Fluidic Sciences

Getting Started Guide

Measuring protein-binding affinities with the Fluidity One-M system

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1. About this Getting Started Guide

This Getting Started Guide provides guidance for users who want to characterize protein interactions by generating affinity binding curves with their proteins of choice. The guide provides a generic protocol to be used on Fluidity One-M systems that can be easily adapted for any protein and includes helpful tips and tricks to successfully analyze protein interactions on the Fluidity One-M systems.

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 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.

Measuring binding affinity is of importance for:

- Understanding 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

2.2 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 binding affinities of two generic proteins on Fluidity One-M systems by measuring the protein-complex formation 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.

3. Measuring binding affinity on Fluidity One-M systems

The Fluidity One-M systems are microfluidic devices that measure changes in hydrodynamic radius (R_h) directly in solution using microfluidic diffusional sizing (MDS). MDS exploits the well understood relationship between molecule 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, Fluidity One-M systems measure 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 non-linear 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 free, 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.

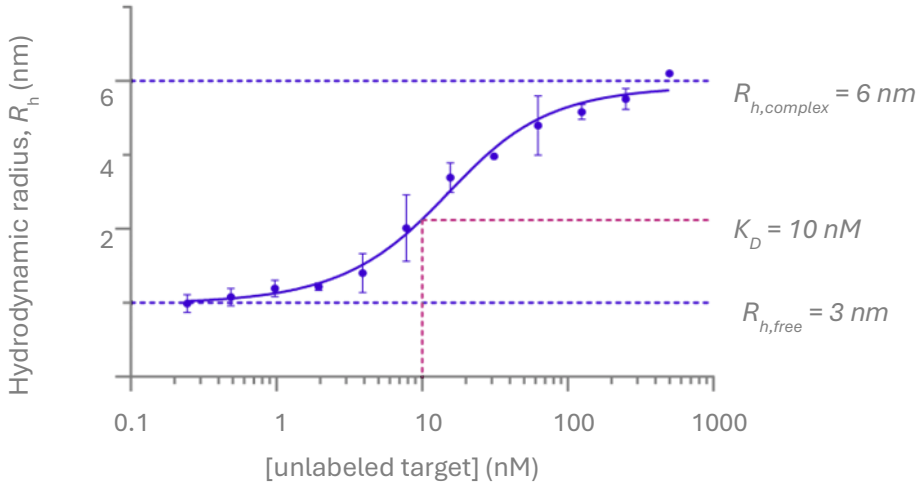


Figure 1. General features of an equilibrium binding curve measured by Fluidity One-M systems. In this example, the fluorescently labeled probe (protein A) in the free, unbound form displays an R_h of 3 nm whereas the AB complex shows an R_h of 6 nm. Each of the 12 data points has a constant concentration of fluorescently labeled probe of 10 nM and various concentrations of unlabeled target. The K_D value can be deduced from the equilibrium binding curve by non-linear least squares fit. The Fluidity One-M systems 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 Fluidity One-M systems are performed in-solution, the data obtained from these protein–target interaction assays are representative of a near-native state. Fluidic Science's Fluidity One-M technology can analyze proteins in simple aqueous buffers as well as in complex biological backgrounds such as cell lysates or plasma.

4. Equilibrium binding affinity measurements to determine the K_D value of bimolecular protein–protein interactions

Performing equilibrium binding experiments that yield an accurate K_D value requires upfront knowledge of the approximate K_D. In many cases this information might not be available and therefore requires a range finding experiment to identify the concentrations of fluorescently labeled probe and unlabeled target required to ensure that both a pre-transition and a post-transition plateau are well defined in the binding curve.

4.1 Range-finding experiment

Range-finding experiments can be carried out with a limited number of data points to save time and material. The experiments should cover at least three orders of magnitude in concentration of the unlabeled target. The concentration of the labeled probe should be kept as low as possible. This ensures that the binding curve covers both the pre-transition baseline as well as the post-transition plateau, both of which are required for accurate K_D determination.

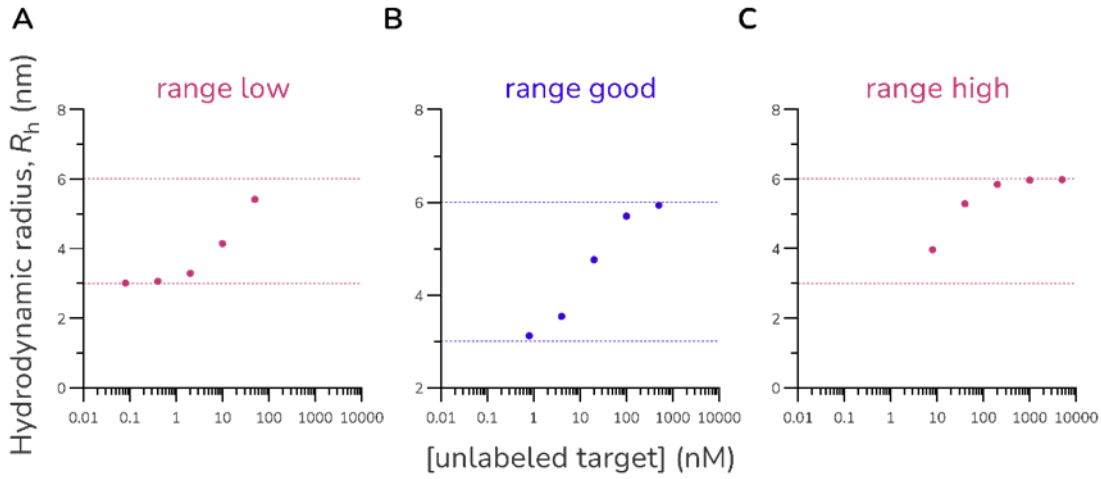


Figure 2. Identifying the correct concentration range for the titration of unlabeled target B. If the K_D is unknown it is best to choose a low concentration of fluorescently labeled probe and test a wide range of concentrations of target to ensure the binding curve covers both the pre-transition baseline as well as the post-transition plateau, both of which are required for accurate K_D determination. For A), the concentration range of the target that is covered is too low, for B) the concentration range is ideal, for C) the concentration range that is covered is too high. Both too low and too high concentration ranges of the target will result in an inaccurate K_D values.

In addition to finding optimal probe and target concentrations, the range finding experiment can also be used to ensure that the binding reaction is fully equilibrated. If equilibrium is established, the first series of measurements should fall in line with the results of the second series and the two curves of the repeat runs will overlap.

4.2 Protein QC

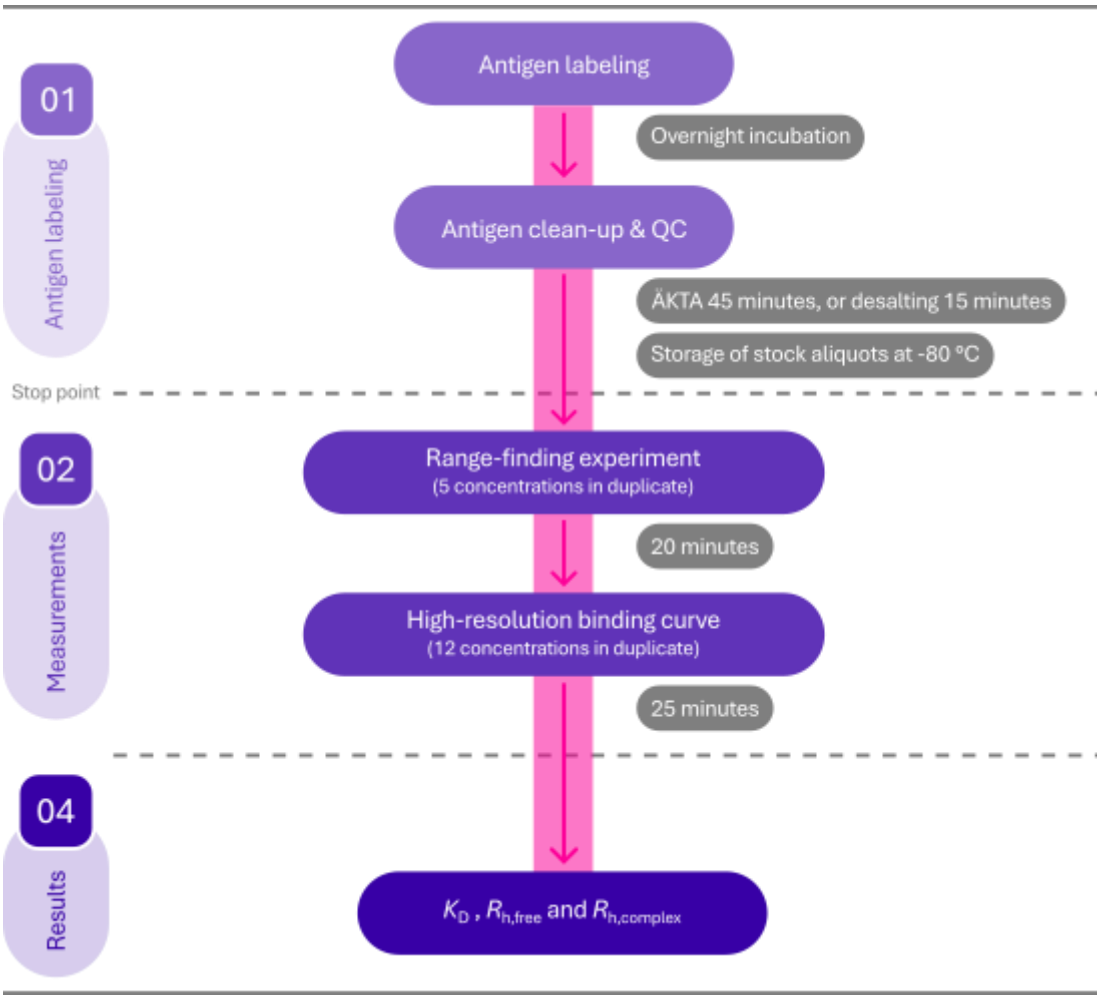
For accurate K_D determination it is essential that the used protein reagents are fit for purpose. Since the Fluidity-One M measures R_h, it can easily be used to quality check proteins during an experiment

(please refer to section 0 for details). For example, a too large R_h can be an indication of protein aggregation while a too small R_h can be a symptom of protein degradation.

4.3 K_D determination

Once concentrations of labeled probe and unlabeled target have been established in the range finding experiment or if the approximate range of K_D is known, a binding curve with a larger number of data points can be measured.

4.4 Suggested experimental workflow



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)
- Zeba™ Desalting Chromatography Cartridges, 7K MWCO, 1 mL (*Thermo Fisher 89934*)
- pH meter (various suppliers)
- Centrifuge 5430R (*Eppendorf 5428000060, or equivalent*)

5.2 Required reagents

- Alexa Fluor™ 488 NHS Ester (*Thermo Fisher, A20000*) *or* Alexa Fluor™ 647 NHS Ester (*Thermo Fisher, A20006*)
- DMSO (anhydrous) (*Invitrogen, D12345*)
- 100 µg probe 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 graduated tips (various suppliers)
- Protein Lo-Bind tubes (*Eppendorf 0030108094* 0.5 mL*; Eppendorf 0030108116* 1.5 mL)
- Sterile filters, 0.22 µm, PVDF (sterile, various suppliers)
- 10 and 50 mL syringes, non-sterile (various suppliers)
- 5 mL Luer Lock syringe (e.g., *HSW HENKE-JECT®, Luer Lock 4050.X00V0*)
- 1 mL Luer syringe (e. g., *HSW HENKE-JECT®, Luer 4010.200V0*)
- 96-well assay plates, flat bottom clear, black polystyrene, non-binding surface (*Corning® 3881*)

- TopSeal™-A PLUS, clear adhesive microplate seal (*PerkinElmer 6050185*)
- 50 mL Falcon tubes (various suppliers)
- Eppendorf rack (various suppliers)
- Disposable needle (various suppliers)

6. Protocol

6.1 Labeling of the probe

6.1.1 Required equipment:

- Fluidity One-M
- Nanodrop
- Pipettes (100 µL, 20 µL, 10 µL)
- Zeba™ Desalting Chromatography Cartridges, 7K MWCO, 1 mL
- pH meter
- Centrifuge 5430R

6.1.2 Required reagents:

- Alexa Fluor™ 488 NHS Ester or Alexa Fluor™ 647 NHS Ester
- DMSO (anhydrous)
- 100 µg probe to be fluorescently labeled
- Sodium bicarbonate NaHCO3
- PBS buffer at pH 7.4
- Tween® 20
- Glycerol
- Ultrapure water
- HCl solution at 1 M for pH adjustment

6.1.3 Required consumables:

- Fluidity One-M chip-plate
- 1000 µL, 200 µL, 10 µL low-retention graduated tips
- Protein Lo-Bind tubes
- Sterile filters, 0.22 µm, PVDF
- 10 and 50 mL syringes, non-sterile
- 5 mL Luer Lock syringe
- 1 mL Luer syringe
- 96-well assay plates, flat bottom clear, black polystyrene, non-binding surface
- TopSeal™-A PLUS, clear adhesive microplate seal
- 50 mL Falcon tubes
- 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 solution

- Immediately before use, dissolve 1 mg of Alexa Fluor™ 488 NHS Ester or Alexa Fluor™ 647 NHS Ester in DMSO (155 or 80 µL respectively) 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 Removal of unbound Alexa Fluor™ label using desalting column

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 either Alexa Fluor™ 488 or Alexa Fluor™ 647 depending on which dye was used)
    - 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™ 488:

$$\text{protein concentration (M)} = \frac{[A_{280\,\text{nm}} - 0.11 \times A_{494\,\text{nm}}] \times \text{dilution factor}}{\text{molar extinction coefficient at 280 nm}}$$

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 K_D-range finding measurements

If the approximate K_D range of the probe–target interaction and suitable buffer conditions are known, this step can be skipped, and users can proceed with section 6.3.

If the range of the K_D is unknown, a range finding experiment can be performed to select appropriate concentrations of labeled probe and unlabeled target for the actual binding curve experiment. Please refer to section 0 for details.

6.2.1 Required equipment:

- Fluidity One-M (Fluidic Sciences F1M0001)
- Pipettes (1000 µL, 20 µL, 10 µL)

6.2.2 Required Reagents:

- PBS buffer at pH 7.4
- Fluorescently labeled probe
- Unlabeled target

6.2.3 Required consumables:

- Fluidity One-M microfluidic chips
- 100 µL, 20 µL, 10 µL low-retention graduated tips
- Protein Lo-Bind tubes

6.2.4 System set-up

- Switch on the system (power button on the back of the system)
- Wait until the system has booted up
- Tap START when the system has completed initialization

6.2.5 Preparation of samples for range-finding experiment

- Step 1: Pipette a 10-fold dilution series of unlabeled target covering a concentration range of 5 µM–0.5 nM (5 samples in total)
  - Prepare 25 µL of unlabeled target at a concentration of 5.55 µM in well A1 of a 96-well plate
  - Add 22.5 µL of PBS-T to wells B1–E1
  - Take 2.5 µL from A1 and transfer to B1, mix by carefully pipetting up and down several times

    - Take 2.5 µL from B1 and transfer to C1, mix by carefully pipetting up and down several times
    - Take 2.5 µL from C1 and transfer to D1, mix by carefully pipetting up and down several times
    - Take 2.5 µL from D1 and transfer to E1, mix by carefully pipetting up and down several times
- Step 2: Prepare 15 µL of labeled probe at a concentration of 100 nM in an Eppendorf tube
- Step 3: Transfer 18 µL of target solutions in wells A1–E1 to A2–E2
- Step 4: Add 2.0 µL of a labeled probe at a concentration of 100 nM to wells A2–E2
- Step 5: Incubate for at least 15 min
- Note: Keep on ice if proteins are unstable at ambient temperature

6.2.6 Measurement of a range-finding experiment

- Measure all 5 samples prepared in section 6.2.5 in duplicate at the appropriate size range setting
    - Press "K_D determination"
    - Complete the fields (replace "probe" and "target" with actual names of proteins):
    - Labeled species – Name: probe
    - Labeled species – Concentration: 10 nM
    - Unlabeled targets – Name: target
    - Unlabeled targets – Titration-dilution: X-fold
    - Unlabeled targets – Start concentration: 5 µM
    - Unlabeled targets – Factor: 10
    - Dilutant (buffer): PBS-T
    - Average sample viscosity: Setting 1
    - Settings – Data points: 5
    - Settings – Repeats: 2
    - Settings – Ex. Wavelength: Red 647 nm or Green 647 nm according to label used
    - Settings – Size range setting: select highest range into which your free probe falls
    - Press "Continue"
    - Select the first unused circuit on the chip plate (hold the plate up to the chip reader below the loading drawer to check which circuits, if any, have already been used) and tap "Continue"
    - Pipette 4 µL of PBS-T into the flow buffer wells of the indicated circuits and tap "Continue"
    - Wait for the 90 s timer to complete
    - Pipette 4 µL of the indicated samples into the shown wells and tap "Continue"
    - Load the plate into the instrument and tap "Start run"

6.2.7 Analysis of range-finding experiment on the Fluidity One-M

Scenario 1: If the range finding curve displays both, a pre-transition and a post-transition baseline, Fluidity One-M systems will analyze the measurement and display an estimate of the K_D value. If this is the case proceed with section 03.

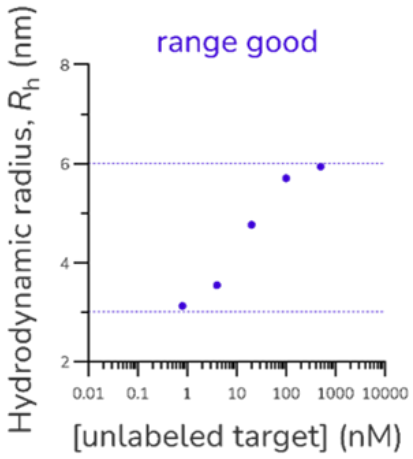


Figure 3. Optimal concentrations identified by a range finding experiment.

Scenario 2: If the range finding curve does not display a pre-transition baseline prepare another sample with a concentration of unlabeled target that is 10-fold lower than the lowest concentration measured in the range finding experiment. Repeat if pre-transition still cannot be detected.

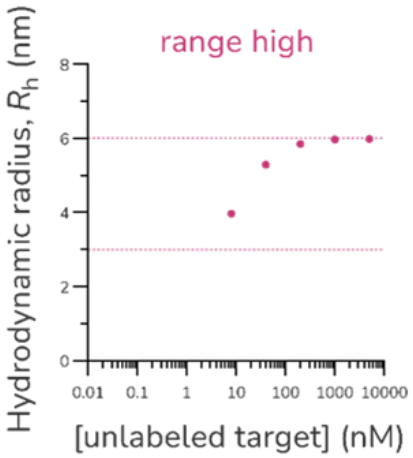


Figure 4. Too high concentrations of unlabeled target were selected and the range finding experiment lacks a pre-transition baseline.

Scenario 3: If the range finding curve does not display a post-transition baseline prepare another sample with a concentration of unlabeled target that is 10-fold higher than the highest concentration measured in the range finding experiment. Repeat if the post-transition baseline still cannot be detected.

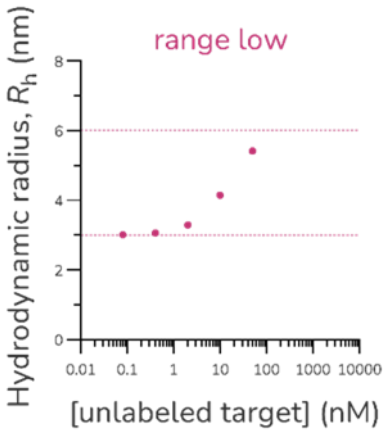


Figure 5. Too low concentrations of unlabeled target were selected and the range finding experiment lacks a post-transition baseline.

6.3 K_D determination experiment

To determine K_D with high accuracy and precision, an equilibrium binding curve with a constant concentration of labeled probe and 12 different concentrations of unlabeled target will be measured.

6.3.1 Concentration of labeled probe protein

For the binding curve with 12 data points, select the concentration of the expected K_D (or the value estimated in the range-finding experiment; Section ?) as the concentration of the labeled probe. For example, if the K_D is expected to be around 10 nM, select 10 nM as the concentration of labeled probe for all 12 data points.

Note: If the concentration of labeled probe cannot be adjusted to the exact value of K_D, select a value that is as close as possible. We do not recommend the concentration of labeled probe to be higher than 5 x the expected K_D. The minimum concentration of fluorophore that can be measured on the Fluidity One-M systems is 100 pM for Alexa Fluor™ 647 labeled probes and 1 nM for Alexa Fluor™ 488 labeled probes.

6.3.2 Concentrations of unlabeled target

As the highest concentration of unlabeled target, select a concentration that is 50 times higher than the expected K_D value (or as estimated in the range-finding experiment; Section ?2). Then perform a 2-fold dilution series of 12 concentrations to yield a concentration of target that is 50 times lower than the expected K_D.

Note: If 50 x K_D cannot be reached, start with the highest possible concentration of unlabeled target. We do not recommend the highest concentration of unlabeled target to be below 20 x expected K_D.

6.3.3 Required equipment:

- Fluidity One-M (Fluidic Sciences F1M0001)
- Pipettes (1000 µL, 20 µL, 10 µL)

6.3.4 Required Reagents:

- PBS buffer at pH 7.4 (*Merck P4417*)
- Fluorescently labeled probe
- Unlabeled target

6.3.5 Required consumables:

- Fluidity One-M microfluidic chips
- 100 µL, 20 µL, 10 µL low-retention graduated tips (various suppliers)
- Protein Lo-Bind tubes (*Eppendorf 022431081* 1.5 mL; *Eppendorf 0030108302* 5 mL)

6.3.6 System set-up

- Switch on the system (power button on the back of the system)
- Wait until the system has booted up
- Tap START when the system has completed initialization

6.3.7 Preparation of samples for K_D measurement

This protocol uses a protein–protein interaction with a K_D value of 10 nM for a labeled probe and an unlabeled target as an example. Concentrations of both labeled and unlabeled proteins will have to be adapted if the expected K_D is different to the 10 nM stated in this protocol.

As a general guideline, the concentration of the labeled probe should be the same as the expected K_D (e.g., determined in the range-finding experiment, section 6.2). For the dilution series of unlabeled target, the lowest concentration should be 1/50 of the expected K_D and the highest concentration should be 50 x K_D. Please refer to section 0 for more information on equilibrium binding experiments.

- **Step 1:** Pipette a 12-step 2-fold dilution series of unlabeled target covering a concentration range of 500 nM–0.24 nM (after addition of labeled binding partner)
    - Use a 96-well assay plate and add 40 µL of a 556 nM solution of unlabeled target in PBS-T to well A1
    - Add 20 µL of PBS-T to wells B1–D2
    - Take 20 µL from A1 and transfer to B1, mix by carefully pipetting up and down several times
    - Repeat the above step for a transfer of solution from wells B1 to C1 and so on until well D2 is reached
- **Step 2:** Prepare 30 µL of labeled probe at a concentration of 100 nM in an Eppendorf tube
- **Step 3:** Add 2.0 µL of labeled probe at a concentration of 100 nM to wells A3–D4
- **Step 4:** Transfer 18 µL of the unlabeled target dilutions from wells A1–D2 to wells A3–D4
- **Step 5:** Incubate for at least 30 min (or use the incubation time as determined during the range finding experiment)
- **Note:** Keep on ice if proteins are known to be unstable

6.3.8 Measurement of binding experiment on a Fluidity One-M

- Measure all 12 samples prepared in section 6.3.7 in duplicate at the appropriate size range setting

- Press "K_D determination"
- Complete the fields (replace "probe" and "target" with actual names of proteins, and the concentration fields with the relevant values for your experiment):
  - Labeled species – Name: probe
  - Labeled species – Concentration: 10 nM
  - Unlabeled targets – Name: target
  - Unlabeled targets – Titration-dilution: X-fold
  - Unlabeled targets – Start concentration: 500 nM
  - Unlabeled targets – Factor: 2
  - Dilutant (buffer): PBS-T
  - Average sample viscosity: Setting 1
  - Settings – Data points: 12
  - Settings – Repeats: 2
  - Settings – Ex. Wavelength: Red 647 nm or Green 647 nm according to label used
  - Settings – Size range setting: select highest range into which your free probe falls
  - Press "Continue"
- Select the first circuit on the chip plate (you will need a new, unused plate as this experiment will use a full plate) and tap "Continue"
- Pipette 4 µL of PBS-T into the flow buffer wells of each circuit and tap "Continue"
- Wait for the 90 s timer to complete
- Pipette 4 µL of the indicated samples into the sample wells and tap "Continue"
- Load the plate into the instrument and tap "Start run"

6.3.9 Display of K_D on Fluidity One-M systems during the experiment

When the run is complete the K_D, $R_{h,free}$ and $R_{h,complex}$ of the protein–protein interaction will be displayed on the Fluidity One-M screen.

6.3.10 Analyze K_D experiments on Fluidity Insight

The results (K_D, $R_{h,free}$ and $R_{h,complex}$) are also displayed in the binding assay application of Fluidity Insight. See Fluidity Insight user guide for more information.

7. Troubleshooting

7.1 Adjustment of experimental design

The binding curve does not display a pre-transition baseline (Figure 4).

- This can happen if the lowest concentration in the dilution series of unlabeled target is too high. To the existing experiment, add three more 2-fold dilutions of unlabeled target and mix with labeled probe as described in section 6.3.7. Rerun the experiment exchanging previously run high concentration samples for the new lower concentration samples.

The binding curve does not display a post-transition baseline (Figure 5).

- This can happen if the highest concentration in the dilution series of unlabeled target is too low. Prepare 40 µL of unlabeled target at a concentration 10-fold higher than initially used. From this sample, prepare three 2-fold dilutions and mix with probe as described in section 0. Rerun the experiment exchanging previously run low concentration samples for the new higher concentration samples.

The binding curve displays a sharp "kink" rather than a smooth transition before saturation is reached (Figure 6).

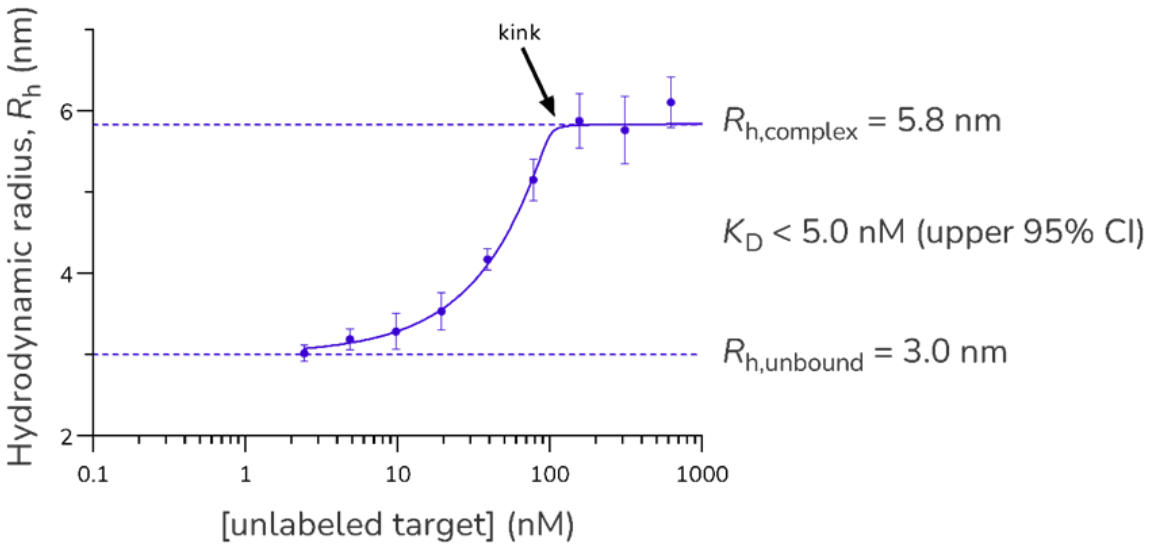


Figure 6. Binding curve for which K_D is considerably lower than the concentration of labeled protein. This suboptimal experimental setup should be avoided as it will only allow an estimation of a maximum K_D value.

- As shown in Figure 6, the concentration of labeled probe is too high. Reduce the concentration of labeled probe by a factor of 10 and repeat the experiment. Note that in some cases also the concentrations in the dilution series of unlabeled target must be lowered to ensure a pre-transition plateau is observed in the binding curve (Please refer to section 0 for more details).

7.2 Suboptimal sample quality

Since the Fluidity One-M measures R_h, it has an inbuilt sample-quality control to detect off-target binding, protein aggregation, and protein degradation.

7.2.1 Off-target binding

A measured complex size that is considerably smaller or larger than expected might 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 systems, check binding of the labeled probe to a negative control that features the same background but does not contain the unlabeled target
- On Fluidity One-M systems, use an unlabeled ligand that competes with the labeled probe for binding to the unlabeled target to check specificity of the probe
- Analyze target purity by SDS-PAGE or size-exclusion chromatography