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Multiplex Mini Y Short Tandem Repeat Haplotyping using Fluorescence Energy Transfer Labeled Primers

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Abstract

A fluorescence energy transfer (ET) cassette labeled mini Y short tandem repeat (STR) genotyping system is presented. A capillary electrophoretic (CE) microdevice with a cross-injector design is used to determine the fluorescence intensities of ET and single dye-labeled STR amplicons, demonstrating a 2-12 fold higher fluorescence signal of ET cassette labels than the corresponding single dye-labeled ones. Eleven extended haplotype mini Y-STRs using ET cassette labeled primers are constructed, and sensitivity and mixed sample studies are performed. Due to the improved spectroscopic properties of ET labels, multiplex mini Y-STR typing is successful with as low as 30 pg of genomic male DNA, and in the high background of female DNA. These results indicate the practical advantage of ET cassette labels for low copy number and poor-quality DNA STR genotyping to be applied for criminal investigations, paternity testing, and evolutionary studies.

Keywords: Short tandem repeat, Capillary electrophoretic microdevice, Fluorescence energy transfer, Multiple polymerase chain reaction, Mini Y STR haplotyping

Introduction

Short tandem repeat (STR) typing is widely accepted as the gold standard in modern forensic societies for human identification[1,2]. Commercial STR kits such as Powerplex 16 and AmpFlSTR Identifiler are designed to genotype 16 autosomal STR loci including the 13 Combined DNA Index System (CODIS) STR loci, providing high discrimination power[3]. Likewise, Y chromosome-specific STR typing kits (PowerPlex Y and AmpFlSTR Yfiler) are developed specifically for analyzing male DNA. Y-STR is valuable, particularly in sexual assault cases, since a vast majority of crimes involve males as the perpetrator, and a sample could contain high levels of female DNA in the presence of minor amounts of male DNA[4,5]. The challenging issue in forensic laboratories is the analysis of low copy number DNA or a degraded DNA sample that often cause an allelic drop-in, allelic drop-out, and heterozygous peak imbalance[6]. To overcome these issues, mini STR typing systems have been developed to reduce the STR amplicon size below 200 bp for more efficient and uniform amplification. Another approach is to use a fluorescence energy transfer (ET) as a fluorescent label[7]. The commercial STR typing kits employ three or four single fluorescent dyes that emit at distinctive maximal wavelengths. However, these dyes do not have equivalent molar absorbances at a single excitation wavelength, resulting in imbalanced emission signals. To compensate for this effect, a higher concentration of long wavelength absorbing fluorescent dye labeled primers are added into the multiplex PCR reaction to achieve balanced fluorescence signals of PCR amplicons. ET cassette labels have proven to provide more balanced multicolor emission signals, as well as an increased sensitivity of DNA detection[1].

Here, we present the combination of an 11 extended haplotype mini Y-STR system and ET labeling technology for the advanced Y-STR genotyping. We modified the primer sequence to generate reduced-size amplicons of multiplex mini Y-STR loci and attached the ET cassette labels to improve the spectroscopic properties. The fluorescent intensities of ET -labeled single-locus PCR products were compared with those of corresponding single dye-labeled ones on a capillary electrophoretic microchip. We conducted a limit-of-detection analysis to evaluate the capability of ET multiplex for low copy number male genomic DNA typing, and a male-to-female mixture study to mimic real samples of sexual assault cases.

Results and Discussion

The ET dye-labeled primers were synthesized ac-

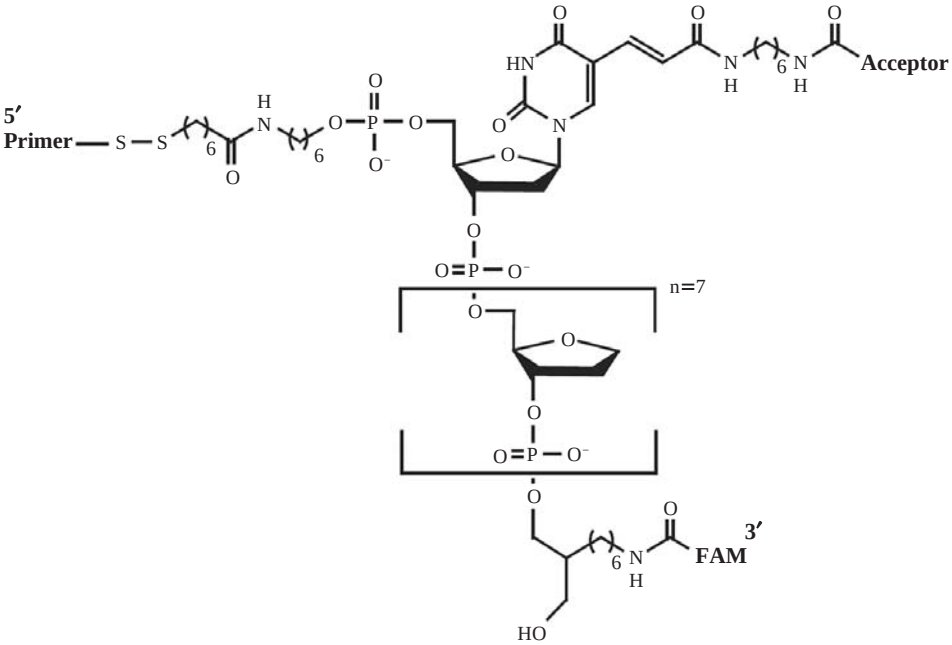


Figure 1. Structure of an ET dye-labeled primer. FAM, a donor, is separated from an acceptor (FAM, R6G, TMR, or ROX) by seven 1′,2′-dideoxyribose phosphate spacers. The acceptor is linked at the modified T base, and the ET dye is connected to the primer by a disulfide bond.

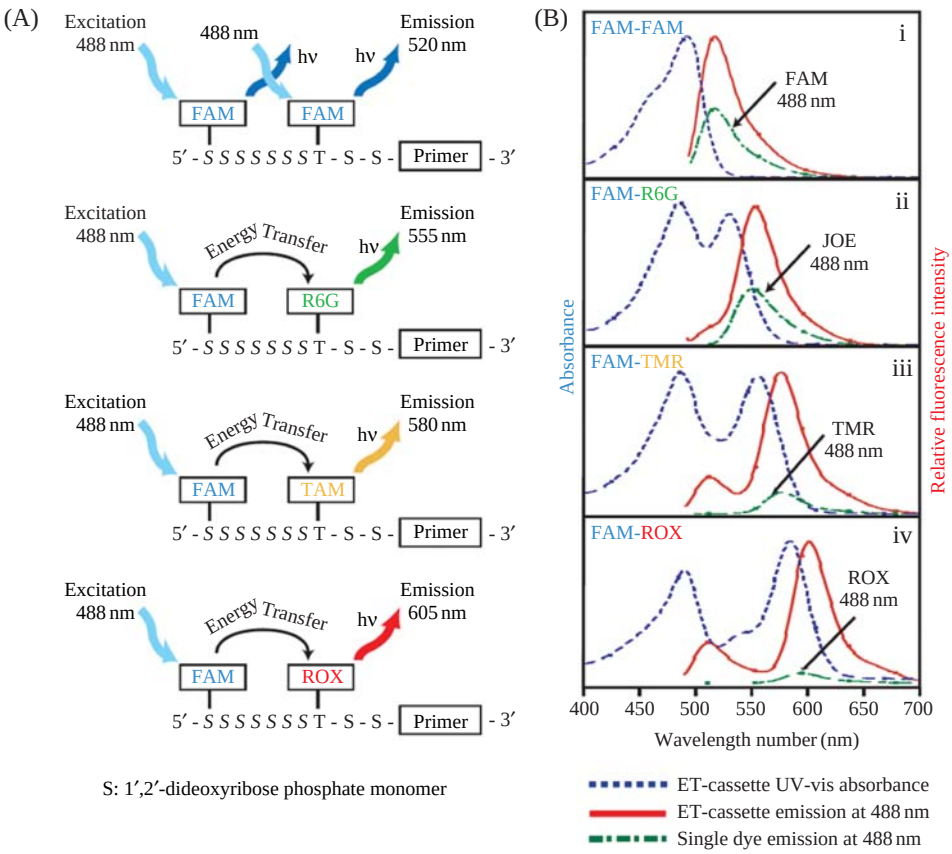


Figure 2. (A) A schematic of the ET process. A single laser of 488 nm irradiates a common donor, FAM, whose excited energy is transferred to the neighboring acceptor (FAM, R6G, TMR or ROX). Each acceptor exhibits a unique fluorescence signal with emission wavelength at 520, 555, 580 and 605 nm, respectively. (B) Absorption (blue line) and fluorescence emission (red line for ET and green line for single dye) spectra of four ET labels. The comparison of fluorescence emission is (i) FAM-FAM *vs.* FAM (ii) FAM-R6G *vs.* JOE (iii) FAM-TMR *vs.* TMR, and (iv) FAM-ROX *vs.* ROX.

cording to Berti *et al.*[7] (Figure 1). FAM, which absorbs maximally at 497 nm, was used as a common donor, and FAM, R6G, TMR, and ROX, which emit 525, 555, 580, and 605 nm respectively, were the acceptors.

Table 1. Information of multiplex PCR set for 11 extended haplotype mini Y-STRs.

Locus	ET label	Sequences (5′ to 3′)[a]	Conc. (μM)	Allele range[b]	PCE size
DYS391	FAM-FAM	FWD: TTCAATCATACACCCATATCTGTC REV : GATAGAGGGATAGGTAGGCAGGC	0.35 0.35	9-12	101-113
DYS389I/II	FAM-FAM	FWD: CCAACTCTCATCTGTATTATCT REV : GTTATCCCTGAGTAGTAGAAGAAT	0.60 0.60	11-15/ 27-32	151-167/ 267-287
DYS19	FAM-FAM	FWD: CTACTGAGTTTCTGTTATAGT REV : GATGGCCATGTAGTGAGGACA	0.50 0.50	13-17	189-205
DYS392	FAM-R6G	FWD: AAAAGCCAAGAAGGAAAACAAA REV : GAAACCTACCAATCCCATTCCTT	0.10 0.10	10-15	106-121
DYS390	FAM-R6G	FWD: CTGCATTTTGGTACCCCATA REV : GCAATGTGTATACTCAGAAACAAGG	0.06 0.06	21-26	160-180
DYS393	FAM-TMR	FWD: GTGGTCTTCTACTTGTGTCAATAC REV : AACTCAAGTCCAAAAAATGAGG	0.10 0.10	12-15	120-132
DYS385	FAM-TMR	FWD: GAAGGAAGGAAGGAAGGGAAA REV : TAAGGGCTGCTGACCAGATT	0.15 0.15	9-22	176-228
DYS438	FAM-ROX	FWD: TGGGGAATAGTTGAACGGTAA REV : GGCAACAAGAGTGAAACTCCA	0.20 0.20	9-13	105-125
DYS439	FAM-ROX	FWD: ACATAGGTGGAGACAGATAGATGAT REV : GCCTCAAGTGATCCACCCAAC	0.17 0.17	10-14	184-200

[a]The underlined 5′ guanine residue was added to promote adenylation.
[b]The common alleles are from the YHRD database (http://yhrd.org).

The distance between the donor and acceptor is critical to determine the efficiency of an energy transfer. A larger spacer distance enhances signal strength but suffers from more donor leakage, while a shorter spacer distance is more prone to quenching effect of the donor but with less cross-talk between the color channels. The (1′,2′-dideoxyribose) phosphates were used as spacers since a sugar phosphate chain maintains the charge and electrophoretic properties characteristic of DNA, and it is easy to control the length of the spacers to optimize the energy transfer efficiency. Previous studies have shown that seven spacers are optimal to maximize the spectral overlap between donor emission and acceptor absorbance, thereby resulting in high energy transfer along with reduced color cross-talk[7].

A 488 nm single laser was used to excite the common donor dye, FAM, which transfers the absorbed energy to an acceptor, producing high fluorescence intensities at a distinctive wavelength (Figure 2A). The synthesized ET cassette labels were confirmed by measuring the UV-vis absorption to detect the unique maximal absorption wavelength for each dye (FAM: 490 nm, R6G: 530 nm, TMR: 555 nm, and ROX: 587 nm) (Figure 2B, blue line). Compared with the fluorescence intensity of a single dye, ET cassette labels (FAM-FAM, FAM-R6G, FAM-TMR, and FAM-ROX) show 2, 3, 5, and 12 fold enhancement, respectively (Figure 2B, red vs. green line), with a residual fluorescence from FAM. Therefore, the ET cassette labeled primers can offer the advantages of excitation at a common wavelength for efficient multicolor detection and higher acceptor fluorescence emission over single dye-labeled primers.

To take advantage of the increased fluorescence intensity of ET cassette labeled primers for forensic DNA genotyping, we constructed an ET cassette labeled mini Y-STR typing system and evaluated its capability to detect mini Y-STR amplicons with low copy number DNA and in a high background of female genomic DNA. The primer sequences are redesigned to produce smaller-sized amplicons than those of the AmpFlSTR Yfiler kit by moving the primers as close as possible to the STR region. The single dye labeling was replaced by the ET cassette labels shown in Table 1.

Microfabricated capillary elelctrophoresis devices provide many advantages over current state-of-the-art technology in terms of speed, high-throughput, and sensitivity for genetic analysis[3]. We conducted electrophoretic separation and fluorescence detection of STR amplicons on a microdevice with a cross-injector design (Figure 3A). The equal amount of single and ET dye-labeled STR monoplex amplicon was mixed and loaded in the sample reservoir and electrophoretically injected into the microchannel toward the waste reservoir at an electric field strength of 250 V/cm for 30 s while floating the cathode and anode. A separation field of 250 V/cm was then applied between the

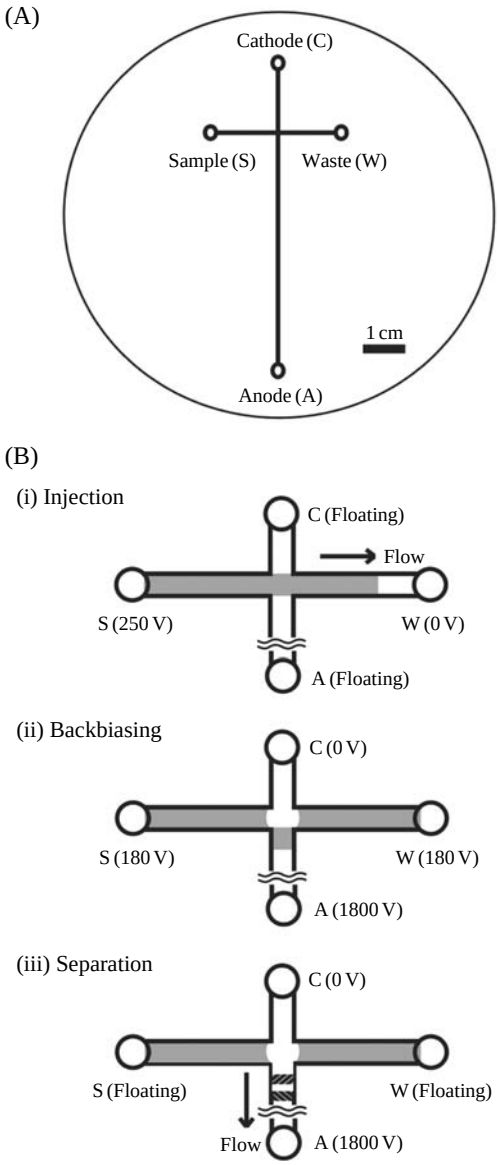


Figure 3. (A) A capillary electrophoresis microchip to separate PCR products by cross injection process. This 10 cm dia. microdevice consists of a glass-glass layer and is etched to form a cross fluidic microchannel and four reservoirs (an anode, a cathode, a sample, and a waste). (B) CE operation on a microdevice. (i) sample injection step (ii) backbiasing step and (iii) separation step (▨: ET dye-labeled PCR product, ▩: Single dye-labeled PCR product).

cathode and anode. During the first 5 s of the separation, a 180 V/cm backbias was applied to define a 1.6 nL plug at the injection cross. After that time, the sample and waste were floated. An ET dye-labeled STR amplicon migrates slower than the single dye-labeled one due to the additional acceptor dye and spacers of the ET cassette labels (Figure 3B).

We prepared monoplex Y-STR PCR products amplified with ET dye-labeled primers and the corresponding single dye-labeled primers under identical PCR conditions, and the equimolar mixture was separated on a CE microchip. DYS391, DYS392, DYS393, DYS439 loci were selected as representatives of FAM -FAM, FAM-R6G, FAM-TMR, and FAM-ROX labeling, respectively. The ET cassette labeled amplicons displayed higher fluorescence intensities than the single dye-labeled PCR products as shown in Figure 4. A FAM-FAM labeled DYS 391 allele displays an ~2-fold higher peak area of the FAM labeled allele (A). FAM-R6G labeled DYS 393, FAM-TMR labeled DYS 393, and FAM-ROX labeled DYS 439 give 3.2 (B), 4.2 (C), and 12.4 (D) fold enhancements in intensity versus corresponding JOE, TMR, and ROX labeled alleles, demonstrating a better sensitivity provided by the ET cassette labels.

Genotyping at 11 extended haplotype mini Y-STR markers (DYS19, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS385, DYS438, and DYS439) labeled with ET-fluorescence dyes was performed and analzyed on an ABI PRISM 3100 Avante sequencer. The electropherogram of multiplex STR amplicons shows well-balanced peaks as shown in Figure 5A. The sensitivity of ET STR typing was evaluated using serially-diluted DNA templates from 1,000 pg to 10 pg (Figure 5B). All the amplicons of the extended haplotype Y-STRs were detected without an allele drop-in or drop-out with 30 pg of the DNA template, while such phenomenon existed with 10 pg of DNA template, the results of which are consistent with Park *et al.*[6]. It is common in forensic laboratories to encounter mixed samples, particularly in sexual assault cases. We genotyped five samples that consists of male and female DNA at ratios of 1 : 0, 1 : 1, 1 : 10, 1 : 100, and 1 : 1,000. Even with the highest background of female genomic DNA, the 11 mini Y-STRs were successfully identified without unexpected peaks (Figure 5C). These results demonstrated that ET mini Y-STR systems provide improved DNA genotyping efficiency and excellent specificity for male genomic DNA. In addition, the increased fluorescence intensities of ET cassette labels can be successfully applied for multiplex STR typing in a conventional automatic DNA sequencer as well as an electrophoretic microchip system.

Conclusion

A key issue in forensic laboratories is the analysis of touch evidence since it contains very little biological material. A feasible approach to tackle challeng-

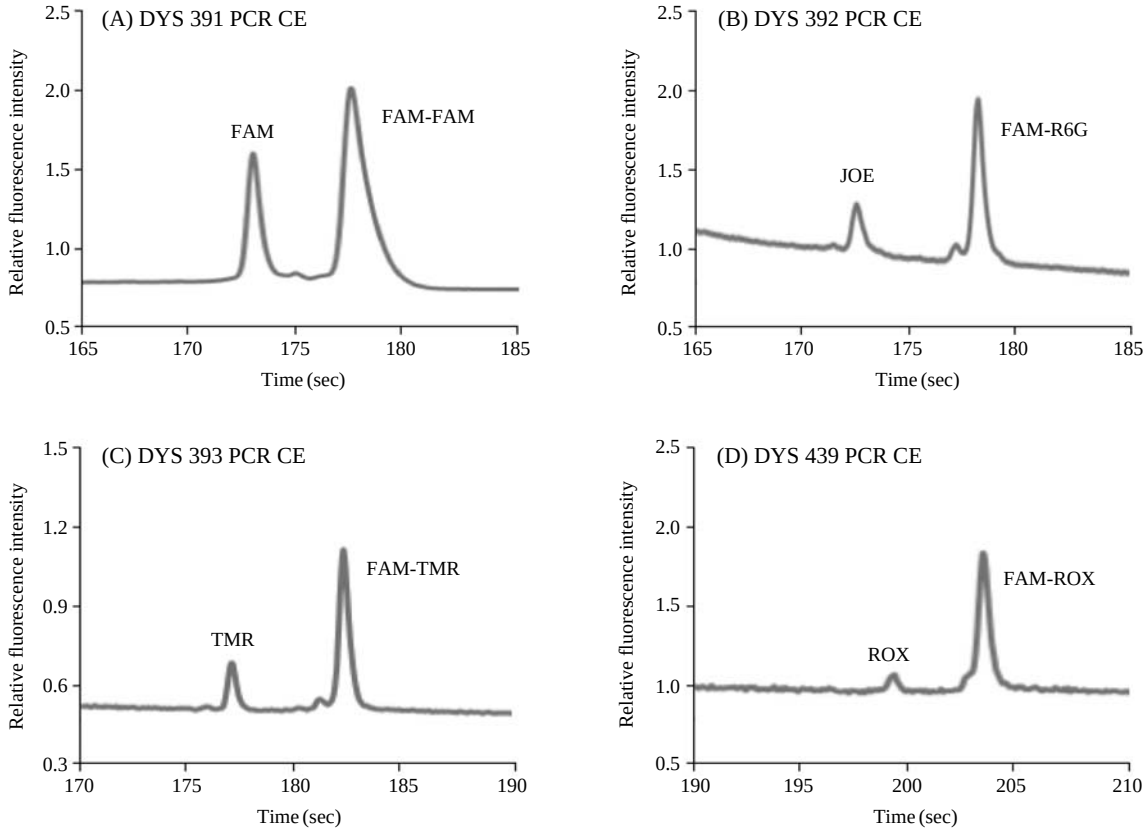


Figure 4. Electropherograms of monoplex mini Y-STR PCR product that are produced by using single and ET dye-labeled primers. Equal amounts of single and ET dye-labeled PCR products are mixed and co-injected into the CE microchip for separation. A comparison of the fluorescence intensity of (A) FAM *vs*. FAM-FAM labeled DYS 391 PCR product, (B) JOE *vs*. FAM-R6G labeled DYS 393 PCR product, (C) TMR *vs*. FAM-TMR labeled DYS 393 PCR product, and (D) ROX *vs*. FAM-ROX labeled DYS 439 PCR product.

ing touch-evidence sample typing is to increase the sensitivity of DNA detection as well as PCR efficiency. The ET cassette labeled mini Y-STR offers enhanced signals, distinct multiplex fluorescence bands, and better PCR efficiency that are beneficial to analyzing low-level and mixed DNA samples for human identification. Therefore, the combined mini Y-STR system and ET technology can contribute to increasing a high success rate and improved DNA profiling.

Materials and Methods

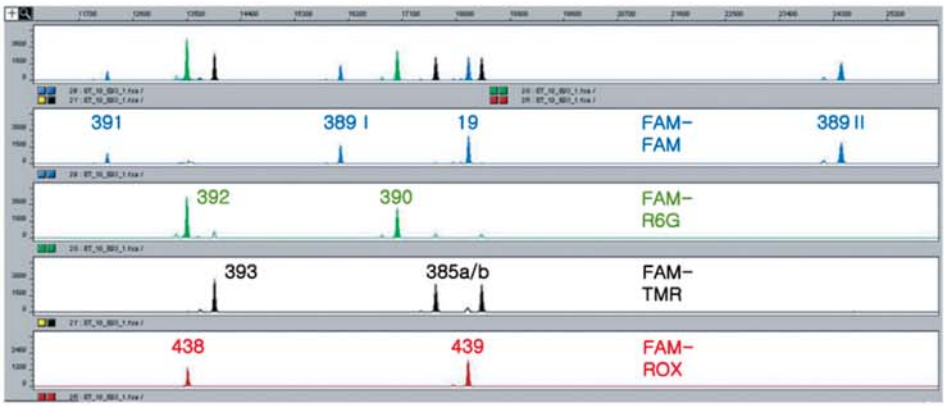
ET Cassette Synthesis and Characterization

Synthesis of ET cassette-labeled primers was followed by Berti *et al.*[7]. Briefly, the precursor of ET consisting of FAM at the 3′ end, seven 1′,2′-dideoxyribose spacers, an amino-modified thymine (T) nucleotide, and monomethoxytrityl (MMT) protecting group at the 5′ end was ordered from Midland Co. NHS esters of the acceptors (FAM, R6G, TMR, and ROX) was reacted with an amino group on the T. After deprotection of MMT, Sulfo-LC-SPDP (Pierce) was linked to the 5′ end to produce a reactive ET intermediate that is subsequently coupled with a 5′-thiol modified primer through a disulfide exchange reaction. The resultant ET cassette-labeled primer was purified by RP-HPLC, confirmed by MALDI-TOF mass spectrometry, and quantified by measuring the absorbance at 260 nm with correction of donor and acceptor contributions in a 0.1 M TEAA buffer using a JASCO V-540 spectrophotometer. Fluorescence spectra were recorded using a JASCO FP-750 fluorimeter at a concentration of 10 M in a 0.1 M TEAA buffer with excitation at 488 nm.

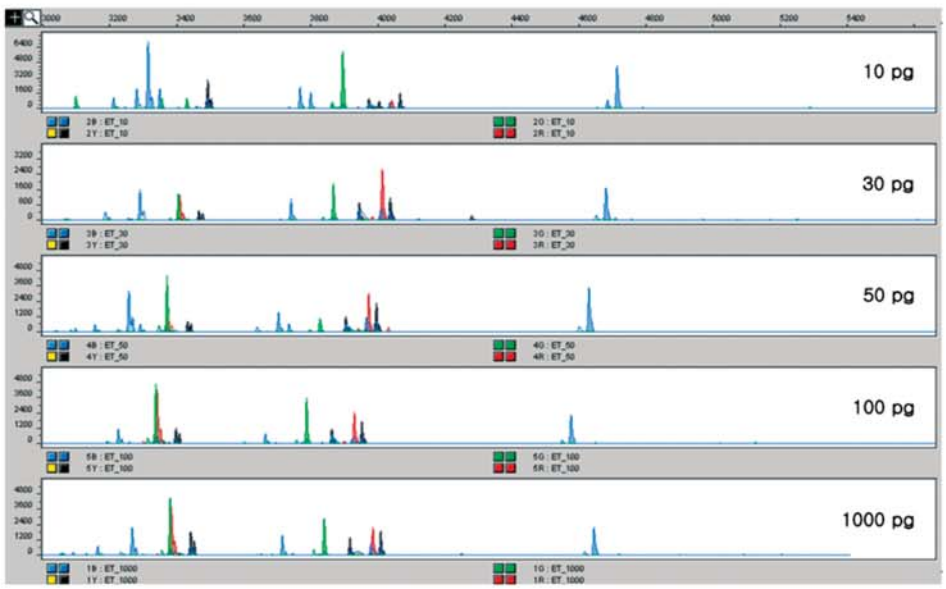
CE Microchip Fabrication and Operation

Electrophoretic analysis of an STR PCR product on a microdevice was performed as previously described[2]. A 100 mm dia. D263 glass wafer (1.1 mm thick) was coated with 2,000 Å amorphous silicon, and the microfluidic channel pattern was photolitographically

(A) Electropherogram of Y-STR PCR product of 9948 male DNA



(B) Limit of detection test



(C) Mixed sample study

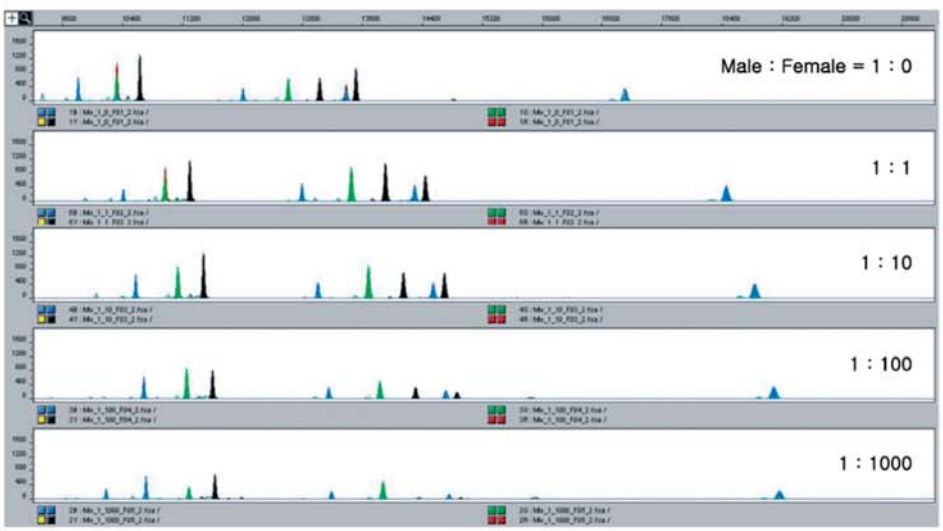


Figure 5. (A) Electropherogram of 11 mini Y-STR PCR products of 9948 male DNA, (B) limit of detection test and (C) male and female mixed sample study. Multiplex mini Y-STR genotyping was performed using ET dye-labeled primers, demonstrating that high sensitivity and good discrimination of Y-STR genotyping even with high background of female genomic DNA.

transferred to the silicon. The sacrificial silicon was etched with SF_6 in a parallel-plate reactive ion etching system, and the exposed glass was subsequently etched to a depth of 38 μm in 49% hydrofluoric acid. After etching, the photoresist and silicon were removed using acetone and SF_6, respectively. Four reservoir holes were drilled using a CNC mill, and the CE patterned wafer was thermally bonded with a blank glass wafer at 580˚C for 6 h. The CE chip incorporates 200 μm wide and 38 μm deep channels in a cross-injector design coupled to a 5-cm effective length separation channel. To minimize electroosmotic flow during CE, the channel was treated with a 50% dynamic coating (The Gel Co.) for 1 min and then loaded with a 5% w/v solution of linear polyacrylamide (LPA) in 1 × Tris TAPS-EDTA (TTE). The STR amplicons were electrophoresed using 1 × TTE as a running buffer in the cathode, waste, anode, and sample wells with injection, backbiasing, and separation steps as in Figure 3. The 1.6 nL plug at the injection cross was loaded into the separation channel and dye-labeled STR amplicons were detected within 4 min at the end of the separation channel with a 488 nm laser-induced fluorescence system. The peak quantitation in the electropherogram was calculated using GRAMS/ 32 software.

Multiplex PCR Conditions

A multiplex PCR set for 11 extended haplotype Y-STRs (DYS19, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS385, DYS438, and DYS439) was constructed. To minimize the size of the PCR products, a primer design suggested from previous reports was used[2,6]. PCR amplification was carried out in a final volume of 10 μL containing 1 ng of 9948 male DNA (Promega, Madison, USA), 1.5 μL of Gold ST*R 10X buffer (Promega), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and the appropriate concentration of ET dye-labeled primers or single dye-labeled primers (Table 1). Thermal cycling was conducted on a PTC-200 DNA engine (MJ Research, Waltham, MA, USA) under the following conditions: 95˚C for 11 min, 96˚C for 1 min; 10 cycles of 94˚C for 30 s (then ramped 0.5˚C/s to 58˚C), 58˚C for 30 s (then ramped 0.2˚C/s to 70˚C), 70˚C for 45 s (then ramped 1.0˚C/s to 94˚C); 20 cycles of 90˚C for 30 s (then ramped 0.5˚C/s to 58˚C), 58˚C for 30 s (then ramped 0.2˚C/s to 70˚C), 70˚C for 45 s (then ramped 1.0˚C/s to 90˚C) and a final extension at 60˚C for 45 min.

Elctrophoresis and Data Analysis

One μL of the amplified product was added to 19.0 μL of deionized Hi-Di formamide (Applied Biosystems), denatured at 95˚C for 3 min, and then chilled on ice for 3 min. Samples were injected and electrophoresed on an ABI PRISM 3100 Avante (Applied Biosystems). The data were collected using the ABI PRISM 3100 data collection software, and electrophoresis results were analyzed using ABI PRISM GeneScan analysis software.

Limit of Detection

The 9948 male DNA standard was diluted to a concentration of 10 pg/μL, 30 pg/μL, 50 pg/μL, 100 pg/μL, and 1,000 pg/μL for sensitivity study. Five replicates were tested for each concentration of DNA with appropriate amplification cycles: 30 cycles for 1 ng of template DNA, 33 cycles for 100 pg and 50 pg, 35 cycles for 30 pg and 10 pg. Peaks of each dilution were compared to the "expected" peak determined using 1 ng of 9948 male DNA. PCR failure was decided when no peaks were observed above the interpretational threshold of 100 relative fluorescent units (RFUs).

Mixed Sample Study

Commercial standard human genomic male and female DNA was purchased from Promega. Mixed sample studies were performed on five sets of male and female DNA at 1 : 0, 1 : 1, 1 : 10, 1 : 100, and 1 : 1,000. The amount of 9948 male DNA was kept constant at 100 pg, and the amount of female DNA was varied from 0 to 100 ng. Thirty-three amplification cycles were used.

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