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Short Communication

Inert splint-driven oligonucleotide assembly

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Abstract

In this study, we introduce a new *in vitro* method for oligonucleotide fragment assembly. Unlike polymerase chain assembly and ligase chain assembly that rely on short, highly purified oligonucleotides, our method, named *Splynthesis*, uses a one-tube, splint-driven assembly reaction. Splynthesis connects standard-desalted "contig" oligos (~150 nt in length) via shorter "splint" oligos harboring 5′ and 3′ blocking modifications to prevent off-target ligation and amplification events. We demonstrate the *Splynthesis* method to assemble a 741-bp gene fragment. We verify the assembled polymerase chain reaction product using standard molecular biology techniques, as well as long-read Oxford Nanopore sequencing, and confirm that the product is cloneable via molecular means, as well as Sanger sequencing. This approach is applicable for synthetic biology, directed evolution, functional protein assays, and potentially even splint-based ligase chain reaction assays.

Keywords: gene assembly; gene fragments; oligos; DNA splints

Introduction

The ability to precisely construct synthetic DNA sequences is central for many emerging applications [1, 2]. Limitations in the length of *de novo* synthesized DNA requires the use of oligonucleotide fragment assembly for the construction of larger genes and genomes. For example, phosphoramidite synthesis struggles with synthesizing long stretches of DNA (>200 bp) due to a relatively low (99%–99.4%) coupling efficiency [1, 3, 4]. Enzymatic approaches hold greater promise in regard to coupling efficiency (99.7%–99.9%) [5–8]. However, secondary structure formation during *in situ* synthesis limits elongation cycle efficiency [9, 10]. Therefore, when long sequences of synthetic DNA are required, short oligos are used as precursors for larger assemblies [11, 12].

In vitro assembly of short, single-stranded DNA (ssDNA) fragments into long, contiguous, double-stranded (dsDNA) fragments can be achieved through various means. Polymerase cycling assembly (PCA), overlapping extended polymerase chain reaction (OE-PCR), and ligase chain assembly (LCA) use the annealing properties of short semi-overlapping complementary primers and PCR, or ligation, to construct desired products [13–17]. Recent advances in assembly methods have improved upon PCA and LCA by utilizing short ssDNA antisense "splint" oligos in combination with ligation, plus selective amplification to reduce overlap between the sense and antisense oligos, thereby saving costs and simplifying the overall workflow [18–20]. Despite advances, these splint-based approaches thus far still require purification prior to selective amplification or the use of microfluidics to perform ligation in series due to their use of extension-capable antisense splint oligos. Furthermore, all methods prefer the use of short sense oligos for assembly to increase the probability of correct assembly.

Here we present an improved method for oligonucleotide fragment assembly based on the short antisense splint oligo approach. This simple and efficient approach resembles the newer versions of splint-based PCA and LCA approaches, but benefits from a few subtle improvements; chiefly, the use of blocking modifications on the splints to facilitate the one-tube approach and the ability to use long, standard-desalted oligos as contig oligos. We call this method *Splynthesis*, owing to its reliance on the short, inert splint oligos to assemble the contig oligos into longer gene fragments.

Materials and methods

Molecular methods

Input oligos The superfolder GFP (*sfGFP*) amino acid sequence was obtained from FPbase [21] and the amino acids were converted to DNA sequences and optimized for *Escherichia coli* K-12. All oligos were ordered from IDT DNA (Coralville, IA, USA) with standard desalt purification. Contig oligos were ordered as Ultramers™. Splint oligos as well as selection primers were ordered as standard ssDNA oligos. Supplementary Table S2 lists all oligos ordered, their modifications, as well as the synthesis scale ordered.

Contig oligo libraries Individual libraries were created for each contig oligo utilizing Claret Bioscience's SRSLY® PicoPlus kit along

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with the manufacturer's instructions. Ten nanograms of each contig oligo (based on theoretical molarity concentration) was used as input for each library. Samples were amplified for eight cycles of index PCR and purified using the "short fragment retention" purification protocol. DNA was quantified utilizing both a Qubit™ Fluorometer (Thermo Fisher) and a TapeStation™ (Agilent Technologies).

Splynthesis For ligation, 6 pmols of each contig oligo and 8.5 pmols of each splint oligo were mixed with 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM dithiothreitol (DTT) final in 50 μl to a volume of 47.5 μl in a 0.2 ml PCR tube. Samples were then run in a thermocycler at 95°C for 5 min then cooled to 4°C at a rate of 0.1°C/s. After the reaction reached 4°C, 1000 units of T4 DNA ligase (New England Biolabs) were added, and the sample was incubated at 37°C for 1 h.

For amplification, the 50 μl ligation reaction was brought up to 100 μl utilizing 45 μl of 2× Equinox Master Mix (Watchmaker Genomics) and 0.5 pmols of each PCR selection amplification primer. The reaction was then incubated at 98°C for 3 min, followed by various cycle numbers of 98°C for 20 s → 65°C for 30 s → 72°C for 30 s, followed by a final extension of 72°C for 1 min. Post amplification, the Splynthesis reaction was purified utilizing a 1× ratio of Claret Bioscience's Clarefy™ DNA Purification Beads (all subsequent reactions were purified using Clarefy™ DNA Purification Beads). Post purification, Splynthesis assembled DNA was quantified utilizing both a Qubit™ Fluorometer (Thermo Fisher) and a TapeStation™ (Agilent Technologies).

NanoPore libraries Completed Splynthesis libraries of interest were sent to SeqCenter LLC (Pittsburgh, PA, USA) for Oxford Nanopore Technology (ONT) sequencing, utilizing their Standalone ONT Ligation Sequencing offering. Libraries were prepared using the PCR-free ONT Ligation Sequencing Kit with the NEBNext® Companion Module to the manufacturer's specifications. No DNA fragmentation or size selection was performed.

Cloning and transformation To perform T/A cloning, we first removed the restriction enzyme (RE) sites we engineered into the product by digesting 500 ng of Splynthesis product with 20 units of SphI and 20 units of XmaI in the presence of 1× rCutSmart® buffer (New England Biolabs) in a 50 μl reaction at 37°C for 30 min. Post restriction digest, the Splynthesis DNA was purified utilizing a 0.8× DNA purification bead ratio.

To A-tail, 300 ng of Splynthesis DNA was combined with 7 μl of NEBNext® ER/A tail buffer and 3 μl of NEBNext® ER/A tail enzyme (New England Biolabs) in a 60 μl reaction and incubated at 20°C for 20 min, followed by 65°C for 30 min. Post end repair and A-tailing, the Splynthesis DNA was purified utilizing a 0.6× DNA purification bead ratio, then quantified utilizing both a Qubit™ Fluorometer (Thermo Fisher) and a TapeStation™ (Agilent Technologies).

Splynthesis DNA insert was cloned into the pGEM®-T vector system (Promega) at a ratio of 2:1 (insert to backbone) following the manufacturer's instructions. Transformed vectors were cloned into the provided JM109 *E. coli* high-efficiency competent cells via heat shock at 42°C. Post recovery in SOC media at 37°C, the transformation was plated onto LB/ampicillin/X-gal plates and incubated at 37°C overnight. Colony screening was performed the subsequent day.

Plasmid extraction and screening White colonies were picked and grown up overnight in 5 ml of sterile LB supplemented with 1× Ampicillin. The next day, plasmids were extracted from pelleted cultures utilizing the Monarch® Plasmid Miniprep kit (New England Biolabs) following the manufacturer's instructions. To assess the success of transformation, 500 ng of each plasmid was digested in a 50 μl reaction with 10 units of EagI-HF (New England Biolabs) in the presence of 1× rCutSmart buffer for 15 min at 37°C. Post digestion, the reaction was cleaned utilizing a 2× DNA purification bead ratio. Inserts were verified using and a TapeStation™ (Agilent Technologies). Plasmids of interest were sent to Molecular Cloning Laboratories (South San Francisco, CA, USA) for Sanger sequencing.

Sequencing methods

Contig oligo libraries Contig oligo libraries were sequenced on an Illumina® MiSeq benchtop sequencer at a read length of 2 × 151 utilizing the MiSeq V2 chemistry. Supplementary Table S1 lists sequencing metrics for the contig libraries.

Splynthesis libraries Splynthesis libraries were sequenced by SeqCenter LLC (Pittsburgh, PA, USA) on an Oxford Nanopore MinION Mk1B sequencer using R10.4.1 flow cells in one or more multiplexed shared flow cell runs. Run design utilized the 400-bp sequencing mode with a minimum read length of 200 bp. Adaptive sampling was not enabled. Guppy1 (v6.5.7) was used for super-accurate base-calling (SUP). Supplementary Table S1 lists sequencing metrics for the Splynthesis libraries.

Sanger sequencing Plasmids of interest were sequenced by Molecular Cloning Laboratories (South San Francisco, CA, USA) utilizing their ABI 3730XL sequencers and pUCR M13 forward and reverse primers.

Informatic methods

Contig oligo libraries Raw fastq reads were trimmed and merged utilizing SeqPrep (https://github.com/jstjohn/SeqPrep) with default parameters. Trimmed and merged fastq reads were then mapped to contig oligo specific fasta files using BWA MEM [22] with default parameters. Mapping rates were determined from samtools flagstats. Insert length distributions of merged reads were parsed from the BAM files of individual libraries using samtools view -q20 -f66. Frequency of reads per length was calculated and plotted as the percent of reads relative to total library. Plots were generated in R utilizing ggplot2.

Splynthesis libraries Raw fastq reads were trimmed with cutadapt [23] to remove the ONT native adapters flanking sequences of CAGCACCT on the 5´ end of the template and AGGTGCTG on the 3´ end of the template. Reads were then mapped to the Splynthesis reference fasta file using BWA MEM [22] with default parameters. Mapping rates were determined from samtools flagstats. Insert length distributions for each library were parsed from the BAM files of individual libraries using samtools view -q20 -F3844. Frequency of reads per length was calculated and plotted as the percent of reads of the total library. To calculate the percent of bases successfully mapped at the expected correct length of 741 bp, reads at length 741 that passed samtools view filter of -F3844 -q20 were passed to a python script that calculated how many mapped bases and unmapped bases are in the cigar string for each read, then output the percent of mapped bases for each cigar string. Bases were binned using the floor function in R for each percentage less than 100%. We assessed the base quality by aligning each sample with BWA MEM and filtering for reads that meet -F3844 -q20 criteria to reach 100 000 reads. We used samtools

with—subsample {frac}—subsample-seed 101 to subsample each sample, extracted reads of length 741, and saved their base quality strings to a file. Finally, we converted the ASCII characters to Phred quality scores in R using utf8ToInt(quality_string)—33. All plots were generated in R utilizing ggplot2.

Sanger reads We converted Sanger sequencing ab1 files to fastq using seqret [24]. The sed command was used to strip the named header for proper read mapping. A reference fasta file was created by integrating the Splynthesis mapping fasta into the pGEM plasmid sequence. Sanger fastq files were mapped to this reference using BWA MEM with default parameters. To improve quality, we trimmed 50 bp beyond the ligation sites from both ends of the mapped reads. Finally, the reads were uploaded to IGV viewer [25] for visualization.

Supplementary Table S2 lists all the input oligos utilized in the assembly. Supplementary Table S3 lists all the fasta files utilized for mapping.

Results

Splynthesis assembly

The idea for Splynthesis originates from the SRSLY (Single Reaction Single-stranded LibraryY) NGS library preparation protocol [26]. Like other ssDNA library prep methods, SRSLY uses splint-adapters to create localized dsDNA interactions, allowing T4 DNA ligase to ligate dsDNA adapters to ssDNA templates [27–29]. We hypothesized that this concept of utilizing short, inert splint oligos could be adapted to chain ssDNA oligos together and drive assembly selection through localized dsDNA ligation followed by PCR.

To test this, we aimed to construct a reporter gene, *sfGFP*, using the Splynthesis method (Fig. 1). We divided the 715 nt gene into five segments (∼145 nt each), added restriction sites at the termini, and ordered the corresponding oligos. All contig oligos, except the most 5′ one, were 5′-phosphorylated. Short 36 nt splint oligos were designed, with 18 nts complementary to the 3′ terminus of 1 oligo and the other 18 nts to the 5′ terminus of the next. These splint oligos, modified with 5′ and 3′ ligation and extension blocking amino groups to render them inert in the reaction beyond annealing, facilitated the correct assembly without participating in the ligation or amplification. Flanking primers matching the 5′ and 3′ ends of the entire gene were used for PCR selection after ligation.

Splynthesis is a one-tube reaction with four steps: annealing, ligation, amplification, and purification. First, contig and splint oligos are combined, with the splints at a 1.4× molar ratio to promote proper annealing. After heating and cooling, T4 ligase is added for ligation. Once ligation is complete, the reaction volume is doubled, and PCR selection primers, polymerase, dNTPs, and buffer are added for amplification. The product is then purified using DNA purification beads. This process takes about 2 h, including 1 h for ligation, 30 min for PCR, and 30 min for purification; with PCR time increasing for longer assemblies.

Assembly assessment

To evaluate the quality of the ∼140 nt standard-desalted phosphoramidite synthesized contig oligos, we created ssDNA NGS libraries with each oligo and sequenced them on an Illumina sequencer (Fig. 2a, Supplementary Table S1). Theoretically, a 140-nt oligo produced by phosphoramidite synthesis should contain about 43% correct length product (99.4^{140}). However, the sequencing results indicate that, except for the poorly synthesized contig

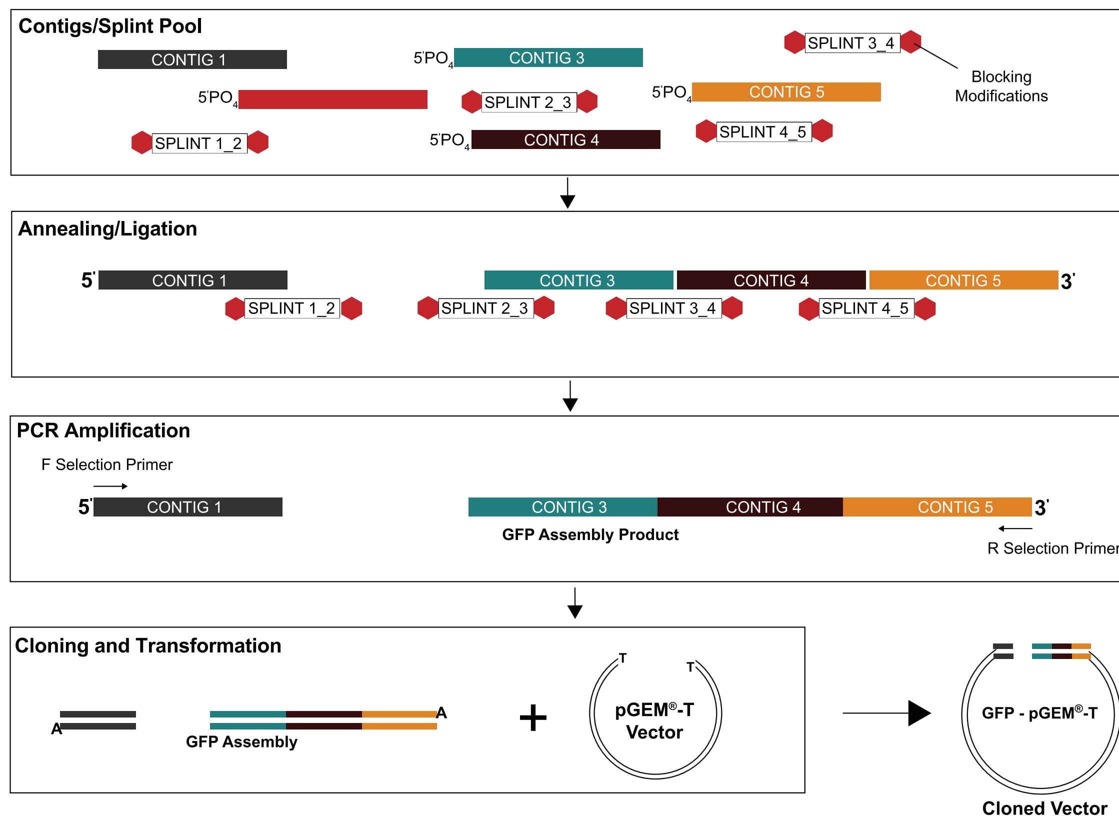

Figure 1. Schematic overview of Splynthesis.

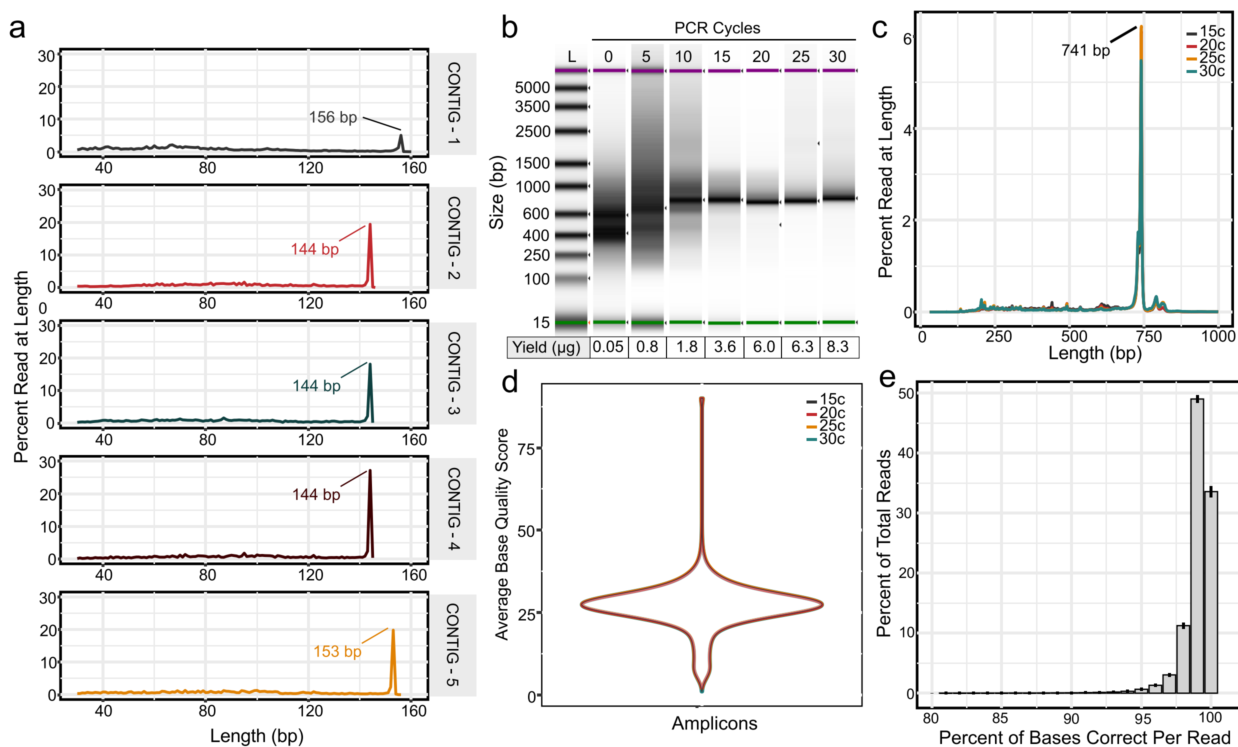

Figure 2. Assessments of the input contigs and the dsDNA assembly products. (a) Insert distribution plots for each individual contig oligo (Illumina sequenced). (b) Gel image from a D5000 TapeStation trace to visualize PCR cycle titration of the assembly product. (c) Insert distribution plots for each assembly amplicon cycles 15–30 (ONT sequenced). (d) Average ONT base quality score at 741 bp for each assembly amplicon cycles 15–30. (e) Aggregated histogram of ONT CIGAR scores per read at 741 bp for each assembly amplicon cycles 15–30.

oligo 1, the oligos we ordered were about 20% correct. This may be because these oligos were not purified beyond standard desalting. Theoretically, purified oligos would contain more full-length product, but would also increase cost.

After assessing the purity of our contig oligos, we focused on streamlining and optimizing the Splynthesis reaction. Since Splynthesis, like its predecessors, relies on a high-fidelity polymerase for amplification and selection, we evaluated the effects of PCR cycle number on the assembly (Fig. 2b). We hypothesized that fewer PCR cycles would minimize amplification-induced mutations. Our results showed that a minimum of 15 cycles was required to preferentially amplify the full-length assembly from the background. We observed no abnormal effects on yield or amplicon size up to 30 cycles under our test conditions. Moreover, product yield increased proportionally with PCR cycle number, starting from about 200 ng of input per contig oligo.

To assess the impact of PCR cycle number on the assembly product, we sequenced the 15–30 cycle products on an Oxford Nanopore (ONT) MinION, as we wanted to sequence every base of our full-length products contiguously. The most striking result was that the sequencing data from all assembly products were nearly identical (Fig. 2c–e). This suggests that a difference of 15 PCR cycles has minimal impact on the mutation rate and the correctness of our assembly product.

We then evaluated the percentage of correct amplicon products. The highest peak in each PCR product library was the expected length, followed closely by the correct length minus one base pair. However, this pattern was reversed in the 30-cycle product (Fig. 2c, Supplementary Fig. S1). At least 30% of the total reads per library were within ± 4 bp of the expected length, consistent with the observation that indels are the most common errors for ONT reads [30–32]. From the reads of correct length, the average base quality score was 25, indicating a high-quality ONT run and good read trimming (Fig. 2d). The CIGAR string results showed that about 30% of reads at the correct length were the correct sequence, and nearly 80% were at least 99% correct (Fig. 2e). These results provided confidence that the assembly was successful and suitable for cloning.

Cloning assessment

To confirm our Splynthesis assembly, we cloned the 25-cycle assembly reaction into an expression vector using T/A cloning. We opted for T/A cloning over RE cloning after realizing we inadvertently introduced a false start site when incorporating an SphI cut site at the 5′ terminus. To correct this, we digested the product with SphI, end-repaired, and A-tailed the assembly before cloning.

We cloned the assembly into an Ampicillin-resistant expression vector and plated the transformation on Ampicillin/X-gal plates for blue/white colony screening. Colony formation and counts indicated that both the Ampicillin and X-gal selections worked well (Fig. 3a). Unfortunately, the colonies did not fluoresce under 480 nm light due to an error during oligo construction, where we mistakenly truncated the last 11 amino acids of the sfGFP gene. Despite this, we proceeded with colony screening.

We picked 20 white colonies, isolated plasmids, and performed RE digests on all 20 plasmid preparations. We checked for the presence of two bands: one at the length of the linearized vector and one at the length of the insert. While two plasmids showed abnormal RE digestion patterns, 18 digests were of the expected length (Fig. 3b). We sent all 20 plasmids for Sanger sequencing to verify the insert sequences. Of the 20 plasmids sequenced, 7 contained

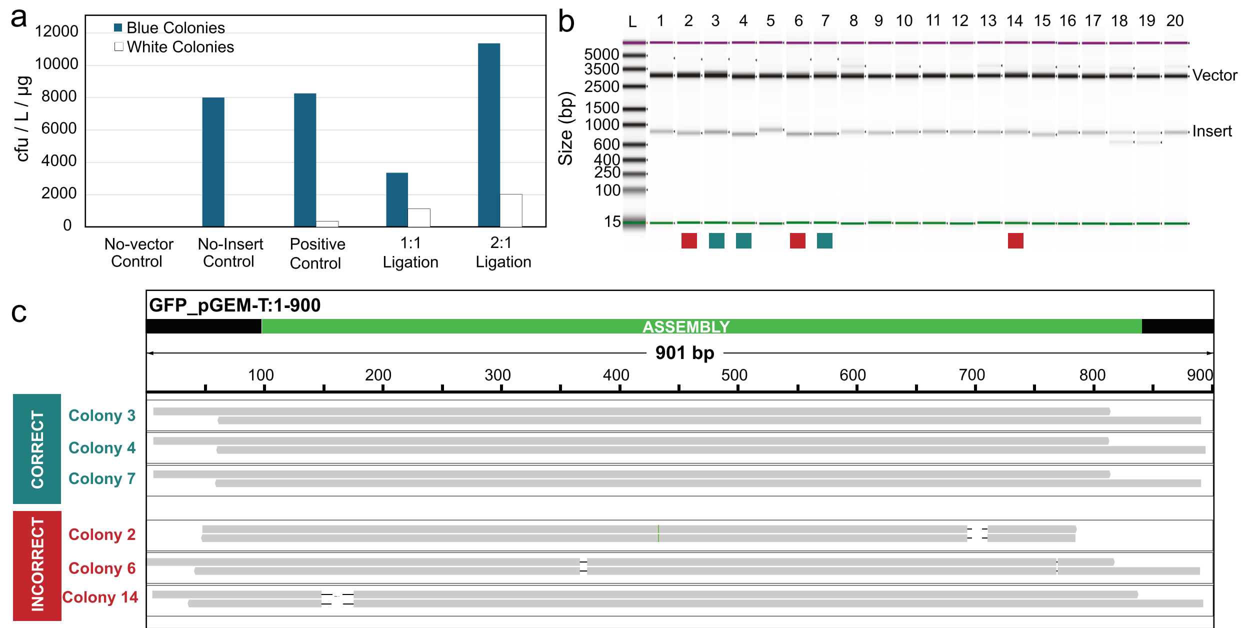

Figure 3. Assessment of the cloned amplicon. (a) Blue/white colony counts for controls and vector ± insert (amplicon). (b) Gel image from a D5000 TapeStation trace to visualize the restriction digest evaluation of minipreps from 20 white colonies. (c) IGV visualization of Sanger sequencing results.

the correct assembly as verified by forward and reverse sequencing primers, 2 were possibly correct but contained errors in one of the Sanger reads, 7 were confirmed incorrect with various indels or point mutations, and 4 were sequencing failures (Fig. 3c, Supplementary Fig. S2). Overall, we observed a 35% success rate after blue/white colony screening (7 correct out of 20 sequenced).

Discussion

We present a proof-of-concept validation of a simple, one-tube, splint-driven assembly reaction by constructing a 741-bp gene fragment from five contig oligos and four inert splint oligos. Unlike similar methods, we have shown that the reaction can be carried from annealing through amplification without the need for purification. Furthermore, we have shown that simple bead purification suffices to select for full-sized assembly products. While this study demonstrates feasibility, we believe the protocol could be further streamlined. For example, T/A cloning could be easily facilitated by using an A-tailing polymerase during PCR. Topoisomerase-based (TOPO) cloning could be achieved by incorporating the TOPO recognition sequence at the 5′ terminus of the first contig oligo. Finally, splint-based LCA for the purpose of point mutation detection might be possible by increasing the melting temperature of splints through either elongation or the utilization of locked nucleic acids. Additionally, splint oligo blocking could be performed via dideoxy nucleotide incorporation with terminal transferase if cost reduction is desired.

Like previous methods that utilize semi-overlapping oligo hybridization and amplification to create gene constructs, we believe the main benefits of Splynthesis will be its amenability to build difficult constructs and its reusability. For instance, varying the location of the splint in repeated sequences could help build repetitive constructs, swapping out single contig oligos, in a Splynthesis assembly set, with those containing strategic point mutations or indels allows for rapid directed evolution and functional protein assays. Taken a step further, contig oligos could even contain random bases internally, provided the termini ends of the contig(s) are such that splints could anneal. Additionally, completed assemblies can be extended by adding more contig oligos to create fusion genes, while whole contigs or gene blocks can be fused via a single splint to elongate or create gene fusions *in vitro*.

Despite its strengths, Splynthesis is not without its drawbacks. The main limitation to Splynthesis is cost. Not only do long oligos cost more per base than short oligos, but blocking modifications increase cost as well. Therefore, the price point of Splynthesis might indeed be higher than other splint-based assembly approaches and on par with the costs of PCA and LCA. Furthermore, due to economies of scale, many large manufacturers can create simple gene fragments for customers at a fraction of the price thereby making Splynthesis and all similar techniques best suited for niche and difficult assemblies currently.

Even though Splynthesis relies on phosphoramidite-synthesized oligos, inert splints, a high-fidelity polymerase, and sequence confirmation through cloning and transformation, we believe it offers many practical benefits for DNA construct assembly. With the advent of benchtop DNA synthesizers, it is possible that in the future, Splynthesis and equivalent methods will become cost-effective for simple gene fragment assemblies as well.

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Author contributions

C.J.T. developed the method with contributions from R.E.G. C.J.T. conceived the investigations and designed the experiments. A.A.M. and T.G. performed experimentation. C.J.T., A.A.M., and T.G. performed data analyses and interpretation. T.G. performed data visualization. C.J.T. wrote the manuscript with contributions from all co-authors. All authors read and approved the final manuscript.

Supplementary data

Supplementary data is available at *SYNBIO* online.

Conflict of interest: C.J.T. and R.E.G. are cofounders, shareholders, advisors and/or officers/consultants of Claret Bioscience LLC, a genomics company that commercializes sequencing and analysis tools for cfDNA and other nucleic acid sources. The described methods are the subject of patent applications of which C.J.T. is listed as an inventor.

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Data availability

Sequencing Data uploaded to SRA under Accession ID: PRJNA1157451.

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