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Review

Advances in forensic DNA quantification: A review

This review focuses upon a critical step in forensic biology: detection and quantification of human DNA from biological samples. Determination of the quantity and quality of human DNA extracted from biological evidence is important for several reasons. Firstly, depending on the source and extraction method, the quality (purity and length), and quantity of the resultant DNA extract can vary greatly. This affects the downstream method as the quantity of input DNA and its relative length can determine which genotyping procedure to use—standard short-tandem repeat (STR) typing, mini-STR typing or mitochondrial DNA sequencing. Secondly, because it is important in forensic analysis to preserve as much of the evidence as possible for retesting, it is important to determine the total DNA amount available prior to utilizing any destructive analytical method. Lastly, results from initial quantitative and qualitative evaluations permit a more informed interpretation of downstream analytical results. Newer quantitative techniques involving real-time PCR can reveal the presence of degraded DNA and PCR inhibitors, that provide potential reasons for poor genotyping results and may indicate methods to use for downstream typing success. In general, the more information available, the easier it is to interpret and process the sample resulting in a higher likelihood of successful DNA typing. The history of the development of quantitative methods has involved two main goals—improving precision of the analysis and increasing the information content of the result. This review covers advances in forensic DNA quantification methods and recent developments in RNA quantification.

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DNA / Quantification / Real-time PCR
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1 Introduction

Several advances in forensic molecular methods for DNA typing of biological evidence have been made over the last 20 years. DNA typing methods started with the restriction fragment length polymorphism analysis (RFLP) of variable number of tandem repeat loci (VNTRs) and have evolved to current methods such as PCR short-tandem repeat (STR) profiling. Additional important advances have also been forged in the quantification of DNA.

Different quantification methods have been utilized in forensic molecular biology laboratories. Quantification methods started with nonnucleic acid evaluations and total genomic methods, progressed to DNA hybridization-based human and higher primate specific DNA methods, and then with the advent of PCR, evolved to real-time PCR, human target specific methods and PCR endpoint assays. These methods are critical as they permit assessment of DNA extraction efficiency, selection of the optimal amount of the DNA extract to utilize and determination of the most appropriate downstream methods of genotyping analysis to implement.

This review covers the evolution of the quantification methods used in forensic DNA analysis as well as recent developments in forensic RNA quantification. The history of the development of quantitative methods in forensic analysis has involved two main goals—improving precision of the analysis and increasing the information content of the result.

This review is divided into the following subsections:

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   b. Intact versus degraded DNA
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For additional information on DNA quantification, see reviews published in 2003, by Nicklas and Buel [1], and Alonso et al. [2], and book chapters published in 2012 by Butler [3] and Barbisin and Shewale in 2013 [4].

2 Nonnucleic acid-based quantification methods

Assessment of biological evidence will usually start with a macroscopic visual examination. Visual examination may be performed using ambient light, additional white light sources, UV lights, and/or alternate light sources in conjunction with filter glasses. Both visual examination and tactile examination (using gloves) may be performed resulting in a crude estimate of the quantity of the amount of biological evidence [5]. Historically, this type of visual and tactile examination was the first type of quantification used on biological evidence. For example, investigators were told that approximately a "dime sized" bloodstain was required for methods that utilized restriction enzymes to detect length polymorphisms called restriction fragment length polymorphisms (RFLP) of variable number of tandem repeat (VNTR) loci. Quantification of the DNA was required not only to determine the amount to utilize for the digestion of the DNA but also to load approximately the same amounts of restricted genomic DNA onto a single gel. This would result in hybridization of probes to target DNA that were as similar as possible in amounts. In this way, autoradiographs would contain approximately equivalent signals (band intensity patterns) for every sample and every probe.

Screening for common biological fluids such as blood, semen, and saliva using chemical, immunological, and/or microscopic examinations of the evidence may also be performed [5]. Although the results of those examinations are mainly qualitative, they can also provide a crude estimate of expected quantity of DNA per unit area of an evidence sample. These procedures are limited since the total number of cells that contain DNA may not always be the same within each sample due to differences in cell counts per volume of biological fluid, and the potential for uneven redistribution of cells during the deposition and drying of the biological fluid on its substrate. Knowledge of the range of cells per unit volume has also been invoked in estimating the amount of DNA deposited on bloodstain cards such as FTA™ paper [6].

Microscopic examination of semen for the detection of spermatozoa, is a common test utilized in forensic analysis. This procedure can assist in determining the expected DNA yield from sexual assault evidence since the amount of DNA per cell is known. For example, analysts commonly extract cuttings of biological evidence into known volumes of buffer, spot a known volume of the resulting solubilized cells on a microscopic slide and then count the number of cells present in the extract [5]. This visual examination may be used to confirm the presence of spermatozoa and estimate the expected amount of male DNA by multiplying by the number of cells observed per unit area by the quantity of DNA present in sperm cells (3 pg).

3 Total genomic methods—DNA based

The first method used in forensic DNA analysis, RFLP of VNTR loci, required a relatively large amount (50 ng) of intact genomic DNA. Methods developed and implemented assessed both the quantity and quality of the genomic DNA.

Early methods that were utilized to determine total nucleic acids were UV Spectrophotometry and PicoGreen™ microtitre plate homogeneous assays.

The use of UV spectrophotometry is most applicable to purified double-stranded DNA, and the measurement of optical density at both 260 and 280 nm ($A_{260/280}$) permits the discrimination between DNA and residual peptides. This technique is not commonly used by crime laboratories as the method has a relatively low sensitivity, is not human specific and does not differentiate between RNA and DNA or between intact versus degraded DNA. The technique also suffers from absorption interference from biomolecules and other materials (dyes from clothing, phenol from extraction solutions, etc.) that coextract with the DNA [7].

Fluorescent assays using intercalating dyes such as PicoGreen™ are more sensitive than UV methods with limits of detection as low as 25 pg/mL [8]. The detection of DNA by intercalating dyes results from a fluorescence enhancement that occurs due to the fixed orientation of the dye following binding to DNA [1]. Although more sensitive than UV spectrophotometry, these methods are also not human specific and have the potential to overestimate the total DNA present due to potential bacterial contamination. For this reason, these methods are generally not utilized in forensic DNA laboratories for casework samples. However, databanking laboratories that process samples from convicted offenders and other known individuals often utilize these methods for rapid quantification of single source samples.

Another quantification approach popular during the RFLP era, allowed the analyst to estimate the amount and quality (in terms of degree of degradation) of extracted DNA.

This assessment could be accomplished by using agarose gel electrophoresis. The sample DNA was loaded on a gel adjacent to quantification standards prepared with high molecular weight DNA. The DNA in the gel was stained with a fluorescent dye such as ethidium bromide and then visualized with a UV light and photographed with an appropriate filter. Intact DNA was observed as a band whose migration matched that of the high molecular weight DNA standards on the gel. Any fluorescent smear present indicated a sample that may have undergone some degree of degradation. The intensity of the sample fluorescence could be compared to the fluorescence of the quantification standards to obtain a quantitative estimate of the DNA in the sample. This approach had a lower limit of about 1 ng of DNA and was not an option for samples with limited amounts of DNA. As the field moved toward PCR analysis as a standard identification technique, smaller sample size became the norm and highly sensitive PCR quantification-based methods quickly replaced the less sensitive agarose gel methods.

4 Human and higher primate specific methods—DNA based

Since biological evidence may contain nonhuman sources, determining the amount of human DNA in the evidence is needed. The methods described above provided the total DNA present in the sample regardless of its origin. In order to assess the total amount of human/higher primate DNA the need arose for a method that could selectively identify the human from "other" DNA present in a sample.

The first method utilized in forensic DNA analysis to determine the total amount of human DNA in a sample was based on the human/higher primate specific probe, D17Z1. Extracted sample DNA was southern blotted to a nylon membrane and subsequently hybridized with the D17Z1 probe [9, 10]. In forensic DNA laboratories, the method was called slot blot hybridization [10] and was available in a kit (QuantiBlot® from Applied Biosystem—See http://www3.appliedbiosystems.com/cms/groups/applied_markets_marketing/documents/generaldocuments/cms_040281.pdf).

Detection was performed using a colorimetric or chemiluminescent enzyme linked assay where the intensities were recorded using X-ray film or a camera [11]. This new procedure had many advantages over previous techniques, however it still had limitations. For example, while the procedure had better sensitivity than UV and gel-based methods and was very specific for higher primate DNA, it was still not as sensitive as the downstream PCR-based typing methods. Thus, most laboratories would still amplify samples even if the quantification method indicated no DNA was present. In addition, interpretation of slot blots was subjective, time consuming, and laborious [9–11].

The membrane hybridization step used in the slot blot procedure required considerable analyst attention. To streamline quantification, procedures were developed that eliminated the need to perform sample hybridization on membranes. One example of this type of system was a human quantification system called the AluQuant™ [12]. AluQuant assay targets the Alu repeats that are in high copy number in the human and primate lineages. In this assay, hybridization of the Alu-based probe to the target DNA initiates a cascade of enzymatic reactions ending in the production of light that can be read by a luminometer. The assay has a dynamic range from 0.1 to 50 ng and is putatively sensitive down to 50 pg of DNA [12]. Another highly sensitive hybridization-based assay was developed using Quantum Dots by Tak et al. 2012 [13]. The assay is reported to detect as little as 2.5 fg of human DNA, however the reactions were not capable of detecting inhibitors and were relatively time consuming when compared to the real time qPCR methods (qPCR).

The Slot Blot, the AluQuant, and the Quantum Dot assays provided total human/primate DNA amounts but did not provide information on the "quality" or the amount of intact DNA present. One approach that was often used to determine the amount of human intact versus degraded DNA was a simple southern analysis of the yield gel using a D17Z1 probe that was called a yield gel blot. Detection was by radioactivity or chemiluminescence, and therefore, was more tedious and less sensitive than real-time PCR methods.

5 Real-time PCR, DNA-based human target-specific methods

Current methods using PCR to amplify autosomal STRs and other loci require an optimal range of input DNA amounts in order to minimize problems with low input-based stochastic amplification and high input-based peak overload. Because forensic samples can vary greatly in the relative amounts of DNA present, quantification methods with a wide dynamic range are needed. In addition, because the PCR reaction is so sensitive, the optimum quantification method should also be extremely sensitive. Real-time qPCR using a 5′-nuclease fluorogenic or TaqMan® assays are currently the preferred method to achieve these two goals [14]. Real-time qPCR has several advantages over the other methods in that it produces a linear response that is proportional to the quantity of input DNA over a dynamic range of up to 5 orders of magnitude and it occurs in a closed-tube system, reducing the potential for carryover or contamination. The procedure compares favorably with the previously used slot blot techniques both in terms of sensitivity and dynamic range. It also is much easier to automate as qPCR systems utilize automated fluorescence detection techniques. Real-time qPCR systems quantify DNA based on the measurement of the rate of accumulation of dsDNA product during the exponential phase of the reaction. Input DNA levels are correlated with the cycle threshold (C_t), a measure of the number of amplification cycles required to cross a fluorescent baseline threshold. Using these techniques, a forensic biologist can monitor and quantify the accumulation of PCR products over a wide range of input levels [14]. Important criteria for real-time qPCR assays were recently reviewed in detail by Barbisin and Shewale (2013) [4].

Several qPCR human-specific assays are now available that target autosomal, Alu repeats, Y chromosome, and mtDNA targets [2, 15–23]. The assays may be performed on single targets or in multiplexes and a detailed report of the chemistries utilized in the assays is provided in a recent review [4].

The early forensic real-time assays were designed using repeats in the Alu family. One of the main reasons is that the high copy number of these Alu elements in the human genome provides for sensitivity levels equivalent to the detection of single cells. The targets utilized in these assays included Yb8, Yd6, and Ya5 and inter-Alu sequences [17, 18, 24–26].

Real-time PCR assays have also been developed for mtDNA. mtDNA is a highly useful target for highly degraded, low template samples due to its high copy number in eukaryotic cells. In 2002 a mtDNA assay was developed by von Wurmb–Schwark [16] that targeted the NADH dehydrogenase subunit 1 (ND1) gene. An assay for mtDNA degradation was then developed by Alonso (2004) [19] utilizing two separate HV I regions of 113 and 287 bp. Kavlick et al. (2011) generated a synthesized DNA standard for mtDNA, enhancing sensitivity down to 10 copies of mtDNA [27].

Multiplex assays designed to detect multiple targets were next to appear. One such assay called Quantifiler®, targeted a human telomerase reverse transcriptase gene (hTERT) and a synthetic internal positive control (IPC) that was used to monitor inhibition [19]. The procedure utilized a TaqMan assay resulting in a quantification range from 46 pg (23 pg/μL) to 100 ng (50 ng/μL).

Another duplex multiplex developed was the Investigator Quantiplex™, a procedure that targets a 4NS1C gene at 146 bp along with an IPC of 200 bp [28]. Since the 4NS1C is a multicopy gene, the assay produced detection limits below 4.9 pg/μL. Nicklas and Buel (2006) developed a duplex TaqMan assay to simultaneously determine both human and male DNA quantities by amplifying a Ya5 subfamily of the Alu marker and DYZ5 [29].

Degradation and inhibition are two important variables to assess for downstream DNA typing success [3]. qPCR assays have also been designed to detect degraded versus intact human DNA and PCR inhibitors [30–35]. Opel et al. [34] designed an assay that uses three Alu targets of different length to estimate degradation in hair with a sensitivity down to 60 pg [34]. This assay was further improved by Nicklas et al. [35] to produce a multiplex based upon a common forward primer and two reverse primers with different fluorophores to produce a 63 and 246 bp amplicon from an Alu target. In these assays, the relative amounts of PCR amplicons of different length targets provide a measure of the level of degradation in the samples [34, 35]. Another quantitative PCR assay was developed by Swango et al. [30] that amplifies two human nuclear DNA target sequences of different lengths to assess DNA degradation and a third amplification target, a synthetic oligonucleotide internal PCR control (IPC), to allow for the assessment of PCR inhibition [31].

Hudlow et al. [30] developed an assay that simultaneously amplifies four targets: a ~170–190 bp TH01 STR locus (nuTH01), a 137 bp region within SRY locus (nuSRY), a 67 bp target sequence flanking the CSF1PO STR locus (nuCSF), and a 77 bp synthetic DNA template as the IPC target. The quantity of human and male DNA is determined from the nuTHO1 and SRY loci, respectively, while the IPC enables detection of PCR inhibitors [30]. Finally, the ratio of the small nuCSF: to the larger nuTH01 provides a measure of DNA degradation. It was observed that for degraded samples, there is better amplification of the small target resulting in a higher degradation ratio [30].

A similar approach to evaluating a sample for degradation was more recently developed by Sinha et al. [36]. The new qPCR kit called Innoquant™, utilizes two independent genomic targets. Primers and TaqMan probes were designed using two independent intra retrotransposon insertions targets. The 80 bp "short" target sequence is from an Alu insertion whereas the 207 bp "long" target sequence is from a separate retrotransposon element. The primers and probes for the two targets are selected such that they have no interaction among themselves and are completely independent. The ratio of the quantity of long targets versus short targets provides a useful assessment of the quality of DNA. This quality index (QI) can have applications in predicting the profiling success of forensic samples. The use of a synthetic target as an IPC provides an additional assessment for the presence of PCR inhibitors in the test sample. The new assay resulted in sensitivities down to 3 pg as determined in interlaboratory studies [37].

In addition to degradation, the assessment of PCR inhibition in forensic DNA samples is important as inhibitors may be either intrinsic or extrinsic to the sample and may copurify with the template DNA during extraction. Recent reviews discuss the characteristics, proposed mechanisms, and detection of PCR inhibition [38, 39]. The use of qPCR to detect inhibitors has been reported by Kontanis and Reed [40]. In this study, they use a computational method permitting the detection of inhibition by comparing the qPCR amplification efficiencies of unknowns versus clean standards [40]. Opel et al. proposed an assay to assist in the determination of the potential mechanism of inhibition [41]. In general, PCR inhibition can be measured by examining the effect of the inhibitor on the internal control sequence cycle threshold, curves, and their characteristics [41]. Changes that modify the slope may indicate a decrease in amplification efficiency and, therefore, a potential inhibition of the DNA polymerase. A shift of the curve to later cycles with no change in slope (modification of the "take-off" cycle) may indicate competitive template binding inhibition [41].

Finally, a multiplex was described by Walker et al. [42] that amplifies three targets: a region on Yb8 in the Alu Y family, a human mtDNA region, and a human X-chromosome region. The assay provides data for downstream selection of genotyping with autosomal, Y, or mtDNA [42].

More recent qPCR kits that have been widely adopted in forensic DNA laboratories include Quantifiler Duo®

and Plexor HY®. Barbisin et al. (2009) [43] assessed a commercially available product called the Quantifiler Duo DNA Quantification Kit. This kit targets a 140 bp region of the ribonuclease P RNA component H1 (RPPH1), a 130 bp region of the sex-determining region Y (SRY), and a synthetic 130 bp oligonucleotide IPC. Another widely adopted qPCR kit was developed by Krenke et al. (2008) [44] and is also commercially available called the Plexor HY System. This kit targets a multicopy, human RNU2 locus that encodes a small nuclear RNA at 99 bp; the human male target is a multicopy TSYP locus on the Y-chromosome at 133 bp and a 150 bp synthetic IPC. Since both kits contain human nuclear and male-specific targets as well as IPCs, a relative ratio of female to male DNA and inhibition may be determined.

Further advances have been made in the development of four new quantification kits in 2014. Two of these, the Quantifiler Human Plus® (HP) DNA Quantification kit and the Quantifiler Trio® DNA Quantification kits (Life Technologies, Foster City, CA) detect a small 80 bp and larger 214 bp PCR target. In a degraded sample, the large target is more susceptible to DNA degradation versus the small target and is thus disproportionately depleted. The ratio of the small to large target reflects the level of degradation in the sample (See http://www.forensicmag.com/articles/2013/08/development-innovative-dna-quantification-and-assessment-system-streamlining-workflow-using-intelligent-tools and https://tools.lifetechnologies.com/content/sfs/manuals/4485354.pdf). Similarly, the PowerQuant® Kit (Promega, Madison, WI) amplifies a small and a large target and the resulting ratio provides a predictive measure of the degradation present in the sample (https://www.promega.com/resources/webinars/worldwide/archive/informed-casework-sample-decisions-for-downstream-str-typing-using-the-powerquant-system/). Finally, another multiplex quantification kit was described by Pineda et al. [45] that targets two different sized Alu intra retrotransposon insertions targets repeat regions, a Y chromosome target and an IPC [45]. All four of these kits are capable of assisting analysts in determining not just the amount of nuclear and Y chromosome DNA within a sample, but also whether a sample is inhibited and/or degraded.

6 End-point PCR DNA quantification and alternate DNA detection methods

End point PCR is a quantification assay that does not make use of a real-time qPCR instrument but rather uses a thermocyler to conduct the PCR. The method conducts PCR for a certain number of cycles, after which an aliquot of the amplified product is removed for further testing. This testing could involve mixing the aliquot with a fluorescent dye and measuring the fluorescence intensity. An estimate of the starting template amount can be derived from a comparison to DNA standards treated the same way. Alternatively, fluorescent dyes can be attached to primers and quantification conducted via a CE analysis of the product with comparison standards. The end point approach eliminates the need for an expensive real-time PCR instrument but it typically suffers from a reduced analytical quantification range and the need to open the tubes containing amplicons to obtain a result that may increase the risk of contamination over real-time qPCR methods [46, 47].

Alternative DNA detection methods utilize electrochemical methods [48–50]. In addition, alternate optical detection methods are based on hybridization between target DNA and substrate modified with radioactive, fluorescent, chemiluminescent, or nanoparticle tags [51–53]. The use of gold nanoparticles (nAu) as labeling tags have resulted in highly sensitive detection assays [51, 54, 55], and can reach attomolar and high zeptomolar sensitivity [56–58]. Such sensitivity might permit direct detection of genomic DNA and according to the authors, may bypass the need for PCR amplification [55].

7 RNA-based quantification methods

Different genetic expression patterns (mRNAs) exist in different tissue types. Body fluid identification has been reported based on their mRNA profiles [59–62]. In addition, the time elapsed since the deposition of a bloodstain was reported using analysis of mRNA: rRNA ratios [63]. This information may be useful in establishing the tissue origin and time the stain was deposited at the crime.

Advantages of the mRNA-based approach, versus the conventional biochemical tests, include greater specificity, simultaneous, and semi-automated analysis, rapid detection, decreased sample consumption, and compatibility with DNA extraction methodologies. The quantification of the amounts of the mRNA species relative to housekeeping genes is a critical aspect of the assays [59, 64].

Broadly speaking, one can split RNA quantification into two approaches. One strategy measures the total RNA in a sample and the other determines the relative amount of mRNA transcript or a noncoding RNA molecule such as micro-RNA. Classical approaches measure the UV absorption [65] of the extract to obtain a total quantity of RNA. However, UV spectrophotometric methods may not meet the demands of forensic analysis since most samples are of limited quantity. To obtain the total RNA extracted from a sample, forensic investigators may employ a fluorometric assay that has a higher sensitivity than UV spectrophotometric methods. One fluorescent method employs the RNA dye RiboGreen® (Life Technologies, Carlsbad, CA, USA) [66]. This dye can be used in microplate readers as well as specific micro-assay instruments such as of the NanoDrop® (Thermo Scientific, Waltham, MA, USA), Qubit® (Life Technologies) or Agilent 2100 Bioanalyzer® (Agilent Technologies, Santa Clara, CA, USA). The dye also binds to dsDNA and an increase in assay specificity can be obtained by treating samples with DNase prior to sample measurement

(http://tools.lifetechnologies.com/content/sfs/manuals/mp11490.pdf). The product literature for NanoDrop claims a 5 ng/mL sensitivity with RiboGreen® (http://www.nanodrop.com/Library/ND-3300-RiboGreen-Performance-Data.pdf). Imbeaud et al. [67] discussed measuring the quality of an extract RNA via microcapillary analysis. They examined two software programs, an open source [68] and the other available from Agilent Technologies [69] to yield an estimate of RNA quality.

The estimation of the total quantity and quality of the RNA extract can be useful for some applications. In forensic analysis, an estimate of the level of a particular mRNA transcript can aid in the prediction of tissue type. Bustin [70–72] provide reviews of techniques used to quantitate mRNA along with the need to use suitable standards to allow comparison of results. The nature of the selected standard and how it should be employed is discussed. Coamplification of a standard that meets certain requirements with the target is recommended (i.e. a cellular RNA expressed at constant levels between tissues during all stages of cellular development and expressed at similar levels to the target). The use of this cellular RNA for forensic applications is discussed by Moreno et al. [64]. The authors provide an assessment of a number of housekeeping gene transcripts that could be used as a suitable reference, allowing the level of mRNA extracted to be evaluated. A possible housekeeping gene, B2M (beta-2 microglobulin), was identified from a number of candidates but the authors recognized the variations in B2M levels and recommend that normalization could be assessed with additional housekeeping markers.

A number of authors have examined the use of micro-RNA as a possible marker for the identification of tissues. Micro-RNA (miRNA), is a small RNA molecule with a length of 18–24 nucleotides. The small size of the molecule makes it ideal for forensic use where sample degradation is a concern. Courts and Madea [73] used the Qubit fluorometer and Agilent Bioanalyzer to determine RNA concentration and quality of their extracted samples. The authors also measure the level of miRNA in blood and saliva with quantitative PCR using RNA 6B (RNU6b) for normalization. Wang et al. [74] have also used RNU6b to determine relative levels of miRNA, but additional work by Davoren et al. [75] indicates that other controls may be more stable.

Quantitative methods for body fluid typing based on epigenetic methylation of DNA have also been developed [76]. These procedures measure the relative levels of methylation at CpG islands in and around gene promotor regions responsible for cell-based gene expression. Procedures using methylation-specific restriction enzymes and bisulfite-modified PCR and pyrosequencing have been developed [76, 77].

8 Sources of variation in quantification methods

Quantification methods whether used in the past or present are estimates, as the accuracy and precision of the methods may be affected by several factors. Among these are the stability of the quantification standards, pipetting variability, and kit reagent stability. In addition, variation in the storage conditions and tube types as well as the extraction method used may result in variability.

A series of interlaboratory quantification studies has been conducted by the US National Institute of Standards and Technology (NIST) on quantification [78–80]. In one study conducted by NIST [79], a tenfold range of reported concentrations was observed for the same sample among different forensic DNA laboratories. Variation was attributed to several sources including differences in pipetting, quantification method used, DNA sample stability, and variation in quantification standards. Errors due to variations in DNA standards can result in estimates of the quantities of DNA for the "same" sample to vary between laboratories using similar methods [80]. This issue has spurred the development of a NIST human DNA quantification standard, SRM 2372 [81, 82]. This certified quantification standard helps to eliminate the interlaboratory differences that result from variations with in-house standards. The certified reference standard is typically loaded and analyzed adjacent to newly purchased in-house standards to permit better quality control and more consistent results [81]. For years the forensic community struggled with the comparison of quantification data due to the lack of an established quantification standard. The availability of a NIST standard should allow better review of data within laboratories, between laboratories, and across assays and instrument platforms.

9 Summary and future trends

Several different methods have been utilized in forensic laboratories for the quantification of DNA. Table 1 contains a summary of the DNA quantification methods covered in this review. These include UV spectrophotometry, fluorometry, southern hybridization, end-point PCR, and quantitative or real-time PCR. Sensitivity of these assays has been reported down to the picogram level. Single source or reference samples, such as samples being entered into DNA databases, may be quantified utilizing UV spectrophotometry and fluorescent dye-based assays, such as Pico-Green.

However, most forensic laboratories are utilizing real-time PCR methods for the detection and quantification of biological evidence that utilize intercalating dyes to target DNA, TaqMan probes, and/or Plexor chemistry.

Development of new multiplex qPCR assays targeting multiple loci of different sizes facilitates the simultaneous quantification of human nuclear, Y chromosome, and/or mtDNA and DNA quality assessment in a single tube. The incorporation of internal-positive controls provides the ability to measure inhibition and in some cases, determine potential mechanisms of inhibition in forensic samples. Data from these multiplex assays provide analysts with information to determine the amount of template and the most appropriate genotyping system to utilize. In addition, the

Table 1. Summary of DNA quantification methods

Category	Method	Advantages[a)]	Limitations[a)]	Sensitivity	Ref(s)
Nonnucleic acid	Macroscopic	Low cost, easy, scalable	NSp, NoD, NoI	Crude	[5]
	Microscopic	Low cost, easy	Time, NoD, NoI	Fair	[5]
Total genomic DNA	UV Spec	Low cost, easy, fast	NSp, NoD, NoI	2.5 ng/μL	[7, 65]
	Pico-Green	Low cost, easy, fast	NSp, NoD, NoI	25 pg/mL	[8]
	Agarose Gel	Low cost, easy, DD	NSp, NoI	1 ng	[65]
Human/primate-hybe	Slot blot	Human specific-D17Z1	Subjective, NoD, NoI	150 pg	[9]. [10]
	Alu Quant	High copy, sensitive	NoD, NoI	50 pg	[12]
	QuantumDot	Human specific, sensitive	Time, NoD, NoI	2.5 fg	[13]
	Agarose Gel Blot	Low cost, human specific.	Subjective, time, NoI		[65]
Real-time PCR	Alu repeats	High copy, Sp, Sn Yb8, Yd6, Ya5	Time	pg (variable)	[17, 18], [24–26], [34, 35, 42]
	mtDNA	High copy, Sn	Only mt detection	10 copies [27]	[16, 19, 27]
	Quantifiler	Sp, Sn, DI,	False positives	32 pg	[20]
	Quadruplex	Sp, Sn, DNu/Y, DI, DD	Noncommercial	44 pg	[30]
	Investigator	Sp, Sn, multicopy target	NoD	9.8 pg	[28]
	Quantifiler Duo	Sp, Sn, DNu/Y, DI	NoD	46 pg	[43]
	Plexor HY	Sp, Sn, DNu/Y, DI,	NoD	3.8 pg	[44]
	Innoquant & IHY	Sp, Sn, DI, DD & DNu/Y (IHY only)	Not yet available (IHY only)	3 pg	[36, 37, 45]
	PowerQuant	Sp, Sn, DNu/Y, DI, DD	Not yet available	2–4 pg	b)
	Quantifiler HP/Trio	Sp, Sn, DNu/Y, DI, DD	Just released, LV	10 pg	c)
End point PCR	Tho1 microplate	Sp, rapid, scalable, low cost	NoD, NoI, NoNu/Y	200 pg	[46]
	QTaT	Sp, DX/Y, rapid, low cost	NoD	50 pg	[47]

a) Sp, specific; NSp, nonspecific; DD, detection of degradation; NoD, no detection of degradation; DI, detection of inhibition, NoI, no detection of inhibition; Sn, sensitive; DNu/Y, detects both autosomal and Y chromosome targets; DX/Y, detects both X and Y chromosome targets; LV, limited validation studies.
b) https://www.promega.com/resources/webinars/worldwide/archive/informed-casework-sample-decisions-for-downstream-str-typing-tusing-the-powerquant-system/.
c) http://www.forensicmag.com/articles/2013/08/development-innovative-dna-quantification-and-assessment-system-streamlining-workflow-using-intelligent-tools and https://tools.lifetechnologies.com/content/sfs/manuals/4485354.pdf.

information may assist in determining the need for further dilution, concentration, purification, or addition of amplification enhancers to provide the most likely success of DNA typing, enhancing the first pass success rates, thereby preserving evidence, and saving time and cost.

The potential for enhancing first pass success rates has inspired the development of new, highly informative multiplex qPCR assays. This, in turn, has spurred the testing of these new commercial real-time PCR kits in crime laboratories. The near term future will likely include validation and implementation of these newly developed qPCR kits leading to enhanced genotyping success on challenging forensic samples.

Further advances in sensitivity, information content, and improvement of the qPCR assays with implementation of standardized controls and advances in technology will likely be achieved in the near future. One method of improvement is already underway at NIST utilizing digital PCR to improve the SRM 2372 (http://www.cstl.nist.gov/biotech/strbase/pub_pres/Haynes-DNA-quantitation-digital-PCR.pdf). In addition, implementation of new technologies such as the use of gold nanoparticles (nAu) as labeling tags have the potential to reach exquisite sensitivity that might permit direct detection of genomic DNA bypassing the need for PCR amplification. Qualitative and quantitative assessment on DNA from evidentiary samples that may contain low quality, low quantity DNA, and coextracted inhibitors provides useful data for deciding which downstream steps to implement for successful genotyping.

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