DNASCRIPT

TECHNICAL NOTE

Enzymatic DNA Synthesis

Access to synthetic DNA has been a key driver of the biological revolution over the past four decades. Almost every routine and emerging genomics and life science application requires oligonucleotides—the bulk of which is currently sourced from a handful of commercial suppliers. In this Technical Note we describe DNA Script's novel and disruptive, fully enzymatic method for oligo synthesis. Enzymatic DNA Synthesis (EDS) offers significant advantages with respect to accessibility and convenience, transforming the pace of and control over iteration and innovation, with reduced environmental impact.

IT IS TIME FOR A PARADIGM SHIFT

Today the vast majority of oligos consumed by academic, non-profit and government research institutions, biotech and biopharma companies, and in molecular diagnostic assays are supplied by a limited number of manufacturers. These oligos are produced in dedicated, centralized facilities using 40-year old phosphoramidite chemistry. Although many scientists currently enjoy next-day access to primers, this is not always the case, depending on location and oligo length or complexity. And, as experienced during the COVID-19 pandemic, when extraordinary circumstances overburden global manufacturing capabilities and logistics.

To truly democratize access to custom DNA, a complete paradigm shift in synthesis chemistry was needed. It was imperative to eliminate hazardous organic chemicals that require specialized infrastructure and technical skills. Our template-free, fully enzymatic EDS technology does just that, making oligo synthesis as simple and safe as everyday molecular biology.

THREE CORE COMPONENTS MAKE EDS POSSIBLE

To develop a method for producing DNA on-demand in a highly controlled manner, our R & D efforts have focused on three core components: a purpose-engineered enzyme, reversibly terminated nucleotides, and solid synthesis supports.

- ### Highly Engineered Terminal Deoxynucleotidyl Transferase (TdT)

  TdT is a specialized X-family DNA polymerase that introduces diversity during antibody gene recombination by adding nucleotides to the 3'-termini of V, D and J exons in a random, template-independent manner.[1] The unique ability of TdT to create genomic material de novo has long been leveraged for the labeling of oligonucleotides in a wide range of applications. It also renders TdT the enzyme of choice for EDS.

  Our proprietary EDS enzymes have been engineered to rapidly and selectively add our reversibly-terminated nucleotides to the 3'-end of any single DNA strand (irrespective of the sequence context), with high fidelity and high coupling efficiency.

- ### Reversibly Terminated Nucleotides

  To achieve full control over the EDS process—i.e. ensure that the desired base, and that base only, is added to each oligo during a synthesis cycle—a mechanism was needed to effectively pause synthesis after a single base addition. As in other applications (e.g. Illumina® sequencing), this is achieved through the use of nucleotides with a modified 3'-hydroxyl group that prevents extension of the growing DNA chain in one cycle, but can be removed to permit synthesis to continue in the next.

  The reversibly terminated deoxy-nucleotide triphosphates (dNTPs) used in our EDS process are exclusively

licensed from Firebird Biomolecular Sciences, and contain a simple aminooxy (ONH_2) group on the 3'-carbon of the nucleotide. Compared to other reversible terminators used in the industry, ONH_2 groups are relatively small, permitting TdT to effectively utilize the modified bases. Critically, a functional 3'-hydroxyl group (ready for the next phosphodiester bond) is rapidly and efficiently restored using mild, acidic conditions. An additional advantage of our nucleotides is that deprotection leaves no "scars," which means that EDS yields completely "natural" (native), molecular biology-ready DNA.

Solid Supports and iDNA

The third core component of a highly predictable and controlled DNA synthesis technology is the solid support on which oligos are produced. This support allows us to control synthesis scale, and manipulate the growing oligo strands throughout the EDS process.

The solid support used in our SYNTAX™ System enables yields akin to those obtained from phosphoramidite-based methods. The physical properties and chemical functionalization of the solid support have been specifically optimized to enable the synthesis of full-length DNA at nanomolar scale to support molecular biology and genomics applications.

The solid support is covalently coated with predefined DNA linkers, called the "initiator DNA" (iDNA). The iDNA has four important functions:

- It provides the footprint needed by the TdT enzyme to initiate template-free DNA synthesis.
- It contains a cleavage site, which enables enzymatic release of the complete oligo from the linker.
- The iDNA coating density determines synthesis scale.
- The iDNA itself has been optimized for maximum synthesis yield by minimizing steric hindrance during enzymatic elongation.

THE EDS PROCESS

The three core EDS components (engineered TdT enzyme, reversibly terminated dNTPs, and solid support with iDNA) are utilized in a simple, cyclic, two-step synthesis process to synthesize oligos (Figure 1).

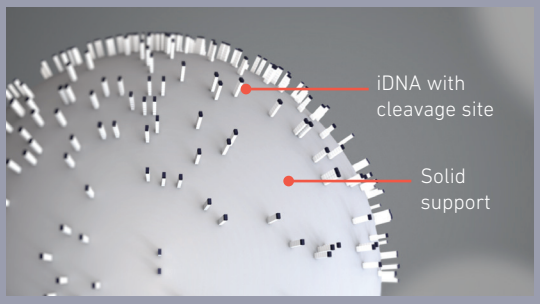
In our fully automated SYNTAX System, synthesis is performed in plate format. Each well is individually addressed, allowing for the production of a unique sequence and oligo of different length (up to the maximum supported by the system version) in each well. The number of synthesis cycles, and total synthesis time, is determined by the longest oligo in the batch.

Once synthesis has been completed, the plate is subjected to post-synthesis processing, which involves (i) enzymatic cleavage of the custom oligo sequence from the solid support, (ii) desalting, (iii) on-board quantification using UV spectrophotometry, and (iv) normalization. The entire process yields normalized, ready-to-use oligos, and currently takes less than standard workday (≤30-mers), or overnight (for oligos up to 60 nt).

UNIQUE FEATURE: EXTENDING EXISTING DNA

Unlike phosphoramidite-based methods, EDS does not require harsh and complicated organic chemistry that necessitates base protection. As a consequence, EDS offers the unique ability to add sequence to the 3'-end of existing, native, single-stranded DNA.

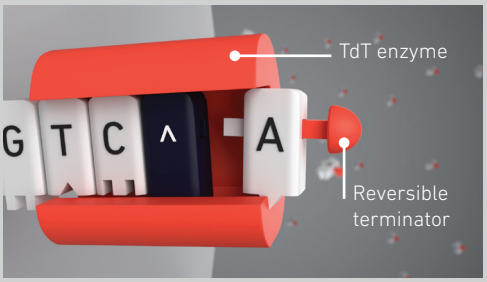
This feature can be leveraged to include a pre-defined 5'-motif—such as a primer or protein binding site, promoter sequence, restriction enzyme recognition site, adapter, or barcode—in all or a subset of oligos in a batch. This feature is enabled via custom synthesis plates, designed to include additional sequence downstream from the cleavage site in the iDNA. De novo synthesis commences directly after the last base of your pre-defined motif, which is included in the cleaved, full-length oligo and is taken into account during quantification and normalization.



PRE-SYNTHESIS

The solid support with covalently linked iDNA in each well of the synthesis plate is ready to accept the first base, which is incorporated directly after the cleavage site. When a custom synthesis plate is used, de novo synthesis starts directly after the pre-defined, fixed 5'-motif contained within the iDNA (downstream from the cleavage site).

SYNTHESIS



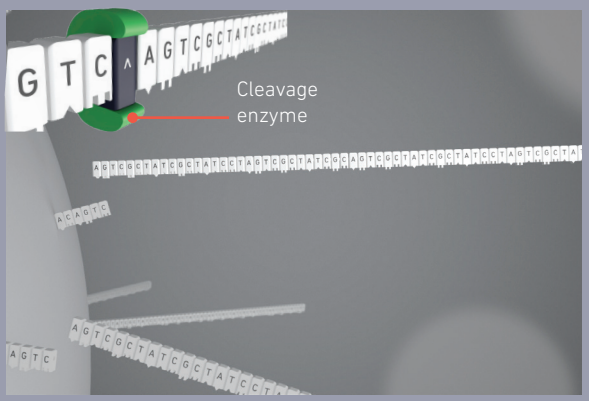
STEP 1: ELONGATE

The engineered TdT enzyme catalyzes the addition of the first base to the iDNA. The acid-labile, reversible terminator group ensures that only a single base is added. Enzyme and unincorporated nucleotides are washed away.

STEP 2: DEPROTECT

The newly added base is deprotected with a mild acidic solution. This restores a functional 3'-hydroxyl for the addition of the next base. The deprotection solution is removed with a brief wash.

Enzyme and nucleotides are dispensed for the next elongation cycle. Steps 1 and 2 are repeated until the longest oligo on the plate is completed. Shorter oligos undergo the same number of wash steps but do not receive reactive reagents.



POST-SYNTHESIS

Following completion of the last synthesis cycle, enzymatic cleavage is performed to release all bases downstream from the cleavage site. The resulting oligos are desalted, quantified, and normalized. Molecular biology-ready oligos are collected, and the system may be prepared for another run within just 30 min.

Figure 1. Overview of the EDS process. A sequence file is uploaded to the fully automated SYNTAX benchtop DNA printer. The run is prepared by loading cartridge-based reagents (enzymes, buffers and reversibly terminated dNTPs), and consumables. Each synthesis cycle consists of two simple steps: elongation and deprotection. The last cycle is followed by post-synthesis processing. Oligos are synthesized in the 5'- to 3'-direction and are delivered by default with a 5'-phosphate.

CONCLUSION

DNA Script's novel Enzymatic DNA Synthesis (EDS) process employs three core components—a highly engineered TdT enzyme, reversibly terminated 3'-ONH_2 dNTPs, and a solid support with customizable iDNA—to enable fully automated, same-day, in-house production of DNA oligos on a nanomolar scale. Independence from centralized suppliers and third-party logistics enables broader access and full control over proprietary sequence information and oligo supply, and accelerates the pace of iteration and innovation. The EDS technology has been shown to support genomics and life science applications, including PCR, qPCR, Sanger sequencing, hybridization- and amplicon-based[2] targeted next-generation sequencing, FISH and CRISPR-Cas9 workflows.

REFERENCES

1. Delarue M. et al. EMBO J. 2002; 21(3):427-439. doi: 10.1093/emboj/21.3.427.
2. Derrien B. et al. AGBT 2021 poster presentation.

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