Early kinetic screening of hybridomas for confident antibody selection using Biacore A100

• Rapid, kinetic screening of mAbs direct from hybridoma samples
  - efficient screening rate of \( \approx 800 \) hybridomas/day
  - effective selection of mAbs with desired kinetic profiles

• High resolution kinetic characterization of selected mAbs
  - affinity and kinetic parameters determined directly from crude samples
  - dedicated evaluation software processes kinetic data in minutes
  - selection of candidates based on therapeutically relevant binding properties
  - parallel analysis under different buffer conditions for characterization of stability of antibody performance

• More confident mAb selection earlier in the development process compared to end-point assays such as ELISA

“"The Biacore A100 provides a substantial throughput increase for accurate determination of binding kinetics and affinities to help make lead choices much earlier in the discovery process”"
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Introduction
Monoclonal antibodies (mAbs) represent an important class of proteins, which impact across the whole biomedical spectrum. They function as essential research tools in the understanding of disease processes, as precision diagnostic reagents and increasingly, as biotherapeutics against a wide range of diseases. Technological advances now enable the production of large numbers of antibodies directed against a chosen biological target, creating an increasing demand for screening methods that supply the high quality binding data required for confident candidate selection.

In contrast to standard label-dependent, end-point screening methods such as ELISA, protein interaction analysis systems from Biacore enable label-free, real-time analysis of antibody performance, facilitating selection based on functionally relevant kinetic properties.

Acknowledgements
We gratefully acknowledge Scott Klakamp & Andy Drake (Abgenix Inc.) and David Myszka (University of Utah) for their invaluable contributions in making this collaboration possible.
Appropriate assay formats enable rapid analysis, direct from crude hybridoma samples, and antigen consumption is relatively low, around 50% compared to a typical ELISA. Biacore A100 enables kinetic screening of around 800 hybridoma samples per day, with 8 mAbs analyzed simultaneously during each cycle. Selected samples showing the desired binding profiles can then undergo comprehensive kinetic characterization, in terms of quantitative affinity and kinetic rate constant data. This information facilitates confident selection of candidate mAbs, based on a thorough understanding of mAb-antigen interactions. Parallel processing in Biacore A100 enables simultaneous analysis of interactions in up to four different buffer environments, to assess the robustness of mAb performance under different conditions, such as salt concentration and pH. This type of data may provide a valuable additional selection parameter for therapeutic antibodies, which must function in the complex and potentially variable biochemical environment of human patients.

**Purpose of study**

A total of 384 crude hybridoma samples (including a number of negative controls) were supplied by Abgenix Inc. The initial strategy was to undertake a rapid kinetic screening of all samples, using a single concentration of the target antigen (identity confidential), leading to selection of a subset of mAbs with a good range of binding profiles. Selected mAbs were then analyzed using a concentration series of antigen, providing comprehensive characterization to determine kinetic rate constants. The aims of the collaborative study were as follows:

- evaluate Biacore A100 for rapid kinetic screening of Abgenix hybridoma samples with required throughput rate (= 800/day)
- assess the data quality of rapid kinetic screening for selection of mAbs with required kinetic profiles
- obtain high-resolution, quantitative kinetic and affinity data for selected mAbs
- explore the potential for parallel examination of mAb performance in multiple biochemical environments

**Results**

**Rapid kinetic screening of 384 hybridomas**

As illustrated in Figure 2, mAbs from two hybridoma samples were captured (using a goat anti-human IgG antibody) within each of four independent, parallel flow cells. A single concentration of antigen was injected simultaneously over the 8 mAbs. After recording the interaction, the bound antigen and captured mAb were removed to regenerate the sensor surface in preparation for the next cycle. In this way, 256 hybridomas were analyzed within an 8 hour run, meeting the required throughput rate of around 800 per 24 hours. Antigen consumption was of the order of 3.5 nanomoles per run (= 256 mAbs), saving approximately 50% compared to a typical ELISA.

**PRODUCTION OF HUMAN mAbs USING XENOMOUSE® TECHNOLOGY**

The antibodies used in this study were obtained from Abgenix Inc, using their XenoMouse technology, which enables the production of fully human IgGk and IgG1 antibodies from mice that are transgenic for the human IgG loci. Unlike traditional methods of “humanizing” antibodies, which involve the grafting of complementarity determining regions (CDRs) of mouse mAbs into a human IgG framework, these antibodies from Abgenix are 100% human in sequence. This greatly reduces the risks of unwanted immunogenic responses in patients, significantly improving the potential of such mAbs as successful therapeutics.

**Figure 2.** Assay setup for rapid kinetic screening. Capturing antibody (goat anti-human IgG) was immobilized onto 4 spots in each flow cell, providing an adjacent reference for each captured mAb.
Evaluation of screening results using report point values

Protein interaction analysis produces a real-time plot of binding response against time (the sensorgram) over the entire course of binding and dissociation. Binding responses at specific selected times (report points) can be taken, to rapidly plot binding characteristics of interest (see Monitoring Protein Interactions With Biacore at the end of this note). Figure 3 shows a plot of responses from the late association phase (binding_late report point) and late dissociation phase (stability_late report point) from each mAb-antigen interaction recorded in the screening assays. Since the binding_late response is related to the association rate of the interaction and stability_late is inversely related to the dissociation rate, these two values provide a good overview of the kinetic profile of each mAb.

![Image](336x298 to 530x446)

**Figure 3.** Rapid kinetic screening of hybridomas, evaluated by report point plot (responses normalized to capture level). Areas corresponding to low/non-binders and highest-affinity, as well as the 16 mAbs selected for further characterization are indicated on the figure.

As seen in Figure 3, mAbs from the 384 hybridoma samples displayed a wide range of kinetic profiles. A number of negative hybridomas were supplied blind by Abgenix, and all of these were determined to be non-binders by the kinetic screening assay (i.e. no known false-positives were detected). Using this plot, 16 hybridomas were selected for comprehensive kinetic characterization, covering a wide range of association and dissociation properties among the positive samples.

Estimation of affinity & kinetic parameters from rapid kinetic screen

Single binding curves can be used to derive approximate $k_a$ and $k_d$ values and hence, estimate affinities, although fully quantitative, high-resolution kinetic characterization requires analysis of multiple sensorgrams derived from a concentration series of analyte. To obtain an estimate of the affinity/kinetic parameter ranges corresponding to the profiles observed in the report point plots, sensorgrams were therefore fitted to a 1:1 interaction model and the approximate rate constants used to generate an off/on-rate map (Figure 4). From this log-scale plot of estimated $k_d$ against estimated $k_a$, a generally good correlation in kinetic profiles was observed compared to those indicated by the report point plot (Figure 3). The off/on-rate map also showed that many mAbs lay within an approximate affinity range of 1-10 nM, and that the three highest affinity examples lay in the sub-nanomolar range.

![Image](355x355)

**Figure 4.** Rapid kinetic screening of hybridomas, evaluated by estimated rate constants. Log-scale plot of estimated $k_d$ against estimated $k_a$. The isometric lines corresponding to 10, 1 & 0.1 nM affinities are indicated by the red diagonals. Negative hybridomas and lower-level binders were excluded from this plot.
**Kinetic characterization of selected mAbs – evaluation of rate constants within minutes**

The 16 hybridomas selected from the rapid kinetic screen were subjected to a comprehensive, high-resolution kinetic characterization using a similar assay setup to that described in Figure 2. In this case, however, longer injections of hybridoma supernatant were used in order to maximize the amount of mAb captured. A concentration series of antigen (from 3.3 to 270 nM) was then injected over each mAb. Using this approach, all mAbs were characterized in an overnight run (in two sets of eight). An automatic quality control (QC) software function was used to approve data sets, which were fitted to a 1:1 interaction model and used to derive kinetic rate constants using the dedicated evaluation software.

Figure 5 shows the fitted sensorgram data from the kinetic characterization of all 16 mAbs, along with the binding affinities that were derived from the rate constant ratios in each case. The dedicated evaluation software in Biacore A100 enabled this analysis to be completed within 10 minutes from opening the results file to determination of rate constants. The affinities of the mAbs ranged from 62 to 1 nM, with a wide range of association and dissociation profiles. This endorsed the selection criteria used in choosing the 16 mAbs from the kinetic screening results.

**Figure 5.** Fitted sensorgrams from the kinetic characterization of 16 selected mAbs. The small number of non-optimal curves identified by the QC function in the software were excluded from the data fitting procedure.

**Kinetic characterization – high resolution selection among mAbs of similar affinities**

The 16 mAbs covered a total affinity range of around 60-fold, although the majority (11 of 18) fell within an approximately 10-fold range (21-2 nM). The high resolution kinetic characterization provided by Biacore A100 enabled these mAbs to be clearly differentiated according to their specific association and dissociation rate constants (Figure 6). This analysis showed that the variations seen in mAb affinity were principally due to differences in off-rates rather than on-rates, since the $k_d$ values varied 55-fold compared to a $k_o$ variation of only 11-fold. This degree of kinetic characterization resolves fundamental differences in binding properties between mAbs of near-equal affinity. While two mAbs highlighted in Figure 6 differ in affinity by only 25% (21 & 16 nM, respectively), both their on-rate and off-rate kinetics vary by around 10-fold. These marked differences in kinetic behavior can have great significance for eventual therapeutic use, where for example, a slow off-rate may be a crucial property for clinical success.

**Figure 6.** A) Off/on-rate map for 16 selected mAbs. B) Kinetic resolution of very similar affinities. The two mAbs highlighted in panel A (1 & 2) differ by only around 25% in their affinities, but exhibit completely different kinetic profiles.
Kinetic screening as a predictor of true kinetic properties

For the 16 fully-characterized mAbs, it was possible to directly compare their true kinetic rate constants with those estimated from single antigen concentration sensorgrams during the kinetic screening assay. Figure 7 shows an off/on-rate map in which the two sets of rate constants are compared for each mAb. There was a generally good correlation between the high resolution and estimated rate constants, demonstrating that kinetic screening assays are a very effective tool for rapid selection of candidates with the required affinity and kinetic ranges from amongst large numbers of hybridoma samples. This comparison also showed, however, that the predictive accuracy of the kinetic screening was variable and was significantly reduced for interactions with fast kinetics. The optimal approach for efficient and effective mAb selection may therefore involve full kinetic characterization of a limited number of candidates with appropriate binding profiles selected from kinetic screening. As shown, Biacore A100 strongly supports both of these functions.

Figure 7. Off/on-rate map comparing approximate rate constants estimated from kinetic screening and rate constants derived from high resolution kinetic characterization. Examples of mAbs showing more significant differences between the equivalent values are circled.

Parallel analysis of mAb performance in different biochemical environments

While conditions for screening and selection of antibodies must obviously be optimized and standardized for good assay performance, a biotherapeutic must operate in the much more complex and variable environment of patients. The robustness of mAb performance under varied biochemical conditions may therefore be of interest when selecting therapeutic candidates. Using Biacore A100, the same interaction partners may be analyzed using four different buffers in parallel. In these studies, four mAbs were selected for kinetic characterization under eight different buffer conditions (four different salt concentration and four different pH variants of the running buffer).

Since changing buffer composition could affect mAb-antigen and/or mAb-capturing antibody interactions, a direct immobilization strategy was used (Figure 8). A rapid purification of hybridoma supernatants was performed (protein A affinity resin tips) to avoid immobilization of unwanted hybridoma proteins to the sensor surface, as well as to increase mAb concentrations to achieve optimal immobilization levels.

Figure 8. Assay setup for kinetic characterization of mAbs in parallel, under different buffer conditions.

Increasing salt concentration from 50 to 600 nM reduced affinity by between 2 to 5-fold for three out of four mAbs (Figure 9). For mAb12, however, very little effect of salt concentration was seen on affinity. This indicates that mAb12 has the least salt-sensitivity of the antibodies examined and also suggests that electrostatic interactions may be of relatively less importance in overall binding affinity compared to the other three candidates.
Four different salt concentrations examined. Affinity changes from sensorgrams for the kinetic characterizations are shown for each of Figure 9.

Avenue in antibody characterization.

\[ k \]

Using an off/on-rate map to plot the \( k \) pH studies provides an overall view of how the different changes can be visualized in proportion to the area of an ellipse drawn around the eight data points. Using this method, it was observed that mAb12 showed the least sensitivity to buffer conditions, while mAb18 was the most sensitive. This ability of Biacore A100 to examine binding in parallel under multiple buffer conditions opens up new avenues in antibody characterization.

All four mAbs also showed affinity changes in relation to pH (between 8 to 20-fold over a pH range of 5.5 to 8.5). Combining the kinetic characterization data for the salt and pH studies provides an overall view of how the different mAbs perform in relation to variations in biochemical environment. Using an off/on-rate map to plot the \( k_d \) and \( k_a \) coordinates for all eight buffer conditions corresponding to each mAb (Figure 10), the degree of sensitivity to buffer changes can be visualized in proportion to the area of an ellipse drawn around the eight data points. Using this method, it was observed that mAb12 showed the least sensitivity to buffer conditions, while mAb18 was the most sensitive. This ability of Biacore A100 to examine binding in parallel under multiple buffer conditions opens up new avenues in antibody characterization.

**Figure 9.** mAb binding under different salt conditions. Overlay sensorgrams for the kinetic characterizations are shown for each of four different salt concentrations examined. Affinity changes from 50 to 600nM NaCl are shown in parenthesis for each mAb.

**Figure 10.** Off/on-rate map showing kinetic characterization data for 4 mAbs in eight different buffer conditions.
Summary

- Rapid, high-quality screening of mAbs direct from hybridomas
  - valuable kinetic profile data without compromising throughput

- High-resolution kinetic characterization of candidates
  - discriminate between equal-affinity mAbs with therapeutically important differences in key binding properties

- Screening & characterization assays with one versatile instrument

- Parallel analysis in different buffers opens new avenues for antibody characterization

Methods

Assays were run on a Biacore A100 prototype, using Series S Sensor Chip CM5. Hybridoma supernatants and antigen were kindly supplied by Abgenix Inc. For kinetic screening and kinetic characterization assays, goat anti-human IgG (Caltag Laboratories) was immobilized using amine coupling for high-affinity capture of mAbs. In the assays examining buffer conditions, mAbs were rapidly purified from hybridoma supernatants using PhyTip™ columns (PhyNexus Inc.), and immobilized using amine coupling.

MONITORING PROTEIN INTERACTIONS WITH BIACORE

Biacore systems monitor protein interactions in real-time using a label free detection method. One of the interacting molecules is immobilized onto a sensor surface, while the other is injected in solution and flows over the sensor surface. As molecules from the injected sample bind to the immobilized molecules, this results in an alteration in refractive index at the sensor surface that is proportional to the change in mass concentration. Using the phenomenon of surface plasmon resonance (SPR), these changes are detected in real time and data is presented as a sensogram (SPR response plotted against time). Sensograms display the formation and dissociation of complexes over the entire course of an interaction, with the kinetics (association and dissociation rates) revealed by the shape of the binding curve.

The sensogram provides real-time information about the entire interaction, with binding responses measured in resonance units (RU). Binding responses at specific times during the interaction can also be selected as report points.
Reference

More information about protein interaction analysis can be found at www.biacore.com