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2 **On-chip fraction collection for multiple selected ssDNA**
3 **fragments using isolated extraction channels**

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22 **High efficiency and high-purity fraction collection is highly sought in analysis**
23 **of fragments-of-interest from selective Polymerase Chain Reaction (PCR)**

1 products generated by High Coverage Gene Expression Profiling (HiCEP)
2 methods. Here we demonstrate a new electrophoretic chip device enabling
3 automatic high-efficient fractionation of multiple ssDNA target fragments
4 during a run of separation. We used thoroughly isolated extraction channels
5 for each selected target to reduce the risk of cross-contamination between
6 targets due to cross-talk of extraction channels. Fragments of 35, 108 and 138
7 bases, were successfully isolated, then the recovery was PCR-amplified and
8 assessed by capillary electrophoresis (CE) analysis. Total impurity level of the
9 targets due to unwanted fragments of 0.7%, 2% and 6% respectively, was
10 estimated. Difficulties in collecting multiple target factions are due to band
11 diffusion and DNA adsorption to the walls for the fragments in the separation
12 channel, which is generated by transferring the DNA target fraction from the
13 extraction section to the target reservoir. Therefore, we have carefully
14 measured band broadening and analyzed its influence on the separation
15 resolution due to the delay.

16

1 **1 Introduction**

2 Transcriptome studies of gene expression and regulation mechanisms are among
3 the most important areas of post-genome research [1-6]. Gene expression analysis
4 is usually performed using DNA microarrays. This technique uses an array of
5 oligo-nucleotides derived from cDNA synthesized from mRNA templates, and can
6 only be used to profile known genes. In general, sensitivity of this technique is low,
7 and differences in mRNA expression less than a factor of two or three can not be
8 detected. To overcome these difficulties, a method based on cDNA-Amplified
9 Restriction Fragment Length Polymorphism (AFLP), called High Coverage Gene
10 Expression Profiling (HiCEP) has been developed [7]. Because HiCEP data is
11 based on cDNA fragments obtained using restriction enzymes, it can be used to
12 profile unknown transcripts as well as known genes. However, like all the AFLP
13 methods, it requires isolation of fragments-of-interest from selective polymerase
14 chain reaction (PCR) products in order to accomplish the sequencing. The
15 conventional fraction collection technique based on slab gel electrophoresis is
16 time-consuming with their typical requirement of overnight pre-run time, and has
17 insufficient resolution of the separation. Moreover, its reproducibility is poor due to
18 the subjectiveness of human operator, and inaccuracy of the pickup tools used,
19 whose selectivity exceeds the band spacing.

20 Liquid chromatography technology provides the means for separation and
21 fraction collection of organic molecules and DNA, or peptides and proteins. K. Lim,
22 et al., has collected eluted fractions during denaturing high-performance liquid
23 chromatography analysis of mitochondrial DNA mutation [8]. Capillary
24 electrophoresis (CE) has become widely used in molecular diagnostic laboratories,

1 mainly because of its single base resolution and ability to automate both loading
2 and analysis. CE is not only an analytical technique but it can also be used for
3 collection [9-12]. M. Lin, et al., designed and constructed an alternative block for
4 the ABI 310 CE instrument, capillary electrophoresis was stopped before the
5 desired molecules migrating to the end of the capillary, and original standard gel
6 block was then replaced by a reconfigured gel block holding a collection tube. The
7 reconfigured system allows collection with a resolution of as high as three bases
8 [13]. However, the collection resolution of both methods is limited by their 1D
9 nature and absence of real-time detection capability. Microfluidic chips available
10 due to advances in 2D fabrication methods [14-23] have enabled replacement of the
11 capillaries by more modern techniques of separation and fraction collection. Two
12 novel on-chip structures for continuous-flow fraction collection [17, 18] have been
13 proposed to obtain much more recovery. However, it is difficult to apply to
14 high-resolution DNA fraction collection. Earlier, we have shown high-resolution
15 and high-fidelity fraction collection of ssDNA fragments differing in size by one
16 base on a spiral-channel glass chip [20]. The combination of a long spiral channel
17 with a small microchannel cross-section, a short injection plug, and automated
18 fragment selection and extraction algorithm was instrumental in achieving the
19 record-high resolution at low impurity levels. In addition to improving the
20 resolution and reducing the cross-contamination, high throughput is also essential
21 in the collection of multiple target fragments for lower sample and reagent
22 consumption, shorter running time and lower running costs. We have shown a
23 method for multiple fraction collection during a run of separation, in which four
24 transfer sub-channels are connected to an extraction channel [23]. In this case, all

1 targets have to share the same extraction channel, which increases the risk of
2 cross-contamination.

3 Here, we present high-efficiency and high-fidelity fraction collection with a
4 novel three-parallel isolated extraction-channel structure on a $3 \times 10 \times 0.2$ cm
5 quartz chip. The isolated extraction channel for each target may reduce cross
6 contamination between collections due to sharing one extraction/transferring
7 channel. We monitored fluorescence flux at the extraction intersections and
8 optimized conditions for collection procedures, and with which we achieved
9 collection of three selected targets with low ratio of impurities during one run of
10 separation. Difficulties in collecting of multiple target factions are due to delay time
11 necessary for transferring the DNA target fraction from the extraction section to the
12 target reservoir. The delay may result in band broadening of the fragments in the
13 main separation channel, degrading the resolution and even overlapping the
14 neighboring bands. As a result, subsequent collections may become difficult.
15 Therefore, we have carefully measured band broadening and analyzed its influence
16 on the resolution of separation due to the delay time.

17

18 **2 Materials and methods**

19 POP-4 matrix, 10×running buffer, Hi-deionized formamide solution, GeneScan
20 500 ROX, and BigDye Terminator v3.1 Cycle Sequencing Kit for separation in
21 capillaries and microchannels from PE Applied Biosystems (USA) were used. All
22 other required chemicals were supplied by Wako Pure Chemical Industries (Japan).
23 Water was purified by reverse osmosis followed by treatment by Milli-Q Plus ion

1 exchange and organic adsorption cartridges (Millipore). HiCEP method and
2 determination of impurities in the recovery by PCR and CE analysis were the same
3 as described in our previous work [7].
4

5 **2.1 Chip Design and fabrication**

6 The chips were fabricated using standard photolithography and wet-etching
7 techniques [20]. Photomask for pattern transfer was designed using AutoCAD
8 (AutoDesk, Inc., USA) and subsequently fabricated by Micro Electronic
9 Technology Development Application Corp. (Beijing, China). The microfluidic
10 structure (Fig. 1) fabricated on a 30×100×0.5mm quartz wafer, consist of nine
11 3-mm-diameter reservoirs, a typical injector unit, a 87-mm-long straight separation
12 channel, and three parallel channels for the extraction and retrieval of the target.
13 The channels were 55 μm-wide and 25 μm-deep. The interval between parallel
14 channels was 100 μm. Access holes serving as reservoirs were drilled into the
15 1.5-mm-thick cover plate with diamond-tipped drill. The channel plate and the
16 cover plate were thermally bonded at the glass transition temperature of $T_g =$
17 1070°C for 3 h.
18

19 **2.2 Electrophoretic separation**

20 First, POP-4 matrix was injected into the channels. An aliquot of 2.0 μl of the PCR
21 products labeled with three different fluorescent dyes or 0.2 μl of GeneScan 500
22 ROX (Applied Biosystems), and 15 μl of formamide was then mixed, denatured by
23 incubation at 95.0°C for 2 min and chilled on ice for 5 min. The mixed solution was
24 loaded on the chip. The sample was loaded from C to B at 200 V/cm for 100 s (Fig.

1 2(a)), and then injected into the separation channel for 1 s (Fig. 2(b)). Injection and
2 separations for ssDNA fragments were performed at 200 V/cm, with the injection
3 plug approx. 150 μ m long (Fig. 2(b)). The samples were then driven to the anode
4 (Reservoir D, Fig. 1(a)). The above described separation and fractionation
5 procedures were performed at room temperature.

6 An inverted microscope (IX71, Olympus, Japan) with a fluorescence filter unit
7 (Dichronic mirror: 505nm; Emission: 510nm~IF) and a 12-bit digital CCD camera
8 (Hamamatsu: ORCA-EG, Japan) were used for fluorescence imaging. A mercury
9 lamp was used as the source of fluorescence excitation in order to monitor and
10 optimize collection conditions (Fig.2 and 3). A 488-nm argon ion laser illumination
11 (177G-UK01, Spectra Physics Laser, USA) with 50mW output power was used to
12 improve S/N ratio of segment recognition in the application of resolution
13 measurement and multiple collections. The rest of experimental setup was the same
14 as described in our previous works [20, 23].

15

16 **2.3 Monitoring of the band broadening**

17 The fluorescently labeled single-stranded DNA ladder (GeneScan 500 ROX,
18 35~500 bases) was separated in a length of 87 mm (K-H), and was stopped close to
19 the detector (H) by turning off the electric field. Then the DNA fragments were
20 allowed to diffuse in the POP-4 matrix in absence of the electric field. After a delay,
21 the separation was restarted and the peak was detected during passage through the
22 detector [24]. Full width at half-height of the obtained peaks was then compared
23 with the separation in absence of delay.

24

1 **3 Results and discussion**

2 fraction collection of multiple selected ssDNA fragments successively with only
3 one run of electrophoretic separation may become a way for high-throughput
4 fraction collection, which is often desired during HiCEP analysis. However, sharing
5 of the extraction channel results in cross-contamination easily. Furthermore, bands
6 of the fragments in the separation channel may diffuse during the delay time
7 between two collections. This will affect resolution of the separation negatively.
8 These issues will be discussed below.

9

10 **3.1 Procedures of multiple target collections**

11 For high-efficiency fraction collection, we have developed a structure containing
12 three parallel channels instead of single extraction channel. Each channel
13 corresponds to one extraction, and the extraction channel order for Target 1, 2 and 3
14 is from left to right (Fig. 1(b)). Figure 2 and figure 3 shows the separation and
15 collection procedure at the injection intersection K and the extraction intersections
16 H. Here, fluorescence flux of SYBR Green II stained 100-base fragment
17 (Invitrogen Life Technologies, Japan) was used in order to monitor any sensitive
18 variance during the collection. In this study, in order to suppress the contamination,
19 same potential is applied to the reservoir E and F to block unselected fragments
20 entering into extraction channels (hereafter called focusing, the focusing method
21 was introduced to decrease the volume of injection plug on microfluidic devices
22 [25]). As shown in Fig. 2(e), the bands therefore become narrowed by focusing;
23 they then pass through the three intersections retaining their narrow shape.
24 Separation without focusing or with insufficient focusing (Fig. 2(d)) may result in

1 a problem, namely, contamination due to unwanted passing fragments which may
2 enter the extraction channels. Line profile analysis (Fig. 2(g-i)) shows the
3 fluorescence-intensity distribution at the three intersections, illustrating that there is
4 no leakage into the extraction channel. It should be noted that too low focusing
5 potential (hereafter called over-focusing, Fig. 2(f)) decreases electric field in the
6 separation channel, which leads to separation velocity lower by about 20%.
7 Moreover, over- focusing also results in low recovery volume.

8 For each target extraction, all separation electrodes were isolated from the
9 electric field, while bias was applied to corresponding extraction channels (Fig. 3(a,
10 d, g)). Finally, transfer operation was carried out for 60 s in order to drive the
11 extracted target to the target reservoir G/N/F respectively. During the transfer of
12 Target 1, we found a band distortion occurring near the middle intersection (Fig.
13 3(b)) due to halt of the focusing, and partial sharing of channels M-G and M-N. If
14 not moved out of the middle extraction channel, this band will absolutely
15 contaminate subsequent Target 2. Hence, a cleaning procedure as shown in Fig.
16 3(c) is essential to guarantee non-contamination in the channel before the next
17 target extraction. The cleaning involves similar manipulations as the focusing, with
18 the only difference that the corresponding electrodes are grounded during the
19 cleaning. Although a very small band generated by cleaning can be detected at the
20 entrance of the down-most extraction channel for Target 1 (Fig. 3(c, and h)), Target
21 1 is already transferred into the target reservoir G, and therefore can not be
22 contaminated. The transfer of Target 2 also generates a band distortion on the first
23 extraction channel (Fig. 3(e)), but it can be ignored due to the same reason as
24 described above. Before the extraction of Target 3, a cleaning (Fig. 3(f)) is used to

1 remove the contamination from two sides of the arrow at the up-most extraction
2 intersection. The procedure thus enables on-chip multiple fraction collection in
3 succession with one run of separation.

4

5 **3.2 Application of three-target collections for HiCEP analysis**

6 Fragments of 35, 108 and 138-base long in HiCEP products of mouse embryonic
7 stem (ES) cell E14 (E14-inc-H(-)-1-1-P1_B01) were selected for collection with
8 one separation run on the chip. Before fractionation a run of electrophoretic
9 separation was carried out in order to detect features such as position and peak
10 width of the selected targets. The electrophorogram of the
11 E14-inc-H(-)-1-1-P1_B01 separated on the chip is shown in Figure 4(a). The three
12 dashed lines on the target peaks in Fig. 4(a) show the positions of collection where
13 they are close to the center of each peak. Using the above procedures, the targets
14 were extracted and transferred into the target reservoirs G, N and F in succession.
15 Below, we discuss the evaluation of the discovery. Any peak purity determination
16 can only confirm the presence of impurities and never prove absolutely that the
17 peak is pure. The likelihood of discovering an impurity increases with the
18 resolution between target and impurity, as well as with absolute and relative
19 concentration of the sample. Therefore, we initially amplified the recovery by 28
20 cycles of PCR to increase the concentration, and then performed CE analysis using
21 PRISM3100 genetic analyzer with a 47 cm long (36 cm at the detector) fused silica
22 capillary. The electrophorograms are shown in Figure 4(b).

23 All the baselines in Fig. 4(b) are very flat, and no obvious contamination in any
24 of the three electrophorograms was observed. In order to assess the existence of

1 small contamination, all the peak profiles were magnified and analyzed. The ratio
2 of peak area of the unselected peaks to the target peak (I_u/I_t) according to the CE
3 analysis was used to evaluate the contamination. The leftmost peaks in all checks,
4 which come from primer dimer and primers during PCR, were not considered in the
5 contamination calculation. Thorough analysis has revealed that purity of the
6 recovery of 35 b was very satisfactory, the contamination rate was only about 0.7%
7 which was even close to the standard deviation of the baseline. According to the
8 magnified peak profiles (Fig. 4(b)), some very small peaks before the targets were
9 identified in the recovery of 108 b and 138 b. Furthermore, these contamination
10 peaks were in a certain range before the targets, and there was no contamination
11 from much smaller size DNA fragment than the targets. The contamination rates of
12 the recovery of 108 b and 138 b were 2.1% and 6.3% respectively. By comparison
13 of the total peak area of the unselected peaks in the recovery of 108 b and 138 b, we
14 found that the absolute area of them is roughly similar. However, the contamination
15 evaluation in our work is a relative value to the target peak area. One can see that
16 the target peak of 138 b was smaller than 108 b (Fig. 4(a)), therefore the
17 contamination rate of the recovery of 138 b fragment was higher than that of 108 b
18 fragment.

19 Although impurity of the recovery was very low, and did not degrade the
20 quality for the subsequent biotesting, it is relevant to discuss the possible sources of
21 contamination. It may also explain why the absolute impurities to 108 b and 138 b
22 are higher than that to 35 b.

23

24 **3.3 Influence of the delay during multiple collections**

1 Halting the migration of fragments in the separation channel for the target
2 extraction and transferring the target to the destination generated a delay which
3 definitely led to band diffusion of these fragments [26, 27]. Figure 5 shows series
4 of fluorescence images of SYBR Green II stained 100-base fragment (Invitrogen
5 Life Technologies, Japan) over a delay time from 0 to 10 min by turning off the
6 electric field. One can see that the band width became wider and wider over time
7 due to band diffusion. In the first image (no delay), the band was observed to have a
8 flat front due to DNA molecules moving evenly toward the anode under very low
9 EOF condition (the electroosmotic mobility $\mu_{\text{eof}} : \sim 10^{-5} \text{cm}^2 \text{V}^{-1} \text{s}^{-1}$). It is well-known
10 that DNA adsorption leads to reduced signal levels, strongly asymmetric peaks, and
11 surface charge on the wall. From the sequent images the flat front became parabolic
12 after turning off the electric field, and the parabolic front became obvious as delay
13 time increased. This phenomenon may be explained by that DNA molecules
14 adsorbed at the channel walls and then it led to slow diffusion along the wall. The
15 peak tailing in Figure 5 may confirm the existence of DNA adsorption during delay
16 time. Furthermore, the adsorbed molecules may be pulled and exfoliated by
17 electrophoretic force and/or scratched off the wall by migrating DNA molecules in
18 the continuous separation over time. This may explain why impurities are more
19 likely to originate from the neighboring peaks.

20 Below, we would like to discuss influence of the delay on separation resolution
21 during multiple collections. Band broadening due to the delay will degrade the
22 identification resolution of the separation system and even overlap the neighboring
23 peaks. As a result, subsequent collections may become difficult. Separation

1 resolution per base (R_b) as obtained by Eq. 1, is defined by separation between two
2 peaks, ΔX , and the average peak width, W as follows:

$$3 \quad R_b = \Delta X / [N(W_{h1} + W_{h2})] \quad (1)$$

4 where W_h is the peak width measured at half height, and N is the base number
5 difference between the two neighboring peaks [28]. In a measurement of the
6 diffusion coefficient, same fragment was allowed to diffuse over different delay
7 times. However, multiple-target collections involve same transfer time after each
8 collection. This means that delay time for the final target is therefore much longer
9 than that for the first peak.

10 A total of seven peaks from 50 bases to 450 bases were delayed one by one
11 before passing through the detector, and delay time between two fragments was 0, 1,
12 2, and 3 min. The first peak of 50 bases was also delayed in the measurement. Thus,
13 the final peak of 450 bases was delayed for 7, 14 and 21 min. The delay time was
14 taken into account when calculating peak spacing. The achieved resolution,
15 calculated by Eq. 1 is shown in Figure 6. In order to collect a sample without
16 contamination by electrophoresis, the target peak desires no overlap with neighbor
17 peaks theoretically ($R_b \geq 1$ for one-base difference in size from neighbor fragment).
18 Such resolution is twice higher than that required for sequencing ($R_b \geq 0.5$). Fig. 6
19 depicts the decrease of resolution with delay time. Comparing with the delay time
20 of 0 min, the resolution of the fragments which are bigger than 200 bases in case of
21 1 min delay decreases very little. For both the delay time of 0 and 1 min, our
22 method can perform collection for 8 target fragments less than ~450 bases with the
23 resolution of more than a four-base difference in size from neighbor fragments (R_b
24 ≥ 0.25). When the delay time is up to 3 min, the available range of fragment size

1 reduces to ~300 bases in case of four-base difference in size from neighbor
2 fragments. Hence, in a high-throughput application of fraction collection the
3 available range may reduce significantly due to the delay, a higher separation
4 resolution or faster transfer are therefore desired in the future work.

5

6 **4 Concluding remarks**

7 We have proposed a method which can realize on-chip successive collections of
8 selected ssDNA fragments using isolated extraction channels. Using isolated
9 extraction channels decreases the possibility of cross-contamination from targets
10 due to sharing the same extraction channel. Monitoring the fluorescence flux during
11 focusing and extraction was helpful in finding ways to reduce or avoid the
12 contamination. The successful application of this method is evidenced by very low
13 impurity levels assessed via CE separation of the PCR-amplified recovery.
14 Analysis on the influence of the delay on multiple collections demonstrated that
15 high-throughput may be possible by further improvement of transfer. The
16 demonstrated high performance of fractionation-integrated electrophoretic
17 microfluidic device may become a new powerful analytical tool for bioanalysis and
18 related applications.

19

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5

6

7 Figure Captions

8

9 Figure 1. 3D schematic drawing of the microfabricated device with the microfluidic
10 channels (a), 87 mm effectively long straight separation channel (K-H), focusing
11 channel (E-H/F-H), and extraction channel (M-G, M-N and E-F). 9 reservoirs of the
12 buffer (A), sample waste (B), sample (C), buffer waste (D), focusing (E and F), and
13 target (G, N and F) are marked. Microscopic images of the intersections of
14 extraction (b) and injection (c), and photograph of the microchip (d). The interval
15 distance between the parallel extraction channels is 100 μm . All the channels are 55
16 μm wide and 25 μm deep.

17

18 Figure 2. Fluorescence images of the procedures: sample loading (a), sample
19 injection into the separation channel (b), Pull-back to cut off the leakage (c) at the
20 injection intersection. Comparison of insufficient- focusing (d), appropriate
21 focusing (e) and over- focusing (f) by applying a different bias potential 2000 V,
22 1500 V and 1200 V on the reservoir of E and F. The line-profile analysis on each
23 extraction intersection is shown under the fluorescence images (g, h and i).

24

1 Figure 3. The operating flow of the collection procedure: extraction, transfer and
2 cleaning of three-target collections in succession. Four line-profile analysis for the
3 marked area in (c) and (f) are shown in (h).

4
5 Figure 4. Electrophoretograms of the HiCEP analysis product (E14-inc- H(-)-1-1-
6 P1_B01) separated on a 8.7 cm long channel chip (a) and CE analysis of the
7 on-chip fractionated samples (35b, 108 b and 138 b) on a PRISM3100 genetic
8 analyzer with a 47 cm long (36 cm to the detector) fused silica capillary (b).

9
10 Figure 5. Fluorescence images of 100 base fragment diffusing in POP-4 polymer
11 over time in a microchip. A mercury lamp and SYBT Green II were utilized. 2 µl of
12 10 bp dsDNA ladder (Invitrogen Life Technologies, Japan) in 14 µl of formamide
13 was denatured at 95 °C for 2 min and chilled on ice for 5 min. Then the sample was
14 stained by SYBR Green II (1000:1) for 15 min. The fragment migrated to anode
15 (from left to right in the images) before the electric field was turned off. According
16 to a reference defect in the channel, the band broadening and the band-shape
17 changing can be clearly confirmed.

18
19 Figure 6. The resolution per base of GeneScan-500 ROX ssDNA markers separated
20 on the chip with the delay time of 0, 1, 2, and 3 min before each band passed
21 through the detector.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6