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# Forensic application of a rapid and quantitative DNA sex test by amplification of the X-Y homologous gene amelogenin

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**Summary.** Gender identification of forensic samples was determined by amplifying a segment of the X-Y homologous gene amelogenin. Using a single pair of primers spanning part of the first intron, 106 bp and 112 bp PCR products were generated from the X and Y homologues respectively, which were then resolved by agarose gel electrophoresis. This test enabled as little as 20 pg of DNA from severely degraded bones to be amplified and typed in a single tube reaction. Furthermore, using dye-labelled primers, it was possible to quantitate, by automated fluorescence detection, the relative yields of X and Y-specific PCR products generated from mixtures of male and female DNA. The versatility of this sex test was further demonstrated by co-amplifying with the HLA-DQA1 Amplitype kit in a combined gender/identity DNA test.

**Key words:** Sex test – Amelogenin – PCR – HLA-DQA1

**Zusammenfassung.** Durch Amplifikation eines Segments des X-Y-homologen Gens Amelogenin wurde eine Geschlechtsidentifikation forensischer Proben durchgeführt. Mit einem einzigen Primerpaar, welches einen Teil des ersten Introns überspannt, wurden PCR-Produkte mit 106 bp und 112 bp von den homologen Anteilen des X- und des Y-Chromosoms generiert, welche dann mit Hilfe der Agarosegelelektrophorese aufgetrennt wurden. Dieser Test erlaubte es, daß so geringe Mengen wie 20 pg DNA von stärkergradig degradierten Knochen amplifiziert und in einer Einzelreaktion typisiert wurden. Durch Benutzung von farbstoffmarkierten Primern war es ferner möglich, durch automatisierte Fluoreszenzdetektion die relative Ausbeute von X- und Y-spezifischen PCR-Produkten zu quantifizieren, wie sie von Mischungen männlicher und weiblicher DNA generiert wurden. Die Vielseitigkeit dieses Sex-Tests wurde ferner dadurch nachgewiesen, daß eine Co-Amplifikation mit dem HLA-DQA1 „Amplitype"-Kit in einem kombinierten Geschlechtsbestimmungs- und Identifizierungs-DNA-Test möglich war.

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**Schlüsselwörter:** Sex-Test – Amelogenin – PCR – HLA-DQA

## Introduction

Many techniques have been proposed for gender identification of DNA including direct visualisation of restriction digestion patterns on agarose gels [1], hybridisation of restricted DNA with Y-specific probes [2] and various PCR-based methodologies. PCR analysis offers the advantages of greater speed and sensitivity compared with the aforementioned techniques and can be achieved by amplifying either multiple copy or single copy sequences. Multicopy repeat sequences on the X and Y chromosomes can be amplified separately or in the same reaction mix using two pairs of primers [3–9]. This provides a highly sensitive assay by which single copies of DNA have been detected in some instances [10]. However, although the X chromosome product acts as a positive control, quantitation of the relative concentrations of X and Y chromosomal DNA is not possible due to differences in the copy number of the repeated sequences in the sex chromosomes, and in the efficiency of their amplification with different primer pairs. Alternatively, single copy X-Y homologous regions such as amelogenin [11, 12] can be amplified to provide a rapid sex test [13, 14]. Only one pair of primers is required to amplify both X and Y regions and both sequences are of equal copy number. Although this is theoretically less sensitive than typing multiple copy loci, optimisation of conditions and amplification of short segments of the amelogenin gene have enabled less than 1 ng of template DNA to be analysed [15]. By performing 2 rounds of nested amplification of amelogenin, single DNA molecules have been typed [16].

We describe here a simple, rapid and ultra-sensitive sex test for the analysis of forensic samples by amplifying part of the amelogenin gene. Blood, degraded muscle and bone samples were readily typed from as little as 20 pg of template DNA. Furthermore, mixtures of DNA could be

analysed by tagging the PCR products with a dye and measuring the relative fluorescence of the X and Y products. This enabled 1 ng of male DNA to be detected in a 100-fold excess of female DNA. Development of a combined identification/gender assay by co-amplification of HLA-DQA1 is also discussed.

## Materials and methods

Blood samples were taken from 50 male and 50 female donors; the DNA was extracted using a protocol described previously [17]. DNA samples extracted from degraded muscle tissue were recovered from the scene of a mass disaster. DNA from severely degraded bones believed to have been buried for more than 70 years was extracted using a modified previously published method [18]: the outer surfaces of a bone fragment were removed by sanding with emery paper, and the remaining bone, approximately 1 g, was frozen in liquid nitrogen, then ground into a fine powder using a Spex freezer mill. This powder was mixed thoroughly in 2 ml 0.5 M EDTA (pH 8.0) containing 1 mg of Proteinase K, plus 0.5% Tween 20, and incubated overnight at 37°C. This mixture was then extracted twice in phenol, twice in phenol/chloroform and once in chloroform before spinning in a Centricon 30 microconcentrator for 1 h. The DNA was quantitated by fluorimetry in most instances but because of low DNA content, extracts from bones were quantitated in a hybridisation assay with a human-specific DNA probe kit (Gibco BRL, New York, USA, cat. no 4220 SA) [19]. DNA from muscle and blood samples was quantitated fluorometrically with a Hoefer TKO 100 Mini Fluorometer with Hoechst dye 334258 (Hoefer Scientific Instruments, San Francisco, CA, USA). Primers used in amplification were designed using the OLIGO computer program [20]: 5'CCCTGGGCTCTGTAAAGAATAGTG3' (Amel-A) and 5'ATCAGAGCTTAAACTGGGAAGCTG3' (Amel-B) were synthesized by Oswel Ltd, Edinburgh, UK. These primers flank a 6 bp deletion within intron 1 of the X homologue resulting in 106 bp and 112 bp PCR products from the X and Y chromosomes respectively. PCR conditions were as follows: extracted DNA samples ranging from 10 pg to 100 ng were amplified in a 50 µl reaction mix comprising 0.2 µM each primer, 0.2 mM each dNTP (Boehringer Mannheim GmbH, Mannheim, FRG), 1.25 U Ampli-Taq DNA polymerase (Perkin-Elmer, Norwalk, CT) and 5 µl PARR buffer (Cambio Ltd, Cambridge). Samples were amplified through 35 cycles comprising 1 min at 94°C, 1 min at 60°C and 1 min at 72°C in a Perkin Elmer 480 thermal cycler.

Following amplification, 20 µl aliquots of PCR products were loaded in a 4% agarose gel and separated by electrophoresis for 90 min at 100 V, then visualised by ethidium bromide staining with UV transillumination.

*Automated fluorescence analysis and multiplex PCR.* Dye labelled PCR products were generated using primer Amel-A coupled with fluorescent dye 'FAM' via a 5' aminolinker (Oswel Ltd, Edinburgh, UK) in conjunction with unlabelled Amel-B. These products were analysed on either an ABI 362A GeneScanner or 373A Sequencer in conjunction with 672 software (Applied Biosystems, Foster City CA, USA): 1 µl of each PCR product was combined with 2.5 µl formamide and 6 fmol internal size standard comprising PstI restriction fragments of bacteriophage lambda DNA labelled with dye 'ROX', then denatured for 2 min at 90°C and loaded in a 6% denaturing acrylamide gel. Samples were electrophoresed for 4 h at 800 V in the GeneScanner or 1650 V in the 373A Sequencer. The fragment sizes were automatically determined by the software using the method of second-order regression to establish a curve of best fit generated from the internal standard in each lane. From this analysis, electrophoretograms were generated in which the DNA segments were depicted as coloured peaks. Peak area was automatically estimated as a measure of product yield.

Amelogenin was co-amplified with the HLA-DQA1 locus to provide a combined gender/identity test. This was achieved by adding primers Amel A and B at a final concentration of 0.4 µM to the stock mix provided in a standard Amplitype amplification kit (Perkin Elmer, Norwalk, CT, USA). Amplification was performed in a Perkin Elmer 9600 thermal cycler under the following conditions: 94°C for 3 min then 32 cycles comprising 94°C for 20 s, 60°C for 20 s and 72°C for 20 s followed by a final extension of 10 min at 72°C. Dot-blot analysis of the HLA DQA1 PCR products was performed according to the manufacturers instructions. Sex was determined by running 15 µl of the products in an agarose gel as described previously.

## Results and discussion

A total of 50 male and 50 female DNA samples extracted from liquid blood stocks were sexed with this test: all gave the expected results of a single band (106 bp) in females and a doublet (106/112 bp) in males. PCR products generated from as little as 1 ng of male and female template were readily visible on an agarose gel after ethidium bromide staining followed by UV transillumination (Fig. 1).

DNA extracted from muscle samples that had been recovered from the scene of a mass disaster and were therefore of unconfirmed origin, gave clear-cut results with the sex test, using 2 ng of template DNA, and this assisted in the identification of the remains (Fig. 2). Control samples of known sex from the same investigation were analysed "blind" and were typed correctly (results not shown).

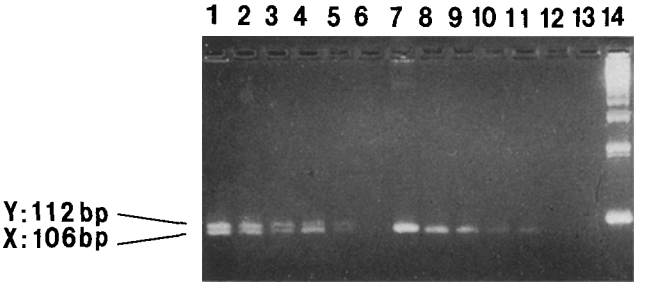


**Fig. 1.** Agarose gel electrophoresis of PCR products generated from different concentrations of male and female DNA templates. Lanes 1–6, products from 100 ng, 10 ng, 5 ng, 2 ng, 1 ng and 100 pg, respectively of male genomic DNA; lanes 7–12, products from 100 ng, 10 ng, 5 ng, 2 ng, 1 ng and 100 pg respectively of female genomic DNA; lane 13, negative control water blank. Lane 14, 123 bp ladder size standard

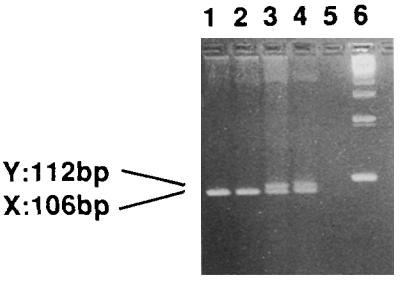


**Fig. 2.** Agarose gel separation of PCR products generated from muscle samples retrieved from the scene of a mass disaster. Lane 1–4, questioned specimens, with 1 and 2 identified as female, 3 and 4 identified as male; lane 5, negative control water blank; lane 6, 123 bp ladder size standard. Sizes of PCR products are shown

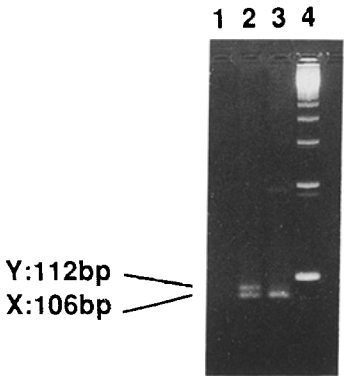


**Fig. 3.** Sexing of bone material exhumed 74 years after burial. Lane 1, negative control water blank; lanes 2 and 3, PCR products from bone specimens, identified as male and female respectively; lane 4, 123 bp ladder size standard

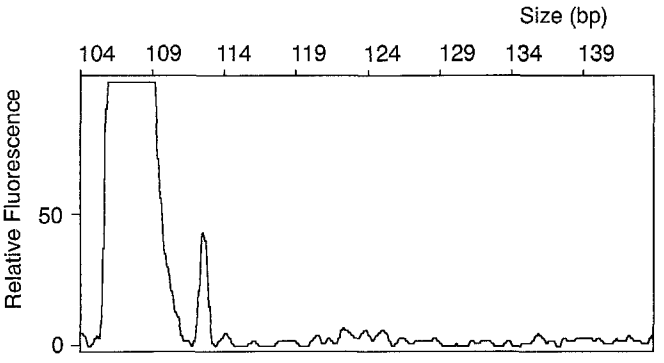


**Fig. 4.** Electophoretogram of male/female mixed DNA derived PCR products generated by an Applied Biosystems 373 A Gene-Sequencer. Amplification products are depicted as peaks but restriction fragments of the internal size standard are not shown. The male specific peak (112 bp) is clearly distinguishable from the larger female peak (106 bp). Size in bp, plus peak height and area in arbitrary units are determined for each fragment. Time (min) and scan number of band detection from the start of electrophoresis are also given. Male/female DNA template ratio 1/100

| Peak/ lane | Min. | Size bp | Peak height | Peak area | Scan # |
|---|---|---|---|---|---|
| 1B, 11 | 209 | 107.20 | 7122 | 46374 | 1047 |
| 2B, 11 | 216 | 112.87 | 54 | 203 | 1084 |

The extracted DNA from the bone samples was severely degraded and of low concentration. Approximately 20 pg from each of two samples were amplified, and the number of cycles was increased to 39. The products were clearly visible following electrophoresis in an ethidium bromide-stained agarose gel (Fig. 3), and the results confirmed conclusions drawn from physical examination of the bones, regarding their sex.

Allelic dropout due to stochastic variation is a major concern when amplifying such low copy numbers of DNA template, but this did not appear to affect these particular samples because repeating the amplifications yielded the same results. For this reason we do not advocate amplifying under a large number of cycles without recourse to replicate analyses due to the extreme sensitivity of the test, and the subsequent risk of mistyping due to stochastic fluctuation in allelic copy number. This problem is avoided by limiting the cycle number to 35. Otherwise, contamination becomes more problematic as the sensitivity of detection is increased, and requires extra care to be taken in manipulation of the samples. In one instance we observed random contamination of a negative control in which only the Y-specific product had been amplified (data not shown). This indicated that 39 cycles of amplification enabled very few or even single DNA molecules to be typed which means that sensitivity of the protocol described in this work may be close to the theoretical limits.

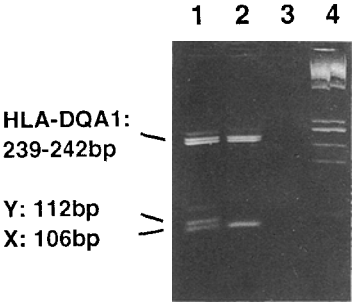


**Fig. 5.** Amelogenin/HLA-DQA1 coamplification product evaluation gel. Lanes 1 and 2, 15 µl of PCR product from male and female DNA samples respectively; lane 3, negative control water blank; lane 4, size standard phage Phi X174 digested with HaeIII. Faint heteroduplex bands are visible above the HLA DQA1 products and the male amelogenin products

Such a high sensitivity and quantitation potential implicit to the test [15] in conjunction with advantages of automated fluorescence dye-detection technology provided a means to successfully type difficult mixed samples, e.g. detecting trace amounts of male DNA in a vast excess of female DNA template. Figure 4 illustrates clear detection of Y-specific product in a particular mixture that contained 100 ng of female DNA and 1 ng of male. The expected ratio of peak areas is 201:1, this compares with the observed ratio of 228:1. To achieve this result, PCR products were analysed on an ABI 373 A DNA Sequencer to take advantage of its high resolving power. This method could potentially be a useful tool to detect the presence of sperm (or white cells in case of azoospermy) on vaginal swabs.

The versatility of this sex test was further demonstrated by co-amplifying with the HLA-DQA1 locus, thereby providing a combined gender/identity DNA test. When 0.1 µM concentrations of the Amelogenin primers were used in combination with the Perkin Elmer HLA-DQα Amplitype Kit, the 239–242 bp products from HLA primers were preferentially amplified compared with the smaller 106/112 bp products from primers Amel A and B. This effect was compensated for by increasing Amelogenin primer concentrations up to 0.4 µM in the standard HLA-DQA1 PCR reaction mix (Fig. 5). Comparisons were made between the HLA DQA1 results generated both with and without dual amplification of amelogenin. No differences were observed in the genotyping results, and in the intensity of the hybridisation signals (results not shown). Thus, we anticipate that this combined gen-

der/identity test may prove to be a valuable tool in forensic individualisation and clinical practice.

For forensic PCR applications, it is recommended that the investigation of X and Y sequences should be carried out in parallel or simultaneously, and the distinction between male and female DNA cannot be made based solely on the absence of a band [21]. Although several PCR-based tests have been developed for gender identification, some do not meet these criteria and are, therefore, not ideal for forensic casework. A major advantage of the test described in this paper is that both X and Y sequences are detected together in the same reaction using a single primer pair. The main drawback with using separate reactions for X and Y template is that results can be difficult to interpret in some situations. Lack of a Y-specific product could be due to 3 alternatives: presence of female template only, presence of male DNA of insufficient concentration to yield a visible PCR product, or failure of the PCR reaction. Our test greatly simplifies interpretation of results, especially when only the X-specific product has been generated, because this serves as an in-built positive control for the amplification reaction. Interpretation of results is further facilitated by the X and Y-specific products being only 6 bp different in size: even severely degraded samples can be typed without the risk of amplifying only the smaller of the 2 alleles. This "allelic drop-out" problem can afflict amplification and typing of loci with wider ranges of allelic sizes such as VNTRs [22].

Another problem which may cause false sex identification is different copy number of target X and Y-specific templates. It is a potential disadvantage of, for example, α-satellite based systems [7, 8]: alphoid blocks on the Y chromosome are repeated only 100 times whereas alphoid blocks on the X chromosome are present in about 5000 copies [5], this may bias the result of the analysis, under some conditions. Obviously such a problem is circumvented when amplifying single copy sequences like amelogenin.

Copy number of target molecules is not the only parameter that determines the overall specificity and sensitivity of the PCR reaction: primer design and optimisation of PCR parameters also have a profound effect. Results of the present work demonstrate that the primers utilised in this test (Amel A and B) provide robust and highly efficient amplification, which is of comparable sensitivity to the amplification of multicopy alphoid repeat sequences [7]. It is envisaged that this test will prove to be an advantageous addition to other methods of forensic DNA analysis.

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