BIOCATALYSIS

An enzyme cascade enables production of therapeutic oligonucleotides in a single operation

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Therapeutic oligonucleotides have emerged as a powerful drug modality with the potential to treat a wide range of diseases; however, the rising number of therapies poses a manufacturing challenge. Existing synthetic methods use stepwise extension of sequences immobilized on solid supports and are limited by their scalability and sustainability. We report a biocatalytic approach to efficiently produce oligonucleotides in a single operation where polymerases and endonucleases work in synergy to amplify complementary sequences embedded within catalytic self-priming templates. This approach uses unprotected building blocks and aqueous conditions. We demonstrate the versatility of this methodology through the synthesis of clinically relevant oligonucleotide sequences containing diverse modifications.

Nucleic acid therapeutics (NATs) are short oligonucleotide sequences [typically ~20 nucleotides (nt)] that selectively bind to target mRNAs to modulate the production of disease-related proteins. Drug specificity is defined by the base sequence, and chemical modifications to the phosphate backbone, ribose sugar, and nucleobases confer improved efficacy, metabolic stability, and bioavailability (Fig. 1A) (*1*, *2*). Until recently, marketed NATs were limited to the treatment of rare diseases (*3*, *4*). However, the recent approval of inclisiran as a therapy for atherosclerotic cardiovascular disease, which affects ~30 million people in the US alone, marks a transition in the field from low-volume drugs to oligonucleotide therapies for the treatment of population-based disorders (*5*). This emergence of NATs for common diseases creates a substantial manufacturing challenge because existing methods of chemical synthesis are not suitable for large-scale applications.

Traditional approaches to oligonucleotide synthesis rely on solid-phase phosphoramidite chemistry, which involves iterative coupling, capping, oxidation, and deprotection steps for each nucleotide addition (*6*). Although powerful, this approach suffers from fundamental limitations that make it unsuitable for large-scale manufacturing. The use of solid supports limits production to <10-kg batches (*7*). An excess of densely protected monomers (Fig. 1B) is needed to ensure efficient coupling, which compromises atom efficiency and leads to the formation of by-products that must be separated by washing steps. Moreover, final products require chromatographic purification, which necessitates the use of prohibitively large volumes of acetonitrile (1000 kg per kilogram of oligonucleotide) and contributes to large process mass intensity (PMI) ratios (~4000 kg waste per kilogram of product for a 20-nt sequence) (*8*, *9*). Owing to the limitations of existing synthetic methods, currently marketed therapies are produced in <50% yield with modest purities (~90%) and phosphorothioate (PS)–modified sequences are produced as complex mixtures of stereoisomers. Given these challenges, several elegant approaches to oligonucleotide production have been developed, including the use of nucleoside 3′-oxazaphospholidine derivatives (*10*), phosphorus(V) reagents (*11*–*13*), chiral phosphoric acid catalysts (*14*), and enzymatic approaches that use terminal deoxyribonucleotidyl transferases (*15*–*19*). Although these strategies offer improvements in stereocontrol, step economy, and/or reduced solvent consumption, they all share the same fundamental approach of stepwise chain extension by sequential coupling and deprotection steps. Alternative strategies to construct oligonucleotides in a convergent fashion by ligating smaller fragments have been developed by Biogen, Almac, GSK, and Ajinomoto (*20*–*24*). These methods have great potential for improving the speed of oligonucleotide synthesis and generating products with improved purity, but they currently rely

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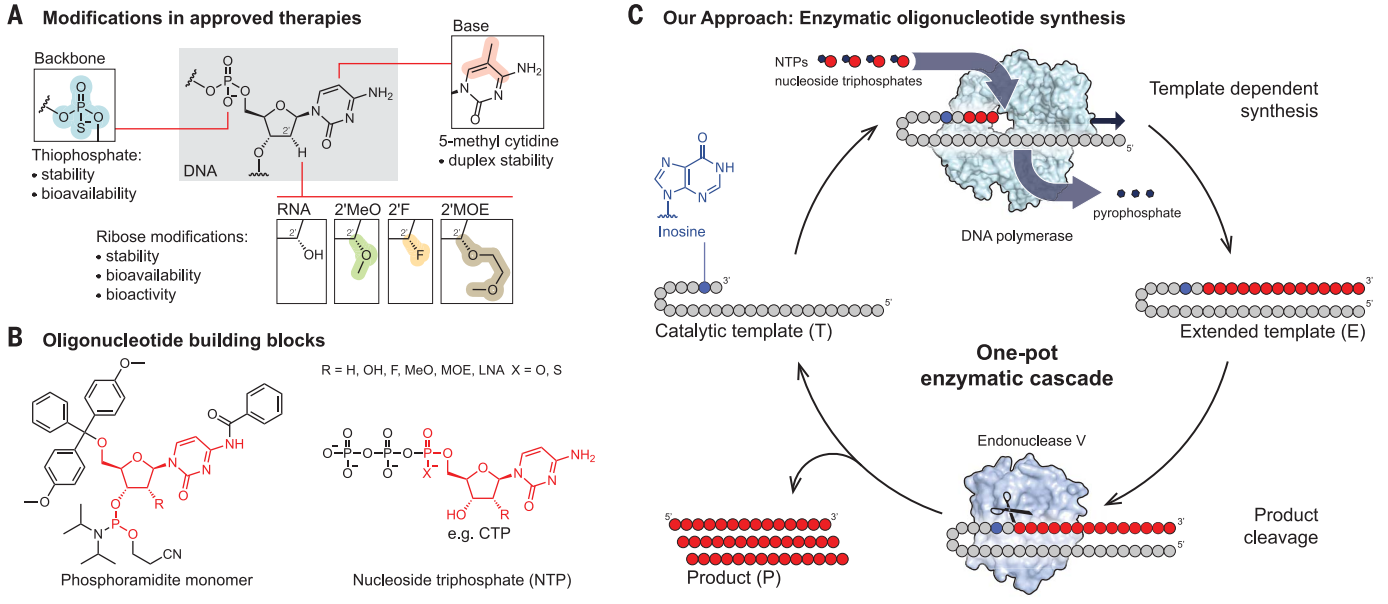


Fig. 1. Nucleic acid therapeutics (NATs). (**A**) Pharmaceutically relevant oligonucleotide modifications and their biological effects. (**B**) Phosphoramidite and nucleoside triphosphate building blocks used in chemical and enzymatic synthesis, respectively. (**C**) This work: a one-pot enzymatic cascade for oligonucleotide synthesis.

on fragments produced by using phosphoramidite chemistry. Here, we report a biocatalytic platform to produce modified oligonucleotides from unprotected nucleoside triphosphate (NTP) building blocks in a single operation. The process operates under aqueous conditions and does not require solid supports or large volumes of acetonitrile, thus addressing scalability and sustainability challenges associated with existing methods.

An enzyme cascade for scalable oligonucleotide synthesis

Our strategy uses a DNA polymerase and NTP building blocks to extend a catalytic self-priming hairpin template (Fig. 1C). An endonuclease V (EndoV) then selectively cleaves a single strand of the resulting duplex DNA downstream of an inosine base built into the hairpin sequence, releasing the product and regenerating the template. Given that the polymerase is larger than the extended DNA, the endonuclease is unable to bind to the template until the polymerase is released, thus avoiding premature cleavage of partially amplified sequences. The process is designed to operate under isothermal conditions above the product melting temperature (T_m) to facilitate scale-up and to ensure effective dissociation of the cleaved product from the template. In this way, repeated cycles of polymerase extension and endonuclease cleavage result in accumulation of the desired oligonucleotide product. Our approach has some similarities to alternative DNA amplification methods, such as those used in biosensing, bioimaging, and genome sequencing (*25*, *26*), but is specifically tailored toward the large-scale production of short, modified oligonucleotides typical of those used as therapeutics. Unlike alternative methods, our system does not require stoichiometric primers (*27*), delivers unscarred products, and does not generate oligonucleotide by-products that complicate purification and compromise process efficiency (*26*, *28*).

To evaluate the potential of the proposed cascade, we first assessed the extension and cleavage steps in isolation. The B-family polymerase from *Thermococcus kodakarensis* KOD1 (KOD) efficiently extended a self-priming template (T1) with deoxynucleotide triphosphates (dNTPs) to afford the double-stranded product (E1) in quantitative conversion (fig. S1A). The cleavage reaction performed with an endonuclease from *Thermotoga maritima* (*Tm*EndoV) also proceeded to completion, providing the 18-nt product (P1) and template (T1) (fig. S1B). With the individual steps working as intended, we performed a one-pot cascade using KOD polymerase, *Tm*EndoV endonuclease, T1 (20 μM), and dNTPs (250 μM each). Encouragingly, under these conditions the target sequence (28 μM) was the sole product, which correlates with 78% consumption of the limiting 3′-deoxythymidine 5′-triphosphate (dTTP) reagent (fig. S1C).

To intensify the process, we next investigated the effect of increased substrate (dNTP) loading on the individual steps. Although KOD polymerase was able to efficiently extend T1 in the presence of 30 mM dNTPs (fig. S2A), *Tm*EndoV endonuclease–mediated hydrolysis was inhibited at dNTP concentrations >2.0 mM (fig. S2B). To address this limitation, we evaluated a panel of thermophilic *Tm*EndoV homologs and identified an endonuclease from *Thermotoga neapolitana* (*Tn*EndoV) that operates effectively at high dNTP concentrations, catalyzing complete hydrolysis of E1 (20 μM) in the presence of 20 mM dNTPs (fig. S2C). After optimization of buffer conditions, hairpin sequences, and reaction temperature (fig. S3), 330 cycles of template extension and product cleavage were achieved in analytical-scale reactions (20 μl) under isothermal conditions, affording 0.33 mM P1 as the sole product (fig. S4). Next, we performed a reaction on a 5-ml scale, which provided 0.44 mM P1 (equivalent to 2.5 g/l), consuming 69% of the available dNTP starting materials. After removal of the proteins and membrane filtration, we isolated P1 in 88% purity (11.5 mg, 2.1 μmol) without chromatographic purification (fig. S5). Although in this instance we did not attempt to separate the catalytic template (<1 mol %) from P1, there are numerous opportunities for template removal and/or recycling (see below) (*29*–*31*). Finally, to demonstrate that our processes can be readily scaled without compromising efficiency, we performed a biotransformation on a 50-ml scale, delivering 123 mg of P1 (22.2 μmol) with 89% purity after protein precipitation and membrane filtration (Fig. 2, A to C, and fig. S6).

Using the metrics of our 50-ml scale reaction, we performed a PMI analysis to evaluate the material efficiency of the process (Fig. 2D). Even at this early stage of development, the enzymatic approach compares favorably to traditional phosphoramidite chemistry [530 kg compared to ~4000 kg of raw material per kg

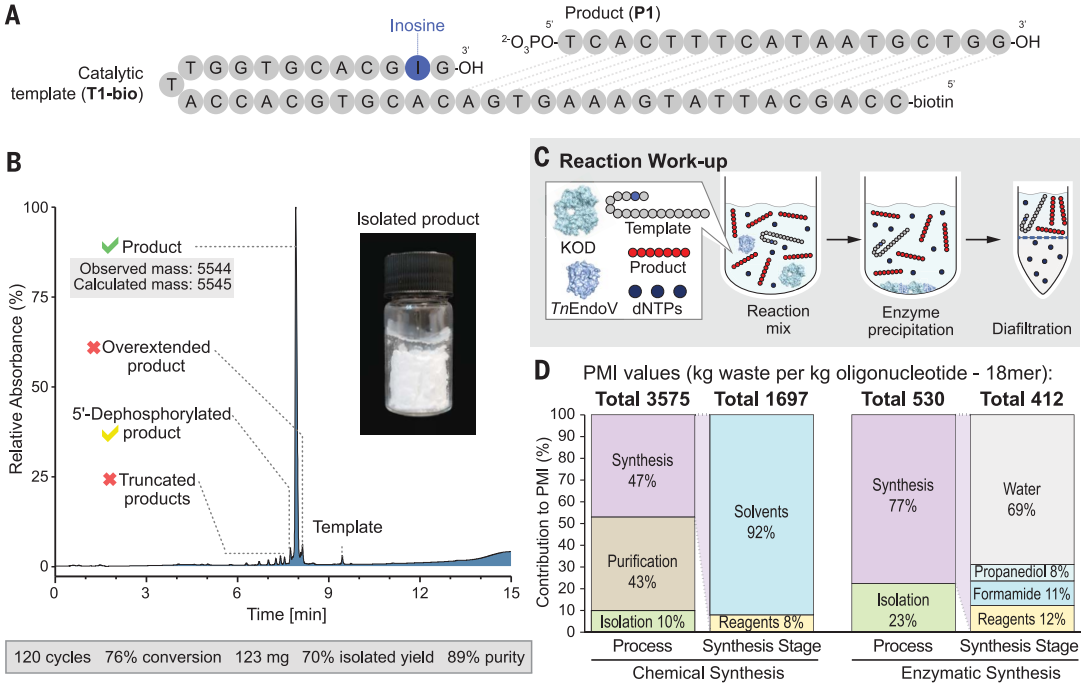


Fig. 2. 123-mg scale synthesis of oligonucleotide P1. (**A**) P1 nucleotide sequence. (**B**) LC-MS trace and image of the isolated product. Minor impurities include overextended product and truncated sequences. (**C**) Scheme showing the product isolation steps. (**D**) PMI analysis (kilogram of waste per kilogram of oligonucleotide product) for the production of an 18-nt sequence by using traditional phosphoramidite chemistry (*8*) versus our enzymatic process. The bar chart shows how each stage of the manufacturing process (synthesis, purification, and isolation) contributes to the overall PMI and provides a breakdown of the raw materials used in the synthesis stage.

of product (*8*)]. This improvement in PMI primarily comes from alleviating the need for multiple washing steps and chromatographic purification. The template that we used in our enzymatic approach is currently produced by using phosphoramidite chemistry, but it is only required in catalytic quantities (<1%) and thus has substantially reduced economic and environmental impacts compared with producing large quantities of DNA products chemically. Moreover, there is the potential to generate catalytic templates by using enzymatic methods in the future, which would provide further improvements in green chemistry metrics.

Biocatalytic synthesis of modified sequences

To manufacture therapeutic oligonucleotides, we require polymerases and endonucleases with activity toward modified nucleic acids. To this end, eight thermophilic polymerases were selected for evaluation and used to extend a hairpin template encoding the sequence TCAGTCAG (T2) with a mixture of three unmodified dNTPs and one modified NTP (Fig. 3A). Polymerases that were able to transcribe the template and incorporate two copies of a modified NTP resulted in full-length extension. We also performed control reactions in the absence of modified NTP. From this preliminary screen, we identified several polymerases with good activity toward NTP substrates containing S_p-PS [(S_p)-**2**]2′-fluoro (**3**), and a combination of S_p-PS and 2′-fluoro (**4**) modifications, including KOD and TfPol* polymerases, as evidenced by polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 3B and fig. S7). We observed more modest activity toward 2′-MeO-NTP substrates (**5**) with several polymerases including SFM4-6 (*32*), TfPol*, and Dpo4*, whereas KOD^{DGLNK} (*33*) and SFM4-6 displayed modest activity with locked nucleic acid (LNA)–modified substrates (**6**). We observed no activity toward R_p-stereoisomers of α-PS–modified dNTPs (R_p-2) with any of the polymerases tested. 2′-O-Methoxyethyl (2′-MOE)–modified NTPs are not commercially available and were not evaluated in this study; however, a variant of Tgo polymerase has recently been reported that has activity toward 2′-MOE-NTPs, making us optimistic that our methodology will be extendable to this class of substrate (*34*). Similarly, therapeutic oligonucleotides containing nucleobase modifications (e.g., 5′-methylated pyrimidines) should also be accessible with our technology, as polymerases have been shown to accept a wide range of base-modified NTPs (*35*–*37*).

Having identified promising polymerases, we explored sequential incorporations of modified NTPs by extending templates (T3 to T6) that encode eight copies of a single nucleobase (Fig. 3C). Consistent with the initial screen,

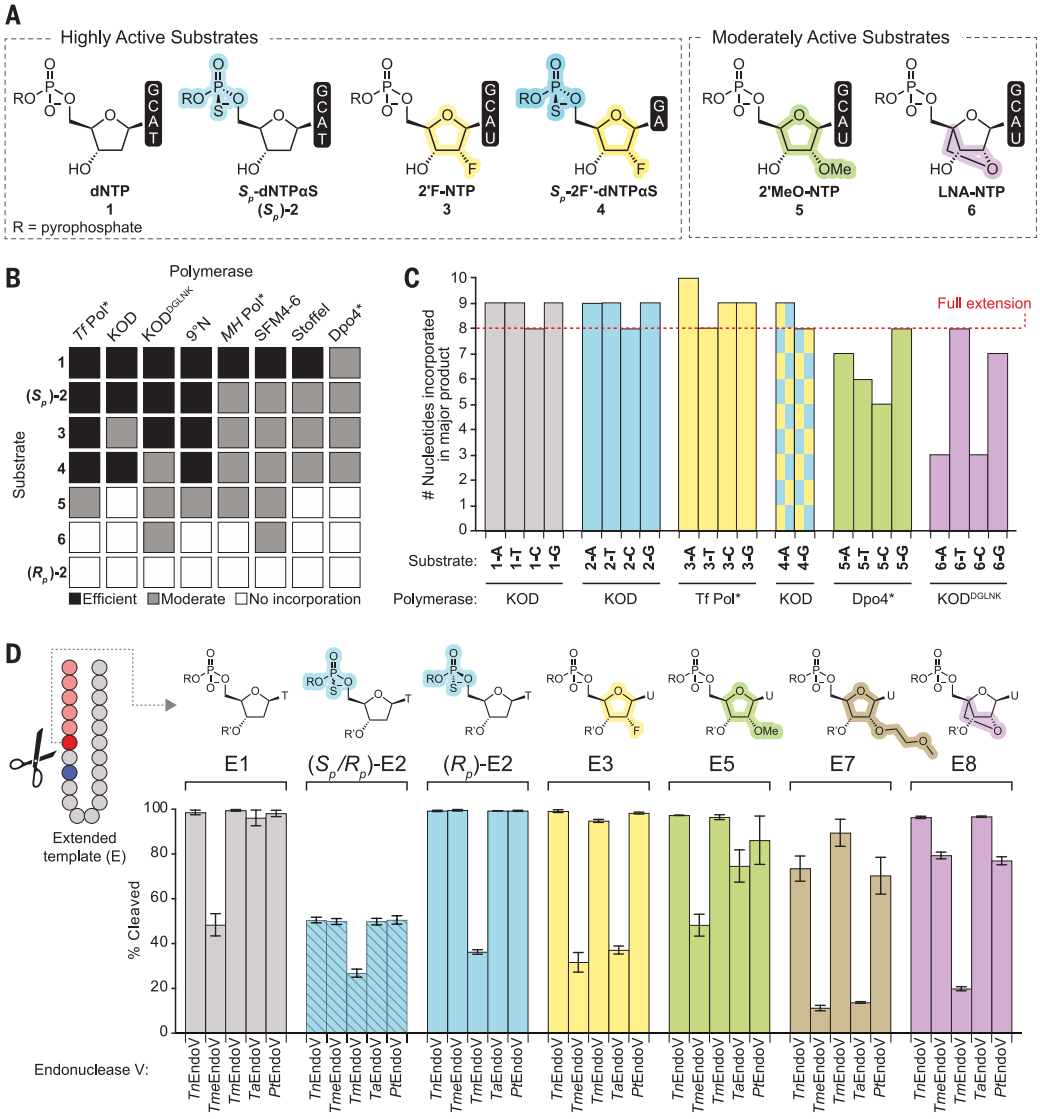
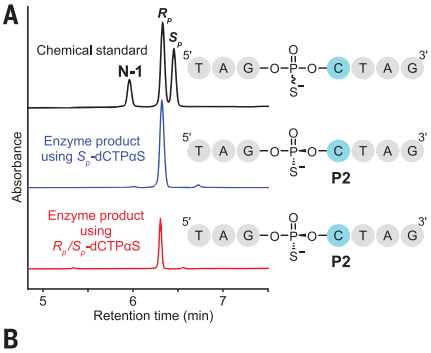


Fig. 3. Substrate profiling of polymerases and endonucleases.
(**A**) NTP substrates accepted by polymerases from the panel. (**B**) Heat map showing polymerase activity with a mixture of three unmodified dNTPs and one modified NTP. Polymerases that accept all four nucleobases of a specified NTP and yield the extended product in >50% conversion are shown in black. Polymerases that extend sequences with at least one nucleobase of a specified NTP are shown in gray. (**C**) Polymerase-catalyzed extension of templates that encode eight copies of a single nucleobase. The graph shows the number of nucleotides incorporated in the major product as determined by LC-MS analysis (fig S8). (**D**) Endonuclease-catalyzed cleavage of modified extended templates E1, E2, E5, E7, and E8 after 2 hours of incubation and E3 after 4 hours of incubation. Cleavage of an all-LNA modified 18-mer (E6) gave low conversion and was omitted from the graph (fig. S9). Extended template E8 contains a single LNA 3′ of the cleavage site and was successfully hydrolyzed. Extended template sequences are presented in table S3. Error bars represent the standard deviations of reactions performed in triplicate. Biotransformations were analyzed by PAGE, and product identity was confirmed by LC-MS analysis (figs. S9 and S10).

liquid chromatography–mass spectrometry (LC-MS) analysis shows that selected polymerases were highly active toward substrates **1** to **4**, with reactions proceeding to completion within 1 hour (fig. S8). Although single-nucleotide overextension products are often observed with these substrates, overextension is notably reduced in cascade processes in the presence of EndoV endonucleases (see below). Reactions with each of the four 2′-MeO (**5**)– and LNA (**6**)–modified NTPs gave rise to mixtures of partially extended products (fig. S8), consistent with modest polymerase activity toward these modifications. We anticipate that efficient production of these sequences can be achieved through further enzyme engineering.

We next evaluated the panel of EndoV endonucleases for activity toward extended templates containing 2′-fluoro (E3), 2′-methoxy (E5), and 2′-O-methoxyethyl (E7) ribose modifications; LNAs (E6 and E8); and PS linkages (E2) (figs. S9 and S10). Hydrolysis of E3, E5, and E7 proceeded to >90% conversion, and endonuclease *Tn*EndoV, which tolerates elevated NTP concentrations, performed consistently well across the substrate range (Fig. 3D). Although cleavage of an LNA-modified 18mer (E6) was sluggish, sequences containing a single LNA 3′ of the cleavage site (E8) were efficiently hydrolyzed. The modest activity observed with E6 likely arises from distorted secondary structure of the LNA-DNA duplex (*38*). However, therapeutic oligonucleotides rarely contain more than three sequential LNAs in their sequence. Endonuclease-catalyzed cleavage of stereorandom PS-modified DNA R_p/S_p-(E2) stalled at 50% conversion. The most likely explanation for this behavior is that the endonucleases are sensitive to the stereochemistry at the phosphorothioate center undergoing hydrolysis. To test this hypothesis, we synthesized a stereodefined extended template using KOD polymerase and S_p-dNTPαS. Several polymerases have been shown to incorporate S_p-dNTPαS into oligonucleotides with inversion of stereochemistry (*39*–*42*), meaning that the oligonucleotide product is expected to contain R_p linkages. In this case, endonuclease *Tn*EndoV–mediated cleavage proceeded to completion (Fig. 3D and fig. S11), suggesting that endonucleases are R_p-selective and can be used to cleave enzymatically synthesized products.

Next, we turned our attention to one-pot cascade reactions to produce modified sequences. First, we took advantage of polymerase specificity to produce 6-mers and 7-mers containing a single PS linkage as single diastereomers [>99% diastereomeric excess (d.e.)]. These sequences can be produced by using either pure S_p-dNTPαS or R_p/S_p-dNTPαS mixtures (Fig. 4A and fig. S12). To highlight the versatility of our approach, we successfully amplified a range of 8-nt products (P2 to P16) (Fig. 4B and fig. S13) with different base sequences and a variety of 2′F, 2′-MeO, R_p-PS, R_p-2′F-PS, and LNA modifications. In favorable cases, we achieved up to 238 cycles of template extension and product cleavage (238 moles of product per mole of template), which consumed 76% of the NTP starting materials. The NTP concentrations that we used in these reactions are lower than those used earlier with unmodified dNTPs owing to the lower concentrations of modified NTPs currently available from commercial suppliers.



	Products	Cycles	% conv.	Pol.	[NTPs] (mM)
P3	TCAGTCAG	190	38	KOD	16
P4	TCAGTCAG	42	34	KOD	4
P5	UCAGUCAG	49	39	*Tf*Pol*	4
P6	CAUGCAUG	37	30	KOD^{DGLNK}	4
P7	CCUAGGUA	56	45	KOD^{DGLNK}	4
P8	AUGCAUGC	30	24	Dpo4*	4
P9	TCAGTCAG	238	76	KOD	10
P10	TCAGTCAG	235	75	KOD	10
P11	UCAGUCAG	104	33	*Tf*Pol*	10
P12	TCAGTCAG	138	44	*Tf*Pol*	10
P13	TCAGTCAG	134	43	*Tf*Pol*	10
P14	TCAGTCAG	43	34	KOD	4
P15	UCAGUCAG	76	61	*Tf*Pol*	4
P16	UCAGUCAG	22	18	*Tf*Pol*	4
P17	TCAGTCAG	85	68	*Tf*Pol*	4

Fig. 4. Biocatalytic synthesis of oligonucleotide sequences. (**A**) Comparison of stereopure oligonucleotides produced by our enzyme cascade and either S_p-CTPαS or a mix of R_p/S_p-CTPαS to chemically synthesized standards. Cascade reactions were performed with KOD and *Tn*EndoV. (**B**) Oligonucleotides with varying sequence and chemical modifications were produced in one-pot biotransformations with the most suitable polymerase (Pol.) and *Tn*EndoV. For comparison, under optimized conditions, the unmodified product P1 was produced following 330 cycles (37% conv.). The percentage conversion (% conv.) describes the conversion of NTP starting material to product. Cycles refers to the number of moles of product formed per mole of template. Specific reaction conditions are presented in table S1.

We anticipate that substantially improved turnovers will be achieved by increasing NTP concentrations and through specific optimization of reaction conditions for the target sequence.

Production of clinically relevant oligonucleotides

To further showcase the utility of our methodology, we produced a range of clinically relevant oligonucleotide sequences (Fig. 5A and fig. S14). First, we synthesized prexigebersen, an unmodified liposome-incorporated oligonucleotide currently under evaluation in phase 2 clinical trials for the treatment of acute myeloid leukemia. Prexigebersen was produced in 0.44 mM (equivalent to 2.4 g/l), which is comparable to the product titers achieved in earlier experiments with P1, demonstrating the transferability of the technology to different sequences. We also produced selected fragments of the Food and Drug Administration–approved aptamer pegaptanib (2′-MeO and 2′-F modifications) and gapmers mipomersen (PS modifications) and inotersen (PS modifications). These fragments could potentially be integrated into convergent ligation-based oligonucleotide assembly strategies (*20*–*24*).

Finally, we synthesized the PS-modified oligonucleotide therapies fomivirsen, alicaforsen, trabedersen, and aganirsen as single stereoisomers. Subsequent production of fomivirsen, a 21-nt sequence that was the first approved antisense oligonucleotide therapy (Fig. 5B), on a preparative scale (0.75 ml) demonstrates how reaction intensification can deliver improved conversions. After concentration of commercial S_p-dNTPαS stocks and optimization of reaction parameters, the target sequence was produced (0.26 mM, equivalent to 1.7 g/l, 195 nmol) after 65 cycles of template extension and product cleavage, which correlates to consumption of 90% of the available S_p-dNTPαS starting materials. We achieved product isolation after protein precipitation, deoxyribonuclease (DNase)–catalyzed template degradation, and filtration steps to provide fomivirsen (0.73 mg, 108 nmol) as an all-R_p single stereoisomer in 87% purity (fig. S15). We compared the biophysical and biochemical properties of enzymatically synthesized fomivirsen to stereorandom material produced by chemical synthesis (*10*, *43*). Thermal denaturation studies showed that the all-R_p stereoisomer has higher binding affinity to the target RNA compared with the stereorandom mixture (T_m of 69° ± 0.8°C and 64° ± 0.3°C, respectively). Assays with ribonuclease H (RNase H) nonspecific endonucleases show that *Escherichia coli* RNase H activity is unaffected by oligonucleotide stereochemistry, whereas the human enzyme is about twofold more active with stereorandom fomivirsen compared with the all-R_p stereoisomer (fig. S16). Investigations into fomivirsen stability showed that

phosphodiesterase from *Crotalus adamanteus* preferentially degraded the all-R_p oligonucleotide, whereas stereorandom fomivirsen was more rapidly degraded by S1 nuclease from *Aspergillus oryzae* (fig. S17). A third endonuclease from *Staphylococcus aureus* showed comparable activity with either sample, showing that the rate of oligonucleotide degradation is strongly dependent on the specific nuclease.

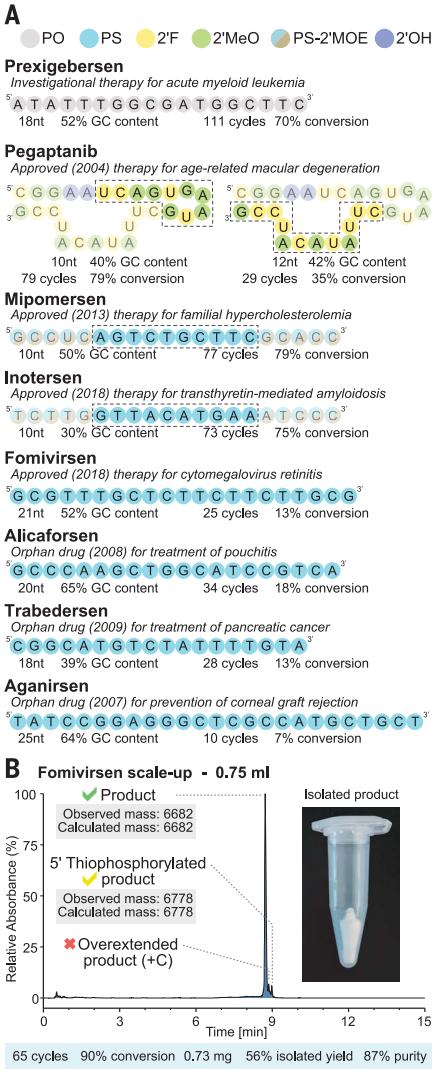


Fig. 5. Enzymatic synthesis of clinically relevant oligonucleotide sequences. (**A**) Oligonucleotide sequences were produced on an analytical scale. Mipomersen and inotersen contain cytosine and not 5′-methylcytosine because the required NTP monomers are not commercially available. The number of nucleotides (nt) in the sequences and percent GC content are provided. The percentage conversion describes the conversion of NTP starting material to product. Cycles refers to the number of moles of product formed per mole of template. The crude HPLC traces are shown in figure S14. Specific reaction conditions are presented in table S2. (**B**) Preparative-scale synthesis of fomivirsen as a single stereoisomer. LC-MS trace and image of the isolated product. Minor impurities include overextended product and 5′-thiophosphorylated product.

Conclusions

This study offers a distinct paradigm in therapeutic oligonucleotide manufacturing: Iterative rounds of chain extension, oxidation, capping, and deprotection are replaced by a single biotransformation. Our approach provides access to a diverse array of oligonucleotide sequences and modifications, which will be further expanded through the discovery and/or engineering of biocatalysts with extended substrate scope (*32*, *44*). For example, the development of polymerases with activity toward R_p-dNTPαS will enable production of libraries of stereochemically defined PS-modified oligonucleotides for biological evaluation, ultimately leading to therapeutics with improved safety and efficacy. Previous studies have shown that the stereopreference of polymerases and cyclic guanosine-adenosine synthase (cGAS) can be altered by exchanging the Mg^{2+} cofactor and protein engineering (*45*, *46*). Similarly, directed evolution will generate more robust biocatalysts specifically engineered to meet target process parameters. Translation of our approach into oligonucleotide manufacturing processes will also be facilitated by the development of scalable and low-cost routes to the required NTP building blocks. These NTPs will likely be accessed from the corresponding nucleoside, a common intermediate in phosphoramidite production, by using enzymatic phosphorylation methods that have been previously demonstrated at scale (*46*). Thus, the biocatalytic platform described here has the potential to transform the way that nucleic acid therapeutics are manufactured, addressing imminent scalability and sustainability challenges associated with the production of this emerging class of therapeutics.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S17
Tables S1 to S3
Protein Sequences
References (*47*–*49*)
MDAR Reproducibility Checklist

View/request a protocol for this paper from *Bio-protocol*.

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