Rapid and simultaneous multiple genotyping of human Y chromosome from a human blood sample by on-chip enzymatic digestion and microchannel array electrophoresis coupled with blood sample pretreatment, and microscale PCR

Lihua Zhang a,b,d, Toshikatsu Shinkae e, Yutaka Nakahori e, Noritada Kaji c, Manabu Tokeshi c, Yoshinobu Baba a,c,f,*

a Department of Molecular and Pharmaceutical Biotechnology, Graduate School of Pharmaceutical Sciences, The University of Tokushima, CREST, Japan Science and Technology Corporation (JST), Tokushima 770-8505, Japan
b National Chromatographic R. & A. Center, Dalian Institute of Chemical Physics (DICP), Chinese Academy of Sciences (CAS), Dalian 116023, China
c Department of Applied Chemistry, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan
d Furuno Electric Co. LTD., Nishinomiya 662-8580, Japan
e Department of Public Health, School of Medicine, The University of Tokushima, Tokushima 770-8505, Japan
f Health Technology Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Takamatsu 761-0395, Japan.

Available online 17 October 2006

Abstract

In this paper, with distinct six DNA markers, 47z, SRY, YAP, 12f2, TB4Y and UTY, as the judgement standard, a combined technique for the rapid haplotyping (multiple genotyping) of human Y chromosome was developed by a DNA analysis system. By Generation capture disk, 5 min were enough to extract and purify DNA from five samples with only 4.5 L blood for each one. Consequently, LightCycler was chosen for the amplification of the six fragments of each sample. Under the optimized conditions, the simultaneous amplification of DNA extracted from five samples could be finished within 25 min. Among the six genomic fragments of each sample, the polymorphisms of YAP and 12f2 could be distinguished directly from PCR products. The other four were single nucleotide polymorphisms. Therefore, microchip-based restriction fragment length polymorphism analysis was carried out. With a 12-channel microchip, in 8 min, two blood samples could be analyzed at the same time through on-chip digestion and the subsequent separation of DNA fragments in the channel. In summary, with the above-mentioned technique, microscale haplotyping of human Y chromosome could be finished at the average speed of 10 min/sample with only 4.5 µL blood.

Keywords: Gene analysis; Microchip; Haplotyping; Human Y chromosome

1. Introduction

Human Y chromosome has unique characteristics in genetics because it is a single haploid unit in human genome that is passed only from father to son. Thus it represents the patrilineal contribution to the male genome. DNA markers residing in the non-recombining portion of the human Y chromosome have been shown useful for tracing male-specific gene flow and also

* Corresponding author at: Department of Applied Chemistry, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan.
Tel.: +81 52 789 4664; fax: +81 52 789 4666.
E-mail address: babaymt@apchem.nagoya-u.ac.jp (Y. Baba).
such traditional methods are not only time-consuming, but also require a relatively large amount of samples. Therefore, the development of new techniques to solve these problems is necessary.

Miniaturized analytical and bioanalytical instruments have been developed very rapidly in the past 10 years [10–17], which could not only reduce the consumption of samples and reagents, but also offer high throughput analysis. Accordingly, in this paper, to the best of our knowledge, for the first time, microchip-based haplotyping of human Y chromosome was performed by a fast DNA analysis system, which was composed of the DNA extraction and purification by Generation capture disk, amplification by capillary PCR, microchip-based restriction fragment length polymorphism analysis and the subsequent confirmation of DNA fragments by microchip array electrophoresis. By this method, not only the required amount of blood samples could be reduced, but also the total consumed time could be shortened greatly, which showed its potential as a modern technique in studying the human tracing and evolution, especially when a large number of samples are under examination.

2. Materials and methods

2.1. Instrumentation

Capillary PCR was performed on LightCycler (Roche Diagnostics, Mannheim, Germany). Block Incubator BI-525 (ASTEC CO. LTD., Fukuoka, Japan) was used for the on-chip digestion of PCR products. The analysis of amplified products was carried out by microchip array electrophoresis with a Hitachi SV 1210 system (Hitachi Electronics Engineering Co., Tokyo, Japan).

2.2. Materials

Primers for TB4Y and UTY with customer-designed sequence were synthesized by Kurabou Industries Ltd. (Osaka, Japan). Primers for the rest genomic fragments were obtained from Invitorgen Japan K.K. (Tokyo, Japan). Z-Taq polymerase (2.5 U/µL), 10 × Z-Taq Buffer (Mg²⁺ plus, 30 mM) and dNTP Mixture (2.5 mM each) were ordered from TaKaRa Shuzo Co. Ltd. (Shiga, Japan). Bovine serum albumin (BSA) (2.5 mg/mL) was purchased from Idaho Technology Inc. (Salt Lake City, USA). All reagents for PCR were kept at −20°C. Before usage, they were thawed and kept on ice. Restriction endonuclease, Fnu4HI and StuI, were bought from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan), while MluI and MaeI were from Roche (Mannheim, Germany). DNase- and RNase-free water used throughout experiments were from ICN Biomedicals Inc. (Aurora, USA).

Generation capture disk kits (Gentra Systems) were ordered from Funakoshi Co. (Tokyo, Japan). Thermowell sealers were obtained from Corning Incorporated (Corning, USA). Polymethylmethacrylate (PMMA) microchips with 12-channel of 100 µm width, 30 µm depth and an effective length of 42 mm were bought from Hitachi Electronics Engineering Co. (Tokyo, Japan).

2.3. Procedure

The procedure for the rapid haplotyping of human Y chromosome by DNA analysis system is shown in Fig. 1, including the DNA extraction and purification by Generation capture disk, amplification by capillary PCR, microchip-based restriction fragment length polymorphism analysis and the subsequent confirmation of DNA fragments by microchip array electrophoresis.

3. Results and discussion

Based on our previous study, the polymorphisms of 47z (Y1 and Y2), SRY (C and S), YAP (YAP+ and YAP−), UTY (UTYin17+ and UTYin17−), 12f2 (12f2 del+ and 12f2 del−) and TB4Y (TB4Y+ and TB4Y−) could be employed together to classify the haplotype of human Y chromosome (Fig. 2), and the detailed information of the polymorphisms is shown in Table 1. The probe 47z detected a polymorphism on the short arm of the Y chromosome and the long arm of the X chromosome. The alleles could be distinguished by the digestion with Stul. The YAP
Table 1
Details of polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXYS5Y (47z)</td>
<td>AGCCCT</td>
<td>Y1: SfiI digest: 370 bp</td>
</tr>
<tr>
<td></td>
<td>AGGCCT</td>
<td>Y2: SfiI digest: 100, 270, 370 bp</td>
</tr>
<tr>
<td>SRY</td>
<td>AGCCGC</td>
<td>C: Fnu4HI digest: 50, 100 bp</td>
</tr>
<tr>
<td></td>
<td>AGTCGC</td>
<td>Fnu4HI digest: 150 bp</td>
</tr>
<tr>
<td>DYS287 (YAP)</td>
<td>Alu repeat YAP+: 455 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>YAP−: 150 bp</td>
</tr>
<tr>
<td>12f2</td>
<td>Fragment deletion 12f2del+: 112 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12f2del−: 88, 112 bp</td>
</tr>
<tr>
<td>TB4Y</td>
<td>AATGCT</td>
<td>TB4Y+: MluI digest: 61, 83 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TB4Y−: MluI digest: 144 bp</td>
</tr>
<tr>
<td>UTY</td>
<td>CACGTA</td>
<td>UTYin17+: MaeII digest: 78, 99 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATGTA UTYin17−: MaeII digest: 177 bp</td>
</tr>
</tbody>
</table>

at locus DYS287 was a simple polymorphism resulting from the insertion of Alu element on the long arm of the Y chromosome. YAP+ was of 455 bp, and YAP− was of 150 bp, which could be distinguished directly by the PCR products. 12f2 marker was located on the long arm of the Y chromosome on the AZFa region. Two primer sets were used, one to detect the absence or deletion of the 88 bp of the 12f2 marker, whereas the second set to amplify a Y specific marker, tat, with its 112 bp to serve as an internal control for each sample. Furthermore, polymorphisms of SRY, TB4Y and UTY could be identified by the digest of Fnu4HI, MluI and MaeII, respectively.

3.1. DNA extraction and purification from human blood

To extract and purify DNA from human blood, Generation capture disk kit was employed according to the following steps. (1) 3 μL blood was released on a 3 mm disk, which was stored in the insert of a 2 mL spin tube, and adsorbed at room temperature for at least 1 min. (2) 200 μL Generation DNA purification solution was added to the insert and incubated at room temperature for 1 min. (3) The tube was centrifuged at 4 °C at a speed of 15 000 rpm for 10 s. (4) Steps 2 and 3 were repeated for three washes. (5) The wash solution in the receiver tube was discarded and the immobilized DNA was ready for amplification. From this procedure it could be seen that Generation capture disk has advantages such as low consumption of samples (3 μL) and ease of operation (no mixing, no pre-lysing and no precipitation). Based on our previous study, 1 min was enough for the absorbance of blood on the disk, which could be further used as the template in the consequent PCR [17]. Under such conditions, DNA extraction and purification of five samples could be finished within 5 min, which was much faster than the traditional or even MEMS-based methods.

3.2. Capillary PCR

The amplification solution was prepared at 4 °C by adding the reagents in the order of water, Z-Taq buffer, dNTP mixture, primer and BSA. After the solution was mixed completely, it was transferred into the capillary reaction chamber by a pipette. Then DNA template, immobilized on disk, was added. Finally, after the bubbles in the reaction solution were removed by a short time centrifuging, the capillary tube was put into LightCycler for amplification. With such an instrument, up to 32 samples could be amplified simultaneously.

Primers used to flank six genomic fragments were custom designed and synthesized (Table 2). In our experiments, it was found that 1/4 disk was enough to amplify a certain DNA fragment in the capillary reaction chambers. Therefore, one and a half pieces of disk, totally 4.5 μL blood, were enough to amplify the six probes in one sample. The effects of the reaction solution composition on the amplification yields were studied, and the optimal conditions were as follows: 1/4 piece capture disk with immobilized DNA, 1 μM of the appropriate primers, 200 μM

![Fig. 3. Scheme for the microchip-based restriction fragment length polymorphism analysis.](image-url)
dNTPs, 1× Z-Taq buffer, 5 U/100 μL Z-Taq polymerase, and 2.5 μg/100 μL BSA, with a total volume of 10 μL. Furthermore, systematic study on the amplification conditions was carried out. Under the optimized conditions, 30 fragments from five samples could be amplified simultaneously within 24 min 58 s, performed by 0 s at 94°C, followed by 30 cycles of 0 s at 94°C, 15 s at 62°C, and 15 s at 72°C.

3.3. On-chip enzymatic digestion and microchip electrophoresis-based analysis

To distinguish the polymorphisms of 4 DXYS5Y, SRY, TB4Y and UTY with only single nucleotide difference, microchip-based restriction fragment length polymorphism analysis was carried out. All channels of the microchip, together with wells G1–G3 and g1–g12, were first filled with the sieving matrix offered by i-DNA 12 kit (Hitachi). The amplification products of DXYS5Y, SRY, TB4Y and UTY were mixed with proper restriction endonuclease, respectively, as shown in Table 3, and added into wells of s1, s2, s5 and s6. Wells s3 and s4 were just filled with PCR products of DYS287 and 12f2. s7–s12 were well with the same solution except from the second blood sample. After all samples wells were sealed with Thermowell sealers, the microchip was put between two aluminum plates of the incubator at 37°C for several min, as shown in Fig. 3. Under the optimized conditions, DNA markers from two samples could be digested within 5 min.

Subsequently, microchip electrophoresis-based analysis for both enzymatic and PCR products was carried out with the sieving matrix offered by i-DNA 12 kit. In the sample loading procedure, 350 V was applied to wells g1–g12 for 120 s while the rest were grounded. During separation, G2 and G3 were grounded and a fixed 350 V was applied to s1–s2 and g1–g12. The voltage applied to G1 was 1100 V. A diode laser detector with the detection wavelength over 640 nm was employed for the system.

Since haplotype II was very rare, all the other six haplotypes of human Y chromosome were analyzed by such a DNA analysis system, and the results are shown in Fig. 4, from which we could see that the fingerprint electrophorograms of each haplotype was quite obvious. Furthermore, only 3 min were enough for the simultaneous analysis of two samples on a 12-channel microchip.

4. Concluding remarks

With the combination of DNA extraction by Capture generation disk, capillary PCR with LightCycler, on-chip enzymatic digestion of amplification products, and the analysis of DNA fragments by microchip array electrophoresis, rapid haplotyping for human Y chromosome by DNA analysis system was achieved at the average speed of 10 min/sample, which showed the advantages of high speed, low consumption of samples, and ease of operation. Therefore, such a method might be promising in the haplotype analysis of human disease related genes.

Acknowledgements

The present work is partially supported under the CREST program of the Japan Science and Technology Corporation (JST), a Grant from the New Energy and Industrial Technology Development Organization (NEDO) of the Ministry of Economy, Trade
and Industry, Japan, a Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare, Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Technology, Japan. L. Zhang is grateful for the financial support from Chinese Academy of Sciences.

References


Biographies

Lihua Zhang obtained PhD degree from Chinese Academy of Sciences in 2000 and is currently a professor at Dalian Institute of Chemical Physics, Chinese Academy of Sciences. Her research interest is focused on the development of novel separation and detection methods for complex samples.

Toshikatsu Shinka obtained MD, MSc degrees from Tohoku University, Japan in 1998 and is currently a staff at Medical & Nursing Care Group, Health & Hygiene Promotion, Division, Social Health & Welfare Department Social Affairs Department, Fukushima Prefectural Government. His research interest is Public Health.

Yutaka Nakahori obtained MD, PhD degrees from The University of Tokyo, Japan in 1987 and is currently a professor, at Department of Human Genetics and Public Health, Graduate School of Medical Science, The University of Tokushima. His research interests are the Y chromosome polymorphism in people of Japan, and the prevention of lifestyle oriented disease.

Noritada Kaji obtained PhD degree from The University of Tokushima, Japan in 2004 and is currently an assistant professor, at Department of Applied Chemistry, Graduate School of Engineering, Nagoya University. His research interests are nano-bio-devices for DNA analysis, single molecule biophysics, and biological process on microTAS.

Manabu Tokeshi obtained PhD degree from Kyushu University, Japan in 1997 and is currently an associate professor, at Department of Applied Chemistry, Graduate School of Engineering, Nagoya University. His research interests are micro- and nano-system for chemical and biochemical applications.

Yoshinobu Baba obtained PhD degree from Kyushu University, Japan in 1986 and is currently a professor at Department of Applied Chemistry, Graduate School of Engineering, Nagoya University and also a director of Nanobio Research at Health Technology Research Center, National Institute of Advanced Industrial Science and Technology (AIST). His research interests are analytical chemistry, nanoscience, nanotechnology, biotechnology, bioscience.