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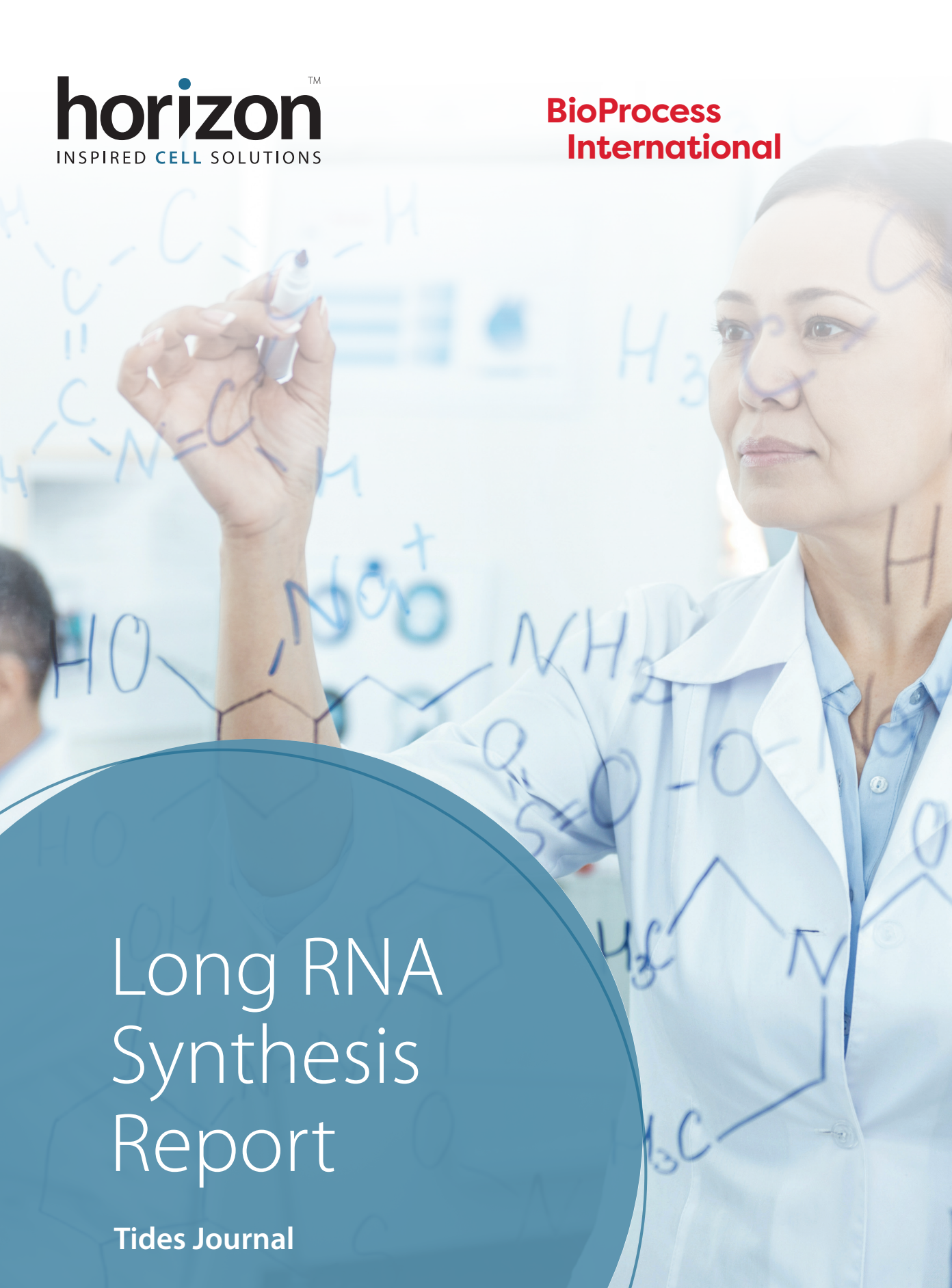


Long RNA Synthesis Report

Tides Journal

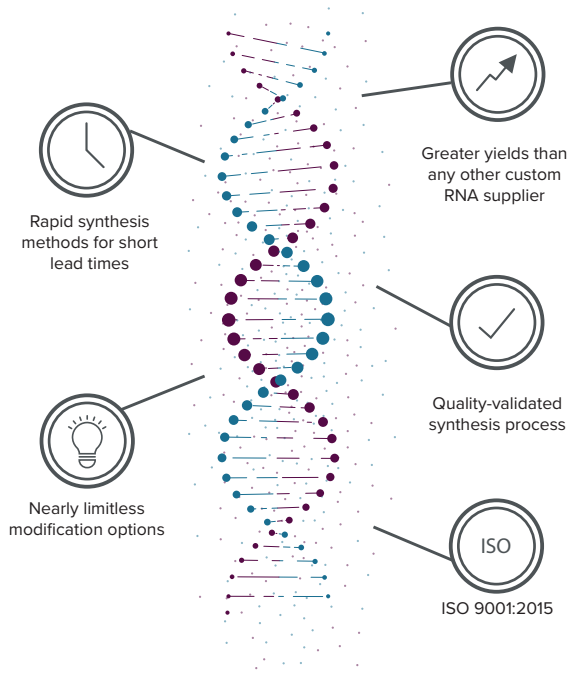
Solutions for Long and Challenging Synthetic Oligonucleotides

Amanda Haas

Long-RNA oligonucleotides have grown increasingly important for genomic pathway research and drug discovery. But long, single-stranded RNAs can be difficult to synthesize because of their sequence composition, secondary structure, and applied modifications. Here I explore how Horizon Discovery is solving the challenge of synthesizing long oligonucleotides to address unmet customer needs.

The Dharmacon custom oligonucleotide synthesis laboratory has spent over 20 years perfecting the process of reliably producing and purifying long, unique, and difficult-to-synthesize RNA oligonucleotides. The company's chemists routinely synthesize RNA up to 120 bases long, working side by side with researchers to determine the proper scale, sequence feasibility, and purification requirements necessary for reliable, high-yield production.

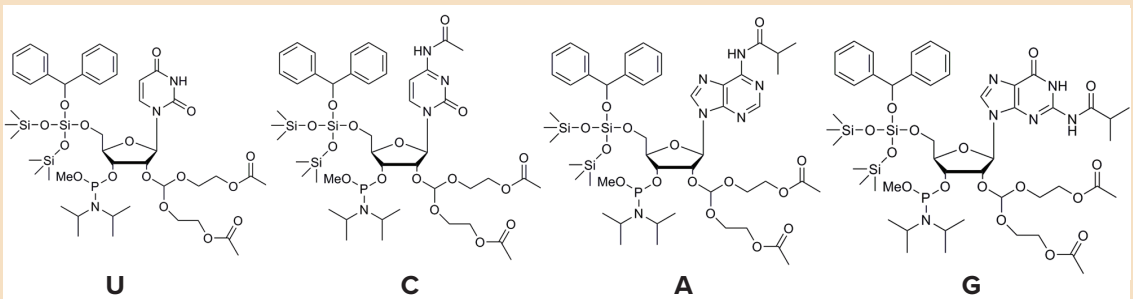
Since acquiring Dharmacon in 2017, Horizon has leveraged that expertise in long-RNA manufacturing to develop a diverse offering of unique research reagents and innovative custom synthesis options.



2′ ACE Oligo Synthesis

Horizon's synthetic oligonucleotide manufacturing is based on Dharmacon's proprietary 5′-silyl-2′–acetoxyethylorthoester (2′-ACE) chemistry for fast and efficient coupling rates resulting in more full-length product. This guarantees higher yields of single-stranded RNA while significantly reducing

Figure 1: Protected RNA nucleoside phosphoramidites for Dharmacon 2'-ACE synthesis chemistry



SYNTHESIS of sgRNAs often can be difficult and time consuming because of their 100-base length. A proprietary Dharmacon method reduces synthesis time and increases throughput to allow for faster delivery of higher quality products.

per-nanomole oligonucleotide prices. The (2′-ACE) RNA synthesis chemistry is based on a novel protecting group scheme. A novel class of silyl ethers protects the 5′-hydroxyl, and an acid-labile orthoester protects the 2′-hydroxyl. Then these protecting groups are used with standard phosphoramidite solid-phase synthesis technology (Figure 1).

RNA oligonucleotides are synthesized stepwise using the nucleotide-addition reaction cycle (Figure 2). Each nucleotide is added sequentially (in the 3′ to 5′ direction) to a solid support-bound oligonucleotide. The first nucleoside at the 3′ end of the chain is attached covalently to a solid support. Then the nucleotide precursor, a ribonucleoside phosphoramidite, and an activator are added (Step 1 in Figure 2) by coupling the second base onto the 5′ end of the first nucleoside. After the support is washed, any unreacted 5′-hydroxyl groups are capped with acetic anhydride to yield 5′-acetyl moieties (Step 2). Then the P(III) linkage is oxidized to a more stable and ultimately desired P(V) linkage (Step 3). At the end of the nucleotide addition cycle, the 5′-silyl group is cleaved with fluoride (Step 4). This cycle repeats for each subsequent nucleotide, resulting in a synthesis process that reliably provides high-quality, long, and fully modifiable RNA oligonucleotides.

CRISPR-Cas9 sgRNA Synthesis

One example of a long-RNA synthesis project that Horizon has developed into a commercial product line is CRISPR-Cas9 single guide RNA (sgRNA). Synthesis of sgRNAs often can be difficult and time consuming because of their 100-base length. Horizon sgRNAs are synthesized using a proprietary Dharmacon synthesis method to reduce synthesis time and increase throughput, allowing for faster delivery of higher quality products. Synthetic sgRNAs are column-purified, deprotected, and desalted to provide highly reliable, ready-to-use

Figure 2: Outline of the RNA synthesis cycle

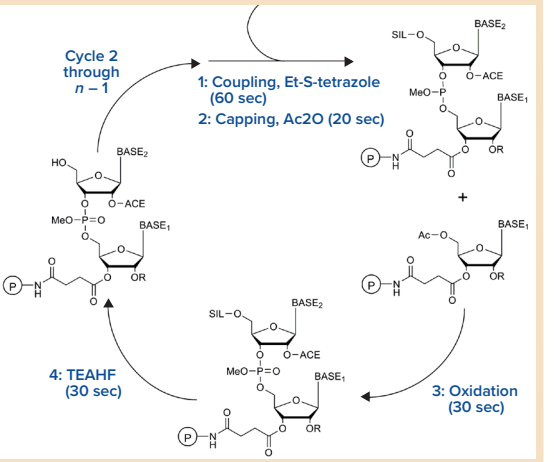
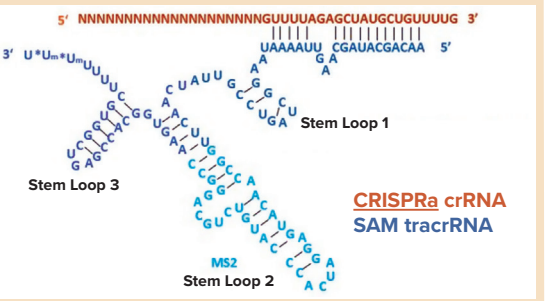


Figure 3: Synthetic CRISPRa system



Guide RNAs in the CRISPR-Cas9 System

CRISPR-Cas9 is a popular gene editing tool commonly used for gene knockout. In addition to a Cas9 nuclease, the CRISPR-Cas9 system requires a specific RNA guide to recruit and direct the nuclease activity. This guide can be used in one of two ways:

- in a two-part format consisting of a transactivating CRISPR RNA (tracrRNA) with a chemically synthesized CRISPR RNA (crRNA)
- in single guide (sgRNA) format that consists of both the crRNA and tracrRNA as a single 100-base construct.

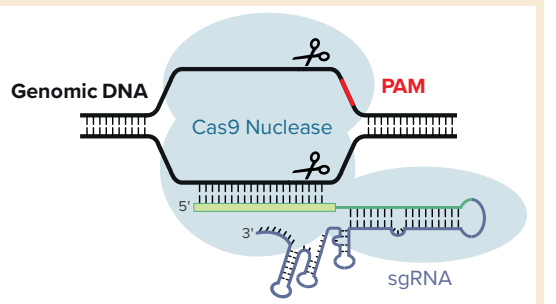
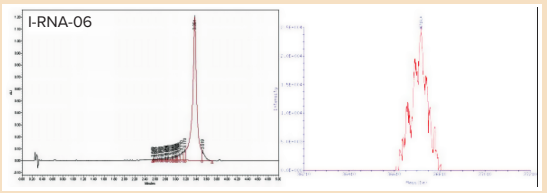


Illustration of Cas9 nuclease programmed by the sgRNA complex cutting both strands of genomic DNA upstream of the protospacer adjacent motif PAM

Figure 4: Ultraperformance liquid chromatography (UPLC) and mass spectrometry (MS) data for a synthetic 114-base oligonucleotide

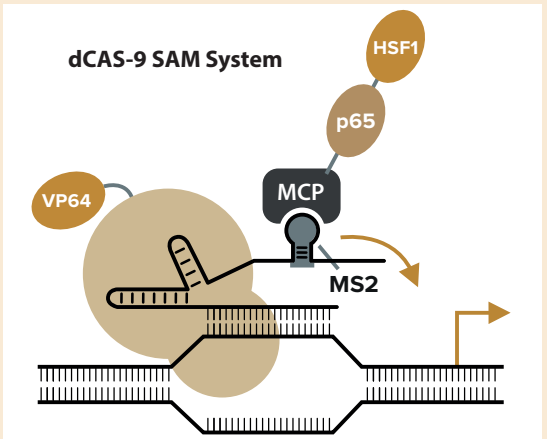


The SAM tracrRNA System Explained

The CRISPR-Cas9 Synergistic Activation Mediator (SAM) system is an engineered protein complex for transcriptional CRISPR activation (CRISPRa) of endogenous genes.

SAM CRISPRa requires three components:

- the SAM complex, consisting of an inactive Cas9 (dCas9) protein fused to the transcriptional activator VP64
- a SAM guide RNA to direct the complex onto the promoter site of a target gene.
- a MS2-p65-HSF1 fusion protein to recruit more transcriptional factors to the target site.



Synthesizing the 104-base SAM tracrRNA can be a CHALLENGE because of the secondary structure and oligonucleotide length. Horizon's experience in optimizing synthetic protocols has allowed it to scale up this manufacturing process and deliver SAM tracrRNA as a catalog product.

molecules for gene-editing experiments. This process allows for high-throughput production of thousands of guides per week, enabling Horizon to create library scale guides in far less time than any other manufacturing source. Horizon offers custom synthetic sgRNAs for *Streptococcus pyogenes* or any other species of Cas9 using its CRISPR design tool (https://dharmacon.horizondiscovery.com/gene-editing/crispr-cas9/crispr-design-tool).

CRISPRa SAM tracrRNA Synthesis

Another long-oligo project that Horizon released recently is the synergistic activation mediator (SAM) tracrRNA for CRISPR activation studies. SAM guide RNAs either can be expressed on a plasmid as a single guide or can be chemically synthesized in two parts: a crRNA guide and a SAM tracrRNA (Figure 3). Two-part synthetic guides offer greater experimental flexibility, with the same SAM tracrRNA used for multiple synthetic crRNA guides. However, synthesizing the 104-base SAM tracrRNA can be challenging because of the secondary structure and oligonucleotide length. Horizon's experience in optimizing synthetic protocols has allowed it to scale up this manufacturing process to deliver SAM tracrRNA as a catalog product.

Very Long Oligonucleotides

Very long RNA oligonucleotides are used increasingly to explore the critical structural, functional, and regulatory roles of RNA in biology. Therefore, a rapid, reliable, and cost-efficient method of synthesizing these very long oligonucleotides is needed to supply that experimental demand. However, traditional methods of RNA synthesis based on TBDMS or TOM 2′-silyl protection strategies are limited in their ability to construct longer oligonucleotides. The 2′-ACE chemistry approach offers a significant improvement in synthesis of very long RNAs, resulting in faster coupling rates, higher yields, greater purity, and superior ease of handling.

Using 2′-ACE chemistry, Horizon has developed and optimized protocols to synthesize efficiently and purify long RNA sequences longer than 120 bases. Some examples include spike-in controls, modified transfer RNAs, and long RNA with dual-labeled fluorescent dyes. The resulting yield and purity data clearly demonstrate that 2′-ACE chemistry is the method of choice for long-RNA synthesis applications (Figure 4).

Table 1: Modifications available through web ordering

Standard RNA Bases	Backbone Modifications	Spacer Modifiers	Labeling
A, C, G, U	Phosphorothioate	C18	3′-biotin
2′-omethyl RNA Bases	**Phosphorylation**	C3	3′-cholesterol
2′-OMe-(A, C, G, U)	5′-phosphate	C9	3′-Cy3
Standard DNA Bases	**2' Modifications**	dSpacer	3′-Cy5
2′-deoxy-(A, C, G, T)	2′-amino-butyryl-pyrene-uridine	rSpacer	3′-Cy5.5
Base Modifications	2′-amino-cytidine	**Chain Terminators**	3′-DY547 (Cy3 alternate)
1-methyl-guanosine	2′-amino-uridine	3′ inverted abasic	3′-fluorescein
2,6-diaminopurine	2′-deoxy-uridine	3′ inverted deoxy-thymidine	3′-biotin LC
2-methyl-adenosine	2′-fluoro-adenosine	3′-terminal 3′-deoxy-guanosine	3′-biotin LC LC
2-aminopurine	2′-fluoro-cytidine	3′-terminal dideoxy-cytidine	3′-puromycin
4-thio-uridine	2′-fluoro-guanosine	5′-inverted deoxy-thymidine	3′-TAMRA
5-bromo-uridine	2′-fluoro-uridine	5′-terminal 5′-deoxy-ribo-adenosine	5′-biotin
5-fluoro-cytidine	2′-OMe-inosine	5′-terminal 5′-deoxy-ribo-cytidine	5′-cholesterol
5-fluoro-uridine	**Amino Modifiers**	5′-terminal 5′-deoxy-ribo-guanosine	5′-Cy3
5-iodo-uridine	3′-amino modifier C12	5′-terminal 5′-deoxy-ribo-uridine	5′-Cy5
5-methyl-cytidine	3′-amino modifier C6	**Degenerate Bases**	5′-Cy5.5
5-methyl-deoxycitidine	3′-amino modifier C3	dN	5′-dabcyl
5-methyl-uridine	5′-amino modifier C12	dS	5′-DY547 (Cy3 alternate)
inosine	5′-amino modifier C3	dW	5′-DY647 (Cy5 alternate)
N2-methyl-guanosine	5′-amino modifier C5	mN	5′-DY677 (Cy5.5 alternate)
N3-methyl-uridine	5′-amino modifier C6	rN	5′-fluorescein
N6, N6-dimethyl-adenosine	5′-aminohexylacylamino-uridine	rS	5′-pyrene
N6-methyl-adeonsine	**Thiol Modifiers**	rW	5′-TAMRA
O6-methyl-guanosine	3′-disulfide thiol modifier		5′-TET
pseudo-uridine	5′-disulfide thiol modifier		
purine ribonucleoside			
pyrrolo-cytidine			
ribavirin			

Table 2: Deprotection and purification recommendations

When you need	Protected RNA	Unmodified siRNA Short, unmodified RNA (<42 nt)	Longer, unmodified RNA (>43 nt) RNA for highly sensitive applications such as X-ray crystallography	3′, 5′ internal or dual modifications	Short, unmodified RNA (<42 nt) for in vivo work	Longer, unmodified RNA (>43 nt) for in vivo work
Horizon recommends	**Unprocessed**	**Desalt/ Deprotect**	**PAGE**	**HPLC**	**In Vivo Preparation**	**In Vivo HPLC**
Because	Unprocessed RNA retains 2′ACE protection groups	85% purity is routinely achieved without purification for these types of oligonucleotides	PAGE purification provides greater purity but lesser yield than HPLC	Ion-exchange purification	Counter-ion (Na+) exchange, desalting, sterile filtration, and endotoxin testing prepares oligos for in vivo use	In vivo processing with HPLC purification provides purified oligonucleotides for in vivo use

PAGE = polyacrylamide gel electrophoresis **HPLC** = high-performance liquid chromatography

The ability to **SCALE UP** synthetic oligonucleotide production while maintaining quality standards has allowed Horizon to commercialize many long-oligonucleotide projects into novel catalog products.

Custom Modifications

Horizon offers a broad portfolio of modifications that can be applied to both small interfering RNA (siRNA) and single-strand RNA (Table 1). These typically can be made at any location (5′, 3′, or internal), which expands experimental capabilities and provides greater flexibility for researchers. Horizon's custom synthesis laboratory also has extensive capabilities for custom dyes, amidites, succinates, locked nucleic acids, postsynthesis NHS esters, and more.

Purification, Yield, and Quality

Horizon's synthetic RNA oligonucleotides come with several purification options for a variety of applications and uses (Table 2). The company also offers larger scale synthesis options up to multiple-gram amounts. This was designed to help move projects seamlessly from research into preclinical phases, with additional support for rapid scale-up to clinical studies.

Conclusion

Dharmacon has pioneered synthetic RNA oligonucleotide manufacturing and purification processes for over 20 years. Beginning with single-stranded RNA (20–25 nt) in the 1990s, then moving on to siRNA (21 nt), shRNA (80 nt) and sgRNA (100 nt), Dharmacon chemists continue to push the boundaries of commercial oligonucleotide synthesis to supply reliable research tools for a range of experimental applications.

Since acquiring the company in 2017, Horizon Discovery has leveraged its RNA synthesis expertise in manufacturing long and difficult-to-synthesize oligonucleotides to offer a new generation of synthetic products for use in CRISPR gene editing and CRISPRa endogenous gene activation.

The proprietary 2′-ACE chemistry offers an innovative solution to RNA synthesis and modification problems, producing reliable oligonucleotides for a broad range of research needs. The ability to scale up synthetic oligonucleotide production while maintaining quality standards has allowed Horizon to commercialize a number of long-oligonucleotide projects into novel catalog offerings.

Taken together, this combination of proprietary chemistry, available chemical modifications, experience manufacturing oligonucleotides up to 120 nt in size, and high production and purification quality make Horizon's custom oligonucleotide synthesis service a trusted source for your long, challenging or unique synthetic oligonucleotide projects.

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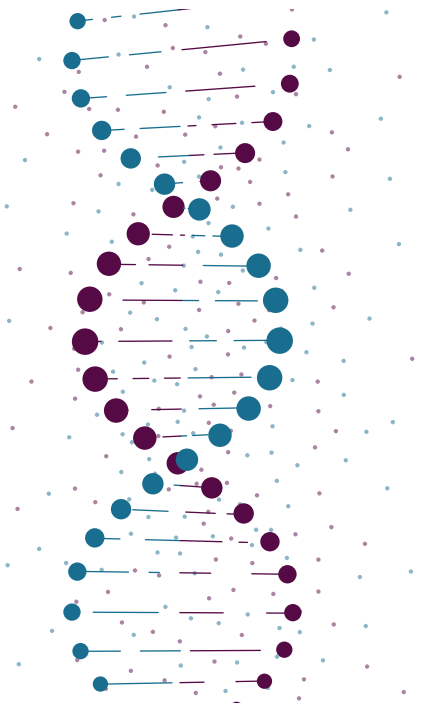

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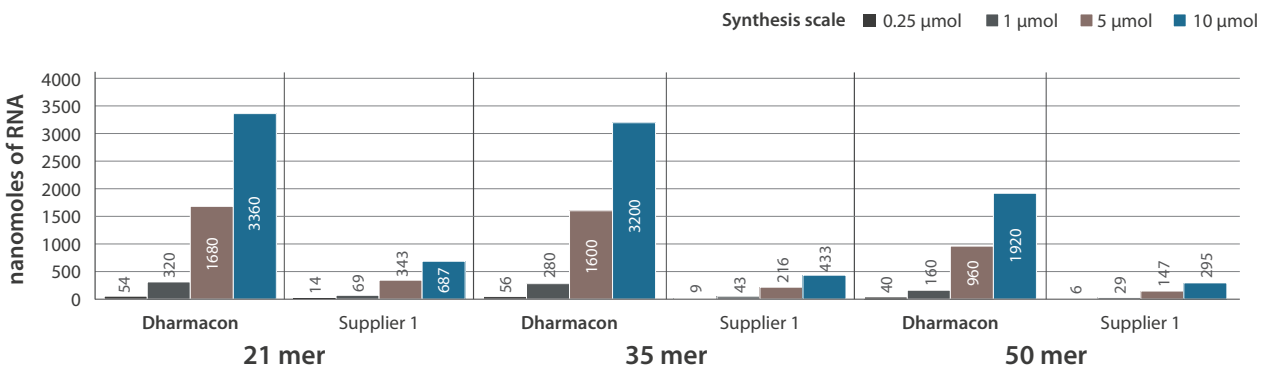
Meet your custom RNA requirements with our nearly limitless synthetic options

If your NMR, crystallography, or RNA binding study requires a non-standard chemical modification or a commercially unavailable modified nucleobase, we can help. For more than 20 years our chemists have utilized Dharmacon™ proprietary 2′ ACE chemistry to synthesize RNA with superior yield and quality giving you the flexibility you need.

Better RNA yields give you more for your money

RNA yield (HPLC-purified)



The yield for RNA oligos of three different lengths was compared for all available synthesis scales between Dharmacon and a competitor.

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