International Journal of Legal Medicine (2021) 135:23–41
https://doi.org/10.1007/s00414-020-02314-z

ORIGINAL ARTICLE

Validation of the Microreader 40Y ID System: a Y-STR multiplex for casework and database samples

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Received: 29 August 2019 / Accepted: 5 May 2020 / Published online: 9 June 2020

Abstract

Y-chromosome-specific short tandem repeat loci (Y-STRs) are commonly analysed in forensic science for paternity testing, familial searches, and, in sexual assault cases, to determine male DNA identity from mixed sources with high background female DNA content. The Microreader 40Y ID System is a six-dye multiplex amplification kit that contains 17 Y-STR loci from the Yfiler Plus PCR Amplification Kit and the powerplex Y23 system (DYS19, DYF385a/b, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS549, DYS635(Y GATA C4), DYS643, Y GATA H4, DYS460, DYS481, DYS533, DYF387S1, DYS449, DYS518, DYS570, DYS576, and DYS627), plus six high polymorphic loci (DYS444, DYS447, DYS557, DYS596, DYS527 a/b) as well as 4 additional candidate Y-STR loci (DYS593, DYF404S1, DYS645) and a Y-Indel loci (Rs2032678), thereby providing greater efficiency, compatibility, and accuracy. The Microreader 40Y ID System can directly amplify markers from blood or saliva on filter paper or FTA cards, without template extraction or purification, and can also be used for extracted DNA templates. To verify the efficiency and accuracy of the kit, the Microreader 40Y ID System was validated by investigating sensitivity, amplification conditions, male-male and male-female mixtures, PCR inhibition, species specificity, reproducibility, and efficacy with degraded samples. The Y-STR loci were also tested using 437 male samples from Tibet, Han, and Yi. The Microreader 40Y ID System was able to compensate for some of the shortcomings of Y-STR markers in practical applications, such as cost and profile interpretation, and fully meets the domestic Y chromosome database construction specifications and requirements.

Keywords Y-STR validation · Haplotype diversity · Microreader 40Y ID System

Yuqing Liu and Meili Lv contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00414-020-02314-z) contains supplementary material, which is available to authorized users.

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Introduction

Recently, Y-chromosome research has become more important in forensic laboratories. In actual cases, especially in sexual assault cases when the male component of samples is largely dominated by a high background concentration of female component, it is difficult to detect minor male contributors by relying on autosomal short tandem repeat loci (STRs) [1, 2]. In contrast, Y-chromosome-specific STRs (Y-STRs) are more efficient for targeting minor male DNA components. Furthermore, the rapid mutation Y-STRs targeted by the Microreader 40Y ID System are useful for kinship identification, and the kit also contains most of the loci found in other common commercial kits, thereby making the kit more efficient and stable when analysing the samples. The Microreader 40Y ID System contains 20 core loci, 15 preferred loci, and four common candidate loci. Among these loci, DYS570, DYS449, DYS518, DYS576, DYS404S1, DYS387S1, and DYS627 are hypermutant loci which use six-dye fluorescent chemistry. Except for the size standard fluorescence, the blue channel (FAM) includes Y-Indel, DYS393, DYS570, DYS19, DYS392, DYS549, Y GATA H4, DYS444, and DYS593. The green channel (HEX) includes DYS460, DYS458, DYS481, DYS635, DYS438, DYS447, and DYS596. The yellow channel (TAMRA) includes DYS456, DYS389I, DYS448, DYS533, and DYS449. The red channel (ROX) includes DYS391, DYS439, DYS437, DYS385a/b, DYS643, and DYS518. The purple channel (PURP) includes DYS576, DYF404S1, DYF387S1, DYS627, DYS527a/b, DYS557, and DYS645 (Table 1). With the mature six-dye fluorescence system, the selection of loci with higher compatibility can effectively realize the family identification and achieve the precise location of the target family. Meanwhile, in the case of family system determination, accurate individual identification can be achieved effectively, and the overall solution of public security database construction and case analysis can be created [3]. It provides greater identification capabilities for Y database applications.

The aim of the present study was to verify the efficiency and accuracy of the Microreader 40Y ID System. This was achieved by investigating sensitivity, amplification conditions, male-male and male-female mixtures, PCR inhibition, species specificity, reproducibility, and efficacy with degraded samples. For the forensic genetic analysis study, the Y-STRs were also tested using 437 male samples from Tibetan, Han, and Yi populations. In this study, the validation analysis of the Microreader 40Y ID System was performed as the guidelines for DNA analysis methods published by the Scientific Working Group on DNA Analysis Methods (SWGDAM) [4, 5].

Materials and methods

The Microreader 40Y ID System was validated for forensic use under conditions recommended by the manufacturer (Beijing Microread Genetics Co., Ltd.) [6] and several control DNA samples, namely M308 (male 2 ng/μl), F312 (female 2 ng/μl), and 2800 M (male 10 ng/μl). Whole-genome DNA was extracted using the new rapid extraction kit (centrifugal column; Beijing BioTeke Co., Ltd.) from human blood. The blood samples used for direct amplification were collected using 1.2-mm FTA blood cards, whereas the semen samples were collected and extracted using the Chelex 100 method (5% Chelex, w:v).

Samples for genetic analysis

The samples used for genetic analysis were obtained from 437 individuals, including 169 Sichuan Han people, 168 Tibetans, and 100 Yi people. Human blood samples were collected with the approval of the Ethics Committee at the Institute of Forensic Medicine, Sichuan University (K2015008) [7]. DNA was extracted from the Yi samples using the Chelex 100 method (1 ml Chelex, 5%, and 15 μl proteinase K, 20 mg/ml) and from the Han and Tibetan samples using the new rapid extraction kit for whole blood genomic DNA.

All the participants in this study gave their written informed consent and the approval of the study was granted by the Ethical Committee of the Sichuan University. The samples were processed in anonymous form, and the confidentiality of personal information of each study participant was assured.

DNA amplification

All the samples were amplified using the GeneAmp PCR System 9700 (Thermo Fisher Scientific, Waltham, MA USA), according to the Microreader 40Y ID System instructions. Briefly, the 25-μl reactions mixtures included 10 μl Microreader 2.5× Master Mix III, 5 μl Microreader 40YD 5× Primer Mix, 1 μl DNA (0.5–2 ng) or 1.2 mm FTA card, and 9 μl water [4] and were amplified under the following conditions: 95 °C for 5 min, followed by 28 cycles of 94 °C for 20 s and 59 °C for 90 s, 60 min at 60 °C, and a final temperature of 4 °C [8]. A series of gradient experiments were performed to determine the effects of Master Mix, primer concentration, cycle number, reaction volume, annealing temperature, and final extension temperature. Different inhibitors were also used to determine inhibitor tolerance.

To determine the minimum and maximum effective Master Mix concentrations, five concentrations (0.5X, 0.75X, 1X, 1.25X, and 1.5X) were tested, and to determine the minimum and maximum effective Primer Mix, five concentrations (0.5X, 1X, 1.25X, and 1.5X) were tested, and the experiments

were all repeated three times. The gradient of the number of cycles was set to 27, 28, 29, 30, and 31, of which 28 cycles were the recommended number of cycles. To investigate the effect of PCR reaction conditions, various reaction volumes (5, 10, 12.5, and 25 μl), annealing temperatures (57, 59, 60, 61, and 63 °C), final extension temperatures (56, 58, 60, 62, and 64 °C), and cycle numbers (27, 28, 29, 30, and 31) were tested.

Amplification analysis

The Microreader 40Y ID System is a six-dye multiplex amplification kit. The kit provides microreader size standard (QD 550) and allelic ladder. The 6-Dye Matrix standard can be used to spectral collected for correcting all the types of fluorescent dyes used in the kit. The separation of amplification products was performed using a 3500XL (Thermo Fisher Scientific, Waltham, MA, USA). And the instrument was used as the default for most studies. Sample setup for capillary electrophoresis (CE) was performed using a 36-cm capillary array and the performance optimized polymer-4 (POP-4 polymer), and the following run condition for a 3500XL:3.0kv for 10 s, input of 1 μl amplification product to 9 μl Hi-Di formamide. The electrophoresis results were analysed using GeneMapper ID-X v1.2 and a threshold of 150 RFU [9].

Sensitivity study

For sensitivity analysis, a serial dilution of male control DNA (M308) was established (1 ng, 500 pg, 250 pg, 125 pg, and 62.5 pg), and the average peak heights of the resulting Y-STR profiles were calculated for each dilution.

DNA mixtures

Male-male DNA mixtures were prepared using two male control DNAs, namely M308 (Beijing Microread Genetics) and 2800M (Life Technologies, Applied Biosystems); the two DNAs were selected to minimize the number of coincident loci types. And male-female DNA mixtures were prepared using M308 in the presence of a high female DNA (F312) background. For amplification, the total male input DNA was maintained at 1 ng, whereas the total female input DNA was maintained at 400 pg. Mixture ratios used for the male-male DNA mixtures were 19:1, 9:1, 3:1, 1:1, 1:3, 1:9, and 1:19. The total input DNA remained at 1 ng for the male-female DNA mixtures, whereas the different concentration gradients of male DNA corresponds to 1 ng, 500 pg, 250 pg, 125 pg, and 62.5 pg input range for the minor male component [8, 10].

Detection and genotyping (sizing accuracy and stutter calculation)

The average base size and standard variance for each locus were determined using the kit's allelic ladder, and 437 samples were amplified and analysed using a 3500 series Genetic Analyzer and a threshold of 150 RFU. Peaks that were one repeat smaller or larger than the true allele (± 0.5 bases) were considered stutter peaks, and the peak height threshold of stutters was 20 RFU [11].

Species specificity

Total DNA was extracted from *Escherichia coli*, *Mycobacterium tuberculosis*, and whole-blood samples from goat, rat, rabbit, chicken, dog, cat, duck, and pig using the new rapid extraction kit for whole blood genomic, and 1 ng of DNA was used for amplification.

PCR inhibition

Because the real world, from which samples are obtained, is relatively complex, a large portion of forensic samples are affected by chemical reagents or other factors. Therefore, to evaluate the effort of PCR inhibitors on the efficacy of the Microreader 40Y ID System, three inhibitors were tested, including humic acid, which binds to DNA, and both haemoglobin and EDTA, which inhibit the action of Taq polymerase. The three inhibitors that were used for testing were humic acid (50, 75, or 100 ng/μl), haemoglobin (250, 500, 750, or 1000 μM), and EDTA (0.25, 0.5, 0.75, or 1 mM).

Effects of ultrasound on DNA

Ultrasound can damage DNA and break it into small fragments, eventually destroying even long DNA fragments. DNA was degraded using an ultrasonic instrument as follows: 200 watts for 10 s per cycle, with 4 s between cycles and cycles repeated 100, 200, 300, or 400 times. The resulting DNA samples then subject to amplification and electrophoresis.

Reproducibility

In actual cases, many different types of body fluid spots are collected. Therefore, the ability of the kit to type the three most common types of body fluids (i.e. blood and DNA from buccal swabs, and semen) were tested. The kit can directly type blood samples without extraction. The detection of DNA samples from different types of body fluids can be used to assess the consistency of genotyping. In the present study, three male DNA samples were extracted from three sampling types: oral swab, whole blood, semen samples, and the fourth type blood sample without

Table 1 Y-chromosome-specific short tandem repeat loci targeted by the Microreader 40Y ID System. The kit targets 20 core loci, 15 preferred loci, and four common candidate loci, including seven rapidly mutating loci

Locus	Chromosome location*	Dye	Allelic	Rapidly mutating loci	Core loci	Optimizing loci	Alternative loci
Rs2032678	ChrY:15,508,700-15,508,711	FAM	1-2				1
DYS393	ChrY:3,263,107-3,263,185	FAM	9-16		1		
DYS570	ChrY:6,993,138-6,993,271	FAM	15-19	√		1	
DYS19	ChrY:9,684,267-9,684,503	FAM	9-20		2		
DYS392	ChrY:20,471,958-20,472,129	FAM	7-17		3		
DYS549	ChrY:19,358,225-19,358,438	FAM	12-15			2	
Y GATA H4	ChrY:18,743,725-18,743,751	FAM	9-13		4		
DYS444	ChrY:17,114234-17,114422	FAM	10-16			3	
DYS593	ChrY:16,473,816-16,474,000	FAM	15-17				2
DYS460	ChrY:18,888,772-18,889,046	HEX	6-14			4	
DYS458	ChrY:7,999,821-7,999,958	HEX	14-22		5		
DYS481	ChrY:8,558,301-8,558,409	HEX	19-27		6		
DYS635	ChrY:12,258,755-12,258,975	HEX	17-27		7		
DYS438	ChrY:12,825,876-12,825,984	HEX	9-14		8		
DYS447	ChrY:15,278,883-15,278,902	HEX	23-29			5	
DYS596	ChrY:8,519,457-8-519640	HEX	14-17			6	
DYS456	ChrY:4,402,915-4,403,029	TAMRA	13-18		9		
DYS389I	ChrY:12,500,448-12,500,495	TAMRA	10-15		10		
DYS390	ChrY:15,162,996-15,163,228	TAMRA	17-27		11		
DYS389II	ChrY:12,500,448-12,500,611	TAMRA	24-34		12		
DYS448	ChrY:22,218,904-22,219,084	TAMRA	17-24		13		
DYS533	ChrY:16,281,244-16,281,458	TAMRA	9-13		14		
DYS449	ChrY:8,217,908-8,217,930	TAMRA	24-35	√		7	
DY8391	ChrY:11,982,064-11,982,195	ROX	6-13		15		
DYS439	ChrY:12,403,456-12,403,587	ROX	9-15		16		
DYS437	ChrY:12,346,255-12,346,431	ROX	13-17		17		
DYS385a/b*	ChrY:18,639,701-18,639,898, ChrY:18,680,490-18,680,699	ROX	7-24		18, 19		
DYS643	ChrY:15,314,074-15,314,226	ROX	8-14			8	
DYS518	ChrY:15,207,972-15,208,220	ROX	33-42	√		9	
DYS576	ChrY:7,185,281-7,185,449	PURP	14-21	√	20		
DYF404S1*	ChrY:23,807,887-23,808,094, ChrY:25,861,995-25,862,194	PURP	12-17	√			3, 4
DYF387S1*	ChrY:23,785,347-23,785,516, ChrY:25,884,565-25,884,738	PURP	33-42	√		10, 11	
DYS627	ChrY:8,781,939-8,782,152	PURP	16-25	√		12	
DYS527a/b*	ChrY:23,739,580-23,739,783, ChrY:25,930,301-25,930,496	PURP	20-25			13, 14	
DYS557	ChrY:21,072,729-21,072,969	PURP	12-18			15	
DYS645	ChrY:21,165,932-21,166,195	PURP	8-9				5
Total					20	15	5

Primer with "*" marked loci have two binding sites, so there are two copies of this loci; "a" and "b", producing amplification products with corresponding two fragment size

*According to GRCh38/hg38 (https://www.ncbi.n1m.nih.gov/nucleotide/)

extraction. The male oral swab samples used in the experiment were collected using cotton swabs.

Forensic genetic analysis

To evaluate the value of the Microreader 40Y ID System for forensic genetics, the 437 samples were subject to population genetics studies and mutation analyses. Single-copy loci were calculated using an online tool [7] (STRAF, STR Analysis for Forensics, http://www.cmpg.iee.unibe.ch/services/shiny/index_eng.html), and the forensic parameters of the multi-copy loci (DYS385a/b, DYF404S1, DYF387S1, DYS527a/b) were calculated separately. The samples contained three populations: Tibet (168), Han (169), Yi (100). Haplotype diversity (HD), discrimination capacity (DC), haplotype match probability (HMP), and distinct haplotypes were calculated [12].

Results and discussion

Y-STR loci information

All the Y-STR data are presented in Table 1. The kit contained 20 core loci, 15 preferred loci, and four common candidate loci. Among these loci, DYS570, DYS449, DYS518, DYS576, DYS404S1, DYS387S1, and DYS627 are rapidly mutating Y-STRs loci.

PCR reaction component study

Many factors affect PCR amplification, and changes in the composition and conditions of PCR reactions are important factors.

Microreader 2.5× Master Mix III

When the concentration of the master mix increased or decreased to a certain degree, the loci called suffered a corresponding decreased. At 0.75X, 92.59% of the Y-STRs were typed successfully, and only eight loci were not accurately classified. At 0.5X, only 22.22% of the Y-STRs were typed successfully. However, all the Y-STRs were successfully typed at 1X and above (Fig. 1).

Microreader 5X Master primer

When the concentration of master primer was 0.5X, 84.26% of the Y-STRs were typed successfully, and all the Y-STRs were successfully typed at 1X and above (Fig. 2).

PCR reaction conditions

The recommended reaction conditions were 25 μl, 59 °C, 60 °C, and 28 cycles, respectively. Reaction volume had no effect on the success of Y-STR typing (figure not shown). In regard to annealing temperature, all the Y-STRs were successfully typed at 57, 59 (figure not shown), and 60 °C, whereas only 98.15 and 83.33% could be typed at 61 and 63 °C, respectively (Fig. 3). Neither final extension temperature nor cycle number affected the success of Y-STR typing either. However, unrelated peaks were observed when using 30 cycles, with peak heights that exceeded the detection threshold of 150 RFU, and when using 31 cycles, the unrelated peaks increased significantly, with even greater peak heights (figure not shown).

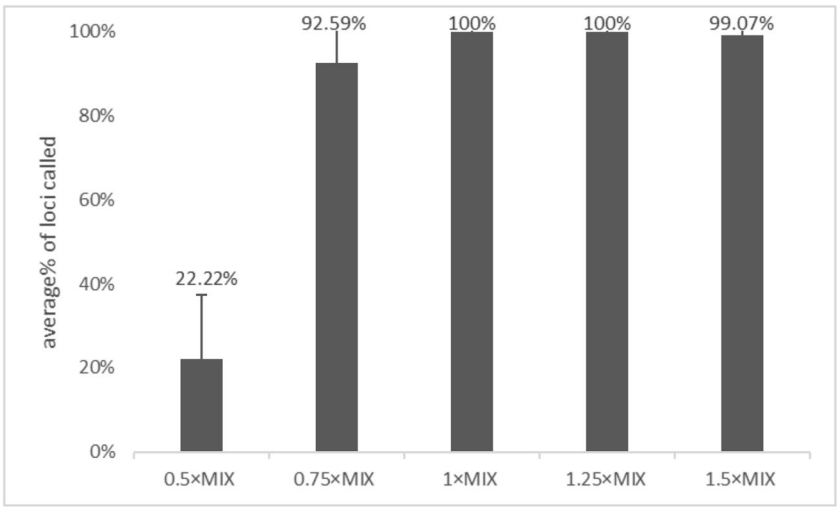

Fig. 1 Effect of Master Mix concentration on the mean percent of successfully typed Y-chromosome-specific short tandem repeat loci

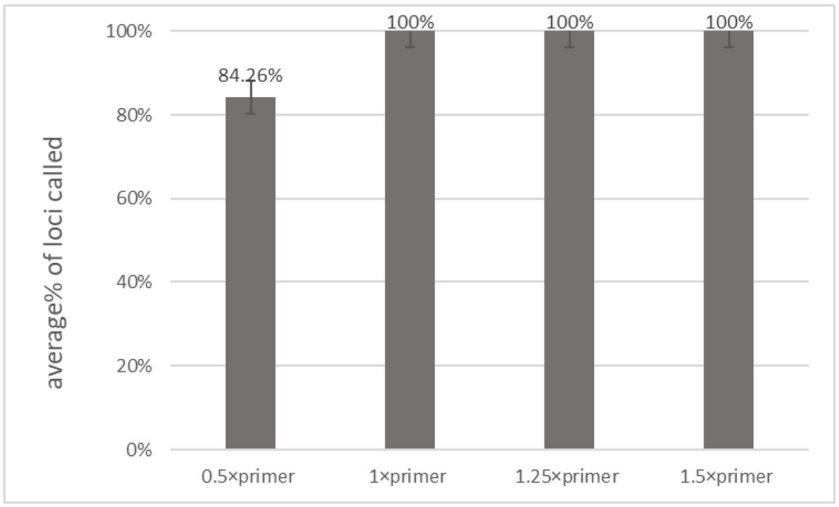

Fig. 2 Effect of Primer Mix concentration on the mean percent of successfully typed Y-chromosome-specific short tandem repeat loci

Sensitivity studies

The control male DNA from the kit (M308) was used to evaluate the kit's sensitivity, with 31.25, 62.5, 125, 250, 500, or 1 ng DNA in 25-μl reaction volumes, and three replicates were performed for each concentration. All the Y-STRs were successfully typed at DNA concentrations of 125 pg and above, and mean peak height increased with increasing DNA concentration, from 443.46 RFU (31.25 pg) to 4002.31 RFU (1 ng), with a peak height threshold of 80 RFU (Fig. 4). At 62.5 pg, the Y GATA H4 locus could not be typed, and at 31.25 pg, only 50% of loci could be typed.

DNA mixture study

Because the real world is relatively complex and mixed spots are often obtained from actual situations, verification of the Microreader 40Y ID System is crucial for ensuring the accurate identification of secondary components in mixed spots. For Y-STR kits, in particular, it is important to identify the performance of male minor contributors in a high female background. Therefore, the ability of the kit to type Y-STRs from both male-male and male-female mixtures was investigated.

Male-male mixture

When the M308:2800M ratio was 1:19, 96.83% of the minor contributor Y-STRs could be typed successfully, and only DYS627 (24), DYS385a/b (11/14), DYS391 (11) DYS449 (type 30), and Y GATA H4 (12) were not typed. When the M308:2800M ratio was 9:1, 99.47% of the Y-STRs could be typed successfully, and only DYS635 (21) was not typed. When the M308:2800M ratio was 19:1, 97.35% of the Y-STRs could be typed successfully, and only DYS635 (21),

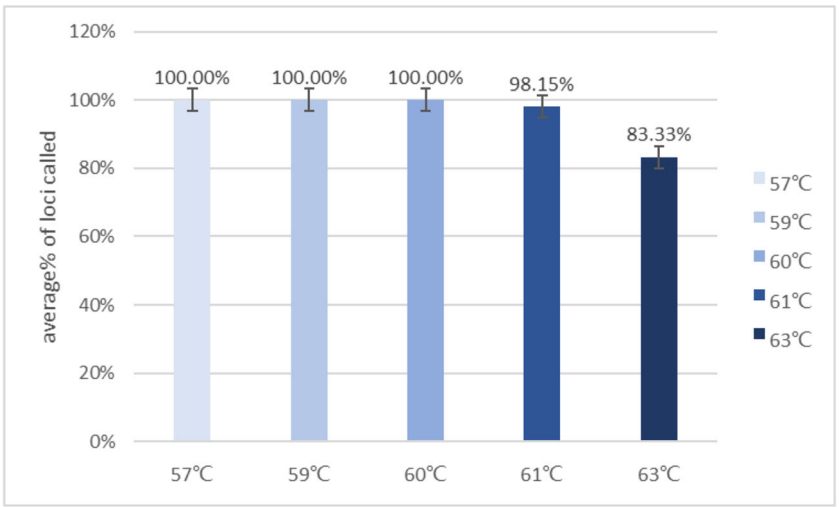

Fig. 3 Effect of PCR annealing temperature on the mean percent of successfully typed Y-chromosome-specific short tandem repeat loci. Values and error bars indicate mean ± standard (n = 15)

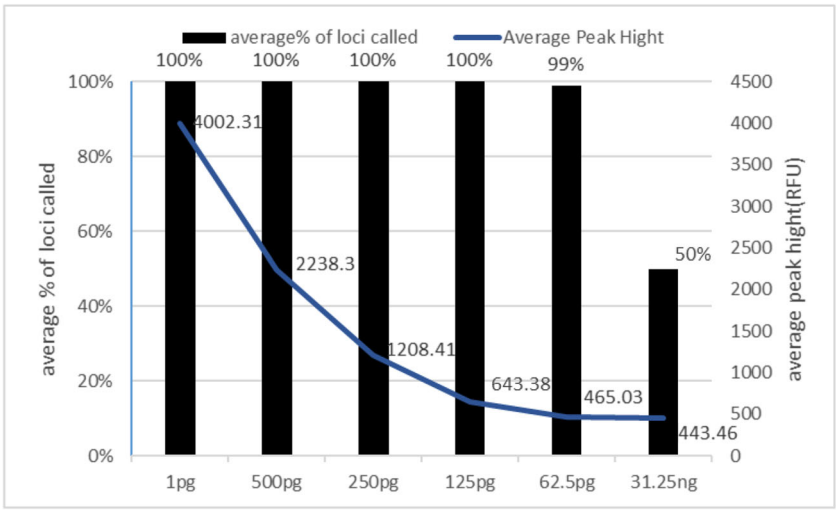

Fig. 4 Microreader 40Y ID System sensitivity. Allele recovery is expressed as a percentage. Blue line indicates the average peak height of each DNA concentration

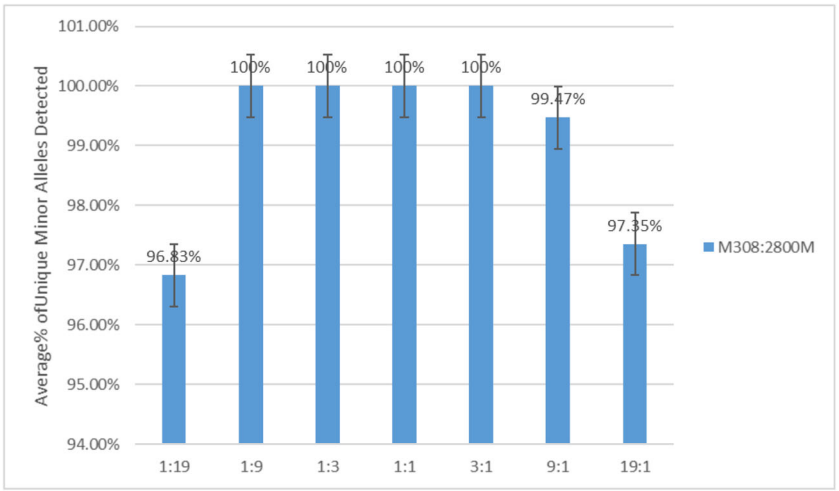

Fig. 5 Successful typing of minor contributors in male:male DNA mixtures. Values and error bars represent means ± SD (n = 3)

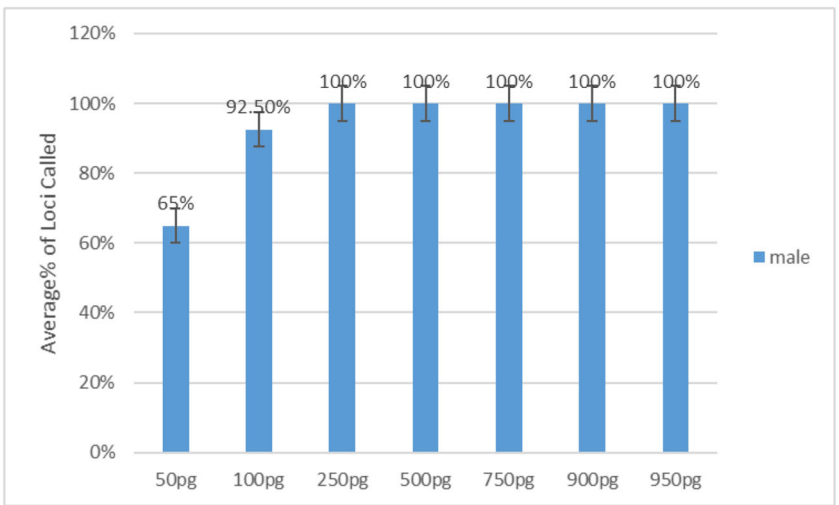

Fig. 6 Successful typing of minor contributors in male:female DNA mixtures. Values and error bars represent means ± SD (n = 3). X-axis represents the absolute amount of male DNA

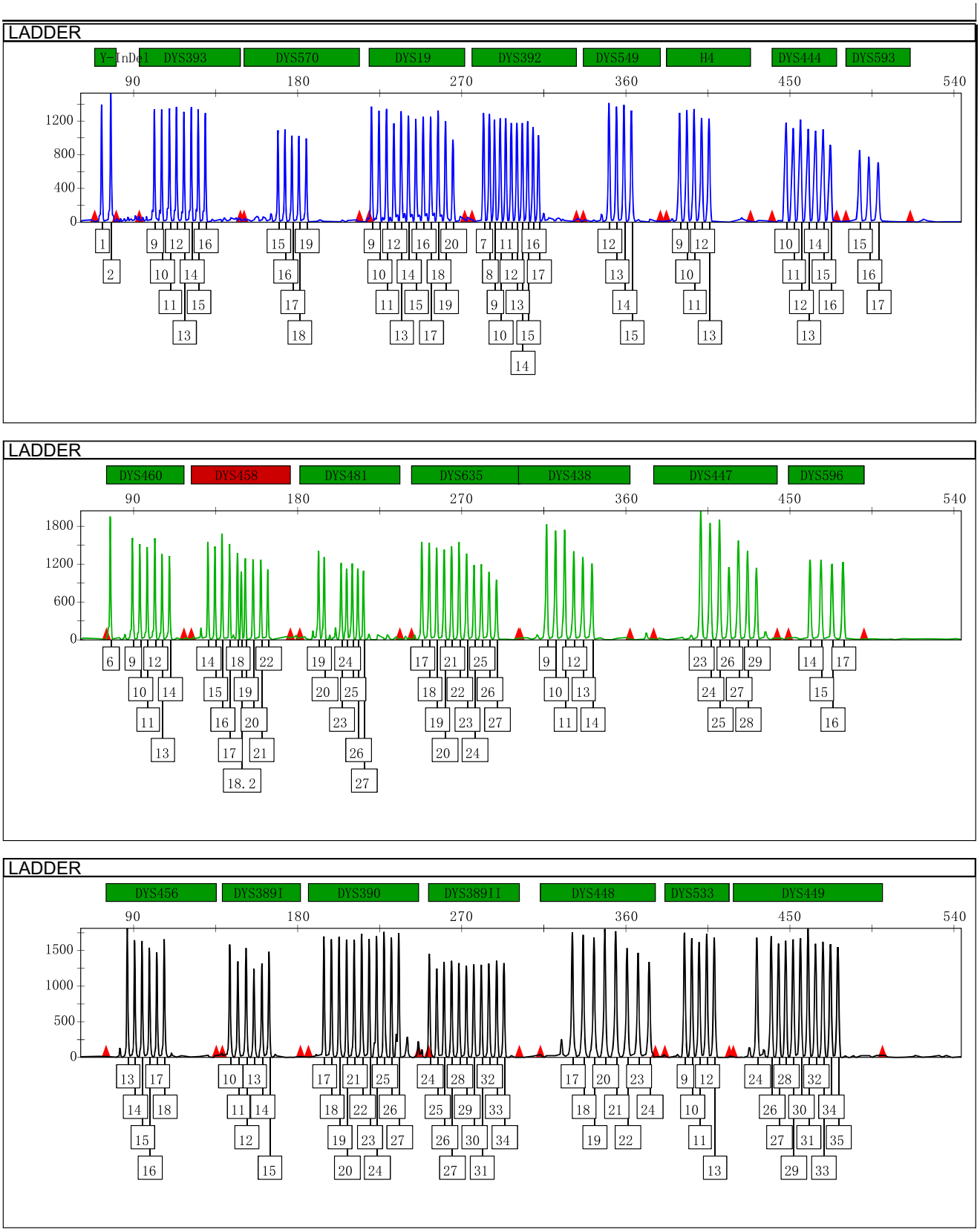

Fig. 7 Electropherogram of the Microreader 40Y ID System allelic ladder

DYS389I (14), DYS527 (type 19), and DYS438 (type 9) were not typed (Fig. 5).

Male-female mixture

The M308 and F312 DNAs were mixed at ratios of 1:19, 1:9, 1:3, 1:1, 3:1, 9:1, and 19:1 using 50, 100, 250, 500, 750, 900, and 950 pg of M308 DNA, respectively, in a total of 1 ng DNA. When the M308:F312 ratio was 1:9 (100 pg M308), 92.5% of the M308 Y-STRs could be typed successfully, and only DYS533 (12), DYS527a/b (21), and DYS19 (type 14) were not typed. However, when the M308:F312 ratio was reduced to 1:19 (50 pg M308), only 65% loci of the M308 Y-STRs could be typed successfully (Fig. 6).

Sizing accuracy, precision, and stutter effects

The allelic ladder profile is presented in Fig. 7. Twenty-one allelic ladder samples were run on the 3500XL (Life Technologies; Fig. 8). The standard deviation (SD) of the allelic ladder ranged was from 0.031 (DYS390) to 0.115 (DYS449), which indicated that the detection system was suitably precise (Table 2).

Species specificity

Capillary electrophoresis (3500xL Genetic Analyzer, Thermo Fisher Scientific, Waltham, MA, USA) results of eight species of non-primate (Fig. 9a). The animal DNA yielded few peaks larger than 200 RFU, but the peaks could not be typed. Meanwhile, the two bacteria yielded no peaks larger than 200 RFU (Fig. 9b).

PCR inhibition

The performance of the Microreader 40Y ID System was compared against three PCR inhibition models. The experimental reactions were performed using 1 ng of control DNA (M308). The humic acid (Fig. 10a), haemoglobin (Fig. 10b), and EDTA (Fig. 10c) yielded expected results. When the concentration of humic acid was increased to 200 ng/μl, all the Y-STRs could be typed successfully, which indicated that the inhibitory effect of humic acid on the kit's efficacy was

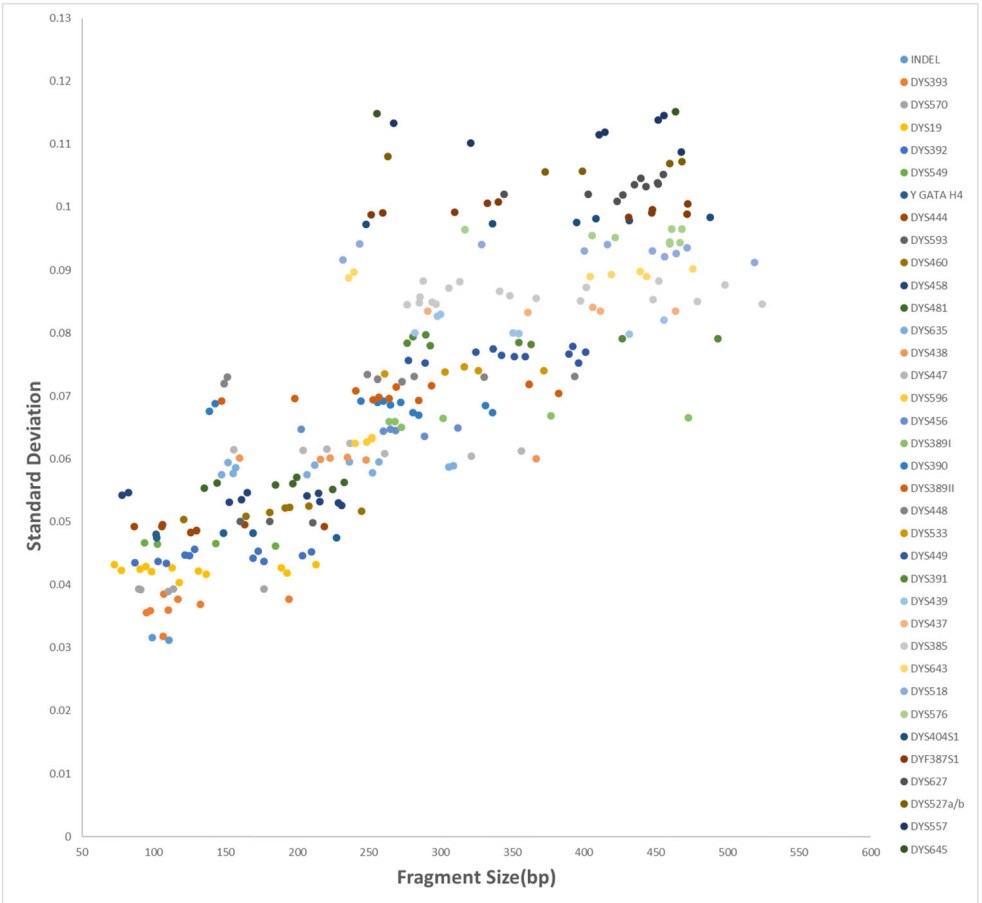

Fig. 8 Sizing variation of all allelic ladders. Ladders were analysed using a 3500xl Genetic Analyzer ($n = 21$)

insignificant. At 250 μM haemoglobin, all the Y-STRs could be typed successfully, and when the concentration was increased to 500 μM, 99.07% of the Y-STRs could be typed successfully. However, at 750 μM haemoglobin, only 32.41% of the Y-STRs could be typed successfully, and at 1000 μM haemoglobin, PCR amplification was completely inhibited. At 0.75 mM EDTA, all the Y-STRs could be typed successfully, and at 1 mM, 90.65% of the Y-STRs could be typed successfully. However, when the concentration was increased further, to 1.5 mM EDTA, PCR amplification was completely inhibited (Fig. 11).

Effects of ultrasound on DNA

To investigate the effect of sample degradation, three samples were irradiated using an ultrasonic apparatus. The samples were fresh Han male DNA. The DNA was exposed to either 100, 200, 300, or 400 cycles of ultrasound. After the DNA was exposed to 100 cycles of ultrasound, all the Y-STRs could be typed successfully. After 200 cycles, the DYS645 locus was not typed, and after 300cycles, even more loci were not typed or yielded type errors. After the DNA was exposed to 400 cycles of ultrasound, even more loci were not typed (Fig. 12). Detailed typing results are presented in Supplementary Table 1.

Table 2 Stutter values for the Microreader 40Y ID System markers. The average value plus SD value*3 obtained as the stutter threshold

Locus $n = 200$ Y-ilnDel*	Repeat unit	Minimum	Maximum	Average	SD	Stutter threshold	Count	Occurrence (%)
Y-InDel								
DYS393	4	0.05105	0.15767	0.09075	0.02801	0.17478	192	96.00
DY5570	4	0.01351	0.15781	0.10851	0.02033	0.16950	199	99.50
DYS19	4	0.03976	0.11936	0.07259	0.01553	0.11920	192	96.00
DYS392	3	0.02040	0.19880	0.10296	0.04209	0.22923	190	95.00
DYS549	4	0.02729	0.11249	0.07462	0.01513	0.12002	186	93.00
YGATA4	4	0.00779	0.05552	0.08585	0.01538	0 11199	189	94.50
DYS444	4	0.02618	0.12593	0.08084	0.02266	0.12883	178	89.00
DYS593	5	0.00563	0.07015	0.021465	0.01669	0.07152	39	19.50
DYS480	4	0.03367	0.13862	0.07802	0.02007	0.13822	197	98.50
DYS458	4	0.01430	0.14052	0.09903	0.01751	0.15156	197	98.50
DYS481	3	0.01625	0.26811	0.17683	0.03448	0.28028	200	100.00
DYS635	4	0.01177	0.11381	0.07818	0.01518	0.12372	198	99.00
DYS438	5	0.01508	0.05793	0.02709	0.01146	0.06147	178	89.00
DYS447	5	0.01154	0.08094	0.04542	0.01332	0.08539	181	90.50
DYS596	6	0.00853	0.05873	0.02174	0.01698	0.07268	40	20.00
DYS456	4	0.01093	022541	0.12444	0.03258	0.22219	197	98.50
DYS389I	4	0.01843	0.15044	0.07527	0.02617	0.15380	194	97.00
DYS390	4	0.04978	0.14149	0.09298	0.01808	0.14723	195	97.50
DYS389II	4	0.09670	0.20363	0.14647	0.01985	0.20602	198	99.00
DYS448	6	0.00742	0.05297	0.02968	0.00889	0.05635	166	83.00
DYS533	4	0.02611	0.08916	0.05504	0.01251	0.09258	181	90.50
DYS449	4	0.09754	0 21329	0.15515	0.02160	0.21995	198	99.00
DYS391	4	0.00916	0.16336	0.08190	0.02929	0.16979	197	98.50
DYS439	4	0.04510	0.12487	0.07620	0.01618	0.12473	196	98.00
DYS437	4	0.01031	0.08243	0.04425	0.01447	0.08767	188	94.00
DYS3285a	4	0.00566	0.12088	0.07203	0.01613	0.12043	189	94.50
DYS385b	4	0.06913	0.16811	0.12032	0.02040	0.18152	110	55.00
DYS843	5	0.00944	0.06252	0.02615	0.01289	0.06481	155	77.50
DYS518	4	0.01293	0.22061	0.16272	0.02265	0.23066	198	99.00
DYS578	4	0.07053	0.15728	0.10670	0.01960	0.16552	197	98.50
DYF404S1	4	0.01450	0.11468	0.06521	0.01660	0.11500	185	92.50
DYF404S1	4	0.01257	0.13938	0.07515	0.02802	0.15921	50	25.00
DYF387S1	4	0.05712	0.15985	0.09850	0.02217	0.16502	194	97.00
DYF387S1	4	0.00742	0.16680	0.11398	0.02532	0.18993	95	47.50
DYS627	4	0.01813	0.11860	0.07351	0.01798	0.12744	193	96.50
DYS527a	4	0.02978	0.10374	0.06406	0.01583	0 11155	177	88.50
DYS527b	4	0.01924	0.10974	0.08035	0.01517	0.12587	70	35.00
DYS557	4	0.02931	0.13508	0.08643	0.01621	0.13505	197	98.50
DYS845	5	0.00660	0.02300	0 01174	0.00858	0.03748	27	13.50

*Y-Indel is an insert-deletion polymorphism loci

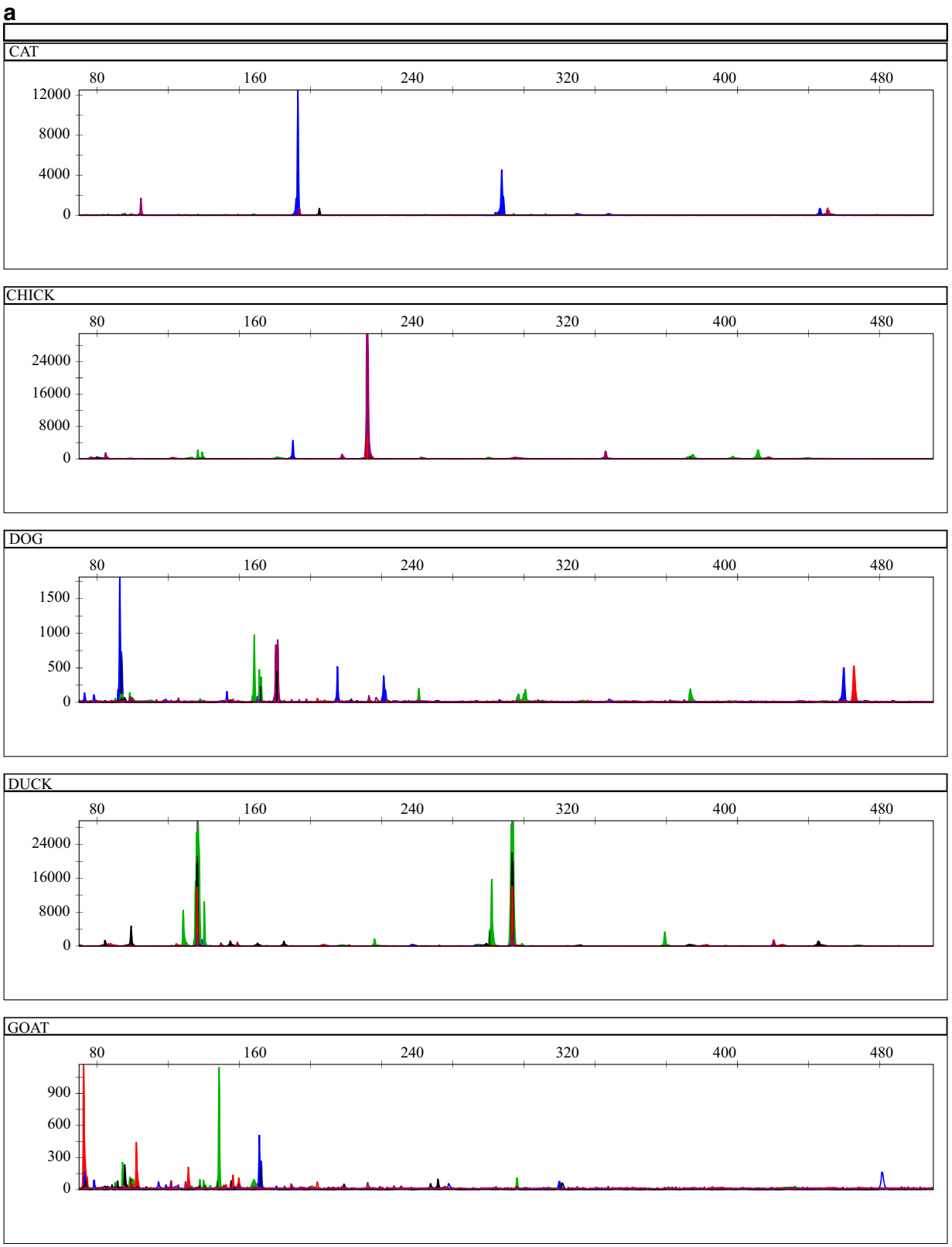

Fig. 9 **a** Species specificity of the Microreader 40Y ID System. Few peaks were higher than 150 RFU. **b** Species specificity of the Microreader 40Y ID System. Using the standard protocol, 1 ng of bacterial DNA was amplified

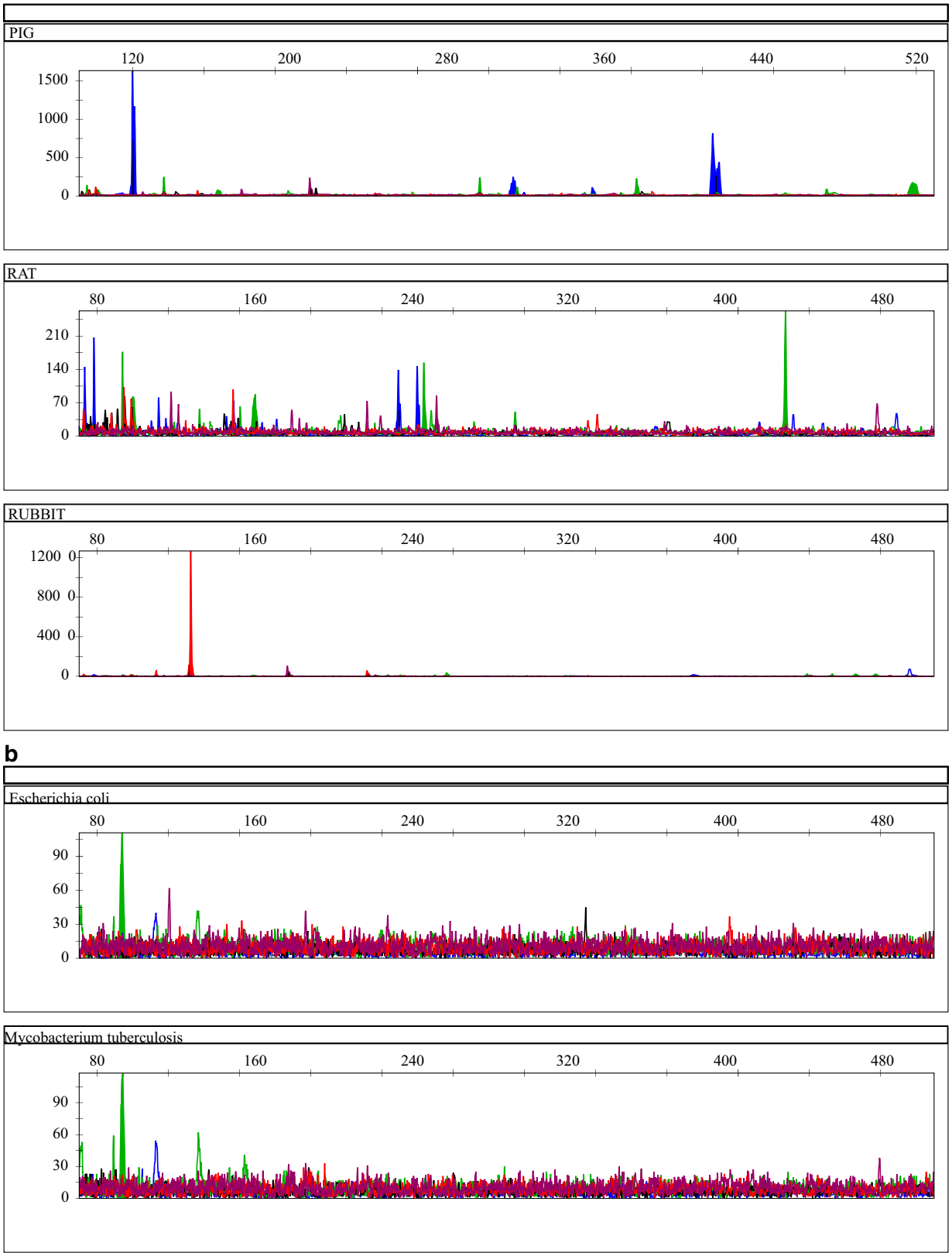

Fig. 9 (continued)

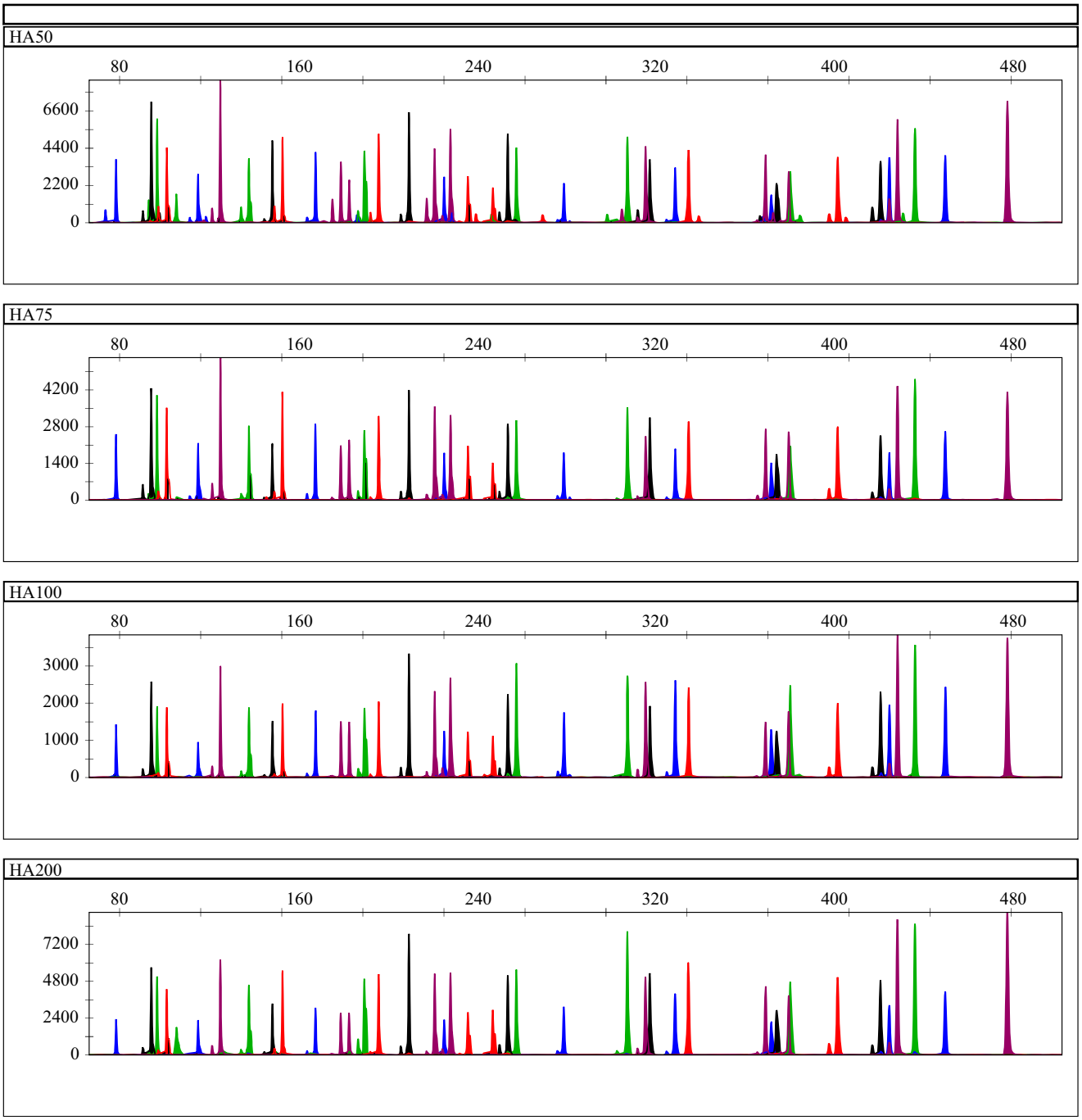

Fig. 10 **a** Effect of humic acid (HA) on the successful typing of male DNA. **b** Effect of haemoglobin (HE) on the successful typing of male DNA. **c** Effect of EDTA on the successful typing of male DNA

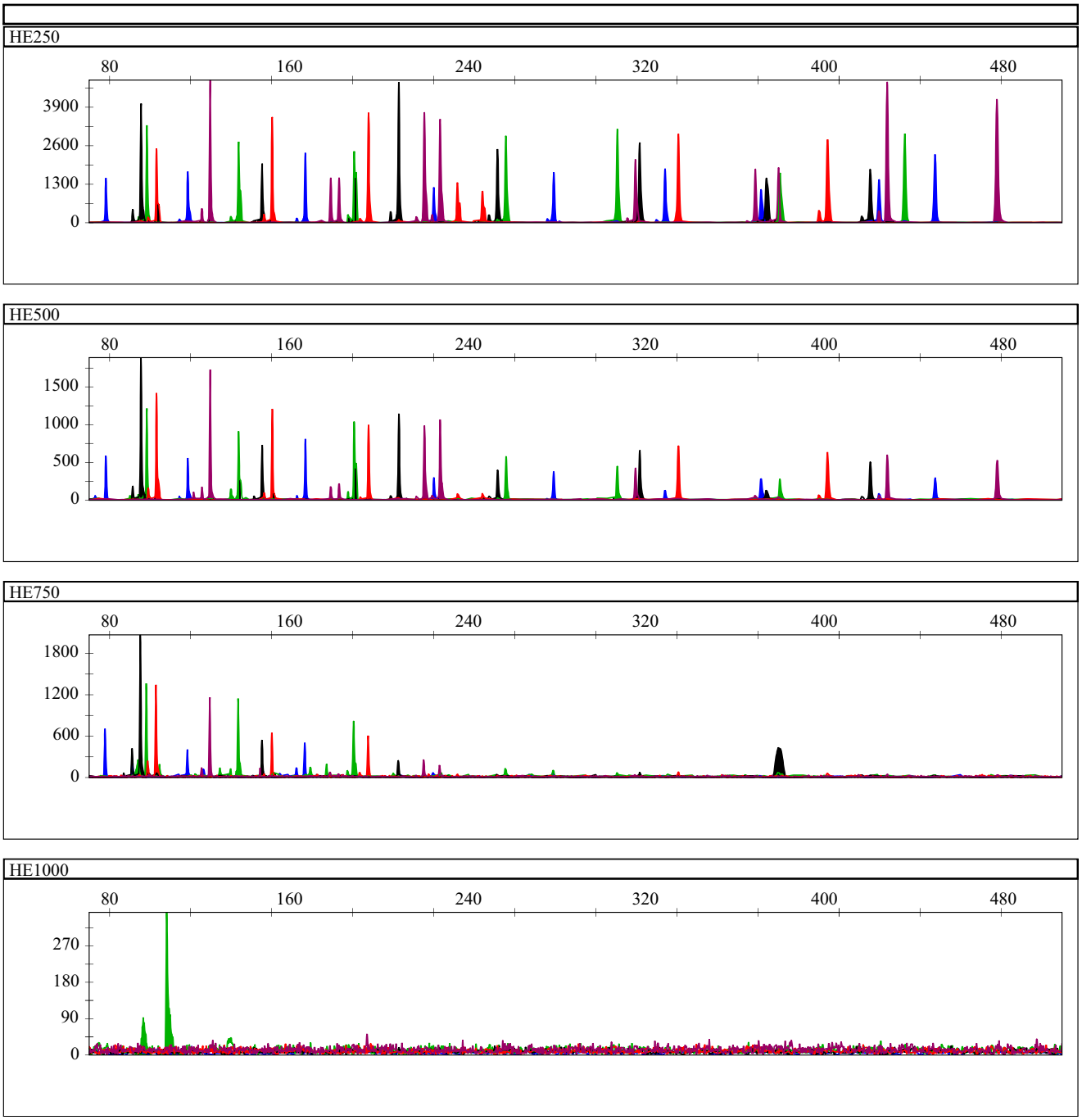

Fig. 10 (continued)

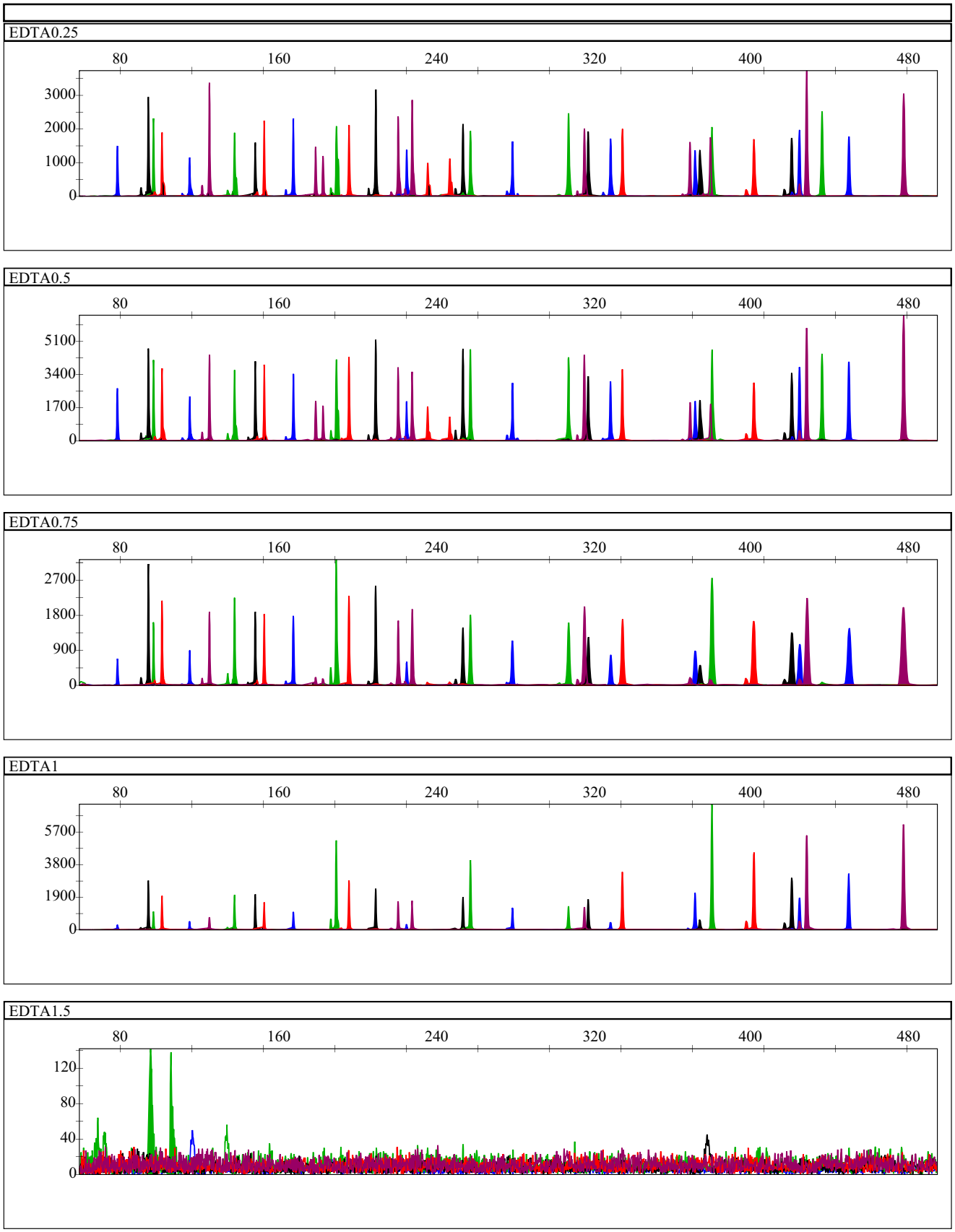

Fig. 10 (continued)

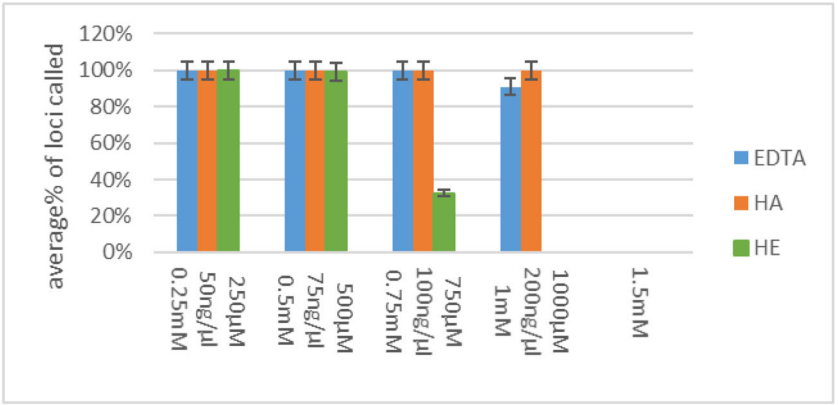

Fig. 11 Effect of three PCR inhibitors. EDTA (blue, five concentrations), humic acid (orange, four concentrations), and haemoglobin (green, four concentrations)

Case samples

As shown, profiles were obtained for all the samples, which indicated that the kit is suitable both for direct typing and for typing DNA extracted from different types of samples (figure not shown).

Forensic genetic analysis

In addition to the Yfiler plus loci, additional loci (DYS549, DYS643, Y-Indel, DYS447, DYS444, DYS557, DYF404S1, DYS527a/b, DYS596, DYS593, DYS645) were also highly polymorphic. The sample types are presented in Supplementary Table 2.

Genetic diversity (GD), or haplotype diversity (HD), was calculated using as follows: $GD = HD = \frac{N\left(1-\sum_{i=1}^{k} p_i^2\right)}{N-1}$, where p_i represents the frequency of the ith haplotype, N represents sample size, and k represents haplotype number. Analysis was performed on 437 samples, which included samples from 169 unrelated Han males, 168 unrelated Tibetan males, and 100 unrelated Yi males. DYS385a/b, DYF404S1, DYF387S1, and DYS527a/b were each considered two separate single-copy markers (Table 3). Other relevant forensic parameters were calculated using an online tool (STRAF, STR Analysis for Forensics, http://www.cmpg.iee.unibe.ch/services/shiny/index_eng.html). Genetic diversity was > 0.5 for all the Microreader 40Y ID System markers, except DYS645, Y-Indel, DYS438, DYS437, DYS391, and DYS593 (Fig. 13). Other parameters included polymorphism information content (PIC), match probability (MP), and power of discrimination (PD; Supplementary Table 3).

Conclusion

The Microreader 40Y ID System includes 40 polymorphic Y-STR loci that can be used for the sensitive and rapid analysis of samples from a variety of real-world situations. The kit contains all the loci included in the Yfiler Plus PCR Amplification Kit and PowerPlex Y23 System, as well as others. Therefore, the Microreader kit is more accurate and efficient. The present study verified the sensitivity, reaction volume, PCR conditions, and inhibitor effects of the kit, as well as the kits ability to handle mixes or degraded samples and usefulness in local populations. Many factors influencing kit performance in real cases, and the standard system is often not suitable for the analysis of imperfect samples. The Microreader 40Y ID System can generate complete DNA profiles in reaction volumes as low as 6.25 µl, and male minor contributors can be accurately detected when the ratio of either male to female components or of male to male components is as low as 1:19. The most noteworthy characteristic of the kit is its anti-interference ability. In particular, humic acid failed to inhibit PCR amplification, even at 200 ng/µl humic acid. Haemoglobin partially inhibited PCR amplification at concentrations up to 750 µM and completely inhibited amplification at 1000 µM. The inhibitory effect of EDTA was obvious and increased with its concentration. Furthermore, ultrasound significantly damaged the DNA samples, and the number of missing loci increased with the number of ultrasound cycles.

This kit can also be applied to different types of body fluids and, in some cases, can be used for direct amplification, which ultimately saves time (no DNA extraction) and facilitates the progress of actual cases. This kit with more loci also possesses greater recognition capabilities for Y-STR applications compared to other common commercial kit and is compatible with

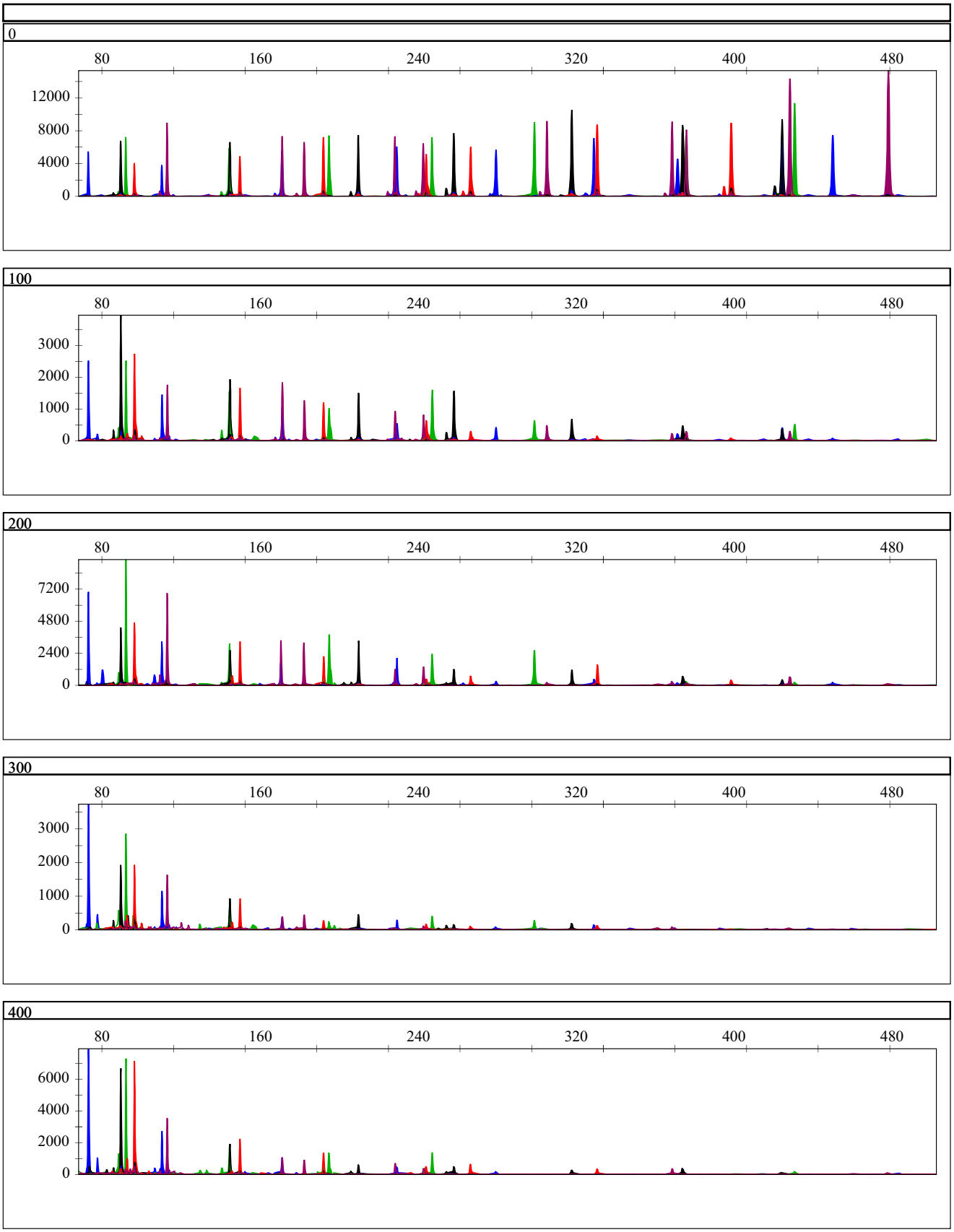

Fig. 12 Effects of ultrasound on DNA. DNA was degraded using an ultrasonic instrument as follows: 200 watts for 10 s per cycle, with 4 s between cycles and cycles repeated 0 (top), 100, 200, 300, or 400 (bottom) times

Table 3 Forensic parameters and diversity values for Y-chromosome-specific short tandem repeat data from three populations

Sample	Times haplotype observed			Distinct haplotypes	HD	MAP	DC
	1	2	3				
Han (n = 169)	150	6	1	159	0.99904	0.006687	0.94083
Tibet (n = 168)	127	16	3	146	0.99223	0.00772	0.86905
Vi (n = 100)	89	4	1	94	0.98849	0.01140	0.94000
Overall (n = 437)	366	28	5	399	0.99955	0.00274	0.91304

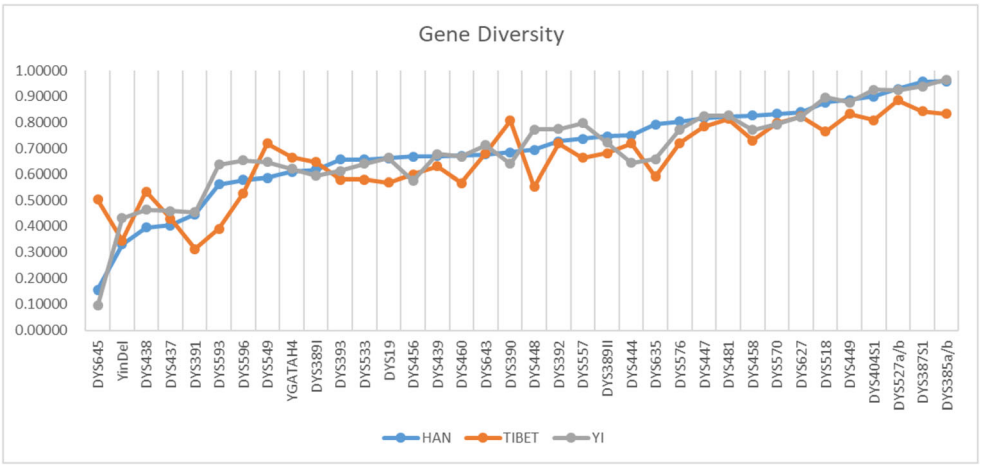

Fig. 13 Variability of the Microreader 40Y ID System markers within three populations. Loci are ranked by genetic diversity

other Y-STR ID systems, thereby ensuring maximum data capacity and consistency, as well as database comparison. In summary, the Microreader 40Y ID System is a powerful, sensitive, and balanced typing tool for forensics.

Funding Information This study was funded by the National Nature Science Foundation of China (No. 30801317).

Compliance with ethical standards

All the participants in this study gave their written informed consent and the approval of the study was granted by the Ethical Committee of the Sichuan University.

Conflict of Interest The authors declare that they have no conflict of interest.

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