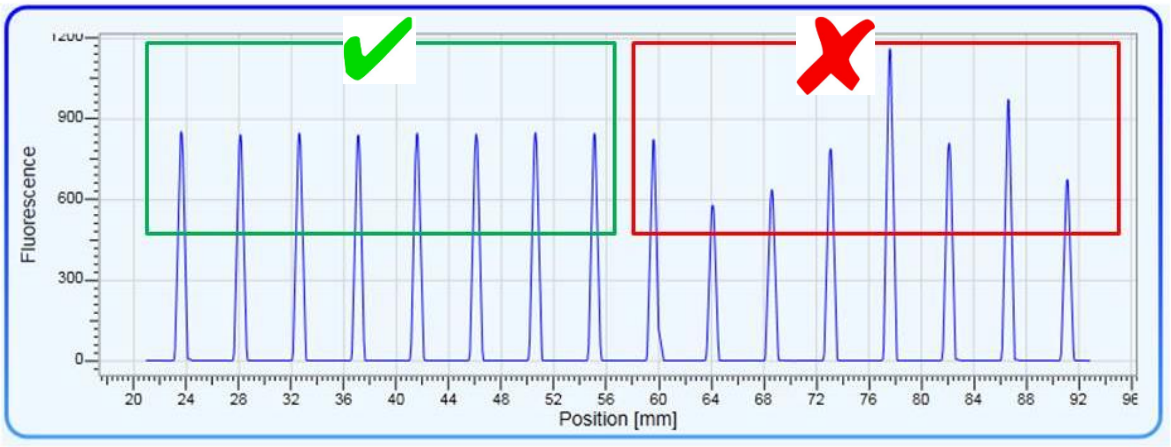
FAQ: Fluorescence changes are observed in the capillary scan using the Monolith NT.115.

Different causes of fluorescence changes
How can I try to improve the assay?
What control experiments do I have to perform?
Can I use the data if a binding event results in a fluorescence change?

Fluorescence should be identical in all capillaries since you add the same amount of fluorescent molecule to each binding reaction. However, two types of fluorescence changes can occur:

Random fluorescence changes
Ligand dependent fluorescence changes

Random fluorescence changes:



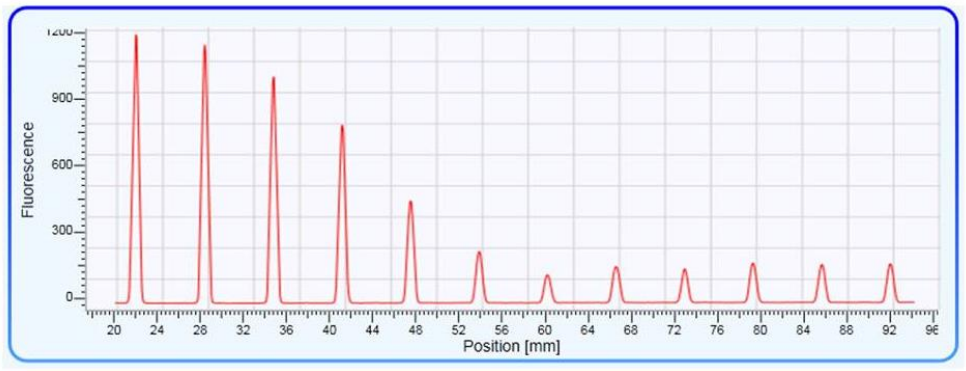
≤ ±10 % **> ±10 %**

What are the reasons for these fluctuations in the fluorescence signal of more than 20%?

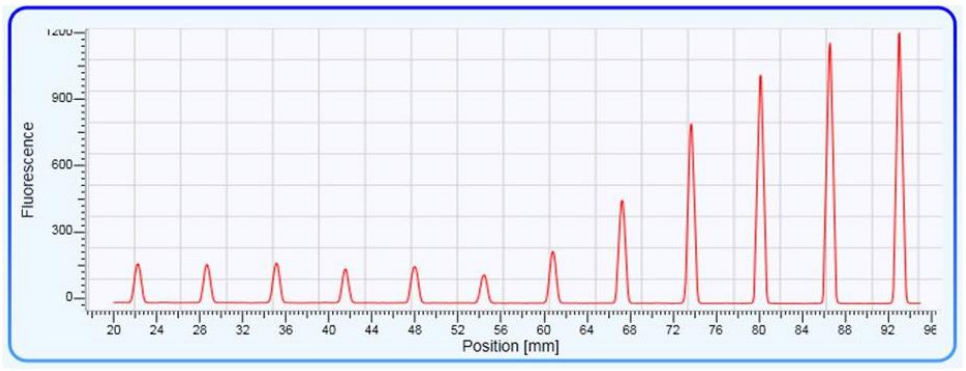
1. Poor sample quality*: The buffer is not optimal for the protein leading to slight aggregation, varying amount of sample (material might be lost during sample preparation due to adsorption to pipet tips and plastic micro reaction tubes) or partial unfolding.
2. Poor mixing and/or pipetting:
   - Make sure that your pipettes are working properly and recently calibrated.
   - Mix your samples thoroughly by pipetting several times up and down with at least half of the total volume.
   - Do not vortex or snip/flick your sample.
   - Ensure that no liquid is lost inside and outside of the tip.
   - Avoid air bubbles.
   - Do not prepare less than a 10 µl ligand + 10 µl fluorescent molecule reaction*.

*) For assay optimization please also refer to the Monolith NT.115 Starting Guide.

Ligand dependent fluorescence changes (the fluorescence signal changes with the concentration of the unlabeled molecule):



high concentration low concentration



high concentration low concentration

What are the reasons for a ligand dependent change in fluorescence?

- Quenching or enhancement of the fluorescence intensity upon binding.
- Unspecific adsorption to capillaries and/or plastic micro-reaction tube walls
- Aggregation of the fluorescent molecule upon addition of the ligand

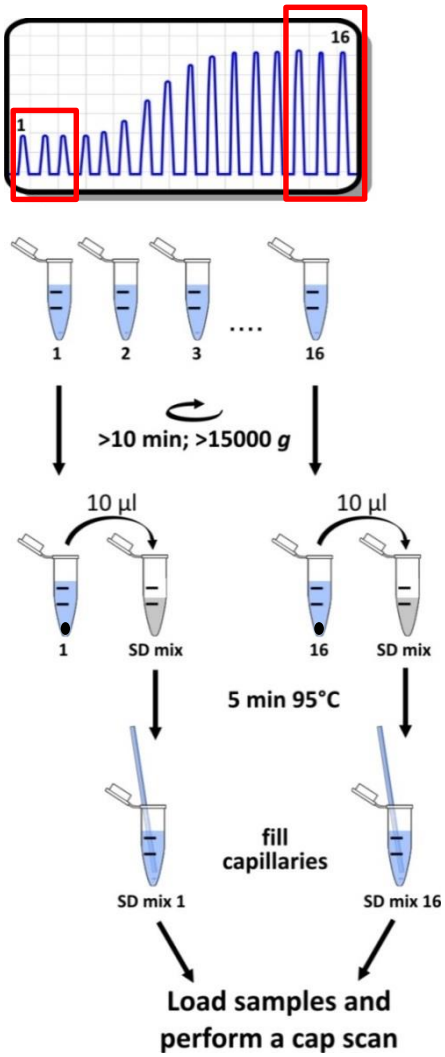
If you observe ligand dependent fluorescence changes, you need to rule out any material loss caused by unspecific adsorption at capillary/tube walls or due to aggregation, since this causes false positive results.

- If there are any indications for sticking of your sample to the capillaries (split peak or shoulders of the peaks in the capillary scan), you should try a different type of capillary.*
- If there is no sticking to the capillaries or the fluorescence changes persist even after changing to a different capillary type, please perform the SDS-denaturation test (SD-test) as described to confirm the reasons for the fluorescence changes observed.

IMPORTANT: The SD-test does not work for fluorescent fusion proteins such as GFP or YFP!

*) For assay optimization please also refer to the Monolith NT.115 Starting Guide

SD-Test Protocol:



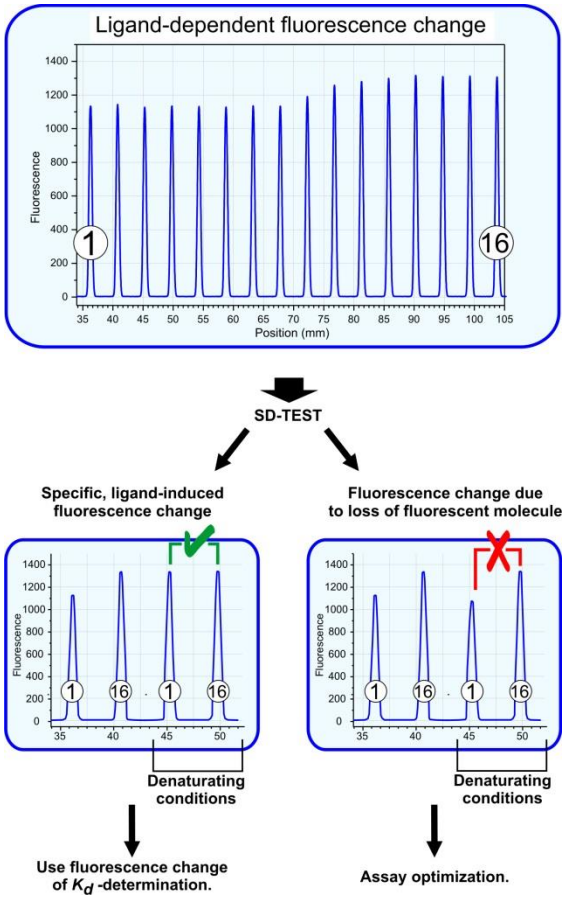
1. **Centrifuge tubes 1-3 and 14-16 for at least 10 min at ≥15,000 *g***
2. **Prepare six tubes, each containing 10 µl of a 2 x SD mix (4 % SDS, 40 mM DTT).**
3. **Carefully remove 10 µl* of supernatant from assay tubes 1-3 and 14-16 and transfer to the tubes containing the SD-mix. Mix well. Incubate for 5 minutes at 95 °C to denature the samples.**
4. **Briefly centrifuge the tubes to ensure all the sample is at the bottom of the tube.**
5. **Fill all samples into 2 standard capillaries each and measure the fluorescence intensity.**

Note: *It is essential to ensure that none of the pellet after centrifuging is transferred to the tubes with SD mix. If the pellet is disturbed, centrifuge again for at least 10 min at ≥15,000g.*

Note*: The SD test cannot be performed with samples containing potassium (200 mM or more) because the SDS will precipitate.*

Note: *If less than 10 µl remains, use equal volumes of supernatant and SD mix for the test.*

Interpretation of SD-test Results:



Capillary scans of the SD-test to discriminate between ligand induced quenching and sample loss due to aggregation/adsorption.

After denaturing the samples from tube 1-3 and 14-16, the fluorescence intensity is analyzed by performing a capillary scan.

If the fluorescence intensities for both capillaries (containing samples from tubes 1-3 and 14-16) are identical, it can be concluded that the previously observed fluorescence changes were induced by a binding event. The denaturation process disrupts the binding of the ligand to the fluorescent molecule, thus its fluorescence is restored. In this case you can analyze your data by directly using the binding information deduced from the fluorescence intensity changes.

If you observe a difference in fluorescence intensity for tubes 1-3 and 14-16, material was lost either by aggregation and subsequent centrifugation or by unspecific adsorption to the tube walls. In this case, you cannot use your data and you need to optimize your assay conditions.

To avoid unspecific adsorption of the sample to tube walls, you should use our MST optimized buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.05 % Tween-20). Alternatively, add passivating agents such as BSA (0.5 mg/ml) or different detergents such as Pluronic-F127 (0.1 %) to your assay buffer. Low binding tubes and pipette tips can also help to avoid sticking of molecules to tube walls/tips.

To prevent the formation of aggregates, improve your buffer conditions by adding detergents or additives that stabilize your molecules, by changing the pH or by changing the ionic strength*.

*) For assay optimization please also refer to the Monolith NT.115 Starting Guide.

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