DNASCRIPT

APPLICATION NOTE

Gene Assembly

Synthetic gene constructs are required for personalized medicine, vaccines, and industrial synthetic biology. In this Application Note, we demonstrate polymerase cycling assembly (PCA) of two reporter genes from relatively short (<60 nt) overlapping oligos. Oligos produced with DNA Script's Enzymatic DNA Synthesis (EDS) technology and benchtop SYNTAX System supported high success rates and faster turnaround times than commercially sourced oligos.

INTRODUCTION

Recombinant genes are indispensable in life sciences research, medicine, and biotechnology, with rapidly growing applications in fields such as personalized therapeutics, vaccine development, and industrial synthetic biology. Synthesis of full-length constructs enables broad-based experimentation, particularly with genes that are difficult to isolate from biological sources or do not exist in nature at all.

Several strategies for the assembly of synthetic DNA fragments into full-length gene constructs have been developed. These include Gibson assembly,[1] polymerase cycling assembly (PCA),[2,3] and various ligation-based approaches.[4] PCA provides a simple, robust method for the assembly of relatively short (<100 bp) building blocks into kilobase-sized DNA fragments or short genes. The same methodology may be used to join such fragments into more complex genes or constructs.

To date, DNA synthesis has relied on phosphoramidite-based chemistry,[5] which requires large volumes of harsh, organic chemicals as well as specialized skills and infrastructure. As a result, commercial oligo production has become highly centralized, with supply chains reliant on multiple third-party service providers. In contrast, DNA Script's novel Enzymatic DNA Synthesis (EDS) technology[6] enables on-demand, in-house production of DNA oligos with a benchtop instrument[7] in a standard laboratory environment.

In this study, we employed PCA to qualify EDS oligos for gene assembly. As proof of concept, we successfully assembled two widely used reporter proteins: enhanced green fluorescent protein (EGFP) and monomeric red fluorescent protein (mRFP) (Figure 1). Expression of fully functional fluorescent proteins confirmed that EDS oligos are of the required quality to support gene assembly for a variety of applications. Additionally, the study demonstrated that on-demand printing of custom oligos with the SYNTAX System in less than a day enabled greatly accelerated experimental timelines. The full workflow was reduced to just three days—with oligo printing on Day 1, assembly on Day 2, and cloning and sequence verification on Day 3. This represents time savings of at least 1 – 3 days as compared to ordering genes from a commercial vendor.

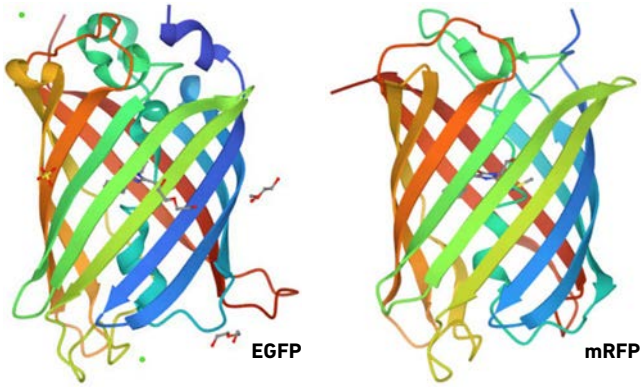

FIGURE 1. GRAPHICAL REPRESENTATION OF THE FLUORESCENT PROTEINS expressed in this study: enhanced green fluorescent protein (EGFP; PDB ID: 4EUL) and monomeric red fluorescent protein (mRFP; PDB ID: 7RY2). The unique cylindrical structures of these proteins confer their ability to form an internal chromophore without any cofactors other than molecular oxygen.

Images from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (https://www.rcsb.org).

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EXPERIMENTAL DESIGN

A typical polymerase cycling assembly (PCA)-based gene assembly workflow (Figure 2) consists of three phases:

- Oligo design and synthesis
- Gene assembly (including error correction)
- Cloning and sequence verification

In this study, traditional or "2-step PCA"[2] and a streamlined, "1-step integrated PCA-PCR" protocol[3] were employed to assemble the coding sequences (CDS) of EGFP and mRFP, respectively. The two workflows are compared and contrasted in Figure 3. In short,

- EGFP assembly was performed with the 2-step method, using enzymatically synthesized oligos (EDS oligos) or chemically synthesized oligos from a commercial supplier (CS oligos). Each set of oligos was produced four times (EDS1 – EDS4, CS1 – CS4), and assemblies were performed in duplicate with the first set from each source

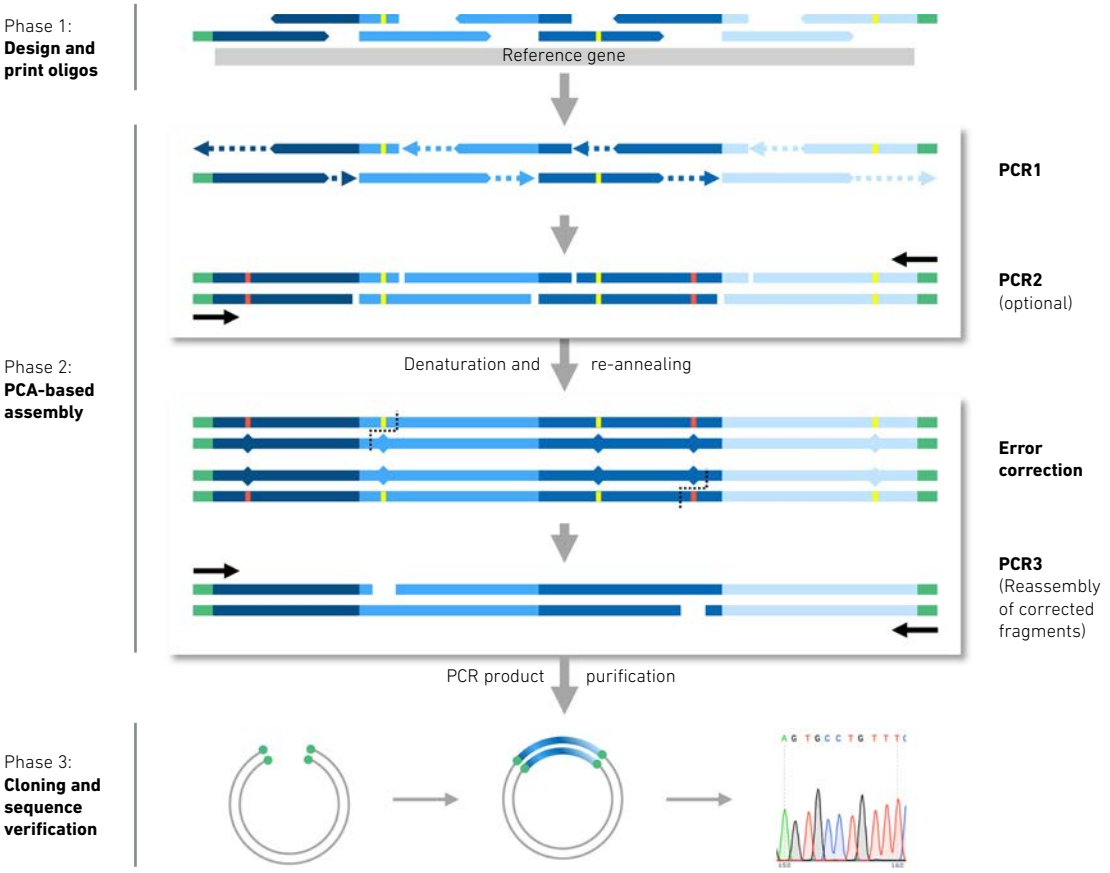


FIGURE 2. GRAPHICAL ILLUSTRATION OF THE POLYMERASE CYCLING GENE ASSEMBLY (PCA) WORKFLOW. Blue bars represent overlapping oligos designed to cover the gene or gene fragment to be assembled. Green regions designate optional overhangs for downstream manipulation of the assembled construct; these may be included in terminal assembly oligos (as shown here), or in amplification primers used for PCR3. Black arrows represent amplification primers. Errors resulting from oligo synthesis are depicted in yellow and errors from polymerase extension in red (for illustrative purposes only, not based on true error rates). PCR1 or PCR2 products are denatured and re-annealed to create mismatches at error sites. An enzyme, such as the CorrectASE enzyme (ThermoFisher Scientific), is used to recognize and excise these mismatches. For the sake of simplicity, not all error correction events are shown. Corrected fragments are reassembled in PCR3, thus increasing the likelihood of obtaining error-free clones. Assembled gene constructs are cloned, and sequences are verified by Sanger (or next-generation) sequencing.

(EDS1A/B, CS1A/B). EGFP constructs were cloned (i) into a sequencing vector for sequence verification of randomly selected constructs using Sanger sequencing, and (ii) into an expression vector for expression of green fluorescent protein in *E. coli.*

- Assembly of the mRFP gene was accomplished with the 1-step method using a set of EDS oligos. Constructs were cloned into a single vector that supports constitutive protein expression in *E. coli*. Red fluorescent and white colonies were picked for sequencing verification by Sanger sequencing.

FIGURE 3. WORKFLOWS USED FOR THE ASSEMBLY OF EGFP AND MRFP. Full experimental details are provided in *Materials and Methods*.

RESULTS AND DISCUSSION

Gene Assembly

Representative reaction products generated at different stages of the EGFP assembly process are shown in Figure 4. Each stage yielded the expected profile:

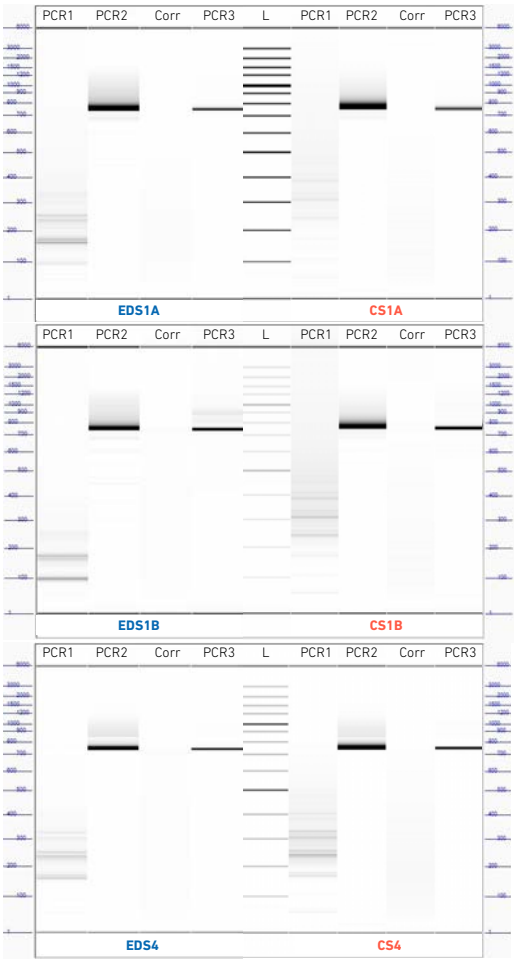


FIGURE 4. REACTION PRODUCTS GENERATED DURING DIFFERENT STAGES OF EGFP GENE ASSEMBLY performed with enzymatically synthesized (**EDS**) or commercial (**CS**) oligos. The dominant band in PCR2 and PCR3 products correspond to the full-length (739 bp) EGFP construct. Lower-molecular weight bands in PCR1 products correspond to incomplete constructs/unextended oligos. Reaction products were analyzed by capillary electrophoresis using a 5300 Fragment Analyzer System (Agilent Technologies). L = Molecular weight ladder.

- In PCR1 products, bands at the bottom of the lane correspond to unextended oligos. The upward smear represents intermediate assembly products (shorter than the full-length construct). The dominant band in PCR2 products corresponds to the full-length 739-bp construct.
- Lanes labeled "Corr" contain the products of the error correction reaction. Disappearance of the 739-bp band indicates that error sites were efficiently excised.
- All lanes labeled PCR3 contain final, full-length assembly products, which were cloned into a sequencing and expression vector, respectively (as outlined in Figure 3).

Individual stages and the overall process were found to be efficient and reproducible across DNA synthesis chemistry, synthesis batches, and assembly replicates.

Select reaction products generated using the 1-step integrated PCA-PCR approach are shown in Figure 5. The full-length (718 bp) assembly product was visible in the denatured/re-annealed PCR1 product and clearly amplified in the PCR3 product.

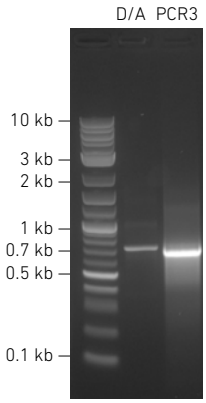


FIGURE 5. REACTION PRODUCTS GENERATED DURING THE mRFP GENE ASSEMBLY performed with EDS oligos. The denatured/re-annealed PCR1 product (lane D/A) was subjected to error correction and amplified (PCR3) to yield the full-length (718 bp) mRFP construct. Reaction products were analyzed by agarose gel electrophoresis. The PCR3 band was extracted from the gel, purified, and cloned.

Expression of Functional Fluorescent Proteins

Assembled EGFP genes were expressed in *E. coli* BL21(DE3) under control of the T7 promoter. Protein expression was induced with IPTG, and colonies were viewed under UV illumination. A representative example of the results is shown in Figure 6A. The vector in which assembled mRFP constructs were cloned allows for constitutive protein expression. Transformants were grown overnight at 37°C, but plates were incubated for an additional 24 hours at 4°C to confirm colony phenotypes (Figure 6B).

Since fluorescence of both EGFP and mRFP is dependent on the three-dimensional protein structure (correct folding), fluorescent green and red colonies are assumed to contain assembled genes that are either error-free or harbor mutations (originating from oligo synthesis and/or polymerase extension) that do not impact protein expression, folding, or function.

Sequence Verification of Cloned mRFP Constructs

A total of 75 colonies (67 red and 8 white) were recovered from the mRFP experiment. Plasmid DNA was isolated from all colonies. As indicated in Figure 7, all of the white colonies proved to be "empty," indicating that the white phenotype was caused by cloning failure rather than deleterious mutations.

Of the cloned constructs recovered from the 67 red colonies:

- Sixty (89.5%) contained a full-length mRFP CDS that aligned perfectly to the reference.
- Four contained a full-length mRFP CDS with a single SNP, which appeared to have a non-material impact on protein folding (not shown).
- Three failed sequencing or did not yield complete coverage of the mRFP CDS (not shown).

With at least 80% of the total number of clones from the experiment confirmed to contain a full-length, perfect mRFP construct, these results demonstrate that EDS oligos are capable of supporting very high success rates in PCA-based gene assembly.

Sequence Verification of Cloned EGFP Constructs

Unlike the mRFP experiment, the EGFP verification strategy was designed to mimic gene assembly projects in which a phenotypic assay is not available. Instead of sequencing fluorescent and non-fluorescent clones, assembly products were cloned into a small sequencing vector and transformed into an *E. coli* strain that does not support expression of the recombinant gene. For each of the ten assemblies (two oligo sources x five replicates each), 96 colonies

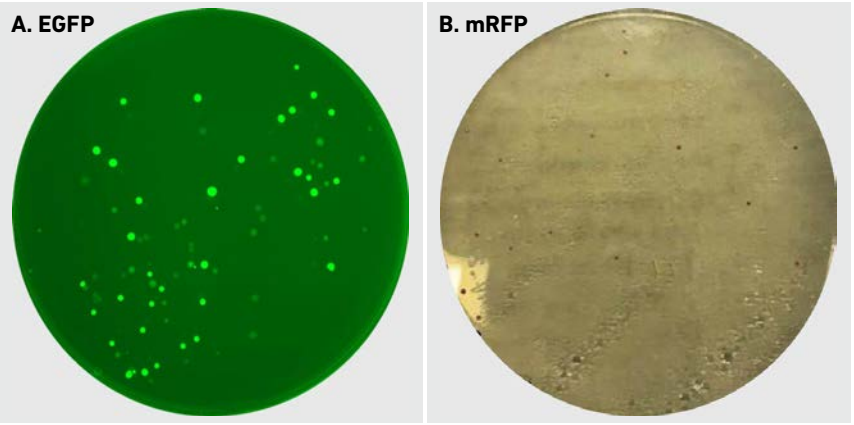


FIGURE 6. RECOMBINANT BACTERIA EXPRESSING ASSEMBLED EGFP OR mRFP GENES. The plate on the left was derived from the EDS1B EGFP assembly and was visualized under UV light. mRFP colonies on right were visualized in standard laboratory lighting.

Red colonies (60) containing a full-length, perfect mRFP CDS

White colonies (8) lacking a cloned mRFP insert

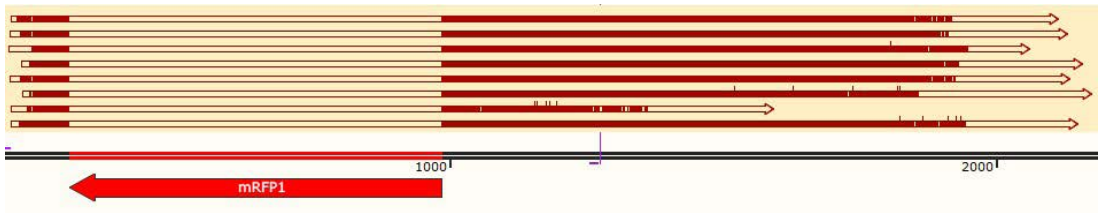


FIGURE 7. GRAPHICAL REPRESENTATION OF SEQUENCE ALIGNMENTS FOR ASSEMBLED MRFP GENES RECOVERED FROM RED AND WHITE COLONIES. Alignments were done with the SnapGene software (Dotmatics). Arrows represent untrimmed Sanger reads. Red bars correspond to the portion of each read that aligns to the pU6-pegRNA-GG-acceptor_ mRFP plasmid sequence. The alignment at the top represents the 60 red colonies that contain a full-length, perfect mRFP CDS. Plasmids recovered from the eight white colonies (bottom) lacked an insert, as indicated by the gap in the alignment.

were randomly selected and subjected to a "crude-sample" sequencing approach that does not require purification of plasmid DNA. Results are given in Figure 7.

Of the 96 colonies from each assembly submitted for sequencing, between 63 (66%) and 87 (91%) were successfully processed, with no significant difference in the average success rate for EDS vs CS clones (79% and 82%, respectively). The percentage of clones that returned perfectly aligned, merged EGFP reads were higher for EDS oligos than CS oligos in three of the five replicates, but statistically comparable overall (average of 55% ±16% for EDS oligos vs. 42 ±33% for CS oligos). Due to relatively short read lengths for some clones, not all perfect alignments covered the entire EGFP CDS. The percentage of successfully sequenced clones for which perfect, full-length coverage was obtained (i.e., clones that can be assumed to express fully functional fluorescent protein) ranged between 16% and 45% (average of 24% ±16%) for EDS oligos and between 8% and 31% (average of 18% ±9%) for CS oligos. For EDS oligos, both metrics (% perfectly aligned merged reads and % perfect full-length reads) correlated with sequencing success across all replicates, whereas this was not the case for CS oligos—suggesting higher batch-to-batch variability in the quality of CS oligos.

When comparing the experimental approaches and results of the EGFP and mRFP experiments, two interesting observations can be made:

- The availability of a phenotypic assay can greatly reduce the number of clones that need to be screened to identify a perfectly assembled construct. In the mRFP experiment, 4 of 5 red (80%) clones were confirmed to be perfect, compared to 1 in ~4 from the EGFP experiment, in which phenotype was not considered in the selection of clones for sequence verification.
- Although the success rate of crude-sample Sanger sequencing used for the verification of EGFP constructs was lower (~80%) than the conventional approach followed in the mRFP experiment (96% success rate), it eliminated the time and effort associated with preparing overnight cultures and isolating plasmid DNA from transformants. In similar fashion, the choice of cloning vectors, methods, and strains, as well as colony screening and sequencing strategies (e.g., Sanger

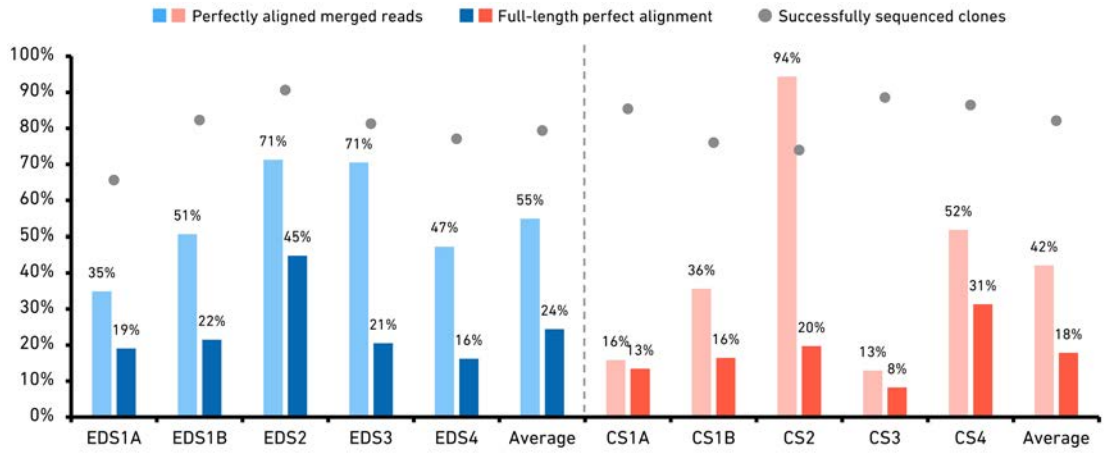


FIGURE 8. RESULTS FROM SEQUENCE VERIFICATION OF EGFP CONSTRUCTS. Sequencing reads were generated and merged as described in *Materials and Methods*. Of the 96 colonies from each assembly submitted for sequencing, between 63 (66%) and 87 (91%) (average of 81%) were successfully processed (gray dots). The percentage of clones that yielded perfectly aligned, merged reads and full-length perfect EGFP alignments was generally higher and less variable across the five replicate assemblies performed with EDS vs. CS oligos.

vs. next-generation sequencing) can be tailored to optimize the time-to-results in same-day gene assembly workflows enabled by PCA and EDS oligos.

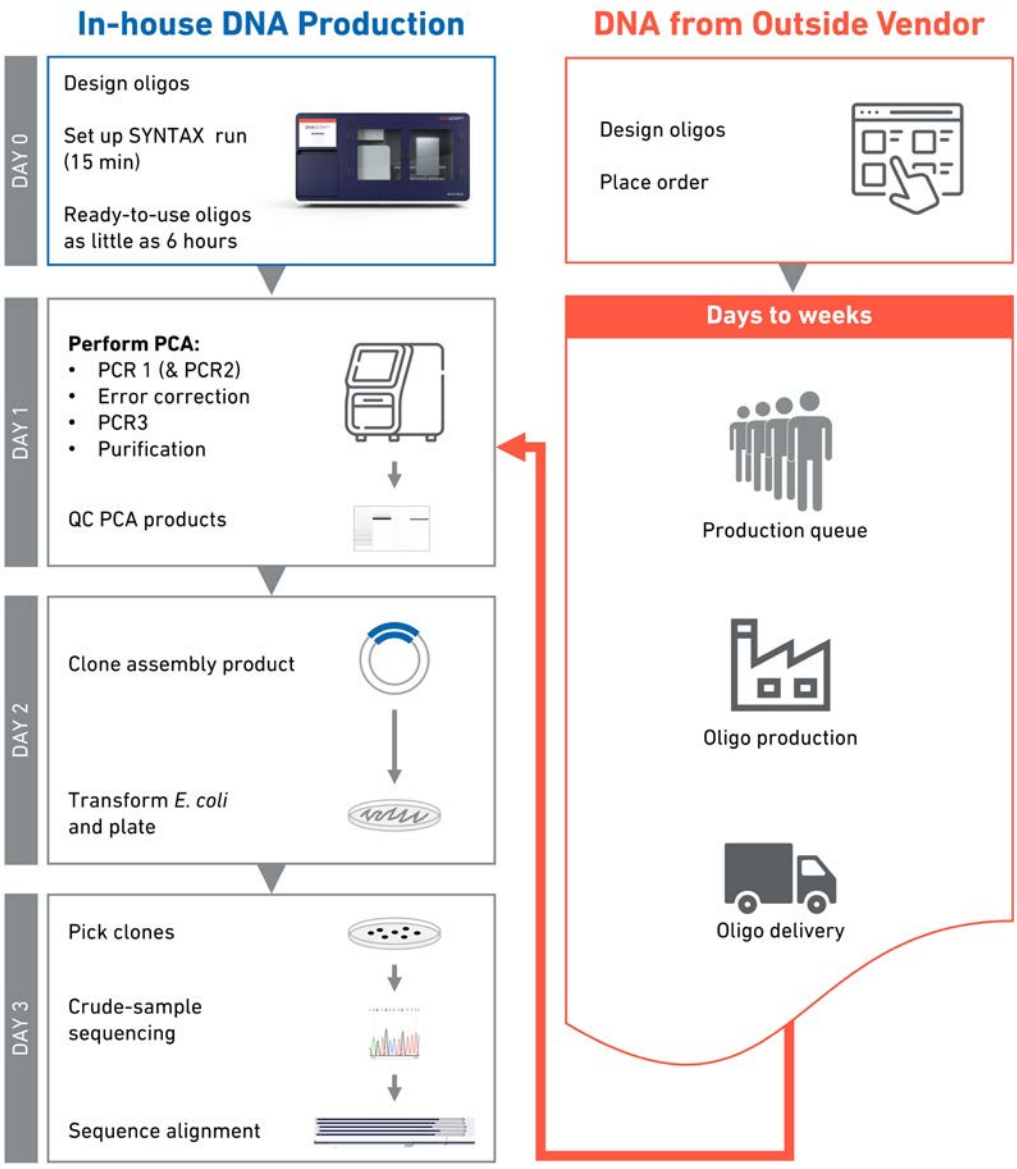
CONCLUSION

PCA provides a simple and convenient strategy for the assembly of genes and other DNA constructs from relatively short building blocks. Success rates depend on oligo quality, polymerase error, and the efficiency of cloning and screening procedures. As demonstrated through the successful assembly of EGFP and mRFP, DNA Script's EDS technology and SYNTAX System supports:

- Efficient, same-day PCA-based assembly of constructs <1 kb with overlapping oligos ranging from 25 to 58 nt in length.
- High success rates—as measured by the probability of recovering full-length perfect clones that express functional protein. In the phenotype-driven mRFP screening process, 80% of total clones and 90% of red clones were confirmed to contain full-length perfect constructs. In the phenotype-agnostic EGFP verification process, 1 in ~4 clones were confirmed to be full-length perfect. The percentage of full-length perfect clones would likely be in the range of 33 – 50% if success rates were prioritized over the shorter turnaround time enabled by crude-sample Sanger sequencing.

The data presented here indicates that EDS oligos perform as well as those from an industry-leading producer of chemically synthesized oligos, with potentially lower batch-to-batch variability. In addition, in-sourced oligo production with DNA Script's SYNTAX System offers four key advantages over DNA as a service in low- to mid-throughput PCA-based gene assembly pipelines for a wide range of applications:

- **Simplicity.** With the SYNTAX System, DNA oligos can be produced in any standard laboratory environment, by anyone trained in basic molecular biology. This makes DNA writing as simple and accessible as DNA reading (sequencing).
- **Certainty.** PCA offers a robust method for same-day gene assembly, but this workflow can be leveraged only when oligo supply is certain. With the SYNTAX System, a new batch of ready-to-use assembly oligos can be prepared overnight for next-day use. This capability enables efficient planning and on-schedule completion of assemblies, irrespective of local or global factors impacting oligo production or delivery.
- **Speed.** With the predictability that the SYNTAX System affords, sequence-verified clones can be achieved in as little as three days (Figure 9)—depending on laboratory resources, cloning procedures, and screening strategies. As demonstrated in this study, turnaround time can be reduced at the expense of screening more colonies, or *vice versa*. On-demand access to oligos further reduces the length of iteration cycles and recovery time when assemblies have to be repeated as a result of complications downstream.
- **Security.** In the highly competitive field of synthetic biology, the SYNTAX System offers full control over proprietary sequences—thereby eliminating potential risks related to data security and contamination associated with centralized processing.



- Additional days are needed if the preferred workflow includes a phenotypic assay or requires overnight incubation of clones for any other reason (e.g., DNA purification for sequencing).
- With sufficient resources and automation, multiple genes can be assembled in parallel and pooled for verification using next-generation sequencing.

FIGURE 9. EDS AND PCA ENABLE SAME-DAY GENE ASSEMBLY AND SEQUENCE-VERIFIED CLONES IN AS LITTLE AS THREE DAYS. This 3-day workflow was compiled from methods used in this study, but does not represent the actual workflow or turnaround time for the EGFP or mRFP experiments, and assumes that the necessary laboratory resources and equipment are available to support the desired throughput. The amount of time needed to complete the workflow from the start of the PCA protocol to sequence-verified clones is the same, irrespective of how oligos are synthesized. Nevertheless, the services of at least two vendors are needed to obtain chemically synthesized oligos from centralized commercial oligo houses. Even though next-day delivery is supported in some parts of the world, this is typically not the norm for ready-to-use, plated oligos, and can never be guaranteed. Unlike in-sourced oligo production with the SYNTAX System, outsourced oligo supply adds days to weeks (or even months, as was the case during COVID-19 pandemic) to project schedules.

MATERIALS AND METHODS

Oligo Design

Primers for the EGFP assembly were designed using the open source DNAWorks software (v3.2.4) with the following custom parameters:

- Organism: *E. coli* standard
- Annealing temperature: 62 – 65°C
- Oligo length: 59 – 60 nt

This yielded a set of 20 oligos tiling the 239-amino acid EGFP ORF (720 bp). Restriction enzyme recognition sites were included at the 5'-end of oligos 1 and 20 (19 nt in total), resulting in an assembly product of 739 bp.

Oligos for the mRFP assembly were designed using the open source Gene Design software (target assembly oligo length = 50). This yielded a set of 24 oligos tiling the 225-amino acid mRFP ORF (678 bp). Twenty nucleotides were included at the 5'-end of the forward and reverse primers used in PCR3 to create regions of homology between the error-corrected assembly product and the cloning vector. This resulted in a final assembly product of 718 bp.

Oligo Synthesis

EDS oligos were produced using the SYNTAX System (DNA Script part number 100296 or 100094) and SYNTAX 60-cycle kit, 96 oligos (DNA Script part number 100134) with standard synthesis plates according to the manufacturer's instructions. The four sets of EDS oligos for replicate EGFP assemblies and the EDS oligo set for the mRFP assembly were synthesized independently (on different days, different instruments, and by different users). The SYNTAX System yields ready-to-use, desalted, quantified and normalized oligos. Oligos were normalized to a concentration in the range of 3 – 10 µM (depending on the synthesis batch).

CS oligos and all amplification primers were ordered from an industry-leading oligo supplier with operations in US and Europe. The four sets of CS oligos for replicate EGFP assemblies were from four separate orders placed with the same vendor over the course of a three-month period.

Gene Assembly and Error Correction

As outlined in *Experimental Design*, a total of ten EGFP assemblies were performed using a traditional 2-step PCA protocol. The following parameters were used for individual stages of the process:

- All oligo extension and amplification steps were performed with Q5 High-fidelity DNA Polymerase (New England Biolabs). Reactions were set up according to manufacturer's instructions, except as indicated below.
- An oligo pool containing each of the 20 EGFP assembly oligos at a concentration of 0.15 µM was created. Of this, 10 µL was used in the 50-µL PCR1 reaction. After initial denaturation (30 sec at 98°C), oligo extension was performed for 15 cycles of 10-sec denaturation at 98°C, 15-sec annealing at 55°C, and 20-sec extension at 72°C. A final extension of 2 min at 72°C was included.
- For PCR2, 1.25 µL of each amplification primer (EGFP-F and EGFP-R, both diluted to 10 µM) was added to the PCR1 product, and the reaction was made up to 50 µL with the appropriate volumes of other reaction components. After initial denaturation (30 sec at 98°C), oligo extension was performed for 30 cycles of 10-sec denaturation at 98°C, 10-sec annealing at 57°C, and 20-sec extension at 72°C. A final extension of 2 min at 72°C was included.
- Error correction was performed with the CorrectASE Enzyme (ThermoFisher Scientific). PCR2 products were diluted to 25 ng/µL in 50 µL of 1X CorrectASE Buffer, and denatured and re-annealed according to the manufacturer's instructions. Each re-annealed product (10 µL) was incubated with 1 µL of CorrectASE Enzyme for 15 min at 25°C.
- PCR3 was performed as described for PCR2 (25 cycles only), with 2 µL of the CorrectASE reaction product in a final volume of 50 µL.

The mRFP gene assembly was performed with a 1-step integrated PCA-PCR protocol:

- PCR1 was performed with KOD Hot Start Master Mix (2X, MilliporeSigma). The 24 assembly oligos (0.5 µL each) were included in a 50 µL-reaction (for a final oligo concentration of approximately 0.094 µM each). After initial denaturation (30 sec at 94°C), oligo extension was performed for 30 cycles of 2-sec denaturation at 94°C, 10-sec annealing at 56°C, and 10-sec extension at 72°C. A final extension of 15 sec at 72°C was included.
- PCR2 was omitted. PCR1 product denatured, re-annealed, and error corrected as outlined in the CorrectASE protocol. Re-annealed product was incubated with the CorrectASE enzyme for 15 min. The reaction product was amplified in PCR3 and visualized by agarose gel electrophoresis.
- PCR3 (final amplification) of the mRFP construct was also performed with KOD Hot Start Master Mix. The CorrectASE reaction product (10 µL) was included in a 100 µL-reaction containing the forward and reverse primers (mRFP-F and mRFP-R, with 5'-overhangs) at a final concentration of 0.3 µM each. After initial denaturation (2 min at 95°C), extension was performed for 30 cycles of 20-sec denaturation at 95°C, 10-sec annealing at 56°C, and 30-sec extension at 70°C. A final extension of 5 min at 70°C was included.

EGFP Protein Expression

The purified 739-bp EGFP construct (PCR3 product) from each of the ten assemblies was cloned into the pET-28a(+) vector (Novagen/MilliporeSigma), using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) with a 2:1 insert:vector ratio in a 20-µL reaction volume. Reactions were incubated for 15 min at 50°C. One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen/Thermo Fisher Scientific) were transformed with 2 µL of reaction product and plated on LB-agar containing 50 µg/µL kanamycin. After overnight incubation at 37°C, all of the colonies recovered from an assembly condition/replicate were pooled and resuspended in 5 mL of liquid LB medium containing 50 µg/µL kanamycin. Cultures were grown overnight at 37°C, after which plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (QIAGEN).

Chemically competent BL21(DE3) Competent *E. coli* cells (New England Biolabs) were transformed with plasmid DNA pools (250 pg per transformation; one transformation per gene assembly condition). Bacteria were plated on LB-agar containing 50 µg/µL kanamycin and 0.1 mM IPTG to induce T7-driven expression of the assembled EGFP gene. After overnight incubation at 37°C, plates were observed under UV light and green fluorescent colonies were counted.

mRFP Protein Expression

For mRFP expression, the 718-bp band from the amplified 15-min CorrectASE incubation product was extracted from the agarose gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research) according to manufacturer's instructions. Purified DNA was quantified using a Qubit 4 Fluorometer and Qubit dsDNA HS Assay Kit (Invitrogen/Thermo Fisher Scientific) and cloned into a linearized pU6-pegRNA-GG-acceptor plasmid (Addgene plasmid # 132777)[8] from which the original mRFP ORF had been deleted. Cloning was performed with the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs), using a 2:1 insert:vector ratio in a 20-µL reaction volume. Reactions were incubated for 25 min at 50°C. NEB 10-beta Competent *E. coli* (High Efficiency) cells (New England Biolabs) were transformed with 1 µL of the reaction product and plated on LB-agar containing 100 mg/L ampicillin. Plates were incubated overnight at 37°C, followed by 24 h at 4°C. Plates were inspected under normal laboratory lighting to record the number of red and white colonies.

mRFP Plasmid DNA Extraction and Sanger Sequencing Verification

All 75 colonies from the mRFP experiment (67 red and 8 white) were picked for sequence verification. Overnight cultures were made in liquid 2XYT medium containing 50 mg/L ampicillin. Plasmid DNA was purified using the CosMCPrep system (Beckman Coulter) according to the manufacturer's instructions.

Sanger sequencing of inserts in recombinant mRFP plasmid DNA was performed at the Stowers Institute for Medical Research using standard protocols and sequencing primer U6-seqF1 (5'-TGGACTATCATATGCTTACCGTA ACTTGA-3'; binds to the vector backbone

upstream of the cloning site). Sequencing data were analyzed using SnapGene.

Sequence Verification of EGFP Constructs

EGFP PCR3 products were purified using the Monarch PCR and DNA Cleanup Kit (5 µg) (New England Biolabs) according to the manufacturer's instructions. Purified DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

For each of the ten assemblies, the purified 739-bp construct was cloned into the pJET1.2/blunt Cloning Vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen/Thermo-Fisher Scientific) were transformed with 2 µL of ligation product and plated on LB-agar containing 100 µg/µL ampicillin. Plates were incubated overnight at 37 °C.

For each transformation, 96 colonies were picked for verification by Sanger sequencing. Plates were submitted to a third-party provider (GENEWIZ/Azenta Life Sciences) for Direct Colony Sequencing[9] using the pJET1.2 forward and reverse sequencing primers. Raw data were analyzed with a custom bioinformatics pipeline designed to merge forward and reverse reads and align merged reads to the EGFP reference sequence.

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