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Specific Quantification of Human Genomes from Low Copy Number DNA Samples in Forensic and Ancient DNA Studies

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We reviewed the current methodologies used for human DNA quantitation in forensic and ancient DNA studies, including sensitive hybridization methods based on the detection of nuclear alpha-satellite repetitive DNA regions or more recently developed fluorogenic real-time polymerase chain reaction (PCR) designs for the detection of both nuclear and mitochondrial DNA regions. Special emphasis has been put on the applicability of recently described different real-time PCR designs targeting different fragments of the HV1 mtDNA control region, and a segment of the X-Y homologous amelogenin gene. The importance of these quantitative assays is to ensure the consistency of low copy number DNA typing (STR profiling and mtDNA sequencing).

Key words: *DNA; DNA fingerprinting; DNA mitochondrial; evolution; forensic medicine; polymerase chain reaction; tandem repeat sequences*

The specific quantification of human DNA molecules has gained great importance in forensic (1-3) and ancient DNA studies (4,5) as aid in the interpretation of the consistency and the reliability of polymerase chain reaction (PCR)-based short tandem repeat (STR) profiling and mitochondrial DNA (mtDNA) sequence analysis from low copy number DNA samples (6-10). First, an estimation of the quantity of human genome is a recommended procedure in forensic casework (11,12) that will aid to decide whether the isolated DNA is suitable for nuclear or mitochondrial DNA analysis and to adjust the DNA input to improve the performance of subsequent end-point PCR-based DNA typing approaches. Second, classification of low copy number DNA samples by DNA content allowed decision to be made on the number of DNA typing repetitions required for statistically reliable results (13). In particular, it is important to consider three types of errors that could affect DNA typing reliability when low copy number DNA samples are analyzed: allele drop-out, stutter false alleles, and the influence of low levels of DNA contamination (8-10). Third, DNA quantification ensures the optimal use of the limited amounts of DNA found in many forensic evidences ensuring that the DNA is not wasted with expensive repetitive endpoint PCR typing analysis performed with inappropriate amounts of DNA template. Furthermore, some DNA quantification designs could also provide additional information about DNA degradation (14,15), the presence of PCR inhibitors (3), or specific information about the DNA content of X and Y sexual chromosomes (14-17).

We reviewed the most recent methodologies used for human DNA quantitation in forensic and ancient DNA studies, including sensitive hybridization methods based on the detection of nuclear alpha-satellite repetitive DNA regions (1,18) or more recently developed fluorogenic real-time PCR designs for the detection of both single-copy nuclear genes and mtDNA regions (3,14-17). Special emphasis has been put on the applicability of different real-time PCR designs recently described by the authors targeting different fragments of the hypervariable (HV)1 mtDNA control region, and a segment of the X-Y homologous Amelogenin (AMG) gene (14,15).

Nuclear DNA Quantification

Detection of Highly Repetitive Human DNA Sequences by Molecular Hybridization

At present, the most popular method for quantification of picogram quantities of the nuclear human DNA in forensic genetics is the slot-blot hybridization approach (1,18) This method allows detection of highly repetitive satellite DNA regions with the primate-specific alpha-satellite probe to the D17Z1 locus (19). Early development of a commercial chemiluminescent human DNA quantification kit (20) to tar-

get this satellite locus has clearly contributed to a valuable worldwide standardization of this quantitation method in forensic genetics. The kit is easy to perform, has a high specificity for primate DNA samples, and offers a sensitive standard detection limit of 30 pg/μL that correspond to approximately 10 DNA copies/μL if we assume 3 pg as the haploid human genome content of a single cell (21). Although the method is generally used as a semi-quantitative assay (eye-based interpretation of the film), a CCD camera image system with similar range of sensitivity can capture the chemiluminescent signal of the D17S1 probe, providing enhanced capability of data interpretation (22). More recently, a new human DNA quantitation kit with similar sensitivity has been developed to target alpha-satellite primate DNA sequences by using a new luciferase chemiluminescent detection system based on the Readit technology (23,24).

Perhaps the main limitation of these hybridization procedures for human DNA quantification is that their detection limit is above the limit of the PCR profiling range obtained with different commercial kits for multiplex STR typing. Therefore, a proportion of low copy number DNA samples that gave negative results for human DNA quantitation can still be genotyped by PCR-based methods. We have recently reported that a total of 30% of 4-5 years old bone samples that gave negative chemiluminescent signal after slot-blot hybridization with the D17Z1 probe yielded reliable genotyping results when analyzed with two different kits for PCR multiplex of STR markers (15). It has also been described that the performance of the slot-blot hybridization assay could be affected by the state of DNA degradation (25) or by the presence of high amounts of microbial DNA on the DNA extracts retrieved from skeletal remains (2). On the other hand, one interesting advantage of these molecular hybridization methods in comparison with the PCR-based methods is that the former is not sensitive to Taq polymerase inhibitors. Therefore, a PCR failure with a DNA sample that yielded a positive signal after hybridization with the D17Z1 probe could alert to the presence of inhibitors of the Taq polymerase activity on the DNA extract.

End-point PCR Methods

Different fluorogenic end-point PCR methods have been developed for quantification of nuclear human DNA from forensic samples, including an Alu-based quantitation protocol using a fluorescently labeled primer (26) and a procedure based on specific amplification of the TH01 locus followed by Pico Green dye detection (27). The main limitation of PCR end-point measurements is that they are generally made after a fixed number of cycles, when the reaction is beyond the exponential phase and some reaction components act as a limiting factor. Therefore, a wide variation on the final amount of PCR-amplicons could be generated among different replicates with the same starting DNA copies. The linear correlation between the accumulated amplicon and initial DNA template amount typically observed in end-point assays is just for one or two orders of magnitude.

Real-time PCR Designs to Estimate the DNA Content of Specific Human Chromosomes

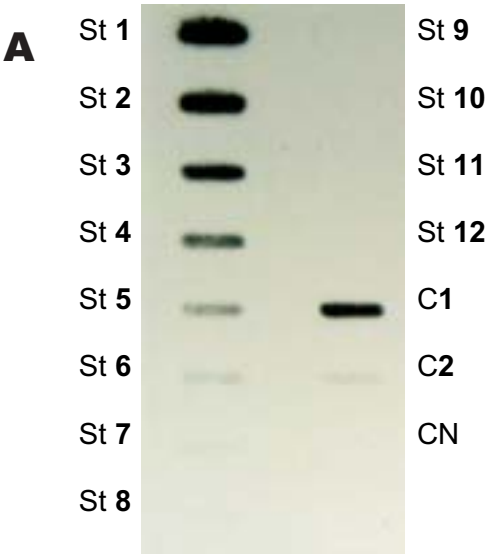
There are two general fluorogenic methods to monitor the real-time progress of the PCR. The first one is by measuring Taq polymerase activity with double-stranded DNA binding dye chemistry (SYBR Green or ethidium bromide) (28), and the other is by measuring the 5-nuclease activity of the Taq DNA polymerase to cleave a target-specific fluorogenic probe (an oligonucleotide, complementary to a segment of the template DNA, with both a reporter and a quencher dye attached, which only emits its characteristic fluorescence after cleavage) (29). Real-time analysis of the fluorescence levels at each cycle of the PCR (amplification plot) allows obtaining a complete picture of the whole amplification process for each sample. In the initial cycles of PCR, a baseline is observed without any significant change in fluorescence signal. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. The higher the initial input of the target genomic DNA, the sooner a significant increase in fluorescence is observed. The cycle at which fluorescence reaches an arbitrary threshold level during the exponential phase of the PCR is named threshold cycle (Ct). A standard curve can be generated by plotting the log of the starting DNA template amount of a set of previously quantified DNA standards against their Ct values. Therefore, an accurate estimation of the starting DNA amount from unknown samples is accomplished by comparison of the measured Ct values and the Ct values of the standard curve. Compared with end-point PCR quantification methods, the use of Ct values is a more reliable quantification assay. This is mainly due to the fact that Ct determination is performed during the high precision exponential phase of the PCR when none of the reaction components is limiting contrary to PCR end-point measurements.

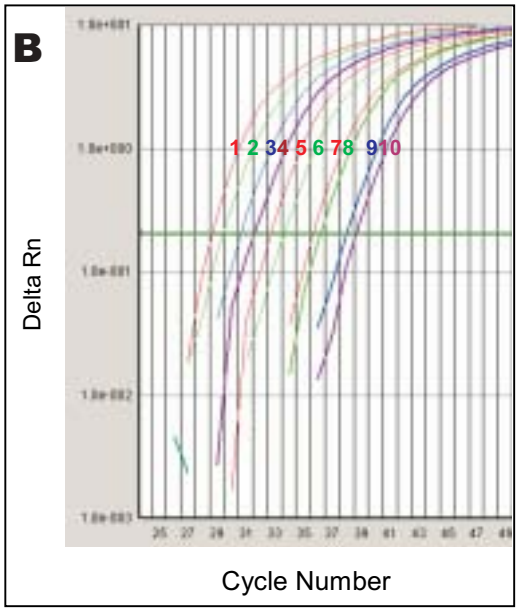
SYBR Green-based real-time detection has been used to target Alu sequences (30,31), or the amelogenin gene (16) from forensic specimens. One limitation of SYBR Green-based detection is that non-specific amplifications (primer-dimer or non-human products) cannot be distinguished from specific amplifications. On the other hand, the amplicon-to-dye ratio varies with amplicon length. Obviously, SYBR Green can only be used in singleplex reactions.

The use of probe-based real-time PCR to quantify human nuclear DNA in forensic analysis has been recently described by Andréasson et al (3). A 78-bp region of the human retinoblastoma susceptibility gene (RB1), a nuclear-encoded single copy gene located on chromosome 13, was target in a multiplex-PCR quantification assay that was also designed to amplify an mtDNA target. The system has been shown to detect down to nuclear DNA single copies in the dilution series of the standard curve and has been applied to quantify nuclear DNA from different forensic specimens, such as skin debris, saliva stains, hair, and bloodstains. Altered amplification plots were observed during the analysis of 50 years old saliva stains on stamps and enveloped due to the presence of in-

hibitors. The addition of bovine serum albumin (BSA) and extra Taq amounts has proven efficient in overcoming the effects of the inhibitors. Other probe-based real-time PCR designs to target Alu sequences (31), STR markers (32), or the amelogenin gene (14, 16,17) were also reported in different forensic meetings.

We have recently described a method for nuclear DNA quantification based on the real-time PCR-amplification of a segment of the X-Y homologous amelogenin (AMG) gene (33,34), which allowed the simultaneous estimation of a Y-specific fragment (AMGY: 112-bp) and a X-specific fragment (AMGX: 106 bp), making possible not only DNA quantitation, but also sex determination (14,15). Detection of the specific AMGX-fragment (106 bp) and AMGY-fragment (112 bp) was achieved by using the primer pair sequences (34) and two newly designed fluorogenic minor groove binder probes that specifically detect the AMGX-fragment (TATCCCAGAT GTTTC, FAM-labeled) or the AMGY-fragment (CATC CCAAATAAAGTG, VIC-labeled) (14,15). The minor groove binder probes were designed to target the 6-bp X-deletion / Y-insertion segment within the AMG second intron fragment (33). Two different standard curves can be generated with this design – the X standard curve by plotting the log of the starting X chromosome copies from a female or a male DNA standard against the Ct values measured by the AMGX-FAM detector, and the Y standard curve by plotting the log of the starting Y chromosome copies from a male DNA standard against the Ct values measured by the AMGY-VIC detector. The sensitivity of the AMGX-FAM design was slightly better than the





C

Standards	DNA amount pg	DNA amount log (pg)	Ct values mean	Ct values SD
St 1	10,000	4	29.48	±0.404
St 2	5,000	3.699	30.34	±0.320
St 3	2,500	3.398	31.58	±0.381
St 4	1,250	3.097	32.51	±0.236
St 5	625	2.796	33.83	±0.332
St 6	312	2.494	34.88	±0.762
St 7	156	2.193	36.69	±0.270
St 8	78	1.892	37.72	±0.622
St 9	39	1.591	38.74	±0.014
St 10	19.5	1.290	39.15	±1.193

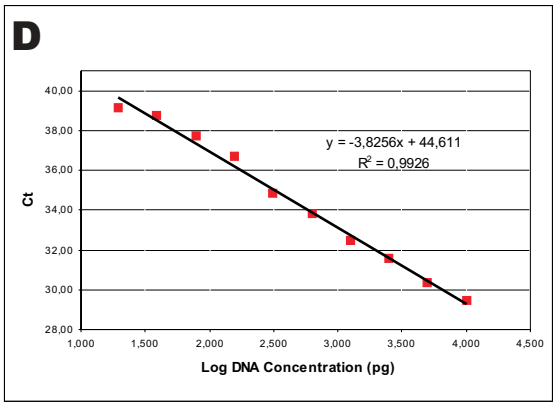
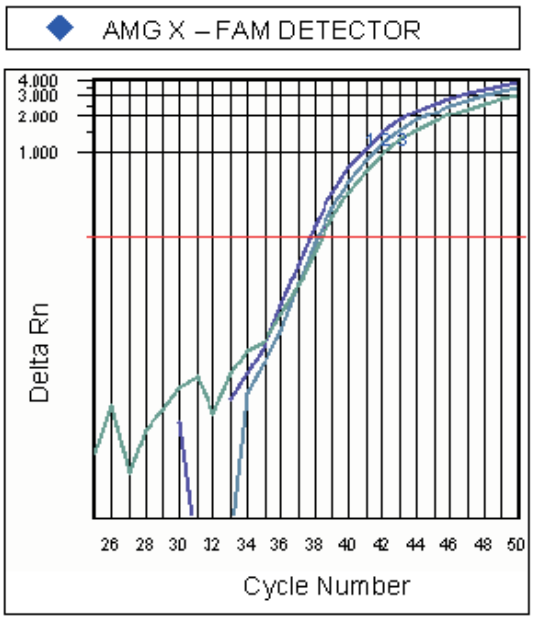
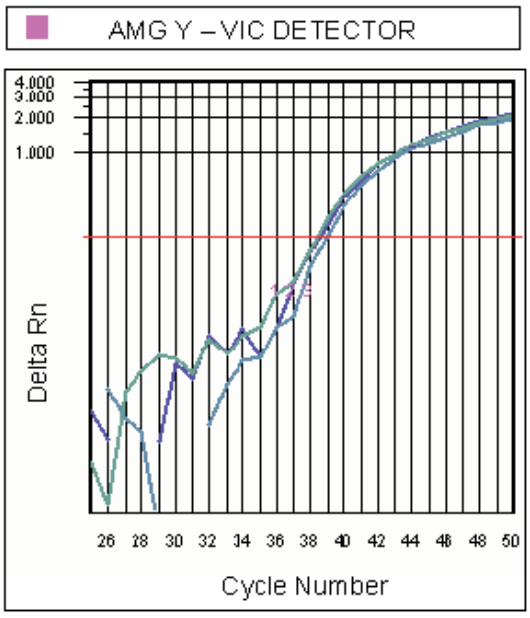


Figure 1. Nuclear DNA quantification – a comparison between molecular hybridization and real-time polymerase chain reaction (PCR). **A**. Quantiblot results of 2-fold serial dilutions of female DNA standards (from St 1:10,000 pg to St 12:4.9 pg), with a detection limit of 156 pg (St 7:31 pg/µL), two positive controls (C1 and C2), and a negative control (CN). **B**. Amplification plot of the same female DNA standards (see A) as monitored by amelogenin (AMG)-X specific Taqman probe. The detection limit in this experiment was 19,5 pg (St 10:4 pg/µL); St 11 and St 12 were undetectable. **C**. Average threshold cycle (Ct) values and standard deviations (SD) from three independent AMG-X real-time PCR experiments performed with the female DNA standards shown in A and B. **D**. Standard curve (average Ct values against log DNA concentration) of the data showed in C.

A



B



C

Bone sample	Observed Ct values		Sex determination	[DNA] Total (pg/µL) (AMG-X detection) (× 2)
	AMG-X detector	AMG-Y detector		
1	37.59	38.44	male	28
2	38.09	38.85	male	20
3	38.32	38.30	male	18

D

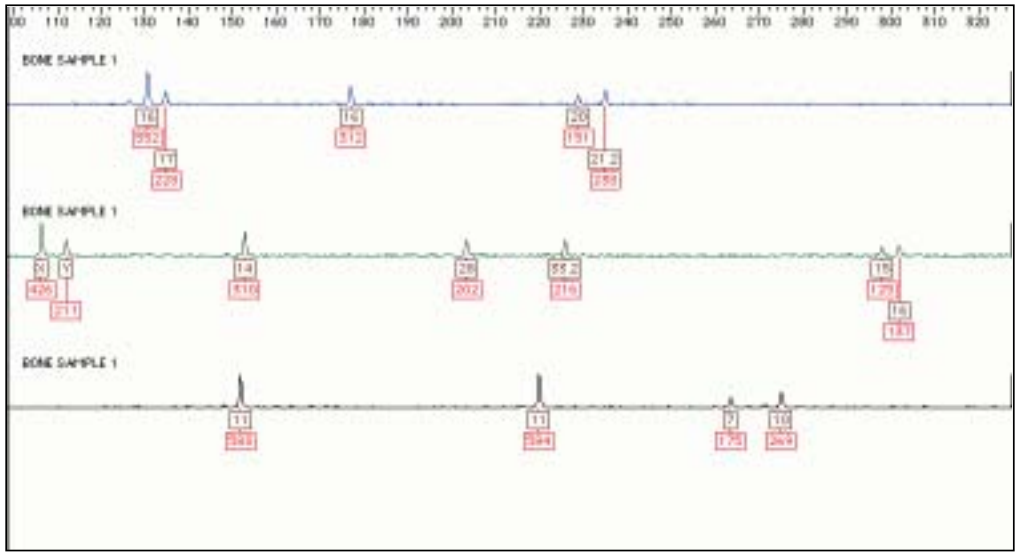
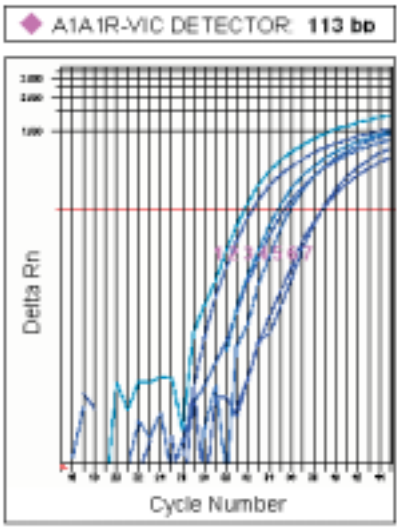
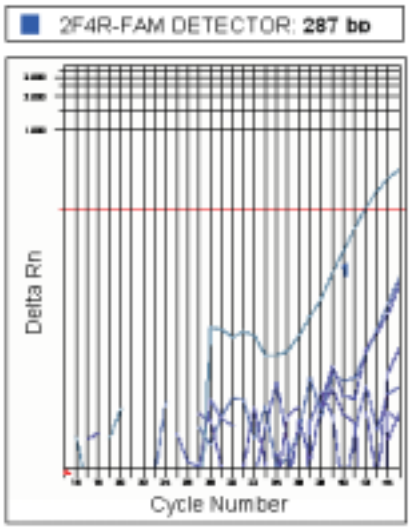


Figure 2. Nuclear DNA quantification and sex determination from three bone DNA samples that rendered negative Quantiblot results. **A**. Amplification plot of three bone DNA samples (1, 2, and 3) as monitored by the amelogenin (AMG)-X Taqman probe. **B**. Amplification plot of three bone DNA samples (1, 2, and 3) as monitored by the AMG-Y Taqman probe. **C**. AMG-X and AMG-Y Ct values, sex determination results, and calculated DNA amount (by using the AMG-X detector and the trendline showed in fig. 1D) for the three bone DNA samples displayed in A and B. **D**. Low quality short tandem repeat (STR) profile (AmpflSTR profiler Plus) obtained from bone sample 1 after 30 polymerase chain reaction (PCR) cycles by using a calculated amount of 18 DNA starting copies for heterozygote loci or 36 DNA starting copies for homozygote loci. Note the extensive allele imbalance for several loci (including AMG).

A





B

C

		Ancient teeth (T) samples (1500-500 bp)						
Detector	Parameter	T1	T2	T3	T4	T5	T6	T7
113 bp	Ct*	31.73	32.49	34.79	35.40	35.89	38.77	38.86
	DNA copies/µL	>500	500	125	86	64	<50	<50
287 bp	Ct	41.89	ND†	ND	ND	ND	ND	ND
	DNA copies/µL	3	neg‡	neg	neg	neg	neg	Neg

*Threshold cycle value.
†Not detected.
‡Negative.

Figure 3. Mitochondrial DNA (mtDNA) quantification and degradation state evaluation from seven ancient teeth samples (1500-500 BP) by using real-time polymerase chain reaction (PCR) to target two different fragment sizes (113 bp and 287 bp) within the HV1 region of the human mtDNA. A. Amplification plot of seven ancient DNA samples with the A1A1R-VIC detector (113 bp). B. Amplification plot of seven ancient DNA samples with the 2F4R-FAM detector (287 bp). C. Observed threshold cycle (Ct) values and calculated mtDNA copy number from each ancient DNA sample.

AMGY-VIC design, but in both cases determination of single nuclear DNA copies was possible in the standard dilution series. However, amplification dropout has been observed for both the AMG-X and AMG-Y detectors when DNA input was under 60 pg, which corresponded to approximately 20 AMG-X copies in female DNA, and 10 AMG-X and 10 AMG-Y copies in male DNA. A comparison between the molecular hybridization method to target the D17Z1 locus and the AMG-X real-time PCR design for human DNA quantitation is shown in Figure 1. This real-time PCR design is highly sensitive and specific method for both nuclear DNA quantitation and sex determination from forensic bone samples (15). A total of 30% (17/57) of bone samples that gave negative chemiluminescent signal after slot-blot hybridization with the D17Z1 probe resulted positive by this real-time PCR design. Obviously, a high incidence of allele dropout was observed from these low copy number samples when STR typing was attempted, especially for the larger STR markers; in some cases only reliable partial STR profiles were obtained after multiple amplification reactions with different DNA input. On the other hand, all the bone samples analyzed offered AMGX/AMGY real-time PCR results that were in agreement with the results of sex determination by amelogenin typing performed with the AmpflSTR Profiler Plus and Cofiler kits (15). Figure 2 shows the results of nuclear DNA quantification and sex determination from three of these bone DNA samples that rendered negative Quantiblot results.

At present, we are interested to assess the utility of this sensitive technology to the quantification of nuclear DNA from other types of low copy number DNA samples and especially from imbalanced male/female mixtures of body fluid and shed hairs.

Mitochondrial DNA Quantification

Competitive PCR Methods

Mitochondrial DNA quantification has not been performed in a routine way in forensic analysis. Competitive PCR methods were first used to quantify the number of mtDNA molecules from bone samples in ancient DNA studies (5). Different dilutions of a competitor DNA template with known concentration (a PCR amplicon of 398 bp was constructed encompassing positions 16190 to 16517 of the mtDNA control

region but with 22 bp deletion from positions 16210 to 16231) were mixed with a constant amount of DNA extract and were PCR-amplified. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining to determine the ratio of target to competitor on each dilution. The system, however, has important limitations. First of all, ethidium bromide staining after agarose gel electrophoresis does not offer an accurate measurement of target and competitor amplicons ratio. Furthermore, the dynamic range of the competitive assay is limited to a target/competitor ratio of about 1:10 to 10:1. Another drawback is that several samples of the valuable DNA extract obtained from each evidence material are wasted to perform this competitive PCR assay, with the risk of exhausting the sample material before DNA profiling is completed.

Real-time PCR of Mitochondrial DNA

The use of real-time PCR for quantification of mitochondrial human DNA from forensic specimens has been recently reported (3). The mitochondrial target is a 142 bp region spanning over the genes for tRNA Lysine and ATP synthase 8 that can be amplified in a single PCR reaction or in combination with a nuclear DNA target. The target can be detected down to single mtDNA molecules from the DNA standards and a high reproducibility has been demonstrated from five independent real-time PCR experiments. The assay was tested on 236 forensic specimens (hair, bloodstains, fingerprints, skin debris, saliva stains, and others) containing from 0 to >100,000 mtDNA copies. The mtDNA sequence analysis (HV2 region of the D-loop) on these evidentiary materials was successful when at least 80 mtDNA copies were used for HV2 end-point PCR.

We have recently reported the specific quantification of human mtDNA by monitoring the real-time progress of the PCR-amplification of two different fragment sizes (113-bp and 287-bp) within the hypervariable region I (HV1) of the mtDNA control region (35), using two fluorogenic probes to specifically determine the mtDNA copy of each fragment size category (14,15). This additional information (number of copies in each size category) could be very helpful to evaluate the mtDNA preservation state from ancient bone samples. Both targets, 113 bp and 287 bp, could be detected down to single copies in the dilution series of the mtDNA standards. However, an increased incidence of PCR failures was observed when the starting DNA amount was less than 60 copies. The mtDNA real-time PCR design was applied to estimate both DNA content and DNA degradation state from ancient DNA samples retrieved from 500 to 1,500 years old skeletal remains. The majority of samples only yielded low or very low DNA starting copies (500-10) when analyzed with the 113-bp design, whereas negative results were obtained from these samples with the 287-bp design (Fig. 3). These results clearly indicated that a high proportion of mtDNA samples were highly degraded. This valuable information about DNA copy number and degradation state was crucial in making decision about the better strategy to succeed with the HV1 sequence analysis from these samples. All the ancient DNA samples that yielded positive real-time PCR results for the 113 bp target also rendered enough PCR products when analyzed by end-point PCR (36 cycles) of short amplicons (around 100 bp) for subsequent HV1 sequencing analysis (36). Those DNA samples with the lower mtDNA copy number as determined by real-time PCR were also the samples that offered more difficulties to obtain high quality (free from nucleotide ambiguities) sequencing results.

Criteria for Low Copy Number DNA PCR-profiling Accuracy Based upon Sensitive and Specific Quantitation of Human DNA Template

Low copy number DNA profiling has been defined as typing samples containing less than 100 pg of starting DNA template (8) and is generally associated with stochastic variation. In practice, this fixed threshold could vary within and between laboratories, depending on several methodological aspects, including variation in both estimates of DNA quantity and PCR efficiency. Therefore, low copy number typing has also been defined as the analysis of any results below the stochastic threshold for normal interpretation (10). Low copy number typing has several limitations, especially when profile reliability and reproducibility cannot be investigated. Allele dropout, stutter false alleles, and the influence of sporadic background contamination should at least be considered when interpreting low copy number DNA profiles (8-10). Increasing the number of PCR cycles (from 28 to 34) has been used to enable better detection of low copy number DNA profiles (8). An increased number of artificial STR profiles (ie, increase of stutter false alleles) has also been observed when using 34 PCR cycles (8). Furthermore, there is a higher risk of PCR amplification from low levels of background DNA contamination, because of the extreme sensitivity. Therefore, under certain circumstances (possibility of adventitious transfer) and especially for certain non-discrete forensic samples (body fluid stains or cellular debris), a low copy number DNA profile cannot be used for exculpatory purposes (10). Other strategies have been described to enable better detection of STR profiles. These included different methods to increase the sensitivity of detection of current capillary electrophoresis instruments without increasing the cycle number (10).

The first strategy that should be attempted to improve the sensitivity of PCR low copy number profiling is to quantify the amount of DNA template to adjust the DNA input to obtain an acceptable stochastic threshold. Obviously, increasing the DNA input is not always possible for different reasons (limited DNA amount or presence of inhibitors) and sometimes, estimation of DNA quantity is not accurate or sensitive enough, rendering false negative quantification results from low copy number DNA samples. However, the recent development of sensitive real-time PCR methods (with a theoretical detection limit of a single DNA copy) for human DNA quantification offers a very interesting tool to classify low copy num-

ber DNA samples by DNA content or degradation state, providing crucial information for subsequent DNA typing analysis and also for the final interpretation of low copy number profiles. If DNA input is below the stochastic threshold for an unambiguous result, the reproducibility of the DNA profile should be investigated from DNA extraction replicates (if possible) or just from PCR amplification replicates with different DNA inputs where only one DNA extraction can be performed (minimal stains or shed hairs). Although reproducibility of PCR replicates from a single DNA extract would be obtained, the significance of the result (depending on the case and on the sample type) should be interpreted with caution, especially if adventitious transfer cannot be excluded (9,10). Independent replication from a second DNA extract is a usual procedure to ensure DNA typing reliability from low copy number DNA samples isolated from bones and teeth. Independent replication between two or more labs is also required for certain ancient DNA studies (4).

It is important to note that a negative PCR-based DNA quantification results could be due to the absence of DNA or to the presence of Taq inhibitor compounds on the DNA extract. A relatively high incidence of PCR inhibitors could be predicted when analyzing bone and ancient DNA samples and other forensic samples. Partial inhibition of both Taq polymerase and 5-nuclease activities could produce a situation similar to that of low concentration DNA samples (13). Therefore, the use of BSA to prevent Taq inhibition should be a standard practice on quantitative real-time PCR assays in forensic and ancient DNA studies. On the other hand, the development of an internal standard control to determine the PCR efficiency from each individual sample could be of great help to interpret negative real-time PCR results.

Low copy number profiling is frequently more complicated due to the presence of a minor low copy number profile mixed with a high copy number profile. Imbalanced mixtures of low copy number male DNA and high copy number female DNA are very often detected during the analysis of sexual assault cases. The use of real-time PCR designs to specifically target the number of X and Y sexual chromosome copies (14,15,17) could be of great help for an accurate detection of each contribution from these imbalanced male/female mixtures, helping to decide the initial DNA input to succeed with the subsequent DNA analysis of the low copy number male component.

Another cause that could lead to a low copy number DNA situation is extensive DNA degradation. This is very frequently observed in DNA samples obtained from skeletal (forensic and ancient) remains that have suffered extensive DNA damage, because of the nucleases attack and the effect of oxidative and hydrolytic processes (4). The development of a mtDNA real-time PCR quantitative assay to target specifically two different fragment sizes (113 bp and 287 bp) (14,15) has been demonstrated as effective in evaluation of both mtDNA copy number and degradation state from ancient bone and teeth samples.

In conclusion, the current methods for highly sensitive quantification of human DNA retrieved from forensic and ancient DNA samples are important because they ensure the consistency of low copy number DNA typing (STR profiling and mtDNA sequencing). Real-time quantitative PCR offers several advantages with respect to other current (hybridization or end-point) methods, including higher sensitivity and dynamic range of quantitation, unnecessary post-PCR processing, automation feasibility and high throughput, and the possibility to simultaneously perform different qualitative analysis (sex determination, mtDNA degradation, Taq inhibition rate). Further validation studies within and between laboratories are needed to evaluate the potential applications of this technology in forensic and ancient DNA studies.

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