Procedure

Steady-state affinity analysis using Biacore systems

The affinity of an interaction can be determined from the dependence of steady-state binding levels on analyte concentrations, or calculated as the ratio between kinetic rate constants. Steady-state analysis is particularly suited to measurement of weak to moderate interactions. For strong interactions it is generally more suitable to determine affinity via kinetic experiments. An overlay plot of sensorgrams from a typical steady–state affinity analyse can be seen in Figure 1.

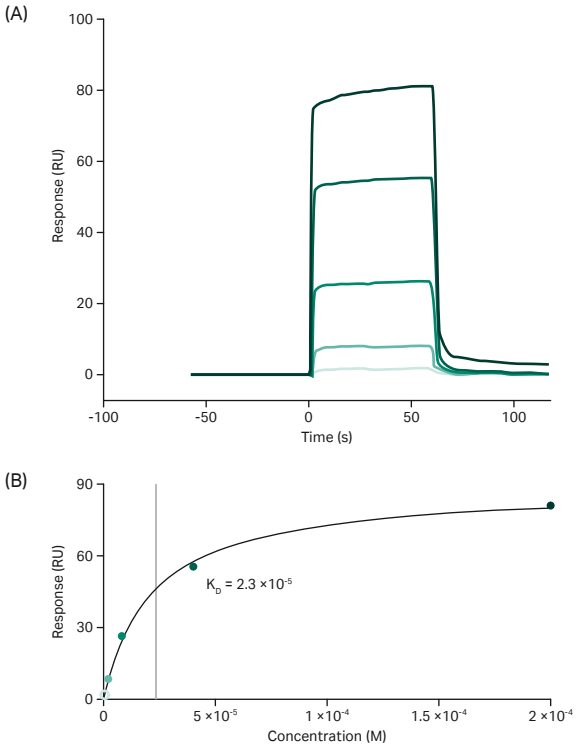

Fig 1. (A) Overlay plot of sensorgrams from a typical steady-state affinity analyse with five sample injections. The sensorgrams are reference subtracted and blank subtracted. Sample injections include five samples from a range of different concentrations, two zero-concentration samples and one sample concentration in duplicate. (B) Response versus concentration plot for the five samples of different concentration. From the plot the affinity (K_D) is read.

Experimental design

1. Aim for an ligand attachment level that gives clearly measurable responses over the whole range of analyte concentrations.
2. It is important that concentrations of analyte can be accurately determined. If it is difficult to determine the concentration of one of the interactants, use this one as the ligand.
3. Check activity, specificity and non-specific binding.
4. The maximum binding capacity of the surface (response at saturation) can be theoretically calculated using the formula (assuming 100% activity):

$$R_L = (MW_L/MW_A) \times (R_{max}/S_m)$$

R_L (RU) = Attachment level
R_{max} (RU) = Maximum binding response
MW_A (Da) = Molecular weight of analyte
MW_L (Da) = Molecular weight of ligand
S_m = Stoichiometric ratio (number of binding sites per ligand)

5. Determine steady-state binding levels over a range of analyte concentrations. The concentration range should cover at least from 20% to 80% saturation of the surface.
6. Design a reference surface and use reference-subtraction. An unmodified surface is often adequate as reference surface for affinity measurements.
7. Include at least five different analyte concentrations.
8. Include at least one concentration in duplicate to check the stability of the assay.
9. Include one or two zero-concentration samples (using running buffer as blanks) for double referencing.
10. Match sample and running buffer refractive index as closely as you can. Dilution into running buffer may be sufficient if the sample is stored as a concentrated stock solution.

Important considerations

- How does the concentration range affect the affinity value?
  - Rule of thumb: do not trust K_D values (affinity) that are greater than half the highest concentration used. If the R_{eq} (response at equilibrium) versus C (concentration) plot does not flatten out sufficiently, curve fitting to the steady-state model will be uncertain.
- Can I trust the affinity value if steady-state was not reached?
  - The response must be close to steady-state to give a reliable value.
  - If steady-state is reached at some, but not all concentrations, the value will be close but not exact. This premature steady-state evaluation underestimates the K_D since the true affinity is stronger than calculated.
  - The fitted R_{max} parameter should not be significantly larger (more than two times) than the theoretically calculated response.

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CY17751-07Dec20-PD