From Digital Sequence to Fully Assembled Gene in Less than Three Days Using Enzymatically Synthesized Oligos

Gene synthesis is a key part of the biomedical toolkit used in protein engineering, molecular biology research, and rapid vaccine development. In this Application Note, we demonstrate the use of DNA Script's Enzymatic DNA Synthesis (EDS) technology and benchtop SYNTAX™ STX-200 Platform to synthesize oligos for polymerase cycling assembly (PCA) of genes. Using the 1,698 bp hemagglutinin (HA) gene as a model, we compared PCA-based assemblies using 60-mer and 120-mer oligos printed using the SYNTAX platform. The HA gene was successfully assembled using both sets of starting oligos. Incorporating error correction with CorrectASE yielded an 18.5% and 21.9% rate of producing a perfect sequence with 60-mer and 120-mer oligos respectively, meaning that one in six clones had a perfect sequence. Thus, we can start from a digital sequence and successfully assemble a gene in less than three days with oligos synthesized using EDS technology and the SYNTAX platform.

INTRODUCTION

Gene synthesis involves building an entire gene from synthetic precursors, in contrast with molecular cloning, which copies existing DNA sequences. Producing short DNA fragments in vitro — essential for DNA amplification and molecular cloning — has been the workhorse of molecular biology for decades. As technology has improved with the ability to build longer DNA constructs, synthetic gene synthesis has become more common.[1] Gene synthesis is used regularly in molecular biology research[2] and in the pharmaceutical sector, providing the building blocks for multiple applications such as gene therapy, antibody development, and more (**Figure 1**). The technique has been instrumental in vaccine development: it took 66 days from downloading the newly sequenced SARS-CoV2 genome to a clinician administering the first vaccine dose.[3]

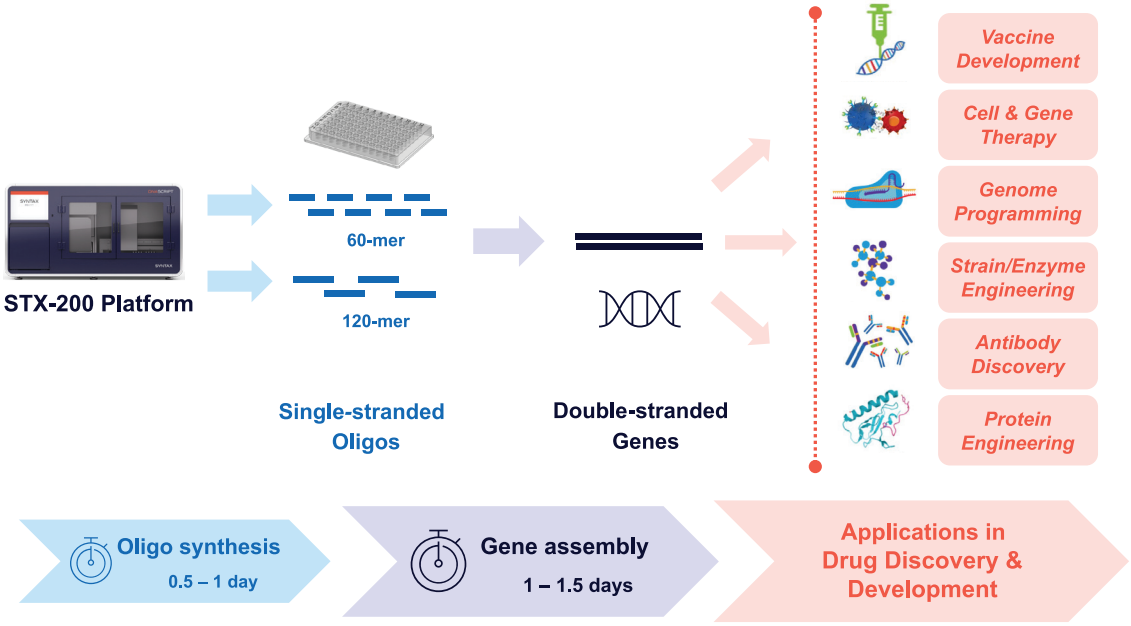

Figure 1. Gene synthesis process and downstream applications. Fully assembled genes can be obtained in less than three days from digital sequence, enabling significant time savings in multiple downstream applications.

The starting point for all these applications is creating oligonucleotides (oligos). Oligos have been synthesized for more than four decades using phosphoramidite chemistry technology, where a cycle of chemical reactions using chemically modified nucleotides assemble oligos one base at a time. Due to technical and cost limitations, oligos synthesized this way are typically limited to about 60-mer or shorter oligos for routine use and must be obtained from third-party service providers.

Enzymatic DNA synthesis (EDS) is a more recent technology that uses an engineered DNA Terminal Deoxynucleotidyl Transferase (TDT) enzyme to catalyze the addition of nucleotides to a growing oligo rather than non-enzymatic chemistry using modified bases.[4] This is more aligned with natural DNA synthesis and can produce longer oligos — up to 120-mer — without an increase in cost per base. EDS also produces about 40 times less hazardous organic waste than phosphoramidite chemistry,[1,5] making it more tractable for a benchtop setting.
DNA Script's proprietary EDS technology and innovative SYNTAX Platform[4,6] allow the production of oligos up to 120-mers in a matter of hours, speeding up the production of oligos and moving this typically outsourced step in-house. With the capability to synthesize 60-mer oligos in 11 hours or 120-mer oligos in 21 hours, researchers could start the gene assembly process on the same day they design their oligos (**Figure 1**).

This Application Note describes designing and assembling a gene by Polymerase Cycling Assembly (PCA) in less than three days, using EDS oligos printed on the SYNTAX STX-200 platform, with the influenza hemagglutinin (HA) gene as a model. The HA protein is one of two proteins the influenza virus uses to infect cells. It is encoded by a 1,698 bp long gene with 42% GC content and has no anticipated synthesis difficulty, such as long repeats or secondary structure. See **Figure 2** for the experimental workflow. **Figure 3** shows a more detailed representation of the PCA, and error correction processes used in this Application Note.

MATERIALS AND METHODS

Oligo design

The three blocks for the HA assembly were designed using the NEBuilder Assembly Tool (https://nebuilder.neb.com/#!/), with 40bp overlaps between blocks. The DNAWorks oligo design software (https://helixweb.nih.gov/dnaworks/) was then used to design primers to build individual blocks by PCA, using both the 60-mer and 120-mer approaches. Forward and reverse amplification primers were also designed to extend individual blocks in the PCR2 step of the PCA process, and primers to create regions of homology between the assembly product and the cloning vector.

Oligo synthesis

All oligos were synthesized by EDS technology using the SYNTAX STX-200 Platform (DNA Script part number 101773 using the SYNTAX DNA Synthesis Kit (DNA Script part number 101867) and SYNTAX 96 Hi-Fidelity Kit (DNA Script part number 101868) according to the manufacturer's instructions. The SYNTAX platform yielded ready-to-use, desalted, quantified, and normalized oligos (at 4µM).

Gene assembly and error correction

The gene assembly process is outlined in Figure 1, following the traditional 2-step PCA protocol to which an error correction step was added (Figure 2). For all PCR steps, the Q5 High-Fidelity DNA Polymerase (New England Biolabs, Cat No M0491S) was used, and PCR experiments (including thermal cycling conditions) were performed according to the manufacturer's instructions. To assemble each individual block, corresponding oligos were pooled at a 150nM final concentration and used in the first oligo extension PCR (PCR1) of 15 cycles. Using the PCR1 product as the template with block amplification primers, a second PCR (PCR2) of 30 cycles was performed. The PCR2 product was visualized using agarose gel electrophoresis to verify block sizes. Error correction on the assembled blocks was then performed with the CorrectASE enzyme (Thermo Fisher Scientific, Cat No A14972) following the manufacturer's protocol. Finally,

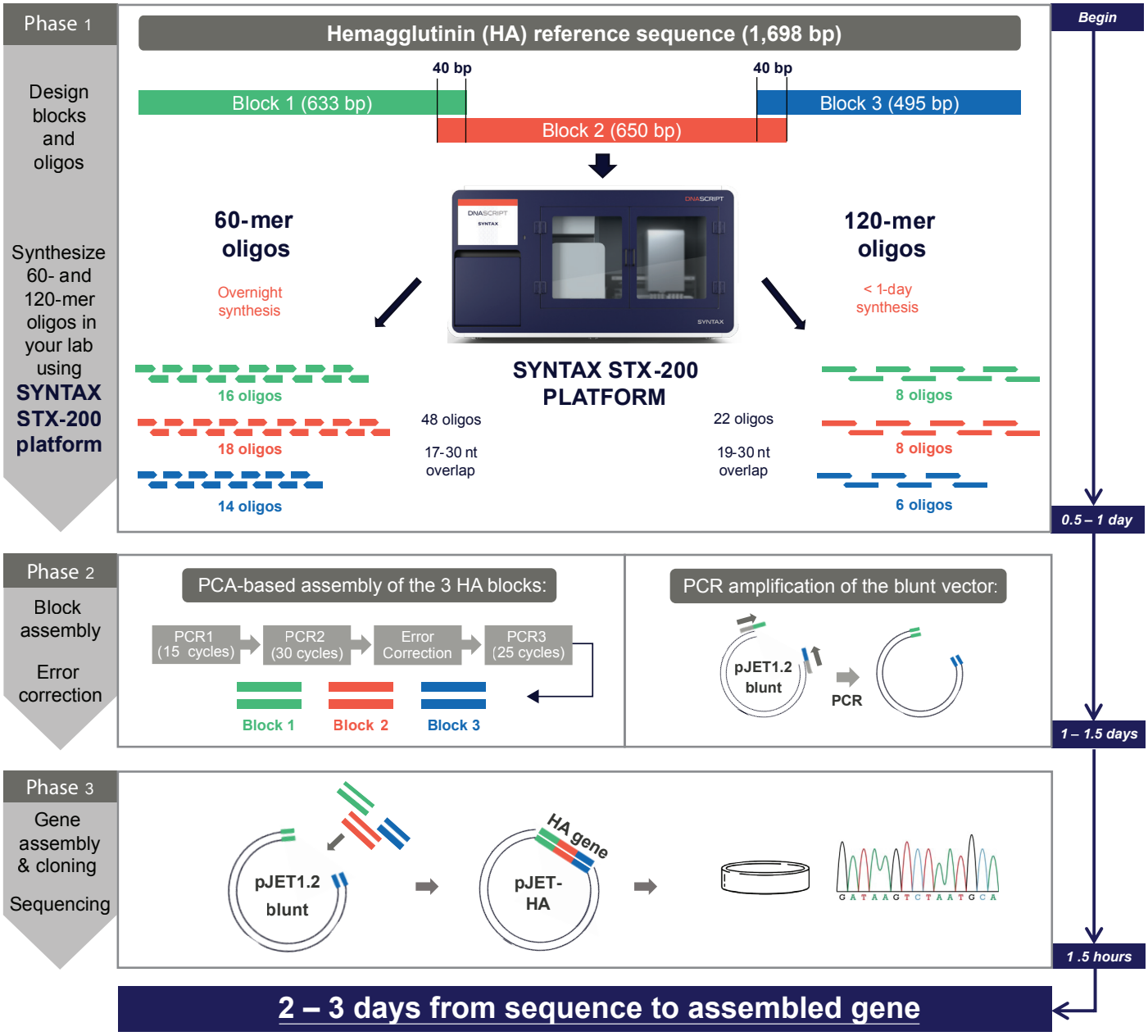

Figure 2. Experimental workflow for assembly of the HA Gene by Polymerase Cycling Assembly. **Phase 1:** the 1,698 bp HA gene sequence was split into 3 overlapping blocks (green, orange, and blue) and overlapping oligos of either 60nt or 120nt in length were designed. Oligos were synthesized using the SYNTAX STX-200 Platform, overnight or in less than one day. **Phase 2:** ssDNA oligos were used as starting material for PCA assembly into dsDNA blocks. This PCA-based assembly included three PCR steps and an error correction step between PCR2 and 3 using the CorrectASE enzyme (Thermo Fisher Scientific). The pJET1.2 blunt vector was PCR-amplified with gene-overlapping primers designed to contain 25nt-holomogy with blocks 1 and 3. **Phase 3:** purified blocks were assembled into the HA gene and cloned into the pJET vector for transformation and sequence verification by rolling circle amplification (RCA) – Sanger sequencing. See Figure 2 for the experimental workflow, and Figure 3 for a detailed representation of the PCA and error correction processes used in this Application Note.

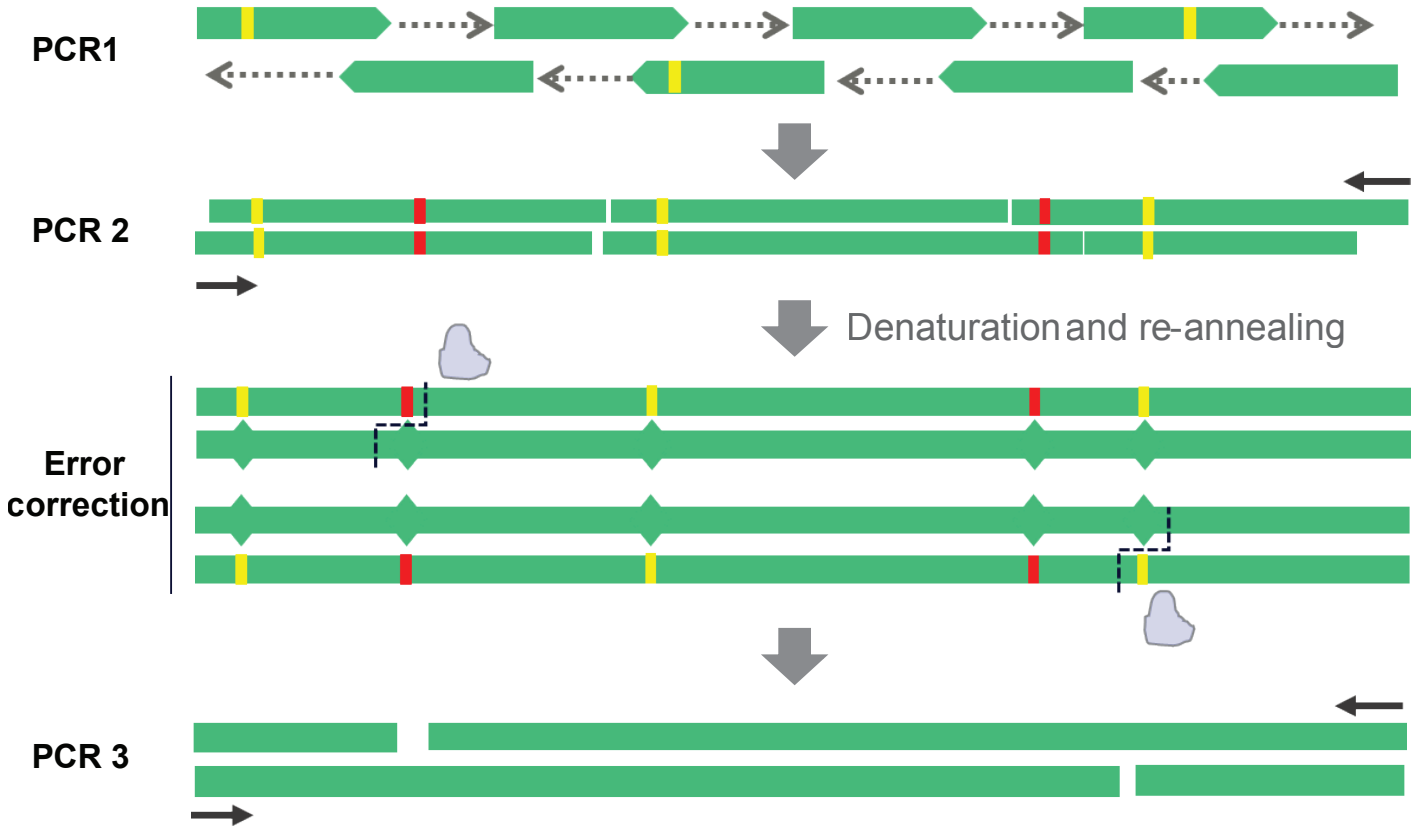

Figure 3. PCA process used to assemble EDS oligos into double-stranded DNA fragments. Overlapping oligos of either 60nt or 120nt in length were designed, and then synthesized on the SYNTAX Platform, to be used as starting material for PCA assembly into blocks. Each dsDNA block was generated by PCA assembly in three PCR steps. Potential errors resulting from oligo synthesis and from polymerase extension are depicted in yellow and red respectively (for illustrative purposes only). PCR2 products were denatured and re-annealed to create mismatches at error sites. Mismatches were then identified and excised using the CorrectASE enzyme (Thermo Fisher Scientific) in an error correction step prior to PCR3 reassembly of blocks, increasing the likelihood of obtaining error-free clones.

corrected blocks were re-amplified in a final PCR step (PCR3) of 25 cycles. Purified block fragments were assembled into the HA full gene and into the pJET1.2 cloning vector, using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Cat No E2621X) according to the manufacturer's instructions.

HA gene sequence verification

Using the four assembled pJET-HA gene constructs (with or without error correction performed, starting from either 60nt or 120nt oligos), One Shot TOP10 chemically competent E. Coli cells (Thermo Fisher Scientific Cat No C404010) were transformed, and plated on LB-agar plates containing ampicillin. After overnight incubation, 59 to 76 colonies per assembly condition were picked, inoculated in 300µL LB containing 100µg/mL ampicillin in a 2mL 96-deep well plate and grown for 8 hours. 10µL of each culture was transferred to agar plates, and plasmid DNA was sequenced by automated rolling circle amplification (RCA) – Sanger sequencing.

For a detailed, extensive protocol from DNA sequence to assembled gene, refer to the Quick Guide – DIY Gene Assembly by PCA (available on demand).

RESULTS AND DISCUSSION

Output from the NEBuilder Assembly tool led us to a synthesis strategy of three blocks of 633, 650, and 495 bp in length with a 40bp overlap between blocks (**Figure 2**). DNAWorks oligo design software output led to 48 oligos for 60-mer assembly and 22 oligos for 120-mer assembly, with 17–30 nucleotide overlaps. PCA oligos were synthesized on two 96-well plates using the SYNTAX STX-200 platform. Printing time was less than 12 hours for the 60-mer oligos and less than 24 hours for the 120-mer oligos.

Figure 4 demonstrates the successful assembly of the three HA blocks by PCA, showing the profile of products obtained at each PCR and error correction steps (refer to **Figures 2 and 3** for a visual guide of steps included), for both the 60-mer and 120-mer assembly approaches.

- PCR1 products shows DNA fragments of varying length, representing intermediate assembly products, with lower molecular weights corresponding to unextended oligos.

- Each full-length block was assembled in the second PCR step (PCR2). DNA electrophoresis yielded a single major band with the molecular weight showing the expected value for each full-length construct (633 bp, 650 bp, 495 bp) for both 60-mer and 120-mer assembly.

- Error correction with CorrectASE produced a visible smear compared to the untreated blocks. This is expected, as the CorrectASE enzyme excises sites of mismatches, which can produce lower molecular weight fragments. PCR3 is the final assembly step, where a proofreading polymerase fixes gaps introduced by the error correction step. The major products corresponded to the expected molecular weights for all three blocks for all four assembly conditions (60-mer and 120-mer with or without correction).

In a single-step reaction, the three blocks were assembled into the full-length HA gene and cloned into the pJET1.2 blunt vector. Assembled constructs were used to transform competent bacteria cells. From these:

- 10 bacteria colonies (six from the 60-mer assembly, four from the 120-mer assembly) were randomly selected and subjected to plasmid DNA extraction. PCR amplification of all 10 HA inserts yielded amplicons of the expected molecular weight (1,698 bp), demonstrating that all picked colonies had taken up the fully assembled gene (**Figure 5**). This was the case with and without error correction.

- 59 to 76 individual colonies were picked per assembly condition and subjected to Rolling Circle Amplification (RCA) – Sanger sequencing. Of those, 55 to 74 were correctly sequenced. Results are shown in **Figure 6**.

Sequencing of isolated clones identified a perfect sequence rate of 18% (10 perfect clones / 55 total clones) and 22% (16/74 clones) for 60-mer and 120-mer assembly respectively with error correction, compared to 5%for 60-mer (3/65 clones) and 120-mer (3/58 clones) without error correction (**Figure 6A**). Starting with 60-mer oligos, one in six clones on average had the perfect HA sequence (**Figure 6B**). With 120-mer starting oligos, the ratio increased slightly with one perfect clone in five clones when using error correction. Without error correction, this rate dropped to one in 22 for 60-mers and one in 19 for 120-mers.

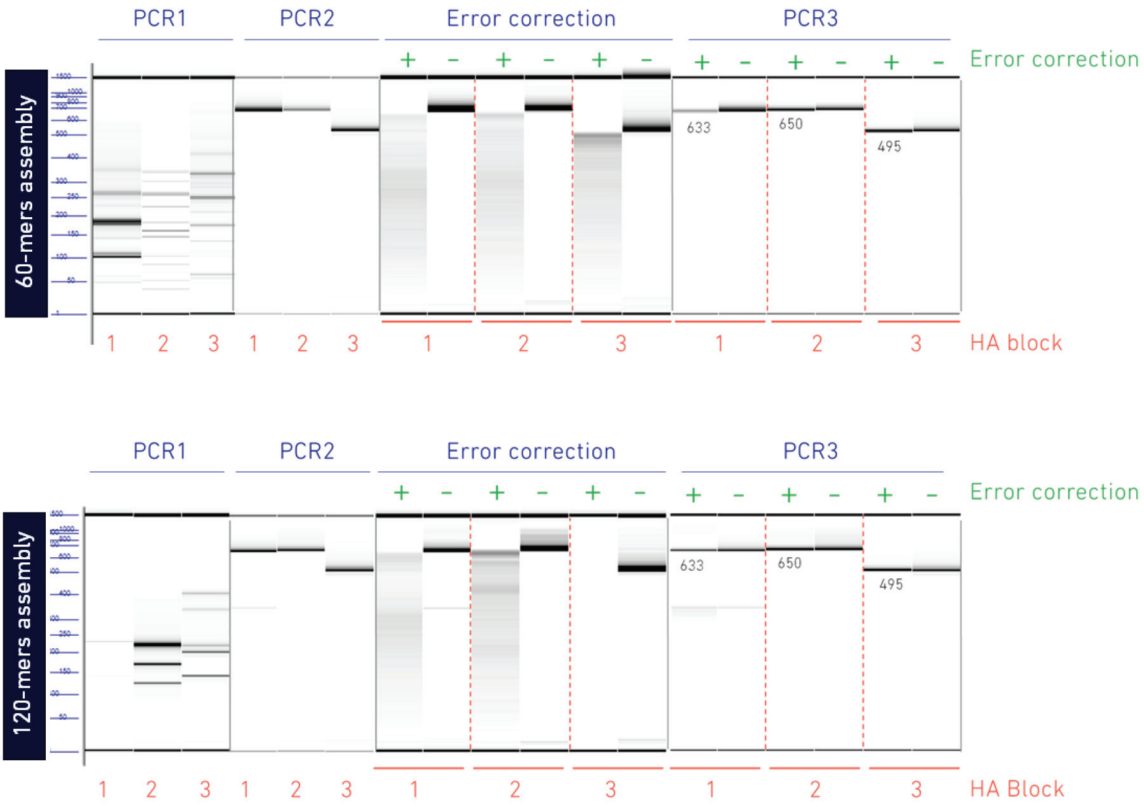

Figure 4. Reaction products generated during different stages of PCA assembly of oligos into blocks. The dominant bands in PCR2 and PCR3 products correspond to the 3 full-length block constructs (633, 650 and 495 bp) building the HA gene. Lower molecular weight bands in PCR1 products correspond to incomplete constructs/unextended oligos. Error correction was performed after PCR2, resulting in a smear for CorrectASE-treated samples. Reaction products were analysed by capillary electrophoresis using a 5300 Fragment Analyzer System and the HS Small Fragment kit (Agilent Technologies).

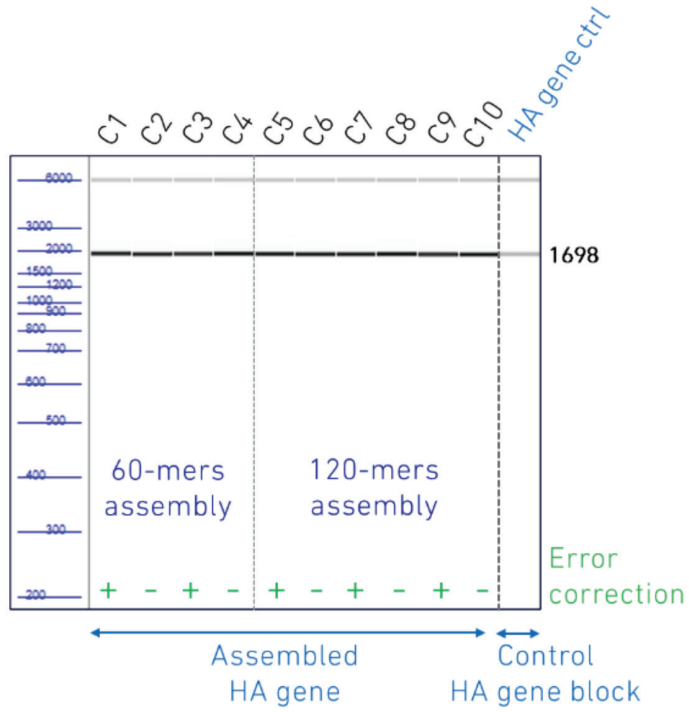

Figure 5. HA gene assembly product visualization. The PCR amplicon product was visualized by capillary electrophoresis using a 5300 Fragment Analyzer System. Plasmid DNA from 10 randomly picked colonies (C1-C10) was extracted, and the assembled HA gene (with or without error correction) was PCR-amplified using primers complementary to HA gene extremities, yielding a 1,698 bp product. A positive control (HA gene block obtained from a commercial vendor) was also included.

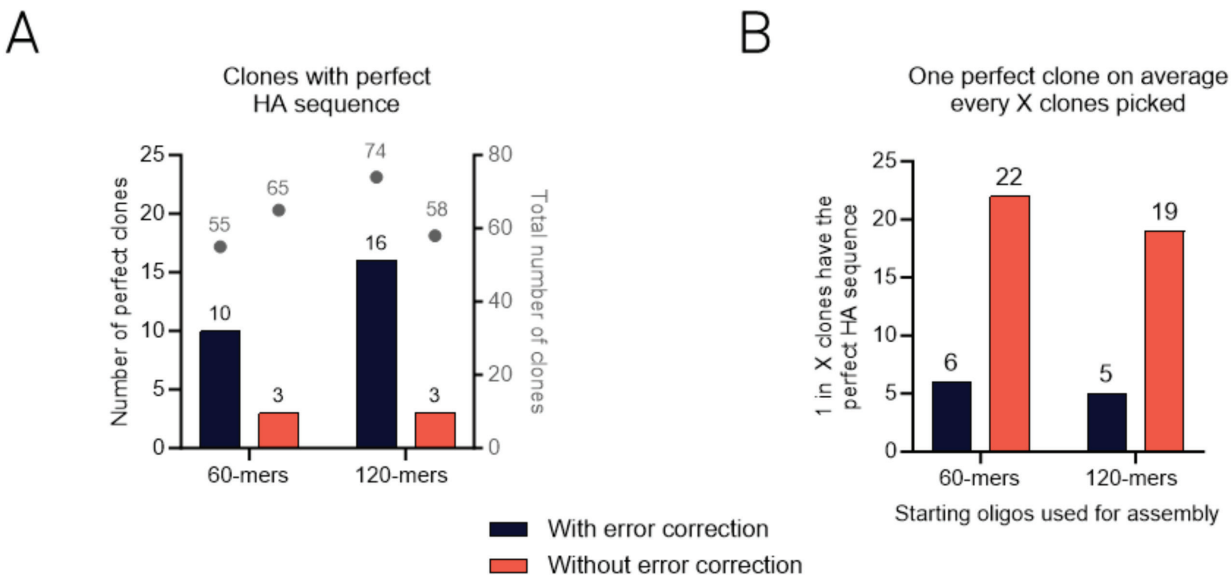

Figure 6. HA gene assembly sequence verification. Sequence verification of assembled HA product, starting from 60-mer or 120-mer oligos, with or without error correction (EC) step (blue: with EC; orange: without EC). **A**: The number of perfect clones (i.e., clones for which the HA sequence completely matches the expected reference sequence) are indicated as bars, while the total number of correctly sequenced clones in each condition is represented by a grey dot. **B**. Number of clones that were sequenced on average to observe one perfect HA sequence.

CONCLUSION

The data presented here demonstrates the successful assembly of the 1,698 bp HA gene from oligos synthesized using the SYNTAX STX-200 platform. We demonstrated that each step of our workflow functioned as intended and yielded the correct sequence with a high success rate, especially if error correction was used. With 120-mer starting oligos and CorrectASE error correction in the protocol, one in five sequenced clones resulted in a perfect HA sequence, reducing the required labor and sequencing costs.

The SYNTAX STX-200 platform provided several advantages. With the SYNTAX platform synthesizing 120-mer oligos, the oligos could be designed on commercially available tools, synthesized, and the gene assembly complete and verified in the time it would take to order and receive oligos from a commercial source. This faster process also keeps all gene synthesis in-house, removing potential intellectual property concerns and eliminating the need for external commercial manufacturers.

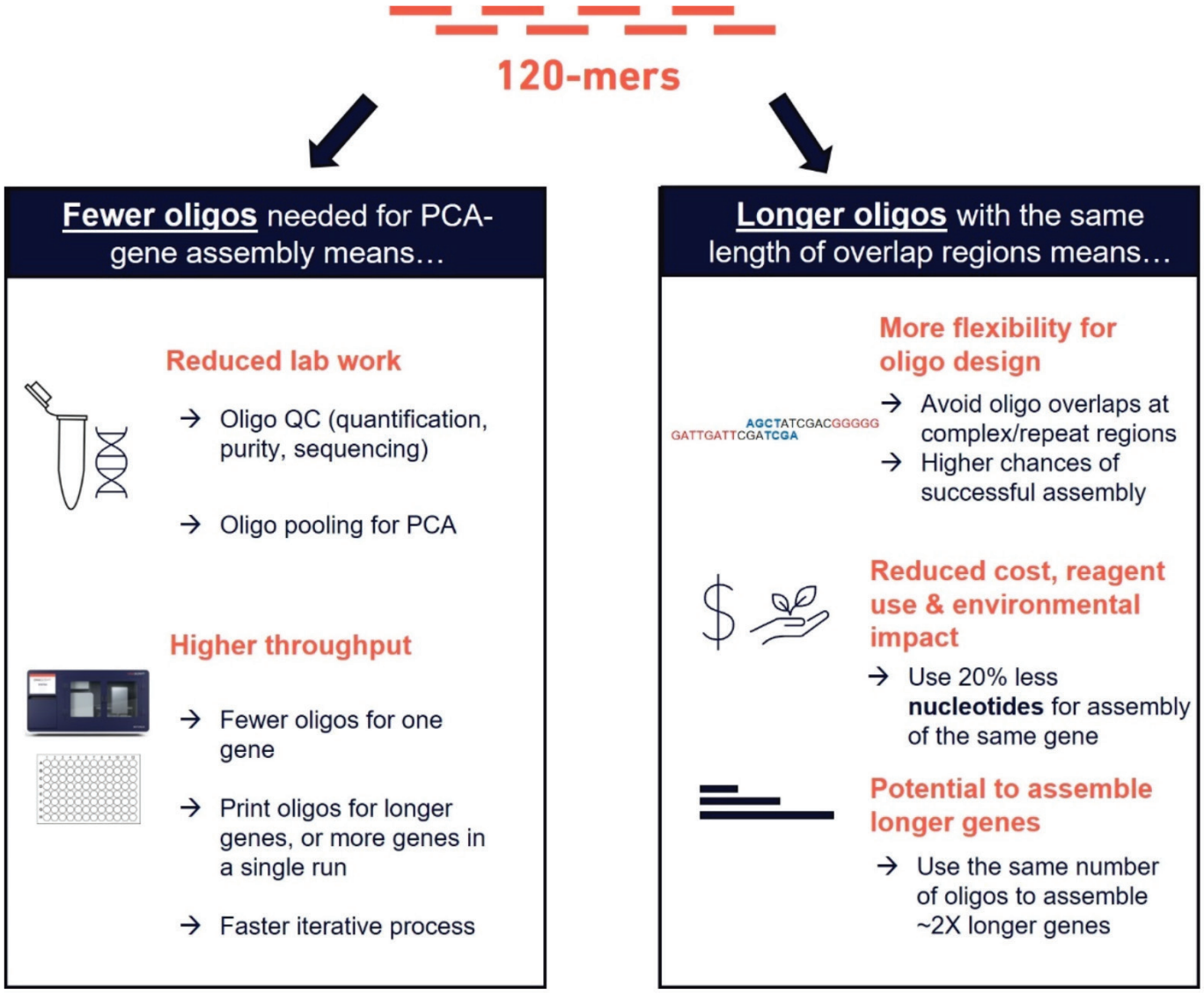

Figure 7. Advantages of PCA-based gene assembly starting from 120-mer oligos instead of 60-mer oligos typically used in gene assembly currently.

This experiment has shown clear advantages to using 120-mer oligos to build a gene versus 60-mers (**Figure 7**). The capability of the SYNTAX platform to synthesize 120-mer oligos means fewer individual oligos are needed for a single 96-well workflow. This allows labs to synthesize oligos for multiple genes in a single synthesis run, increasing throughput, and providing options for iteration. In addition, an independent analysis found that synthesizing 120-mer oligos using EDS could be more cost-effective per base than using 60-mers synthesized by either EDS or phosphoramidite chemistry.[5]

Start your next gene assembly project today with the **Quick Guide** (provided upon request), this Application Note and freely available commercial tools. With benchtop SYNTAX platform for oligo production and the PCA approach for assembly, you can go from design to assembled gene in 2–3 days.

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Technical Support
support@DNAScript.com
USA & Canada: +1 888-693-4022
Europe: +33 1 84 88 02 42