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Sexing of forensic samples using PCR

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Summary. A rapid protocol has been established for sexing forensic samples by the Polymerase Chain Reaction method. Three sets of primer were used, two specific for Y chromosome repetitive sequences and one specific for X chromosome repetitive sequences. Detailed procedures of experiments, the controls and the applications to testing bloodstains and a vaginal swab are presented. The sensitivity of the test and problems due to contamination are discussed.

Key words: DNA – PCR – Y chromosome – X chromosome – Sexing

Zusammenfassung. Wir haben ein Schnellprotokoll zur Geschlechtsbestimmung forensischer Proben mit Hilfe der „Polymerase Chain Reaction" etabliert. Hierzu werden 3 Primer-Paare verwendet; 2 hiervon sind spezifisch für repetitive Sequenzen auf dem Y-Chromosom, ein Paar ist spezifisch für repetitive Sequenzen auf dem X-Chromosom. Die Methode wird detailliert beschrieben, ebenfalls die durchgeführten Kontrollen. Die Anwendungsmöglichkeiten an Blutflecken und Vaginalabstrichen werden vorgestellt. Erwähnung findet auch die Empfindlichkeit des Tests und die Problematik, welche durch Verunreinigungen der Spur entsteht.

Schlüsselwörter: DNA – PCR – Y-Chromosome – X-Chromosom – Geschlechtsbestimmung

The Polymerase Chain Reaction (PCR) [1, 2] is a very efficient and sensitive method for analysing DNA [2–5]. It may be particularly useful in forensic medicine, where only degraded DNA or small amounts of DNA are available. Some VNTR (variable number of tandem repeats) sequences [6, 7] and HLA genes [8] have already been used for genotyping samples by PCR. In this paper, a protocol for sexing of forensic samples has been established using two sets of specific primers [9, 10] for Y chromosome repetitive sequences [11] and one set of specific primers [12] for X chromosome repetitive sequences [13].

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Materials and methods

DNA extraction of bloodstains. Bloodstains were cut into small pieces and incubated in 400 µl of extraction buffer (0.01 *M* tris pH 8.0; 0.01 *M* EDTA; 0.1 *M* NaCl; 0.039 *M* DTT; 2% SDS) in the presence of 100 µg proteinase K for 15 h at 56°C. After 2 extractions in phenol/chloroform (v/v), DNA was precipitated in 2 volumes of ethanol in the presence of 0.2 *M* NaCl. After centrifugation (10.000 g, 30 min at 4°C) the pellet was resuspended in 40 µl 10 m*M* tris (pH 7.5); 0.1 m*M* EDTA.

DNA extraction of vaginal swabs. Swabs were incubated in 400 µl phosphate buffered saline (PBS) in the presence of 2% sarkosyl for 15 h at 4°C with gentle shaking. Swabs were placed in the lid (holed) of an Eppendorf tube and centrifuged for 3 min at 10.000 g. The supernatant was removed (fraction V1) and both pellet and swab were placed in 400 µl 100 m*M* tris-HCl pH 8.0; 100 m*M* NaCl; 1 m*M* EDTA. After addition of 100 µl 5% SDS, incubation was carried out for 2 h at 37°C in the presence of 100 µg proteinase K. The swab was placed in a lid (holed) and centrifuged at 10.000 g for 3 min. The supernatant was removed (fraction V2) and the swab discarded. The pellet (fraction V3) was incubated for 2 h at 37°C in 400 µl 2.5% sarkosyl; 40 m*M* tris-HCl pH 8.0; 40 m*M* NaCl; 0.1 m*M* EDTA; 40 m*M* DTT in the presence of 100 µg proteinase K. The 3 fractions were extracted twice in phenol/chloroform and DNA was precipitated and resuspended as previously described. Fractions V1 and V2 were rich in female DNA (vaginal cells), fraction V3 in male DNA (spermatozoa) [14].

Amplification of DNA by the polymerase chain reaction. The primers used for X-amplification were [12]:

X1: 5′ ATT CAT CAA ATG GAG ATT TG 3′
X2: 5′ GTT CAG CTC TGT GAG TGA AA 3′

The primers used for Y-amplification were:

For the first primer set [9]:

Y1-1: 5′ TCC ACT TTA TTC CAG GCC TGT CC 3′
Y1-2: 5′ TTG AAT GGA ATG GGA ACG AAT GG 3′

For the second primer set [10]:

Y1-3: 5′ ATT ACC CTA CAT TCC CTT CCA 3′
Y1-4: 5′ AGT GAA ATT GTA TGC AGT AGA 3′

Amplifications were performed using the conditions set out in the Gene Amp DNA Amplification Reagent Kit (Cetus Corporation Emeryville, CA, USA) and a Perkin Elmer Cetus thermocycler. Amplification with each primer set was performed independently using 1 µg of each primer in a final reaction volume of 100 µl.

After denaturation for 7 min at 94°C, 20–40 cycles (usually 30) were performed as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The reaction

was ended by a final extension of 7 min at 72°C. This protocol was adapted from Witt and Eriksen [12] and was chosen because it allowed specific amplification with the 3 primer sets in parallel using the same thermocycler programme.

Electrophoresis. From the final PCR product, 10 µl were run in 4% agarose gel (2% Agarose Na, Pharmacia Uppsala Sweden and 2% NuSieve GTG, FMC Rockland USA) in TAE buffer [15] or 8% acrylamide gel electrophoresis in TBE buffer [15].

When only small amounts of template DNA were available the PCR product was concentrated by ethanol precipitation and all the DNA loaded in the gel. After electrophoresis DNA was visualised by ethidium bromide.

Results

Two blood stains and one vaginal swab were analysed simultaneously with the following controls (see Fig. 1): one amplification was performed in a tube without DNA, one with male DNA and one with female DNA. In the experiment, the three primer sets were used. Male DNA gave positive signals in the two Y-specific and in the X-chromosome amplifications, whereas female DNA was only positive in the latter test. The X chromosome PCR was important to control false negative results on the Y chromosome tests. Absence of a Y chromosome specific signal but presence of an X-specific signal, allowed the diagnosis of female DNA. No positive results either on Y chromosome or on X chromosome tests were due to absence of DNA. Therefore sample S2 and the 3 fractions of the DNA from the vaginal swab contained Y chromosome sequences (Fig. 1). Thus, it could be concluded that the bloodstain S2 was of male origin and the presence of male cells on the vaginal swab could be diagnosed. On the other hand, sample S1 was negative for Y chromosome sequences not because of absence of DNA, but because of the presence of female DNA.

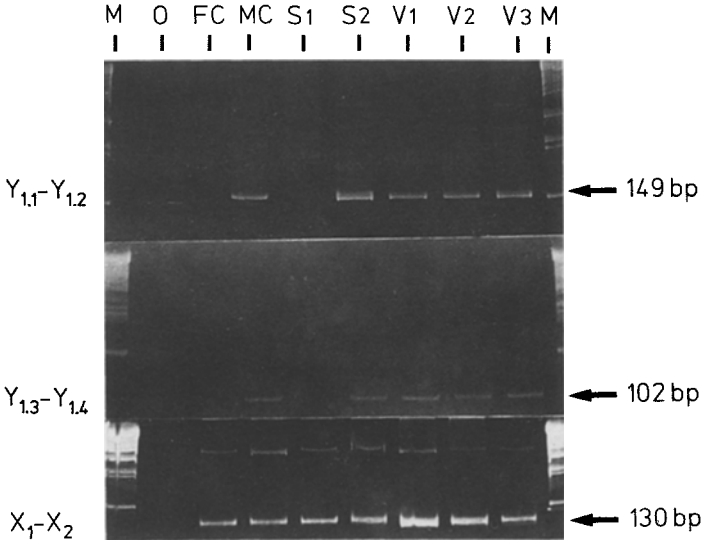

Fig. 1. PCR was performed using primer sets Y1-1/Y1-2 (9), Y1-3/Y1-4 [10] and X1/X2 [12] as described in materials and methods. Ten µl of the final PCR product were loaded onto an 8% acrylamide gel. M = molecular weight marker; O = control PCR without DNA; FC = female DNA control; MC = male DNA control; S1 and S2 = bloodstains; V1, V2, V3 = vaginal swab DNA fractions [14]. *Arrows* indicate the specific amplified band, for each PCR, with its molecular weight in base pairs. Samples containing male DNA (S2, V1, V2, V3) show specific amplification for Y and X chromosomes, whereas female DNA (S1) gives only the X chromosome specific signal

Discussion

This method for sexing forensic samples is quite simple (a single PCR reaction for the 3 tests), very quick (one day from DNA extraction to reading of electrophoresis), safe (no radioactive probes are used) and quite sensitive. For example, using 40 amplification cycles, a signal could be obtained from 0.5 ng of total genomic DNA, as illustrated in figure 2, using primers Y1-3 and Y1-4. Amplification of repetitive sequences means a greater amount of template for PCR than a single sequence, therefore increasing the sensitivity of the test.

After having tested the best PCR conditions allowing amplification of both X and Y chromosome specific sequences on about 30 unknown samples, our current experience can be summarized as follows: results were obtained from 72 vaginal swabs or sperm stains, of which 12 were tested for both X and Y sequences. The other 60 samples were tested only with Y chromosome specific primers. We have also tested 35 bloodstains or whole blood samples, of which 7 have been analyzed using only the Y chromosome specific primers. Some PCR experiments were classified as invalid because the control tube without DNA was positive (36 samples rejected in Y chromosome specific tests and 7 samples rejected in X chromosome specific tests) or because the female DNA control alone was positive for Y chromosome PCR (3 samples rejected). This was particularly true in the first experiments carried out and therefore we had to dramatically improve each step of the experiment to avoid and to control DNA contamination, as discussed below.

False positive results are due to contamination which can appear at several levels:

– The most common contamination is seen in the preparation of the reaction mixture, especially in the primer stock solutions which are regulary opened and pipetted. It is absolutely necessary to use clear pipettes dedicated only to PCR, autoclaved tips or solutions and to divide the primer stock solutions into aliquots. All these precautions may be sufficient, but in our hands, safer results are obtained when preparing batches of 50–100 reaction tubes. Such tubes, containing primers, incubation buffer and nucleotides can be easily stored at −70°C or −20°C for long periods of time and it is only necessary to add the DNA to be tested and the Taq polymerase. The batch is checked at the date of preparation and at every experiment for the presence of contaminants by making an amplification without DNA. Another way to avoid false positive results due to contamination of the reaction mixture is to preincubate the aliquoted tubes, before PCR assay, with the restriction enzyme *Eco*RI as described by Handyside et al. [5] and Lo et al. [10] as this enzyme cuts the Y chromosome target in two pieces. Therefore any contaminant target is cut, and amplifica-

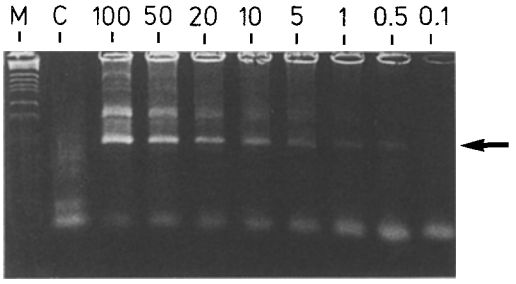

Fig. 2. Different concentrations (0.1–100 ng) of human genomic DNA were analysed by PCR, using primers Y1-3 and Y1-4 [10] and 40 amplification cyles. The final PCR product was concentrated in 10 µl loading buffer after ethanol precipitation. The total sample was loaded onto a 4% agarose gel. M = molecular weight marker. C = female DNA control

tion cannot be performed as the specific primers will bind to two different DNA fragments. Finally, another method consisting of UV exposure (312 nm for 3–5 min) of the reaction tubes, before performing PCR, can be proposed. This will break contaminant DNA, but it is not easy to obtain reproducible results and it can also break the primers.

– During extraction, DNA can be contaminated by using solutions which have been pipetted many times for other DNA extractions. For example we obtained a Y chromosome specific amplification of the female DNA control, while the control without DNA and other known female DNA were negative. A new extraction of this DNA with clean solutions gave the expected result. Such problems are easily avoided by using aliquoted and freshly autoclaved solutions for DNA extraction.

– The sample under test may itself be contaminated. False positive results could be observed with bloodstains which are a mixture from 2 sources or which have been extensively handled by investigators. However this is not usually seen on vaginal swabs and therefore this protocol has been used as the first test for rape cases.

As we have been careful in extraction of the DNA and in preparation of reaction mixtures, we rarely reject results because of false positive signals in our controls, even when using more than 30 cycles (For example 40 cycles as shown in Fig. 2) of amplification as is our normal practice.

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