Putting DNA Synthesis in the Hands of Every Researcher

Previously, DNA synthesis methods were outsourced to third parties, but growing interest in this field is bringing about the commercialisation of these technologies and making them more commonplace in labs

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There is an ever-increasing need to discover and develop new medicines that could potentially provide major improvements in healthcare across the globe. One technology that is expected to contribute significantly to this is the emerging field of synthetic biology, an approach that combines the use of synthetic DNA with a growing knowledge of genomics, enabling researchers to quickly assemble novel DNA constructs and genomes (1). With the advent of synthetic biology, the ability to explore, select, and optimise the biology around us is greatly enhanced. This has led to great anticipation of significant and broad impact in the drug discovery and development field, building on some notable successes already achieved, including the development of novel vaccines (e.g., antigen genes or nucleic acid vaccines), gene therapy (with its requirement for synthetic DNA), CRISPR-Cas9, and other cellular engineering techniques.

For those researchers working in synthetic biology labs, the ability to rapidly synthesise and access high-fidelity DNA is crucial. DNA synthesis is a powerful technique that requires the joint expertise of chemists, biologists, and engineers to offer researchers the power to better understand and manipulate the fundamental building blocks of life.

Molecular cloning techniques form the foundation of much of modern molecular biology research, and most bioscience labs today are equipped with their own polymerase chain reaction machine. Molecular cloning relies on the synthesis of short DNA sequences that are inserted into genomic DNA; however, this process to synthesise the oligonucleotides required to make double-stranded DNA is typically outsourced to specialist companies and seldom performed by the individuals that actually publish the data. DNA synthesis remains inaccessible to conventional researchers across the globe; with this comes myriad limitations. As such, there is an increasing demand for high throughput DNA synthesis technologies to be brought in-house, replacing the current outsourcing model.

DNA Synthesis – An Outdated Chemistry?

The current paradigm of DNA synthesis uses phosphoramidite chemistry to build single-stranded oligonucleotides, which are later annealed to generate double-stranded DNA (usually 5-50 oligonucleotides provide the raw substrate for constructing 200-3,000 bp double-stranded DNA). The phosphoramidite-based methods for DNA synthesis were first developed in the 1980s, and have since remained largely unchanged (2).

The typical phosphoramidite process relies on a four-step cycle that adds nucleotides one by one to a growing single strand, attached to a solid support of controlled pore glass or polystyrene beads (see **Figure 1,** page 10). In most commercial DNA synthesisers, the solid support is packed into a continuous flow column or reaction well, into which the reagents needed to build the sequence are added. This process works for synthesising short DNA sequences, which in the past have made up a large proportion of the demand within the DNA synthesis market; however, this is now changing as the research community begins to understand and unlock the opportunities of synthetic biology.

Nucleotides are added in a 3'-5' manner, with the 5'-hydroxy (OH) group lying at the end of the chain and the 3'-end attached to the surface via a cleavable linker. The 5' terminus must be kept protected to prevent uncontrolled addition until the next nucleotide is ready to be added, and the 5'-protecting group must be removed under conditions that are orthogonal to the cleavage of the completed oligo from the surface at the end of the synthetic process. Once

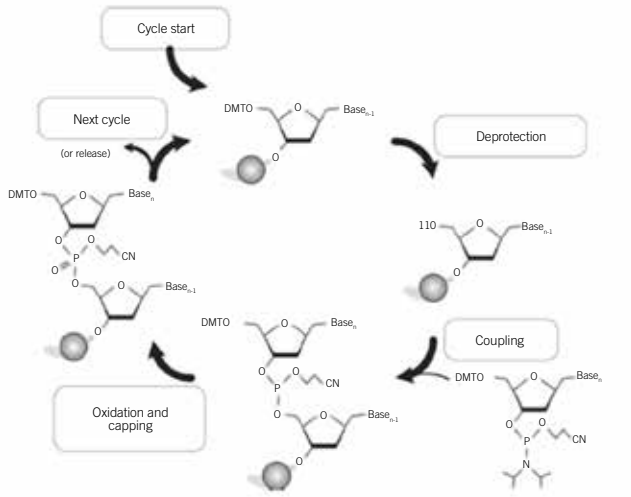


Figure 1: The phosphoramidite method – the current gold standard for oligonucleotide synthesis

the protecting group is removed under acidic conditions, the exposed 5'-OH can then couple to the next nucleotide, which is attached to a phosphorous (P)(III) group to provide electrophilic reactivity. Before proceeding further, any unreacted 5'-OH groups are capped to prevent the growth of chains missing a nucleotide (shortmers), producing truncated strands, which are easier to remove. At this stage, the unstable P(III) is also oxidised to P(V). Once this is complete, the next nucleotide in the sequence can be added, and the process continues.

Following stepwise synthesis of the desired oligonucleotide, the product is cleaved from the solid support and the remaining exocyclic P(V) protecting groups are removed in a single step, using a strong base such as ammonium hydroxide. To make a DNA product from these individual oligonucleotides, they must be purified to remove errors and then ligated to give the complementary strands which are hybridised to generate double-stranded DNA.

Limitations of Outsourcing to Conventional Synthesis Providers

The first major limitation posed by the majority of conventional DNA synthesis providers is that the process is not amenable to extremely high-throughput applications as the number of strands that can be sequenced in parallel is fairly low. This is because every reaction well remains in the same chemical environment, therefore the number of different sequences that can be produced at any one time is directly determined by the number of reaction wells available. This places a limit on the scale to which DNA can be synthesised.

There is also the issue of accuracy as the likelihood of introducing errors into the DNA strand increases as more nucleotides are added. Even though each nucleotide coupling proceeds with around 99% accuracy, the yield of the full-length DNA sequence reduces by the accuracy of each coupling, raised to the power of the number of nucleotides added. This means that by 100 bases, the typical yield is only 37%, and purification to remove the error-containing oligonucleotides is an extra task for the supplier, and an extra cost for the lab (3). This imposes a limitation on the length of double-stranded DNA achievable, and the maximum length offered by most providers is around 5,000 base pairs.

Not only is there the direct cost of buying the DNA itself, but there is also the indirect cost of delaying projects due to the potential time constraints of outsourcing, contributing to the significant turnaround time often experienced by researchers. Although efforts are being made to make the process more cost-effective, and competitive pricing between suppliers is contributing to this, recently prices have stopped declining at the rates they once were (4-5). The primary reason for this is most likely down to the margins between the cost of manufacturing and shipping the DNA fragments and the cost of the actual product. One way to further reduce these costs is to redesign the current phosphoramidite method, which will require a fundamental rethinking of the chemistry involved.

Building a Desktop DNA Synthesiser

There is now a new wave of technologies being developed that are compatible with both chemical and enzymatic DNA synthesis methods, all designed to overcome the limitations discussed above and to be sold to labs as desktop instruments with minimal infrastructure requirements. To remove current service-based bottlenecks and restrictions, in-house technologies must have rapid prototyping capabilities, providing a fast turnaround time and random access for the end-users as required for their experimental goals.

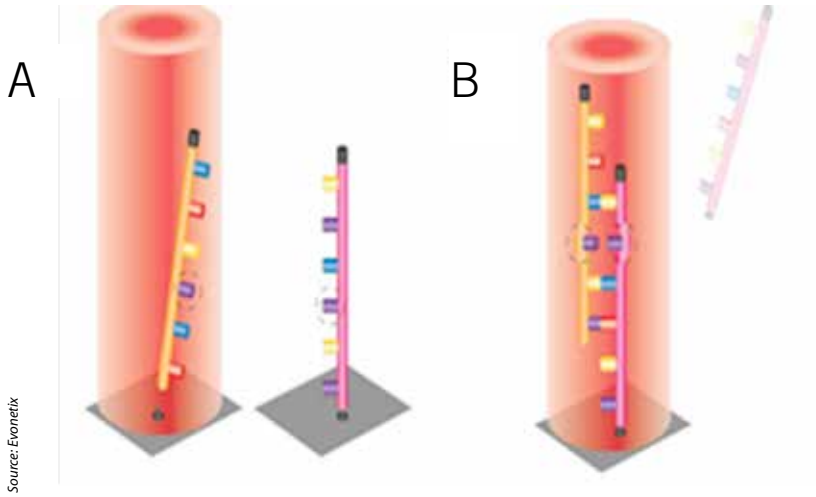
One particular method being pioneered in Cambridge, UK, is modifying the phosphoramidite cycle to become thermally controllable. By using a thermosensitive protecting group at the 5'-position, the base addition is thermally controlled, and researchers can distinguish between reaction sites, which otherwise see exactly the same chemical conditions. Although this method remains in development, preliminary results have indicated that the temperature control achieved is highly accurate, allowing the researchers to carry out DNA synthesis at scale.

Thermal control of oligonucleotide synthesis and subsequent assembly into double-stranded DNA offers further significant advantages as it allows errors to be detected and removed during assembly, as opposed to post-synthesis (see **Figure 2**). Using this method, DNA mismatching is easily identified as once oligonucleotides are annealed the site is heated to a precise, sequence-dependent temperature at which dissociation will be faster for imperfect matches, allowing the population of oligonucleotides to be purified by discarding imperfect sequences. This error purification allows for longer sequences with high accuracy to be assembled, preventing bad sequences from diluting the population of correct molecules (6).

> *Bringing such technologies in-house will change how DNA is currently accessed, made, and used*

More accurate and accessible DNA synthesis technology will allow researchers to use biology on a scale not previously possible. While many are focusing on high-throughput techniques, others are seeking to massively reduce the time it would take to make DNA at scale by assembling oligonucleotides in parallel rather than one by one, vastly shortening the time it takes to produce a single large molecule.

Bringing such technologies in-house will change how DNA is currently accessed, made, and used. Not only will it present individual laboratories with the ability to engineer biological systems with high accuracy, scale, and complexity, it will accelerate the evolution of new genes and pathways, and make biological engineering as accessible and widespread as next-generation sequencing. The in-house synthesis of large libraries of sequences or modified DNA sequences could open opportunities to design structural variants of proteins such as biotherapeutics, and could give scientists the ability to select for proteins with enhanced properties and desired phenotypic outcomes using directed evolution methods that would not be feasible otherwise.



Source: Evonetix

Figure 2: Schematic of the thermal error-correction process. (A) Oligonucleotides are heated to selectively remove them from the surface. (B) Oligonucleotides hybridise to complementary strands – these sites are heated, causing strands with mismatches to dissociate

Conclusions

The restrictions of standard DNA synthesis methods currently in use have meant that in most labs, synthesising DNA has been outsourced to third-party providers. The increasing momentum for synthetic biology is moving DNA writing technologies forward, and the new wave of technologies could finally change this, making DNA synthesis affordable and simple enough to be performed at the lab benchtop. This could significantly reduce the time it takes to obtain a DNA sequence needed for an experiment, giving researchers more flexibility and accelerating workflows. Commercialisation of such technologies and the continued development of other efficient DNA synthesis techniques for scientists to use in their own labs will further enhance the availability of increasingly inexpensive synthetic DNA.

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