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Detection System of Compound Amplification of Autosome and Y Chromosome Loci: Establishment and Practical Application

Fang Si[1], Longfei Ma[1] and Xiangyang Liu[2,3,4]*

[1]*Department of Criminal Science and Technology, Henan Police College, Zhengzhou, 450046, China*
[2]*Key Laboratory of Growth Regulation and Translational Research of Zhejiang Province, School of Life Sciences, Westlake University, Hangzhou 310024, China*
[3]*Westlake Laboratory of Life Sciences and Biomedicine, Hangzhou, Zhejiang310024, China*
[4]*Institute of Basic Medical Sciences, Westlake Institute for Advanced Study, Hangzhou, Zhejiang310024, China*

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FS and XL conducted the experiments in this study. LM and XL contributed to the design and interpretation of the current study and wrote the article. All authors read, revised, and approved the final manuscript.

Key words
Autosomes, Y Chromosome, Gene Loci, Detection system, Compound amplification, Forensic medicine

ABSTRACT

The objective of this study was to establish a new compound amplification detection system by using the loci of autosomes and Y chromosomes to achieve the classification of DNA, so as to identify forensic samples. A total of two hundred fifty blood samples were collected from Chelex-100 method and direct amplification method, respectively. Twenty five animal blood samples were used to prepare mixed DNA samples for males and females, and 29 criminal case samples were collected. By being used six color fluorescence labeling technology, gene loci on 21 autosomes and 34 Y chromosomes were detected and directly amplified to establish a composite amplification detection system. The stability, sensitivity, direct expansion feasibility, species specificity, and anti-inhibitor properties of the system should be examined. After being tested Chelex samples and direct expansion samples, the detection system showed good accuracy and stability, and could produce clear and complete the classification of DNA. When the template DNA concentration is ≥ 0.125ng, the detection system can accurately identify all gene loci. When the ratio of male DNA concentration is ≥ 1:4, all loci can be accurately classified. And then by being added to a certain concentration of PCR inhibitors (heme ≤ 150 μmol/L, hemoglobin ≤ 500 μmol/L, humic acid ≤ 10 ng/μL), all loci can be clearly classified. The detection system of compound amplification of autosomal and Y-chromosome loci is suitable for DNA identification in forensic samples. It has good stability in template DNA concentration and inhibitors within a certain range, and can accurately and stably identify multiple loci on the autosomal and Y chromosomes, providing a practical application form for DNA quantitative identification.

INTRODUCTION

Complete mitochondrial genome of individuals, Y chromosome single nucleotide polymorphism and human genome include 597573 SNPs. So, forensic researchers are unable to fully match all loci of the sample in their work. Y chromosome insertion- deletion polymorphism (Y-InDels), like Y chromosome single nucleotide polymorphism (Y-SNPs), has genetic stability and therefore carries mutations that can accumulate over generations (Zhou *et al*., 2023). Y chromosome STR (Y-STR) is commonly used in the field of forensic medicine. It exhibits low haplotype diversity in inbred populations and cannot distinguish male relatives from the same lineage, resulting in relatively low differentiation rates (Nazir *et al*., 2022). Y-STR and Y-SNP are genetic markers on the male Y chromosome, which can be used for individual identification, forensic application, and paternal genetic history analysis (Zhang *et al*., 2022). In the newly generated Y-STR haplotype data, there is a strong correlation between the prevalent haplotype groups in the Mongolian population and some observed micro variations (Wang *et al*., 2021). The six different nuclear genetic markers and mtDNA supervariant regions have

* Corresponding author: liuxiangyang2018@126.com
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shown sufficient efficiency and are expected to become testing tools for forensic DNA typing applications (Lan *et al*., 2022). There is a high demand for bloodline search to narrow down the scope of criminal investigations (Liu *et al*., 2021). Therefore, DNA typing should choose more targeted chromosomal loci accordingly to reduce the workload of locus matching in the DNA typing process and simplify work procedures, so as to save time and economic costs.

With the development of third-generation high-throughput sequencing technology (Köksal *et al*., 2022), it can be used for the process of NRY haplogroup classification with high accuracy (García-Olivares *et al*., 2023). A high-resolution Y-SNP panel contains the main advantages of Y-genealogy among Chinese populations of different ethnicities and geographical regions, which can serve as a powerful tool for forensic practice (He *et al*., 2023). A study has accurately identified relatives of remains by typing the STR of autosomes and Y chromosomes (Zupanič Pajnič, 2021). The purpose of this study is to establish a forensic DNA direct amplification PCR method for determining the accuracy of DNA typing. This method can simultaneously detect gene loci on both autosomes and Y chromosomes, and has high sensitivity and specificity. This study designed a new series of primers that can simultaneously amplify gene loci on both autosomes and Y chromosomes. We applied the primer to 500 forensic samples and 29 DNA samples, and analyzed the results. This study assumes that the composite amplification detection system can effectively determine the DNA typing of samples, and has high sensitivity and specificity.

MATERIALS AND METHODS

Samples

Five hundred unrelated blood samples from the DNA database of our court's scientific laboratory were selected. It was divided into 250 samples, respectively. One was extracted by the Chelex 100 method (Chelex samples), while the other was extracted by the direct amplification method (direct amplification samples). Additionally, 25 animal blood samples (pigs, chickens, sheep, fish) were taken as species-specific testing samples (specific samples). The female DNA (9947A) and male DNA (9948) were regarded as the testing standards. Male and female DNA were mixed in concentrations of 1:1, 1:2, 1:4, 1:9, and 1:19 (mixed samples). Fifteen samples of blood stains, six samples of rib cartilage, four samples of seminal plaques, and 29 samples of cigarette butts were taken from the criminal case scene as template DNA samples, all of which contained inhibitors such as heme and humic acid (template samples). The samples are all tested on the day of submission or within 7 days of being placed at room temperature.

Chelex 100 extraction method of sample

To 1-5 ml of the sample in 1.5ml of centrifuge tube 1ml of pure water was added, shaken and mixed at room temperature for 15 min and centrifuged at 10000 rpm for 2 min. The supernatant was discarded whereas pellet was washed with 1-2 times with pure water. Ultimately pellet was suspended in 200pl 5010 Chelex 100 solution in a centrifuge tube and placed in water bath at 56℃ for 30 min in order to shake the solution for 5-10 sec. Later temperature may be increased to 100℃ for 8 min and the solution be shaken at high speed for 5-10 sec. Later it is centrifuged at 12000 rpm for 3 min at room temperature. Lastly, the supernatant is the DNA sample which is used for PCR reaction.

Samples of direct amplification

The Power Plex 16HS system was used to implement the direct amplification, and all samples were taken to be placed in 0.2 ml amplified tubes. The volume is similar with the sesame seed.

Compound amplification system

For compound PCR amplification system 20μL of total reaction volume included 8.0μL of PCR reaction solution (usually containing necessary buffer and dNTPs), 0.6μL of specific primer solution for amplification of target DNA fragments, 0.6μL of hot start Taq DNA polymerase for DNA synthesis at higher temperatures, and 10.8μL of template DNA solution (containing H_2O). The amplification program followed specific thermal cycling: the initial denaturation at 95°C for 11 min, followed by 33 cycles, each of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min, and extension at 72°C. Finally, a 60 min thermal cycle at 60°C completes amplification.

The detection and analysis of PCR products was performed at ABI 3500 genetic analyzer and GeneMapper ID v3.2 software.

Performance index of compound amplification system

Chelex samples were taken for PCR amplification and DNAF typing detection. Meanhile, 250 direct amplification samples were taken for direct PCR and DNA typing test. The consistency and success rate of its determination results were compared with the Sinofir kit, Identifiler plus kit, Y-filer kit, and AGCU Y-24 kit, respectively.

Female DNA (9947A) and male DNA (9948) are mixed in ratios of 1:1, 1:2, 1:4, 1:9, and 1:19 to ensure consistency of total DNA for each ratio. Each mixing ratio

is prepared for PCR reaction mixtures, including PCR buffer, dNTPs, specific primers, Taq DNA polymerase, and DNA template. Corresponding amounts of male and female DNA standards are added to the PCR reaction mixture according to the predetermined ratio. Such PCR thermal cycling conditions as initial denaturation, annealing, elongation, and final elongation steps can be set. Two parallel experiments on each mixing ratio should be done to increase the reliability of the data. Electrophoresis was carried out at an appropriate voltage of 50V (gel length of 10 cm) and 30 min~60 min to separate DNA fragments in the presence of EB or SYBR Green. The DNA samples under different mixing ratios were analyzed. By being compared with the results of different mixing ratios, as well as the consistency of the two parallel experiments, it was possible to assess the effect of mixing male and female DNA standards on PCR amplification and DNA typing.

Male DNA 9948 is prepared, and then the standard is diluted at specified concentrations of 1.0ng, 0.5ng, 0.25ng, 0.125ng, 0.0625ng, and 0.031ng to prepare DNA template at different concentrations. Specific samples were selected for PCR amplification and DNA classification.

Twenty nine samples were selected for PCR amplification and DNA classification.

Heme with concentrations of 100, 150, and 200μmol/L, hemoglobin with concentrations of 500, 750, and 1000 μmol/L, and humic acid with concentrations of 5, 10, and 20ng/μL were regarded as inhibitors to be added to 29 template samples. Then, PCR amplification and DNA typing test were conducted to determine their tolerance.

RESULTS

The results of PCR amplification of 250 Chelex samples indicate that the compound amplification system in this study has stable results, clear and complete spectra. It can be seen that the results of 250 direct amplification samples are similar to those of Chelex samples. The DNA typing results of the two samples are similar to those of the corresponding loci in Sinofile, Identifile plus, Y-filer, and AGCU Y-24 assay kits, with good accuracy and consistency (Fig. 1).

According to mixed sample test in 1.4.2, it was found that accurate identification of all gene loci can be achieved when the concentration is ≥ 0.125ng. When the concentration of template dropped to 0.0625ng, it had effects on three STR loci of autosome and one Y gene loci. However, when the concentration decreased to 0.0312 ng, 6 STR loci and 4 Y loci were not displayed (Table I).

As shown in Figure 2, when the concentration ratio of DNA 9948 and 9947A samples is more than 1:4, male DNA can be accurately classified at both autosome and Y-chromosome loci. However, when the concentration ratio is less than 1:4, only male DNA loci can be fully detected. When the concentration drops to 1:19, DNA loci cannot be detected; No corresponding DNA classification was examined in the specific samples after PCR amplification.

Table I. Test results of compound amplification of mixed samples with different concentrations.

Concentration	Results of amplification
≥0.125ng	All loci can be clearly classified
0.0625ng	Three STR loci, D3S1358, D16S539 and vWA and one Y locus, DYS444) were not accurately identified
0.0312ng	Six STR loci of autosome, D3S1358, CSD-1PO, 04S1173, D12S391, D8S1179, TPOX and five Y loci, DYS385, DYS392, DYS444, DYS695, DYS417 were not displayed

Twenty-nine samples were extracted and then PCR amplification was done. The results are stable, the integrity of the atlas is good, clear and accurate. Compared with such four different test kits as Sinofiler and Identifiler-plus kits, the results of the loci of DNA classification were identical, indicating good accuracy and consistency of the results.

The results in Table II shows that when heme with a concentration of ≤150μmo/L is added to the DNA template, all loci can be clearly classified; When adding hemoglobin with a concentration ≤500μmo/L to the DNA template, all loci can be clearly classified; When humic acid with a concentration of ≤10ng/μL is added to the DNA template, all loci can be clearly classified; The results of other tests are not examined or partial loci were incomplete.

Table II. DNA classification of inhibitors with different concentrations.

Inhibitors	Concentrations	Results
Heme (μmol/L)	100	All loci can be clearly classified
	150	All loci can be clearly classified
	200	No genotyping was detected in all loci
Hemoglobin (μmol/L)	500	All loci can be clearly classified
	750	No genotyping was detected in 2 STR loci of D3S1358 and D16S539
	1000	No genotyping was detected in all loci
Humic Acid (ng/μL)	5	All loci can be clearly classified
	10	All loci can be clearly classified
	20	No genotyping were not detected in 2 STR loci of D6S1043 and D13S317 and 1 Y chromosome loci

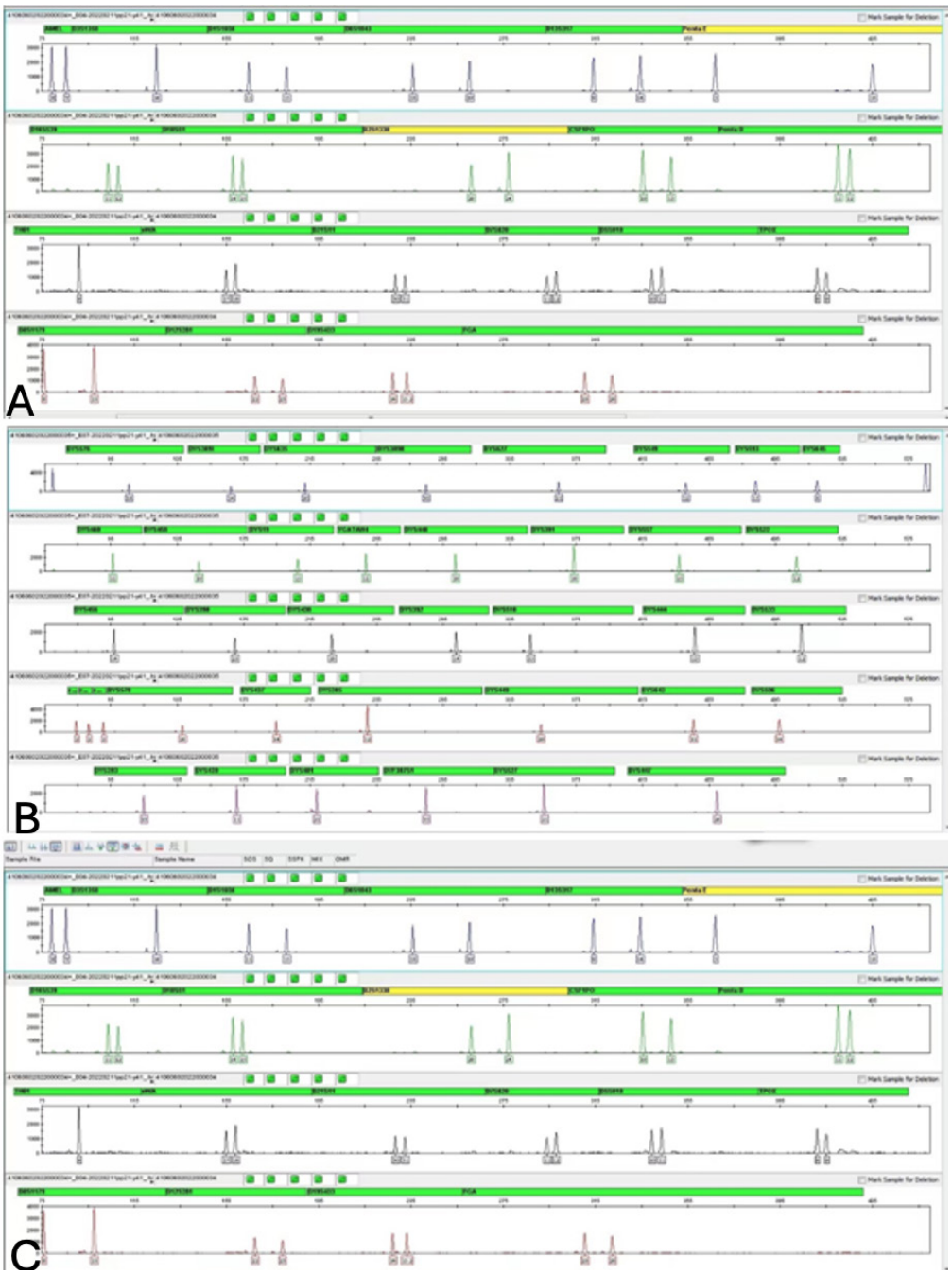

Fig. 1. Atlas of the test results of compound amplification system in Chelex and direct amplification samples. A, atlas of autosome locus; B, atlas of Y chromosome locus; C, atlas of Y chromosome locus.

DISCUSSION

In the context of forensic science, if trace amounts of biological staining samples are found, DNA typing can be chosen to provide clues for tracing the truth (Di Candia *et al*., 2023). Usually, both autosomes and Y chromosomes need to be measured separately, which limits their detection range and recognition ability. DNA identification techniques rely on restriction fragment length polymorphism analysis and PCR techniques, but these techniques depend on a large number of DNA samples (Jordan and Mills, 2021). In recent years, the development

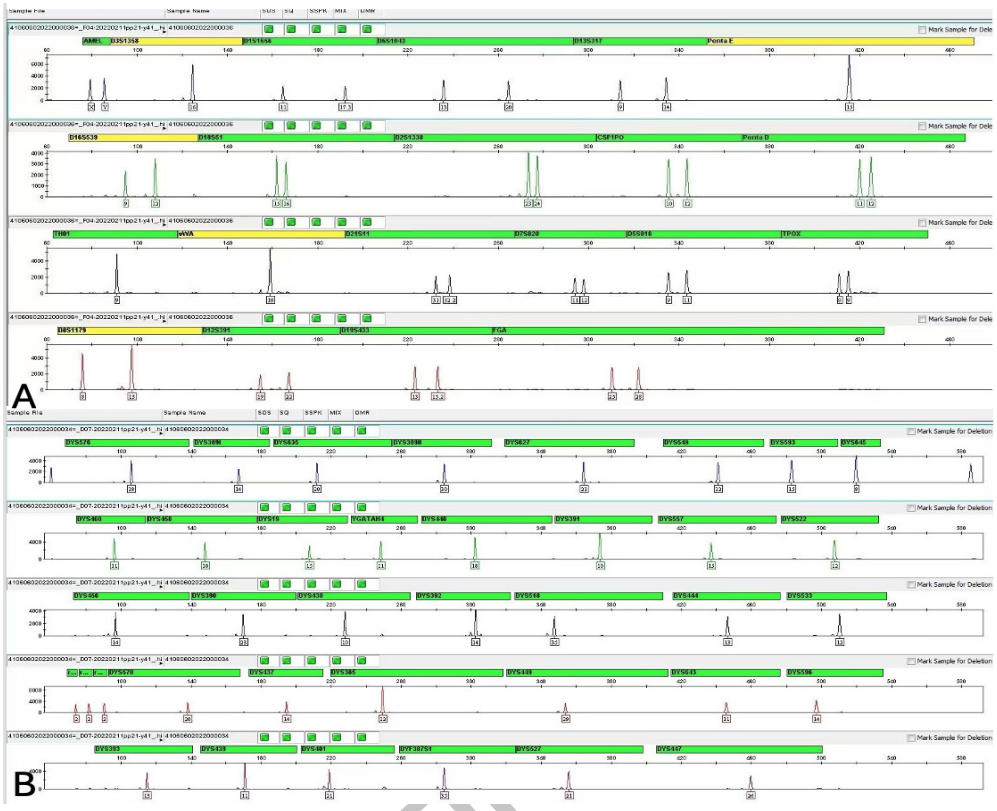

Fig. 2. Atlas of test results of mixed samples with more than the concentration ratio of 1:4 between DNA 9948 and 9947A samples. A, atlas of autosome locus; B, atlas of Y chromosome locus.

of nanoporous sequencing technology has been rapid, with its excellent ability to measure long continuous DNA fragments and obtain phase information, making it possible to detect longer haploid markers (Liu *et al*., 2023). A compound amplification detection system was designed for simultaneous determination of autosome and Y-chromosome loci based on existing equipment and technology, which can better meet the practical needs of forensic science for DNA recognition.

The establishment of the compound amplification system in this study revealed potential advantages in improving DNA identification sensitivity and recognition ability. Through extensive experiments, the system has demonstrated good stability and repeatability, laying the foundation for its application in forensic work. The detection of mixed samples also showed their value in case restructuring. In the past decades, Y chromosome and mitochondrial DNA profiles have been used as evidence in court, but the problem has not been fully resolved in terms of evaluating evidence weight. Y-STRs have a unique role in forensic research (Chai *et al*., 2023). Both are genetic markers that have more sufficiently evidential power than that of the DNA atlas of standard autosome (Andersen and Balding, 2021). DNA extraction and typing methods have become important research directions in the United States and worldwide (Butler, 2023). The Amelogenin sex test in forensic DNA typing kits has the potential to identify congenital diseases, such as sexual developmental differences or diseases (Gabriele *et al*., 2023). Forensic DNA phenotyping, a type of evidence based on the appearance of chromosomal DNA, has become important evidence in routine crime scene investigation (Diepenbroek *et al*., 2023). In this study, the direct amplification technique sequenced autosome and Y chromosome loci successively, reducing the detection time and the need for blood sample volume. It decreases the steps of DNA extraction, and the results show consistency compared with the Chelex-100 method.

Compared with current domestic and international research, the results of this study show that the autosome and Y-chromosome loci are mainly based on typing, with high sensitivity and specificity. This result is similar to that of 8-dyne fluorescent labeling system of 82 locus designed by Liu *et al*. (2023). Tan *et al*. (2023) found that NGS technology can better identify insertion and deletion polymorphisms and their combination with adjacent single

nucleotide polymorphisms to form compound markers, so as to improve the efficiency of mixed DNA analysis. Scholars have found that a large amount of parallel sequencing allow to obtain more information from short tandem recurrence analysis, which not only combines with capillary electrophoresis features, but also applies them to sequences. With the development of parallel sequencing technology, the typing results of related STR genes are completely consistent with those of capillary electrophoresis (Soldati *et al*., 2023).

The combined detection of DNA methylation markers (CpG) and single nucleotide polymorphism markers (SNP) has been shown to be a promising tool for identifying semen and its donors (Li *et al*., 2023). Based on the significant differences in genetic diversity among some Y chromosome loci (Fu *et al*., 2023), further analysis of Y chromosome loci in more samples can be made to provide assistance for forensic evidence identification. However, there are some shortcomings in this study. For example, compared with the traditional method of detecting only autosome or Y chromosome, the Y locus of compound amplification system in this study has more obvious advantages. It not only expands the recognition range by detecting autochromosomes, but also improves the discrimination ability by detecting Y chromosomes, so it has obvious advantages in the use of information. Compared with the results of multi-color PCR retrieval abroad, this study has some innovations in optimizing the reaction conditions of multiple PCR and realizing automatic analysis results, and the system design and experimental process are more mature and thoughtful. Finally, the in-depth research of the effects on template DNA concentration and PCR abnormal factors was made, which enrichs the theoretical basis for the practical application of this new DNA identification technology.

CONCLUSION

This study preliminarily established a compound amplification detection system by six color fluorescent labeling technology to simultaneously detect multiple loci, effectively reducing detection time and requirements for sample size. The stability of concentration of DNA template and common PCR inhibitors has expanded the applicability of this technology in practical applications. Compared with the detection results of Chelex method and direct amplification method, Chelex method meets the DNA detection standards and provides a simple and fast method for forensic sample analysis. The results of mixed sample reveal the application value of this method in DNA mixed samples, which is beneficial for DNA identification in complex cases. Compared with existing commercial reagents, this method has better consistency in results and more stable reactions, providing an independent and controllable composite direct amplification technology measure for relevant fields in China. Although further optimization is needed in terms of sensitivity and species specificity, the overall performance of this method can meet the basic requirements of forensic DNA identification. This has laid the foundation for China's independent innovation and development in the field of forensic DNA technology.

DECLARATIONS

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IRB approval

This study was approved by the Advanced Studies Research Board of School of Life Sciences, Westlake University, Hangzhou 310024, China.

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board committee of School of Life Sciences, Westlake University, China. The official letter would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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