

UV Talk Letter

Vol.7





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BioSpec-nano Life Science UV-VIS Spectrophotometer

Life science research involves the handling of extracted and purified nucleic acids (DNA, RNA) in conducting PCR (Polymerase Chain Reaction), quantitative PCR, cloning, base sequence determination, DNA micro-array analysis, etc. Nucleic acid analysis using ultraviolet-visible spectrophotometers is widely conducted to successfully guide these processing operations. This document introduces the BioSpec-nano UV-VIS spectrophotometer, which provides simple and quick analysis of micro-level nucleic acids.

1. BioSpec-nano Features

The BioSpec-nano, unlike conventional spectrophotometers, adopts an optical system that does not rely on the use of cells (cuvettes). The optical system comprises a xenon flash lamp, an upper window, a target platform (fiber optic element housing), holographic grating, and photodiode array detector.

Measurement with the BioSpec-nano is conducted by automatically sandwiching the sample between the upper window and target to achieve a fixed pathlength (0.2 mm or 0.7 mm). This type of optical system allows measurement of ultra-small sample volumes in the range of 1 – 2 μ L, as well as undiluted high-concentration samples, greatly facilitating the analysis operation.

Cell washing and drying with conventional instruments is an extremely time-consuming, tedious task, which greatly impedes the analysis operation. With the BioSpec-nano, however, cleaning the liquid-contact surfaces (upper window and target) is accomplished by a simple wiping of the wet surfaces. The automated mounting mechanism, which establishes the appropriate pathlength, and the automated liquid-contact surface wiping feature allow true drop-and-click analysis. Thus, large numbers of samples can be analyzed in a smooth progression, with the entire cycle, including automatic wiping of the wet surfaces, taking approximately 15 seconds.



Fig. 1 BioSpec-nano

2. Drop-and-Click Analysis

Fig. 2 shows the analysis procedure used with the BioSpec-nano. First, 1 μL (pathlength 0.2 mm) or 2 μL (pathlength 0.7 mm) of sample is deposited on the tip of the fiber optic element housed in the target platform. Then, by clicking the [start] button on the instrument or via the software, the upper window is lowered automatically to sandwich the sample between the upper window and target at the pathlength set beforehand using a simple setting lever. At this point, the xenon lamp flashes, and a UV-VIS absorption spectrum (220 – 800 nm) is acquired within 3 seconds to generate the results, which The analysis results are displayed via the software, as shown in Fig. 3. Upon completion of the analysis, the liquid-contact surfaces (target and upper window) are wiped clean by the action of the automatic wiping mechanism. A piece of cloth specially developed for wiping these surfaces is used for the automatic wiping. The wiping cloth has an ultra-fine fiber structure displaying excellent wiping performance thanks to its high water-absorbing properties. A single piece of the cloth can be used for up to 100 wiping operations. Carryover (residual liquid on target) is suppressed to a very low level as the wiped solution is trapped internally by capillary action. In testing of wiping performance using purified dsDNA (double-stranded DNA) as the sample, carryover was 0.3 % or less over 120 continuous wiping operations¹⁾.

3. Superb Analysis Performance

With an optical pathlength of 0.7 mm or 0.2 mm, quantitation is possible over wide concentration ranges, from 0.3 – 21 OD* (15 – 1,000 ng/ μL dsDNA) and 1 – 75 OD (50 – 3,700 ng/ μL), respectively. A stable optical path is formed with the automatic mounting system, allowing high-precision, accurate data to be obtained even with 1 - 2 μL samples¹⁾.

* OD (Optical Density) here refers to the absorbance at a wavelength of 10 mm.

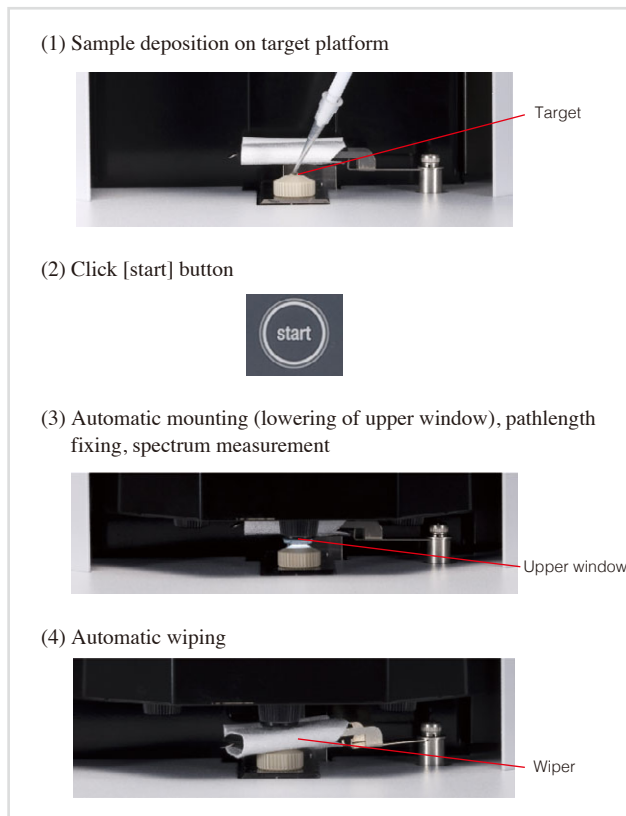


Fig. 2 Drop-and-Click Analysis

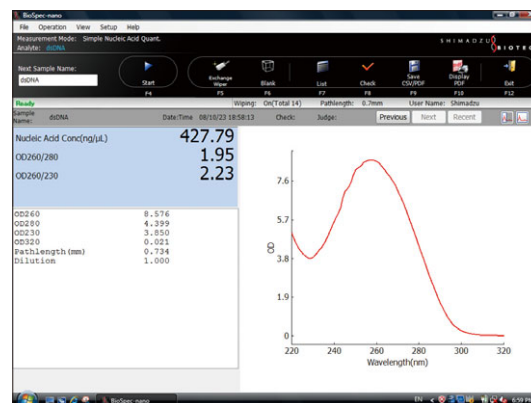


Fig. 3 dsDNA Analysis Results

4. Nucleic Acid, Protein Analysis Using BioSpec-nano

The BioSpec-nano offers three modes of quantitation, including modes for Simple Nucleic Acid Quantitation, Labeled Nucleic Acid Quantitation, and Protein Quantitation (OD280 nm method). Table 1 presents the types of analysis output according to these quantitation modes.

4.1 Nucleic Acid Analysis

The Simple Nucleic Acid Quantitation Mode can be used for analysis of purified RNA, dsDNA, ssDNA (single-stranded DNA) and OligoDNA. The analysis results include the nucleic acid concentration (ng/μL) and the OD ratio (OD260/280, OD260/230). OD260/280 is an index that is used to express the purity of DNA and RNA. Protein displays absorption at 280 nm, so contamination will reduce the OD260/280 value. High-purity DNA and RNA will generate an OD260/280 value of 1.8 or greater. OD260/230 is an index that is used to express the purity of RNA, and becomes lower when contaminated with protein, EDTA, phenol and other organic substances. High-purity RNA will generate an OD260/230 value of 2.0 or greater.

4.2 Labeled Nucleic Acid Analysis

Applicable targets for the Labeled Nucleic Acid Quantitation Mode include Cy3, Cy5 and other fluorescent labels used in nucleic acids in DNA microarrays, northern blot, southern blot, fluorescence in situ hybridization (FISH) and in functional analysis. The labeling rate is detected as the number of picomoles (pmol) per 1 μg of nucleic acid. The labeling reaction efficiency can be checked using the labeling rate and nucleotide/labeling ratio.

4.3 Analysis of Proteins and Labeled Proteins

The Protein Quantitation Mode is used for analysis of highly purified proteins and labeled proteins for conducting structural analysis, functional analysis and molecular interaction analysis, in addition to analysis of antibodies and labeled antibodies for immunostaining, flow cytometry and western blot. Protein quantitation is conducted using ultraviolet absorption (UV absorption spectrometry, OD280 nm method) at 280 nm. When the protein molar absorption coefficient (ϵ_{280}) is known, the value is entered directly. If the value of ϵ_{280} is unknown, it can be calculated from the tryptophan (Trp), tyrosine (Tyr), cysteine (S-S) residue number³⁾. For quantitation using a short optical pathlength, a high-concentration sample greater than BSA (Bovine Serum Albumin)-converted 0.45 mg/mL (pathlength 0.7 mm) must be used.

Table 1 Calculation Items for Each Mode

Quantitation Mode (Analysis Target)	Calculation Items	Calculation Formula
Simple Nucleic Acid Quantitation (Nucleic Acids)	Nucleic acid concentration (ng/μL)	Dilution rate * Nucleic acid Conc. Coefficient * (OD260 - OD320)
	OD260/280	(OD260 - OD320) / (OD280 - OD320)
	OD260/230	(OD260 - OD320) / (OD230 - OD320)
Labeled Nucleic Acid Quantitation (Labeled Nucleic Acids)	Nucleic acid concentration (ng/μL)	Dilution rate * Nucleic acid Conc. Coefficient * (OD260 - Label OD * CF260)
	Label concentration (M)	Label concentration (M) = Dilution rate * Label OD / Label ϵ
	Nucleotide concentration (M)	Dilution rate * Nucleic acid Conc. Coefficient * 0.001 * (OD260 - Label OD * CF260) / Nucleotide molecular weight
	OD260/280	(OD260 - Label OD * CF260) / (OD280 - Label OD * CF280)
	Labeling rate (pmol/μg)	Label Conc. (M) * E9 / Nucleic acid Conc. (ng/μL)
	Nucleotide/labeling ratio	Nucleotide Conc. (M) / Label Conc. (M)
Protein Quantitation (Proteins)	Protein concentration (M)	Dilution rate * OD280 / ϵ_{280}
	Protein concentration (μg/mL)	Protein concentration (M) * MW * 1,000
	ϵ_{280} (M ⁻¹ cm ⁻¹ , residue number input)	5,500 * [Trp residue No.] + 1,490 * [Tyr residue No.] + 125 * [S-S residue No.]
Protein Quantitation (Labeled Proteins)	Protein concentration (M)	Dilution rate * (OD280 - Label OD * CF280) / ϵ_{280}
	Protein concentration (μg/mL)	Protein concentration (M) * MW * 1,000
	Label concentration (M)	Dilution rate * Label OD / ϵ_{Label}
	Labeling rate	Label Conc. (M) / Protein Conc. (M)

Nucleic acid concentration coefficient²⁾: RNA (40.0), dsDNA (50.0), ssDNA (37.0), OligoDNA (33.0)

Nucleotide Molecular Weight: RNA (321.5), dsDNA (309.0), ssDNA (309.0), OligoDNA (309.0)

Label OD: OD associated with label wavelength

ϵ_{Label} : Molar absorption coefficient (M⁻¹cm⁻¹) associated with label wavelength

CF260, CF280: Coefficient for correction of OD contribution for label 260 nm and 280 nm

MW: Molecular weight of protein

References

- 1) Shimadzu Application News No. A420
- 2) Ausubel, F., et al., Short Protocols In Molecular Biology, 4th edition 1999, WILEY.
- 3) Pace, C.N., et al., Protein Sci., 4, 2411(1995).

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Nucleic Acid Thermal Stability Analysis (Tm Analysis)

Techniques such as PCR and DNA microarray that are based on hybridization of nucleic acids are indispensable to life science research. In addition, functional nucleic acids, including nucleic acid drugs and siRNA (small interference RNA), are receiving considerable attention. Thermal stability analysis (Tm analysis) is indispensable for the research, development, and as well as application of nucleic acids.

DNA typically has a double-stranded helix structure. The double strand comprises a sense strand and an antisense strand made up of the complementary bases, G (guanine) and C (cytosine), and A (adenine) and T (thymine), respectively, and these complementary base pairs are joined by a hydrogen bond. When the temperature is raised, the hydrogen bond is broken, the double strand dissociates, and a single-stranded structure is formed. This phenomenon is referred to as "melting."

Nucleic acids display an ultraviolet absorption peak at 260 nm, and their absorbance at 260 nm increases when melting occurs. The data curve acquired when measuring UV absorbance with respect to temperature rise is called a UV absorbance melting curve. In addition to UV absorbance measurement, melting curves are also obtained in other types of measurement, such as circular dichroism spectroscopy and thermal measurement. At the melting temperature (T_m), the ratio of the double strands to the single strands becomes equal. T_m is an index of the thermal stability of a nucleic acid, and is dependent on such conditions as the base sequence, base number, nucleic acid concentration, solvent conditions (salt composition, organic solvent composition, pH), mismatch (non-complementary base pairs), nucleic acid analog (artificial nucleic acid) structure, etc.

In contrast to the melting process, lowering of the temperature will cause a single-stranded nucleic acid to adopt a double-stranded structure through the reinstatement of the hydrogen bonds between the complementary base pairs. The phenomenon is referred to as hybridization. Hybridization of nucleic acids is utilized in PCR, DNA microarray, southern hybridization, northern hybridization, and in situ hybridization, etc. In addition, nucleic acids (functional nucleic acids) which possess functionality associated with siRNA, antisense nucleic acids, antigene nucleic acids, ribosomes, etc., are applied in various fields, including drug discovery research, drugs, and diagnostics. Thermal stability of nucleic acid is an important factor that controls the structure, hybridization, and functions of nucleic acids; therefore, thermal stability analysis of nucleic acid (Tm analysis) is indispensable in these fields.

Below is a simple description of nucleic acid thermal stability analysis (Tm analysis). Here, it is assumed that there is a state of equilibrium between the 2 different single-stranded (A_1 , A_2) and double-stranded (A_1A_2) states, and that the concentrations of A_1 and A_2 are the same.



If the double-stranded molar fraction is α , and the total nucleic acid concentration is C_t , the equilibrium constant K for the system is expressed as follows.

$$K = [A_1A_2] / ([A_1][A_2]) = 2\alpha / ((1 - \alpha)^2 C_t) \quad (2)$$

The observed absorbance (A_{obs}) at 260 nm is expressed as follows.

$$A_{obs} = (\epsilon_{ds}\alpha + \epsilon_{ss}(1 - \alpha)) C_t L \quad (3)$$

ϵ_{ds} , ϵ_{ss} and L express the double-strand molar absorption coefficient, single-strand molar absorption coefficient, and pathlength, respectively.

As shown in the melting curve of Fig. 1 obtained from actual measurement, ϵ_{ds} and ϵ_{ss} are temperature-dependent. Since the absorbance changes linearly to a greater extent in the low-temperature, pre-transition region (region prior to large absorption change) and high-temperature post-transition region as compared to during the transition region itself (region of large absorption change), ϵ_{ds} and ϵ_{ss} are expressed as follows, where T represents temperature.

$$\epsilon_{ds} = a_{ds} + b_{ds}T \quad (4)$$

$$\epsilon_{ss} = a_{ss} + b_{ss}T \quad (5)$$

The temperature becomes T_m when $\alpha = 1/2$, and this is what is used to determine T_m (melting temperature) from the melting curve. As shown in Fig. 1, the baseline is determined with respect to the pre-transition region and post-transition region. Next, the median of the 2 baselines is drawn, and the temperature (T_m) at which this line intersects with the melting curve is obtained.

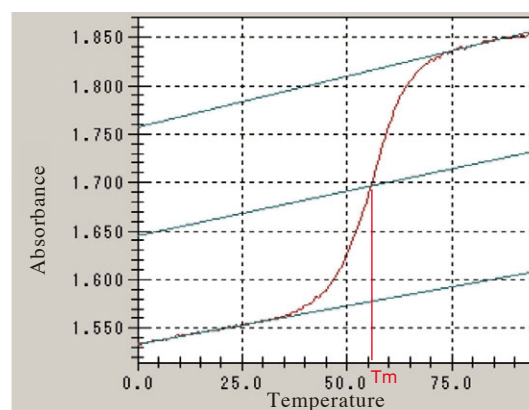


Fig. 1 Example of Melting Curve, Tm Determination Method

Gibbs change in free energy, change in enthalpy and change in entropy, ΔG , ΔH , and ΔS , respectively, are used to express the basic principle of thermodynamics, in which the following expression is obtained when the change in molar specific heat (ΔC_p) is zero. R is the gas constant.

$$\Delta G = \Delta H - T\Delta S = -RT \ln K \quad (6)$$

Substituting expression (2) for expression (6), the following expression is obtained when $\alpha = 1/2$ at temperature T_m .

$$1/T_m = (R/\Delta H) \ln(C_t/4) + \Delta S/\Delta H \quad (7)$$

The samples are prepared at the various concentrations (C_t), the respective T_m values are obtained, $1/T_m$ is plotted with respect to $\ln(C_t/4)$, and ΔH and ΔS are determined from the obtained slope of the straight line and the y intercept.

Shimadzu offers a T_m analysis accessory option for its wide array of double-beam UV-VIS spectrophotometers (UV-1800/UV-2450/UV-2550, etc.) that ensures stable thermal analysis of nucleic acids. The T_m analysis system (TMSPC-8) comprises a thermoelectrically temperature-controlled 8-series micro cell holder, a temperature-controller, an 8-series micro multi-cell (pathlength 10 mm, 1 mm), and specially designed T_m analysis software. The 8-series cell allows simultaneous thermal melting analysis for up to 8 samples.

Shown below are examples of T_m analysis data and the results of thermal stability performance analysis.

Using a sample consisting of GCGAAAAGCG/CGCTTTTCGC, data was acquired from measurement of 10 samples having different concentrations. The concentrations are listed in Table 1. Cells with an optical pathlength of either mm or 1 mm were used depending on the absorbance of the sample. The obtained data are shown in Fig. 2 and Fig. 3, and the measurement conditions are shown in below.

Buffer: 100 mM Sodium Phosphate, 1 M NaCl, 0.5 mM EDTA, pH7.0
Wavelength: 260 nm, 320 nm
Temperature Range: 0 to 95 °C
Ramp Rate: 1 °C/min
Sampling Interval: 0.5 °C
Wait Time: 30 sec
Post-Process Annealing (95 °C, 2 min)

The T_m analysis software was used to determine the T_m value at each concentration, and calculate $1/T_m$ and $\ln(C_t/4)$. The data of Table 1 were plotted, and the linearity of Fig. 4 was obtained. Using the slope and y intercept of this straight line, -74.6 kcal/mol and -202.9 cal/(molK) were obtained for ΔH and ΔS , respectively.

In addition to the above-mentioned ΔH and ΔS determination method, the melting curve nonlinear fitting method can also be used for determination of ΔH and ΔS .

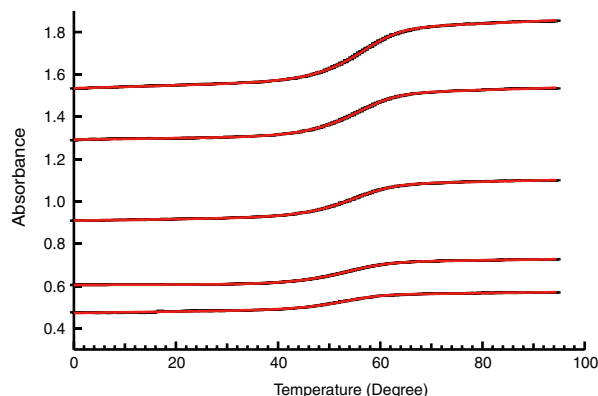


Fig. 2 Measurement Results with 10 mm Optical Pathlength

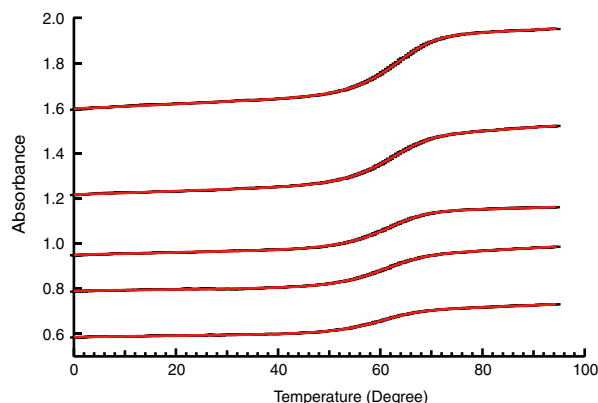


Fig. 3 Measurement Results with 1 mm Optical Pathlength

Table 1 Analysis Results at Each Concentration

C_t (μ M)	T_m (°C)	$\ln(C_t/4)$	$1/T_m$ (1/K)
196.4	62.4	-9.9217	0.002980
157.1	61.1	-10.1448	0.002992
117.8	60.8	-10.4325	0.002995
98.2	59.7	-10.6148	0.003004
78.6	58.9	-10.8379	0.003012
19.6	55.6	-12.2242	0.003042
15.7	54.2	-12.4474	0.003055
11.8	53.8	-12.7351	0.003058
7.9	52.8	-13.1405	0.003068
5.9	51.8	-13.4282	0.003077

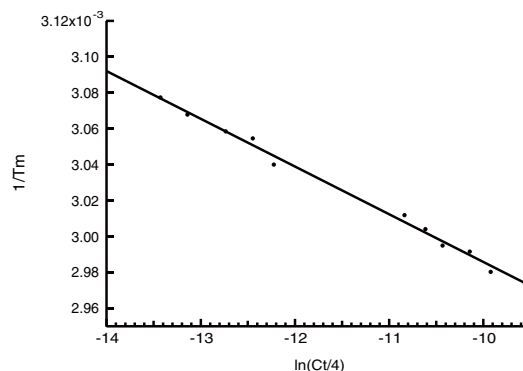


Fig. 4 Plot of $\ln(C_t/4)$ vs $1/T_m$



What types of colorimetric determination methods are there for proteins?



Many types of protein colorimetric determination reagent kits are available on the market. Described below are the typical methods employed in these kits.

(1) Bradford (CBB: Coomassie Brilliant Blue) Protein Assay

A Coomassie Brilliant Blue G-250 (CBBG) solution displays maximum absorption at 470 nm, but displays a shift in the maximum absorption to 595 nm due to binding with protein, with the color of the solution changing from its reddish form to a more bluish form. CBBG binds strongly to the arginine residue, but it is also known to bind with tryptophan, tyrosine, histidine, and phenylalanine residues. The Bradford assay is characterized as a method that is quick and easy, with few interfering substances. However, its shortcomings include wide-ranging coloration depending on the type of protein, and its inhibited activity in the presence of solubilizers and detergents. Its quantitation range is 1 µg/mL – 1.5 mg/mL. The Bradford assay is a method that is frequently used along with the BCA method, described below.

(2) Biuret Test

When an alkaline solution consisting of copper sulfate and potassium sodium tartrate (Biuret reagent) is mixed with a protein solution, Cu^{2+} and nitrogen atoms in the polypeptide chain form a violet-colored complex (Biuret reaction). The advantages of the Biuret test are that it is easy to conduct and generates little variation of coloration when reacting with proteins. However, analytical sensitivity with the Biuret test is low, requiring protein concentrations in the order of mg/mL. The following Lowry protein assay and BCA assay are variations of the Biuret test and provide greatly improved sensitivity.

(3) Lowry Protein Assay

In the Lowry protein assay, the protein solution is first mixed with Biuret reagent to form Cu^{2+} -protein complexes. When this is mixed with Folin-Ciocalteu reagent (Folin's phenol reagent), which consists of a mixture of phosphomolybdic acid and phosphotungstic acid, the phosphomolybdic acid-phosphotungstic acid is reduced due to the tyrosine, tryptophan and cysteine residues, with the solution finally presenting an indigo blue color. Although the Lowry assay provides high analysis sensitivity, there are several drawbacks, including the labor-intensive operation, considerable variation in coloration among proteins, and the many types of substances that can interfere with the reaction (reducing agents, surfactants, EDTA, etc.). The quantitation range is 1 µg/mL – 1.5 mg/mL.

(4) BCA (Bicinchoninic Acid) Assay

In the BCA assay, the Cu^{2+} -protein complexes are formed due to the Biuret reaction, and the bluish purple-colored chelate consisting of Cu^{+} and 2 molecules of BCA formed due to reduction of the tyrosine, tryptophan and cysteine residues is detected. The BCA assay is quick and easy to conduct, displays high analysis sensitivity, and little fluctuation of coloration due to the types of proteins present. In addition, it is subject to inhibited activity by few interfering substances, which consist primarily of reducing agents. The quantitation range is 0.5 µg/mL – 1.2 mg/mL.

References

- 1) Current Protocols in Protein Science, 3.4.1-3.4.29, John Wiley & Sons (2007).
- 2) From Easy Principles, a Protein Experimental Method! Make Proteins – Extraction, Purification and Synthesis, Kagaku-Dojin (2008).

BioSpec-nano

Life Science UV-VIS Spectrophotometer

The BioSpec-nano is equipped with a cell (cuvette)-free optical system, a groundbreaking automatic sample mounting mechanism, and an automatic wiping mechanism, offering fast, simple nucleic acid analysis of 1 – 2 μL samples. A single analysis can be completed in approximately 15 seconds, delivering true, high-throughput analysis.



Drop-and-Click Analysis

The nucleic acid concentration and purity (OD ratio) can be verified by depositing a drop of sample on the target site, and clicking the [start] button. All measurement and wiping operations are handled automatically by the instrument.

Nucleic Acid Quantitation with 1 – 2 μL

Analysis can be conducted with sample volumes of 1 μL (pathlength 0.2 mm) and 2 μL (pathlength 0.7 mm).

Simple and Quick Analysis

Basic operations like blank measurement, sample measurement, PDF output of reports, and CSV output are accomplished simply and quickly by button-click operations.

Support for a Variety of Analyses

Nucleic acid quantitation, quantitation of labeled nucleic acid microarrays, protein quantitation by OD280 nm method, and labeled protein quantitation are all supported.

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