Fluidic Sciences

Getting Started Guide

SeroAffinity and Concentration (SAffCon) assay on the Fluidity One-M

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1. About this Getting Started Guide

This Getting Started Guide provides guidance for users who want to profile antibodies in plasma or serum of their choice. The guide provides a generic protocol to be used on the Fluidity One-M that can be easily adapted for any protein and includes helpful tips and tricks to successfully analyze protein interactions on the Fluidity One-M.

2. Protein-protein interactions

Proteins play critical roles in the human body, maintaining and regulating the structure and function of tissues and organs. Ultimately, proteins are responsible for nearly every task of cellular life. To deliver this task, more than 75% of proteins interact with at least one other. Understanding these interactions on a quantitative level is therefore essential to predict biological function, understand the effects of the disruption of normal cellular functions in human disease and subsequently help develop and optimize successful vaccines, drugs and treatments.

2.1 Why measure binding affinity?

While the identification of protein interactions is certainly important, understanding how strong these interactions are and how they impact biological function can be even more critical. Researchers therefore typically measure binding affinity to develop a more comprehensive understanding of the intermolecular interactions that drive biological processes and cellular pathways Determination of binding affinities is also important to researchers studying structural biology and structure-function relationships. In addition, understanding affinity and determining whether candidate drugs bind their target with high selectivity and specificity is crucial in the drug development process to guide the selection of drug candidates for further investigation. When is measuring binding affinity of importance:

- Investigating antigen/antibody interactions
- Characterizing binding epitopes
- Evaluating and ranking drug candidates
- Characterizing protein complexes
- Assessing the effect of buffers and other biological solutions on affinity

3. SeroAffinity and Concentration (SAffCon) assay

Conventionally, antibody response has been measured using immunoassays such as ELISA (enzyme-linked immunosorbent assay). These assays provide antibody titers which are a combination of antibody concentration and affinity. It is not possible to decouple the concentration or affinity of the antibodies from a titer value. Moreover, immunoassays suffer from the difficulty of controlling the surface concentration and activity of an immobilized ligand, which makes distinguishing between affinity and avidity difficult. Being able to directly measure these parameters can give a better understanding of antibody maturation and persistence of immunity. Surface-based technologies such as Surface Plasmon Resonance (SPR), Biolayer Interferometry (BLI) and immunoassays cannot be performed directly in serum because the high protein content in serum causes nonspecific binding which may result in false positive results.

The SeroAffinity and Concentration (SAffCon) assay can determine, directly in solution, the affinity of an antibody to antigen as well as the concentration of antibody binding sites without the need for ligand immobilization or antibody purification.

3.1 The dissociation constant (K_D) quantifies binding affinity

Binding affinity is a measure of how tightly two molecules bind to each other and is typically reported by the equilibrium dissociation constant (K_D). The smaller the K_D value, the greater the strength with which the two binding partners bind to each other and vice versa.

This Getting Started Guide describes how to determine the binding affinities of an antigen and antibody in serum or plasma on the Fluidity One-M by measuring the formation of complex according the equilibrium A + B ⇌ AB and determining the K_D value

$$K_D = \frac{[A]_{eq}[B]_{eq}}{[AB]_{eq}} \qquad \text{Equation 1}$$

in which $[A]_{eq}$, $[B]_{eq}$, and $[AB]_{eq}$ are the equilibrium concentrations of the unbound proteins A and B and the AB protein complex respectively.

Antibody affinity impacts disease severity and retained immunity. It is therefore a crucial parameter for a comprehensive understanding of an immune response.

3.2 Why concentration of target binding sites is important

The titer determined from immunoassays such as ELISA can either be derived from a large number of weak-binding antibodies or a small number of strong-binding antibodies. It's not only the K_D of antibodies that vary between individuals but also the concentrations, which may also vary during disease progression. Being able to determine the concentration of binding sites (i.e., antibodies) means that the efficacy of vaccines and functional immunity can be determined more effectively.

4. Measuring binding affinity by MDS

The Fluidity One-M measures changes in hydrodynamic radius (R_h) directly in solution using microfluidic diffusional sizing (MDS). MDS exploits the well understood relationship between molecular size and diffusion rate to enable absolute size measurements. If two proteins bind to each other, the absolute size of the complex is larger than the size of the individual binding partners, which is what is being detected by MDS. In practice, the Fluidity One-M measures the size increase of a fluorescently labeled probe protein when it binds to an unlabeled target protein. Thus, by mixing a constant concentration of fluorescently labeled probe with the unlabeled target at increasing concentrations, an equilibrium binding curve will be generated (Figure 1). From the binding curve, a K_D value can be obtained using nonlinear least squares fitting to the following equation:

$$R_h = R_{h,free} + \left(R_{h,complex} - R_{h,free}\right) \times \frac{K_D + [B] + [A] - \sqrt{K_D + [B] + [A]^2 - 4[B][A]}}{2[A]} \qquad \text{Equation 2}$$

Here, R_h is the measured R_h value, $R_{h,free}$ is the R_h of the unbound labeled probe, $R_{h,complex}$ is the R_h of the complex, and [A] and [B] are the total concentrations of labeled probe and unlabeled target, respectively

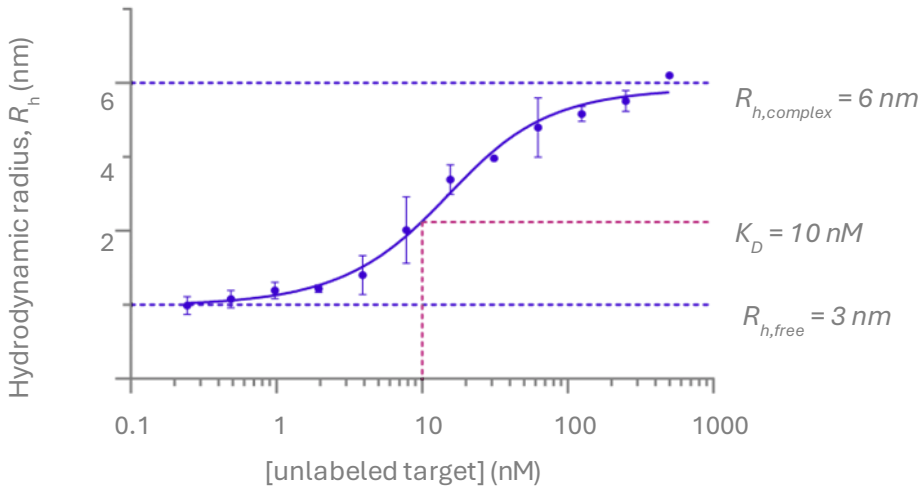


Figure 1. General features of an equilibrium binding curve measured by MDS using the Fluidity One-M. In this example, the fluorescently labeled probe (protein A) in the unbound form displays an R_h of 3 nm whereas the AB complex shows an R_h of 6 nm. Each of the 12 data points has a constant concentration of fluorescently labelled probe of 10 nM and various concentrations of unlabeled target. The K_D value can be deduced from the equilibrium binding data by non-linear least squares fit. The Fluidity One-M will provide K_D values as well as a fitted binding curve automatically once data acquisition is complete. Visually, the K_D can be estimated from the inflection point of the binding curve.

As measurements on the Fluidity One-M are performed in solution, the data obtained from these protein–target interaction assays are representative of a near-native state Fluidic Analytic's technology can analyze proteins in simple aqueous buffers as well as in complex biological backgrounds such as cell lysates, serum, or plasma.

Multi-dimensional data is needed to determine the concentration of antibody binding sites

Determination of K_D based on the model described in section 4 is straightforward if the total concentrations of both binding partners are known, as shown in Figure 1. In the case of SAffCon assays, the concentration of binding sites (e.g., antibodies) in the sample is not known which makes the use of equation 2 less straightforward to determine K_D. As shown in Figure 2, when attempting to fit K_D and binding site concentration from single binding curves, the two parameters are cross-correlated leading to inaccurate results covering several orders of magnitude.

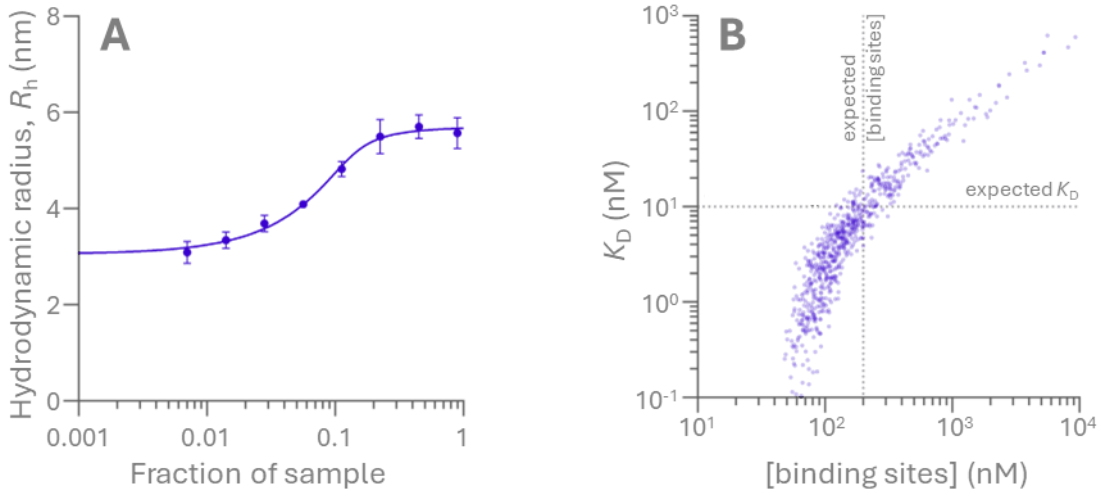


Figure 2. Determination of K_D and binding site concentration from a single binding curve leads to inaccurate results covering several orders of magnitude. (A) Example of a binding curve for which both K_D and binding site concentration are unknown. Note that the x-axis changed to fraction of sample as compared with Figure 1 in which the concentration of target is known. The curve is simulated for a K_D of 10 nM and a binding site concentration of 200 nM. The line represents a fit in terms of equation 1 treating both K_D and [B] as unknown, adjustable parameters. (B) Monte Carlo simulation of 1000 fits to the data shown in (A). Each fit was analyzed in terms of Equation 2 using K_D and [B] = [binding sites] as unknown, adjustable parameters. The plot shows the cross-correlation of the resulting best-fit parameters.

To better constrain K_D and binding site concentration, a modification of the experimental protocol is required. The simplest way to achieve that is to measure additional binding data using a second concentration of the labeled probe, as depicted in Figure 3 overpage.

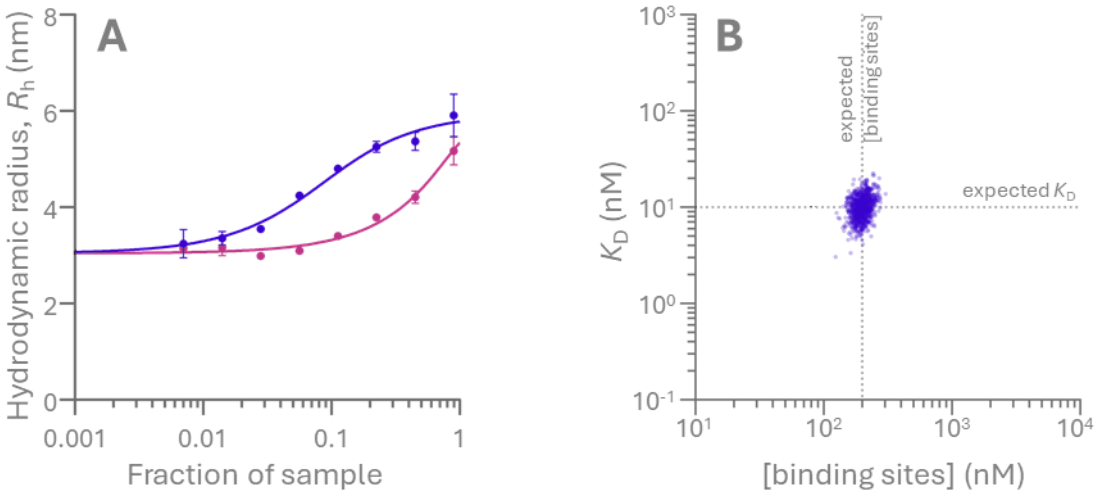


Figure 3. Determination of K_D and binding site concentration from two binding curves leads to accurate results. (A) Example of two binding curves of the same sample measured at two concentrations of labeled probe. Both K_D and binding site concentration are unknown in this example. The curves are simulated for a K_D of 10 nM and a binding site concentration of 200 nM. The line represents a fit in terms of equation 1 treating both K_D and [B] as unknown, adjustable parameters under the conditions that both sets of data must share K_D and binding site concentration. (B) Monte Carlo simulation of 1000 binding curve pairs with the same model parameters as shown in (A). Each fit was analyzed in terms of Equation 2 using K_D and [B] = [binding sites] as unknown, adjustable, global parameters. The plot shows the resulting best-fit parameters.

For K_D, a statistical analysis of the results generated by the Monte Carlo simulations using the two-curve approach shows that 95% of the best-fit values are within a factor of 1.6 of the expected K_D. For the binding site concentration, 95% of the best-fit values fall within a factor of 1.3 of the expected value demonstrating the accuracy of this approach.

While the example in Figure 3 makes a SAffCon assay look straightforward, in reality different combinations of probe concentration and sample dilution will be most suitable to yield highly precise results in the shortest amount of time (e.g., low probe/high sample, high probe/low sample, high probe/high sample, etc.).

The challenge lies in knowing which combinations of probe and sample are most suitable to achieve highly precise affinity and concentration values.

For this reason, Fluidic Sciences has developed Fluidity Insight as a data analysis tool which allows non-expert users to benefit from the advanced statistical analysis technique Bayesian inference. The SAffCon application on the Fluidity Insight platform allows users to combine measurements composed of different combinations of probe and sample to determine affinity and binding site concentration including experimental uncertainty. In addition to providing best fit parameters, Fluidity Insight then also checks the data quality and recommends further experiments in the form of additional combinations of probe and sample that will render the greatest improvement in fit quality.

5. Required equipment, reagents and consumables

All reagents and equipment described in this Getting Started Guide have been validated for the Fluidity One-M system. While the use of reagents and equipment of similar quality is likely to achieve similar results, these have not been tested.

5.1 Required equipment

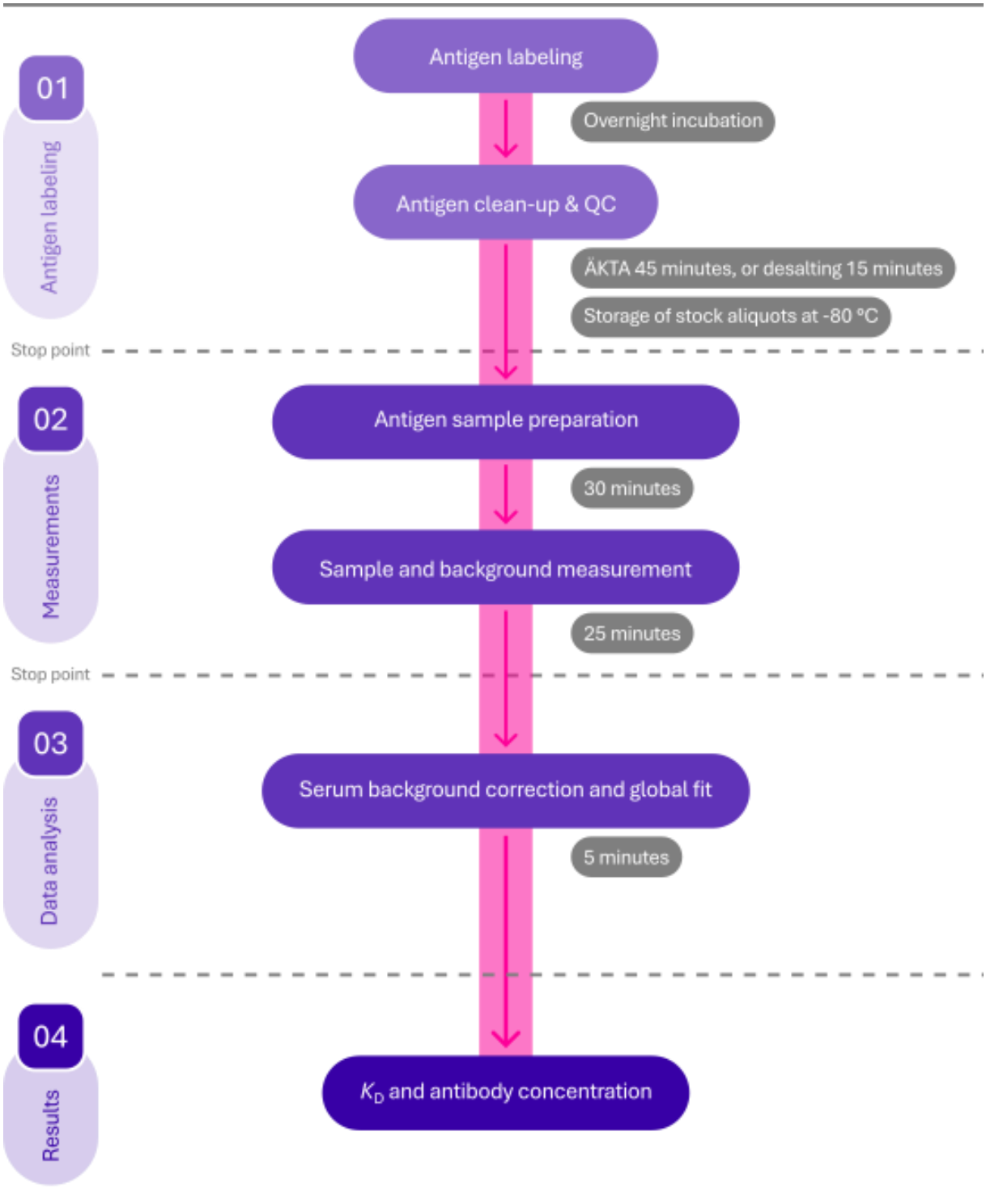
- Fluidity One-M (*Fluidic Sciences*)
- Nanodrop (*Thermo Fisher ND-ONE-W, or equivalent*)
- Pipettes (1000 µL, 200 µL, 10 µL; various suppliers)
- Zeba™ Desalting Chromatography Cartridges, 7K MWCO, 1 mL (*Thermo Fisher 89934*)
- pH meter (various suppliers)
- Centrifuge (*Eppendorf 5428000060, or equivalent*)

5.2 Required reagents

- Alexa Fluor™ 647 NHS Ester (*Thermo Fisher, A20006*)
- DMSO (anhydrous) (*Invitrogen, D12345*)
- 100 µg probe protein to be fluorescently labeled
- 50 µg unlabeled target protein
- Sodium bicarbonate $NaHCO_3$ (*Merck S6014*)
- PBS buffer at pH 7.4 (*Merck P4417*)
- Tween® 20 (*Merck P7949*)
- Glycerol (*Sigma G9012*)
- Ultrapure water (various suppliers)
- HCl solution at 1 M for pH adjustment (various suppliers)

5.3 Required consumables

- Fluidity One-M chip-plate (*Fluidic Sciences*)
- 1000 µL, 200 µL, 10 µL low-retention pipette tips (various suppliers)
- Protein Lo-Bind tubes (*Eppendorf 0030108094* 0.5 mL*; Eppendorf 0030108116* 1.5 mL)
- Sterile filters, 0.22 µm, PVDF (various suppliers)
- 10 and 50 mL syringes, non-sterile (various suppliers)
- 5 mL Luer Lock syringe (e.g., *HSW HENKE-JECT®, Luer Lock 4050.000V0*, or equivalent)
- 1 mL Luer syringe (e.g., *HSW HENKE-JECT®, Luer 4010.200V0*, or equivalent)
- 50 mL Falcon tubes (various suppliers)
- Eppendorf rack (various suppliers)
- Disposable needle (various suppliers)


01
Antigen labeling
Antigen labeling
Overnight incubation
Antigen clean-up & QC
ÄKTA 45 minutes, or desalting 15 minutes
Storage of stock aliquots at -80 °C
Stop point
02
Measurements
Antigen sample preparation
30 minutes
Sample and background measurement
25 minutes
Stop point
03
Data analysis
Serum background correction and global fit
5 minutes
04
Results
K_D and antibody concentration

6 Protocol

6.1 Labeling of the probe

6.1.1 Required equipment

- Fluidity One-M
- Nanodrop
- Pipettes (1000 µL, 200 µL, 10 µL)
- Zeba™ Desalting Chromatography Cartridges, 7K MWCO, 1 mL
- pH meter
- Centrifuge 5430R

6.1.2 Required reagents

- Alexa Fluor™ 647 NHS Ester
- DMSO (anhydrous)
- 100 µg probe to be fluorescently labeled
- Sodium bicarbonate $NaHCO_3$
- PBS buffer at pH 7.4
- Tween® 20
- Ultrapure water
- HCl solution at 1 M for pH adjustment

6.1.3 Required consumables

- 1 Fluidity One-M microfluidic chip-plate
- 1000 µL, 200 µL, 10 µL low-retention pipette tips
- Protein Lo-Bind tubes
- Sterile filters, 0.22 µm, PVDF
- 10 and 50 mL syringes, non-sterile
- 1 and 5 mL Luer Lock syringes
- Eppendorf rack
- Disposable needle

6.1.4 Prepare 6-fold concentration labeling buffer

- Dissolve 0.84 g of sodium bicarbonate ($NaHCO_3$) in 9.5 mL of ultrapure water

Note: This might take 10 – 15 min depending on stirring speed and temperature

- Adjust pH to 8.3 with 1 M HCl if required
- Add ultrapure water to a final volume of 10 mL
- Filter using a pore size of 0.22 µm
- Store at -20 °C in 20 x 500 µL aliquots. If stored at -20 °C, 6-fold concentration labeling buffer can be used for up to 12 weeks

6.1.5 1% PBS-T (stock solution)

- Add 0.5 mL of Tween® 20 to 49.5 mL PBS and stir for at least 30 min at room temperature to obtain a stock solution of 1% Tween 20 in PBS pH 7.4
- Filter sterilize the solution using sterile filters (0.22 µm) and store at 4 °C
- 1% PBS-T can be used for up to 6 months

6.1.6 0.05% PBS-T (working solution)

- Mix 4.75 mL of filter sterilized PBS (pH 7.4) with 0.25 mL of 1% PBS-T stock solution
- Vortex for 10 sec
- Store at ambient temperature
- 0.05% PBS-T can be used for up to 8 weeks

6.1.7 Prepare Alexa Fluor™ label stock solutions

- Immediately before use, dissolve 1 mg of Alexa Fluor™ 647 NHS Ester in 80 µL of DMSO to prepare a 10 mM solution; use a fresh, unopened vial of DMSO and do not re-use once opened
- Aliquot into 5 µL portions and store at -20 °C
- Labeled stock solution can be used for at least 12 weeks if stored at -20 °C
- Do not re-freeze aliquots once thawed

6.1.8 Labeling reaction

- Use 100 µg of probe protein at a minimum concentration of 0.5 mg/mL in PBS buffer at pH 7.4

Note: If the probe is dissolved in a buffer that contains primary amines (e.g., Tris-buffer), buffer exchange into PBS buffer (pH 7.4) is required prior to labeling

- Add labeling buffer as 6 x fold stock (i.e., 20 µL per 100 µL protein solution) and mix carefully by pipetting up and down 10 times. Do not vortex
- Add Alexa Fluor™ label stock solution at a molar ratio of 3:1 (label:protein) to the probe solution; refer to the Fluidic Sciences Labeling Calculator to determine required volumes

Note: As the label is usually dissolved in DMSO, check that the final concentration of the label stock solution in the labeling reaction does not exceed 1.8 % to prevent DMSO interfering with the structural integrity of the probe.

- Carefully pipette up and down 10 times; do not vortex
- Incubate the labeling reaction at 4 °C overnight protected from light

6.1.9 Purification

If an ÄKTA or other FPLC system is available, the highest quality reagents can be achieved by purifying the labeled antigen using SEC according to the recommended protocol of the system and column used. If purification by SEC is not possible the following quick benchtop protocol is suitable for most antigens with a molecular weight above 20 kDa:

- Connect a 5 mL Luer Lock syringe to a desalting column and equilibrate with 5 mL of 0.05% PBS-T (pH 7.4)
- Place 10 Eppendorf tubes in a rack, leave the lids open
- Connect a disposable needle to a 1 mL Luer syringe
- Draw the labeling mixture into the 1 mL Luer syringe

- Remove the needle from the syringe and dispose appropriately
- Remove trapped air from the syringe before connecting it to the desalting column
- Push the sample onto the column

Note: It is not necessary to collect the flow-through at this stage

- Fill a second, unused 1 mL Luer syringe with 1 mL of 0.05% PBS-T (pH 7.4) buffer
- Elute the sample by collecting fractions of approximately 100 µL in the prepared Eppendorf tubes. Read the fraction volumes using the scale of the syringe
- The protein will likely elute in fractions 2 – 4, and later eluting fractions will likely contain unbound label

6.1.10 Labeling QC

- Measure yield of labeled probe as well as labeling ratio
    - To determine the yield as well as labeling ratio, measure the absorbance of all fractions at a wavelength of 280 nm and the absorbance of the conjugated label on a Nanodrop (select Alexa Fluor™ 647)
    - Disable sloping dye correction and Analysis correction functions on the Nanodrop

Note: If using a different model of UV/Vis spectrometer the measured absorbance needs to be corrected manually. Correction factors are 0.11 and 0.03 for Alexa Fluor™ 488 and Alexa Fluor™ 647 respectively.

The following equations yield the corrected protein concentration (required only when not using the automated correction on a Nanodrop).

Alexa Fluor™ 647:

$$\text{protein concentration (M)} = \frac{[A_{280\,\text{nm}} - 0.03 \times A_{650\,\text{nm}}] \times \text{dilution factor}}{\text{molar extinction coefficient at 280 nm}}$$

- The labeling ratio should be between 0.5 and 2 dye molecules per protein
    - If considerably higher label-to-probe ratios are measured (e.g., >3), unbound label was not completely removed, and the desalting procedure should be repeated
- Check the R_h of the fluorescently labeled probe on a Fluidity One-M
    - Dilute the labeled probe to a concentration of 50 nM in a volume of 15 µL using PBS-T buffer (pH 7.4)
    - Measure the size of the purified labeled protein in triplicate on a Fluidity One-M as instructed in the user guide.
- The measured Rh should fall within ±10% of the expected value (consult the Fluidic Sciences hydrodynamic radius calculator)
    - If an R_h lower than expected is measured, unbound label was not completely removed, and the desalting procedure needs to be repeated
- Typical yields of labeled probe are ≤50 µg
- Store the labeled probe at an appropriate buffer condition and temperature

Note: Use of a cryoprotectant (e.g., 10% glycerol) and flash-freezing in liquid nitrogen is recommended for storage at -80 °C

6.2 Sample measurements

6.2.1 Required equipment

- Fluidity One-M
- Table-top centrifuge
- Pipettes (1000 µL, 20 µL, 10 µL)

6.2.2 Required reagents

- PBS buffer at pH 7.4
- Glycerol
- Fluorescently labeled probe
- Sample containing the target (e.g., serum)

6.2.3 Required consumables

- Fluidity One-M chip-plates
- 100 µL, 20 µL, 10 µL low-retention pipette tips
- Protein Lo-Bind tubes

6.2.4 Prepare ViscoMatch buffer

- Prepare a 16% (w/w) glycerol solution by weighing out 1.6 g of glycerol; add PBS pH 7.4 to a final mass of 10 g

6.2.5 Prepare samples Alexa Fluor™ 647-antigen (labeled probe preparation)

- Thaw one aliquot of Alexa Fluor™ 647-labeled antigen and determine the concentration if not known
- Thaw 100 µL of serum slowly on ice and centrifuge for 5 min at 14,000 × g at 4 °C

6.2.6 SAffCon sample preparation

This guide provides instructions for sample preparation in microtubes.

Prepare all samples in 0.5 mL protein Lo-bind tubes. This protocol requires 51 µL of serum.

- **Step 1**: Label 4 Lo-bind tubes with “1000 nM”, “100 nM”, “20%”, and “4%”. If working with multiple samples, add sample identifier labels. Label a further 7 tubes with “10 nM / 50%”, “10 nM / 10%”, “10 nM / 2%”, “10 nM / 0%”, “100 nM / 50%”, “100 nM / 10%”, and “100 nM / 2%”
- **Step 2**: In the “1000 nM’’ tube prepare 10 µL of 1000 nM labeled probe, diluting from stock with ViscoMatch buffer
- **Step 3**: From the 1000 nM stock, prepare 20 µL of 100 nM labeled probe in ViscoMatch buffer by mixing 2 µL of 1000 nM stock with 18 µL of ViscoMatch buffer in tube labelled 100 nM
- **Step 4**: In the “20%’’ tube prepare 20% serum by mixing 6 µL of 100% serum with 24 µL of ViscoMatch buffer
- **Step 5**: In the “4%’’ tube prepare 4% serum by mixing 6 µL of 20% serum with 24 µL of ViscoMatch buffer

- **Step 6**: In pre-labeled tubes prepare reaction mixtures according to the following table:

Sample Name	Volume of Antigen	Volume of ViscoMatch Buffer	Volume of Serum
10 nM / 50%	2 µL of 100 nM	8 µL	10 µL of 100%
10 nM / 10%	2 µL of 100 nM	8 µL	10 µL of 20%
10 nM / 2%	2 µL of 100 nM	8 µL	10 µL of 4%
10 nM / 0%	2 µL of 100 nM	8 µL + 10 µL	Not Applicable
100 nM / 50%	2 µL of 1000 nM	8 µL	10 µL of 100%
100 nM / 10%	2 µL of 1000 nM	8 µL	10 µL of 20%

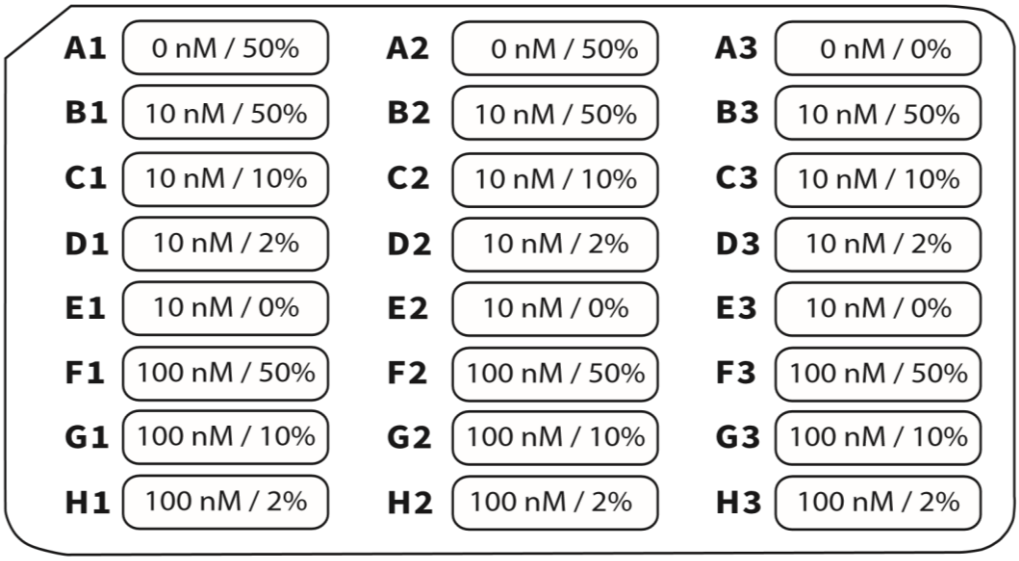
- **Step 7**: Incubate reaction mixtures for 1 h at 4 °C
- **Step 8**: While reactions equilibrate prepare flow buffers. Label 4 Lo-bind tubes with "50% FB", "10% FB", 2% FB", "0% FB"
- **Step 9:** Prepare flow buffers according to the following table:

Flow Buffer Concentration	Volume of Serum (add 100%)	Volume of ViscoMatch Buffer
50% FB	20 µL	20 µL
10% FB	4 µL	36 µL
2% FB	1 µL	49 µL
0% FB	Not Applicable	40 µL

- **Step 10**: Switch on the instrument (power button on the back of the instrument)
- **Step 11**: When the instrument has booted up, log into guest account
- **Step 12**: Prepare the custom template for your experiment. Open the provided SAffCon template excel file (SAffCon template for UG-001.xlsx). Ensure that instrument settings (viscosity, wavelength, and size range) are appropriate for your experiment. Insert probe and sample names. Open the output tab and copy the top two rows of the sheet. Open a new excel file and paste as values into the new sheet. Save the sheet as .csv format – this is the template for your experiment

Instructions:	**Edit pale blue cells. Copy top two rows from Output sheet to a new excel document (pasting as values) and save as *.csv.**
	Edit probe concentrations as appropriate - default values are as per WI-0080
Viscosity	SETTING 3 - serum default
Wavelength	Red 647nm
Size range	2.0 - 9.3 nm (SMALL)
Probe	**Sample**
Probe	Sample
Probe concentrations:	
10	
100	

- **Step 13:** Transfer samples from microtubes to the chip-plate according to the following map:



- **Step 14**: Insert the chip-plate into the Fluidity One-M
- **Step 15**: Measure the samples as described in the User Manual using the template created in step 12

6.2.7 Analysis of SAffCon experiments on Fluidity Insight

The results (K_D, binding site concentration, $R_{h,free}$, and $R_{h,complex}$) are obtained via the SAffCon assay application of Fluidity Insight. For analysis, measurements need to be exported from the Fluidity One-M and processed on Fluidity Insight as described in the User Guide for Fluidity Insight.

7. Troubleshooting

Adjustment of experimental design

Scenario 1: Complex formation is observed at every sample dilution

- Typically caused by high levels of tightly binding antibodies in the sample
    - To resolve this issue, dilute the sample by at least a factor of ten and repeat the experiment using the 10-fold dilution as the highest concentration of the dilution series

Scenario 2: All measurements display the same size as free labeled probe

- The antibody affinity or concentration is too low to measure binding
    - If possible, reduce the concentration of labeled probe

Scenario 3: The binding curves display a flat line at a complex size that is considerably smaller or larger than expected. It may indicate that the probe is binding to a different target. If that is the case, we recommend additional experiments to ensure that the fluorescently labeled probe is indeed binding to the correct target.
Specifically, we suggest the following controls:

- On Fluidity One-M, check binding of the labeled probe to a negative control that features the same background but does not contain the target antibodies
    - On Fluidity One-M, use an unlabeled ligand that competes with the labeled probe for binding to the target/ antibody to check specificity of the probe.

About us

It's not just the proteins that make life, it's the interactions among them. Here at Fluidic Sciences, we make protein interaction analysis easy and robust by developing transformative in-solution technologies and accessible instruments that help scientists quickly and accurately understand how proteins truly interact.

For more information about us, please visit our website www.fluidic.com.