# Software Manual
# MO.Affinity Analysis 3

# Contents

The MO.Affinity Analysis 3 software allows straightforward analysis and evaluation of MicroScale Thermophoresis data. It allows quantification of binding parameters such as dissociation constants ($K_d$) or $EC_{50}$ values and easy comparison of results e.g. for one target protein binding to different compounds.

The MO.Affinity Analysis 3 software guides the user through all important steps from data selection to evaluation by using a clearly organized submenu layout in the task bar. Creating an analysis file will retain the chosen settings for data analysis. Additionally, the software allows inspection and exporting of both raw and processed data at any step during data analysis.

This manual explains the main functions integrated in the MO.Affinity Analysis 3 software.

# 1. System Requirements

If the necessary licenses have been purchased, MO.Affinity Analysis 3 software can be installed on computers meeting the following requirements:
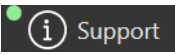
| | |
|---|---|
| Operating system: | Windows 7/10 (x86 or x64) |
| CPU: | Intel Core i5, 6th generation or higher |
| RAM: | 8 GB or higher |
| Hard disk: | 20 GB available space, SSD recommended |
| Display resolution: | Full HD @ 125 % |
| Software: | Microsoft .NET Framework 4.5.1or higher (included in installer of MO.Affinity Analysis 3 software) |
| Operating system language: | English or German |

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

# 2. Installation, License Activation, and Software Updates

The software can be installed on any computer and will automatically start in trial mode, when opened for the first time. The trial version offers full functionality and has to be activated with a license key purchased from NanoTemper Technologies after 30 days.

To activate the license, navigate to the main menu and select Licensing Status. Please follow the instructions provided. One software license is valid for one account per computer and licenses can be transferred between computers. To transfer a license, please deactivate it first via the Licensing Status dialogue and reactivate it on the new computer.

When a software update is available, a green button will appear next to  button at the top right corner of the software window. This feature is accessible only if your device is connected to a network.

# 3. Term Definitions

**Target:** The fluorescent molecule. The concentration of the target molecule is constant throughout a dilution series.

**Ligand:** Non-fluorescent binding partner. The ligand concentration is varied by serial dilution.

**MST Trace**: MST fluorescence signal over time. A typical MST trace contains an initial detection of the sample fluorescence (by default recorded for 3-5 seconds), followed by activation of the MST power to induce the temperature gradient and subsequent detection of thermophoretic changes in fluorescence (by default recorded for 20-30 seconds). Finally, MST power is deactivated and fluorescent recovery is monitored (recorded for a short period only).
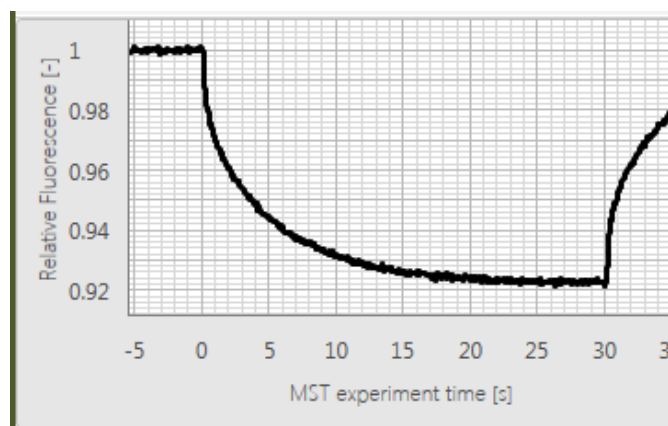


**MST Run**: A run includes a series of MST traces, typically of a fluorescent target molecule versus a serial dilution of a ligand.
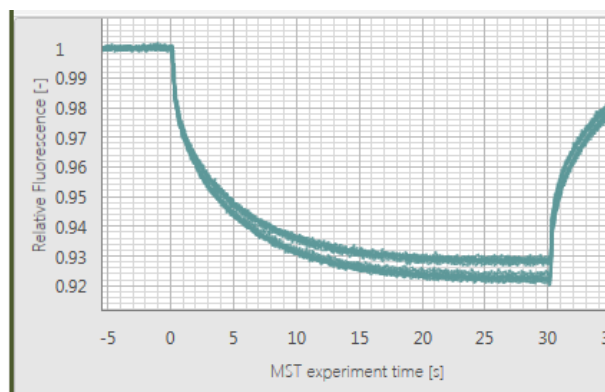


**Merge Set**: A series of replicates of MST runs, with identical MST power, LED/excitation power as well as target concentrations. Data within one *Merge Set* will be averaged and error bars will be calculated and displayed. Note that the ligand concentrations do not necessarily have to be identical and can vary between merged MST runs.
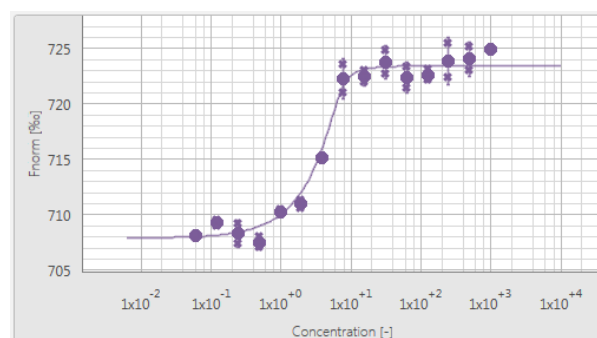


**Analysis Set**: A complete dataset consisting of a number of *Merge Sets* or single MST runs, for parallel analysis and direct comparison. Dose response curves of runs and *Merge Sets* in *Analysis Sets* can be compared in the same charts with the MO.Affinity Analysis 3 software. All runs contained in one Analysis Set are analyzed with the same evaluation parameters.

**Analysis (file)**: A single *Analysis Set*, or a collection of *Analysis Sets*. The analysis can be saved at any time. An analysis file can be used to integrate a larger number of MST experiments for a comprehensive and systematic data analysis.

**Raw Data**: All fluorescence data recorded by the Monolith instrument: MST traces, capillary scans and shapes, initial fluorescence values and bleaching rates. All raw data can be viewed in the Raw Data Inspection tool (see Figure 1 and section 4, point 6).
Please note that the capillary scan displayed is the scan recorded *before* the MST measurement, not afterwards (applicable to MST measurements performed with MO.Control 2 software).
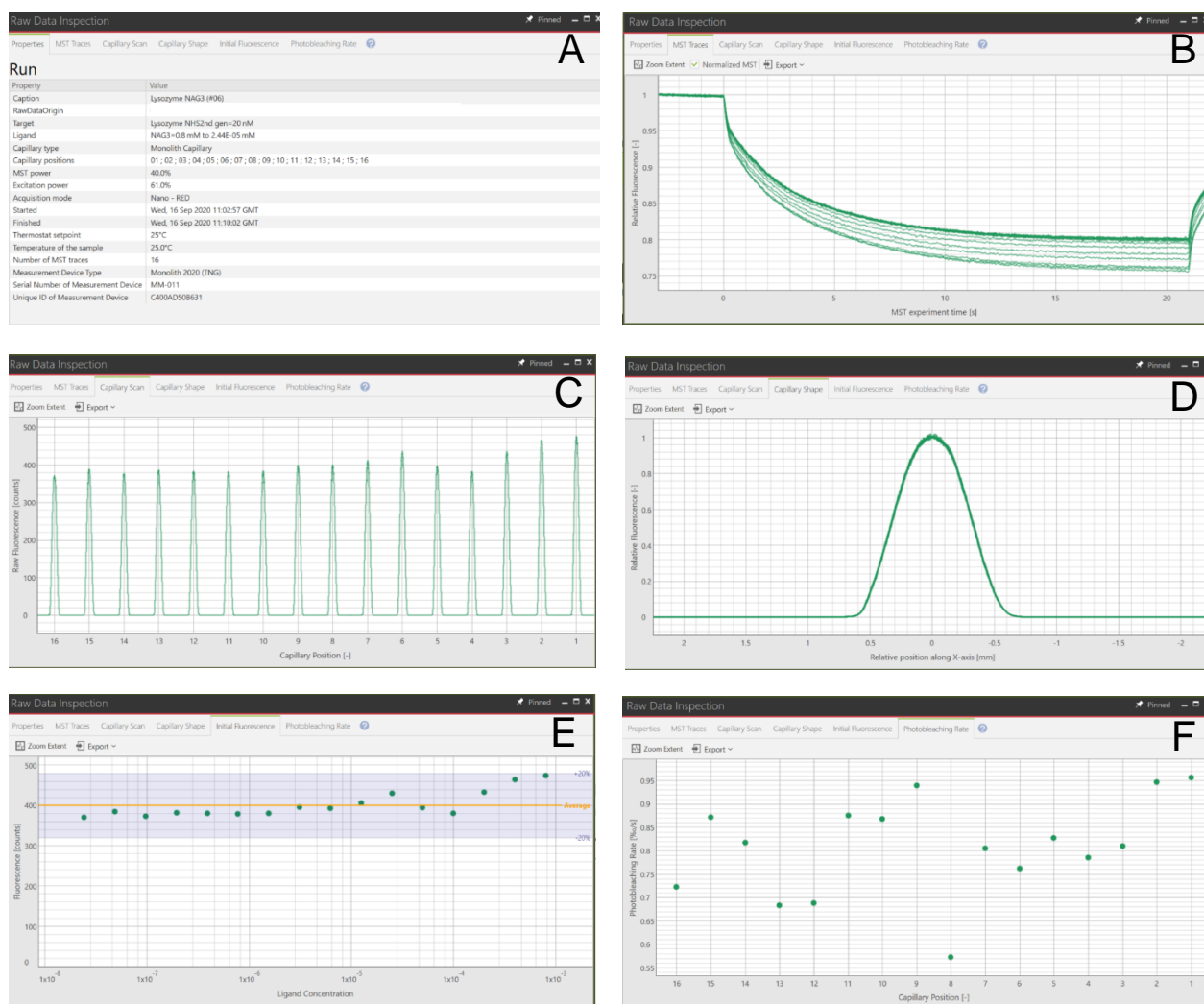


**Figure 1: MST Raw Data Inspection**: (A) Properties, (B) MST Traces, (C) Capillary Scan, (D) Capillary Shape, (E) Initial Fluorescence, (F) Bleaching Rate. Please note that the capillary scan displayed is the scan recorded *before* the MST measurement, not afterwards (applicable to MST measurements performed with MO.Control 2 software).

# 4. General Layout

All major functions of the MO.Affinity Analysis 3 software are organized in the task bar:
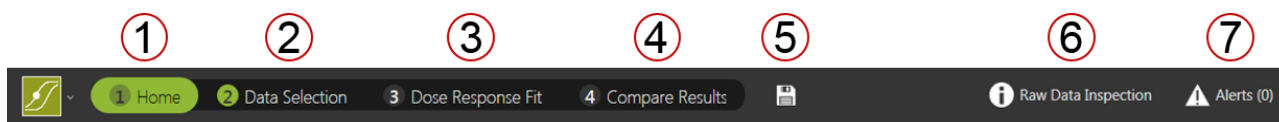


**Four tabs** guide the user through the process of MST data analysis:

1. **Home**
2. **Data Selection**
3. **Dose Response Fit**
4. **Compare Results**

It is recommended to complete all four steps in this order to ensure proper documentation and analysis of MST experiments. Movement between tabs during the analysis process is possible, e.g. to add additional files, edit names of Analysis sets, etc.

Additional buttons in the task bar are:

5. **Quick saving** of the analysis file.

6. **Raw Data Inspection** is available at any time during the analysis process by selecting the *Raw Data Inspection* button on the top right of the window. This will open a separate window which displays all experiment-associated settings and meta-data, as well as detailed charts of raw MST traces, capillary scans, overlays of capillary shapes, initial fluorescence values and bleaching rates. Selected runs and traces will be highlighted in both, the MO.Affinity Analysis 3 main window as well as in the *Raw Data Inspection* window. For detailed views of *Raw Data Inspection* options, see Figure 1.

7. **Alerts** will be displayed on the top right of the main window. Alerts include experimental inconsistencies as well as warnings about potential inconsistencies during data processing and fitting.

**Context-related supporting information**, such as term definitions and equations, can be found when clicking the ⓘ buttons located on each page.

Anything you do in the software can be undone by pressing Ctrl + Z.

# 5. Saving and Exporting Data

The MO.Affinity Analysis 3 software allows for saving the current analysis at any time, using the drop-down menu in the top left (click ), the quick save button in the task bar  or navigation back to the *Home* tab.

Moreover, chart and tabular data can be exported where indicated using the export buttons (click  Export ), which are located in the *Dose Response Fit* and *Compare Results* submenus as well as in the *Raw Data Inspection* section. Available image formats for export are .svg, .pdf and .png. Note

that .svg and .pdf contain vector graphics which can be processed by graphic editing software. Tabular data are saved in .xlsx or .csv format. Results can also be saved as a condensed report in .pdf format on the *Dose Response Fit* and *Compare Results* screens.

# 6. Analysis Setup in the Home Screen

In the *Home* screen, create a new MST analysis or load a preexisting analysis file. Analysis files are saved in the .nta3 format. Changes in an analysis can be saved at any time.

When creating a new analysis, enter an analysis name and optionally add comments, e.g. purpose of the analysis, assay conditions etc. Recently opened analysis files are listed chronologically. Start adding raw data to your analysis here, or in the next tab *Data Selection*.

# 7. Data Selection

See Figure 2 for a summary of the options you have for *Data Selection*.

To add MST runs to the analysis, use the drag-and-drop function for .moc2 files, or use the load function to browse folders and select single or multiple files. MST runs of the selected file will appear in the *Data Selection* window as thumbnails of normalized *MST Traces* with a description of name, experiment settings and date. By changing the *View* option in the top panel, you can alter the presentation of the MST data thumbnails to *Dose Response*, *Capillary Shape* or *Initial Fluorescence*.

Before data analysis is performed, choose the type of analysis. To analyze binding by MST, click on the *MST* button in the *Choose Analysis Type* panel. In cases of ligand-induced fluorescence changes where the fluorescence values of each capillary are used to determine binding constants, click the *Initial Fluorescence* button.

**Note:** *Use the* Initial Fluorescence *analysis if there is a ligand-concentration dependent change in sample fluorescence >±20 %. Refer to the User Starting Guide or the MO.Control 2 software for more information.*

**Note:** *The data points presented in the "Dose Response" thumbnail view correspond to the $\Delta F_{norm}$ values determined after the MST power dependent time intervals (see section 8).*
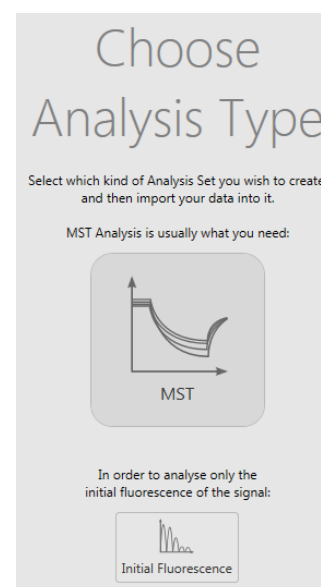
For further analysis and determination of binding parameters, MST runs are combined into *Analysis Sets*. Clicking the "*Auto-Append*" button will create a single *Analysis Set* which contains all loaded MST runs as independent single runs. Alternatively, MST runs can be added by clicking the ➕ symbol on the bottom right of each MST run thumbnail. This automatically creates a new *Merge Set*.

To add replicate runs to a *Merge Set*, drag-and-drop them there directly. Merged runs will be displayed with average values and error bars in the *Dose Response Fit* screen. The software allows the merging of runs if the runs were collected using the same

- LED/excitation power
- MST power
- Capillary type
- Acquisition mode (fluorescence channel) and optics module



**Figure 2:** Create *Analysis* and *Merge Sets* from MST runs in the *Data Selection* menu.

Depending on the software used to perform the measurement, not all of these criteria may be saved in the file. As a result, it may not be possible to merge data collected with different software. When

the user tries to add an incompatible run to a *Merge Set*, the software will reject the run and display an incompatibility message. To create a custom number of different *Merge* and *Analysis Sets*, drag-and-drop the MST run thumbnails.

**Hint:** Analysis Sets and Merge Sets can also be rearranged by simple dragging and dropping.

Please note that names of *Merge* and *Analysis Set* can be edited for better description and documentation by clicking the pen symbol on the respective flyout. The flyout appears upon mouse-over in the respective *Analysis Set* field (see screenshot below). Also, after an *Analysis Set* was created the analysis mode can still be switched between MST- and initial fluorescence analysis by selecting the respective button. Another button allows to switch to the expert mode for analysis (see next section for more details). Once *Analysis Sets* are created, binding data can be quantified.
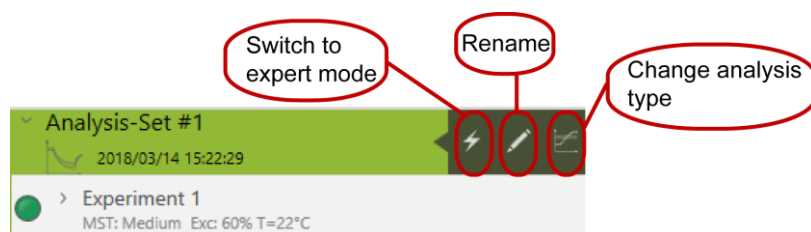


# 8. Dose Response Fit

The *Dose Response* fit window allows for fitting MST data to obtain either dissociation constants ($K_d$s, using the law of mass action) or $EC_{50}$ values (using the Hill equation). In the window, normalized MST traces as well as corresponding dose response plots of the selected MST data are shown.

Figure 3 summarizes the data analysis and fitting workflow.

By selecting either an *Analysis Set* or a *Merge Set* on the left, the respective MST traces and their dose response plots are displayed. By default, $F_{norm}$-values in the dose response plot are calculated from the ratio of normalized fluorescence $F_0/F_1$, where $F_0$ corresponds to the normalized fluorescence prior to MST activation. $F_1$ is by default determined after an optimal MST power-dependent time interval which yields the best signal-to-noise ratio.

Use the mouse or the arrow keys to navigate through the analysis tree in the left panel. The right arrow key expands an *Analysis Set* or *Merge Set*, while the left arrow key collapses it.
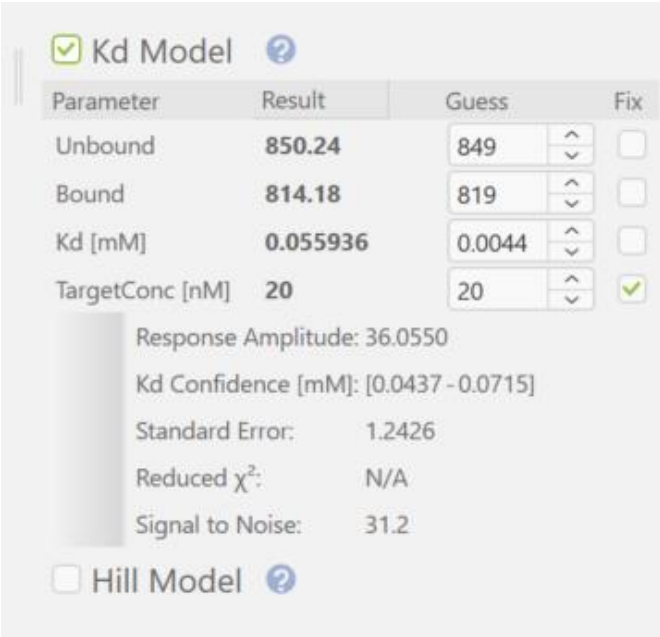
Data fitting is performed instantly after selecting the respective fit routine ($K_d$ Model or Hill Model). Fitting requires initial values, which are determined automatically by the software (shown as *Guess* values in the fit model). Known parameters, such as target concentration, need to be fixed by checking the *Fix* checkbox. In some cases, it may be required to guide the fitting algorithm by manually entering initial *Guess* values.

***Note:*** *The Hill fit should only be used if the interaction involves a cooperative binding mode. A 1:1 interaction should always be fitted using the $K_d$ Model.*

After a fit is performed, a range of statistical parameters is automatically calculated and displayed. For definitions, fit equations and more information, click the 🔵❓ button.

Replicates within one *Merge Set* are displayed as average values and error bars representing the standard deviation. Fits are applied to the average values. To get an error estimation on the resulting $K_d$, fit the replicates individually and use this data to perform statistics.

For in-depth data evaluation and fit refinement, single runs and MST traces can be highlighted either by selection on the left, or by clicking on the respective MST trace or data point in the graphs. After highlighting, outliers can be excluded from the fit (either greyed out 🔲 or invisible 🔲).

| Kd Model ❓ | | | |
|---|---|---|---|
| Parameter | Result | Guess | Fix |
| Unbound | **850.24** | 849 ⌃⌄ | ☐ |
| Bound | **814.18** | 819 ⌃⌄ | ☐ |
| Kd [mM] | **0.055936** | 0.0044 ⌃⌄ | ☐ |
| TargetConc [nM] | **20** | 20 ⌃⌄ | ☑ |

Response Amplitude: 36.0550
Kd Confidence [mM]: [0.0437 – 0.0715]
Standard Error:     1.2426
Reduced χ²:     N/A
Signal to Noise:     31.2

☐ Hill Model ❓

**Analysis Set selected**
**(Overview +**
**set global parameters)**

**Merge Set selected**
**(Obtain binding affinities)**

**Single Run selected**
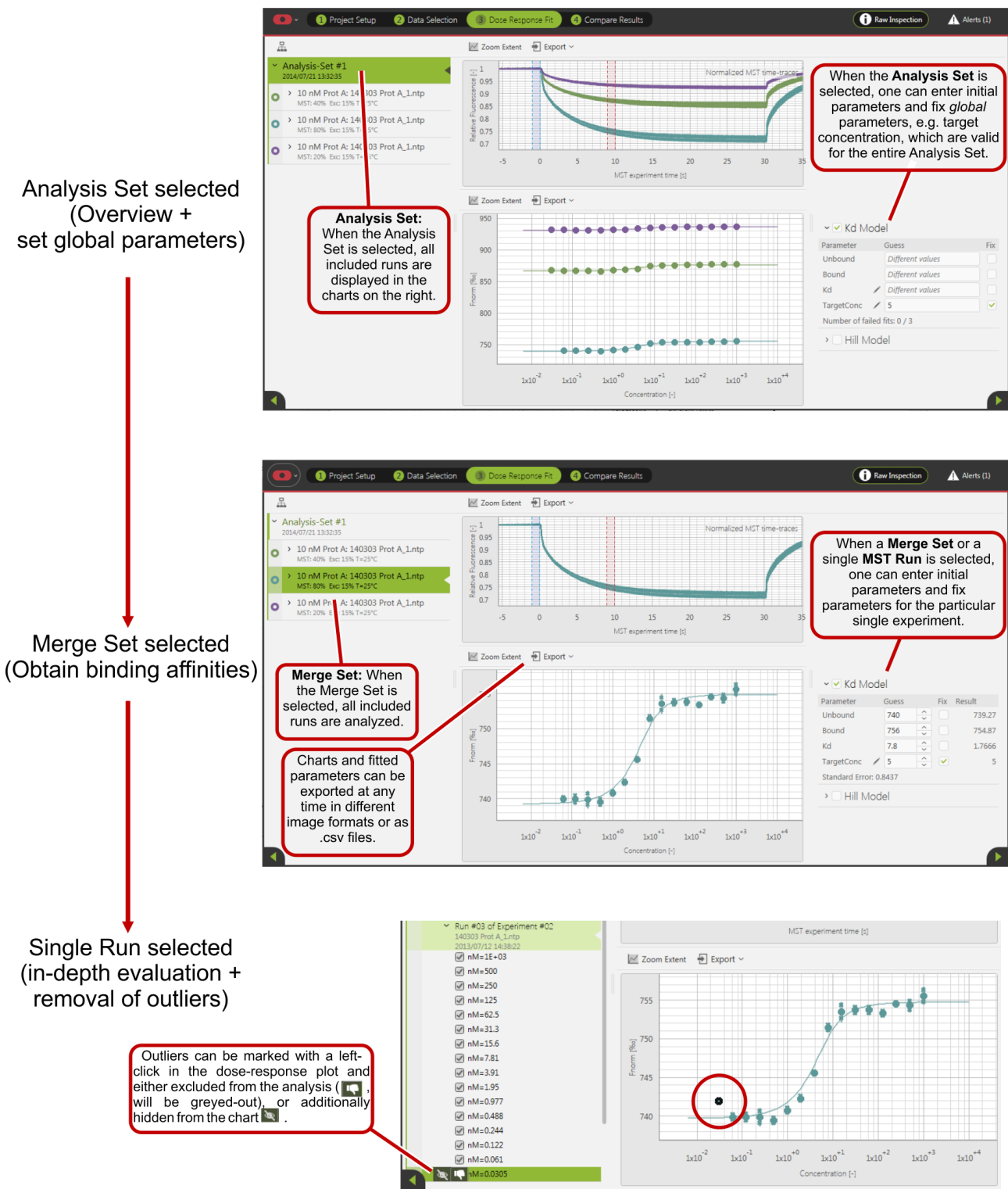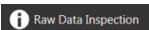**(in-depth evaluation +**
**removal of outliers)**

**Figure 3:** Analysis of binding constants and $EC_{50}$-values.

For a more in-depth evaluation of MST data, activate the *Raw Data Inspection* [🛈 Raw Data Inspection]. Here, effects such as sample aggregation, adsorption to capillary walls or fluorescence intensity variations in the titration series can be easily identified. Please see the MO.Control 2 software for more information on sample quality and assay optimization.

When preparing *Merge Sets* for presentation that contain non-binding negative control interactions, move the mouse cursor over the name of the *Merge Set*. A flyout will appear with a chain link symbol. Clicking this symbol initializes all enabled fit parameters as nonbinder, which means a horizontal line will be drawn through the points at their average value. This allows the comparison of nonbinders with binders in the *Compare Results* view. To revoke non-binder status, navigate to the data fitting section of the respective run and untick all unneeded checkboxes.
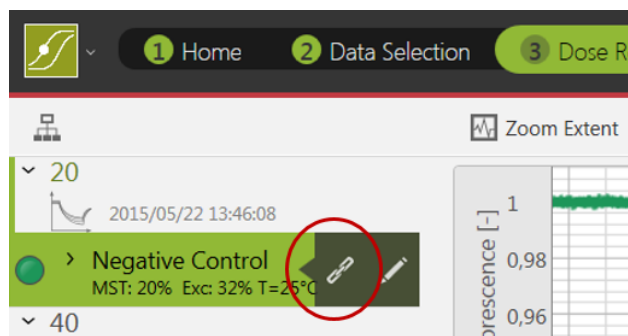


As an alternative to using the default analysis settings, the positions of $F_1$ and $F_0$ can be manually adjusted after enabling the *Expert Mode* for the *Analysis Set* (see Figure 4). Using this mode, the $F_1$ and $F_0$ cursors can be placed anywhere along the MST time traces. The *Expert Mode* should only be used if the default analysis procedure did not yield satisfying results.
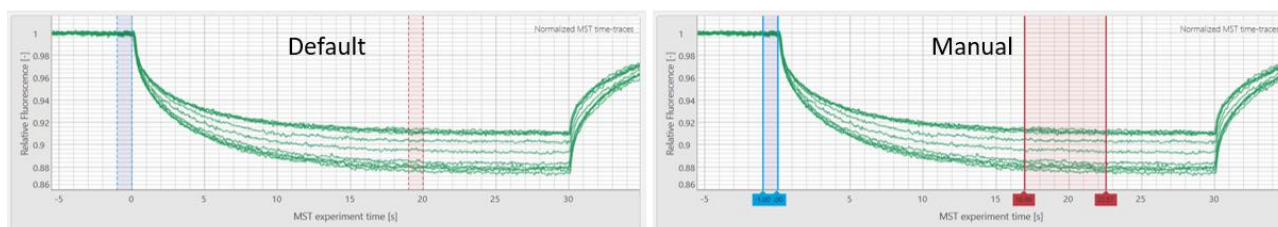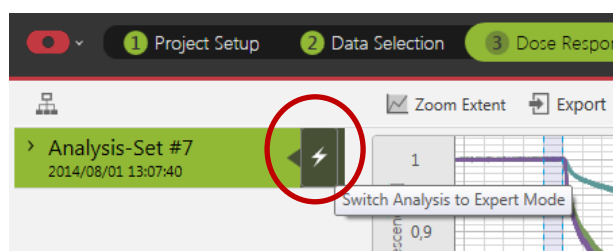




**Figure 4:** Activation of the Expert Mode and visualization of different cursor settings.

Similarly, when working with an *Initial Fluorescence Analysis Set*, the Expert Mode can be enabled to analyze ligand-dependent photobleaching effects (bleaching rate). Please contact your NanoTemper Technologies Support for more information.

**Chart visuals:** Chart colors can be changed in the *Data Selection*, *Dose Response Fit* and *Compare Results* sections. All charts in the *Dose Response Fit* and *Compare Results* sections can be zoomed and adjusted for optimal visualization. Use the *Zoom Extent* button to adjust all data in the chart to the chart size. Zooming in-and-out of the chart is performed by scrolling the mouse wheel. Horizontal or vertical zooming can be performed by pressing shift or control on the keyboard while scrolling, respectively. Click and hold the mouse wheel and move the mouse to drag the chart (see Figure 5).
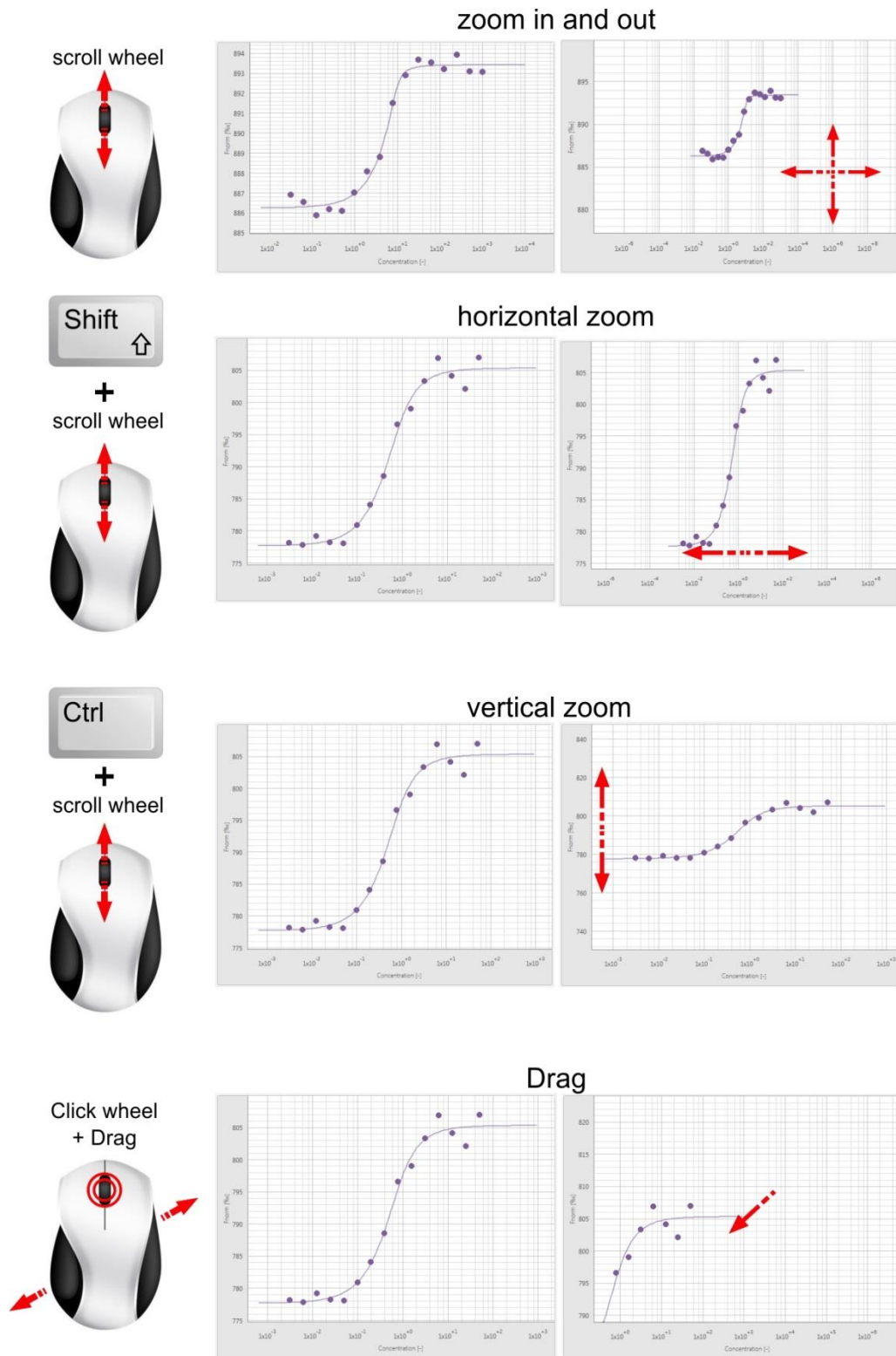
**Figure 5:** Mouse control of chart visualization.

# 9. Compare Results

The *Compare Results* tab allows for a side-by-side comparison of MST runs and *Merge Sets* within an *Analysis Set*. In this tab, data and fitting results can also be exported in tabular and graphic format, including all binding data and the algorithms used. By selecting an *Analysis Set* on the left, all included data are plotted in the same chart. Selection of a *Merge Set* or a single MST run will highlight the selected experiments and grey-out the remaining experiments in the *Analysis Set*.

Dropdown menus to change the *Fit Model* (*$K_d$* or *Hill*) and display type *($F_{norm}$, $\Delta F_{norm}$, Fraction Bound)* are located on the top of the dose response chart. While the *Fraction Bound* normalization is best suited for a direct comparison of binding affinities, the *$\Delta F_{norm}$* normalization provides additional information about amplitude size and direction (please contact your NanoTemper Technologies Customer Support for more information). Both charts and chart raw data can be exported as an image file (.svg, .png or .pdf) or text file (.csv or .xlsx) for further use and external analysis.

In addition to the visualization options, the *Compare Results* menu also includes a table summarizing number of averaged experiments (n), fit parameters, affinities and fit quality.

Finally, the *Generate Full Report* button summarizes all charts and tables into one single PDF. Click the *Generate Full Report* button in the *Dose Response Fit* view to obtain a report even with unfitted data.
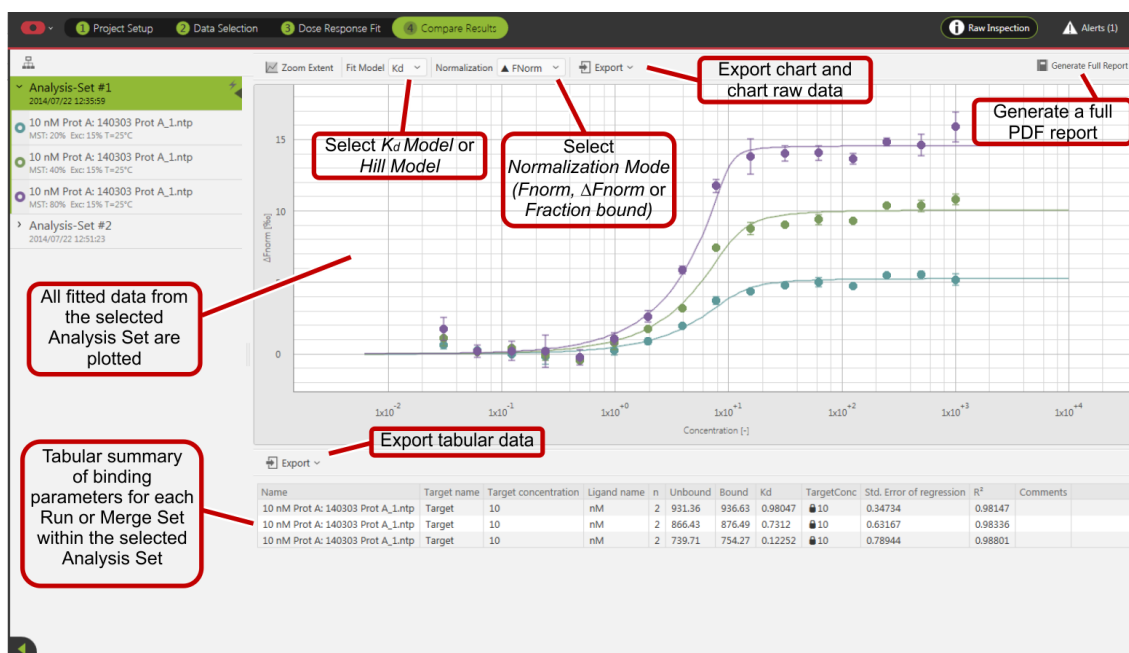


# 10. Patents and intellectual property

Monolith, MST and TRIC technology are patent protected, especially by the following patents, US8431903B2, US8853650B2, US10345312B2, US8741570B2 including their application and registration in different other countries.

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