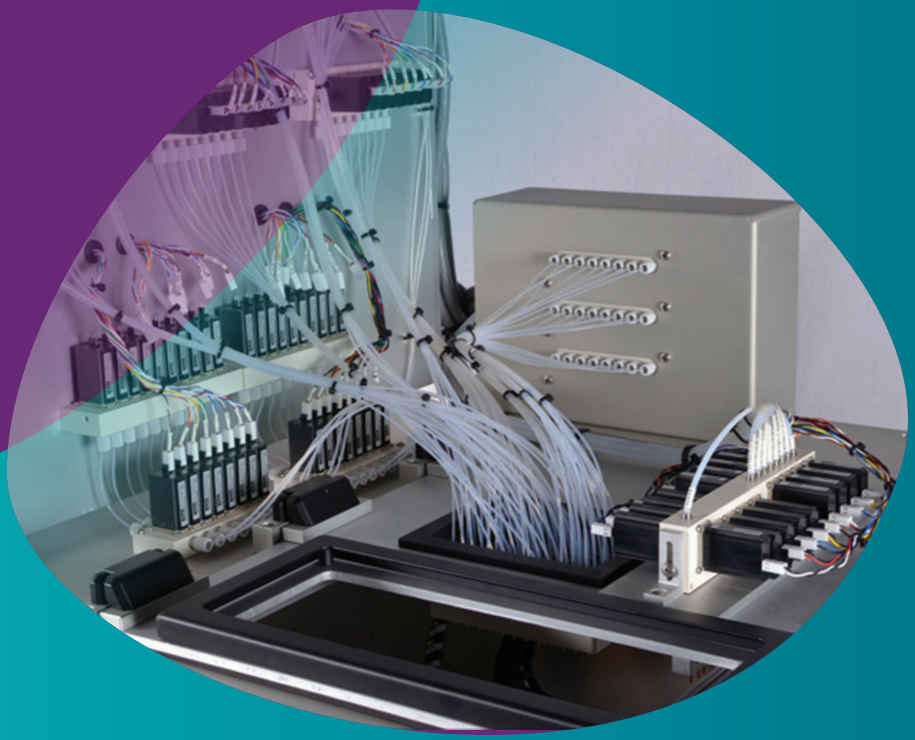
Bringing oligonucleotide synthesis in-house



A guidebook for molecular diagnostic labs, assay developers and assay manufacturers

BIOSEARCH™ TECHNOLOGIES
GENOMIC ANALYSIS BY LGC

Enjoy the benefits of synthesising your own oligos

Whether your goal is to secure your supply chain, speed turnaround time, protect intellectual property, control costs, or a combination of all of the above, establishing in-house oligonucleotide synthesis capabilities can be an effective solution. But there are many factors to consider beyond simply selecting and ordering an instrument. In this guide, we'll highlight what these factors are, from space and throughput considerations to tips for organisations that have low to no experience conducting organic chemistry in their facility, to help you ensure a smooth and successful oligo synthesis production setup.

Our technical support team has extensive experience in oligonucleotide synthesis and system installation. If you have additional questions, feel free to get in touch via the details in the contact us section.

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In-house or outsource?

Does it make sense for my organisation to synthesise our own oligonucleotides?

While the focus of this guidebook is on how to bring oligonucleotide synthesis in-house, you may still be wondering if this is the best option for your organisation. The challenge is that the cost-benefit analysis of supply chain security, intellectual property protection, and faster turnaround times will vary depending on your unique situation. However, if you think synthesising in-house could be a good fit for you, we're here to help.

We're ready to commercialise our kit and need scaled-up, GMP oligo production – should I outsource again?

While in-house production can make sense during assay development, implementing and managing the quality systems and facilities for oligonucleotide synthesis in a GMP-compliant environment can dilute the focus of your internal teams. We find that companies often choose to outsource when rigorous quality standards are required, internal resources limit production capabilities, or there is a need for very high throughput or to synthesise the same oligo consistently.

Learn more about our oligo synthesis services

Consideration 1:
List the critical parameters that will guide instrument selection

Addressing the following key questions can help you identify the best oligo synthesizer to meet your organisation's projected needs:

What is your expected throughput?

- How many different oligos will you need to produce during each run?
- How many different oligos will you need to produce each week?
- Can you dedicate entire runs to a single oligo or will you need each run to produce multiple different oligos?

What are your expected scales?

- How much of each oligo will you typically need to produce?
- What are the largest scales you might need to produce?
- What are acceptable turnaround times?

What will you be synthesising?

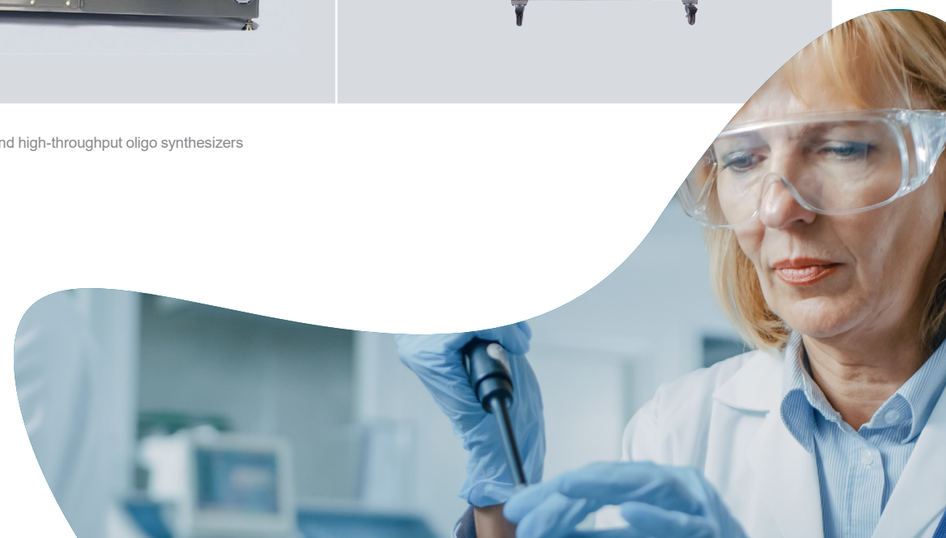
- DNA? RNA? LNA? A combination of all of the preceding?
- How long are the oligos you will typically need to produce?
- What are the longest oligos you will need to produce?
- Will you need to add non-standard or modified nucleosides?

Answering these questions can help you understand whether you need a plate-based or single column instrument, the volume of the synthesis columns, and how many amidite positions you need – these are the spots for individual nucleobases, so typically one spot each for A, G, C, and T and additional spots for each modification that will be added.

See examples of oligo synthesizers in figure 1.

A low/medium throughput synthesizer	A high-throughput oligo synthesizer
The MerMade™ 6 synthesizer from LGC Biosearch Technologies	The MerMade 192X synthesizer from Biosearch Technologies
	

Figure 1. Examples of low/medium- and high-throughput oligo synthesizers



Comparing the MerMade family of oligo synthesizers

Biosearch Technologies offers oligonucleotide synthesis instruments that support production at a range of scales, flexibility, and throughputs.

Oligo synthesizer	Throughput	Columns format	Max Amidite positions (standard)	Columns format	Scale	Benefit
MerMade 4	low	4	10	20mer primers in 3.5 hours on each column	50 nmole to 5 µmol	Each of the four columns can be assigned a different protocol to synthesise oligos of different quality and yield within the same run.
MerMade 6	medium	6	10, 16, 20 or 24	20mer primers in 3.5 hours on each column	50 nmole to 200 µmol	Capable of making a combination of standard, degenerate and modified oligos in the same run. Unmatched flexibility, upgrade ability, dependability, efficiency and ease of use.
MerMade 12	medium	12	10, 16, 20 or 24	20mer primers in 3.5 hours on each column	50 nmole to 200 µmole	Ideal for labs requiring medium throughput with maximum flexibility. Capable of making a combination of standard, degenerate and modified oligos in the same run.
MerMade 48X	high	4 plates of 12 columns	20 or 24	20mer primers in 3.5 hours	50 nmole to 5 µmol	A fast oligo synthesizer that is capable of producing high quality oligos – even those with extensive modifications. Allows for hot swapping plates as the synthesis is completed to facilitate a continuous, efficient operation.
MerMade 96E	high	96 well format of columns	12	20mer primers in 3.5 hours	50 nmole to 1 µmol	Ideally suited for labs that need a fast synthesizer that can be configured for all types of chemistry and is capable of producing high-quality oligos.
MerMade 192E	high	2x 96 well format of columns	8	20mer primers in 3.5 hours	50 nmole to 1 µmol	Produces a very high-quality product with very minimal reagent usage. Ideal for those who need a fast synthesizer that is capable of producing high-quality oligos that do not require extensive modifications.
MerMade 192X	high	2x 96 well format of columns	12-64	20mer primers in 3.5 hours	50 nmole to 5 µmol	This oligo synthesizer adds even more flexibility, configurability and speed compared to models listed above. It can be configured to synthesise all types of chemistries and produces high-quality oligos.

Oligonucleotide synthesis in brief

Automated oligonucleotide synthesis instruments use the phosphoramidite chemistry method introduced more than 35 years ago.[1] DNA phosphoramidite nucleosides are coupled together on a solid support to build a growing oligonucleotide chain through a four-step cycle that consists of deblocking, activation, capping and oxidation. After the entire oligonucleotide is built, it is cleaved from its solid support, deprotected, and further processed by desalting and purification.

4 key takeaways for each step of the synthesis cycle

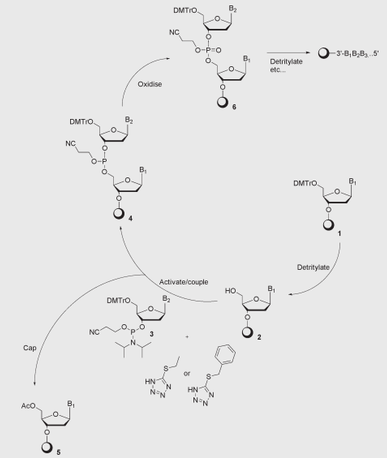


Figure 2: The oligonucleotide synthesis cycle using phosphoramidite chemistry.

Deblocking (detritylation)

Starting with the support bound nucleoside or modifier, the first step of the oligonucleotide synthesis cycle is deblocking or detritylation. This involves removing the dimethoxytrityl protecting group from either the 5′ position of the nucleoside or from the modifier backbone with an acid treatment to afford a reactive OH group. The 4,4′-dimethoxytrityl (DMTr) protecting group is required, specifically on phosphoramidites, to prevent self coupling during the coupling step.

DMTr is commonly used for the protection of OH groups because it is easily removed with a dichloroacetic acid (DCA) or trichloroacetic acid (TCA). However, prolonged contact with acid can result in side reactions such as depurination, especially when assembling oligonucleotides that contain polyA sequences or sensitive nucleosides.

Some examples of bases vulnerable to depurination include N(6)-benzoyl-2-deoxyadenosine, 8-aryl derivatives of 2-deoxyguanosine and to a lesser extent, N(2)-isobutyryl-2-deoxyguanosine.[2]

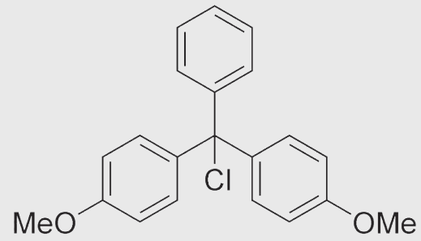
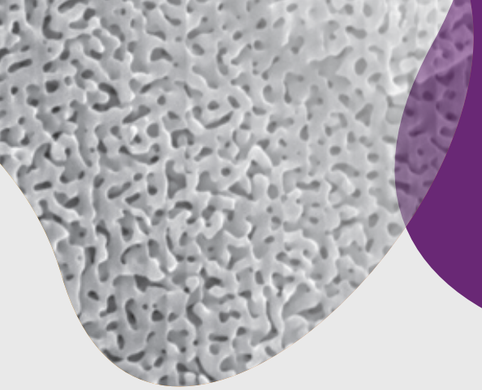


Figure 3: 4,4-Dimethoxytrityl Chloride (DMTr-Cl), a reagent for creation of reactive DMT functionality on nucleosides.

Depurination can lead to chain cleavages under basic conditions, such as during the final cleavage and deprotection steps to cleave the oligonucleotide from the support and remove the nucleobase and phosphorus protection. In some cases, using weaker acids during detritylation or reducing acid contact times can be beneficial to maintain low levels of depurination.[2] DCA is a milder acid than TCA, making it a good option for detritylation of long oligonucleotides and tends to result in better synthetic yields than TCA.[3] However, using TCA for deblocking allows for faster reactions. When 3% TCA was used in the detritylation step, studies show that acid delivery time as short as 10 seconds (versus 110 seconds) did not significantly compromise the yield of the full-length product.[4] A deblocking step that completes in less than one minute is ideal for reducing the oligonucleotide's exposure to acid.[5]

Another option is to alternate the detritylation step with wash steps, this enables full deprotection but minimises the contact time with acid.



Key takeaway:
Use the mildest effective deblocking acid and shortest contact to reduce the risk of depurination.

Activation/coupling

After the deblocking step is complete, the 5′-OH group is free for reaction. The phosphoramidite corresponding to the second base in the oligonucleotide sequence is activated.

The diisopropylidene group is protonated by an activating agent such as 5-ethylthio-1H-tetrazole (ETT) or 5-benzylthio-1H-tetrazole (BTT). This is delivered to the column and reacts with the free 5′-OH group, displacing the diisopropylidene group and forming a phosphite linkage.

4,5-dicyanoimidazole (DCI) is an alternative activator that may be used. DCI is less acidic than ETT or BTT but is a more nucleophilic activator, reducing coupling times.[6] It is highly soluble in acetonitrile and thus allows for higher effective concentrations of nucleoside phosphoramidites during solid-phase synthesis. A higher effective concentration, in turn, allows for lower phosphoramidite excess during coupling.[6]

Coupling efficiency is fundamental for optimising yield, oligonucleotide quality and for ease of the downstream purification process, especially for longer oligonucleotides. Many factors can affect the coupling efficiency of amidites including coupling time and acetonitrile dryness. In addition to using the most effective activator for the application, effective coupling efficiency can be achieved by:

- Eliminating moisture in reagents involved in the coupling step. Use anhydrous acetonitrile (ACN) as a diluent and wash and keep an anhydrous environment when dissolving phosphoramidites. We suggest pre-treating ACN with molecular sieves before adding it to the amidite.
- Considering oligo length, complexity and loading requirements when choosing a solid support. Starting with an appropriate solid support based on the oligonucleotide's characteristics is critical to maintaining a high coupling efficiency throughout the synthesis cycle.
- Increasing the phosphoramidite concentration to enhance the coupling efficiency for long oligonucleotides.
- Adjusting reagent amounts and coupling time.
- For long oligos, using a cap/ox/cap cycle can improve coupling. This is thought to be a result of the second capping drying the support after the oxidation step.

Key takeaway:
Take steps to enhance coupling efficiency by optimising the coupling time and ensuring an anhydrous environment during the coupling process.

Key takeaway:
Even with the most efficient chemistry and purest reagents, it's not reasonable to expect 100% coupling efficiency.

Capping

Following phosphoramidite coupling, a capping step is introduced to acetylate any unreacted 5′-OH groups. While solid-phase phosphoramidite coupling usually proceeds to approximately 99% efficiency, any reactive 5′-OH groups left uncapped can result in undesirable side-products. Unless blocked, these truncated oligonucleotides can continue to react in subsequent synthesis cycles, creating near full-length oligonucleotides with internal deletions.

Capping is generally achieved using a solution containing acetic anhydride (Cap Mix A) and the catalyst N-methylimidazole (Cap Mix B), which acts as an activator for Cap Mix A. The 5′ acetyl ester cap is unreactive in all subsequent synthesis cycles and is removed during the final base deprotection step.[5]

Capping is a critical process in the synthesis cycle to reduce the accumulation of deletion mutations that are difficult to purify and could render the oligonucleotide ineffective for subsequent applications. Additional acetonitrile washing following capping may increase synthetic yield.[5]

Oxidation

In the final step of the oligonucleotide synthesis cycle, the unstable phosphite triester linkages are oxidised to a more stable pentavalent phosphotriester using iodine in a THF/water/pyridine or THF/water/lutidine solution. Because the oxidiser contains water, the support is rinsed several times with acetonitrile following this reaction.[5] The most effective way to remove residual water is to perform an additional capping step. The pyridine or lutidine in the capping reagents remove the water from the support more efficiently than MeCN.

After oxidation is complete, the entire 4-step cycle is repeated, beginning with detritylation of the 5′-terminus of the support bound oligonucleotide. The process continues until each base of the oligonucleotide is coupled to the growing chain.

Key takeaway:
While oxidation is the final step in the synthesis cycle, further processing of the oligonucleotide is required to make it a biologically active molecule.

Consideration 2:

Ensure you have enough space for the instrument and the required ventilation system

Critical consideration for labs with low- or no previous organic synthesis on site

Once you have selected an instrument that will meet your needs, you will need enough properly ventilated space for the instrument, associated reagents, and post-production processing steps. Because oligo synthesis relies heavily on organic solvents, you will either need to place the instrument in a fume hood, use two ventilation snorkels, or use a dropdown canopy.

Some post-production processing steps, such as cleavage of the oligo from the solid support and removal of the protecting groups, are often performed off of the synthesizer. These steps will need to be conducted in a fume hood or similarly ventilated space.

What's the right ventilation solution for your lab?

The technical support team at Biosearch Technologies can provide recommendations on a ventilation solution for your lab space prior to delivery of your synthesizer.

> To learn about cleavage, deprotection and purification options for oligonucleotide synthesis, read our key takeaways for post-synthesis processing blog post.

Consideration 3:

Ensure you have proper organic solvent storage and disposal systems

Critical consideration for labs with low- or no previous organic synthesis on site

As mentioned in Consideration 2, oligonucleotide synthesis requires the use of organic solvents. Depending on the scale of production, you may need to store several dozen litres of material in appropriately ventilated flammable storage cabinets. You will also need to arrange for hazardous waste collection and disposal.

What's the best way to store solvents, and who can help with the disposal of solvent waste?

The technical support team at Biosearch Technologies can provide recommendations on storage cabinets as well as vendors who provide disposal of organic solvent waste.

Consideration 4:

Verify that your proposed setup will comply with local fire codes

Critical consideration for labs with low- or no previous organic synthesis on site

Because of the use of organic solvents, many of which are highly flammable, you will likely need to consult your local fire codes to ensure compliance with regulations. These regulations may govern the amounts of solvents you may store on site, how those solvents are stored, and the type of signage required.

Consideration 5:
Obtain permits for controlled substances, if required

Depending on your country and location, some of the reagents used in oligonucleotide synthesis may be considered controlled substances for which you will need to obtain a permit. For example, if you use ammonium hydroxide/40% aqueous methylamine 1:1 v/v (AMA) for post production processing, the methylamine is considered a controlled substance by the US Drug Enforcement Agency and will require permitting.

Plan ahead and consult with your chemical supplier to ensure you can obtain all necessary permits for any controlled substances you will be using.

Consideration 6:
Ensure you have the required accessories

Because oxygen and water can negatively impact oligonucleotide synthesis, you will need to ensure you have the right accessories to support your instrument.

Inert gas

To maintain the right environment for oligonucleotide synthesis you will need to supply your system with an inert gas. The Biosearch Technologies team recommends ultra-high purity (<1 ppm H_2O) argon. If argon is unavailable then nitrogen can be substituted, but argon is preferred as it provides the most inert conditions for your reactions.

You will also need a regulator at the right specifications for your instrument to ensure consistent pressurisation of your system.

Reagent bottles

You will also need reagent bottles with the right thread standard to attach to your system. Depending on your supplier, they may provide bottles with GL38, GL45, or 28-400 threads.

Figure 4. Bottles have different types of threads. You will need to make sure you obtain bottles with the compatible thread standard.

Consideration 7:
Verify that you have other necessary equipment

Additional equipment that your lab may need, especially if your lab does not typically conduct organic synthesis, include:

- Oven or heating block
- Additional vacuum source
- Evaporator
- Drying reagents
- Nucleic acid quantification instrument such as a Nanodrop or spectrophotometer
- A quality assurance method such as LC-MS.

Consideration 8:

List the types of oligos you will be synthesising and identify supplier partners that can reliably deliver the raw materials at the scales you need

Modified and unmodified nucleoside phosphoramidites

To keep your synthesizers operating at full capacity, you'll need to ensure that you'll have a steady supply of the raw materials needed for the oligos you will be producing. We recommend thinking ahead over the course of 6-months to a year and determining what types of phosphoramidites and solid supports you'll need to keep in stock.

Here are a few questions to guide your assessment process:

What are all the applications you will be using the oligos for?

qPCR reactions will need both fluorophores and quenchers. Therapeutic and *in vivo* detection applications may need:

- Backbone modifications
- Cell delivery modifications
- Nuclease protection modifications
- Spacers or conjugation reagents for adding functional groups.

If you will be using oligos for detection purposes, what are the wavelengths that your instruments are capable of detecting?

What quality systems does your supplier adhere to and are they compatible with your regulatory and quality needs?

Among the many advantages of choosing Biosearch Technologies as your source for modified and unmodified oligonucleotide reagents and solid supports are:

- Our ability to provide a wide variety of nucleosidic and non-nucleosidic modifications
- Our ability to provide reagents at the full range of scales you may need, from initial discovery and pilot studies to manufacturing
- Our consistent quality
- Our ability to customise our offering to meet your specific needs, whether it's adherence to a specific quality system, quality control using a specific analytical technique, and more.

Learn more about modified and unmodified oligo synthesis starting on page 27 of our Oligonucleotide synthesis reagents catalogue, 3rd edition. Download the catalogue

Solid supports

Selecting the best solid support for your oligo synthesis program is critical for ensuring the efficiency, purity, and reproducibility of your synthesis.

Our range of controlled pore glass (CPG) products are designed to suit nearly any oligonucleotide synthesis application. Your ideal CPG support should possess the following parameters optimised for your application:

- Particle size and shape
- Pore size
- Pore volume
- Specific surface area

The support should also be uniform and consistently produced to maximise the desired solution exchange behaviour, ligand loading and distribution, and reaction kinetics.

Selecting the right pore size

The length, complexity and application of the oligo will guide your choice of pore size. The following guidelines can help you match the pore size to application:

- 500 Å CPG
  - ≤30mers medium to large scale oligo synthesis
  - High yields of product are required such as therapeutic oligos
  - High loaded support is required. 500 Å can load up to ~100 µmol/g.
- 1000 Å CPG
  - >20mers or highly modified oligonucleotides
  - The loading is typically 25-40 µmol/g and most of our modifiers are functionalised onto this pore size as standard.
- 2000 Å CPG
  - >80mers, used in CRISPR
  - The loading is typically 15-35 µmol/g
- 3000 Å CPG
  - >80mers
  - The loading is typically 10-20 µmol/g
  - With a few exceptions, it is possible to have any of our 1000 Å products with a 3000 Å pore size.

In general, large scale oligo synthesis for therapeutic applications requires high loaded 500-600 Å and small to medium scale synthesis for diagnostic or research use require higher pore sizes.

Learn more about how Biosearch Technologies supports the full oligonucleotide synthesis workflow from synthesizers and solid supports to modified nucleosides, locked nucleic acid phosphoramidites, fluorophores, and quenchers – visit our webpage

Contact us

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North America, Latin America	Genomics.americas@lgcgroup.com
Europe, Middle East and Africa	Genomics.emea@lgcgroup.com
Asia Pacific, excluding China	Genomics.apac@lgcgroup.com
China	Genomics.china@lgcgroup.com

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