

# **MST Starting Guide – Monolith NT.115**

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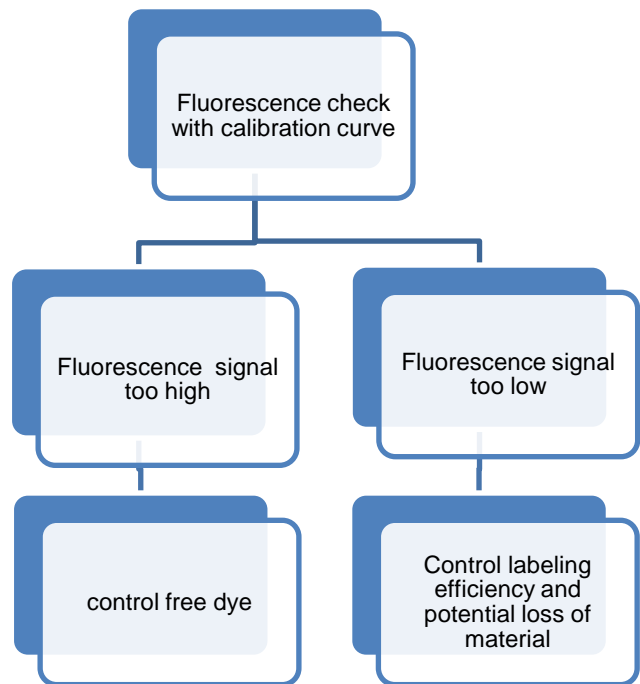
## (1) How to design an Experiment

The Monolith Systems measure equilibrium binding constants between varieties of molecules, with almost no restriction to molecular size or mass.

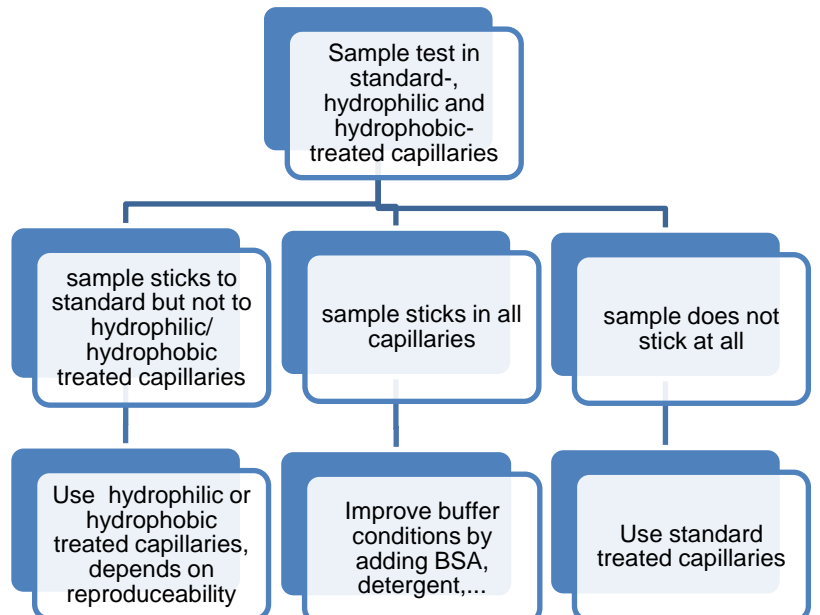
Although the system is easy to handle, you should follow this guide when using the instrument for the first time. This guide is designed to help you, to get reliable results as quick as possible. More detailed information is available in the Monolith.NT115 User Manual.

## Flow Chart Assay Setup

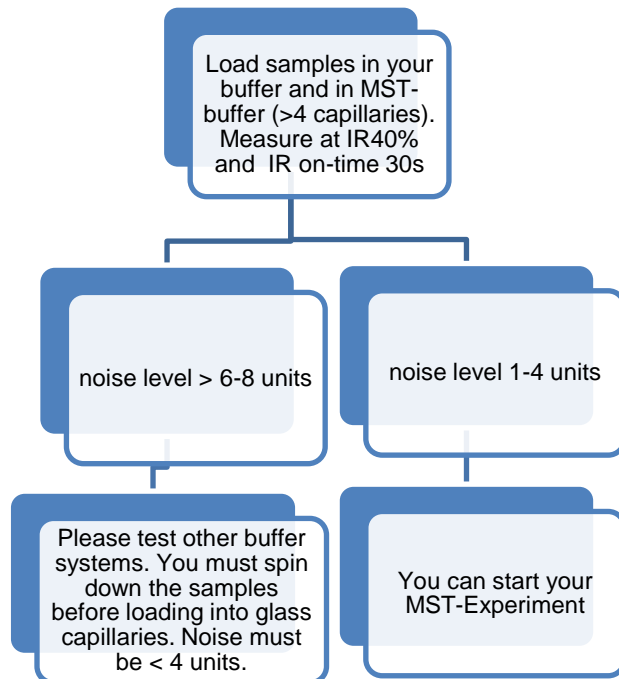
### Step 1: Fluorescence Check



### Step 2: Capillary Check

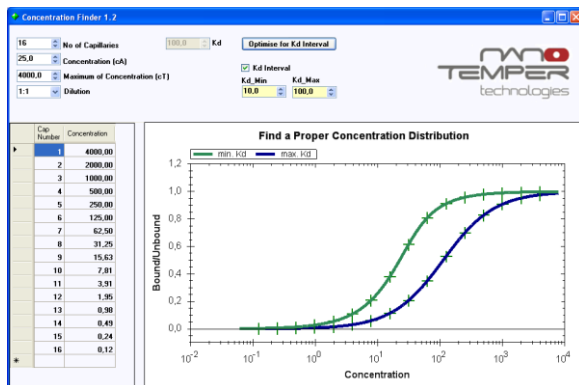


**Step 3: Buffer / Sample Quality Check**



## 2) Before you start

### 2.1) Design of Experiment



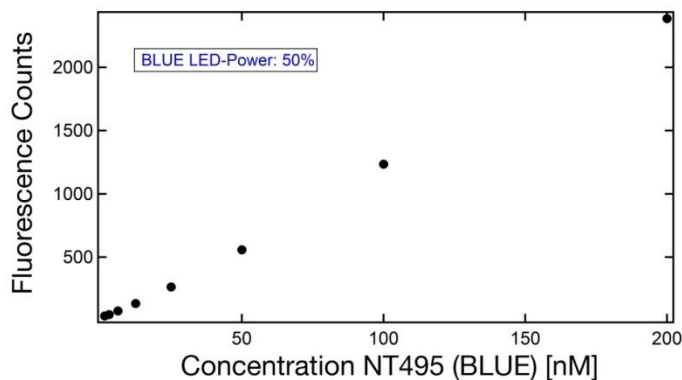
**Figure 1:** Use the “Concentration finder” tool in order to design your MST-Experiment

Check before 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 (KD)**. For details refer to the “Concentration Finder”-Tool in the Monolith 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 is a method that uses very small quantities and volumes of material. The capillaries only need 4µl of your sample. 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.  
*Optional: if your protein sticks to surfaces, you may use detergent or low binding reaction tubes to stabilize the sample.*
- 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 microwell plates. You can also obtain MST tested tubes from NanoTemper Technologies.

## 2.2 Quality of your labeling procedure



**Figure 2:** Example of a calibration curve using NT-647 dye at 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 labeled molecule preparations, use NanoTemper Labeling Kits ([www.nanotemper.de](http://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 with a fluorescent dye, the protein preparation you are using has to be as pure as possible. For the same reason avoid the use of carrier proteins as BSA in the protein stock that you want to label. Other proteins that get labeled as well will reduce your signal to noise ratio as the free dye does.

**Always spin down the stocks of labeled and unlabeled molecules for 5min at 13.000 rpm in a table top centrifuge.** This step will remove big aggregates, which is one of the main sources for noise.

**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. 200nM, 100nM, 50nM, 25nM, 12.5nM, 6.25nM, 3.12nM 1.56nM). Use your interaction buffer. You should use 50 % LED – Power for measuring. Fill the samples in standard capillaries (K002) and start a capillary scan and a measurement using 50% LED-Power. Plot or note the fluorescence intensity in the peak of each capillary.
- 2) **Determine the concentration of your labeled molecule.** Prepare a 100µl dilution of 50 nM of the labeled molecule in your interaction buffer and fill it into a single capillary and place it on the tray. Insert the tray in the instrument and start a scan at the capillary position at 50% LED power of the suited LED. Use the fluorescence value from the dye calibration curve (step 1) in order to estimate the concentration of your labeled molecule.

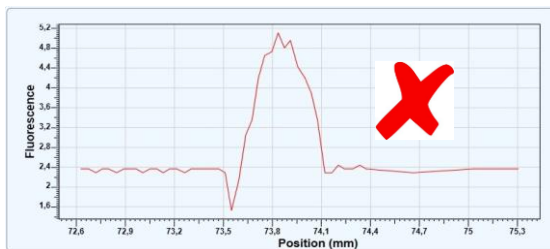
**Caution:** If the fluorescence counts of your sample deviates more than a factor of two or three from the calibration curve, you either have to optimize labeling efficiency or you have less sample material than you thought. Also rule out that there is free dye in the sample. Characterize your labeling efficiency and sample concentration as good as possible before you proceed with assay development. A labeling ratio of 1:1 is optimal, but not necessary. A strong deviation from the expected fluorescence might indicate a general problem of the sample.

**Note:** You can test the degree of labeling by measuring the absorbance of the dye and of your protein (280 nm) using a photometer.

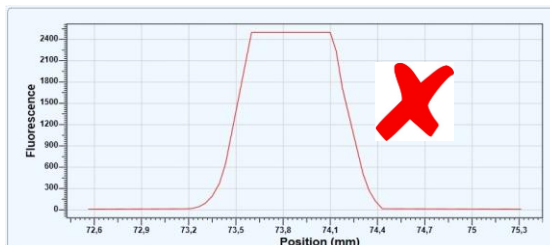
## 3) Assay Setup Pretests

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 in which your sample is homogeneous.

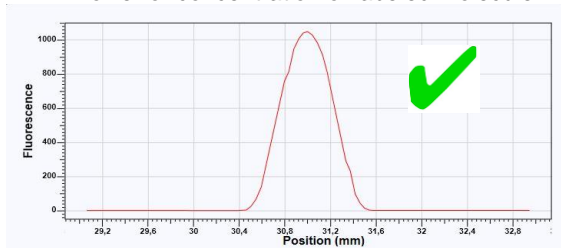
### 3.1) Fluorescence Check



**Figure 3.1:** Fluorescence signal too low. Increase LED-Power or concentration of labeled molecule.



**Figure 3.2:** Fluorescence signal too high. Decrease LED-Power or concentration of labeled molecule.



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

How much of the fluorescently labeled molecule should I use?

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

**Note:** It is important to measure at 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 NanoTemper Standard Treated Capillary (K002) and start a Capillary Scan with 50% LED Power with the LED that matches your dye.
- 3) Compare the intensity to the dye calibration curve you prepared previously.

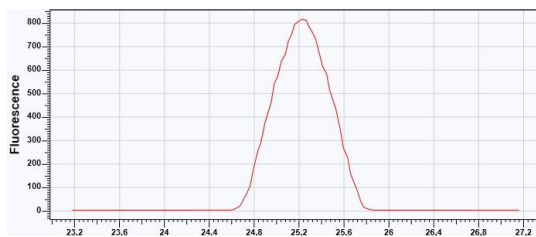
**Note:** If the fluorescence intensities do not match by a factor of 2-3 then either labeling efficiency is low or protein/sample concentration is not in the expected range. It is not necessary to have a labeling ratio of 1:1 (typically 0.5 to 1.1 is used), but a very low labeling efficiency might also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

- 4) Never work at 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 should be varied between 15% and 95%.

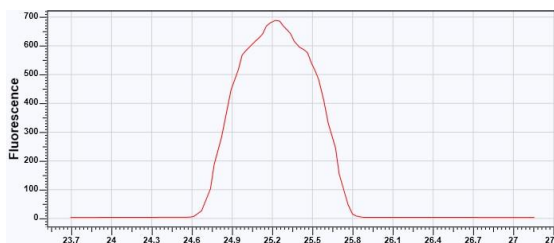
**Note:** For high affinity interaction ( $KD < 10$  nM) the concentration of the molecule should be on the order of the KD or below. If the KD is lower as the detection limit of the dye you are using, use the lowest possible concentration of the labeled molecule, in which you get 200 fluorescence counts at 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 10nM 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 before.

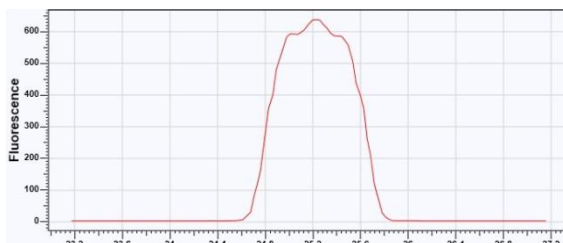
### 3.2) Capillary Check: Which MST capillary type should I use?



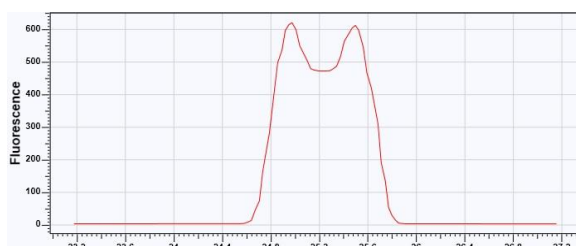
**Figure 4.1: No Sticking, symmetrical fluorescence peak.** You can use this capillary type for your MST-Experiment.



**Figure 4.2: Slight Sticking, shoulders in fluorescence peak.** Please note that it might take a 2-3 minutes to observe a sticking effect, since it is a diffusion limited process. If you are not sure, start a capillary scan after your measurement. Repeat the Scan after 2-3 minutes.



**Figure 4.3: the labeled molecule sticks to the capillary you are using.** You MUST test another capillary format or improve the buffer composition (go to step 3.3).



**Figure 4.4: Very Strong and clear double peak behavior of a sticking sample.** 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 the capillary. The resulting MST-signal has a poor quality. NanoTemper offers different types of covalently coated capillaries to avoid any unspecific sticking to the glass surfaces. Depending on the type of polymer used for the coating, the capillaries are referred to as either hydrophilic (K004) or hydrophobic (K003). For more information visit our homepage ([www.nanotemper.de](http://www.nanotemper.de)). NanoTemper also offers an Assay Development Kit, which contains all important capillary types.

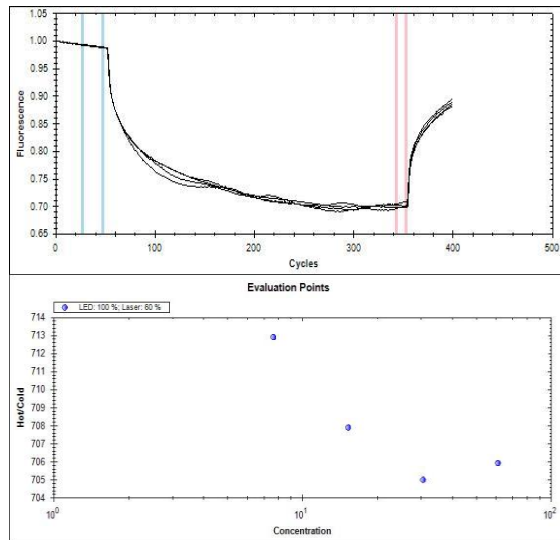
**To test the best capillary type, please follow the following steps:**

1. Prepare 120µl of the labeled molecule at the concentration you want to use in the assay (as determined in step 2 and 3).
2. Fill 4 standard treated capillaries (K002), 4 hydrophilic capillaries (K004) and 4 hydrophobic capillaries (K003) with the same sample taken directly from the 120µl stock.
3. Put the 12 capillaries on the tray, insert it into the instrument and start a capillary scan using the LED settings determined in step A. Please note that 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.

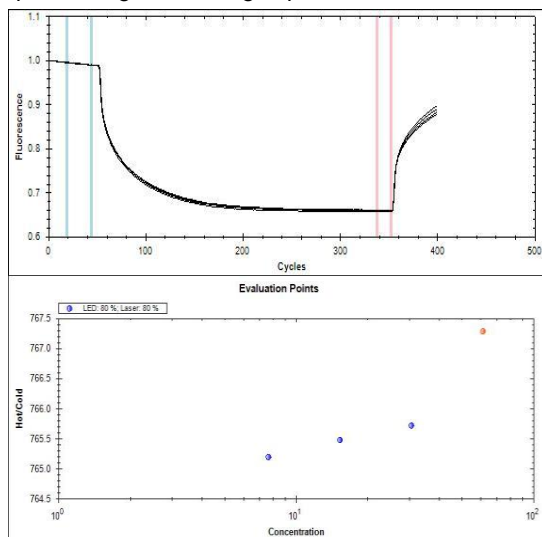
**Note:** In the unlikely case that the sample is sticking to all types of capillaries, you can also try different buffers (e.g. containing BSA, detergent, Casein or other additives). Adding detergent to the buffer might make a sample work, even in standard treated capillaries.



### 3.3) Sample Quality: How can I find the best buffer



**Figure 5.1:** The graph shows 4 times the same sample measured with 40% IR power. The sample quality is very poor. The inhomogeneity of sample is clearly seen by the “bumpyness” of the MST-curves (aggregation). The “Thermophoresis and T-Jump” result shows a minimum to maximum noise of 10 units. It is strongly recommended to improve sample quality, before performing the binding experiment.



**Figure 5.2:** The graph shows 4 times the same sample measured with 40% IR power. The sample quality is very good. There are no “bumps” in the curves. The time traces almost perfectly overlap. (bottom) The “Thermophoresis and T-Jump” result shows a minimum to maximum noise of 1 unit. You are ready to start an experiment. Please note that the sample in Fig. 5.1 and 5.2 is the same. The sample in Fig.5.2 was measured in MST optimized buffer and was spinned down at 13.000 rpm for 5min.

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 stable in solution. In this chapter you will learn how to find the most suited buffer for your MST-Experiment. The most important criteria for an optimal buffer: Good reproducibility of MST results, meaning time traces are well overlapping for the same sample. The most straight forward test for the quality is, to compare the results obtained in >4 capillaries filled with exactly the same sample. To do so, please follow the following steps:

1. Prepare 100µl of the sample in your binding buffer **and** 100µl in MST optimized buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 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% IR-Laser Power with an on-time of 30 seconds.

Load the results in the analysis software; select the “normalized fluorescence” tab for time traces and the “Thermophoresis with T-Jump” tab for analysis. The data should have an average minimum to maximum noise of 4 units or less.

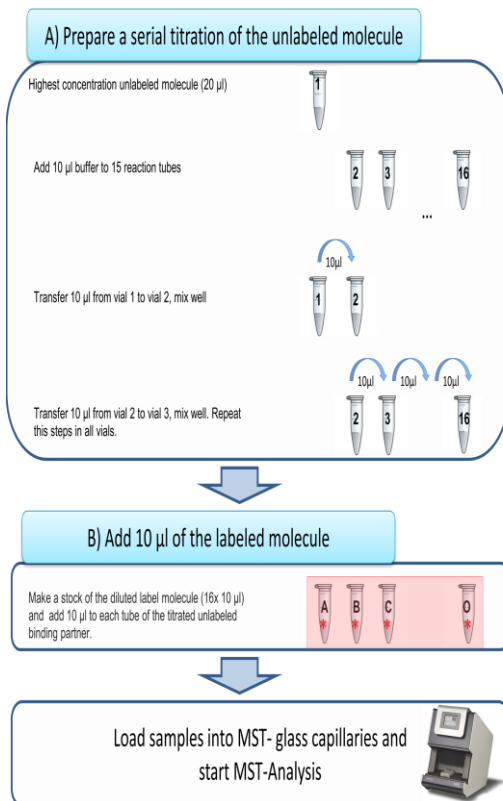
**IMPORTANT:** If the noise is more than 8 units, we strongly recommend testing different buffers to improve the result (see figure 5.1 and 5.2). As a rule of thumb: when a decimal appears on the left axis of the “Thermophoresis with T-Jump” plot, the quality of the sample is good.

**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. It is a good practice to centrifuge solutions of conjugates in a microcentrifuge for 5 min at 13000 rpm before use; only the supernatant should then be used in the experiment. This step will remove any aggregates that may have formed during storage.

**Note:** Standard buffer recommendation: MST Optimized Buffer: 50mM Tris pH 7.4, 150mM NaCl, 10mM MgCl<sub>2</sub>, 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.

## 4) Assay Setup



**Figure 6:** A schematic overview how to prepare a MST-Experiment

Now you are ready to start your interaction experiment. You have a stable sample that has a low base-line noise, which will allow you to detect even minute changes of your molecule of interest.

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

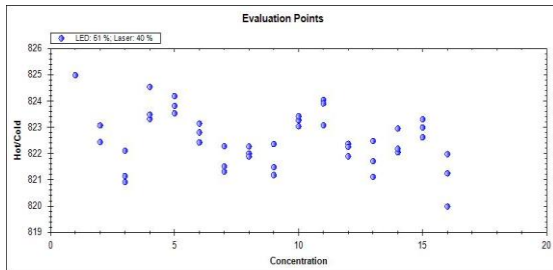
**Note:** Avoid any buffer dilution effects. The buffer in tube number one and the buffer in the other tubes must be the same. Otherwise you get a gradient in salt, DMSO, glycerol or other additives. This interferes with the MST measurement.

- 4) Transfer 10µl of tube number one to tube number two and mix very well by pipetting up and down several times.

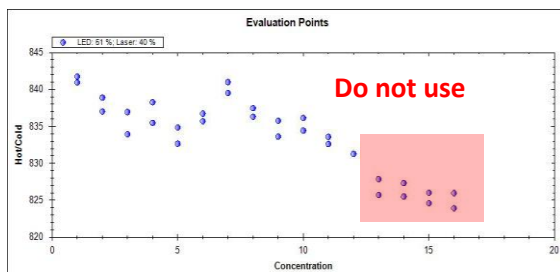
**Note:** Do not vortex these small volumes, since that will not mix efficiently and may lead to denatured protein.

- 5) Repeat this 15 times and remove 10µl from tube number 16 after mixing.
- 6) Mix 10µl of fluorescently labeled sample at double the concentration determined at step 3.1 with the 10µl 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 minutes incubation at room temperature are sufficient.

## 5) MST-Experiment using Temperature Control



**Figure 7.1:** No Baseline Drift – All tray positions can be used



**Figure 7.2:** Baseline Drift – Do not use capillary positions highlighted in red

The NanoTemper Monolith NT.115 and Monolith NT.LabelFree are equipped with a temperature controlled sample tray (20-50°C). We do not recommend that the temperature control is used by inexperienced users or for assay setup. In these cases measure with the temperature control turned off. The affinity will be determined at ambient temperature of your laboratory.

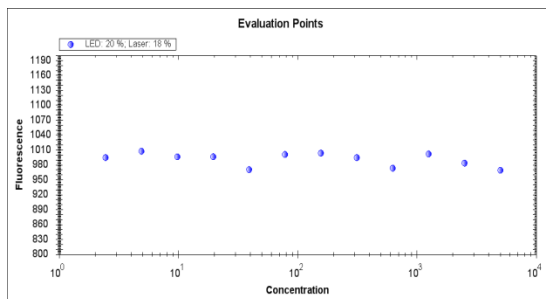
When you want to work with a temperature controlled samples, we strongly recommend you to do the following experiment before you do the actual binding experiment.

- 1) Prepare a solution of the fluorescently labeled molecule for 16 capillaries. Prepare it in the concentration you intend to use in the assay (i.e. as determined previously)
- 2) Turn the temperature control of the instrument “on” and adjust it to the respective temperature you want to use.
- 3) Fill 16 capillaries with the same sample, start a capillary scan, and wait for at least 5 minutes before you start the measurement. This is the time it takes for the tray and capillaries to equilibrate to the new temperature.
- 4) Measure at 40% IR Laserpower with 30 seconds Laser-on time
- 5) Analyze the data with the “Thermophoresis and T-Jump” settings and look for a potential base line drift effect (see Figure 7.2)

## 6) Data interpretation

This section gives you some hints to access the quality of your MST data. Please refer to the User guide manual in order to learn more how to use the MST analysis software.

### 6.1 Fluorescence



**Figure 8:** the fluorescence intensity should not vary more as 10 % between different capillaries.

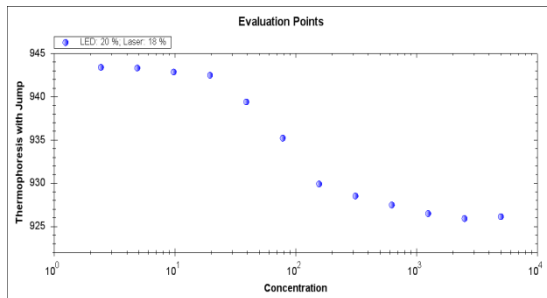
This is the first parameter of your results that you should check.

Typically the intensity should only vary by about 10%. If there are stronger random variations, either the mixing of the sample has to be optimized, or labeled sample is lost during sample preparation (pipetting, micro reaction tube, and the like). One way to test this is using Tween-20 or BSA in the buffer. If it increases counts and/or stabilizes the variations, then the loss of material was an issue.

The intensity should, in general, be constant within +/-10% throughout the whole serial dilution. If there is a concentration dependent shift in intensity (i.e. constant, not random, increase or decrease) there are some reasons for a fluorescence change that you should be aware of:

- Change in fluorescence yield 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). **Note:** *No need to improve the assay.*
- Either the bound or unbound state is lost during sample preparation, which leads to a concentration dependent change in fluorescence throughout the serial dilution. In most case this effect may only generate a false positive response at very low fluorescence counts. Improve by working at a constant level of BSA (e.g. 0.1-0.5mg/ml) or Tween-20 (up to 0.05%).

## 6.2) Thermophoresis and T-Jump



**Fig. 12:** the MST-amplitude should be significantly higher as the noi.

Depending on the interaction of interest, Thermophoresis and Temperature-Jump analysis tabs may all report the binding event (individual as well as in combination).

**Note:** The MST T-Jump does not yield a result for every interaction. It rare case it might deviate from Thermophoresis results since it is sensitive to the local surrounding of the dye. E.g. if you have a mixture of monomers and dimers, the T-Jump might only report binding to monomers.

If more than just one setting shows a result, they yield the same affinity constants.

The standard setting for evaluation is the tab "Thermophoresis and T-Jump"

**There are different rules to access the quality of a signal:**

- 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 at 3 different laser powers and compare the results.

**IMPORTANT:** Start always from the lowest IR-Laser power (20%, 40%, 80%). This allows you to get the best signal to noise and you learn if laser power has an effect on the KD you determined. Always choose the lowest possible laser power for you analysis, which give in a good signal to noise ratio.