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Parallel gene synthesis in a microfluidic device

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ABSTRACT

The ability to synthesize custom *de novo* DNA constructs rapidly, accurately and inexpensively is highly desired by researchers, as synthetic genes and longer DNA constructs are enabling to numerous powerful applications in both traditional molecular biology and the emerging field of synthetic biology. However, the current cost of *de novo* synthesis—driven largely by reagent and handling costs—is a significant barrier to the widespread availability of such technology. In this work, we demonstrate, to our knowledge, the first gene synthesis in a microfluidic environment. The use of microfluidic technology greatly reduces reaction volumes and the corresponding reagent and handling costs. Additionally, microfluidic technology enables large numbers of complex reactions to be performed in parallel. Here, we report the fabrication of a multi-chamber microfluidic device and its use in carrying out the syntheses of several DNA constructs. Genes up to 1 kb in length were synthesized in parallel at minute starting oligonucleotide concentrations (10–25 nM) in four 500 nl reactors. Such volumes are one to two orders of magnitude lower than those utilized in conventional gene synthesis. The identity of all target genes was verified by sequencing, and the resultant error rate was determined to be 1 per 560 bases.

INTRODUCTION

It has long been recognized that the capacity to design and synthesize genes and longer DNA constructs can be enabling to a broad cross-section of applications within molecular biology (1), including the study of large sets of single genes (2), the design of genetic circuitry (3), the engineering of entire metabolic pathways for target molecule manufacture (4), and even the construction and re-engineering of viral and bacterial genomes (5–7).

The core technology for custom DNA synthesis centers on the assembly of pools of oligonucleotides (oligos), typically less than 50 nt in length, into increasingly larger DNA molecules. These oligos, hereafter referred to as 'construction oligos', are synthesized by variations of phosphoramidite chemistry (8), and are the building blocks for the different gene synthesis techniques developed thus far. The most widely reported methods for building long DNA molecules involve variations of the polymerase-mediated assembly technique shown in Figure 1, collectively termed polymerase construction and amplification (PCA) (9–10). Here, much like in the more conventional polymerase chain reaction (PCR), three temperature steps are employed to denature, anneal and elongate the various overlapping construction oligos until, after multiple rounds of thermocycling, the desired full-length DNA construct is obtained. Furthermore, assembly and amplification can be performed in a single reaction with the introduction of amplifying primers (11). Thus, once a minute quantity of full-length product is assembled, this product is amplified as per PCR. Using such polymerase-mediated techniques, researchers have successfully synthesized DNA constructs as large as 12 (12) and 15 kb (11). A PCA process was also employed as the first step in generating a 32 kb DNA construct by Santi and co-workers (13). In addition, significant progress has been made in correcting synthesis errors, which originate primarily from the phosphoramidite synthesis of initial oligonucleotide building blocks. The use of protein-mediated error correction has been effective in increasing the accuracy of synthetic DNA (14–16), with error rates as low as 1 per 10 000 bp reported (14).

Despite these promising results, significant challenges remain, most significantly the cost and time of synthesizing long constructs. Currently, while conventionally synthesized oligos are available at a cost on the order of USD0.1 per nucleotide, the cost for custom gene synthesis services is significantly higher, at USD0.65–USD1.10 per base pair, with the major expenditure components for such long syntheses being attributable to reagent and sample handling. Microfluidic technology provides an elegant means to overcome these limitations. By scaling reactions down to volumes of less than a microliter,

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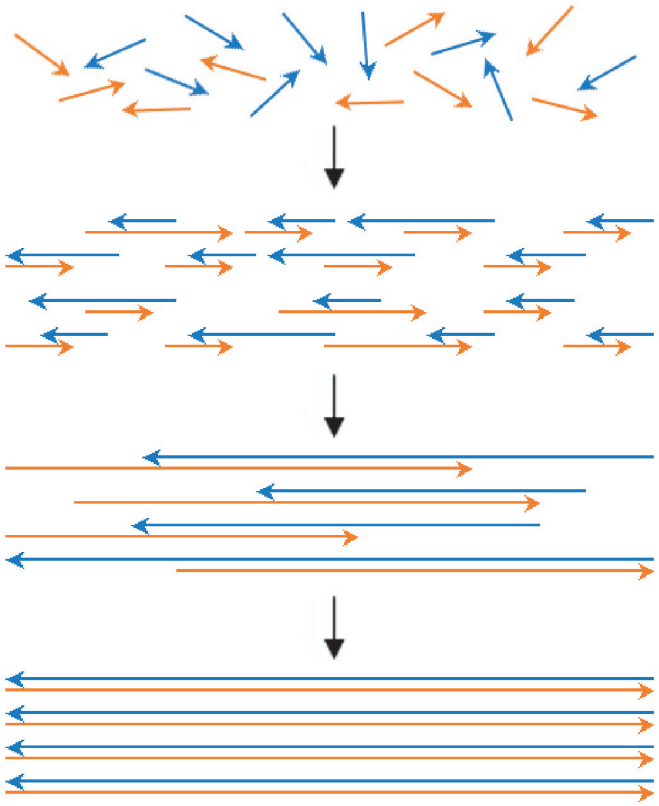

Figure 1. Schematic for gene synthesis by polymerase construction and amplification (PCA). Multiple rounds of oligo annealing and extension by DNA polymerase generate successively longer DNA assemblies from a starting pool of construction oligos, typically <50 nt, until the full-length gene is produced. The pool of heterogeneous DNA products is enriched for the full-length species by amplification in a separate subsequent reaction, or in the same reaction by including amplifying primers in the reaction mixture.

reagent costs can be substantially reduced (17). Furthermore, microfluidic technology enables highly parallelized synthesis along with the potential for automated sample handling and process integration.

In this article, we report what is, to our knowledge, the first gene synthesis conducted in a microfluidic environment. We have successfully conducted synthesis and amplification in a single reaction for a variety of genes and gene segments, including GFP, OR128-1, DsRed, ble (bleomycin resistance), a Holliday junction cleavase (hjc) gene from the bacteriophage *SIRV-1*, and a variant alba gene from *Sulfolobus solfataricus*. The identities of all synthetic genes were verified by sequencing, and extensive sequencing of DsRed enabled the determination of an error rate for genes synthesized in a microfluidic environment, along with a comparison of error rates for genes synthesized in standard PCR tubes. In other reports, construction oligos were synthesized on the microscale, cleaved from the surface and subsequently assembled in macroscopic (≥5 µl) reactions (11,18–19). In contrast, we have synthesized these DNA constructs in parallel within four 500 nl reactors of a poly(dimethylsiloxane) (PDMS) based microfluidic device. Furthermore, the minute construction oligo concentrations utilized (10–25 nM each oligo) are significantly lower than concentrations attainable (without amplification) from high-density oligonucleotide microarrays. Thus, such a microfluidic approach should be compatible with DNA microarray-derived oligonucleotides (11), further reducing the cost of this crucial reagent.

MATERIALS AND METHODS

Master mold fabrication

Devices utilized in this work employed 'push-down' valve geometries for fluidic valve actuation (20). Two master molds were fabricated, one from which the fluidic 'flow layer' could be cast, the other from which the fluidic 'control layer' could be cast. The flow layer master was fabricated by first rinsing a 4'' silicon wafer (WaferNet) in acetone and isopropyl alcohol, followed by wafer dehydration at 200°C on a hot plate. Next, hexamethyldisilizane (HMDS, Sigma) was spun on the wafer at 4000 r.p.m. to promote adhesion of the photoresist. A layer of AZP 4620 positive photoresist (AZ Electronic Materials) was then coated at 1500 r.p.m. for 40 s followed by a 1 h soft-bake at 90°C. Upon completion of the soft-bake, the wafer was then exposed for 20 s at 50% intensity using a UV floodlight (Uvitron, Int.), followed by development. Next, the resist was placed on a hot plate at 150°C for 1 min to reflow the resist and achieve rounded fluid channels, thus enhancing sealing during valve actuation.

The control layer master was fabricated by again employing a solvent wash followed by wafer dehydration. A layer of SU-8 50 negative photoresist (MicroChem) was then coated at 1000 r.p.m. followed by pre-exposure bake steps of 65°C for 10 min and 95°C for 30 min. The resist was then exposed for 40 s at 50% intensity and post-exposure baked at 65°C for 1 min and 95°C for 10 min before being developed.

Finally, both flow layer and control layer masters were briefly exposed to chlorotrimethylsilane (Sigma) vapors for several minutes to promote release of the elastomer from the master molds. All transparency masks used for the various exposure steps were designed in Adobe Illustrator and printed by PageWorks (Cambridge, MA).

Microfluidic device fabrication

Approximately 30 g of liquid PDMS pre-polymer (GE, RTV 615) at a component A to B ratio of 5:1 was poured onto the control layer master to a thickness of ~1 cm, followed by partial curing in a convection oven at 80°C for 45 min. Liquid PDMS pre-polymer at a component A to B ratio of 20:1 was coated onto the flow layer master, at 2000 r.p.m. for 60 s and also partially cured at 80°C for 45 min. The PDMS control layer was then peeled from its master, and individual devices were cut out with a razor blade. Holes for control line inlet ports were cored with an 18 G needle whose tip had been beveled and sanded down for clean coring. Next, control layer devices (typically six per wafer) were aligned and bonded to the PDMS-coated flow layer master, followed by additional curing for 45 min

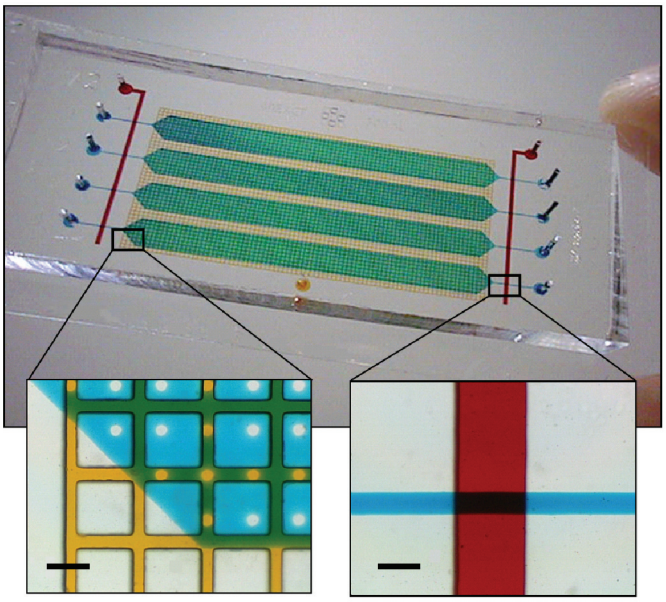

Figure 2. Optical images of a microfluidic device capable of conducting four parallel 500-nl reactions with various features emphasized with food coloring. Left inset: gene synthesis chamber (blue and green) and water jacket (yellow) layers. Right inset: fluid inlet channel (blue) overlaid with valve channel (red). Scale bars correspond to 200 μm.

at 80°C. These two-layer devices were then cut and peeled off the flow-layer molds, cored, and bonded overnight at 80°C to 1 mm thick glass cover slips coated with a thin layer of partially cured PDMS (typically spun on at 2000 r.p.m. for 40 s, with a 20:1 polymer-to-curing-agent ratio, and cured at 80°C for 45 min).

An example of a three-layer PDMS device capable of parallel gene synthesis is shown in Figure 2. Colored food dyes are used to emphasize various features of the device, with red indicating actuation lines in the PDMS control layer, blue (and green) indicating the four gene synthesis reactors and yellow indicating a mesh of fluid lines in the control layer, hereafter referred to as a 'water jacket', placed above the reactors to minimize sample evaporation during thermocycling.

Parsing of genes

Several genes and gene segments were selected for synthesis and parsed utilizing the program DNAWorks (21) to generate the desired oligonucleotide sequences for assembly and amplification. The genes selected for synthesis were: (1) a randomized amino acid sequence of the alba gene from *S. solfataricus* (total length 327 bp, 16 oligos); (2) a Holliday junction cleavase (hjc) gene from the bacteriophage *SIRV-1* (total length 390 bp, 16 oligos); (3) ble (bleomycin resistance, total length 461 bp, 16 oligos); (4) DsRed (total length 733 bp, 26 oligos); (5) OR128-1 (total length 942 bp, 32 oligos); and (6) a GFP construct including a promoter and regulatory elements (total length 993 bp, 42 oligos), using the same sequence reported in Carr *et al.* (14). All genes were parsed in protein-mode, utilizing codon optimization with the exception of GFP, which was parsed in DNA-only mode. Relevant parameters for the parses selected from DNAWorks for all synthesized gene and gene segments are summarized in Supplementary Table I. Complete DNAWorks output files can be found in Supplementary Tables IIa–f.

PCA reaction mixtures

PCA reaction mixtures for each desired gene or gene segment were prepared for utilization with the microfluidic device. Each reaction mixture contained the following concentration of reagents: 1 mM dNTPs (250 μM each), 0.15 U/μl of Pfu Turbo Hotstart DNA Polymerase (Stratagene), ×1 cloned Pfu Buffer (Stratagene), 0.1% *n*-Dodecyl-β-D-maltoside (Sigma), 10 or 25 nM of each construction oligo depending on the construct and 500 nM of each amplifying outside primer. The addition of amplifying outside primers enabled the synthesis and amplification of the desired DNA construct in a single reaction. For synthesis of the full GFP construct and DsRed, 10 nM of each construction oligo was utilized, while for all other genes and gene segments, 25 nM of each construction oligo was used.

Two segments of the GFP gene were also synthesized; for these experiments, the first pool consisted of oligonucleotides 1–22, with 1 and 22 used as the primers to amplify segment 1, which was 531 bp in length. The second pool consisted of oligonucleotides 21–42, with 21 and 42 used as the primers to amplify segment 2, which was 529 bp in length. Oligonucleotides were purchased from Integrated DNA Technologies and Operon Biotechnologies without additional purification.

PDMS microchannel preparation

While PDMS has a number of superb characteristics that make it, in many cases, an ideal material from which automated biological platforms can be built, its hydrophobicity has inhibited certain biological processes due to a strong tendency for non-specific protein adsorption. PCR in μl and nl volumes generally suffers from such surface effects for a variety of materials because of the high surface-area-to-volume ratio of reactors (22), thus mandating some type of surface passivation. To address this problem in PDMS, we have successfully employed a nonionic surfactant, *n*-Dodecyl-β-D-maltoside (DDM), as a passivating agent (23). DDM adsorbs strongly to hydrophobic surfaces and, when included in reaction mixtures, is capable of successfully eliminating the majority of protein adsorption. Reaction mixtures that did not include DDM or any other passivating reagent failed to generate desired synthesis products.

Additionally, we found that devices exhibited the most robust, reliable performance after having been extensively thermocycled prior to conducting gene synthesis reactions. While the mechanism for this is not yet clear, experiments have shown a substantial increase in product yields when devices were first thermocycled with reactors containing a mixture of 0.1% DDM, ×1 Pfu Buffer and water for 100 cycles utilizing the following program: 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s (data not shown).

An Eppendorf Mastercycler Gradient thermocycler with an *in situ* adapter that facilitated thermal contact between the heating block and the glass slide was utilized for all thermocycling of microfluidic devices in this work.

Sample evaporation

Because of the high porosity of PDMS, during the course of thermocycling significant sample evaporation can occur, thus altering reactant concentrations and subsequently reducing reaction efficiency, and in some cases completely inhibiting synthesis. It has been found that the addition of fluid reservoirs in the vicinity of reaction chambers can reduce sample evaporation (17); thus, a water jacket composed of a mesh of fluid lines 50 μm wide with 300 μm spacing was designed in the control layer above the four reactors. When filled with water and actuated during thermocycling, the water jacket substantially decreased reactor evaporation as observed qualitatively.

Device design and operation

The microfluidic device was designed with individual reactor volumes of 500 nl to facilitate analysis of reaction products by polyacrylamide gel electrophoresis (PAGE). The overall device architecture is quite simple, with only three control lines necessary: a single valve to address all reactor inputs, a single valve to address all reactor outputs, and a control line for water jacket actuation. An array of 50 μm diameter posts present in each reactor prevented chamber ceiling collapse. Reactor input and output channels were 100 μm wide, while control lines were 300 μm wide, thus ensuring a strong seal to prevent sample evaporation from the reactor inlets and outlets during thermocycling. Without such valving, evaporation occurs almost instantaneously upon reaching the denaturation temperature.

All control lines were dead-end loaded with water by backing with pressurized air to force any air initially within the control lines out through the porous bulk PDMS. PCA mixes were introduced into the device by first actuating the reactor output valve at 15 psi and then dead-end loading the four reaction mixes at 5–10 psi into the reactor. Once all air bubbles were pushed out of the device, the inlet valve was closed to seal the reaction mix for thermocycling. All control valves, including the water jacket, were actuated and maintained at 15 psi for the duration of the synthesis reaction. Fresh devices that had been extensively thermocycled as described were used for each experiment.

Upon completion of sample loading, the device was placed on the *in situ* adapter of the Eppendorf Mastercycler Gradient and adhered with a small volume of mineral oil. Thermocycling commenced by heating first at 94°C for 2 min to activate the polymerase, followed by either 35 or 45 cycles of the subsequent program: 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. For synthesizing GFP and DsRed, 35 cycles were utilized, while for synthesizing all other gene and gene segments 45 cycles were used. Upon completion of cycling, a final 2 min extension at 72°C was conducted. Samples were collected by flushing with 5 μl of deionized water in preparation for analysis by PAGE.

It should be noted that while steel pins (New England Small Tube Corp.) were utilized to interface polymer control line tubing to the fluidic device, for all reaction mix introduction and collection steps, only polymer pins were utilized to interface to device inlets and outlets, as it has been reported that prolonged contact between reaction mixes and steel can inhibit PCR (24).

All fluid manipulations, including valving and pressure-driven flows, were controlled by individually actuated solenoid valves (The Lee Co.) connected through a custom-printed circuit board to a National Instruments DAQ card. A LabVIEW software interface allowed control over individual valves and fluid lines, while air flow for pressure-driven fluid manipulation was controlled by standard pressurized air regulators (McMaster).

Control experiments

Several sets of control experiments were conducted. For each PCA reaction mix, synthesis reactions were performed both within the fluidic and also *in vitro* in standard 0.2 ml PCR tubes to compare the performance of fluidic versus *in vitro* synthesis. Additionally, negative controls were conducted where construction oligos for synthesis reactions were omitted from the mixes. These 'primers-only' negative controls were run side-by-side in the microfluidic device with synthesis mixtures containing construction oligos. *In vitro* positive control experiments were conducted in an MWG Primus 2500 thermocycler utilizing the same thermocycle programs described above. All 'in fluidic' control experiments were similarly conducted with the 100-thermocycle microchannel treatment, discussed previously.

Polyacrylamide gel electrophoresis

Reaction mixtures collected from the four 500 nl reactors for all 'in fluidic' syntheses and negative control experiments along with 0.5 μl of each positive *in vitro* control were analyzed by PAGE (4–12% gradient TBE gel, Invitrogen) and visualized by SYBR Gold staining (Molecular Probes). Band intensities for synthesized genes and gene segments were approximated utilizing AlphaEaseFC software from Alpha Innotech Corporation.

DNA sequencing

Gene synthesis products were sequenced to confirm the identities of the six target genes. Upon completion of microfluidic gene synthesis and visualization by PAGE, reaction mixtures that demonstrated successful synthesis along with successful 'in fluidic' negative controls were further PCR amplified for 25 or 30 cycles to produce larger quantities of DNA for sequencing. 'Primers-only' negative controls were again conducted alongside this amplification step to verify that only gene products from the original microfluidic synthesis reaction and not a contaminant species were amplified. Upon completion of PCR, the resultant reaction mixtures were visualized by PAGE to verify successful amplification and the absence

of product in the negative controls. PCR products were purified using QIAquick PCR Purification Kits (QIAGEN) prior to sequencing. It was subsequently demonstrated (with the alba and DsRed genes) that gene assembly products taken directly from the microfluidic devices provided sufficient material for DNA sequencing, after first using ethanol precipitation to remove salts and enzymes.

The GFP gene product was sequenced using internal sequencing primers as in Carr *et al.* (14). All other gene products were sequenced (top and bottom strands) using the amplifying primers as sequencing primers, by the MIT Biopolymers Laboratory.

To quantify the errors present in these synthetic genes, one gene was chosen for further analysis. DsRed gene synthesis products were cloned (without purification or secondary amplification) into vector pCR4Blunt-TOPO (Invitrogen) and transformed into chemically competent DH5α cells. Individual colonies were picked and grown in Luria-Bertani broth. Glycerol stocks of these cultures were sent to Cogenics for plasmid extraction and sequencing. One 96-well plate of samples was sequenced (48 from cloned microfluidic-synthesized DsRed genes, 48 from the positive control synthesis reactions performed in standard 0.2-ml PCR tubes). All sequence reads were analyzed using the sequence-alignment tool ClustalX, and each error was verified by direct visual confirmation of electropherograms using Chromas (Technelysium).

RESULTS

Parallel gene syntheses were successfully conducted in a PDMS-based microfluidic device, as visualized in the gel shown in Figure 3 and ultimately verified by DNA sequencing. Here, parallel synthesis of four gene and gene segments, namely GFP segment 1 (531 bp), GFP segment 2 (529 bp), the hjc gene from bacteriophage *SIRV-1* (390 bp) and the randomized alba gene from *S. solfataricus* (327 bp), is demonstrated. Successful assembly was also achieved for the four positive *in vitro* controls, while successful 'primers-only' negative controls were conducted both 'in fluidic' and *in vitro* to confirm that the presence of desired-length product was not a consequence of amplification of contaminant species (not shown). Strong, dominant bands are evident for the desired products of all four fluidic syntheses, with product yields greater than 50% relative to the positive *in vitro* controls (i.e. in PCR tubes). The lower molecular weight species below the product bands indicate normal levels of assembly intermediates for a single reaction PCA.

Additionally, the synthesis of four additional constructs, the full-length GFP construct (993 bp), OR128-1 (942 bp), DsRed (733 bp), and ble (461 bp) was also accomplished, thus demonstrating the generality and robustness of microfluidic gene synthesis. Lower oligonucleotide concentrations (10 nM) were required for the longer genes (GFP, OR128-1 and DsRed), as it is hypothesized that, at higher construction oligo concentrations, all dNTPs are consumed generating intermediate products.

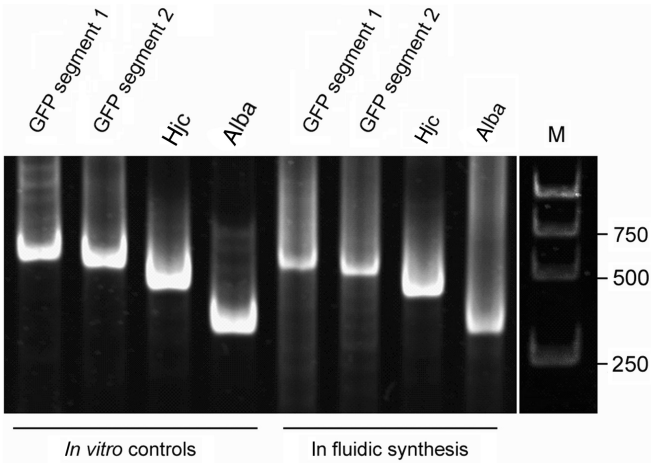

Figure 3. Polyacrylamide gel electrophoresis (PAGE) showing successful parallel synthesis of four gene and gene segments: GFP segment 1 (531 bp), GFP segment 2 (529 bp), the hjc gene from *SIRV-1* (390 bp), and a variant alba gene from *S. solfataricus* (327 bp). Positive *in vitro* controls are shown side-by-side. Molecular weight markers are shown (M) with 250, 500, and 750 bp positions indicated.

The results of the parallel syntheses of these four genes along with their respective negative controls are shown in Figure 4. Again, strong, dominant product bands are observed for all four assemblies, while the negative controls exhibit no discernable product bands. To obtain successful negative controls as shown in Figure 4, significant care must be taken to eliminate all contamination, as the presence of even minute quantities of template molecules can lead to undesired amplification—and thus erroneous results—in both PCA and PCR. These negative controls have yet to fail when appropriate care is taken to avoid contamination (fresh reagents and thorough cleanliness of all lab surfaces and equipment—pipettors and tips, PCR tubes, fluidic tubing, etc.).

In all cases, direct sequencing of microfluidic gene synthesis products unambiguously confirmed the identity of each target gene. However, such sequencing does not effectively report on the rate of error in the product material, as errors in individual molecules are effectively averaged out in the ensemble of products. Thus, one gene product (DsRed, 733 bp) was cloned, and the resultant clones sequenced to quantify error rates. For DsRed sequencing, purification (by length or secondary amplification) was deliberately omitted to prevent the addition or masking of errors in such processing. For the same reason, clones were not screened prior to sequencing other than blue/white screening to confirm successful insertion into the cloning vector. Thus, gene synthesis products (which include the desired full-length species along with other incomplete, intermediary products, as seen in Figure 4) were cloned directly from the microfluidic device or PCR tube upon verification of synthesis by PAGE. The results of this sequencing are shown in Table 1. Forty eight clones for both 'in fluidic' and *in vitro* DsRed synthesis yielded

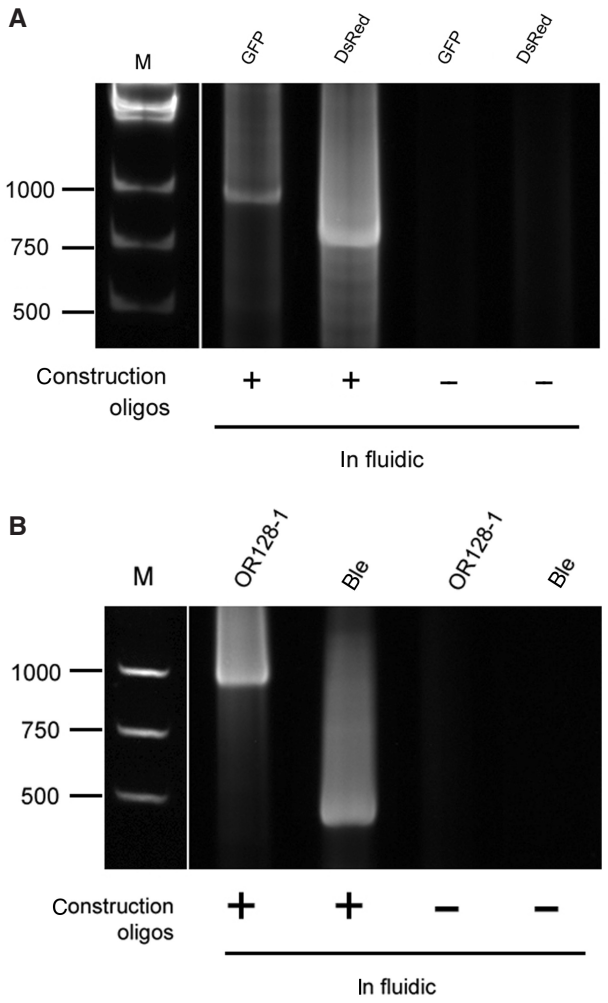

Figure 4. Polyacrylamide gel electrophoresis (PAGE) showing successful parallel synthesis of genes along with negative controls. In the presence of construction oligos, DNA constructs GFP and dsRed (993 and 733 bp, respectively; Figure 4A) and OR128-1 and ble (942 and 461 bp, respectively; Figure 4B) are synthesized and amplified. Without construction oligos, no product bands are generated. Molecular weight markers are shown (M) with 500, 750 and 1000 bp positions indicated.

Table 1. Summary of errors for synthesis of DsRed in the microfluidic device as compared to in a standard PCR tube

Error type		Microfluidic device	PCR tube
Deletion	Single-base	19	16
Deletion	Multiple-base	5	5
Transition	G/C to A/T	3	6
Transition	A/T to G/C	0	2
Transversion	G/C to C/G	0	0
Transversion	G/C to T/A	1	1
Transversion	A/T to C/G	1	0
Transversion	A/T to T/A	0	0
Total errors:		29	30
Bases sequenced:		16 250	13 389
Error rate (per base):		0.0018	0.0022

16 250 and 13 389 bases of sequence information, respectively. A total of 29 and 30 errors were identified for the 'in fluidic' and *in vitro* DsRed syntheses, thus generating error rates, per base, of 0.0018 and 0.0022, respectively, with an overall per-base error rate for all sequence reads of 0.0020. These values correspond well with the 0.0018 per-base error rates for the unpurified synthesis products reported by Carr *et al.* (14) and Hoover *et al.* (21), along with the 0.0027 per-base error rate reported by Kodumal *et al.* (13) Given the 0.0018 per-base error rate for 'in fluidic synthesis', as calculated in Carr *et al.* (14), ~9 DsRed clones are required for sequencing to have a high probability (95%) of at least one that is error-free. Ultimately, 12.5% of full-length clones were error-free, in agreement with theoretical expectations. For detailed tabulation of sequencing results, see Supplementary Table III.

DISCUSSION

Currently, the cost and time required to generate long, high-fidelity DNA molecules prevents such synthesis technology from being an extensively utilized resource. For example, at current oligo costs of approximately 1×10^{-1} dollars per base, applications such as the *de novo* synthesis of bacterial genomes 10^6 bp in size become prohibitively costly, requiring on the order of USD100 000 in oligos alone. Similarly, the ability to generate sets of hundreds or thousands of single genes is restricted. The costs of expensive reagents such as polymerase and oligonucleotides can be significantly reduced by utilizing microfluidic technology to minimize reaction volumes to a fraction of a microliter as compared to tens of microliters required in conventional syntheses.

Further reductions in oligonucleotide costs by several orders of magnitude can be achieved by utilizing the oligos synthesized from DNA microarrays (11,18–19). In such arrays, large numbers of distinct oligos are synthesized massively in parallel [10^4–10^5 or more for a single high-density array (25–26)] but in minute quantities (femtomoles or less). Thus, each oligo in a microarray can cost as little as 1×10^{-5} to 1×10^{-3} dollars per base, depending on the array, which typically cost between a few hundred to a few thousand dollars (e.g. USD489 for a 244 000 spot Agilent microarray). These costs per base are orders of magnitude less than for conventional oligo synthesis. Thus, the current significant contribution of oligo costs to the overall price of gene synthesis could be reduced to an almost trivial amount if the wealth of raw building material provided by microarrays could be successfully utilized. If maximally employed, oligo costs for building a 10^6 bp genome could potentially be reduced to tens of dollars. To achieve this goal, two difficulties must be addressed: (1) conducting synthesis from the low yields of each oligo in a microarray; and (2) problems that arise from manipulating highly complex pools of oligonucleotides ($>10^4$ distinct sequences). In this work, successful gene synthesis from minute oligo quantities (femtomoles) utilizing a microfluidic device architecture has been demonstrated, while such an architecture employed in conjunction with a microarray has the potential to overcome the limitations associated with complex pool manipulation.

Table 2. Calculations for expected oligonucleotide yields from a typical DNA microarray for 16 oligonucleotides, sufficient to build a 400 bp gene. Values for spot area and spot spacing are for commercially available Agilent and Nimblegen DNA microarrays

	Agilent	Nimblegen
Area of spot	$1.4 \times 10^4\ \mu m^2$	$2.56 \times 10^2\ \mu m^2$
Oligo density	0.1 pmol/mm^2	0.1 pmol/mm^2
Maximum expected yield per spot	1.4 fmol	0.0256 fmol
Dimensions of spot spacing	212 μm by 188 μm	25 μm by 25 μm
Minimal footprint of 16 oligo spots	$6.4 \times 10^5\ \mu m^2$	$1 \times 10^4\ \mu m^2$
Minimal chamber volume (10 μm height)	6.4 nl	100 pl
Estimated concentration of each oligo	220 nM	256 nM

In prior applications, oligos synthesized in microarray format have been cleaved from the arrays and collected in 'large' volumes (e.g. 5 μl or more) (11,18–19). The resulting low concentrations of oligo have been below the minimum needed to perform gene synthesis. Thus, additional process steps such as DNA concentration and/or amplification by PCR were required in order to assemble genes from this raw material. Direct gene synthesis of microarray oligos in microfluidic reactors such as the ones presented here can circumvent these requirements by confining synthesis reactions to individual chambers, thus maintaining oligo concentrations at levels sufficient for synthesis. Table 2 indicates the concentrations of construction oligos expected for two different microarrays (25,26) (Agilent, Nimblegen) for a reactor enclosing 16 oligo spots, sufficient to build a 400 bp gene. Using a reasonable estimate for oligo yields as function of spot area [0.1 pmol/mm^2, as in Richmond *et al.* (18); as much as 4 pmol/mm^2 have been estimated (27). See also Pirrung (28) for further discussion of oligo density], the spot size and spacing for the two microarrays, and assuming a chamber with the same height as the reactors used in this work (~10 μm), we estimate that construction oligos can be confined to yield concentrations in excess of 200 nM each. This is substantially larger than the 10–25 nM per oligo utilized for microfluidic synthesis reported here. Thus, ample room for error is provided to account for low oligo synthesis and/or cleavage yields, as well as chambers enclosing more oligo spots to synthesize larger genes. Employing such direct synthesis without concentration or an initial amplification step not only reduces the time and cost of the overall synthesis protocol, but also eliminates the possibility that additional errors will be generated during the amplification procedure. The oligonucleotide building blocks themselves are currently the greatest source of error in synthesized products; therefore, reducing the likelihood of further inaccuracies is crucial for obtaining high-quality synthetic DNA.

Resolving hundreds of thousands of oligos into reactions generating thousands of genes is a non-trivial challenge. For example, while multiplex gene synthesis utilizing bulk sample handling has been impressively demonstrated from an oligo pool containing ~600 distinct oligonucleotides (11), we expect such amplifications to become unfeasible for pools of higher complexity. Just as multiplex PCR suffers from inconsistencies such that each template may not be equally amplified (29), similarly the simultaneous amplification and subsequent assembly of 10^5 or more sequences is unlikely to proceed evenly. For gene synthesis, this is expected to be limiting; if the pool becomes dominated by a few DNA species, the required pool diversity would be lost, rendering assembly impossible. The absence of a single oligo prevents the assembly of its corresponding gene, so that losses even as low as 0.1% could interfere with the production of dozens or hundreds of genes. Correspondingly, other reagent concentrations become impacted by the complexity of oligo pools. For example, if only 1 nM of each construction oligo were required for synthesis (a low estimate), for a pool of 10^5 oligos the starting material would be 0.1 mM, meaning that virtually all the required deoxynucleotide (dNTP) precursors used by DNA polymerase would be consumed in the first cycle of a PCA reaction, terminating the reaction before generating the desired product. Use of a microfluidic device architecture such as the one presented in this work to enclose sets of oligo spots for gene synthesis would maintain reagent concentrations at desired levels while eliminating unwanted interference between sets of oligonucleotides in a complex pool. In the case of parallel synthesis of genes with related sequences (e.g. many variants of the same gene), avoiding undesired oligo annealing events during assembly will be crucial.

In this work we report, to our knowledge, the first gene synthesis in a microfluidic environment. Genes and gene segments with sizes as large as a kilobase were assembled in four parallel reactors in a single device. Reactions were conducted in 500 nl chambers, which are reaction volumes one to two orders of magnitude smaller than those used in conventional gene synthesis, thus achieving substantial reductions in reagent costs. This work also demonstrates the feasibility of utilizing such device architecture in conjunction with high-density oligonucleotide microarrays to potentially further reduce costs by several orders of magnitude. Microfluidic syntheses were successfully conducted at low construction oligonucleotide concentrations of 10–25 nM, values substantially lower than the anticipated concentration attainable from microarrays. By enclosing microarray oligos in microfluidic chambers, the currently required complex pool handling would be eliminated while enabling researchers, in principle, to maximally harness the high density of oligonucleotides present on a microarray. The effective use of such architecture in combination with high-density oligo microarrays would constitute a major step toward realizing the goal of low cost *de novo* gene synthesis.

While this work utilized four parallel 500 nl chambers to facilitate analysis of reaction products via PAGE, both the number and volume of reactors can be scaled substantially. Previous work has demonstrated PCR in volumes as small as 86 pl (30), and a 100 pl chamber with dimensions of 100 μm × 100 μm × 10 μm capable of enclosing groups of 16 oligonucleotides (described in the calculations in Table 1) can be fabricated with ease

using existing techniques. Extension of current work to microfluidic devices containing a high density of reactors for massively parallel gene synthesis is being currently investigated.

While fusion of microfluidic handling with oligo microarrays will provide the first step in making gene synthesis more available to researchers, integration with further microfluidic functions will allow this technology to mature. These advances will include:

(1) Incorporation of existing DNA error correction techniques (11,14–16) on-chip to improve the quality of the synthesis products. This will help minimize the need for another substantial contribution to the cost and time of gene synthesis: quality control (i.e. typically cloning and sequencing). While the device described in this work does not integrate on-chip error correction, it can be used readily with existing DNA error correction techniques both before and after synthesis. For example, construction oligos can first be gel-purified, as demonstrated by Hutchinson *et al*. (6), prior to conducting gene synthesis in the microfluidic device, or alternatively the MutS error-filter described by Carr *et al*. (14) could be performed on reaction mixtures collected from the device upon completion of synthesis. Thus, the microfluidic device can complement these bench-top error correction methods while providing the associated benefits of reduced reagent costs during synthesis. For certain *in vitro* applications, cloning will not necessarily be required.
(2) A second application will be the integration of *in vitro* protein expression using high quality synthetic DNA as a template.
(3) Finally, assembly of constructs larger than single genes can be achieved with microfluidic devices, employing the same types of hierarchical *in vitro* assembly reactions used to create 12 kb and larger segments (11–12).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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