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Review

Next generation gene synthesis: From microarrays to genomes

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Similar to the incredible advances in DNA sequencing, the de novo synthesis of DNA is subject to innovations and fast progress in terms of synthesis speed and cost. We will discuss novel techniques that are expected to enable high-throughput synthesis of oligonucleotides on microarrays and the subsequent assembly into longer fragments, up to whole genomes. Especially, the inherent disadvantages of microarray-derived oligonucleotide pools for gene synthesis will be discussed in detail, and also the different approaches to still render these oligonucleotides useful for gene assembly. These so-called next-generation techniques will lead to a significant cost reduction of gene synthesis and to the possibility of much larger projects, such as whole genome synthesis.

Keywords: Cloning / Gene synthesis / Genome synthesis / Microarrays / Microfluidics / Oligonucleotides

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1 Introduction

A major prerequisite for modern biotechnology and synthetic biology, in particular, is the availability of low-cost de novo gene synthesis [1]. This not only allows liberalization from natural sources of genetic material and unrestricted flexible design of DNA sequences, but also adapting coding sequences to the genetic requirements of the target organism. Today, most biotechnological projects make good use of gene synthesis and profit on safety, availability, reliability, throughput, flexibility, and last but not least, total cost [2]. Its application not only completely changed the way in which scientists think, when designing appropriate DNA constructs, but also facilitates outsourcing of related experimental steps in order to concentrate on less trivial scientific operations. Researchers can electronically access DNA sequences through comprehensive databases, redesign constructs in silico to specifically fit given requirements, and order the designed genes online being synthesized and shipped within a matter of days [2].

Current protocols for gene synthesis are based on the ability of chemically synthesized single-stranded oligonucleotides (oligos) to self-assemble into larger contiguous sequences (see Fig. 1). The

assembly process itself is usually a multiplex primer extension reaction under controlled temperature cycling conditions.

Unfortunately, template-less chemical production of oligos is still a significant cost factor. Herein, we will discuss new techniques that are expected to enable high-throughput synthesis of oligo and the subsequent assembly into fragments. We will further discuss new methods for assembly and cloning to further assemble these fragments into longer constructs, up to whole genomes. These so-called next-generation techniques will lead to a significant cost reduction of gene synthesis and will therefore pave the way for the field of synthetic biology and for whole genome synthesis.

2 Oligonucleotide synthesis and microarrays

The phosphoramidite method, which was introduced in the 1980s by Caruthers and coworkers [3, 4], is still commonly used to synthesize oligos in synthesis columns, which is a major bottle neck in gene synthesis, with respect to costs and throughput.

Because the number of different oligos is too low for high-throughput gene synthesis and each oligo is produced in vast excess, a switch from columns to microarrays would be very attractive in terms of costs and parallelization (Fig. 1).

Microarrays, based on mask-based lithography, were developed for the parallel analysis of DNA sequences by Fodor et al. in the early 1990s [5, 6], starting the success of Affymetrix. The flexibility of this process was later improved by Nimblegen by using a digital micro-mirror array [7]. Later, Gao et al. used

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Abbreviations: bp, base pair; CPEC, circular polymerase extension cloning; NGS, next generation sequencing; oligo, oligonucleotide; TypeIIS-RE, type IIS restriction enzyme; USER, uracil-specific excision reagent

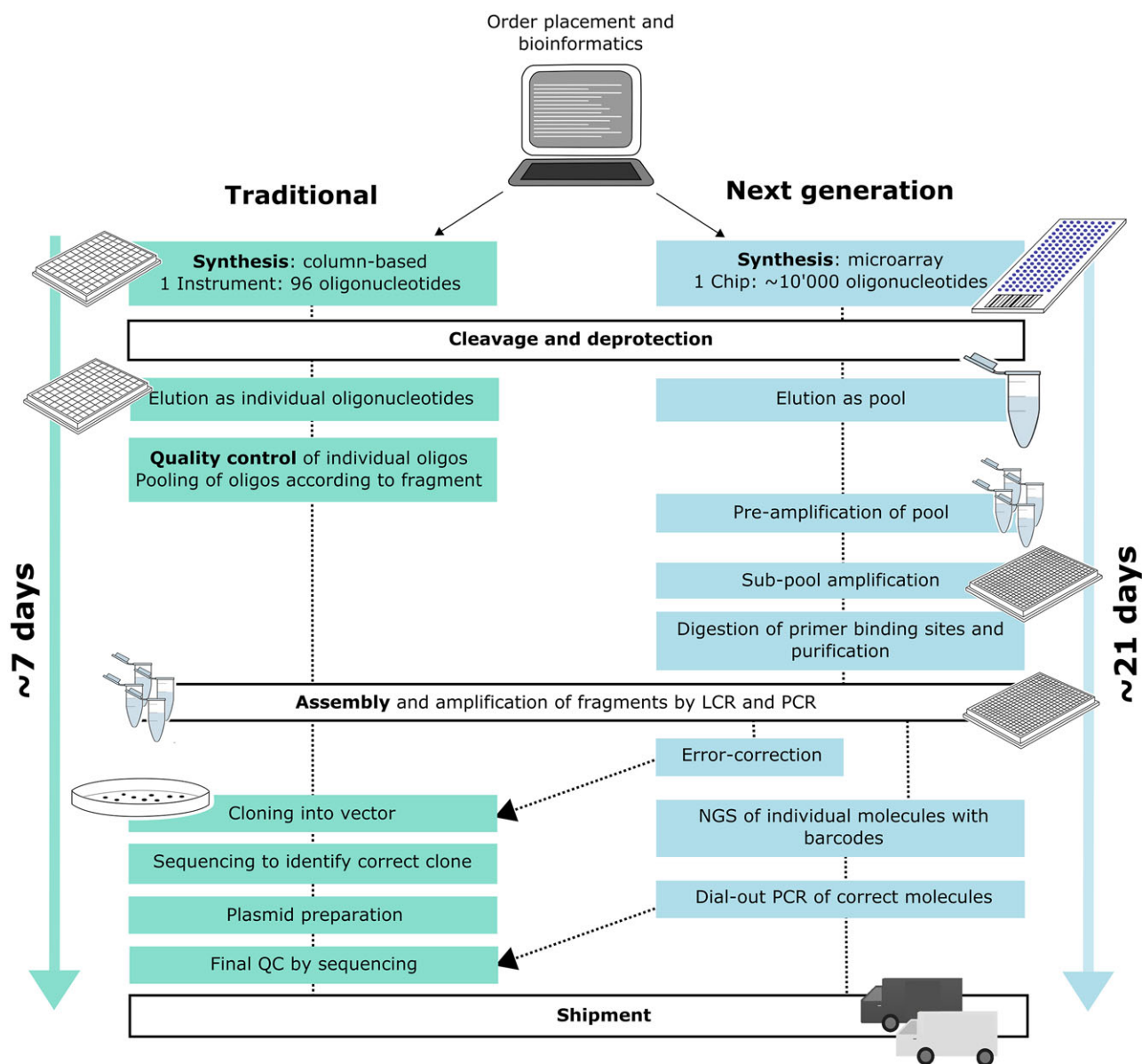


Figure 1. Comparison of traditional and next generation gene synthesis workflows (for <1 kb genes/fragments). In the traditional gene synthesis, one synthesizer produces oligonucleotides for about 3–5 fragments, while a microarray synthesis can yield hundreds of fragments. All the numbers and steps are examples and do not represent a certain workflow by one company or by one single publication.

photogenerated acid to synthesize microarrays with standard phosphoramidites [8], which was commercialized by LC Sciences, and the Southern group developed a similar technique based on electrochemical deprotection on microchips [9, 10], today available by Customarray.

In general, all of the above methods control the deprotection step and have the disadvantage that a full cycle for all four amidites is necessary to couple one base, leading to longer synthesis time and higher error rates. For this reason, controlling the sequence by adding the correct amidite (in analogy to the standard column-based synthesis) is favorable for speed and quality of the synthesis. Miniaturization of this process was achieved by

adapting ink-jet technology [11] and was later commercialized by Agilent [12].

Even with all these improvements over the last 25 years, microarray-based oligo synthesis still comes with several disadvantages. Microarrays yield a low-concentrated pool of thousands of oligos and are usually prone to more errors than column-synthesized oligos, due to edge effects including escaping protons, light beam drift, or droplet misalignment [13]. Additionally, a quality control of the single oligos is nearly impossible, and definitely impracticable with current techniques, which can lead to oligos being over- or underrepresented in the pool [14]. Because of these shortcomings, microarray-derived oligos

cannot be used for gene synthesis without adapting the workflow, or the microarray.

3 Microarrays for gene synthesis

Many different strategies and methods have been developed during the last years to overcome these problems and pave the way for a truly next generation technique. Although all different disadvantages typically have to be solved to render a certain approach fully functional, we will discuss them separately here, i.e. concentration, pool complexity, and error rates.

3.1 Low concentration of oligonucleotides

The concentrations of oligo mixtures from microarrays are typically 1–20 pmol for the whole pool, which means that only atto- to femtomol quantities of every oligo are available for gene assembly reactions [15]. Unfortunately, this is several orders of magnitudes less than the required concentration for a successful gene assembly in standard volumes (1 pmol) [16].

The most obvious solution to this shortcoming would certainly be to simply synthesize oligos in higher quantities. Lee et al. showed that when columns of silica particles are integrated on a microfluidic chip, different oligos can be synthesized in almost nanomolar quantities for direct assembly into genes [17]. However, this approach was limited to 16 oligos, and is not easily scalable to thousands of synthesis columns.

Another way to achieve higher concentrations is to miniaturize the volume of the assembly reaction. It was shown that assembly is possible in microfluidic chambers of 500 nL volumes with no negative influence on assembly efficiency [18]. To be truly compatible with microarray yields, the volume would need to be further decreased to about 10 nL, which would render the retrieval of the assembled genes very difficult, as these small volumes are difficult to handle without loss and a subsequent amplification of the assembled gene would be needed. As discussed by the authors, all of this would only be possible if the chamber encapsulates the microarray to avoid loss of the synthesis product. The first step in this direction, although with larger volumes, was shown by Quan et al. [19], who used a custom-built printer to synthesize oligos in microwells. Recently, Twist Bioscience has started to develop a similar approach by using microfabricated nanowells that capture oligos in small volumes [20].

However, the approach which is most commonly used by groups working with microarrays for gene synthesis is amplification of the oligos by PCR. It was first shown in 2004 in several publications [21–23], that a PCR of microarray-derived oligos can yield concentrations that are useful for gene assembly. Although this method seems simple and is compatible with biomolecular workflows, it suffers from (i) amplification bias [24], leading to concentration differences in the amplified pool, and therefore to high percentages of unsuccessful assembly reactions; (ii) even higher error rates of oligos as primer binding sites need to be chemically synthesized, and errors correlate with length [25]; and (iii) additional preparative steps for the removal of primer binding sites [16, 25]. To overcome amplification bias, linear amplifications have been successfully

implemented [19], but only with a fourfold concentration increase, and therefore a miniaturization of the assembly volume was still needed. Borovkov et al. proposed to amplify after an assembly of small fragments of less than 300 bp, which reduced amplification bias by tenfold in their experiments [15]. As we will discuss in Section 3.2, direct assembly is limited to low complexity pools and therefore will not allow to completely exploit the full potential of commercial microarrays.

3.2 Complex mixtures of oligonucleotides

Current microarray systems are typically used to produce 10 k–1 M oligos per chip for a wide range of different applications. The high density of synthesis positions available on commercial microarrays entails that the oligos can only be cleaved and eluted together in a highly complex pool. Hence, for DNA synthesis most users have focused on a density in the range of thousands different oligos per chip in an effort to balance yield per oligo (see above) and complexity. As all oligos are present, the probability for cross hybridizations among different fragments increases and chances of successful assembly decreases accordingly. It has been shown that the one-pot assembly of many different genes is only possible for low-complexity mixtures, and not for the amount of different oligos that are produced on commercial microarrays [15].

One possible solution for this problem is physical separation, i.e. to synthesize subsets of microarrays in different locations and/or volumes. This possibility was first discussed by Kong et al. [18], and was later shown experimentally by Quan et al. to be a viable option to keep oligos separated [19]. Different subsets of oligos were synthesized in small pots that were embossed into plastic slides. Each pot contained all oligos belonging to one fragment and had no fluidic contact during the assembly with the other reactions. Unfortunately, commercial microarrays cannot be used for this method as they have been optimized for high density. Another disadvantage is that expensive microstructures need to be used (compared to the flat surface of a common microarray).

Other groups have therefore developed protocols to directly use the pools that can be derived from commercial microarrays. Tian et al. assembled a multitude of genes from one pool, which was also achieved by others at the same time [21–23]. As discussed before, these approaches proved difficult to scale and could not use all the possible oligos on highly parallel arrays. Kosuri et al. showed that the selective amplification of barcoded oligos belonging to one fragment (subpool) can decrease the complexity of the pool and leads to more successful assemblies [25]. Their approach, which was also used by Kim et al. [16], involved synthesizing additional primer binding sites barcoding different fragments, which could be used for amplification with the corresponding primer set. Both groups showed that the incomplete removal of primer sites by enzymatic cleavage leads to failures in assembly. In another experiment, Kosuri et al. preamplified larger mixtures of oligos before the sub-pool amplification, with better assembly results. However, since they needed even longer oligos (200 mers) for this approach, the error rates were substantially higher (1/250 bp) in the assemblies [25]. The advantage of selective amplification is that standard

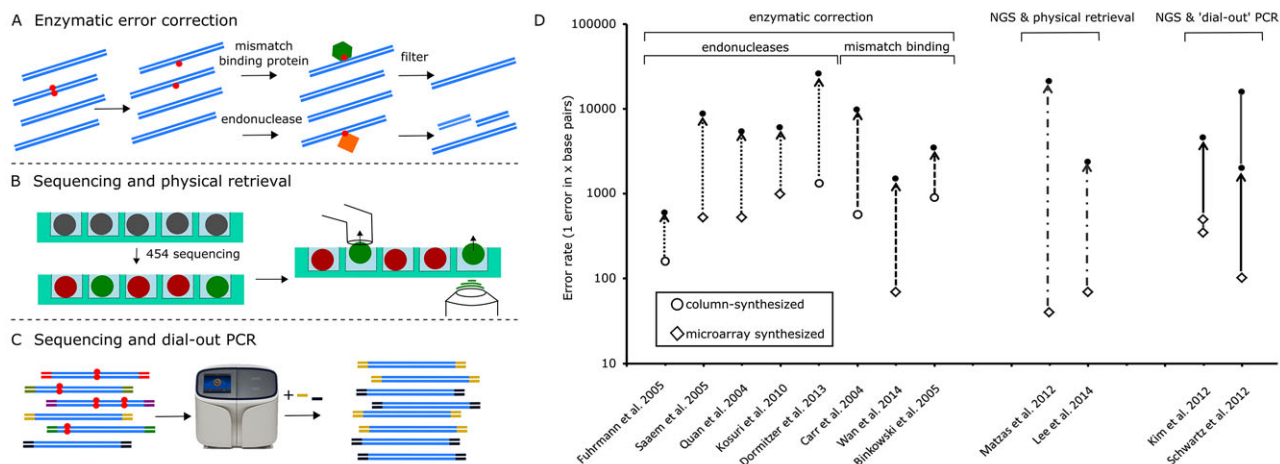


Figure 2. Different approaches for error correction. (A) Enzymatic error correction uses either mismatch binding proteins with filtration to remove DNA with errors, or enzymes that cleave erroneous DNA. (B) DNA that has been sequenced can be located and physically removed by micropipettes [31], or lasers [32]. (C) Next generation sequencing of barcoded molecules can identify correct molecules that are then amplified with the barcode as a primer [33]. (D) Comparison of the different error correction methods.

laboratory equipment and consumables can be used, and it works directly with pools from (commercial) microarrays. However, primers need to be designed very carefully to be orthogonal and to keep amplification bias to a minimum. As the removal of the primer sites needs an additional gel isolation, the process is difficult to automate and expensive. Klein et al. recently showed the multiplexed co-assembly of up to 2271 small fragments, and omitted the enzymatic cleavage site by using uracil-containing pool specific primers, which could later easily be removed by uracil specific excision reagents [14]. In summary, all these approaches show very good potential to make use of microarrays, but significantly increase the time to synthesize a gene (see Fig. 1).

3.3 Error rate

Currently, conditions for mass production of genes are chosen to have a >95 % chance of picking at least one correct fragment with a single screen after transformation (Fig. 1), limiting the size of the initial fragment to <3 kb. Thus, to not further extend gene synthesis time, DNA fragments with low error rates are important to reduce the screening effort, as a resynthesis is needed if no correct clone can be found. Besides using more stringent assembly conditions [15], special procedures are necessary to compensate for the higher error rates of microarray-derived oligos (Fig. 2); the most common ones being (i) the removal of errors by mismatch binding proteins or mismatch cleaving enzymes and (ii) Sequencing-based methods to identify error free DNA with subsequent physical retrieval of correct DNA, or (iii) its selective amplification.

Removal of errors with error correcting enzymes, e.g. mismatch cleaving enzymes such as T7 endonuclease I, CEL1, T4 endonuclease VII, and *Escherichia coli* endonuclease V is probably the most commonly used approach [25–27]. Mismatch cleaving enzymes recognize mismatched base pairs (bp) in double

stranded DNA and cleave 2–5 bp downstream of the mismatch. The resulting overhangs, which contain the mismatched base, are then removed by 3′-5′ exonucleases or 5′-3′ exonuclease activity of proof reading polymerases. Error-free fragments are then assembled via PCR [26]. Mismatch binding proteins such as MutS capture error containing fragments and only the purified, error-free DNA will be used for further assembly [28].

Error correction methods using only mismatch binding proteins are not efficient for error removal in long synthetic DNA fragments where virtually all fragments contain errors [29]. For longer DNA fragments, a combination of restriction digestion with subsequent MutS-filtration led to error-reduced long genes [29]. Unfortunately, not all types of errors are corrected equally, e.g. some enzymes preferentially correct insertions and deletions, but are less efficient for substitutions. Thus, enzyme choice depends on the expected error type in the oligos or synthetic DNA fragments.

In a very recent publication, solid-state nanopores were used to discriminate between correct and incorrect fragments by detecting MutS binding [30]. If nanopore-based monitoring of DNA quality could be used to purify the error-free DNA, it might lead to a novel strategy for high-throughput preparation of synthetic DNA for gene synthesis [30].

Use of massive parallel sequencing enabled by next-generation sequencing (NGS) platforms offers another approach to identify correct sequences rapidly and efficiently. These methods can be applied both to select correct oligos prior to assembly and error-free larger fragments after assembly. This was first used to identify correct oligos with the so-called “mega cloning” prior to assembly (Fig. 2B) [31]. Here, oligos synthesized on a microarray platform were sequenced on a Roche 454 GS FLX platform. Single beads containing the correct oligos were selected from the picotiter plate, and transferred with a micropipette into a 96-well plate. The sequence verified oligos were then amplified and used for the assembly of larger fragments resulting in a significantly reduced error rate.

In a faster and more automated approach, the so-called “sniper cloning” [32], Lee et al. transferred the beads with a laser pulse from the sequencing plate to a 96-well plate. Both retrieval methods require highly specialized equipment and are not compatible with all NGS platforms.

An NGS-based method that does not require special equipment and can be implemented on any NGS-sequencer is tag-directed retrieval, also termed dial-out PCR [33]. It is also applicable for complex pools of DNA such as libraries, multiplex assembly reactions, or error-rich sequences, where enzymatic error correction methods are often not sufficient [27, 33, 34]. In dial-out PCR, either single molecules of oligonucleotides [33] or up to 300 bp [14, 34] are barcoded with primer tags (see Fig. 2B). Correct fragments are then identified by NGS, and amplified using PCR primers specifically recognizing the tags. The sequence verified fragments can then be used to assemble larger constructs [34]. Ninety percent error-free fragments of up to 252 bp in length could be retrieved from a 250plex assembly reaction by using this technique [15]. Due to steadily decreasing costs for NGS, dial-out PCR is a very cost-effective method, but also adds significantly to the overall gene synthesis time (Fig. 1) [33].

4 Assembly into longer constructs

After assembly and error reduction, the linear gene synthesis product may be used as such, e.g. directly for *in vitro* transcription/translation, or is inserted into a vector using classical restriction endonuclease cloning, seamless assembly techniques (Fig. 1) [35–37] or any other cloning method. As discussed before, compiling synthetic constructs exceeding 3 kb requires advanced error reduction protocols and/or sequence-independent and scar-less assembly of cloned and sequence-verified building blocks.

We will thus discuss novel assembly techniques that enable scar-less assembly of several fragments in one step. They can broadly be classified into TypeIIS restriction enzyme (TypeIIS-RE) based methods and homology-based methods.

TypeIIS-RE based assembly methods take advantage of the fact that TypeIIS-REs cut indiscriminately at a defined distance from their recognition site creating an overhang which is not part of the recognition sequence. Appended to the sequence ends in the correct orientation, they can be used to create any desired overhang at any position without leaving behind the recognition sequence. The Golden Gate method uses TypeIIS restriction sites at the ends of fragments and vector to generate unique overhangs for the scar-less assembly of several fragments in a defined order [38]. Golden Gate cloning allows the assembly of up to nine fragments in one reaction but is limited by the necessity to avoid or remove the relevant TypeIIS restriction sites within the fragments [39]. MASTER (methylation-assisted tailorable ends rational [40]) removes this limitation through the use of a methylation-dependent TypeIIS-RE and PCR fragments generated with methylated primers such that only restriction sites at the fragment ends are recognized and cut.

Homology-based assembly methods require the incorporation of short stretches of homologous sequence at the fragment

and vector ends. Methods of this type were first described in 1990 [41–43] but only recently protocols were developed that enable highly efficient, sequence independent assembly of multiple fragments.

These protocols create single stranded overhangs from the homologous stretches; assembly is then achieved by annealing complementary single strands. In sequence and ligase independent cloning (SLIC [44]), the 3' exonuclease activity of T4 DNA polymerase in the absence of dNTPs is used to chew-back one strand from 3' to 5'. Exonuclease activity is halted by the addition of dCTP, and the overhangs are used to assemble up to five fragments into a construct with single-stranded gaps. These are repaired *in vivo* after transformation into *E. coli*. Gibson assembly [45] uses T5 exonuclease for 5' to 3' chew-back, Phusion DNA polymerase for gap filling, and a ligase to seal the nicks, resulting in a covalently closed vector from a one-cup *in vitro* reaction. Five fragments are efficiently assembled using Gibson assembly, and constructs up to 318 kb can be generated in multiple steps. Single-stranded overhangs can also be formed through the activity of uracil-N-glycosylase and endonuclease VIII on DNA fragments that were generated using uracil-containing primers as in uracil-specific excision reagent (USER) cloning and USER fusion [46]. USER fusion assembles up to three fragments in a single reaction.

Circular polymerase extension cloning (CPEC [47]) utilizes a high-fidelity DNA polymerase and temperature cycling to allow the homologous ends to anneal to and extend each other, forming the nicked target construct. CPEC has been used to assemble four fragments with a total size of 8.4 kb but entails possible introduction of sequence errors by the DNA polymerase. CPEC and USER cloning require *in vivo* repair in *E. coli* to generate the resulting plasmid, analogous to SLIC.

Several methods use the intrinsic homologous recombination activity of cells for *ex vivo* or *in vivo* assembly: Seamless Ligation Cloning Extract (SLiCE [48]) employs a bacterial cell extract to assemble up to six fragments of the same type as the *in vitro* approaches described above.

The budding yeast *Saccharomyces cerevisiae* possesses considerable homologous recombination activity, which has been used for the assembly of large constructs from overlapping fragments for decades, e.g. by transformation associated recombination [49] or the DNA-assembler method [50]. *Saccharomyces cerevisiae* has been used to assemble 25 fragments *in vivo* [51], and assembly of constructs up to 1.1 Mb has been achieved [51]. More recently, it has been shown that also certain *E. coli* strains possess sufficient homologous recombination activity to allow for transformation associated recombination of up to six fragments that create a 6.5 kb plasmid by simple transformation of fragments and vector [52].

In combination with microarray-based next-generation gene synthesis workflows that provide sequence-correct DNA fragments at very low cost, these techniques allow for the efficient and cost-effective production of large to very large constructs, up to the synthesis of complete genomes from scratch. In 2010, Gibson et al. published the first completely synthetic bacterial genome of 1.1 Mbps that made use of a hierarchical combination of seamless assembly methods, i.e. Gibson assembly and homologous recombination in yeast [53]. More recently, the

first synthetic eukaryotic yeast chromosome was published by the Sc2.0 consortium [54].

The goal here is to eventually build a completely synthetic variant of the yeast genome of ~12 Mb including first approaches to redesign the natural wild-type sequence, therefore, expanding the engineering to the content and architecture of the genome. Further, in 2016 Boeke et al. announced the human “Genome Project-Write,” with the aim of synthesizing the entire human genome of ~3000 Mb [55]. With this undertaking it is to be expected that the development of high-throughput next generation gene synthesis technology is further propelled and consequently gene synthesis costs will significantly drop over the next years. In addition, new and innovative DNA assembly and DNA propagation techniques will be needed and are likely to emerge from this ambitious moonshot project.

5 Concluding remarks

The progress of writing DNA has been truly impressive since the first automatic oligo synthesizer was developed in the 1980s [56]. The herein discussed processes show the potential for a massive parallelization and cost reduction. The reliability could be greatly improved by coupling them with conventional techniques as a backup for complex genes and when the new approach fails. This all assumes that a scientist ordering a gene is willing to sacrifice fast delivery times for decreasing costs. For researchers with very large orders, e.g. working on whole genome synthesis, this is probably necessary to keep within their budgets. For customers ordering an individual gene, which is crucial to advance the project in time, it might be more difficult to accept.

Nonetheless, many reports have already shown that microarray-derived genes can advance the understanding of analysis of promoter and enhancer functions in an unprecedented multiplexed manner [57,58]. More exciting experiments can be expected as large projects become a routine tool in biomolecular research.

As a result, it will be even more important that the according bioinformatics tools are developed to design DNA. In analogy to the design of computer chips, where the individual transistor is usually not designed anymore, new levels of abstraction will help to design, and maybe simulate, parts that will later be synthesized [59].

In either setting, tangible DNA molecules, genes, and genomes, are needed to build and create Synthetic Biology's innovations and makings. Like in any other engineering discipline, easy access to these building blocks is a major driver of the advancement of the technology itself. Simple, low-cost access to synthetic DNA therefore will accelerate biotechnology and life-science research and open new opportunities in the new era of a bio-based economy.

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6 References

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