Assessment of protein concentration using a calibration-free method

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Introduction

In development and production of biotherapeutics it is important to determine the concentration of functionally active molecules in a given sample. Many established methods for protein concentration measurement do not distinguish between active and inactive molecules, or rely on comparison with a standard that may or may not be a mimic of the actual sample. The method discussed here, Calibration-Free Concentration Analysis (CFCA), provides a highly sensitive and robust biosensor-based determination of the protein concentration and does not require a standard. The method relies on measurement of analyte binding to a target immobilized on a sensor surface at varying flow rates, under conditions where the observed rate of binding is partially or completely limited by transport of analyte molecules to the sensor surface. This transport is a diffusion controlled process that depends on the analyte concentration. The concentration is obtained by running Biacore™ binding experiments at different flow rates and fitting the binding data to the model describing the process.

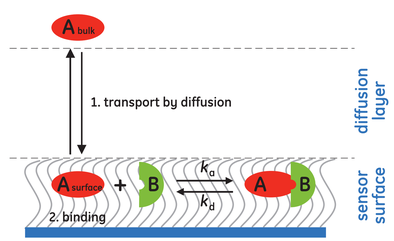
In Biacore instruments samples are injected in a micro flow system and transported in laminar flow to the sensor surface. Molecules reach the sensor surface from bulk solution by a diffusion-controlled transport process. In addition to the concentration of analyte molecules, factors influencing the transport rate include the diffusion coefficient, flow cell dimensions, and flow rate. The balance between the transport rate and the binding rate determines whether the observed binding will be transport limited or reaction limited. For successful CFCA, the observed binding rate must be at least partially limited by transport. In this poster, we discuss how suitable flow rates, immobilization levels, and binding data are selected for successful implementation of CFCA.

A_{bulk} is the concentration of injected analyte

$A_{surface}$ is the concentration of analyte at the sensor surface

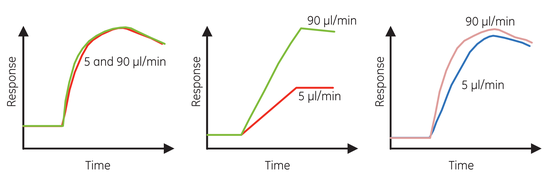
B is the target immobilized on the surface

k_a and k_d are association and dissociation rate constants, respectively



How to recognize transport limited data?

Sensorgrams obtained at two flow rates



- Reaction limited data
- Binding rates are not affected by flow rate variation
- Data not suitable for CFCA

- Transport limited data
- Binding rates increase with increasing flow rate
- Data suitable for CFCA

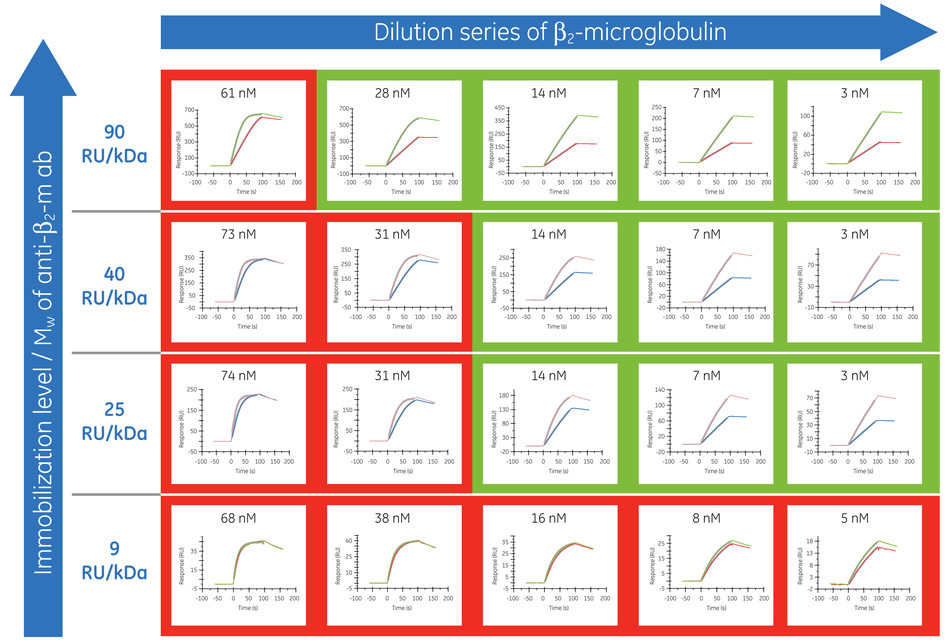
- Partially transport limited data
- Initial binding is affected by flow rate variation
- Late binding is less affected by the flow rate
- Data potentially suitable for CFCA

Immobilization level and concentration range

Calibration-free concentration analysis of 2-fold dilution series of β_2-microglobulin was performed on four surfaces immobilized to increasing levels of anti-β_2-microglobulin antibody. Immobilization levels are expressed as response units, RU, divided by the molecular weight of interaction partner, kDa, to compensate for the difference in response between differently sized molecules. The protein solutions were injected at two flow rates, 5 and 90 µl/min.

The concentration determined by the method is shown in each panel. Sensorgrams framed in green are obtained under experimental conditions that return the same calculated concentration after adjustment for the dilution factor. The concentration given by sensorgrams framed in red are overestimated.

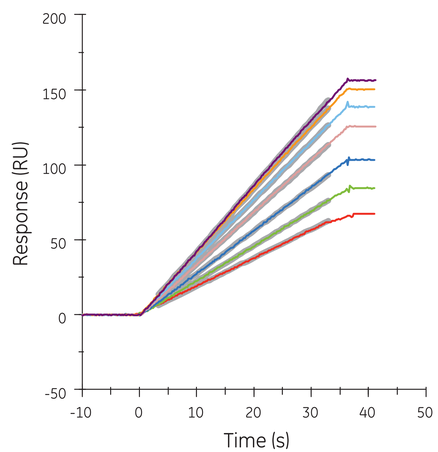
The most apparent visual indicator of data suitable for CFCA is a significant separation of the binding curves obtained at high and low flow rates.

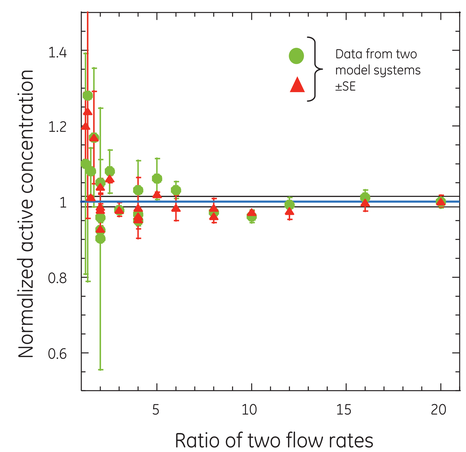


Flow rates

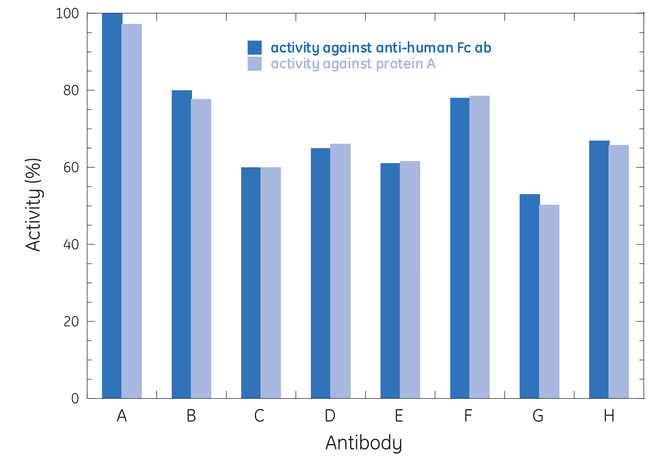
Concentrations were determined for two independent antibody model systems using binding data obtained at flow rates of 5, 10, 20, 40, 60, 80 and 100 µl/min. The binding data was analyzed in all 21 pairwise combinations of flow rates, as well as in the whole data set of 7 flow rates. Results were normalized to the value obtained from 7 flow rates (shown as value 1 with thinner lines to indicate +/- SE) to allow comparison between the model systems.

The results indicate that reliable concentration data is obtained when using two flow rates that differ by a factor of 10 or more.





Concentration of 8 commercial antibodies



The CFCA of eight commercial antibodies, denoted as A to H, was performed under transport limited conditions using anti-Fc antibody and protein A. The concentration values were compared to the concentration declared by manufacturers. Activity is given as the ratio of measured and specified concentrations, respectively. The concentrations of all antibodies were in good agreement when measured against two different interaction partners although could differ as much as 50% when compared to the concentration specified by manufacturers.

Conclusions

- Calibration-free concentration analysis on Biacore systems open new possibilities for concentration measurements that relate directly to determination of the concentration of a particular binding site.

  Measurements are direct, label-free, and do not require a calibration standard.

  Robust and reliable results are obtained when experiments are performed using:

  – at least two flow rates, where the ratio between flow rates is > 10

  – a high surface concentration, typically ≥ 25 RU/kDa (immobilized level/M_w of interaction partner)

  – protein dilutions that give concentrations ≤ 30 nM

- In the example, the concentrations of eight antibodies were in good agreement when measured against two interaction partners targeting the antibody Fc part. However, concentrations determined by CFCA differed by up to 50% from the values specified, depending on the method used. More work is required to explain the discrepancies.

References

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