Short tandem repeat typing of cells transferred via micromanipulation with whole-genome amplification

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Abstract: This study attempted to determine the minimum number of cells required to conduct DNA analyses effectively. Oral mucosal cells obtained from eight persons were suspended and individually collected by using micromanipulation technique. DNA was extracted and amplified by whole-genome amplification (WGA). Nuclear DNA was extracted to evaluate the feasibility of autosomal short tandem repeat (STR) polymorphism and Y-chromosomal STR polymorphism analyses. Tests were conducted with 20 and 30 cells, to determine the minimum number of cells required for each DNA analysis. Tests with 20 cells were repeated 5 times, to examine reproducibility. When five or 10 cells were used, loci could not be identified for most alleles. Furthermore, DNA polymorphism analyses of a single cell transferred directly to a polymerase chain reaction solution were unsuccessful. The present findings suggest that, in forensic analyses of a single cell transferred directly to a polymerase chain reaction, could not be identified for most alleles. Furthermore, DNA polymorphism and Y-chromosomal STR polymorphism analyses. Tests were conducted with 20 cells were repeated 5 times, to examine reproducibility. When five or 10 cells were used, loci could not be identified for most alleles. Furthermore, DNA polymorphism analyses of a single cell transferred directly to a polymerase chain reaction solution were unsuccessful. The present findings suggest that, in forensic identification, 20 or more cells are required in order to obtain clear results from autosomal and Y-chromosomal STR polymorphism analyses. Furthermore, the feasibility of sample preservation and reexamination was also confirmed by DNA amplification with WGA.

Keywords: forensic science, identification, short tandem repeat, transferred cell, whole-genome amplification

Introduction

The quality of evidence in forensic science is often poor. Some materials undergo degradation or decomposition or are received in trace amounts, and the amount of DNA collected from samples is thus miniscule, which leads to difficulties in DNA analyses. Some studies used whole-genome amplification (WGA) to analyze denatured DNA [1-8]; however, very few studies have reported the minimum amount of DNA required for such analysis. The ability to collect and test DNA from minute samples would be extremely beneficial for identification in forensic medicine. In fact, the possibility of conducting DNA analysis of a single cell collected from 1 sample would be ideal, as it would enable identification of 1 or more persons from a single sample. However, because there is only 1 nucleus in a cell, detection of a Y-short tandem repeat (STR) polymorphism in autosomal and sex chromosomes is extremely difficult. Moreover, the quantity of obtained samples is usually minute, even when samples are fresh. Thus, development of a detection method with high specificity and sensitivity is of utmost importance in forensic medicine. At present, original DNA is amplified during DNA testing of trace samples [5-8].

WGA is an alternative to DNA extraction and purification and yields large quantities of DNA from small amounts of sample [1-8]. In the present study, the number of cells required for DNA analysis was determined by suspending cells obtained from dried spot samples and securely collecting them individually with a micromanipulation technique. The collected cells were amplified by WGA and identified by autosomal and Y-chromosomal STR polymorphism analyses.

Materials and Methods

Samples

Oral mucosal cells from eight volunteers (four males and four females) were obtained by stripping the surface of the oral mucosa with Sterile Foam Tipped Applicators (Whatman, Buckinghamshire, UK). Informed consent was obtained from all participants. This study was approved by the Ethics Committee of the Nihon University School of Dentistry, Japan (EP2011-18).

The applicator tips were dried at room temperature for 30 min, immersed in 1 mL of phosphate-buffered saline (pH 7.4), and spotted on a culture dish. As shown in Fig. 1, floating cells were captured with a micro-injector (CellTram Vario, Eppendorf, Hamburg, Germany) and transferred to a polymerase chain reaction (PCR) tube containing phosphate-buffered saline by using a micromanipulator (TransferMan NK2, Eppendorf) and microscope CKX41-31PHP (Olympus, Tokyo, Japan). DNA samples purified from peripheral blood were used as controls.

WGA

The cells were amplified with a REPLI-g Single Cell Kit (Qiagen, Limburg, Netherlands), in accordance with the manufacturer’s instructions. The final yield was 50 μL.

Purification

From the samples amplified by WGA, 40 μL was purified with a QIAquick PCR Purification Kit (Qiagen, Limburg, Netherlands), for a final yield of 50 μL.

Quantification of DNA

DNA quantity before and after purification was measured with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

DNA genotyping

Autosomal and Y-chromosomal STR loci were examined in samples containing 30, 20, 10, and 5 cells after purification.

Multiplex PCR with template DNA (10 ng) was performed with a GlobalFiler PCR Amplification Kit (Thermo Fisher Scientific) [2,6,9] (Izawa H et al., Allele frequencies of Japanese using GlobalFiller PCR amplification ki. DNA polymorphism, 23, 121-124, 2015) and PowerPlex Y23 System (Promega Corporation, Madison, WI, USA) [7,8,10,11], in accordance with the manufacturers’ instructions.

PCR-amplified fragments were separated and analyzed with an ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific) and typed by using the allelic ladder contained in the kit and the GeneScan (version 3.1, Thermo Fisher Scientific) and Genotyper (version 2.5; Thermo Fisher Scientific) software packages. The STR type determination of each locus was analyzed if it had a peak relative fluorescence unit (RFU) value of 50 or higher.
Table 1 Comparison of autosomal STR and Y-STR polymorphism analyses of 20 and 30 cells, before and after DNA purification

| Sample | Male | A | B | C | D | E | F | G | H |
|--------|------|---|---|---|---|---|---|---|---|
| Before |    |    |   |   |   |   |   |   |   |
| Autosomal STR | Discriminative | 24 | 24 | 24 | 24 | 21 | 21 | 17 | 17 |
| Only one of the allele | 0 0 0 0 0 0 0 0 0 |
| Undetected | 0 0 0 0 0 0 0 0 0 |
| Y-STR | Discriminative | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 |
| Undetected | 0 0 0 0 0 0 0 0 0 |

| After | Male | A | B | C | D | E | F | G | H |
|-------|------|---|---|---|---|---|---|---|---|
| Sample |    |    |   |   |   |   |   |   |   |
| Autosomal STR | Discriminative | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Only one of the allele | 0 0 0 0 0 0 0 0 0 |
| Undetected | 0 0 0 0 0 0 0 0 0 |
| Y-STR | Discriminative | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 |
| Undetected | 0 0 0 0 0 0 0 0 0 |

Numbers represent the number of loci obtained with the GlobalFiler PCR Amplification Kit and PowerPlex Y23 System. Only 1 allele: number of loci observed as hetero despite homozygosity. RFU, 50

Table 2 Reproducibility of results of autosomal STR and Y-STR polymorphism analyses of 20 cells

| Sample | Male | A | B | C | D | E | F | G | H |
|--------|------|---|---|---|---|---|---|---|---|
| Autosomal STR | Discriminative | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Only one of the allele | 0 0 0 0 0 0 0 0 0 |
| Undetected | 0 0 0 0 0 0 0 0 0 |
| Y-STR | Discriminative | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 |
| Undetected | 0 0 0 0 0 0 0 0 0 |

| Sample | Female | E | F | G | H |
|--------|--------|---|---|---|---|
| Autosomal STR | Discriminative | 20 | 20 | 20 | 20 |
| Only one of the allele | 0 0 0 0 0 0 0 0 0 |
| Undetected | 0 0 0 0 0 0 0 0 0 |
| Y-STR | Discriminative | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 |
| Undetected | 0 0 0 0 0 0 0 0 0 |

Numbers represent the number of loci obtained with the GlobalFiler PCR Amplification Kit and PowerPlex Y23 System. Only 1 allele: number of loci observed as hetero despite homozygosity. RFU, 50

Table 3 Amount of DNA (ng/μL) before and after DNA purification

| Sample | A | B | C | D | E | F | G | H |
|--------|---|---|---|---|---|---|---|---|
| Before | 20 | 3,240 | 1,590 | 3,120 | 1,450 | 928 | 624 | 4,500 |
| After | 30 | 916 | 2,160 | 2,200 | 5,200 | 532 | 625 | 3,760 |

Results

Minimum number of cells required for reliable testing and the effects of purification

Table 1 shows the results of autosomal STR polymorphism analysis with 20 and 30 cells. All alleles were determinable when 20 cells were used in six samples. In contrast, no locus (or only 1 locus, in some cases) was detected before purification in samples such as C, whereas, after purification, the locus type of samples could be identified (Fig. 2a). The heights of peaks in electropherograms were uniform, which facilitated reliable determination of loci after purification, in samples such as C (Fig. 2b). Nevertheless, 1 sample exhibited non-uniform peak heights after purification (Fig. 2c). In Y-chromosomal STR polymorphism analysis, the 23 alleles were determinable when 20 cells were analyzed, even before purification. However, the number of artifacts was lower after purification, which yielded unambiguous data.

A search of the Y Chromosome Haplotype Reference Database (http://www.yhrd.org) confirmed the findings for sample 4 but not for samples 1-3. Among samples in which all alleles were determinable with 20 cells, 8 (A-H) were retested 5 times by PCR amplification, to confirm the reliability of the results (Table 2). Determination of an allele with autosomal STR polymorphism analysis was possible in sample A; however, in samples C-F and H, alleles were undetectable, or only 1 could be identified, in some instances. Most alleles remained undeterminable. Only 1 of
the 2 alleles were detected in 7 of 24 loci and 5 of 22 loci in samples B and G, respectively, although they were predominantly hetero types. All alleles in samples A and D were undetectable under Y-chromosomal STR polymorphism analysis. Most alleles were determinable in samples B and C; however, 1 of the 2 alleles of the DYS385 marker (i.e., DYS385a and DYS385b) remained undetected in the 2 samples.

Table 3 shows the results of DNA measurement of samples before and after purification. A high volume of DNA was maintained, although the volume was 30% to 70% lower after purification.

**Evaluation of 5 and 10 cells**

In autosomal STR polymorphism analyses of 5 cells, most loci in all samples remained undetenable (or only 1 allele was detected) (Table 4). When 10 cells were used, 23 loci were determinable in samples D, F, and G. Moreover, the number of determinable alleles was slightly higher in Y-chromosomal STR polymorphism analyses. Although 22 of 23 loci were determinable in sample D, only 2 were detected in sample C. Thus, the results of the analyses varied considerably in relation to the sample tested.

**Direct PCR analysis of 1 cell**

The above findings showed that it was almost impossible to conduct STR polymorphism analyses with 5 or fewer cells. DNA type analyses were therefore conducted by directly transferring 1 cell to a PCR solution, without using WGA (Table 5). Almost all alleles remained undetected in autosomal and Y-chromosomal STR polymorphism analyses. Moreover, in some cases the peaks detected did not correspond with the alleles in the provided samples.

**Discussion**

The three methods generally used for DNA type analyses in forensic identification are mtDNA type, autosomal STR polymorphism, and Y-chromosomal STR polymorphism analyses. DNA type testing is difficult because cells have only 1 nucleus and because only trace amounts of sample are usually available in forensic cases. All 3 DNA type analyses are sometimes necessary. The present study thus aimed to determine the minimum numbers of cells required to conduct STR type analyses for forensic identification. In addition, the possibility of improving the reliability of identification was investigated by purifying amplified DNA with WGA.

When 20 cells were obtained from samples, all alleles were detected via autosomal and Y-chromosomal STR polymorphism analyses, and the findings were consistent with the blood data of the participants. Although only 1 nuclear DNA exists in a cell, analysis of 20 cells is sufficient to detect all alleles in a sample. An increase in the number of determinable alleles, along with the presence of uniform peak heights, was noted after purification, which indicates that purification after WGA is effective in determining DNA type (Fig. 2). Nonetheless, peak heights remained non-uniform in some samples requiring careful approaches for type determination. Because of the small amounts of the original samples, alleles may not have been uniformly amplified during amplification, which suggests a need for modifications of the WGA method.

The amount of DNA in the samples was 30% to 70% lower after purification. However, for example, in sample C in Table 1, determination of DNA type was impossible even when large quantities of DNA were available, perhaps because the entire DNA was not amplified in a stable manner owing to the miniscule amount of the original sample. The amount of DNA may have decreased following purification after WGA, which might have affected the precision of the analyses. Nevertheless, no negative effects in identification were noted.

To confirm the reliability of the results, 20 cells from 5 samples (A-H) were tested 5 times, under the same conditions, by PCR amplification. In sample A, all loci were determinable in autosomal STR polymorphism analysis; however, 1 locus exhibited uneven peak heights, indicating a need for careful additional analysis. In samples C-F and H, the results for loci 1-3 and the alleles of only 1 side were undeterminable. However, this did not greatly complicate identification. Among the 24 loci tested in the current study, only 1 of the 2 alleles was detected in 7 loci from sample B. Moreover, 5 of 22 loci, except for Y indel and DYS391, were detected in sample G, although they were hetero. However, two thirds of all loci were detected, which was sufficient for identification. In contrast, because one third of loci were identified as homo types, the possibility of samples with low reproducibility should be considered. The present results suggest that forensic identification can be performed effectively with STR polymorphism analyses of at least 20 cells.

In Table 3, the amount of DNA purified from 30 cells was less than that purified from 20 cells, because of the presence of contaminants such as food residues in saliva when the cells are transferred. Buffer intake is not constant when cells are transferred; thus, the amount of buffer present may be affected by the technique used by the operator. A Qubit 3.0 Fluorometer was used to measure the amount of DNA and inhibitors. Increased amounts of buffer may have been included during the transfer of the 20 cells in this study, which might explain why greater amounts of DNA were measured in these 20 cells than in the 30 cells that were transferred.

Subsequently, autosomal STR polymorphism analyses were conducted with 5 to 10 cells. Only 3 samples had a sufficient number of loci for identification when 10 cells were used, and less than half of loci were detected when 5 cells were used for the analyses. A few unstable samples exhibited a decrease in the number of determinable loci, even when the number of cells was increased from 5 to 10. This may be attributable to technical issues in collection or purification, or to an uneven increase in DNA after WGA, because of the minute amounts of samples used.

To evaluate the possibility of conducting DNA type analyses with a single cell, 1 cell was directly transferred to a PCR solution, without using conventional WGA. Almost no alleles were detected, and the few that were detected appeared to differ from those identified by autosomal and Y-chromosomal STR polymorphism analyses of corresponding participants. STR polymorphism analyses are extremely challenging because cells have only 1 nuclear DNA. Moreover, although the risk of contamination was low, the collected samples were entirely depleted while performing this procedure.

Tsuchumi et al. (Tsuchumi H et al., STR typing on Y chromosome from saliva with the Single-Tube PCR kit. DNA polymorphism, 6, 64-67, 1998) reported that STR type analyses on Y chromosomes from saliva were con-
ducted with a Single-Tube PCR Kit (TaKaRa Bio, Kusatsu, Japan). This kit is used for DNA extraction, and the reagent for direct PCR amplification is added to the extract. This is a simple method of DNA extraction, and PCR amplification is accomplished with low levels of contamination. However, as mentioned earlier, the disadvantage of this procedure is that it consumes the entire extracted DNA sample.

Forensic identification sometimes requires multiple DNA analyses, such as autosomal STR polymorphism analyses, Y-STR polymorphism analyses, and mitochondrial DNA polymorphism analyses, for a single case. Therefore, new methods of amplifying DNA are needed, to enable investigators to perform various DNA type tests on miniscule samples during forensic identification. In WGA, the reagent from the kit is added to the tube, thus lowering contamination risk. Moreover, reexamination and preservation of samples is possible because 50 µL of the sample is collected.

This study conducted autosomal polymorphism and Y-chromosomal STR polymorphism analyses of DNA samples obtained from buccal mucosa cells after WGA. Cells from dried spot samples obtained from multiple people were transferred individually, and DNA polymorphism analyses were conducted after DNA amplification with WGA. The results confirmed that identification was effective when 20 or more cells from the samples were used. In addition, preservation and reexamination of samples was feasible. This new approach appears beneficial for accurately identifying mixed and dried spot samples and may improve forensic identification. Nonetheless, analysis with fewer than 20 cells is extremely challenging. Moreover, because of the minute amount of sample available, contamination remains an important concern.

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Conflict of interest
The authors declare no conflict of interest.

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