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A Solid-Phase Platform for Combinatorial and Scarless Multipart Gene Assembly

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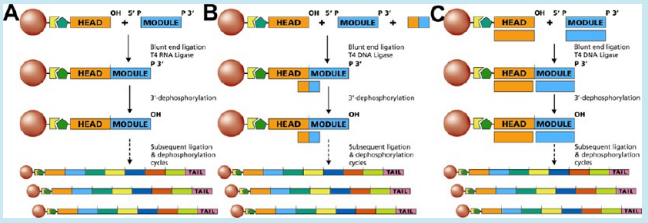
Supporting Information

ABSTRACT: With the emergence of standardized genetic modules as part of the synthetic biology toolbox, the need for universal and automatable assembly protocols increases. Although several methods and standards have been developed, these all suffer from drawbacks such as the introduction of scar sequences during ligation or the need for specific flanking sequences or *a priori* knowledge of the final sequence to be obtained. We have developed a method for scarless ligation of multipart gene segments in a truly sequence-independent fashion. The big advantage of this approach is that it is combinatorial, allowing the generation of all combinations of several variants of two or more modules to be ligated in less than a day. This method is based on the ligation of single-stranded or double-stranded oligodeoxynucleotides (ODN) and PCR products immobilized on a solid support. Different settings were tested to optimize the solid-support ligation. Finally, to show proof of concept for this novel multipart gene assembly platform a small library of all possible combinations of 4 BioBrick modules was generated and tested.



KEYWORDS: *standardized, scarless, multipart DNA assembly, solid-phase, SEC*

The field of synthetic biology heavily relies on robust methods to enable the construction of genes, pathways, and genomes.[1−5] All of these methods differ in both mechanism and scale. This offers the user methods for self-assembly of many parts in a single reaction (parallel assembly) and assembly of constructs with a predefined physical arrangement (ordered assembly) and allows the use of multiple parts simultaneously (combinatorial assembly).[6]

The ideal assembly method should be based on a modular assembly strategy, allowing seamless and sequence-independent fusion of modules in a fast, simple, and combinatorial way. A modular strategy enables the use of standardized parts and simplifies the generation of combinatorial gene libraries. Fusion of the modules must be seamless to avoid unwanted sequences that could have undesired effects and should be sequence-independent in order to allow for the creation of combinatorial gene libraries. Furthermore, the fusion method should be fast and simple to increase the number of generated constructs. However, current methods do not possess all of these characteristics.

Almost all currently used modular strategies (including BioBrick, BglBrick, and Golden Gate) that are often based on BioBrick assembly standards require restriction endonuclease digestion, which results in scar formation and is sequence-dependent.[4,5,7−10] Also, most modular strategies require multiple ligation, transformation, overnight growth, and plasmid extraction steps in order to align multiple modules in a construct.

Methods for seamless fusion, including SLIC, Gibson, and CPEC, rely on synthetic oligonucleotides or PCR-generated modules with complementary regions, which are annealed and then fused by using either ligases or polymerases.[5,8,11−15] Using these methods, large constructs can be created in single-step assembly reactions, which improves construction speed.[13,16] However, due to the use of complementary regions, fusion is sequence-dependent and hinders simultaneous assembly of different modules for the construction of combinatorial gene libraries. Also, repetitive and GC-rich sequences are problematic.[6]

Here we present a modular assembly strategy for seamless, sequence-independent and fast combinatorial gene construction at the gene/pathway level. The assembly strategy is based on the ligation of single- or double-stranded oligonucleotides (ssODN or dsODN) using a solid-phase platform, as initially described by Dietrich et al.[17] Their method is further optimized by a decreased ligation time and increased amount of ligated dsODNs and expanded by including the assembly of ssODN as well as using PCR-generated dsODN. The use of a solid-phase system facilitates purification of the obtained products and enables automation for high-throughput gene construction.

Our strategy includes three different assembly methods: (I) solid-phase assembly of single-stranded oligonucleotides, (II) solid-phase assembly of single-stranded oligonucleotides using

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linkers, and (III) solid-phase assembly of double-stranded oligonucleotides, either obtained by PCR or by annealing complementary ssODN. To demonstrate the feasibility of our methods, a protein expression device containing a small RBS library taken from the BioBrick parts registry and a combinatorial library encoding multidomain peptide vectors for gene delivery were created.

RESULTS

Solid-Phase Assembly. In our attempt to construct combinatorial gene libraries in a sequence-independent manner without using restriction endonucleases, 3 different ligation methods were tested and compared: (A) ligation of ssODN using RNA ligase, (B) ligation of ssODN using DNA ligase in the presence of linker ODN, and (C) blunt-end ligation of dsODN using DNA ligase. For all three strategies ligation was performed on DNA immobilized on magnetic beads to facilitate and accelerate iterative ligations rounds.

For the ligation of ssODN using RNA ligase (Figure 1A), a universal single-stranded oligodeoxynucleotide (ssHead) modified with 5′-biotin was immobilized on streptavidin-coated magnetic beads. Using RNA ligase, 5′-phophorylated single-stranded oligodeoxynucleotides (ssModules) were ligated stepwise to the ssHead. To ensure proper orientation of the ligated ssModules and to prevent multiple additions of ssModules, the ssModules were 3′-phosphorylated. In the final ligation step, a universal single-stranded oligodeoxynucleotide (ssTail) was added. One ligation/dephosphorylation cycle took 16 h.

The ligation of ssODN in the presence of linker ODN with DNA ligase makes use of linkers to stabilize the association of 3′-hydroxyl group and 5′-phosphate group of two ssModules in order to enhance ligation efficiency (Figure 1B). The used linkers were 12 nucleotides long and were complementary to the last 6 nucleotides of the immobilized ssModule and the first 6 nucleotides of ssModule to be ligated. The linkers supported the assembly of a preligation complex, which can be recognized and joined by DNA ligase. One ligation/dephosphorylation cycle took 1 h.

Blunt-end ligation of dsDNA made use of double-stranded oligodeoxynucleotides (dsModules) and DNA ligase (Figure 1C). One ligation/dephosphorylation cycle took 1 h.

Comparison of the Three Assembly Methods. In order to compare the three different assembly methods, three different sized ss- or dsModules (30, 60, and 90 nucleotides) were ligated onto a ss- or dsHead (36 nucleotides) in one assembly cycle. After ligation, equal amounts of magnetic beads were loaded onto a 10% TBE-urea gel (Figure 2).

Only faint bands were visible at the height of the expected ligation products (boxes) when using the ssODN assembly method, where the use of thermostable RNA ligase (Epicenter) resulted in higher ligation efficiencies compared to T4 RNA ligase. Clear bands were visible for both the ssODN linker and dsODN assembly methods.

In order to improve ssODN assembly, several different parameters were screened, which included the use of different ligation temperatures and the addition of hexamminecobalt(III) chloride (HCC), betaine, and spermidine. Ligation reactions performed with thermostable RNA ligase (Epicenter) at 60 °C or T4 RNA ligase (NEB) at 22 °C showed the highest ligation efficiency, compared to other RNA ligases (data not shown). Also, the addition of DMSO, HCC, betaine, or spermidine did not enhance ligation efficiency (data not shown).

Ligation efficiency of the three assembly methods was determined by size-exclusion chromatography (SEC). Three different sized ss- or dsModules (45, 60, and 90 nucleotides) were ligated in one assembly cycle to a ss- or dsHead module (36 nt) to create Head-module constructs. The obtained ligation efficiencies are shown in Table 1 and were in line with the observed ligation efficiencies on gel (Figure 2). The ssODN linker and dsODN assembly methods displayed the highest ligation efficiencies with 93% and 55%, respectively. The ssODN assembly method displayed lower ligation efficiencies. The use of thermostable RNA ligase resulted in higher ligation efficiencies compared to normal RNA ligase. The difference in ligation efficiency between the three differently sized modules is less for the ssODN linker and dsODN assembly methods, compared to the ssODN assembly methods.

Creation of Multipart Combinatorial Libraries by ssODN Ligation in the Presence or Absence of Linker ODN. For some applications, such as the generation of gene libraries encoding TALEs,[18] elastin-like polymers,[19] or multidomain DNA carrier proteins,[20] a technique to ligate different combinations of DNA modules in a combinatorial fashion is highly desired. To demonstrate our technology we have tested two conditions to ligate two different ssODN modules in a sequence-independent and/or scarless fashion. Ligation of ssODN using RNA ligase and ligation of dsODN using DNA ligase was used for the seamless fusion of modules. For the ligation of ssODN in the presence of linker ODN using DNA ligase, ssModules were flanked with linker sequences GGTTCT at the 5′ and GGTGGC at the 3′. Both assembly methods used the same Head module and Tail module. Both Head and Tail module contain restriction sites for cloning purposes.

First, specific constructs were created using both assembly methods for a proof of principle. Two different sized ssModules, module T (45 nucleotides) and module L (90 nucleotides), were ligated onto the ssHead module (36 nucleotides). Multiple assembly cycles were used to ligate multiple T and L modules to each other, creating Head-T_n and Head-L_n where n represents the number of assembly cycles. All constructs were terminated with a Tail module (45 nucleotides). In addition, an alternating construct of both T and L modules, Head-$(T\text{-}L)_3$, was created using ODN linkers. The constructs formed were analyzed on a 6% TBE-urea gel, after loading equal amounts of magnetic beads (Figures 3a and 4a).

For the determination of the ligation efficiency of the subsequent assembly cycles, specific constructs were created using all three assembly methods. In six subsequent assembly cycles, three different sized ss- or dsModules (module T, module S (60 nucleotides), and module L) were ligated onto a ss- or dsHead module. The constructs created by ssODN assembly and ssODN linker assembly were terminated with a Tail module in the seventh subsequent assembly cycle. The constructs formed were analyzed by SEC and loaded on a 6% TBE-urea gel (Supplementary Figures 1−7).

The Head-T_2-Tail and Head-L_2-Tail constructs generated using the solid-phase assembly of ssODN with RNA ligase showed expected bands; however, the bands were only faintly visible (Figure 3a, lanes 2 and 4). In contrast Head-T_2-Tail and Head-L_2-Tail constructs, generated using the solid-phase assembly with linker ODN showed the expected bands clearly (Figure 3a, lanes 1 and 3). SEC analysis confirmed the low yield of ligation products with more than 2 modules.

In order to verify product length and attachment of the Tail module, constructs were extracted from gel, purified and amplified by PCR. Only faint products bands could be

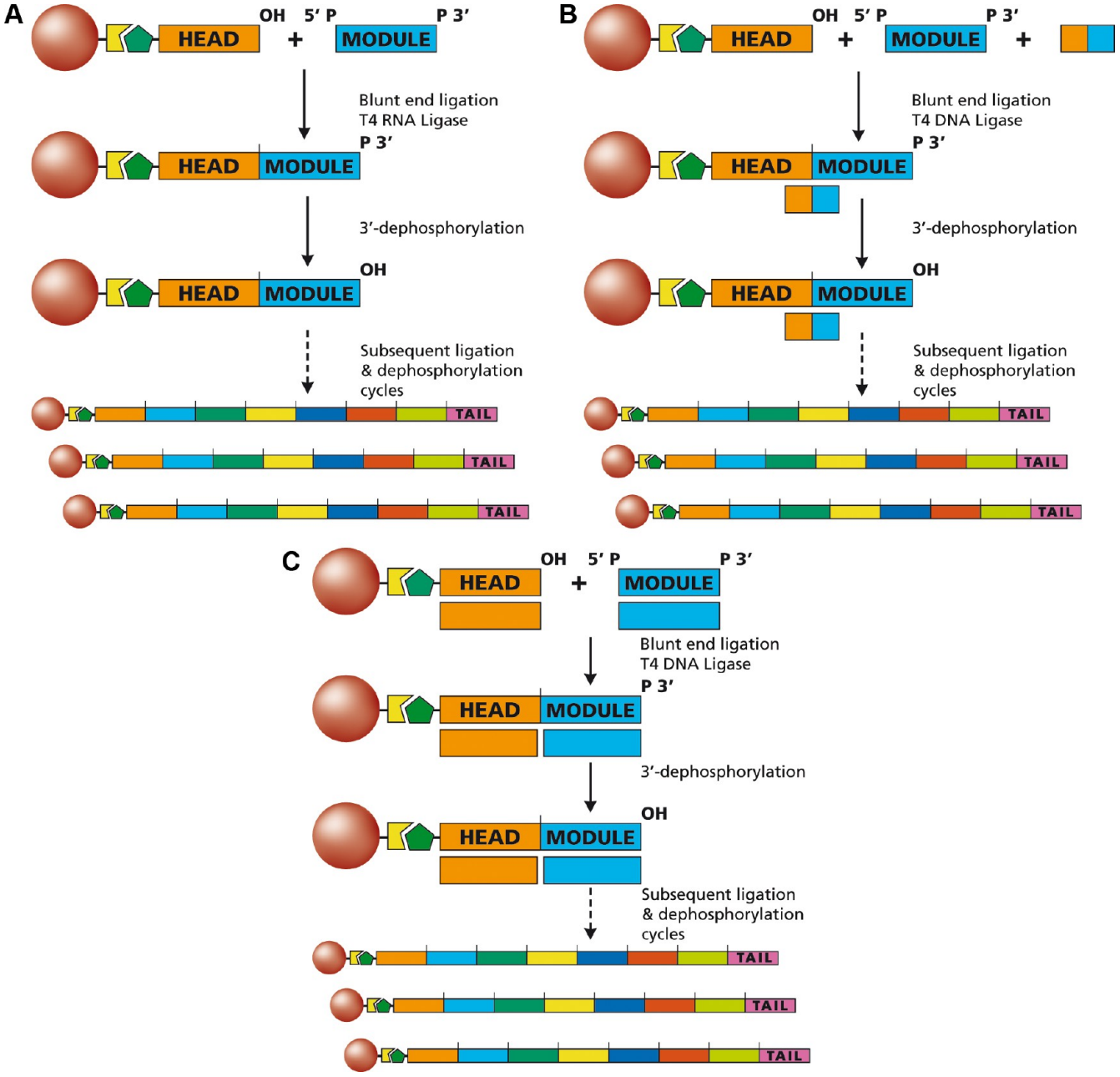

Figure 1. (A) Solid-phase assembly of single-stranded oligodeoxynucleotides. 5′-Biotinylated ssHeads were immobilized on streptavidin-coated magnetic beads. Using RNA ligase, 5′-phophorylated ssODN were ligated to the ssHeads. To ensure proper orientation and to prevent multiple additions of ssModules, the ssModules were 3′-phosphorylated. By using repetitive ligation/dephosphorylation cycles, it is possible to assemble products of any length and composition. In the final ligation step, a ssTail containing a double stop codon was added. (B) Solid-phase assembly of single-stranded oligodeoxynucleotides using linkers. 5′-Biotinylated ssHeads were immobilized on streptavidin-coated magnetic beads. Using linker ODN, the 3′-hydroxyl group and 5′-phosphate group of two ssModules were brought in close proximity. The linkers are complementary to the last 6 nucleotides of the immobilized ssModule and the first 6 nucleotides of ssModule to be ligated. The linkers support the assembly of a preligation complex, which can be recognized and joined by DNA ligase. By using repetitive ligation/dephosphorylation cycles, it is possible to assemble products of any length and composition. In the final ligation step, a ssTail containing a double stop codon was added. (C) Solid-phase assembly of dsODN using blunt-end ligation. 5′-Biotinylated ssHeads were immobilized on streptavidin-coated magnetic beads, and ssModules were made double-stranded by hybridization with complementary ODN. Using DNA ligase, 5′-phophorylated dsModules can be liagted to the ssHeads. To ensure proper orientation and to prevent multiple additions of ssModules, the ssModules were 3′-phosphorylated. By using repetitive ligation/dephosphorylation cycles, it is possible to assemble products of any length and composition. In the final ligation step, a dsTail containing a double stop codon was added.

detected for constructs generated in the absences of linker ODN (Figure 3b).

Figure 4a shows the Head-Tail, Head-T_n-Tail, Head-L_n-Tail (n = 1, 2, 3, and 6), and Head-$(T\text{-}L)_3$-Tail constructs generated using the solid-phase assembly with linker ODN. All constructs showed the expected band pattern. Only the band of Head-T_6-Tail is not clearly visible. SEC analysis confirmed the distribution of ligation products and corresponds to the observed products on gel. Where subsequent ligation cycles with module T yielded ligation products with mostly 2, 3, and 4 modules,

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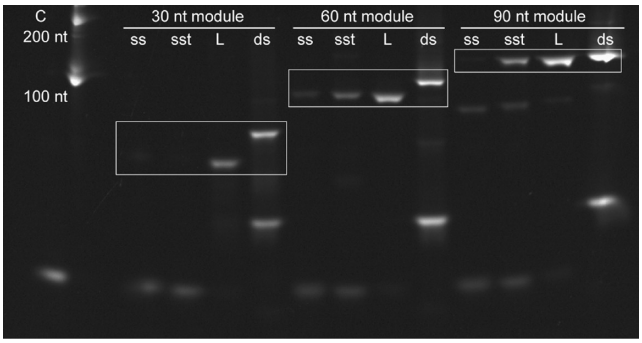

Figure 2. Comparison of the three assembly methods: assembly of ssODN's using RNA ligase (ss); assembly of ssODN's using thermostable RNA ligase (sst); assembly of ssODN using linker ODN (L); assembly of dsODN (ds). Ligation reactions were performed with ss- or dsModules 30, 60, and 90 nucleotides long. Equal amounts of magnetic beads were loaded on a 10% TBE-urea gel. Ladder: low range RNA ladder. C: 3.1 pmol Head.

Table 1. Ligation Efficiencies of the Three Assembly Methods Determined by SEC. ND:

	ligation efficiency (%)			
assembly method	45 nt module	60 nt module	90 nt module	average
ssODN assembly	ND[a]	5	17	11
ssODN assembly using thermostable RNA ligase	ND[a]	30	75	53
ssODN linker assembly	ND[a]	93	93	93
dsODN assembly	59	57	50	55

[a]Not determined.

subsequent ligation cycles with module s and L yielded ligation products with mostly 5, 6, and 7 modules (Supplementary Figures 1, 3, 5 and 7).

In order to verify product length and attachment of the Tail module, constructs were extracted from gel (boxes Figure 4a), purified, and amplified by PCR (Figure 4b). Constructs with up to 3 repeating modules and a Tail module were amplified successfully and showed the expected lengths. Product bands could be clearly identified after amplification of constructs generated by assembly using linker ODN. However, amplification of Head-T_6-Tail, Head-L_6-Tail, and Head-$(T\text{-}L)_3$-Tail yielded multiple bands, all shorter in size than the expected products. Product bands corresponding to Head-T_1-Tail through Head-T_5-Tail and Head-Tail could be detected after amplification of Head-T_6-Tail construct. Only products corresponding to Head-L_1-Tail and Head-Tail could be detected after amplification of Head-L_6-Tail. After amplification of Head-$(T\text{-}L)_3$-Tail, multiple product bands could be detected corresponding to constructs with multiple T and/or L modules.

Gel electrophoresis and SEC analysis showed the expected band pattern of the constructs generated by solid-phase assembly of dsODN with DNA ligase, in six subsequent assembly cycles with three different sized dsModules (Supplementary Figures 2, 4, 6, and 7).

After the creation of specific constructs, two random libraries were assembled using the solid-phase assembly of ssODN with linkers. A mix of nine different ssModules was ligated onto a ssHead. Three or six assembly cycles were performed with this mix, generating two libraries, Head-MIX_3 and Head-MIX_6. This would theoretically yield libraries with $9^3 = 729$ and $9^6 = 531441$ different constructs. Both libraries were terminated with a

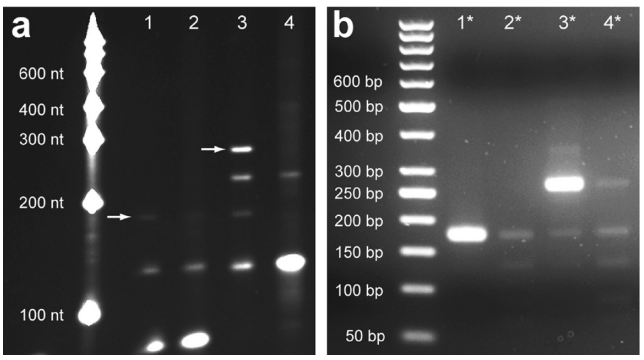

Figure 3. Ligation of ssODN using RNA ligase (a) and subsequent amplification of purified ligation products by PCR (b). (a) 6% TBE-urea gel showing the ligation products after ligating 3 modules with either DNA ligase in the presence of linker ODNs (lanes 1 and 3) or with RNA ligase (lanes 2 and 4). Formed products are Head-T_2-Tail (lanes 1 and 2) and Head-L_2-Tail (lanes 3 and 4). For size estimation, a low range RNA ladder was loaded. (b) PCR products loaded on 2% w/v agarose gel with a 50 bp DNA ladder. * lanes correspond with lanes in panel A. Lane 1* and 3*: Head-T_2-Tail and Head-L_2-Tail using the assembly of ssODN in the presence of linker ODN using DNA ligase, respectively. Lanes 2* and 4*: Head-T_2-Tail and Head-L_2-Tail using the assembly of ssODN using RNA ligase, respectively.

Table 2. Theoretical Length of Ligation Products[a]

product	length	product	length
Head-T-Tail	128	Head-L-Tail	173
Head-T2-Tail	173	Head-L2-Tail	263
Head-Tail	83		

[a]In number of nucleotides.

Tail module. The formed constructs were analyzed on a 6% TBE-urea gel, after loading equal amounts of magnetic beads.

Both generated libraries were clearly visible and appeared as smears (Figure 5a). Separate constructs could still be detected in the lower regions but with increasing size the separate construct bands became gradually obscured in a diffuse smear. By increasing the number of assembly cycles from three to six, the absolute size of the constructs and the average size of the library increased.

After extraction from gel and purification, both libraries were amplified by PCR (Figure 5b). Also after PCR amplification, smears were detected indicating that all formed constructs were amplifiable. PCR smears were denser at the bottom of the gel (smaller constructs) than at the top of the gel (larger constructs).

The PCR-amplified Head-MIX_6-Tail library was excised from the smallest theoretical 6 module construct (±350 bp) to the largest theoretical 6 module construct (±700 bp), purified, and cloned into the *Bam*HI/NcoI-sites of vector pIVEX-EJ. *E.coli* strain DH5α was transformed with the cloned library. To check for cloning bias, all colonies of a single plate were pooled, and pDNA was harvested. After restriction enzyme analysis using *Bam*HI/NcoI, the same smear pattern was observed as with the initial PCR amplification (results not shown).

To check for insert size, restriction analysis using *Bam*HI/NcoI was performed on pDNA obtained from 20 colonies after overnight culturing. Two colonies contained Head-Tail inserts, all other 18 colonies contained inserts corresponding to at least one peptide module, and no empty vectors could be detected (Figure 5c). Sequence analysis of 10 clones confirmed the

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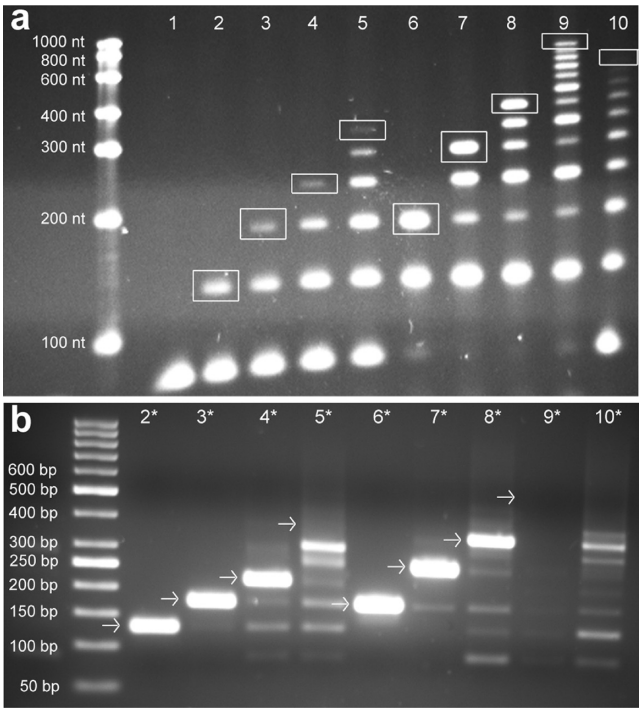

Figure 4. Ligation of ssODN in the presence of linker ODN (a) and subsequent amplification of purified ligation products by PCR (b). (a) 6% TBE-urea gel showing the ligation products after ligating 1, 2, 3, 4, and 7 modules with DNA ligase in the presence of linker ODNs. Formed products are Head-Tail (lane 1); Head-T_n-Tail, with n = 1, 2, 3, and 6 (lanes 2–5); Head-L_n-Tail, with n = 1, 2, 3, and 6 (lanes 6–9) and 10 Head-$(T\text{-}L)_3$-Tail (lane 10). For size estimation, a low range RNA ladder was loaded. Boxed constructs were extracted from gel, purified, and amplified. (b) PCR products loaded on 2% w/v agarose gel with a 50 bp DNA ladder. * lanes correspond with lanes of A. Lanes 2*–5*: Head-T_n-Tail with n = 1, 2, 3, and 6 respectively. Lanes 6*–9*: Head-L_n-Tail with n = 1, 2, 3, and 6 respectively. Lane 10*: Head-$(T\text{-}L)_3$-Tail. Arrows indicate expected product heights.

Table 3. Theoretical Length of Ligation Products[a]

product	length	product	length
Head-T-Tail	128	Head-L-Tail	173
Head-T_2-Tail	173	Head-L_2-Tail	263
Head-T_3-Tail	218	Head-L_3-Tail	353
Head-T_6-Tail	353	Head-L_6-Tail	623
Head-Tail	83	Head-$(T\text{-}L)_3$-Tail	488

[a]In number of nucleotides.

error-free ligation and random insertion of all used modules in the multipart constructs (Table 4).

Assembly of a Protein Expression Device Containing a Small RBS Library. A goal in synthetic biology is identification of the precise level of activity of specific parts. As an example, in metabolic engineering the expression levels of individual enzymes are modulated through, e.g., manipulating promoter strengths and ribosome binding sites (RBS).[21] Solid-phase assembly of dsODN modules is ideal for modular screenings, as it allows for seamless, fast, and modular assembly.

Using solid-phase assembly of dsODN modules, a small library of RBSs was screened on translation efficiency. From the Registry of Standard Biological Parts catalog,[22] the Tet promoter sequence (BBa_J72005), four RBS sequences (BBa_B0034, BBa_B0035, BBa_J61141, and BBa_K376001) and the

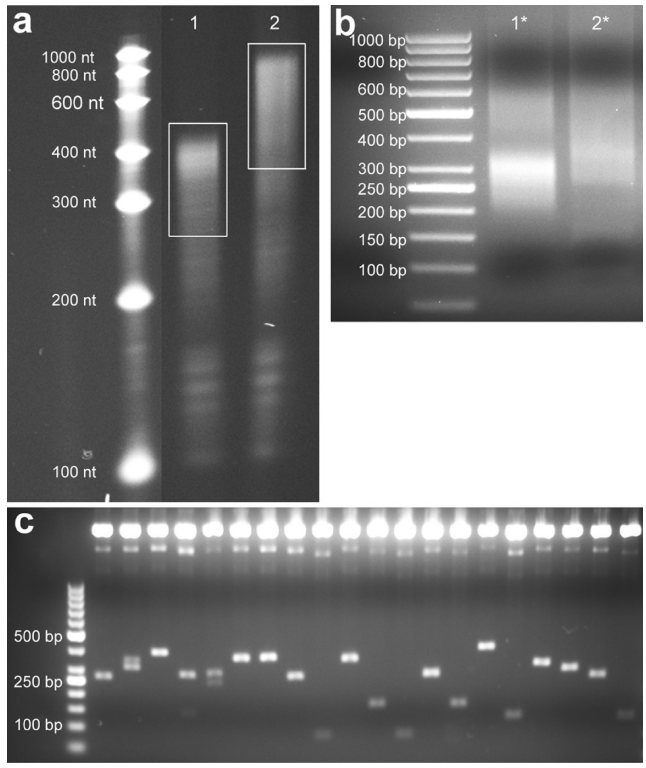

Figure 5. Random multidomain peptide libraries constructed with the assembly of ssODN using linker ODN. (a) Initial ligation products were loaded on a 6% TBE-urea gel and purified at final product height as indicated. Ladder: low range RNA ladder. Lanes 1 and 2: H-MIX3-T and H-MIX6-T, respectively. (b) Purified ligation products were amplified using PCR and loaded on a 2% agarose gel. Ladder: 50 bp DNA ladder. Lanes 1* and 2*: PCR-amplified ligation products corresponding to lanes 1 and 2. (c) Plasmid DNA isolated from 20 colonies was digested with *Bam*HI/NcoI and loaded on 0.7% agarose gel. Eighteen out of 20 colonies contained inserts corresponding to at least one peptide module.

Table 4. Modules Present in the 10 Sequenced Constructs

construct	module present
1	EB1-NLSV402
2	H5WYG-NLSV402
3	EB1-NLSV402-H9.2
4	NLSV402-EB1
5	EB1-ppTG20-SPKR4
6	SPKR4-SPKR4
7	H9.2-NLSV402
8	EB1-ppTG20
9	NLSV402-Pr18-TAT
10	SPKR4-SPKR4

LuxICDABEG terminator sequence (BBa_B0011) were selected to create an expression device. As a reporter, Enhanced Green Fluorescent Protein (EGFP) was chosen (Figure 6). To all four RBS sequences, a 5 nucleotide spacer was added to create a spacer between the RBS sequence and the start codon of the EGFP (Figure 6). The Head module contains the promoter sequence, and the Tail module contains a stop codon and the terminator sequences. Both Head and Tail module contain restriction sites for cloning purposes.

First, a mix of the four different ds-modules encoding the four different RBSs was ligated onto the dsHead. Then, the

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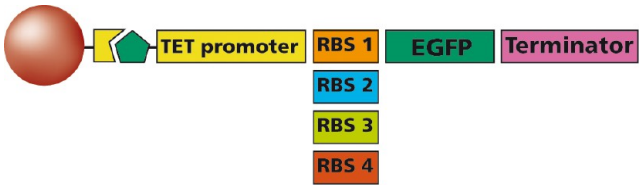

Figure 6. Using the solid-phase assembly of dsODN using DNA ligase, a series of EGFP expression devices was generated. A small library was created with the possibility of 4 different ribosome binding sites coding sequence parts between the promoter and EGFP coding sequence parts.

dsEGFP and dsTial modules were sequentially ligated. The formed constructs were analyzed on a 6% denaturing polyacrylamide TBE-urea gel, after loading equal amounts of magnetic beads.

Figure 7a shows the generated Promoter-RBS$_{mix}$-EGFP-Terminator constructs using the solid-phase assembly of dsODN.

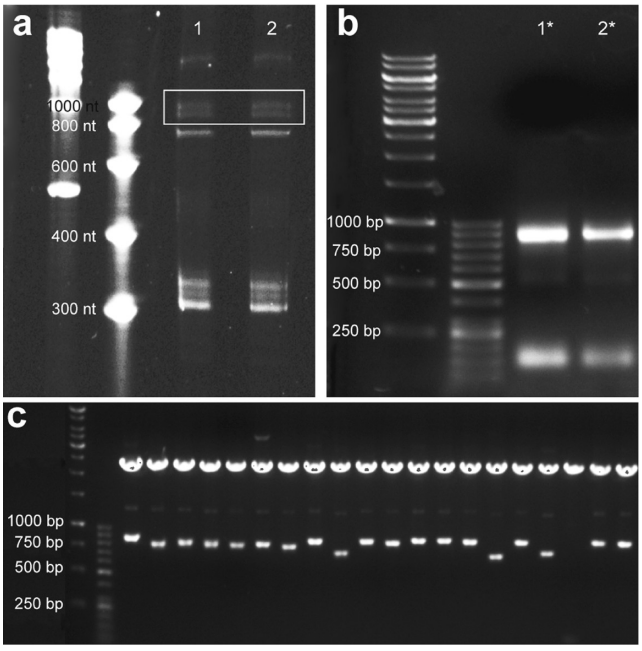

Figure 7. EGFP expression devices containing a small RBS library were created using solid-phase assembly of dsODN using DNA ligase. (a) Initial ligation products were loaded on a 6% TBE-urea gel and purified at final product height as indicated. First ladder: high range RNA ladder. Second ladder: low range RNA ladder. Lanes 1 and 2: Promoter-RBS$_{mix}$-EGFP-Terminator in duplo. (b) Purified ligation products were amplified using PCR and loaded on a 2% agarose gel. Ladder: 50 bp DNA ladder. Lanes 1* and 2*: PCR-amplified ligation products corresponding to lanes 1 and 2. (c) Plasmid DNA isolated from 20 colonies was digested with *Bgl*II/*Hin*dIII and loaded on 0.7% agarose gel to confirm the presence of promoter-RBS-EGFP-Terminator coding sequence.

The theoretical size of the library constructs ranges from 858 to 870 nucleotides and on gel product bands of these sizes were clearly visible. Also bands of intermediate products were visible.

After extraction and purification of the product bands between 800 and 1000, the library constructs were amplified by PCR. The library constructs were amplified successfully and showed bands with expected lengths (Figure 7b).

The PCR-amplified Promoter-RBS$_{mix}$-EGFP-Terminator library was excised, purified, and cloned into the *Bgl*II/*Hin*dIII-sites of the vector pIVEX-LacZ. *E.coli* strain XL-1 blue was transformed with the cloned library, and the number of colonies obtained per transformation was approximately 200.

To check for insert size, restriction analysis using *Bgl*II/*Hin*dIII was performed on pDNA obtained from 39 colonies after overnight culturing, of which 20 are shown in Figure 7c. Two colonies contained empty vectors. In total, 25 colonies contained pDNA with inserts corresponding to the size of the Promoter-RBS$_{mix}$-EGFP-Terminator constructs.

To check for construct integrity, fluorescence of the same overnight cultured colonies was determined. A total of 30 overnight cultured colonies showed fluorescence levels above background. Within this group, large differences in fluorescence levels were observed.

To confirm seamless ligation and identification of the different RBSs, 21 out of the 39 obtained constructs were sequenced. Two constructs did not contain an RBS module, and the corresponding colonies did not display fluorescence. One construct contained a correctly insert but did not display fluorescence. The other constructs contained seamless and correct ligated modules that displayed fluorescence (Figure 8). All four different RBSs were found in the constructs and had similar occurrences. The differences between activity of the four RBSs is clearly visible in Figure 8; constructs with the K376001 RBS displayed the highest level of fluorescence, and constructs with the J61141 RBS displayed the lowest level of fluorescence.

Although many different methods have been developed for the assembly of multipart gene segments, these methods either introduce scar sequences during ligation or rely on the use of predefined flanking regions for seamless ligation. In theory, the solid-phase assembly of ssODN using RNA ligase is the ideal method for the creation of gene constructs. It is simple, requires only (modified) single-stranded oligodeoxynucleotides and RNA ligase, allows for seamless and sequence-independent ligation, and uses a modular approach. Also, through the use of a solid support platform, this method is suited for high-throughput usage.

Compared to methods based on restriction endonucleases, the advantages of solid-phase assembly of ssODN using RNA ligase is the ability for scarless ligation, whereas methods using restriction endonucleases introduce at least a 2 bp scar. Moreover, in contrast to methods based on complementary overhangs, solid-phase assembly of ssODN using RNA ligase is truly sequence-independent. Also, ssModules with repetitive or identical sequences can be used, whereas methods based on complementary overhangs have difficulties with sequences homology.[23] Also, without the need of restriction sites or flanking regions, module design is straightforward and modules can be reused. However, methods based on complementary overhangs can assemble multiple parts in one step and most restriction endonuclease methods are cheaper. Although our proposed ligation method requires 5′ and 3′ modifications of ODNs, which makes this method more expensive, this can be compensated by using ssODNs and the general applicability of the ODNs.

Direct oligosynthesis methods, such as developed by Blue Heron Biotechnology, also use a solid support-based ligation-mediated assembly process to synthesize DNA.[12,24] In each ligation round, the next section of dsDNA anneals to the previously assembled dsDNA, which is attached onto a solid support, through designed sequence overhangs, and the new section of dsDNA can be ligated using DNA ligase. Using this technology, DNA fragments of 50 bp to 25 kb have been synthesized with high accuracy, repetitive sequences, and complex secondary structures.[12] However, by using overhangs, reuse of dsDNA

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Table 5. Name, Function, and Sequence of Used Modules

module name	function	sequence (nt)
Head_pTET_J72005	TET promoter	AGCCAGATCTTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCAC
BBa_B0034	RBS	AAAGAGGAGAAACACAT
BBa_B0035	RBS	ATTAAAGAGGAGAACACAT
BBa_J61141	RBS	TGGCTAACTGAGGATCACAT
BBa_K376001	RBS	TGGCTAACATAGGGTCACAT
Tail_Term_B0011	terminator	AAAGAGAATATAAAAAGCCAGATTATTAATCCGGCTTTTTTATTATTTAAGCTTCACGC

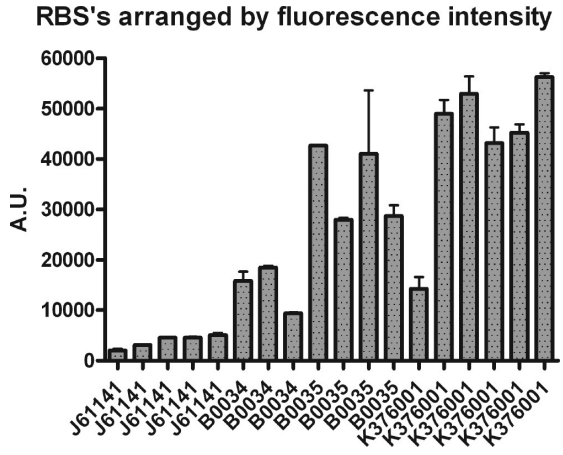

Figure 8. EGFP fluorescence of colonies containing the RBS library constructs, sorted by RBS.

modules is not possible and the method becomes sequence-dependent.

Using solid-phase assembly of ssODN with RNA ligase, we were able to ligate 3 ssModules onto an ssHead in 3 separate assembly cycles. However, the ligation efficiency of the solid-phase assembly of single-stranded modules method is low, with an average of 9%. Due to overlapping retention times of the nonligated ssModule and the ssHead, the ligation efficiency of the 45 nt ssModule could not be determined (Supplementary Figure 1). The low ligation efficiency may have several causes.

One is the formation of secondary structures in the used modules, which can restrict the accessibility of the 5′- and 3′-ends of the ssODN for ligation. In fact, we experienced that if the secondary structure involves the 5′- or the 3′-end of the oligonucleotide, ligation is impaired. This can also explain the observed differences in ligation efficiencies between the different modules. The secondary structure formation can be diminished by addition of DMSO, HCC, or betaine.[25,26] However, the addition of these reagents did not increase ligation efficiency in our hands (data not shown). Another strategy to diminish secondary structure formation is increasing the reaction temperature to >50 °C. At present there is only one thermophilic RNA ligase commercially available (Epicenter, TRL8101K). Using this thermostable RNA ligase at 60 °C, ligation efficiency increased (with an average of 53%), as do the costs. Secondary structure can also be diminished by altering the nucleotide sequence. Modules encoding proteins can be sequence-optimized using different codons. However, the sequences of RBSs, promoters, or other regulatory elements cannot be altered without changing their activity.

Besides the formation of secondary structures as a cause for the low ligation efficiency, it is known that RNA ligases have a lower intrinsic activity compared to DNA ligases. Because of this, one assembly cycle took 16 h. In order to assemble a construct containing 5 different modules, it takes 4 assembly cycles and thus 4 days. By increasing the assembly cycle time, ligation efficiency could be improved, but also the total assembly time is further increased.

To increase the ligation efficiency of solid-phase assembly of ssODN modules, other parameters may be investigated. Such parameters should be generally aimed at decreasing the random molecular motion in solution of the 5′ phosphate and 3′ hydroxyl groups and increasing the association of both groups. One could think of the use of nucleotide binding proteins or polymers and enhancement of molecular crowding by addition of PEG, dextrans, or BSA. The addition of spermidine, a protein that binds nucleic acids, did not result in enhanced ligation efficiency (results not shown).

To improve the ligation efficiency of the solid-phase assembly of ssODN, a 12 nucleotide linker was introduced. The linker is complementary to the last 6 nucleotides of the immobilized ssModule and the first 6 nucleotides of ssModule to be ligated. The linkers support the assembly of a preligation complex, which stabilizes the 3′-hydroxyl group and 5′-phosphate group of two ssModules and can be ligated by DNA ligase.

The use of linkers greatly improved ligation efficiency, up to an average of 93%, and therefore the assembly cycle time could be decreased to 1 h. Compared to Dietrich et al., the ligation efficiency almost doubled, from 35−70% to 80−90%.[17] As mentioned earlier, the ligation efficiency of the 45 nt ssModule could not be determined (Supplementary Figure 1). However, by using linkers, the assembly method either becomes sequence-dependent or introduces scars. By using linkers containing the last 6 nucleotides of module A and the first 6 of nucleotides of module B, the construct AB can be joined seamlessly. For the seamless joining of construct BA, a different linker is needed. If one specific construct of 5 modules is needed, 4 different linkers are required. For generating a random library containing 5 different modules, 25 different linkers are required. However, the 12 nt linker is shorter than the minimal 20 bp overhang/known sequence required in methods for seamless fusion (including SLIC, CPEC, and Gibson).[13−15]

To circumvent this problem universal linkers can be used and random libraries can be constructed using only one linker. However, this will create a 12 nucleotide scar, which becomes a problem for libraries containing RBSs or other regulatory elements, which can be hampered by the introduction of scars, or if the ligated modules are part of a coding gene. Although the 12 nt scar is longer than scars introduced by the BioBrick (8 bp), BglBrick (6 bp), and Golden Gate (4 bp) assembly methods, the 12 nt scar is sequence-independent and can be reused.[7,9,10]

Also, with the linker assembly method, secondary structures can hinder ligation. If the secondary structure involves the 5′- or the 3′-end of the oligodeoxynucleotide, the linker cannot bind to its complementary sequence and ligation is impaired.

In this paper we assembled multipart constructs using all three solid-phase assembly methods. Seven ssModules could be

ligated onto a ssHead module using solid-phase assembly of ssODN with linkers, and 6 dsModules could be ligated onto a dsHead module using solid-phase assembly of dsODN. Dietrich et al. only demonstrated the ligation of 4 dsModules onto a Head module.[17]

For ssODN assembly, the low ligation efficiency hinders the formation of constructs with more than 3 ssModules (Figure 3a; Supplementary Figures 1, 3, 5, 7; Supplementary Tables 1, 3, 5).

For ssODN assembly using linkers, after seven subsequent ligation cycles, a distribution is expected of 12%, 37%, and 48% for the Head-M5, Head-M6, and Head-M7, respectively (based on 90% ligation efficiency). If the ligation efficiency decreases during the subsequent cycles, the distribution will shift toward lower ligation products. For the S and L module, 52% and 34% of the products contain at least 6 modules after seven ligation cycles, respectively (Figure 4a; Supplementary Figures 3, 5, 7; Supplementary Tables 3, 5). This demonstrates that the decrease in ligation efficiency for subsequent steps is limited. However, for the T module, only 10% of the products contain at least 5 modules after seven ligation cycles, which shows a great loss in ligation efficiency during subsequent ligation steps (Figure 4a; Supplementary Figure 1; Supplementary Table 1). The loss of ligation efficiency seems module specific, as the 'intrinsic' ligation efficiency of the T module is already lower compared to the S and L module. The resolving power of the used SEC system was too low to visualize all expected products (7 assembly cycles could result in 13 different ligation products) and made it impossible to determine ligation efficiency for each individual cycle.

For dsODN assembly, the obtained distribution after six subsequent ligation cycles corresponded to the 50−60% ligation efficiency (Supplementary Figures 2, 4, 6, 7; Supplementary Tables 2, 4, 6). Again, the resolving power of the used SEC system was too low to visualize all expected products.

After gel extraction, the created constructs could be amplified using PCR. A bias toward the amplification of smaller constructs is observed. Separation of all ligation products is required in order to obtain only full length constructs. Here we used gel purification and extraction to obtain full length products, which is laborious and limits automation/high throughput. However, DNA separation techniques using chip-based nanostructures/nanomaterials or microfluidics allow automation and high-throughput analysis.[27,28] In order to avoid gel extraction, which costs about 16 h, excised bands can directly be used in PCR. Alternatively, desired constructs can be 'picked' from TBE-urea gel using a needle and inserted into the PCR mix. Both techniques resulted in the amplification of the desired products (Supplementary Figure 8). Also, by using a needle, the bias toward the amplification of smaller constructs can be decreased.

After demonstrating the creation of specific constructs, a library containing nine different ssModules was created using solid-phase assembly of ssODN modules with linkers (Figure 5). After assembly and amplification, a smear pattern was visible indicating the creation of many constructs with different sizes. Sequence analysis confirmed the ligation via the linker site and the presence of different modules. With the solid-phase assembly of ssDNA modules using the linkers method, in only 3 days the library was created, amplified, ligated, and transformed into bacteria.

To create a truly seamless, sequence-independent and fast solid-phase assembly method, we explored the use of double-stranded modules. The advantage of using dsODN is that secondary structures cannot hinder or impede the ligation reaction, which is a problem with methods based on complementary overhangs. The assembly of dsModules using a solid-phase platform was previously described by Dietrich et al., and we optimized the ligation efficiency and assembly cycle time of this method.[17]

Using our method, it took only 1 h to ligate two dsModules, whereas the previous method required 5 h. The ligation efficiency of the solid-phase assembly of dsModules (50−60%) is comparable to the method of Dietrich et al. (35−70%). We also demonstrate the use of PCR generated modules, which makes it possible to assemble large (>500 bp) modules. We were successful in ligating a PCR generated module of 3100 bp onto a dsHead module (results not shown).

To show the possibilities of the solid-phase assembly of dsModules, a small library of RBSs was screened on translation efficiency (Figure 6). Sequence analysis confirmed the seamless ligation of the used modules and the presence of different RBSs in the constructs. Also, the translation efficiency of the four different RBS could be reviewed, where K376001 > B0035 > B0034 > J61141.

With the solid-phase assembly of dsModules, in only 4 days the RBS library was created in a truly seamless and sequence-independent manner, amplified, ligated, transformed into bacteria, and screened on fluorescence.

In conclusion, we have introduced a sequence-independent ligation method for combinatorial and scarless assembly of multipart gene constructs, which allows fast ligations of multiple fragments in less than a day and will be particularly useful for generation of combinatorial libraries of proteins with a repetitive nature, such as TALEs or DARPins. This versatile ligation method will be a useful addition to the toolbox of molecular and synthetic biologists.

MATERIALS AND METHODS

Materials. All chemicals and reagents were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands), unless stated otherwise. Dynabeads M-280 Streptavidin were purchased from Dynal (Oslo, Norway). Restriction enzymes, FastAP (Thermosensitive Alkaline Phosphatase), Generuler DNA and Riboruler RNA ladders, *Pfu* polymerase, Rapid DNA Ligation Kit, T4 RNA ligase, and GeneJET Plasmid Miniprep Kit were purchased from Thermo Fisher Scientific (St. Leon-Rot, Germany). Thermostable RNA ligase was purchased from Epicenter Biotechnologies (Madison, WI, USA). Precast denaturing polyacrylamide TBE-urea gels (6% and 10%) were purchased from Invitrogen (Breda, The Netherlands).

Synthetic Oligodeoxynucleotides. All oligodeoxynucleotides were synthesized by Eurogentec S.A. (Seraing, Belgium). ssODN were synthesized with 5′- and 3′-phosphates and PAGE purified at the 40 nmol scale or with 5′-biotin-TEG and PAGE purified at the 200 nmol scale. ssODN linkers, primers and complementary strands were synthesized unmodified and SePOP desalted at a 40 nmol scale, except for the primer fw_EGFP, which was 5′-phosphorylated and PAGE purified. Oligodeoxynucleotides were dissolved in nuclease-free water and stored at −30 °C as 100 μM stocks.

Oligodeoxynucleotides encoding peptides were codon-optimized for bacterial expression and flanked with linker sequences GGTTCT (GS) at the 5′ (all except Head modules) and GGTGGC (GG) at the 3′ (all except Tail modules). The possible formation of secondary structures (checked with Oligocalc software) was reduced by adjustment of any self-complementary oligonucleotide sequence.[29]

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Table 6. Used Oligodeoxynucletides

module name	sequences
Head_GG	5′-Biotin-TEG-AGCCCATGGTTATGGAAAACCTGTATTTTCAGGGTGGC
GS_EB1_GG	5′ P-GGTTCTCTGATTCGTTTATGGAGCCATCTGATTCACATTTGGTTTCAGAACCGTCGTCTGAAATGGAAA-AAAAAGGGTGGC-P 3′
GS_H5WYG_GG	5′ P-GGTTCTGGTCTGTTTCACGCGATTGCCCATTTCATCCACGGTGGTTGGCATGGTTTAATTCACGGTTG-GTATGGCGGTGGC-P 3′
GS_H92_GG	5′ P-GGTTCTAAGACACCGAAAAAGGCCAAAAAGCCAAAAACCCCGAAAAAGGCGAAAAAACCAGGTGGC-P 3′
GS_LAH_GG	5′ P-GGTTCTAAAAAGGCACTGCTGGCACTGGCACTCCATCACTTAGCACACCTTGCTCATCATCTTGCGTTA-GCGCTGAAAAAGGCTGGTGGC-P 3′
GS_NLSV402_GG	5′ P-GGTTCTCCAAAAAAGAAACGTAAAGTTCCAAAAAAAAAGCGCAAAGTCGGTGGC-P 3′
GS_ppTG20_GG	5′ P-GGTTCTGGCTTATTTCGTGCGCTGTTGCGTCTGTTACTGTCTCTGTGGAGATTACTTTTACGTGCGGGTGGC-P 3′
GS_Pr18_GG	5′ P-GGTTCTTCTCGTAGTCGGTATTACCGTCAGCGCCAACGTTCTCGCCGTCGCCGGCGTAGAGGTGGC-P 3′
GS_SPKR4_GG	5′ P-GGTTCTAGCCCGAAACGTAGCCCTAAGCGCAGCCCAAAAAGATCTCCTAAACGTGGTGGC-P 3′
GS_TAT_GG	5′ P-GGTTCTTATGGCCGCAAGAAGCGTCGTCAAAGACGTCGTGGTGGC-P 3′
GGGS linker	GGTGGCGGTTCT
GS_Tail	GGTTCTTAATAAGGATCCCATAAGTATGAC
Fw_Head	AGCCCATGGTTATGGAAAA
Rev_Tail	GTCATACTTATGGGATCCTTATTAA
Head_pTET_J72005	5′-Biotin-TEG-AGCCAGATCTTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCAC
Complement Head_pTET_J72005	GTGCTCAGTATCTCTATCACTGATAGGGATGTCAATCTCTATCACTGATAGGGAAGATCTGGCT
BBa_B0034	5′ P-AAAGAGGAGAAACACAT-P 3′
BBa_B0035	5′ P-ATTAAAGAGGAGAAACACAT-P 3′
BBa_J61141	5′ P-TGGCTAACTGAGGATCACAT-P 3′
BBa_K376001	5′ P-TGGCTAACATAGGGTCACAT-P 3′
Complement BBa_B0034	ATGTGTTTCTCCTCTTT
Complement BBa_B0035	ATGTGTTCTCCTCTTTAAT
Complement BBa_J61141	ATGTGATCCTCAGTTAGCCA
Complement BBa_K376001	ATGTGCTCTCCTTAAATTTGTGTGAATTT
Fw_EGFP	5′ P-ATGGTGAGCAAGGGCGAGGAG
Rev_EGFP	CTTGTACAGCTCGTCCATGCCG
Tail_Term_B0011	5′ P-TAAAGAGAATATAAAAAGCCAGATTATTAATCCGGCTTTTTTATTATTTAAGCTTCACGC-P 3′
Complement Tail_Term_B0011	GCGTGAAGCTTAAATAATAAAAAAGCCGGATTAATAATCTGGCTTTTTATATTCTCTTTA
Fw_Head_pTET	AGCCAGATCTTCCCTATCAGTGA
Rev_Tail_Term	GCGTGAAGCTTAAATAATAAAAAAGC

Promoter, RBS and terminator sequences were deduced from the Registry of Standard Biological Parts.[22]

METHODS

Solid Support. As a solid support M-280 streptavidin-coated magnetic beads were used, and biotinylated universal single-stranded oligonucleotides (ssHead) were bound to the beads according to manufacturer's protocol. To 1 mg of M-280 beads 400 pmol ssHead was added, and the mixture was incubated in an Eppendorf incubator (thermomixer comfort; Eppendorf, Nijmegen, The Netherlands) for 30 min at 22 °C and 800 rpm. To remove unbound ssHead, coated magnetic beads were washed 3 times with 1x B&W buffer (5 mM Tris-base (pH 7.5), 0.5 mM EDTA, 1 M NaCl) using a 96-well magnetic rack (Life Technologies, Bleijswijk, The Netherlands) to capture the beads during buffer replacements.

Solid-Phase Assembly of Single-Stranded Oligodeoxynucleotides Using RNA Ligase. Ligation reactions (20 μL) with thermostable RNA ligase (Epicenter) contained 12.5 pmol ssHead coated on M-280 magnetic beads, 1x Thermostable RNA Ligase buffer, 20% (v/v) poly(ethylene glycol) (PEG) 4000, 50 μM ATP, 100 pmol ssODN, and 10−100 U thermostable RNA ligase. After vortexing briefly, ligation was performed by incubation for 16 h at 60 °C.

Ligation reactions (20 μL) with T4 RNA ligase (New England Biolabs) contained 12.5 pmol ssHead coated on M-280 magnetic beads, 1x T4 RNA Ligase 1 Reaction buffer, 20% (v/v) PEG 4000, 100 pmol ssODN, and 20 U T4 RNA ligase. After briefly vortexing, ligation reactions were performed at 22 or 37 °C for 16 h.

After ligation, samples were washed 2 times with 50 μL 1x B&W buffer and 3′-dephosphorylated in 50 μL reactions containing 1x FastAP Buffer and 1 U FastAP Alkaline Phosphatase for 10 min at 37 °C. Three washes with 50 μL 1x B&W buffer were performed, after which a new ligation cycle could be initiated. After ligation of the final ssODN, the products were washed 3 times with 1x B&W and stored at 4 °C in 20 μL 1x B&W. This protocol is schematically shown in Figure 1A.

Solid-Phase Assembly of Single-Stranded Oligodeoxynucleotides in the Presence of Linker Oligodeoxynucleotides Using DNA Ligase. Ligation products were assembled in a stepwise protocol. Ligation reactions (20 μL) contained 12.5 pmol ssHead coated on M-280 magnetic beads, 50 pmol linker, 100 pmol ssODN, 1x Rapid Ligation Buffer (part of Rapid DNA Ligation Kit), and 5 U T4 DNA ligase (Thermo Fisher Scientific, St. Leon-Rot, Germany). After vortexing briefly, annealing and ligation reactions were performed in a PCR thermocycler. The annealing/ligation PCR program used the following settings: 10 min at 42 °C, stepwise decrease from 42 to 16 °C in −1 °C/min steps, 30 min at 16 °C. After ligation, samples were washed 2 times with 50 μL 1x B&W buffer and 3′-dephosphorylated in 50 μL reactions containing 1x FastAP Buffer and 1 U FastAP Alkaline Phosphatase for 10 min at 37 °C. Three washes with 50 μL 1x B&W buffer were performed, after which a new ligation cycle was initiated. After ligation of the final ssODN, the products were washed 3 times

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with 1x B&W and stored at 4 °C in 20 μL 1x B&W. This protocol is schematically shown in Figure 1B.

Solid-Phase Assembly of Double-Stranded Oligodeoxynucleotides Using DNA Ligase. First, single-stranded oligodeoxynucleotide modules were made double-stranded. For hybridization, equal amounts of ssODN and its complementary oligodeoxynucleotide were mixed and heated to 70 °C for 10 min following a cooling down to room temperature within 1 h. Hybridization of ssHead was performed after coupling to the magnetic beads.

Ligation products were assembled in a stepwise protocol. Ligation reactions (20 μL) contained 12.5 pmol dsHead coated on M-280 magnetic beads, 100 pmol dsON, 1x Rapid Ligation Buffer, and 5 U T4 DNA ligase (Fermentas, part of Thermo Fisher Scientific, St. Leon-Rot, Germany). After vortexing briefly, ligation reactions were performed at 16 °C for 1 h. After ligation, samples were washed 2 times with 50 μL 1x B&W buffer and 3′-dephosphorylated in 50 μL reactions containing 1x FastAP Buffer and 1 U FastAP Alkaline Phosphatase for 10 min at 37 °C. Three washes with 50 μL 1x B&W buffer were performed, after which a new ligation cycle could be initiated. After ligation of the final dsODN, the products were washed 3 times with 1x B&W and stored at 4 °C in 20 μL 1x B&W. This protocol is schematically shown in Figure 1C.

Ligation Efficiency Determination of the Three Different Solid-Phase Assembly Methods by SEC. Ligation efficiency of the 3 different solid-phase assembly methods was determined using size exclusion chromatography (SEC) on an Acquity UPLC with a BEH400SEC column (Waters, Dublin, Ireland). Ligation products were prepared using the described protocols. After ligation of the final ssODN or dsODN, ligation products were washed with 1x B&W and resuspended in 16 μL SEC sample buffer (0,1 M Tris pH 8.0, 1 mM EDTA, 0,1% SDS). To break the biotin-streptavidin bond, samples were incubated for 7 min at 95 °C, and magnetic beads were removed by a 96-well magnetic rack. Supernatants containing ligation products were eluted with 100% eluent A (0.1 M Tris pH 8.0, 1 mM EDTA) in 15 min at a flow rate of 1 mL/min. Ligation products were detected at 260 nm.

Ligations products were identified using controls (the used ss- and dsHead and ss- and dsModules). Ligation efficiency was determined by dividing the peak area of the ligation product by the combined peak area of nonligated ss- or dsHead and the ligation product(s).

Purification and PCR Amplification of Ligation Products. Of the obtained ligation products (20 μL), 2−5 μL was loaded on 6% and 10% denaturing polyacrylamide TBE-urea gels, and the desired ligation product bands were excised. The ligation products were eluted from the gel by passive elution in elution solution (0.3 M sodium acetate, 2 mM Na_2EDTA, pH 7.6) overnight. Eluted ligation products were precipitated by the addition of 2.5 vol 96% ethanol, cooling down to −80 °C for 45 min, and centrifugation for 12 min at 17000 × *g*. Purified ligation products were redissolved in 25 μL nuclease-free H_2O, of which 5 μL was used in high-fidelity polymerase chain reactions (PCRs). PCRs (50 μL) contained 1x *Pfu* Buffer with 20 mM $MgSO_4$, 0.2 mM dNTP mix, 0.5 μM Fw-primer, 0.5 μM Rev-primer, and 2 U *Pfu* polymerase. Primer sequences are listed in Table 1. Reactions were performed in a PCR thermocycler with the following cycling parameters: 2.30 min at 95 °C, 30x (0.30 min at 95 °C, 0.30 min at 54 °C, 3.00 min at 72 °C), 10.00 min at 72 °C and stored at 4 °C. Reaction products were analyzed on 2% w/v agarose gels stained with ethidium bromide. Bands were visualized with UV illumination at 302 nm.

Creation of Multipart Combinatorial Libraries by Assembly of ssODN in the Presence of Linker ODN. Using the described method for the assembly of ssODN in the presence of linker ODN, a random polypeptide library was assembled. Ligation reactions (20 μL) contained 12.5 pmol ssHead (Head_GG) coated on M-280 magnetic beads, 50 pmol GGGS linker, 1x Rapid Ligation Buffer, 5U T4 DNA ligase, and 100 pmol of a mix of 9 different ssODN (10 pmol of GS_Pr18_GG; GS_SPKR4_GG; GS_H9.2_GG; GS_H5-WYG_GG; GS_TAT_GG; GS_ppTG20_GG; GS_EB1_GG; GS_NLSV402_GG and 20 pmol of GS_LAH_GG). Up to six ligation steps with this composition were performed. One additional ligation step was performed for the ligation of the reaction products to a closing single-stranded oligonucleotide, an ssTail (GS_Tail). Ligation products were loaded on a 6% denaturing polyacrylamide TBE-urea gel, and the area between the smallest theoretical product (353 bp) and the largest theoretical product (623 bp) was excised, purified, and PCR amplified as described. PCR products were analyzed on a 2% w/v agarose gel, and the same area was excised and purified.

The obtained library was *Bam*HI/NcoI digested and purified, and 40 ng of the digested products was ligated in 10 ng of dephosphorylated pIVEX_EJ (*Bam*HI/NcoI digested and purified from an agarose gel). Half of the reaction mixture was directly used to transform *E.coli* strain DH5α. Bacteria were plated out on LB agar plates containing ampicillin (100 μg/mL) and grown overnight at 37 °C.

From the obtained LB-agar plates, 20 colonies were inoculated overnight in 5 mL LB culture medium containing ampicillin (100 μg/mL), and plasmid DNA (pDNA) was harvested. Constructs were *Bam*HI/NcoI digested and analyzed on a 2% w/v agarose gel. In addition, the obtained library constructs were partially sequenced (BaseClear, Leiden, The Netherlands).

Assembly of a Protein Expression Construct Containing a Small RBS Library. Using the described dsODN assembly method, a protein expression construct containing a small RBS library was assembled. To obtain the 5′-phosphorylated EGFP module, PCR was performed, using a 5′-phosphorylated Fw-primer and an unmodified Rev-primer (Table 1) with 1 ng pCMV-EGFP vector as a template. PCRs (50 μL) contained 1x *Pfu* Buffer with 20 mM $MgSO_4$, 0.2 mM dNTP mix, 0.5 μM Fw-primer, 0.5 μM Rev-primer, and 2 U *Pfu* polymerase. Products were purified from a 1% agarose gel.

First, the assembly of a double-stranded Head and four double-stranded RBS was realized. Ligation reactions (20 μL) contained 12.5 pmol dsHead (Head_pTET_J72005) coated on M-280 magnetic beads, 1x Rapid Ligation Buffer, 5 U T4 DNA ligase, and 100 pmol of a mix of the four different RBS dsODN (25 pmol of BBa_B0034, BBa_B0035, BBa_J61141, and BBa_K376001). For the assembly of the obtained double-stranded EGFP module and the formed Head_RBS products, ligation reactions (20 μL) contained 6.25 pmol dsHead_RBS, 1x Rapid Ligation Buffer, 5 U T4 DNA Ligase (Fermentas), and 6.25 pmol 5′-phosphorylated ends of EGFP modules. Constructs were finished by the ligation with the double-stranded terminator. Ligation reactions (20 μL) contained 6.25 pmol dsHead_RBS_EGFP ligation product, 100 pmol dsTail (Tail_Term_B0011), 1x Rapid Ligation Buffer, and 5 U T4 DNA Ligase. Constructs were analyzed and purified from 6% denaturing polyacrylamide TBE-urea gel at the final product height of 800−1000 nt and PCR amplified as described previously.

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PCR products were purified from a 2% w/v agarose gel, *Bgl*II/*Hin*dIII digested, and purified. Subsequently, 40 ng of the purified product was ligated in 10 ng dephosphorylated *Bgl*II/*Hin*dIII digested pIVEX-LacZ vector. Half of the reaction mixture was used to directly transform *E. coli* strain XL-1 blue, which were grown overnight at 37 °C on LB-agar plates, containing ampicillin (100 μg/mL). From the obtained LB-agar plates, 40 colonies were inoculated overnight in 5 mL LB culture medium containing ampicillin (100 μg/mL). pDNA was harvested from 2 mL of these cultures and analyzed by *Hin*dIII/*Bgl*II restriction analysis. The remainder of the bacterial cultures was used for screening of fluorescence. From each overnight culture, 1.15×10^8 bacteria were transferred to a black 96-well plate and bacteria were lysed using 100 μL Cellytic B. Fluorescence was determined using a fluorescence well-plate reader (FLUOstar OPTIMA; BMG-Labtech, Offenburg, Germany) with excitation 488 nm, emission 520 nm filters.

Also, fluorescence was determined by fluorescence microscopy with excitation at 460−500 nm and an EGFP filter (510−560 nm). In addition, the obtained library constructs were partially sequenced.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ss, single-stranded; ds, double-stranded; ODN, oligodeoxynucleotides

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