

## SEVA 3.1: enabling interoperability of DNA assembly among the SEVA, BioBricks and Type IIS restriction enzyme standards

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### Summary

**Robust synthetic biology applications rely heavily on the design and assembly of DNA parts with specific functionalities based on engineering principles. However, the assembly standards adopted by different communities vary considerably, thus limiting the interoperability of parts, vectors and methods. We hereby introduce the SEVA 3.1 platform consisting of the SEVA 3.1 vectors and the Golden Gate-based ‘SevaBrick Assembly’. This platform enables the convergence of standard processes between the SEVA platform, the BioBricks and the Type IIS-mediated DNA assemblies to reduce complexity and optimize compatibility between parts and methods. It features a wide library of cloning vectors along with a core set of standard SevaBrick primers that allow multi-part assembly and exchange of short functional genetic elements (promoters, RBSs) with minimal cloning and design effort. As proof of concept, we constructed, among others, multiple *sfGFP* expression vectors under the control of eight RBSs, eight promoters and four origins of replication as well as an inducible four-gene operon expressing the biosynthetic genes for the black pigment proviolacein. To demonstrate the interoperability of the SEVA 3.1**

vectors, all constructs were characterized in both *Pseudomonas putida* and *Escherichia coli*. In summary, the SEVA 3.1 platform optimizes compatibility and modularity of inserts and backbones with a cost- and time-friendly DNA assembly method, substantially expanding the toolbox for successful synthetic biology applications in Gram-negative bacteria.

### Introduction

Synthetic biology is a fast-growing field that incorporates biological and engineering principles to extend or modify the capabilities of organisms or biological systems towards new applications (Andrianantoandro *et al.*, 2006). To increase the speed and predictability of any synthetic biology application, it is critical for it to be designed in accordance with the principles of standardization, decoupling, abstraction and modularity (Andrianantoandro *et al.*, 2006; Way *et al.*, 2014). Often, these applications require the combination of multiple genetic elements such as promoters, ribosomal binding sites (RBSs) or coding sequences (CDSs) into new and complex DNA-encoded molecular devices (Ellis *et al.*, 2011). To this end, DNA parts need to be modular, standardized and based on rational design in order to minimize redundancy and unpredictability (Kelly *et al.*, 2009). Evolution has driven biological systems to be inherently dynamic and even though complex, having a very well-balanced behaviour (Purnick and Weiss, 2009). Disturbing this balance using poorly designed, incompatible or uncharacterized DNA parts could disrupt the whole biological system with unpredictable and often detrimental effects (Gardner *et al.*, 2000). Although *de novo* DNA synthesis could become a solution to the problem, as parts can be directly ordered and synthesized in a matter of days, double-stranded DNA synthesis is still relatively expensive and at times impossible when it comes to complex DNA structures (Ma *et al.*, 2012; Hughes and Ellington, 2017; Perkel *et al.*, 2019). Consequently, synthetic biology still relies on robust DNA assembly methods to avoid *de novo* DNA synthesis of long and complex parts and to facilitate high-throughput exchange of DNA parts at low cost and within a reasonable short time. Moreover, the design of the DNA parts to be assembled requires adherence to standard rules

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independently of each specific part, thereby allowing interchangeability between laboratories and automation of construction. In addition, given that a wide range of hosts are used in synthetic biology applications, with new organisms being used as *chassis*, a holistic approach towards the interoperability of parts and vectors has become imperative (Adams, 2016).

Currently, DNA parts can be assembled using several *in vitro* and *in vivo* methods. Popular protocols to this end include, inter alia: (i) homology-based *in vitro* methods [Overlap Extension PCR (Higuchi *et al.*, 1988), Gibson assembly (Gibson *et al.*, 2009), USER (Bitinaite *et al.*, 2007)], (ii) Golden Gate-based (Engler *et al.*, 2008) methods [MoClo (Weber *et al.*, 2011), Golden-Braid (Sarrion-Perdigones *et al.*, 2011; Sarrion-Perdigones *et al.*, 2013), Mobius (Andreou and Nakayama, 2018), Loop (Pollak *et al.*, 2019)] and (iii) *in vivo* methods [Yeast DNA assembly (Gibson *et al.*, 2008; Chandran and Shapland, 2017), *Bacillus subtilis* DNA assembly (Tsuge *et al.*, 2003; Itaya *et al.*, 2018), recombineering in *E. coli* (Sharan *et al.*, 2009)]. Even though these methods can result in highly functional constructs, they require specific restrictions and/or design per part. Regarding homology-based and *in vivo* methods, each part needs to be prepared in advance with individualized primers every time the specific part has to be assembled in a new construct. Consequently, this process could hamper the concept of standardization and interoperability. On the other hand, mainstream Type IIS (Golden Gate-based) assembly methods require the use of at least two restriction enzymes whose restriction sites must be mutated. Additionally, parts need to be cycled between different vectors during the assembly process in order to result in complex constructs. Such diverse requirements often limit the throughput of constructs and the collaboration in time and space among synthetic biology communities.

In this context, the BioBricks™ platform (Shetty *et al.*, 2011) was one of the initial attempts to standardize the assembly process of interchangeable DNA parts using standardized flanks which enable a simple and universal restriction/ligation. By using BioBricks, it became possible to store and share DNA parts which could be easily assembled by the synthetic biology community. As a result of the popularity of the iGEM competition, the Registry of Standard Biological Parts has become one of largest public DNA libraries with more than 20,000 parts ([http://parts.igem.org/Main\\_Page](http://parts.igem.org/Main_Page)). However, despite the high simplicity of the standardized assembly, the proposed 3A assembly (Shetty *et al.*, 2011) is a laborious and time-consuming process, while backbone availability is limited and poorly characterized.

In addition to standardizing DNA parts, vectors also require corresponding standardization and robustness.

The key elements of an engineered vector that make it important for biotechnological purposes are the origin of replication (Ori), the antibiotic resistance (AR) and the cloning module. Various vector collections have been developed in recent years, with unique features for specific applications (e.g. high gene expression, reporter genes). The Standard European Vector Architecture (SEVA) (Silva-Rocha *et al.*, 2013; Martínez-García *et al.*, 2015, 2020) is a well-characterized and curated public platform of vectors for use in Gram-negative bacteria. This platform contains a large number of vectors, combining seventeen Ori and eight AR genes, which follow the same format and nomenclature. However, the proposed assembly method of SEVA vectors is based on traditional restriction/ligation in which each part has to be individually amplified with specific primers, digested and purified for any particular application (Martínez-García *et al.*, 2015). Therefore, although SEVA vectors are widely preferred for their reliability and variety, their performance as assembly vectors for multiple DNA constructs is not optimal.

To combine the unique features of SEVA vectors and BioBricks, eliminating their drawbacks, we developed the SEVA 3.1 platform. The concept of the SEVA 3.1 vectors arose as an attempt to bridge (i) the backbone (Ori and AR) flexibility of the SEVA vectors, (ii) the part abundance and interchangeability of BioBricks and (iii) the practicality of the Type IIS restriction enzymes. The SEVA 3.1 vectors consist of any SEVA backbone (Ori, AR, terminators) merged with the standard BioBrick cloning site (prefix, suffix) resulting in the new SEVA 3.1 cloning site called MCS 2.0 (Modular Cloning Site). The end-product named pSEVA<sub>Ab</sub> carrying SevaBricks is a vector with maximized host flexibility, compatible with the BioBrick assembly protocol (RFC10). The SEVA standard was the ideal candidate due to its modular design, lack of *Bsa*I restriction sites, well-curated modules, multi-origin compatibility, SBOL (Galdzicki *et al.*, 2014) compatibility and widespread scientific acceptance (Kuepper *et al.*, 2015; Calero *et al.*, 2016; Kim *et al.*, 2019). In addition, we present the SevaBrick Assembly which emulates the 3A Assembly by further simplifying and upgrading the assembly process. This Golden Gate-based assembly method consists of standardized protocols and primers that enable simple and straightforward one-step assembly of single or multiple BioBricks into the SEVA 3.1 backbones. A critical characteristic of the method is the use of a sole restriction enzyme (*Bsa*I) for the whole process. To our knowledge, the proposed method is the first Golden Gate-based method which allows direct construction of vectors with different standardized elements such as promoters, RBSs, CDSs, ARs and origins of replication using one restriction enzyme and a single ligation step.

In this study, the efficiency and flexibility of the newly developed SEVA 3.1 platform was showcased through the construction and characterization of several genetic constructs with varying degrees of complexity. Furthermore, in order to prove the broad host nature of the methodology, all constructed expression vectors were tested in both *E. coli* DH $\alpha$  and the considerably promising as microbial chassis *P. putida* KT2440 (Poblete-Castro *et al.*, 2012; Calero and Nikel, 2019), with successful observation of reporter molecules.

## Results and discussion

### *Design and construction of the SEVA 3.1 platform*

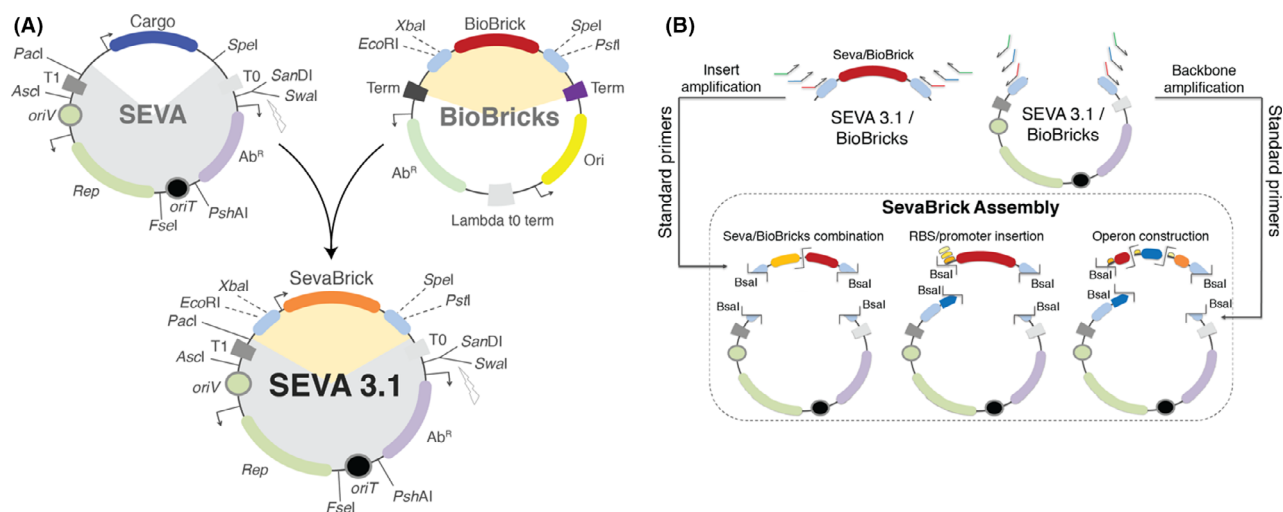
The herein described SEVA 3.1 platform converges existing standards of vectors, DNA parts and assemblies into one universal standard which can be used for efficient downstream synthetic biology applications. The required elements of the SEVA 3.1 platform are the SevaBrick Assembly (Fig. 1B) and the SEVA 3.1 vectors (Fig. 1A). The SevaBrick Assembly is a method where all the parts and backbones to be assembled are PCR amplified from any SEVA 3.1 or BioBrick vector, using a core set of standard long primers. All SevaBrick primers (Table 1) anneal on standard sequences of the SEVA 3.1 or BioBrick vectors, introducing *Bsal* recognition sites for directional multipart assembly via Golden Gate. The unique characteristic of the process is an in-house biphasic PCR protocol, same for all primer sets, which enables these long primers to successfully amplify any part or backbone of the SEVA 3.1 and BioBricks platforms (see Experimental procedures). A SEVA 3.1 vector is constructed through the assembly of three modules, the SEVA 3.1 cloning site module (MCS 2.0), the AR module and the Ori module. The construction of the entire vector library is based on PCR amplification of all modules (MCS 2.0, AR, Ori) using three sets of standard primers with subsequent SevaBrick Assembly (Fig. 2). The MCS 2.0 module is amplified from any BioBrick, preferably a reporter transcription unit (TU), using the Ep/Pp insert primers. The remaining two sets are used to amplify the AR (AR-F/AR-R) and Ori (Ori-F/Ori-R) modules from any SEVA vector, while adding extra DNA sequences which after the assembly with the BioBrick amplicon reconstruct the BioBrick prefix and suffix. Additionally, the Ori-R and AR-F primers carry 23 nt and 27 nt of the upstream and downstream BioBrick terminators respectively (Fig. 2). These additional DNA sequences are essential elements of the new MCS 2.0 as they function as annealing sequences for the SevaBrick Assembly primers. All three primer sets anneal on standard sequences of the template vectors and are therefore compatible with all available SEVA and BioBrick plasmids, allowing any possible combination of all

modules. In terms of design, the new vectors have to be fully compatible with the BioBricks assembly protocol and due to this prerequisite, the SEVA AR module had to be modified during PCR amplification in order to remove the *SpeI* restriction site within it. For the construction of any possible combination, the method requires only a selected number of the template SEVA plasmids carrying all modules (AR, Ori) and one BioBrick. Therefore, we amplified all modules (Fig. S1) required for this study and constructed a wide set of SEVA 3.1 vectors (Table S1) which we make available through the SEVA database (<http://seva.cnb.csic.es>).

The nomenclature of the SEVA 3.1 vectors complies with SEVA's (Silva-Rocha *et al.*, 2013). However, as the configuration of the SEVA 3.1 vectors is not identical with SEVA's (*SpeI* restriction site has been moved to the BioBrick suffix), the vector's name has changed from pSEVA to pSEVAb. Thus, all SEVA 3.1 plasmids are named pSEVAb, while the nomenclature of the standard modules was maintained. For instance, vector pSEVAb23 carries the AR kanamycin [2] and the Ori pBBR1 [3].

### *Convergence of new DNA parts*

A critical step of the SEVA 3.1 platform is the convergence of new DNA parts. This is the only step of the platform where individualized primers have to be designed, using standard rules and flank sequences (Table S2). In terms of structure, all new parts have to comply with the RFC10 protocol of BioBricks, which means being cloned into a BioBrick repository vector and flanked with the appropriate prefix and suffix sequences (<http://parts.igem.org/Help:Prefix-Suffix>). Although any SEVA 3.1 vector could serve as repository vector, we chose the BioBrick vector pSB1C3, as it is the main repository vector of the iGEM registry ([http://parts.igem.org/Help:2019\\_DNA\\_Distribution](http://parts.igem.org/Help:2019_DNA_Distribution)). During convergence, the repository vector is amplified using the standard backbone linearization primers, Ev/Pv, while the part under construction is amplified with primers which add either the CDS or non-CDS BioBrick prefix and suffix along with *Bsal* recognition sites (Fig. 3). The generated sticky ends are compatible with the sticky ends of the linearized vector making the parts of the assembly compatible. Therefore, the only requirement for a part to be SEVA 3.1 compatible is to be free of *Bsal* recognition sites. *Bsal* recognition sites can be mutated during the convergence process through a multipart domestication assembly of the new part. Domestication primers are designed to implement the mutation through a loop on the one of the two primers and concurrently add compatible *Bsal* restriction sites between amplified parts. This method is an iteration of the method proposed in GoldenBraid (Sarrion-Perdigones *et al.*,



**Fig. 1.** The SEVA 3.1 platform.

A. The SEVA 3.1 vectors are generated by merging: (i) the backbone of the SEVA vectors (grey shade), containing the antibiotic resistance module (purple), the origin of replication module (green), origin of transfer (black) and two transcriptional terminators (dark and light grey) and (ii) the cloning site of the BioBrick vectors (orange shade) comprised by: the BioBrick prefix and suffix (light blue) and a BioBrick (red). B. Graphical overview of the SevaBrick Assembly. Inserts and backbones are amplified using a core set of standard primers which introduce compatible *BsaI* sites, allowing the construction of expression vectors with varying degrees of complexity via Golden Gate.

**Table 1.** SevaBrick Assembly standard primers.

Purpose	Insert primers <sup>a</sup>	Backbone primers <sup>b</sup>
Basic assembly		
Convergence	Brk-F/Brk-R <sup>c</sup>	Ev/Pv
Single part	Ep/Pp	Ev/Pv
Double part	Ep/Sp, Xp/Pp	Ev/Pv
Extended assembly		
Single-gene constitutive TU	OP1-F/Pp	Ev <sub>promoter</sub> /Pv
Double-gene constitutive TU	OP1-F/OP1-R, OP2-F/Pp	Ev <sub>promoter</sub> /Pv
Triple-gene constitutive TU	OP1-F/OP1-R, OP2-F/OP2-R, OP3-F/Pp	Ev <sub>promoter</sub> /Pv
Quadruple-gene constitutive TU	OP1-F/OP1-R, OP2-F/OP2-R, OP3-F/OP3-R, OP4-F/Pp	Ev <sub>promoter</sub> /Pv
n-gene inducible TU <sup>d</sup>	Ep/Sp, OPn-F/OP(n-1)-R + Pp	Ev/Pv

a. Templates for insert primers: Seva/BioBricks for Basic Assembly, Seva/BioBrick CDSs for Extended assembly, converged inducible expression systems for inducible TUs of Extended Assembly.

b. Templates for backbone primers: SEVA 3.1 and BioBrick vectors.

c. Individualized primers, based on standard rules, for part convergence.

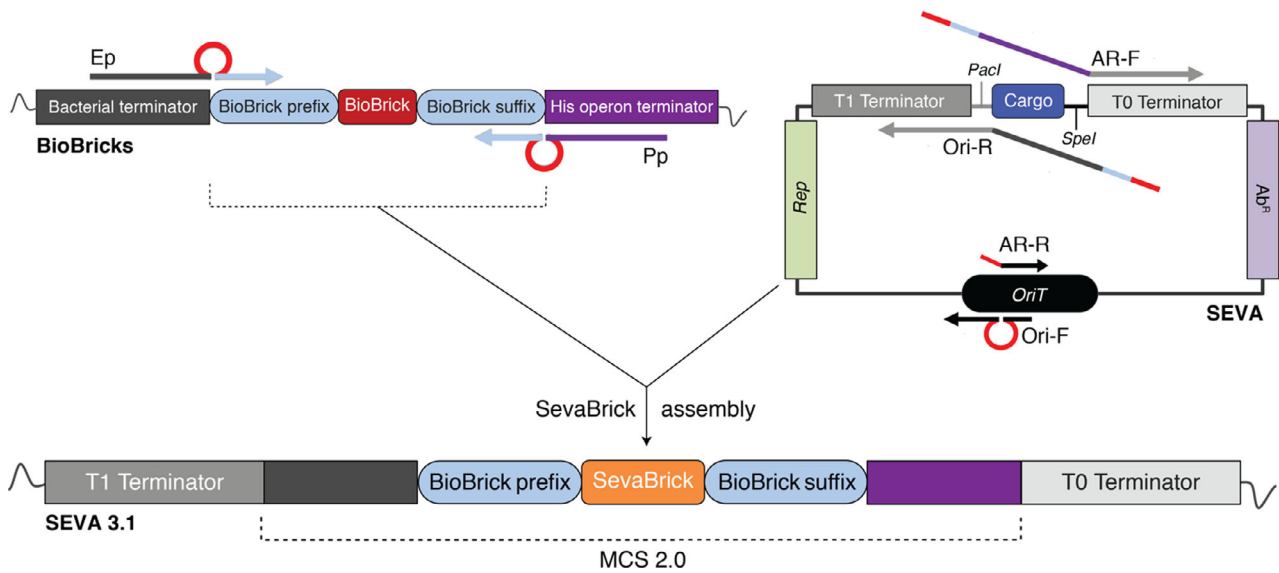
d. Ep/Sp for the amplification of the inducible expression system.  $n$  = number of genes in TU.

2011). However, instead of adding the mutation via sticky-end manipulation on both primers, the change is performed through one primer (Fig. S2). In the case of DNA synthesis, the part can be suitably designed to be pre-domesticated and ready for convergence by carrying the required flanks and sticky ends prior to ordering. After the preparation and final assembly of all parts, the

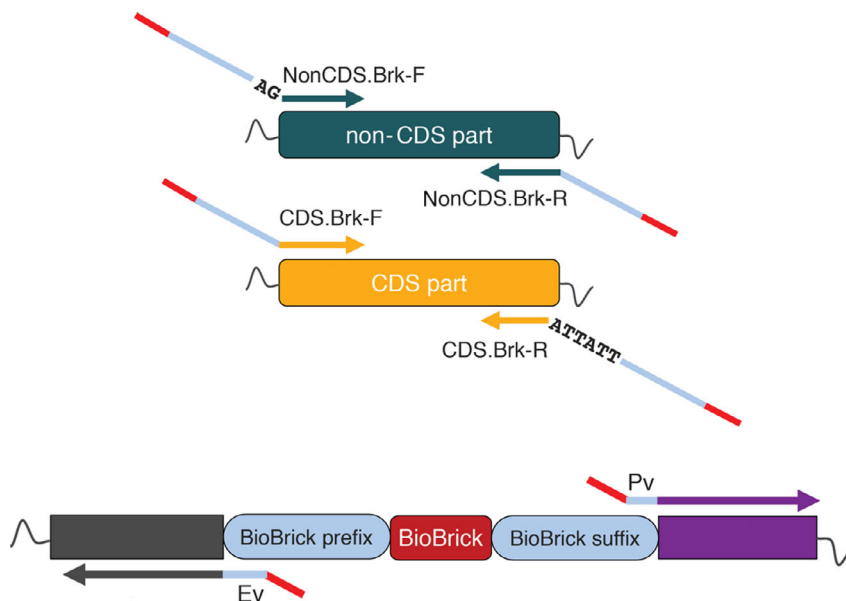
final product is a scarless and domesticated BioBrick. As proof of concept, we have converged, inter alia (Table S3), the (i) *E. coli* rhamnose-induced expression system RhaRS/*P<sub>rhaB</sub>* (Egan and Schleif, 1993), (ii) the *sfGFP* CDS from the BBa\_K515105 BioBrick and (iii) the *XylS/P<sub>m</sub>* expression system from the SEVA collection (Marques *et al.*, 1999). All parts were stored in the pSB1C3 repository vector as most iGEM parts and further used in downstream constructs. In particular, in the case of the *XylS/P<sub>m</sub>* expression system, which carries a *BsaI* recognition site on its *XylS* regulator, the domestication process was carried out in parallel with convergence. All primers used for the convergence process are listed in Table S4.

#### Basic SevaBrick Assembly for Seva/BioBricks

The SevaBrick Assembly for BioBricks is an alternative method to the 3A Assembly proposed for BioBrick construction. Since all converged parts of the SEVA 3.1 platform are stored into pSB1C3, we sought to engineer a method that could assemble Seva/BioBricks together while removing the complexity of the 3A assembly. The construction of repository parts, as described in the convergence process, can be considered as the simplest single part SevaBrick Assembly application, equivalent to constructing basic BioBricks. To construct composite parts, in addition to the backbone and insert amplification primers (Ev/Pv and Ep/Pp, respectively), two additional standard primers (Sp and Xp) were designed to allow double part assembly. Each of these primers



**Fig. 2.** Construction of SEVA 3.1 vectors. The Ep/Pp insert primers anneal on the standard BioBrick prefix and suffix, respectively, amplifying any BioBrick. The AR-F/AR-R and Ori-F/Ori-R primers sets anneal on the SEVA transcriptional terminators (T0 and T1, respectively) and on the origin of transfer (OriT), amplifying any AR and Ori module, respectively, when pSEVA is used as template. The amplified parts contain two *Bsa*I recognition sites (red loop or line) required for SevaBrick Assembly. The AR-F and the Ori-R primers carry overhang sequences that reconstruct the BioBrick prefix and suffix after SevaBrick Assembly. In addition, they carry nucleotides of the upstream and downstream BioBrick terminators which are essential sequences of the new MCS 2.0.



**Fig. 3.** Convergence of DNA parts into SEVA 3.1 platform. A non-CDS part is converged using the non-CDS.Brck-F and the non-CDS.Brck-R primers. The non-CDS.Brck-F carries the non-CDS BioBrick prefix (blue) while the non-CDS.Brck-R carries the BioBrick suffix (blue). A CDS part is converged using the CDS.Brck-F and CDS.Brck-R primers. The CDS.Brck-F carries the CDS BioBrick prefix while the CDS.Brck-R carries the BioBrick suffix and a double stop codon (TAATAA). All primers carry a *Bsa*I recognition site (red) at the 5'- or 3'-end which enables assembly with the amplified with Ev/Pv primers repository vector (pSB1C3). After the assembly the BioBrick suffix and prefix are reconstructed.

anneals on the standard cloning sequence of SEVA 3.1 (MCS 2.0) and BioBrick compatible vectors (Fig. 4A). The unique amplicon overhangs were designed so that after the final assembly, the plasmid contains a regenerated prefix and suffix and a newly generated BioBrick

scar between the assembled parts as it occurs after the 3A assembly. The fidelity of the method was tested by assembling two TUs of the reporter proteins amilCP, mRFP and counting the blue-purple (only amilCP), light red (mRFP), dark red (amilCP, mRFP) and white (vector

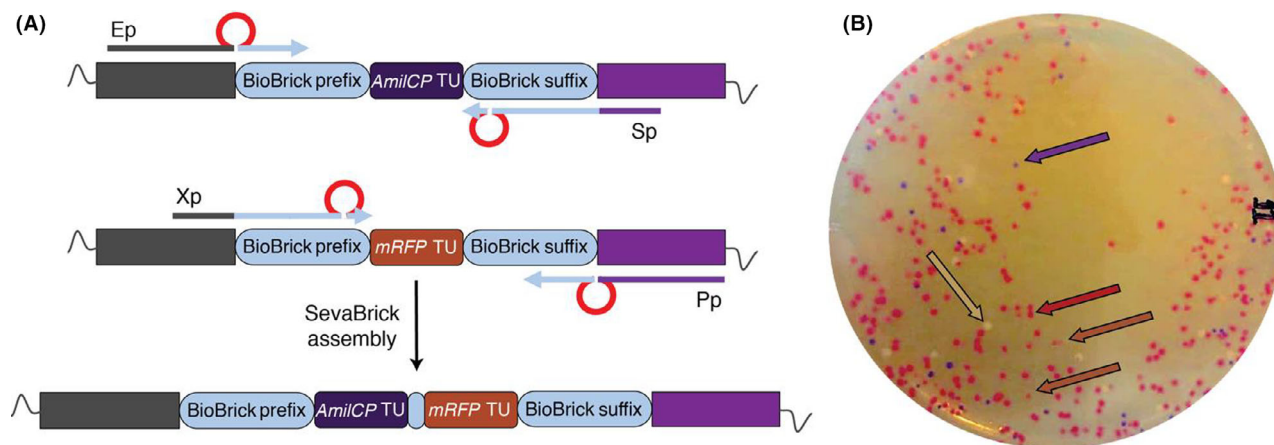
self-assembly) colonies (with dark red expected to be produced by the correct composite part). Initially, the *amiCP* and *mRFP* TUs were amplified from two in-house vectors (the construction of both vectors is described below) using the Ep/Sp and Xp/Pp primers, respectively, and later cloned into the pSEVAb23 vector linearized with the Ev/Pv primers. Both TUs consist of the BBa\_BJ23100 promoter, the BBa\_B0034 RBS and the corresponding reporter. The assembly procedure was repeated three times, and the ligation efficiency was calculated based on the dark red colonies to 90% (Fig. 4B). Thus, SevaBrick Assembly enables the construction of double TU cassettes using three standard primer sets and one restriction enzyme (*Bsal*), whereas 3A assembly requires at least four restriction enzymes (*EcoRI*, *XbaI*, *SpeI*, *PstI*) and the individual digestion of each part.

#### Introduction of short functional genetic elements via PCR amplification

The 'Registry of Standard Biological Parts' contains a plethora of genes (metabolic, reporters, regulators etc.) which are stored as CDSs starting at the 5'-end by a start codon (ATG) and ending at the 3'-end with a double stop codon (TAATAA). They are stored as basic parts which encode for proteins, without additional functional elements such as promoters or RBSs. This set-up allows the user to combine BioBricks with the functional genetic elements of choice (RBSs, promoters) in order to achieve the desired transcription-translation levels. Specifically, in synthetic biology and metabolic engineering approaches, the right combination of RBSs and

promoters has a significant impact on the required performance. The process of constructing and selecting optimal promoter-RBS-CDS combinations can be very complicated and often requires high-throughput strategies in which random combinations need to be constructed and screened. To simplify and standardize the process of introducing short functional DNA sequences into any BioBrick, we engineered two primers that introduce to the parts such functional sequences within a single-step PCR amplification. These two long standard primers are (i) the CDS amplification primer (OP1-F<sub>RBS</sub>) and (ii) the promoter-backbone amplification primer (EV<sub>promoter</sub>). In the first case, an RBS is introduced to the amplified CDS BioBrick, while in the second case, a short constitutive promoter is introduced to any SEVA 3.1 or BioBrick backbone (Fig. 5A). As shown in Figure 5A, the OP1-F<sub>RBS</sub> primer consists of one standard and one modular sequence region (yellow) in which any RBS of choice is introduced. The primer anneals on the standard MCS 2.0 sequence and carries a loop with the RBS, flanked by a *Bsal* recognition site followed by standard sticky ends. The EV<sub>promoter</sub> primer anneals on the standard non-CDS BioBrick prefix sequence while carrying the promoter sequence at its 5'-end along with the required sticky ends and a *Bsal* recognition site.

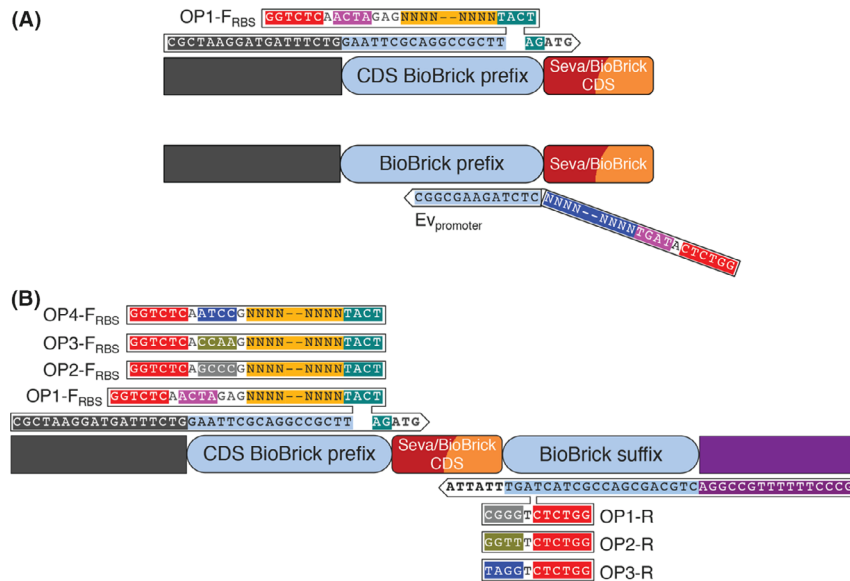
**Introduction of RBSs and short promoters.** Using the standard primers described above, we amplified several chromoproteins and fluorescent proteins registered as CDSs in the iGEM registry and constructed functional TUs in the pSEVAb23 vector. All reporter proteins were amplified using the OP1-F<sub>BBa\_B0034</sub> primer, for introducing the RBS BBa\_B0034 and the reverse insert



**Fig. 4.** Illustration of Basic SevaBrick Assembly.

A. Design of the Basic SevaBrick Assembly primers. Each primer anneals on the standard MCS 2.0 sequence and they all carry *Bsal* recognition sites (red) which allow directional cloning of two parts after digestion with *Bsal*. Primer sets i) Ep/Sp: amplification of first part, ii) Xp/Pp: amplification of second part.

B. Representative picture showing the efficiency of the Basic SevaBrick Assembly to ligate two parts (*amiCP* TU, *mRFP* TU). White colonies: vector self-assembly, blue-purple colonies: *amiCP* TU, light red: *mRFP* TU, dark red: *amiCP* TU + *mRFP* TU.



**Fig. 5.** Structure of the OP1-F<sub>RBS</sub> and Ev<sub>promoter</sub> primers.

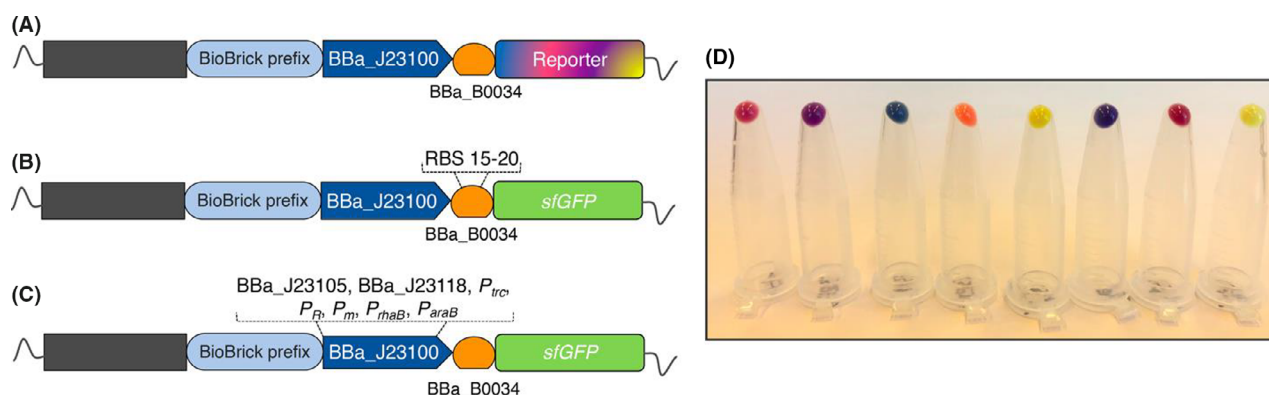
A. The OP1-F<sub>RBS</sub> primer consists of one standard and one modular sequence region; Standard sequence region: annealing sequence [black, light blue, green (AG), start codon (ATG)], *Bsa*I recognition site (red), sticky ends (pink), RBS scar [green (TACTAG)]. Modular sequence region: RBS sequence (yellow). The Ev<sub>promoter</sub> primer also consists of one standard and one modular sequence region; Standard sequence region: annealing sequence (light blue), *Bsa*I recognition site (red), compatible with the OP1-F<sub>RBS</sub> sticky ends (pink). Modular sequence region: promoter sequence (dark blue).

B. Graphical representation of four OP primer sets: all the forward and reverse primers share the same annealing sequence, which is represented by the OP1-F<sub>RBS</sub> and OP1-R primers, respectively. The OP-F<sub>RBS</sub> primers use the ATG of the stored CDSs for annealing to the 3'-end while the OP-R utilizes the standard double stop codon (TAATAA) used in all BioBrick CDSs. Unique sticky ends are introduced to each primer enabling the directional assembly of each amplified CDS. The sticky ends of the OP1-F<sub>RBS</sub> and Pp (used to amplify the last gene in the operon) are compatible with the sticky ends of the backbone linearization primers, Ev<sub>promoter</sub>/Pv.

primer Pp. The backbone of choice, pSEVA23b, was linearized using the forward Pv primer and the reverse Ev<sub>BBa\_J23100</sub> primer which introduces the BBa\_J23100 promoter allowing directional cloning of any RBS-CDS module. Following the PCRs, all amplified CDSs were cloned into vector pSEVAb23<sub>BBa\_J23100</sub> through a one-step SevaBrick Assembly resulting in the TU structure shown in Figure 6A. In this way, we constructed expression vectors for the reporter proteins mCherry (BBa\_J06504), gfasPurple (BBa\_K1033919), aeBlue (BBa\_K1864401), mOrange (BBa\_E2050), amil-GFP (BBa\_K592010), amilCP (BBa\_K592009), mRFP (BBa\_E1010) and amilLime (BBa\_K1033916) which were later transformed in *P. putida*. All reporter proteins were successfully expressed and matured in *P. putida* giving colour in < 24 h in rich LB medium (Fig. 6D). Consequently, the SevaBrick Assembly allowed the construction of eight expression vectors for both *E. coli* and *P. putida* using only two sets of standard primers and a single ligation step. In addition, although we did not conduct additional experiments to further evaluate these reporter proteins, Liljeruhm *et al.* (2018) and Shaner *et al.* (2004), demonstrated their potential as quantitative reporters of gene expression and promoter strength in *E. coli*. It was shown that most of them have

a comparable maturation time with the widely used fluorescent protein, mRFP.

*Modular exchange and screening of functional genetic elements.* Next, to demonstrate the ability of the OP1-F<sub>RBS</sub> and Ev<sub>promoter</sub> primers to easily exchange genetic functional elements, we constructed several plasmids expressing the *sfGFP* under the control of various RBSs or promoters. At first, we predicted RBS sequences suitable for the translation of the *sfGFP* protein in *P. putida* by using the freely available tool 'Salis RBS calculator' (Salis, 2011). Six RBSs with different predicted strengths, from high to low (RBS.15 > RBS.20), were selected and variants of the OP1-F<sub>RBS</sub> primer were synthesized. The standard sequence region of the OP1-F<sub>RBS</sub> primer was maintained and only the modular RBS sequence region was exchanged by each individual predicted RBS. Using as template a pSB1C3 plasmid carrying the *sfGFP* as CDS BioBrick, we amplified the *sfGFP* with all OP1-F<sub>RBS.15-20</sub> primers separately, resulting in multiple RBS-*sfGFP* amplicons. In parallel, backbone pSEVAb23 was linearized with Ev<sub>BBa\_J23105</sub> and Pv primers. In a single SevaBrick Assembly step, we constructed *sfGFP* expression vectors under the control of the BBa\_J23105 promoter and six *in silico* predicted RBSs. RBS



**Fig. 6.** Graphical representation of constructed TUs using SevaBrick Assembly and the standard primers for introduction of short functional genetic elements.

A. Each reporter CDS, amplified from the iGEM registry, was assembled with the constitutive promoter BBa\_J23100 and the RBS BBa\_B0034. B. sfGFP was assembled with BBa\_J23105 promoter and either the BBa\_B0034 RBS or six in silico predicted RBSs (RBS 15–20). C. sfGFP was assembled with either five short constitutive promoters or three inducible expression systems and the RBS BBa\_B0034. D. *P. putida* transformed with eight different reporter expression vectors. From right to left: mCherry, gfasPurple, aeBlue, mOrange, amilGFP, amilCP, mRFP and amajLime.

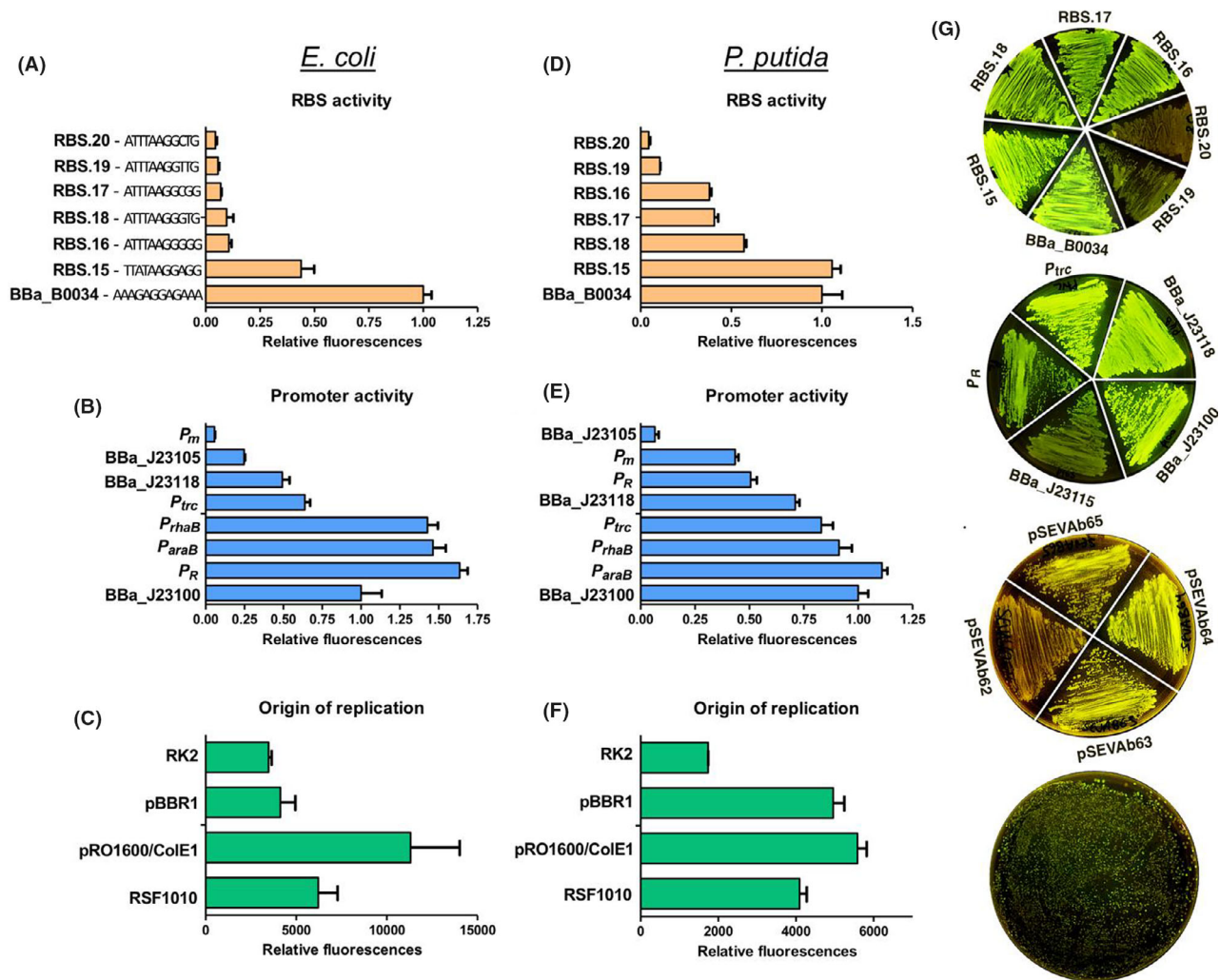
BBa\_B0034 was used as an internal reference for the normalization of the fluorescence levels. *In vivo* fluorescence assays in *P. putida* showed correlation between the predicted RBSs strength and the fluorescence levels. The predicted as strongest RBS.15 provided the highest fluorescence levels at 8 h, similar to the internal standard RBS BBa\_B0034, while the predicted as the weakest RBS.20 was considerably the least strong (Fig. 7D,G). Moreover, to highlight the interoperability of the SEVA 3.1 vectors, all constructs were subsequently tested in *E. coli* (Fig. 7A) where, unlike *P. putida*, RBS BBa\_B0034 exhibited  $\approx 2.5$ -folds higher fluorescence levels than RBS.15. Last, we sought to randomize the RBS sequence and construct a pooled library of sfGFP expression vectors. To do this, primer OP1-F<sub>RBS.15</sub> was synthesized with two random nucleotides within the RBS sequence (-TTATAAGGNNG-) resulting in a new CDS amplification primer (OP1-F<sub>RBS.NN</sub>). Following the PCR, the random RBS-sfGFP amplicon was assembled with pSEVAb23<sub>BBa\_J23105</sub> and transformed into *E. coli* (Fig. 7G). Ten obviously high fluorescent colonies were selected and sent for sequencing. Out of the ten sequenced colonies, we were able to recover five different RBS sequences (Fig. S6) highlighting the feature of the SevaBrick Assembly primers to construct random RBS libraries within a PCR step.

A similar strategy was followed for the characterization of constitutive and inducible promoters. In this case, the vector pSEVAb23 was linearized using variants of the Ev<sub>promoter</sub> primer in which the modular promoter sequence region was exchanged by three constitutive promoters of the iGEM Anderson collection as well as the well-characterized constitutive promoters  $P_{trc}$  (without the lac operator) and  $P_R$  (without the cl repressor,

BBa\_R0051). The sfGFP was amplified with OP1-F<sub>BBa\_B0034</sub>/Pp and later cloned into pSEVAb23<sub>promoter</sub>. Moreover, three inducible expression systems [Xyls/ $P_m$ , RhaS/ $P_{rhaB}$ , AraC/ $P_{araB}$  (Guzman *et al.*, 1995)] were cloned upstream the RBS-sfGFP module. All three inducible expression systems, previously converged to pSB1C3 repository vector, were amplified using the Ep/Sp primers and cloned directly with the RBS-sfGFP amplicon into a pSEVAb23, pre-linearized with Ev/Pv primers. All expression vectors were then characterized in both *E. coli* and *P. putida* (Fig. 7B,E,G). As shown in Figure 7B,E, all Anderson promoters showed similar expression patterns with the previously reported levels starting from the BBa\_J23100 as the strongest to BBa\_J23105 as the weakest (Kelly *et al.*, 2009). The highest expression levels were achieved by the  $P_R$  promoter for *E. coli* and the AraC/ $P_{araB}$  expression system for *P. putida*. However, in our hands and in contrast to previously published work in *P. putida*, the Xyls/ $P_m$  expression system showed less than the half expression level of the AraC/ $P_{araB}$  (Calero *et al.*, 2016).

Finally, the applicability of the Ev<sub>promoter</sub> primer to easily amplify and introduce short promoters to any SEVA 3.1 backbone was showcased by assembling the sfGFP into four different expression vectors with either the RK2, pBBR1, pRO1600/ColE1 or RFS1010 origins of replication (Silva-Rocha *et al.*, 2013). Vectors pSEVAb62, pSEVAb63, pSEVAb64 and pSEVAb65 were linearized using Ev<sub>BBa\_J23105</sub>/Pv and assembled with the sfGFP harbouring the RBS.20. All vectors were transformed in *P. putida* and *E. coli*, and the sfGFP fluorescence levels were measured. As expected, vector pSEVAb62 (low copy number) showed the least sfGFP fluorescence, while the highest fluorescence levels were achieved from pSEVAb64 for





**Fig. 7.** Part characterization in *E. coli* and *P. putida*. (A, D) A selected range of predicted RBS, (B, E) constitutive and inducible promoters, (C, F) origins of replication. The BBa\_B0034 and BBa\_J23100 used for normalization of promoter and RBS levels respectively. For the RBS screening, *sfGFP* was placed under the control of the BBa\_B0034 RBS. For the origins of replication screening the *sfGFP* was placed under the control of the BBa\_J23105 promoter and the predicted RBS.20. (G) Visual appearance of *sfGFP* production on LB plates. From top to bottom RBSs (*P. putida*), constitutive promoters (*P. putida*), origins of replication (*P. putida*) and random RBSs (*E. coli*).

both *E. coli* and *P. putida* (Fig. 7C,F,G). The observed fluorescence levels in *E. coli* are similar to those presented by Jahn and colleagues (2016).

Therefore, the modular design of the OP1-F<sub>RBS</sub> and E<sub>V<sub>promoter</sub></sub> primers allows the user to easily introduce and exchange RBS or promoter sequences, respectively, within one standard PCR step and with reasonably minimal design.

#### Extended SevaBrick Assembly for operon construction

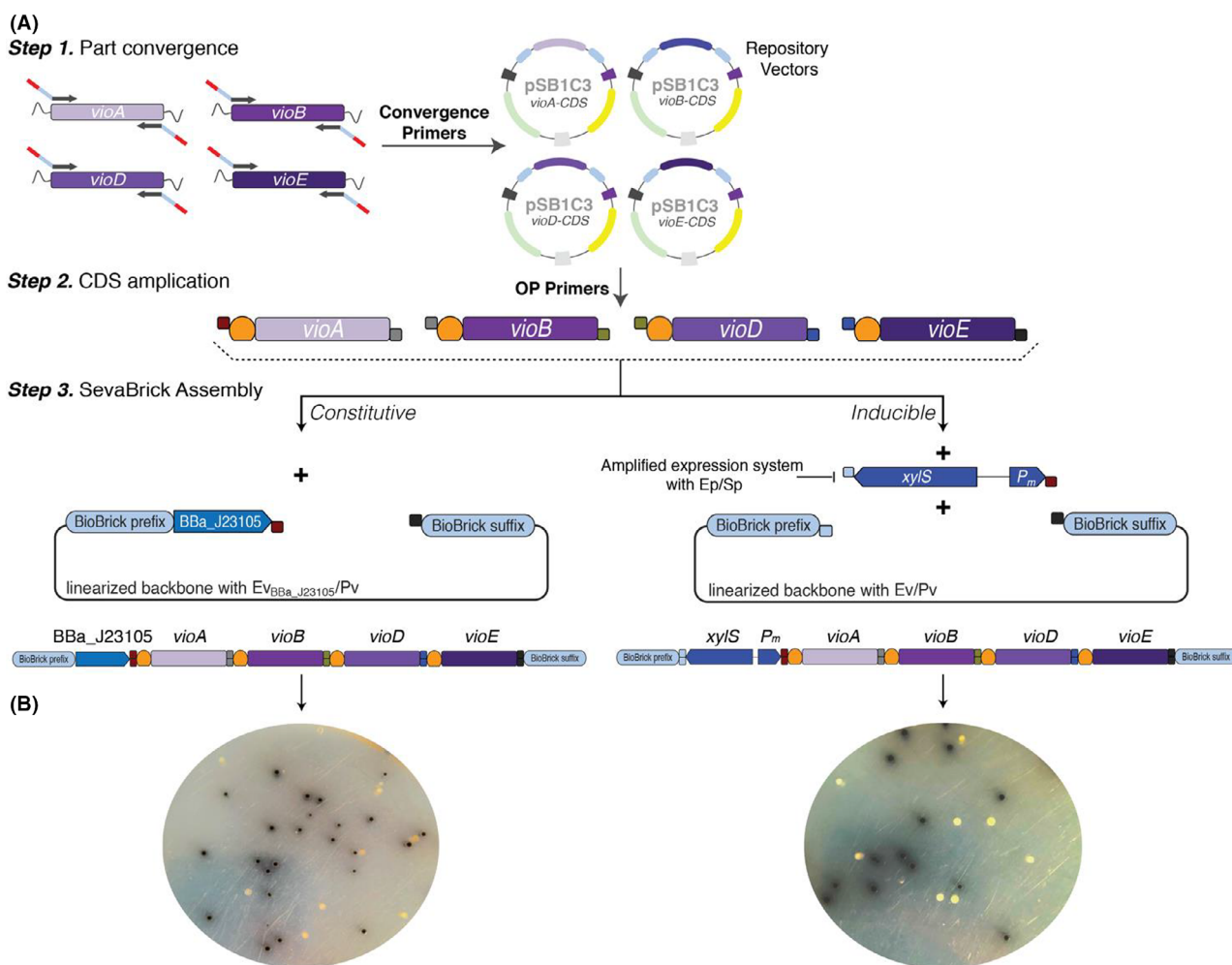
The basic version of the SevaBrick Assembly, as explained above, is a quick and easy method of assembling parts, however its main limitation is the small number of fragments that can be simultaneously assembled;

two parts at a time. To further extend the capabilities of SevaBrick Assembly to construct more complex genetic designs, such as multigene operons, we propose another set of standard primers based on the structure of the OP1-F<sub>RBS</sub> primer (Fig. 5B). Using the sequence of the OP1-F<sub>RBS</sub> primer as the basic template for the forward primer, we designed four primer sets (OP1 to OP4) that allow amplification of any BioBrick CDS while introducing RBSs and compatible sticky ends for sequential assembly of up to four CDSs. To maximize assembly efficiency, the sticky ends for this process were selected from a high-fidelity library proposed by New England Biolabs (NEB) (Potapov *et al.*, 2018). These unique sticky ends allow directional cloning of each particular amplicon into a final operon through SevaBrick

Assembly. For instance, the OP2-F<sub>RBS</sub> primer introduces sticky ends compatible with the sticky ends of the OP1-R primer, while the OP2-R introduces sticky ends compatible with the OP3-F<sub>RBS</sub>. In this way, the position of each particular gene can be easily determined using the corresponding OP primer set (OP1/position 1, OP2/position 2 etc). The last gene in the operon has to be amplified with the OP-Fw<sub>n</sub> (being n the number of the genes in the operon) and the basic assembly primer Pp. The sequence between the RBS and the ATG was kept constant (TACTAG) for standardization purposes since this sequence has a significant impact on the translation initiation. Based on this specific design, constitutive

promoters can be introduced to the operon via PCR amplification of the backbone, as it is described above. In the case of inducible operons, the inducible expression system (stored in repository vector) and the backbone of interest have to be amplified using the primers Ep/Sp and the Ev/Pv respectively. The Ev<sub>promoter</sub> and Sp primers carry identical sticky ends allowing the construction of either constitutive or inducible operon by using the same RBS-CDS amplicons.

To validate the functionality of the OP primers and SevaBrick Assembly to construct multigene operons, we assembled the protoviolacein acid biosynthesis operon by introducing the RBS of choice to each particular



**Fig. 8.** Overview of the *vioABDE* operon construction via SevaBrick Assembly.

A. *Step 1*: Convergence of each CDS to the repository vector pSB1C3. Each CDS was amplified with unique convergence primers, following the strategy described in the part convergence paragraph, and later cloned into pSB1C3. *Step 2*: PCR amplification of each particular CDS using the OP primers (OP1-OP4) to introduce the RBS BBa\_B0034 and unique sticky ends for directional cloning of all CDS in the order of *vioA-vioB-vioD-vioE*. *Step 3*: All RBS-CDS amplicons were assembled with either (i) a linear pSEVAb23<sub>BBa\_J23105</sub> vector resulting in the constitutive *vioABDE* operon or (ii) a pre-linearized with Ev/Pv pSEVAb23 vector and the pre-amplified with Ep/Sp *XylS/P<sub>m</sub>* expression system, resulting in the inducible *vioABDE* operon.

B. Cells transformed with the constitutive *vioABDE* operon (left) and the inducible *XylS/P<sub>m</sub>-vioABDE* operon (right) form black colonies due to the production of protoviolacein.

gene. Protoviolacein is an intermediate of the violacein biosynthesis pathway, originated from the soil bacterium *Chromobacterium violaceum* (August *et al.*, 2000). Violacein and its precursors have notable antimicrobial activity, while the genes involved in its biosynthesis are arranged in an operon consisting of *vioA*, *vioB*, *vioC*, *vioD* and *vioE*. L-tryptophan is converted to protoviolacein acid via four steps catalysed by the VioA, VioB, VioD, VioE. Protoviolacein acid is then converted to either violacein via VioC or is being oxidized to proviolacein, a black pigment. Therefore, we reconstructed the pathway towards proviolacein and assessed the efficiency of the extended SevaBrick Assembly by counting the black versus the white colonies. Initially, plasmid BBa\_K598020 was used as template to amplify and converge each particular gene into the repository vector pSB1C3, following the convergence strategy described above and the primers listed in Table S4. As all CDSs were stored in the repository vectors, a second PCR step was performed to introduce the RBS BBa\_B0034 using the OP primers. The order of the CDSs into the operon was determined as *vioA-vioB-vioD-vioE* by using the primer sets OP1-F<sub>BBa\_B0034</sub>/OP1-R, OP2-F<sub>BBa\_B0034</sub>/OP2-R, OP3-F<sub>BBa\_B0034</sub>/OP3-R and OP4-F<sub>BBa\_B0034</sub>/Pp respectively. Finally, all RBS-CDS amplicons were assembled into a linear pSEVAb23<sub>BBa\_U23105</sub> vector resulting in the operon structure shown in Fig. 8A. The assembly was performed three times and the efficiency was calculated by measuring the ratio of black to white colonies. Appearing black colonies (Fig. 8B) were to 60–65% of the total colonies, indicating the high efficiency of the SevaBrick Assembly to assembly up to five parts in a single ligation step. Several black colonies were selected for plasmid sequencing, which resulted in the correct operon sequence.

A notable characteristic of the SevaBrick Assembly is the high modularity and reusability of the amplicons to be assembled. To highlight this feature and additionally evaluate the efficiency of the method to assemble six parts at once, we constructed an inducible version of the protoviolacein operon. To do this, the [*vioABDE* + RBS] amplicons were assembled with the XylS/*P<sub>m</sub>* inducible expression system into vector pSEVAb23. This time, vector pSEVAb23 was pre-linearized with the standard Ev/Pv primers, while the XylS/*P<sub>m</sub>* cassette was amplified with Ep/Sp. Both parts were already prepared and utilized in previous applications. All parts were assembled via SevaBrick Assembly and the transformed *E. coli* cells were plated on LB agar with the XylS/*P<sub>m</sub>* inducer (3-methylbenzoate). Based on the ratio of the black to white colonies, as shown in Figure 8B, the efficiency of the 6-part assembly calculated to 53%. The overall procedure of the operon construction is summarized in Figure 8A. Due to the modular design of the SevaBrick

Assembly primers which allows the use of multiple sets of ligation sites and the numerous high-fidelity combinations provided by NEB, the number of the OP<sub>RBS</sub> primer sets could be further increased. In addition, although in this work we used the standard BBa\_B0034 RBS for all four genes, the OP primer design allows the use of different or even random RBSs as previously described.

## Conclusions

The SEVA 3.1 platform was developed to provide a modular and versatile Golden Gate-based assembly method in combination with the part abundance of BioBricks and the flexibility of SEVA vectors. To this end, the SEVA 3.1 platform offers a newly engineered vector standard consisting of the SEVA backbone (AR, Ori, terminators) and the standard BioBrick cloning site. Any SEVA 3.1 vector is compatible with any available BioBrick part either via the BioBrick 3A Assembly or the proposed SevaBrick Assembly. The SevaBrick Assembly comprises a set of standard PCR primers which anneal to any SEVA 3.1/BioBrick compatible vector and enables Golden Gate assembly of single or multiple parts with minimal cloning effort and design. This was underscored by the fact that only fourteen primer sets had to be individually designed per part (convergence primers) for all 52 plasmids constructed in this study, while all assemblies performed using exclusively one restriction enzyme, *BsaI*. The capabilities of the SEVA 3.1 platform, were demonstrated by constructing several genetic constructs of various complexities. Using the standard primer sets and protocols (same for all applications) of the SevaBrick Assembly, we successfully cloned and expressed single and multigene TUs both in *P. putida* and *E. coli*. Additionally, to highlight the modularity of the SevaBrick primers, we cloned the *sfGFP* under the control of multiple genetic functional elements such as promoters, RBSs and origins of replication. Thus, the newly engineered SEVA 3.1 vectors, along with its proposed assembly, extend and simplify the applicability of the SEVA vectors and BioBricks, while maintaining their unique features and standards.

## Experimental procedures

### *Bacterial strains and growth conditions*

*E. coli* and *P. putida* were routinely grown in lysogeny broth (LB) medium with kanamycin (50 µg ml<sup>-1</sup>), chloramphenicol (35 µg ml<sup>-1</sup>) or gentamycin (10 µg ml<sup>-1</sup>) as needed at 37 °C and 30°C respectively. Solid media additionally contained 1.5% (w/v) agar. 1 mM of 3-methylbenzoate, 3 mM rhamnose and 10 mM arabinose were used as inducers of the *P<sub>m</sub>*, *P<sub>rhaB</sub>* and *P<sub>araB</sub>* promoters respectively. M9 minimal medium (6 g l<sup>-1</sup>

Na<sub>2</sub>HPO<sub>4</sub>, 3 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1.4 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g l<sup>-1</sup> NaCl, 0.2 g l<sup>-1</sup> MgSO<sub>4</sub>, 2.5 ml l<sup>-1</sup> trace elements solution (Nikel and de Lorenzo, 2014) supplemented either with 2% of glycerol (*E. coli*) or 2% glucose (*P. putida*) was used for the fluorescence assays.

#### Transformation

*E. coli* DH5 $\alpha$  chemical competent cells were prepared and transformed as described by Green and Rogers (2013). For transforming *P. putida*, 10 ml overnight LB culture was washed three times with 1 ml of 300 mM sucrose (filter-sterilized) and resuspended in 400  $\mu$ l of 300 mM sucrose. Later, 100 ng plasmid was electroporated into 100  $\mu$ l cell suspension aliquots with a voltage of 2.5 kV, 25  $\mu$ F capacitance and 200  $\Omega$  resistance.

#### DNA manipulation

Plasmids used in this study are listed in Table S5. General cloning procedures, such as endonuclease restriction digest, ligation and PCR, were performed with enzymes and buffers from New England Biolabs<sup>®</sup> (NEB; Ipswich, MA, USA) or ThermoScientific<sup>™</sup> (Waltham, MA, USA) according to the respective protocols. Q-5 hot start<sup>®</sup> polymerase was used for PCR if the resulting fragment was further used, otherwise for colony PCR, Phire<sup>®</sup> was the polymerase of choice. PCR purification was performed with the Macherey-Nagel purification kit. All primers were synthesized by IDT. Primer sequences are provided in the Table S4.

#### Biphasic PCR protocol with standard SevaBrick Assembly primers

For the preparation of the parts to be assembled and due to the complexity and length of the engineered primers, the standard PCR protocol had to be modified to a biphasic one: 98 °C, 2 min; Phase 1: (98 °C, 20 s; 50 °C, 20 s; 72 °C, part dependent)  $\times$  10; Phase 2: (98 °C, 20 s; 76 °C, 20 s; 72 °C, part dependent)  $\times$  25; 72 °C, 2 min. The final product is obtained using an initial amplification at low annealing temperature (50 °C) for 10 cycles and a subsequent amplification at an annealing temperature of 76 °C (higher than the standard annealing temperature range) for another 25 cycles. Each primer consists of a short 3'-end annealing sequence, a modular sequence in a non-annealing loop and optionally another standardized 5'-anchor sequence. The short 3'-end sequence requires a low annealing temperature (Phase 1) to be correctly hybridized with the template, while the anchor sequence, which optimizes the annealing fidelity elevates the T<sub>m</sub>. High annealing temperature leads to negative results, while the low temperature

cycling provides PCR amplified DNA but at a low yield. This low DNA concentration is due to the structural complexity of the long primers at this temperature. Running Phase 1 for 10 cycles provides enough PCR product which carries the additional sequences to be used as the template for Phase 2. At Phase 2, 76 °C is used for 25 additional cycles providing enough DNA to be used downstream in the process. 25  $\mu$ l reactions followed by agarose gel purification are recommended for optimal results.

#### SevaBrick Assembly

All DNA assemblies were performed via Golden Gate in 8  $\mu$ l reaction comprised of 2  $\mu$ l of the standard SevaBrick Assembly mix [12  $\mu$ l *Bsal*, 10  $\mu$ l T4 ligase (NEB), 18  $\mu$ l T4 ligase buffer (NEB), 1  $\mu$ l *DpnI* (NEB), 1  $\mu$ l of 20 mg ml<sup>-1</sup> BSA (NEB)] and 1  $\mu$ l of entry parts and backbone with final concentration of 1nM. An Excel file was programmed to automatically calculate DNA amounts and dilutions (Appendix S1). The SevaBrick Assembly protocol follows the thermocycling condition 37 °C, 20 min; (16 °C, 4 min; 37 °C, 3 min)  $\times$  30; 50 °C, 10 min; 80 °C, 10 min. 8  $\mu$ l of the assembly reaction mix was transformed into 50  $\mu$ l of chemically competent *E. coli* DH5 $\alpha$  cells using heat shock transformation. After recovery in 500  $\mu$ l of SOC medium, cells were plated onto LB antibiotic plates and grown at 37 °C overnight.

#### sfGFP fluorescence and growth measurements

All the genetic functional elements such as RBS, promoter and origin of replication parts were characterized in *P. putida* and *E. coli*. Single colonies were picked in triplicate and grown overnight at 30 °C (*P. putida*) or 37 °C (*E. coli*) in 10 ml LB with antibiotics. Cell density was measured with IMPLEN OD600 photometer at 600 nm and cells were diluted into 200  $\mu$ l of M9 medium with antibiotics, and inducers where needed, in a 96-well Greiner plate to a starting OD600 of 0.1. The plate was incubated at 30 °C or 37 °C for 12 h in a Synergy plate reader (Biotek). OD600 and sfGFP measurements were recorded every 10 min. Fluorescence was determined with the following settings: Ex. 467 nm, Em. 508 nm and the levels were corrected with the fluorescence signal of a blank sample. Relative sfGFP production was quantified after 8 h for *P. putida* or 5 h for *E. coli* from an average of triplicate data.

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### Conflict of interest

The authors declare no conflict of interest.

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### Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** List of available pSEVAb vectors.

**Table S2.** Standard flanks of the Convergence, OP1-FRBS and Evpromoter primers.

**Table S3.** List of converged parts (BioBricks).

**Table S4.** List of oligonucleotides used in this study.

**Table S5.** Plasmids used and constructed in this study.

**Fig. S1.** PCR amplified SEVA modules.

**Fig. S2.** Graphical representation of XylS/Pm domestication primers.

**Fig. S3.** Graphical representation of SevaBrick primers attached on MCS 2.0

**Fig. S4.** DNA sequencing summary of predicted RBSs + promoter BBa\_J23105 + sfGFP variants from Figure 7.

**Fig. S6.** DNA sequencing summary of random RBSs + sfGFP variants from the last plate of Figure 7G.

**Fig. S7.** DNA sequencing summary of constitutive promoters + RBS BBa\_0034 + sfGFP variants from Fig. 7.

**Appendix S1.** Calculation of DNA parts concentrations for SevaBrick assembly.