Fidabio

ASSAY OPTIMIZATION MANUAL

BY FIDABIO

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Introduction

Fida 1 is a powerful tool which delivers a range of biophysical parameters from a single assay format. It provides measurements of absolute hydrodynamic radius (R_h), affinity constants (K_D) and insights into complex bindings, ternary complex formation, stability, oligomeric state, and polydispersity. In addition to these measurements, the raw FIDA data also provide critical supplementary information, including details about unreacted fluorophore, protein aggregation, buffer mismatch, viscosity changes, and protein stickiness. Unlike other technologies where such sample attributes cause difficulties in assay designing and data interpretation, FIDA spots these issues and thereby provides users with the opportunity to strategically resolve them.

This document provides a compilation of tips and tricks that enable the user to get the most out of their Fida 1.

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1. The FIDA solution to sticky proteins

Techniques involving the use of low protein concentrations often face the challenge of sample adsorption or stickiness to the apparatus surface. FIDA is recognized as a method where the stickiness of proteins -when present- will not go unnoticed and can be accounted for.

1.1 Detection of indicator adsorption

Assays performed at low nanomolar protein concentrations are often impacted by sample loss due to passive adsorption to glass/plasticware, ultimately leading to a reduction in fluorescence signal. FIDA is a great tool to detect this tendency of molecules and thus enables the user to take appropriate measures to prevent it.

Below is a suggested method that can be used to spot adsorption of the indicator:

01

Step 1

Determine the degree of labelling (DoL) of the sample being tested. DoL should ideally be less than 0.8.

02

Step 2

Prepare three different test samples of the indicator at 25 nM diluted in:
a) standard buffer (e.g., PBS, HEPES, Tris)
b) standard buffer containing 0.01-0.03% pluronic acid F127 (or Tween-20 0.02%)
c) standard buffer containing BSA 0.5 mg/ml

03

Step 3

Perform FIDA measurements with all 3 samples and monitor the signal-to-noise (S/N) ratio. A significant difference in intensity between a, b and c may indicate the tendency of the indicator to undergo passive adsorption.

Note: Reagents like pluronic acid, Tween-20 and BSA act as surfactants and aid in preventing sample adsorption to the surface. These reagents may also diminish the tailing effect of FIDA taylorgrams (discussed in paragraph 1.2).

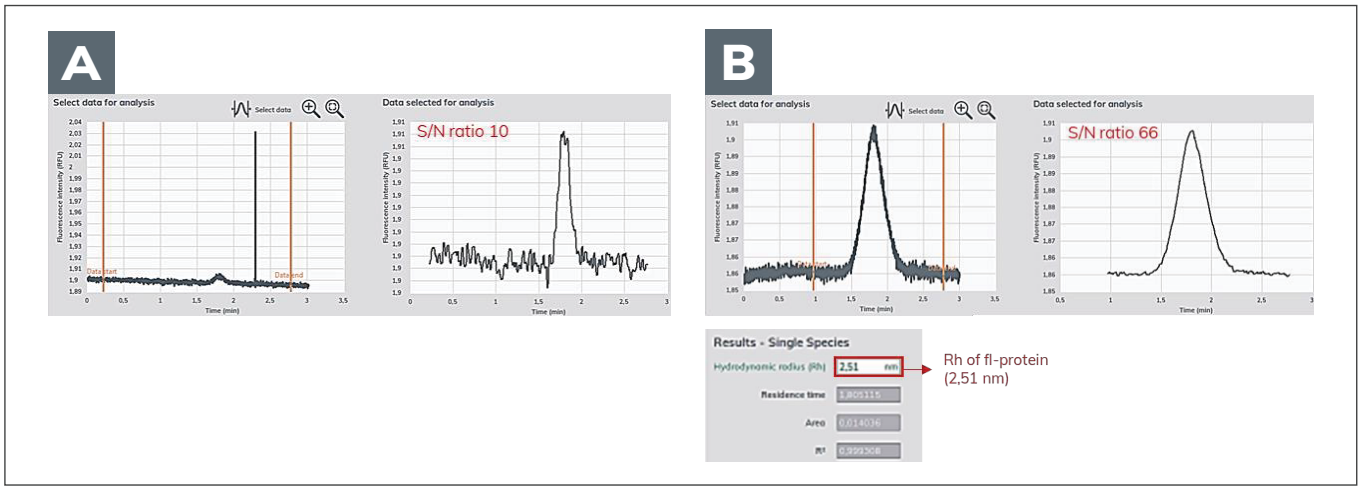


Figure 1. A. An example of a labelled protein demonstrating adsorption effect.
B. Addition of pluronic acid to the assay buffer prevented sample loss due to adsorption.

1.2 Observation and elimination of surface adsorption

In FIDA, the radial diffusion of the sample leads to a symmetric Gaussian distribution of the indicator which is fitted with a Gaussian function during analysis. In cases where the indicator/complexes stick to the wall of the glass capillary, a clearly visible tailing effect is observed at the end of the taylorgram peak (Table 1). In the Fidabio data analysis method, 75% of the raw signal peak is used by default to perform size estimation enabling tolerance towards mild stickiness/tailing effect. In cases of stronger stickiness/tailing, Fidabio offers dynamic and permanently coated capillary solutions to counteract the adsorption.

Coated and Uncoated capillaries must be tested empirically to determine which conditions work best for a specific system (Figure 2).

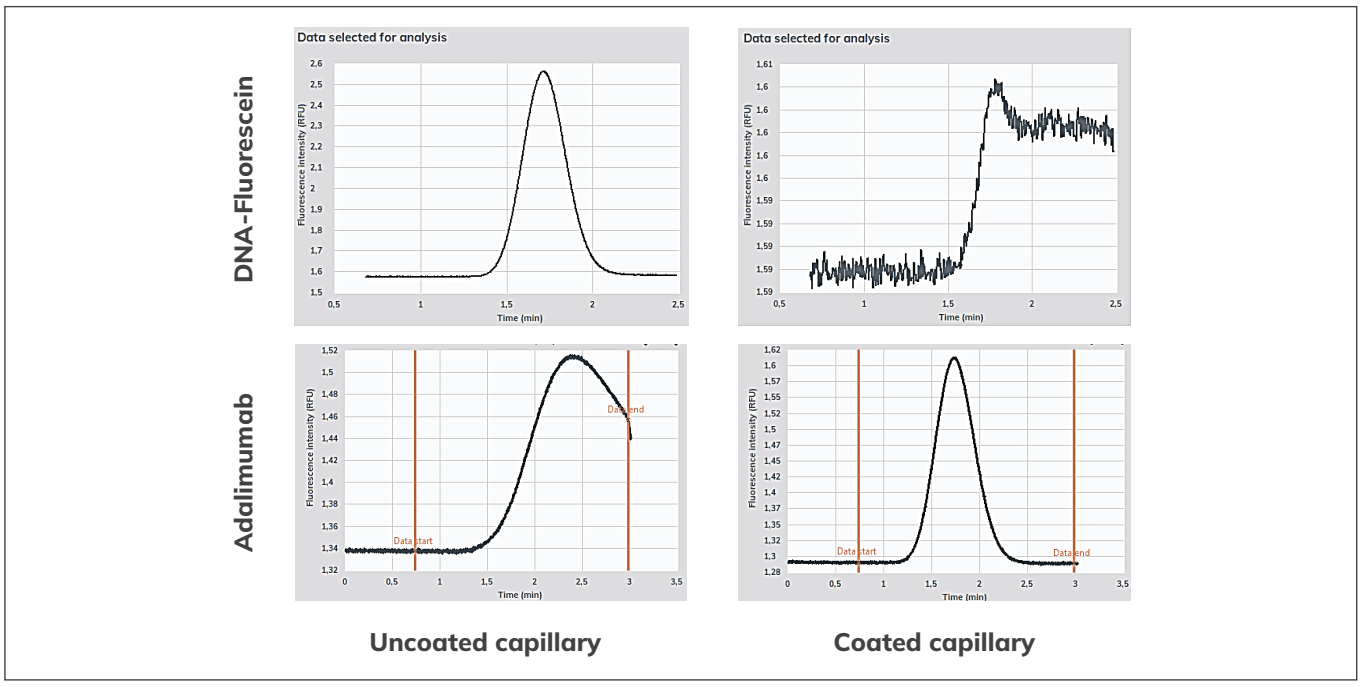
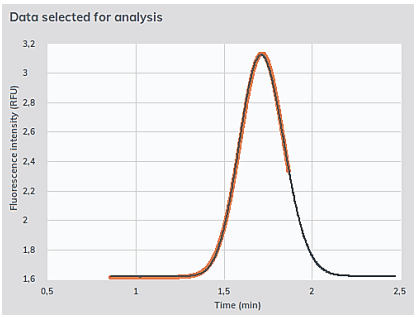
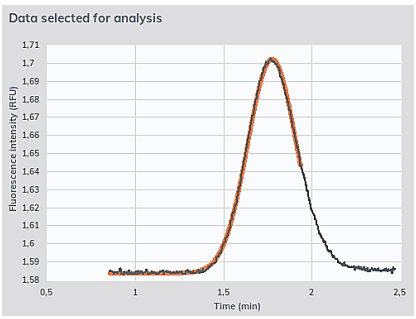
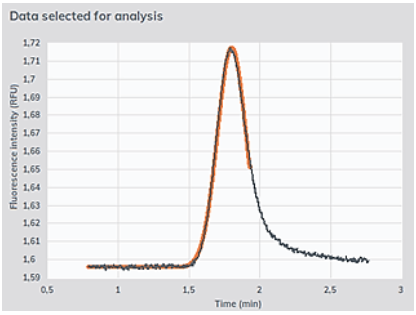
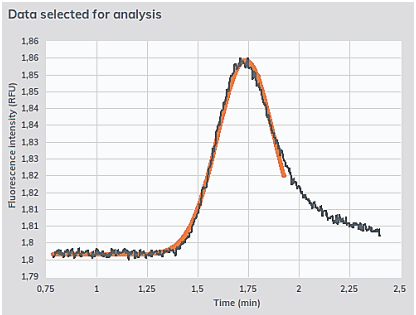
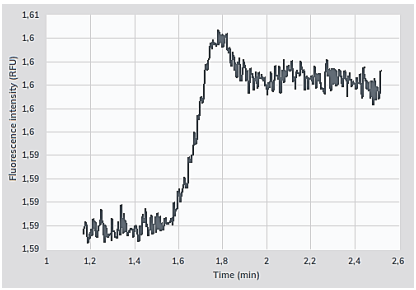


Figure 2. Signal differences of the same sample in Coated and Uncoated capillary.

Table 1 highlights a few examples of commonly encountered stickiness/tailing scenarios and suggestions on how these can be effectively resolved.

Table 1. Frequent cases related to sample stickiness.

Taylorgram	Signal	Solution
	**Perfect signal**	No action needed. Can be used for accurate size estimation.
	**Nearly perfect signal** **Minor tailing**	No action needed. Can be used for accurate size estimation.
	**Mild tailing**	R_h calculation is still reliable. **Optional:** • Coat the capillary • If you use a coated capillary, strip it with NaOH 1M • Use assay buffer containing 0.02% pluronic F127 or 0.05% Tween-20
	**Moderate tailing** The sample stickiness affects the fitting of the data and the Rh calculation. **Signal optimization needed**	• Coat the capillary • If you use a coated capillary, strip it with NaOH 1M • Use assay buffer containing pluronic F127 (0.03-0.01%), 0.05% tween-20 or BSA (0.5mg/ml)
	**Strong tailing** The sample stickiness affects the fitting of the data and the Rh calculation. Data cannot be used as standard fitting conditions are not met.	

In addition to the solutions above, further measures can be taken to avoid adsorption.

01

1. Usage of low-binding Eppendorf tubes for sample storage and preparation of dilutions, especially if the solution is in the nanomolar concentration range.

02

2. Perform the final dilutions directly in the assay vial or plate well to minimize the exposure of proteins to multiple surfaces.

03

3. Buffer pH can be a critical factor for determining the overall surface charge distribution, hence the tendency of a protein to adsorb. Always consider the isoelectric point (pI) of proteins under examination.

1.3 R_h measurement for highly sticky samples

In rare cases, the FIDA peak/taylorgram might exhibit strong tailing, even after implementing methods to avoid passive sample adsorption to the capillary surface. Such a signal can be still analyzed to obtain the R_h by applying the "minimal fitting parameter". As shown in Figure 3, this fitting accounts for only 50% of the peak excluding the tailed region.

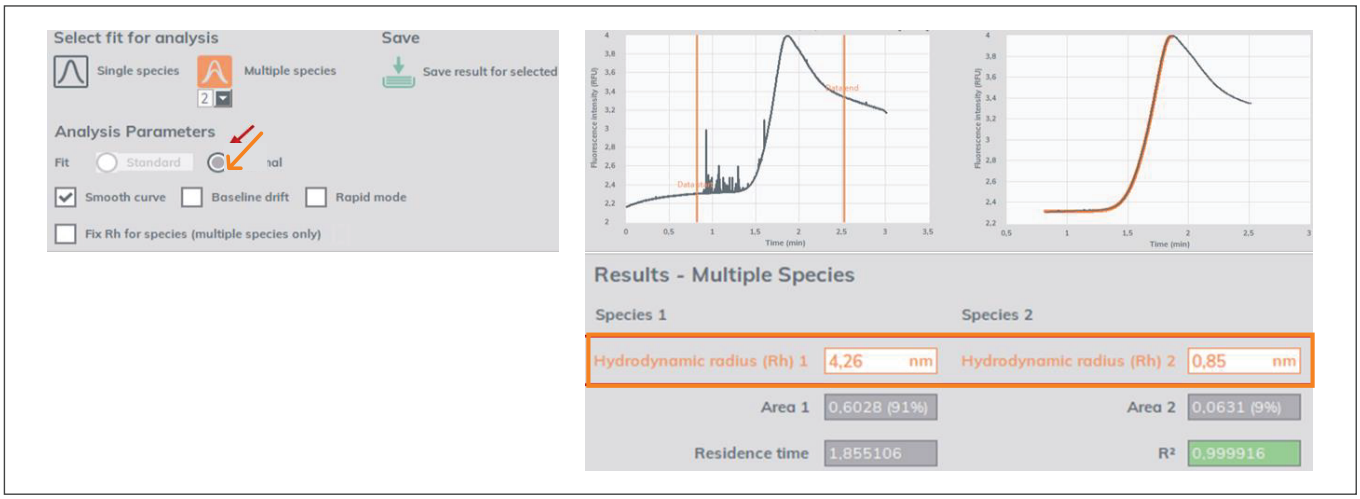


Figure 3. Applying minimal fitting to achieve size measurement from a taylorgram that exhibits a significant tailing effect.

2. Optimal Signal-to-Noise ratio

To achieve precise R_h measurement, it is important to maintain an optimal signal-to-noise (S/N) ratio. As a rule of thumb, it is recommended that the indicator must be measured with a minimum S/N of 30. If the indicator has S/N <20, the signal is considered sub-optimal for vvR_h measurement.

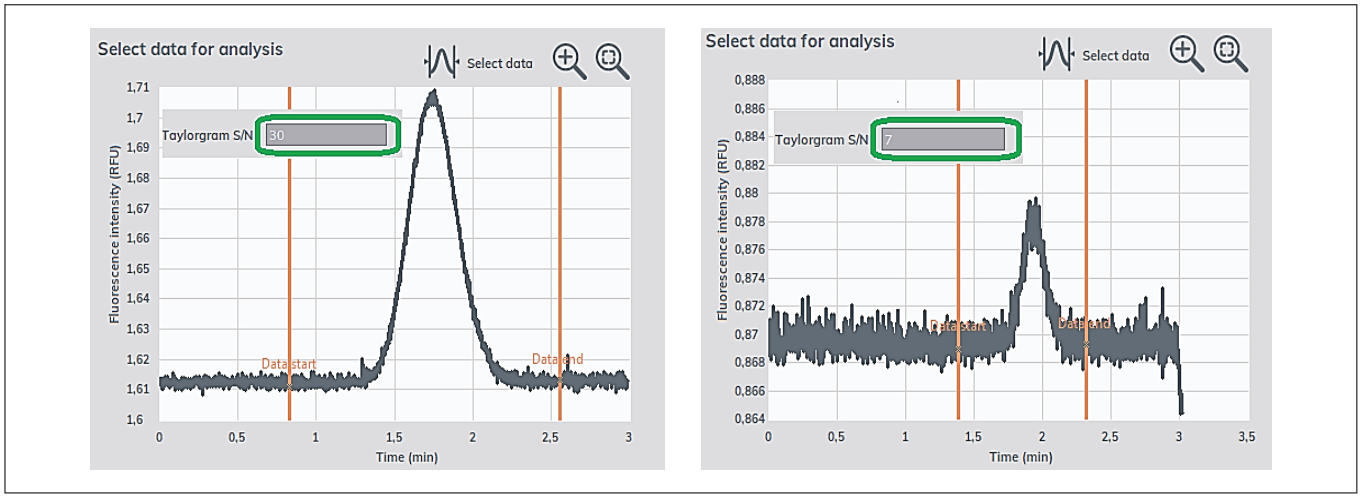


Figure 4. Comparison of a signal of a protein at 10 nM with S/N 30 (left) and a low signal with S/N 7 (right).

When working with labelled proteins (using 480 or 650 nm detector), a low signal can be due to a low Degree of Labelling (DoL). When DoL is below 0.5, a higher indicator concentration can be used to achieve an accurate R_h measurement. The lower the DoL, the higher protein concentration must be used.

When working label-free (using a 275 nm detector), a low signal can be due to either a low number of tryptophan residues or local buffer/pH conditions. Tryptophan fluorescence is typically sensitive to environmental conditions and hence can be largely impacted by improper solutions. Therefore, in case of a poor signal, the buffer conditions should be optimized. When the fluorescence of the POI is generally low, it is recommended that a higher protein concentration is used.

Note: If the optimized concentration of the indicator is higher than the expected K_D of the two binders, the binding assay can be still obtained by adopting the analysis approach in paragraph 9.

3. The FIDA solution to unreacted fluorophore

The failure of standard fluorescence-based bioassays to detect the presence of unreacted free fluorophore usually affects the binding measurements dramatically. Often, even after standard cleaning steps of the commercial labelling kits, some free fluorophore is co-eluted with the protein. The excess amount of free fluorophore in a sample can induce non-specific binding causing a potential underestimation of protein-protein binding affinity. FIDA can detect the presence of free fluorophore in a sample, providing an explicit measurement of respective fractions of free versus conjugated fluorophore.

Paragraph 3.1 describes how FIDA detects the unreacted fluorophore. Paragraph 3.2 explains how FIDA quantifies the fraction of conjugated versus unreacted fluorophore and presents the corresponding actions to be taken.

3.1 Detection of unreacted fluorophore

FIDA works on the principle of molecular diffusion, and this enables the detection of species of different sizes (i.e., of distinct diffusivities) by simple analysis of the taylorgram signal. A double species signal is the sum of the fluorescence detected from two distinct fluorescent molecules (Figure 5). The signal appears to be a combination of two Gaussian distributions with a wider base and a narrow peak.

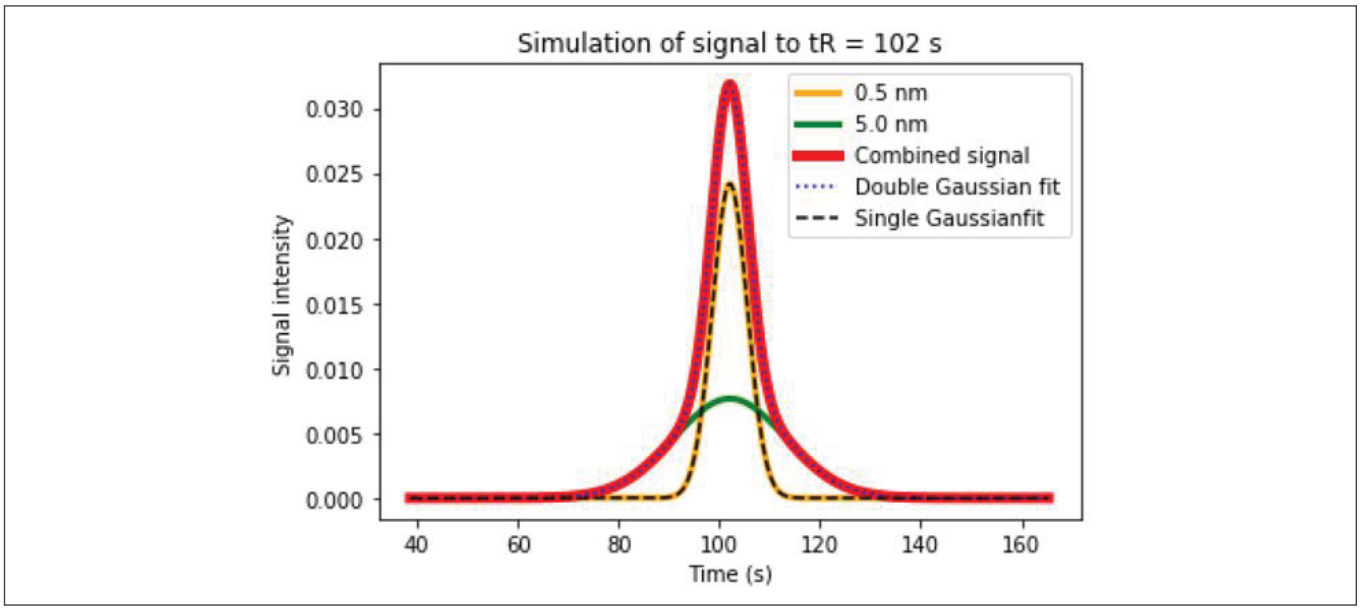


Figure 5. Double species taylorgram distribution. The observed signal (red line) is the sum of the free-fluorophore (black-yellow dashed line, R_h 0.5 nm) and the labelled protein (green line, R_h 5 nm).

The presence of two or more species in a sample can be easily detected by automatic fitting of the FIDA signal. Upon loading the acquired dataset into the software, apply the double (or multi) species fit. If double (or multi) species are detected in a sample, the respective R_h values will be reported under species 1 and species 2 (Figure 6A, green rectangle). In case a sample consists of only one species, the two calculated R_h values will be identical (Figure 6B). Applying a single species fitting would be more appropriate in this case.

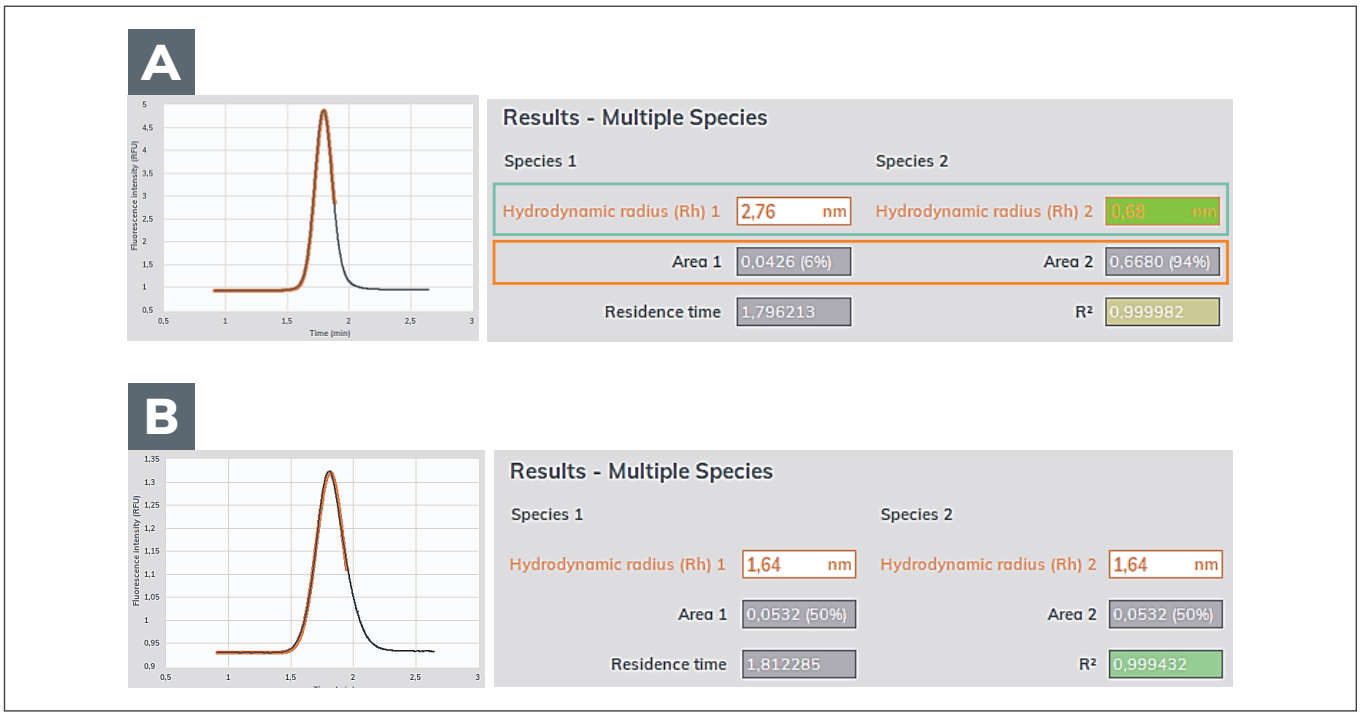


Figure 6. Comparison of **A.** a double species peak composed of a mixture of labelled protein (6%) and free fluorophore (94%) and, **B.** a successfully labelled protein without any free fluorophore.

3.2 Quantification of unreacted and conjugated fluorophore

In FIDA measurements, the peak area readout is proportional to the intensity of the fluorescence signal and is used to estimate the fractions of free and conjugated fluorophore. This is automatically performed using the FIDA analysis software. In the example reported in Figure 6, upon detection of multiple species the respective area readouts are also obtained (orange rectangle). In Figure 6A, Area 1 corresponds to the fluorophore conjugated to the protein of interest (R_h = 2.76 nm) and makes up 6% of total fluorophore. Area 2 corresponds to the free fluorophore (R_h = 0.68 nm) in the sample which is in 94% abundance. Table 2 presents different free fluorophore fractions and the corresponding measures to be taken.

Table 2. Unreacted-conjugated fluorophore fractions and corresponding solutions.

Case	Solution
Free-fluorophore 5-30%	Data can be processed normally. The presence of free fluorophore will not affect the size calculation of the target molecule.
Free-fluorophore 30-50%	Data can be processed. Optional: Additional dye removal step when free fluorophore approaches 50%. The R_h standard deviation might be slightly higher when free fluorophore is close to 50%.
Free-fluorophore 50-70%	At these percentage, the free fluorophore can dominate the protein signal. R_h standard deviation is likely higher. Dye removal step is strongly recommended.
Free-fluorophore 70-90%	Dye removal step must be done. The R_h calculation of the protein of interest will not be accurate.

4. Sample quality assessment with every measurement

Besides providing information on hydrodynamic radius and affinity constant, FIDA offers sample quality assessment with every measurement. When proteins aggregate, they often form big insoluble particles which cause high light scattering and are detected as spikes in the FIDA signal. The spikes can be counted using the "spike counter tool" integrated into the FIDA analysis software and thereby provide an indication of the amount of aggregates in a sample.

The position of the spikes in the FIDA signal dictates where and when the aggregates are formed: in the indicator vial, the analyte vial or upon binding.

Figure 7 illustrates the Fidabio CapMix assay and a Gaussian peak with no aggregation.

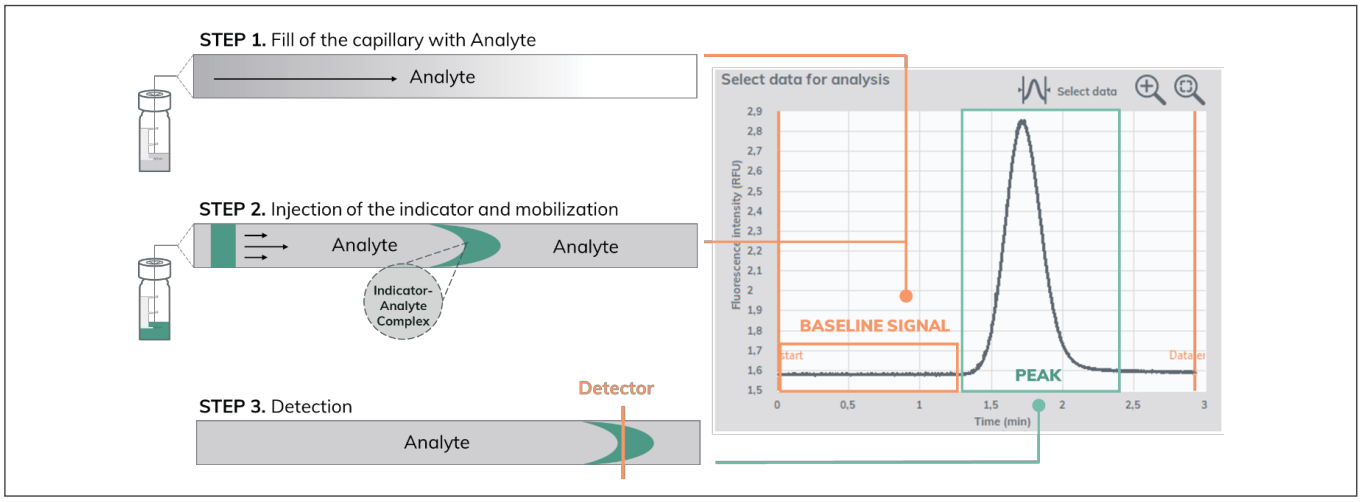


Figure 7. CapMix process (left) and the generated FIDA signal with no aggregates (right).

The following paragraphs present cases where the FIDA signal exhibit spikes at different sections of the curve deviating from the "normal" profile shown in Figure 7. The paragraphs explain how to distinguish between aggregation that occurs in the indicator vial, analyte vial or upon binding and suggest specific solutions for each case.

4.1 Aggregation of particles in the analyte vial or buffer

As shown in Step 1 of Figure 7, the Analyte solution is used to equilibrate the capillary before injection of the indicator, and it generates the baseline signal. Therefore, spikes in the baseline signal indicate aggregation in the Analyte vial.

Figure 8 is an example of aggregation in the analyte vial, as spikes are distributed throughout the total recording time frame.

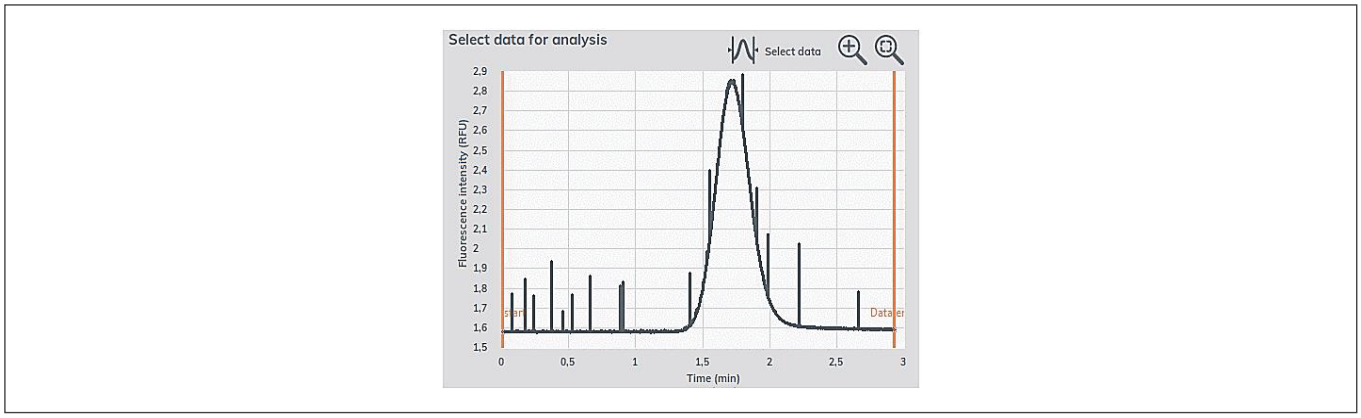


Figure 8. Spikes distributed throughout the run - both in the baseline and the Gaussian peak- indicate the presence of aggregates in the Analyte vial.

Suggested solution: If the analyte is a protein/premix of multiple proteins, a centrifugation of the stock solutions is suggested. If the analyte is a buffer, it is recommended to perform a buffer filtration step.

4.2 Aggregation of particles in the indicator vial

In Figure 9, signal spikes mainly appear after the baseline signal, at the bottom of the Gaussian peak. Spikes appearing with this "time delay" i.e., not present in the baseline signal, exclude the possibility of aggregation in the analyte vial. The spike profile of Figure 9 rather indicates the presence of aggregates in the indicator vial.

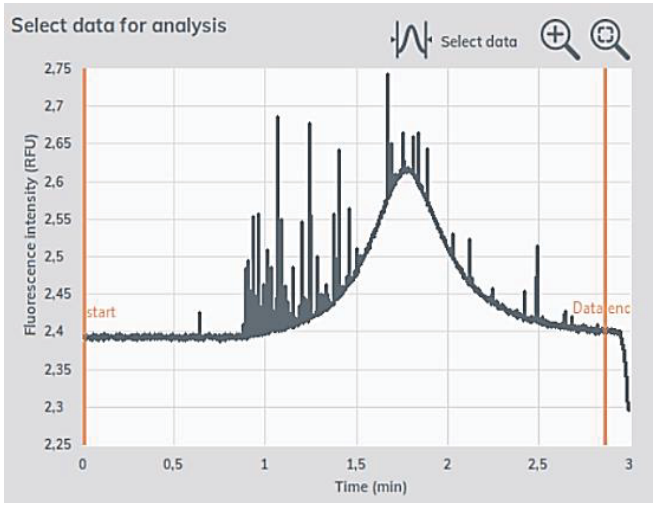


Figure 9. Spikes concentrated at the bottom of the Gaussian peak, and after the baseline signal, indicate the presence of aggregates in the indicator vial.

Aggregates in the indicator vial that are >1 μm in diameter tend to flow in the "highest velocity lane" of the laminar flow (see Appendix Figure 1) and are eluted early in the run, hence appearing mostly before the Gaussian peak. The indicator size can be still measured by choosing the fitting region as the Gaussian peak only (eliminating the spikes).

Suggested solution: Centrifuge the indicator stocks and re-run the measurement. If the appearance of aggregation persists, refer to paragraph 4.3.

4.3 Aggregation upon analyte-indicator binding

As stated in section 4.2, spikes at the bottom of the Gaussian peak indicates the presence of aggregates in the indicator vial. Usually, a test-run with indicator only is carried out to evaluate its quality. When the test-run shows no aggregation, but signal spikes appear upon addition of the analyte, it can be inferred that the aggregation occurs upon binding between the analyte and indicator. Figure 10A-D demonstrates different cases where aggregates appear upon binding. Note that spikes appear after the baseline signal, ruling out the aggregation in the analyte vial.

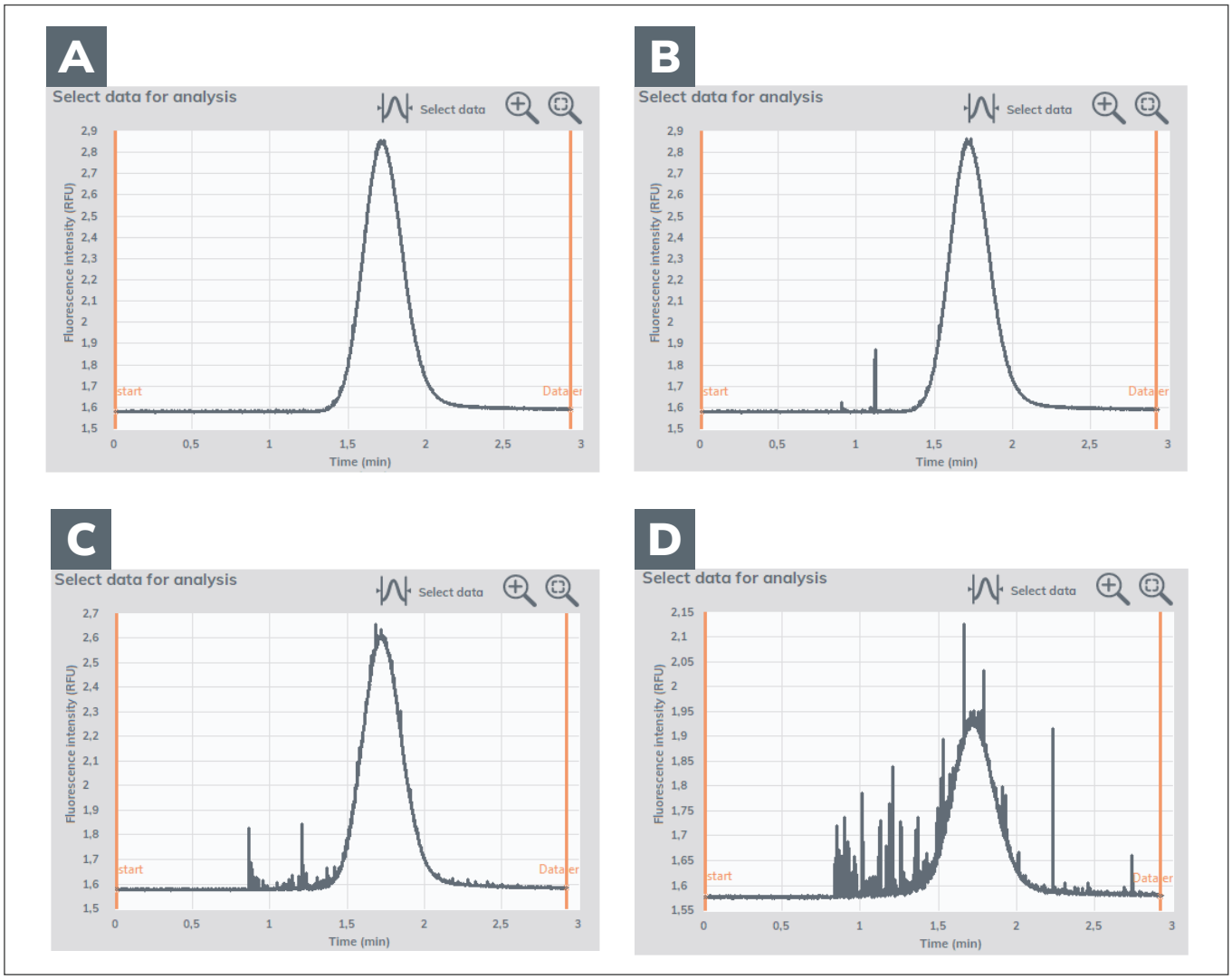


Figure 10. Appearance of signal spikes with low to high (**A** to **D**) analyte concentration.

R_h measurements can still be performed, while the signal spikes are valuable in assessing conditions that induce aggregation. As an example, the protein of interest can be screened for various buffer conditions to obtain an ideal buffer for storage or functional assays.

Suggested solution: If aggregation upon binding must be eliminated, it is suggested that the running buffer is reconsidered to define better conditions for the binding assay.

5. Calculation of optimal pressure

To get the optimal Taylor dispersion profile, the right pressure must be chosen in the final "mobilization step". The FIDA signal enables the user to know whether the pressure is optimal for the molecule in question.

High pressure leads to high flow rates and shorter run time while lower pressure leads to low flow rates and longer run time. The standard FIDA method employs a pressure of 400 mbar and works well for molecules with R_h <13 nm.

Very large molecules or complexes (such as liposomes, exosomes, and protein soluble aggregates) require more time to diffuse and therefore, a lower mobilization pressure. For example, with the standard FIDA method (400 mbar) the signal from molecules larger than standard proteins i.e., >20nm, would typically look like the one in Figure 11A. The observed "premature peak", indicated by the arrowhead, indicates that the chosen FIDA conditions are not optimal for the molecules in question, which are unable to diffuse efficiently.

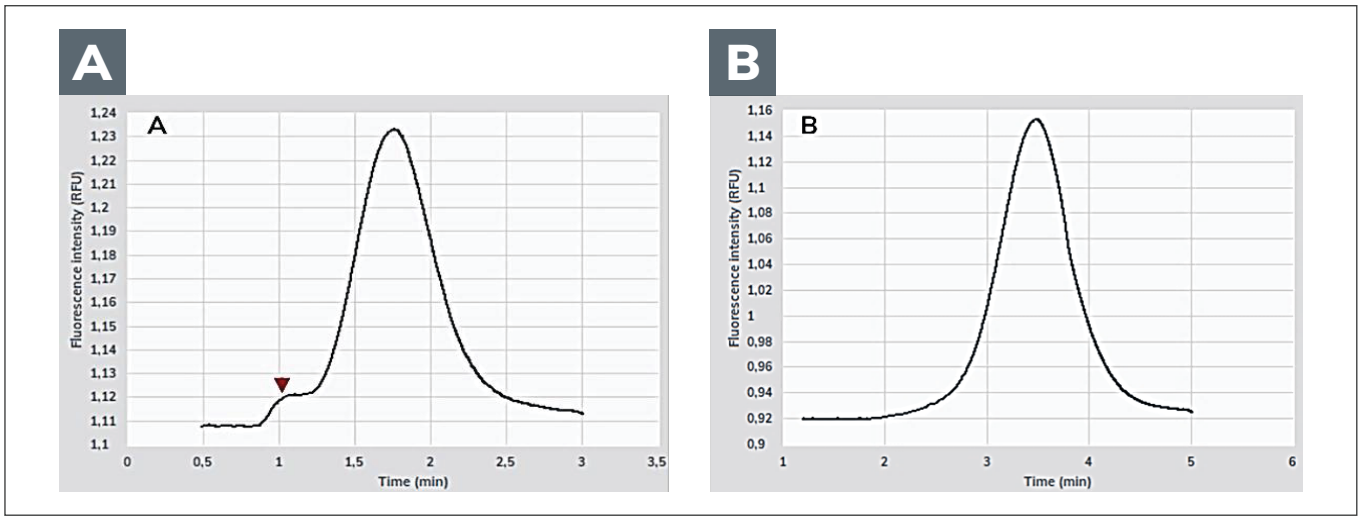


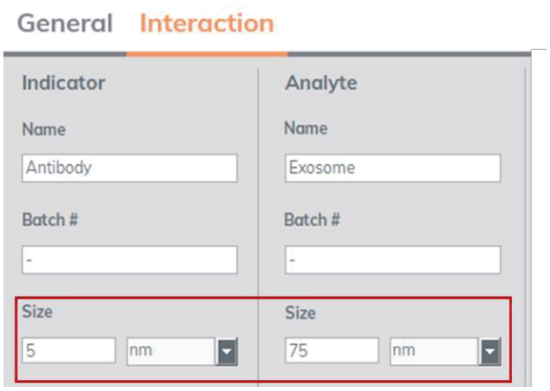
Figure 11. A. The suboptimal Gaussian signal shows that the indicator size is too large for the pressure conditions chosen (400 mbar). **B.** Same sample measured using a lower mobilization pressure at 300 mbar (i.e., longer mobilization time) displays an optimal signal.

Suggested solution: To obtain the optimal pressure conditions, use the FIDA analysis software to automatically design a suitable method:

01

Step 1

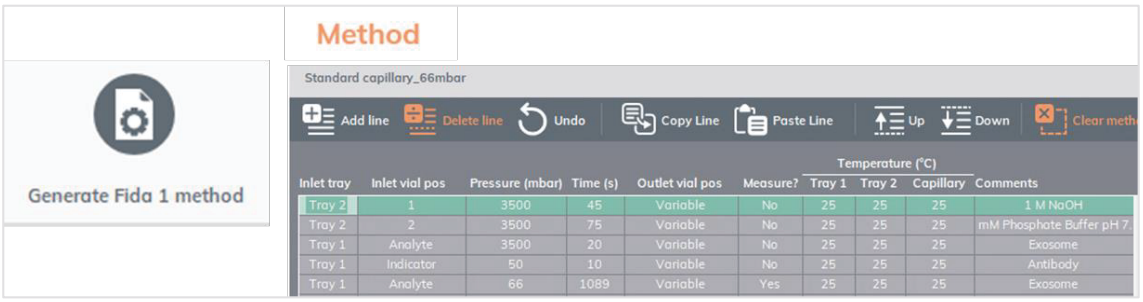
Open the Analysis software, choose the “Assay Design” window and click on the “Interaction” tab. Type a rough estimate of indicator/analyte size in nm in the Indicator or Analyte Size slot.



02

Step 2

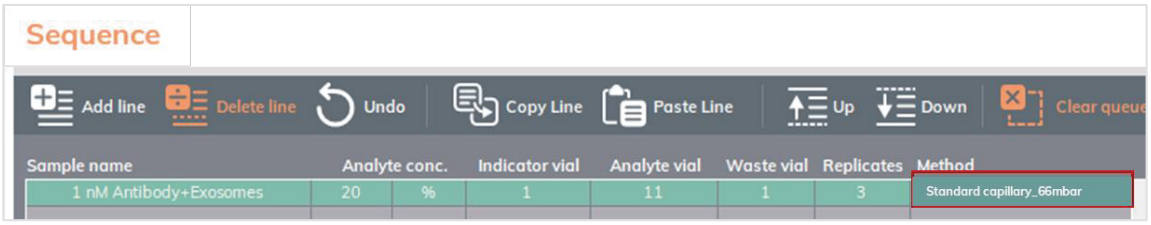
Click the “Generate Fida 1 method” button (top right corner) and save the customized method file (.met file) under the pre-defined Methods folder.



03

Step 3

Use the new method file while designing the assay sequence.



6. Optimal buffer conditions

The FIDA signal assists users in defining the optimal buffer conditions for each experimental setting by revealing any potential buffer mismatch between the indicator and analyte. Below, different scenarios of buffer mismatch are presented followed by simple optimization solutions.

Case 1. Double peak

As shown in Figure 12, a double peak typically arises when the indicator solution has a significantly lower background compared to the analyte. An example where this behaviour might become prominent is when working with complex matrices and the analyte is in 20% plasma while the indicator is in 10% plasma. The R_h measurements in this case might not be accurate.

Suggested solution: Ensure that the indicator and analyte are diluted in the same matrix/buffer.

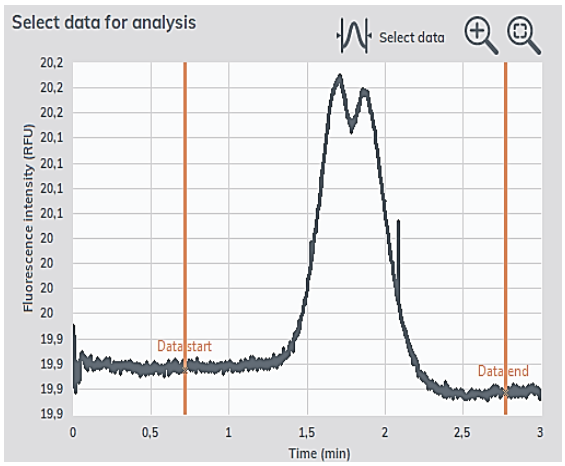


Figure 12. Buffer/baseline mismatch between the indicator and analyte solutions.

Case 2. Misaligned baselines before and after the peak

The baseline misalignment usually does not impact the FIDA size measurements, since the fundamental factor used to obtain the R_h value is the width of the peak. An optional optimization step for misaligned baselines is to double-check that the indicator and analyte are diluted in the same buffer/matrix. Occasionally, complex matrices like E.Coli supernatant and high plasma concentration can produce baseline irregularities but this do not significantly impact the size measurement.

7. Viscosity compensation

Viscosity significantly affects the size measurements and can lead to over- or underestimation of the molecular size if it is not taken into account. Unlike most biophysical technologies, FIDA accounts for viscosity (η) in the R_h calculations (see Eq.1) and offers the possibility to compensate for potential fluctuation. Thereby, it provides unbiased results regardless of viscosity changes that can occur due to concentration and/or temperature variation.

$$R_{\mathrm{h}} = \frac{k_{\mathrm{B}}T}{6\pi\eta D} \qquad \textbf{(Eq.1)}$$

7.1 Viscosity compensation for binding curves

To obtain a FIDA binding curve, the indicator is initially mobilized in neat buffer (control), followed by a titration with increasing concentrations of analyte (Figure 13).

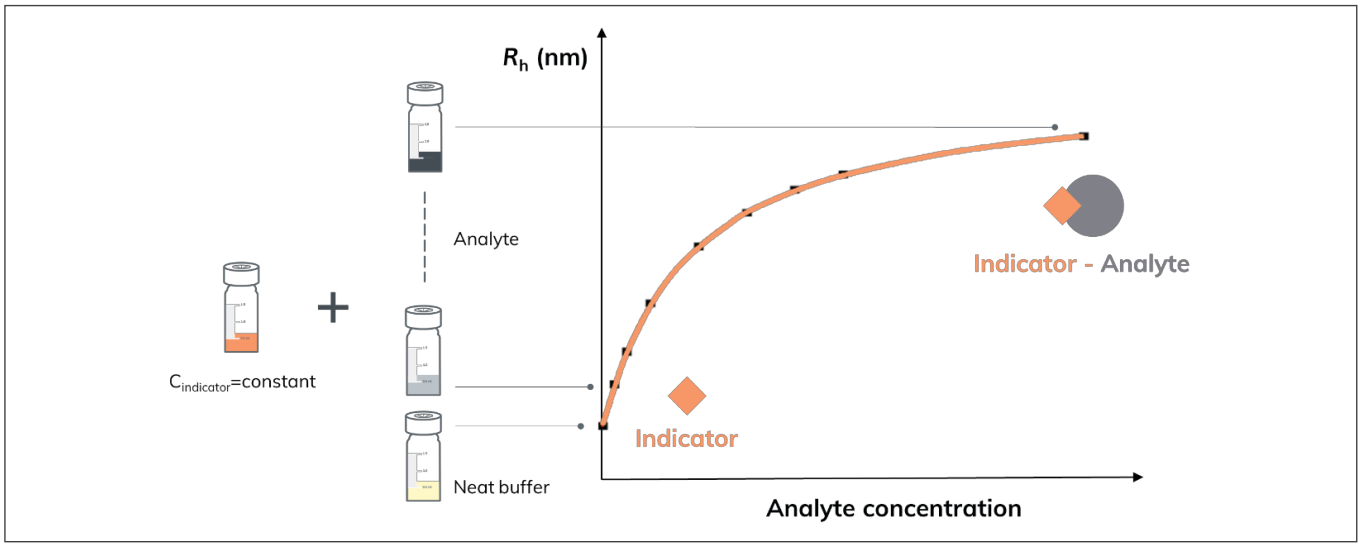


Figure 13. Experimental set-up for obtaining a standard FIDA binding curve.

The residence time (t_R) obtained in each measurement is used for the calculation of the R_h. Given that the capillary diameter and temperature are constant throughout measurements, the residence time only depend on the viscosity of the sample which often rises with increasing analyte concentration. For this reason, FIDA uses the reference residence time from the neat buffer condition to compensate for the concentration-dependent viscosity increase throughout a titration.

When creating a FIDA binding curve, the Rh is automatically adjusted for viscosity increase by simply clicking the checkbox “Viscosity compensation” (Figure 14A). This option enables the software to fetch the reference t_R from the neat buffer datapoint (Figure 14B) and perform R_h correction for all other datapoints being analyzed.

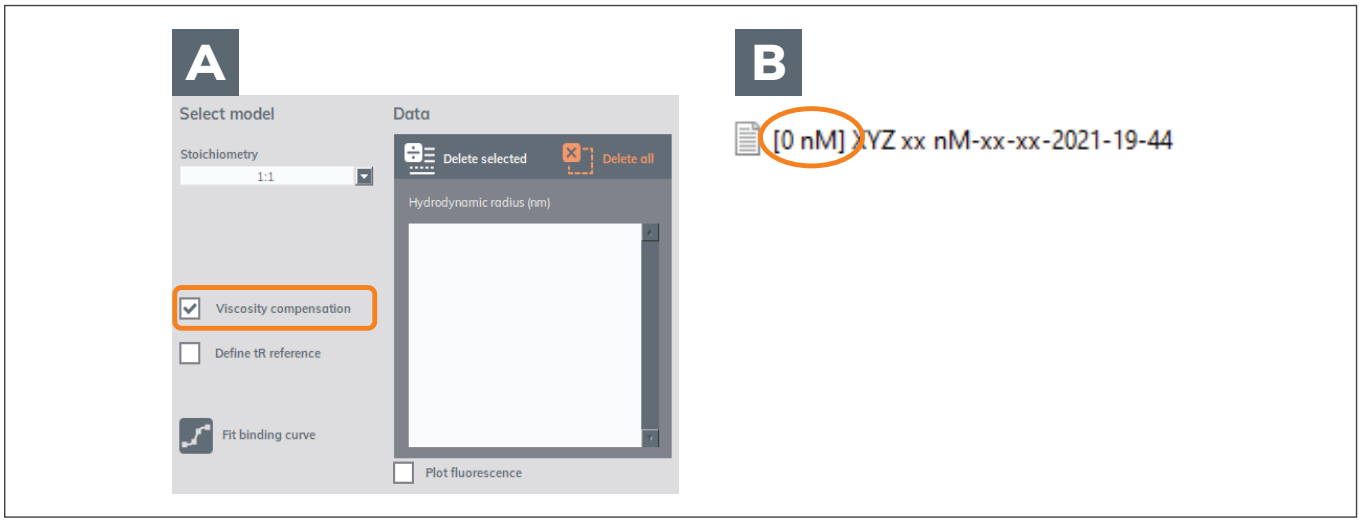


Figure 14. A. Viscosity compensation checkbox in Binding Curve section. **B.** An example of a datapoint label used to fetch reference t_R.

7.2 Viscosity compensation for single data points

For single data points, the reference t_R is defined manually by checking the box "Define t_R reference". When using this option, an independent measurement of the indicator in neat buffer must be done first to obtain the reference t_R. Then, the steps below are followed to perform R_h correction (Figure 15):

01

1. Click on the "Viscosity" tab located on the top-left corner of the FIDA data analysis window.

02

2. Open the "Viscosity adjusted Rh calculator" tool.

03

3. Type the calculated R_h of the indicator in the "Enter apparent R_h" slot.

04

4. Type the residence time of the same sample in the "Enter t_R sample" slot.

05

5. Type the t_R of the indicator measured in neat buffer in the "Enter t_R reference" slot and get the new viscosity-adjusted R_h for the single datapoint.

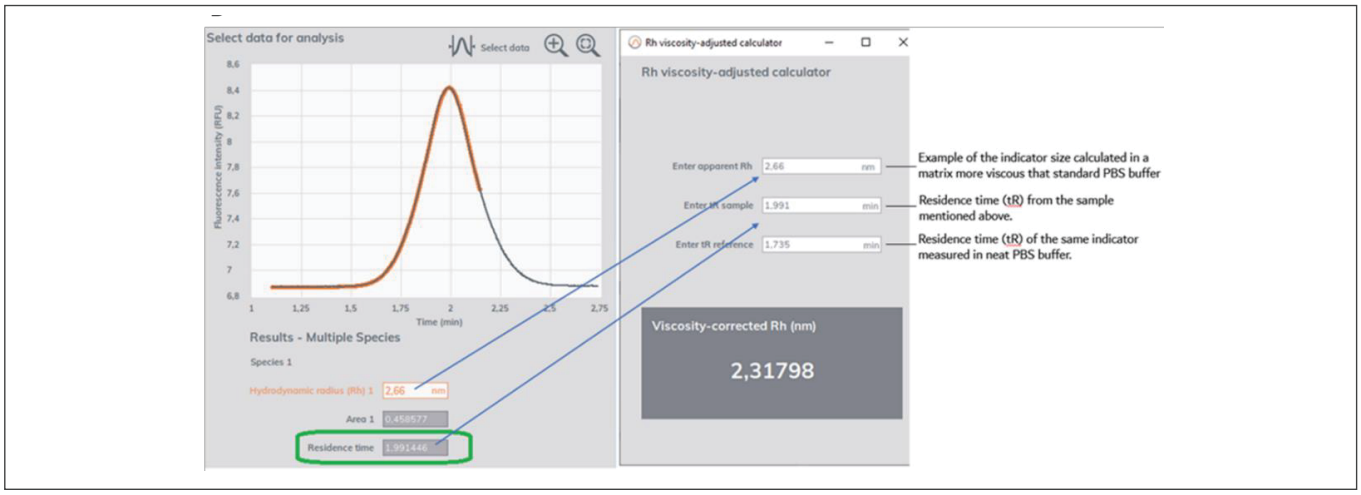


Figure 15. Performing viscosity-adjusted Rh calculation for a sample measured at 37 °C using the FIDA analysis software.

7.3 FIDA indications for optimal method design

Besides viscosity compensation, FIDA offers viscosity-related indications that delineate the adjustments which can be done to achieve optimal method design. More specifically, certain FIDA signal profiles indicate the need to prolong the mobilization or analyte filling time. The mobilization or analyte filling time dictates the time span and the pressure by which the indicator will be mobilized from the injection point to the detection window. The paragraphs below present two FIDA signal profiles showing that time adjustment is needed.

7.3.1 Right-shifted peak

If the standard FIDA method (400 mbar, 180 sec) generates a peak curve that looks shifted or incomplete (Figure 16A), it is an indication that the viscosity of the sample is high, and the mobilization time is short.

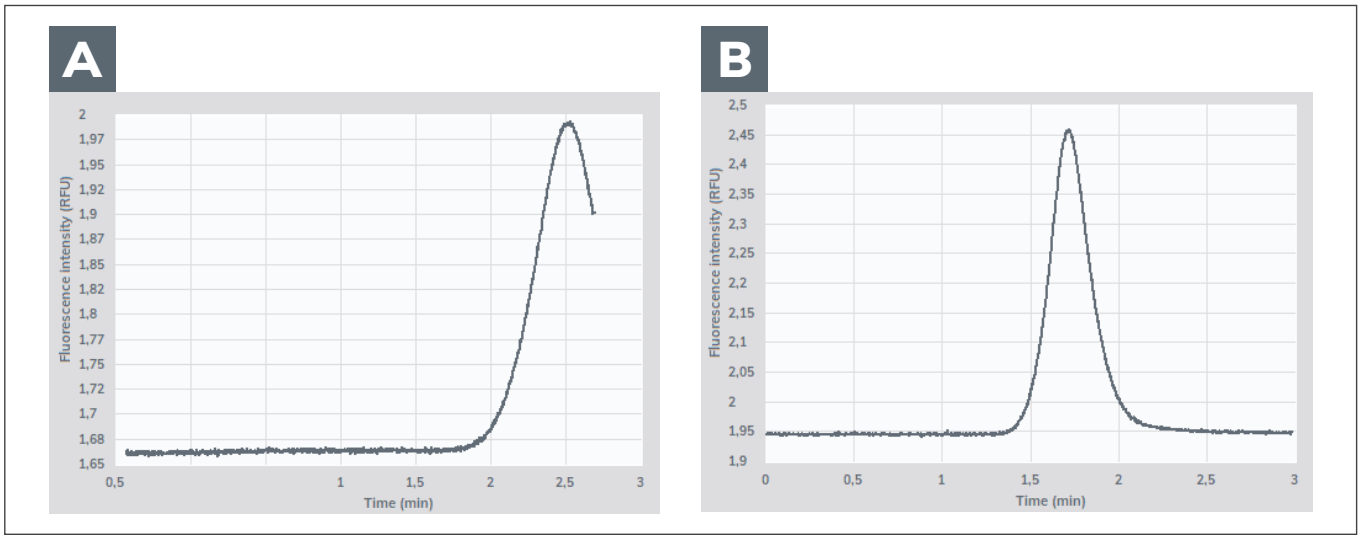


Figure 16. A. Incomplete fluorescence signal due to high sample viscosity and short mobilization time. **B.** Complete peak with optimized mobilization time.

FIDA offers an easy fix allowing for modifications of the mobilization time. Increase the mobilization time by following the next steps:

01

Step 1

Open the Method file in the FIDA software (under “Method” tab).

02

Step 2

The mobilization time can be changed “mobilize and measure step” by double clicking on the cell.

03

Step 3

Change the time (for example, from 180 seconds to 240 seconds) and save the new method.

04

Step 4

Run the sample again using the new method and check for the indicator signal through the mobilization time (Figure 16B).

7.3.2 Dis-equilibrated baseline

Sometimes, high analyte concentration leads to autofluorescence and an elevated baseline. These effects are visible in the acquired FIDA signal as a dis-equilibrated baseline (Figure 17A).

The solution is to prolong the analyte filling step. This can be done by editing the desired method and increasing the run time of the "Analyte filling" step from 20 to 30 sec (Figure 17B, orange rectangle).

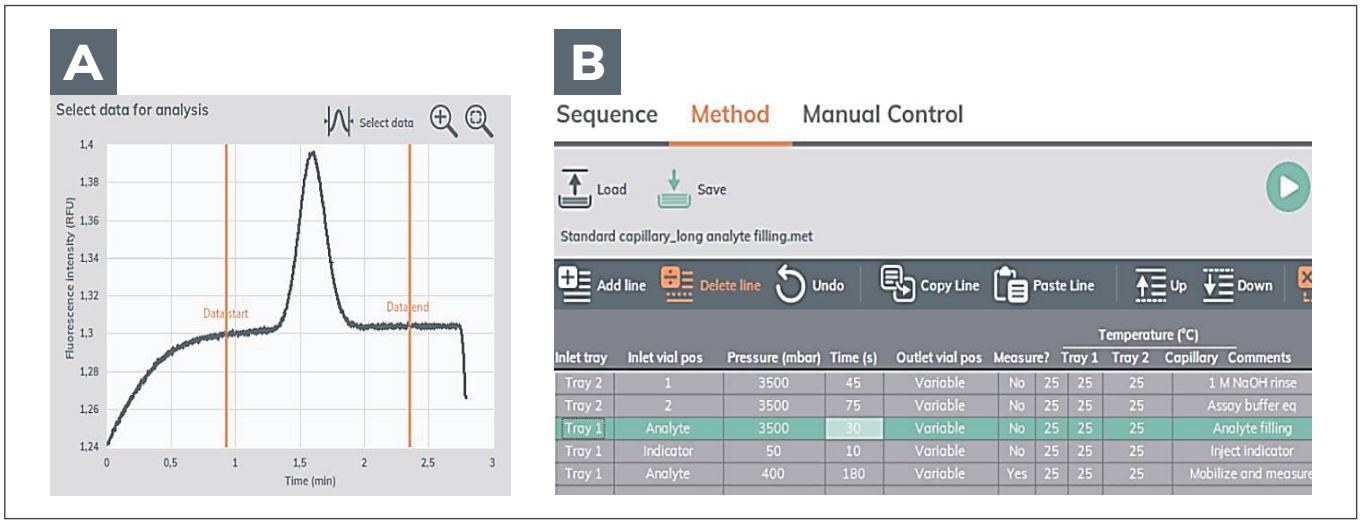


Figure 17. A. Elevated baseline signal due to a high analyte concentration. The capillary is flushed with the analyte prior to the indicator injection. When the analyte concentration is very high, an elevated baseline is observed, **B.** Baseline equilibration issues can be resolved by prolonging the "Analyte Filling" step (orange rectangle).

8. Temperature control

8.1 Temperature control during measurements

In some cases, like when analyzing unstable proteins, a specific assay temperature is required to perform reliable measurements. The Fida 1 instrument offers independent temperature control of the two sample trays as well as the capillary chamber. The default temperature is pre-set at 25 °C. However, this can be easily modulated from 5 °C up to 55 °C by editing the method files (Figure 18).

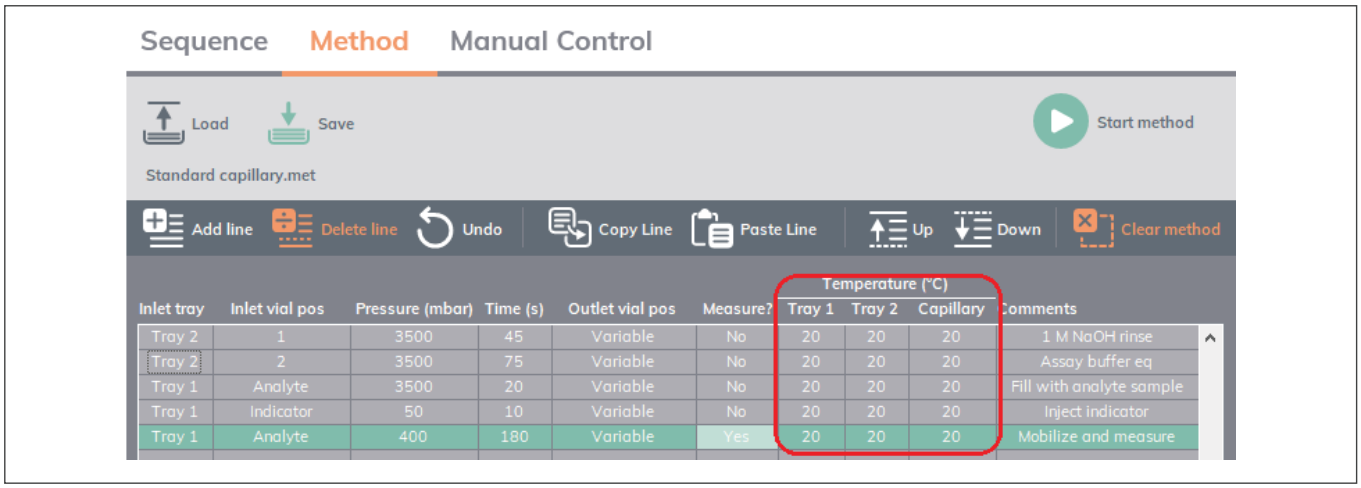


Figure 18. Example of changing the temperature to 20 °C.

Important! Always remember to save a method after making the necessary changes and select the right method in the sequence.

8.2 Temperature-correction during data analysis

Once measurements have been done at a designated temperature, it is critical to account for it during the Data Analysis. The correct assay temperature must be entered in the FIDA data analysis software as shown in Figure 19.

Additionally, the reference viscosity must be calculated for the respective temperature. This can be done by entering the right temperature value in the “Water’s viscosity calculator” integrated in the Fidabio Data Analysis software (Figure 19).

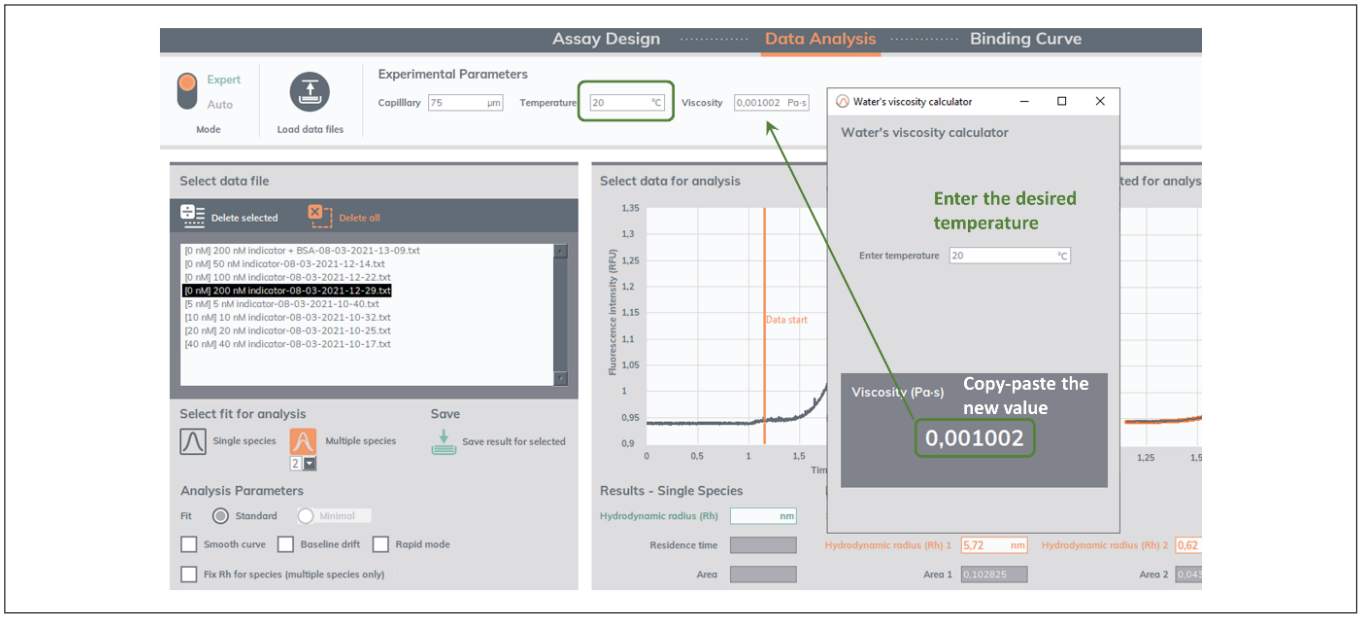


Figure 19. Example of analyzing datasets measured at 20 °C.

9. Measurements at low K_D

As a rule of thumb, the concentration of the indicator in a binding assay should be around or below the K_D of the system. However, when characterizing a system with strong affinity (picomolar or low nanomolar range K_D), it might be challenging to measure the indicator at such concentrations due to the low signal. The “excess indicator fitting model”1, available in the Data Analysis software, offers the solution in cases where the concentration of indicator and/or analyte is higher than the K_D.

In the example below (Figure 20), the indicator (ProteinX-Alexa488) can be detected at a minimum concentration of 20 nM, while the analyte (ProteinY) has a binding K_D of 0.3 nM. The same binding curve was fitted with "1:1 binding model" (Figure 20A) and with the "Excess indicator model" (Figure 20B). Clearly, the fitting in Figure 20B is more accurate, in terms of both the obtained curve and the R^2 value (regression coefficient).

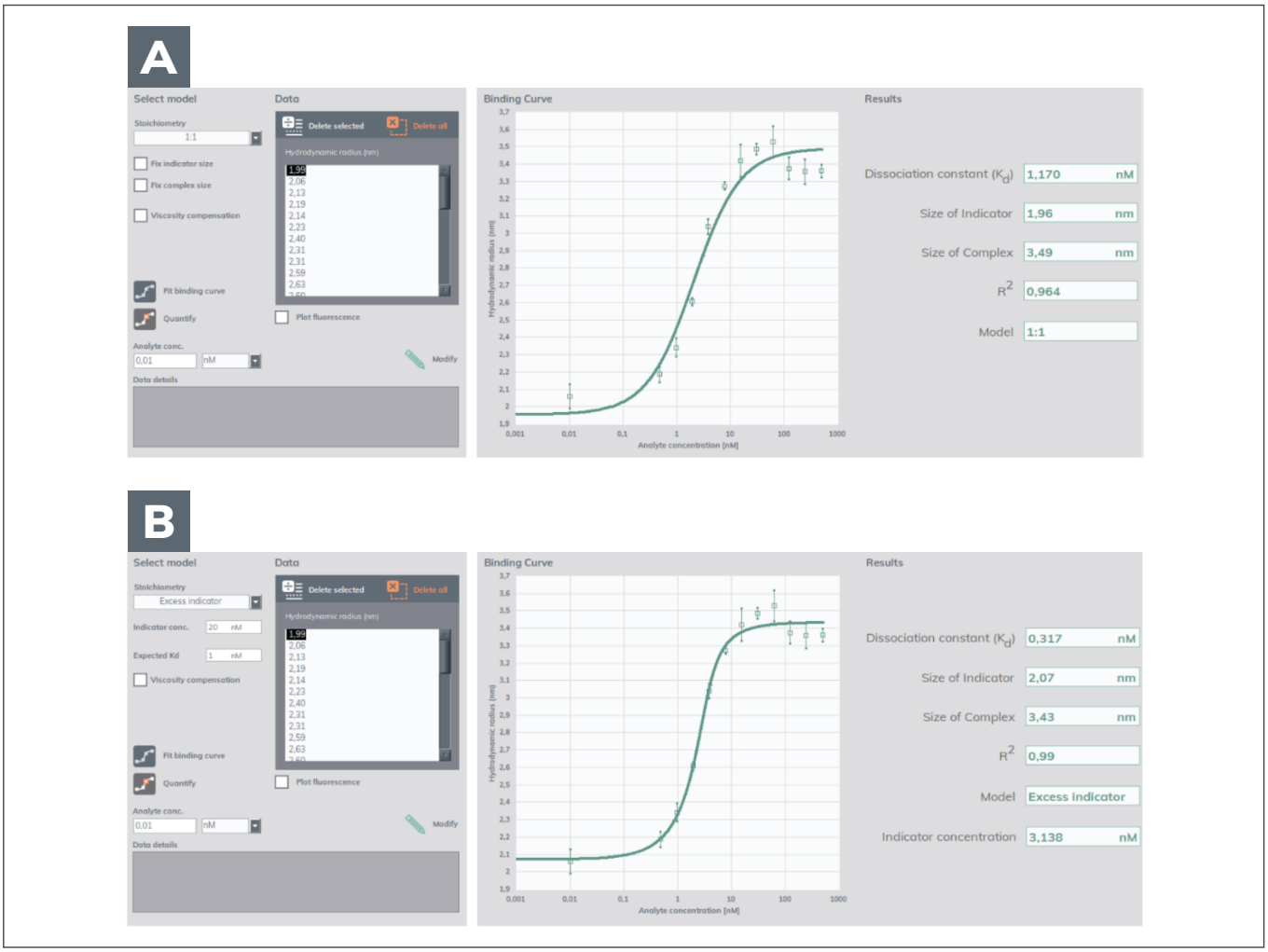


Figure 20. Example of the same data set fitted with **A.** 1:1 binding model and **B.** excess indicator model.

10. Troubleshooting when no peak is detected

If no fluorescence signal is detected at the end of the run and the raw signal looks like a flat baseline (Figure 21), there are several possible explanations and solutions which are described below.

[1] Detailed description of excess indicator isotherm can be found in the analysis software under “Help > Description of Fida models”.

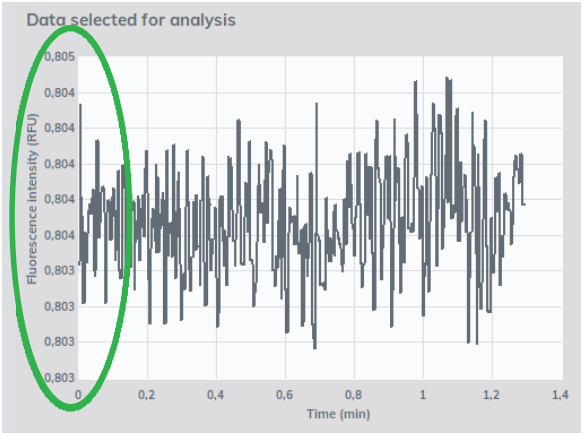


Figure 21. Example of signal at baseline level equal to ~0.8.

Scenario 1. Improper connection between the detector head and detector plug

Check if the baseline RFU is below 0.1. If yes, this could indicate an improper connection between the detector head and the detector plug (inside the capillary chamber). When the connection between the two detector components is properly done, there is a visible blue excitation light, as shown in Figure 22 (blue arrows).

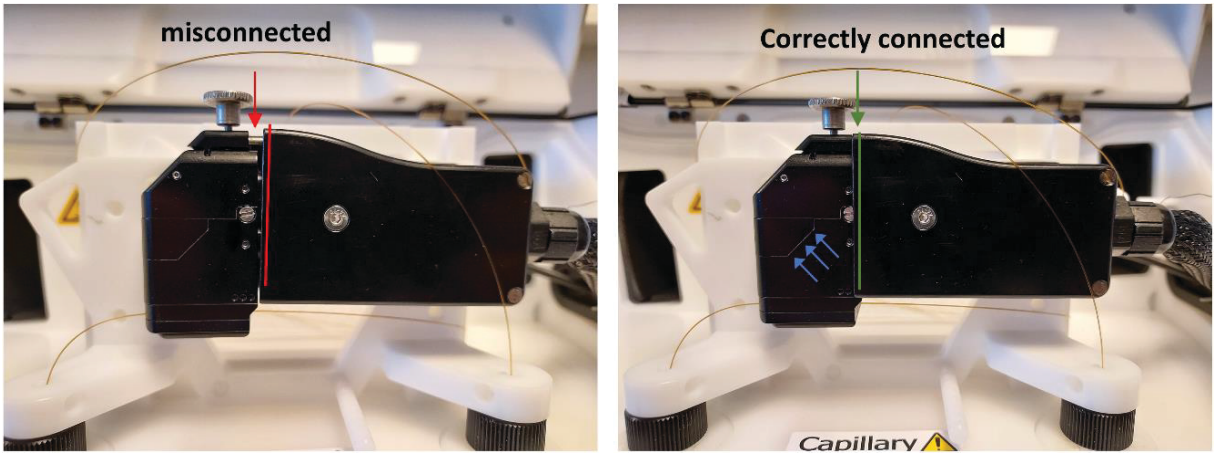


Figure 22. Detector head and plug inside the capillary chamber. Detector head misconnected (left picture-red line); Detector-head correctly connected (right picture-green line).

Scenario 2. Wrong position of analyte and indicator vials

Check if the vial positions for the analyte and indicator indicated in sequence are correctly stated.

Scenario 3. Clogged capillary

Check for capillary clogging by testing a known indicator sample (for example, 10nM Fluorescein) with a buffer analyte. Ideally, the signal peak should be observed at 80-95 seconds of the mobilization time. In case that no peak is detected, set the inlet position on a milliQ vial (at least 1ml) and the outlet on a new empty vial. Apply 3,500 mbar pressure for 120 seconds (this can be done using the “Manual Control window”) and check if there’s any liquid eluted in the new outlet vial. If there is no eluted volume, it is, of course, an indication that there is no capillary flow or that the capillary is clogged. Thus, a new capillary must be installed.

Precautionary steps to avoid capillary clogging:

- Perform a milliQ wash at the end of an automated sequence. This prevents the drying out of the capillary inlet and/or outlet and prevents the deposition of salt crusts from buffers at the capillary surface.
- Filter the wash buffers and spin the stock solutions prior to use. This can significantly prevent large particles from clogging the capillary.

11. Troubleshooting for pressure build-up

Pressure build-up is typically flagged with the pop-up error messages “Syringe leakage detected” or “Error building pressure”. Such errors most likely indicate one or more of the following:

- **A sequence is not correctly defined.** The inlet vial is set at a position where there are no vials in respective wells. Check if the samples are positioned correctly, in accordance with the sequence.
- **The vial cap is not properly closed.** Check if the vial/well number flashed on the error message is sealed properly. Take the vial/plate out and firmly push down the sealing cap/mat and initiate the run again. If the error persists, replace the cap/mat. Also, check for any cracks on the vials/plates.

- **The black capillary knob is not sufficiently tight.** Check if the black knob (Figure 23) on the inlet side of the capillary chamber is thoroughly tight.
- **The capillary is not aligned.** Click on the “Capillary align” option in the top right corner of the software. When both inlet & outlet positions are set on “Capilla.”, open the capillary chamber. Loosen the black knob on the inlet side, and gently pull up and down the capillary. Push the capillary all the way down until you feel resistance from a surface underneath. Hold the capillary in this position and tighten back the black knob. Repeat the same with the black knob on the outlet side of the capillary.

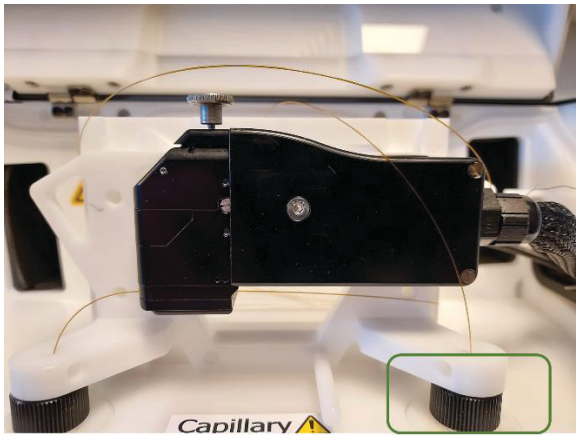


Figure 23. Capillary black knob.

Appendix

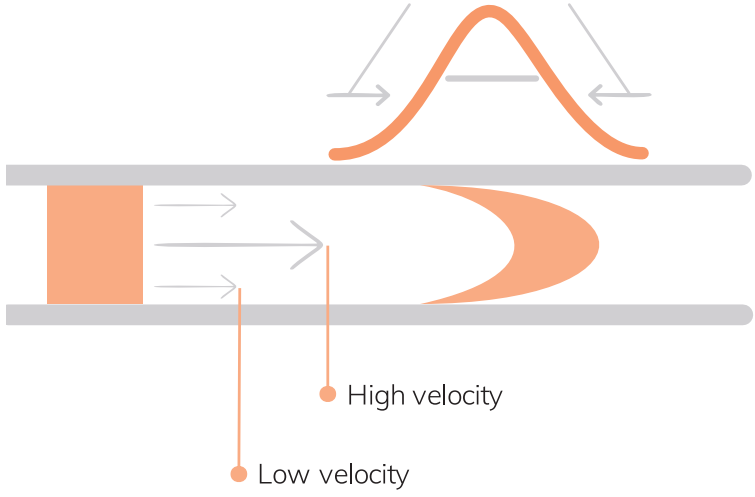


Figure 1. Representation of laminar flow-induced dispersion of the sample in the FIDA capillary. The flow rate is higher in the capillary center compared to the regions closer to capillary walls.

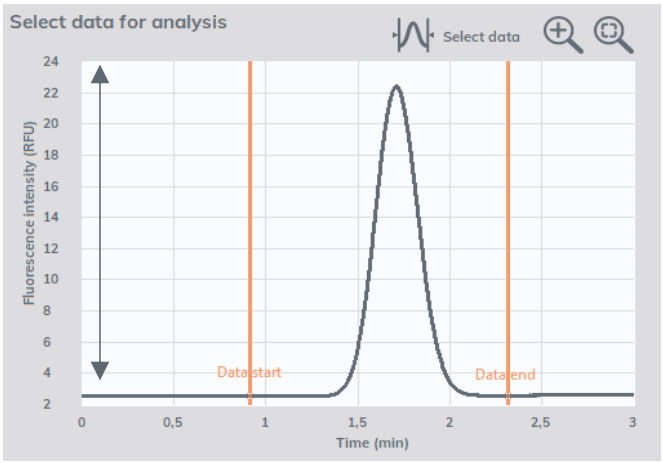


Figure 2. Protein signal (labelled) at 1 µM concentration[1].

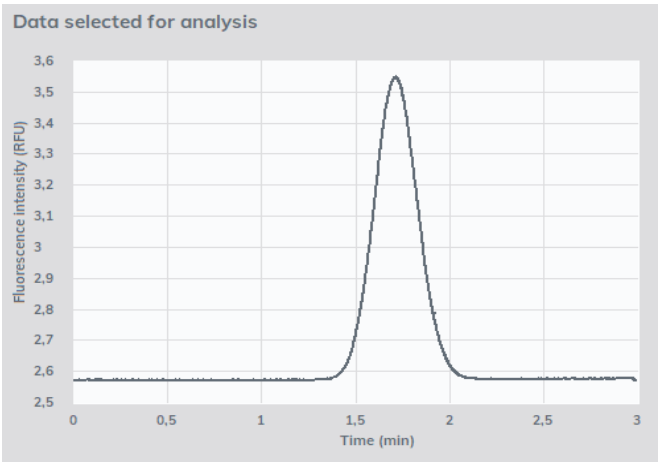


Figure 3. Protein signal (labelled) at 100 nM concentration[1].

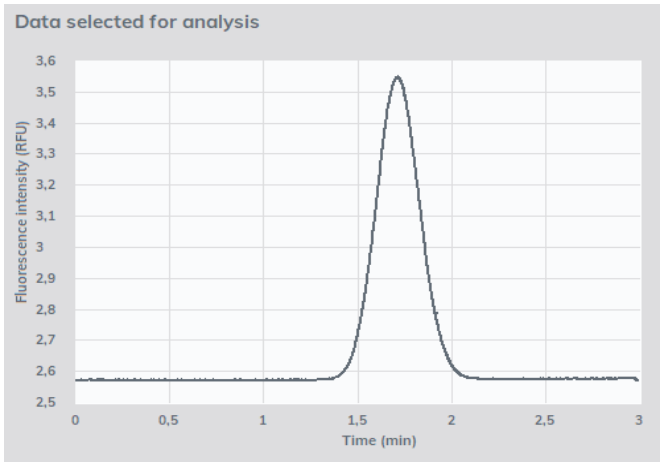


Figure 4. Signal overload (S/N 12k). Excess fluorophore creates tailing and peak fronting asymmetries.

[1] A small variation of signal intensity can vary from protein to protein, depending on fluorophore quantum efficiency, and labelling degrees (i.e., number of fluorophore molecules conjugated to one molecule of protein)

Customer Support

E-mail: support@fidabio.com
Tel.: +45 53 72 78 70
Fida Biosystems ApS
Generatorvej 6
2860 Søborg
www.fidabio.com


Fidabio