, J., Du, G., Ledesma-Amaro, R., Liu, L., 2020. Synthetic metabolic channel by functional membrane microdomains for compartmentalized flux control. Metab. Eng. 59, 106–118. https://doi.org/10.1016/j.ymben.2020.02.003.

Ma, X., Liang, H., Cui, X., Liu, Y., Lu, H., Ning, W., Poon, N.Y., Ho, B., Zhou, K., 2019. A standard for near-scarless plasmid construction using reusable DNA parts. Nat. Commun. 10, 1–12. https://doi.org/10.1038/s41467-019-11263-0.

Madsen, C., Goni Moreno, A., Palchick, Z., P, U., Roehner, N., Bartley, B., Bhatia, S., Bhakta, S., Bissell, M., Clancy, K., Cox, R.S., Gorochowski, T., Grunberg, R., Luna, A., McLaughlin, J., Nguyen, T., Le Novere, N., Pocock, M., Sauro, H., Scott-Brown, J., Sexton, J.T., Stan, G.-B., Tabor, J.J., Voigt, C.A., Zundel, Z., Myers, C., Beal, J., Wipat, A., 2019. Synthetic biology Open Language visual (SBOL visual) version 2.1. J. Integr. Bioinform. 16 https://doi.org/10.1515/jib-2018-0101.

Mann, D.G.J., Bevan, S.A., Harvey, A.J., Leffert-Sorenson, R.A., 2019. The use of an automated platform to assemble multigenic constructs for plant transformation. In: Methods in Molecular Biology. Humana Press Inc., pp. 19–35. https://doi.org/10.1007/978-1-4939-8778-8_2

Martella, A., Matjusaitis, M., Auxillos, J., Pollard, S.M., Cai, Y., 2017. EMMA: an extensible mammalian modular assembly toolkit for the rapid design and production of diverse expression vectors. ACS Synth. Biol. 6, 1380–1392. https://doi.org/10.1021/acssynbio.7b00016.

Martínez-García, E., Gõni-Moreno, A., Bartley, B., Mclaughlin, J., Sanchez-Sampedro, L., Pascual Del Pozo, H., Prieto Hernández, C., Marletta, A.S., De Lucrezia, D., Sanchez-Fernández, G., Fraile, S., De Lorenzo, V., 2020. Seva 3.0: an update of the Standard European Vector Architecture for enabling portability of genetic constructs among diverse bacterial hosts. Nucleic Acids Res. 48, D1164–D1170. https://doi.org/10.1093/nar/gkz1024.

McCarty, N.S., Ledesma-Amaro, R., 2019. Synthetic biology tools to engineer microbial communities for biotechnology. Trends Biotechnol. 37, 181–197. https://doi.org/10.1016/j.tibtech.2018.11.002.

Meyer, A.J., Segall-Shapiro, T.H., Glassey, E., Zhang, J., Voigt, C.A., 2019. *Escherichia coli* "Marionette" strains with 12 highly optimized small-molecule sensors. Nat. Chem. Biol. 15, 196–204. https://doi.org/10.1038/s41589-018-0168-3.

Minssen, T., Rutz, B., van Zimmeren, E., 2015. Synthetic biology and intellectual property rights: six recommendations. Biotechnol. J. 10, 236–241. https://doi.org/10.1002/biot.201400604.

Misirli, G., Beal, J., Gorochowski, T.E., Stan, G.B., Wipat, A., Myers, C.J., 2020. SBOL visual 2 ontology. ACS Synth. Biol. 9, 972–977. https://doi.org/10.1021/acssynbio.0c00046.

Misirli, G., Nguyen, T., McLaughlin, J.A., Vaidyanathan, P., Jones, T., Densmore, D., Myers, C.J., Wipat, A., 2019. A computational workflow for the automated generation of models of genetic designs. ACS Synth. Biol. 8, 1548–1559. https://doi.org/10.1021/acssynbio.7b00459.

Mitchell, L.A., Chuang, J., Agmon, N., Khunsriraksakul, C., Phillips, N.A., Cai, Y., Truong, D.M., Veerakumar, A., Wang, Y., Mayorga, M., Blomquist, P., Sadda, P., Trueheart, J., Boeke, J.D., 2015. Versatile genetic assembly system (VEGAS) to assemble pathways for expression in *S. cerevisiae*. Nucleic Acids Res. 43, 6620–6630. https://doi.org/10.1093/nar/gkv466.

Moazami, E., Perry, J.M., Soffer, G., Husser, M.C., Shih, S.C.C., 2019. Integration of world-to-chip interfaces with digital microfluidics for bacterial transformation and enzymatic assays. Anal. Chem. 91, 5159–5168. https://doi.org/10.1021/acs.analchem.8b05754.

Moore, S.J., Lai, H.-E., Kelwick, R.J.R., Chee, S.M., Bell, D.J., Polizzi, K.M., Freemont, P. S., 2016. EcoFlex: a multifunctional MoClo kit for *E. coli* synthetic biology. ACS Synth. Biol. 5, 1059–1069. https://doi.org/10.1021/acssynbio.6b00031.

Moore, S.J., MacDonald, J.T., Wienecke, S., Ishwarbhai, A., Tsipa, A., Aw, R., Kylilis, N., Bell, D.J., McClymont, D.W., Jensen, K., Polizzi, K.M., Biedendieck, R., Freemont, P. S., 2018. Rapid acquisition and model-based analysis of cell-free transcription–translation reactions from nonmodel bacteria. Proc. Natl. Acad. Sci. U. S.A. 115, 4340–4349. https://doi.org/10.1073/pnas.1715806115.

Moser, F., Espah Borujeni, A., Ghodasara, A.N., Cameron, E., Park, Y., Voigt, C.A., 2018. Dynamic control of endogenous metabolism with combinatorial logic circuits. Mol. Syst. Biol. 14, e8605 https://doi.org/10.15252/msb.20188605.

Motohashi, K., 2015. A simple and efficient seamless DNA cloning method using SLiCE from *Escherichia coli* laboratory strains and its application to SLiP site-directed mutagenesis. BMC Biotechnol. 15, 47. https://doi.org/10.1186/s12896-015-0162-8.

Naseri, G., Behrend, J., Rieper, L., Mueller-Roeber, B., 2019. COMPASS for rapid combinatorial optimization of biochemical pathways based on artificial transcription factors. Nat. Commun. 10, 2615. https://doi.org/10.1038/s41467-019-10224-x.

Naseri, G., Koffas, M.A.G., 2020. Application of combinatorial optimization strategies in synthetic biology. Nat. Commun. 11, 2446. https://doi.org/10.1038/s41467-020-16175-y.

Nielsen, J., Bubela, T., Chalmers, D.R.C., Johns, A., Kahl, L., Kamens, J., Lawson, C., Liddicoat, J., McWhirter, R., Monotti, A., Scheibner, J., Whitton, T., Nicol, D., 2018. Provenance and risk in transfer of biological materials. PLoS Biol. 16, e2006031 https://doi.org/10.1371/journal.pbio.2006031.

Nora, L.C., Westmann, C.A., Martins-Santana, L., Alves, L. de F., Monteiro, L.M.O., Guazzaroni, M.E., Silva-Rocha, R., 2019. The art of vector engineering: towards the construction of next-generation genetic tools. Microb. Biotechnol. 12, 125–147. https://doi.org/10.1111/1751-7915.13318.

Nyerges, Á., Bálint, B., Cseklye, J., Nagy, I., Pál, C., Feher, T., 2019. CRISPR-interference-based modulation of mobile genetic elements in bacteria. Synth. Biol. 4 https://doi.org/10.1093/synbio/ysz008.

Oberortner, E., Cheng, J.F., Hillson, N.J., Deutsch, S., 2017. Streamlining the design-to-build transition with build-optimization software tools. ACS Synth. Biol. 6, 485–496. https://doi.org/10.1021/acssynbio.6b00200.

Occhialini, A., Piatek, A.A., Pfotenhauer, A.C., Frazier, T.P., Stewart, C.N., Lenaghan, S. C., 2019. MoChlo: a versatile, modular cloning toolbox for chloroplast biotechnology. Plant Physiol. 179, 943–957. https://doi.org/10.1104/pp.18.01220.

Okegawa, Y., Motohashi, K., 2015. A simple and ultra-low cost homemade seamless ligation cloning extract (SLiCE) as an alternative to a commercially available seamless DNA cloning kit. Biochem. Biophys. Reports 4, 148–151. https://doi.org/10.1016/j.bbrep.2015.09.005.

Oliveira, T.L., Stedman, A., Rizzi, C., Dorneles, J., da Cunha, C.E.P., Junior, A.S.V., Dellagostin, O.A., McFadden, J., 2019. A standardized BioBrick toolbox for the assembly of sequences in mycobacteria. Tuberculosis 119, 101851. https://doi.org/10.1016/j.tube.2019.07.002.

Ortiz, L., Pavan, M., McCarthy, L., Timmons, J., Densmore, D.M., 2017. Automated robotic liquid handling assembly of modular DNA devices. J. Vis. Exp., e54703 https://doi.org/10.3791/54703.

Palluk, S., Arlow, D.H., De Rond, T., Barthel, S., Kang, J.S., Bector, R., Baghdassarian, H. M., Truong, A.N., Kim, P.W., Singh, A.K., Hillson, N.J., Keasling, J.D., 2018. De novo DNA synthesis using polymerase-nucleotide conjugates. Nat. Biotechnol. 36, 645–650. https://doi.org/10.1038/nbt.4173.

Park, J., Yu, B.J., Choi, J. Il, Woo, H.M., 2019. Heterologous production of squalene from glucose in engineered *Corynebacterium glutamicum* using multiplex CRISPR Interference and high-throughput fermentation. J. Agric. Food Chem. 67, 308–319. https://doi.org/10.1021/acs.jafc.8b05818.

Patron, N.J., Orzaez, D., Marillonnet, S., Warzecha, H., Matthewman, C., Youles, M., Raitskin, O., Leveau, A., Farré, G., Rogers, C., Smith, A., Hibberd, J., Webb, A.A.R., Locke, J., Schornack, S., Ajioka, J., Baulcombe, D.C., Zipfel, C., Kamoun, S., Jones, J. D.G., Kuhn, H., Robatzek, S., Van Esse, H.P., Sanders, D., Oldroyd, G., Martin, C., Field, R., O'Connor, S., Fox, S., Wulff, B., Miller, B., Breakspear, A., Radhakrishnan, G., Delaux, P.M., Loqué, D., Granell, A., Tissier, A., Shih, P., Brutnell, T.P., Quick, W.P., Rischer, H., Fraser, P.D., Aharoni, A., Raines, C., South, P.F., Ané, J.M., Hamberger, B.R., Langdale, J., Stougaard, J., Bouwmeester, H., Udvardi, M., Murray, J.A.H., Ntoukakis, V., Schäfer, P., Denby, K., Edwards, K.J., Osbourn, A., Haseloff, J., 2015. Standards for plant synthetic biology: a common syntax for exchange of DNA parts. New Phytol. 208, 13–19. https://doi.org/10.1111/nph.13532.

Perkel, J.M., 2019. The race for enzymatic DNA synthesis heats up. Nature 566, 565. https://doi.org/10.1038/d41586-019-00682-0.

Pervouchine, D., 2018. Towards long-range RNA structure prediction in eukaryotic genes. Genes (Basel). 9, 302. https://doi.org/10.3390/genes9060302.

Plesa, C., Sidore, A.M., Lubock, N.B., Zhang, D., Kosuri, S., 2018. Multiplexed gene synthesis in emulsions for exploring protein functional landscapes. Science 359, 343–347. https://doi.org/10.1126/science.aao5167.

Poliner, E., Clark, E., Cummings, C., Benning, C., Farre, E.M., 2020. A high-capacity gene stacking toolkit for the oleaginous microalga, *Nannochloropsis oceanica* CCMP1779. Algal Res 45, 101664. https://doi.org/10.1016/j.algal.2019.101664.

Pollak, B., Cerda, A., Delmans, M., Álamos, S., Moyano, T., West, A., Gutiérrez, R.A., Patron, N.J., Federici, F., Haseloff, J., 2019. Loop assembly: a simple and open system for recursive fabrication of DNA circuits. New Phytol. 222, 628–640. https://doi.org/10.1111/nph.15625.

Pollak, B., Matute, T., Nuñez, I., Cerda, A., Lopez, C., Vargas, V., Kan, A., Bielinski, V., von Dassow, P., Dupont, C.L., Federici, F., 2020. Universal loop assembly: open, efficient and cross-kingdom DNA fabrication. Synth. Biol. (Oxford, England) 5, ysaa001. https://doi.org/10.1093/synbio/ysaa001.

Popp, P.F., Dotzler, M., Radeck, J., Bartels, J., Mascher, T., 2017. The *Bacillus* BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with *Bacillus subtilis*. Sci. Rep. 7, 1–13. https://doi.org/10.1038/s41598-017-15107-z.

Potapov, V., Ong, J.L., Kucera, R.B., Langhorst, B.W., Bilotti, K., Pryor, J.M., Cantor, E.J., Canton, B., Knight, T.F., Evans, T.C., Lohman, G.J.S., 2018a. Comprehensive profiling of four base overhang ligation fidelity by T4 DNA ligase and application to DNA assembly. ACS Synth. Biol. 7, 2665–2674. https://doi.org/10.1021/acssynbio.8b00333.

Potapov, V., Ong, J.L., Langhorst, B.W., Bilotti, K., Cahoon, D., Canton, B., Knight, T.F., Evans, T.C., Lohman, G.J.S., 2018b. A single-molecule sequencing assay for the comprehensive profiling of T4 DNA ligase fidelity and bias during DNA end-joining. Nucleic Acids Res. 46, e79–e79 https://doi.org/10.1093/nar/gky303.

Presnell, K.V., Flexer-Harrison, M., Alper, H.S., 2019. Design and synthesis of synthetic UP elements for modulation of gene expression in *Escherichia coli*. Synth. Syst. Biotechnol. 4, 99–106. https://doi.org/10.1016/j.synbio.2019.04.002.

Prielhofer, R., Barrero, J.J., Steuer, S., Gassler, T., Zahrl, R., Baumann, K., Sauer, M., Mattanovich, D., Gasser, B., Marx, H., 2017. GoldenPiCS: a Golden Gate-derived modular cloning system for applied synthetic biology in the yeast *Pichia pastoris*. BMC Syst. Biol. 11, 123. https://doi.org/10.1186/s12918-017-0492-3.

Quan, J., Saaem, I., Tang, N., Ma, S., Negre, N., Gong, H., White, K.P., Tian, J., 2011. Parallel on-chip gene synthesis and application to optimization of protein expression. Nat. Biotechnol. 29, 449–452. https://doi.org/10.1038/nbt.1847.

Quan, J., Tian, J., 2011. Circular polymerase extension cloning for high-throughput cloning of complex and combinatorial DNA libraries. Nat. Protoc. 6, 242–251. https://doi.org/10.1038/nprot.2010.181.

Quan, J., Tian, J., 2009. Circular polymerase extension cloning of complex gene libraries and pathways. PLoS One 4, e6441. https://doi.org/10.1371/journal.pone.0006441.

Rajakumar, P.D., Gowers, G.-O.F., Suckling, L., Foster, A., Ellis, T., Kitney, R.I., McClymont, D.W., Freemont, P.S., 2019. Rapid prototyping platform for *Saccharomyces cerevisiae* using computer-aided genetic design enabled by parallel software and workcell platform development. SLAS Technol 24, 291–297. https://doi.org/10.1177/2472630318798304.

Rajkumar, A.S., Varela, J.A., Juergens, H., Daran, J.-M.G., Morrissey, J.P., 2019. Biological parts for *Kluyveromyces marxianus* synthetic biology. Front. Bioeng. Biotechnol. 7, 97. https://doi.org/10.3389/fbioe.2019.00097.

Rebatchouk, D., Daraselia, N., Narita, J.O., 1996. NOMAD: a versatile strategy for in vitro DNA manipulation applied to promoter analysis and vector design. Proc. Natl. Acad. Sci. U.S.A. 93, 10891–10896. https://doi.org/10.1073/pnas.93.20.10891.

Reed, K.B., Wagner, J.M., d'Oelsnitz, S., Wiggers, J.M., Alper, H.S., 2019. Improving ionic liquid tolerance in *Saccharomyces cerevisiae* through heterologous expression and directed evolution of an ILT1 homolog from *Yarrowia lipolytica*. J. Ind. Microbiol. Biotechnol. 46, 1715–1724. https://doi.org/10.1007/s10295-019-02228-9.

Ren, J., Lee, J., Na, D., 2020. Recent advances in genetic engineering tools based on synthetic biology. J. Microbiol. 58, 1–10. https://doi.org/10.1007/s12275-020-9334-x.

Richardson, S.M., Mitchell, L.A., Stracquadanio, G., Yang, K., Dymond, J.S., DiCarlo, J.E., Lee, D., Huang, C.L.V., Chandrasegaran, S., Cai, Y., Boeke, J.D., Bader, J.S., 2017. Design of a synthetic yeast genome. Science 355, 1040–1044. https://doi.org/10.1126/science.aaf4557.

Richter, D., Bayer, K., Toesko, T., Schuster, S., 2019. ZeBRα a universal, multi-fragment DNA-assembly-system with minimal hands-on time requirement. Sci. Rep. 9, 1–16. https://doi.org/10.1038/s41598-019-39768-0.

Rodrigues, J.L., Gomes, D., Rodrigues, L.R., 2020. A combinatorial approach to optimize the production of curcuminoids from tyrosine in *Escherichia coli*. Front. Bioeng. Biotechnol. 8, 59. https://doi.org/10.3389/fbioe.2020.00059.

Roehner, N., Young, E.M., Voigt, C.A., Gordon, D.B., Densmore, D., 2016. Double Dutch: a tool for designing combinatorial libraries of biological systems. ACS Synth. Biol. 5, 507–517. https://doi.org/10.1021/acssynbio.5b00232.

Rondon, R.E., Groseclose, T.M., Short, A.E., Wilson, C.J., 2019. Transcriptional programming using engineered systems of transcription factors and genetic architectures. Nat. Commun. 10, 1–13. https://doi.org/10.1038/s41467-019-12706-4.

Ros, E., Torres, A.G., Ribas de Pouplana, L., 2020. Learning from nature to expand the genetic code. Trends Biotechnol. https://doi.org/10.1016/j.tibtech.2020.08.003.

Rugbjerg, P., Myling-Petersen, N., Porse, A., Sarup-Lytzen, K., Sommer, M.O.A., 2018. Diverse genetic error modes constrain large-scale bio-based production. Nat. Commun. 9, 787. https://doi.org/10.1038/s41467-018-03232-w.

Saleski, T.E., Kerner, A.R., Chung, M.T., Jackman, C.M., Khasbaatar, A., Kurabayashi, K., Lin, X.N., 2019. Syntrophic co-culture amplification of production phenotype for high-throughput screening of microbial strain libraries. Metab. Eng. 54, 232–243. https://doi.org/10.1016/j.ymben.2019.04.007.

Sarrion-Perdigones, A., Falconi, E.E., Zandalinas, S.I., Juárez, P., Fernández-del-Carmen, A., Granell, A., Orzaez, D., 2011. GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. PLoS One 6, e21622. https://doi.org/10.1371/journal.pone.0021622.

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J., Castelijns, B., Forment, J., Ziarsolo, P., Blanca, J., Granell, A., Orzaez, D., 2013. Goldenbraid 2.0: a comprehensive DNA assembly framework for plant synthetic biology. Plant Physiol. 162, 1618–1631. https://doi.org/10.1104/pp.113.217661.

Schefer, Q., Hallmann, S., Grötzinger, C., 2014. Knockin' on pHeaven's door: a fast and reliable high-throughput compatible zero-background cloning procedure. Mol. Biotechnol. 56, 449–458. https://doi.org/10.1007/s12033-014-9736-2.

Schmid-Burgk, J.L., Xie, Z., Benenson, Y., 2014. Hierarchical ligation-independent assembly of PCR fragments. Methods Mol. Biol. 1116, 49–58. https://doi.org/10.1007/978-1-62703-764-8_4.

Schmid-Burgk, J.L., Xie, Z., Frank, S., Virreira Winter, S., Mitschka, S., Kolanus, W., Murray, A., Benenson, Y., 2012. Rapid hierarchical assembly of medium-size DNA cassettes. Nucleic Acids Res. 40, e92.

Schmidt-Dannert, C., Lopez-Gallego, F., 2016. A roadmap for biocatalysis – functional and spatial orchestration of enzyme cascades. Microb. Biotechnol. 9, 601–609. https://doi.org/10.1111/1751-7915.12386.

Schulz, P., Piepenburg, K., Lintermann, R., Herde, M., Schöttler, M.A., Schmidt, L.K., Ruf, S., Kudla, J., Romeis, T., Bock, R., 2020. Improving plant drought tolerance and growth under water limitation through combinatorial engineering of signalling networks. Plant Biotechnol. J. pbi 13441. https://doi.org/10.1111/pbi.13441.

Segall-Shapiro, T.H., Sontag, E.D., Voigt, C.A., 2018. Engineered promoters enable constant gene expression at any copy number in bacteria. Nat. Biotechnol. 36, 352–358. https://doi.org/10.1038/nbt.4111.

Seifert, S., Brakmann, S., 2018. LOV domains in the design of photoresponsive enzymes. ACS Chem. Biol. 13, 1914–1920. https://doi.org/10.1021/acschembio.8b00159.

Selma, S., Bernabé-Orts, J.M., Vazquez-Vilar, M., Diego-Martin, B., Ajenjo, M., Garcia-Carpintero, V., Granell, A., Orzaez, D., 2019. Strong gene activation in plants with genome-wide specificity using a new orthogonal CRISPR/Cas9-based programmable transcriptional activator. Plant Biotechnol. J. 17, 1703–1705. https://doi.org/10.1111/pbi.13138.

Seo, S.W., Yang, J.S., Kim, I., Yang, J., Min, B.E., Kim, S., Jung, G.Y., 2013. Predictive design of mRNA translation initiation region to control prokaryotic translation efficiency. Metab. Eng. 15, 67–74. https://doi.org/10.1016/j.ymben.2012.10.006.

Sgobba, E., Wendisch, V.F., 2020. Synthetic microbial consortia for small molecule production. Curr. Opin. Biotechnol. 62, 72–79. https://doi.org/10.1016/j.copbio.2019.09.011.

Shao, Z., Zhao, Hua, Zhao, Huimin, 2009. DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Res. 37, 16. https://doi.org/10.1093/nar/gkn991.

Shapland, E.B., Holmes, V., Reeves, C.D., Sorokin, E., Durot, M., Platt, D., Allen, C., Dean, J., Serber, Z., Newman, J., Chandran, S., 2015. Low-cost, high-throughput sequencing of DNA assemblies using a highly multiplexed Nextera process. ACS Synth. Biol. 4, 860–866. https://doi.org/10.1021/sb500362n.

Shen, R., Yin, J., Ye, J.W., Xiang, R.J., Ning, Z.Y., Huang, W.Z., Chen, G.Q., 2018. Promoter engineering for enhanced P(3HB- co-4HB) production by *Halomonas bluephagenesis*. ACS Synth. Biol. 7, 1897–1906. https://doi.org/10.1021/acssynbio.8b00102.

Shetty, R., Lizarazo, M., Rettberg, R., Knight, T.F., 2011. Assembly of BioBrick standard biological parts using three antibiotic assembly. In: Methods in Enzymology. https://doi.org/10.1016/B978-0-12-385120-8.00013-9.

Shih, S.C.C., Goyal, G., Kim, P.W., Koutsoubelis, N., Keasling, J.D., Adams, P.D., Hillson, N.J., Singh, A.K., 2015. A versatile microfluidic device for automating synthetic biology. ACS Synth. Biol. 4, 1151–1164. https://doi.org/10.1021/acssynbio.5b00062.

Shilling, P.J., Mirzadeh, K., Cumming, A.J., Widesheim, M., Köck, Z., Daley, D.O., 2020. Improved designs for pET expression plasmids increase protein production yield in *Escherichia coli*. Commun. Biol. 3, 214. https://doi.org/10.1038/s42003-020-0939-8.

Shola, D.T.N., Yang, C., Kewaldar, V.-S., Kar, P., Bustos, V., 2020. New additions to the CRISPR toolbox: CRISPR-CLONInG and CRISPR-CLIP for donor construction in genome editing. Cris. J. 3, 109–122. https://doi.org/10.1089/crispr.2019.0062.

Silverman, A.D., Akova, U., Alam, K.K., Jewett, M.C., Lucks, J.B., 2020. Design and optimization of a cell-free atrazine biosensor. ACS Synth. Biol. 9, 671–677. https://doi.org/10.1021/acssynbio.9b00388.

Sleight, S.C., Bartley, B.A., Lieviant, J.A., Sauro, H.M., 2010. Designing and engineering evolutionary robust genetic circuits. J. Biol. Eng. 4, 12. https://doi.org/10.1186/1754-1611-4-12.

Smanski, M.J., Bhatia, S., Zhao, D., Park, Y.J., Woodruff, L.B.A., Giannoukos, G., Ciulla, D., Busby, M., Calderon, J., Nicol, R., Gordon, D.B., Densmore, D., Voigt, C.A., 2014. Functional optimization of gene clusters by combinatorial design and assembly. Nat. Biotechnol. 32, 1241–1249. https://doi.org/10.1038/nbt.3063.

Srinivasan, P., Smolke, C.D., 2020. Biosynthesis of medicinal tropane alkaloids in yeast. Nature 585, 614–619. https://doi.org/10.1038/s41586-020-2650-9.

Staal, J., Alci, K., Schamphelaire, W. De, Vanhoucke, M., Beyaert, R., 2019. Engineering a minimal cloning vector from a pUC18 plasmid backbone with an extended multiple cloning site. Biotechniques 66, 254–259. https://doi.org/10.2144/btn-2019-0014.

Stephens, K., Pozo, M., Tsao, C.Y., Hauk, P., Bentley, W.E., 2019. Bacterial co-culture with cell signaling translator and growth controller modules for autonomously regulated culture composition. Nat. Commun. 10, 1–11. https://doi.org/10.1038/s41467-019-12027-6.

Storch, M., Casini, A., Mackrow, B., Ellis, T., Baldwin, G.S., 2017. BASIC: a simple and accurate modular DNA assembly method. In: Methods in Molecular Biology. https://doi.org/10.1007/978-1-4939-6343-0_6.

Storch, M., Casini, A., Mackrow, B., Fleming, T., Trewhitt, H., Ellis, T., Baldwin, G.S., 2015. BASIC: a new biopart assembly standard for idempotent cloning provides accurate, single-tier DNA assembly for synthetic biology. ACS Synth. Biol. 4, 781–787. https://doi.org/10.1021/sb500356d.

Storch, M., Haines, M.C., Baldwin, G.S., 2020. DNA-BOT: a low-cost, automated DNA assembly platform for synthetic biology. Synth. Biol. 5, ysaa010. https://doi.org/10.1101/832139.

Szymanski, E., Scher, E., 2019. Models for DNA design tools: the trouble with metaphors is that they don't go away. ACS Synth. Biol. 8, 2635–2641. https://doi.org/10.1021/acssynbio.9b00302.

Tarozzi, A., Angeloni, C., Malaguti, M., Morroni, F., Hrelia, S., Hrelia, P., 2013. Sulforaphane as a potential protective phytochemical against neurodegenerative diseases. Oxid. Med. Cell. Longev., 415078 https://doi.org/10.1155/2013/415078.

Taylor, G.M., Heap, J.T., 2020. Combinatorial metabolic engineering platform enabling stable overproduction of lycopene from carbon dioxide by cyanobacteria. bioRxiv 3 (11), 983833. https://doi.org/10.1101/2020.03.11.983833.

Taylor, G.M., Mordaka, P.M.M., Heap, J.T., 2019. Start-Stop Assembly: a functionally scarless DNA assembly system optimized for metabolic engineering. Nucleic Acids Res. 47, e17. https://doi.org/10.1093/nar/gky1182.

Torella, J.P., Boehm, C.R., Lienert, F., Chen, J.H., Way, J.C., Silver, P.A., 2014. Rapid construction of insulated genetic circuits via synthetic sequence-guided isothermal assembly. Nucleic Acids Res. 42, 681–689. https://doi.org/10.1093/nar/gkt860.

Tóth, E., Huszár, K., Bencsura, P., Kulcsár, P.I., Vodicska, B., Nyeste, A., Welker, Z., Tóth, S., Welker, E., 2014. Restriction enzyme body doubles and PCR cloning: on the general use of Type IIS restriction enzymes for cloning. PLoS One 9, e90896. https://doi.org/10.1371/journal.pone.0090896.

Trubitsyna, M., Liu, C.K., Salinas, A., Elfick, A., French, C.E., 2017. Paperclip: a simple method for flexible multi-part DNA assembly. In: Methods in Molecular Biology. Humana Press Inc., pp. 111–128. https://doi.org/10.1007/978-1-4939-6343-0_9

Trubitsyna, M., Michlewski, G., Cai, Y., Elfick, A., French, C.E., 2014. PaperClip: rapid multi-part DNA assembly from existing libraries. Nucleic Acids Res. 42, e154. https://doi.org/10.1093/nar/gku829.

Tschirhart, T., Shukla, V., Kelly, E.E., Schultzhaus, Z., Newringeisen, E., Erickson, J.S., Wang, Z., Garcia, W., Curl, E., Egbert, R.G., Yeung, E., Vora, G.J., 2019. Synthetic biology tools for the fast-growing marine bacterium *Vibrio natriegens*. ACS Synth. Biol. 8, 2069–2079. https://doi.org/10.1021/acssynbio.9b00176.

Umenhoffer, K., Draskovits, G., Nyerges, Á., Karcagi, I., Bogos, B., Tímár, E., Csörgő, B., Herczeg, R., Nagy, I., Fehér, T., Pál, C., Pósfai, G., 2017. Genome-wide abolishment of mobile genetic elements using genome shuffling and CRISPR/Cas-assisted MAGE allows the efficient stabilization of a bacterial chassis. ACS Synth. Biol. 6, 1471–1483. https://doi.org/10.1021/acssynbio.6b00378.

Van Dolleweerd, C.J., Kessans, S.A., Van De Bittner, K.C., Bustamante, L.Y., Bundela, R., Scott, B., Nicholson, M.J., Parker, E.J., 2018. MIDAS: a modular DNA assembly system for synthetic biology. ACS Synth. Biol. 7, 1018–1029. https://doi.org/10.1021/acssynbio.7b00363.

Van Hove, B., Guidi, C., De Wannemaeker, L., Maertens, J., De Mey, M., 2017. Recursive DNA assembly using protected oligonucleotide duplex assisted cloning (PODAC). ACS Synth. Biol. 6, 943–949. https://doi.org/10.1021/acssynbio.7b00017.

Vasudevan, R., Gale, G.A.R., Schiavon, A.A., Puzorjov, A., Malin, J., Gillespie, M.D., Vavitsas, K., Zulkower, V., Wang, B., Howe, C.J., Lea-Smith, D.J., McCormick, A.J., 2019. Cyanogate: a modular cloning suite for engineering cyanobacteria based on the plant MoClo syntax. Plant Physiol. 180, 39–55. https://doi.org/10.1104/pp.18.01401.

Vazquez-Vilar, M., Quijano-Rubio, A., Fernandez-Del-Carmen, A., Sarrion-Perdigones, A., Ochoa-Fernandez, R., Ziarsolo, P., Jos, J., Blanca, J., Granell, A., Orzaez, D., 2017. GB3.0: a platform for plant bio-design that connects functional DNA elements with associated biological data. Nucleic Acids Res. 45, 2196–2209. https://doi.org/10.1093/nar/gkw1326.

Vazquez-Vilar, M., Gandía, M., García-Carpintero, V., Marqués, E., Sarrion-Perdigones, A., Yenush, L., Polaina, J., Manzanares, P., Marcos, J.F., Orzaez, D., 2020. Multigene engineering by GoldenBraid cloning: from plants to filamentous fungi and beyond. Curr. Protoc. Mol. Biol. 130, e116. https://doi.org/10.1002/cpmb.116.

Vecchione, S., Fritz, G., 2019. CRIMoClo plasmids for modular assembly and orthogonal chromosomal integration of synthetic circuits in *Escherichia coli*. J. Biol. Eng. 13, 92. https://doi.org/10.1186/s13036-019-0218-8.

Volke, D.C., Turlin, J., Mol, V., Nikel, P.I., 2020. Physical decoupling of XylS/*Pm* regulatory elements and conditional proteolysis enable precise control of gene expression in *Pseudomonas putida*. Microb. Biotechnol. 13, 222–232. https://doi.org/10.1111/1751-7915.13383.

Walsh, D.I., Pavan, M., Ortiz, L., Wick, S., Bobrow, J., Guido, N.J., Leinicke, S., Fu, D., Pandit, S., Qin, L., Carr, P.A., Densmore, D., 2019. Standardizing automated DNA assembly: best practices, metrics, and protocols using robots. SLAS Technol. Transl. Life Sci. Innov. 24, 282–290. https://doi.org/10.1177/2472630318825335.

Wang, J., Xu, R., Liu, A., 2014. IRDL cloning: a one-tube, zero-background, easy-to-use, directional cloning method improves throughput in recombinant DNA preparation. PLoS One 9, e107907. https://doi.org/10.1371/journal.pone.0107907.

Wang, R.Y., Shi, Z.Y., Guo, Y.Y., Chen, J.C., Chen, G.Q., 2013. DNA fragments assembly based on nicking enzyme system. PLoS One 8, e57943. https://doi.org/10.1371/journal.pone.0057943.

Weber, E., Birkenfeld, J., Franz, J., Gritzan, U., Linden, L., Trautwein, M., 2017. Modular Protein Expression Toolbox (MoPET), a standardized assembly system for defined expression constructs and expression optimization libraries. PLoS One 12, e0176314. https://doi.org/10.1371/journal.pone.0176314.

Weber, E., Engler, C., Gruetzner, R., Werner, S., Marillonnet, S., 2011. A modular cloning system for standardized assembly of multigene constructs. PLoS One 6, e16765. https://doi.org/10.1371/journal.pone.0016765.

Werner, S., Engler, C., Weber, E., Gruetzner, R., Marillonnet, S., 2012. Fast track assembly of multigene constructs using Golden Gate cloning and the MoClo system. Bioengineered 3, 38–43. https://doi.org/10.4161/bbug.3.1.18223.

Wiltschi, B., Cernava, T., Dennig, A., Galindo Casas, M., Geier, M., Gruber, S., Haberbauer, M., Heidinger, P., Herrero Acero, E., Kratzer, R., Luley-Goedl, C., Müller, C.A., Pitzer, J., Ribitsch, D., Sauer, M., Schmölzer, K., Schnitzhofer, W., Sensen, C.W., Soh, J., Steiner, K., Winkler, C.K., Winkler, M., Wriessnegger, T., 2020. Enzymes revolutionize the bioproduction of value-added compounds: from enzyme discovery to special applications. Biotechnol. Adv. 40, 107520 https://doi.org/10.1016/j.biotechadv.2020.107520.

Windram, O.P.F., Rodrigues, R.T.L., Lee, S., Haines, M., Bayer, T.S., 2017. Engineering microbial phenotypes through rewiring of genetic networks. Nucleic Acids Res. 45, 4984–4993. https://doi.org/10.1093/nar/gkx197.

Wong, L., Engel, J., Jin, E., Holdridge, B., Xu, P., 2017. YaliBricks, a versatile genetic toolkit for streamlined and rapid pathway engineering in *Yarrowia lipolytica*. Metab. Eng. Commun. 5, 68–77. https://doi.org/10.1016/j.meteno.2017.09.001.

Woodruff, L.B.A., Gorochowski, T.E., Roehner, N., Mikkelsen, T.S., Densmore, D., Gordon, D.B., Nicol, R., Voigt, C.A., 2017. Registry in a tube: multiplexed pools of retrievable parts for genetic design space exploration. Nucleic Acids Res. 45, 1553–1565. https://doi.org/10.1093/nar/gkw1226.

Wu, Y., Zhu, R.Y., Mitchell, L.A., Ma, L., Liu, R., Zhao, M., Jia, B., Xu, H., Li, Y.X., Yang, Z.M., Ma, Y., Li, X., Liu, H., Liu, D., Xiao, W.H., Zhou, X., Li, B.Z., Yuan, Y.J., Boeke, J.D., 2018. In vitro DNA SCRaMbLE. Nat. Commun. 9, 1935. https://doi.org/10.1038/s41467-018-03743-6.

Wu, Z., Zhao, D., Li, S., Wang, J., Bi, C., Zhang, X., 2019. Combinatorial modulation of initial codons for improved zeaxanthin synthetic pathway efficiency in *Escherichia coli*. Microbiologyopen 8, e930. https://doi.org/10.1002/mbo3.930.

Xia, Y., Li, K., Li, J., Wang, T., Gu, L., Xun, L., 2019. T5 exonuclease-dependent assembly offers a low-cost method for efficient cloning and site-directed mutagenesis. Nucleic Acids Res. 47, e15. https://doi.org/10.1093/nar/gky1169.

Yamazaki, K., de Mora, K., Saitoh, K., 2017. BioBrick-based 'quick gene assembly' in vitro. Synth. Biol. 2, ysx003. https://doi.org/10.1093/synbio/ysx003.

Yan, P., Zeng, Y., Shen, W., Tuo, D., Li, X., Zhou, P., 2020. Nimble Cloning: a simple, versatile, and efficient system for standardized molecular cloning. Front. Bioeng. Biotechnol. 7, 460. https://doi.org/10.3389/fbioe.2019.00460.

Yang, H., Qin, J., Wang, X., Yu, B., 2020. Production of plant-derived anticancer precursor glucoraphanin in chromosomally engineered *Escherichia coli*. Microbiol. Res. 238, 126484 https://doi.org/10.1016/j.micres.2020.126484.

Yehezkel, T. Ben, Rival, A., Raz, O., Cohen, R., Marx, Z., Camara, M., Dubern, J.-F.F., Koch, B., Heeb, S., Krasnogor, N., Delattre, C., Shapiro, E., Ben Yehezkel, T., Rival, A., Raz, O., Cohen, R., Marx, Z., Camara, M., Dubern, J.-F.F., Koch, B., Heeb, S., Krasnogor, N., Delattre, C., Shapiro, E., Yehezkel, T., Ben Rival, A., Raz, O., Cohen, R., Marx, Z., Camara, M., Dubern, J.-F.F., Koch, B., Heeb, S., Krasnogor, N., Delattre, C., Shapiro, E., 2016. Synthesis and cell-free cloning of DNA libraries using programmable microfluidics. Nucleic Acids Res. 44 https://doi.org/10.1093/nar/gkv1087 e35–e35.

Yim, S.S., Johns, N.I., Park, J., Gomes, A.L., McBee, R.M., Richardson, M., Ronda, C., Chen, S.P., Garenne, D., Noireaux, V., Wang, H.H., 2019. Multiplex transcriptional characterizations across diverse bacterial species using cell-free systems. Mol. Syst. Biol. 15, e8875 https://doi.org/10.15252/msb.20198875.

Young, E.M., Zhao, Z., Gielesen, B.E.M., Wu, L., Gordon, D.B., Roubos, J.A., Voigt, C.A., 2018. Iterative algorithm-guided design of massive strain libraries, applied to itaconic acid production in yeast. Metab. Eng. 48, 33–43. https://doi.org/10.1016/j.ymben.2018.05.002.

Yu, M., Li, Y., Banakar, S.P., Liu, L., Shao, C., Li, Z., Wang, C., 2019. New metabolites from the co-culture of marine-derived actinomycete *Streptomyces rochei* MB037 and fungus *Rhinocladiella similis* 35. Front. Microbiol. 10, 915. https://doi.org/10.3389/fmicb.2019.00915.

Zampini, M., Stevens, P.R., Pachebat, J.A., Kingston-Smith, A., Mur, L.A.J., Hayes, F., 2015. RapGene: a fast and accurate strategy for synthetic gene assembly in *Escherichia coli*. Sci. Rep. 5, 11302. https://doi.org/10.1038/srep11302.

Zeng, F., Zang, J., Zhang, S., Hao, Z., Dong, J., Lin, Y., 2017. AFEAP cloning: a precise and efficient method for large DNA sequence assembly. BMC Biotechnol. 17, 81. https://doi.org/10.1186/s12896-017-0394-x.

Zhang, J., Wang, Y., Chai, B., Wang, J., Li, L., Liu, M., Zhao, G., Yao, L., Gao, X., Yin, Y., Xu, J., 2020. Efficient and low-cost error removal in DNA synthesis by a high-durability MutS. ACS Synth. Biol. 9, 940–952. https://doi.org/10.1021/acssynbio.0c00079.

Zhang, S., Zhao, X., Tao, Y., Lou, C., 2015. A novel approach for metabolic pathway optimization: oligo-linker mediated assembly (OLMA) method. J. Biol. Eng. 9, 23. https://doi.org/10.1186/s13036-015-0021-0.

Zhang, Y.-H.P., Evans, B.R., Mielenz, J.R., Hopkins, R.C., Adams, M.W.W., 2007. High-yield hydrogen production from starch and water by a synthetic enzymatic pathway. PLoS One 2, 456. https://doi.org/10.1371/journal.pone.0000456.

Zhou, H., Vonk, B., Roubos, J.A., Bovenberg, R.A.L., Voigt, C.A., 2015. Algorithmic co-optimization of genetic constructs and growth conditions: application to 6-ACA, a potential nylon-6 precursor. Nucleic Acids Res. 43, 10560–10570. https://doi.org/10.1093/nar/gkv1071.

Zhou, K., Qiao, K., Edgar, S., Stephanopoulos, G., 2015. Distributing a metabolic pathway among a microbial consortium enhances production of natural products. Nat. Biotechnol. 33, 377–383. https://doi.org/10.1038/nbt.3095.

Zong, Y., Zhang, H.M., Lyu, C., Ji, X., Hou, J., Guo, X., Ouyang, Q., Lou, C., 2017. Insulated transcriptional elements enable precise design of genetic circuits. Nat. Commun. 8, 52. https://doi.org/10.1038/s41467-017-00063-z.

Zou, R., Zhou, K., Stephanopoulos, G., Too, H.P., 2013. Combinatorial engineering of 1-deoxy-D-xylulose 5-phosphate pathway using cross-lapping in vitro assembly (CLIVA) method. PLoS One 8, e79557. https://doi.org/10.1371/journal.pone.0079557.