Integrated two-step gene synthesis in a microfluidic device†

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Herein we present an integrated microfluidic device capable of performing two-step gene synthesis to assemble a pool of oligonucleotides into genes with the desired coding sequence. The device comprised of two polymerase chain reactions (PCRs), temperature-controlled hydrogel valves, electromagnetic micromixer, shuttle micromixer, volume meters, and magnetic beads based solid-phase PCR purification, fabricated using a fast prototyping method without lithography process. The fabricated device is combined with a miniaturized thermal cycler to perform gene synthesis. Oligonucleotides were first assembled into genes by polymerase chain assembly (PCA), and the full-length gene was amplified by a second PCR. The synthesized gene was further separated from the PCR reaction mixture by the solid-phase PCR purification. We have successfully used this device to synthesize a green fluorescent protein fragment (GFPuv) (760 bp), and obtained comparable synthesis yield and error rate with experiments conducted in a PCR tube within a commercial thermal cycler. The resulting error rate determined by DNA sequencing was 1 per 250 bp. To our knowledge, this is the first microfluidic device demonstrating integrated two-step gene synthesis.

Introduction

The design and manufacture of custom genes or long DNA biomolecules is fast becoming an indispensable tool in synthetic biology1 and protein engineering.2,3 The DNA biomolecules with man-made sequences are constructed by assembling pools of oligonucleotides into larger DNA using de novo synthesis methods. As the DNA structures can be custom designed and chemically synthesized, this technology has enabled broad applications on the engineering of proteins with novel functions,4 artificial gene networks,5 and synthetic genomes.6–8 Researchers have successfully utilized the de novo gene synthesis methods to assemble a viral genome6 (7.5 kb) in 2002, bacteriophage genome7 (5.4 kb) in 2003, and a gene cluster9 as large as 32 kb in 2004. The longest synthetic DNA reported to date is 582 kb, the genome of a bacterium (Mycoplasma genitalium) by Venter and co-workers8 in 2008. Furthermore, DNA synthesis has been successfully combined with high-density DNA microarray technologies,10,11 providing millions of unique oligonucleotides at a significantly lower cost (on the order of 1 cent per oligonucleotide) compared to the conventionally synthesized oligonucleotides (USD 0.2 per base). DNA biomolecules as large as 15 kb12 has been successfully constructed with oligonucleotides from a DNA microarray thus far.

Fig. 1 shows the concept of the two-step overlapping gene assembly method13 to create a synthetic gene. A pool of short oligonucleotides is first assembled into long double-stranded DNA (called a template) with the desired length and sequence information using the polymerase cycling assembly (PCA).17 The quantity of the assembled template DNA is then amplified by the PCR step. The PCR assembly utilizes the DNA polymerase to extend the oligonucleotides. As the mixed oligonucleotide is subjected to PCR, the overlap at their 3' ends are extended to generate longer double-stranded products. This process is repeated for the double-stranded products until the full-length gene is obtained. Synthesis via PCR can be performed either as a one-step process, combining assembly PCR and amplification PCR into a single stage, or as a two-step process with separate stages for assembly and amplification.
Numerous integrated microchip-based PCRs have been constructed using lab-on-a-chip technologies. These technologies and micro-PCR applications have been reviewed.18–21 Most of the reported microPCRs are designed for genetic analysis with integrated components, such as sample pre-treatment, microvalves, on-chip heaters and sensors, and various detection schemes. Kong et al. demonstrated the only polydimethylsiloxane (PDMS) device for one-step gene synthesis.22

In this article, we reported an integrated microfluidic device integrating PCRs with in situ hydrogel valves and a micromixer for constructing short oligonucleotides into a long DNA sequence. The baseline protocols on the concentrations of oligonucleotide and primer were first developed in a PCR tube, and then applied to microfluidic syntheses. Synthesis in microfluidic environments was successfully demonstrated in constructing a 760 bp GFPuv gene segment from a pool of 39 oligonucleotides using both the one-step and two-step synthesis processes. The accuracies of microfluidic gene synthesis were determined by DNA sequencing, and compared along with control experiments performed in standard PCR tubes within a commercial thermal cycler.

Along with the integrated gene synthesis chip, a microfluidic design to purify the synthesis product and prepare buffer solution for downstream application was described. Silica-coated magnetic beads were employed for the solid-phase PCR purification. The DNA extraction efficiency was tested. A short heat shock was applied to enhance the extraction efficiency.

Materials and methods

Microfluidic device fabrication

Instead of using the SU-8-based lithography process to create the PDMS casting mold, we have adopted a three-dimensional (3D) rapid prototype method that printed a 3D structure using photosensitive resin.23 The 3D structure was designed in SolidWorks and transferred to the Eden 350 (Objet Geometries), which printed photopolymer material (FullCure 720) and support material (FullCure 705) layer by layer. The photo-polymer layer was cured by UV light immediately after it was printed. Upon completion, the fabricated structure was soaked in 25% tetramethylammonium hydroxide (TMAH) solution for 3 h to remove the support material designed for supporting the printed geometries. The microfluidic mold was soaked in water for 1 h to wash away TMAH. This method provided a resolution of 42 μm in the x-axis and y-axis, and a resolution of 16 μm in the z axis, well-suited for generating thick and multilevel structures without lithographic process. Conversely, other rapid prototype methods such as liquid phase photopolymerization24 and contact liquid photolithographic polymerization25 utilized photomasks to facilitate construction of structures with superior resolution. Two-level mold was designed with different heights for connection channels (height: 0.2 mm; width: 0.2 mm) and chambers (height: 0.5 mm) to minimize the dead volume of connection channels.

The PDMS precursor was prepared by mixing Sylgard 184 base and Sylgard 184 curing agent in a 10 : 1 volume ratio. The precursor was poured into the mold, degassed in a vacuum chamber for 30 min, and cured in a convection oven at 75 °C for 3 h. The 3 mm thick PDMS slab was then peeled off from the mold, and connection holes were pierced. The microfluidic device was assembled by bonding PDMS and silicon substrate (500 μm-thick). Both the PDMS and silicon substrate were treated with electrical discharges (Model BD-10AV, Electro-Technic Products) for 30 s, and the two surfaces were brought together immediately after treatment.26 Finally, the device was cured in an oven at 75 °C for 2 h to ensure irreversible bonding between PDMS and the silicon substrate.

To prevent sample evaporation, the bonded device was deposited with a 2 μm-thick Parylene C using the PDS 2010 Parylene Deposition System (SCS, USA). The vapor deposited Parylene C created a barrier to control the water vapor diffusion. Parylene also passivated the inner surface of the device in preventing unwanted protein adsorption.27,28

Preparation of hydrogel valves

Thermosensitive hydrogel valves were selected for fluidic regulation and confining PCR reaction. Hydrogel was synthesized following the method suggested by H. J. van der Linden et al.29 Temperature-sensitive monomer N-isopropylacrylamide (NIPAAm, 286 mg), N,N’-methylene bisacrylamide (BIS, 7.88 mg) crosslinker and 2,2’-dimethoxy-2-phenyl acetophenone (DMPAP, 18.86 mg) photoinitiator were mixed in 500 μL of dimethylsulfoxide (DMSO), generating a precursor solution containing 2% BIS crosslinker. The precursor was purged with nitrogen to remove oxygen, and wrapped with aluminium foil to avoid unwanted photopolymerization. All the chemicals were purchased from Sigma-Aldrich (Singapore).

The mixture was then injected into the fabricated chip using a 1 mL syringe through the access holes, and photopolymerized in situ at 32 °C with a chromium mask defining the exposed area. The sample was then irradiated at a wavelength of 365 nm (dose: 252 mJ/cm²) using OmniCure Series 2000 UV illumination system (EXFO, Canada). After ultraviolet exposure, the device was placed on a hotplate at 60 °C to keep the hydrogel valves open, and the unpolymerized precursor was removed with de-ionized water at a flow rate of 500 μL/min for 40 min using a syringe pump (74900 Series, Cole-Parmer Instrument Company). Finally, the device was baked at 75 °C for 3 h in an oven to dry its inner surface and hydrogel valves.

The NIPAAm-based hydrogel is thermosensitive with a lower critical solution temperature (LCST) of 32 °C.29 The hydrogel would swell at temperatures below 32 °C, blocking the fluidic channel. At a temperature above 32 °C, the polymer chains became hydrophobic, causing the hydrogel to shrink and allowing fluid to flow through. The opening and closing of valves were controlled by varying the temperature between 4 °C and 60 °C. An example of the integrated hydrogel valves in the microfluidic device fabricated with the printed photosensitive resin mold is shown in Fig. 2.

PCR thermal cycling

The PCR was performed by using a home-made thermal cycler, which included a fan, a thermoelectric (TE) heater/cooler (9501/127/030, FerroTec) and a thermoelectric control kit (FerroTec, USA) consisting of a FTA600 H-bridge amplifier, FTC100...
A published gene segment of GFPuv with a total length of 760 bp (sequence 261–1020 with T357C, T811A and C812G base substitutions) was selected for synthesis. It was assembled using (sequence 261–1020 with T357C, T811A and C812G base substitutions) was selected for synthesis. It was assembled using an algorithm to optimize the temperature response time.

**Gene assembly and amplification**

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**Solid-phase buffer exchange**

Solid-phase buffer exchange was conducted using the magnetic beads based PCR purification method (ChargeSwitch PCR clean-up kit, Invitrogen) on microfluidic devices, and in standard 0.2 mL PCR tubes (as control) with the synthesized PCR product and 100 bp DNA ladder (New England, 170 ng/μL) as control.

For the control experiment performed in the PCR tube, the 100 bp DNA ladder or PCR-synthesized product (7 μL) was mixed with 5 μL of beads and 11 μL of purification buffer (Invitrogen), and incubated for 1 min. The beads were then captured by a magnet to remove the supernatant. We washed the beads with 150 μL of washing buffer (Invitrogen) three times, and loaded 7 μL of elution buffer (10 mM of Tris-HCl, pH 8.5) to the washed beads. The elution buffer and beads were incubated at different conditions (25–80 °C for 2–3 min) to optimize the elution efficiency of bound DNA. The concentrations of the original and eluted DNA samples were measured and compared by a UV-Vis spectrophotometer (ND-100, NanoDrop Technologies).

A similar process was conducted on the two-step microfluidic device (Fig. 2c). The 100 bp ladder or PCR product (7 μL) and magnetic beads (5 μL) in purification buffer (11 μL) were first loaded into M3 and M4, respectively, with a volume defined by the meter chambers. These two solutions were mixed using an external syringe pump (Cavro XLP 6000), pushed to the bead chamber (C3), and incubated for 1 min. The impurities in the bead chamber were then washed with washing buffer introduced from A5 at a flow rate of 200 μL/min for 15 min with beads captured by a permanent magnet (M1219–5, Assentech). After washing, elution buffer (10 mM of Tris-HCl, pH 8.5, 5 μL) was introduced into the bead chamber, and incubated with the beads at 25–80 °C for 2–3 min to release the bound DNA. The magnetic beads were actively mixed at a rate of 0.5 Hz before elution using external electromagnets with the setup shown in Fig. 4b. A permanent magnet was mounted on a flexible and suspended metal arm located between two electromagnets (GMHX, Magnet-Schultz Ltd). Alternate magnetic forces were applied to the metal arm when 180° out-of-phase voltages were supplied to the electromagnets, which swung the metal arm and the mounted
magnet. The electromagnets were powered through the solid-state relays (ODCM-5, Tyco) and a DC power supply (HY3003, Digimess). Electromagnetic forces were regulated through the relays using an analog voltage output board (PCI-6713, National Instruments), and a computer with a LabVIEW program (National Instruments).

**Agarose gel electrophoresis**

Synthesized products were analyzed by 1.5% agarose gel (NuSieve® GTG®, Cambrex Corporation), stained with ethidium bromide (Bio-Rad Laboratories), and visualized using a Typhoon 9410 variable imager (Amersham Biosciences). Gel electrophoresis was performed at 100 V for 45 min with 100 bp ladder (New England) and 5 µL of DNA samples collected from a commercial thermal cycler and devices.

**DNA sequencing**

One-step and two-step overlapping synthesis products were sequenced to check the error rate. GFPuv gene synthesis products (without further PCR purification) were cloned into vector pCR®2.1-TOPO® (Invitrogen) and transformed into chemically competent TOP10 cells. After overnight growth on a 1× Luria-Bertani (LB) agar plate (with 100 µg/mL of ampicillin), individual colonies were picked and grown in 1× LB media (with 100 µg/mL of ampicillin). The plasmid DNA was extracted by using QIAprep Spin Miniprep Kit (QIAGEN), and sequenced by Research Biolabs (Singapore). In total, 150 individual samples were sequenced using M13 forward and reverse sequencing primers (for one-step process: 96 from microfluidic device and 48 from 0.2 mL PCR tube; for two-step process: 54 from microfluidic device and 48 from 0.2 mL PCR tube). All sequence results were analyzed using sequence analysis tool Vector NIT, and the errors were verified by visual confirmation of the electrophoregrams of the ABI PRISM® 3100-Avant Genetic Analyzer.

**Results and discussion**

**Device operation**

A precision syringe pump with multi-position valve (Cavro XLP6000, Tecan Systems) was used to manipulate reagents inside the microfluidic device. This syringe pump is capable of withdrawing and dispensing reagents with a volume resolution of better than 10 nL, as controlled by a LabVIEW program (National Instruments). To control the hydrogel valves and thermal cycling simultaneously and separately, two TE modules with individual temperature controllers were used (Fig. 2). One TE module (TE 1) was located under the PCR chambers to perform the temperature cycling, and the other TE module (TE 2) was located under the hydrogel valves to control their action.

The overall device operation of the gene synthesis device was illustrated in Fig. 3 with the volume defined by each chamber. Oligonucleotide and PCR mixture was first loaded into the PCA chamber through the inlet port (A1). The solution was then sealed by the hydrogel valves (V1 and V2) and thermally cycled with the thermoelectric heater to assemble oligonucleotides. After PCA, the hydrogel valves (V1 and V2) were opened, and the solution was pumped into meter chamber M1, and simultaneously mixed with an equal volume of fresh PCR mixture containing outer primers from meter chamber M2. To enhance mixing, this mixture was shuttled between two mixing chambers (C1 and C2) five times (flow rate = 120 µL/min) with the precision syringe pump at inlet port B2, and then moved to PCR chamber with hydrogel valves (V3 and V4) kept open. After PCR amplification, the hydrogel valves (V3 and V4) were open again, and the solution was moved to meter chamber M3 (through inlet port A3), and simultaneously mixed with the magnetic beads solution defined by meter chamber M4 in the beads chamber (C3). With the DNA-adsorbed magnetic beads captured by a permanent magnet, the impurities solution was washed out. Finally, the elution buffer was loaded and mixed with the magnetic beads; the DNA was then released into the elution buffer. To control the flow direction, unused inlets and outlets were plugged with metal pins. For example, to direct a PCA mixture to a PCR chamber, the inlets (A4-A7) for solid-phase PCR purification were plugged.

Two micromixers were developed to effectively mix the PCA product with fresh PCR mixture for PCR amplification, and mix the magnetic beads with DNA solution and elution buffer for solid-phase PCR purification. The gene synthesis chip was to be developed as a bench-top instrument to perform automatic gene synthesis. To control the cost and simplify the fabrication...
process of these disposable chips, mixing approaches utilizing simple fluidic structures and methods were desired. Fig. 4 shows our approaches using shuttle mixing and electromagnetic mixing. In shuttle mixing, solution was shuttled between two chambers connected by a narrow channel. This narrow channel reduced the diffusion distance of two mixing reagents, and the abrupt opening at channel-chamber junctions created chaotic advection at the junctions and recirculated the flow. Both of these features were reported to enhance mixing. Fig. 4a demonstrated the performance of the shuttling micromixer. Two colored food dyes (blue and red) were well mixed after being shuttled three times between two chambers at a flow rate of 150 μL/min, pumped by a precision syringe pump. This method was effective with compact and simple fluidic structures as compared to other reported methods. Mixing was completed within 1 min in our application with a fluid volume of 19 μL. No visible air bubbles were trapped inside the solution.

Permanent neodymium rare earth magnet was utilized to capture magnetic beads in the microfluidic device, as it provided a strong magnetic force. However, this strong magnet could also cause the aggregation of beads and hinder the beads from full contact with the desired biomolecules in solution. To make sure that the beads were well mixed with solution, we have developed an approach to agitate the solution inside the chamber (Fig. 4b). A permanent magnet was mounted on a flexible metal arm that was sandwiched by two electromagnets. When out-of-phase voltages were applied to the electromagnets, alternating magnetic forces were generated, which swung the metal arm and the permanent magnet simultaneously. The swinging magnet dragged the magnetic beads and agitated the solution. This simple approach was employed to mix the elution buffer with DNA-bound magnetic beads in the final step of PCR purification at a mixing rate of 0.5 Hz.

**In situ hydrogel valve**

Microvalves are critical to the successful integration of the PCR process into a microfluidic device, which has to be able to withstand at least 6.8 psi to ensure successful sealing of the PCR mixture within the chamber. During the PCR process, the air solubility variation from 4 °C to 94 °C could create a pressure of ~3.1 psi, and potential trapped air bubbles would contribute to an additional pressure of 3.7 psi at 94 °C.

The hydrogel valves were tested prior to use on the single-chamber device (Fig. 2b) with a liquid flow meter (SLG1430, Sensirion) connected between a constant pressurized water reservoir (8 psi) and the device. The flow rate variation was monitored as the valve was subjected to repetitive cooling and heating by a thermoelectric heater underneath the device. As indicated in Fig. 5, the valve functions were highly repeatable with valve’s opening and closing times of ~5 s and ~20 s, respectively (see inset in Fig. 5), limited by the ramping rate of the heater underneath, and the water diffusion rate in the hydrogel swelling/de-swelling process. The closed valve exhibited no leakage (zero flow rate) at 8 psi, showing that it was strong enough to seal the PCR chamber. Yu et al. reported that an in situ photopolymerized NIPAAm-based valve could withstand a pressure of up to 200 psi. Wang et al. also described the successful integration of a chemically polymerized NIPAAm hydrogel valve with PCR by manual insertion of pre-synthesized hydrogel in the flow paths.

![Fig. 4](image1.png) (a) Photographs of micromixer. Colored dyes (blue and red) were well mixed after being shuttled three times between two chambers. (b) Schematic illustration of the experimental arrangement with a syringe pump, electromagnetic mixer, thermoelectric heaters and data acquisition.

![Fig. 5](image2.png) The thermal response of in situ photopolymerized hydrogel valve. The valve functions were highly repeatable. The insets show the transitions of valve functions.
PCR thermal cycling

The gene synthesis process was integrated into a chip composed of a PDMS fluidic structure on a silicon substrate. Although PDMS has a number of interesting material properties that make it superior for constructing highly integrated biological micro-systems, its non-specific protein adsorption\(^{3,4}\) and permeability to water vapor\(^{44}\) could pose problems in performing PCR in a microfluidic environment, which has a small volume and a high surface-to-volume ratio. To address these problems, we have coated the fabricated devices with 2 \(\mu\)m-thick parylene, which created a barrier against water vapor diffusion and improved the surface compatibility with PCR mixture.\(^{47}\)

A thermoelectric module with heat sinks and fans was utilized for thermal cycling. Fig. 6 shows the temperature profiles of the thermal cycler obtained from a calibration chip, which has identical dimensions as the actual device, but has a thermocouple embedded within the PCR chamber. Temperatures at the heater surface and within the PCR chamber were measured. The temperature difference between these two locations indicated that the 500 \(\mu\)m-thick silicon substrate could cause a temperature drop of >5 \(^\circ\)C, which was compensated during the operation of thermal cycling. The heating and cooling rates estimated from Fig. 6 were 2.4 \(^\circ\)C/s and 4.3 \(^\circ\)C/s, respectively, which were faster than those in the commercial thermal cycler (DNA Engine PTC-200).

To generate enough quantity of synthesized products for further process, the PCR chamber was designed with a volume of 7 \(\mu\)L. Genes synthesized by PCR methods contained both full-length DNA and intermediaries with shorter lengths. After synthesis, gel electrophoresis was usually conducted to confirm the success of the synthesis, and to separate the full-length product, which was then extracted from the gel by using gel extraction kits. Some DNA could be lost due to these steps and the pipetting process. The PCR mixture was introduced into the PCR chamber through the hydrogel valves that were kept opened by a thermoelectric heater at 60 \(^\circ\)C. Once the PCR chamber was filled with the solution, the hydrogel valves were cooled to 4 \(^\circ\)C, sealing the chamber. Since silicon with high thermal conductivity was used as the device substrate, the PCR chamber and hydrogel valves were positioned apart to minimize thermal interference between the PCR thermal cycling and the valves’ operation. The hydrogel valve has to be kept below the transition temperature to seal the PCR chamber during thermal cycling, which could reach a temperature as high as 95 \(^\circ\)C. One way to suppress the thermal interference and reduce the dead volume between the PCR chamber and valves was to use a polymer substrate (such as polycarbonate)\(^{48}\) or an isolation trench to suppress the lateral heat flow along the substrate, as reported by Wang et al.\(^{44}\) and Yang et al.\(^{46}\)

Comparison of one-step and two-step gene syntheses

The thermal cycler’s requirement for PCR assembly was the same as that for the standard PCR amplification. However, the number of oligonucleotides involved in PCR assembly was much larger than in the standard PCR amplification. Full-length DNA was constructed from a pool of solution containing tens of oligonucleotides with various melting temperatures. The efficiency of successful gene synthesis relied on several important factors including the polymerase, concentrations of assembly oligonucleotides and amplification primers, and structure and properties of oligonucleotides.\(^{3,47}\)

To identify the baseline of oligonucleotide and primer concentrations, a segment of GFPuv (760 bp) was synthesized from a pool of short oligonucleotides (40 bases) using a two-step PCR process by varying oligonucleotide concentration from 5 to 25 nM, and primer concentration from 0.1 to 0.4 \(\mu\)M; this was conducted on the commercial thermal cycler. Desired full-length product was first assembled from oligonucleotides without outer primers (PCA assembly), and then amplified by adding these primers at the second PCR (PCR amplification). To match the microfluidic device design (Fig. 2c), the PCR amplification was performed with the PCA product diluted with an equal volume of fresh amplification reaction mixture. Gel electrophoresis results for PCA assembly (Fig. 7a) and PCR amplification (Fig. 7b) were illustrated for the indicated oligonucleotide and primer concentrations. The PCA has smearing gel results, indicating that the assembled product contained a spectrum of DNAs, the majority of which possessed lower molecular weights than the desired target (760 bp). For products assembled from oligonucleotide concentrations of <10 nM, the quantity of full-length DNA (760 bp) was very low and invisible in the PCA gel images, but this was effectively boosted with PCR amplification. PCR gel images show samples 1–1 to 1–3 synthesized with an oligonucleotide concentration of 5 nM and a primer concentration of 0.1 \(\mu\)M, 0.2 \(\mu\)M and 0.4 \(\mu\)M, respectively. Other samples were the same as samples 1–1 to 1–3, except that the oligonucleotide concentrations used were 10 nM, 15 nM and 25 nM. Syntheses with an oligonucleotide concentration of >10 nM and a primer concentration of 0.1 \(\mu\)M and 0.2 \(\mu\)M failed to provide the desired full-length (760 bp) product. In contrast, syntheses performed with 0.4 \(\mu\)M of primer all successfully produced the target 760 bp DNA. Of the four samples, sample 2–3 with an oligonucleotide concentration of 10 nM and a primer concentration of 0.4 \(\mu\)M produced the most full-length product, even though samples 3–3 (15 nM, 0.4 \(\mu\)M) and 4–3 (25 nM, 0.4 \(\mu\)M)
have more full-length DNA generated initially from the PCA step. These results corresponded well with those reported by Kong et al.\textsuperscript{22} and Wu et al.\textsuperscript{47} for the one-step gene synthesis process. Based on these findings, an oligonucleotide concentration of 10 nM and a primer concentration of 0.4 mM were selected for gene synthesis on a microfluidic device.

With the optimized oligonucleotide and primer concentrations, GFPuv (760 bp) was successfully synthesized from a pool of short oligonucleotids (40 bases) by using either one-step (single-chamber chip) or two-step microfluidic devices. A strong, dominant band of the desired products was obtained in the gel images (Fig. 8). The visually estimated yields of microfluidic devices were \(rac{50\%}{\text{of the controls performed in PCR tubes with a commercial thermal cycler. These were limited by the dead volume (2.87 \mu L) in the channels between the PCR chamber (7 \mu L) and the valves. The oligonucleotides mixture within the dead volume did not assemble, but contributed to \~30\% of the eluted solution. The gel results also demonstrated that parylene was compatible with PCR reaction mixture, and effectively blocked the reagents against evaporation from the water vapor-permeable PDMS.}

Compared to the one-step process, the two-step process generated much more full-length product from the same amount of initial oligonucleotides. In the one-step process, the assembly and amplification were conducted simultaneously, which competed for the fixed amount oligonucleotides and monomers (dNTPs), and rendered intermediary products with lower molecular weights (Fig. 8a). The process competition was minimized in the two-step process, resulting in more full-length product.\textsuperscript{14} The two-step process was reported to be more reliable than the one-step process, which sometimes failed to generate full-length DNA.\textsuperscript{12,13} Gene synthesis with the two-step process also allowed for different annealing temperatures to optimize the assembly and amplification processes separately.

The assembled sequence was identified by DNA sequencing. Synthesized products from the microfluidic devices and PCR tubes were cloned directly without further purification using PCR\textsuperscript{2.1-TOPO} cloning vector (Invitrogen). Full-length target along with intermediary products were all cloned to reflect the real composition of the synthesized products. Table 1 shows the sequencing results. The error rates per kilobase (kb) calculated from full-length clones were 3.45 in device and 4.36 in PCR tube for the one-step process, and 4.01 in device and 4.10 in PCR tube for the two-step process. These values were within the range of the error rates reported (1.8–6 per kb).\textsuperscript{10,13,48,49} Most errors (>85\%) were associated with single-base insertion, deletion and mutation. The indifference in error rates implied that they were independent of the synthesis methods (device versus PCR tube).
and processes (one-step versus two-step). Hoover et al.66 and Tian et al.19 pointed out that the greatest errors were attributed to the quality of synthetic oligonucleotides, not from the fidelity of polymerase enzyme. Oligonucleotides were chemically synthesized base-by-base with a step yield of \( \sim 98.5\% \).60 The overall yield of full-length oligonucleotides decreased as the oligonucleotide length increased. For example, only 54.6% of oligonucleotides were full-length in a targeted 40 base-long synthesis product. The building blocks of synthetic oligonucleotides containing both perfect match sequence and impurities with mismatch (single base and multiple bases) could all have participated in the PCR process and generated products of incorrect sequence. In contrast, the DNA polymerase has a replication error rate of \( \sim 10^{-6} \) base/duplication,41 which was 3–4 orders lower than the error rate of synthetic gene products. Performing gene synthesis in a microfluidic device might not improve the accuracy of synthesis products, as demonstrated by Kong et al. in microPCR one-step gene synthesis.22 However, it would reduce the handling time and reagents costs, and eliminate human process factors.

PCR product cloning and DNA sequencing were required to ensure that an accurate synthesis product was obtained. These processes involved substantial laboratory efforts. To obtain an error-free gene, many randomly selected clones were sequenced,30,55 which might contain either undesired truncated DNAs or the desired full-length DNA. The greater full-length yield of the two-step process increased the possibility in obtaining effective full-length clones, and in achieving an error-free gene. About three out of four clones (35/47 in PCR tube) produced by the two-step process contained full-length products, which was greater than that produced in the one-step process (about one out of three clones (16/47 in PCR tube)) (Table 1). Therefore, the two-step process would be preferred in minimizing the number of colony sequencing required to obtain an error-free gene, and the effort of cloning and DNA sequencing, especially for long DNAs.

Thermally enhanced solid-phase PCR purification

For applications such as cell-free protein synthesis (which directly use synthetic genes for protein expression) and integration of enzymatic error filtering methods on chip to reduce the error rate of synthesized products, a solid-phase buffer exchange process was integrated with the two-step microfluidic device utilizing magnetic beads-based PCR purification method (ChargeSwitch PCR clean-up Kits, Invitrogen). This process was intended to purify the assembled product from short primers and dNTPs, and to prepare the buffer solution for downstream application. Silica-coated magnetic beads could help simplify the device integration as compared to other nucleic acid extraction methods reported by Jemere et al.,58 West et al.,59 and Breadmore et al.60

ChargeSwitch utilized the same approach as other reported methods.59,60 DNA was first adsorbed onto the silica surface under high ionic strength conditions. The unbound impurities were washed away, and then the adsorbed DNA was released into solution with a higher pH (10 mM of Tris-HCl, pH 8.5). The ChargeSwitch Kit was first optimized in standard PCR tubes using a 100 bp DNA ladder with a known DNA quantity (1.19 µg) as the control following the approach and protocol suggested by the manufacturer. The reagents volume was modified to match the design of the microfluidic device. After the baseline protocol was established using the PCR tube and the 100 bp ladder, the procedure was applied to the microfluidic device for the 100 bp DNA ladder and PCR synthesized product. The total amount of 100 bp DNA ladder (1.98 µg) or PCR product (1.98 µg) was less than the binding capability of the ChargeSwitch beads loaded. Based on the manufacturer’s protocol, the ChargeSwitch beads would bind double-stranded DNAs with lengths of >90 bp; thus, the 100 bp DNA ladder was selected as the control. The DNA extraction included three steps—DNA capture, impurities wash, and DNA elution. The DNA elution conditions (time and temperature) were investigated to increase the extraction efficiency.

The extraction efficiency, defined as the percentage of DNA captured and released, is shown in Fig. 9. The quantities of the original and eluted DNA samples were determined by a UV-Vis spectrophotometer. Every measurement was repeated three times. The average extraction efficiency of 100 bp DNA ladder was 65.4% in PCR tube and 42.2% in microfluidic device, eluted at 25 °C for 3 min in 7 µL of Tris-HCl buffer (10 mM of Tris-HCl, pH 8.5). When the adsorbed DNA was subjected to increasing elution temperature, the release of bound DNA was enhanced. The extraction efficiency increased effectively to 86% in PCR tube and 70% in the microfluidic device when incubated at 60 °C for 3 min. A further increase in temperature did not improve the extraction efficiency. The extraction efficiency was slightly improved (<10%) with increasing incubation time (2 min to 3 min). The thermally enhanced DNA elution could be due to either the temperature effect of pH variation in Tris-HCl buffer or the increased thermal momentum of bound DNA;41 the details are being investigated.

For PCR synthetic product, the extraction efficiencies were 76.6% (+3.38%, −5.66%) in PCR tube and 61.3% (+3.51%, −2.56%) in microfluidic device when incubated at 60 °C for 3 min. These efficiencies were lower than the 86% and 70.1% achieved for 100 bp DNA ladder, respectively. The differences could be due to the short primers and monomers (dNTPs), which also absorbed UV light at a wavelength of 260 nm like dsDNA.

![Fig. 9](https://example.com/fig9.jpg)
The drop in extraction efficiency indicated that the impurities (short primers and monomers) in the PCR product were removed. This thermally enhanced DNA extraction was simple, and provided an extraction efficiency that was close to that achieved with sol-gel derivized silica particles (65%).66 monolithic sol-gel microchip (85%)64 and densely packed microfabricated silicon structure (75%).65 It could be easily integrated with most microfluidic devices without extra fabrication steps or modification, and allowed successful extraction of microgram quantities of DNA in 7 μL of elution buffer in <10 min. The high loading capacity (micrograms) was particularly desirable for extracting PCR-synthesized products. Most solid-phase DNA extraction chips69-42 were designed for DNA purification from biological samples, having a binding capacity of nanograms only. The short heat shock (3 min) effectively increased the extraction efficiency from 42.2% (25 °C) to 70% (60 °C) in the microfluidic device.

Conclusion

In conclusion, we have successfully integrated the gene synthesis and DNA extraction processes into a chip using a fast prototyping method without lithography process. The PDMS/silicon chip was fabricated by utilizing a printed three-dimensional mold of photopolymerized resin. The protein adsorption and PCR mixture evaporation in PDMS were eliminated by coating the device with a thin layer of parylene. The fluidic control was realized with a precision syringe pump, and thermally activated hydrogel valves. PCR reaction mixture was sealed during thermal cycling by in situ hydrogel valves, which were tested and capable of withstanding pressures of ≥8 psi without visible leakage.

Microfluidic syntheses were successfully attained with low oligonucleotide concentration of 10 nM and primer concentration of 0.4 μM using one-step and two-step PCR-based gene synthesis processes. More full-length products were generated by the two-step process, but the resulting error rates of both processes were not very different. The synthesized products were verified by DNA sequencing to have an error rate of ~1 per 250 bases, comparable to the control experiments conducted in PCR tube with a commercial thermal cycler. Although performing gene synthesis in a micro-environment might not improve the accuracy of synthesis products, it would reduce the handling time and reagents costs, and eliminate human process factors.

A magnetic beads-based PCR purification was also developed to separate the synthesized product from the PCR reaction mixture for further applications. A 70% extraction efficiency and microgram-level DNA loading capacity were obtained by applying a short heat shock (60 °C for 3 min) before DNA elution. This key component helped to prepare the synthesized gene in a suitable buffer solution for in vitro cell-free protein synthesis,53,54 or integrate DNA error correction methods,30,52,55,56 on chip to improve the accuracy of synthesized products, which is critical for successful gene synthesis. The process takes ~2 h including two PCRs (30 cycles each) and the PCR purification (<10 min), producing ~2 μg of DNA products (752 bp).

The device reported herein would be useful for constructing a more comprehensive system for fully automated gene synthesis. To achieve this goal, several issues need to be addressed to make the device ready for wide-spread use. One of those is the cost of device and synthesis reagents. The current cost per device is ~$5 (mainly from the silicon substrate) and can be reduced to a feasible cost of pennies per device if plastic substrates and hot embossing technology64 are applied for mass fabrication. While this work was demonstrated with fluidic design in microlitre scale to directly compare with experiments in PCR tube, the volume of reactors and the dimensions of structures can be scaled down substantially to provide more cost-effective gene synthesis. Previous work by Kong et al.25 has demonstrated the feasibility of one-step gene synthesis in 500 nL chambers. The design of hydrogel valves, reaction chambers, micromixers, and PCR purification are flexible and can be scaled down without significant design modification.

While the device reported here provides significant progress toward the end goal, substantial efforts are needed for system integration of the entire unit, such as chip-system fluidic interfacing and precision fluid delivery.

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Notes and references

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