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# Comparison of Methods for Accurate Quantification of DNA Mass Concentration with Traceability to the International System of Units

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**Accurate estimation of total DNA concentration (mass concentration, e.g., ng/μL) that is traceable to the International System of Units (SI) is a crucial starting point for improving reproducible measurements in many applications involving nucleic acid testing and requires a DNA reference material which has been certified for its total DNA concentration. In this study, the concentrations of six different lambda DNA preparations were determined using different measurement platforms: UV Absorbance at 260 nm ($A_{260}$) with and without prior sodium hydroxide (NaOH) treatment of the DNA, PicoGreen assay, and digital polymerase chain reaction (dPCR). DNA concentration estimates by $A_{260}$ with and without prior NaOH treatment were significantly different for five of the six samples tested. There were no significant differences in concentration estimates based on $A_{260}$ with prior NaOH treatment, PicoGreen analysis, and dPCR for two of the three samples tested using dPCR. Since the measurand in dPCR is amount (copy number) concentration (copies/μL), the results suggest that accurate estimation of DNA mass concentration based on copy number concentration is achievable provided the DNA is fully characterized and in the double-stranded form or amplification is designed to be initiated from only one of the two complementary strands.**

Accurate estimation of total DNA concentration (mass concentration) is a critical component for many analytical processes involving nucleic acids, including various DNA manipulations (e.g., digestion, ligation, cloning, sequencing, gene expression, etc.) and molecular analyses (e.g., polymerase chain reaction (PCR), real-time PCR). However, several studies[1−3] have highlighted the lack of standards which leads to difficulties in comparing results from different laboratories or between different methods. DNA reference materials certified for their DNA mass concentration, with an estimated measurement uncertainty and traceable to the International system of units (SI), are currently unavailable, yet are necessary for effective comparison of quantitative measurements, method validation, and quality control in routine analysis.

Measuring absorbance of light at a wavelength of 260 nm ($A_{260}$) remains the simplest and most familiar way of estimating DNA mass concentration,[4] based on the Beer−Lambert's law ($A_{260} = \varepsilon cl$), where $\varepsilon$ is the molar absorption coefficient, $c$ is the concentration, and $l$ is the path length of the cuvette.[5] The presence of nucleotides, RNA, single stranded DNA (ssDNA), and impurities such as proteins and phenols may significantly affect the final $A_{260}$ reading[5,6] and could lead to a bias in DNA concentration estimates. When the molar absorption coefficient for double-stranded DNA (dsDNA) is used, the presence of significant amounts of ssDNA could lead to an overestimation of the DNA concentration.[5,7] For this reason, ISO21571 "Foodstuffs−methods of analysis for the detection of genetically modified organisms and derived products−nucleic acid extraction"[8] recommends denaturing dsDNA to its single stranded form using NaOH prior to measuring the $A_{260}$ and using the molar absorption coefficient for ssDNA to estimate the DNA concentration. The $A_{260}$ method is applicable to DNA concentrations in the range of 2−50 μg/mL.[1,8−10]

More recently, fluorescent dyes have been used for quantifying total dsDNA. One such dye is PicoGreen which is more sensitive than the $A_{260}$ assay, so suitable for quantifying lower concentrations of dsDNA. The dye fluoresces upon intercalating into dsDNA, with limited binding to RNA and ssDNA.[11] However, the PicoGreen assay relies on an external standard (e.g., lambda DNA supplied by manufacturers) which has been quantified using $A_{260}$ and is not currently traceable to the SI.[2,4,6]

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Because of the potential bias associated with ultraviolet (UV) absorbance measurements of nondenatured DNA, DNA concentration estimates based on $A_{260}$ readings of nondenatured DNA are often higher than those based on either intercalating fluorescent dyes or $A_{260}$ readings of denatured ssDNA.

In 2007, the measurement of phosphorus content of nucleotides and DNA by high-performance inductively coupled plasma optical emission spectroscopy (HP-ICP-OES) was reported for quantifying total DNA. This method can provide an accurate measurement which is traceable to the SI[2] provided that the material contains no other sources of phosphorus, such as RNA. However, this approach requires large amounts of DNA (1 to 2 mg).[10]

Digital PCR (dPCR) is a relatively new technology which can measure both absolute and relative copy numbers of template DNA independent of external calibrators and, hence, has the potential to be used as a reference method for quantifying DNA amount (copy number) concentration (copies/$\mu$L) required for certification of reference materials.[12,13] Digital PCR requires only small amounts of material when compared to phosphorus analysis, although the cost of dPCR analysis is higher than other techniques. Basically, dPCR involves splitting the PCR solution into a very large number of individual assays. The concentration of DNA in the PCR assay is adjusted so that, when the DNA molecules are delivered across a large number of individual partitions, not all partitions will contain a DNA molecule. Following amplification of the DNA in the partitions, the number of partitions that contain amplified DNA is counted. On the basis of the proportion of positive partitions, statistics can then be used to estimate the number of DNA molecules present in the original solution.[12,14] This technique is ideally suited to resolving subtle effects on PCR analysis[12] which should lead to significant improvements in the reproducibility and quality of quantitative PCR data generally. Digital PCR is increasingly being adopted to undertake studies on copy number variation.[12,15]

While dPCR can potentially be utilized as a reference method for determining copy number concentration of a specific target DNA sequence, it could also be applicable as a reference method for determining mass concentration of total DNA in solution. This study compared DNA mass concentration estimates (ng/$\mu$L) derived from dPCR copy number concentration estimates (copies/$\mu$L) with the mass concentration estimates obtained using the traditional methods of measurements: $A_{260}$ of either nondenatured or denatured DNA and PicoGreen fluorescence. Six different lambda DNA samples, purchased from five different manufacturers, were chosen for this study.

## EXPERIMENTAL SECTION

**Lambda DNA Solutions.** Unmethylated lambda DNA solutions were purchased from five different manufacturers (Promega, Sigma, Fermentas, Biolabs and Invitrogen) at a stated concentration of 329, 300, 536, 350, and 500 ng/$\mu$L, for samples A, B, C, E, and F, respectively. The order of the stated concentration does not correspond to the order of manufacturers listed. Methylated lambda DNA (sample D) at a stated concentration of 383 ng/$\mu$L was purchased from the same manufacturer as the unmethylated lambda solution C. None of the suppliers provided an uncertainty or standard deviation with the stated concentration.

**UV Absorbance Measurements.** The absorbances of both undigested and *Taq*I (NEB, Arundel, Australia) digested DNA were measured at 230, 260, 280, and 320 nm wavelengths under nondenaturing and denaturing conditions. For the denatured samples, the absorbance was measured after treating the samples with NaOH to convert the DNA to the single-stranded form, as detailed in ISO21571.[8] The denatured DNA was prepared by diluting stock lambda DNA 1 to 10 in 1× $TE_{0.1}$ buffer (10 mM Tris, 0.1 mM EDTA buffer, pH 8.0) and adding 2 mol $L^{-1}$ NaOH to the diluted DNA solutions to produce a final concentration of 0.2 mol $L^{-1}$ NaOH prior to reading the absorbance. The UV absorbance measurements at each wavelength were recorded from replicates of five independent dilutions of each lambda DNA sample, using an Eppendorf Biophotometer.

To estimate the DNA concentration in the nondenatured and denatured samples, the molar absorption coefficients of 50 and 37 ng/$\mu$L were used for dsDNA and ssDNA, respectively.[8] The final mass concentration, $\beta$DNA, of nondenatured and denatured DNA was calculated using eq 1,

$$\beta\text{DNA} = F \times (A_{260} - A_{320}) \times \text{molar absorption coefficient (ng}/\mu\text{L)} \quad (1)$$

where $F$ is the dilution factor of DNA solution from the stock tube prior to reading, and $A_{260}$ and $A_{320}$ are the absorbance readings at 260 and 320 nm, respectively. The $A_{320}$ is used since nucleic acids do not absorb at this wavelength, and the reading is informative in determining the background absorption due to light scattering and other UV-active compounds.[8]

**PicoGreen Assay Measurements.** The concentrations of the five independent dilutions of each lambda genomic DNA preparation were estimated using the dsDNA quantification kit (Quant-iT PicoGreen dsDNA assay, Invitrogen P7589) in accordance with manufacturer's instructions. The lambda DNA standard supplied in the Quant-iT kit was diluted in 1× $TE_{0.1}$ to a final concentration of 2 ng/$\mu$L. Further volumetric dilution of the DNA standard in 1× $TE_{0.1}$ was performed to produce five standards (0.1, 0.05, 0.025, 0.0125, and 0.00625 ng/$\mu$L) that were used in the preparation of a standard curve. The contents in each tube were mixed thoroughly by vortexing for 30 s and pulse centrifuged to collect prior to subsequent dilution. The PicoGreen dsDNA quantification reagent was diluted on the day of the experiment by making a 10−20 fold dilution in 1× $TE_{0.1}$ buffer. For PicoGreen analysis, 50 $\mu$L of each dilution of the standard or 1× $TE_{0.1}$ used as no template controls (NTCs) were pipetted into a 96 well PCR plate in duplicate. To each well, 50 $\mu$L of the prepared PicoGreen solution was added using a multipipette dispenser. The wells were sealed with strip caps, and the plate was briefly centrifuged. The plate was placed in the Stratagene Mx3005P and incubated at room temperature for 5 min before the fluorescence was measured using the quantitative plate read setting. Fluorescence data for each standard was collected using the FAM filter which allows optical excitation at 480 nm and measurement at 520 nm. A linear standard curve was plotted using the duplicate data from the standards. On the

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**Table 1. Primers and Probes Used in This Study**[a]

| assay | primer/probe | sequence 5′ to 3′ | concentration (nM) | product length (bp) |
|---|---|---|---|---|
| #1 | F | TGCAATGACCCCGCTGATG | 500 | 147 |
| | R | CGGAACGTGCCGGACTTG | 500 | |
| | P | 6-FAM-CTGGTCTGGTCAGCAGCAACCGCAA -BHQ1 | 100 | |
| #2 | F | TGCGCTGTATGCCGGTATG | 500 | 188 |
| | R | GTTGTTCGGGTCAATCCAGTTC | 500 | |
| | P | 5-HEX-CCTCAACGGCATTATGGCGGTCCTT -BHQ1 | 300 | |
| #3 | F | CTTCATCTCTGTTCATTATCATCGC | 500 | 173 |
| | R | TTTAACTCGTGAGGTGTTTTTACTTG | 500 | |
| | P | 6-FAM-ACCACCACTTATTATCGCAGTCCGT -BHQ1 | 40 | |
| #4 | F | TGATTCTGTTCCGCATAATTACTCC | 500 | 134 |
| | R | CACCAATGCTGAGATAGCTGAAG | 500 | |
| | P | 5-HEX-TCCTTAACTTTGCCCACCTGCCTTT -BHQ1 | 500 | |
| #5 | F | CCCAGCAACAGCACAACCC | 500 | 76 |
| | R | GCCGCAGCGTAACTATTACTAATG | 500 | |
| | P | 6-FAM-ACTGAGCCGTAGCCACTGTCTGTCCT -BHQ1 | 40 | |

[a] The entire lambda genome used for this study is a forward sequence: (F) forward primer, (R) reverse primer, (P) probe, (6-FAM) 6-carboxyfluorescein, (BHQ-1) Black Hole Quencher 1, and (5-HEX) hexachloro-6-carboxyfluorescein.

basis of the concentration of DNA estimated from the $A_{260}$ measurements, each lambda DNA solution was diluted in 1× $TE_{0.1}$ to approximately 0.08 ng/$\mu$L and analyzed in duplicate using the same process as described above for the standards. The DNA mass concentration of each lambda DNA solution was calculated relative to the prepared standard curve. To determine the effect of DNA digestion on PicoGreen binding efficiency, lambda DNA digested with *Taq*I restriction enzyme was used in some cases.

**Digital PCR Measurement.** Using sequence information retrieved from Genbank for lambda genome AJ02459 and the lambda DNA restriction map, five different real-time PCR assays (Assays #1−#5) were designed using Beacon Designer 7.5 software (Table 1). The assays were located at different positions along the length of the 48 502 base pair (bp) genome to determine if the copy number concentration estimate was independent of the target region selected by the assay.

End-point gradient PCR was performed to verify the optimal annealing/extension temperature for each assay. The concentrations of the primer and probe sequences were then optimized by real-time PCR on the Stratagene Mx3005P prior to use in the dPCR (Table 1). The primer concentration was the same for both forward and reverse primers in all five assays. However, the optimal probe concentration varied depending on the assay. The real-time PCR thermocycling conditions on the Mx3005P consisted of a 20 s activation period at 95 °C, followed by 45 cycles of a two-step thermal profile involving 2 s at 95 °C for denaturation, and 20 s at 60 °C, for combined annealing and extension.

The dPCR analysis was performed on the BioMark System (Fluidigm, South San Francisco) using the 12.765 digital arrays (Fluidigm). Lambda DNA samples A, B, and C were tested as a proof of concept. To improve PCR amplification efficiency,[12] the DNA was digested with restriction endonuclease prior to gravimetric dilution. On the basis of PicoGreen estimated values, 2 or 20 $\mu$g of lambda DNA was digested in a total volume of either 50 or 500 $\mu$L, respectively, of the restriction digestion reaction mix containing the appropriate restriction enzyme buffer, 20 ng/$\mu$L RNase A, and 40 units of either *Taq*I or *Msp*I. The final volume was made up with nuclease-free water (Promega, Sydney, Australia), and the digest was incubated for 2 h at either 65 °C (*Taq*I) or 37 °C (*Msp*I) for digestion. The enzymes were inactivated by incubating at 80 °C for 10 min. Five $\mu$L of digested DNA was analyzed on a 1% TBE (0.89 M Tris−0.89 M borate−20 mM EDTA, pH 8.0) agarose gel to confirm complete digestion. Three independent gravimetric dilutions of both the *Taq*I and the *Msp*I digested lambda DNA samples were prepared. Assays #1 and #2 both had an *Msp*I site between the two primer binding sites, hence *Taq*I digested DNA was used. Likewise, Assay #3 had an internal *Taq*I site; hence, *Msp*I digested DNA was used, while Assays #4 and #5 had neither *Taq*I nor *Msp*I restriction sites. The three *Taq*I digested and diluted DNA preparations were each analyzed in quadruplicate using both Assays #1 and #2. The three *Msp*I digested and diluted DNA preparations were each analyzed in quadruplicate using Assays #3, #4, and #5. NTCs containing 1× $TE_{0.1}$ buffer in place of DNA were only used in the preliminary runs and were analyzed in single or duplicate panels for each assay. The reactions were carried out either in simplex or in duplex conditions. The final reaction mix for each digital panel composed of *Taq*I or *Msp*I digested DNA, 1× Taqman FAST Universal PCR Mastermix with no UNG AmpErase (Applied Biosystems Melbourne, Australia), 1× Digital Array sample loading reagent (Fluidigm, South San Francisco), and relevant forward and reverse primers and probe (Table 1). This digested lambda DNA template contained between 150 and 550 copies of the target sequence per $\mu$L of final reaction mix, based on the DNA concentrations estimated using the Quant-iT PicoGreen dsDNA assay. The range of 150−550 copies was chosen to minimize the uncertainty associated with the dPCR copy number concentration estimates.[12] To minimize the uncertainty from pipetting, all PCR components excluding DNA were premixed and, then, the final reaction mix was prepared gravimetrically by combining the DNA and PCR components. Ten $\mu$L of reaction mix was aliquoted into each sample inlet on the digital array with approximately 4.6 $\mu$L of the reaction mix distributed throughout the partitions within each panel using an automated NanoFlex IFC Controller (Fluidigm, South San Francisco). The digital array thermocycling conditions on the BioMark System PCR were identical to those used on the Stratagene Mx3005P.

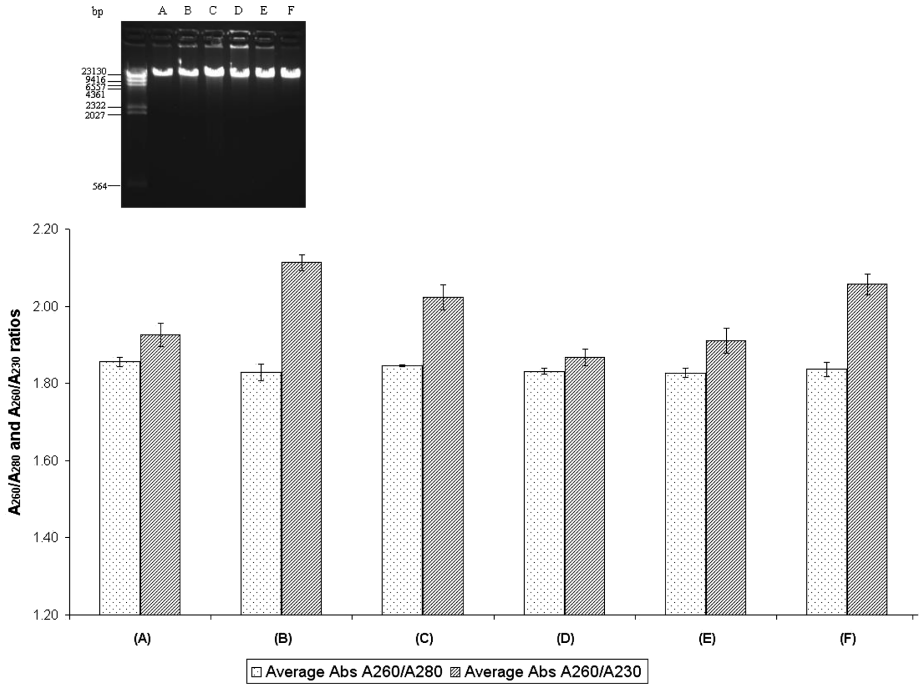


**Figure 1.** Measurement of six different undigested lambda DNA samples (A, B, C, D, E, and F) at 260, 280, and 230 nm to evaluate the purity of the samples: (speckled) $A_{260}/A_{280}$ and (diagonal bar) $A_{260}/A_{230}$ ratios. The error bars represent standard deviation ($n = 5$) for each sample. Inset shows ethidium bromide stained 1% TBE agarose gel image of the same lambda DNA samples.

## RESULTS AND DISCUSSION

**Characterizing the Integrity and Purity of the Lambda DNA Preparations Using Absorbance Measurements and Gel Electrophoresis.** The integrity and purity of each lambda DNA sample were estimated based on $A_{260}$, $A_{280}$, and $A_{230}$ and by agarose gel electrophoresis. On the basis of the banding pattern following agarose gel electrophoresis (Figure 1, inset), the DNA in all of the lambda samples was free of RNA and relatively intact, with little fragmentation of the DNA observed. The $A_{260}/A_{280}$ ratio is indicative of the purity of DNA. A ratio of approximately 1.8 is generally considered as "pure" for DNA. If the ratio is lower than 1.8, it may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. The $A_{260}/A_{230}$ ratio for "pure" DNA is often higher than the respective $A_{260}/A_{280}$ value. Expected $A_{260}/A_{230}$ values are commonly in the range of 2.0−2.2.[16,17] If the ratio is lower than expected, it may suggest the presence of contaminants which absorb at 230 nm, such as EDTA, carbohydrates, and phenol.[16–18] The $A_{260}/A_{280}$ ratios ranged between 1.83 and 1.86 for all nondenatured lambda DNA samples. (Figure 1). The $A_{260}/A_{230}$ ratios were more variable, with lambda DNA solutions ranging from 1.87 to 2.11.

**Estimating the Concentration of Lambda DNA Preparations Using UV Absorbance.** The DNA concentration in each lambda DNA preparation was estimated using UV absorption spectroscopy of dsDNA samples and compared to the supplier's estimation using a two-tailed $t$ test (eq 2) to determine if the estimated mean from five replicate measurements, $\bar{x}$, is significantly different from the value supplied by the manufacturer. The manufacturer's supplied value was taken as the hypothesized population mean, $\mu_0$, and the critical value for $t$ was based on 4 degrees of freedom $(n - 1)$.

$$t = \frac{\bar{x} - \mu_0}{(s/\sqrt{n})} \tag{2}$$

where $s$ is the standard deviation of the five replicate measurements and $n$ is the sample size. The measured concentration for all samples was significantly different ($p < 0.05$) from the value provided by the supplier (Figure 2).

The estimated DNA concentration for NaOH treated samples was significantly lower ($p < 0.05$) than the value obtained from nondenatured DNA in each case, with the exception of sample F where there was no significant difference in the estimated concentration between the nondenatured and the NaOH treated sample ($p = 0.307$). This suggests a possible overestimation of the DNA concentration for samples A−E when nondenatured samples are used.

**Estimating the Concentration of Lambda DNA Preparations Using the PicoGreen Assay.** The DNA standard, supplied with the Quant-iT PicoGreen dsDNA assay kit (Invitrogen P7589 lambda DNA), was used to estimate the DNA concentrations of the other five samples using the PicoGreen assay. For three of the undigested lambda DNA samples (C, D, and F), quantification based on $A_{260}$ of nondenatured DNA was not significantly different from quantification based on the PicoGreen assay ($p > 0.05$). Samples A and B did show a significant difference in quantification estimates using $A_{260}$ of nondenatured DNA and PicoGreen measurement techniques with $p$-values of 0.037 and 0.007, respectively (Figure 2). Subsequently, when *Taq*I digested DNA samples were analyzed using the $A_{260}$ and PicoGreen

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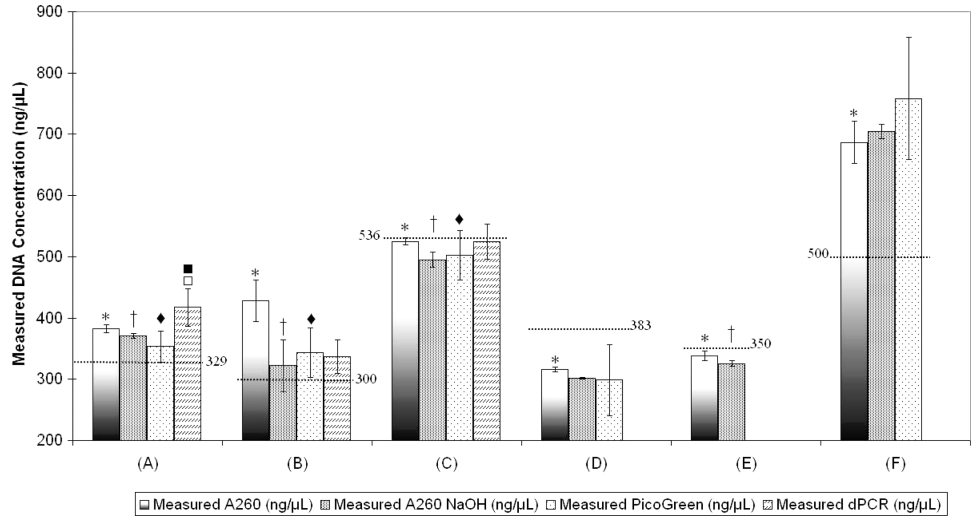


**Figure 2.** Comparison of four different DNA measurement methods: (shaded) $A_{260}$ nondenatured, (concentrated spots) $A_{260}$ denatured, (speckled) PicoGreen, and (diagonal lines) digital PCR. The error bars represent the standard deviation for $A_{260}$ nondenatured ($n = 5$), $A_{260}$ denatured (NaOH) ($n = 5$) and PicoGreen ($n = 5$), and pooled standard deviation of dPCR data obtained from Assays #1 to #5 for Samples A and C and from Assays #1, #2, #4, and #5 for Sample B. The dotted lines correspond to the manufacturer's stated concentration (ng/$\mu$L) estimated from the $A_{260}$ value. Significant differences observed between measurements ($p$-values <0.05) are denoted in symbols above the bars for each sample, *: supplier versus measured $A_{260}$ (untreated); †: measured $A_{260}$ (NaOH versus untreated); ♦: PicoGreen versus $A_{260}$ (untreated); □: digital PCR versus PicoGreen; ■: digital PCR versus NaOH treated.

**Table 2. Summary Table Representing DNA Concentrations (ng/$\mu$L) Estimated from Four Different DNA Measurement Methods**

| supplier | supplier value (ng/$\mu$L) | mean ($n = 5$) estimates concentration (ng/$\mu$L) (standard deviation) | | | mean ($n = 15$) estimates concentration (ng/$\mu$L) (standard deviation) |
|---|---|---|---|---|---|
| | | $A_{260-}$ nondenatured | PicoGreen | $A_{260-}$ NaOH denatured | digital PCR |
| A | 329 | 383 (6.7) | 354 (25) | 371 (4.1) | 417(28) |
| B | 300 | 428 (34) | 343 (40) | 322 (43) | 385[a] (108)<br>337[b] (14) |
| C | 536 | 525 (6.0) | 503 (40) | 495 (13) | 523 (33) |
| D | 383 | 316 (3.9) | 299 (58) | 302 (1.4) | |
| E | 350 | 338 (7.3) | | 325 (4.7) | |
| F | 500 | 687 (35) | 758 (100) | 705 (12) | |

[a] With assay#3. [b] Assay#3 excluded.

methods, no significant difference was observed for samples D ($p = 0.284$) and F ($p = 0.069$), while a significant difference ($p < 0.05$) was observed for all other samples. *Taq*I digested DNA was utilized for the PicoGreen measurement to evaluate whether digestion of DNA would enhance PicoGreen binding and increase fluorescence. Recently, Holden et al.[10] reported that supercoiled plasmid DNA does not bind PicoGreen to the same degree as linearized plasmid. The results presented in this study comparing undigested and *Taq*I digested lambda genomic DNA are similar to those observed by Ahn et al.,[18] who noticed no significant difference in PicoGreen fluorescence between intact DNA versus DNA digested with restriction endonucleases. They further concluded that neither size nor complexity of DNA influenced the assay and that PicoGreen binds with the same intensity and efficiency regardless of fragment size, in agreement to our findings. This observation can vary with different templates.

**Estimating the Concentration of Lambda DNA Preparations Using Digital PCR.** Lambda DNA samples A, B, and C were tested as a proof of concept to compare estimation of DNA mass concentration (ng/$\mu$L) by dPCR with values obtained using UV absorbance and PicoGreen assay measurements (Table 2). Prior to actual analysis, we first evaluated template amplification in digital panels by comparing results with undigested genomic DNA and genomic DNA digested with either *Taq*I or *Msp*I restriction endonuclease. Enhancement of amplification efficiency has been observed previously following fragmentation of target genomic DNA with restriction enzymes.[19] The results confirm that digestion of lambda genomic DNA with either *Taq*I or *Msp*I improves efficiency of target amplification and reduces the proportion of positive partitions with a Ct >30 compared to undigested DNA. For Assay #3, 16.5% of positive partitions had a Ct >30 for the undigested sample, whereas only 0.1% of positive partitions had a Ct >30 for the digested sample (Figure 3A,B). A similar result (data not shown) was observed for Assays #1, #2, #4 and #5. This observation suggests that single molecule amplification from some nonlinearized genomic DNA molecules may be unsuccessful in the initial cycles. A similar result was obtained previously[12] using nonlinearized plasmid DNA molecules. On the basis of a two-tailed $t$ test, there was no significant

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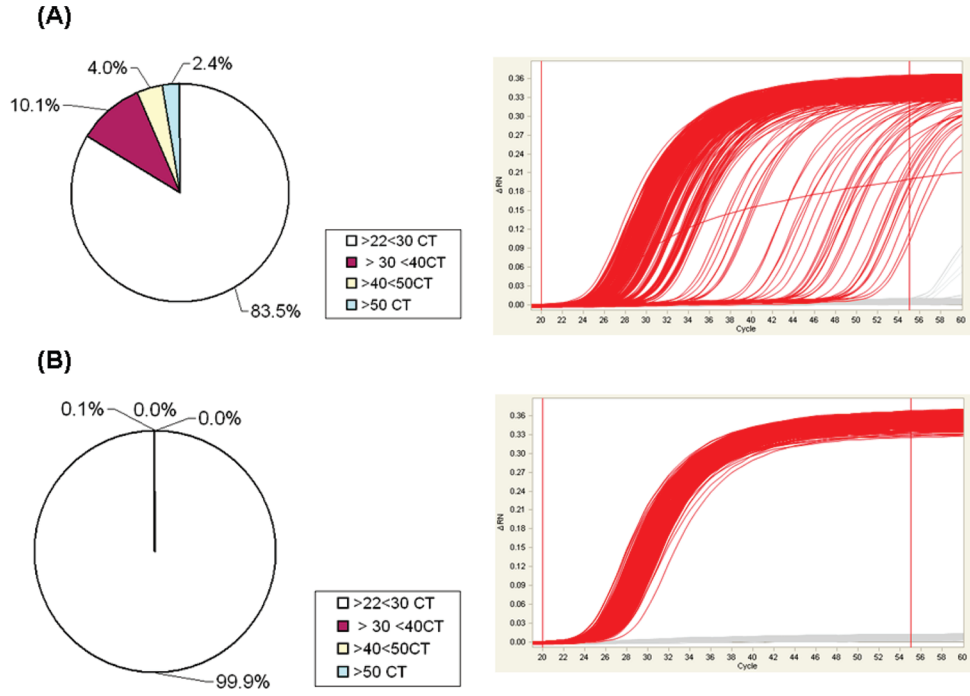


**Figure 3.** Template amplification using Assay #3 in digital panels. Digital PCR pie-charts of percentage of positive partitions within a defined Ct range and amplification plots of undigested (A) and *Msp*I digested (B) phage lambda genomic DNA showing distribution of Ct values as a result of fragmentation of total DNA compared to intact DNA.

difference in the estimated DNA concentration ($p > 0.05$) between *Taq*I (Assay #1/ #2) and *Msp*I (Assay #3, #4, #5) digested DNA using three independent gravimetric dilutions ($p = 0.234$) and the values obtained were close to the $A_{260}$ measured value (NaOH denatured or nondenatured) (data not shown). However, a significant difference ($p < 0.001$) in the estimated DNA concentration between Assays #1/#2 and Assays #3, #4, #5 for undigested DNA was observed. Therefore, *Taq*I and *Msp*I digested lambda genomic DNA was used for dPCR analysis of samples A, B, and C. Further, we tested the lambda DNA purchased from different manufacturers using the *E. coli* 16S ribosomal DNA (rDNA) primer/probe sets. Since Lambda DNA is isolated from infected *E. coli*, it was essential to determine that no background *E. coli* genomic DNA remained after preparing the pure Lambda DNA sample as this would create an overestimation of $A_{260}$ (intact and NaOH treated) and PicoGreen measurements. Thus, *E. coli* 16S rDNA primer/probe sets were used and no amplification was observed when lambda DNA samples were used as template (data not shown), suggesting that the lambda DNA purchased from different manufacturers were all free of any *E. coli* genomic DNA.

ANOVA (Analysis of variance) showed no significant difference in estimated DNA concentration ($p > 0.05$) from all five dPCR assays tested for both samples A and C; however, on the basis of the Grubbs' test,[20] the concentration for sample C estimated using Assay #3 was close to being an outlier ($p = 0.064$). With sample B, a significant difference in the estimated DNA concentration ($p = 0.00009$) was observed between results from Assay #3 and the other four assays. Assay #3 was less reproducible than the other assays, as demonstrated by the larger standard error of the mean for this assay (Figure 4). A Grubbs' test[20] determined that the concentration for sample B estimated using Assay #3 was an outlier ($p = 0.001$) (Figure 4). Therefore, in order to estimate the accurate concentration of sample B in the original solution, data from Assay #3 was excluded. The concentrations of the original lambda DNA solutions using dPCR analysis and Assays #1−#5 were calculated to be 417 ng/$\mu$L with a pooled standard deviation of 28 ng/$\mu$L for sample A and 523 ng/$\mu$L with a pooled standard deviation of 33 ng/$\mu$L for sample C (Figure 4). The concentration of sample B using data from Assays #1, #2, #4, and #5 was calculated to be 337 ng/$\mu$L, with a pooled standard deviation of 14 ng/$\mu$L. On the basis of *t*-statistics, there was no significant difference ($p > 0.05$) between the PicoGreen-based and dPCR-based DNA concentration estimates for samples B and C while the dPCR estimate was significantly higher than the PicoGreen-based measurement for sample A ($p < 0.05$) (Figure 2).

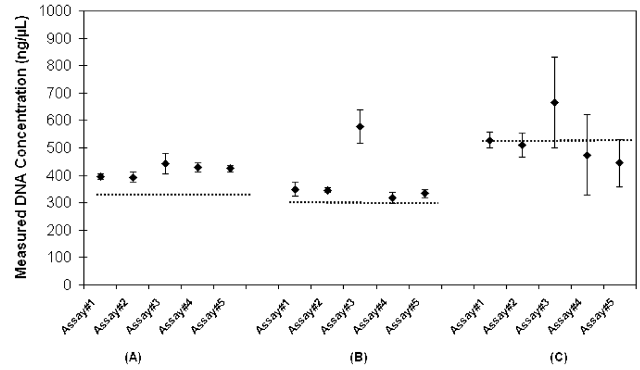


**Figure 4.** DNA concentration estimated by digital PCR for three different *Taq*I or *Msp*I digested lambda DNA samples (A, B, and C). The dPCR value was derived from quadruplicate analysis on each of three independently prepared gravimetric dilutions. The error bars represent standard error of the means ($n = 3$).The dotted lines correspond to the manufacturer's stated concentration (ng/$\mu$L) estimated from the $A_{260}$ value.

(20) Grubbs, F. E. *Technometrics* **1969**, *11*, 1–21.

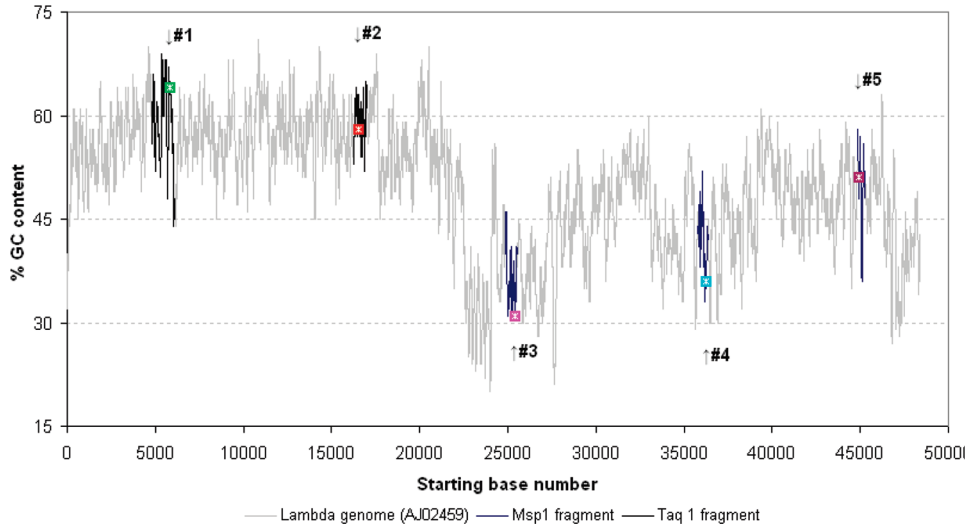


**Figure 5.** GC content within a moving window of 100 bases for lambda DNA AJ02459 determined using the DAN program (http://www.cbib.u-bordeaux2.fr/pise/dan.html). Starting base number indicates the initial base in the group of 100 bases. The position of Assays #1−#5 are shown by the labeled star symbols, and the associated *Taq*I and *Msp*I fragments are as indicated.

On the basis of the genome sequence retrieved from Genbank for lambda DNA (Accession no: J02459), the regional GC content for 100 bp segments across the entire length of the genome varies from 20% to 70% (Figure 5). The 779 bp *Msp*I digested fragment containing the target sequence for Assay #3 has a GC content of 37%. We speculated that the low GC content of this fragment could result in a shift in the equilibrium between dsDNA and ssDNA forms of this fragment in the PCR mix prior to amplification. If some complementary strands are in single stranded form during the process of loading the sample onto the digital chip, it is possible that the two strands are distributed into separate partitions. This would create two independent amplifiable units in the dPCR format and could account for an increase by a factor of up to two in estimated copy number concentration when Assay #3 is used. For dPCR measurement, the physical form of the DNA (single-stranded vs double-stranded) is critical since target copy number is estimated on the basis of the proportion of partitions containing amplified DNA regardless of whether the amplifiable unit was a ssDNA or a dsDNA template.[12] For accurate copy number estimation, either the DNA must be in the double-stranded form or the amplification process must be designed so that amplification can only be initiated from one of the two complementary strands.

To further verify whether the overestimation of concentration by dPCR using Assay #3, as seen in Figure 4 for both samples B and C, is due to ssDNA fragments, sample B DNA was heat-treated at 95 °C for 30 s and immediately chilled on ice to limit reannealing of the ssDNA, and the results were compared with sample DNA that had not been heat-treated. In this experiment, there was no significant difference ($p > 0.05$) in the estimated DNA concentration when Assays #3 and #5 ($p = 0.153$) were used for sample B that had not been preheated. Heat treatment of sample B at 95 °C for 30 s resulted in an exact doubling (2.0 fold increase) of the estimated number of copies/$\mu$L of the target molecule using either assay compared to the equivalent sample that had not been heat treated. There was no significant difference in the estimated concentration of the heat-treated sample using Assays #3 and #5 ($p = 0.144$). This observation confirms that dPCR amplification can occur from either dsDNA or ssDNA templates. It also supports the possibility that the lack of reproducibility of Assay #3 may be due to the low GC content of the fragment containing the Assay #3 template region and the resultant variability in the proportion of these fragments in the ssDNA conformation.

We have previously shown[12] that the combined relative uncertainty for the concentration of template molecules in the PCR solution, $T_p$, considering the factors, $M$ (template molecules per panel) and the $V_p$ (partition volume) can be under 6% when analyzing five replicate panels. The combined relative uncertainties of the mass concentration estimated using dPCR for the three lambda samples, A, B, and C, were between 5.2 and 5.3%. This is slightly lower than our previous estimate since, for each lambda solution, 24 digital panels were analyzed using duplex assays and 12 panels were analyzed using simplex assays, thus providing a total of 60 digital assay data points for each lambda DNA solution. Such a large number of digital assays reduces the uncertainty associated with $M$, leaving $V_p$ as a major contributor to the uncertainty. The combined relative uncertainty based on $M$ and $V_p$ is slightly lower than the relative standard deviation derived from precision data, which varies from 5.8 to 8.5% depending on the assay. However, the current estimate of the combined uncertainty does not include the bias component for the proportion of ssDNA in a dsDNA preparation and, therefore, can be underestimating the uncertainty. Improvements in accuracy of the partition volume measurements together with development of methodology for amplification from a single strand of the dsDNA will reduce this uncertainty further.

## CONCLUSION

Accurate and precise quantification of DNA is vital for various nucleic acid analyses. In this study, DNA concentration estimated by UV absorbance at 260 nm (intact and NaOH denatured), PicoGreen based fluorometric quantification, and dPCR analysis were compared. For accurate dPCR measurement, intact dsDNA is critical, since dPCR involves splitting the reaction mix across a

large number of individual partitions prior to amplification and alterations in the physical state of the DNA can lead to bias in copy number estimates. As with $A_{260}$ measurements, a mixture of single-stranded and dsDNA can lead to an overestimation of DNA concentration by dPCR. Denaturing dsDNA using the method described in ISO21571[8] before measuring the $A_{260}$ and using the molar absorption coefficient for ssDNA will eliminate the bias created by ssDNA in a dsDNA preparation. However, presence of other contaminants such as proteins, RNA, and salts can contribute toward overestimation of DNA concentration by $A_{260}$ and, therefore, can affect the PicoGreen estimate as the dye fluoresces upon intercalating into dsDNA, with limited binding to RNA, ssDNA, and other contaminants.[11] The measured $A_{260}$ value obtained by denaturing the dsDNA using NaOH is an effective approach for direct comparison of values obtained with dPCR. Alternative methods such as measurement of the phosphorus content of nucleotides and DNA are useful in quantification of nucleic acids and can be traceable to SI. However, phosphorus analysis requires large amounts of material and purity of DNA is critical.[10] On the basis of the observed results in this study, the choice of the method used for accurate quantification of DNA is crucial. Potential sources of error associated with each method should be considered to minimize bias and to maximize accuracy and precision. Digital PCR has the potential to be used as a primary method for accurate quantification of both target DNA copy number and DNA mass concentration independent of an external calibrator. Development of methods for accurate measurement of the digital chip partition volume and for digital amplification from a single strand of the target will address two of the major sources of uncertainty that are currently associated with dPCR.

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## NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on August 6, 2010 with an error in the amount of lambda DNA specified in the experimental discussion of the digital PCR measurement. The corrected version was reposted on August 12, 2010.

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