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TECHNICAL NOTE

Y-STR analysis of degraded DNA using reduced-size amplicons

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Abstract To increase the success rate of Y-STR genotyping for degraded DNA, we have developed two multiplex PCR sets for 21 Y-STR loci. Besides the 17 Y-STR loci of DYS19, DYS385, DYS389-I, DYS389-II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and GATA H4.1 contained in a commercial Y-STR kit, AmpFlSTR® Yfiler™, the other four loci of DYS388, DYS446, DYS447, and DYS449 were also included in the multiplexes to increase the discrimination capacity. Among a total of 21 Y-STR loci, the primers for eight loci (DYS385, DYS390, DYS438, DYS446, DYS448, DYS449, and DYS635) were newly designed in the present study and nine loci (DYS385, DYS390, DYS391, DYS392, DYS438, DYS439, DYS448, and DYS635) have PCR amplicons smaller than those of the AmpFlSTR® Yfiler™ kit. A sensitivity test using serially diluted standard 9948 male DNA showed that all the values of Y-STR loci in the Y-miniplexes are reliable at template concentrations as low as 30 pg. We compared the effectiveness of the two multiplexes with the AmpFlSTR® Yfiler™ kit by using both enzymatically degraded DNA and 30 samples of 50-year-old skeletal remains. This comparison demonstrated that the new Y-miniplex sets can produce a better signal from degraded DNA than the AmpFlSTR® Yfiler™ kit.

Keywords Y chromosome · STR · AmpFlSTR Yfiler · Miniplex · Degraded DNA

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Introduction

Typing of Y chromosome-specific short tandem repeat (Y-STR) markers based on PCR amplification is widely used in human identification, criminal investigations, and lineage and inheritance studies [1–6]. In the forensic field, typing of Y-STR markers was mostly performed using commercially available multiplex kits. A multiplex PCR system has the advantage of low DNA sample consumption with reduced time and costs. Application of such multiplex kits usually results in complete Y-STR profiles from a variety of substrates with high quality DNA. DNA samples from forensic cases, however, are often degraded and/or tainted by environmental contaminants. When such low quality DNA is used for STR profiling, loss of signal is typically observed with larger-sized amplicons [7–11]. One approach for recovering information from degraded samples is to reduce the PCR amplicon sizes by moving primers as close as possible to the STR repeat region. The observation that smaller-sized amplicons from autosomal STR markers produce a higher success rate with degraded DNA was confirmed by a number of recent studies [12–21]. In this context, we expect that using reduced-size amplicons to type Y-STRs could also produce a high success rate with highly degraded forensic or anthropological samples.

Since 2000, a major effort was undertaken in Korea to identify and return the remains of victims of the Korean

War (1950–1953) to their bereaved families. Some of the missing casualties were identified and returned to their families on the basis of circumstantial evidence and matching results of mitochondrial DNA (mtDNA) genetic profiles. Because mtDNA testing is not so powerful for positive identification due to the haploid and maternal transmission characteristics, there is a need for additional genetic analyses to further identify individuals with greater scientific exactitude. Unfortunately, many of the missing casualties do not have descendants, and their parents are usually deceased. In these circumstances, the analysis of Y-STRs, which are inherited without recombination, is better than autosomal STR analysis for solving these cases because unlike autosomal STR analysis, siblings and paternal relatives can be used for Y-STR analysis.

Therefore, in the present study, two mini Y-STR multiplex PCR sets (Y-miniplexes) were developed by reducing the amplicon sizes of 21 Y-STR loci. The two Y-miniplexes included 17 Y-STR loci (DYS19, DYS385, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and GATA H4.1) from a commercial multiplex kit, AmpFlSTR® Yfiler™, and another four Y-STR loci (DYS388, DYS446, DYS447, and DYS449) to increase the haplotype diversity. To examine the amplification efficiency of the Y-miniplexes, the effect of DNA template concentration on signal intensity was investigated and the amplification results were also compared with the AmpFlSTR® Yfiler™ kit on enzymatically degraded DNA and 30 DNAs extracted from 50-year-old skeletal remains.

Materials and methods

Primer design and selection for Y-miniplex

Primers for 21 Y-STRs were gathered based on information from previous reports [4, 22–28] and Y chromosome STR fact sheets at the STRbase (http://www.cstl.nist.gov/div831/strbase/ystr_fact.htm). Additional candidate primers were also designed against the reference sequences obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) using web-based Primer 3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The typical default parameters for primer design were as follows: primer size range from 10 to 27 nucleotides with 20 nucleotides as the optimum, primer T_m values from 57°C to 63°C with 60°C as the optimum, and the primer GC% range from 20% to 80%. The amplicon size was made as small as possible by moving primers as close as possible to the STR core repeat. The independently selected candidate primer pairs were screened for potential secondary structure using the AutoDimer software (http://www.cstl.nist.gov/biotech/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm) [29]. Also, whether they were selected from previous reports or were newly designed, the total length of the amplified products was limited to less than 250 bp. The highest amplification efficiency in a single PCR was used as a first-level screen for primers, and the final set of primers were selected from a multiplex PCR condition with no interference from the other primers. Tables S1 and S2 (see Electronic supplementary material) show the GenBank accession number, observed allele range, and size reduction information for each 21 Y-STR locus and the final set of selected primers, respectively. Fluorescent dye was used to label the forward or reverse primer of each STR locus (Applied Biosystems, Foster City, CA). Unlabeled primers were purchased from Genotech (Seoul, Korea). An additional guanine base was added to the 5′ end of each unlabeled primer to promote the adenylation of PCR products (Table S2) [30].

Multiplex PCR conditions and electrophoresis

A total of two multiplex sets were constructed for 21 Y-STR loci. Y-miniplex I consisted of DYS19, DYS389I/II, DYS392, DYS437, DYS439, DYS448, DYS449, DYS456, DYS458 and DYS635. Y-miniplex II consisted of DYS385, DYS388, DYS390, DYS391, DYS393, DYS438, DYS446, DYS447, DYS635 and GATA H4.1. In the two Y-miniplexes, DYS635 was commonly used to check for sample switching (Figs. S1 and S2). PCR amplifications were carried out in a final volume of 10 μl containing template DNA, 1.6 μl of Gold ST*R 10X buffer (Promega, Madison, WI), 2.0 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems) and appropriate concentration of primers (Table S2). Thermal cycling was conducted on the GeneAmp® PCR System 9600 (Applied Biosystems) under the following conditions: 95°C for 11 min, 96°C for 1 min; 10 cycles of 94°C for 30 s (then ramp 68 s to 60°C), 60°C for 30 s (then ramp 50 s to 70°C), 70°C for 45 s; 20–23 cycles of 90°C for 30 s (then ramp 60 s to 58°C), 58°C for 30 s (then ramp 50 s to 70°C), 70°C for 45 s and a final extension at 60°C for 45 min. The PCR products of the multiplexes were mixed with GeneScan® 400HD (ROX) size standard (Applied Biosystems) and analyzed by capillary electrophoresis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and GeneScan software 3.1 (Applied Biosystems).

Construction of allelic ladders and genotyper macros

Except for DYS385, all the allelic ladders for the 16 Y-STRs, which are included in the AmpFlSTR® Yfiler™ kit were created by amplifying each locus using new primers with 17 cycles of the above PCR conditions after a 1:500

dilution of the kit allelic ladders. The allelic ladders for DYS385, DYS388, DYS446, DYS447 and DYS449 were constructed by combining all observed alleles from each locus using previously studied DNA [31]. Genotyper macros were constructed for each of the multiplex sets to perform allelic designation with allelic ladders using the Genotyper 2.5 software (Applied Biosystems).

PCR amplification with serially diluted DNA

A commercial standard 9948 male DNA and 9947A DNA were purchased from Promega. The 9948 male DNA was serially diluted to a concentration of 1 ng–15 pg/μl for the sensitivity study, and the 9947A DNA was used for the male specificity test. Ten replicates were tested for each concentration of DNA with appropriate amplification cycles: 30 cycles for 1 ng and 500 pg of template DNA, 31 cycles for 250 and 125 pg, 32 cycles for 62 pg, and 33 cycles for 30 and 15 pg. Genotype results at each dilution were compared to the "correct" genotype, which was determined using 1 ng of the standard DNA. Genotyping failure was decided when no peaks were observed above the interpretational threshold of 100 relative fluorescent units (RFUs).

PCR amplification with artificially degraded DNA

Degraded DNA was prepared by digesting 3.0 μg of blood DNA with 0.01 units/μl of DNase I (NEB, Ipswich, MA) for time periods of 2, 5, 10, 15, 20 and 30 min and DNA fragmentation was confirmed by agarose gel electrophoresis. Two Y-miniplexes amplifications were carried out under the same conditions as described above and the AmpFlSTR® Yfiler™ kit was utilized according to the AmpFlSTR® Yfiler™ PCR amplification kit user's manual with 30 cycles of amplification.

PCR amplification with DNA extracted from old skeletal remains

Long bones obtained from 30 skeletal remains of victims from the Korean War (1950–1953) were also used to compare the amplification efficiency of the Y-miniplexes with that of the AmpFlSTR® Yfiler™ kit (Applied Biosystems). Long bones were cut into slices of approximately 1×3 cm size using a dental diamond disk. The outer surface of the bone specimens were removed with a dental drill and irradiated with UV light. Bone pieces were frozen in liquid nitrogen and ground in a SPEX Mill 6700 (SPEX CertiPrep, Metuchen, NJ). DNA extraction from bone powder was carried out following the method modified by Yang et al. [32]. Bone powder was incubated in 1 ml of extraction buffer (0.5 M EDTA pH 8.0, 0.5% SDS) with proteinase K (50 μL of 20 mg/ml) at 56°C for 1–2 days. After incubation, samples were purified and concentrated using a commercially available QIAquick PCR purification kit (Qiagen, Hilden, Germany). The DNA extraction procedure was performed from each skeletal remain sample at least twice.

PCR amplifications were carried out under the same conditions as described above except for the use of 3.0 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems) and 10/25 amplification cycles. The AmpFlSTR® Yfiler™ kit was utilized with amplification of 35 cycles. For accuracy, the Y-STR genotyping results were decided by obtaining consensus allelic scores from duplicate analyses using two independent DNA extracts.

Results and discussion

Primer design and selection for Y-miniplex

The newly designed multiplex PCR systems included the 17 Y-STR loci of AmpFlSTR® Yfiler™ kit and an additional four Y-STR loci. Of the 17 Y-STR loci of AmpFlSTR® Yfiler™ kit, nine Y-STR loci (DYS385, DYS390, DYS391, DYS392, DYS438, DYS439, DYS448, and DYS635) had amplicon sizes smaller than those of the AmpFlSTR® Yfiler™ kit in the Y-miniplexes, but primers for eight Y-STR loci (DYS19, DYS389I/II, DYS393, DYS437, DYS456, DYS458 and GATA H4.1) could not be redesigned or selected from the previous reports to generate amplicons smaller than those of the AmpFlSTR® Yfiler™ kit due to the innately small amplicon sizes obtained from existing PCR primers (DYS393, DYS456, DYS458 and GATA H4.1) or due to the inability to design other primers for smaller amplicons (DYS19, DYS389-I, DYS389-II, and DYS437) (Table S2). Out of a total of 21 Y-STR loci, primers for DYS385, DYS390, DYS438, DYS446, DYS448, DYS449, and DYS635 were newly designed in the present study (Table S2) and all these STRs produced amplification products smaller than any others from previous reports. Primers for DYS388 and DYS437 could have been designed to produce amplicon sizes smaller than those of the present study, but they were selected from previous publications [24, 25] due to the convenience of multiplex PCR construction or product size similarity. Forward primers for DYS385 was designed to be located four bases into the repeat region, and primers for DYS446 were designed not to amplify a homologous region on the X-chromosome (GenBank accession no.: AL133512.10) by locating the 3′ end of each primer at the specific nucleotide position that reveals the difference between the two loci (Fig. S3).

The resulting primers were evaluated for potential interactions with each other using Autodimer software with a score threshold of seven. Autodimer screening results indicated significant complementarity between the forward primers for DYS392 and DYS449 with a score of nine. However, they empirically did not show significant interference for each other and could be used together in the Y-miniplex I. The resulting amplification product sizes obtained from the final set of primers ranged from 91 to 299 bp.

Sensitivity test

To evaluate the newly developed Y-miniplexes, sensitivity tests were conducted using ten replicates of DNAs in various concentrations (Fig. S4). Correct genotypes were observed at concentrations as low as 30 pg of DNA in most replicates tested. However, DYS388 and DYS389I often showed allele drop-out at 30 pg of template DNAs. Samples containing less than 30 pg of template started to show allele drop-out or allele drop-in, which causes an incorrect genotype result. At 15 pg of template concentration, only four (in Y-miniplex I) or three (in Y-miniplex II) out of ten diluted samples showed correct genotypes at all loci. DYS19, DYS389I/II, DYS388, DYS391 and DYS635 showed allele drop-out most commonly among all loci, and DYS635 also frequently displayed allele drop-in.

To some extent, the increasing the number of PCR cycles and the addition of more DNA polymerase can produce more useful profiles of lower levels of DNA [33]. However, low copy number DNA (LCN) template coupled with an increase in amplification cycle number is known to increase the rate of both allelic drop-out and allelic drop-in [34], and possibly creates the potential for misinterpretation of genetic data [33, 35]. Since, with the exception of DYS385, allele drop-out at Y-STR loci does not provide any information, Y-STR analysis with low quantities of template coupled with an increase in amplification cycle number and DNA polymerase concentration can be more useful than autosomal STRs. Nevertheless, it is important to be attentive to the potential misinterpretation caused by increases in drop-in due to the influence of DNA contaminants and false stutter alleles.

Efficiency test

We tested the efficiency of Y-miniplexes for blood DNA, which had been treated with DNase I for several time periods. An experiment on artificially degraded DNA showed that DNase I treatment for 20 min or more caused the loss of genotype results for higher molecular weight loci when using the AmpFlSTR® Yfiler™ kit. However, almost all these loci were successfully genotyped using the Y-miniplexes except for DYS389II which has a similarly large amplicon size in both systems. Even after 30 min of DNase I treatment, partial profiles were obtained using the Y-miniplexes (Fig. S5).

The forensic usefulness of the two multiplexes was also evaluated on 30 samples of skeletal remains, which showed complete profiles of the mtDNA control region sequence in our previous analysis (unpublished). Although six samples did not produce consensus results of duplication in any locus from a total of 30 bone samples, Y-miniplexes gave more useful profiles in most samples tested compared to the AmpFlSTR® Yfiler™ kit. The success rate at each Y-STR locus also revealed that Y-miniplexes are much more efficient for the genotyping of STRs, which have reduced-size amplicons. Specifically, the PCR product sizes of four Y-STRs were reduced by 80 bp or more, and they showed the greatest improvement in the success rate of the Y-miniplexes (26.7% increase for DYS438 and 20.0% for DYS392, DYS439 and DYS635). Among the four additional Y-miniplex loci, DYS388 and DYS449 displayed relatively high success rates (Table 1). The mean numbers of successfully genotyped loci also demonstrated that Y-miniplexes could produce more reliable genotype results

Table 1 Success rate of Y-STR genotype determination using the AmpFlSTR® Yfiler™ kit and the Y-miniplexes from old skeletal remains (n=30)

Locus	Size reduction	AmpFlSTR® Yfiler™ kit*	Y-miniplex*
DYS19	–	4 (13.3%)	7 (23.3%)
DYS389I	–	11 (36.7%)	10 (33.3%)
DYS389II	–	5 (16.7%)	6 (20.0%)
DYS393	–	12 (40.0%)	13 (43.3%)
DYS437	–	8 (26.7%)	7 (23.3%)
DYS456	–	13 (43.3%)	16 (53.3%)
DYS458	–	11 (36.7%)	8 (26.7%)
GATA H4.1	–	17 (56.7%)	10 (33.3%)
DYS385	75 bp	8 (26.7%)	10 (33.3%)
DYS390	49 bp	7 (23.3%)	10 (33.3%)
DYS391	59 bp	12 (40.0%)	15 (50.0%)
DYS392	197 bp	6 (20.0%)	12 (40.0%)
DYS438	123 bp	6 (20.0%)	14 (46.7%)
DYS439	87 bp	9 (30.0%)	15 (50.0%)
DYS448	62 bp	4 (13.3%)	8 (26.7%)
DYS635*	95 bp	5 (16.7%)	11 (36.7%)
DYS388	–	–	13 (43.3%)
DYS446	–	–	6 (20.0%)
DYS447	–	–	6 (20.0%)
DYS449	–	–	10 (33.3%)

*The Y-STR genotyping results were decided by obtaining consensus allelic scores from twice-performed analyses. At DYS635, genotype results of Y-miniplexes reflect consensus allelic scores only from Y-miniplex I.

Table 2 Mean number of loci, which were successfully genotyped with the AmpFlSTR® Yfiler™ kit and the Y-miniplexes from old skeletal remains (n=30)

Sample quality[a]	Number of samples	9 Y-STR loci[b]		17 Y-STR loci[c]		4 Y-STR loci[d]	21 Y-STR loci[e]
		AmpFlSTR® Yfiler™ kit	Y-miniplex	AmpFlSTR® Yfiler™ kit	Y-miniplex		
Very low	7	0.0	0.4	0.0	1.0	0.3	1.3
Low	8	0.5	1.3	1.4	2.3	0.5	2.8
Medium	9	2.9	4.4	6.6	8.1	1.3	9.4
High	6	5.2	7.8	12.0	13.2	2.8	16.0
Total	30	2.0	3.3	4.7	5.9	1.2	7.1

[a] Sample qualities classified into four groups according to the number of successfully genotyped loci when using the AmpFlSTR® Yfiler™ kit; very low (0 loci), low (1–2 loci), medium (3–9 loci) and high (10–17 loci).
[b] Mean number of loci typed successfully among nine Y-STR loci with reduced-size amplicons in Y-miniplexes.
[c] Mean number of loci typed successfully among 17 Y-STR loci, which consist of the AmpFlSTR® Yfiler™ kit.
[d] Mean number of loci typed successfully among four Y-STR loci (DYS388, DYS446, DYS447, DYS449) in Y-miniplexes.
[e] Mean number of loci typed successfully among 21 Y-STR loci including 17 Y-STRs and additional four Y-STRs.

than the AmpFlSTR® Yfiler™ kit (Table 2). These better results of the Y-miniplexes might be explained by the fact that the number of loci in the multiplexes is smaller than that of the AmpFlSTR® Yfiler™ kit (10–11 loci vs 17 loci). However, it rather seems to have been attributed to more small sized amplicons in the Y-miniplexes compared with the AmpFlSTR® Yfiler™ kit, and it was also demonstrated in many previous reports [15–17, 20, 21].

On the other hand, the Y-miniplexes and the AmpFlSTR® Yfiler™ kit produced allele drop-in or stutter in seven and five samples, respectively. The Y-miniplexes showed single occurrences of allele drop-in or stutters at five loci (DYS385, DYS388, DYS392, DYS449, and DYS458) and two at DYS456. The AmpFlSTR® Yfiler™ kit also displayed allele drop-in or stutters once for three loci (DYS393, DYS389, and DYS456) and twice for one locus (DYS458). Except for DYS449, which belongs only to the Y-miniplexes, the occurrences of allele drop-in or stutters in Y-miniplexes are comparable to those in the AmpFlSTR® Yfiler™ kit (six samples vs five samples). Also from determined consensus genotypes of DYS385, allele drop-out was suspected from single peaks in five out of ten samples genotyped using the Y-miniplexes and in five out of eight samples genotyped using the AmpFlSTR® Yfiler™ kit.

Conclusions

Our developed Y-miniplex sets provide an effective analysis tool for degraded forensic samples as demonstrated by sensitivity and efficiency testing using diluted or enzymatically digested DNA or 50-year-old bone samples. The genotyping results of these sets seemed to be reliable and sensitive when at least 30 pg of template DNA was present. The usefulness of the nine reduced-size Y-STRs (DYS385, DYS390, DYS391, DYS392, DYS438, DYS439, DYS448, and DYS635) in the degraded DNA was also confirmed by comparing the success rates with those of the AmpFlSTR® Yfiler™ kit using enzymatically degraded DNA and 50-year-old bone samples under the same PCR amplification conditions. The Y-miniplexes with reduced-size amplicons proved to be a useful tool that can produce a better signal from degraded DNA than the commercial Y-STR kit. Accordingly, the Y-miniplexes of the present study will increase the overall number of successfully analyzed loci, increasing the discrimination power of the Y-STR profiles produced. Since the two Y-miniplexes were, however, not fully forensically validated, a primer concordance study and a study of non-human samples and of mixed stains (male/female) need to be followed-up for further evaluation.

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