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Understanding Your Biochemical Analysis Software

Agilent Technologies

Notices

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Software Revision

This handbook is for B.02.xx revisions of the Agilent ChemStation software, where xx is a number from 00 through 99 and refers to minor revisions of the software that do not affect the technical accuracy of this handbook.

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CAUTION

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In This Guide...

This handbook contains full details of the operation of the kinetics and thermal denaturation modes of the biochemical analysis software of the Agilent ChemStation for UV-visible spectroscopy. It describes the acquisition of time-based and temperature-based data, the processes of data transformation and the calculations used in the evaluation of the data. The manual is designed to enable you to follow good laboratory practice (GLP) guidelines. Using the information in the manual, you will be able to understand the data processing calculations from beginning to end and perform the data evaluation manually.

1 Kinetics

This chapter describes kinetics mode. It contains details of the acquisition parameters, and explains the special calculations used in the data processing and evaluation of time-based data for kinetics experiments.

2 Thermal Denaturation

This chapter describes thermal denaturation mode. It contains details of the acquisition parameters, and explains the special calculations used in the data processing and evaluation of temperature-based data for thermal denaturation experiments and DNA melts.

Contents

1 Kinetics

The Kinetics mode acquires and processes time-based data. Spectra are acquired at regular or increasing time intervals over a period of time; wavelength data are extracted from the acquired spectra and a plot of wavelength result against time is constructed.

Acquisition

The Kinetics mode allows two types of experiment to be constructed, based on the sampling system that is configured.

Manual Sampling System

The manual sampling system acquires data from a single cell. In this case, up to six wavelengths can be defined; the wavelength results are processed individually.

Multicell Transport System

The multicell transport system allows up to eight samples to be measured. In this case, measurements are restricted to a single wavelength, which is the same for each cell. The results from all cells are processed individually.

NOTE If only one cell is used in a multicell transport system, it behaves as a manual sampling system, and allows up to six wavelengths to be defined.

Table 1 shows the acquisition parameters for acquisition in Kinetics mode

Table 1 Acquisition Parameters for Kinetics

Parameter	Range	Defaults
Data type	Absorbance	Fixed
Integration time	0.1–25.5	0.5
Std. deviation	Off	Fixed

Data Recovery

In the case of a time-based acquisition that has terminated prematurely, the data up to the point of termination is not destroyed. It is recoverable by loading the data file, and can be treated as a partially-completed experiment.

Data Processing

For single-cell analyses with multiple wavelengths, all wavelengths are processed individually. The wavelength result at the specified wavelength is calculated for each acquired spectrum to give a trace of absorbance against time. The absorbance value at the specified wavelength is first corrected by any specified background correction, which subtracts a calculated value from the wavelength result. There are three methods of calculating the background values:

- single reference wavelength
- subtract average over range
- three-point drop line

Single Reference Wavelength

The single reference wavelength method subtracts the absorbance at a specified wavelength, as in Figure 1 on page 11. The reference wavelength is usually selected at a point on the baseline beyond the sample absorbance.

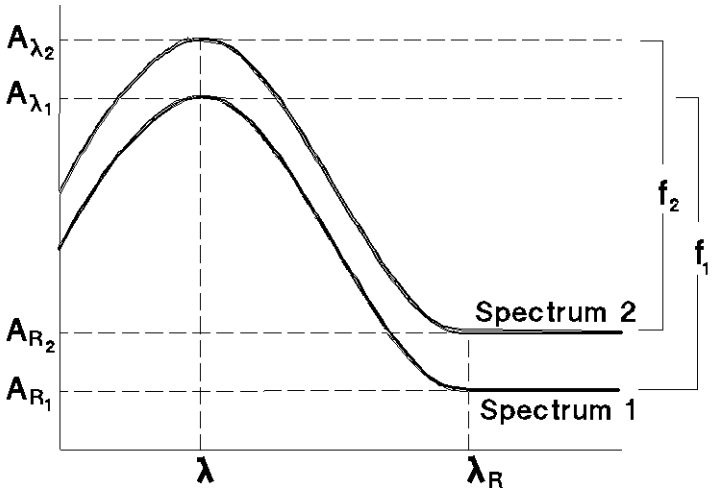

Figure 1 Internal Referencing

Subtraction is carried out using Equation 1:

$$f_\lambda = A_\lambda - A_{R_\lambda} \quad \textbf{(1)}$$

where

f_λ is the function result at wavelength λ

A_λ is the absorbance at wavelength λ

A_{R_λ} is the absorbance at reference wavelength R_λ

Subtract Average Over Range

The subtract average over range method is an extension of the single reference wavelength method that replaces the single wavelength absorbance value, A_{R_λ} in Equation 1, with the average absorbance value over a wavelength range.

Three-Point Drop Line

If a three-point drop line is specified, then the absorbance values from the two reference wavelengths, A_{R_1} and A_{R_2} define a straight line (see Figure 2) which is used to calculate the absorbance at the reference wavelength, A_{R_λ}, using Equation 2.

$$A_{R_\lambda} = \frac{1}{\lambda_{R_2} - \lambda_{R_1}} \{(\lambda_{R_2} - \lambda)A_{R_1} + (\lambda - \lambda_{R_1})A_{R_2}\} \quad \textbf{(2)}$$

where the terms are the same as for a single reference wavelength (see "Single Reference Wavelength" on page 11).

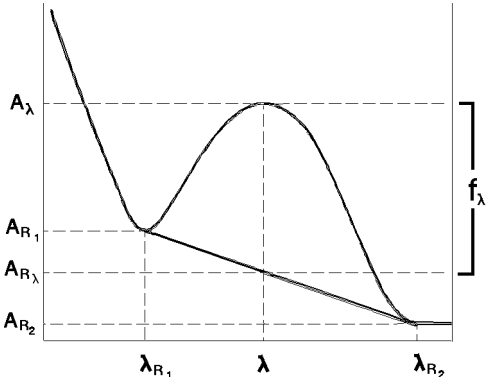

Figure 2 Three-point Drop Line Background Correction

Evaluation

Four calculation methods are available:

- Initial Rate calculates the rate using a quadratic fit.
- Zero Order calculates the rate using a linear fit.
- First Order calculates the rate using an exponential fit.
- Delta AU calculates an absorbance difference.

In all cases, the calculation is made over the calculation time range specified in the Rate Calculation group of the Time & Calculations Parameters dialog box. If no calculation time range is specified, the full time range of the data is used.

Initial Rate

The Initial Rate calculation uses a quadratic fit to calculate the rate, k, by linear regression using Equation 3

$$A_t = A_i + kt + ct^2 \quad \textbf{(3)}$$

where

A_t is the background-corrected absorbance at time t

A_i is the background-corrected absorbance at the start of the calculation time range

Zero Order

The Zero Order calculation uses a linear fit to calculate the rate, k, by linear regression using Equation 4:

$$A_t = kt + A_i \quad \textbf{(4)}$$

where A_t and A_i are as in Equation 3.

First Order

The First Order calculation uses an exponential fit to calculate the rate, k, using Equation 5:

$$A_t = A_\infty + (A_0 - A_\infty)e^{-kt} \quad \textbf{(5)}$$

where k is calculated by linear regression of

$$\frac{A_{t_{i+1}} - A_{t_i}}{t_{i+1} - t_i} = kA_\infty - k\frac{1}{t_{i+1} - t_i}\int_{t_i}^{t_{i+1}} A_t dt = kA_\infty - \frac{1}{2}k(A_{t_{i+1}} + A_{t_i}) \quad \textbf{(6)}$$

Delta AU

The Delta AU is the difference between the absorbance at the start of the calculation time range and the absorbance at the end. The calculation (Equation 7) is very simple:

$$\text{delta AU} = A_e - A_s \quad \textbf{(7)}$$

where

A_s is the background-corrected absorbance at the start of the time range

A_e is the background-corrected absorbance at the end of the time range

Conversion to Different Rate Units

The rate calculations produce a result in absorbance units per second (AU/s), which can be converted to a specified rate unit by multiplication by a factor. This facility is included in the result calculation only when the Multiply Rate check box in the Rate Calculation group of the Time & Calculations Parameters dialog box is selected.

Data Flow and Registers in Kinetics Mode

The Kinetics mode uses five registers, three of which are specific to time-based data (see Table 2).

Table 2 Kinetics Mode Registers

Register	Contents	View
Kinetics Parameters	Time-based method parameters	Time & Calculations dialog box
Blank Spectrum	Last blank spectrum	Last Blank Spectrum window
Samples	Spectrum of a single measurement	Sample Spectra window
Sample Cell n	Spectra from cell n of a multicell acquisition	Sample Spectra window
Traces	Generated time traces	Time Traces window

During time-based acquisition, the spectra from individual cells are stored in matrix objects in the Sample Cell n register. The time traces generated during data processing are stored as matrix objects in the Traces register.

Interactive Math Functions

The Kinetics mode provides several math functions which can be used to manipulate spectra interactively. The results of interactive math processing are placed in the Math Result register and displayed in the Math Result window. There are two types of math function:

- **Unitary functions** operate on individual spectra; they can be used to process several spectra in the same operation.
- **Binary functions** operate on two spectra; any attempts to operate binary functions on a number of spectra other than two results in an error message.

Derivative

Derivative is a unitary function; it calculates the derivative of the data points (y-values) in the spectrum using a Savitsky-Golay algorithm and places the resulting spectrum in the math result register.

Savitsky-Golay Algorithm

The Savitsky-Golay algorithm uses the Derivative Order, Filter Length and Polynomial Degree from the Derivative Parameter dialog box. For each data point in the trace, the calculation takes a set of data points equal to the filter length around the current data point and fits a curve of the specified polynomial degree, using a least squares fit. The fitted curve is then used to calculate the new value for the current data point, and the derivative of that point.

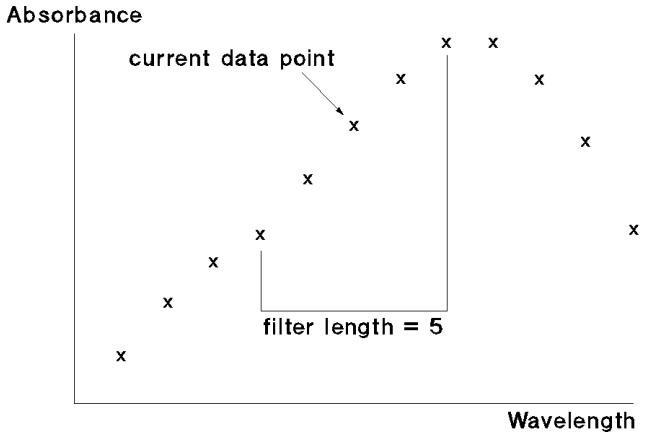

Figure 3 Filter Length in the Savitsky-Golay Algorithm

For each y-value, y_i,

$$\text{deriv}(y_i) = \sum C_{kj} \cdot y_{i+j-1} \quad \textbf{(8)}$$

where

i is *1 ... data points-(filter length-1)*

j is *1 . . . filter length*

k is 2 for derivative

deriv(y_1) corresponds to the $\left(\frac{\text{(filter length-1)}}{2} + 1\right)$ th value. For example, if *filter length* is 5, then *deriv(y_1)* becomes y_3. For this reason, the length of the spectrum is reduced by *(filter length-1)* values; $\left(\frac{\text{(filter length-1)}}{2}\right)$ values at the beginning of the spectrum and $\left(\frac{\text{(filter length-1)}}{2}\right)$ values at the end of the spectrum are not processed. In Equation 8, C is the coefficient matrix:

$$C = N^{-1} \cdot F^T \quad \textbf{(9)}$$

where

N-1 is the inverse of N, the product of F^T and F

F^T is the transpose of F, the matrix of the powers of the polynomials

The matrix of the powers of the polynomials is generated using Equation 10:

$$F_{ij} = k^{(j-1)} \quad \textbf{(10)}$$

where

i is $1 \ldots filter\ length$

j is $1 \ldots degree + 1$

k is $i - \frac{\text{filter length} - 1}{2} - 1$

At the end of the calculation, the y-values are multiplied by the reciprocal of the step:

$$y_i = \frac{y_i}{\text{step}} \quad \textbf{(11)}$$

where

step is the increment of the equidistant x-axis.

All additional information, such as annotations, are preserved unchanged in the derivative spectrum.

Logarithm (ln)

Logarithm is a unitary function; it calculates the natural logarithm of each of the data points (y-values) in the spectrum or trace and places the result in the math result register.

$$y_{\log} = \ln(y) \quad \textbf{(12)}$$

All additional information, such as annotations, are preserved unchanged in the logarithmic spectrum or trace.

Scalar Multiply

Scalar Multiply is a unitary function; it multiplies each of the data points (y-values) in the spectrum or trace by a constant value and places the result in the math result register.

$$y_{\text{new}} = y \times C \quad \textbf{(13)}$$

The scalar multiply function can be used to divide by a constant value by using the reciprocal of the desired divisor, $1/C$.

All other additional information, such as annotations, are preserved unchanged in the resulting spectrum or trace.

Scalar Add

Scalar Add is a unitary function; it adds a constant value to each of the data points (y-values) in the spectrum or trace and places the result in the math result register.

$$y_{\text{new}} = y + C \quad \textbf{(14)}$$

The scalar add function can be used to subtract a constant value by using a negative constant.

All other additional information, such as annotations, are preserved unchanged in the resulting spectrum or trace.

Add

Add is a binary function; it adds two spectra or traces together and places the result in the math result register. The spectrum or trace selected first (item A) is taken as the model and provides the x-value range (or list) and resolution for the resulting spectrum or trace. Item A is first copied into the math result register, then the y-values (for example absorbance) from the second selected spectrum or trace (item B) are added to the y-values of the item in the math result register as follows:

Items of Different X-Value Resolutions

If the x-values of item A do not completely agree with those of item B (for example spectra with different wavelength resolutions):

- The y-values of item B are interpolated before adding them to the y-values of the result when the interval of the x-value in item A is less than that of item B.
- The y-values at intermediate x-values in item B are ignored when the interval of the x-value of item A is greater than that of item B, and only the y-values from item B at the x-values corresponding to item A are added to the y-values of the result.

Items of Different X-value Ranges

If the x-range of item A is greater than the x-range of item B (for example spectra with different wavelength ranges), the extra y-values in item A remain unchanged in the result.

If the x-range of item A is less than the x-range of item B, the extra y-values in item B are ignored.

If there is no overlap between the x-ranges of the items, item A remains unchanged.

Additional information, such as annotations, are taken solely from item A; all such items from item B are ignored.

Subtract

Subtract is a binary function; it subtracts one spectrum or trace from another and places the result in the math result register. The spectrum or trace selected first (item A) is taken as the model and provides the x-value range (or list) and resolution for the resulting spectrum or trace. Item A is first copied into the math result register, then the y-values (for example absorbance) from the second selected spectrum or trace (item B) are subtracted from the y-values of the item in the math result register as follows:

Items of Different X-Value Resolutions

If the x-values of item A do not completely agree with those of item B:

- The y-values of item B are interpolated before subtracting them from the y-values of the result when the interval of the x-value in item A is less than that of item B.
- The y-values at intermediate x-values in item B are ignored when the interval of the x-value of item A is greater than that of item B, and only the y-values from item B at the x-values corresponding to item A are subtracted from the y-values of the result.

Spectra of Different X-Value Ranges

If the x-range of item A is greater than the x-range of item B, the extra y-values in item A remain unchanged in the result.

If the x-range of item A is less than the x-range of item B, the extra y-values in item B are ignored.

Additional information, such as annotations, are taken solely from item A; all such items from item B are ignored.

2 Thermal Denaturation

The Thermal Denaturation mode acquires and processes temperature-based data. Spectra are acquired at regular temperature intervals according to the defined temperature ramps; wavelength data are extracted from the acquired spectra and a plot of wavelength result against temperature is constructed.

The Thermal Denaturation mode is designed to perform classical DNA melt experiments. However, the diode-array detection system of the Agilent 8453 spectrophotometer enables the classical method to be enhanced by the collection and storage of full spectra, rather than the single-wavelength (260 nm) used in the classical method.

Acquisition

The Thermal Denaturation mode operates using manual sampling only. The system configuration *must* include the Agilent 89090 Peltier Temperature Controller to operate in Thermal Denaturation mode.

Table 3 shows the parameters for acquisition in Thermal Denaturation mode

Table 3 Acquisition Parameters for Thermal Denaturation

Parameter	Range	Defaults
Data type	Absorbance	Fixed
Integration time	0.1–25.5	0.5
Std. deviation	Off	Fixed

Registers and Data Flow in Thermal Denaturation Mode

The Thermal Denaturation mode uses five registers, three of which are specific to temperature-based data (see Table 4).

Table 4 Thermal denaturation mode registers

Register	Contents	View
Thermal Parameters	Temperature-based method parameters	Temperature & options dialog box and Temperature Ramping Menu
Blank Spectrum	Last blank spectrum	Last Blank Spectrum window
Samples	Spectrum of a single measurement	Sample Spectra window
Spectra of Current Trace	Spectra from the current time trace	Sample Spectra window
Traces & Results	Generated heating and cooling traces	Heating and Cooling Traces windows

During temperature-based acquisition, the heating and cooling traces generated during data processing are stored as matrix objects in the Traces & Results register.

Data Processing

The wavelength result at the specified wavelength is calculated for each acquired spectrum to give a trace of absorbance against temperature. The absorbance value at the specified wavelength is first corrected by any specified background correction (see "Data Processing" on page 10), and then, if the Volume correction check box in the Calculation Parameters dialog box has been selected, the background-corrected absorbance value is corrected for the thermal expansion of an aqueous buffer. The default equation for volume correction is:

$$\text{Volume(T)} = 0{,}99829 + 104{,}5 \times 10^{-6} T + 3{,}5 \times 10^{-6} T^2 \quad \textbf{(15)}$$

where T is the temperature in °C.

If the results contain both heating and cooling ramps, the heating and cooling traces are separated before undergoing further data processing. Each trace then undergoes a three-step processing operation:

1. a linear interpolation to yield an equidistant temperature axis with a 0.1° interval
2. if the resulting number of data pairs is less than 500, the data are filled up using a spline algorithm to obtain a minimum of 500 data points.

Spline algorithm

The spline function constructs a cubic splined curve through the data points (y-values) in the spectrum. The spline function does not smooth the trace; the splined curve passes through all the original data points, and the spline process inserts additional points between the original ones to produce a continuous curve. The additional data points are calculated by a two-stage process:

Stage 1: Calculating the Second Derivative

The algorithm for calculating the second derivative originates in the following tri-diagonal system of linear equations:

$$(x_i - x_{i-1})y''_{i-1} + 2(x_{i+1} - x_{i-1})y''_i + (x_{i+1} - x_i)y''_{i+1} \quad \textbf{(16)}$$

$$= 6\left(\frac{y''_{i+1} - y''_i}{x_{i+1} - x_i} - \frac{y''_i - y''_{i-1}}{x_i - x_{i-1}}\right)$$

or, in simplified form,

$$\text{diff1} \cdot y''_{i-1} + 2(\text{diff1} + \text{diff2} \cdot y''_i) + \text{diff2} \cdot y''_{i+1} = 6(\text{quot2} - \text{quot1}) \quad \textbf{(17)}$$

where

diff1 is $x_i - x_{i-1}$

diff2 is $x_{i+1} - x_i$

quot1 is $\dfrac{y''_i - y''_{i-1}}{\text{diff1}}$

quot2 is $\dfrac{y''_{i+1} - y''_i}{\text{diff2}}$

The solution is achieved in a two-step process:

1. decomposition and forward substitution
2. back substitution

Stage 2: Calculating the Splined Values

The new y-values to be inserted between the original y-values are calculated according to Equation 18:

$$y_x = ((A \cdot \text{diffX} + B)\text{diffX} + C)\text{diffX} + D \quad \textbf{(18)}$$

where

diffX is the distance between current x-value and original x-value, x-x_i

In Equation 19, the coefficients A, B, C, and D are given by:

$$A = \frac{y''_{i+1} - y''_i}{6 \cdot \text{interval}} \quad \textbf{(19)}$$

where

interval is x_{i+1} - x_i

i is the current index, starting at 1 and ending at one less than the number of original values

$$B = \frac{y''_i}{2} \quad \textbf{(20)}$$

$$C = \frac{y_{i+1} - D}{\text{interval}} - \frac{1}{6} \cdot \text{interval} \cdot (y''_{i+1} + 4B) \quad \textbf{(21)}$$

where

interval is x_{i+1} - x_i

i is the current index, starting at 1 and ending at one less than the number of original values

$$D = y_i \quad \textbf{(22)}$$

The data are smoothed by a Savitsky-Golay moving average calculation (see "Savitsky-Golay Algorithm" on page 16) using a filter length of 99 points.

Evaluation

Data evaluation has up to four stages, depending on the parameters set in the Calculation Parameters dialog box.

Stage 1: Normalization

If the Normalization check box has been selected in the Absorbance Ratio group of the Calibration Parameters dialog box, and a normalization temperature has been specified, the whole trace is divided by the absorbance at the specified temperature.

Stage 2: Calculation of Delta Absorbance

The percent change in absorbance between the start and end of the trace is calculated for each trace:

$$\text{Heating trace: \% Absorbance} = 100\left(\frac{A_{end} - A_{start}}{A_{start}}\right) \quad \textbf{(23)}$$

$$\text{Cooling trace: \% Absorbance} = 100\left(\frac{A_{start} - A_{end}}{A_{end}}\right) \quad \textbf{(24)}$$

If a Calculation Range has been specified in the Calculation Parameters dialog box, then A_{start} and A_{end} correspond to the starting and ending absorbances of the calculation range, otherwise they correspond to the stating and ending absorbances of the whole trace.

Stage 3: Calculation of Melting Temperature, T_M

Two methods are available for the calculation of T_M, selected in the TM Calculation group of the Calculation Parameters dialog box:

1 Using the average absorbance (mean value)

In this case, T_M is the temperature at the absorbance value $A = \frac{1}{2}(A_{start} + A_{end})$ in the melting curve, where A_{start} and A_{end} correspond to the starting and ending absorbances of the calculation range (see Figure 4).

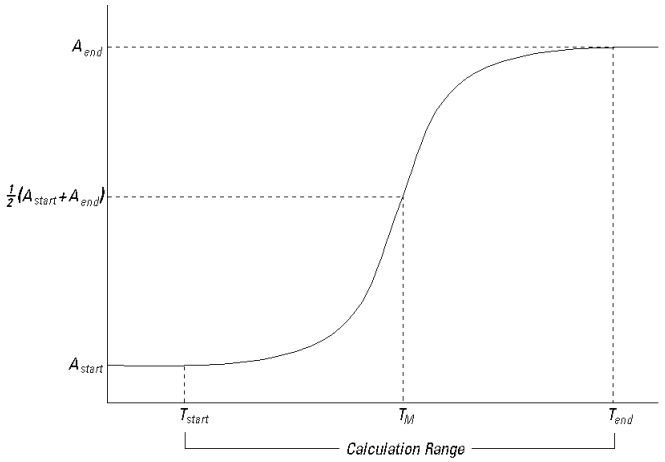

Figure 4 Calculation of Using Average

2 Using the first derivative

The first derivative of the trace is calculated using the Savitsky-Golay algorithm described in"Interactive Math Functions" on page 16 with a filter length as specified in the TM Calculation group of the Calibration parameters dialog box and a polynomial degree of 2 (quadratic).

The first derivative trace is then searched for peaks. The trace is first differentiated using the specified filter length (see "Savitsky-Golay Algorithm" on page 16) and a polynomial degree of 3. The differentiated y-values are examined for transitions (change of sign); each transitional y-value is compared with its neighboring values, and if the neighboring values are more extreme, the transitional y-value is compared with the previous transitional y-value. If the difference of both values is greater than or equal to the sensitivity threshold as specified in the TM Calculation group of the Calibration parameters dialog box, then the previous transitional point is stored as a melting temperature; if the difference is less than the sensitivity threshold, then neither point is stored.

If no sensitivity threshold is specified, only melting temperatures with an absolute value of greater than 1/1000 of the difference between the maximum and minimum values are reported.

Stage 4: Calculation of Melting Range and %G-C Content

The melting range is calculated within the specified calculation range by defining the low temperature as that where the slope begins to increase steadily, and the high temperature as that where the slope approaches zero again.

The the default equation for the calculation of %G–C (Guanine–Cytosine) base pairs is

$$\%\text{G–C} = 2{,}44 \times (T_M - 81{,}5 - 16{,}66 \times \log(M)) \quad \textbf{(25)}$$

where M is the molarity in mol/l.

If a DNA base pair length (K) is entered in the Sample Information dialog box at run time, then the equation becomes

$$\%\text{G–C} = 2{,}44 \times \left(T_M - 81{,}5 - 16{,}66 \times \log(M) + \frac{500}{K}\right) \quad \textbf{(26)}$$

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In This Book

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