In-solution affinity determination using Gyrolab systems

Key benefits of Gyrolab systems for affinity determinations:

- **Determines K_D values down to low picomolar levels**
- **Rapid affinity determination** – up to 14 interacting pairs per CD per hour
- **In-solution measurement mimics the biological system** – unmodified interactants in free solution
- **Active concentration** – stoichiometrically-controlled measurement is valuable for QC and batch release
- **No equilibrium shift during measurement** – short contact time between capture column and complexes formed under equilibrium conditions
- **Multi-Curve Analysis** increases precision and accuracy of K_D and activity measurements

Affinity characterization in the development of biotherapeutics

Effective biotherapeutics often have a high affinity to the target, which enables the dosage to be lowered to improve healthcare and reduce the cost of treatment. Determining affinity plays a key role throughout development – from early screening of hybridomas or recombinant antibodies to affinity maturation and antibody engineering to improve the efficacy, safety and manufacturability of the final antibody drug product. Affinity characterization is also important when selecting reagents for quantitative analytical assays or assessing activity of the drug product in manufacturing release tests. Therapeutic antibodies now have affinities down to the femtomolar level, which places particular demands on analytical methods.

In-solution affinity measurement using Gyrolab systems enables rapid and accurate determination of affinity and activity

The affinity constant, K_D (equilibrium dissociation constant) is commonly used to describe the binding strength between two interactants. The equilibrium

$$A + B \Leftrightarrow AB$$

describes the reversible formation of AB complex and its dissociation into the molecular interactants A and B. By determining the concentration of the interactants at equilibrium K_D can be assessed.

$$K_D = [A][B]/[AB] \text{ (unit: M)}$$

Gyrolab automated nanoliter-scale immunoassay platforms is sufficiently sensitive to determine affinity constants down to low picomolar K_D (Figure 1). The active concentration of one of the interactants, which is valuable for QC and batch release, can also be determined under stoichiometrically limited conditions.

The flow-through affinity column format in Gyrolab CDs is ideal for measuring free interactants in equilibrated solutions since the interaction between sample and capture column of only a few seconds minimizes shifts in equilibrium. Parallel processing enables simultaneous determination of up to 14 interactions using the same CD.

In this Technical Note, we present how Gyrolab systems simplify the accurate and rapid analysis of high affinity ligands to determine affinity and activity, illustrated with a case study.
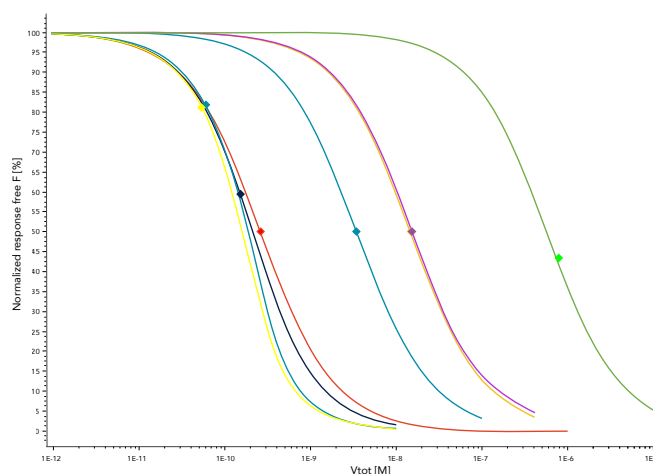

Figure 1. *Screening of anti-idiotypic antibodies with affinities ranging from µM to double digit pM*

The simplicity of measuring high affinity interactions in solution with Gyrolab system

The determination of affinity and active concentration in solution involves measuring interactions between an interactant at a Fixed concentration (F) and an interactant at a range of concentrations (V) that are allowed to reach equilibrium. High affinity interactions involve rapid formation (k_{on}) of complex FV and slow dissociation (k_{off}) of FV to interactants F and V. At equilibrium, the concentrations of unbound interactants F and V are very low.

Accurately measuring the low concentrations of free interactant F at equilibrium in a large background of bound complex FV is simple using a sensitive Gyrolab immunoassay format.

The total amount of Fixed interactant (F_{tot}) can be set relative to the estimated K_D to determine either the K_D itself or the active concentration of the Fixed interactant.

$$F + V \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} FV$$

$$K_D = [F][V]/[FV]$$

Guidance throughout – from setting up the experiment to data analysis

a) The Affinity module in Gyrolab Control Software guides you by defining an affinity series, run method, and a loading list that helps you to set up the affinity samples in the microplate.

a)

b) The affinity samples consist of one interactant that is diluted to form a concentration series [V] and the second interactant that is added at a fixed concentration [F] to each sample in the affinity series.

b)

c) The affinity samples are allowed to reach equilibrium on the bench. Proper affinity measurement should be done at controlled temperatures. This may take hours or days depending on affinity properties of the interaction.

c)

d) The response from free Fixed interactant [F] is measured in Gyrolab Bioaffy CD run using an automated immunoassay procedure on Gyrolab xPand or Gyrolab xPlore. Up to 14 interacting pairs can be processed per CD in parallel in just one hour.

d)

e) Gyrolab Evaluator software plots the response from free [F] interactant against the molar concentration of the variable [V] interactant and fits the affinity curve according to a selected interaction model.

When F_{tot} is below 10xK_D or desirable below K_D the curve is controlled by the affinty of the interaction. This type of curve will give a reliable K_D value for the interaction.

Activity measurement: If the single-curve experiments are stoichiometrically controlled (i.e. the concentration of fixed interactant is much higher than the K_D) then curve fitting will give reliable determinations of the active concentration of the fixed interactant (F_{tot}).

e)

f) *Multi-Curve Analysis*

Since several curves originating from the same interaction have the same K_D value these curves can be analyzed together using Multi-Curve Analysis to improve estimates of K_D and active concentration.
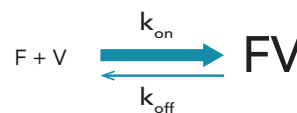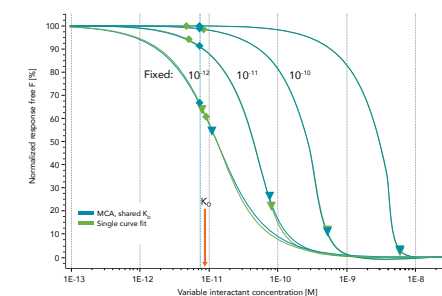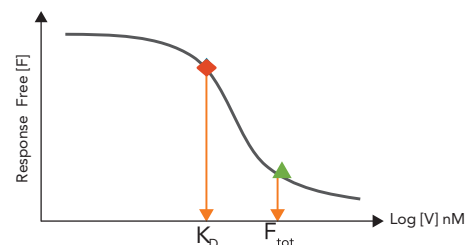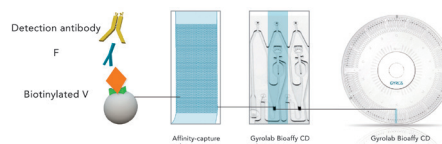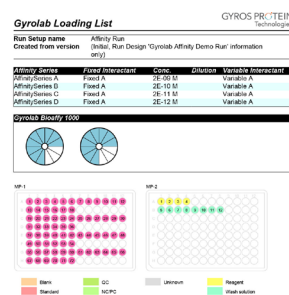
f)

Case study: Determination of K_D and active concentration of five leading therapeutic antibodies

Tumor necrosis factor (TNFα) is a cytokine involved in systemic inflammation and is associated with autoimmune disorders. Treatment involves established antagonists such as monoclonal antibodies or Fc-fusion proteins. These include 1st generation antibodies Remicade® (infliximab), Humira® (adalimumab), and Enbrel® (etanercept), together with the biosimilar Remsima™ (infliximab) and the 2nd generation antibody Simponi® (golimumab).

Experimental Design

The affinity series were created by mixing:

1. TNFα as the variable interactant in the concentration range 20 nM – 2 pM

2. TNFα-antagonist as fixed interactant at binding concentrations of 10 pM and 500 pM for K_D and stoichiometrically controlled curves, respectively.

The affinity samples were incubated at room temperature for 72 hours. Free TNFα antagonist was determined using a Gyrolab affinity method with biotinylated TNFα as capture reagent and fluorescently labeled anti-human IgG as detection reagent.
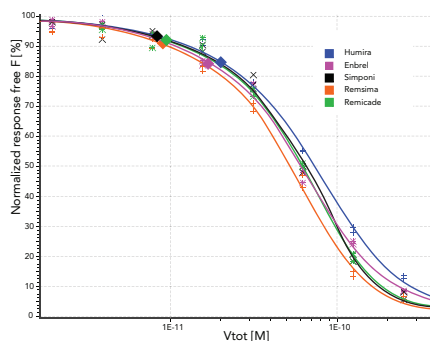

Figure 2. K_D-controlled affinity series to determine the affinity of five TNFα antagonists for TNFα.
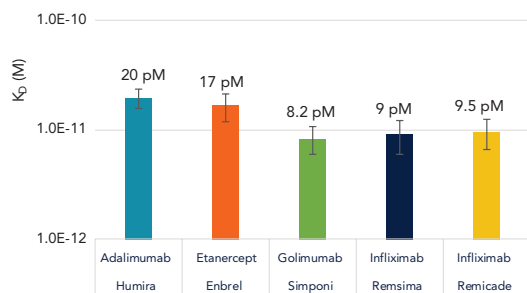

Figure 3. K_D values for TNF antagonists in solution

The results for the 1st generation antibodies, biosimilar and 2nd generation antibodies from the K_D-controlled affinity series were fitted to the model to obtain K_D values (Figure 2). All antagonists were determined to have affinities in the low picomolar range (Figure 3). Both infliximab antagonists (Remicade and Remsima) had K_D values of around 9 pM. The 2nd generation antibody Simponi had a slightly lower K_D.

This approach showed that several binders in the picomolar range could be easily determined in one Gyrolab affinity run.

Measurement of active concentration

Using a stoichiometrically controlled affinity series, the binding sites for the TNFα antagonist were titrated until complete inhibition and the equimolar concentration representing the active binding site concentration was obtained by curve fitting (Figure 4). The active concentrations of the TNFα antagonists were measured in seven independent runs on different CDs, giving coefficients of variation of less than 5%. All active concentrations were close to the expected value of 500 pM.
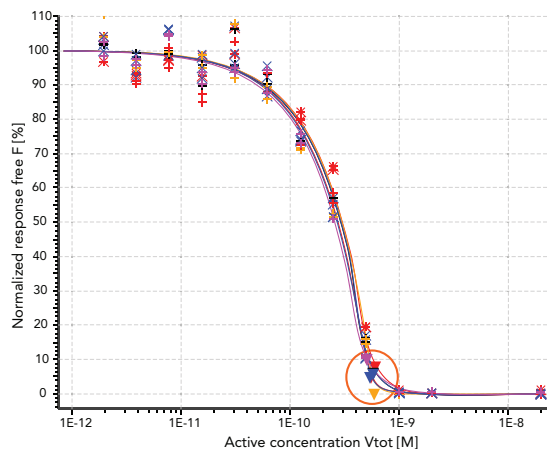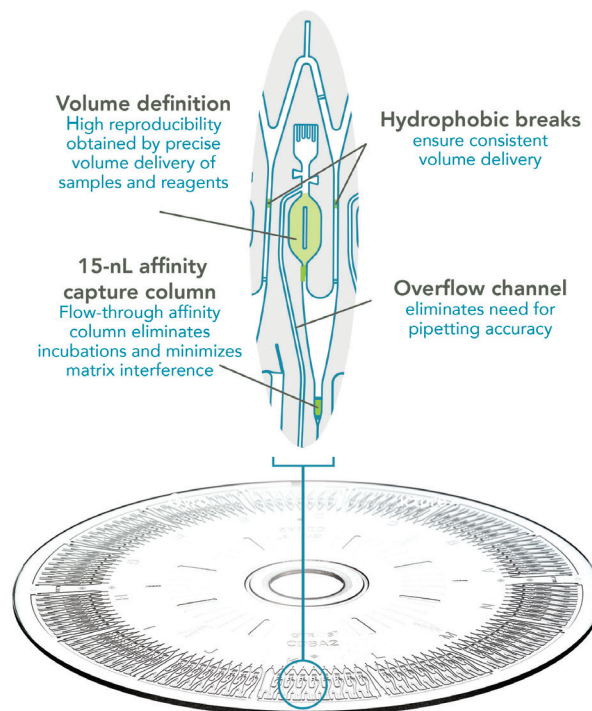

Figure 4. Stoichiometrically controlled affinity series to determine the active concentrations of five TNFα antagonists.

Automated immunoassays at nanoliter-scale

Gyrolab technology delivers high-quality immunoassay data with broad dynamic ranges that enable you to save time (assay time and manual, hands-on time), sample and reagents. Using an affinity flow-through format, this technology simplifies assay workflows by eliminating incubations and shortening run times. It starts with Gyros Protein Technologies' proprietary CD technology engineered with highly reproducible nanoliter microfluidics integrated with Gyrolab platforms, which automate immunoassays with parallel processing using laser-induced fluorescence detection. This is possible through precise, automated control of centrifugal and capillary forces which steer liquid flow through nanoliter-scale microfluidic structures contained within the CD in which the assay workflow is automated.

Volume definition
High reproducibility obtained by precise volume delivery of samples and reagents

Hydrophobic breaks
ensure consistent volume delivery

15-nL affinity capture column
Flow-through affinity column eliminates incubations and minimizes matrix interference

Overflow channel
eliminates need for pipetting accuracy

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