Anal Bioanal Chem (2009) 394:1183–1192
DOI 10.1007/s00216-009-2782-0

ORIGINAL PAPER

Production and certification of NIST Standard Reference Material 2372 Human DNA Quantitation Standard

Margaret C. Kline · David L. Duewer · John C. Travis · Melody V. Smith · Janette W. Redman · Peter M. Vallone · Amy E. Decker · John M. Butler

Received: 9 February 2009 / Revised: 27 March 2009 / Accepted: 30 March 2009 / Published online: 18 April 2009

Abstract Modern highly multiplexed short tandem repeat (STR) assays used by the forensic human-identity community require tight control of the initial amount of sample DNA amplified in the polymerase chain reaction (PCR) process. This, in turn, requires the ability to reproducibly measure the concentration of human DNA, [DNA], in a sample extract. Quantitative PCR (qPCR) techniques can determine the number of intact stretches of DNA of specified nucleotide sequence in an extremely small sample; however, these assays must be calibrated with DNA extracts of well-characterized and stable composition. By 2004, studies coordinated by or reported to the National Institute of Standards and Technology (NIST) indicated that a well-characterized, stable human DNA quantitation certified reference material (CRM) could help the forensic community reduce within- and among-laboratory quantitation variability. To ensure that the stability of such a quantitation standard can be monitored and that, if and when required, equivalent replacement materials can be prepared, a measurement of some stable quantity directly related to [DNA] is required. Using a long-established conventional relationship linking optical density (properly designated as decadic attenuance) at 260 nm with [DNA] in aqueous solution, NIST Standard Reference Material (SRM) 2372 Human DNA Quantitation Standard was issued in October 2007. This SRM consists of three quite different DNA extracts: a single-source male, a multiple-source female, and a mixture of male and female sources. All three SRM components have very similar optical densities, and thus very similar conventional [DNA]. The materials perform very similarly in several widely used gender-neutral assays, demonstrating that the combination of appropriate preparation methods and metrologically sound spectrophotometric measurements enables the preparation and certification of quantitation [DNA] standards that are both maintainable and of practical utility.

Keywords Certified reference material (CRM) · Decadic attenuance · Forensic · Human identity · Interlaboratory comparison · Short tandem repeat (STR) multiplex assay · Standard Reference Material (SRM) · UV/visible absorbance spectrophotometry

M. C. Kline (✉) · J. C. Travis · M. V. Smith · J. W. Redman · P. M. Vallone · A. E. Decker · J. M. Butler
Biochemical Science Division,
National Institute of Standards and Technology,
100 Bureau Drive, MS 8311,
Gaithersburg, MD 20899-8311, USA
e-mail: Margaret.Kline@nist.gov

D. L. Duewer
Analytical Chemistry Division,
National Institute of Standards and Technology,
100 Bureau Drive, MS 8390,
Gaithersburg, MD 20899-8390, USA

Introduction

Modern highly multiplexed short tandem repeat (STR) assays used by the forensic human-identity community require rather tight control of the initial amount of sample DNA amplified in the polymerase chain reaction (PCR) process [1]. Attempts to amplify too little or too much DNA may waste time, expensive reagents, and potentially critical samples. Efficient control of the amount of DNA used in the analysis requires the ability to reproducibly measure the mass concentration of human DNA, [DNA], in an extract.

The National Institute of Standards and Technology (NIST) has been an active member of the forensic human identity community since 1991 [2]. As part of its

involvement, NIST has conducted a series of interlaboratory studies intended in part to monitor the state of practice in human DNA quantitation methods [3–5]. Among-participant quantitation reproducibility was not found to be a major issue in three studies conducted from 1997 to 2002: the STR assay systems then in common use were relatively simple (four to ten simultaneously amplified genetic loci), the available quantitation methods were relatively insensitive, and the samples distributed had relatively high [DNA]. In contrast, the 2004 NIST Quantitation Study (QS04) results indicated a multiplicative one-standard deviation (SD) among-participant reproducibility for sub-ng/μL [DNA] of just less than a factor of two—about equivalent to ±1 PCR amplification cycle. Most significantly, much of this variation arose from apparent biases among the then-recently introduced quantitative PCR (qPCR) methods. These systematic differences are of the same magnitude as the recommended allowable range for the initial amount of DNA used in higher-plexity (ten to 16 loci) STR assays [6].

While NIST staff have given considerable thought and discussion over the years to design, packaging, and certification challenges involved in creating a reference material in support of DNA quantitation (and the NIST interlaboratory studies were used to explore these challenges), the systematic differences observed in QS04 were our first evidence that a NIST-provided quantitation standard could have practical impact. Further, at about the time the QS04 results were being compiled, other members of the community informed us that they had observed factor-of-two differences among different lots of commercially prepared quantitation standards from the same producer and among materials from different producers. The weight of the evidence thus indicated that a well-characterized, stable human DNA quantitation certified reference material (CRM) could benefit the forensic community.

The greatest challenge requiring resolution prior to our initiating active development of a DNA quantitation standard was “What is a nanogram of DNA?” Numerous indirect techniques have been developed, assaying phosphorous, sugar, or individual nucleotides and thus “counting” the total number of nucleotide base-pairs present in a given mass of DNA [7]. In contrast, qPCR techniques assay not the number of base-pairs but the number of intact and accessible stretches of DNA having a specified nucleotide sequence. Depending on the nature of these sequences and the sample material, these measurements can be related to the number of specific chromosomes and this in turn related to the number of complete genomic complements. While related, the quantity intended to be measured in the different assays could well differ among different genomic sources.

However, the number of base-pairs, chromosomes, or genomic complements does not need to be known for a DNA quantitation standard to be useful. A sufficiently large pool of reasonably intact DNA from a well-considered source that is sufficiently stable, homogenous, and representative of real-world samples could be successfully used as a primary reference [8,9]. However, such unique reference materials are of little use should they become degraded, contaminated, or consumed. To ensure that the stability of the material can be monitored over time and that equivalent materials can be prepared as needed, there must be some measureable property of the material that is directly related to [DNA] but that does not itself require calibration to a DNA reference material.

Although originally developed for characterizing protein solutions lightly contaminated with nucleic acids, UV/visible absorbance spectrometry has long been used to characterize nucleic acid solutions lightly contaminated with proteins [10]. It is now conventional to assert that an aqueous solution of double-stranded DNA with an optical density (OD) of 1.0 at 260 nm in a 1 cm pathlength cuvette has a [DNA] of 50 μg/mL (50 ng/μL) [11]. “Optical density” is the logarithm of the reciprocal of transmittance—the ratio between light intensities with and without the presence of the sample in a beam of light—at a specified wavelength and pathlength [12]. While of intellectual interest, establishing the metrological validity of this assertion is not necessarily relevant to the development of a standard intended for reducing quantitation variance. Fairly routine spectrophotometric measurements provide a conventional measure of [DNA] that is widely accepted in the analytical bioscience communities, that is fit for the purpose of monitoring material stability, and that provides an unambiguous metric for preparing replacement materials.

The following sections of this manuscript present and discuss the design, packaging, production, and certification issues related to the recently issued NIST Standard Reference Material (SRM) 2372 Human DNA Quantitation Standard [13]. SRMs are CRMs, issued under the NIST trademark, that are well-characterized using state-of-the-art measurement methods and/or technologies for the determination of chemical composition and/or physical properties [14].

Design considerations

Previous studies established that extraction of DNA from solid supports and even solubilization of lyophilized DNA contributes to quantitation variability [3]. Seeking to minimize such ancillary sources of variability, the SRM 2372 components were prepared as DNA solutions directly suitable for spectrophotometric and qPCR assay. NIST

research (data not shown) has documented that the spectrophotometric characteristics of bulk solutions of DNA stored in Teflon at 2 °C to 8 °C are stable over several years. Gel electrophoretic studies confirmed that these spectrophotometrically stable DNA solutions showed little or no DNA degradation.

DNA can be degraded and/or contaminated during mechanical as well as chemical manipulations. Whenever possible, processes were used that minimized the potential for degradation and contamination. For example, air-drying DNA solutions under laminar flow may reduce fragmentation and does avoid potential contamination from UV-active compounds that may be present in vacuum/refrigerant systems.

Early NIST interlaboratory studies identified DNA loss to sample vial walls as a probable source of variation, with the relative loss increasing as [DNA] declined [5]. However, aqueous solutions with very high [DNA] are viscous and hard to handle. The SRM 2372 component materials were therefore designed to have the highest [DNA] compatible with routine pipetting practice. Given that accurate UV/vis absorption measurements become problematic for OD readings much larger than 1, the assertion that an aqueous solution of double-stranded DNA of 1.0 OD at 260 nm in a cuvette of pathlength 1 cm (OD_{260}) has a conventional [DNA] of 50 ng/μL, and that qPCR calibration curves typically are prepared with an upper limit of 10 to 50 ng/μL, 50 ng/μL was chosen as the target [DNA]. Total sample volumes of about 110 μL were used to minimize sample concentration from evaporation, facilitate sample handling, and enable use of commercially available 80 μL spectrophotometer cuvettes.

The nature and quantity of salts and solvents present in a sample can influence qPCR processes. While RNA contributes to OD_{260} measurements, it does not contribute to qPCR signal. To enable users to evaluate the sensitivity of their measurement processes to small differences in methods used to prepare DNA standards, somewhat different methods and materials were used for each of the SRM 2372 components. Since forensic DNA human identity analyses routinely deal with male-only, female-only, and mixed-source samples, the component materials were designed to represent these sample types.

Materials and methods

Component materials

SRM 2372 has three components, arbitrarily labeled A, B, and C. All three components are human genomic DNA, each of quite different nature.

Component A was prepared at NIST from human Buffy coat white blood cells from a single-source anonymous male. Genomic DNA was isolated from the Buffy coats using a modified "salting out" procedure [15]. The extract was treated with bovine pancreatic ribonuclease A (RNase A) to remove any RNA components that would contribute to the OD [11]. The RNAse-treated DNA was then re-extracted to assure purity. The extracted DNA was air-dried in a laminar flow hood and then stored in a Teflon container at 2 °C to 8 °C.

Component B was prepared (twice—the initial version is designated as component $B_{initial}$) at NIST from Buffy coat white blood cells from multiple anonymous female donors. To more closely mimic DNA from routine extraction protocols, these materials were not treated with RNAse but were otherwise prepared as above. The Buffy coat cells were obtained from a commercial source.

Component C was purchased as a lyophilized, purified unsheared genomic human DNA (Sigma-Aldrich Co., St. Louis, Mo); this material has both male and female donors and contains more salt (NaCl) and metal chelator (EDTA) than do components A and B.

The dried component materials were each solubilized in UV-irradiated Teflon containers with 210 to 250 mL of autoclaved 10 mmol/L 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris–HCl) and 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (disodium EDTA) in deionized water adjusted to pH 8.0 (TE^{-4} buffer). The containers were placed on an orbital shaker and gently rotated for several hours at room temperature prior to refrigerated storage at 2 °C to 8 °C. After storage for several weeks to allow more complete solubilization, a preliminary assessment of OD_{260} nm for each of the materials was made at room temperature using a commercial spectrophotometer. Each material was, if needed, diluted with autoclaved TE^{-4} buffer until an OD_{260} of approximately 1.0 was obtained. The diluted materials were returned to storage at 2 °C to 8 °C. As shown in Fig. 1, gel electrophoresis confirmed that all solutions were of relatively unfragmented (greater than 4,000 base-pairs) double-stranded DNA.

For each solution, the Teflon container containing bulk material was removed from the refrigerated storage and placed on a slow rotary shaker for 30 min. A magnetic stir-bar was then placed in the Teflon container, the container placed on a magnetic stir plate in a laminar flow hood, and the solution gently stirred. Empty, sterile, labeled 2.0 mL screw cap vials were opened and placed in a laminar flow hood in 96-well boxes. Eight vials were filled simultaneously with approximately 110 μL per vial of component solution using a variable-width pipette. Vials were closed and transferred to 100-unit storage boxes. A total of 1,700 vials were prepared for each component. Filled boxes were refrigerated at 2 °C to 8 °C. All materials were allowed to settle in their vials for three weeks before beginning the characterization studies.

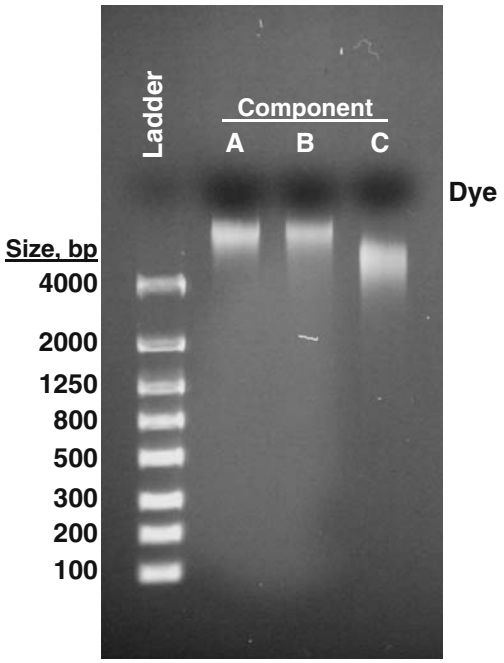

Fig. 1 Exemplar gel image. This image shows the distribution of DNA fragment masses for the three components (**A**, **B**, and **C**) relative to a standard sizing ladder (*Ladder*), with molecular mass expressed as the number of nucleotide base-pairs (bp). There is no evidence for low-mass fragments (i.e., less than a few 1,000 bp) in any of the materials

Due to limited solution volume (210 mL), stirring of component $B_{initial}$ became increasingly inefficient as the solution volume declined. As described below, this version eventually proved insufficiently homogenous for use in SRM 2372. A replacement version was produced exactly as above, only using a greater amount of extracted DNA solubilized in a larger solution volume.

Interlaboratory assessment

Thirty-two forensic DNA testing facility participants from the U.S., Canada, and Australia contributed to an interlaboratory assessment of the relative [DNA] of candidate SRM 2372 components A, $B_{initial}$, and C. Each participant received one unit of each component. Table 1 lists the assays, the number of unique sites reporting each, and the total number of sets of data from all participants.

The study materials were shipped overnight by commercial carrier on cold packs to the participants just before the NIST in-house characterization analyses were begun. Participants were asked to use the same dilution scheme on all three components: the neat solution, a 1+4 volumetric dilution (where the "1+4" indicates one volume of the first component and four volumes of the second), and six 1+1 volumetric serial dilutions from the initial dilution. This scheme provides eight conventional [DNA] concentrations: 50, 10, 5, 2.5, 1.25, 0.62, 0.31, and 0.16 ng/μL. Prepared by an experienced analyst using properly functioning equipment, the volume delivery uncertainty for the first dilution is expected to be just larger than the pipette imprecision and increases steadily to about twice the imprecision at the final dilution. The imprecisions of the 10- and 20-μL pipettes used in the NIST studies were determined to be better than 0.30%, providing relative preparation uncertainties of ≤0.35% for the 10-ng/μL solution and ≤0.62% for the 0.16-ng/μL solution.

Participants were to (1) use the component C dilutions as the calibration standard for their quantitation assay(s); (2) assume that the "true" [DNA] of component C was exactly 50 ng/μL; (3) calculate the apparent [DNA] for all of the dilutions made for components A and B; and (4) to also report all associated cycle threshold (Ct) values if qPCR method(s) were used. The Ct is the interpolated fractional PCR cycle at which reporter fluorescence is estimated to first detectably increase above the baseline signal.

UV/vis measurements

Value assignment UV/vis OD measurements were made at the five wavelengths (230, 260, 270, 280, and 330 nm) that are traditionally used in the assessment of DNA quality as well as quantity [11]. Traceable transmittance measurements, four runs on one day, were made using the second-generation NIST High-Accuracy (Reference) Spectrophotometer (HAS-II) [26]. Measurements utilized six SRM 932 Quartz Cuvette for Spectrophotometry (discontinued) cuvettes containing ≈ 2.5 mL of either a component of SRM 2372 or TE^{-4} as the blank. The cuvettes were acid-washed, water-rinsed, and dried prior to the measurement. SRM 2031 Metal-on-Quartz Filters was used as a control for these transmittance measurements. In addition to the five certification wavelengths, measurements were taken at 250 nm and 340 nm to facilitate use of this control. The 280 nm wavelength was common to both SRM 2031 and SRM 2372 certification. All measurements were made at 22 °C±0.5 °C with a spectral bandwidth of 0.8 nm.

Homogeneity Optical homogeneity for all components was evaluated using a commercial spectrophotometer. Seventeen randomly selected vials, 1% of each production run, were assayed for each component using 80-μL cuvettes with a 1-cm pathlength. Within a component, the run order of the selected vials was randomized. Wavelength scans included 220 nm through 345 nm. The temperature of the cuvettes was 22 °C±1 °C. SRM 2031 and SRM 2034 holmium oxide solution wavelength standard from 240 nm to 650 nm (discontinued) were used to validate the transmittance and wavelength axis calibration of the

Table 1 Interlaboratory assessment summary

Code	#Labs	# Data sets			Assay	Ref
		A	B_{inital}	C		
Quantifiler	20	75	75	78	qPCR, gender-neutral	[16]
Sybr_ALU	5	11	11	13	qPCR, gender-neutral	[17]
CFS	3	16	16	16	qPCR, gender-neutral	[18]
monoTH01	1	4	4	4	qPCR, gender-neutral	[19]
Probe_ALU	1	4	4	4	qPCR, gender-neutral	[20]
3TH01	1	3	3	3	qPCR, gender-neutral	[19]
3CSF	1	3	3	3	qPCR, gender-neutral	[21]
Plexor_auto	1	2	2	2	qPCR, gender-neutral	[22]
Gender	1	4	0	4	qPCR, male specific	[20]
Plexor_Y	1	2	1	2	qPCR, male specific	[22]
ALUQuant	3	11	11	0	Direct, gender-neutral	[23]
Quantiblot	2	7	7	0	Direct, gender-neutral	[24]
Picogreen	1	4	4	4	Direct, gender-neutral	[25]

instrument before and after evaluating each SRM 2372 candidate material.

Temperature dependence The change in OD with temperature for all components was evaluated with a commercial spectrophotometer using 0.9-mL cuvettes and residual bulk solution. Spectra were acquired from 220 to 345 nm at sample temperatures from 15 °C to 30 °C in increments of 5 °C. At least two independent replicate spectra were obtained at each temperature; one while warming the cuvette and the second while cooling.

qPCR measurements

Homogeneity The homogeneity of all components was confirmed using a qPCR assay, Quantifiler Human DNA Quantification Kit (Applied Biosystems, Foster City, CA) [16] in conjunction with an ABI 7900 real time PCR instrument (Applied Biosystems). A second 1% of the production materials were randomly selected. The selected materials were diluted 1+9 volumetrically prior to analysis to obtain a [DNA] that was in the middle of the calibration curve developed for the assay. The diluted components were randomly distributed across two duplicate 96-well plates and analyzed separately. Each plate also contained duplicate aliquots of the undiluted material and three serial volumetric dilutions with TE^{-4} buffer: 1+4, 1+1, and 1+1. The relative preparation uncertainties for these dilutions are all ≤0.5%.

Commutability The [DNA] of four commercially available DNA quantitation standards (coded Std1, Std2, Std3, and Std4) were evaluated relative to each of the candidate SRM 2372 components with three exemplar qPCR assays: Quantifiler Human Assay [16], CFS [18], and Sybr_ALU [17]. The nominal [DNA] was 200 ng/μL for Std1, Std2, and Std3 and 262 ng/μL for Std4. The commercial standards were initially volumetrically diluted 1+9 with TE^{-4} buffer followed by serial volumetric dilutions of 1+4, 1+1, and 1+1. The SRM components were used undiluted, diluted 1+4 with buffer, and six 1+1 volumetric serial dilutions from the initial dilution. All seven dilution series (Std1, Std2, Std3, Std4, A, B, and C) were run in duplicate together on one 96-well plate per assay. The relative preparation uncertainties for these dilutions are all ≤0.7%

Results and discussion

Interlaboratory assessment

The interlaboratory study was designed to assess (1) the acceptability of the above design decisions to practicing forensic analysts and (2) the *relative* [DNA] of the three components determined with the then-current DNA quantitation techniques. While some analysts found that the vial label obscured the liquid meniscus, all found the design acceptable.

Figure 2 is a Youden-style plot displaying results for gender-independent qPCR assays of components A and $B_{initial}$ as calibrated against component C. Regardless of the absolute [DNA], if all three components have the same [DNA] then all of the paired results for all data sets would ideally be superimposed at unity along both axes. The 1.04 median of both the A/C and $B_{initial}$/C ratios are gratifyingly similar and adequately close to unity, although this suggests that by these qPCR assays the [DNA] of component C is about 4% less than that of components A and $B_{initial}$.

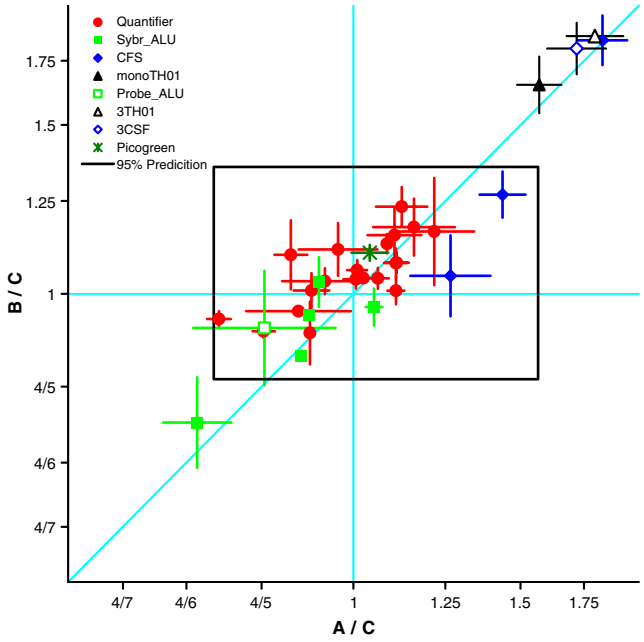

Fig. 2 Interlaboratory qPCR assessment of relative [DNA]. Each symbol represents the average qPCR [DNA] results reported for a given method by one participating laboratory. The *x*-axis presents component A relative to the full calibration curve for component C (A/C); the *y*-axis presents component $B_{initial}$ relative to component C ($B_{initial}$/C). The cross at each {A/C, B/C} pair represents approximate 95% confidence intervals for the two ratios for the method as implemented at that laboratory. The vertical and horizontal lines represent unit A/C and B/C ratios, the ideal if all components were of identical [DNA]. The diagonal line represents the expected behavior when measurement deviations from the consensus value are consistently biased. The central rectangle encloses an approximate 95% confidence region about the ratio medians

The multiplicative within-participant SDs, estimated from the median absolute deviation from the median (MADe) [27], are likewise quite similar: 1.14 for A/C and 1.11 for $B_{initial}$/C. The among-participant multiplicative imprecision for $B_{initial}$/C of 1.13 is quite similar; however, that for A/C at 1.23 is about twice that of the within-participant value. The strong correlation (greater than 0.92) between the A/C and $B_{initial}$/C ratios suggests that component C presents relatively small but quite systematic measurement challenges for some qPCR methods. Combined with the larger among-participant imprecision of the A/C ratio, these differences are greater for the single-source, male, RNased DNA of component A than for the multi-source female DNA of component $B_{initial}$. These among-method biases are not unexpected since different qPCR methods exploit qualitatively different molecular targets.

Material homogeneity

UV/vis measurements The among-vial SDs for the OD_{260} measurements were: A 0.4%, $B_{initial}$ 10%, and C 0.3%. The unacceptable $B_{initial}$ heterogeneity was most pronounced in the final 400 units, but incompletely solubilized material was discovered in several units produced earlier in the production run. This discovery was, unfortunately, made after the interlaboratory samples had been distributed.

The interlaboratory assessment did not detect the UV/vis heterogeneity. Follow-up studies at NIST suggest that this was not just the "luck of the draw" but reflects a profound difference between the two assay systems: the OD_{260} measurements effectively use the entire sample volume whereas each qPCR assay of amplifiable sequences uses only a few percent of the sample. Any incompletely solubilized material in the sample will likely be present in the spectrophotometer beam and thus increase the apparent [DNA] above that of the bulk solutions. Given normal micropipetting practice, it is most probable that only clear bulk solution would be transferred into the qPCR vial—at least for the first several assays.

A second component B was prepared with an among-vial SD for the OD_{260} of 0.8%. With the exception of the interlaboratory assessment described above, all further studies were done (or redone) using this second component B material.

qPCR measurements Table 2 lists the mean Ct, method repeatability, and material homogeneity precision estimates for the three components (17 units of each component run in duplicate). The precision estimates are expressed both in terms of the measured Ct and in conventional [DNA]. The repeatability imprecision of the method, s_{repeat}, is estimated as the pooled SD of replicate Ct measurements for each component. The among-box heterogeneity, s_{hetero}, is estimated from the SD of the means of the replicate pairs and the s_{repeat} estimate [28]. Since each PCR amplification cycle approximately doubles the target [DNA], the SDs of the Ct results are converted to approximate multiplicative SDs of [DNA] by exponentiation: $s([DNA])=2^{s(Ct)}$. Since these multiplicative imprecision values are relatively close to unity, they can in turn be approximated as relative SDs, expressed in percent: $\%RSD=100\times(s[DNA]-1)$.

The estimated repeatability and heterogeneity %RSDs are both roughly 5% for all three components, accounting for about half of the variability observed in the interlaboratory assessment. The three mean Cts overlap within the method repeatability, implying that the [DNA] of the components are the same within about 5%. The mean Ct is smallest for component C, indicating that [DNA] as determined with this one qPCR assay in the hands of one analyst is greatest for this component. Although this differs from the inference drawn from the results of the interlaboratory study, the differences are within the variability of the assays.

Table 2 qPCR homogeneity summary

		[DNA]					
		Ct Additive SD		Multiplicative SD		%RSD	
Component	Ct	s_{repeat}	s_{hetero}	s_{repeat}	s_{hetero}	s_{repeat}	s_{hetero}
A	26.946	0.058	0.073	1.041	1.052	4.1	5.2
B	27.018	0.061	0.077	1.043	1.055	4.3	5.5
C	26.899	0.075	0.064	1.053	1.045	5.3	4.5

UV/vis certification measurements

While it is routine practice to term the observed OD of solutions as "absorbance", the correct designation for colloidal solutions that significantly scatter as well as absorb light is "attenuance" [12]. Scattering results in the removal of light from the spectrophotometer beam and thus decreases the measured transmittance. However, the degree to which scattered light is removed from the beam is somewhat dependent on the optics of the instrument, as forward scattering predominates and some forward-scattered light may remain in the detected beam. This is especially true of the focused-beam design common in analytical spectrophotometers and in the HAS-II National Reference Spectrophotometer used for the certification measurements. The certified values for SRM 2372 are therefore stated as decadic attenuance (D_{10}), where "decadic" distinguishes the base-10 logarithmic transformation from the "Naperian" or "natural" base-e transformation often used by the physics community. The certified D_{10} values are corrected for reflections from the cuvette faces and for absorption and scattering in the TE^{-4} pH 8.0 buffer by subtracting blank D_{10} measurements of the buffer in the cuvettes used for the DNA measurements.

The certified D_{10} values for the three components are listed in the SRM 2372 Certificate [13]. Figure 3 displays these certified values; the determination uncertainty on each value is smaller than the symbols used to represent the values. An exemplar D_{10} spectrum of component A is displayed for visual reference.

The change in attenuance with temperature was found to be uniformly less than 0.0001 D_{10} per°C for all materials in the range of 15 °C to 30 °C.

qPCR calibration and commutability

The presence of systematic biases among different qPCR methods was verified at NIST for three of the assays used in the interlaboratory assessment. Figures 4–6 display the relationships between the conventional [DNA] and Ct for Quantifiler, Sybr_ALU, and CFS. For the Quantifier data shown in Fig. 4, the data agree well with the usual $Ct=\beta_0+\beta_1\times\log_2([DNA])+\varepsilon$ calibration model over the entire 320-fold range; the least squares regression lines for these data are very similar for the three SRM components. For the Sybr_ALU assay in Fig. 5, the data appear to deviate from the log-linear model over part of the range but the regression lines for the imperfect model are quite similar. For the CFS assay of Fig. 6, the data are each quite consistent with the model but material C is systematically biased by about one- half of an amplification cycle relative to A and B.

The close agreement between the qPCR results for components A and B indicates that the RNAse treatment of component A did not significantly influence the OD_{260} and conventional [DNA] relationship. While it is likely that the treatment and repeated extraction was not required, it appears to have done little harm.

Within model error, the β_1 slopes for all of the calibration curves are all −1.0. This is the theoretical slope for PCR reactions where the amount of target DNA is exactly doubled during each cycle.

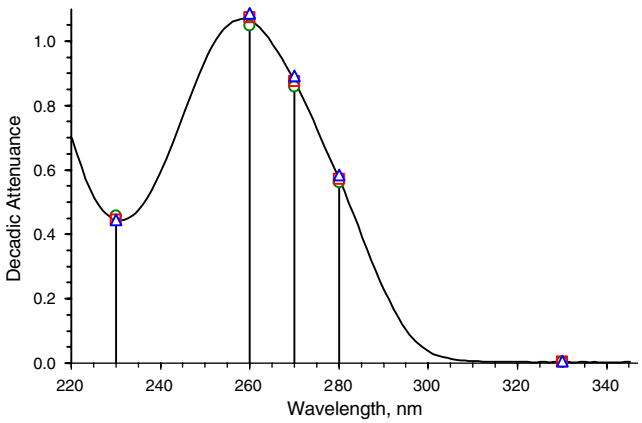

Fig. 3 Certified decadic attenuance and exemplar UV/Vis spectrum. Each symbol represents a certified decadic attenuance at a specific wavelength for one of the components of SRM 2372; *open circle* component A, *open square* component B, and *open triangle* component C. The approximate 95% uncertainties on the certified values are not visible at this scale. All certified values were obtained using the NIST HAS-II reference spectrophotometer. The continuous curve is an exemplar spectrum of component A obtained using a commercial scanning spectrophotometer. The vertical lines are for visual reference only, connecting the wavelength axis to the exemplar spectrum at the certification wavelengths

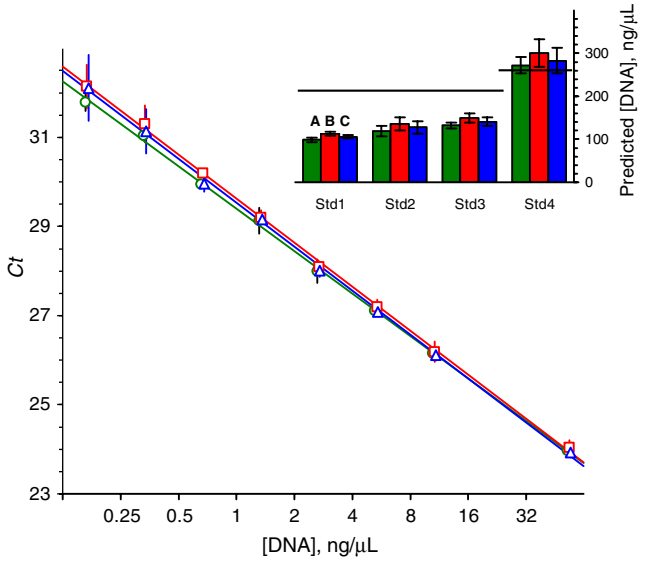

Fig. 4 Quantifiler qPCR calibration curves and commutability assessment. The main panel displays the mean Ct of duplicate assays of dilutions for each of the three SRM 2372 components as functions of their conventional [DNA], estimated as 50 times the certified D_{10} at 260 nm. *Error bars* represent approximate 95% confidence intervals on mean Ct values. The *lines* are least squares regressions of the data to the usual linear model: $Ct=\beta_0+\beta_1\times\log_2([DNA])+\varepsilon$ using all eight elements of the dilution series. The equation parameters are: Component A (*open circle*) β_0=29.398±0.031, β_1=-0.952±0.011, ε=0.081; Component B (*open square*) β_0=29.618±0.033, β_1=-0.988±0.012, ε=0.086; Component C (*open triangle*) β_0=29.528±0.033, β_1=−0.986±0.012, ε=0.087. The *inset* displays the predicted [DNA] of four commercially available DNA quantification materials, labeled "Std1" to "Std4", relative to each of the three SRM 2372 components. The predicted values are calculated using the inverted prediction equation for each component: $[DNA]=2^{(Ct-\beta 0)/\beta 1}$. The thin horizontal lines within the inset represent the claimed [DNA] of the materials: Std1 to Std3=200 ng/µL DNA and Std4=262 ng/µL DNA. *Error bars* represent approximate 95% confidence intervals on the mean predicted [DNA] of replicate evaluations of tenfold, 50-fold, 100-fold, and 200-fold volumetric dilutions of each of the commercial materials

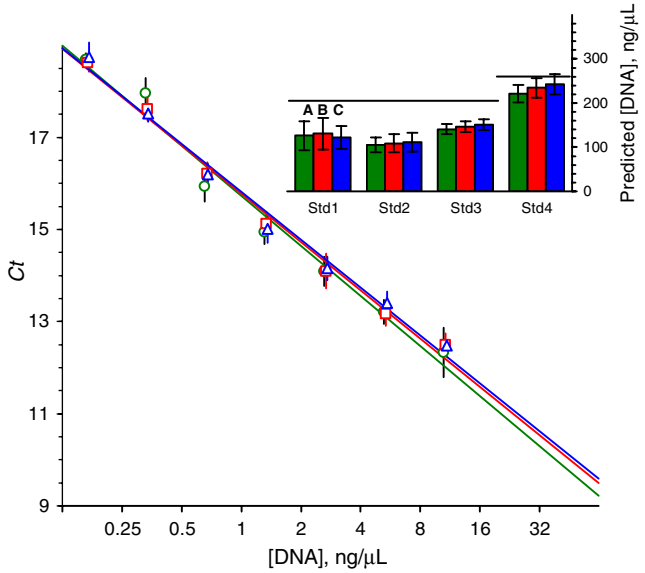

Fig. 5 Sybr_ALU qPCR calibration curves and commutability assessment. The main and inset panels display results for the Sybr_ALU assay in the same format as Fig. 3. The parameters of the three calibration equations, derived from the seven dilutions of the components, are: Component A β_0=15.733±0.143, β_1=−1.086±0.070, ε=0.37; Component B β_0=15.776±0.089, β_1=−1.048±0.044, ε=0.23; Component C β_0=15.814±0.101, β_1=−1.038±0.049, ε=0.26

The inset in each figure displays the [DNA] of four commercial DNA quantification materials predicted from the calibration curves for the three SRM 2372 components. As expected, the predicted values for the four samples are nearly the same across calibration curves for the Quantifiler and Sybr_ALU assays. The predicted conventional [DNA] for Std4 agrees well with the value assigned by its producer; the predicted [DNA] for Std1, Std2, and Std3 are considerably less than the value assigned by their producer. This same pattern holds with the CFS assay for the component A and B predictions, however the component C predictions are relative overestimates. This is similar to the interlaboratory assessment results for these qPCR assays displayed in Fig. 2. It is plausible that the CFS assay may be particularly sensitive to small variations in salt and/or EDTA content of the qPCR reaction mixture. Dialyzation of commercially obtained DNA could reduce the levels of these materials: we anticipate evaluating the costs and benefits of this pretreatment before designing any new or replacement DNA quantitation standards.

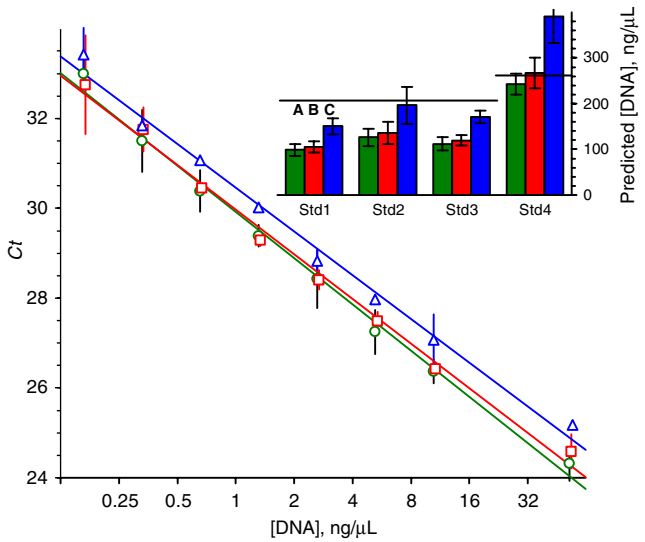

Fig. 6 CFS qPCR calibration curves and commutability assessment. The main and inset panels display results for the CFS assay in the same format as Fig. 3. The parameters of the three calibration equations are: Component A β_0=29.913±0.090, β_1=1.030±0.032, ε=0.23; Component B β_0=29.974±0.091, β_1=−0.995±0.032, ε=0.24; Component C β_0=30.461±0.102, β_1=−0.974±0.037, ε=0.27

Further characterization

The documented multi-year stability of DNA solutions when storaged in bulk in teflon at 4 °C does not necessarily speak to the stability of the SRM 2372 units. NIST has and will continue to monitor the materials for changes in OD, gel electropherographic pattern, and qPCR results relative to those of a suite of bulk-stored materials. As is policy for all NIST SRMs, should adverse changes be observed, customers will be notified and the certified values will either be revised or the SRM withdrawn.

Although not required for its intended purposes, evaluation of the number of nucleotides and the effective number of copies of a human genome present in each of the SRM 2372 components could be useful for very low copy-number applications, as well as being intellectually satisfying. There has been considerable effort recently given to the development of methods capable of making metrologically traceable measurements of the number of nucleotides, either directly [29] or indirectly using high-accuracy phosphorous quantitation [30–32]. We anticipate extending certification of SRM 2372 to include new measurands as DNA quantitation technologies mature.

Conclusions and recommendations

Although prepared from quite different sources of human genomic DNA, the three SRM 2372 components have very similar UV/vis spectrophotometric properties. Using the conventional relationship between optical density (properly designated as decadic attenuance) at 260 nm and the quantity of DNA in aqueous solution, the conventional [DNA] of the three components are thus also nearly identical. Although systematic differences among the components are observed with particular qPCR assays, the materials perform very similarly in two widely used gender-neutral systems. While the "true value" of the conventional [DNA] for the materials cannot yet be evaluated, the combination of appropriate extraction protocols and metrologically sound optical measurements enable development of suitable replacement materials when and as needed.

Spectrophotometric value assignment of secondary reference standards

When SRM 2372 is used to validate the D_{10} assignment of secondary DNA quantitation standards prepared in TE^{-4} pH 8.0 buffer, a research-quality dual-beam spectrophotometer should be used that can achieve a spectral bandwidth of 2.0 nm or less (ideally 0.8 nm) and can control the sample temperature at 22 °C±2 °C. The wavelength calibration of the instrument should be verified using a suitable holmium oxide solution standard [33]. The instrument must reliably measure attenuance (indicated as absorbance) in solutions of volume 0.1 mL or less. This capability can be verified using a suitable potassium dichromate absorption standard such as NIST SRM 935a Potassium Dichromate UV Absorbance Standard [34].

Measurement validation is accomplished by evaluating one of more of the SRM components at the five certified wavelengths in the same measurement session as the candidate secondary standard(s). The same TE^{-4} pH 8.0 buffer used to prepare the candidate secondary standards should be used as the reference blank for both the SRM 2372 and candidate standard materials. Since differences between the measured and certified D_{10} at 260 nm could arise from intrinsic differences in spectrophotometer geometries, any observed difference in the SRM materials should be treated as an additional component of uncertainty in the assessment of the D_{10} for the candidate material.

qPCR value assignment of working standards

When used with qPCR assays to value-assign DNA solution working standards, users should calibrate their assays to SRM 2372 using one or more dilution series, each series prepared from one SRM 2372 component. For non-gender-specific qPCR assays, all three components should be used as calibrants to elucidate potential interactions between the materials and the specific assay. Component C should not be used to calibrate gender-specific assays since it is derived from a mixture of unknown proportions of male and female donors. Users should keep in mind that potential for pipetting error exists and should therefore exercise suitable care in preparing the calibration solutions.

Acknowledgements We thank all of the forensic scientists, supervisors, and forensic DNA testing facilities who contributed their expertise, time, and material resources to the interlaboratory assessment. SRM 2372 would not exist except for their encouragement, advice, and willing cooperation with our sometimes seemingly outré requests. The development of SRM 2372 was funded in part by the National Institute of Justice through an interagency agreement with the NIST Office of Law Enforcement Standards.

Disclaimer Certain commercial equipment, instruments, and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments, or equipment identified are necessarily the best available for the purpose.

References

1. Nicklas JA, Buel E (2003) Anal Bioanal Chem 376:1160–1167
2. Butler JM, Coble MD, Decker AE, Duewer DL, Hill CR, Kline MC, Redman JW, Vallone PM (2006) Int Congr Ser 1288:628–635

3. Duewer DL, Kline MC, Redman JW, Newall PJ, Reeder DJ (2001) J Forensic Sci 246:1199–1210
4. Kline MC, Duewer DL, Redman JW, Butler JM (2003) Anal Chem 75:2463–2469
5. Kline MC, Duewer DL, Redman JW, Butler JM (2005) J Forensic Sci 50(3):571–578
6. Krenke BE, Ekenberg S, Frackman S, Hoffmann K, Sprecher CJ, Storts DR (2005) Profiles in DNA (Promega Corporation) 8(1): 3-5; http://www.promega.com/profiles/801/801_03.html
7. Killeen AA (1997) Clinic Lab Med 17(1):1–19
8. Thompson M (1996) Analyst 121(3):285–288
9. Nielsen K, Mogensen HS, Hedman J, Niederstätter H, Parson W, Morling N (2008) Forensic Sci Intl Genetics 2:226–230
10. Warburg O, Christian W (1942) Biochem Z 310:384–421
11. Sambrook J, Russell DW (2001) Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
12. International Union of Pure and Applied Chemistry Commission on Molecular Structure and Spectroscopy, and Commission on Quantities and Units in Clinical Chemistry (1985) Pure Appl Chem 57:105–120
13. SRM 2372—Human DNA Quantitation Standard Certificate https://www-s.nist.gov/srmors/view_detail.cfm?srm=2372
14. NIST Special Publication 260-136—"Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements" (2000); http://ts.nist.gov/MeasurementServices/ReferenceMaterials/upload/SP260-136.pdf
15. Miller SA, Dykes DD, Polesky HF (1988) Nucleic Acids Res 16 (3):1215
16. Applied Biosystems (2003) Quantifiler human DNA quantitation kit and Quantifiler Y human male DNA quantitation kit user's manual, Part Number 4344790 Rev A. Foster City, CA
17. Nicklas JA, Buel E (2003) J Forensic Sci 48:936–944
18. Richard ML, Frappier RH, Newman JC (2003) J Forensic Sci 48:1041–1046
19. Timken MD, Swango KL, Orrego C, Buoncristiani MR (2005) J Forensic Sci 50:1044–1060
20. Nicklas JA, Buel E (2006) J Forensic Sci 51:1005–1015
21. Swango KL, Timken MD, Chong MD, Buoncristiani MR (2006) Forensic Sci Int 158:14–26
22. Knox C, Krenke B (2007) Forensic Magazine April/May 2007; http://www.forensicmag.com/articles.asp?pid=141
23. Mandrekar MN, Erickson AM, Kopp K, Krenke BE, Mandrekar PV, Nelson R, Peterson K, Shultz J, Tereba A, Westphal N (2001) Croat Med J 42:336–339
24. Walsh PS, Varlaro J, Reynolds R (1992) Nucleic Acids Res 20:5061–5065
25. Singer VL, Jones LJ, Yue ST, Haugland RP (1997) Anal Biochem 249:228–238
26. Modeled after the original instrument described in: Mavrodineanu R (1972) J Res Natl Bur Stds 76A:115-125
27. Analytical Methods Committee, Royal Society of Chemistry (1989) Analyst, 114:1693-8 See also: http://www.rsc.org/images/brief6_tcm18-25948.pdf and http://www.rsc.org/ Membership/Networking/InterestGroups/Analytical/AMC/Software/RobustStatistics.asp
28. ISO (1994) ISO 5725-2. Accuracy (trueness and precision) of measurement methods and results. Part 2: basic method for the determination of repeatability and reproducibility of a standard measurement method. ISO, Geneva, Switzerland
29. O'Connor G, Dawson C, Woolford A, Webb KS, Catterick T (2002) Anal Chem 74:3670–3676
30. Yang I, Han MS, Yim YH, Hwang E, Park SR (2004) Anal Biochem 335:150–161
31. English CA, Merson S, Keer JT (2006) Anal Chem 78:4630–4633
32. Holden MJ, Rabb SA, Tewari YB, Winchester MR (2007) Anal Chem 79:1536–1541
33. Travis JC, Acosta JC, Andor G, Bastie J, Blattner P, Chunnilall CJ, Crosson SC, Duewer DL, Early EA, Hengstberger F, Kim C-S, Liedquist L, Manoocheri F, Mercader F, Metzdorf J, Mito A, Monardh LAG, Nevask S, Nilsson M, Noël M, Rodriguez AC, Ruíz A, Schirmacher A, Smith MV, Valencia G, van Tonder N, Zwinkels J (2005) J Phys Chem Ref Data 34:41-56. See also: SRM 2034 - Holmium Oxide Solution Wavelength Standard from 240 nm to 650 nm, available through http://ts.nist.gov/MeasurementServices/ReferenceMaterials/232.cfm
34. Burke RW, Mavrodineanu R (1977) NIST Special Publication 260-54. http://ts.nist.gov/MeasurementServices/ReferenceMaterials/upload/SP260-54.pdf See also: SRM 935a - Potassium Dichromate, UV Absorbance Standard and SRM 1935 - Potassium Dichromate Solution for Use as an Ultraviolet Absorbance Standard. Information on these and other NIST reference materials available throughhttp://ts.nist.gov/MeasurementServices/ReferenceMaterials/232.cfm