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Research Article

A novel DNA selection and direct extraction process and its application in DNA recombination

In the conventional bench-top approach, the DNA recombination process is time- and effort-consuming due to laborious procedures lasting from several hours to a day. A novel DNA selection and direct extraction process has been proposed, integrated and tested on chip. The integrative microfluidic chip can perform the whole procedure of DNA recombination, including DNA digestion, gel electrophoresis, DNA extraction and insert-vector ligation within 1 h. In this high-throughput design, the manual gel cutting was replaced by an automatic processing system that performed high-quality and high-recovery efficiency in DNA extraction process. With no need of gel-dissolving reagents and manipulation, the application of selection and direct extraction process could significantly eliminate the risks from UV and EtBr and also facilitate DNA recombination. Reliable output with high success rate of cloning has been achieved with a significant reduction in operational hazards, required materials, efforts and time.

Keywords:
Chip integration / DNA recombination / DNA selection and extraction / Microfluidics
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1 Introduction

Subcloning, a standard technique by moving a specific gene of interest (insert) from a chosen vector to a destination vector, is the most commonly used method in genetic engineering, but is also broadly considered to be effort- and time-consuming. The subcloning procedure consists of DNA recombination and transformation and the standard flowchart is shown in Fig. 1A. Generally, three categories of cloning are adopted in biology laboratories: the restriction cloning, in which the interested DNA fragments are digested by restriction enzymes (REs) [1]; the polymerase chain reaction (PCR) cloning, in which the target DNA fragments are obtained by PCR amplification [2]; and the entry/gateway® cloning, in which the insert DNA are moved to the destination vector by the entry vector [3]. Among the three bench-top procedures of DNA recombination, the most straightforward, economical and thus commonly adopted is the restriction cloning. The DNA recombination process of restriction cloning composed of the following steps: RE digestion, gel electrophoresis, DNA extraction and insert-vector ligation. The details of the process are listed in Supporting Information 1. The whole process usually takes from several hours to one day. Moreover, there are still imperfectness in the operation processes, e.g. the risks of encountering EtBr and UV hazards in the manual gel-cutting step; the efficiency drop in the gel-dissolving process and the limitations of DNA recombination for the large fragment (longer than 4–5 kb) (http://www.promega.com/enotes/applications/ap0105.htm, L. Litterer, Promega Corporation 2009). Therefore, a high-throughput device without those drawbacks is highly expected.

Several new chip development for genetic analysis have been reported recently, such as the chips which integrated DNA amplification reactions and electrophoresis separations [4, 5] and the system that employed hybridization fluorescence detection to perform sample preparation, various fluidic

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Abbreviations: DNA-SDE, DNA selection and direct extraction**; EW,** extraction well**; LW,** loading well**; TAE,** Tris-acetate-EDTA

Colour Online: See the article online to view Figs. 1–4 in colour.

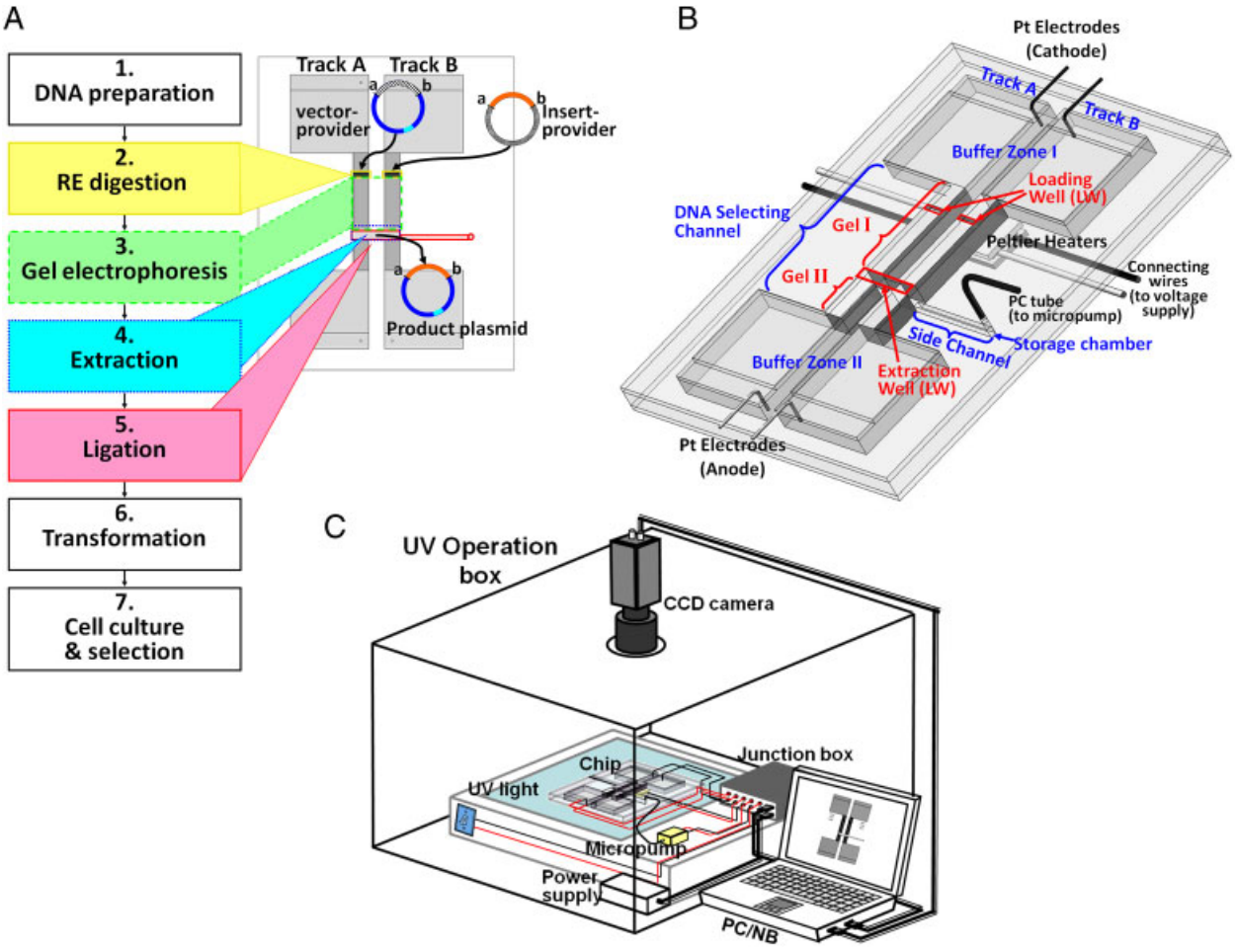

Figure 1. (A) Overview of standard procedure for subcloning (steps 1–7), with the key processes of DNA recombination in steps 2–5, which were integrated as a chip in the present design. (B) The design of integration chip. (C) Integrative system for automatic processing.

manipulation and PCR [6]. However, for DNA preparation, most chips are developed for individual single step involved in DNA recombination procedure. For instance, a microfluidic mixer chip of DNA digestion system was designed for reducing the required reaction time of DNA samples/RE digestion reagents [7]. As for DNA separation and selection, cross shape of gel electrophoresis channels incorporate with direction-adjustable electric field were developed [8, 9]. For the DNA ligation process, the ligase reagent and samples are transported and manipulated by electrowetting [10] or by pneumatic microvalves [11] to significantly reduce the required amount of reagents. Unfortunately, these remarkable improvements of individual bio-chip designs do not seem to provide practical benefit to the majority of users in biological laboratories due to their non-integrality.

As to the chip integration, Pal et al. [4] demonstrated an integrated chip can serially transport DNA sample to different operation zones for serial processes of PCR, RE digestion and electrophoresis. However, more than ten regulated air sources and pneumatic valves with many bulk supporting equipments and control systems were required. The complexity of system design and the need of special assortment of accessories may pose a barrier between engineers and biologists. Therefore, a novel integrative microfluidic chip with simple design philosophy and good adaptation to conventional users for DNA recombination operation has been proposed in this paper. An "in-well" digestion design that combines digestion reactor and loading well (LW) could eliminate the traditional need of product transportation from "outside-well" (micro tube) reaction zone to LW. A brand-new DNA selection and direct extraction (DNA-SDE) method is designed, as shown in Fig. 1B, to simplify the extraction operation processes and enhance its efficiency. Figure 1C illustrates the design of the whole integrative system.

2 Methods and materials

2.1 The DNA-SDE method

Within DNA recombination processes, DNA extraction is the most tedious and hazardous step. In this paper, a new DNA-SDE method was developed by using straight and discontinuous gel channel with extraction well (EW)

in-between, instead of the commonly used direction-adjustable cross-electric-field [8, 9]. When DNA fragments migrated in gel channels from cathodes toward anodes as shown in Fig. 2A (or Fig. 1B), the EW was set in the DNA migration pass to directly collect target DNA fragments with the help of optical monitoring system and program-controlled electric field. The EW was bounded by Gel I and Gel II. Both Gel I and Gel II provided the channel for DNA separation and Gel II offered another extra function to protect the extracted DNA fragments from high temperature and acidic buffer surround the anode electrode in the extraction process. Consequently, the selected DNA fragments could be collected in the EW safely. Within the DNA-SDE process, there is no need of manual gel-cutting process, and those conventional chemical reagent kits for dissolving gel and extracting DNA are no longer required. The risks of UV or EtBr hazard were thus naturally avoided in the DNA-SDE process.

2.2 System design and integration

For an integrated chip, the toughest challenge is not only to offer proper environment of all the individual steps that are usually different and sometimes even in conflict with each other, but also to overcome the interface transport issues that arise. In the present work, the steps of DNA recombination are partly operated in liquid and partly in gel. Therefore, our design has allowed the DNA sample solution to be transferred between liquid-based and gel-based. Moreover, we adopted the less interference carrier buffer and applied the local heating elements to offer suitable environments for two enzymatic reactions and RE digestion processes. With those modifications and improvements, the upstream RE digestion and downstream DNA ligation processes could be successfully integrated in the present design for DNA recombination application.

The design of integrated chip is shown in Fig. 1B. The chip prototype was constructed by three PMMA plates, with thickness of 1, 2 and 1 mm, respectively. Each PMMA plate was fabricated by a precise laser-scribing machine (LasrPro Mercury, 25W) and then stuck together by double-sided tapes (3 M, 8173-305). Two DNA separation channels, denoted as tracks A and B, are symmetric to the plate centerline of the long axis with two buffer zones at each end and have a 2-mm-partition in-between that separated the channels to function independently. In each track, there are two pieces of gels, i.e. Gel I and Gel II. In Gel I, there is a small liquid well, denoted as LW, which is used for DNA

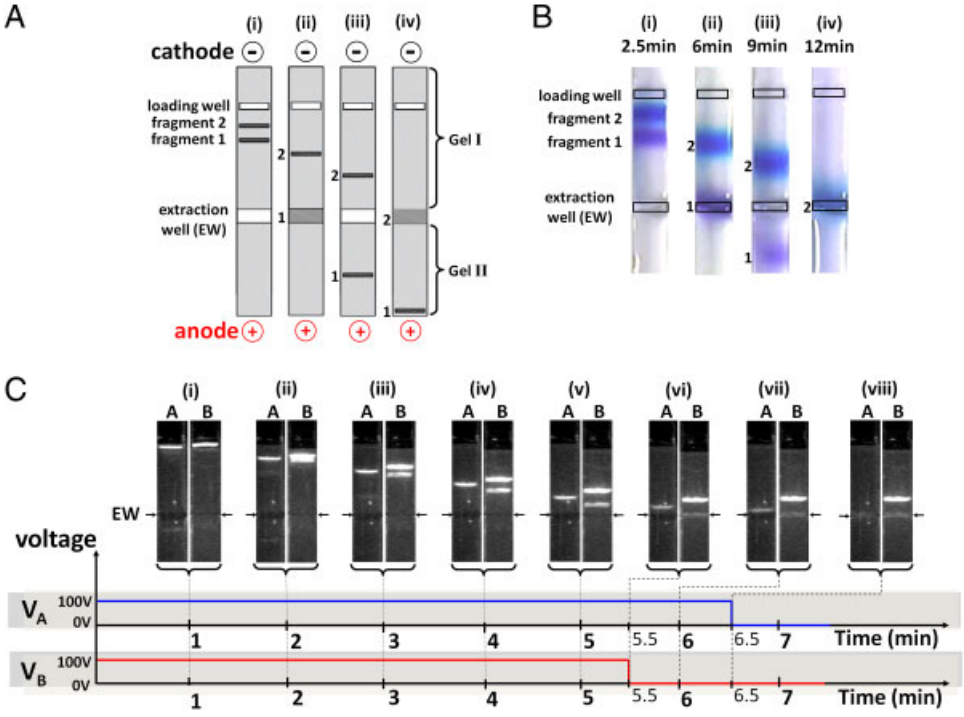

Figure 2. (A) A sketch of DNA-SDE process on single-track chip. The target DNA fragments were selected by an automatic monitor and control system and trapped by an EW. (i) After turning on the electric field, the DNA fragments migrated towards the anode. (ii) The shorter fragment (fragment 1) moved into EW. Provided that fragment 1 was the target fragment, the applied electric field was switched off automatically, the target fragment was trapped in EW and then the selection process was finished. (iii) If the longer fragment (fragment 2) was the target, the electric field would remain to allow both fragments to migrate forward and fragment 1 would escape from EW. (iv) Until the fragment 2 migrated into EW, the electric field was switched off to trap the target fragment. (B) The corresponding experimental photos of on-chip DNA-SDE process using visible DNA loading dye in a single track. (C) Visualization results and sequence control of the DNA selection process on a single-track chip in both A (with vector-provider, pCS2$^+$) and B (with insert-provider, pRSETa-p38a) tracks.

loading and also digestion. The gel length and size/position of the LW could be arbitrarily modified, depending on the user's needs. For small DNA fragment case in this paper, the dimensions of Gel I were 20 mm in length (L), 4 mm in width (W) and 3 mm in height (H) and the LWs, with 1 mm (L), 3.6 mm (W) and 2.8 mm in depth, were located 10 mm ahead of the EW. The dimensions of Gel II were 8 mm (L), 4 mm (W) and 3 mm (H). Two sets of Peltier heating elements (TANDE, TES1-007.39) with dimensions of $8 \times 8\,mm^2$ are attached to the chip bottom under the LW-regions for controlling the reaction temperature during the digestion process. The buffer zones are filled with electrophoresis solution for buffering the variation of pH value and temperature of system. Two pairs of platinum electrodes are installed in the buffer zone I and II, immersed in electrophoresis solution, to offer the needed electric field, about 10 and 13 V/cm (for large and small DNA fragment cases respectively), in each track in the electrophoresis process. EW locates between Gel I and Gel II and is filled with electrophoresis solution. For DNA recombination on-chip process, the EW was designed to link two tracks, with dimensions of 2 mm (L), 10 mm (W) and 3 mm in depth, for extract both desired DNA fragments. Additionally, a shallow side channel – with dimensions of 20 mm (L), 1 mm (W) and 0.2 mm in depth – and a storage chamber – with 2 mm radius and 1 mm in height – were designed to link with the EW. Ligase reagent was stored in the channel and separated from the EW by a hydrophobic valve, which was made by the Teflon treatment on the wall. For ligase reagents transportation, a pneumatic micro pump (KOGE, model KPM14A), linked with side channel by a plastic PVC tube, was used to drive ligase reagents flowing into EW and mix with the target DNA fragments.

The sketch of the automatic control system for on-chip DNA recombination process is shown in Fig. 1C. The DNA recombination chip was connected to a junction box, which was further connected to a computer/laptop. A self-written program by LabVIEW was used to control all active elements of system. A CCD camera, installed above the chip, was used to monitor the operation processes and transfer the images to computer for further processing and control. During the operation, the chip was placed in a UV protection box, in which UV light was provided to excite DNA staining dye (EtBr) for displaying the instantaneous positions of DNA fragments. The power source of UV light was connected to the junction box and illuminated on-demand by the controlling program to minimize the UV exposure time.

2.3 Sample and test materials

Two experiments have been tested for different sizes of DNA fragments to show the capability of this newly designed chip. In the first test, plasmids of small size DNA were chosen, i.e. insert-provider (pRSETa-p38a, 2.9+1.1 kb) and vector-provider ($pCS2^+$, 4 kb). Both plasmids have unique cutting sites by the two chosen REs: KpnI (NEB, ♯R0142S) and HindIII (NEB, ♯R0104S). In the second test, plasmids of large DNA fragment, i.e. insert-provider (pH-dBFVIII, 5.3 kb+4.2 kb) and vector-provider (pEt, 5.5 kb), were used. Both plasmids have unique cutting sites by two chosen REs: NcoI (NEB, ♯R0193S) and XhoI (NEB, ♯R0146S) was used. Tris-Acetate-EDTA (TAE) buffer, instead of Tris-Borate-EDTA (TBE) buffer, was adopted as the electrophoresis buffer to maintain constant pH value. The gel was made by 1% agarose powder dissolved in 0.5 × Tris-Acetate-EDTA buffer. EtBr (10 mg/mL) was added into the gel solution after heating and before gelling. For ligation process, T4 DNA Ligase (NEB ♯ M0202) and ligation buffer were used for reaction. The volume of the materials are listed in Supporting Information 2.

3 Results and discussions

3.1 Demonstration of DNA-SDE on-chip process

Before using DNA for test, a visualization experiment by using visible DNA loading dye was utilized to simulate the DNA migration progress in a single track of the discontinuous gel. Figure 2A and B show the sketched and dye-loaded experimental result of the DNA-SDE on-chip process at different operation instants, respectively. At instant (i) after the electric field was turned on, the dye-loaded DNA fragments migrated toward anode and were separated. At instant (ii) the shorter fragment (fragment 1) has moved into EW. Providing fragment 1 were the target fragment, the applied electric field would be switched off to trap the target fragment in EW and the selection process is then finished. But if the target fragment were the longer one (fragment 2), the electric field would be kept applying to allow both (short and long) fragments migrated forward. The fragment 1 would escape from the EW and continually move forward as shown at instant (iii) and finally the fragment 2 has migrated into EW at instant (iv) and the electric field would be switched off to trap fragment 2 in EW. Based on this simple design, the target fragments can be directly and easily caught in EW, no matter what was the order of the fragments.

Then a proof-of-concept test for the DNA-SDE process was conducted on chip. Both DNA fragments of vector and insert selection were independently operated in two single-track gel channels. The power source was applied across the electrodes and manipulated by a programmable control system. Tracks A and B were used for vector-provider ($pCS2^+$) and insert-provider (pRSETa-p38a) separation, respectively. During the experiment, the DNA location was captured by CCD camera from the visible light of UV-excited DNA staining dye (EtBr) and shown clearly in the upper row of Fig. 2C. The DNA fragments moved toward anodes from top to bottom and separated gradually in images (i)–(v). The applied power of track B was turned off for trapping the target DNA fragment (p38a) at about 5.5 min, as shown in images (vi)–(viii). Parallelly in track A, the small size

undesired fragment, 0.35 kb, cut off from $pCS2^+$ was not clearly observed in images (iv)–(viii). The other target DNA fragment ($pCS2^+$) in track A was trapped in EW by turning off the power in image (viii). The duration of whole process was less than 7 min that was much shorter than that normally needed for DNA electrophoresis and extraction processes in laboratory, more than 1 h, since the tedious conventional DNA extraction processes have been eliminated in the on-chip process. Moreover, for the on-chip process, the physical limitations in the in-lab electrophoresis process, e.g. the minimum separation space of DNA fragments for easy handling of gel cutting, could be avoided. The length of gels can be thus shortened and the electric field is significantly enhanced to speed up the time-consuming electrophoresis process.

3.2 SDE application for DNA recombination

To perform the whole procedure of DNA recombination, including DNA digestion, gel electrophoresis, DNA extraction and insert-vector ligation, a lab-on-a-chip system by extending the SDE process has been further developed and tested. The operation processes are graphically illustrated step by step in Supporting Information 3 for easy understanding. Because the activity of RE strongly relies on the operation temperature and buffer condition, a pair of heaters was used to control the reaction temperature at $37 \pm 2^{\circ}C$ during the DNA digestion process. The testing results are shown in Supporting Information 4.

After DNA digestion process, the operation was switched to the DNA-SDE process and both electric fields were applied simultaneously, which is the same as that operated in the single-track chip mentioned above. However, a small modification should be carried out due to the connected EW in the double-track chip. The reason is because, after the first target fragment migrated into EW and although the related electric field has been switched off, the target DNA could not be trapped statically in EW, but kept moving through the connected EW to the anode from the other track with applied electric field. To avoid this unexpected situation, the operational procedure was thus modified as that the leading target DNA fragment should wait in the user-defined warning region before EW in gel, instead of in EW, as shown in Fig. 3A. The electric field was switched off until the arrival of the other target fragment. Afterward, the electric field would be switched on again and both target fragments could be driven into the connected EW at about the same time. The test of modified SDE process with loading dye in double-track chip is shown in Supporting Information 5.

During the gel electrophoresis process, the instantaneous positions of DNA fragments was detected and fed to computer for operation control. The sequential visualization results are illustrated in Fig. 3A. The top (white) squares are DNA LWs, the middle (green) squares reveal the virtual and user-defined warning regions, and the dashed (yellow)

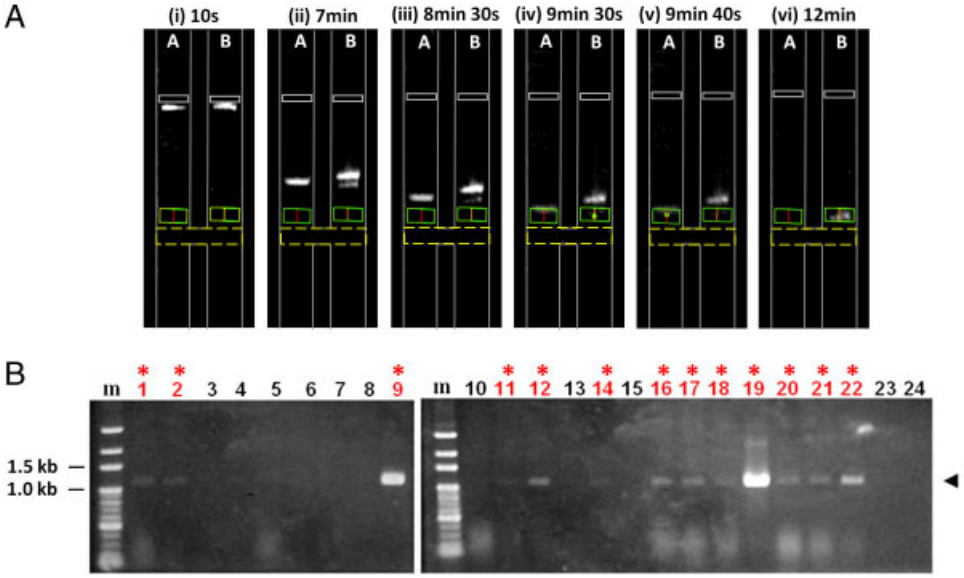

Figure 3. Automatic DNA recombination processes in the integrated dual-track chip: (A) Sequential images of automatic DNA-SDE process. The top (white) squares are DNA LWs, the (green) squares in the middle reveal the virtual and user-defined warning regions, and the dashed (yellow) square below is EW. In images (i)–(iii), the DNA fragments were separated and migrated toward anodes independently. (iv) When the target fragment arrived in the warning region of B track, the power of track B was switched off to trap the fragment. (v) With the same process, the target fragment of track A was also trapped in warming region. Then the electric fields of both tracks were switch on to drive both target fragments moving into the EW. (vi) The target fragments were then trapped in EW by switching off the applied powers. (B) DNA recombination efficiency test: 13 tracks, those numbers marked (in red) with ∗ at the top, from total 24 randomly selected tests have indicated the existence of the target insert fragment (about 1.2 kb, marked by an arrow in the rightmost).

square is EW. After switching on the powers, the DNA fragments migrated into gel channels and were separated in images (i)–(iii). Till the target DNA fragment (p38a) in track B migrated into the warning region, shown as the (green) squares in the middle of image (iv), the image was caught by vision detection system and an alarm signal was triggered to switch off the applied power of track B, then the target DNA fragment (p38a) was trapped in the warning region. With the same process, the other target DNA fragment ($pCS2^+$) of track A was also trapped in the warming region in image (v). And then both applied powers were switched on to drive both target DNA fragments migrated into EW and then switched off to catch both target fragments as shown in image (vi). The duration of DNA selection process was about 12 min, which is slightly longer than that in the single-track chip. This might be caused by the interference of electric field of both tracks due to connected EW. Duration of this process can be still shortened through the optimization of operation parameters.

After two target DNA fragments were collected in EW, the ligation reagents stored in the side channel and storage chamber were transported by a commercial micro pneumatic pump into EW and mixed with the target DNA fragments. The overall ligation process was operated in room temperature for around 30 min.

The following DNA transformation and cell culture processes were then performed off-chip to test the efficiency of recombination. The recombination product was extracted from the chip and transformed into the competent cell of *Escherichia coli* DH5α strain using heat-shock method [12]. Around 20 randomly selected colonies from all the transformed single-cell colonies were examined by PCR-based detection with T3 promoter primer (5′-TTAACCCTCACTAAAGGGA-3′) and p38a reverse primer (5′-TCAAGACTCCATTTCATCCAC-3′) to amplify the target insert fragment [13]. Agarose gel electrophoresis method was utilized to quantify recombination efficiency, which is defined as the proportion of insert-detected colonies to the total selected colonies. Figure 3B shows one of the typical testing results; where 13 of total 24 tracks, which are marked in red and labeled with *, indicated that those colonies indeed contain the target insert fragment (about 1.2 kb, marked by an arrow in the rightmost). The average efficiency of on-chip DNA recombination process was 56%, which was higher than 50% that obtained from the parallel operation in laboratory as shown in Supporting Information 6.

3.3 On-chip DNA-SDE test for large fragments

Numerous techniques using *E. coli* plasmid system have been developed to clone fragments of exogenous DNA in length up to 17 kb [4, 14]. However, it was frequently found that the recombinant plasmid with the insertion of DNA fragment larger than 4–5 kb cannot be consistently produced with the same cloning methods. This is possibly due to the inefficient DNA extraction or the interference by the reagents and manipulations involving in the further steps after gel-cutting, such as gel-dissolving, filtering, centrifuging, washing and DNA precipitating [1]. To address this issue, a set of large-size DNA fragments was chosen to test the capability of this DNA-SDE method.

The procedure of DNA-SDE process for large fragment was the same as that in Section 3.1, except the length of Gel I were extended to 50 mm and the LW for insert-provider was located 20 mm ahead of the EW to effectively separate the DNA fragments. It took about 20 min to separate and collect the target DNA fragments, insert (dBFVIII, 4.2 kb) and vector (pEt, 5.5 kb) in the process. As expected, the duration was about three times longer than that of small size DNA, due to three reasons: (i) the length of gel for DNA electrophoresis was increased (from 10 to 20 mm) to offer enough migration distance to separate those DNA fragments with closer sizes from insert-provider (pH-dBFVIII, 5.3+4.2 kb); (ii) elongation of electrode distance to reduce the magnitude of electric field from 13 to 10 V/cm; and (iii) the decrease of DNA fragment mobility due to the increase of insert fragment size from 1.1 to 4.2 kb. Even so, the time duration is still much shorter than that required in a lab-based test (1–2 h).

After overnight ligation process, we analyzed the recombination efficiency with the similar lab-based DNA transformation process mentioned above. From all survival single-cell colonies, five colonies were randomly selected, amplified and then examined by specific enzyme digestion method. Two sets of enzymes were used for double checking. The first set of enzymes, i.e. NcoI and XhoI, were used to digest the plasmid into two fragments, 4.2 and 5.5 kb, and to ensure the selected colonies containing the target insert fragment. The second set was SalI, which had two cutting sites on the original plasmid (pH-dBFVIII), but only one cutting site on the producing plasmid (pEt-dBFVIII). This process helps checking that the selected colonies do contain the target producing plasmid, pEt-dBFVIII, instead of the original plasmid. The correctly produced plasmid should be digested by SalI into one straight fragment with 9.7 kb. The test result of recombination efficiency is shown in Fig. 4. The test results of NcoI/XhoI and SalI are denoted as tests A and B in the figure, respectively. Three colonies, which are marked in red (1, 3 and 4), in five, were successfully recombined. The average efficiency of on-chip DNA recombination process was 62.5% (see Supporting Information 6).

4 Concluding remarks

This paper presents a simple straight-channel structure with sequential gel–buffer interfaces to integrate different functional features as a total DNA recombination chip. The current design has successfully extended the function of agarose gel from conventional electrophoresis to not only perform as a microreactor for restriction digestion and

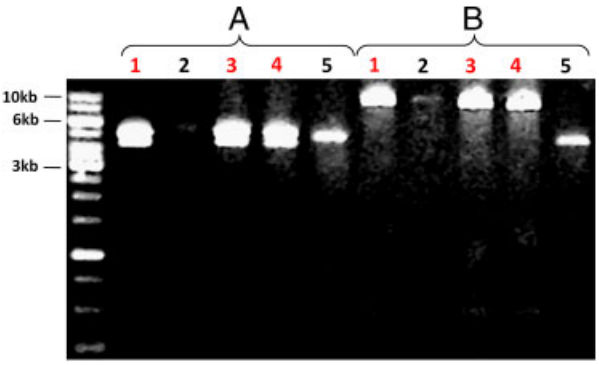

Figure 4. Recombination efficiency test of large DNA fragments: three marked tracks (1, 3 and 4) from five in test (A) indicated that the colonies contain two target DNA fragments (dBFVIII and pEt) of 4.2 and 5.5 kb by NcoI and XhoI digestion enzymes. The double-check in test (B) with one cutting site of SalI showed also one band of 9.7 kb in 1, 3 and 4 track and verified its consistency.

Table 1. Comparisons of in-lab and on-chip DNA recombination process

	In-lab	On-chip
Time consuming	3–24 h	$<$1 h
Manual checkpoints during operation	Many	0
Gel cut	Necessary	Unnecessary
Operator exposure to UV	$\sim$1 min/sample	0
Gel extraction kit	Necessary	Unnecessary
Minimum DNA sample needed	3–5 μg	0.5–1 μg
Parallel operation	Possible with more manpower	Possible with full automation
Large DNA fragment separation and selection	Difficult	Possible (success at least for DNA up to 4–5 kb)

ligation but also as a preserver for selective extraction. This gel-centered design is demonstrated to solve the problems occurring at the gel–buffer interface that impeded the integration of DNA-recombination chip for a long time. Furthermore, by taking the advantages of the new microfluidic design and system integration, the current DNA-SDE design could eliminate DNA loss during the tedious processes in conventional DNA extraction and also yield high-quality product and recovery efficiency for ligation process. This brings another niche of the new chip to extend its capability for the large-sized DNA insertion that could still not be easily operated in the laboratories. The whole DNA recombination process has been conducted in one chip for the first time with its performance clearly demonstrated.

A general comparison of the key concerning issues in conventional laboratories and in an on-chip system is made and listed in Table 1. First of all, the current chip design could significantly reduce the total operation time and efforts. Since the gel-cutting step is no longer needed in the present chip, those conventional chemical reagent kits for dissolving gel and extracting DNA are no more required. Moreover, except the first loading preparation and the last take-out steps, most manual checkpoints in conventional DNA recombination process during the in-lab operations could be eliminated by application of the present automatic system. The risks of UV or EtBr hazard were thus naturally avoided. Moreover, because the high-efficiency on-chip DNA extraction replaces the inefficient steps of gel-cutting and corresponding treatments, the current on-chip operation also reduces the minimum DNA sample needed. Furthermore, with the help of inexpensive automatic optic inspection system, the present design offers a good base to make parallel processing of fully automatic high-throughput DNA recombination on chip possible.

As to the improvement of this new design, e.g. for recombination of much larger size DNA fragments, shortening operation time in each step could be the major key issue to further improve the chip capability and efficiency. In the reaction steps – digestion and ligation processes – methods for increasing molecular interactions and retaining enzyme activity, e.g. built-in micromixers, could be further studied and optimized. It was also found that adding the fluorescence tag, EtBr, caused a decrease of DNA migration rate and the light intensity of DNA bands degrade in the electrophoresis process due to the detachment of fluorescence tags from DNA fragments. The latter is caused by the opposite electric charge of the DNA molecules and bonded fluorescence tags, which migrate in the opposite direction toward the cathode [15]. An alternative DNA detecting method without using EtBr for visualization [16] could solve this problem; however, a special UV-detector should then be needed.

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