Int J Legal Med (2008) 122:333–335
DOI 10.1007/s00414-008-0228-4

TECHNICAL NOTE

Amelogenin sex determination by pyrosequencing of short PCR products

Frank Tschentscher · Ulrich H. Frey · Thomas Bajanowski

Received: 22 October 2007 / Accepted: 22 February 2008 / Published online: 20 March 2008

Abstract We developed an assay, which allows the sex determination of human DNA samples by pyrosequencing of short PCR products. A 48/45-bp stretch including primers of the amelogenin gene with a 3-bp insertion on the Y chromosome was chosen for analysis. In an initial study, we correctly typed 50 male and 50 female DNA samples from unrelated donors. First experiments with forensic samples, which failed in conventional analyses, indicate that this approach might be an advantage when dealing with degraded DNA.

Keywords Sex determination · Amelogenin gene · Short fragments · Pyrosequencing

F. Tschentscher · T. Bajanowski
Institute of Legal Medicine, University of Duisburg-Essen, Essen, Germany

U. H. Frey
Institute of Pharmacogenetics, University of Duisburg-Essen, Essen, Germany

U. H. Frey
Department of Anaesthesiology, University of Duisburg-Essen, Essen, Germany

T. Bajanowski (✉)
Institute of Legal Medicine,
Hufelandstr. 55,
5122 Essen, Germany
e-mail: thomas.bajanowski@uk-essen.de

Present address:
F. Tschentscher
Landeskriminalamt NRW, Kriminaltechnisches Institut, Düsseldorf, Germany

Introduction

The amelogenin sex test has become indispensable in forensic casework not only with respect to rape cases but also in most commercially available polymerase chain reaction (PCR) kits [1, 2]. There are also a number of clinical questions where a reliable sex test is crucial, e.g., for prenatal diagnosis of hemophilia [3] or generally the determination of the copy number of sex chromosomes. Moreover, it is conceivable that when dealing with mass disasters or large burial sites—archaeological or recent—a reliable and straightforward method to distinguish male from female individuals is valuable.

The principle of the commercially available forensic sex tests is based on the fact that numerous polymorphisms are present on the two homologous copies of the amelogenin gene on the X and Y chromosomes (AMELX/AMELY). The primer pairs used for genotyping are mainly those described by Sullivan et al. detecting a 6-bp deletion/insertion on the X/Y chromosomes [4]. The relatively short amplicon length of 106 and 112 bp, respectively, is usually sufficient for routine DNA typing. However, in respect to highly degraded DNA, even this stretch might be too long for sufficient analysis.

Therefore, we developed a PCR test based on the published sequence of the amelogenin loci on chromosomes X and Y. The only prerequisite of the search for suitable regions was a maximum length of about 50 bp including primers. We identified a region of 48 bp that includes a central 3-bp insertion on the Y chromosome (Fig. 1).

Typing of the respective alleles was performed by pyrosequencing. This is a relatively new and rapidly evolving DNA sequencing method based on a chemiluminescent enzymatic reaction. Initially, this was mainly used

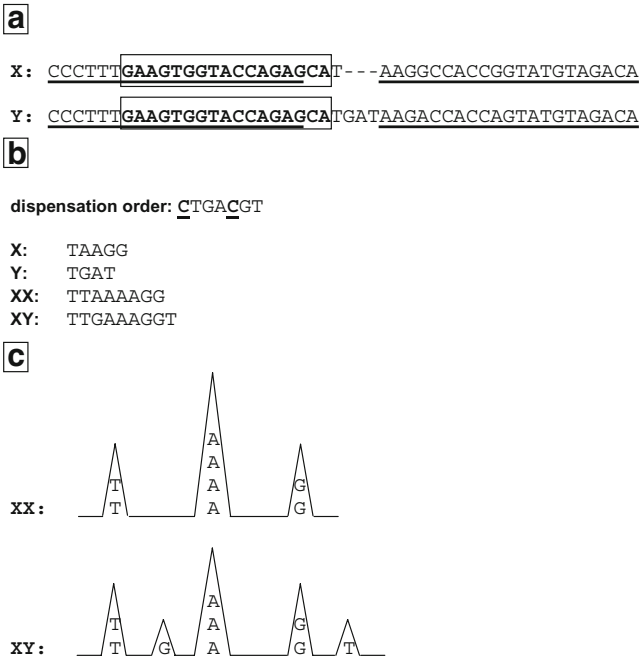

Fig. 1 **a** Analyzed X- and Y-chromosomal sequences of the amelogenin gene; *underlined* are the PCR primers; the primers used for pyrosequencing are *boxed*. **b** Dispensation order for the pyrosequencing reaction, list of the analyzed sequences (X and Y chromosomes) and the expected sequencing result for female and male individuals. **c** Expected pyrogram

for very fast sequence determinations of short DNA fragments, but several applications are at hand today [5–7].

In an initial blind study, we typed 100 randomly chosen DNA samples of healthy donors of known sex. Additionally, we tried to resolve the sex of seven bone samples, which failed in conventional analysis with commercially available PCR kits.

Materials and methods

PCR amplification

The chosen primers flanked a 3-bp GAT insertion on the Y chromosome located at nucleotide position 1499–1501 on the human amelogenin gene (Y chromosome, GenBank accession no.: M55419). The respective location on the X chromosome is 1680 (accession no.: M55418). The primer sequences were F5′-CCCTTTGAAGTGGTACCAGAG-3′, R5′-biotin-TGTCTACATACYGGTGGYCTT-3′. The mixed purine bases (T/C = Y) in the reverse primer were necessary because of two G–A transitions (Fig. 1a). PCR-conditions: Approximately 10 ng of DNA was added to a standard reaction mix in a volume of 50 µl containing 10 pmol of each primer and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). After an initial denaturation for 7 min at 95°C, 35 cycles with 15 s at 95°C, 15 s at 48°C, and 15 s at 72°C were performed followed by a final extension of 10 min at 72°C.

Pyrosequencing

Using a 4-enzyme mixture, this sequencing-by-synthesis method relies on the luminometric detection of pyrophosphate released upon nucleotide incorporation [8]. Each light signal is proportional to the number of incorporated nucleotides. Sample preparations and pyrosequencing reactions were performed as previously described [9]. The following primer was used in the reaction: 5′-GAAGTGGTACCAGAGCA-3′. Figure 1 shows the general setup and the theoretical outcome of the experiments.

Results

In a blind study, we typed 100 DNA samples, 50 from male and 50 from female donors with the described assay. All gave a result corresponding to the recorded sex of the respective donor (Fig. 2). We then tested seven DNA extracts from unearthed bones buried for 1 to 12 years, which failed in conventional short tandem repeat (STR) typing, presumably due to degradation. Six of these samples showed weak but sufficient and reproducible results.

Discussion

Sex determination of human DNA samples by typing of polymorphisms of the amelogenin gene is a routinely applied method in forensic casework and clinical testing. Usually, samples are analyzed with conventional methods such as electrophoresis of PCR products. Although failures in detection of the Y-chromosome-specific allele have been reported [10], typing of the amelogenin locus can still be regarded as a sufficient approach for routine sex determination in forensic DNA testing. However, if the sex is of crucial importance for further investigations or ambiguous results are obtained, additional tests based on other suitable sex specific loci such as the SRY gene or Y-chromosome-specific STRs [11] might be advisable [2, 12]. In clinical diagnostics, e.g., prenatal diagnostics of male specific diseases, additional typing of further loci is imperative [13].

Our test does not solve the above-described problems. However, with the method presented, the reliable amelogenin typing of 100 samples is feasible in ca. 3 h including PCR. It is therefore suitable for routine sex determinations of large sample numbers. The PCR products are of minimal

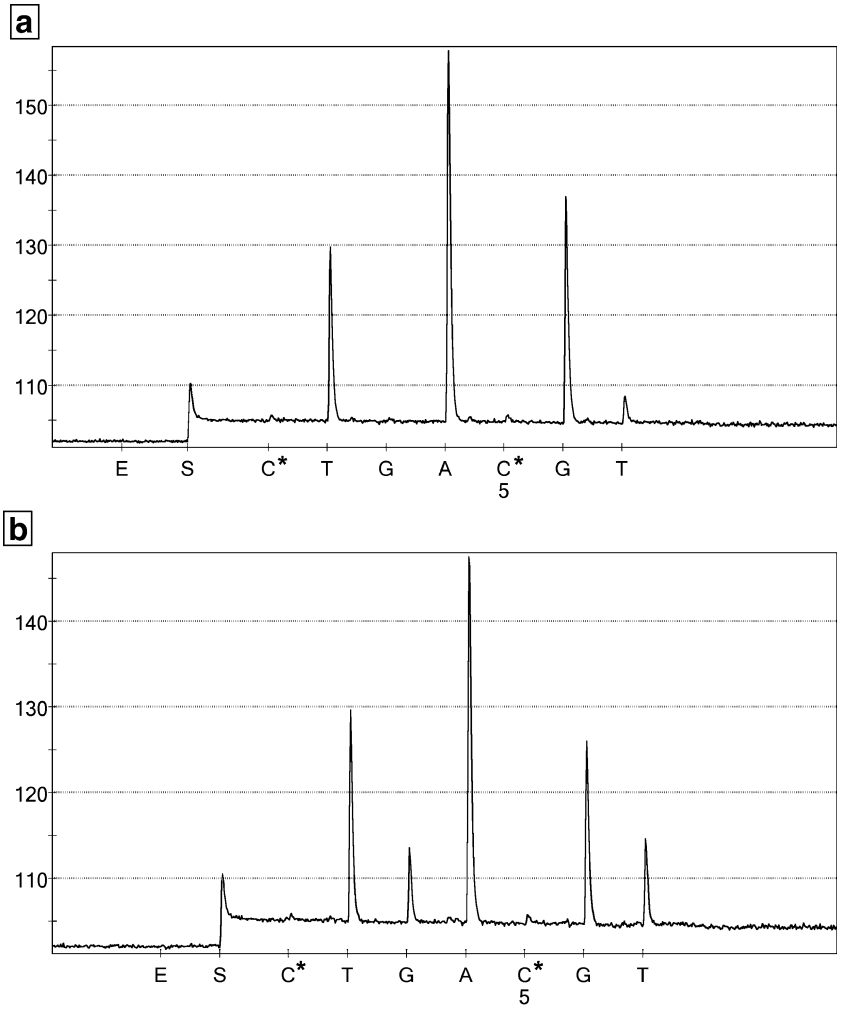

Fig. 2 Typical pyrograms of female (**a**) and male individuals (**b**). The last T peak in the female individual is clearly below the threshold. *Asterisk* spacers

size, and the results were promising regarding degraded samples. Further experiments will show if this approach is especially suitable for degraded or even ancient DNA.

References

1. Kashyap VK, Sahoo S, Sitalaximi T, Trivedi R (2006) Deletions in the Y-derived amelogenin gene fragment in the Indian population. BMJ Med Genet 7:37
2. Steinlechner M, Berger B, Niederstätter H, Parson W (2002) Rare failures in the amelogenin sex test. Int J Legal Med 116:117–120
3. Santacroce R, Vecchione G, Tomaiyolo M, Sessa F, Sarno M, Colaizzo D, Grandone E, Margaglione M (2006) Identification of fetal gender in maternal blood is a helpful tool in the prenatal diagnosis of haemophilia. Haemophilia 12(4):417–422
4. Sullivan KM, Mannucci A, Kimpton CP, Gill P (1993) A rapid and quantitative DNA sex test: fluorescence-based PCR analysis of X–Y homologous gene amelogenin. Biotechniques 15:636–641
5. Ahmadian A, Ehn M, Hober S (2006) Pyrosequencing: history, biochemistry and future. Clin Chim Acta 363(1–2):83–94
6. Balitzki-Korte B, Anslinger K, Bartsch C, Rolf B (2005) Species identification by means of pyrosequencing the mitochondrial 12S rRNA gene. Int J Legal Med 119:291–294
7. Andrèasson H, Nilsson M, Budowle B, Frisk S, Allen M (2006) Quantification of mtDNA mixtures in forensic evidence material using pyrosequencing. Int J Legal Med 120:383–390
8. Frey UH, Nuckel H, Dobrev D, Manthey I, Sandalcioglu IE, Eisenhardt A, Worm K, Hauner H, Siffert W (2005) Quantification of G protein Gaalphas subunit splice variants in different human tissues and cells using pyrosequencing. Gene Expr 12/2:69–81
9. Sun A, Ge J, Siffert W, Frey UH (2005) Quantification of allele-specific G-protein b3 subunit mRNA transcripts in different human cells and tissues by pyrosequencing. Eur J Hum Genet 13:361–369
10. Brinkmann B (2002) Is the amelogenin sex test valid? Int J Legal Med 116:63
11. Gusmao L, Butler JM, Carracedo A, Gill P, Kayser M, Mayr WR, Morling N, Prinz M, Roewer L, Tyler-Smith C, Schneider PM, International Society of Forensic Genetics (2006) DNA Commission of the International Society of Forensic Genetics (ISFG): an update of the recommendations on the use of Y-STRs in forensic analysis. Int J Legal Med 120(4):191–200
12. Santos FR, Pandya A, Tyler-Smith C (1998) Reliability of DNA-based sex tests. Nat Genet 18:103
13. Thangaraj K, Reddy AG, Singh L (2002) Is the amelogenin gene reliable for gender identification in forensic casework and prenatal diagnosis? Int J Legal Med 116:121–123