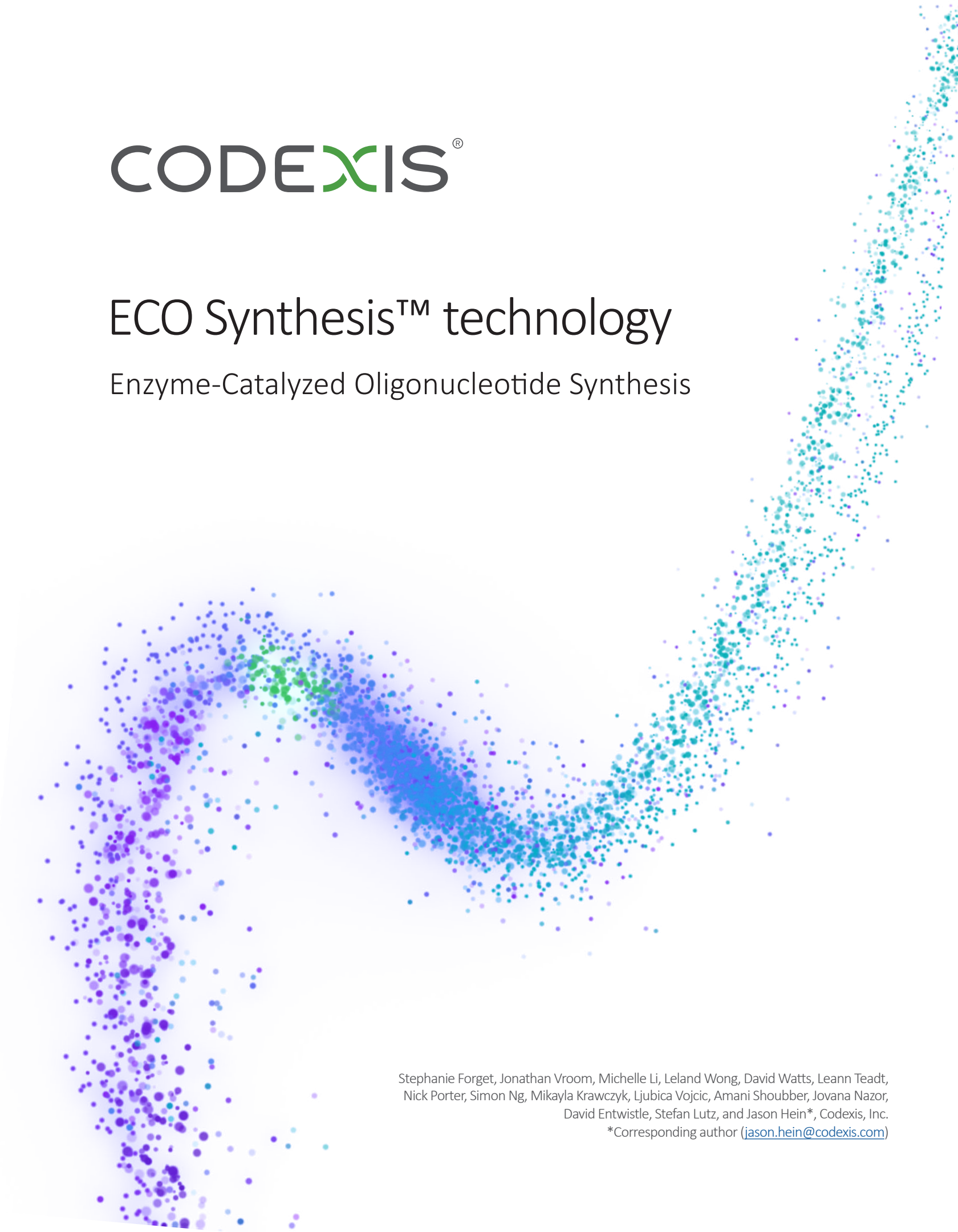
CODEXIS®

ECO Synthesis™ technology

Enzyme-Catalyzed Oligonucleotide Synthesis



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Codexis ECO Synthesis™ technology

A new paradigm in large-scale synthesis of nucleic acid therapeutics

Therapeutic oligonucleotides are a rapidly growing class of drugs comprising of short single or double-stranded fragments of DNA or RNA. Recent advancements in chemical modifications for molecule stability and delivery systems for therapeutic efficacy have opened access to previously "undruggable" targets inaccessible with small molecule drugs or biologics[1].

Current manufacturing methods rely on multi-step iterative phosphoramidite chemical synthesis of immobilized oligonucleotides, pioneered by Marvin Caruthers and colleagues in 1981[2,3]. These methods have been optimized over decades of improvements, driving conversion efficiency with large excesses of reagents. The maturity of technology also means that now only incremental gains are possible in terms of oligo lengths, purity, and costs. Additionally, the existing methods rely on environmentally harmful solvents, such as acetonitrile and pyridine, and chemical reagents such as iodine, lutidine and dichloroacetic acid amongst others. The multiple steps per iteration combined with excess reagents and dilute conditions due to the conspire to generate thousands of kilograms of chemical waste per kilogram API[4]. With over 450 therapeutic candidates in clinical pipelines and indications targeting larger patient populations, the scalable, sustainable, and economical manufacturing of therapeutic oligonucleotides requires a paradigm shift away from current methods that are wasteful of resources and of low throughput per batch.

Codexis ECO Synthesis™ technology (Enzyme-Catalyzed Oligonucleotide Synthesis) is a proprietary new synthesis platform being developed for manufacturing modified-RNA oligonucleotide therapeutics at large-scale. The core iterative oligo extension cycle leverages an immobilized proprietary terminal deoxynucleotidyl transferase (TdT) enzyme engineered to incorporate 3'-blocked common 2'-modified nucleotides, including 2'-F, 2'-OMe, and backbone-modified alpha-phosphorothioate nucleotides. Deblocking is achieved with a phosphatase to close the cyclic process which is envisioned to enable synthesis of multi-kg quantities of modified-RNA in free solution **(Figure 1)**. Running the process with the substrates and products all in aqueous solution brings significant benefits in scalability, throughput, online analysis and improvements in sustainability.

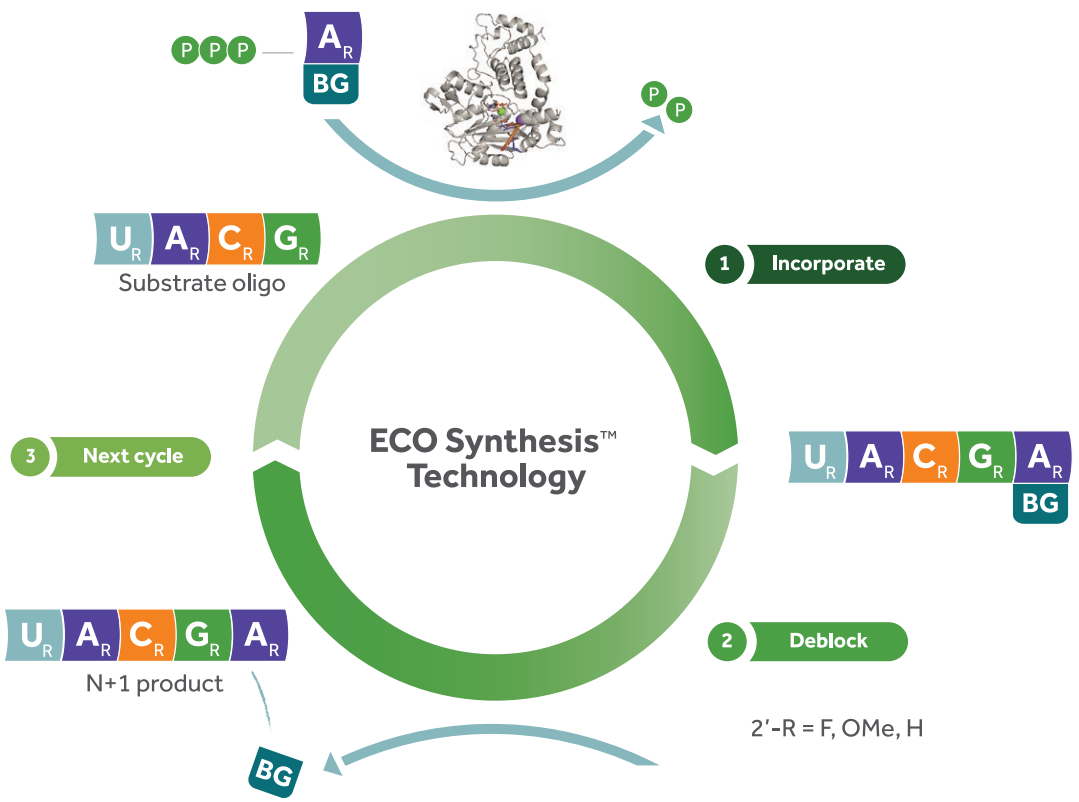

Figure 1: ECO Synthesis™ technology - iterative oligo extension as its core process.
Substrate oligos are extended with 3'-blocked NTP in aqueous conditions with an engineered TdT enzyme. After incorporation, the elongated oligo is de-blocked with an engineered phosphatase to yield N+1 product, ready to proceed to the next cycle of nucleotide incorporation.

Leveraging the proprietary CodeEvolver® platform for transforming enzymes into biocatalysts

Enzymes, by nature's design, are specific for their native substrates and activities. This specificity is critical for biological processes to maintain tightly controlled physiological homeostasis. However, this specificity and often-times reactivity at physiological reaction conditions, requires engineering to achieve performance characteristics required for industrial biology applications. The CodeEvolver® technology platform, Codexis' core enzyme engineering technology, uniquely empowers our scientists to overcome nature's limitations, enabling the substantial enzyme modifications required to meet at-scale process needs of the ECO Synthesis™ technology.

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1 Incorporate

A highly engineered TdT is the key enabling enzyme of ECO Synthesis™ technology by catalyzing the template-independent incorporation of 3'-blocked 2'-modified NTPs onto an oligonucleotide chain. The activity, stability, and promiscuity required of the TdT to facilitate multi-kg scale synthesis of highly pure modified RNA requires changes to almost every facet of the enzyme (Figure 2). Striving for high versatility and synthesis performance, the CodeEvolver® platform allows for optimization of multiple traits in parallel.

Trait	Starting Activity	Targeted Activity
INCORPORATION OF 2'-MOD NTPS	<5%	>99%
Incorporation of αS-2'-mod NTPs	<5%	>99%
Oligo sequence bias	Significant	Minimal to none
Catalytic activity	High	High
Substrate concentrations	Micromolar	Millimolar
Thermostability	<40°C	>60°C

Figure 2: *Table of desired TdT traits*

To meet the targeted activity profile, ongoing TdT engineering is maximizing promiscuity to incorporate required modified nucleotides while eliminating bias with respect to the growing oligonucleotide chain. Figure 3 demonstrates the progression and application of the CodeEvolver® platform to tune the enzyme's activity profile and promiscuity over the course of multiple rounds of evolution.

3'-Terminus sequence	NTP	Starting TdT	Iterative rounds of evolution						
mAmCmU	fATP-3P	0	0	0	0	1	54	91	94
	fUTP-3P	0	0	0	0	1	56	93	94
	mCTP-3P	0	0	0	0	1	54	91	89
	mGTP-3P	0	0	0	0	1	24	87	78
	mATP-3P	0	0	0	0	1	39	88	66
	*mGTP-3P	0	0	0	0	0	0	2	55
	*mUTP-3P	0	0	0	0	0	0	19	24
mGmAmC	fUTP-3P	0	0	0	0	2	33	74	92
	mATP-3P	0	0	0	0	1	16	60	90
	fATP-3P	0	0	0	1	2	34	77	88
	mCTP-3P	0	0	0	0	0	13	66	86
	*mUTP-3P	0	0	0	0	0	0	11	79
	*mGTP-3P	0	0	0	0	0	0	3	64
	mGTP-3P	0	0	0	0	0	0	2	55
AT*mG	mATP-3P	4	1	4	49	75	82	47	56
AmU*mG		0	0	0	12	46	77	75	41
mAmU*mG		0	0	0	0	5	69	82	68
mAmUfG		0	0	0	0	0	2	1	66
mUmGmA	mATP-3P	0	0	0	1	2	38	82	86
mAfUCmC		0	0	0	0	4	58	88	86
mAmG(MOE)C		0	0	0	0	0	4	14	84
mC*mG*mA		0	0	0	0	2	39	75	75
mCmUmG		0	0	0	0	4	82	86	72
mAmUmC		0	0	0	0	2	57	84	63
mAmUfU		0	0	0	0	0	0	0	59
*mA*mA*mC		0	0	0	0	1	30	54	57
mAmUfC		0	0	0	0	0	1	1	51
mCmGmA	fATP-3P	0	0	0	1	4	82	93	92
*fAfGmA		4	2	4	19	50	79	85	85
mC*mG*mA		0	0	0	1	3	47	81	82
*fA*fAfG		0	0	0	0	0	44	56	70
fCfGfA		0	0	0		26	50	55	65
mU*fA*fA		0	0	0	0	3	11	17	34
fGmAfU		6	5	6	3	12	42	52	30
fC*fG*fA		0	0	0	0	4	14	30	14

Figure 3:
Percentage incorporation efficiency of N+1 additions over multiple rounds of enzyme evolution.

***Abbreviations**:*
***A, U, G, C** = ribonucleosides,*
***m** = 2'-methoxy,*
***f** = 2'fluoro,*
***3P** = 3'-phosphate,*
***** = phosphorothioate linkage*

2 Deblock

Efficient enzymatic removal of the 3'-blocking group after the elongation step to yield N+1 oligo product is achieved by phosphatase-catalyzed hydrolysis. Using the CodeEvolver® platform, the engineered phosphatase is being optimized for high productivity and promiscuity to maximize overall extension yield for each round of the oligonucleotide synthesis.

3 Next cycle

Seamless integration of our enzymatic incorporation and deblocking steps are the cornerstone of the success of the ECO Synthesis™ technology. Process development is driving the iterative controlled addition of modified bases to produce full-length oligonucleotides, ensuring that the highly engineered TdT and phosphatase enzymes work as a system for efficient oligonucleotide synthesis. To demonstrate proof of concept for multiple cycles of oligonucleotide extension, still-in-evolution variants of these two enzymes were successfully deployed to catalyze four consecutive incorporation and deblocking steps of an oligo-2'-modified-ribonucleotide primer (Figure 4).

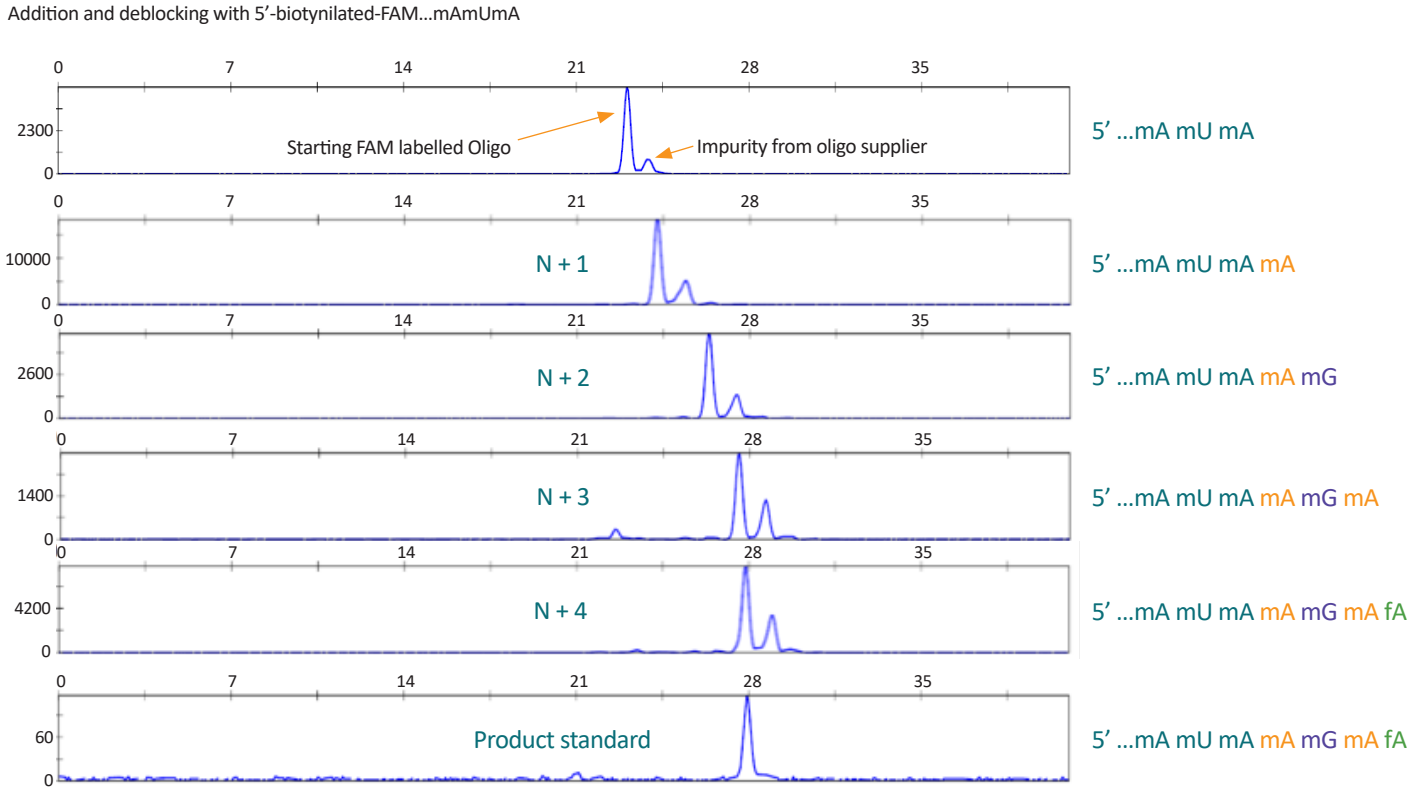

Figure 4: *Capillary electrophoresis traces for four consecutive nucleotide incorporation events.*

ECO Synthesis™ technology

Supply

Enzymatic production of nucleotide reagents

The ECO Synthesis™ technology requires natural and 2'-modified NTPs with a 3'-blocking group as monomers. While 3'-phosphate nucleotides are accessible via traditionally chemical synthesis, their preparation via enzymatic routes from the corresponding nucleoside precursors should be more efficient and selective, due to the elimination of temporary protection groups and benefiting from overall milder, more environmentally benign reaction conditions.

Codexis is leveraging its 15+ years of biocatalysis experience in small-molecule API manufacturing for the pharmaceutical industry to develop a suite of promiscuous enzymes for the synthesis of key nucleotide precursors **(Figure 5)**. The 3'-blocked NTPs that will be produced by scalable multi-enzyme cascades, are the building blocks for our iterative oligo extension process.

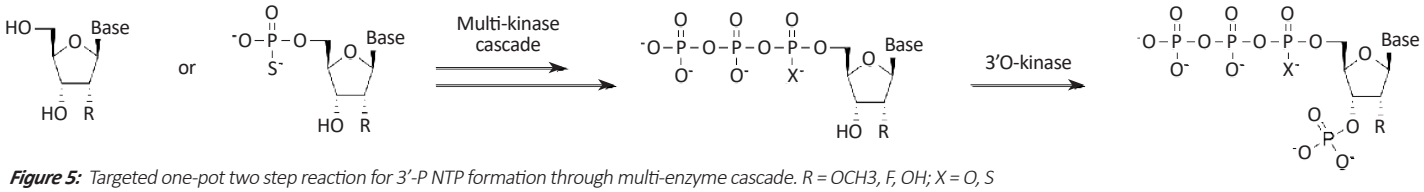

Figure 5: *Targeted one-pot two step reaction for 3'-P NTP formation through multi-enzyme cascade. R = OCH3, F, OH; X = O, S*

To date, significant progress has been made towards the enzymatic synthesis of NTPs from their corresponding nucleosides, as well as the addition of the 3'-blocking group. Together, these successful biocatalytic transformations are driving the end-goal of achieving scalable, economical, and sustainable supply of monomers.

ECO Synthesis™ technology

Impact

Enzymes as transformational solutions for oligonucleotide synthesis

Codexis ECO Synthesis™ technology is envisioned to offer a more sustainable and economically competitive manufacturing process with greater throughput to meet the multi-kg annual demand of RNAi therapeutics. By leveraging the power of immobilized enzymes to operate efficiently and effectively under environmentally benign conditions, the platform offers novel solutions to questions of throughput and sustainability, two of the major drawbacks of the incumbent phosphoramidite chemistry.

It is important the final at-scale process conditions achieve meaningful impacts over existing chemical synthesis methods. The vision for ECO Synthesis™ technology is to achieve improvements in sustainability, scalability, and economics. By using tools, such as the American Chemical Society Green Chemistry Institute Calculator (to compare Process Mass Intensity (PMI) and Life Cycle Analysis (LCA) estimates against phosphoramidite chemical synthesis, the envisioned at-scale processes are poised to substantially improve sustainability metrics for manufacturing by delivering >95% reduction in both CO2 production and water use (**Figure 6**).

Standard scale

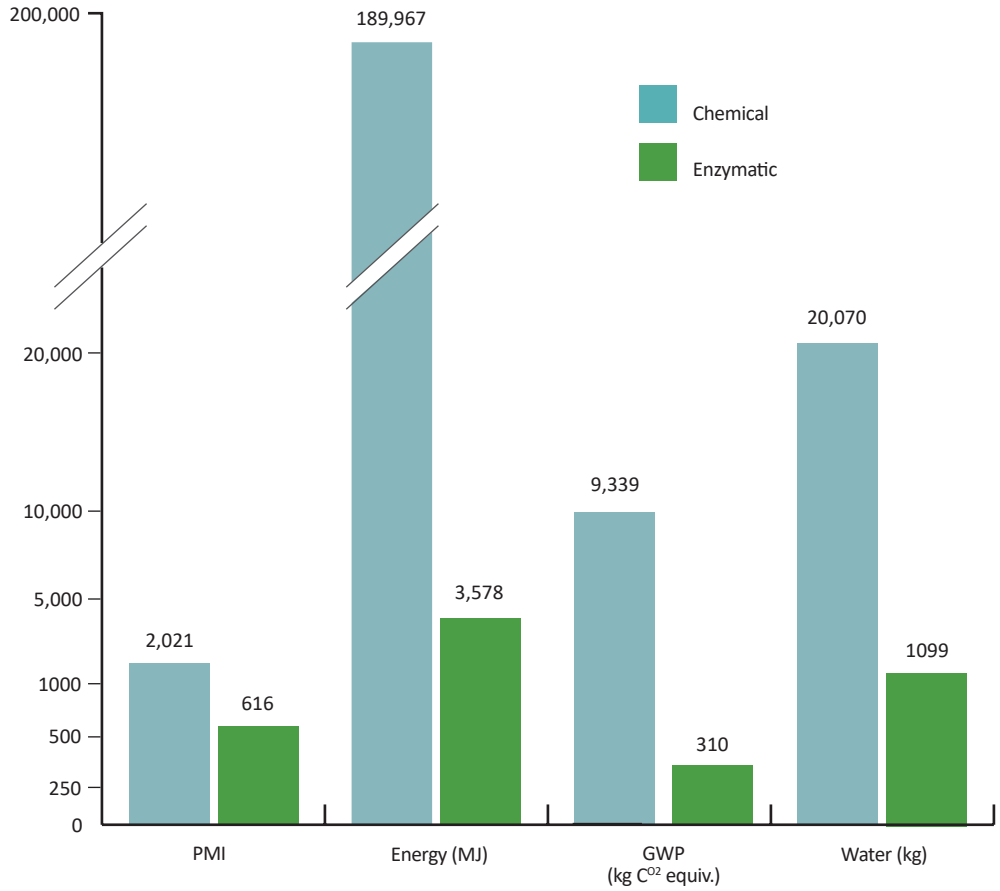

Figure 6: *PMI and selected estimated sustainability metrics from the ACS GCI PT PMI-LCA calculator*

Enzymatic methods for oligonucleotide synthesis have been well researched and reviewed as a means to address the sustainability and scalability challenges of large-scale oligonucleotide manufacturing. The existing enzyme-based methods have, to date, been limiting in at least one dimension – either scalability, technical ability to incorporate modifications, sustainability, or economics. Codexis' CodeEvolver™ platform and vertically integrated enzyme manufacturing, uniquely position Codexis ECO Synthesis™ technology to address every dimension required for large-scale therapeutic oligonucleotide manufacturing.

References

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