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TECHNICAL NOTE

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Rapid Quantification and Sex Determination of Forensic Evidence Materials

ABSTRACT: DNA quantification of forensic evidence is very valuable for an optimal use of the available biological material. Moreover, sex determination is of great importance as additional information in criminal investigations as well as in identification of missing persons, no suspect cases, and ancient DNA studies. While routine forensic DNA analysis based on short tandem repeat markers includes a marker for sex determination, analysis of samples containing scarce amounts of DNA is often based on mitochondrial DNA, and sex determination is not performed. In order to allow quantification and simultaneous sex determination on minute amounts of DNA, an assay based on real-time PCR analysis of a marker within the human amelogenin gene has been developed. The sex determination is based on melting curve analysis, while an externally standardized kinetic analysis allows quantification of the nuclear DNA copy number in the sample. This real-time DNA quantification assay has proven to be highly sensitive, enabling quantification of single DNA copies. Although certain limitations were apparent, the system is a rapid, cost-effective, and flexible assay for analysis of forensic casework samples.

KEYWORDS: forensic science, forensic DNA analysis, sex determination, amelogenin gene, DNA quantification, real-time DNA analysis, dissociation, melting curve, forensic evidence material

Routine forensic DNA analysis, based on short tandem repeat (STR) markers, can be performed successfully on most evidence materials found at a crime scene (1). However, biological material such as shed hair, bone, or teeth are often degraded and contain scarce amounts of DNA, limiting the possibility to perform an analysis based on nuclear markers amplified in large fragments. For such materials, sequence analysis of mitochondrial DNA (mtDNA) is often more successful due to the high copy number of mtDNA molecules per cell (2–6).

In order to determine whether an evidence material contains sufficient amounts of DNA for a multi-locus nuclear STR analysis, or whether an mtDNA analysis is necessary, a sensitive quantification assay is required. A real-time TaqMan® probe system, for simultaneous quantification of mitochondrial and nuclear DNA (nDNA) copies, has been developed previously (7). This dual-color assay, based on specific hybridization of probes to nDNA and mtDNA, is highly sensitive and has proven to be very useful in forensic DNA analysis. In addition to quantification analysis, the real-time DNA assay can be used to access genotype information in a sample. This can be achieved by using two differently labeled probes specific for a certain polymorphism (8–10). Alternatively, the real-time DNA quantification assay can be based on intercalation of the SYBR® Green dye to double-stranded DNA. Since heterologous PCR products sharing the same primer binding sites have different melting temperatures SNPs or insertions/deletions, genotyping can be detected by performing a melting curve analysis in addition to DNA quantification. The main advantages of using the SYBR® Green assay over the probe-based equivalent are that it requires less expensive reagents and equipment, resulting in a reduced overall cost for high-volume routine laboratories. Furthermore, the use of dissociation curve analysis will allow detection of nonspecific amplification, since the melting temperature of a primer-dimer product is significantly lower than the target. The SYBR® Green assay is flexible since there is no need for a specific probe, allowing an easy change of the target in the development phase of a typing system. Once developed, transition to a TaqMan-based probe system might be preferred for a higher specificity and elimination of the melting curve analysis. Most routine forensic laboratories use a quantification assay based on hybridization of a D17Z1 locus-specific probe. This slot blot approach is time-consuming, labor intensive, and less sensitive in comparison to the faster real-time quantification assay (7,11,12).

Sex determination is an important investigative tool, and routine STR analysis kits, such as AmpF*l*STR® Profiler Plus™ kit (Applied Biosystems, Foster City, CA) and PowerPlex™16 System kit (Promega Corporation, Madinson, WI), have a sex determination marker included. At least 0.5 to 1 ng nDNA of input DNA is recommended by manufacturers when using these kits, although smaller quantities of DNA can be typed using the LCN approach (13–15). Nevertheless, for a number of samples, sex determination using these typing kits fails or is not performed. This is apparent, for example, for samples where an mtDNA analysis is carried out but also applies to samples where a sensitive nuclear analysis of other markers such as SNPs is performed on short PCR fragments.

We chose to evaluate the use of the SYBR® Green assay to access genotype and DNA quantity information in forensics by anal-

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ysis of a sex determination marker. Although many typing systems contain a sex determination marker, this assay can be used to access rapid investigative information in forensic cases involving missing persons and no suspect cases where the DNA amounts are scarce. Moreover, it can be used as a first screen to reduce the number of samples that must be typed, as a tool in less well-equipped laboratories, or on degraded samples in ancient DNA studies. Furthermore, sex determination can be used to differentiate between individuals of different gender within a maternal linage in order to exclude matching family members in cases where mtDNA analysis has been performed.

The sex determination relies on melting curve analysis, which in combination with a kinetic analysis, allow quantification of nDNA. The dissociation assay is based on analysis of a 3-bp deletion on the X-chromosome revealing a 70 or 73-bp PCR product (Nucleotide 1470 to Nucleotide 1542) of the human enamel protein amelogenin gene (*Amel*) (16,17). Detection of the deletion is based on intercalation of the SYBR® Green dye to dsDNA, and a dissociation curve analysis displays the different melting temperatures of products from the X and Y chromosome. Furthermore, kinetic analysis of the obtained data can be used for quantification of nDNA in samples when compared with a standard curve. Here, we demonstrate the use of the SYBR® Green assay in forensic analysis as well as the applicability and limitations of a combined genotype analysis (sex determination) and nDNA quantification assay.

Materials and Method

Human male and female blood samples, serving as sex determination controls, were extracted using the Wizard® Genomic DNA Extraction Kit (Promega Corporation, Madison, WI). Male DNA from the Burkitt lymphoma human cell line was used for the quantification standard curve (Applied Biosystems, Foster City, CA). DNA concentrations were converted to copy numbers with the assumption that 1 ng of DNA corresponds to 333 genome equivalents. Forensic evidence materials collected on cotton swabs were extracted using the Wizard® Genomic DNA Extraction Kit (Promega). Forensic samples containing bloodstains were extracted using Chelex® 100 (Bio-Rad Laboratories, Hercules, CA) (18), and hair was extracted using proteinase K and DTT (5,19).

Primers were designed using the Primer Express™ software Version 1.0 (Applied Biosystems). A database search was performed on the PCR primers to evaluate the species specificity of the target. The Nucleotide BLAST was performed as a search for nearly exact matches against viruses, bacteria, and fungi showing high *E*-values for all hits. In addition to the BLAST search, specificity experiments were performed on Saccharomyces cerevisiae, E. Coli (strain W3110), and Mouse (Balb/C) DNA (Sigma, St. Louis, MO). PCR amplifications were performed using the SYBR® Green PCR Core Reagent kit (Applied Biosystems). The PCR reaction contained 5 μL of DNA (1/20 to 1/40 of the extract), 0.2 m*M* of the three dNTPs A, C, and G and 0.4 m*M* of dUTP, 4 m*M* $MgCl_2$, 1 × SYBR® Green PCR Buffer, 0.25 U AmpErase® UNG (uracil *N*′-glycosylase), 0.625 U AmpliTaq Gold® DNA Polymerase, and 0.18 μ*M* of each of the two primers, AmelY-1470F (5′CTCACCCCTTTGAAGTGGTACC) and AmelY-1542R (5′TCAGGGAATAAAGAACAAAATGTCTACA) in a total volume of 25 μL. Samples containing inhibitors were amplified with the addition of 0.16 mg/mL non-acetylated BSA (Sigma) to enhance the PCR efficiency. Amplifications and detections were performed in an ABI Prism® 5700 or 7000 Sequence Detector instrument (Applied Biosystems). Following enzyme activation steps at 50°C for 2 min (AmpErase® UNG) and at 95°C for 10 min (AmpliTaq Gold®), the samples were amplified for 50 cycles at 95°C for 15 s and at 50°C for 1 min. As controls, several no-template reactions were analyzed.

The GeneAmp® 5700 Sequence Detection System software Version 1.3 or GeneAmp® 7000 Sequence Detection System software Version 1.0 (Applied Biosystems) was used for construction of dissociation diagrams showing the melting temperatures of male and female control samples, male DNA in different concentrations, and of male/female mixtures in different ratios (100% male, 80:20, 50:50, 20:80, and 100% female). The software was further used for calculation of the threshold cycle number (C_t) for DNA copy number estimation. Diluted male control DNA was used to generate standard curves, from samples containing between 10^0 and 10^4 copies, by plotting against the log of the copy number. The copy number value for unknown samples was inferred from the regression line of the standard curves.

Results and Discussion

The sex determination assay is based on melting curve analysis demonstrating a 3-bp deletion on the X chromosome within the analyzed fragment. Intercalation of the SYBR® Green dye to the target allows a simultaneous denaturation analysis of fragments from both *AmelY* and *AmelX* to be illustrated in a dissociation diagram. The melting curve for a DNA sample from a male shows a clear difference to that from a female (Fig. 1). A total of 23 control samples of known gender (blood samples from twelve female and eleven male individuals) were analyzed, resulting in correct male or female specific melting curves for all samples (data not shown). In order to evaluate the human specificity of the target, a BLAST search was performed. Of the 1239 BLAST hits within the three groups consisting of viruses, bacteria, and fungi, the best match showed eleven mismatches against the reverse primer sequence (http://www.ncbi.nlm.nih.gov). A search against mammalia showed a low number of hits due to available sequence information from very few organisms (five mammals) in the databases searched (GenBank, EMBL, DDBJ, and PDB). Furthermore, PCR experiments on Saccharomyces cerevisiae, E. Coli, and Mouse DNA did not result in PCR products. However, if applying this marker in routine casework, specificity testing against other species (mammals and higher primates) is required for a full experimental evaluation of the human specificity.

In order to evaluate the sensitivity of the DNA quantification system, male control DNA was diluted in series down to single copies. A linear relationship ($r2 = 0.99760$, based on eight standard curves in four experiments) was found for the different DNA concentrations used in the standard curve (10^0 to 10^4 copies) when plotted on a log scale of DNA copy numbers versus C_t values. Reliable detection and quantification could be performed on control samples containing DNA amounts between 10^0 to 10^4 copies, the highest and the lowest amounts used in the standard curve. The reproducibility of the assay was examined using a set of five different control samples analyzed in six replicates. The observed difference between the replicates fell within the expected range of normal variation (Table 1). During the developmental phase of this system, 50 PCR cycles were used in the quantification assay to evaluate its sensitivity. Using this high cycle number has the advantage of revealing a highly sensitive quantification, allowing determination in the range of one to ten DNA copies between cycles 40 and 50. However, since the system had reached its limit, and low copy targets often reveal varying results, only samples containing at least 10 DNA copies per 5 μL were considered as positive when analyzing actual forensic samples. Moreover, for a final system to

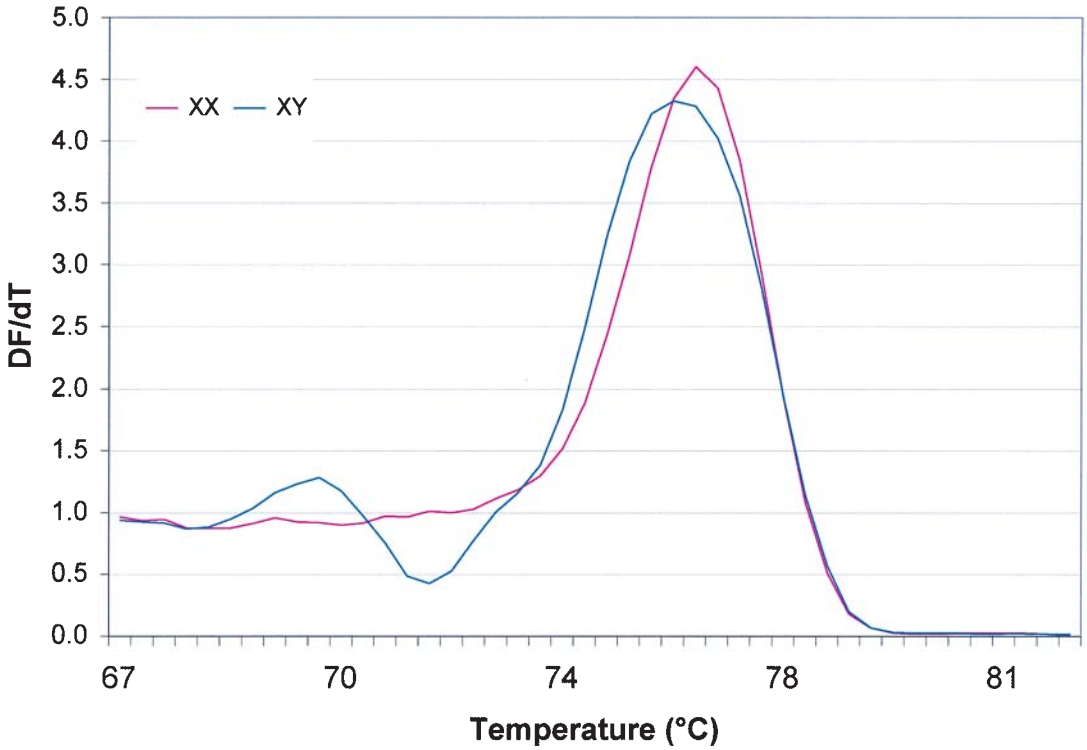


FIG. 1—*Illustration of melting temperature curves as change in fluorescence over time in analysis of male (XY) and female (XX) DNA samples. The analysis was performed in an ABI Prism® 5700 Sequence Detector instrument (Applied Biosystems).*

TABLE 1—*Summary of quantification analysis of 5 different control samples, results are given as average C_t-values and copy numbers for 6 replicates. The quantification results are shown as nDNA copies in 5μL extract. The analysis was performed in an ABI Prism® 7000 Sequence Detector instrument (Applied Biosystems).*

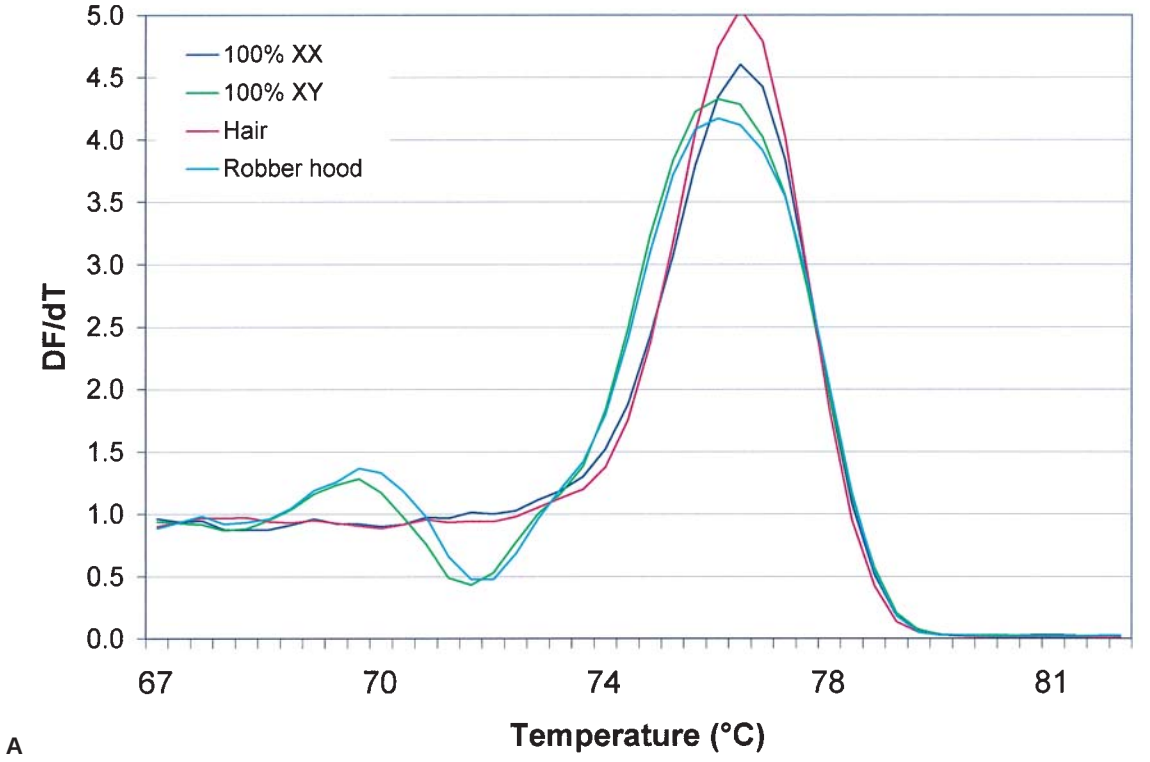
Sample Group	C_t Value	SD	Copies/5 μL	SD
DNA—1000 copies	28.42	0.24	899.83	125.66
DNA—100 copies	32.26	0.26	82.57	12.94
Hair Root 1	30.43	0.21	255.91	34.21
Hair Root 2	25.53	0.10	3088.49	194.33
Hair Root 3	29.88	0.12	227.45	16.83

be used in routine analysis, contamination issues must be considered and fewer cycles are recommended.

DNA quantification was performed on a number of forensic evidence materials that had previously been both mtDNA sequence analyzed and mtDNA/nDNA copy number quantified. The initial quantification was performed using a TaqMan® probe quantification assay, and the data were compared with the quantification obtained in the kinetic analysis used here (7). An overall correlation was apparent in the nDNA quantification using the two different systems. Furthermore, sex determination was accurate for all samples for where the sex of the donor was known (Fig. 2, Table 2). The reason for the discrepancy in the measured DNA copy numbers from the blood sample might be that the datapoint lies outside the standard curve, or that the results are derived from single tests in this experiment. The previously described assay is designed as a multiplex reaction for simultaneous mtDNA and nDNA quantification. It is highly sensitive due to binding of a target specific probe that is cleaved by the 5′ to 3′ exonuclease activity of the Taq polymerase during strand elongation. The cleavage results in increased reporter emission intensity, which is detected by the ABI Prism® 7700 Sequence Detector instrument (Applied Biosystems). Also when the two quantification systems were compared using a larger sample set comprising 40 different forensic materials (Table 3), the TaqMan® probe assay generally revealed higher DNA copy numbers than the SYBR® Green assay for the same evidence material. The generally higher copy numbers achieved by the TaqMan® probe assay are likely to reflect the higher sensitivity of the TaqMan® probe assay due to the different chemistry and detection instruments used in the two approaches.

The dissociation analysis of the male sample used in the standard curve frequently revealed a pattern similar to female samples when using a single starting copy, while ten starting copies always produced a correct sex determination (Fig. 3). The assay will thus require in the region of ten DNA copies for a correct sex determination. Although analysis of the DNA content in a single cell is theoretically possible, random dropout, preferential amplification, or stochastic effects cannot be excluded. This could potentially result in sex determination failure in this assay or incorrect genotype scoring in other assays since these problems can occur for all polymorphic nuclear DNA targets at low DNA copy numbers. The sensitivity of female DNA samples were not tested since the presence of purely X chromosome specific products will not cause the problem apparent in the analysis of *AmelY* and *AmelX* products.

Evidence material in forensic casework often contains a mixture of biological materials from more than one individual. Therefore, the performance of the assay was tested on male and female control samples mixed in different ratios. The dissociation curves were compared and a proportional difference in the melting curve pat-



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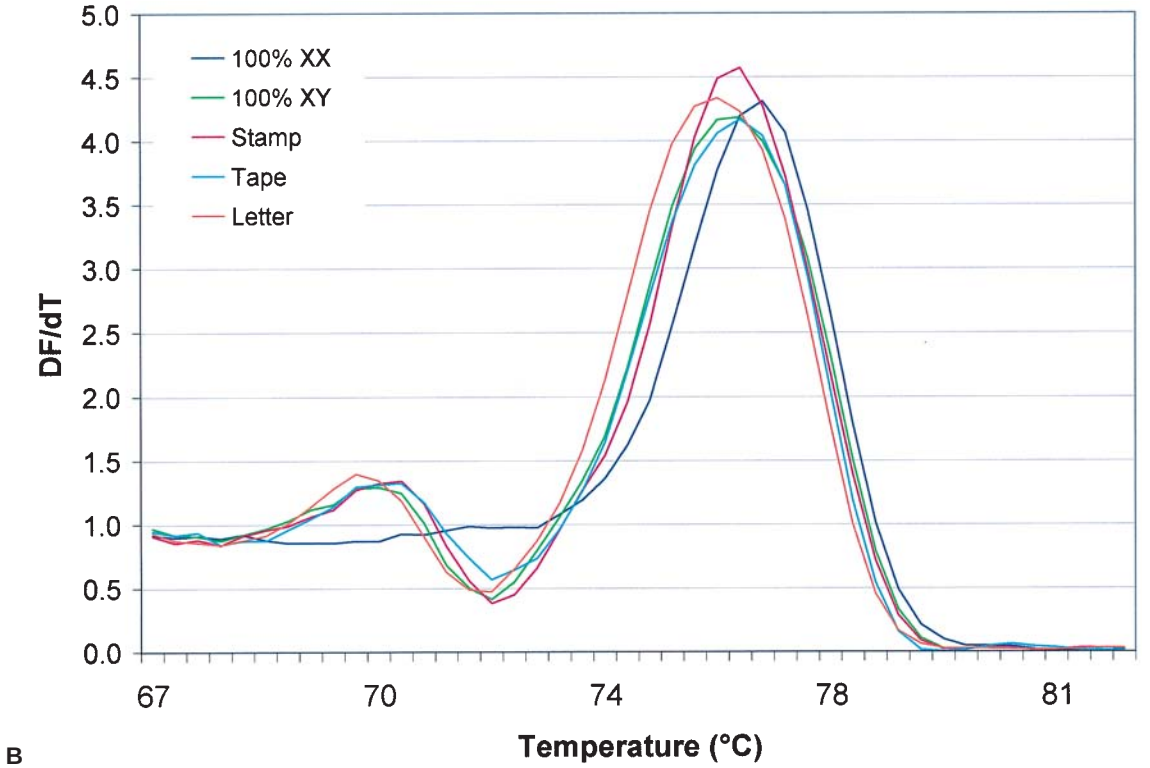

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FIG. 2—(A) *Melting curves obtained from saliva, a hair and a stain on a robber's hood*; (B) *melting curves obtained from skin cells on a piece of a tape and saliva stains on a stamp and a letter. The analysis was performed in an ABI Prism® 5700 Sequence Detector instrument (Applied Biosystems).*

TABLE 2—*Results of sex determination and nDNA quantification for a variety of previously analyzed evidence materials and a comparison with the TaqMan® probe assay (7). The quantification results are shown as total nDNA copies in the extract. The SYBR® Green analysis was performed in an ABI Prism® 5700 Sequence Detector instrument and the TaqMan analysis was performed in an ABI Prism® 7700 Sequence Detector instrument (Applied Biosystems).*

Material	Sex	SYBR® Green*	TaqMan® Probe*
Fingerprint/paper	XY	600	800
Saliva/Robberhood	XY	1 400	1 400
Hair	XX	400	300
Blood	XX	19 200	68 000
Glove	XY	2 000	3 600
Watch	XY	900	800
Pants	XY	700	600
Shoe	XY	800	1 400
Wig	XY	700	1 400

* DNA copy numbers were set to the closest hundred.

TABLE 3—*Average nDNA quantification results, from a total of 40 of previously analyzed different evidence materials, in a comparison between the SYBR® Green assay described here and the TaqMan® probe assay (7). The SYBR® Green analysis was performed in an ABI Prism® 5700 Sequence Detector instrument and the TaqMan analysis was performed in an ABI Prism® 7700 Sequence Detector instrument (Applied Biosystems).*

Material	Number	SYBR® Green*	TaqMan® probe*
Blood	7	1 963 600	3 937 000
Hair	10	26 700	26 500
Skin†/Fingerprints	23	800	1 000

* Average copy numbers were set to the closest hundred.
† Epithelial cells on clothes, jewelry, cell phones, etc.

tern could be seen (Fig. 4). However, it might be difficult to distinguish, for example, a mixed sample of 80% XY and 20% XX from a true male sample (100% XY) by visual comparison and interpretation of the shape of the melting curves using this system. Consequently, a DNA typing assay that allows a more precise quantification of mixtures would be preferred for further analysis of samples containing DNA mixtures. Such an assay can be designed in several different ways using either the TaqMan® probe or the SYBR® Green approach. A combination of a Y-chromosome specific marker and a marker measuring the total nDNA will reveal a ratio of the male DNA content within the total DNA in a sample. Moreover, using a set of several Y-specific SNP markers will further improve the system offering a possibility to distinguish even three donor mixtures. Here, the lack of multiplex capability in the SYBR® Green assay is a clear disadvantage. When fully developed, such assays might greatly improve the possibility to achieve STR-typing data by determining the appropriate amount of DNA required to ensure amplification of DNA from all contributors in a sample. In addition, the lack of multiplex capacity in the SYBR® Green assay makes it impossible to include an internal positive control in the system, while a second probe labeled with a different dye can detect a control target in the TaqMan system.

When analyzing several samples known to contain inhibitors, the majority of them could only be quantified in the presence of 0.16 mg/mL BSA (Table 4). In the analysis of these samples using the TaqMan® probe approach, quantifying mtDNA copies, an altered curve shape was observed (7). In contrast, neither of the nDNA systems revealed this pattern, possibly due to very low nDNA copy numbers in most samples. Moreover, samples containing inhibitors did not usually yield a dissociation curve when the samples were analyzed without BSA, whereas curves could be

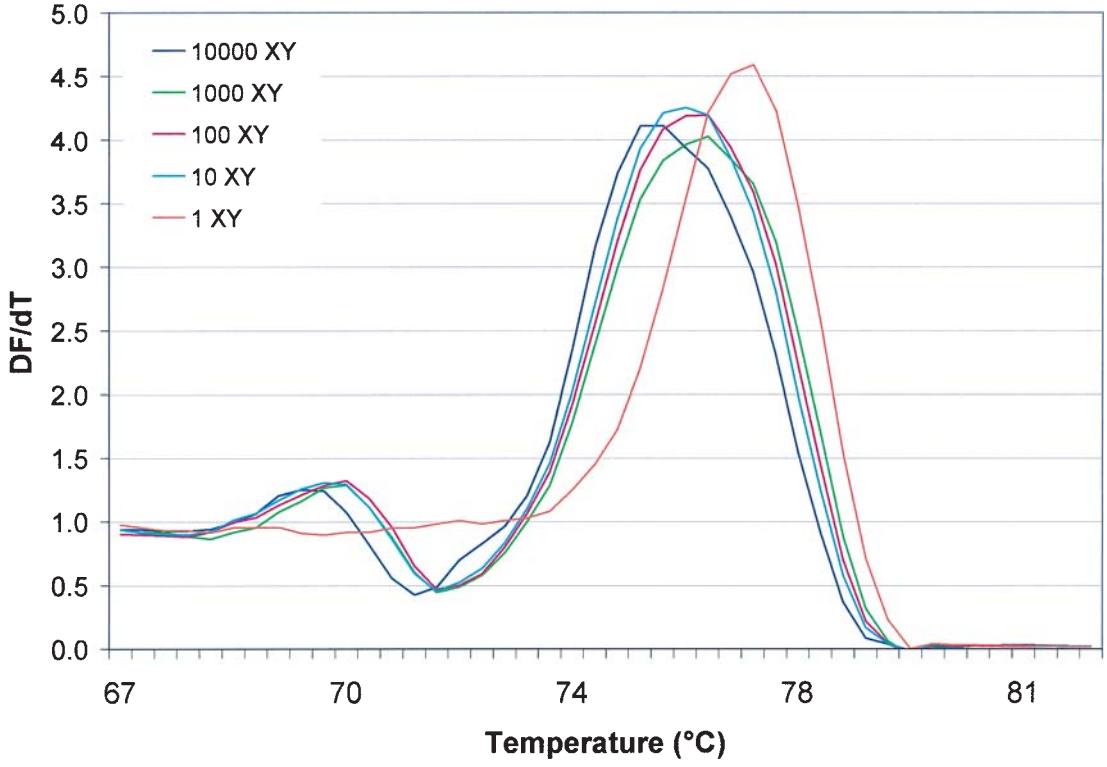

FIG. 3—*Dissociation curves for standard dilutions between 10^0 to 10^4 copies of human male control DNA. Amplification and detection was performed in an ABI Prism® 5700 Sequence Detector instrument (Applied Biosystems).*

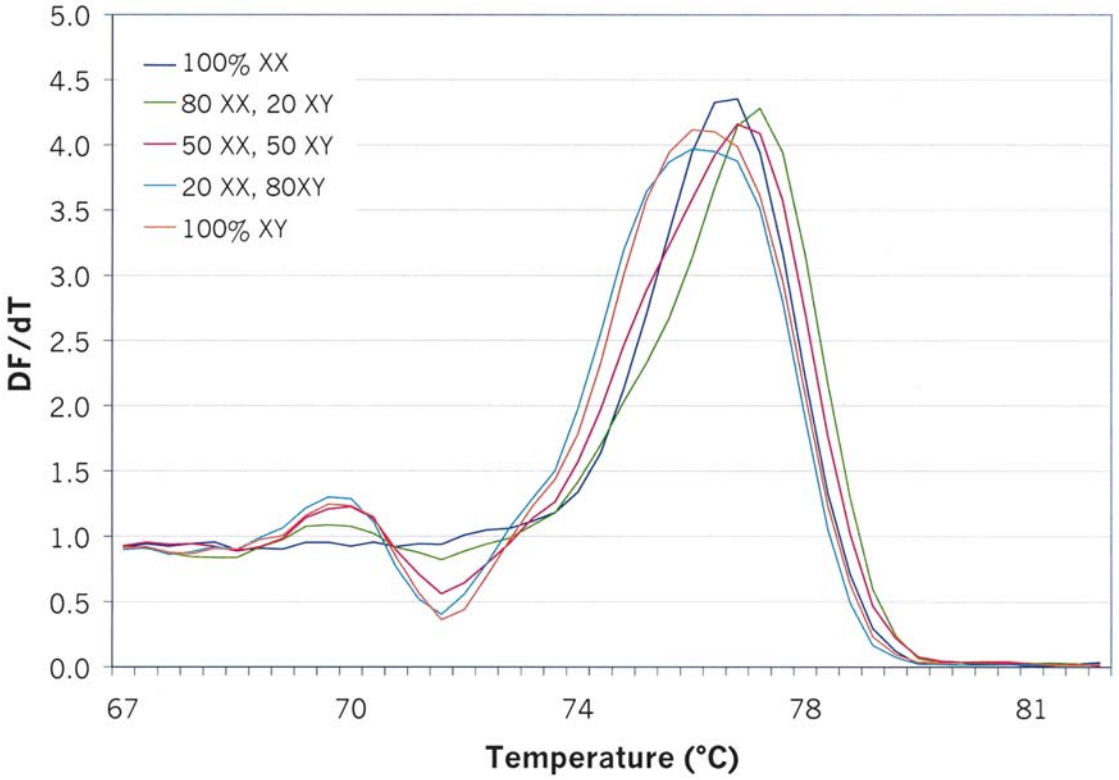

FIG. 4—*Dissociation curves for mixtures of male and female DNA in different ratios. The analysis was performed in an ABI Prism® 5700 Sequence Detector instrument (Applied Biosystems).*

TABLE 4—*Quantification of saliva stains from 1952, with and without addition of BSA and a comparison with the TaqMan® probe assay (7). The quantification results are based on single tests and shown as nDNA copies in 3-μL DNA extract. The analysis was performed in an ABI Prism® 7000 Sequence Detector instrument (Applied Biosystems).*

	nDNA SYBR® Green Assay		nDNA TaqMan®		mtDNA TaqMan®	
Sample	C_t Value	Copies/ 3 μL	C_t Value	Copies/ 3 μL	C_t Value	Copies/ 3 μL
1	0	0	39.36	1	35.47	40
1 + BSA	31.07	50	32.05	90	30.23	1200
2	38.87	0	36.93	5	33.39	160
2 + BSA	34.84	5	35.52	10	32.2	340
3	0	0	0	0	0	0
3 + BSA	31.81	30	33.47	30	29.99	1400
4	0	0	0	0	33.91	110
4 + BSA	32.95	15	34.68	15	30.81	820
5	0	0	0	0	0	0
5 + BSA	34.33	5	35.11	10	30.89	780
6	0	0	0	0	0	0
6 + BSA	30.36	90	31.85	100	29.64	1700

obtained with the addition of BSA (Fig. 5). This illustrates a risk that the actual number of DNA copies in the sample may be underestimated. However, an absolute quantification of a sample is irrelevant; the important issue is to perform the quantification assay and the subsequent PCR for the identification analysis under similar conditions. Therefore, we recommend addition of BSA in the quantification assay if BSA is used in the following typing assay.

Recent reports of mistyping, thought to be due to a deletion in the *AmelY* gene, have initiated a discussion on the reliability of the amelogenin-based sex tests in forensic casework analysis (20–22). In the routinely used multiplex STR typing kits, a 6-bp deletion in the amelogenin gene is determined, but other regions on the Y chromosome (for example *SRY*) or Y chromosome-specific STRs can also be used for sex determination. The sex determination assay described here is based on a different, shorter deletion in the amelogenin gene as well as on a shorter amplified fragment. Although this is a different marker for sex determination, further studies have to be performed in order to exclude problems similar to those described above.

This sex determination test is quicker to perform and more sensitive than previous amelogenin based sex tests (23) or the routinely used STR kits, AmpF*l*STR® Profiler Plus™ (Applied Biosystems) and PowerPlex™16 System (Promega Corporation). This is due to the real-time assay format, eliminating the need for post-PCR processing, and a substantially shorter PCR product, around 70 base pairs compared to approximately 110 base pairs. However, the short amplicon in the assay might be a disadvantage that could result in an overestimation of the template amounts available for amplification of longer targets, especially in degraded samples. Consequently, the assay will not necessarily reflect a true estimation of the number of available molecules for amplification of higher molecular weight targets.

Real-time quantification analysis has proved a reliable, rapid, robust and sensitive technique suitable for forensic casework analysis (7). An important application of a DNA quantification system is the ability to avoid artifact production or allele dropout by estimating the optimal DNA amount to use to ensure a successful downstream analysis. Furthermore, with quantification

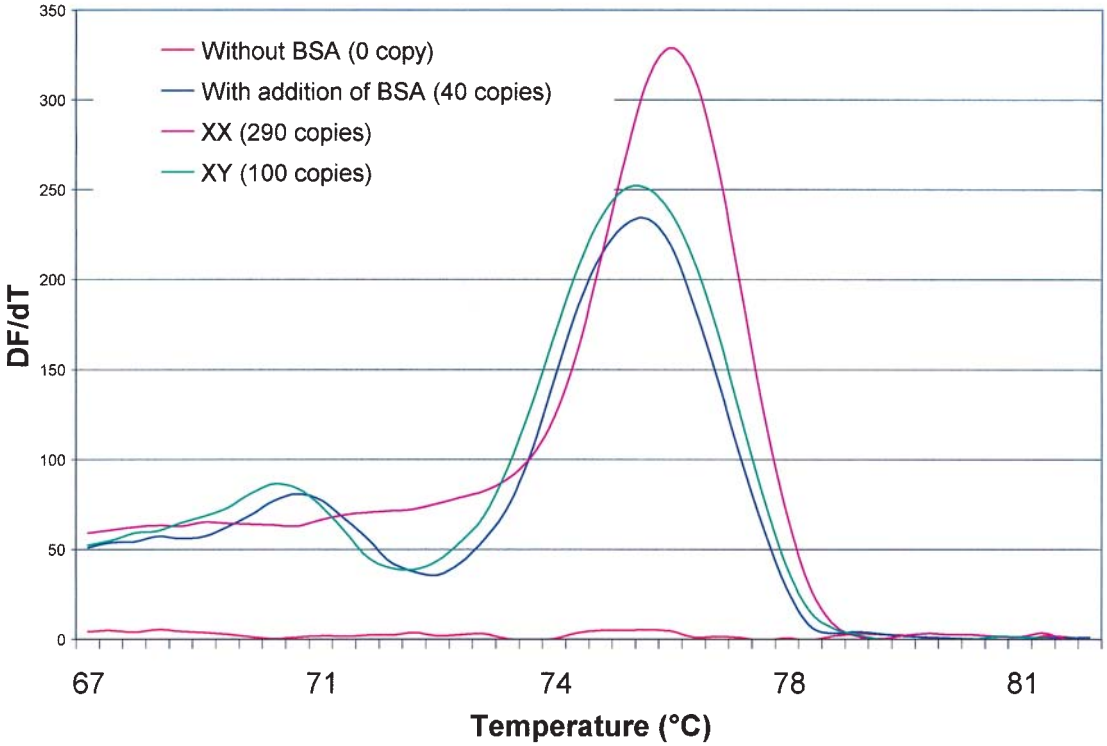

FIG. 5—*Dissociation analysis of saliva stains from 1952, with and without addition of BSA. The analysis was performed in an ABI Prism® 7000 Sequence Detector instrument (Applied Biosystems).*

data at hand, the laboratory can ensure that there will be sample remaining for a repeated in-house analysis or an independent analysis in a second laboratory. Additionally, the assay can be used to determine SNPs, deletions, and insertions using a dissociation curve analysis in the SYBR® Green assay or multiple probes in the TaqMan® probe assay. The flexibility allowing evaluation of different protocols and targets during PCR optimization (PCR efficiency, PCR specificity, product yield, and primer-dimer formation) is specific for the SYBR® Green quantification assay, and specially designed and expensive probes are not needed. Moreover, the SYBR® Green assay provides a cost-effective analysis using less expensive equipment and reagents. The sex determination marker used to evaluate the SYBR® Green assay will be useful in analysis of old and degraded DNA, or samples with minute amounts, where the routine analysis often fails, and has been applied successfully to a number of forensic samples containing limited amounts of DNA. Nevertheless, some limitations of the system are apparent. For example, when only a few starting copies are analyzed, artifacts or stochastic effects can result in incorrect genotyping in the dissociation analysis. Moreover, in analysis of mixed samples the interpretation might be difficult in the present system. In conclusion, the SYBR® Green assay will be useful for PCR optimization, quantification, and DNA typing in forensic analysis of certain samples and may provide a cost-effective alternative to the TaqMan® quantification assay. However, further evaluations and validation of the technology is necessary for use in routine forensic analysis.

Acknowledgments

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