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# Postprint of DNA Synthesis, Assembly, and Transfer Technology

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## Abstract

As a recently and rapidly developing interdisciplinary discipline, synthetic biology has demonstrated increasingly broad application potential in fields such as biomedicine, energy, and new materials. Synthetic genomics, as an important research direction in synthetic biology, focuses on the de novo design and synthesis of novel life systems, supported by core technologies including DNA synthesis, assembly, and transfer. This article elaborates on current genome synthesis-related technologies to systematically understand the existing problems in this technical system, thereby facilitating the identification of breakthrough points and the seizing of development opportunities.

## Full Text

## Abstract

Synthetic biology, as a rapidly developing interdisciplinary field, has demonstrated increasingly broad application potential in biomedicine, energy, new materials, and other domains. Synthetic genomics, a major research direction within synthetic biology, focuses on the de novo design and synthesis of novel life systems, supported by core technologies including DNA synthesis, assembly, and transplantation. This article reviews current developments in genome synthesis technologies, aiming to systematically understand existing challenges in the technical framework, identify breakthrough opportunities, and inform future advances.

**Keywords:** synthetic biology, synthetic genomics, DNA synthesis, genome assembly, genome transplant

With advances in sequencing technologies and decreasing costs, an unprecedented number of species genomes have been sequenced, achieving remarkable

breakthroughs in the "reading" of biological genomes. Concurrently, developments in DNA synthesis and gene editing technologies have made "writing" biological genomes a reality. Genome editing and even whole-genome redesign and synthesis can serve dual purposes: as research tools to explore gene function and advance functional genomics, and as a means to obtain novel organisms for disease treatment, drug production, and other applications benefiting humanity. Whole-genome de novo synthesis represents a current hotspot in synthetic biology research, encompassing genome design, construction, and functional characterization from scratch—an inherently bottom-up biological research strategy.

## 1. DNA Synthesis Technologies

DNA synthesis technology forms the foundation of synthetic genomics and modern molecular biology. While PCR or enzymatic digestion can only obtain naturally existing DNA fragments, de novo DNA synthesis enables acquisition of artificially designed specific DNA fragments through oligonucleotide (Oligo) assembly. Improving Oligo synthesis length and efficiency while reducing costs represents a critical breakthrough for large-scale genome synthesis. Based on synthesis principles, current Oligo synthesis methods can be divided into the well-established and commercialized chemical method and the enzymatic synthesis method still under development.

### 1.1 Chemical Phosphoramidite Method

The synthesis of oligonucleotides dates back to the 1950s, with Michelson and Todd first achieving dinucleotide synthesis [1]. In the 1980s, Beaucage and Caruthers developed the phosphoramidite-based DNA synthesis method [2], which remains the primary approach for automated Oligo production today. This method comprises four steps: deprotection, coupling, capping (optional), and oxidation (Figure 1 [Figure 1: see original paper]). Due to decreasing chemical reaction efficiency, purity, and yield with chain extension, current Oligo synthesis length is generally limited to approximately 200 nucleotides (nt). To increase throughput, microarray chip-based DNA synthesis strategies developed since the 1990s have reduced synthesis costs by several orders of magnitude [3]. However, chip-specific non-uniformity and edge effects result in reduced length and accuracy compared to column-based synthesis. To enhance chip synthesis precision, Kosuri et al. [4] and Matzas et al. [5] employed different technical strategies in 2010 to select correctly synthesized oligonucleotides from chip products. Kosuri used a multiplex PCR strategy to selectively amplify target oligonucleotide fragments, combined with enzymatic error correction, enabling synthesis of oligonucleotide precursors exceeding 200 nt and achieving larger-scale, higher-precision synthesis [4,6]. Matzas et al. utilized 454 sequencing to first identify sequence-correct oligonucleotide products, then massively amplified these products, reducing sample error rates to negligible levels [5,6].

### 1.2 Enzymatic De Novo Synthesis

While the phosphoramidite method dominates commercial Oligo synthesis, its length limitation (~200 nt) and extensive use of toxic chemicals [7] have drawn attention to enzymatic synthesis methods with potential advantages in accuracy and avoidance of toxic compounds. Enzymatic de novo oligonucleotide synthesis was proposed as early as the 1960s [7-9]. Compared with chemical methods, enzymatic methods operate under milder conditions, cause less DNA damage, facilitate accuracy improvement, reduce byproduct formation, and enable synthesis of longer Oligos [7]. However, enzymatic method development has been slow, with commercialization yet to be achieved [9]. According to Jensen and Davis' s summary of DNA enzymatic de novo synthesis development, terminal deoxynucleotidyl transferase (TdT)-mediated enzymatic synthesis represents a promising approach requiring further optimization [9]. Key challenges in TdT-mediated DNA synthesis development include termination efficiency after single dNTP addition and terminal reactivation. Recently, Palluk et al. [7] proposed a solution by linking dNTP to TdT via a photocleavable linker; the resulting dNTP-TdT complex can complete DNA chain extension within 10-20 seconds and can be repeated for synthesis of specific DNA strands. This method lays the foundation for developing practically applicable enzymatic DNA synthesis.

## 2. DNA Assembly Technologies

Although Oligos can be synthesized de novo, they exist as short fragments requiring subsequent stepwise assembly to obtain complete synthetic genomes, followed by transplantation into host cells and functional analysis. DNA synthesis, assembly, and transplantation constitute the core technical system of synthetic genomics and the entire synthetic biology field; breakthroughs in these areas will greatly advance synthetic biology development.

Current DNA splicing technologies can be categorized based on assembly fragment size, sequence characteristics, and tolerance for extraneous sequence residues. Common to all in vitro DNA assembly technologies is the requirement for tool enzymes to achieve DNA cleavage, sticky end generation, double-strand ligation, and gap filling. We classify these methods into five categories based on the tool enzyme system employed.

### 2.1 DNA Polymerase-Based Strategies

This method first synthesizes Oligos with complementary overlapping regions. Using PCR amplification principles, Oligos with complementary overlaps can be extended and ligated to obtain small DNA fragments, which are then progressively connected via overlap PCR to obtain target DNA fragments. These fragments serve as templates for further PCR amplification to mass-produce target sequences (Figure 2a [Figure 2: see original paper]). This polymerase cycling assembly (PCA) method does not require additional DNA ligases, enabling direct assembly from artificially synthesized Oligos with simple, rapid

operation. Stemmer et al. [10] used this method to achieve one-step assembly of genes and plasmids. Smith et al. [11] synthesized the X174 bacteriophage genome by adding a Taq ligase step.

### 2.2 BioBrick and BglBrick Methods Based on Compatible Endonucleases

Compatible endonucleases are restriction enzymes that recognize different DNA sequences but generate identical sticky ends (e.g., XbaI and SpeI); once ligated, these ends no longer form the original restriction sites. This "unidirectional" ligation characteristic enables multi-round connections for DNA fragment assembly. The BioBrick method [12] (Figure 2b [Figure 2: see original paper]) designed based on this principle enables standardized assembly of synthetic biology components. However, the scar sequence left at the junction can encode stop codons, making it unsuitable for fusion protein assembly. To address this limitation, Anderson et al. [13] replaced XbaI and SpeI with BglII and BamHI; the resulting scar sequence encodes a "glycine-serine" linker peptide that minimally affects most fusion proteins, a strategy termed BglBrick.

### 2.3 IIS-Type Restriction Endonuclease-Based Strategies

Although BioBrick and BglBrick methods enable efficient assembly of genetic parts, they inevitably introduce extraneous scar sequences. IIS-type restriction endonucleases represent another enzyme class where recognition and cleavage sites are separate, allowing placement of recognition sites as needed to generate desired sticky ends for seamless multi-fragment ligation. In 2008, Engler et al. [14] designed the Golden Gate assembly method based on this principle, enabling efficient seamless connection of multiple fragments in a single reaction (Figure 2c [Figure 2: see original paper]). Our laboratory developed the YeastFab [15] and EcoExpress [16] assembly systems based on Golden Gate for metabolic pathway optimization and protein expression in engineered cells, achieving DNA assembly efficiencies exceeding 90%.

### 2.4 Multi-Tool Enzyme Combined Systems

Both compatible endonuclease and IIS-type restriction endonuclease methods generate sticky ends with limited base numbers, restricting assembly of larger fragments. The core of in vitro DNA fragment ligation lies in generation and utilization of single-stranded overlap regions. To overcome limitations of endonuclease-generated sticky end length, Gibson et al. [17] abandoned restriction endonucleases entirely, instead employing 5' exonuclease combined with DNA polymerase and DNA ligase to develop Gibson assembly (Figure 2d [Figure 2: see original paper]). This method achieves seamless ligation without scar sequences and can assemble fragments up to several hundred kb (kilobases), dramatically increasing the scale of in vitro DNA assembly.

### 2.5 Standardization of DNA Parts

To maximize reuse of existing DNA fragments and minimize re-synthesis, an important research direction in synthetic biology is promoting standardization of synthetic parts (biological components). The aforementioned BioBrick method represents one of the earliest DNA standardization assembly approaches in synthetic biology. However, BioBrick scars hinder protein component assembly. The BglBrick method emerged to address this limitation. Wang' s research group in China established the iBrick standard using homing endonucleases instead of conventional type II restriction enzymes [18] and developed the C-Brick assembly standard based on CRISPR/Cpf1 technology [19]. Golden Gate-based standards, including MoClo [20] and GoldenBraid 2.0 [21], also enable component assembly through unified approaches. To address limitations of Golden Gate and other standards, Wang' s group established the MASTER ligation method [22] for seamless cloning of larger fragments. Additional methods include HVAS [23], similar to iBrick, and GreenGate [24], similar to MASTER. Standardization of biological components across different laboratories will promote component exchange and sharing, enhancing efficiency in complex life system synthesis.

## 3. In Vivo Assembly in Yeast

Although in vitro assembly can produce fragments up to several hundred kb, yields are often insufficient for subsequent experiments, requiring amplification in *E. coli*. No reports exist for in vitro assembly at the Mb (megabase) level; even if achievable, such assemblies might not be importable into *E. coli* for amplification. *Bacillus subtilis*, yeast, and engineered bacteria (overexpressing T4 DNA ligase or $\lambda$ Red recombinase) can mediate intracellular recombination and ligation [25], allowing direct import of DNA fragments for assembly. Yeast, as a common host for DNA recombination, has demonstrated efficient homologous recombination for over 40 years [26-28]. In 1991, Silverman et al. [29] reported that yeast could assemble artificial chromosomes up to 2 Mb. In 2010, Gibson et al. [30] reported the synthesis of the world' s first artificial life form, whose 1.1 Mb synthetic genome was assembled in yeast. Leveraging yeast' s robust homologous recombination capability, the Sc2.0 project (Synthetic Yeast Genome Project) [31] used the SwAP-In method to replace natural chromosomes with completely synthetic artificial chromosomes [32-37].

In August 2018, Shao et al. [38] reported the consolidation of *S. cerevisiae*' s 16 chromosomes into a single 11.8 Mb chromosome, yielding a functional single-chromosome yeast strain. This single-chromosome yeast serves as a novel research platform to advance our understanding of chromosome recombination, replication, and segregation mechanisms. Additionally, the study demonstrated yeast' s remarkable tolerance for chromosome length (at least 12 Mb), providing theoretical basis for using yeast to construct ultra-long chromosomes of higher organisms and facilitating the Genome Project-write (GP-write) [39].

## 4. Synthetic Chromosome Transfer Technologies

Many organisms targeted for genome synthesis exhibit slow growth, insufficient DNA recombination capacity, or low transformation efficiency, making direct genome assembly in target cells difficult. This necessitates using microorganisms like *E. coli* or yeast for assembly. As synthetic genomics expands from lower to higher organisms, challenges arise not only from assembling larger synthetic chromosomes but also from transplanting ultra-large chromosomes.

### 4.1 Chromosome Clearance Based on Negative Selection

Even when artificial chromosomes are implanted and functional in host cells, the spontaneous loss of corresponding endogenous chromosomes remains extremely rare, requiring screening to obtain such cells. Li et al. [46] reported successful restoration of normal karyotype in trisomy 21 patient-derived induced pluripotent stem cells (iPSCs) by removing one chromosome 21 using a positive-negative selection strategy. They knocked in a bifunctional fusion gene encoding both negative and positive selection markers into chromosome 21; selected cell lines with integrated fusion genes were obtained via positive selection, from which single-copy integrants were further screened. Subsequent culture without positive selection drugs generated cells with loss of the fusion gene-carrying chromosome. Since the negative selection marker converts a non-toxic drug into a toxic substance that kills host cells, negative selection can enrich for cell lines missing one chromosome 21. This positive-negative selection strategy for clearing endogenous chromosomes after synthetic chromosome transfer depends on prior knock-in of selection fusion genes into target chromosomes.

### 4.2 Cre-loxP Mediated Chromosome Elimination

Cre recombinase recognizes loxP sites on DNA and can delete or invert DNA fragments between two loxP sites based on their orientation. This method can also eliminate endogenous chromosomes. Through fusion with mouse embryonic stem cells (ESCs), human somatic cell nuclei can be reprogrammed to pluripotency, but ESC-derived chromosomes must be cleared after reprogramming. To achieve this, Matsumura et al. [47] designed a Cre-loxP recombination system-based strategy called CEC (chromosome elimination cassette). CEC carries a fluorescent reporter and drug resistance marker flanked by two loxP sites in opposite orientations. Cre-mediated sister chromatid recombination generates dicentric and acentric chromosomes, which are eliminated during cell division. However, like the positive-negative selection strategy, this requires prior knock-in of relevant genes into target chromosomes.

### 4.3 CRISPR/Cas9 Mediated Chromosome Clearance

CRISPR/Cas9 is a gene editing technology developed from bacterial type II CRISPR/Cas immune defense systems [48]. Its simplicity and efficiency in mediating precise gene knockout and knock-in have enabled widespread application

across species. Given the complexity and inefficiency of aforementioned methods, Zuo et al. [49] attempted to use CRISPR/Cas9 for targeted chromosome clearance. The CRISPR/Cas9 system comprises two core components: the non-targeting Cas9 nuclease and the single guided RNA (sgRNA) that directs Cas9 cleavage. They found that multiplex CRISPR/Cas9 targeting could selectively eliminate sex chromosomes in cell lines, embryos, and tissues, as well as autosomes in tumor cells. This approach provides new strategies for constructing chromosome deletion animal models and treating related human genetic diseases [49], and represents a potential technical means for targeted endogenous chromosome clearance in synthetic genomics.

## Future Perspectives

Currently, whole-genome synthesis has succeeded in viruses and bacteria, with the first fully synthetic eukaryote—Sc2.0—nearing completion. Synthesis of higher organisms is already on the agenda [39]. However, due to extremely large genome sizes, achieving these syntheses will depend on breakthroughs in the aforementioned technologies and emergence of new methods.

Obtaining non-natural genomes requires DNA synthesis as the only current approach. Synthesis of large genomes demands higher-efficiency, higher-precision DNA synthesis technologies with further cost reductions. Compared with chemical methods, enzymatic DNA synthesis offers numerous advantages, though commercial application remains distant. In large genome design and synthesis, using naturally existing DNA fragments could reduce synthesis costs, but requires corresponding technical support. While conventional-length DNA fragments can be obtained via PCR or enzymatic digestion, these strategies are inadequate for ultra-large fragments. CRISPR/Cas9 technology now enables specific cleavage of large DNA fragments directly from natural genomes. For example, researchers from Tsinghua University and the Institute of Microbiology, Chinese Academy of Sciences jointly developed a CRISPR/Cas9 and Gibson assembly-based cloning strategy to obtain DNA fragments up to 150 kb [50].

For synthetic genome assembly, yeast' s strong DNA recombination capacity makes it an important molecular tool in synthetic genomics. Whether yeast can handle future ultra-large genome synthesis requires further investigation. Additionally, synthetic genome isolation, extraction, and transfer technologies all face reduced efficiency or even inapplicability as target genomes increase in size, necessitating development of novel technologies. Wild-type genome clearance is an essential step for obtaining organisms with completely synthetic genomes. While CRISPR/Cas9 technology shows promise for clearing ultra-large endogenous genomes, cleavage efficiency may vary across different genomic loci and require optimization. Additionally, off-target effects could affect the implanted synthetic genome, issues that must be considered during genome design.

Currently, China has achieved significant accomplishments in synthetic biology, particularly contributing substantially to the Sc2.0 project. As an emerging

interdisciplinary field, synthetic biology faces numerous technical challenges beyond the basic technical system, presenting opportunities for original achievements and interdisciplinary talent cultivation.

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