User Starting Guide for the Monolith NT.115

Content

1. Experiment Design

The Monolith systems measure equilibrium binding constants between a variety of molecules, with almost no restriction to molecular mass.
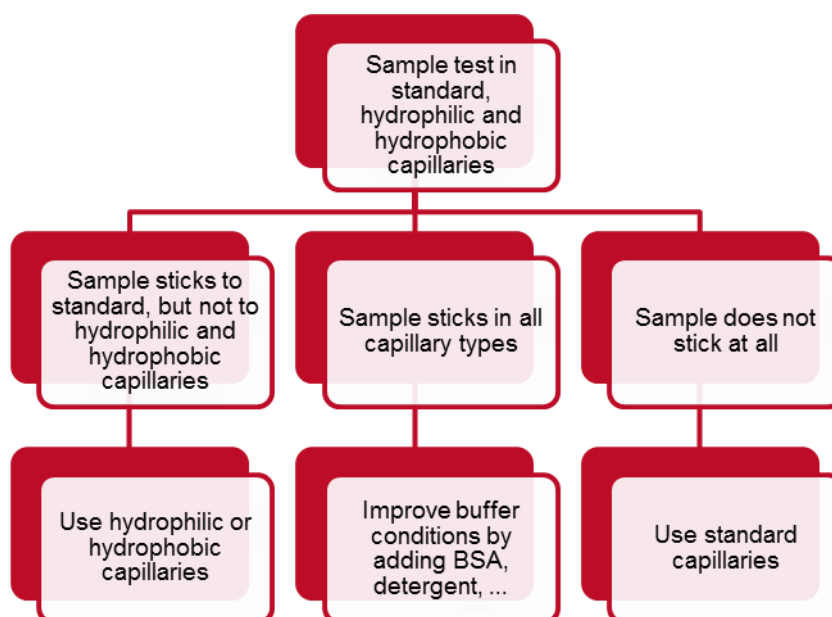
Although the system is easy to handle, you should follow this guide when using the instrument for the first time. This guide is designed to help you to get reliable results as quickly as possible. More detailed information is available in the Monolith NT.115 User Manual.
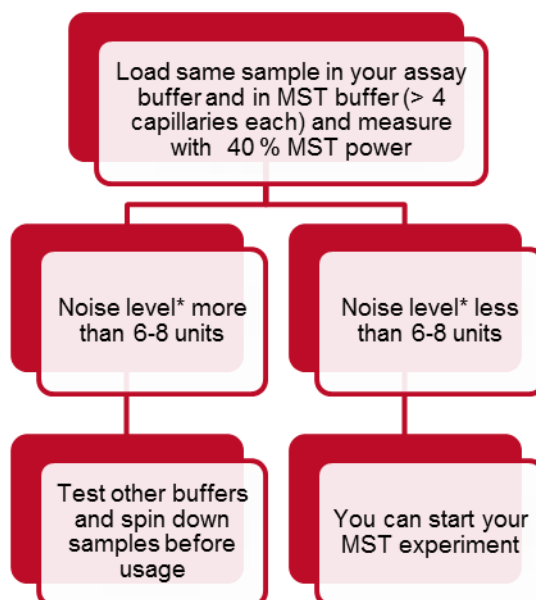
Flow Chart Assay Setup

Fluorescence Check
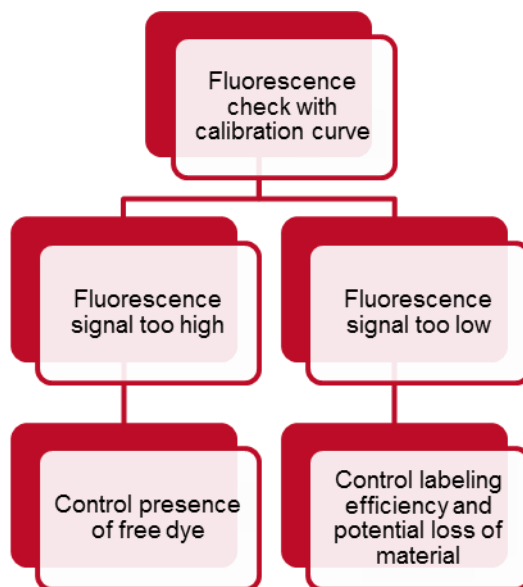
Capillary Type Check

Buffer Composition / Sample Quality Check

Noise level is defined in chapter 3.3

2. Before You Start
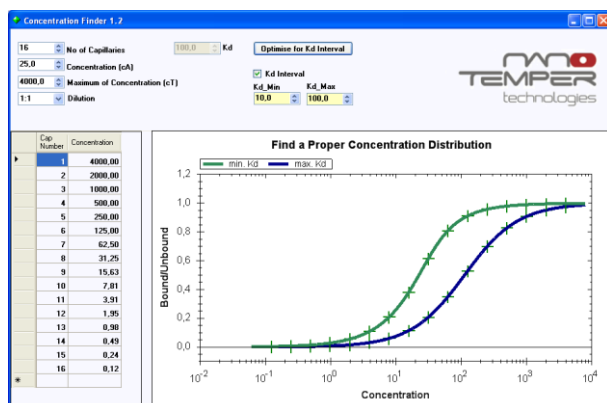
2.1 Design of Experiment

Figure 1: Use the "Concentration Finder" tool in order to design your MST experiments.

Check before you start the experiment if the concentration of the unlabeled molecule is high enough to reach a final concentration **at least an order of magnitude, ideally more above the expected dissociation constant (K_d)**. For details refer to the "Concentration Finder" tool in the NT Analysis Software. You can use this tool in order to simulate the binding event. It will help you to choose an optimal concentration range of the unlabeled molecule.

Microscale Thermophoresis (MST) is a method that uses very small quantities and volumes of material. Only 4 µl of your sample are needed to fill a capillary. However, to ensure optimal results, please follow the following rules:

- Never prepare less than 20 µl of sample. Otherwise you increase the probability to encounter problems due to evaporation, sticking of sample material to the plastic micro reaction tubes and higher pipetting errors.
- Never prepare small volumes (e.g. 20 µl) in large micro reaction tubes (e.g. 500 µl or more). The high surface to volume ratio leads even for well-behaved proteins to a surface adsorption. Always use the smallest micro reaction tubes possible (e.g. PCR tubes) or low volume cone-shaped micro well plates. You can also obtain MST tested micro reaction tubes from NanoTemper Technologies.
- Always spin down the stocks your are using (labeled and unlabeled molecules for 5 min with 13000 rpm). This will remove big aggregates, which is one of the main sources for noise.

Note: *If your protein sticks to surfaces, you may use detergent, additives like BSA or low binding reaction tubes to*
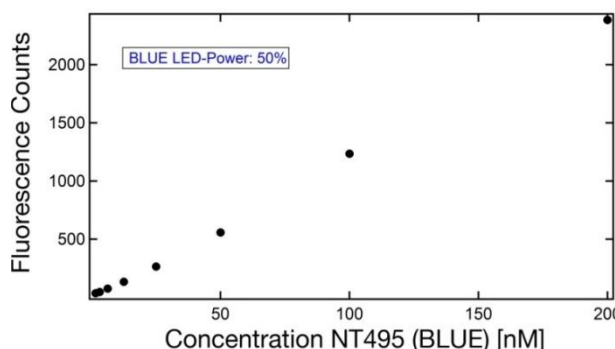
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2.2 Quality of your labeling procedure

Figure 2: Example of a calibration curve using NT495 dye with 50 % LED power.

Make sure that there is no unreacted, free dye in the preparation of your labeled molecule. If you are not sure about the quality of the preparations of the labeled molecule, you can use NanoTemper Labeling Kits (www.nanotemper.de). Free dye molecules will strongly reduce the signal to noise ratio.

Use only highly pure protein samples for labeling. If you intend to label a protein, the protein preparation has to be as pure as possible. For the same reason avoid carrier proteins as BSA in the protein stock. Other proteins that get labeled as well will reduce your signal to noise ratio as the free dye does.

Always test the quality of your labeling procedure before you start.

1. **Prepare a dye calibration curve:** It is best to prepare your own calibration curve for NanoTemper and other dyes on your instrument (e.g. 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.12 nM 1.56 nM). Use your interaction buffer to prepare the serial dilution of the dye, fill these samples in standard capillaries and start a "Capillary Scan" with 50 % LED power. Then check the fluorescence intensity in each capillary.
2. **Determine the concentration of your labeled molecule.** Prepare a 100 µl dilution of 50 nM of the labeled molecule in your interaction buffer and fill it into a single capillary. Then start a "Capillary Scan" at the capillary position with 50 % LED power. Use the fluorescence value from the dye calibration curve (step 1.) in order to estimate the concentration of your labeled molecule.

IMPORTANT: If the fluorescence intensities do not match by a factor of 2-3 then either labeling efficiency is low or protein/sample concentration is not in the expected range. It is not necessary to have a labeling ratio of 1:1 (typically 0.5 to 1.1), but a very low labeling efficiency might also indicate a problem with protein activity. Too much fluorescence might indicate over-labeling or presence of free dye.

Note: You can test the degree of labeling by measuring the absorbance of the dye and of your protein using a photometer e.g. measure absorption at 280 nm for protein and at the wavelength of the dye used e.g. 650 nm.

3. Assay Setup Pretests

Before you start you have to be sure that you are using the optimal concentration of the labeled molecule, the correct capillary type and a buffer composition in which your sample is homogeneous.

3.1) Fluorescence Check

Figure 3.1: Fluorescence signal too low. Increase the LED power or concentration of labeled molecule.
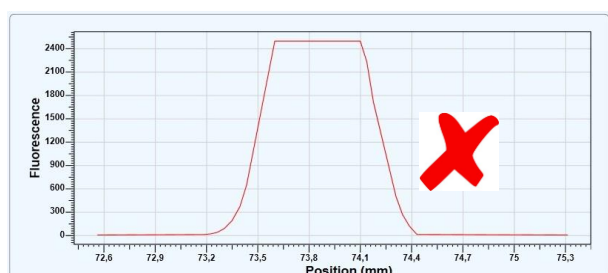

Figure 3.2: Fluorescence signal too high. Decrease the LED power or concentration of labeled molecule.
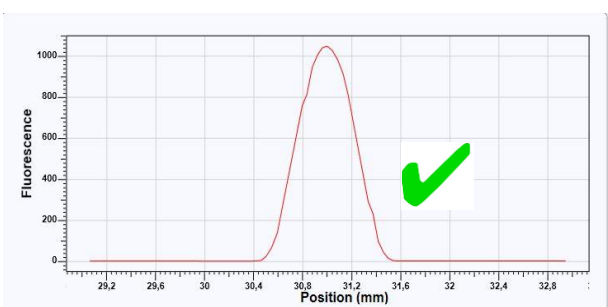

Figure 3.3: Fluorescence intensity is optimal between 200 and 1500 counts.

What concentration of the fluorescently labeled molecule should I use?

1. Choose the concentration of your labeled sample according to the following criteria: It should be lower or in the order of the expected K_d. In a typical experiment 5-100 nM of the fluorescently labeled molecule are used. Do not work with less than 200 fluorescence counts.

Note: It is important to measure with fluorescence intensities that are well above the background of the signal you get from a buffer filled capillary (i.e. without dye).

2. Fill the sample in a standard capillary and start the "Capillary Scan" with 50 % LED power.
3. Compare the intensity to the dye calibration curve you prepared previously.

Note: As described before the fluorescence intensities should match the expectations from the calibration curve by a factor of 2-3. If this is not the case then either labeling efficiency is low or protein/sample concentration is not in the expected range. It is not necessary to have a labeling ratio of 1:1 (typically 0.5 to 1.1), but a very low labeling efficiency might also indicate a problem with protein activity. Too much fluorescence might indicate over-labeling or presence of free dye.

4. Never work with less than 200 fluorescence counts. Never perform MST experiments if the fluorescence intensity is higher than 1500 counts. To achieve this, the sample concentration can be adjusted accordingly, or the LED power should be varied between 15 % and 95 %.

Note: For high affinity interactions (K_d < 10 nM) the concentration of the molecule should be on the order of the K_d or below. If the K_d is lower as the detection limit of the dye you are using, use the lowest possible concentration of the labeled molecule, in which you get 200 fluorescence counts with 95 % LED power. Once your assay is established and you are familiar with the instrument you can also test the system with 100-200 fluorescence counts.

Note: In case you have a low labeling efficiency or your molecule sticks to your plastic micro reaction tubes, the fluorescent counts might be much lower than expected. For a labeling efficiency of 1:1 10 nM of label will give you a sufficient signal for almost any dye. If your fluorescence is much lower than expected, prepare a new dilution, where you add 0.05 % Tween-20 to the buffer. If detergent increases your fluorescence counts, you lost material in the plastic micro reaction tube.

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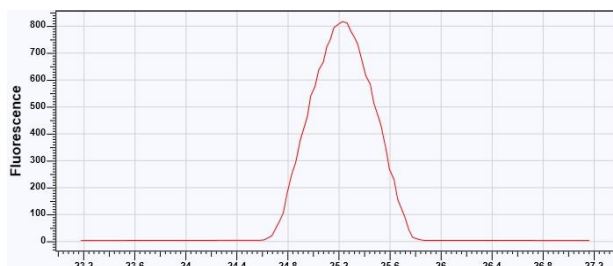## 3.2 Capillary Check: Which MST Capillary Type should I use?

Figure 4.1: No sticking. Symmetrical fluorescence peak. You can use this capillary type for experiments.
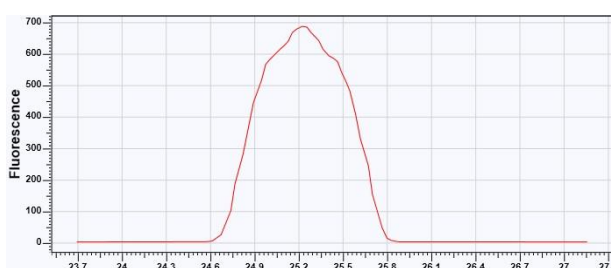

Figure 4.2: Slight sticking. Shoulders in fluorescence peak. Please note that it might take 5 minutes to observe an obvious sticking effect. If you are not sure, repeat the scan after 5 upt to 15 minutes or after the MST measurement.
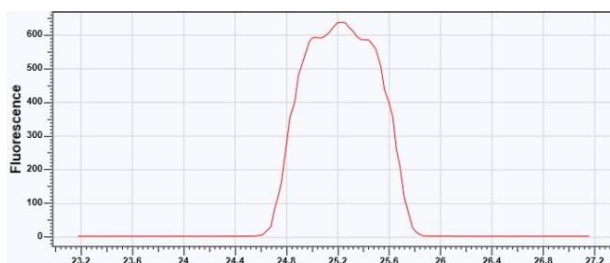

Figure 4.3: Strong sticking. Shoulders in fluorescence peak increase. You MUST test another capillary types or improve the buffer composition (see step 3.3).
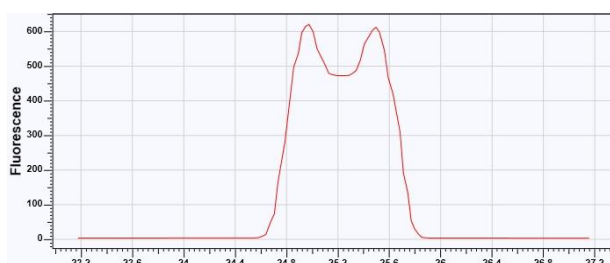

Figure 4.4: Very strong sticking. A clear double fluorescence peak appears. You MUST test another capillary format or improve the buffer composition. You can learn more how to find the best buffer in step 3.3.

IMPORTANT: Some molecules will stick to the surface of capillaries. The resulting MST signal has a poor quality. NanoTemper offers different types of covalently polymer-coated capillaries to avoid any unspecific sticking to the glass surfaces. These are called hydrophilic or hydrophobic capillaries. NanoTemper also offers a Capillary Selection Kit, which contains all important capillary types. For more information visit our homepage (www.nanotemper.de).

To test the best capillary type, please follow the following steps:

1. Prepare 120 µl of the labeled molecule at the concentration you want to use in the assay (determined in step 2 and 3).
2. Fill four standard treated capillaries, four hydrophilic capillaries and four hydrophobic capillaries with the sample from step 1.
3. Put these 12 capillaries on the tray, insert it into the instrument and start a capillary scan using the LED settings determined in step 3.1.

Note: The capillary scan starts at the back of the tray (position 16, or 12 respectively). Take this into account when you choose the type of capillary for your experiment. The following graphs show examples of stable and sticking samples.

4. If the fluorescence peaks of the scan are symmetrical, you can use these capillaries and go on to the next step.

Note: In the unlikely case that the sample is sticking to all types of capillaries, you can also try different buffers (e.g. containing detergent, BSA, casein or other additives). Additionally try to improve buffer conditions by adjusting pH and ionic strength.

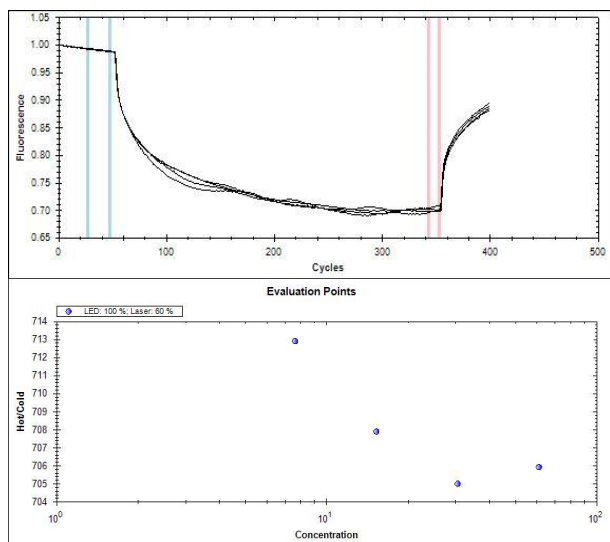## 3.3 Sample Quality: How can I find the Best Buffer Composition?

Figure 5.1: The graph shows 4 times the same sample measured with 40 % MST power. The sample quality is very poor. The inhomogenity of sample is clearly seen by the "bumpyness" of the MST curves (aggregation). The "Thermophoresis with Jump" result shows a noise of ~ 10 units. Sample quality needs to be improved before performing MST experiments.
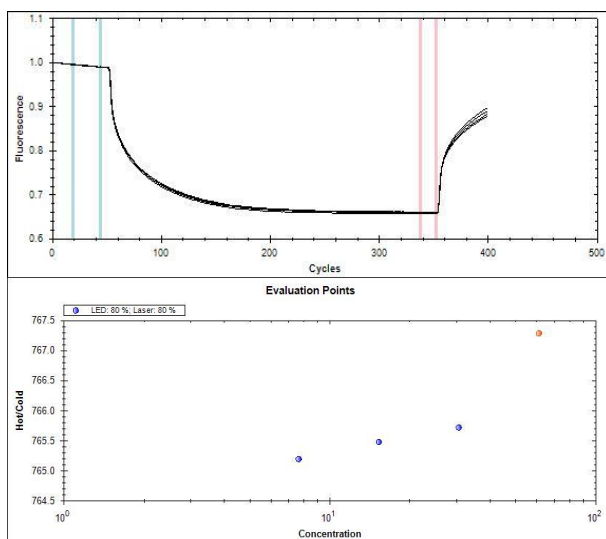

Figure 5.2: The graph shows 4 times the same sample measured with 40 % MST power. The sample quality is very good. There are no "bumps" in the curves. The time traces almost perfectly overlap. The "Thermophoresis with Jump" result shows a noise of less tan 2 units. You are ready to start an MST experiment.

Note: The sample shown in Figure 5.1 and 5.2 is the same. In Figure 5.2 measurement was performed in MST optimized buffer and after a centrifugation step (13.000 rpm for 5 min).

Up to now you have chosen a suitable concentration of the labeled protein/sample and you have tested in which capillary type your sample is stably in solution. In this chapter you will learn how to find the most suited buffer for your MST experiment that guarantees good reproducibility of MST results. This is the case when all time traces are well overlapping for the same sample. The most straight forward test for the buffer quality therefore is to compare the time traces obtained in > 4 capillaries filled with exactly the same sample.

To find the best buffer composition, please follow the following steps:

1. Prepare 100 µl of the sample in your binding buffer **and** 100 µl of the sample in MST optimized buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl$_2$, 0.05 % Tween-20).
2. Fill the type of capillary you determined in step 3.2 using the sample stocks prepared before. Fill at least 4 capillaries with sample in your binding buffer and 4 capillaries with sample in MST optimized buffer.
3. Perform the "Capillary Scan" with the predetermined settings and measure the samples at 40 % MST power. Load the results in the analysis software and select the "Thermophoresis with Jump" tab for analysis. The data should have a noise of 4 units or less.

IMPORTANT: If the noise is more than 8 units, we strongly recommend testing different buffers to improve the results. A rule of thumb: A decimal on the y-axis of the "Thermophoresis with Jump" plot proves a good quality of your sample.

Note: In many cases detergents (e.g. 0.05 % Tween-20) strongly improve the homogeneity of the sample. You can also add BSA, casein or reductive agents to your assay buffer. Also a centrifugation step (13.000 rpm for 5 min) in order to remove aggregates helps to improve sample quality.

Note: Standard buffer recommendation: MST optimized buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM MgCl$_2$, 0.05 % Tween-20. If no improvement could be observed using this buffer, please test different buffers as HEPES, Tris or phosphate buffers. You can add different additives to the buffer. Choose the buffer which gives the best signal to noise ratio.

IMPORTANT: Samples that have the inherent property to aggregate or that show only small thermophoretic amplitudes should be tested in enhanced gradient capillaries as well.
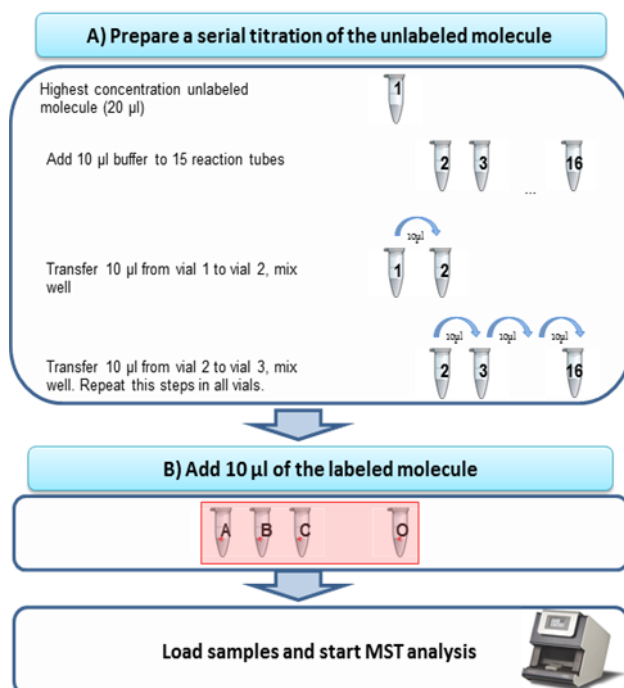
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4. Assay Setup

A) Prepare a serial titration of the unlabeled molecule

Highest concentration unlabeled molecule (20 µl)

Add 10 µl buffer to 15 reaction tubes

Transfer 10 µl from vial 1 to vial 2, mix well

Transfer 10 µl from vial 2 to vial 3, mix well. Repeat this steps in all vials.

B) Add 10 µl of the labeled molecule

Load samples and start MST analysis

Figure 6: **Schematic overview** how to prepare a MST experiment.

Now you are ready to start your MST experiment. You have a homogenous, stable sample that has a low baseline noise. This allows you to detect even minute changes in the thermophoresis of your molecule of interest upon interaction with its partner of interest.

1. Prepare 16 small micro reaction tubes, best suited are tubes with a volume of 200 µl or less. Label them from 1 through 16.
2. Fill at least 20 µl of the highest concentration you intend to use in the first micro reaction tube 1.
3. Fill 10 µl of the optimal assay buffer into the micro reaction tubes 2 to 16.

Note: Avoid any buffer dilution effects. The buffer in tube 1 and the buffer in the tubes 2-16 must be the same. Otherwise you get a gradient in salt, DMSO, glycerol or other additives. This interferes with the results you will obtain from the MST measurement.

4. Transfer 10 µl of tube 1 to tube 2 and mix very well by pipetting up and down several times.

Note: In order to avoid bubbles or droplets etc. we recommend to carefully pipett up and down several times and not to vortex these small volumes, that may also lead to denatured protein or sample.

5. To get a serial dilution repeat step 4 15 times and remove 10 µl from tube number 16 after mixing.
6. Mix 10 µl of fluorescently labeled sample at double the concentration determined at step 3.1 with the 10 µl of the titrated compound and mix well by pipetting up and down several times.
7. Incubate the sample at conditions of your choice before filling it into the capillaries. In most cases 5 min incubation at room temperature are sufficient.

5. Data Interpretation

This section gives you some hints to access the quality of your MST data. Please refer to the Monolith NT.115 User Manual in order to learn in detail how to use the NT Analysis software.
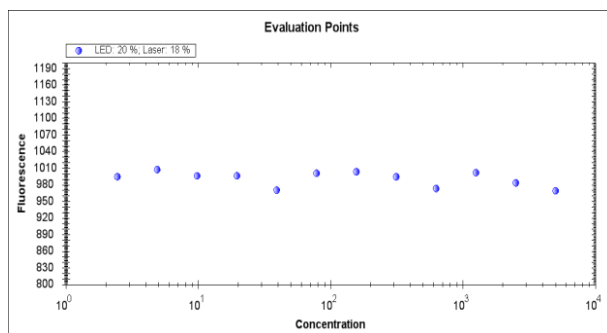
5.1 Fluorescence

Figure 8: The fluorescence intensity should not vary more than ± 10 % throughout the whole serial dilution.

The "Original Fluorescence" is the first parameter of your results that you should check.

A) Random Fluorescence Changes

Typically the intensity should not vary more than 10 %. If there are stronger random variations, either the mixing of the sample has to be optimized or the labeled sample is lost during the sample preparation (pipetting, micro reaction tube, and the like). One way to test this is adding Tween-20 or BSA to the buffer. If this increases counts and/or stabilizes the variations, then the loss of material was an issue.

B) Concentration Dependent Fluorescence Changes

A concentration dependent change in the fluorescence intensity (i.e. non-random, constant increase or decrease) can be explained as follows:

- Change in fluorescence intensity upon **binding**. The electrostatic surrounding of the dye molecule changes upon binding and the intensity changes (typically weak changes, typically not more as 2-3 fold change).
- Either the bound or unbound state is lost due to **unspecific adsorption / precipitation** during sample preparation or when filling the capillary. This leads to a concentration dependent change in fluorescence within the serial dilution. This effect can generate false positive thermophoretic signals.

In order to avoid unspecific adsorption / precipitation supplement the assay buffer with passivating agents like BSA (0.05 mg/ml), detergent (0.05% Tween 20) or test different buffer compositions by varying pH or ionic strength. To rule out sticking and to prove a binding event a mandatory capillary scan > 15 min after filling the capillaries has to be performed for the capillaries with low fluorescence intensities (max. 2 capillaries to obtain a good resolution of peak shape). If sticking (peaks with shoulders or U-shape) is observed the assay has to be optimized (see 3.3) and data cannot be used. In this case improve the assay by working at a constant level of BSA (e.g. 0.1-0.5 mg/ml) or Tween-20 (up to 0.05 %).

Note: *Additional negative controls comprise a non-binding molecule with the molecule of interest or a binding-deficient mutant of the molecule of interest.*
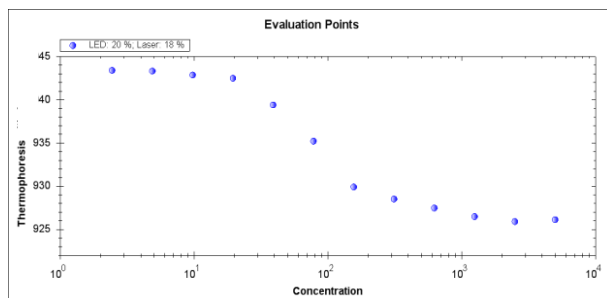
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5.2 Analysis of Thermophoresis Signals

Figure 9: The MST amplitude should be significantly higher than the noise in the baseline and the saturation.

The standard setting for evaluation is the analysis mode "Thermophoresis".

There are different rules to access the quality of your MST data:

- A signal should have more than 5 response units amplitude (amplitude = difference between bound and unbound state)
- The baseline noise should be at least 3 times less than the amplitude
- You should always measure with 2 different MST powers (20 %, 40 %) and compare the results.

IMPORTANT: It is highly recommended to perform MST measurements both with 20 % and 40 % MST power to get the best signal to noise ratio. Always choose the lowest possible laser power for your analysis, which gives a good signal to noise ratio.

IMPORTANT: If you do not obtain any binding curve with 20 % and 40 % MST power, you should use 60 % and 80 % MST power. For measurements performed with 60% and 80 % MST power we recommend to reduce the time settings (5 s Fluo. Before, 15 s MST On, 5 s Fluo. After) in the parameter table in the NT.Control software since the thermophoresis curve approaches the steady state phase earlier compared to measurements at lower MST power.

Depending on the interaction of interest, both analysis modes "Thermophoresis" and "Thermophoresis with Jump" may both report the binding event (individual as well as in combination). If more than just one setting shows a result, they should yield similar affinity constants. Different K_ds obtained by different analysis modes can be explained by:

- Different signal to noise ratios
- Non-homogenous mixture of sample

Note: The analysis modes "Temperature Jump" does not yield a result for every interaction. The "Temperature Jump" result might be different from the "Thermophoresis" result since it is sensitive to the local surrounding of the dye e.g. if you have a mixture of monomers and dimers, the "Temperature Jump" might only report binding of monomers to your molecule of interest.

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