Biomolecular Interaction Analytics using MicroScale Thermophoresis

The Monolith NT.115

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Stefan Duhr

Philipp Baaske

nano TEMPER technologies

2008

Fast thermo-optical particle characterisation
PCT Patent 2008/061706

2010:
Monolith
NT.115

2012:
Monolith
NT.LabelFree

2013:
Monolith
NT.115^{Pico}

2014:
Monolith
NT.Automated

2014:
PROMETHEUS
NT.48

1856:
Carl Ludwig

1879:
Charles Soret

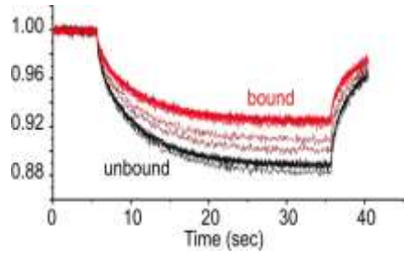
Agenda

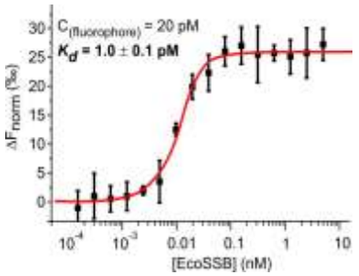
Seminar

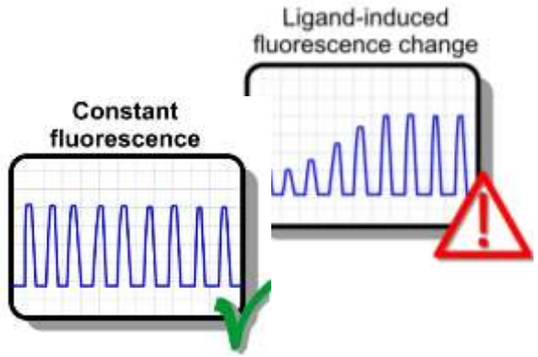
- MicroScale Thermophoresis (MST) Basics
- Assay Setup
- Assay Optimization

Experimental work

- Measurement of positive control
- Sample handling and assay setup
- NT.Control and NT.Analysis Software
- Run experiments with your own samples

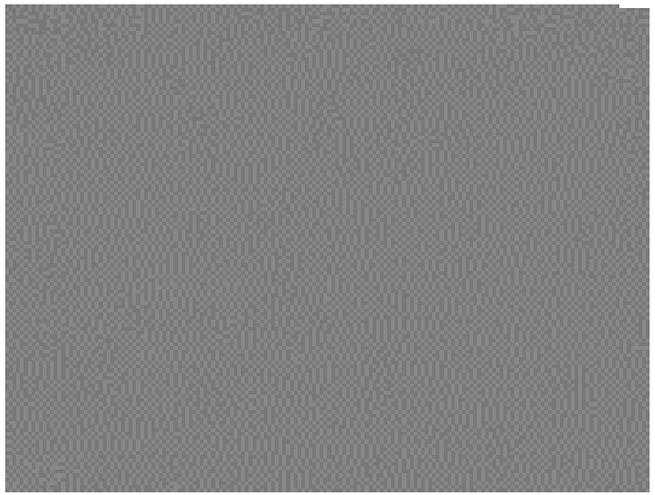




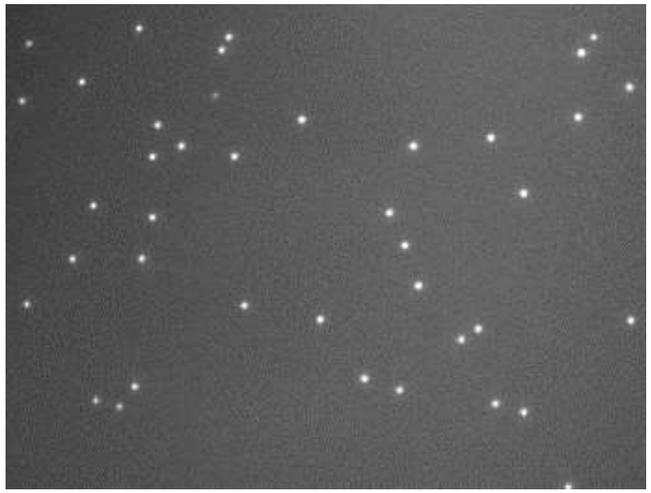


Thermophoresis in Real Time

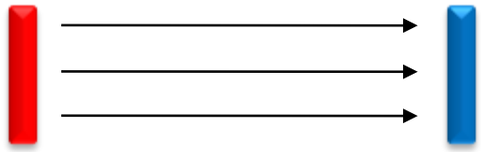
DNA



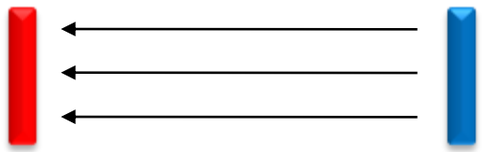
Microbeads



„Positive“ thermophoresis

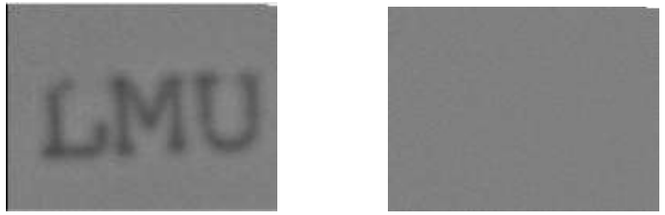


„Negative“ thermophoresis



Theory of Thermophoresis

$c_{hot} / c_{cold} = \exp(-S_T \Delta T)$

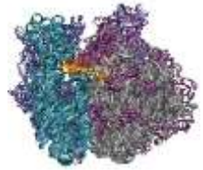


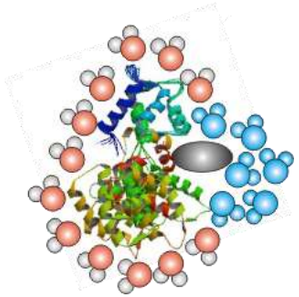
Laser „on“

Laser „off“

$$S_T = \frac{A}{kT}\left(-\Delta s_{hyd}(T) + \frac{\beta\sigma_{eff}^2}{4\pi\varepsilon\varepsilon_0 T} \times \lambda_{DH}\right)$$

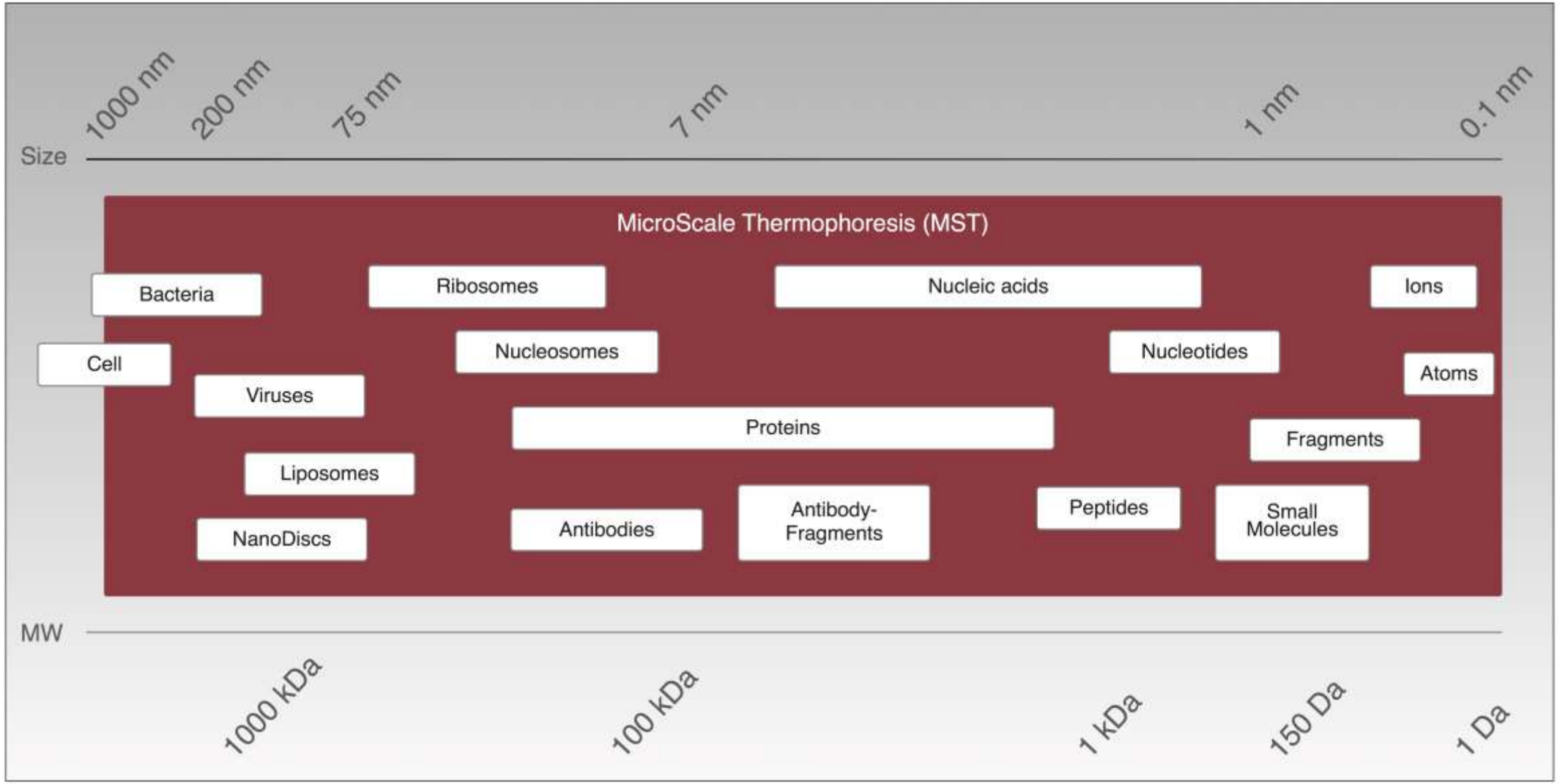
Size | **Hydration shell** | **Charge ²**



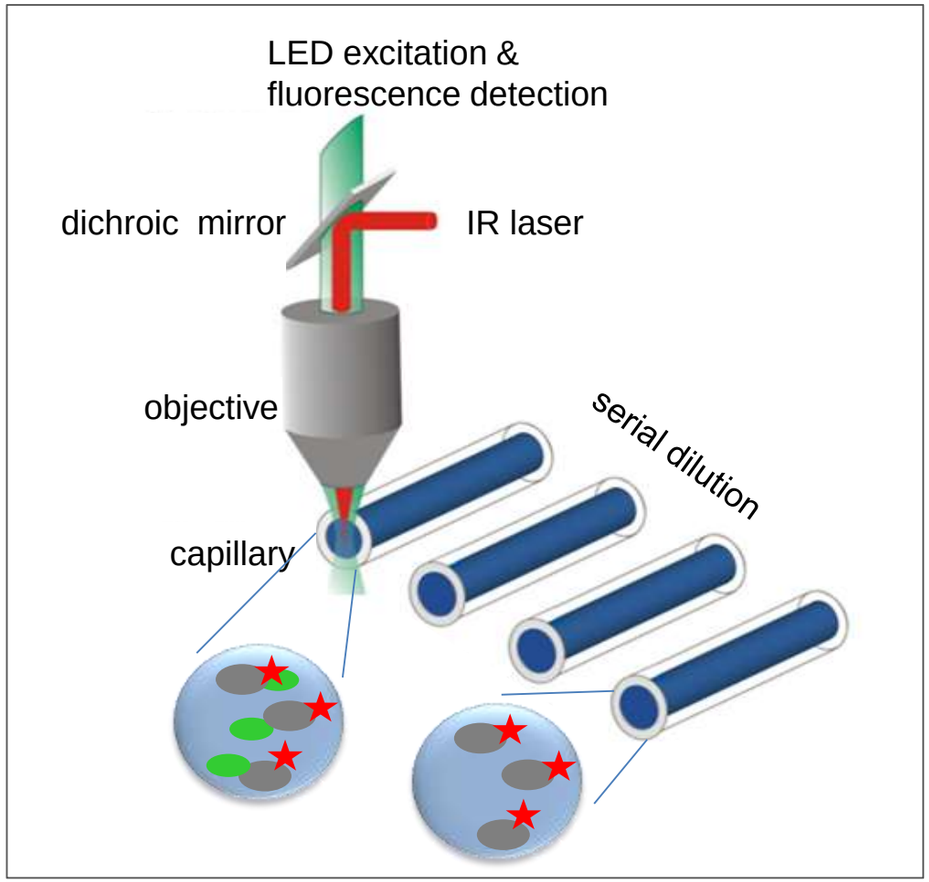


Duhr and Braun PNAS 103, 19678–19682 (2006)
Duhr and Braun PRL 96, 168301 (2006)

Interactions accessible with MST



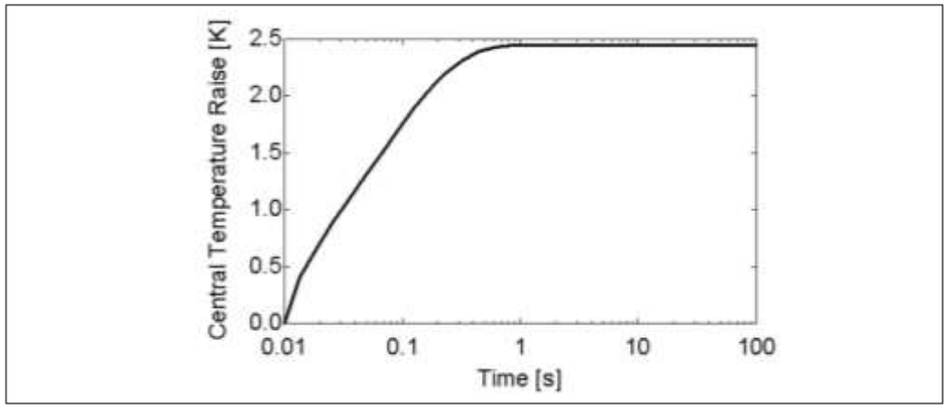
Basic setup of the instrument



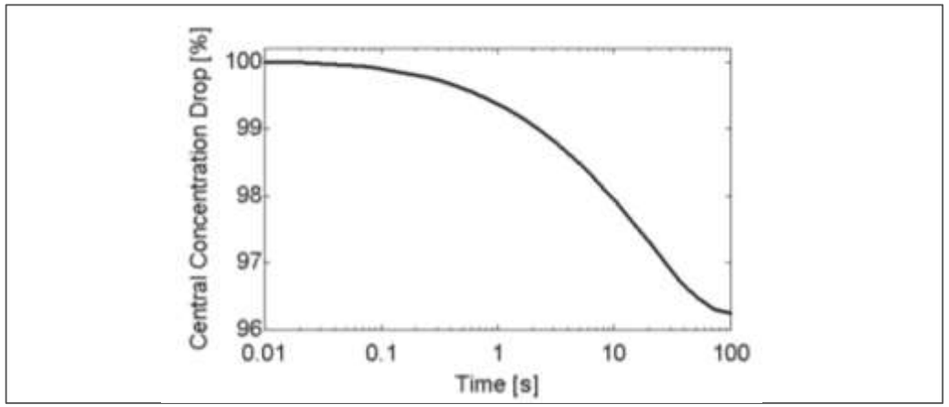
- **Immobilization-free** in buffer or complex bioliquids
- **4 µl** of sample per titration point
- **nM concentrations** of fluorescent molecule
- **40 sec** measurement per titration point

MicroScale Thermophoresis: Physical Processes

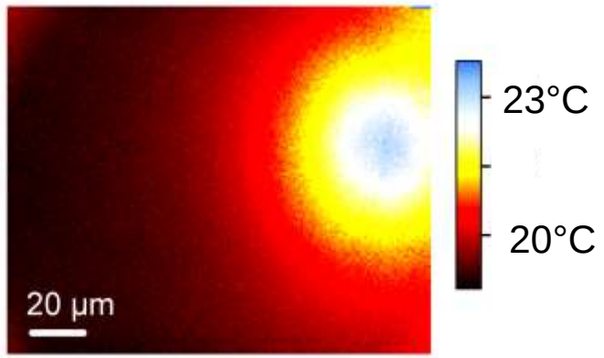
Sample heating < 1 sec



Thermophoresis ~ 30 sec



IR laser induced temperature field

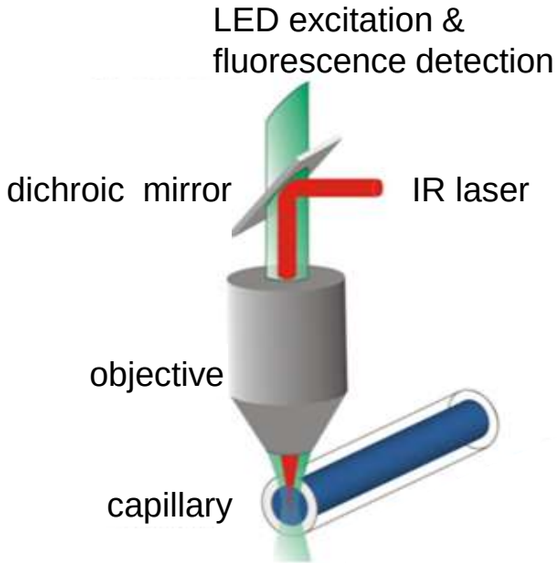


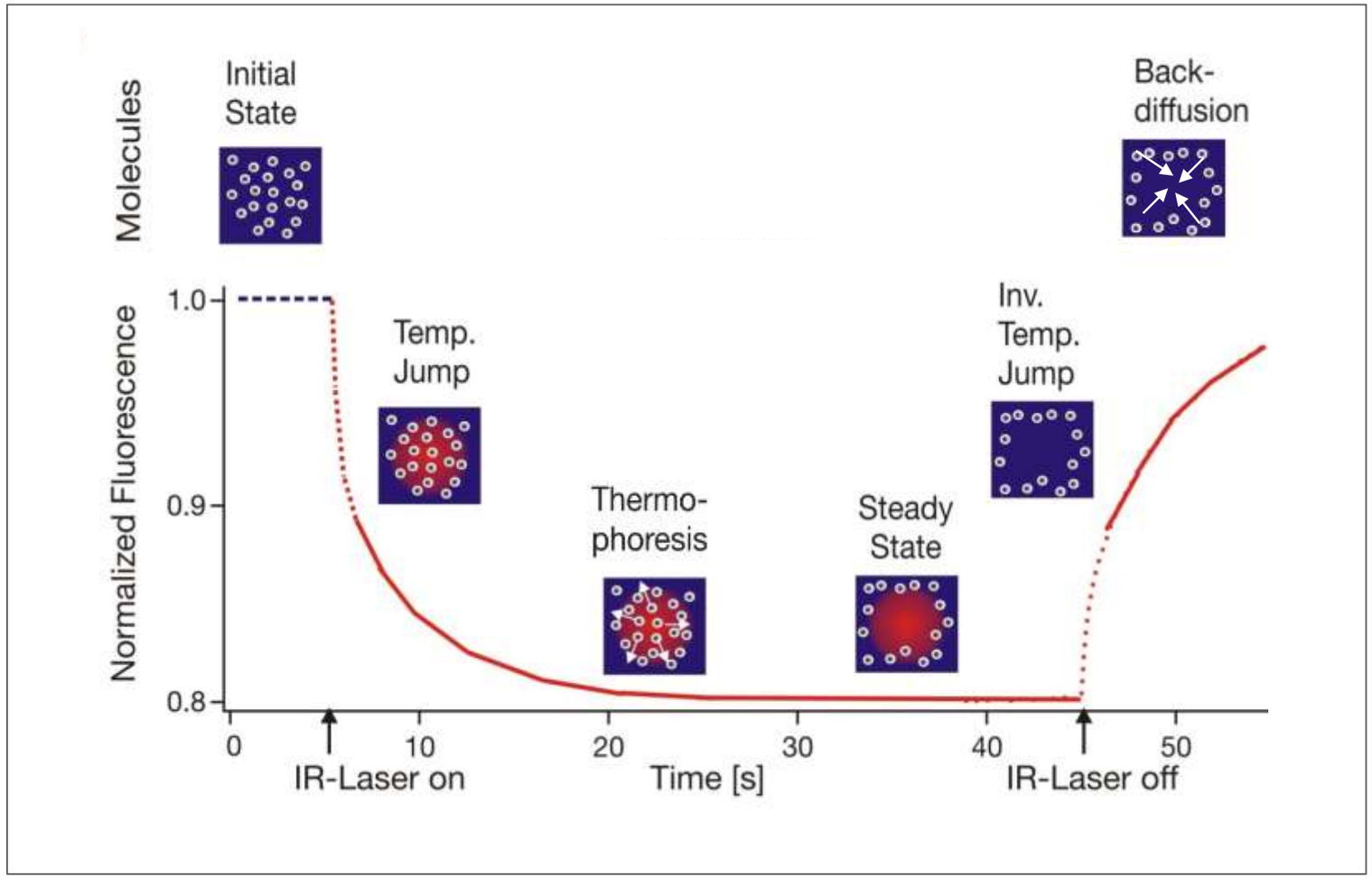
Temperature [°C]

1K/10 µm corresponds to 1000K/cm

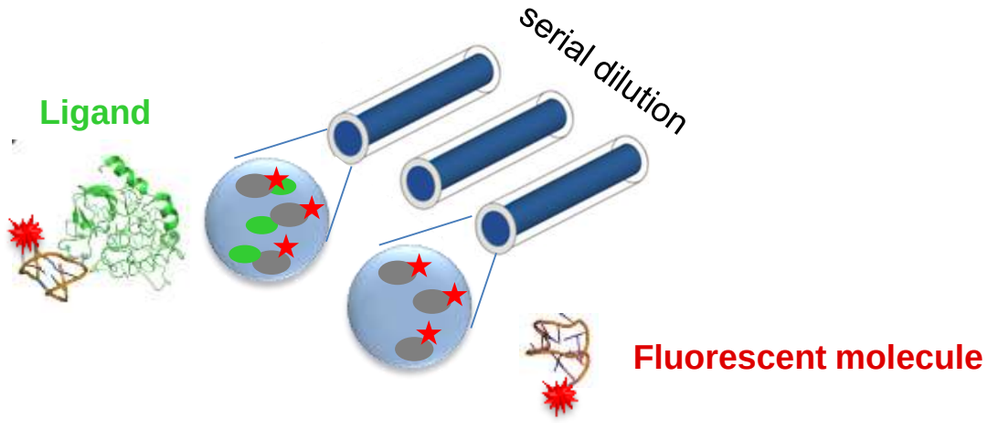
Jerabek-Willemsen et al, ADDT, 2011

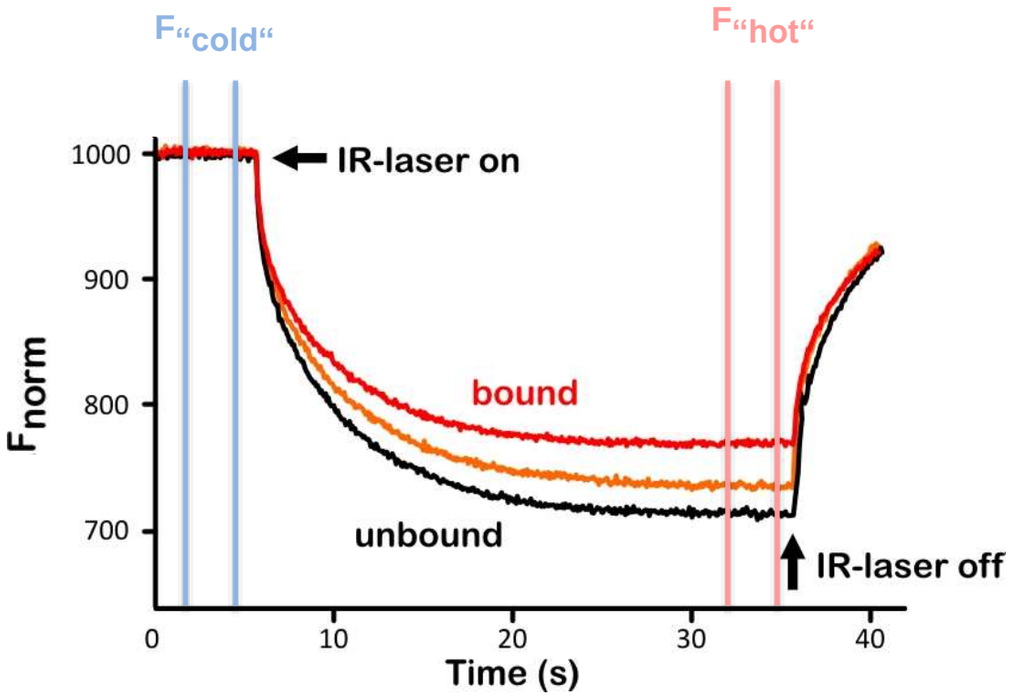
Phases of a MST Signal



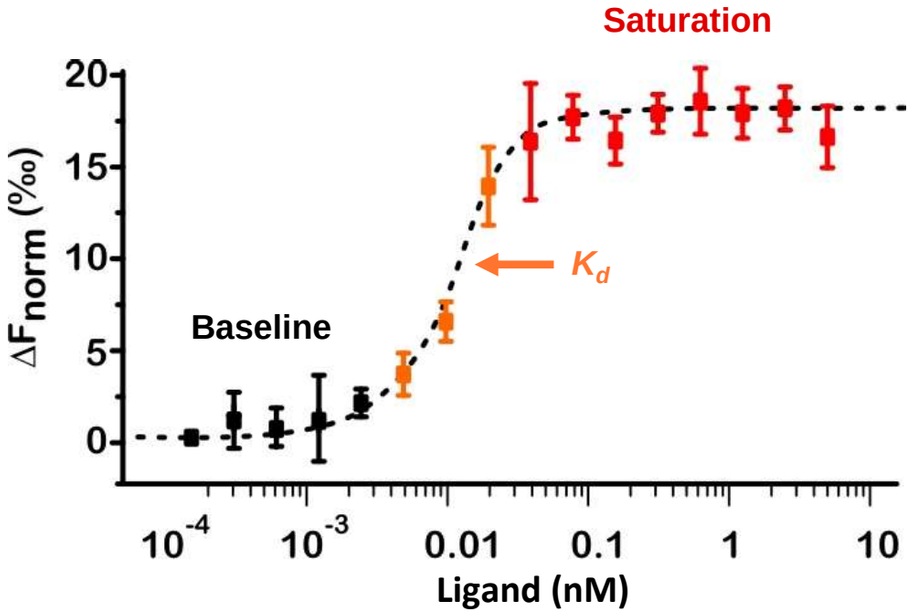


Jerabek-Willemsen et al, ADDT, 2011

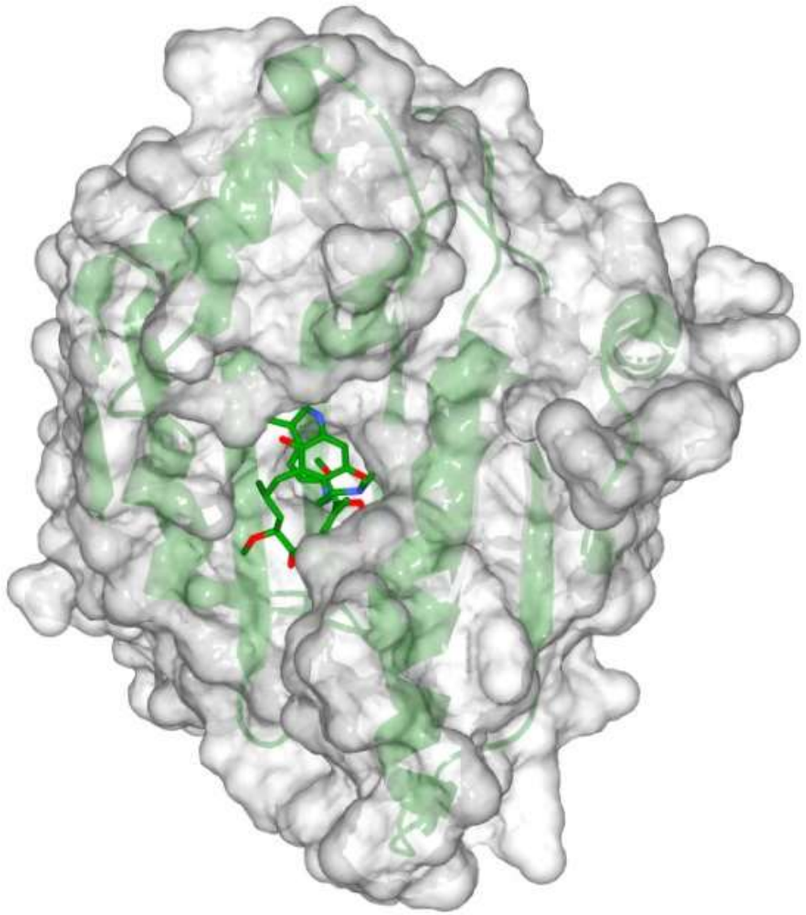

Ligand
serial dilution
Fluorescent molecule

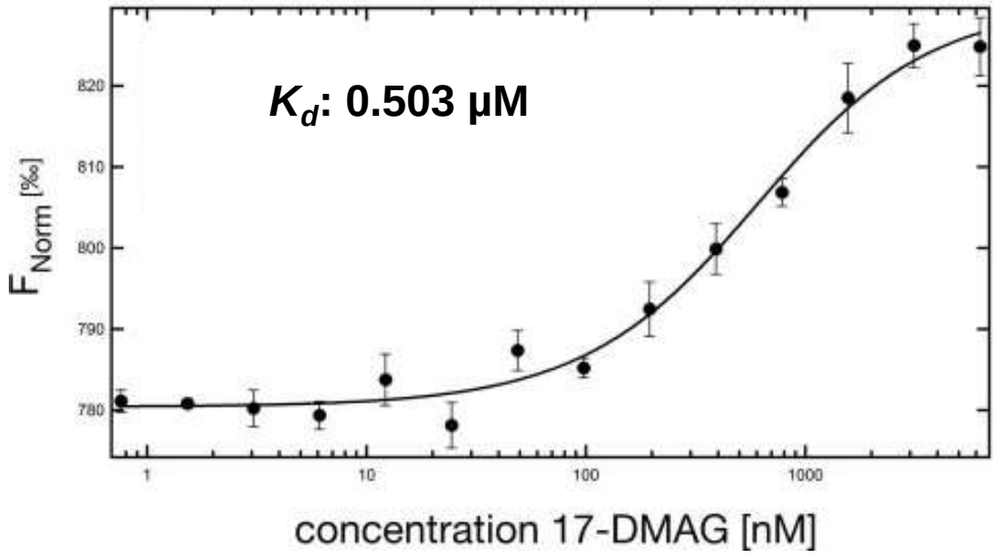

F“cold“
F“hot“
1000
900
800
700
Fnorm
IR-laser on
bound
unbound
IR-laser off
0
10
20
30
40
Time (s)

F“hot“ /F“cold“


Saturation
20
15
10
5
0
ΔFnorm (‰)
Kd
Baseline
10-4
10-3
0.01
0.1
1
10
Ligand (nM)

Interaction of Fluorescently Labeled Hsp90 with 17-DMAG

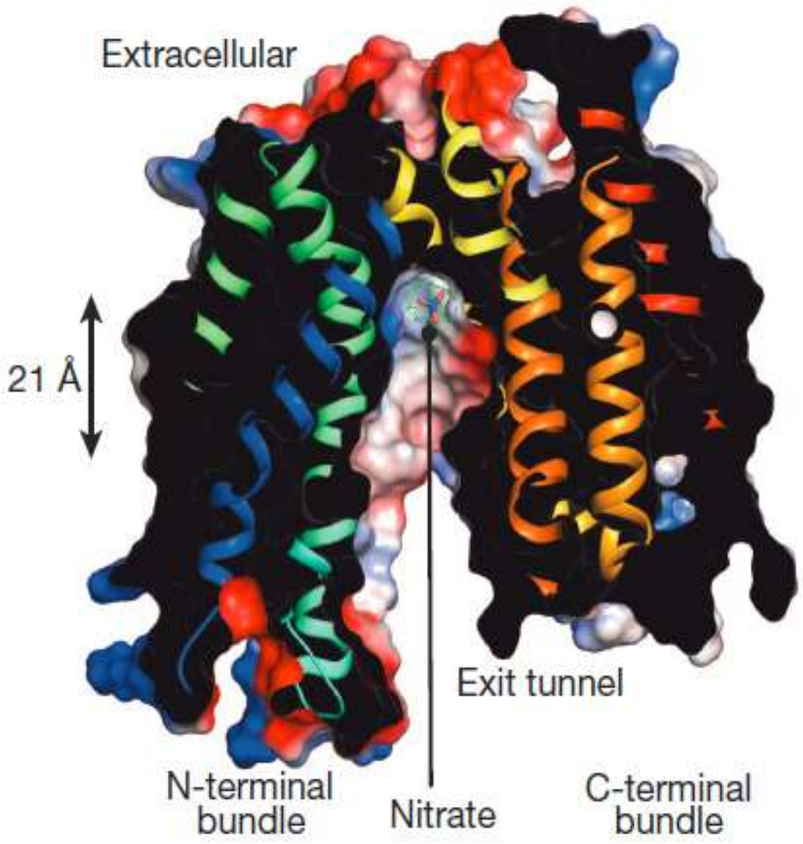


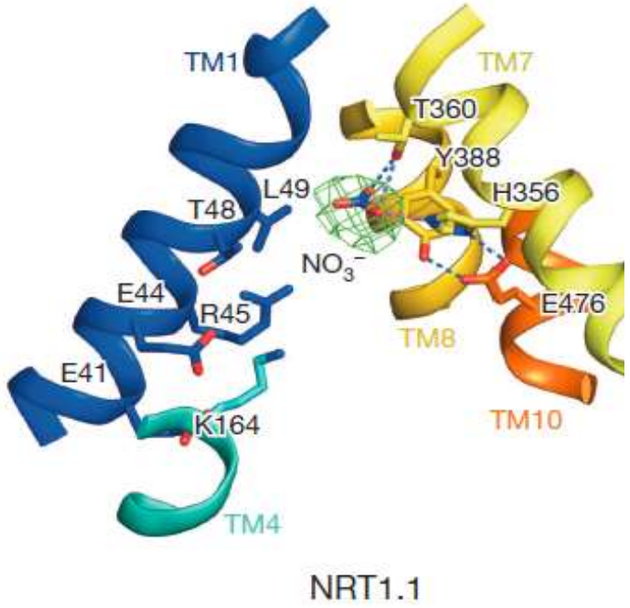


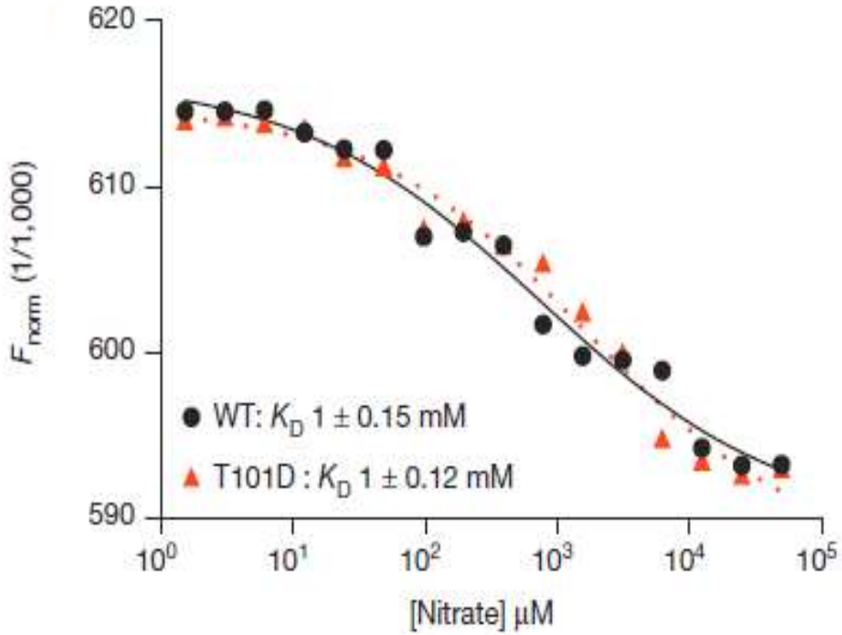
Results were kindly provided by Stephen McLaughlin, MRC-LMB, Cambridge, UK

- **Interactions that do not alter the MW of a protein, only the conformation (hydration shell)**

Nitrate binding to a 12 Transmembrane-Helices Transporter



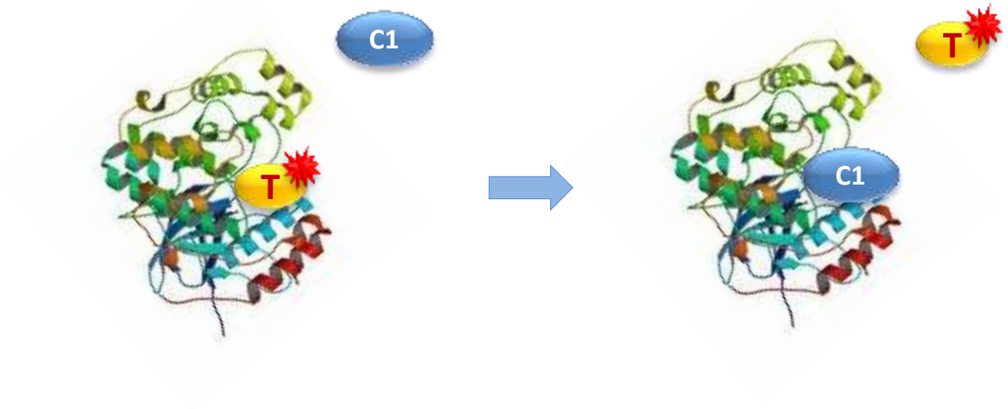




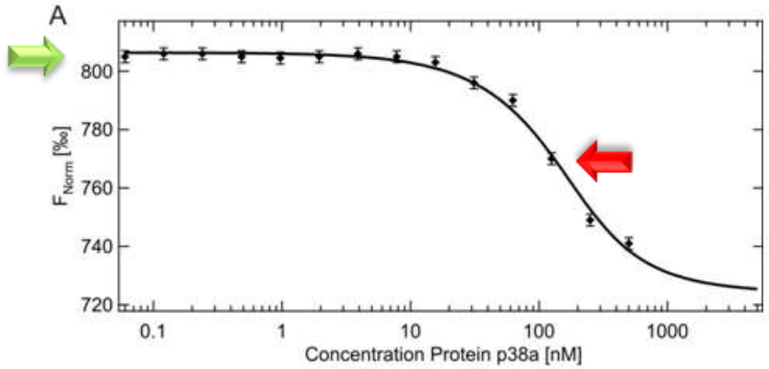
Parker & Newstead, Nature, 2014

► **Unmatched sensitivity of MST: small nitrate molecule binding to a large 12 Transmembrane-Helices Transporter**

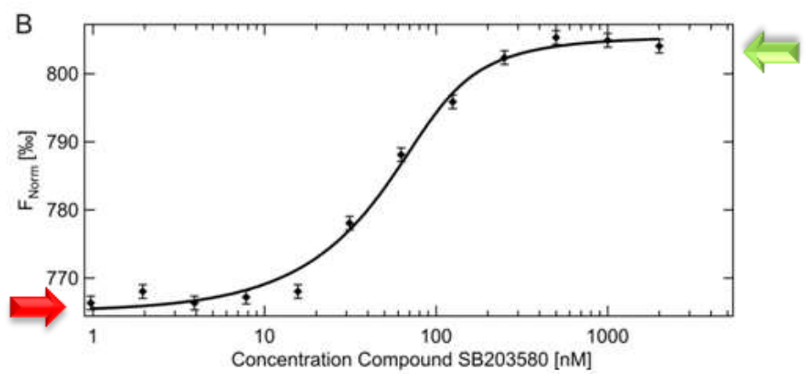
Competitive Approach for p38 using a fluorescent Tracer



Binding of p38 to Tracer



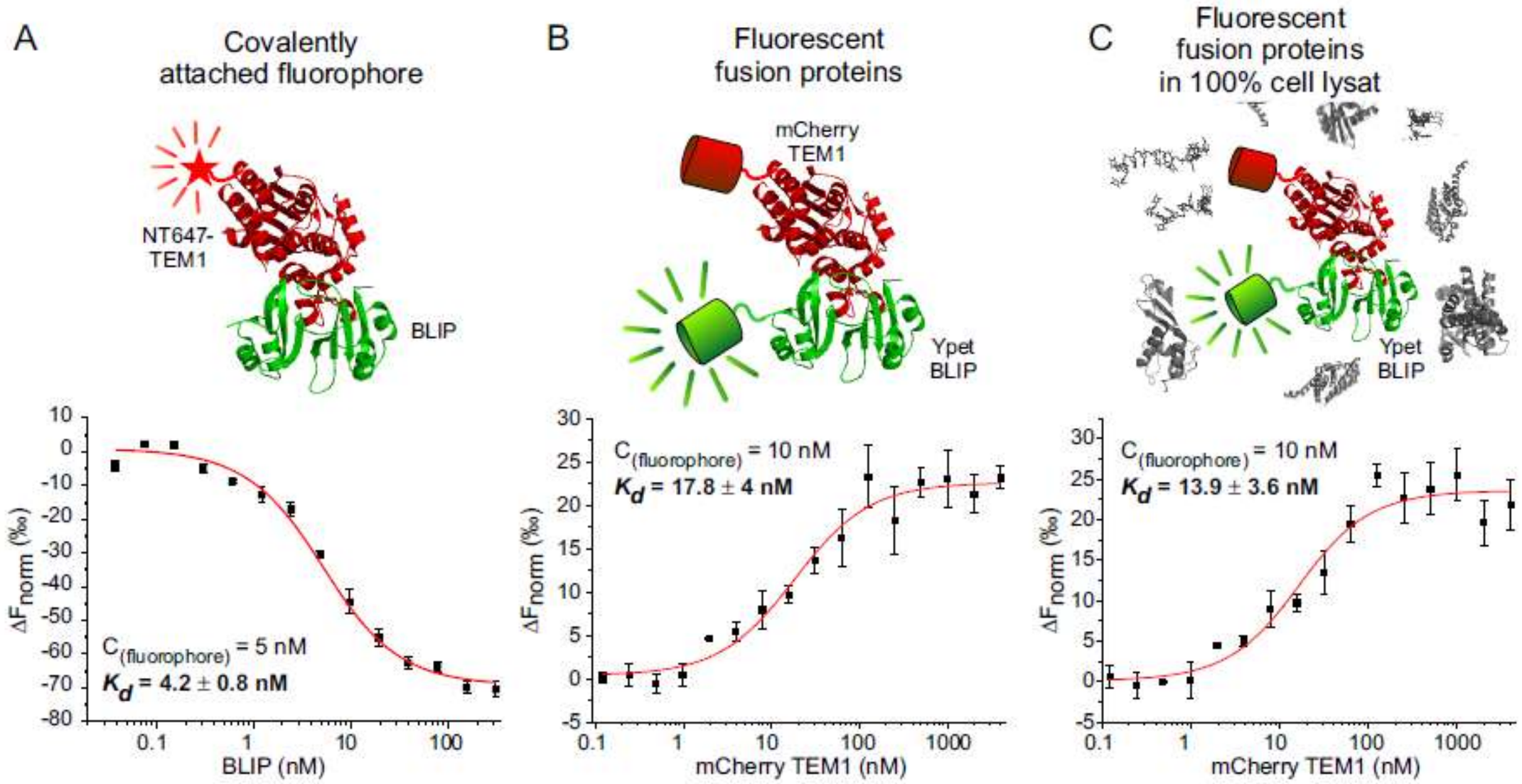
Competitive Assay with Compounds



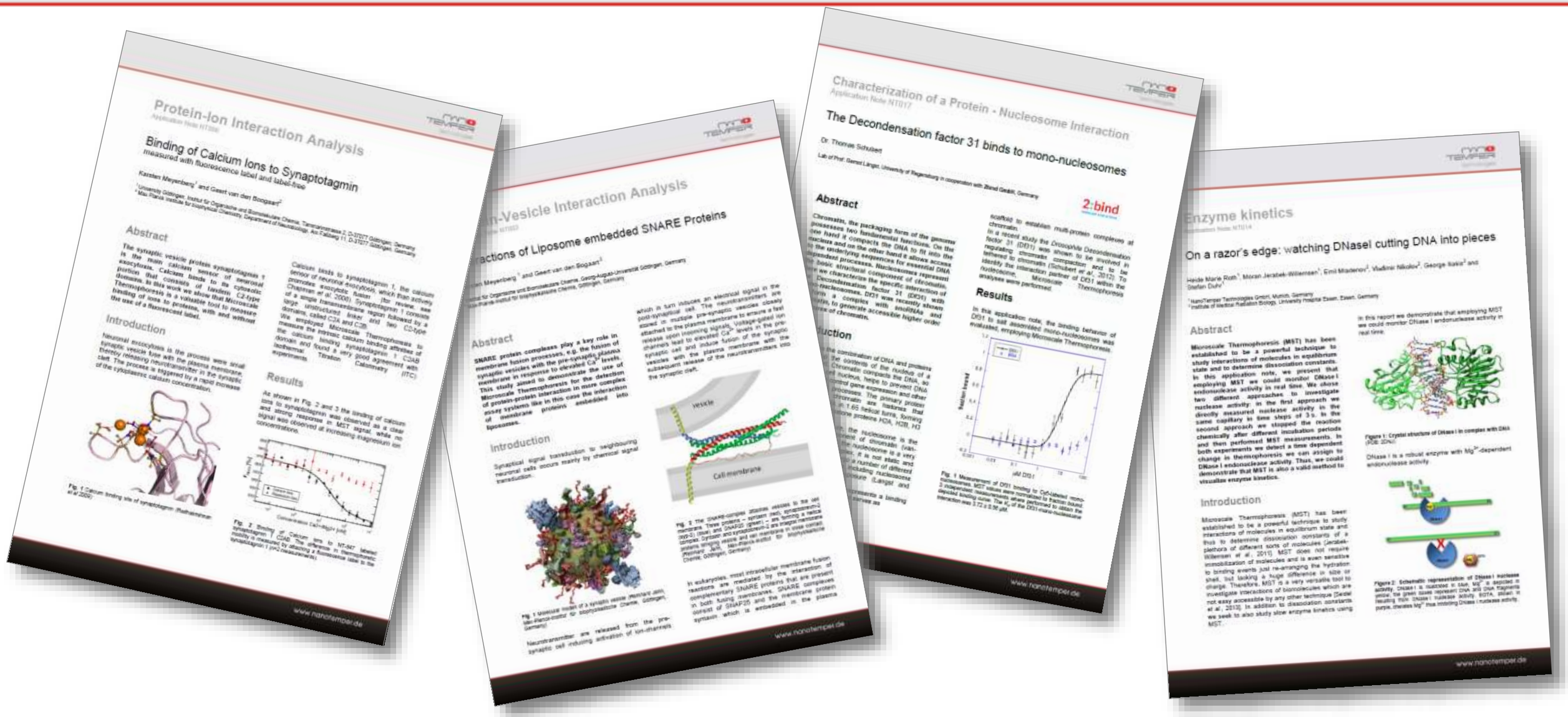
Material was kindly provided by Dr. Krishna Saxena, University Frankfurt, Germany

- **Competition experiments are essentially label-free and site-specific**

BLIP – TEM1 Interaction studies



MST Application Notes



… and many more! www.nanotemper-technologies.com/applications

Selected MST Publications

Nature: “Molecular basis of nitrate uptake by the plant nitrate transporter NRT1.1.” Parker and Newstead, Nature, **2014**, 507, 68-72

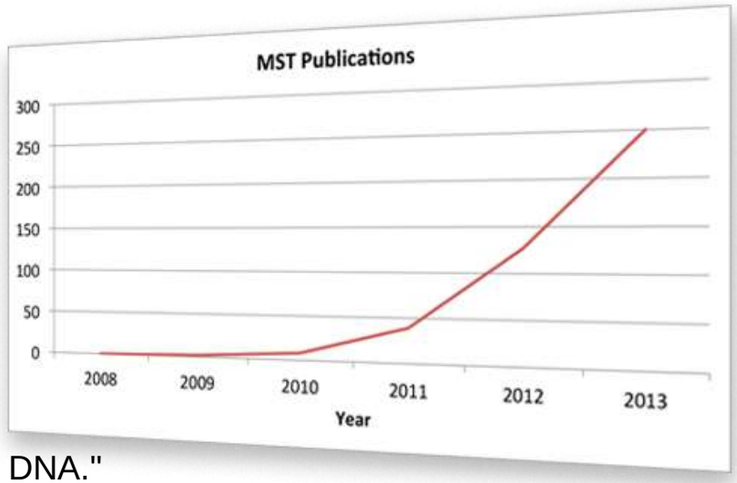
RNA Biology: “Microscale thermophoresis provides insights into mechanism and thermodynamics of ribozyme catalysis.” Gaffarogullari E. C., *et al*. RNA Biology, **2013**, 10, 1-7

Methods: "Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions." Seidel S. A., *et al.* Methods, **2013,** 59, 301-315

Nature: "Receptor binding by a ferret-transmissible H5 avian influenza virus." Xiong X., *et al.* Nature, **2013,** 497, 392-396

Cell: "Inhibition of basal FGF receptor signaling by dimeric Grb2." Lin C. C., *et al.* Cell, **2012,** 149, 1514-1524

J Biol Chem: "Mechanisms of unphosphorylated STAT3 transcription factor binding to DNA." Timofeeva O. A., *et al.* J Biol Chem, **2012,** 287, 14192-14200



… and many more! www.nanotemper-technologies.com/publications/

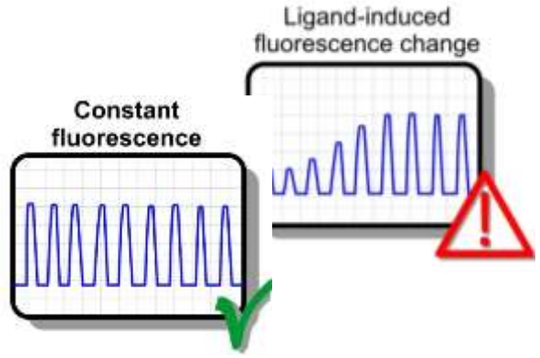
Overview of Topics

Assay Setup

- Sample labeling and important pre-tests
- Serial dilution and loading of capillaries
- NT.Analysis and NT.Control Software

Assay optimization

- Aggregation
- Fluorescence changes
- Noise level



General Remarks on Sample Handling

- Concentration of **fluorescent molecule**: in the same range or lower than the expected K_d
- Highest concentration of **ligand**: ≥ 20 fold above the expected K_d
- Final **sample volume** per titration point: ≥ 20 µl
- Use **small tubes** for the serial dilution: e.g. PCR strips, tubes
- Dilution buffer **should not vary in composition** in serial dilution: e.g. DMSO
- **Accurate pipetting** is essential
- **Mix with pipette** instead of vortexing
- Do not touch **capillaries in the center**

Monolith NT.115:

- Label one binding partner using a NT fluorescent dye
- Expression of a fusion protein (GFP, YFP…)
- Synthetic fluorescent samples (nucleic acids, peptides)
- Different methods: *in vitro* translation, incorporation of unnatural amino acids…

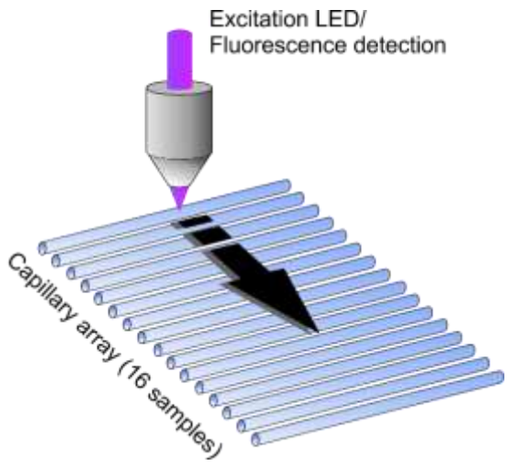
Protein labeling:

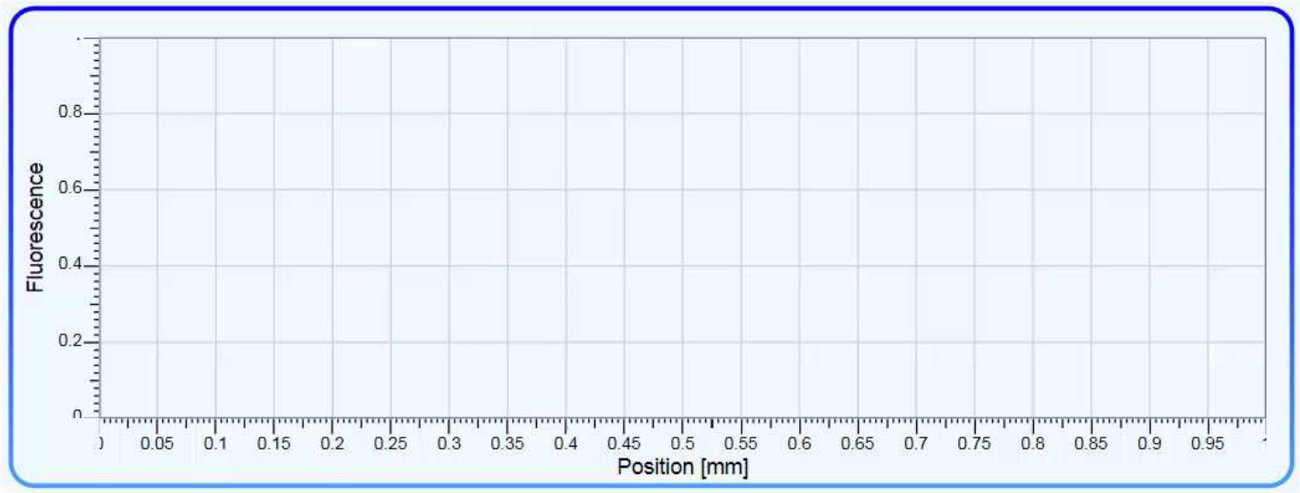
- NanoTemper Protein Labeling Kits are optimized for MST
- Use only high purity samples (>95%)
- Amine-reactive vs. cysteine-reactive labeling
- Efficient removal of free dye

Aim

- Check protein labeling efficiency
- Select proper capillary (Standard treated, Hydrophilic, Hydrophobic and Premium coated)

The Fluorescent Molecule: Cap Scan





Fluorescence intensity: 200 – 1500 counts

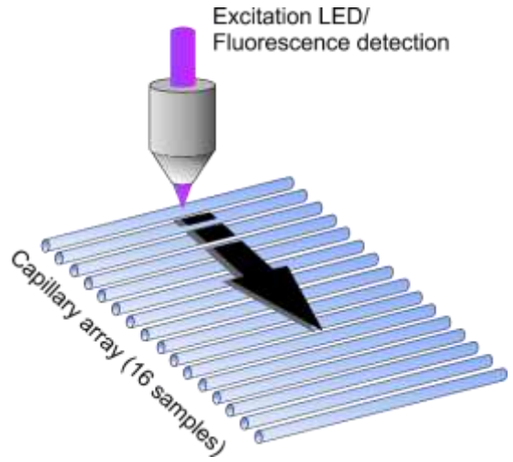
Fluorescence signal < 200 counts:

- Increase LED power
- Increase fluorophore concentration
- Optimize labeling efficiency

Fluorescence signal > 1500 counts:

- Decrease LED power
- Dilute sample
- Check for presence of free dye

The Fluorescent Molecule: Cap Scan



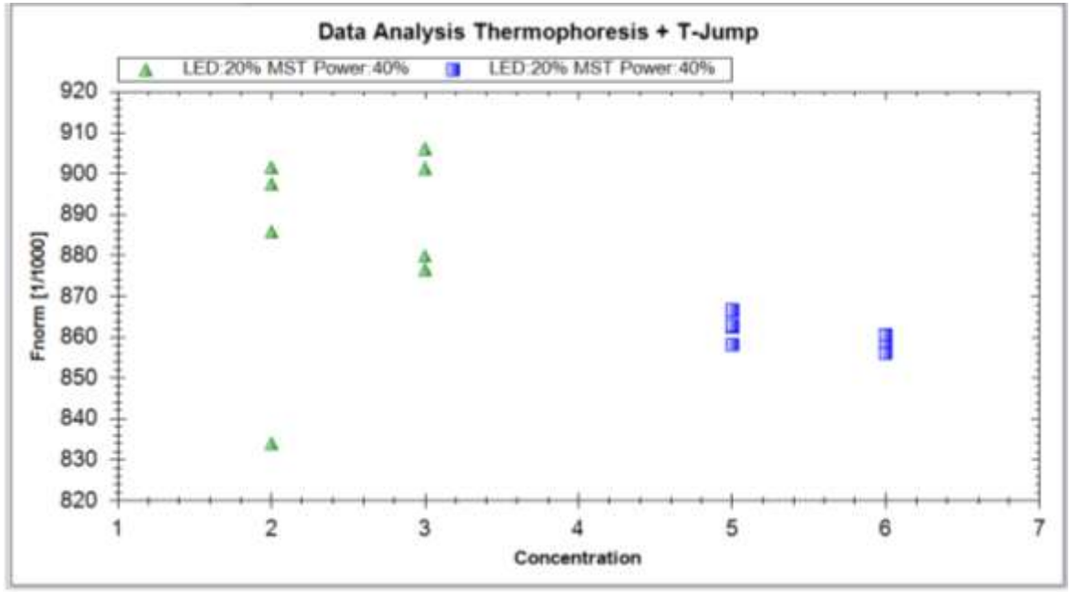
Split peaks or shoulders:

- Change capillary type

(Standard or Premium coated)

- Optimize buffer

Capillary and Buffer Selection



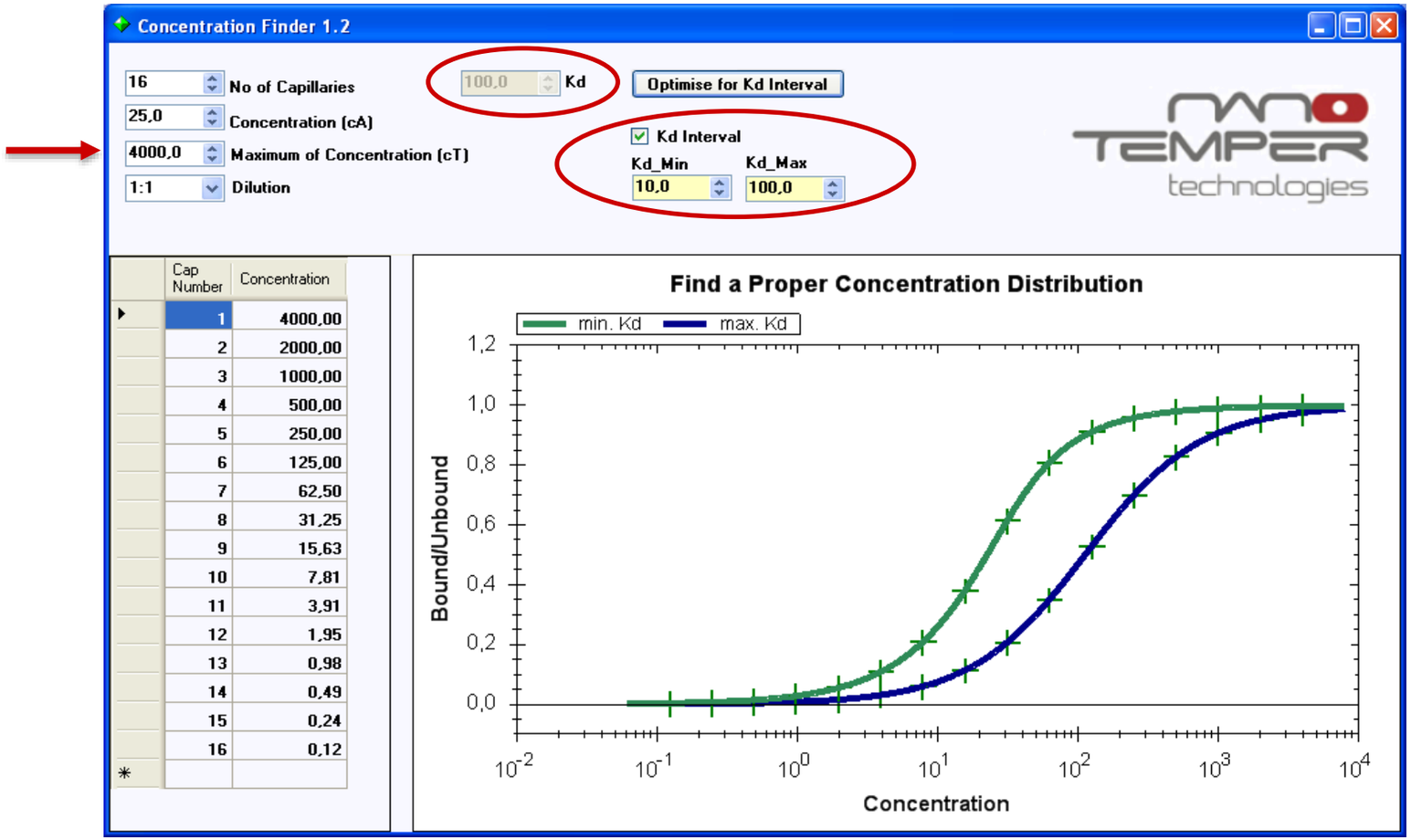
Capillary: std. premium std. premium

Assay buffer

MST optimized buffer

- Load same sample in your assay buffer (4 capillaries each) and measure with 40 % MST power
- Noise level ≤ 6-8 units
- Test different types of capillaries
- Test/improve buffer conditions
  - By adding detergents such as Tween 20, Pluronic-F127,…
  - By changing pH or ionic strength…

The Ligand: Concentration Finder Tool

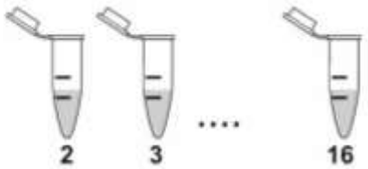


- Note: This tool can be accessed *via* NT.Control Software or NanoTemper website

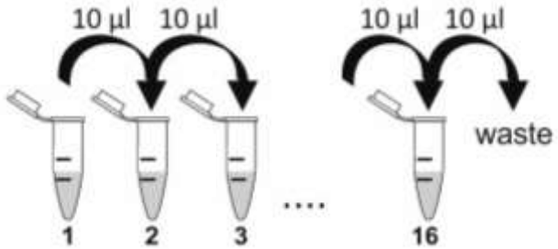
Ligand

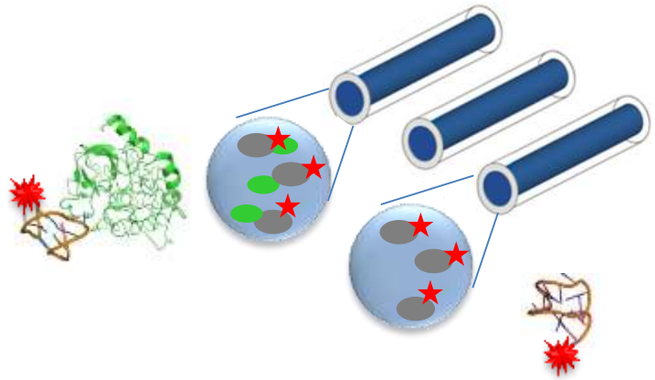
20 µl

10 µl buffer



Ligand dilution

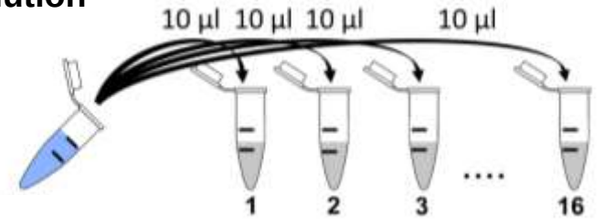




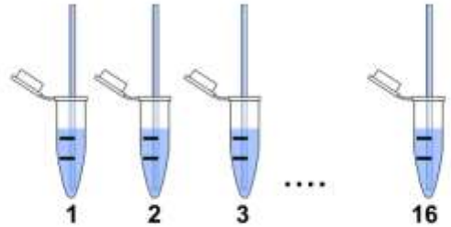
Fluorescent Molecule

200 µl stock

Serial dilution

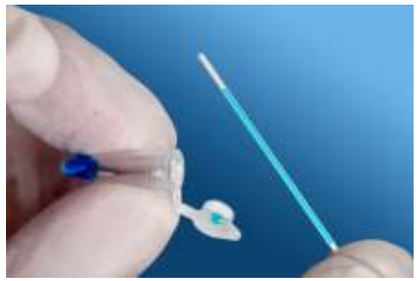
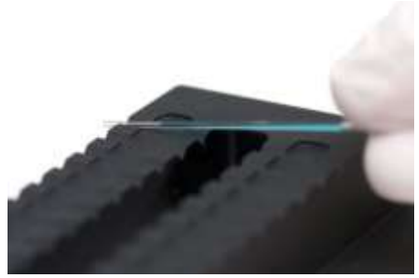


Fill capillary



Sample Loading

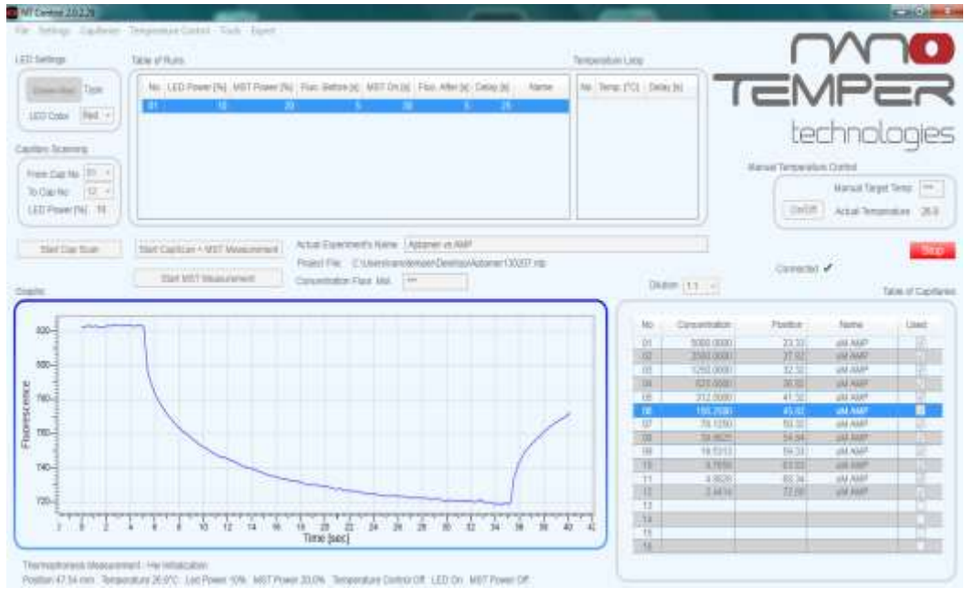




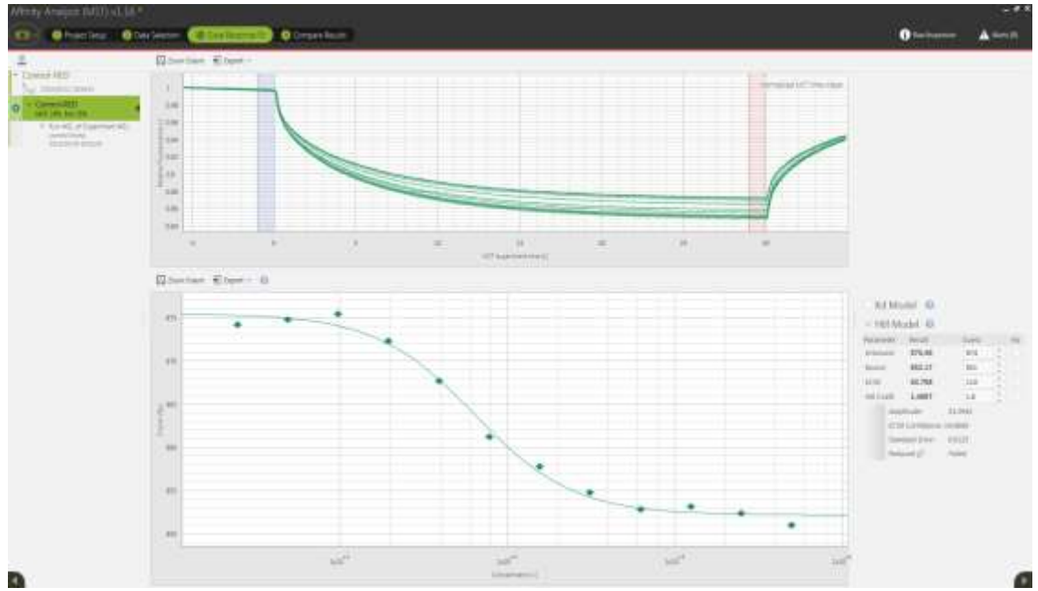
Software and Data Analysis - Overview

Running MST Experiments: NT.Control Software



- Adjust LED power to excite the fluorescent molecule
- Set MST power to induce the temperature gradient

Analyzing MST Data: NT.Analysis Software



- Fit curves to determine K_d or EC_{50}
- Export your data

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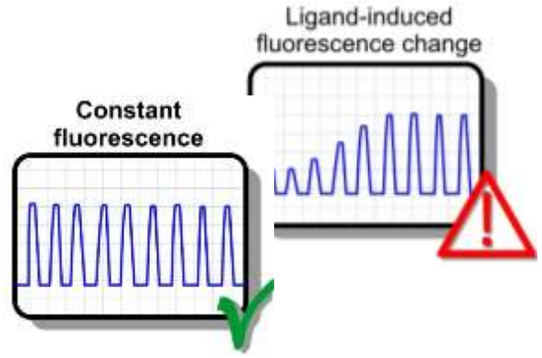
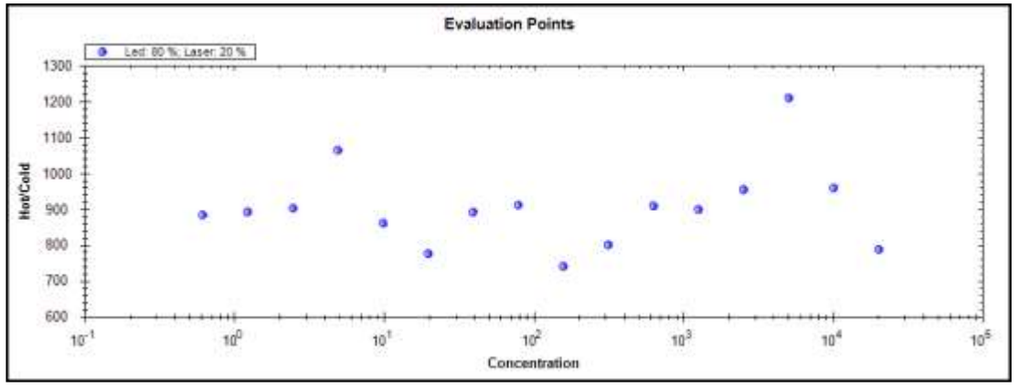
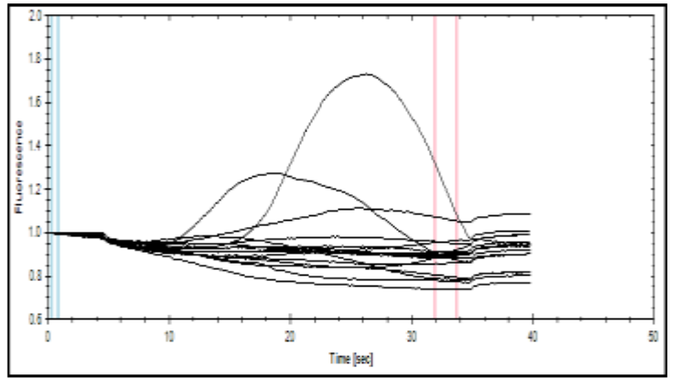
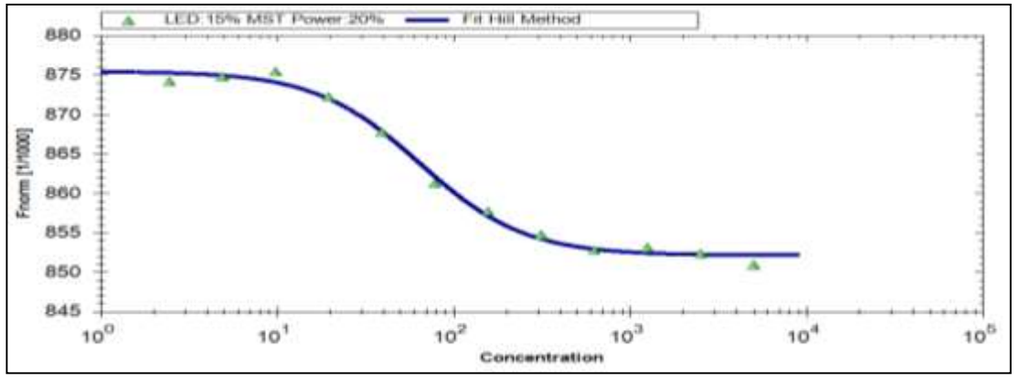
- Aggregation
- Fluorescence changes
- Noise level

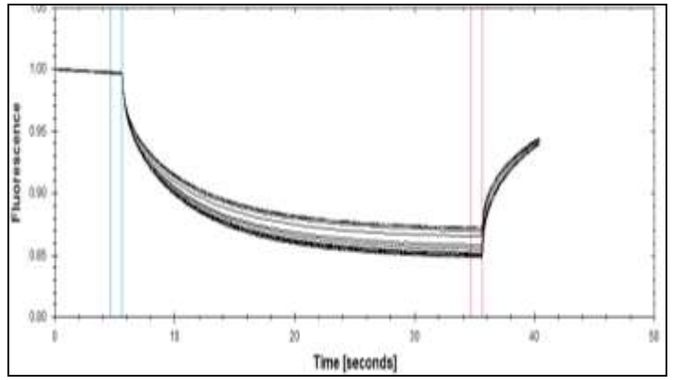


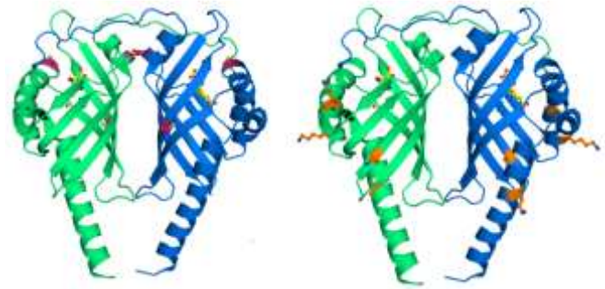
Labeling Strategy and Labeling Position







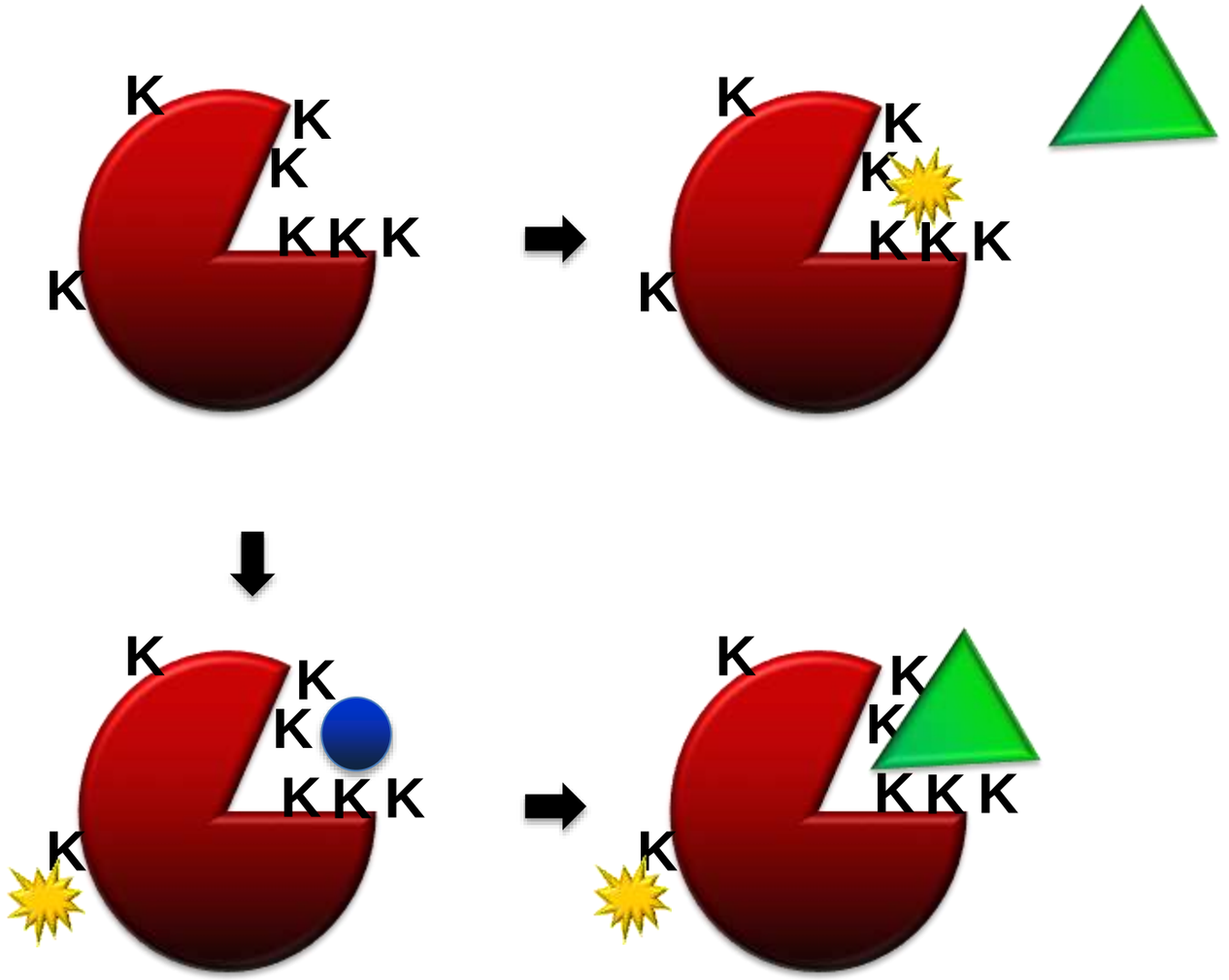




- 4 lysines are involved in binding

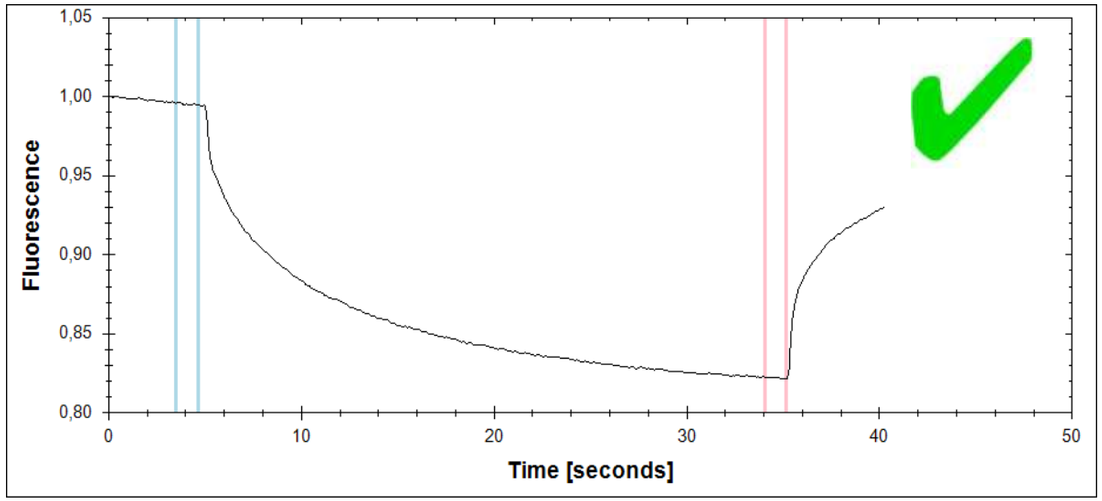
Labeling Strategy and Labeling Position

- Alternative labeling strategies
  - Different coupling chemistry (maleimide labeling)
  - Different dye
  - Fusion protein (GFP, YFP,…)
  - **Protection of binding site**
  - Site-specific label (unnatural amino acids, *in vitro* translation...)
- Alternative assay strategies
  - Competition experiment

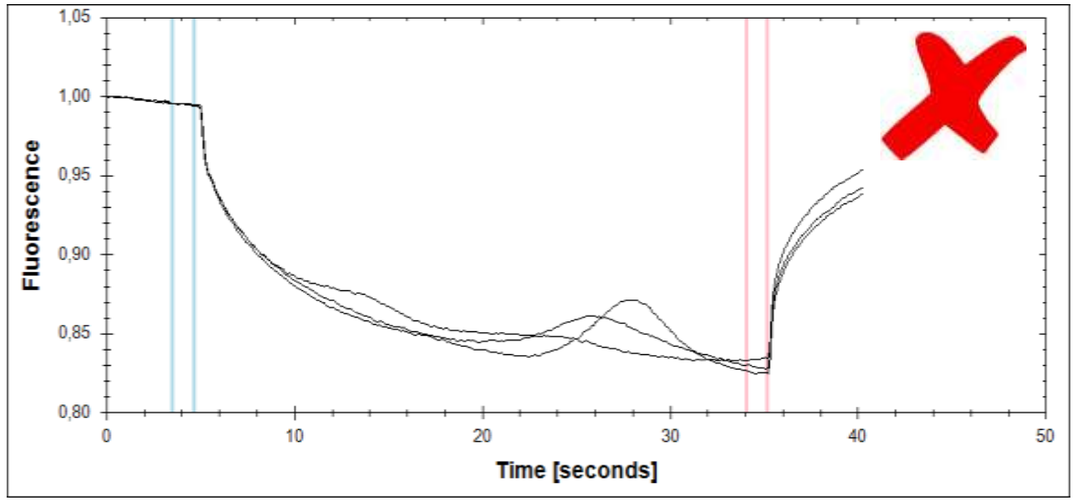


Aggregation

No aggregation



Aggregation



Buffer optimization is necessary:

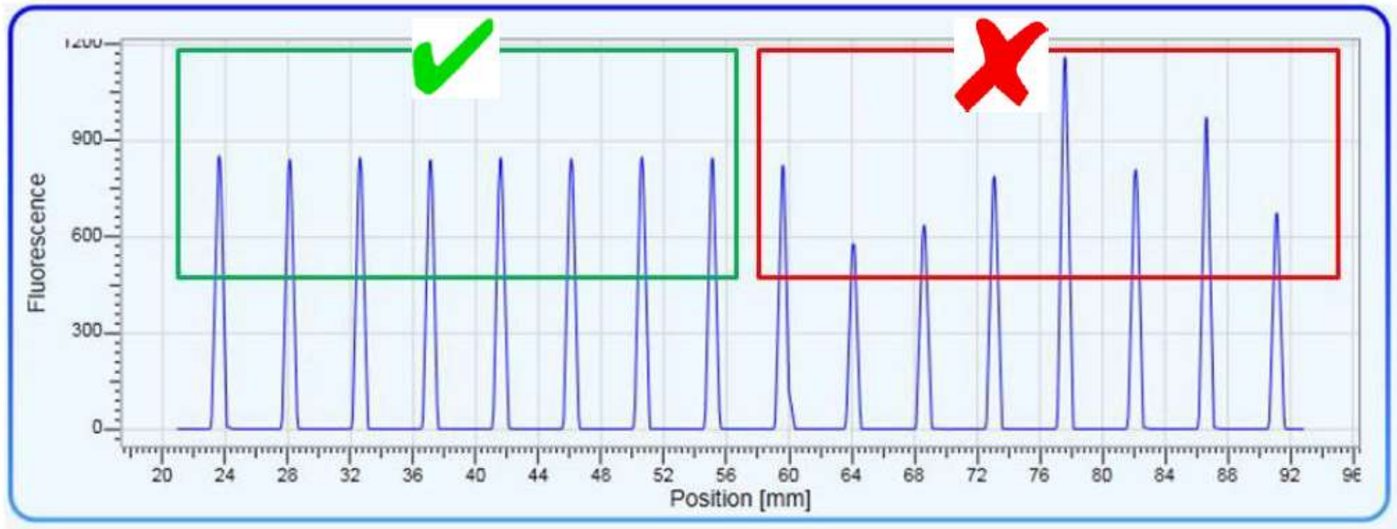
- Detergents and additives (Tween-20, Pluronic F-127, BSA etc.)
- Centrifugation
- pH, ionic strength …
- Concentration of organic solvents (DMSO)

Random Fluorescence Changes

Random fluorescence changes

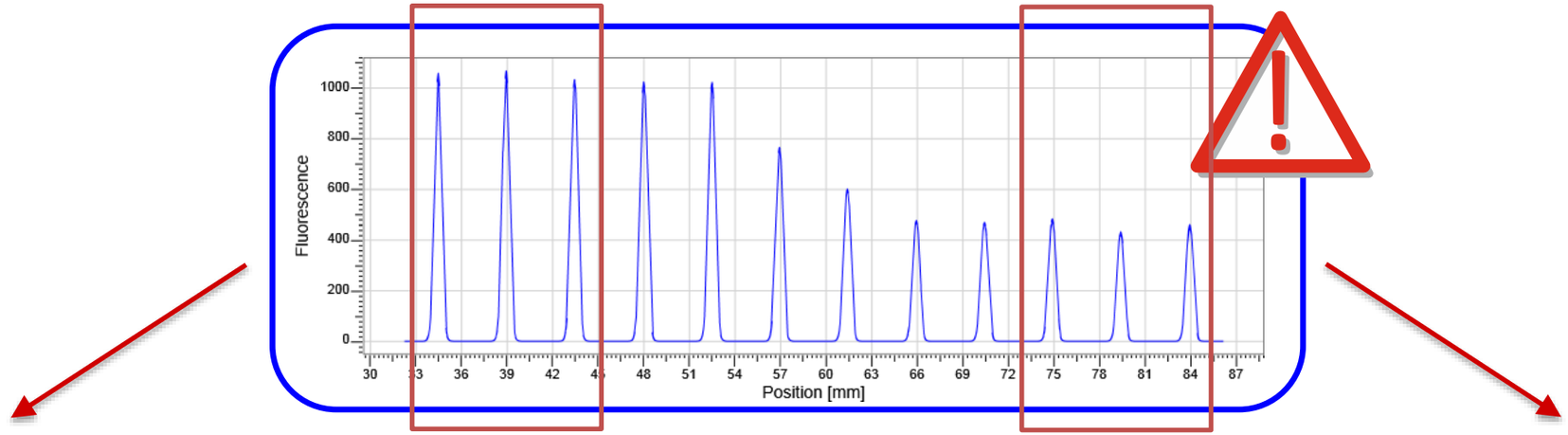
- Inaccurate pipetting
- Low sample quality

- Leads to increased noise level
- Buffer optimization necessary



< ±10 % **> ±10 %**

Ligand-induced Fluorescence Changes

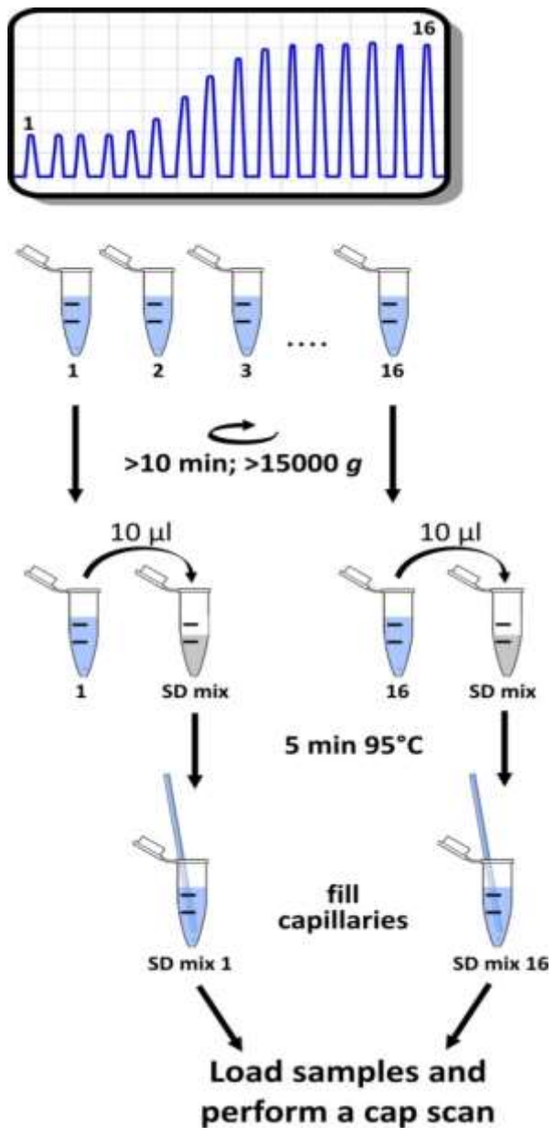


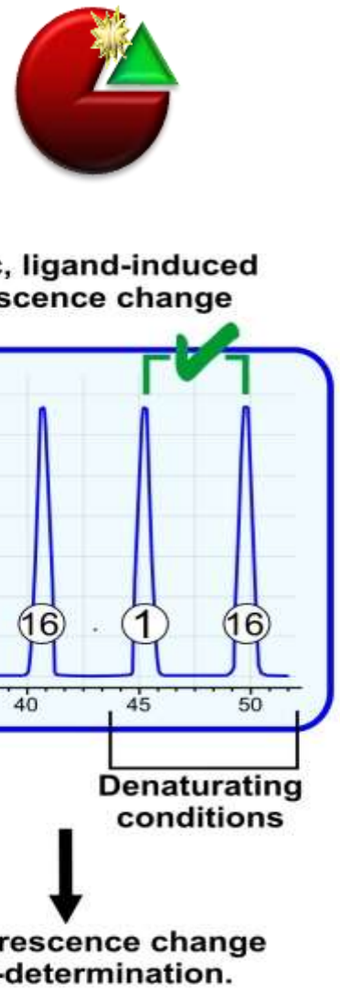
- Fluorescence quenching or enhancement induced by ligand binding

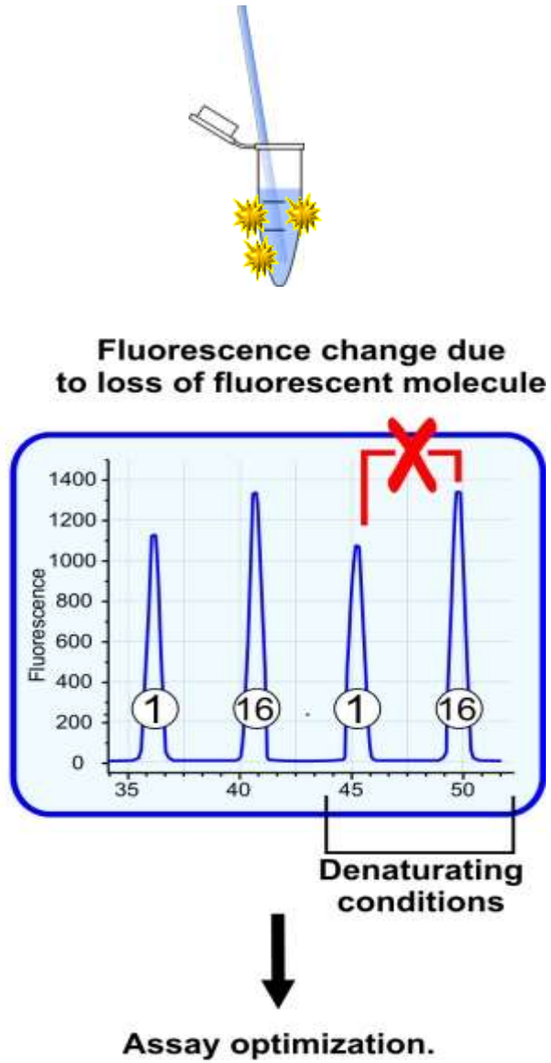
- Ligand-induced surface adsorption or
- Aggregation

→ Perform SDS-denaturation (SD) test

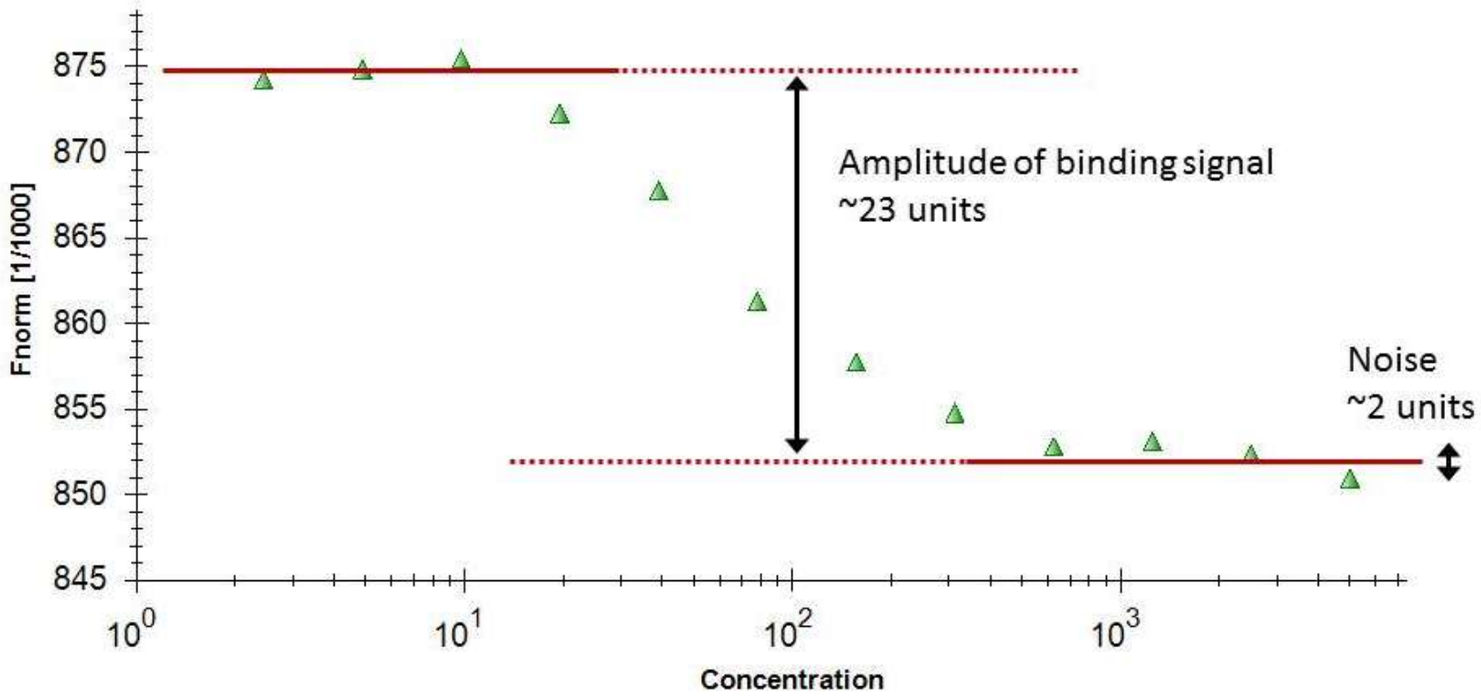
Binding or Material Loss? How to perform the SD-Test





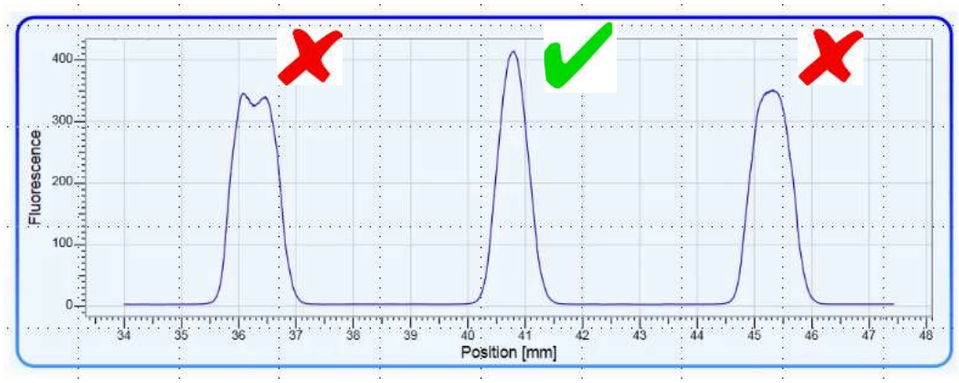


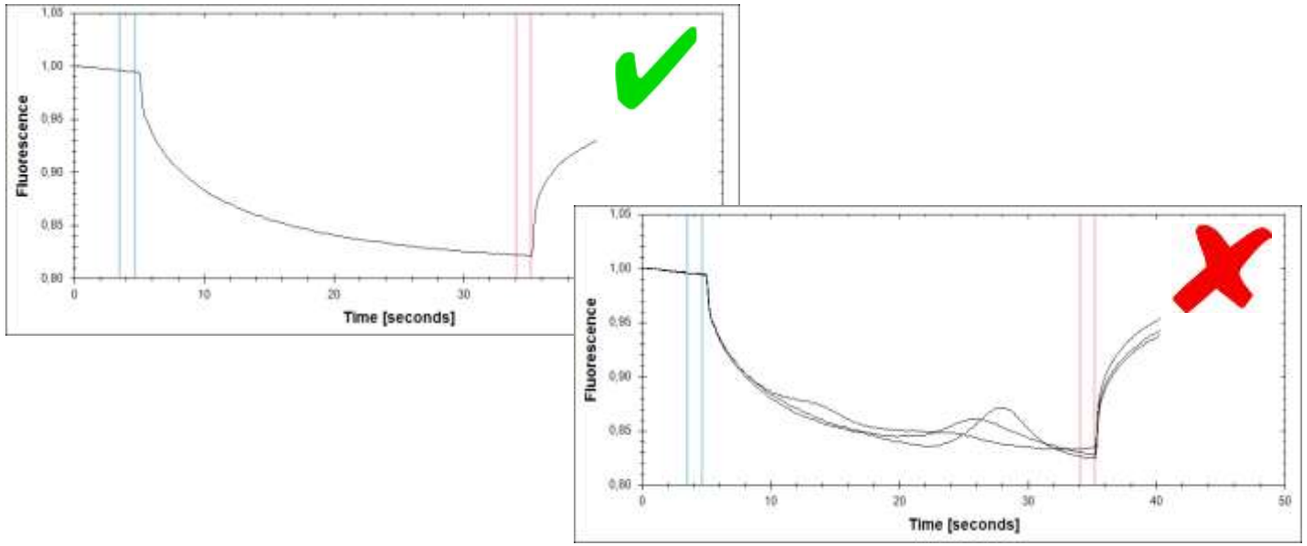
What is an acceptable Noise Level?

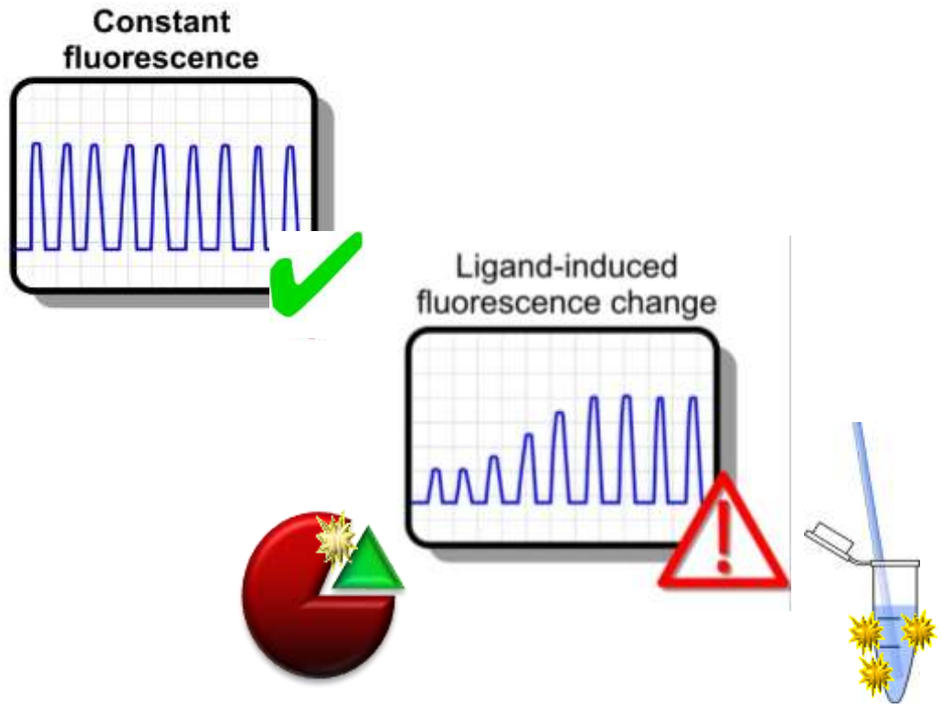


- Amplitude should be at least 3-fold above the noise level

Assay Optimization – Summary

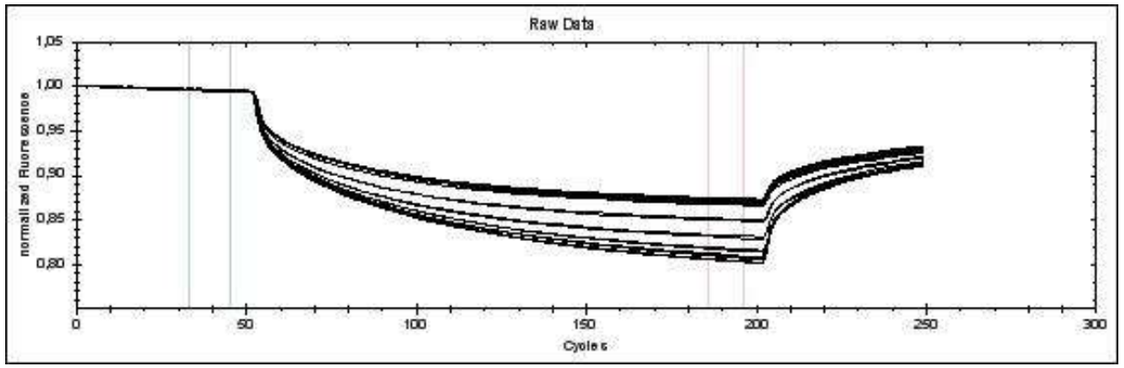




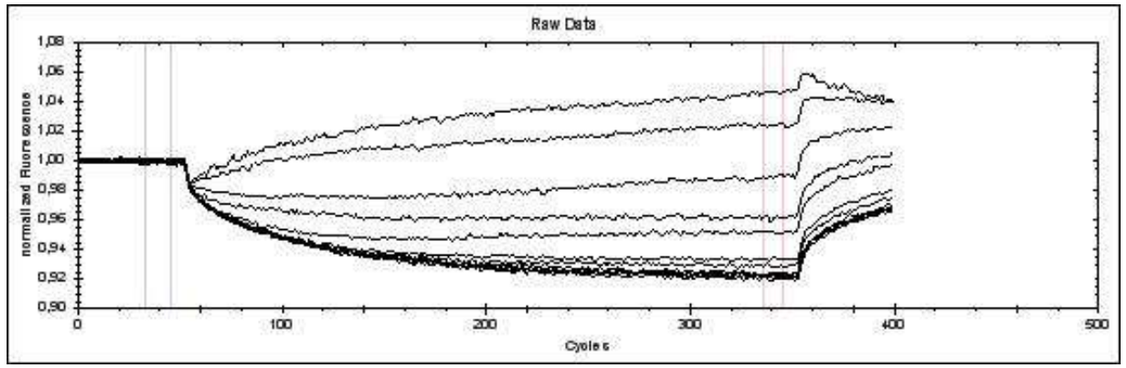


- Easy and straight forward assay optimization

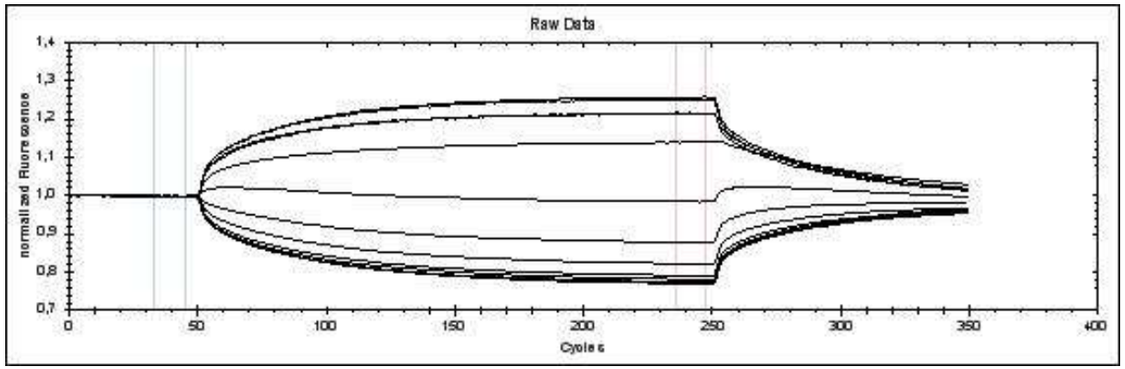
T-Jump (+) and thermophoresis (+)



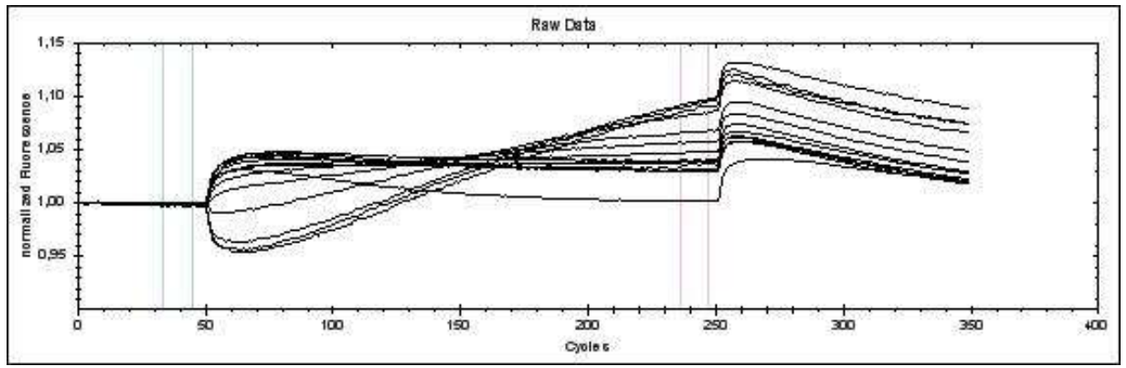
T-Jump (+) and thermophoresis (+/-)



T-Jump (+/-) and thermophoresis (+/-)



T-Jump (+/-) and thermophoresis (+/-)



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