User Manual
PR.TimeControl Software

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NanoTemper® Technologies' Prometheus™ Series instruments detect changes in the fluorescence of the amino acid tryptophan (and fluorophores with equivalent spectroscopic properties) over a wide range of temperatures using nanoDSF™, an advanced Differential Scanning Fluorimetry technology. The instruments can be used to induce thermal unfolding of proteins and to determine thermal unfolding transition temperatures. Furthermore, the instruments are equipped to investigate chemical unfolding and the free energy of unfolding ΔG in an extraordinarily straightforward and fast manner.

The PR.TimeControl software is dedicated to running and analyzing non-linear thermal unfolding and refolding experiments as well as isothermal measurements on Prometheus instruments.

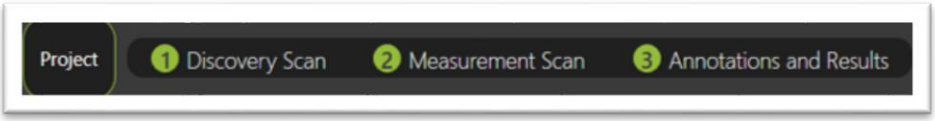
1. System Requirements

If the necessary licenses have been purchased, PR.TimeControl software can be installed on additional computers for convenient data analysis. The computers have to meet the following requirements:

Operating system:	Windows 7 64 Bit or higher
CPU:	Intel Core i5 or better
RAM:	8 GB or more
Hard disk:	20 GB or more free disk space available
Display resolution:	1600 x 900 or better
Software:	Microsoft .NET 4.6.2 framework (included in installer of PR.TimeControl software)
Operating system language:	English or German

An external computer mouse is necessary to access all software features.

2. General Usage



To perform a new measurement, start the PR.TimeControl software. Click *Start New Session* to enter a file name and location, or click *Browse Previous Sessions* to analyze previously acquired data or to add additional measurements. Recently loaded files are listed chronologically on the bottom. Files will be saved in the .prtime format.

After a file has been created or loaded, the software automatically moves to *(1) Discovery Scan*. Three tabs guide the user through running and analyzing nanoDSF measurements:

1. Discovery Scan
2. Measurement Scan
3. Annotations and Results

Proceed from 1 to 3 to set up, run and analyze a nanoDSF measurement. Navigate between all three tabs freely to re-analyze, modify or annotate. More details on each tab follow in the next sections.

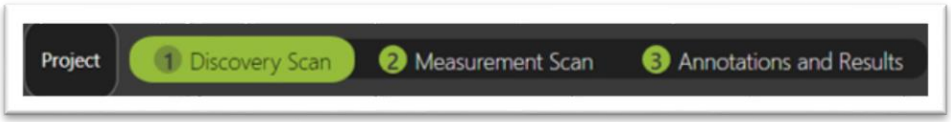
Clicking on *Project* returns the user to the home screen, where optionally, relevant information can be entered into the *Project Name* and *Comments* fields, and the file path is displayed.

Use the *Save* button at any time to save any modifications of the file. An asterisk in the software title bar indicates unsaved changes in the open file. Closing the software will trigger a dialogue box asking whether you want to save the changes.

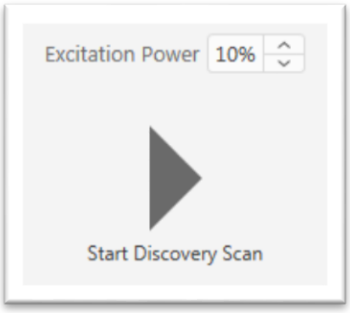
The keyboard shortcut ctrl + z will undo any action, while ctrl + y will redo.

Any graph displayed in the software can be exported by clicking the *Export* button. Options are to copy the graph to the clipboard, to save it as an image (.png or .svg file formats), or to export the raw data needed to recreate the graph in third-party software (.xlsx file format).

3. Discovery Scan



After creating or loading a session, perform a *Discovery Scan* to determine optimal settings for the unfolding experiment. The excitation power can be varied between 1 % and 100 %. Re-scan with different excitation power settings if necessary.



The discovery scan is used to detect the fluorescence intensity and position of each capillary along the entire length of the capillary tray. Each capillary will be visible as a combination of two peaks, which represent the fluorescence intensity at 330 nm and 350 nm, respectively (**Figure 1**).

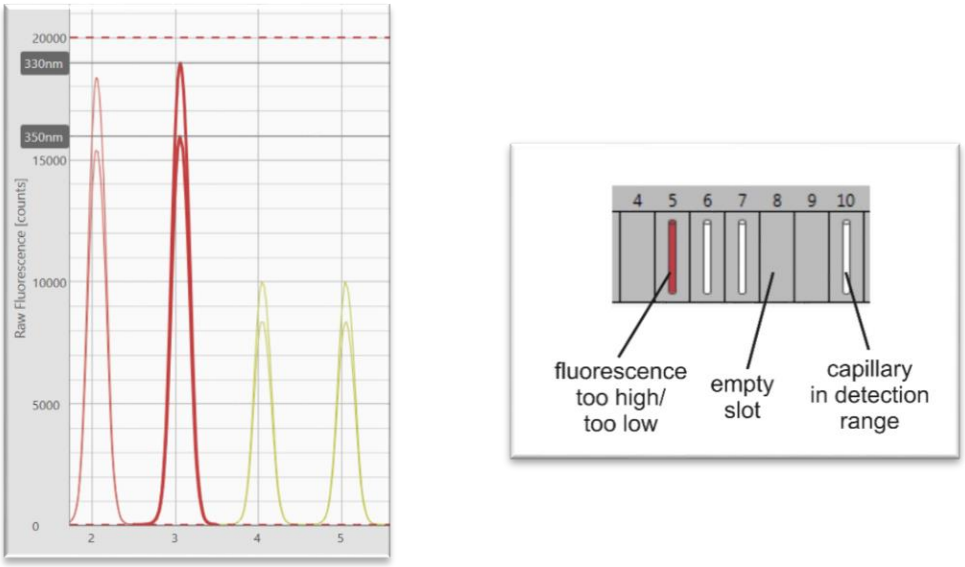


Figure 1: Capillary Scan. The capillary scan provides information about the fluorescence intensities of the samples, and is used to determine optimal measurement settings and the positions of occupied capillary slots. (Left) The fluorescence intensities of the 330 nm channel and 350 nm channel are displayed as two separate, nested peaks for each capillary. Clicking on a specific peak or capillary will highlight this capillary's fluorescence at 330 nm and 350 nm, and also display its values for Peak Fluorescence and Integrated Fluorescence on the right of the screen. Both wavelength peaks should be between the upper and lower detection limits (dotted red lines). (Right) Capillaries with fluorescence intensities within the dynamic detection range are colored white; capillaries with too high fluorescence intensities are colored red.

The upper and lower limits of detection are highlighted by dotted red lines in the discovery scan profile. The upper limit is 20,000 fluorescence counts. Please note that the lower detection limit is dynamically adjusted to the excitation power settings and thus may vary between experiments with different settings.

Zooming into the graph is possible using the scrolling function of the mouse wheel. Holding down shift or ctrl while scrolling will zoom horizontally or vertically, respectively. Holding the mouse wheel also allows to move the graph. To reset the view, click *Zoom Extent*.

Note: *The optimal detection range is between 2,000 and 18,000 counts. Some proteins might require limiting the maximal fluorescence counts to 15,000, since the unfolding might result in an atypical increase in fluorescence intensity.*

Note: *Photobleaching effects are negligible even at high excitation intensities due to the rapid on-the-fly measurement mode.*

Note: *If the capillary fluorescence exceeds the upper limit, the capillary position will be indicated in red and excluded from the measurement. Capillaries with fluorescence intensities below the measurement limit may not be recognized by the software but can be manually chosen to be included in the measurement in the* Measurement scan *submenu (see below).*

4. Measurement Scan

4.1. Measurement Modes

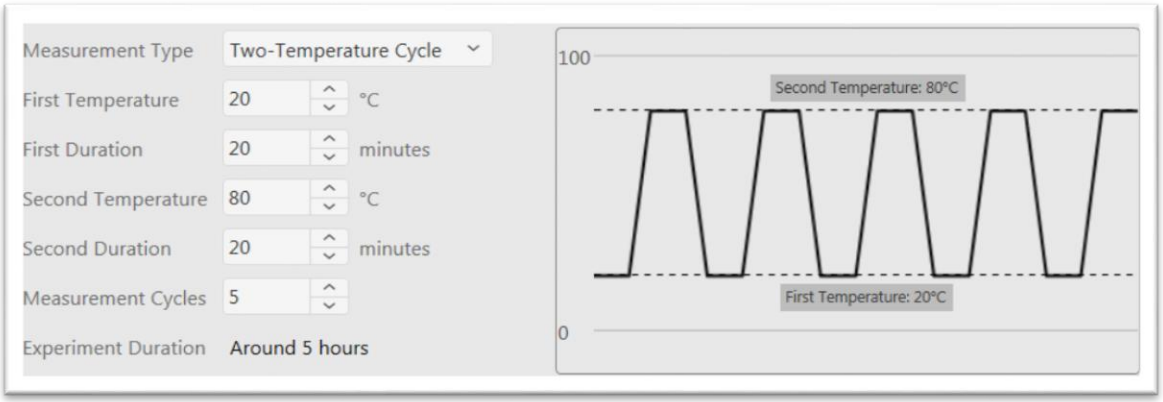
The PR.TimeControl Software provides four different measurement modes which enable a versatile analysis of protein stability properties. When defining temperature settings, a visual representation shows the experimental phases to graphically guide the user.

Note: *Sample evaporation is negligible for measurement times < 3h, temperature ramps ≤ 95 °C, and isothermal measurements < 80 °C and does not affect the results of the measurements. Longer measurements and higher temperatures require sealing of capillaries using the Capillary Sealing Kit (Cat# PR-P001 and PR-P002, NanoTemper Technologies).*

4.1.1. Isothermal

In *Isothermal* measurements, sample fluorescence and aggregation are monitored over time at a constant temperature. The temperature and the duration of the measurement can be adjusted. If the measurement temperature differs from the initial temperature of the Prometheus instrument, a fast heating/cooling at maximal speed (7 °C/min) is used to reach the measurement temperature.

4.1.2. Two-Temperature Cycle



In the *Two-Temperature Cycle* measurement mode, the sample is repeatedly subjected to two different temperatures with defined durations. This measurement mode can be used to evaluate thermal stressing of the sample, e.g. simulating process-related temperature changes. Furthermore, the reversibility of unfolding events can be evaluated regarding both extent and kinetics. The software allows for adjusting the following settings: the first and second temperature, the duration of the temperature cycles and the number of measurement cycles. Fast heating/cooling at maximum speed (7 °C/min) is applied to reach the target temperatures.

4.1.3. Incremental Cycle

In the *Incremental Cycle* measurement mode, the sample is also subjected to temperature cycles. In between cycles, the sample is always cooled back down to the first temperature, while the heating temperature is increased by a defined increment each cycle. This measurement mode is used to evaluate the reversibility and kinetics of unfolding at different temperatures, which is particularly important to determine critical temperatures at which unfolding becomes irreversible, or at which aggregation of the sample occurs. The software allows for setting the temperature, the temperature increase per cycle, as well as the duration and number of measurement cycles. Fast heating/cooling at maximum speed (7 °C/min) is applied to reach the target temperatures.

4.1.4. Temperature Stepping

In the *Temperature Stepping* mode, the sample is subjected to a stepwise increase in temperature. This measurement mode can be used to evaluate the kinetics of unfolding. The software allows for setting the start temperature, the temperature increase per step, as well as the duration and number of measurement steps. Fast heating at maximum speed (7 °C/min) is applied to reach temperature steps.

4.2. Data Acquisition

The PR.TimeControl software automatically identifies the capillaries for unfolding analysis from the discovery scan. By default, only capillaries that lie in the dynamic detection range are pre-selected for the unfolding experiment. However, you can also manually add capillaries that fall below the detection limits.

Note: *You can multi-select capillaries using shift or ctrl + left click.*

Start the thermal unfolding experiment by clicking "*Start Measurement*". After a pre-melting phase in which the start-temperature is reached and the system is equilibrated, continuous scanning of the selected capillaries proceeds while the previously defined temperature settings are applied. Data acquisition can be followed in real time. Select one, more, or all capillaries to view the corresponding data.

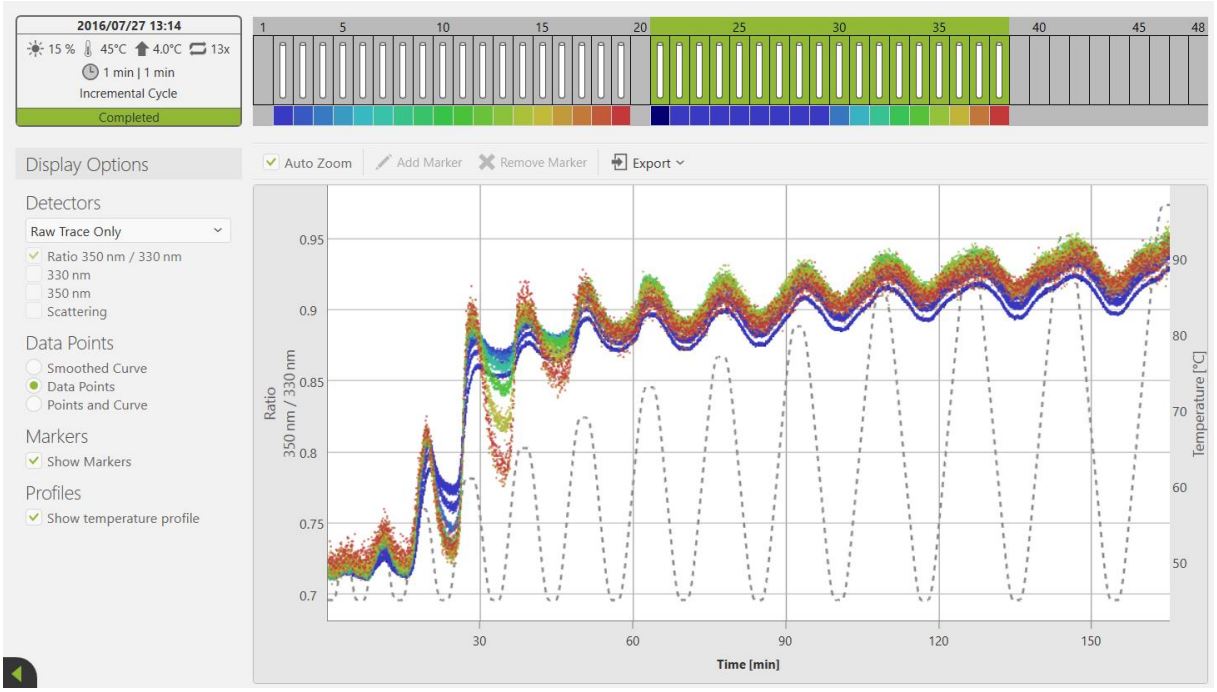
The data graph uses three axes: fluorescence on the left y-axis, time on the x-axis, and set temperatures on the right y-axis (if selected under 'Profiles'). The temperature profile of the measurement is represented by a dotted line in the graph.

Measured fluorescence values can be displayed as smoothed curve, individual data points, or both. For visualization purposes, the raw fluorescence values at 330 nm and 350 nm and the F350/F330 ratio can be displayed either together or separately. The first derivative can also be added to the view after the measurement is finished.

Colored buttons below the capillaries can be used to manually change the color of each curve. Markers (horizontal lines) can be added to individual samples to mark relevant events.

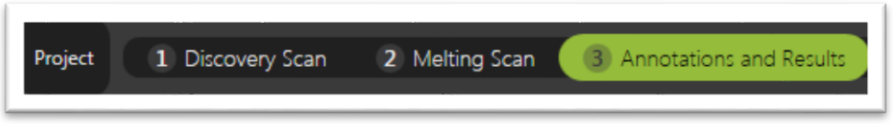
Red bars indicate that one or more samples are exceeding the detection limit.

Zoom into the graph using the scrolling function of the mouse wheel. When zooming, the fluorescence axis always auto-resizes, while the temperature axis remains constant. Check the ‘Auto Zoom’ checkbox to fit everything into view.



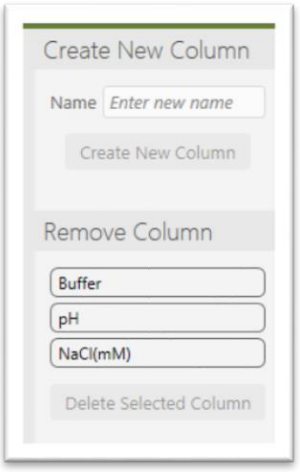
Raw fluorescence and processed data (raw fluorescence and first derivative values) as well as manually annotated markers can be exported in Excel™ format using the *Export* button. Images can be exported as .svg and .png files.

5. Annotations and Results



It is not required to enter sample annotations prior to the experiment. Annotations for each capillary can be entered at any time while the measurement scan is running or after it is finished. Annotations can either be entered by simple copy-and-paste from a spreadsheet software like Excel™ or manually. The annotations table by default includes separate columns for capillary position and sample ID. If Markers are added in the *Measurement Scan* view, they will be displayed as manually annotated points (M) in the annotations table.

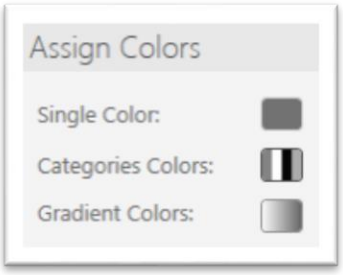
Columns can be added or removed by using the *Create New Column* and *Remove Column* buttons on the right side of the screen:



Annotations can be entered into multiple fields simultaneously after multi-selection, and subsequently sorted in ascending or descending manner by left-clicking on the column header.

Note: *Multi-selections of capillaries applied in the* Measurement Scan *or* Annotations and Results *tabs persist after switching tabs.*

Capillaries can be selected and a single color can be assigned by clicking on the rectangle next to "*Single Color"*. Furthermore, capillaries can be colored by categories or gradients after selecting the cells that are to be used for categorizing. The colors will directly translate into the melting curves in the *Measurement Scan* window. Color assignment will only apply to selected fields. Multi-selection by using shift / ctrl + left click is possible.



Annotation tables can be exported in Excel™ format (.xlsx).

6. Data Export

Each measurement and analysis performed can be exported to be used in third-party software. Throughout this software manual, different export options are mentioned. This section aims to give an overview regarding the file format and the content of each export.

6.1. Discovery Scan

The Discovery Scan tab contains three export options.

Clicking on *Export Raw Data* will create an Excel™ file (.xlsx) containing two sheets. The first sheet gives the major information for each peak, whereas the second one contains the information to reproduce the graph.

The *Copy Chart to Clipboard* button will copy the graph displayed so you can paste it into another software.

The *Export Graph* button will create an image file (.png or .svg) of the zoom extended view displayed by the software.

6.2. Melting Scan

The export button in this tab will display four options.

The *Copy Chart to Clipboard* button will copy the graph exactly how it is displayed in the software (for example only showing selected capillaries, or showing both raw data and first derivative) so you can paste it into another software.

The *Export Graph* button will create an image file (.png or .svg) of the graph as described above.

Clicking on *Export Raw Data* will create an Excel™ file containing four sheets. The first sheet gives an overview of the measurement. The other sheets contain the fluorescence at 330 nm, 350 nm and the ratio as a function of temperature for all capillaries.

Using *Export Processed Data* will create the exact same Excel™ file but including also the data of the first derivative for each fluorescence channel and their ratio as separate sheets.

6.3. Annotations and Results

The export option is located at the bottom right of the screen and it will create an Excel™ file containing the table displayed in this tab.

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