Fluidic Sciences

Getting Started Guide

Neuroaffinity and Stoichiometry (NeSt) 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 characterize the stoichiometry as well as the affinity of an interaction. 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. Neurodegeneration is mediated by complex protein species

Neurodegenerative diseases are a broad and complex group of disorders. The formation of self-templating protein aggregates called amyloid fibrils is widespread amongst neurodegenerative disease. The precise role played by amyloid remains an area of active study, with these species being also important targets for therapeutic development. Amyloid species are heterogeneous, starting from small clusters of monomers and growing into large fibrils that are formed of several interacting strands of aggregated protein. This complexity and heterogeneity makes amyloid difficult to study using traditional techniques.

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. Measuring binding affinity is important when:

- 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. Neuroaffinity and Stoichiometry (NeSt) assay

Determination of binding stoichiometry can be challenging using traditional interaction analysis approaches, particularly for the complex and heterogeneous aggregates implicated in many neurodegenerative diseases. Furthermore, the ratio of species forming a complex can be important for understanding biological function or mechanism of action of pharmaceutical products. The Neuroaffinity and Stoichiometry (NeSt) assay described in this user guide enables the determination of binding affinity of a protein pair as well as the stoichiometry of their interaction, without the need for an external calibrant or any prior knowledge about the interaction. The assay workflow has been optimized to ensure these critical data are obtained as efficiently as possible. The assay takes place entirely in solution and so the results obtained are biologically relevant.

The Neuroaffinity and Stoichiometry (NeSt) assay can determine, directly in solution, the affinity of an interaction between two proteins as well as the stoichiometry of that interaction without the need for calibrants or external reference standards.

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 two interacting proteins on the Fluidity One-M by measuring the formation of complex according to the equilibrium A + B ⇌ AB and determining the K_D value, which is described by Equation 1:

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

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.

Affinity describes how tightly two proteins interact. It is therefore a crucial parameter when understanding biological processes or developing therapeutic agents.

3.2 Why the stoichiometry of interaction is important

The stoichiometry of an interaction describes the relative numbers of each participating species in the product complex. In basic research this is important for understanding the downstream impacts of complexation as it can reveal amplification or suppression of biological signals. In drug development applications stoichiometry can reveal important details of the mechanism of action of a candidate, inform dosing strategies, and provide useful data on possible interaction geometries including any impact of steric occlusion.

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

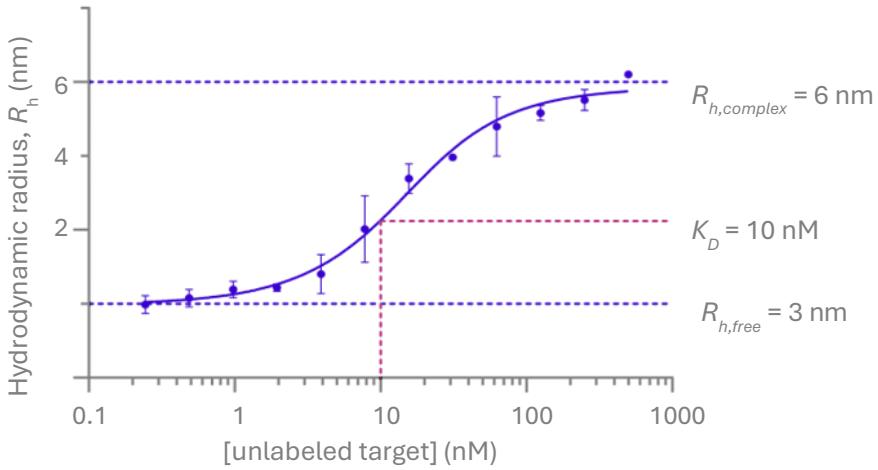


Figure 1. General features of an equilibrium binding curve measured by MDS using the Fluidity One-M. In this example, the free fluorescently labeled probe displays an R_h of 3 nm whereas the complex has an R_h of 6 nm. Each of the 12 data points has 10 nM fluorescently labelled probe and the indicated concentration 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.

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 Science'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 stoichiometry of interactions

Determination of K_D based on the model described in section 4 is straightforward if the concentrations of epitopes of both binding partners are known, as shown in Figure 1. In the case of NeSt assays, where the known molecular concentration of the binding target may differ substantially from that of exposed binding epitopes 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.

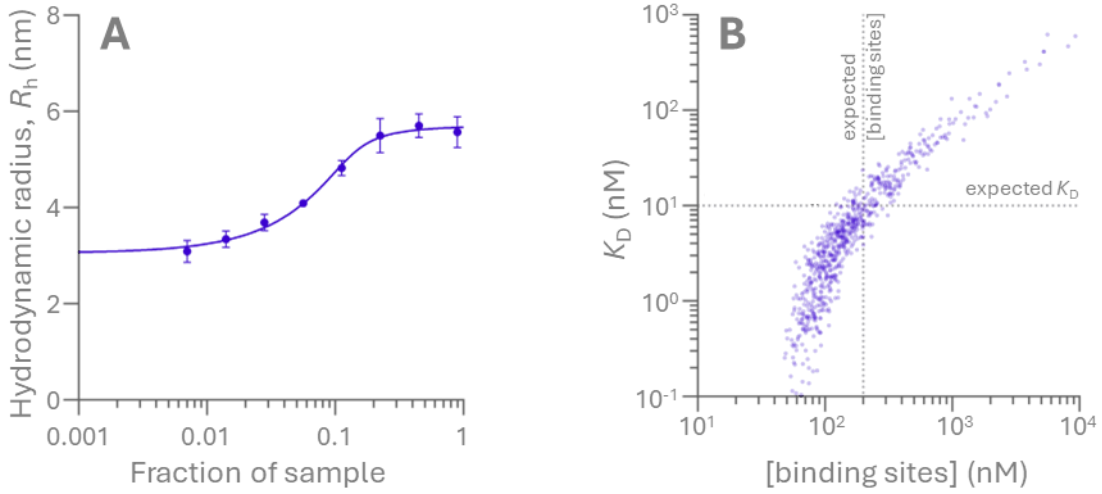


Figure 2. Determination of K_D and binding site concentration from a single binding curve leads to inaccurate results covering several orders of magnitude. (A) Example of a binding curve for which both K_D and binding site concentration are unknown. Note that the x-axis changed to fraction of sample as compared with Figure 1 in which the concentration of target epitopes 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.

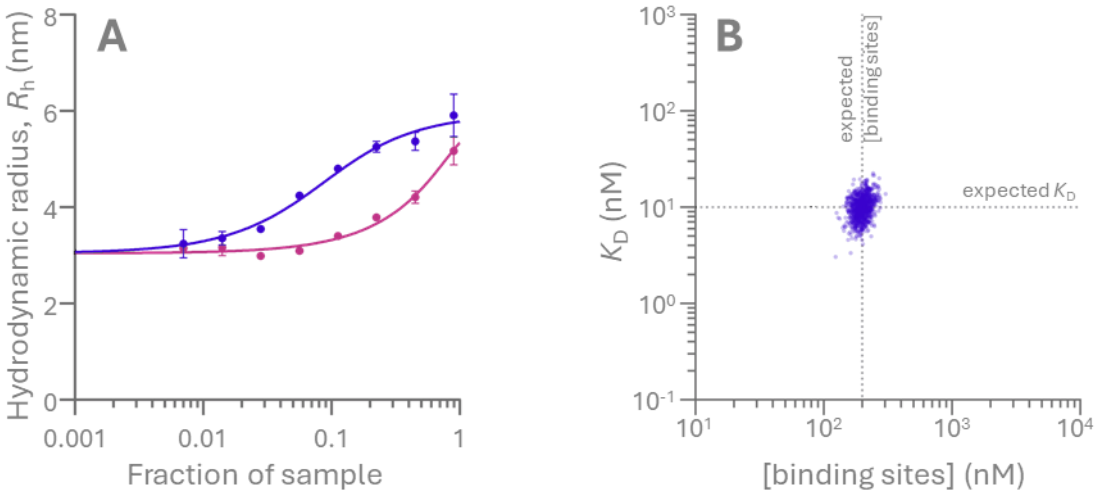


Figure 3. Determination of K_D and binding site concentration from two binding curves leads to fit convergence. (A) Example of two binding curves of the same sample measured at two concentrations of labeled probe. Both K_D and binding site concentration are unknown in this example. The curves are simulated 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 NeSt assay look straightforward, in practice different combinations of probe concentration and sample dilution will be most suitable to yield 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 NeSt application on the Fluidity Insight platform allows users to combine measurements composed of different combinations of probe and sample to determine affinity and binding stoichiometry 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

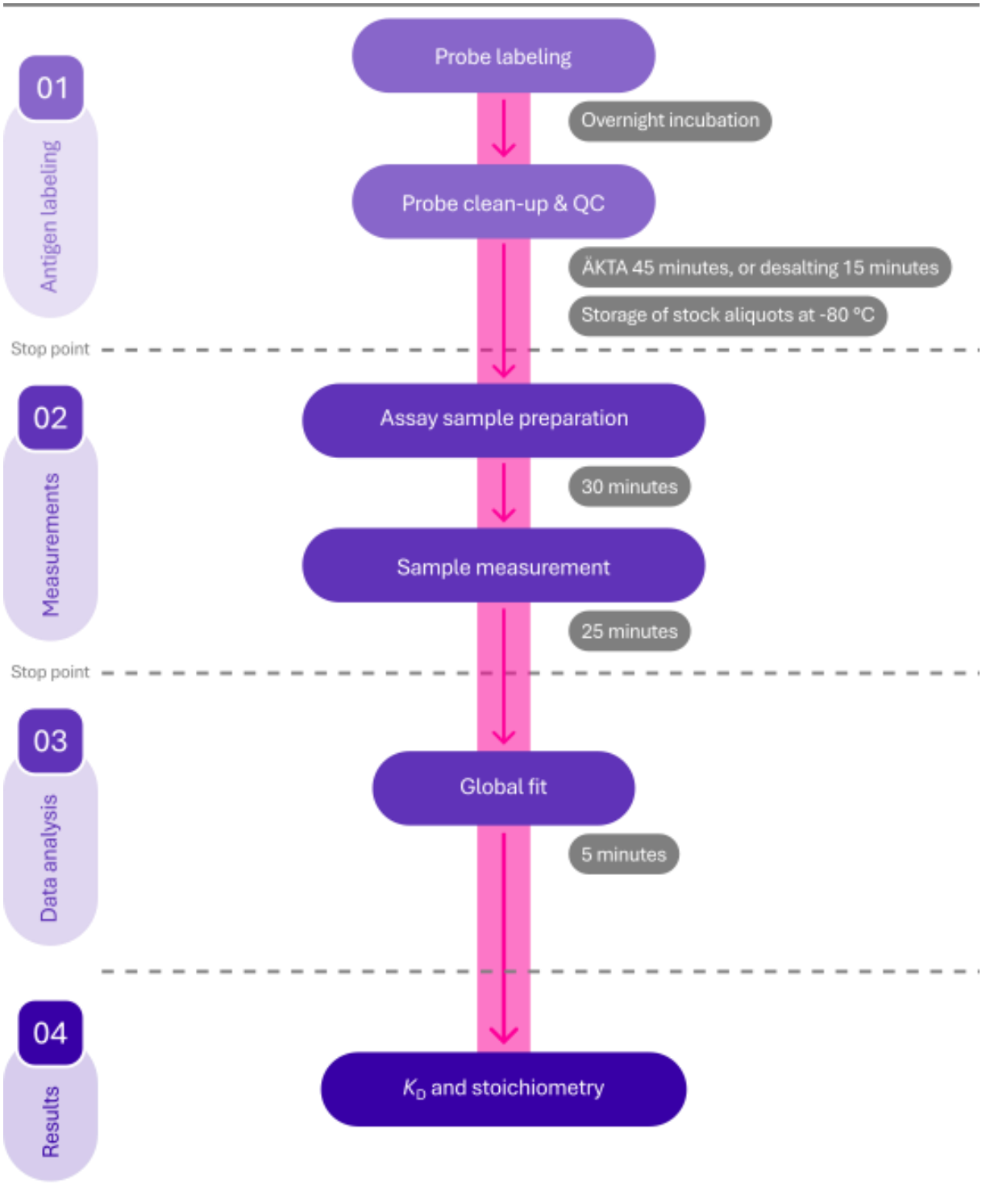
- Fluidity One-M (*Fluidic Sciences*)
- Nanodrop (*Thermo Fisher ND-ONE-W, or equivalent*)
- Pipettes (1000 µL, 200 µL, 10 µL; various suppliers)
- Zeba™ Desalting Chromatography Cartridges, 7K MWCO, 1 mL (*Thermo Fisher 89934*)
- pH meter (various suppliers)
- Centrifuge (*Eppendorf 5428000060, or equivalent*)

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-plates (*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 (Sterile, various suppliers)
- Eppendorf rack (various suppliers)
- Disposable needle (various suppliers)


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

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

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 probe 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 probes 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)

Note: This guide assumes that PBS-T is used for the reaction. If a different buffer is required simply substitute that wherever PBS-T is stated throughout this workflow guide.

- Place 10 Eppendorf tubes in a rack, leaving 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:

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

- 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 R_h should fall within ±10% of the expected value
    - 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
- Target

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 Required samples

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

6.3.5 NeSt 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 target solution.

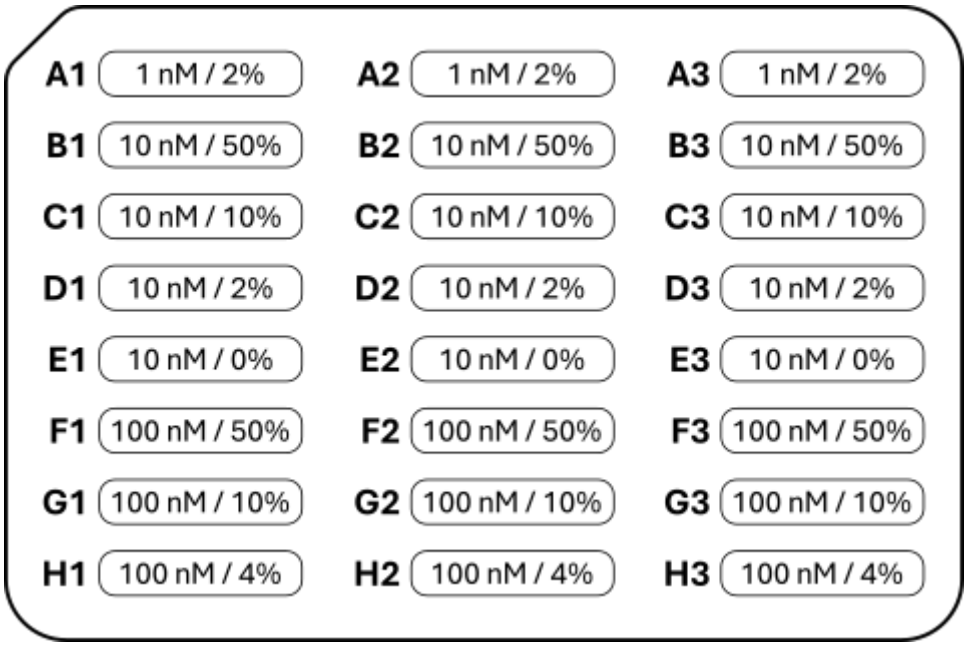
- **Step 1**: Label 4 Lo-bind tubes with “1000 nM”, “100 nM”, “20%”, and “4%”. If working with multiple samples, add sample identifier labels. Label a further 7 tubes with “10 nM / 50%”, “10 nM / 10%”, “10 nM / 2%”, “10 nM / 0%”, “100 nM / 50%”, “100 nM / 10%”, and “100 nM / 2%”
- **Step 2**: In the “1000 nM’’ tube prepare 10 µL of 1000 nM labeled probe, diluting from stock with 0.05% PBS-T buffer
- **Step 3**: From the 1000 nM stock, prepare 20 µL of 100 nM labeled probe in 0.05% PBS-T buffer by mixing 2 µL of 1000 nM stock with 18 µL of 0.05% PBS-T buffer in tube labelled 100 nM
- **Step 4**: In the “10 nM” tube mix 2 µL of 100 nM labeled probe with 18 µL 0.05% PBS-T buffer
- **Step 5**: In the “20%’’ tube prepare 20% target by mixing 6 µL of 100% target with 24 µL of 0.05% PBS-T buffer
- **Step 6**: In the “4%’’ tube prepare 4% target by mixing 8 µL of 20% target with 32 µL of 0.05% PBS-T buffer
- **Step 7**: In pre-labeled tubes prepare reaction mixtures according to the table overpage
- **Step 8**: Incubate reaction mixtures for 1 h at 4 °C

Sample Name	Volume of probe	Volume of 0.05% PBS-T Buffer	Volume of target
1 nM / 2%	2 µL of 10 nM	8 µL	10 µL of 4% stock
10 nM / 50%	2 µL of 100 nM	8 µL	10 µL of 100% stock
10 nM / 10%	2 µL of 100 nM	8 µL	10 µL of 20% stock
10 nM / 2%	2 µL of 100 nM	8 µL	10 µL of 4% stock
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% stock
100 nM / 10%	2 µL of 1000 nM	8 µL	10 µL of 20% stock
100 nM / 2%	2 µL of 1000 nM	8 µL	10 µL of 4% stock

- **Step 8**: Switch on the instrument (power button on the back of the instrument)
- **Step 9**: Prepare the custom template for your experiment. Open the provided NeSt template excel file (NeSt template for UG-005.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 lavender cells. Copy top two rows from Output sheet to a new excel document (pasting as values) and save as *.csv.**
	Edit probe concentrations if needed - default values are as per UG-005 - and input concentration of target stock solution
Viscosity	SETTING 1 - aqueous buffers
Wavelength	Red 647nm
Size range	3.0 - 14 nm (MEDIUM)
Probe	**Sample**
Labeled probe	Target
Probe concentrations:	
10	
100	
Target stock concentration (nM)	
500	

- **Step 10:** Transfer samples from microtubes to the chip-plate according to the following map:



- **Step 11**: Insert the chip-plate into the Fluidity One-M
- **Step 12**: Measure the samples as described in the User Manual using the template created in step 9

6.2.6 Analysis of NeSt experiments on Fluidity Insight

The results (K_D, membrane protein concentration, interaction stoichiometry, $R_{h,free}$, and $R_{h,complex}$) are obtained via the NeSt 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 exposed binding epitope in the target solution
    - To resolve this issue, dilute the target solution 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 target 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 target different to that expected. 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
- On Fluidity One-M, use an unlabeled ligand that competes with the labeled probe for binding to the target 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.