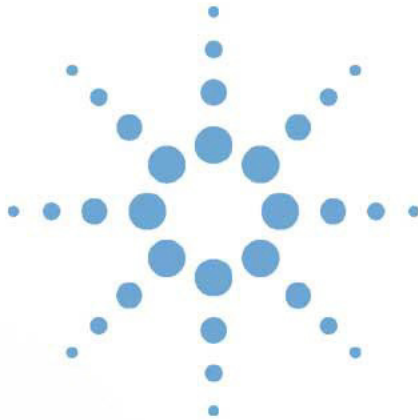


Agilent ChemStation for UV-Visible Spectroscopy



Operator's Manual for Biochemical Analysis Software



Agilent Technologies

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In This Guide...

The biochemical analysis software for Agilent ChemStation adds the kinetic mode and the thermal denaturation mode to the general purpose software. These two modes enable you to perform kinetic experiments and thermal denaturation experiments with the Agilent Cary 8454 UV-Visible spectrophotometer. This includes capabilities to develop analytical methods, real time online monitors to view your ongoing experiments, and tools to evaluate and process the experimental data.

This book gives you instructions for the installation of the software and hardware components needed for kinetic and thermal denaturation experiments. It also describes the most important software features.

This manual is organized in the following three chapters:

1 Installation and Configuration

This chapter describes the installation of the Agilent ChemStation software and the configuration of the Agilent 89090A Peltier temperature controller, which is needed for thermal denaturation experiments.

2 Kinetics Mode

This chapter deals with the configuration of the spectrophotometer and the sampling system, the development of a kinetic method, performing the kinetic measurement and the evaluation of the experimental data.

3 Thermal Denaturation Mode

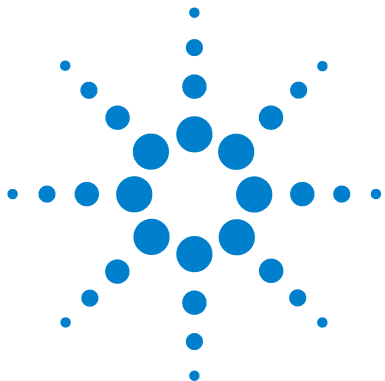
This chapter describes the setup of a temperature ramp and an analytical method for a thermal denaturation experiment.

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1 Installation and Configuration

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Configuring your Agilent 89090A Peltier Temperature Controller 9

Before you can use the biochemical analysis software, you must install the software and configure the spectrophotometer. In this chapter, the tools and the procedures for the installation are described. First, the biochemical analysis software must be installed on your Agilent ChemStation.

To use the thermal denaturation mode of Agilent ChemStation you must also configure the Agilent 89090A peltier temperature controller. This installation is optional for the kinetics mode.



Installing the Biochemical Analysis Software

The biochemical analysis software (G1117A) is an add-on module and is based on the general purpose software (G1115A). For details of the installation please see your UV-Visible Spectroscopy System Installation Guide.

If you want to add the Biochemical Analysis software package please follow the steps below.

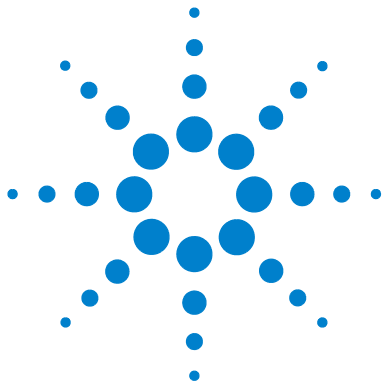
- 1 Insert your Agilent UV-Visible ChemStation DVD.
- 2 Run `setup.exe`.
- 3 Select **Modify** and click **Next**.
- 4 Select **G1117AA/G1123AA Biochemical Add-On** and click **Next**.
- 5 Enter the license number for your Biochemical Analysis software and click **Add License**.
- 6 Click **Next**, wait until the Maintenance Complete dialog is displayed, and click **Finish**.
- 7 After the installation is completed, run **Installation Qualification**, which is available in the UV-Visible ChemStations menu.
- 8 When the Installation Qualification is successfully completed, the biochemical analysis software is ready to use.

Configuring your Agilent 89090A Peltier Temperature Controller

If you are planning to use the thermal denaturation mode of your biochemical analysis software, you must configure the Agilent 89090A Peltier temperature controller.

- 1 Start the **UV-Vis Configuration Editor** from the UV-Visible ChemStations Menu.
- 2 If no UV-Visible spectrophotometer has been configured yet, add a New Instrument as described in your UV-Visible Spectroscopy System Operator's Manual.
- 3 Choose **Instruments...** from the **Configure** Menu.
- 4 Enter the Instrument Name, choose the Initial Screen Window Size and click **OK**.
- 5 From the UV-Vis Modules list select **TemCo**.
- 6 Set an **GPIB address** of 20.
- 7 Click **Add** to add the Peltier Temperature controller to your current configuration.
- 8 Click **OK** to close the Device Configuration dialog box.
- 9 Use the **Exit** command from the File menu to quit the Configuration Editor.
- 10 Click **Yes** to save your configuration and exit.

1 Installation and Configuration



2 Kinetics Mode

Preparing a Time Based Measurement 12
The Kinetic Measurement 23

This chapter describes the most important tasks in the kinetic mode of the biochemical analysis software. The first section, "[Preparing a Time Based Measurement](#)" on page 12, contains information about the steps preceding the kinetic measurement. You will learn how to setup the spectrophotometer, the sampling system and an analytical method.

The second section, "[The Kinetic Measurement](#)" on page 23, describes the actual time based measurement and the options for data processing and data evaluation with the Agilent ChemStation software.



Preparing a Time Based Measurement

Before you perform a time based measurement, you must set up your system according to your application. In this section you will learn to set up your spectrophotometer, to select and set up the sampling system and to develop a kinetic method.

Starting the Kinetics Mode

To start the kinetics mode of the biochemical analysis software choose **Kinetics** in the Mode menu or in the mode drop-down box.

[Figure 1](#) shows the Kinetics mode of the Agilent ChemStation software. The current mode is shown in the mode drop down box on the Toolbar. The Toolbar also includes the method name field, where the name of the actual analytical method is displayed. The letters **Mod** next to the method name field indicate that this method has been modified and that these modifications have not yet been saved. The Toolbar also includes several shortcut buttons, which are described in detail in your UV-Visible Spectroscopy System Operator's Manual.

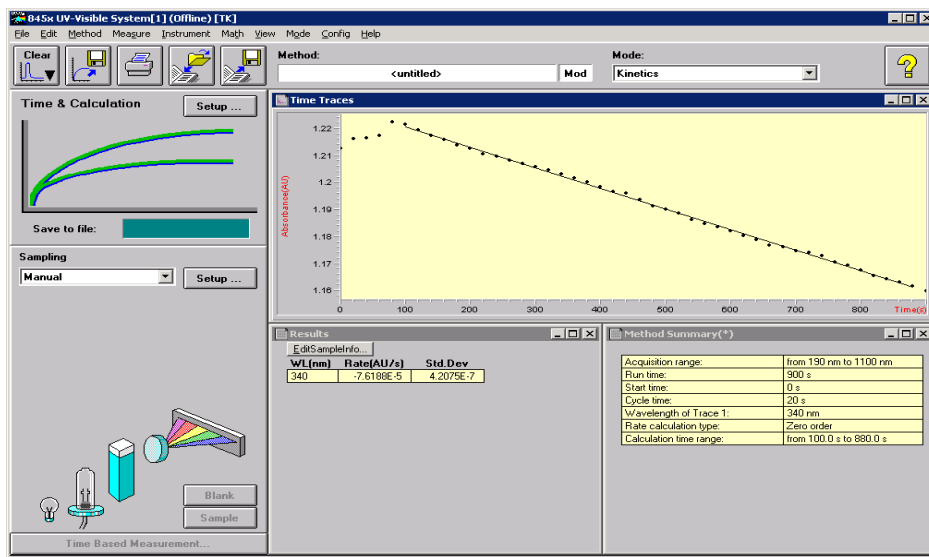


Figure 1 The Kinetics mode

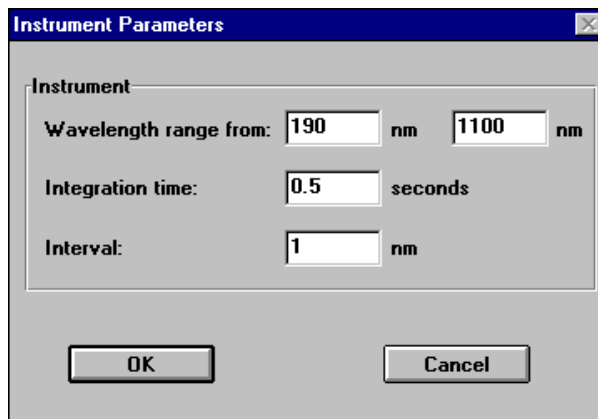
The SideBar is divided into two parts. The (upper) analytical panel includes a Setup... button, which opens the method dialog box. This dialog box includes the most important method parameters. In the (lower) instrument panel you can select the sampling system and switch the lamps of the spectrophotometer.

This view of the Kinetic mode contains the Time Trace window, the Results window and the Method Summary window. In these windows the kinetic time traces, the evaluated kinetic data and the core method parameters are displayed after the measurement.

Setting Up your Spectrophotometer

Select the **Instrument** menu of the biochemical analysis software to setup your UV-Visible spectrophotometer for a time-based measurement.

Choose **Setup Spectrophotometer...** to display the Instrument Parameters dialog box, which allows you to set the run-time parameters for the spectrophotometer. In this dialog box you can set the following parameters.



- You can use the fields to specify lower and upper limits of the range required by your analysis. The From field defines the lower limit of the range, the To field defines the upper limit of the range. The default values are the wavelength range limits of the configured spectrophotometer.

NOTE

Use the maximum wavelength range of your spectrophotometer. This enables you to use the full benefits of the diode array spectrophotometer, for example, post run data evaluation at different wavelengths.

- The value in the Integration time field is the time in seconds over which the signal is collected and integrated. The Integration time must be between 0.1 and 25.5 seconds; the default is 0.5 seconds. Longer integration times improve the signal-to-noise ratio since signal is accumulated while noise is averaged out. The selected integration time has a direct impact on the minimum cycle time for a kinetic measurement. The minimum cycle time cannot be shorter than the integration time. Hence, it might be necessary to minimize the Integration time for fast kinetic measurements.
- The number in the Interval field defines the wavelength measurement interval. The minimum interval is 1 nm, corresponding with the highest resolution.

NOTE

To achieve optimum performance of the spectrophotometer, do not change the default value of 1 nm.

Select **Lamp(s)...** to display the Lamp(s) Parameter dialog box. This is where you can set the parameters of the spectrophotometer lamp(s). The format of the Lamp(s) Parameter dialog box depends upon the spectrophotometer type. With the UV-Visible spectrophotometer you can switch the deuterium lamp and the tungsten lamp On and Off. The lamps can also be switched by clicking on the lamp icons in the Instrument panel of the SideBar in the application screen.

NOTE

If you change the lamp parameters, the current method is modified and the lamp status is changed. In the case of a lamp failure (ignition failure, lamp defect or lamp door open), the lamp(s) parameters may be displayed as On, although the lamp(s) are off. Check the actual status of the lamp(s) in the Spectrophotometer Status window or the Instrument panel of the SideBar of the application window.

The actual status of the spectrophotometer instrument is summarized in the Spectrophotometer Status window. In this window the following actual values are displayed:

Instrument type, wavelength range, wavelength interval, integration time, power on time, the status of the lamp(s) and the overall status of the spectrophotometer (ready/not ready).

Configuring the Sampling System

The sampling system can be selected and setup either in the instrument menu or in the Instrument panel of the SideBar of the graphical user interface.

Select **Sampling System...** to display the Sampling System dialog box, where you can select either Manual operation or the 8-cell multicell transport system. If you want to use the Peltier temperature controller (Agilent 89090A #100), you must select the Manual sampling system.

CAUTION

When you switch from Manual sampling to the multicell transport system, the current single-cell results are discarded.

Setup Sampling System... displays the Setup dialog box that allows you to set the run-time parameters for the selected sampling system.

The Cells dialog box allows you to set up the run-time parameters for the multicell transport.

		Pathlength (cm)
Cell 1:	Blank	1
Cell 2:	Sample	1
Cell 3:	NotUsed	1
Cell 4:	NotUsed	1
Cell 5:	NotUsed	1
Cell 6:	NotUsed	1
Cell 7:	NotUsed	1
Cell 8:	NotUsed	1

OK Cancel

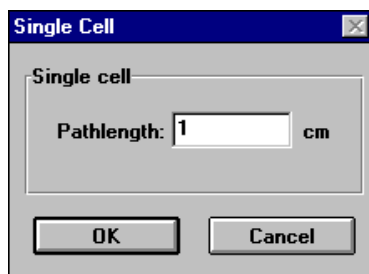
The dialog box shows the used cells as a table with the following columns:

- Cell n: indicates the cell positions in the multicell transport; cell 1 is the cell nearest the front of the spectrophotometer.
- The central column of the table describes the sample in the cell. Choose Sample, Blank or Not Used from the drop-down list.
- The Path Length fields show the path lengths in centimeter of the sample cells in each position of the multicell transport. The default value for each cell is 1 cm.

NOTE

It is good measurement practice to use position 1 for the Blank measurement. Then the Blank spectrum is always renewed at the beginning of a measurement cycle. This is especially important, if you are performing long time kinetic experiments.

The Single Cell dialog box allows you to set up the run-time parameters for manual operation.



The Path Length field is the path length in cm of the sample cell you are using. The default value is 1 cm.

Select **Wait for Temperature Ready** to ensure that the Agilent ChemStation waits for a Ready signal from the Agilent 89090A Peltier temperature control accessory before making a measurement. The default condition is cleared. This check box is only available, when the Agilent 89090A Peltier temperature controller is configured.

Setting Up a Kinetic Method

Method parameters are edited in the Time & Calculation dialog box and the Info & Options dialog box under the Method menu. The Time & Calculation dialog box is also displayed, when Setup button has been pushed. The most important parameters of your method are displayed in the Method Summary window.

In the Time & Calculation dialog box you can define the conditions for the time based measurement.

Time & Calculation Parameters

Wavelengths

Use wavelength: nm

Background correction: nm

Online monitor

Trace monitor: Y-scaling from: to: AU

Monitor spectra: Y-scaling from: to: AU

Timing

Run time: s

Start time: s

Cycle time: s (min 11.9s)

Options

Increment cycle time by: %
after initial time of: s

Rate calculation

Type: Calculation time range from: to: s

Multiply Rate by to convert to Rate unit:

Subtract Rate of cell from all other Rates

OK Cancel

- The **Use Wavelength** field allows you to define the wavelength (for multicell measurements) or up to six wavelengths (for single cell measurements) of your processed data from which the amplitude values are extracted for the wavelength result. The default wavelength is 480 nm. If you enter wavelengths at which no measured data are available (for example non-integer values), the amplitude values are calculated by linear interpolation.
- The Background Correction field allows you to specify any background correction that is applied to the wavelength result to calculate a function result. Choose the down arrow and select a background correction procedure from the drop-down list:

none – specifies that the wavelength result is used as measured without background correction.

single reference wavelength – specifies that the absorbance at a single wavelength is subtracted from the wavelength result. You specify the background wavelength in the field.

subtract average over a range – specifies that the average absorbance over a wavelength range is subtracted from the wavelength result. You specify the upper and lower limits of the background wavelength range in the fields.

three-point drop-line – specifies that a background absorbance calculated using a three-point drop-line is subtracted from the wavelength result. You specify the upper and lower limits of the background wavelength range used for the background calculation in the adjacent left (lower) and right (upper) fields.

- The **Trace Monitor** is a continuously-updated time trace that shows the change of absorbance at the wavelength specified in the Use Wavelength field with time. Define the scaling of the absorbance (y) axis by typing lower and upper limits in the Y scaling from: and to: fields.
- You can choose to monitor either the last acquired spectrum or spectra from a specific cell. Choose the down arrow and select either **Last Spectrum, Cell n** (for multicell measurements) or **All Spectra** (for single cell measurements). Define the scaling of the absorbance (y) axis by typing lower and upper limits in the Y scaling from: and to: fields.
- The **Run Time** is the total time of the analysis. The Run Time range is up to 999999 seconds (11.5 days). The Run Time must be greater than Cycle Time plus Start Time.

- The **Start Time** is the time between initiating measurement and measurement starting, and is optional. The default value is 0.0.
- The **Cycle Time** is the time from the start of one measurement to the start of the next; the default value is 5.0 seconds for multicell operation, 0.5 seconds for single cell operation (minimum acceptable values).
- The minimum cycle time is hardware-dependent, and the value given is an approximation only. To avoid losing data, you must determine the minimum cycle time for your configuration experimentally. The minimum cycle time depends on the sampling system (number of measurements per cycle), the integration time (defined in the Setup Spectrophotometer... dialog box) and the stray light correction (defined in the Info & Options dialog box).
- Select **Increment cycle time** to activate the increase in cycle time. Specify the percentage increase in the % field and a time interval (in seconds) in the after initial time of: field.
- You can choose from five different kinetic evaluation types. Choose the down arrow and select the calculation type from the drop-down list:

None – specifies that no rate calculation is made.

Initial rate – specifies that a quadratic fit is made according to the equation, $a+bt+ct^2$ where b is the initial rate constant.

Zero order – specifies that a linear fit is made according to the equation,
 $a + bt$ where b is the rate constant in AU/s.

First order – specifies that an exponential fit is made according to the equation,
 $a+b\cdot\exp(-kt)$ where k is the rate constant in 1/s.

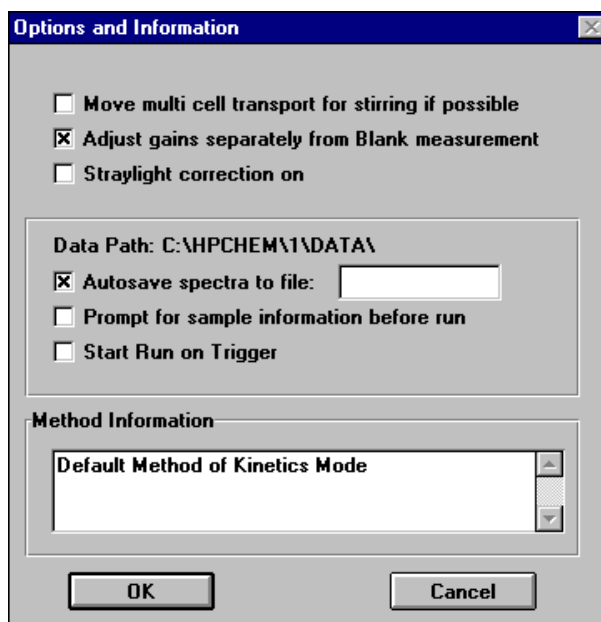
Delta AU – specifies a subtraction of the starting absorbance from the final absorbance:

$$\text{Abs}_{t(\text{end})} - \text{Abs}_{t(\text{start})}$$

- You can specify that the rate calculation is made over a limited time range. Type the start time for the calculation in the from: field and the end time in the to: field.
- Select **Multiply Rate** to activate a conversion factor for the rate result. Specify the factor and the new units in the to convert to Rate unit: field.

Select **Subtract Rate of Cell** to subtract the result of a reference cell from the rate results of all other cells. Choose the down arrow and select the reference cell from the drop-down list. This option is available only when you have configured a multiple-cell measurement.

In the Options & Information dialog box you can define the acquisition conditions for the kinetics experiment.



- **Move multicell transport for stirring if possible** will move the current cell into position for sample stirring during the idle time between two cycles, if the cycle time is sufficiently long (approximately the minimum cycle time plus 4 seconds for each configured cell). This check box is available only when a multicell transport system is selected.
- **Adjust gains separately from blank measurement** adds the Set Gains command into the Measure menu. Set Gains performs a gain adjustment, reference and a measurement; the Blank command performs a reference and a measurement only. The separate gain adjustment on a blank medium with high transmission (for example, water or buffer) sets the gains to a lower value in order avoids an A/D converter overflow and invalid data points due to negative slope time traces.

- **Stray light Correction On** switches stray light correction option. When stray light correction is on, a second measurement is made with the starlight filter in place, and the sample spectrum is corrected for stray light using this spectrum. The stray light correction increases the minimum cycle time by the Integration Time (set in the Instrument Parameters dialog box) plus 2 seconds.
- Select **Autosave spectra to file** to save all acquired spectra to disk. Type a name for the file in the adjacent field. The spectra are stored in the default directory (CHEM32\n\DATA) with the .KD extension. The default directory can be changed in the Config menu
- **Prompt for sample information before run** will display the Sample Information dialog box before measurements are started.
- Select **Start Run on Trigger** to start data acquisition using a closure of contacts 13 and 15 of the GPIO interface. This ensures that minimum time elapses (approximately 0.12 seconds) between the trigger action and the start of data acquisition; it is intended to be used for fast reaction kinetics.
- You can type a description of the method in the **Method Information** field. The field accepts any alphanumeric characters, and the description can be as long as you wish. The text wraps automatically at the end of the line. You can edit an existing description using any of the standard text-processing procedures (for example, Copy, Cut, Paste).

The Kinetic Measurement

Running a Time Based Measurement

Measurements can be started either by using the Measure menu from the menu bar, by pushing the buttons (Blank, Sample, Time Based Measurement) on the SideBar of the graphical user interface or by using selected F keys.

If you have selected the option **Adjust gains separately from blank measurement** in the Options & Info... dialog box, Set Gains is included in the Measure menu. The separate gain adjustment on a blank medium with high transmission (for example, water or buffer) sets the gains to a lower value and avoids an A/D converter overflow. This helps to avoid invalid data points due to negative slope time traces.

- 1 Introduce a cell filled with water or any other low absorbing medium into the cell holder.
- 2 Move the multicell transport to the right position.
- 3 Click on Set Gains in the Measure menu.

With a multicell sampling system you should measure Zero Cell spectra in order to correct for slightly different optical properties of the measurement cells. Initialize the multicell transport with identical blank medium in all cells. The Zero Cells... command measures a true blank spectrum for cell 1, then measures spectra for the remaining cells. The difference spectra obtained by subtracting the blank spectrum from the individual cell spectra are then subtracted automatically from all subsequent measured spectra to correct for any cell-to-cell optical differences.

- 1 Fill all measurement cells with the same blank medium (for example, water).
- 2 Make sure, that there are no bubbles or floating particles in the cells.
- 3 Do a Blank measurement by selecting **Blank** in the measurement menu, by clicking **Blank** on the SideBar of the application window or by using F4.
- 4 Start the Zero Cells measurement from the Measure menu.

The Zero cell spectra are displayed in the Zero Cells Spectra window, which can be activated in the view menu. Make sure that you do not switch the cells after the zero cell measurement.

To measure a single spectrum of the solution in the cell and display it in the Sample Spectra window select Sample (Single Spectrum) in the measure menu. You can also measure a single spectrum by pressing F5 or the Sample Button in the SideBar of the application window.

Pressing F7, or selecting **time based measurement** from the measure menu or from the SideBar of the application window prepares the software for starting the time based measurement and displays the Time Trace window and the Sample Spectra window. Choose **Start** from the new menu bar to start the acquisition or **Abort** to abort an acquisition in progress.

During the Time Based Measurement you can follow the acquisition of the spectra and the time traces in real-time in the Time Trace window and the Sample Spectra window. Left double-click on the mouse in the Time Trace window area or the Sample Spectra window area to autoscale the y-axis.

After the Time Based Measurement has been finished or aborted by the user, the kinetic data are stored as specified in the Options & Info... dialog box. The Time traces are evaluated by the rate type, which has been defined in the Time & Calculation dialog box. These results are displayed in the Results window.

Evaluating your Kinetic Data

After the time based measurement has been finished you can re-evaluate the kinetic data. Since full spectra have been acquired during the measurement, the parameters in the fields Wavelength and Rate Calculation of the Time & Calculation dialog box can be changed. Click **OK** to make the changes active. The new results are displayed in the Results window. If the method parameter has been changed the modified flag Mod will appear.

If manual sampling system has been configured, you can choose **Edit Sample Information** from the Results window to display the Sample Information dialog box. This allows you to include sample information with your results. The Sample Information dialog box contains the following parameters:

- The Sample Name.
- A comment (for example a description of the sample)
- The concentration of a substrate in the [S] (mg/ml) field; this field is present only if you have selected [S] or [S][I] in the Configure Substrate, Inhibitors dialog box.
- The concentration of an inhibitor in the [I](mg/ml) field; this field is present only if you have selected [S][I] in the Configure Substrate, Inhibitors dialog box.
- The concentration of the first of two substrates in the [A](mg/ml) field; this field is present only if you have selected [A][B] in the Configure Substrate, Inhibitors dialog box.
- The concentration of the second of two substrates in the [B](mg/ml) field; this field is present only if you have selected [A][B] in the Configure Substrate, Inhibitors dialog box.

If a multicell transport sampling system has been configured, you can choose **Configure Substrate/Inhibitors** from the Results window to display the Configure Substrate, Inhibitors dialog box. This allows you to include information on substrates and inhibitors with your results. You can also select the Configure Substrate/Inhibitors dialog box by clicking the button in the Edit Sample Information dialog box. The Configure Substrate, Inhibitors dialog box contains the following parameters:

- None specifies that no substrates or inhibitors are included.
- [S] specifies that one substrate is included.
- [S][I] specifies that one substrate and one inhibitor are included.
- [A][B] specifies that two substrates are included.

Enter the units of concentration of the substrates and inhibitors in the Unit field.

NOTE

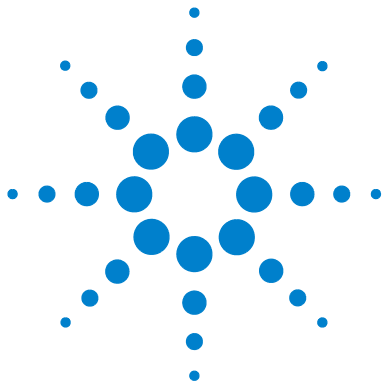
To load and evaluate old data, which have been generated by the HP 89532K software choose *Import from *.MKD...* in the file menu.

Viewing Results

You have several options for viewing your acquired and processed data as well as the tabulated results. In the View Menu:

- **All Spectra** displays all acquired spectra in the Sample Spectra window.
- **Spectra of Cell** allows you to display spectra from a selected cell in the Sample Spectra window. You select the cell number from the submenu.
- **Traces and Results** displays the Time Traces window, the Results window and the Method Summary table.
- **Measured Single Spectra** displays a measured spectrum in the Single Spectra window.
- **Last Blank Spectrum** displays the Last Blank Spectrum window.
- **Zero Cell Spectra** is included in the View menu when you have configured a Multicell Transport system. It displays the Zero Cell Spectra window.
- **Math Results** displays the Math Result window.
- **Tabulate Selected Spectrum/Trace** displays the Tabular Data of Spectra table of the selected spectrum or the Tabular Data of Time Traces table of the selected time trace. These tables are also displayed automatically when you double-click the left mouse button on a spectrum/time trace in a graphical window, or when you press Enter while using the cursor. The title of the table indicates the type of spectrum (for example, Sample, Standard) with the number of the spectrum/time trace in the window in brackets, for example, [1].
- **Logbooks** displays the Logbooks dialog box, from which you can select a logbook to display or print.
- **Load Logbook** displays the Load Logbook dialog box, which allows you to select a previously-stored logbook to load.
- **Next Window** transfers the focus to the next window in the series. If the window is hidden behind other windows, Next Window brings it to the front. If the window is iconized, Next Window displays it with its default size and position. Pressing the F11-key is the keyboard equivalent to choosing Next Window.
- **Previous Window** transfers the focus to the previous window in the series. If the window is hidden behind other windows, Previous Window brings it to the front. If the window is iconized, Previous Window displays it with its default size and position.

- **Reset Current View** resets all windows in the current view to their default sizes and positions.



3 Thermal Denaturation Mode

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This chapter describes the essential tools to develop a thermal denaturation method, to acquire and evaluate the data with the thermal denaturation mode of the biochemical analysis software. The first section, [“Preparing a Thermal Denaturation Measurement”](#) on page 30, contains information about the steps before the measurement. You will learn how to setup the spectrophotometer, the temperature ramp for the Peltier Temperature controller and to choose between different analytical evaluation options.

The second section, [“The Thermal Denaturation Experiment”](#) on page 38, describes the actual thermal measurement, the real-time online monitor and the options for data processing with the Agilent ChemStation software.



Preparing a Thermal Denaturation Measurement

Before you perform a thermal denaturation measurement you must set up your system according to your application. In this section you will learn to set up your spectrophotometer, set up the a temperature ramp with Peltier temperature controller and to develop a thermal denaturation method to analyze DNA or protein sample.

NOTE

The Agilent 89090A Peltier temperature controller must be configured before the thermal denaturation mode is started.

Starting the Thermal Denaturation Mode

To start the thermal denaturation mode of the biochemical analysis software choose **Thermal Denaturation** in the Mode menu or in the mode drop-down box.

Figure 2 shows the thermal denaturation mode screen of the Agilent ChemStation software. The current mode is shown in the mode drop down box on the tool bar. The tool bar also includes the method name field, where the name of the actual analytical method is displayed, and several shortcut buttons which are described in your UV-Visible Spectroscopy System Operator's Manual.

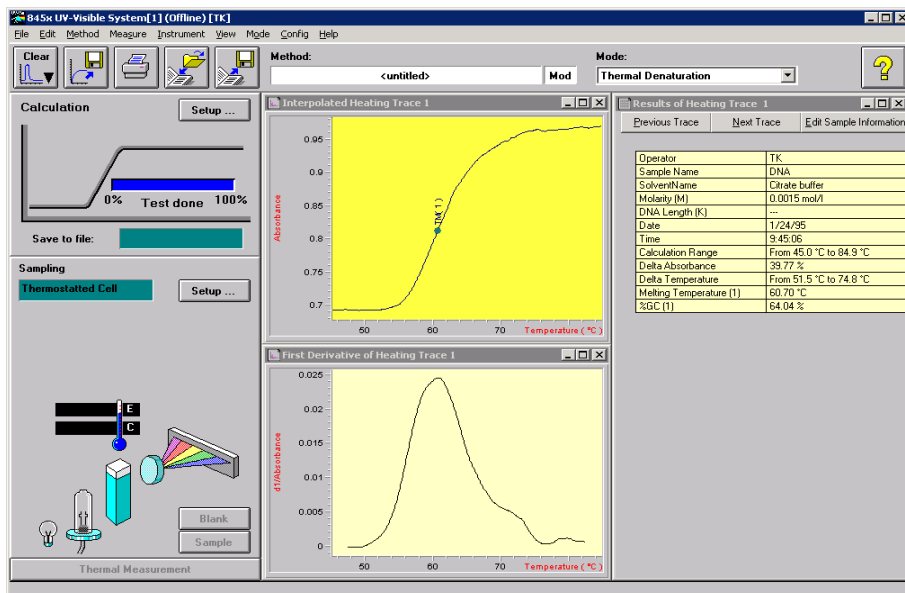


Figure 2 The Thermal Denaturation mode screen

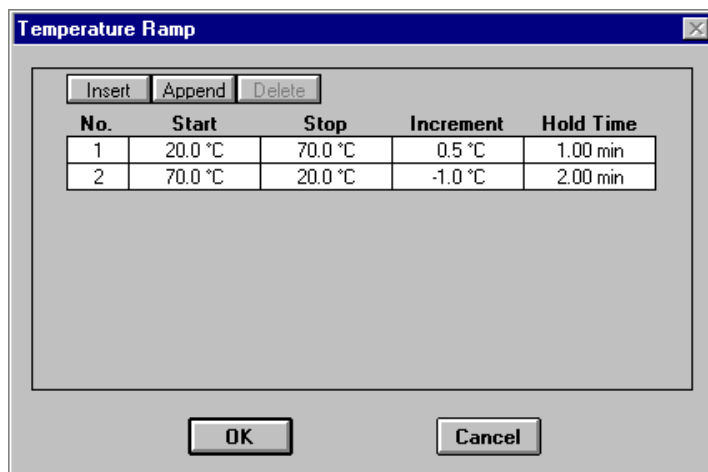
The upper analytical panel includes a shortcut button, which opens the Calculation Parameters dialog box. The blue status bar indicates displays the progress of the thermal denaturation measurement. The sampling panel contains a shortcut button, which opens the Temperature Ramp dialog box and a display for the current temperature.

Setting Up your Spectrophotometer

Select the Instrument menu of the biochemical analysis software to setup your UV-Visible spectrophotometer for a thermal denaturation measurement. The setup of the core spectrophotometer parameters is identical to that in the kinetics mode of your Agilent ChemStation (see "Setting Up your Spectrophotometer" on page 14).

Setting Up the Temperature Ramp

In thermal denaturation mode the Peltier temperature controller must be set as sampling system, which is displayed as Thermostatable Cell in the sampling field of the instrument panel. Click **Setup** next to the sampling field to open the Temperature Ramp dialog box, which allows you to set up the temperature program for the thermal denaturation experiment.



Each line in this dialog box defines a temperature ramp.

- The **No.** column contains the number of the temperature ramp; temperature ramps are numbered in sequence.
- The **Start** column contains the starting temperature for the ramp. When you append a ramp, the default start temperature is the stop temperature of the previous ramp.

- The **Stop** column contains the final temperature for the ramp. When you add a ramp to the program, the stop temperature is undefined. The maximum stop temperature is 100 °C.
- The **Increment** column contains the value by which the temperature is increased step wise. The default increment is 0.5 °C.
- The **Hold Time** column contains the time for which each temperature step is maintained. The default hold time is 1.00 min.

Setting Up a Thermal Denaturation Method

To edit the analytical parameters of your thermal denaturation method, select **Calculation** in the method menu or click **Setup** in the analytical panel of your application screen. This will open the Calculation Parameters dialog box.

Calculation Parameters

Wavelength

Use wavelength at : 260 nm

Background correction : subtract average over range 500 nm 550 nm

Temperature

Use temperature from : Internal sensor

Set calculation range From : °C to : °C

Absorbance ratio Normalize at temperature : °C

TM calculation

Average (mean value)

Derivative Filterlength : 21 Sensitivity :

Equation

%GC = 2.44*(TM-81.5-16.66*log(M))

Equation for thermal expansion

Volume correction

Volume (T) = 0.99829+104.5E-6*T+3.5E-6*SQR(T)

OK Cancel

The **Use Wavelength at:** field allows you to define the wavelength of your processed data from which the amplitude values are extracted for the wavelength result. The default wavelength is 260 nm. If you enter a wavelength at which no measurement is available (for example a non-integer value), the amplitude values are calculated by linear interpolation.

The **Background Correction** field allows you to specify any background correction that is applied to the wavelength result to calculate a function result. Choose the down arrow and select a background correction procedure from the drop-down list. The available options are identical to the procedures in the kinetic mode.

The **Temperature group** allows you to select the source of your temperature measurement. Choose the down arrow and select the temperature source from the drop-down list. If you choose internal sensor the temperature of the thermostatable cell holder, which is measured by the Peltier temperature controller, is used for the data evaluation. If you choose external sensor the temperature of your sample, which is measured by the external temperature sensor, is used for the data evaluation.

The **Set Calculation Range** group allows you to limit the calculation of the results on all traces to a specified temperature range. You specify the lower and upper limits of the temperature range in the From and To fields.

The **Absorbance Ratio** group allows you to specify a temperature at which absorbance data are normalized; the temperature trace is divided by the absorbance value at the specified temperature. Select Normalize at temperature to switch on the normalization, and specify the normalization temperature in the adjacent field.

The **Tm Calculation** group allows you to select the calculation method for the melting temperature.

- Choose *Average (mean value)* to calculate the melting temperature as the temperature at the average value of the absorbance at the start of the calculation range and that at the end of the calculation range.
- Choose *Derivative* to calculate the melting temperature using the first derivative of the temperature trace. Enter a Filter length for the derivative calculation and a Sensitivity for the reporting of Tm values in the adjacent fields.

- The value in the **Filter Length** field sets the number of data points that is used to calculate each point in the derivative trace. The value must be an odd number between 5 and 749; the default value is 21. Increasing the number of points increases the degree of smoothing of the resulting trace.
- Only maxima and minima that are at least the value in the **Sensitivity field** distinct from their neighboring maxima or minima are reported. When the field is left blank, the sensitivity is set to 1/1000 of the difference between the maximum and minimum values.

The **Equation** group allows you to define the equation for the calculation of a result.

- Type a parameter name for the result in the field to the left of the equation and the equation in the field to the right. Use T_m (melting temperature), M (molarity of the sample), and K (DNA length).
- The **Equation for Thermal Expansion** group allows you to select to make a correction for the change in absorbance due to the thermal expansion of the solution on heating. Select Volume Correction to specify that a correction for thermal expansion should be made. Type the equation for the volume correction in the Volume (T) = field. You can use the same operators in the volume correction equation as in the Equation group. Use the variable T for the sample temperature.

3 Thermal Denaturation Mode

Select **Temperature & Options** from the method menu to open the Temperature & Options dialog box. In this dialog box you can choose between several options for your thermal denaturation experiment.

The dialog box is titled "Temperature & Options" and contains the following controls:

- Temperature unit:** Radio buttons for Celsius (selected), Kelvin, and Fahrenheit.
- Stirrer:** Radio buttons for Off (selected) and On. A "Speed" text field is present with "rpm" units.
- Online trace monitor:** Radio buttons for Auto scaling (selected) and Fixed scaling. "From" and "to" text fields are present with "AU" units.
- Idle temperature:** A text field showing "25.00" with a degree Celsius symbol.
- Ramping speed:** Radio buttons for Slow and Fast (selected).
- Save spectra:** A checked checkbox for "Autosave spectra to file:" followed by an empty text field.
- Method Information:** A text area containing "Default Method of Thermal Denaturation Mode".
- Buttons:** "OK" and "Cancel" buttons at the bottom.

- The **Temperature Unit** group allows you to choose the units of temperature measurement and control. You have the options to choose Celsius (°C), Kelvin (K), or Fahrenheit (°F).
- Choose **On** in the **Stirrer** group to switch on the stirrer. When the stirrer is switched on, you can set the speed in rpm by typing it in the Speed field.
- In the **On-line trace monitor** group you can specify the absorbance scale (y-axis) of the online trace monitor. Choose **Auto Scaling** to allow the ChemStation to calculate the absorbance scale based on the signal intensities or choose **Fixed Scaling** to define a fixed absorbance scale. When you select Fixed Scaling, you can define the lower and upper limits of the absorbance scale by typing them in the **From** and **To** fields.

- In the **Idle Temperature** group you can edit the temperature that is set between thermal denaturation experiments.
- The **Ramping Speed** group allows you to select the speed at which the temperature is increased. Choose **Slow** to increase the temperature incrementally to each step of the temperature ramp. Choose **Fast** to increase the temperature ballistically to each step of the temperature ramp.

NOTE

If the steps are large, the temperature may overshoot in the Fast mode.

- Select **Autosave spectra to file** to specify that the acquired spectra are to be saved. Type a name for the data file in the **Autosave filename:** field. The name must comply with DOS naming conventions (maximum 8 alphanumeric characters). When **Autosave spectra to file** is cleared, the spectra are not saved.
- You can type a description of the method in the **Method Information** field. The field accepts any alphanumeric characters, and the description can be as long as you wish. The text wraps automatically at the end of the line.

The Set Individual Calculation Range dialog box is displayed when you choose the **Set Individual Calculation Range** command from the Method menu. It enables you to define a temperature range for the calculation of thermal denaturation results on the currently-displayed trace. Type the lower and upper limits of the temperature range in the from and to fields in the dialog box.

The Thermal Denaturation Experiment

After the temperature ramp has been defined and the method parameters have been edited, you are able to start your thermal denaturation experiment. In this section you will find information about the real-time online monitor of the measurement and the Agilent ChemStation features to process your experimental data.

Running a Thermal Denaturation Experiment

Measurements can be started either by using the measure menu from the menu bar, by clicking the buttons (Blank, Sample, Thermal Measurement) on the sidebar of the graphical user interface or by using selected F keys.

Before you can measure a single spectrum or perform a thermal denaturation experiment you must measure a Blank spectrum by selecting **blank** in the Measure menu, by pressing the F4 key or by clicking **Blank** in the SideBar of the graphical user interface.

To measure a single spectrum of the solution in the cell and display it in the Sample Spectra window select **Sample (Single Spectrum)** in the measure menu. You can also measure a single spectrum by pressing F5 or clicking **Sample** in the SideBar of the graphical user interface.

Pressing F7, or selecting **Thermal Measurement** from the measure menu or from the SideBar of the graphical user interface displays the Sample Information dialog box.

- Type a sample name in the **Sample Name** field. The sample name is included in the table and reports.
- Type a solvent name in the **Solvent** field. The solvent details are included in the Results of Trace table and reports.
- Enter the molarity of the sample in mol/l in the **Molarity (M)** field. The molarity is included in the Results of Trace table and reports and is used in the %G-C calculation.

- Enter the DNA length of the sample in base pairs (bp) in the **DNA Length (K)** field. The DNA length is included in the Results of Trace table and reports and is used in the %G-C calculation.
- You can enter a comment (for example a description of the sample) in the **Comment** field.

Click **Run** to start the acquisition and to display the All Spectra window, the Trace window and the Sample Information window. First the Peltier temperature controller sets the starting temperature at the cell holder. After the hold time which has been defined in the Temperature Ramp dialog box the first spectrum is measured and the Peltier temperature controller sets the next temperature of the ramp.

During the thermal measurement you can follow the acquisition of the spectra and the temperature traces in real-time in the All Spectra window and in the Trace window.

After the thermal denaturation measurement has been finished or aborted by the user, the data are stored as specified in the Temperature & Options dialog box. The traces are evaluated by the procedure, that has been defined in the Calculation Parameters dialog box. These results are displayed in the Result window.

Evaluating your Thermal Denaturation Data

After the thermal denaturation run has been finished you can re-evaluate the experimental data. Since full spectra have been acquired during the measurement, all parameters in the Calculation Parameters dialog box can be changed. Click OK to make the changes active. The new results are displayed in the Results of Heating Trace window. If the method parameter has been changed the modified flag Mod will appear in the tool bar of the graphical user interface.

When you click **Edit Sample Information** in the Results of Heating Trace window, the Sample Information dialog box is displayed. It enables you to include information on your samples for use in results calculations.

Viewing Results

You have several options to view your acquired and processed data as well as the tabulated results. Select the **View Menu** that contains:

- *All Spectra* displays all acquired spectra in the Sample Spectra window.
- *Spectra of Current Trace* displays the spectra of the currently-selected trace in the Spectra of Current Trace window.
- *All Traces* displays all temperature traces in the Temperature Trace window.
- *Traces and Results* displays the Time Traces window, the Results window and the Method Summary table.
- *Measured Single Spectra* displays a measured spectrum in the Single Spectra window.
- *Last Blank Spectrum* displays the Last Blank Spectrum window.
- *Tabulate Selected Spectrum/Trace* displays the Tabular Data of Spectra table of the selected spectrum or the Tabular Data of Time Traces table of the selected time trace. These tables are also displayed automatically when you double-click the left mouse button on a spectrum/time trace in a graphical window, or when you press Enter while using the cursor. The title of the table indicates the type of spectrum (for example, Sample, Standard) with the number of the spectrum/time trace in the window in brackets, for example, [1].
- *Logbooks* displays the Logbooks dialog box, from which you can select a logbook to display or print.
- *Load Logbook* displays the Load Logbook dialog box, which allows you to select a previously-stored logbook to load.
- *Next Window* transfers the focus to the next window in the series. If the window is hidden behind other windows, Next Window brings it to the front. If the window is iconized, Next Window displays it with its default size and position. Pressing F11 is the keyboard equivalent to choosing Next Window.
- *Previous Window* transfers the focus to the previous window in the series. If the window is hidden behind other windows, Previous Window brings it to the front. If the window is iconized, Previous Window displays it with its default size and position.
- *Reset Current View* resets all windows in the current view to their default sizes and positions.

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In This Book

The biochemical analysis software for Agilent ChemStation adds the Kinetic mode and the Thermal Denaturation mode to the general purpose software. These two modes enable you to perform kinetic experiments and thermal denaturation experiments with your UV-Visible spectrophotometer. This includes capabilities to develop analytical methods, real time online monitors to view your ongoing experiments, and tools to evaluate and process the experimental data.

This book gives you instructions for the installation of the software and hardware components needed for kinetic and thermal denaturation experiments. It also provides information about the most important software features, which will help you to solve your analytical problems.

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