

Measurement of Nucleic Acid Concentrations Using the DyNA Quant™ and the GeneQuant™

J.M. Teare, R. Islam, R. Flanagan, S. Gallagher, M.G. Davies¹ and C. Grabau
Hoefler Pharmacia Biotech, San Francisco, CA, USA; ¹Pharmacia Biotech (Biochrom),
Cambridge, England, UK

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ABSTRACT

Molecular biology is now a routine tool in almost all biological research fields. With the exponential growth in the number of molecular biological techniques, there is a recognizable need for sensitive, accurate and precise quantitation of nucleic acids. We present here two complementary instruments designed for the quantitation of nucleic acids, the GeneQuant II and the DyNA Quant 200 Fluorometer. The GeneQuant II can rapidly determine the UV absorbance of a solution and display the calculated DNA, RNA or protein concentration. In addition, the GeneQuant can display calculated melting temperatures for a given DNA oligonucleotide base sequence, a useful feature for primer design. The DyNA Quant 200 quantitates DNA on the basis of the fluorescent Hoechst 33258 dye/double-stranded (ds)DNA assay. Upon binding to dsDNA, the spectral properties of the dye change such that it becomes highly fluorescent at 460 nm when excited at 365 nm. The assay has proven to be a specific and sensitive alternative method for DNA quantitation, particularly for unpurified DNA samples. Together, the GeneQuant II and the DyNA Quant 200 are a cost-effective and convenient solution to the routine protein and nucleic acid quantification needs of the molecular biologist.

INTRODUCTION

Quantitation of nucleic acids is essential in many research procedures in molecular biology. However, accurate determination of DNA or RNA concentrations can be difficult, particularly when the purity of the sample is uncertain. In many cases, the most rapid and accurate approach for determining the concentration of relatively pure nucleic acids and proteins is absorbance spectrophotometry. The amount of light absorbed by a sample is directly proportional to the concentration of protein and/or nucleic acid in the sample. Conversion of absorbance readings to concentration is based on the Beer-Lambert law (3).

Both DNA and RNA absorb maximally at 260 nm, while most proteins absorb strongest at 280 nm. However, nucleic acids also absorb significantly at 280 nm (50%–55% of the absorbance at 260 nm), and most proteins can absorb strongly at 260 nm (the absorbance varies, depending on the protein). Thus, it can be difficult to accurately measure the concentrations of DNA, RNA and protein in complex mixtures. However, measuring absorbance at 260 nm and at 280 nm can provide validation of the purity of nucleic acid samples: A_{260}/A_{280} ratios above 1.8 for DNA or 2.0 for RNA indicate pure samples; lower ratio values indicate the presence of protein or other contaminants (3). The reverse is true for nucleic acid contamination of protein preparations, where very small amounts of nucleic acids can have a significant effect on the absorbance of 280-nm light by a protein sample (2,5,14).

Absorbance spectrophotometry is a rapid and accurate

technique for determining DNA concentration of pure samples. It is less accurate when the DNA sample contains protein, RNA, oligonucleotide primers or nucleotides. RNA, primers and nucleotides absorb strongly at 260 nm and cannot be distinguished from DNA. These contaminants, as well as proteins, can cause an overestimate of the DNA content in mixed samples. This problem can be overcome by using an alternative method to measure only the DNA in the sample. A robust yet sensitive assay for DNA in the presence of other biomolecules is the fluorescent Hoechst dye DNA assay (3,6).

MATERIALS AND METHODS

GeneQuant™ II

The GeneQuant II (Pharmacia Biotech, Piscataway, NJ, USA) is a versatile yet economical instrument for measuring the concentrations of proteins and nucleic acids. The GeneQuant is capable of measuring UV absorbance of samples at 230, 260, 280 and 320 nm simultaneously. Using the specific absorption coefficients for nucleic acids (3), the GeneQuant II can automatically convert the 260-nm absorbance readings into concentrations for double-stranded (ds)DNA, single-stranded (ss)DNA, RNA or oligonucleotides in any of a number of different units (i.e., $\mu\text{g}/\text{mL}$, $\mu\text{g}/\mu\text{L}$, $\text{pmol}/\mu\text{L}$ or mol/mL). The molecular weight is automatically calculated from the base sequence as each base is entered by the user and can be called up and reported. This value is used for the appropriate molar concentrations, which are thus calculated on base units. If the absorption coefficient at 280 nm is known for a given purified or partially purified protein preparation, the GeneQuant II can be programmed to display the protein concentration in mg/mL .

Nucleic acids and proteins absorb minimally at 320 nm. Thus, the reading at 320 nm can be used for background correction to eliminate sample matrix effects. Background subtraction is critical for accurate and reproducible measurement of low nucleic acid concentrations or when sample volumes are low, as when using the capillary and ultra-microvolume cells.

The GeneQuant II can also be used for additional calculations. After entry of the nucleotide sequence and concentration, it will calculate and display the molecular weight, apparent melting temperature (T_m) and theoretical absorbance of a given nucleic acid. The theoretical absorbance is reported as absorbance units/ μmol and, like the T_m , is calculated by the nearest-neighbor method, according to the base sequence entered by the user. This feature is particularly useful for the design and analysis of oligonucleotide primers for polymerase chain reaction (PCR) or sequencing.

Absorbance Measurements Using GeneQuant II

Spectrophotometric measurements of pure or nearly pure nucleic acids and proteins are simple and fast. Simply dilute the sample in an appropriate buffer and read. Measurements of a reference solution at all four wavelengths are stored and used as baseline readings before the samples. The GeneQuant II uses conversion factors that are based on well-established UV data for nucleic acids, nucleotides and proteins (3) so that absorbance measurements are automatically converted to

concentrations. Figure 1 shows an example of the quantitation of calf thymus genomic DNA using the GeneQuant II.

For measurement of samples at very low concentrations or to correct for buffer and/or solution matrix effects, the instrument can be calibrated using appropriate standards. In addition, the GeneQuant II is able to calculate the expected absorbance value for a given nucleotide sequence or sequence composition at a given concentration. This is particularly useful for verifying the quantification of oligonucleotides. A wide range of sample concentrations and volumes can be handled by different cuvettes and capillaries, enabling measurements of a wide dynamic range of DNA concentrations from 35 ng in 3 μL to 100 μg in 2 mL.

The GeneQuant II also calculates and displays the A_{260}/A_{280} ratio of the sample. This reading provides an instant verification of DNA or RNA purity. DNA and RNA may have A_{260}/A_{280} ratios ranging from 1.7–2.0, depending on the concentration of the nucleic acid, the buffer composition and any impurities. Low A_{260}/A_{280} ratios are typically due to the presence of protein, phenol or surfactant. In addition, nucleic acids that are not fully resuspended can scatter light, resulting in low A_{260}/A_{280} ratios. Any precipitates, whether they are silica particles, salt crystals (i.e., guanidine thiocyanate, LiCl or NaI) or surfactant micelles can result in abnormal A_{260}/A_{280} ratios. Light scatter from precipitates is also typified by high background absorbance at 320 nm and very high absorbance at 230 nm. The presence of buffer salts, solvents and other impurities is also indicated by a relatively high 230-nm absorbance.

DyNA Quant™ 200

A complementary method for determining dsDNA concentration is based on changes in the fluorescence of the fluorochrome Hoechst 33258 (bisbenzimidazole) upon binding to DNA (1,3,4,6). When excited with 360-nm light, the 458-nm fluorescence emission of the dye increases in the presence of DNA. The interaction of H33258 with DNA is highly specific. It binds primarily to AT bp sequences in the minor groove of dsDNA (6). In a high-salt, neutral pH buffer, the level of fluorescence is directly proportional to the amount of DNA present (6). This method is easy to use, accurate and avoids errors due to contaminating RNA and protein (1,4,6,9,12). In addition, H33258 fluorescence is much more sensitive than UV absorbance, reducing the amount of DNA sample needed for measurements.

Since the H33258 dye has a preference for AT bp sequences, the fluorescence intensity of a given amount of DNA is dependent on the percent A/T content of the DNA (3,6). Conveniently, an empirically determined relationship between percent A/T content of DNA and H33258 fluorescence can be used to normalize the fluorescence values of genomic DNAs that differ in A/T content (13). Normalization factors, either calculated or determined (10), are useful for measuring the concentration of many different types of DNAs using a fluorometer calibrated with a universal standard such as calf thymus DNA.

The minimum length of dsDNA detectable using the H33258-DNA assay depends on the DNA sequence. For most applications, dsDNA at least 200 bp in length can be detected. Shorter molecules can be detected provided that two or three consecutive AT bp are present.

The Hoefer® DyNA Quant 200 Fluorometer (Hoefer

Table 1. Fluorescence of DNA, RNA and Protein in the H33258-DNA Assay

Solution	Concentration (µg/mL)	Fluorescence Units	Apparent DNA Concentration ^a (µg/mL)
DNA	1.0	1000	1.00
RNA	1.0	41	0.041
Protein	10.0	0	0.00
DNA+RNA+Protein	1.0+1.0+10.0	1037	1.037

^aApparent DNA concentration is calculated by comparing to fluorescence of a known amount of highly purified calf thymus DNA standard.

Solutions of DNA, RNA and protein were measured separately or together in a DyNA Quant 200 Fluorometer. For the fluorescence assay, the manufacturer's recommended sample buffer was 1 µg/mL H33258 dye in 10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4. Macromolecule concentrations were 1 µg/mL calf thymus DNA, 1 µg/mL tRNA or 10 µg/mL bovine serum albumin.

Pharmacia Biotech, San Francisco, CA, USA) was developed for the quantification of DNA with the H33258 fluorochrome. The instrument has a narrow-band excitation light source (365-nm ± 7-nm peak) and a fixed bandpass emission filter (460-nm ± 15-nm peak). The 365-nm long-wave UV excitation light passes through the sample and is absorbed by the DNA-H33258 dye complex, inducing fluorescence in the blue wavelength region (460 nm). Fluorescent light is detected at 90 degrees relative to the excitation light after first passing through the emission filter (Figure 2).

Fluorescence Measurements Using DyNA Quant 200

Because of the specific nature of H33258-DNA binding, the assay is insensitive to relatively high concentrations of protein, RNA, nucleotides and short oligonucleotides, as well as common buffer components (6,9,10). This is illustrated in Table 1, which compares H33258 fluorescence measurements for samples containing DNA, RNA or protein and a mixture

of the three. In the fluorescence assay, the contribution of RNA in such a mixture is only 4%, and protein is not detectable. Thus, for impure DNA samples, the DNA content is more accurately determined by the fluorescent assay than by absorbance spectrophotometry.

The actual numeric value of fluorescence units produced by a sample depends on the instrument design and sensitivity, and requires independent calibration. By calibrating the fluorometer with a known amount of DNA, the results of subsequent measurements of unknown DNA samples can be displayed as actual concentration units.

Fluorometry offers the advantage of high sensitivity and wide dynamic range; the standard H33258-DNA assay using the DyNA Quant 200 can detect as little as 20 ng DNA.

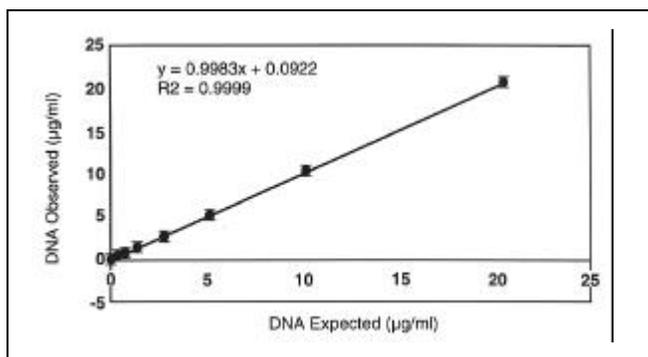


Figure 1. DNA standard quantified by absorption spectrophotometry using the GeneQuant. 20 µL of a 1-mg/mL solution of calf thymus DNA were diluted into 1000 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to give a solution of 20 µg/mL. This DNA solution was diluted serially 1:1 in TE 6 times, resulting in DNA samples at 20, 10, 5, 2.5, 1.25, 0.625 and 0.312 µg/mL. Triplicate sets of DNA samples were made. The absorbance at 260 nm was determined using the GeneQuant in a standard cell, and the resulting observed DNA concentrations were averaged and plotted against the expected values. The reference solution was TE. The data were fitted with linear regression using the relationship: $y = 0.9983x + 0.0922$, $r^2 = 0.9999$. The error bars represent the average percent error: % error = (standard deviation ÷ average DNA concentration) × 100%.

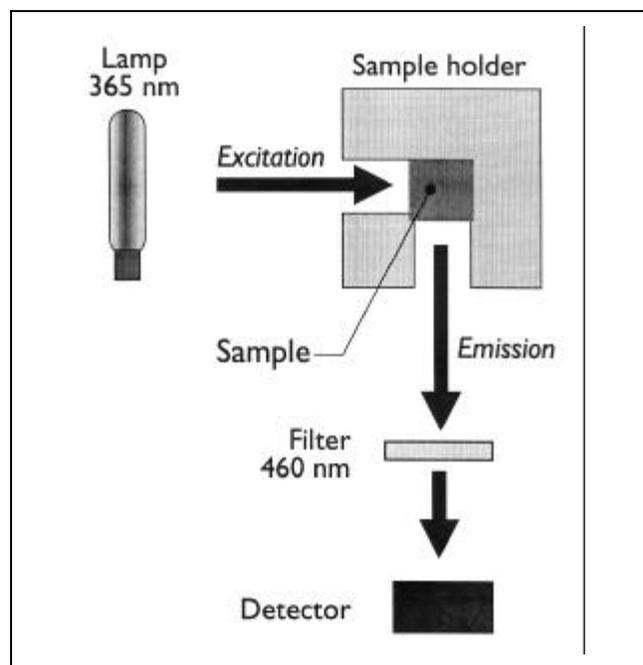


Figure 2. The DyNA Quant 200 Fluorometer uses a typical fluorometer configuration. The excitation light enters the sample at 90° relative to the detector. The DyNA Quant uses optical filters to select for the excitation and emission wavelengths, a design that greatly reduces the complexity and the cost of the fluorometer.

Table 2. A Summary of the Types of Samples that can be Measured by the GeneQuant and the DyNA Quant

Sample	GeneQuant II	DyNA Quant 200
RNA	Yes	No
Genomic DNA	Yes	Yes
Plasmid/Phage DNA	Yes	Yes
PCR Products	Yes	Yes
cDNA	No ^a	Yes
Oligonucleotides	Yes	No ^b
Proteins	Yes	No
Protein Expression	No	Yes ^c

^aThe amount of DNA produced in cDNA synthesis reactions is usually too low to be measured by UV absorbance.

^bSelf-complementary oligonucleotides that contain AATT or 4 contiguous A-T bp can be measured with the DyNA Quant.

^cEnzyme activity assays including β -galactosidase (β -gal), β -glucuronidase (GUS) and nicotinamide adenine dinucleotide (NAD)/reduced nicotinamide adenine dinucleotide (NADH) requiring enzymes.

Typically, 2 μ L of sample containing 20–10 000 ng DNA are added to 2 mL assay dye solution in a standard 4-mL cuvette and measured in the fluorometer. The standard cuvette assay is linear from 10 ng/mL to over 5000 ng/mL DNA. Figure 3 shows results from a typical standard concentration curve.

In addition to the 4-mL cuvette, the DNA assay has been adapted to a variety of capillary formats. The capillary adapter kit is available for measuring DNA concentrations in 100-, 50- and 10- μ L volumes in glass capillary tubes. These cuvettes are useful for measuring DNA in the range of 10 to 1000 ng per tube. Alternatively, the capillary cuvette kit accepts a glass micro-capillary tube that holds 3–9 μ L of sample-dye mixture. The micro-capillary tube is positioned at the focal point of two quartz cylindrical lenses and backed by mirrors to improve the efficiency of excitation and emission light collection. The combined effect of the lenses and mirrors extends the detection limit down to 1 ng DNA in 3 μ L. The DNA sample in both of these capillary systems can be recovered and analyzed by electrophoresis or used in PCR amplification experiments (11).

DISCUSSION

Spectrophotometry is an indispensable, versatile tool that is common to almost every facet of biological, chemical and molecular biological research. The GeneQuant II was designed for rapid quantification of relatively pure DNA, RNA, oligonucleotide and protein samples common to molecular biology laboratories. The ease and speed at which concentrations can be determined can diminish the time required for routine measurements of purified samples. This is particularly important for mRNA isolation for cDNA synthesis and for the quantification of oligonucleotide primers for PCR or sequencing. The instrument is ideal for determining purified plasmid or genomic DNA concentrations before any enzymatic

modifications such as restriction digestion, probe labeling or *in vitro* transcription reactions. Even the final product of genetic analysis, proteins expressed *in vitro*, can be quantified using the GeneQuant II.

Applications for the Hoechst dye/DNA assay are found in all areas of DNA analysis (1–6, 9–12). Unpurified PCR products are easily and accurately quantitated because the proteins, primers, unincorporated nucleotides and other reaction components do not significantly interfere with the H33258 assay. Quantitation of DNA in cell lysates (1,4,6,9,12) permits accurate estimates of the amount of DNA per cell. Rymaszewski et al. (12) extended this to the isolation of intact mRNA and DNA in a single extraction using an acid guanidinium thiocyanate-phenol lysis. DNA can be reliably quantitated fluorometrically from these extracts.

Many procedures benefit by accurate knowledge of the amount of DNA used at the start. For high-quality and reliable results with automated fluorescent sequencing and cycle sequencing protocols, the amount of input template DNA must be consistent and uniform. High-throughput cloning and sequencing is required for the identification of potential candidate genes involved in hereditary diseases (8). Quantitation of hybrid selection clones using a Hoefer DNA Fluorometer and the H33258 DNA assay assures that the appropriate amount of DNA is used in cycle sequencing reactions, yielding strong and clean signal with minimum sample-to-sample variability (7).

DNA quantitation with the H33258-based fluorescence assay brings speed, sensitivity and specificity together in a simple procedure. Because it is virtually unaffected by proteins, RNA and commonly encountered buffer components, this assay is uniquely and ideally suited for DNA quantitation in both pure and crude samples of DNA.

As summarized in Table 2, the GeneQuant II and the

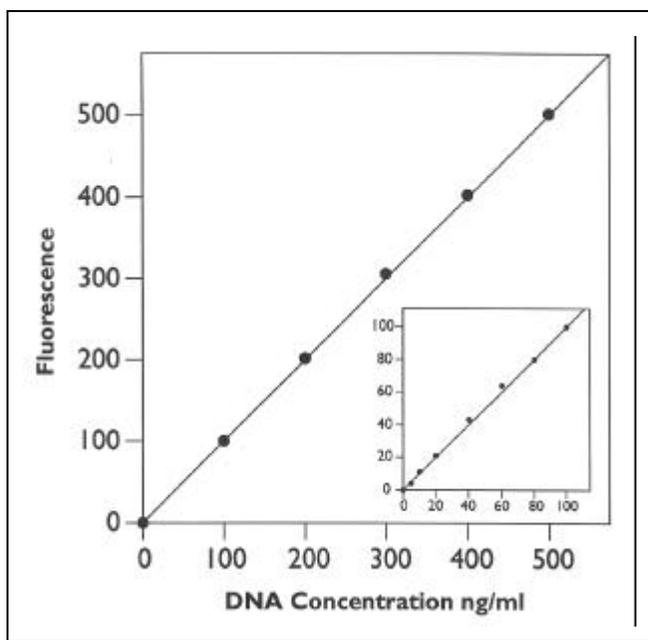


Figure 3. Standard concentration curve for DNA assay using the DyNA Quant 200 Fluorometer. Aliquots (0–10 μ L) of calf thymus DNA standard (100 μ g/mL in TE buffer) were added to 2 mL assay solution (0.1 μ g/mL H33258 in 10 mM Tris-HCl, 1 mM EDTA, 0.2 M NaCl, pH 7.4) to generate the concentration curves shown. The inset indicates the linearity of the assay in the 0–100 ng/mL range.

DyNA Quant 200 can be used to measure the concentrations of biomolecules in a wide variety of different sample preparations for any of a number of different molecular biology experiments. In general, the measurement of DNA in crude preparations or at very low concentrations requires the specificity and sensitivity of the DyNA Quant 200. Routine measurements of protein, RNA and oligonucleotide samples by spectrophotometry can be economically accomplished through the use of the GeneQuant II. Where there is overlap in application, such as genomic DNA preparations, the purity and concentration of the sample will determine which instrument would be the best to use. Together, the GeneQuant II and the DyNA Quant 200 are a cost-effective and convenient solution to the protein and nucleic acid quantification needs of molecular biology laboratories involved in research from gene discovery to gene expression.

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Address correspondence to John M. Teare, Hoefer Pharmacia Biotech, 654 Minnesota Street, P.O. Box 77387, San Francisco, CA 94107, USA. Internet: john.teare@ussfo.pharmacia.se