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# Sex determination and DNA competition in the analysis of forensic mixed stains by PCR

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**Abstract** Sex determination of pure and mixed blood samples and stains was performed by amplification of the X-specific and Y-specific alphoid sequences by PCR (XY-PCR). From mixed blood samples with female DNA present in large excess of male DNA, the Y-specific sequence still amplified efficiently. In the analysis of vaginal secretions in a case of sexual assault, XY-PCR was performed to test the efficiency of the selective lysis procedure in order to investigate whether alleles found with other PCR systems were of male or female origin. Our studies with mixed blood samples revealed pronounced DNA competition in the HLA-DQα and D1S80 PCR systems: the alleles from a minor DNA component could not be detected in the presence of a large excess of DNA from a second person.

**Key words** Sex determination · PCR · Mixed stains · DNA competition · Selective lysis

**Zusammenfassung** Für die Geschlechtsbestimmung (XY-PCR) reiner und gemischter Spuren wurden X-spezifische und Y-spezifische Alphoidsequenzen mittels PCR amplifiziert. Aus gemischten Blutproben, wo die Menge an weiblicher DNA viel größer ist als die der männlichen DNA, wurde die Y-spezifische Sequenz immer noch effizient amplifiziert. Bei der Analyse von Spurenmaterial nach einem Sexualdelikt wurde XY-PCR eingesetzt um die Effizienz der selektiven Lyse zu überprüfen und um zu untersuchen, ob Allele, welche in anderen PCR Systemen nachgewiesen wurden, einer weiblichen oder einer männlichen Person zuzuordnen sind. Diese Untersuchungen mit gemischten Blutproben zeigten, daß in den Systemen HLA-DQα und D1S80 starke Kompetition auftrat, wobei die Allele einer DNA, in Anwesenheit eines großen Überschuß an DNA einer zweiten Person, nicht mehr nachweisbar waren.

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**Schlüsselwörter** Geschlechtsbestimmung · PCR · Mischspuren · DNA Kompetition · Selektive Lyse

## Introduction

Selective lysis is an important method in the analysis of DNA from mixed stains after sexual assaults, where the fraction enriched in sperm cells can be compared with the fraction containing primarily female epithelial cells (Gill et al. 1985). However, depending on the quality of the stain (Schneider et al. 1991; Pötsch et al. 1993b), as well as the ratio of sperm cells to female cells, selective lysis is often only partially successful. But sometimes mixed stains have to be analyzed in other situations too. Mixtures of blood and sweat or saliva on clothing or bedclothes may originate from 2 different persons. A problem in the analysis of mixed stains is DNA competition during PCR which we observed in the HLA-DQα and D1S80 systems and which presumably also occurs in other systems.

Sex determination by PCR can be a valuable tool not only after selective lysis of mixed stains, but also in the analysis of pure stains. Since in many forensic cases persons of both sexes are involved, a rapid and simple sex determination assay may give valuable information on the origin of the stain by identifying the sex of an unknown person involved in the crime.

Several methods for sex determination with PCR have been described which combine PCR with subsequent restriction digest followed by agarose electrophoresis (Aasen and Medrano 1990; Gaensslen et al. 1992) or with dotblot hybridization with Y-specific probes (He et al. 1989; Pötsch et al. 1993a). Amplified regions of the X and Y chromosomes are the alphoid repeat region (Witt and Erickson 1989), the zincfinger protein ZFX/ZFY (Aasen and Medrano 1990), DYZ1/DXS424 (Pfitzinger et al. 1993) or the amelogenin gene (Sullivan et al. 1993; Mannucci et al. 1994). The most rapid methods, however, require only electrophoresis of the amplified products in an agarose gel (Witt and Erickson 1989; Pfitzinger et al.

1993; Mannucci et al. 1994). Amplification of the amelogenin gene from both X- and Y-chromosomes is performed in a single assay with one pair of primers and the products, 106 and 112 bp fragments, are separated in an 4% agarose or 6% polyacrylamide gel (Mannucci et al. 1994). The DYZ1/DXS424 system amplifies an X-specific product (181–199 bp) and a Y-specific product (102 bp) in a single assay using 2 primer pairs and separation of the products is in a 4% Nusieve agarose gel (Pfitzinger et al. 1993).

Here we report on the use of sex determination in the analysis of mixed stains by amplification of chromosome X-and chromosome Y-specific DNA alphoid repeat regions (XY-PCR), using 2 different primer pairs as described by Witt and Erickson (1989). PCR with 2 different primer pairs avoids the problem of DNA competition and allows rapid estimation of the ratio of female to male DNA in mixed stains after selective lysis, even with female DNA in excess.

## Materials and methods

DNA from whole blood was isolated by quick lysis of the cells in TNE buffer (10 mM Tris, pH 7.6, 25 mM Na-EDTA, 75 mM NaCl, containing 1% SDS), followed immediately by phenol extraction and ethanol precipitation. Selective lysis was done in the same buffer, containing proteinase K and DTT as described by Giusti et al. (1987). DNA was quantitated using the TKO100 fluorimeter (Hoefer Scientific Instruments).

Amplification of X- and Y-specific DNA fragments was performed in 2 parallel assays with primers described by Witt and Erickson (1989) in 25 µl reactions in a Bio-Metra Trio-thermoblock with Taq- DNA polymerase (Perkin-Elmer/Cetus) starting with 10 ng DNA, when not indicated otherwise. After 30 cycles (1 min 95°C, 30 s 55°C, 30 s 72°C) the amplified X (130 bp) and Y-products (170 bp) were separated in a 1% agarose gel and visualized by ethidium bromide staining. HLA-DQα amplification and reverse dotblot hybridization, and D1S80 amplification and Detection Gel electrophoresis were performed according to the instructions of the manufacturer (Perkin-Elmer/Cetus) except that the number of D1S80 cycles was increased from 30 to 32. Prior to the hybridization process, HLA-DQα amplification products were analyzed by electrophoresis in a 1% agarose gel. Gels were stained with ethidium bromide and photographed under UV-light.

## Results

In our analyses of mixed blood samples with known DNA concentrations using the HLA-DQα and D1S80 systems, we observed a competition effect during the amplification process. In the presence of concentrations of competitor DNA (HLA-DQα 1.2–4) exceeding 10 ng in an 60 µl assay, 0.5 ng DNA (HLA-DQα 2–4) from a second person was not amplified as efficiently as in the absence of competitor DNA. HLA-DQα allele 2 (Fig. 1) diminished in intensity until it became almost undetectable in the presence of 40 ng genomic competitor DNA. The same phenomenon was observed with the D1S80 system. Increasing amounts of competitor DNA with alleles T21 and T24 drastically reduced the amplification efficiency of the allele T28 from 1 ng genomic DNA from a second person

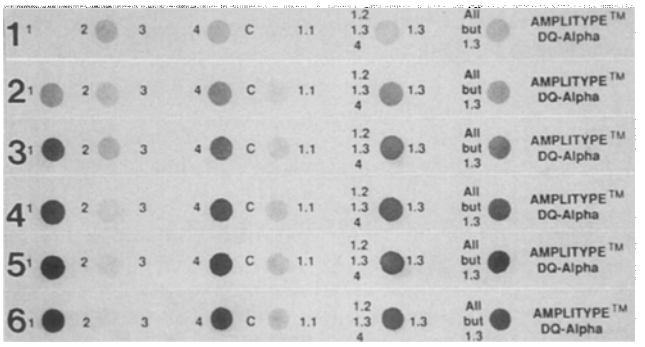


**Fig. 1** HLA-DQα analysis of mixed blood samples. DNA (0.5 ng) of type 2–4 was analyzed in the presence of 0 (strip 1), 0.5, 2, 10, 40 and 100 ng (strip 6) of DNA of type 1.2–4

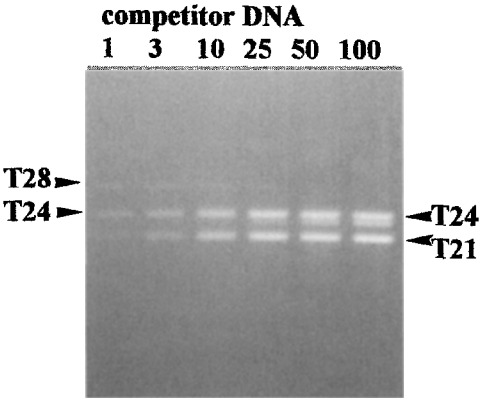


**Fig. 2** D1S80 analysis of mixed blood samples. DNA-1 (T24-T28)(1 ng) was analyzed in the presence of 1, 3, 10, 25, 50 and 100 ng of DNA-2 (T21-T24)(from left to right). The 1.5% agarose gel was stained with ethidium bromide

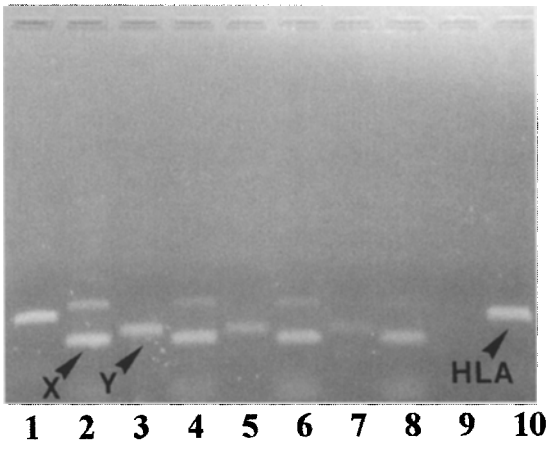


**Fig. 3** Sensitivity of XY-PCR. In 25 µl PCR assays 100 pg (lanes 2, 3), 20 pg (lanes 4, 5), 5 pg (lanes 6, 7) and 1 pg (lanes 8, 9) genomic DNA was added. The 130 bp product of the X-reactions (lanes 2,4,6 and 8) and the 170 bp products of the Y-reactions (lanes 3,5,7 and 9) are shown. HLA-DQα amplified products are shown for comparison (lanes 1 and 10)

(Fig. 2). With template concentrations higher than 25 ng an additional artefact band (comigrating with T23) appeared, probably due to mispriming or misalignment. For these reasons the problem of competition cannot be

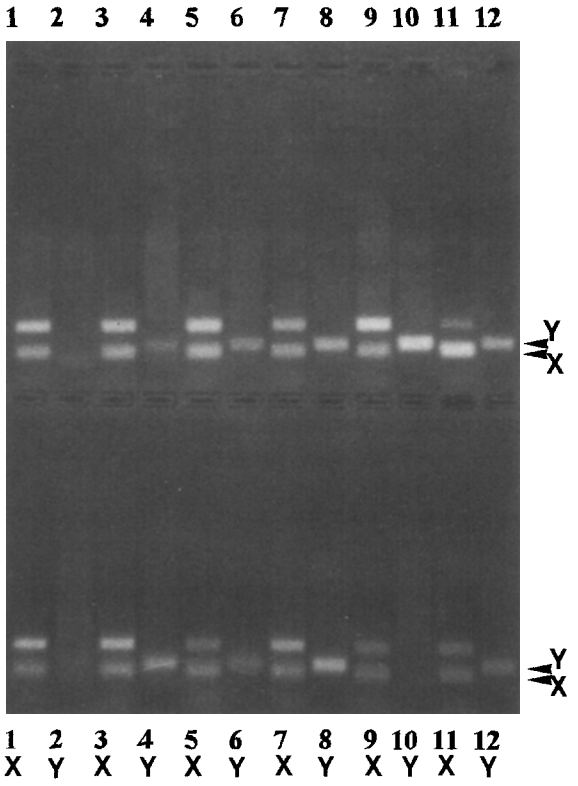


**Fig. 4** upper part: XY-PCR of DNA isolated from pure and mixed blood samples and a cigarette butt. Lanes 1,3,5,7: X-amplification of DNA mixtures: female DNA (total 1 ng) containing none, 50 pg, 200 pg or 500 pg male DNA, respectively. Lanes 2,4,6,8: the corresponding Y-amplifications. Lanes 9, 10: male DNA; lanes 11, 12: DNA from cigarette butt. Lower part: XY-PCR of DNA isolated from mixed stains. Lanes 1, 2: DNA (10 ng) from vaginal secretion on a slide, female fraction; lanes 3, 4: idem, male fraction; lanes 5, 6: DNA from slip, female fraction; lanes 7, 8: idem, male fraction; lanes 9, 10: DNA from victims blood; lanes 11, 12: DNA from blood of suspect. In all odd lanes the X-products (130 bp), in all even lanes the Y-products (170 bp) are shown. The agarose gel is stained by ethidium bromide

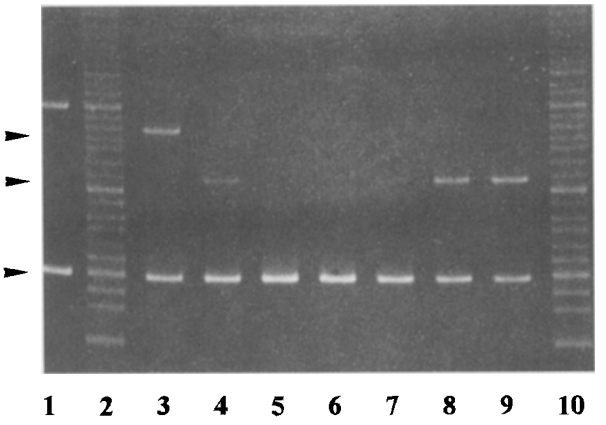


**Fig. 5** D1S80 analysis of DNA (10 ng) of forensic mixed stains. Lane 1: control DNA (T18-T31); lanes 2, 10: allelic ladder; lane 3: blood of suspect (T18-T29); lane 4: vaginal secretion, male fraction; lane 5: slip, male fraction; lane 6: slip; lane 7: slip, female fraction; lane 8: vaginal secretion, female fraction; lane 9: blood of victim (T18-T25). After electrophoresis the detection gel was stained with ethidiumbromide. The alleles T18, T25 and T29 are indicated by the arrowheads

**Table 1** Summary of the results of XY-PCR and D1S80 analysis after selective lysis. Relative intensities of the bands are indicated by the number of (+)-signs; (–): no band visible in the gel

| | D1S80 | | XY-PCR | |
|---|---|---|---|---|
| | T18 | T25 | X | Y |
| Female blood | ++++ | ++++ | ++ | – |
| Vaginal swab (female fraction) | ++++ | ++++ | ++ | – |
| Vaginal swab (male fraction) | ++++ | ++ | ++ | ++ |
| Slip (female fraction) | ++++ | + | ++ | + |
| Slip (male fraction) | ++++ | - | ++ | +++ |

solved by simply raising the DNA concentration in the PCR assay. With the HLA-DQα system no artefact spots appeared even at extremely high (500 ng) template concentrations (data not shown).

Competition can be a drawback in PCR analysis of mixed stains, since the cells from one of the individuals can be in high excess and, in the case of samples containing sperm and other cells, selective lysis is not always 100% effective, depending on the quality of the specimen (Pötsch et al. 1993b). With this in mind we investigated the XY-PCR process with regard to its sensitivity and the effect of competition using DNA mixtures.

The XY-PCR system is very sensitive: 50 pg of genomic DNA per 25 µl assay is sufficient to give clearly visible PCR products after 30 cycles of amplification, with 5 pg genomic DNA per 25 µl assay as the lowest level of detection of the Y-specific product (Fig. 3). Also in the presence of a 41-fold excess of X-DNA, corresponding to a 20-fold higher concentration of female DNA (1 ng), the Y-specific product of the male DNA (50 pg) was easily detected (Fig. 4, upper part, lane 4). PCR products from female genomic DNA are shown in lanes 1 and 2, those from male DNA in lanes 9 and 10. The characteristic 170 bp Y-specific band is absent (lane 2) from the female PCR products, the X-reaction yields the X-specific band (130 bp) plus a non-specific band of higher molecular weight (Witt and Erickson 1989), which is discernable from the Y-specific band (lane 10). XY-PCR with DNA extracted from a cigarette butt showed that this cigarette has apparently been smoked by a male person (lanes 11 and 12).

Figure 4 (lower part) and Fig. 5 show the results of XY-PCR and D1S80 analysis, respectively, in a rape case. These results show a concomitant increase of Y compared to X products and reduction of the female D1S80 allele T25 in comparison to allele T18 and are summarized in Table 1. The victim is of D1S80 type T18-T25 (Fig. 5, lane 9), the accused of type T18-T29 (lane 3). In the male fractions from the slide with vaginal secretions and from the slip the D1S80 allele T18 is clearly visible, allele T25 only very weakly or not at all visible. This suggests, that the offender is homozygous for this allele, although drop-out of a large D1S80 allele cannot be ruled out completely.

## Discussion

Our studies revealed DNA competition during PCR when mixed blood samples were investigated. We observed competition using 2 commercially available kits (HLA-DQα-Alpha, D1S80) used in forensic investigations. Other investigators have also described DNA competition during PCR using DNA mixtures (Patri et al. 1994). Silver staining of the gels instead of staining with ethidium bromide or Southern blotting and subsequent hybridization with labeled probes will be helpful for a better detection of the poorly amplified alleles. We increased the sensitivity of the D1S80 system by increasing the number of cycles from 30 to 32. Also higher primer concentrations may be helpful to reduce the effect of competition, but this increases the risk of artefacts due to mispriming. Moreover, in the increasing number of commercially available PCR systems the primer concentrations cannot easily be varied. We think it important to realize that under optimized conditions, also in other PCR systems, DNA competition may occur even when the amplified products are approximately of the same length (e.g. HLA-DQα, STRs).

Amplification of both X-specific and Y-specific products with PCR is a rapid and easy way to investigate the enrichment of sperm cells through preferential lysis of vaginal secretions and is helpful in the interpretation of the male and female alleles obtained in different PCR systems. When the X-specific and Y-specific reactions are performed with 2 primer pairs, no competition can occur during PCR which would inhibit amplification of the Y-specific product in the presence of excess female DNA, whereas such inhibition could possibly occur if both reactions were done simultaneously using a single primer pair. With 2 parallel assays the X-reaction is a control for the quality of the DNA in the specimens and serves as a positive control. The Y-reaction, in combination with a positive X-result, shows that the DNA originated from a male or female person. In the case of mixed samples, the efficiency of preferential lysis can be tested.

Other PCR systems for sex determination have been described. Some use restriction digest of the amplification products (Aasen and Medrano 1990), others a subsequent hybridization step using specific probes (He et al. 1989; Aasen and Medrano 1990; Pötsch et al. 1993a). Because of its sensitivity, apparent lack of competition effects and easy detection, we consider the method reported here a rapid and valuable method for sex determination in forensic stains.

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