


# Gene Synthesis Handbook



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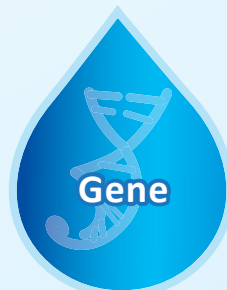
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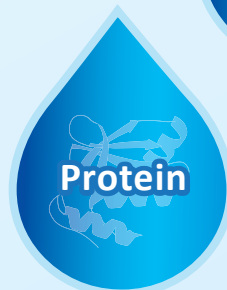
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## What is Gene Synthesis?

Gene synthesis refers to chemically synthesizing a strand of DNA base-by-base. Unlike DNA replication that occurs in cells or by Polymerase Chain Reaction (PCR), gene synthesis does not require a template strand. Rather, gene synthesis involves the step-wise addition of nucleotides to a single-stranded molecule, which then serves as a template for creation of a complementary strand. Gene synthesis is the fundamental technology upon which the field of synthetic biology has been built.

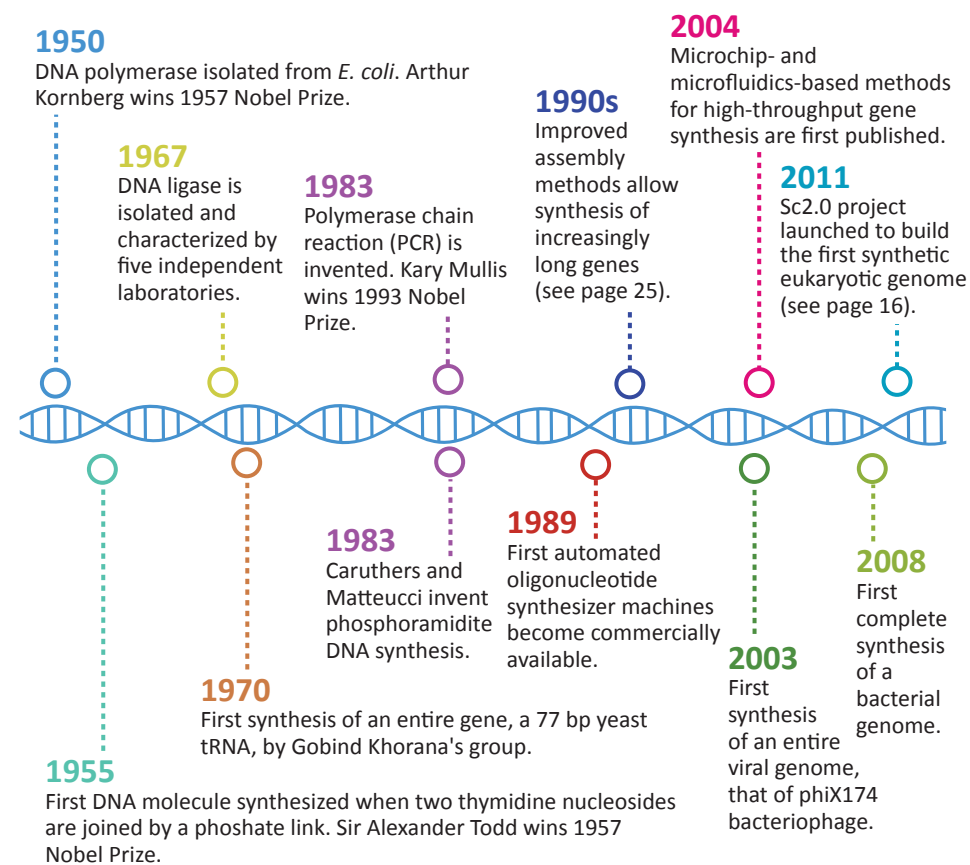
Any DNA sequence can be synthesized, including sequences that do not exist in nature, or variants on naturally-occurring sequences that would be tedious to produce through site-directed mutagenesis, such as codon-optimized sequences for increased heterologous protein expression. Synthetic DNA can be cloned into expression vectors and used in any protocol that requires natural or recombinant DNA. Synthetic genes are used to study all the diverse biological roles that nucleic acids play, from encoding proteins and regulating gene expression in the nucleus, to mediating cell-cell communication and building biofilms from extracellular DNA.

Gene synthesis technologies have revolutionized biology research. Scientists are no longer limited to classical methods of manipulating a single gene at a time; they now have the power to design or reprogram entire genomes and cells. We can quickly synthesize newly identified viral genomes to accelerate vaccine development. We can engineer novel enzymes that fight cancer and produce sustainable biofuels. We can improve crop yield and reduce vulnerability to the common pests and plant diseases that endanger food supplies and contribute to global hunger. We can detect and break down environmental pollutants in the soil, air and water. We can build designer metabolic circuits using interchangeable synthetic parts. We can create synthetic genomes and artificial cells to better understand the basic requirements of life. And life science researchers across many disciplines can do more sophisticated experiments, in less time, on smaller budgets, accelerating advances in healthcare, agriculture, energy, and other fields of human endeavor.

Methods for *de novo* chemical synthesis of DNA have been refined over the past 60 years. Synthetic short oligonucleotides (oligos) serve as custom primers and probes for a wide variety of applications. Longer sequences that serve as genes or even whole genomes can be synthesized as well; these sequences are typically produced by synthesizing 40-200 bp oligos and then assembling them in the proper order. Many methods for oligo assembly have been developed that rely upon a DNA polymerase enzyme for PCR-based amplification, a DNA ligase enzyme for ligation of oligos, or enzymes that mediate homologous recombination *in vitro* or *in vivo*. Most sequences up to 1000 base pairs (1 kb) can be assembled in a standard molecular biology lab, and commercial gene synthesis providers routinely synthesize sequences over 10 kb.

The history of gene synthesis began in 1955, when Sir Alexander Todd published a chemical method for creating a phosphate link between two thymidine nucleosides, effectively describing the first artificial synthesis of a DNA molecule.<sup>1</sup> The first successful synthesis of an entire gene was reported by Gobind Khorana's group in 1970; the 77 bp DNA fragment took 5 years to synthesize.<sup>2</sup> Subsequent improvements in DNA synthesis, sequencing, amplification, and automation have made it possible now to synthesize genes over 1 kb in just a few days, and to synthesize much longer sequences including entire genomes. Gene synthesis can now be easily and cost-effectively outsourced to commercial providers. ***GenScript, a pioneer in gene synthesis, was founded in 2002 and is the largest gene synthesis supplier in the world.***

Figure 1: Timeline of Gene Synthesis Technology Development



## What can be synthesized?

Gene synthesis can generate recombinant, mutated, or completely novel DNA sequences without a template, simplifying the creation of DNA tools that would be laborious to produce through traditional molecular cloning techniques. A wide variety of types of sequences can be produced to aid in diverse research applications (See Table 1). In addition to DNA sequences, RNA and oligos containing modified bases or chimeric DNA-RNA backbones can also be synthesized. However, the most widely used synthetic sequences are customized DNA of the following types:

**cDNA** corresponding to the **open reading frames (ORFs)** of genes can be synthesized for overexpression or heterologous expression. Synthesized genes can encode either naturally occurring transcripts or custom-designed variants such as point-mutants or fluorescently tagged reporters. Gene synthesis accelerates the creation of **recombinant coding sequences** such as fusion proteins or **promoter-reporter constructs** compared to traditional molecular subcloning and mutagenesis techniques. Protein expression levels can be greatly improved through codon optimization (see page 10), which is simple to perform as part of synthetic gene design but practically impossible to perform using traditional recombinant DNA technology.

**Genomic DNA** can be synthesized for the study of gene regulatory elements, mRNA processing, or to create artificial genes or genomes. Synthetic genomic DNA can be studied *in vitro*, inserted into host cells in culture, or used to create transgenic animal lines. Conventional gene targeting of embryonic stem cells with disruption cassettes inserted through restriction enzymes<sup>3</sup> or the Cre-loxp system<sup>4</sup> can be made simpler, more rapid, and less expensive by using synthetic DNA in combination with newly developed methods for genome editing such as CRISPR/Cas-9 nucleases, zinc-finger nucleases (ZFNs) or Transcription Activator-Like Effector Nucleases (TALENs).<sup>5</sup>

**RNA interference (RNAi)** technology can be used to suppress gene expression. Small interfering RNA (siRNA) sequences of 21-25bp block gene expression by targeting complementary sequences within mRNA for degradation or sequestration. siRNA can be synthesized as RNA oligonucleotides annealed to form an RNA duplex, but these molecules are short-lived and often difficult to deliver into cells. Vector-based DNA constructs that encode short hairpin RNA (shRNA) are easier to deliver through viral transduction or plasmid transfection or electroporation, and can stably express shRNA under the control of inducible or tissue-specific promoters. Upon transcription, shRNA sequences form a stem-and-loop structure that is cleaved to produce the biologically active siRNA. siRNA-mediated gene knock-down enables

the study of genes whose complete knock-out would be lethal, and when combined with lentiviral delivery, vector-based siRNA can cost-effectively generate stable cell lines or transgenic animals.

*GenScript's OptimumRNA™ Design tools aid in selecting target sequences and designing scrambled negative control constructs for vector-based RNAi.*<sup>6</sup>

**Extracellular DNA** has recently been shown to play important roles in innate immune responses<sup>7</sup>, thrombosis<sup>8</sup>, cancer metastasis<sup>9</sup>, biofilm formation<sup>10</sup> and other biological functions. Although we normally think of DNA as simply the blueprint specifying the construction of enzymes which do the work of living cells, nucleic acids are in fact versatile biomolecules whose many roles are still being elucidated. Gene synthesis technology can fabricate any DNA sequence to study its roles both inside and outside of the cell.

**Table 1: Types of Synthetic DNA and their Research Applications**

Types of Synthetic DNA	Research Applications
cDNA / ORFs	Over-expression or heterologous expression
Customized coding Sequences	Expressing fusion proteins; high-level protein expression from codon-optimized sequences for purification for enzymatic or structural studies
Promoter-reporter constructs	Monitoring gene expression downstream of manipulations to transcription factors, signaling cascades, etc.
Genomic DNA	Creating synthetic genes or genomes; studying gene structure, regulation, and evolution.
Mutant sequences	Confirming function (promoter-bashing; amino acid substitutions); Protein engineering (rational design or screening/directed evolution)
RNAi constructs (shRNA, miRNA, siRNA)	Regulating (suppressing) gene expression; intercellular communication
Extracellular DNA	Biofilm formation, intercellular signaling as in cancer metastasis



## Gene Synthesis and Molecular Cloning

The *de novo* chemical synthesis of DNA differs from traditional molecular cloning in that it does not require a template. Gene synthesis allows researchers to specify a desired sequence and custom-build it directly. Gene synthesis is frequently far more straightforward, faster, and less costly than using alternative methods of molecular cloning and mutagenesis.

Researchers experienced with molecular cloning know that, despite improvements over the past several decades in recombinant DNA tools tools, such as enzymes and cloning vectors, getting the clone you want is hardly a fool-proof endeavor. Even the seemingly simple task of isolating a gene using PCR cloning or restriction digestion can be tedious and error-prone depending on the sequence and its context in the source material. Numerous cDNAs predicted by genomic or partial cDNA information have proven difficult to clone but simple to synthesize. Recombining several gene cassettes to form fusion proteins can create even more challenges in selecting unique cutters, performing iterative digests and ligations, and ensuring that all assembled portions maintain the correct open reading frame.

Once molecular cloning is successful, constructs designed for heterologous expression often yield protein levels so low that it becomes difficult to do the experiment for which the construct was designed. Codon optimization can overcome this challenge by modifying a sequence to promote efficient heterologous expression (for more details, see p 10). Codon optimization can be performed prior to *de novo* synthesis to yield higher levels of protein expression and facilitate the study of function; this is not possible with traditional molecular cloning which preserves a template found in nature.

Gene synthesis can be combined with molecular cloning to make the synthetic products ready to use in research applications. Placing synthetic genes into a plasmid vector offers several advantages: it protects the DNA against degradation during storage; it allows complete sequence verification using primer sites on the vector that flank the gene insert; it facilitates clonal amplification in a transformation-competent bacterial host; and it facilitates shuttling of the synthetic gene into expression vectors for transfection or electroporation into host cells of interest for transient or stable expression.

Bare DNA fragments can be used directly in certain applications. For example, 10-40bp oligos are used as primers in PCR. Longer sequences may be used as templates for radiolabelled probe synthesis for *in situ* hybridization or other techniques. *In vitro* transcription and translation protocols employ bare DNA fragments as well.

**Table 2: Comparing Gene Synthesis and Molecular Cloning**

Technique	Features
Traditional Molecular Cloning	<ul style="list-style-type: none"><li>• limited to sequences that appear in nature</li><li>• codon optimization is not feasible</li><li>• time- and resource-intensive for end-user</li></ul>
Gene Synthesis of Bare DNA Fragments	<ul style="list-style-type: none"><li>• customizable sequence—no template required</li><li>• error-prone; yields mixed pools of variants</li><li>• time consuming to verify sequences</li><li>• lower cost for “quick and dirty” high-content, high-throughput screens</li></ul>
Gene Synthesis with Subcloning into a Plasmid Vector	<ul style="list-style-type: none"><li>• customizable sequence—no template required</li><li>• uniformly accurate sequences from a clone</li><li>• easy to verify complete sequence using primers that flank gene insert</li><li>• fast and cost-effective</li></ul>

*GenScript delivers all gene synthesis products inserted into the plasmid vector of your choice according to your preferred cloning strategy. Sequence chromatograms covering the entire gene insert provide assurance of 100% sequence accuracy for every gene synthesis order.*

## Why is Gene Synthesis a revolutionary tool for biology research?

Gene Synthesis forms the foundation of the new field of synthetic biology. Gene synthesis is also accelerating research in well-established research fields by providing critical advantages over more laborious traditional molecular cloning techniques. *De novo* gene synthesis is required when template DNA molecules are not available, such as for codon-optimized sequences. In addition, it is frequently a cost-effective alternative to traditional molecular cloning techniques, such as when multiple DNA segments are being recombined. The case studies below illustrate the power of customizable design afforded by gene synthesis.

**Table 3: Gene Synthesis Powers Novel Findings Across Research Disciplines**

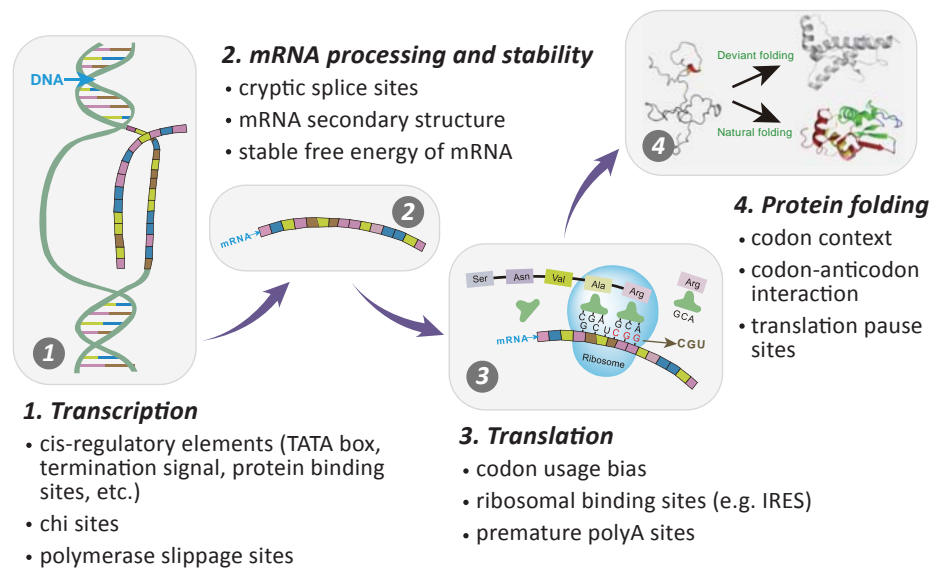
<p><b>Cancer</b></p>	<p><b>Synthetic mutants reveal a novel mechanism for cancer cell metabolic regulation.</b><sup>11</sup> By synthesizing mutated pyruvate dehydrogenase (PDH) genes, O'Mahoney <i>et al.</i> demonstrated a novel phosphorylation of PDH by AMP kinase that mediates breast cancer cell adaptation to varying glucose availability in the tumor microenvironment, and identified PDH inhibition as a new potential therapeutic strategy for breast cancer.</p>
<p><b>Structural Biology</b></p>	<p><b>Structure reveals novel antigenic site for respiratory syncytial virus (RSV), allowing rational design of vaccine antigens.</b><sup>12</sup> Using synthetic codon-optimized heavy and light chains of antibodies that bind the RSV F glycoprotein in its prefusion state, McLellan <i>et al.</i> obtained the first crystal structure of the metastable prefusion conformation of RSV F. This structural characterization allowed them to identify a novel antigenic site, termed "site Ø". Antibodies targeting site Ø are extremely potent, and hold promise to reduce the morbidity and mortality of this ubiquitous childhood virus.</p>

<p><b>Vaccines/ Virology</b></p>	<p><b>Synthetic modified viral sequences produce safer, more effective DNA vaccines.</b><sup>13</sup> Diniz <i>et al.</i> report that codon optimization augmented both the immunogenicity and the therapeutic anti-tumor effects induced by a DNA vaccine targeting human papillomavirus (HPV)-induced tumors. At the same time, introducing specific point mutations into a viral sequence, such as the modification of the E7 protein sequence found in HPV, can abolish residual oncogenic effects rendering the vaccine less likely to cause harm.</p>
<p><b>Neuroscience</b></p>	<p><b>A novel channelrhodopsin variant allows non-invasive optical activation of deep-brain neurons.</b><sup>14</sup> Lin <i>et al.</i> engineered a novel channelrhodopsin (ChR) variant that, when expressed in the brain, can be transcranially stimulated with red light to depolarize neurons, with improved improved membrane trafficking, higher photocurrents and faster kinetics compared to prior ChRs. Several variants of the channelrhodopsin genes, which naturally occur in algae, were codon-optimized for mammalian expression and synthesized to produce this widely applicable new tool for manipulating neuronal activity.</p>
<p><b>Plant Biology</b></p>	<p><b>A fungal plant pathogen induces host cell transdifferentiation and <i>de novo</i> xylem formation.</b><sup>15</sup> In order to study <i>Verticillium</i> -induced developmental reprogramming of the vascular tissues of host plants (including the economically important <i>Brassica</i>), Reusche <i>et al.</i> used a synthetic gene construct containing a pathogen-inducible promoter driving expression of a transcription factor fused to a strong repression motif. This study reveals the molecular mechanism by which plants compensate for detrimental vein clearance caused by common fungal pathogens, and suggests a way to enhance drought stress resistance.</p>

## Codon Optimization to Increase Protein Expression

In order to characterize a gene's function or purify a protein for structural study, researchers frequently need to express a gene in a host cell or model organism different from the one in which it appears in nature. Unfortunately, heterologously expressed genes often suffer from low rates of protein expression. A common cause of low expression levels is the variation in codon usage frequency between different species; this can cause the translation of heterologously expressed genes to stall due to tRNA scarcity, leading to lower protein levels and increased rates of improper protein folding. The degeneracy of the genetic code makes it possible to change the DNA sequence in a way that does not alter the final amino acid sequence, but does have significant effects on the efficiency of transcription, mRNA stability, translation, and protein folding. Aside from codon usage bias, many other features of a DNA sequence affect the efficiency of transcription, the proper splicing and processing of mRNA, and mRNA stability, all of which in turn reduce the ultimate protein yield (Figure 2).

**Figure 2: DNA Sequence Features that Influence Protein Expression Levels**



Modifying a DNA sequence to optimize it for efficient heterologous protein expression while preserving the amino acid sequence is called **codon optimization**. In a study by Burgess-Brown *et al.*<sup>16</sup> that investigated the expression of 30 human genes in *Escherichia coli*, codon optimization increased both the total protein yield and the protein solubility, compared to heterologous expression of native gene sequences. Codon optimization algorithms have improved since this 2008 study, and codon optimization is now widely used by researchers to facilitate the purification of protein for structural studies, enzyme kinetics, and other biochemical investigations.

*GenScript's OptimumGene™ algorithm, patented in 2012, optimizes sequences for over a dozen features known to influence expression, resulting in up to 100-fold higher protein yield than the native sequence and up to 50-fold better results than other codon optimization design tools.*

Functional studies of cell signaling can also benefit from codon optimization. For example, When Yoo *et al.* set out to investigate the physiological role of Lyn, a SRC family kinase, in a zebrafish model of wound healing, they needed to express the Lyn protein in a tractable in vitro cell-based model system for reductionist functional studies. They found that the native zebrafish Lyn sequence was poorly expressed in the HEK293 human cell line, so they turned to GenScript to synthesize a humanized, codon-optimized Lyn sequence. This synthetic construct yielded sufficient heterologous protein expression levels to allow characterization of the Lyn protein. Subsequent experiments identified Lyn as a redox sensor that initiates neutrophil recruitment to wounds. This finding was published in *Nature* and is exciting not only for revealing basic mechanisms of wound healing but also for providing insight on immunotherapeutic strategies for targeting tumors, which are a type of 'unhealed wound' that release H<sub>2</sub>O<sub>2</sub> and thus may be subject to regulation by Lyn.<sup>17</sup>

High-level protein expression is important not only for basic research but also for translational research and drug development, for example, by enhancing the immunogenic response of DNA vaccines. By overcoming the problem of insufficient heterologous expression levels, codon optimization serves as a valuable tool for life science research.

Codon optimization exemplifies the flexibility afforded by gene synthesis. While it is possible to overcome the limits of codon usage bias by expressing the tRNAs that correspond to rare codons, this approach does not address other optimizable features of the gene sequence, and it introduces the confound of altering the translational machinery that regulates all endogenous protein turnover in the cell. It is also possible to achieve codon optimization through site-directed mutagenesis, but given the high number of nucleotide alterations typically desired, this technique is extremely laborious and costly compared to *de novo* gene synthesis.

## Mutagenesis

Gene Synthesis facilitates the discovery or design of new protein variants (mutants) with properties desired for medical, industrial, agricultural, or other applications.

Traditional methods for inducing random mutations include subjecting live cells or animals to UV radiation or chemical mutagens in order to induce DNA damage. DNA repair enzymes can, in some cases, introduce or preserve mutations resulting from damage such as single- or double strand breaks, but this method leads to high rates of lethality and low rates of useful mutant phenotypes. Depending on the type of mutagen used, mutations may be biased, limiting the range of possible mutants to only a fraction of those that are theoretically possible or that can be obtained through other strategies.

Site-directed mutagenesis may be designed based on experimental data such as X-ray crystallography data that identifies residues of interest. Site-directed mutagenesis can be performed using PCR-based methods with mutated primer sequences to create and selectively amplify mutated sequences. This type of mutagenesis must be followed by sequence verification, and can be tedious, error-prone, and costly compared to *de novo* chemical synthesis of the desired mutant sequences.

DNA libraries containing many mutant sequences can be used for unbiased screens to identify novel sequences with desired structural or functional properties. If researchers have a functional assay for a desired protein activity, they can screen a mutant library, sequence the best-performing candidates, and use these sequences to produce new mutant libraries for iterative rounds of screening and improvement. This strategy, called directed evolution, allows researchers to optimize a protein's function even without understanding its mechanistic basis.<sup>18</sup> The mutagenesis strategy and technical method selected critically determine the likelihood of obtaining mutants with the desired properties. Well-prepared libraries can be engineered to contain unbiased point mutations, to maintain the open reading frame when insertions or deletions are present, and to avoid truncations.

*GenScript can deliver sequence-guaranteed mutants of up to 1 kb in length within two weeks. Using standard techniques for site-directed mutagenesis, sequence verification, and error correction in a typical biology research lab can take more than twice as long.*

Gene synthesis can create small or large numbers of systematic or random mutants more efficiently than traditional mutagenesis methods. Carefully designed mutant sequences or libraries can be produced with greater accuracy through gene synthesis than through other methods.

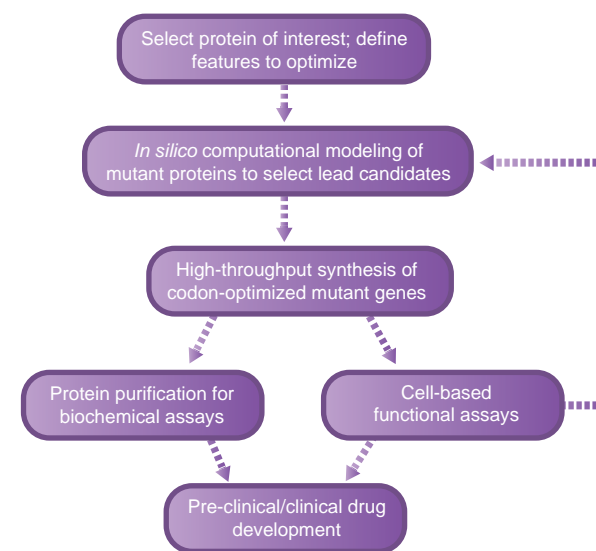
### Case Study: High-Throughput Mutant Gene Synthesis to Accelerate Drug Development

**Challenge:** Speed up R&D process for developing enzyme-based therapeutics.

**Solution:** Synthesize many mutant sequences for rapid screening.

A small company was founded by scientists whose goal is to engineer novel biologic drugs, such as antibodies and growth factors for oncology and immunology, enzyme replacement therapies for genetic disorders, and myriad other therapeutic proteins.<sup>19</sup> The company's expertise is in computational modeling of protein dynamics to design new or improved enzymes. Their virtual simulations allow them to predict which mutations will enhance protein stability, substrate affinity, enzymatic activity, or other key features that contribute to drug efficacy.

**Figure 3: Process for Engineering and Validating Novel Therapeutic Proteins**





To validate their *in silico* mutagenesis, they need to synthesize the mutated sequences, express the proteins for biochemical characterization, and perform cell-based functional assays. Promising candidates can then be licensed to pharmaceutical companies that have the infrastructure to follow through with pre-clinical and clinical trials, bringing new drugs to patients in need. Gene synthesis allows high-throughput, fast, accurate construction of mutant sequences that are then cloned into expression vectors. Gene synthesis also facilitates high-level protein expression through host cell-specific codon optimization, allowing rapid purification of sufficient quantities of protein for biochemical characterization. ***By outsourcing to GenScript, the industry leader in capacity and total automation of gene synthesis, this biotech client was able to speed up their R&D process, meet the deadlines from their pharmaceutical partners, and secure more funding from venture capital investors.***

## Synthetic Biology / Bioengineering

Bioengineers view genes, and even entire metabolic circuits in cells, as machines made up of parts that play standardized functional roles. Once these parts are identified, they can be treated as interchangeable, and rearranged in any way to create new and improved machines. While traditional molecular cloning techniques allow the creation of knock-in genes and fusion proteins (after several steps of digestion, PCR amplification, assembly, and verification), *de novo* gene synthesis streamlines the production of new biomolecular machines with customized sequences.



**Synthetic Biology**  
based on standard parts

***The International Genetically Engineered Machine competition (iGEM) is the premiere Synthetic Biology competition, with separate divisions for high school students, undergraduates, and researchers/entrepreneurs. Teams use standardized biological parts, and new parts of their own design, to build biological systems and operate them in living cells. GenScript is proud to sponsor iGEM teams and to provide gene synthesis services that allow competitors to bring their designs to life!***

## Metabolic Engineering

Metabolic engineering enables the efficient production of naturally occurring compounds that have valuable research, clinical or industrial uses. Before gene synthesis technologies existed, natural products were typically obtained either from plant extracts or from total chemical synthesis of the desired compound. In the case of some compounds, purification from natural sources can be problematic due to the need to resolve complex mixtures of closely related compounds, and our current best methods for chemical synthesis are limited by low yield and low specificity requiring additional painstaking purification. As an alternative approach, metabolic engineering allows biosynthetic pathways to be reconstructed in model organisms (typically microbiological hosts such as *Escherichia coli*) so that useful quantities of the desired product can be harvested.

In one example of metabolic engineering to improve synthesis of a desired biomolecule, Brazier-Hicks and Edwards developed a method for efficient production of C-glycosylated flavanoids for dietary studies by using gene synthesis to re-engineer a metabolic circuit in yeast.<sup>20</sup> They designed synthetic variants of five genes that comprise the flavone-C-glycoside pathway in rice plants, which were subsequently codon-optimized for expression in yeast. These synthetic genes were used to construct a polyprotein cassette that expresses the entire metabolic circuit in a single step.

Metabolic engineering has also been used to create new methods for alternative energy from unconventional sources. Dellomonaco *et al.* showed that fatty acids can serve as biomass for sustainably produced biofuels by using gene synthesis to engineer several native and heterologous fermentative pathways to function in *E. coli* under aerobic conditions.<sup>21</sup> These synthetically engineered bacteria convert fatty acid-rich feedstocks into desirable biofuels (ethanol and butanol) and biochemicals (acetate, acetone, isopropanol, succinate, and propionate), with higher yield than more widely used lignocellulosic sugars.

## Case Study: A New Way to Create Transgenic Animal Lines

**Challenge:** Expand the power of CRISPR/Cas9 nuclease-based genome editing to produce heritable changes in *Caenorhabditis elegans*.

**Solution:** Synthesize novel Cas9 and sgRNA vectors that can be used to efficiently create new transgenic strains.

Transgenic animal models serve as critical tools for many arenas of life science and medical research. CRISPR/Cas9 nuclease-based genome editing is the most recent addition to the repertoire of methods for genetic modification, which also includes TALEN, ZFN, and Cre-lox technologies. Friedland *et al.* sought to optimize the Cas9 gene so it could be used to produce heritable changes in *C. elegans*.<sup>22</sup>

Gene synthesis allowed Friedland and colleagues to boost heterologous expression of the bacterial Cas9 in a *C. elegans* host through codon optimization. It also enabled fast, error-free recombination of the multitude of critical sequences: plasmid vectors to allow efficient delivery into cells, promoters to drive efficient transcription, a nuclear localization sequence fused to the end of the Cas9 sequence to allow it to access the host's nuclear DNA, the sgRNA scaffold to allow precise targeting, and the target sequence they wished to introduce.

These optimized synthetic vectors can be used by other researchers in the future to efficiently bioengineer their own transgenic worm strains, simply by altering the target and sgRNA scaffold sequence to correspond to their gene of interest. The target sequence can include any mutation, insertion, or deletion the researcher desires. **GenScript can synthesize Cas9 and sgRNA sequences to enable efficient genome editing in any host.**

## Case Study: The Synthetic Yeast Genome Project

**Challenge:** Create a functional synthetic genome to support a living yeast cell.

**Solution:** Synthesis of 10 kb chunks for assembly into entire chromosomes.

The Sc2.0 Project, led by Dr. Jef Boeke at the Johns Hopkins University, is the first attempt to synthesize a eukaryotic cell genome, that of *Saccharomyces cerevisiae*. The goal of the Sc2.0 Project is to synthesize the entire yeast genome—about 6,000



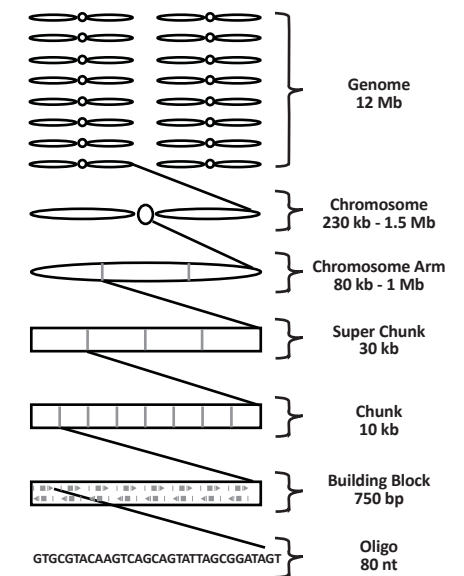
genes— with a built-in diversity generator that will enable researchers to discover how yeast, as a model organism, deals with genetic change and how genomes might be improved to create more robust organisms. This project lays the foundation for future work to design genomes for specific purposes, such as creating new medications or biofuels.

To facilitate gene rearrangement and genome minimization, Boeke and colleagues designed the Synthetic Chromosome Recombination and Modification by LoxP-mediated Evolution (SCRaMbLE) system.<sup>23</sup> More than 5000 loxP sites will be introduced in the final genome, so that pulsatile Cre expression can create innumerable unique deletions and rearrangements. Viable genomes can be sequenced and many variant yeast strains can be studied to deduce the limits of chromosome structure, minimal eukaryotic genome structures, and gene/feature adjacency rules in genomes.

In 2011, the first designer yeast chromosome arm, synIXR, was successfully synthesized and shown to support robust yeast cell growth.<sup>24</sup> To construct the remainder of the synthetic genome, incorporating SCRaMbLE and other designer features, requires DNA synthesis on an unprecedented scale. To this end, in 2012 GenScript synthesized 17 DNA fragments, each ~10 kb in length to become part of synthetic yeast chromosome VI.

**GenScript is proud to be a contributing partner in the Sc2.0 International Consortium whose goal is to build a designer synthetic eukaryotic genome.**

**Figure 4: Building a synthetic genome through assembly of smaller parts**



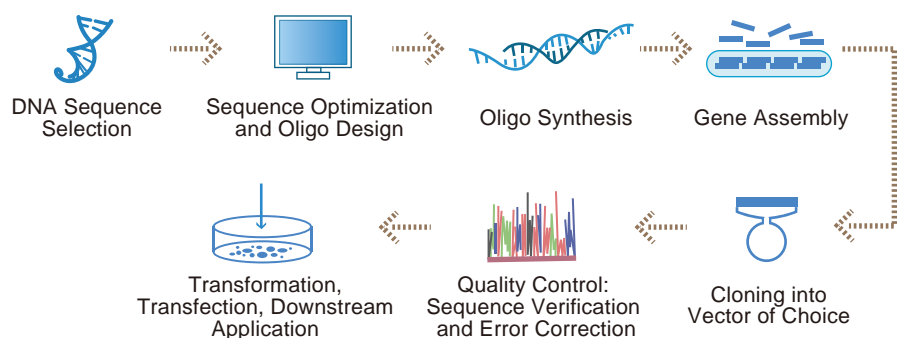
Reprinted, with permission, from Dymond, J. & Boeke, J. The *Saccharomyces cerevisiae* SCRaMbLE system and genome minimization. *Bioengineered Bugs* 3, 5–4 (2012).

## How is Gene Synthesis Performed?

The basic steps of gene synthesis are:

1. sequence optimization and oligo design
2. oligo synthesis
3. gene assembly
4. sequence verification and error correction
5. preparing synthetic DNA for downstream applications

Figure 5: Steps in Gene Synthesis



### Step 1: Sequence Optimization and Oligo Design

#### Sequence Optimization

Once you have selected your gene of interest, you must design the sequence to be synthesized. Keep the end application in mind: for example, codon optimization is appropriate if your goal is to maximize heterologous protein expression levels, but it may not be appropriate if you want to study endogenous regulation of gene expression. For constructs containing multiple segments, make sure your intended reading frame is maintained throughout the entire coding region. Frequently you'll want to add short flanking sequences to facilitate later excision or recombination, via restriction enzymes or similar tools. Be sure your sequence does not include restriction enzyme recognition sites or other sequences that might interfere with your downstream workflow. Be aware of the presence of functional domains such

as *cis*-regulatory elements or RNase splice sites, which may be unintentionally introduced during codon optimization. The presence of biologically functional sequences such as *cis*-regulatory elements or RNase splice site on oligos may hinder *in vivo* assembly, maintenance, or expression.

*GenScript's patented OptimumGene™ algorithm provides free industry-leading sequence optimization for all gene synthesis projects. Other freely available codon optimization tools include JCat and OPTIMIZER.*

Some sequence features make synthesis more challenging, including: extremely high or low GC content; highly repetitive sequences; complex secondary structures such as hairpins; unstable structural elements; polyA stretches; and longer sequences, especially over 1 kb. These are all important features to consider when selecting and optimizing sequences to synthesize, and they will increase the cost and turnaround time of synthesis, whether it is performed in-house or outsourced to a commercial service provider.

#### Oligo Design

After finalizing the sequence that will be synthesized, sequence analysis is required to determine the best way to divide the whole gene into fragments that will be synthesized and then assembled. For very large synthetic genes, you will typically want to divide the complete sequence into chunks of 500-1000 kb to be synthesized separately and assembled later. Numerous oligo design software programs are available to assist in oligo design (See Table 4).

While synthesis platforms utilizing phosphoramidite chemistry have very low error rates, errors do accumulate as strand length increases; therefore, gene synthesis typically uses oligos with lengths of 40-200 bp. The optimal oligo length depends upon the assembly method that will be used, the complexity of the sequence, and the researcher's preferences. As a general rule, shorter oligos may have a lower error rate, but will be more expensive to synthesize because more overlaps will be required. Longer overlaps increase the likelihood of correct assembly by decreasing the rate of nonspecific annealing. Oligos should be adjusted for even lengths and equal melting temperatures. Each oligo design tool has its own default parameters; for example, GeneDesign uses default settings of 60 bp oligos with 20 bp overlaps, values which typically work well with yeast and mammalian sequences which are ~40% GC.

**Table 4: Oligo Design Tools**

Design Tool	Advantages	Limitations
<b>DNA Works<sup>25</sup></b> <a href="http://helixweb.nih.gov/dnaworks/">http://helixweb.nih.gov/dnaworks/</a>	Easy to use. Predicts the potential for oligo mishybridization and secondary structures.	Limited to PCR-based methods. Scores based on simulations may not correlate actual assembly success. <sup>26</sup>
<b>Gene2Oligo<sup>27</sup></b> <a href="http://berry.engin.umich.edu/gene2oligo/">http://berry.engin.umich.edu/gene2oligo/</a>	Simple user interface, designs oligos for both LCR and PCR-based assembly.	Limited to genes less than 1 kb in length.
<b>GeneDesign<sup>28</sup></b> <a href="http://baderlab.bme.jhu.edu/gd/">http://baderlab.bme.jhu.edu/gd/</a>	Breaks multi-kb sequences into ~500 bp blocks for initial assembly in separate pools.	Does not offer mishybridization analysis.
<b>TMPrime<sup>29</sup></b> <a href="http://prime.ibn.a-star.edu.sg">http://prime.ibn.a-star.edu.sg</a>	Designs oligos for both LCR- and PCR-based assembly. Produces the most homologous melting temperatures ( $\Delta T_M < 3^\circ\text{C}$ ) and widest range of annealing temperatures (50–70°C).	Requires thorough understanding of many input parameters for correct submission; less user-friendly interface.

In addition to oligo length, some factors to consider in selecting oligo sequences include GC content, sequence repeats, and the tendency for hairpin formation. GC content determines the stability of DNA strands and thus the melting temperature. All in vitro assembly methods, both ligase and polymerase based, rely upon the melting and annealing of oligos and thus require that oligos have equal melting temperatures. Repeated sequences may pose challenges for correct annealing or ligation of oligos during assembly. Repeats may occur in many forms, including direct, inverted, palindromic, or tandem repeats. Improper hybridization or intramolecular binding such as hairpin structure formation can be avoided through careful design of oligo sequences, including the overhangs or primers used to assemble them.

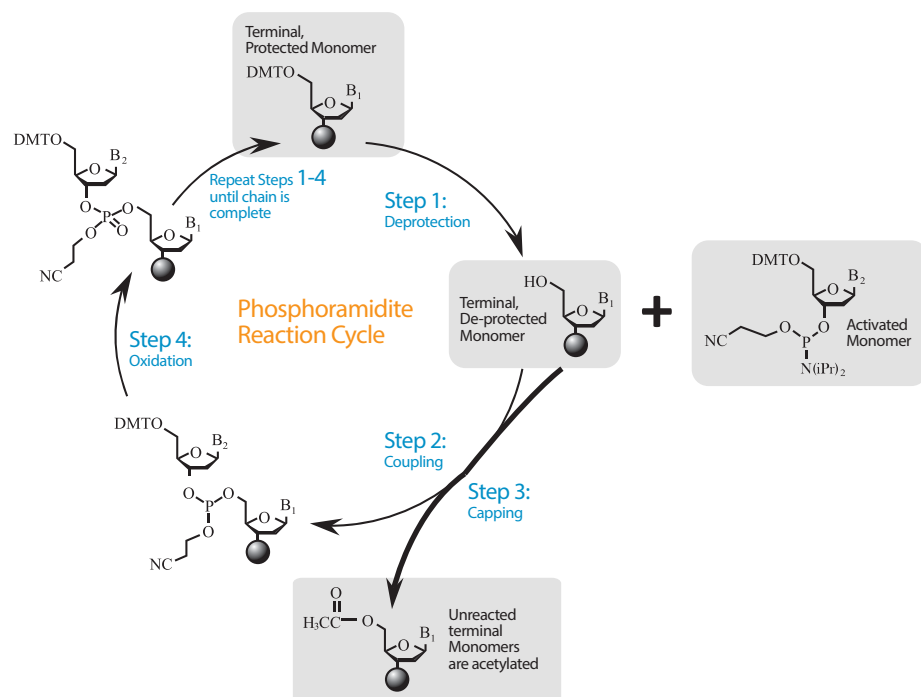
*GenScript's proprietary DSOpt™ Sequence Analysis tool, developed through years of experience as the volume leader in gene synthesis, takes the guesswork out of selecting the best assembly method and oligo design strategy for each project.*

## Step 2: Oligo Synthesis

All DNA fabrication today begins with the step-wise addition of nucleotide monomers via phosphoramidite chemistry to form short oligonucleotides. Oligo synthesis via phosphoramidite chemistry uses modified nucleotides, called phosphoramidites, to ensure that nucleotides assemble in the correct way and to prevent the growing strand from engaging in undesired reactions during synthesis. The phosphoramidite group is attached to the 3' O and contains both a methylated phosphite and a protective di-isopropylamine to prevent unwanted branching. Phosphite is used because it reacts faster than phosphate. Methyl groups are attached to the phosphite, and amino-protecting groups are added to the bases, to protect against unwanted reactions until oligonucleotide synthesis is complete. Although DNA synthesis in living cells always occurs in the 5' to 3' direction, phosphoramidite synthesis proceeds in the 3' to 5' direction. The first monomer is attached via its 3' O to a solid support such as a glass bead, and its 5' O is initially protected from nonspecific reactions by conjugation of a dimethoxytrityl (DMT) group.



**Figure 5: Phosphoramidite Reaction Cycle**



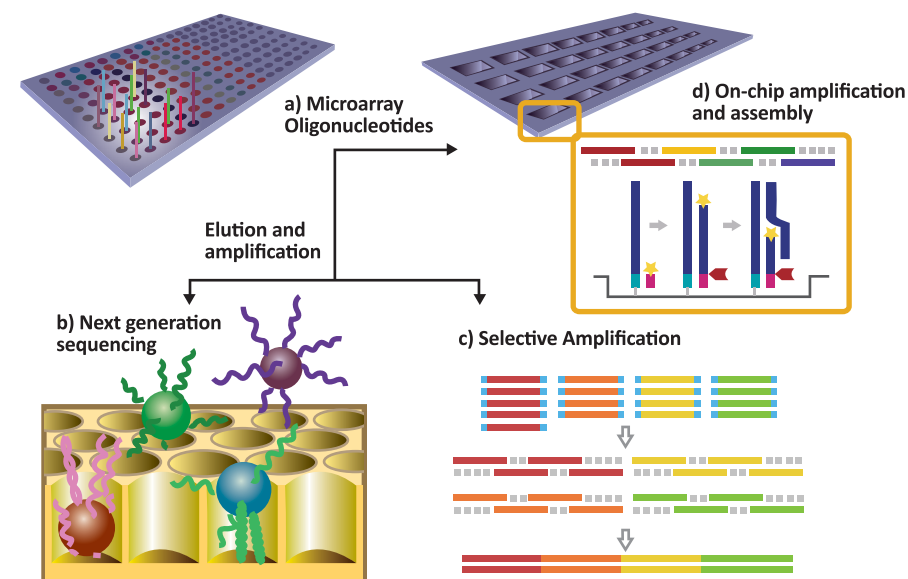
In **Step 1: Deprotection**, DMT is removed by washing with a mild acid such as trichloroacetic acid, exposing the 5' O for reaction. The second nucleotide is introduced to the reaction with its own 5' O protected with DMT, while its 3' O is activated by the conjugated phosphoramidite group. **Step 2: Coupling** occurs when the 3' O of the second nucleotide forms a phosphate triester bond with the 5' O of the first nucleotide. **Step 3: Capping** involves acetylating the 5' OH of any unreacted nucleotides to prevent later growth of an incorrect sequence. Acetic anhydride and dimethylaminopyridine are typically added to acetylate any unreacted terminal 5' OH groups, but will have no effect on terminal nucleotides that are protected by DMT. **Step 4: Oxidation** occurs upon the addition of iodine to convert the phosphate triester bond into the phosphodiester bond that forms the familiar backbone of DNA.

Once the desired chain is complete, all of the protecting groups must be removed, and the 5' end of the oligo is phosphorylated. Prematurely terminated strands can be removed by purifying the eluted product via gel electrophoresis and cutting out the band with the correct length. Further characterization of synthetic oligonucleotides may include sequencing or simply verifying the predicted molecular mass by mass spectrometry.

Oligo synthesis can be done in your lab in a column or plate format with the help of an automated synthesizer, such as a MerMade machine (BioAutomation). Alternatively, oligos may be cheaply and quickly synthesized by commercial vendors, including GenScript. Oligos serve not only as the building blocks to be assembled but also as primers to aid in assembly and PCR amplification of synthetic gene products.

New technologies are emerging to make high-throughput gene synthesis more rapid and affordable, through the use of microfluidics and microarray platforms (Figure 6).<sup>30</sup>

**Figure 6: High-throughput array-based gene synthesis technology**



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Microarray technologies require some method of spatially confining reagents and/or directing reactions with very fine spatial and temporal control. Such methods include: using inkjet printing to dispense picoliter reagents to specific locations on a silica chip; controlling the deblocking step with light-activated photochemistry in a microfluidics system; or using programmable microelectrode arrays to direct redox reactions at desired spots. In practice, these methods can create error through imprecision and can introduce new costs, such as the expense of manufacturing unique photomasks required for photolithography platforms.

Microarray oligo pools provide a cheaper source of oligos, but make gene assembly more challenging and error-prone.<sup>30</sup> To overcome this challenge, highly parallel and miniaturized methods, such as “megacloning,” are being developed that combine the error-prone synthesis of oligo pools with a high-throughput pyrosequencing platform to enable the identification and selective amplification of oligos with the correct sequence prior to assembly.<sup>31</sup> Careful design of oligos for hybridization selection, or of primers for selective amplification of correct sequences, can allow direct assembly from unpurified oligo pools. These emerging technologies will eliminate the expensive and time-consuming oligo purification steps once the design algorithms and array-based platforms become widely available.

### Step 3: Gene Assembly

Many methods of assembling oligos into complete genes or larger genome building blocks have been developed and successfully used (Table 5). For relatively short sequences (up to 1 kb), polymerase-based or ligase-based in vitro assembly methods are sufficient. For longer sequences, in vivo recombination-based methods may be preferred. Correct assembly requires a high-fidelity enzyme (e.g. DNA polymerase or ligase).

**Polymerase chain assembly (PCA)** is a standard technique for polymerase-based oligo assembly in a thermocycler. This reaction is also called templateless PCR. The principle is to combine all of the single-stranded oligos into a single tube, perform thermocycling to facilitate repeated rounds of annealing, extension, denaturation, and then use the outermost primers to amplify full-length sequences. The success of this method depends upon the accurate synthesis of oligos designed to possess sufficient regions of overlap, sufficiently similar melting and annealing temperatures, and minimal opportunities for mishybridization. Protocols for PCA to produce final sequences up to ~750 kb have been published.<sup>32</sup>

**Table 5: Published Methods for Assembly of Oligos into Synthetic Genes or Genomes**

Oligo Assembly Methods	Reference
<b>POLYMERASE-BASED</b>	
Dual-Asymmetric (DA) PCR	Sandhu <i>et al.</i> (1992) <sup>33</sup>
Overlap Extension (OE)	Horton <i>et al.</i> (1993) <sup>34</sup>
Polymerase cycling assembly	Stemmer <i>et al.</i> (1995) <sup>35</sup>
Asymmetric PCR	Wooddell & Burgess (1996) <sup>36</sup>
Thermodynamically-Balanced Inside-Out (TBIO)	Gao <i>et al.</i> (2003) <sup>37</sup>
Two-Step (DA+OE)	Young & Dong (2004) <sup>38</sup>
Microchip-based multiplex gene synthesis	Tian <i>et al.</i> (2004) <sup>39</sup>
One-Step Simplified Gene Synthesis	Wu <i>et al.</i> (2006) <sup>40</sup>
Parallel microfluidics-based synthesis	Kong <i>et al.</i> (2007) <sup>41</sup>
Single Molecule PCR	Yehezkel <i>et al.</i> (2008) <sup>42</sup>
TopDown Real-Time Gene Synthesis	Huang <i>et al.</i> (2012) <sup>43</sup>
<b>LIGASE-BASED</b>	
Shotgun ligation	Eren & Swenson (1989) <sup>44</sup>
Two-Step Ligation and PCR	Mehta <i>et al.</i> (1997) <sup>45</sup>
Ligase chain reaction	Au <i>et al.</i> (1998) <sup>46</sup>
Brick-based	Kelly <i>et al.</i> (2009) <sup>47</sup>
<b>RECOMBINATION-BASED</b>	
Sequence- and Ligation-Independent Cloning (SLIC)	Li and Elledge (2007) <sup>48</sup>
Transformation-associated Recombination	Gibson <i>et al.</i> (2008) <sup>49</sup>
BioBrick assembly in <i>E. coli</i>	Ho-shing <i>et al.</i> (2012) <sup>50</sup>

**Ligase Chain Reaction (LCR)** uses a DNA ligase to join overlapping ends of synthetic oligos in repeated cycles of denaturation, annealing, and ligation. Using a thermostable enzyme such as *Pfu* DNA ligase allows for high-temperature annealing and ligation steps that improve the hybridization stringency and the likelihood of obtaining correct final sequences, which may offer an advantage over PCA. However, this method becomes inefficient as oligo number increases, limiting its usefulness to genes shorter than 2 kb.<sup>51</sup> Further, the use of standard-purity oligonucleotides can introduce point deletions resulting from incorporation of incorrectly synthesized oligos; this can be avoided by using gel-purified oligos, which increases costs substantially, or by performing side-directed mutagenesis to correct errors after assembly, cloning, and sequencing.<sup>55</sup>

**Sequence- and Ligation-Independent Cloning (SLIC)** is a method of in vitro homologous recombination employing a T4 DNA polymerase that allows the assembly of up to five gene fragments via simultaneous incorporation into a plasmid vector.<sup>47</sup> Synthetic oligos are prepared for SLIC by PCR extension to introduce flanking regions of sequence homology; this facilitates recombination of fragments without any sequence restrictions or the introduction of restriction enzyme sites that will produce permanent seams. The exonuclease activity of T4 DNA polymerase generates single-stranded DNA overhangs in the insert and vector sequences. Homologous regions are annealed in vitro and undergo gap repair after transformation into *E. coli*.

**In vivo homologous recombination in yeast** facilitates the assembly of very long synthetic DNA sequences either from “bricks” of 500-1000 bp produced through in vitro methods, or directly from short oligos. Gibson and colleagues have demonstrated that the yeast *Saccharomyces cerevisiae* can take up and assemble at least 38 overlapping single-stranded oligos and a linear double-stranded vector in one transformation event. These oligonucleotides can overlap by as few as 20 bp and can be as long as 200 nucleotides in length to produce kilobase-sized synthetic DNA molecules. A protocol for designing the oligonucleotides to be assembled, transforming them into yeast, and confirming their assembly has been published.<sup>52</sup>

## Step 4: Sequence Verification and Error Correction

Due to the inherent potential for error in each step of gene synthesis, all synthetic sequences should be verified before use. Sequences harboring mutations must be identified and removed from the pool or corrected. Internal insertions and deletions, as well as premature termination, are common in synthetic DNA sequences. The accumulation of errors from phosphoramidite chemical synthesis alone can lead to only about 30% of any synthesized 100-mer being the desired sequence.<sup>53</sup> Improper annealing during oligo assembly can also introduce heterogeneity in the final pool of synthetic gene products.

Cloning newly synthesized sequences into a plasmid vector can simplify the process of sequence verification. Sequencing primers that bind to vector regions flanking the gene insert ensure correct sequencing of the ends of the synthesized gene insert. Further, plasmid DNA can be clonally amplified to create a homogeneous pool of DNA with the correct sequence.

In the event that the correct sequence cannot be obtained and amplified from the pool of synthesized DNA, numerous methods for error detection and correction have been developed and successfully used. These include stringent hybridization using carefully designed oligos; exhaustive purification using electrophoresis, mass spectrometry and other biochemical methods; mismatch-binding or mismatch-cleavage using prokaryotic endonucleases; selection of correct coding sequences via functional assays; and site-directed mutagenesis after sequencing.<sup>53, 54</sup> PAGE or agarose gel purification is the technique in longest use, but it is costly and labor intensive and does not identify or correct for substitutions or for small insertions/deletions. Enzyme-based strategies are limited by the enzyme’s capabilities; for example, the widely used *E. coli* MutHLS is not very effective for substitutions other than G-T, A-C, G-G, A-A.<sup>54</sup> Site-directed mutagenesis after sequencing can introduce point mutations using mutant primers and high-fidelity DNA polymerase followed by selection for unmethylated molecules, but can be an unwieldy technique. Because error correction can be so time-consuming and costly, especially for long or complex sequences, efforts continue to improve the accuracy of oligo synthesis and assembly.

*GenScript’s Gene-on-Demand® gene synthesis platform enables mutation-free DNA synthesis for genes of any length and any complexity. Sequence chromatograms are delivered with all gene synthesis orders, and 100% sequence accuracy is always guaranteed!*

## Step 5: Preparing Synthetic DNA for Downstream Applications

### Cloning

For most applications, synthetic genes need to be cloned into appropriate vectors. These may include plasmid vectors for transfection or electroporation into cells, or viral vectors (such as adenovirus, retrovirus, or lentivirus) for transduction into cells or live animals.

To facilitate cloning, synthetic genes may be designed to include restriction enzyme sites, recombination arms, or other flanking sequences. As an alternative to avoid introducing special flanking sequences, recombination-based methods begin with PCR extension of the gene insert to introduce 15 bases of sequence homology to the linearized vector, facilitating homologous recombination without adding unwanted bases.

*GenScript's CloneEZ™ recombination method uses a high-fidelity enzyme to produce directional cloning of any gene into any position in any vector in only 30 minutes.*

### Propagation of Synthetic Genes

Propagation of synthetic gene constructs may be challenging for several reasons. Some “low copy number” plasmid constructs simply don't amplify well in commonly used bacterial propagation hosts. Long genes are often difficult to maintain and propagate because of the energetic burden on the host cell. Some genes may alter the physiology of their hosts, creating aberrant culture temperature requirements or other conditions that may be difficult to identify and accommodate. Genes may be toxic or unstable in certain host cells but not others. Screening a number of cell lines to find the best host for propagation can be time consuming, but may be necessary for certain difficult sequences.

*GenScript's Elite System™ Host Cell Pool can determine the best bacterial host for propagating difficult genes and deliver a vial of these cells along with the purified plasmid DNA.*

### Biosafety

Throughout the history of genetics research, numerous regulatory boards have addressed the concerns of researchers, citizens, and political leaders regarding the potential for genetic technologies to be used for harm. In 1974, the NIH formed a

Recombinant DNA Advisory Committee (RAC) to review recombinant DNA safety protocols and address public misgivings about newly acquired scientific and technological powers to manipulate genetic materials. Although gene synthesis allows revolutionary flexibility for researchers, it poses no qualitatively different risks than those that have already been regulated for almost half a century since the advent of recombinant DNA technology.



GenScript is proud to be a founding member of the International Gene Synthesis Consortium (IGSC), whose member companies represent more than 90% of the worldwide gene synthesis capacity. This group works closely with the research community and the Presidential Commission on the Study of Bioethical Issues to evaluate and manage biosafety risks. The IGSC

has developed industry-wide protocols to screen all synthetic gene orders against databases maintained by national and private agencies to identify regulated pathogen sequences and other potentially dangerous sequences in order to prevent their misuse.

## The Future of Gene Synthesis

Gene synthesis technology has revolutionized both our understanding of how DNA functions as the blueprint of life and our ability to manipulate DNA for experimental, medical, and industrial purposes. While the capabilities and speed of gene synthesis have steadily increased, its cost has dropped from \$10 per bp to \$0.35 per bp over the last decade, in parallel with advances in DNA sequencing and chip-based bioassays, and consistent with Moore's Law. Further improvements in automation, error correction, and cost-effectiveness will allow gene synthesis to become a valuable tool for a widening range of scientific disciplines and economically important applications. The energy and agriculture industries will increasingly rely upon gene synthesis to solve problems related to environmental protection and food supplies. DNA-based vaccines and bioengineered antibodies will become more widely used in medicine. Designer organisms housing customized metabolic pathways will become commonplace. Tinkering with the cellular circuitry for regulating biosynthesis including synthetic sensors and regulators will continue to be a focus of synthetic biology research. The potential environmental and social impacts of integrating synthetic genes into endogenous systems will kindle ongoing discussion by the research community, biotech industry, and international governmental and regulatory bodies. Without a doubt, the coming decade will present exciting opportunities for gene synthesis to continue to fuel innovation in the life sciences.



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# GenScript's Molecular Biology Service Portfolio

## Gene Synthesis

### Gene Synthesis

*Any DNA sequence you desire*

- Guaranteed 100% sequence fidelity
- Largest capacity at 7.5 million bp/month
- Free OptimumGene™ codon optimization

### Gene-Brick™ Synthesis

*New building blocks for synthetic biology*

- ~10 kb long building bricks
- 100% sequence fidelity guaranteed

### Express Gene Synthesis

*Speed comes from experience*

- 2 flexible options: Rush or Fast
- Delivery in as few as 4 business days
- Fastest turnaround in the market

### Custom Oligos

*Affordable modified/labeled oligos*

- Custom modifications and labeling
- DNA, RNA, or chimeric backbones
- Flexible synthesis scales

## Mutagenesis

### Site-Directed Mutagenesis

*Any variant from your design*

- Unparalleled accuracy
- Large constructs up to 12 kb

### Mutant Libraries

*You design it, we build it*

- Site-directed mutagenesis libraries
- Degenerated libraries
- Randomized libraries
- Customized libraries

### High-throughput Protein Variants

*Get 1,000 protein variants in 30 days*

- Industry-leading capacity
- Cost efficient: Save up to 70%
- Fully customized

## Cloning and Propagation

### GenePool™ ORF Cloning

*Any ORF in any vector*

- 2.5 million ORFs from 186 different species in the database
- ORF genes cloned into any vector of your choice

### Custom Cloning

*Any construct you want to build*

- CloneEZ® seamless cloning technology
- Reliable results with quality assurance

### Plasmid Preparation

*Any amount to fit your needs*

- Flexible scale up to gram level
- ≥ 95% Supercoiled DNA
- ≤ 0.005 EU/μg Endotoxin

### DNA Sequencing (North America)

*Rapid sequencing at low prices*

- 4 free reactions for new sequencing customers
- Bulk discount pricing available
- Convenient online ordering
- Secure online data retrieval

## Custom Vector Construction

### Targeting Vectors for KO/KI

*Fast, economical construction of tools for genetic modification*

- Plasmid and BAC vectors carrying genes up to 20 kb
- Conventional or conditional (inducible/tissue-specific) gene targeting
- Success rate over 95% with typical delivery in under 4 weeks

### Vector-Based RNAi

*Long-lasting, efficient mRNA inhibition in vivo*

- Second-generation siRNA design tools guarantee >75% knock-down efficiency
- Select from over 80,000 pre-designed siRNA sequences for human, mouse, and rat in our Genome-Wide Bio-Reagent Bank (GenPool)
- comprehensive library of miR vectors for pri-miRNA or pre-miRNA sequences