Resolving a bottleneck in screening and characterization of recombinant antibody fragments using Biacore 4000

Antibodies and their derivatives are increasingly important as biotherapeutics, precision diagnostics, and essential tools for biological research. Techniques to screen and characterize large numbers of antibodies and provide confident selection of the best candidates for development are therefore essential.

Biacore 4000 equipped with Biacore 4000 Antibody Extension Package has been used for efficient screening and characterization of antibody fragments for diagnostic purposes. Ten 96-well plates containing avian scFv fragments in crude cell lysates were screened and ranked in less than 20 h. A subset from this screen was selected for analysis with the 2-over-2 kinetics approach. This method provides rapid screening in combination with data-rich selection of fragments with high affinity and the desired kinetic behavior. Kinetic analysis at different temperatures provided additional information on the temperature stability of the selected fragments with respect to affinity.

Chimeric chicken/human Fab fragments were screened and characterized using a preoptimized Human Fab Capture Kit to achieve accurate off-rate determination during initial screening of crude samples. These experiments demonstrate a new dimension for better-informed, more efficient selection of antibody fragments.

Introduction

High-quality antibodies are key components in diagnostic analyses. Today, secondary screening of recombinant antibodies or fragments (e.g., scFv and Fabs) from different selection platforms is often an analysis bottleneck due to large datasets of low quality. In this study, we show how Biacore 4000 equipped with Biacore 4000 Antibody Extension Package is used for rapid screening, characterization, and ranking of antibodies against markers for cardiovascular disease (troponin I) and cancer (prostate-specific antigen, PSA). To serve as high quality diagnostic reagents, ideal antibodies should have a high specificity, and a high affinity that is temperature-stable.

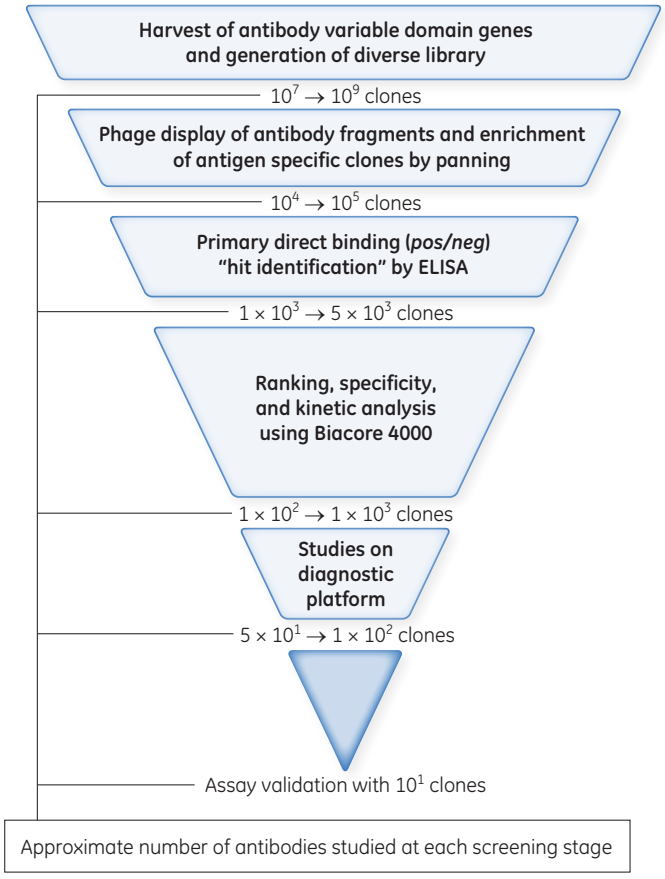


Fig 1. Schematic overview of the process for selecting recombinant antibodies for diagnostic use. Biacore 4000, used for secondary screening, has the throughput required to resolve the analysis bottleneck and provides high quality binding, specificity, and kinetic data.

Materials and methods

An antibody panel consisting of ten 96-well plates of crude bacterial extracts containing avian-derived scFv antibody fragments (HA fusions) were selected from a large immune antibody library of 10^9 clones by phage display.

Biacore 4000, Biacore 4000 Antibody Extension Package, Sensor Chip CM5, Amine Coupling Kit, Human Fab Capture Kit, immobilization buffer (10 mM Acetate-HCl pH 4.2), and running buffer (HBS-EP+) were obtained from GE Healthcare. Troponin I was purchased from Life Diagnostics Inc. and PSA from Lee Biosolutions Inc. Anti-HA was purchased from Affinity BioReagents. Ranking and calculation of kinetic constants was performed with Biacore 4000 Evaluation Software. Data was then exported to Microsoft™ Excel™ or Spotfire™ for further analysis.

Screening of scFv fragments based on stability

A polyclonal anti-HA antibody was covalently immobilized to spot 1, 2, 4, and 5 in all four flow cells on Sensor Chip CM5 with amine coupling to a level of approximately 7000 RU. Ten 96-well plates containing scFv fusion fragments from crude cell lysates were centrifuged, diluted 1:4, and captured onto spots 1 and 5. A solution of troponin I (50 nM) was injected over all spots (Fig 2). With this assay setup, a total of eight different antibody fragments were screened in each cycle. The sensor surface was regenerated using 20 mM NaOH and the surface was used for 120 cycles without any loss of binding activity. Ranking of binding stability was evaluated based on report points, placed early and late during antigen dissociation (Stability_early and Stability_late, respectively).

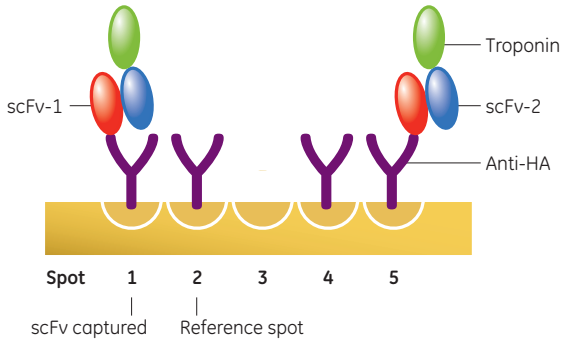


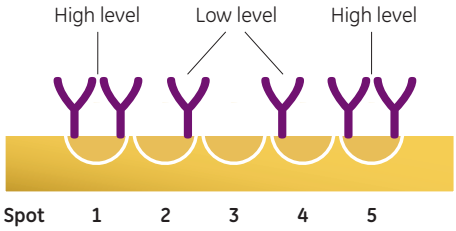
Fig 2. Biacore 4000 assay setup for scFv screening.

2-over-2 kinetics on scFv fragments

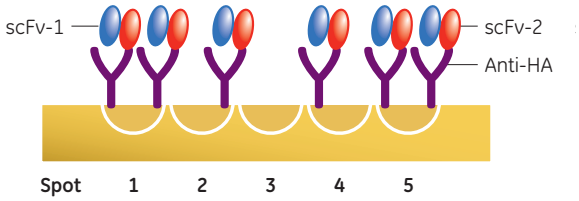
The top 10% binders from the screening experiment above were transferred from the original plates to new 96-well plates and analyzed using 2-over-2 kinetics. Biacore 4000 Antibody Extension Package enables the setup of 2-over-2 kinetics using an additional spot-addressing functionality. Two densities of capturing molecule are immobilized on the spots in each flow cell half (higher density on spots 1 and 5, lower density on spots 2 and 4). This enables capture of two levels of antibody in each flow cell half (Fig 3). The 2-over-2 kinetics analysis requires only 3 cycles: 2 antigen concentrations plus a blank per antibody.

Anti-HA antibody was immobilized at two levels: 4000 RU on spots 1 and 5, and 2000 RU on spots 2 and 4. The scFvs in cell lysate were diluted 1:4 and captured on spots 1+2 and 5+4. In total, only 50 µl of each scFv sample was needed for the kinetic characterization. Troponin I was injected in two subsequent cycles with 25 and 100 nM concentrations, followed by a buffer injection as a blank. Evaluation was performed by combined kinetic fitting of sensorgram data from spots 1-2 and 4-5. The experiments were performed at 25°C and 37°C.

A) Anti-HA immobilized at two densities



B) One scFv captured at two densities in each flow cell half



C) Troponin I at 2 conc. + blank injected over all spots

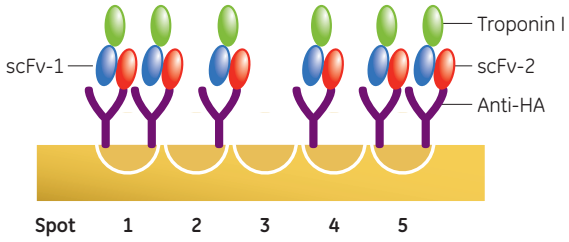


Fig 3. Two densities of capturing molecules are immobilized in each flow cell half. This captures two densities of scFv in each flow cell half, and is followed by injection of troponin I.

Selection of Fab fragments using off-rate ranking

Human Fab Binder from Human Fab Capture Kit was coupled covalently to Sensor Chip CM5 on spots 1, 2, 4, and 5 with amine coupling to a level of 11 000 RU. An antibody panel of 192 Fab fragments were captured on spot 1 or 5, allowing eight samples to be screened per cycle. A 50 nM solution of PSA was injected over the flow cells followed by a buffer blank cycle. The surface was regenerated with Glycine pH 2.1. Evaluation was performed by fitting the sensogram to a 1:1 model after double referencing using the reference spot and blank cycle.

Results and discussion

Screening of scFv antibody fragments based on binding stability

A panel of 960 scFv fragments specific to human cardiac troponin I was screened using the assay setup described in Figure 2. Sensorgrams from 120 samples with the report points indicated as colored bars are shown in Figure 4.

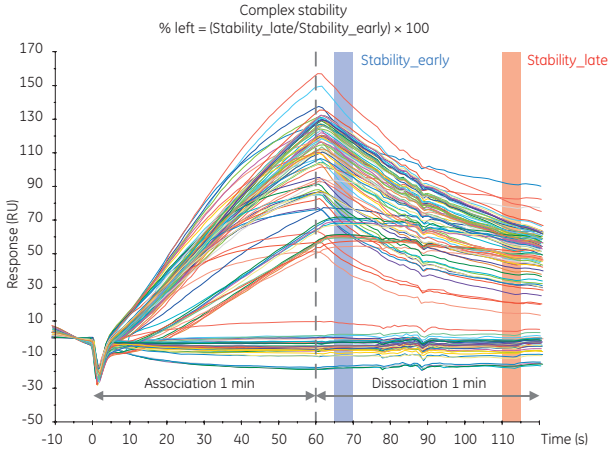


Fig 4. Sensorgrams from 120 samples showing report points used for ranking.

Figure 5 shows a scatterplot of the report points stability_early plotted against stability_late. This type of plot can be used to rank binders based on binding stability. The figure shows two main groups of scFvs: non-binders close to the origin and binders higher along the diagonal. The best binders, with a high binding stability and slow dissociation, are shown as blue squares. In total, 960 samples were analyzed and ranked with respect to binding stability in 18 h. The data evaluation was accomplished in less than one hour.

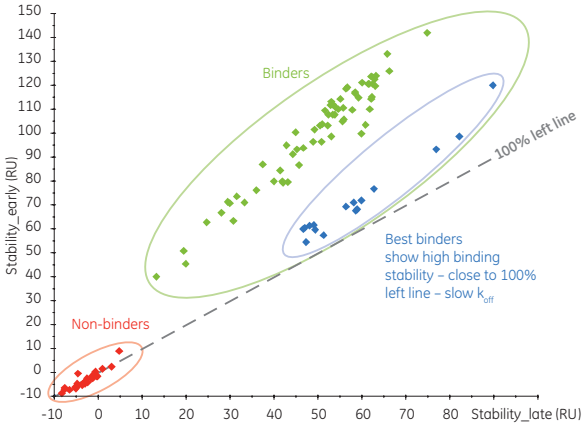


Fig 5. Stability_early vs Stability_late plot for identification of stable binders (best binders shown as blue squares; green binders are not as stable).

Importantly, Biacore analysis of antibodies and their fragments allows both the expression levels of the clones and quality of the antibodies to be measured simultaneously. Figure 6 shows an example of how report point data from the scFv screen are plotted in Spotfire, providing an excellent overview of the performance of different clones.

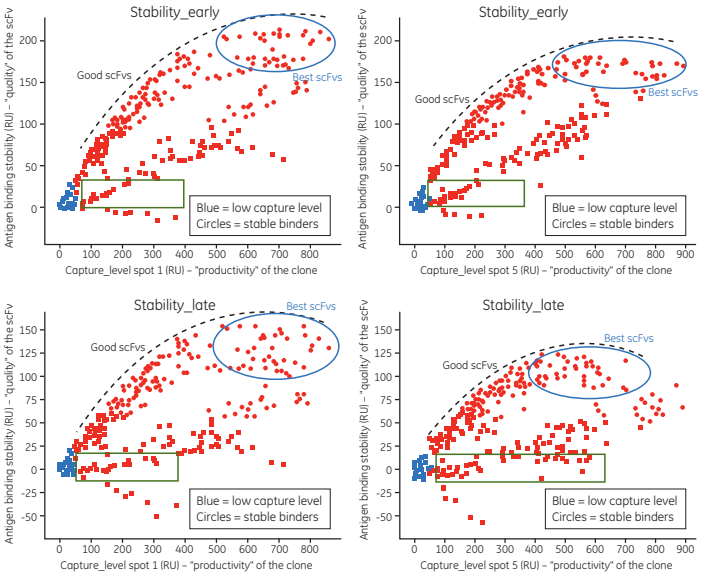


Fig 6. Overview of the scFv screening. Antigen binding stability, stability_early (top) and stability_late (bottom), is plotted against capture level in spot 1 (left) and spot 5 (right). Wells without scFv (blue), with significant capture (red), and scFvs showing a stable antigen response (circles) are displayed. The green box indicates scFvs that are captured, but do not bind to the antigen. The best scFv clones show high capture level and high binding stability (blue circle).

The amount of captured scFv on spot 1 and 5 in the four flow cells is plotted on the x-axis, and indicates the capture level or expression level of the different clones. After scFv has been captured, antigen is injected over the spots. Response levels early and late in antigen dissociation are plotted on the y-axis to show the quality of the interaction of captured scFv with antigen. Uninteresting clones are distinguished from the good clones (close to the dashed lines), and the best clones show both high capture level and binding stability (slow off-rates). These are important criteria when selecting high quality antibodies and antibody fragments for further development, and were applied to the scFvs selected for 2-over-2 kinetic characterization.

Characterization with 2-over-2 kinetics

Obtaining kinetics data early in the development process enables better-informed selection since kinetic properties have consequences for both therapeutic and diagnostic agents. The 2-over-2 kinetics approach is particularly suitable for providing kinetic information already during screening, as the data can be obtained in half the time using significantly less reagents and sample volumes.

Evaluation was performed with the kinetic evaluation tool in Biacore 4000 Evaluation Software, by combined kinetic fitting of the sensorgram data from spots 1-2 and 4-5, respectively. The evaluation combines the different surface densities with the different analyte concentrations in one fit.

To investigate temperature stability of the binding, all samples have been analyzed at 25°C (Fig 7A) and 37°C (Fig 7B). Kinetic constants from the two temperatures were exported to Spotfire for further analysis. Temperature-stable, high-affinity scFvs were identified in Spotfire using on/off-rate maps (Fig 8).

The antibodies displayed in Figure 8 have high affinities in the subnanomolar range. However, two groups of antibodies displaying different kinetic behavior were identified. One group compensated for an increase in off-rate at increased temperature by a parallel increase in on-rate, thereby displaying a temperature-stable affinity, while the other group showed weaker affinity at higher temperature. Because the analyses are typically performed between room temperature and 37°C on this diagnostic platform, suitable reagents must behave similarly at both temperatures.

In less than 11 h, close to 100 samples were characterized with regards to on/off-rates and K_D. Using the 2-over-2 kinetics approach, the best candidates, which showed temperature-stable affinity, were quickly identified and could be further tested on the diagnostic platform.

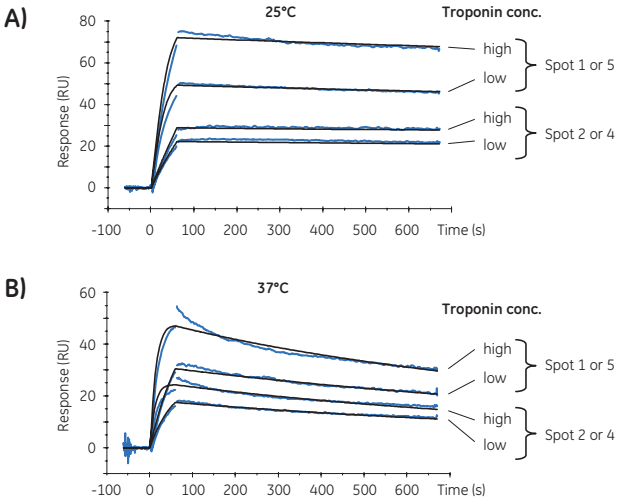


Fig 7. Examples of overlay plots from 2-over-2 kinetics run at two different temperatures to investigate stability of scFv/Troponin binding at **A)** 25°C and **B)** 37°C.

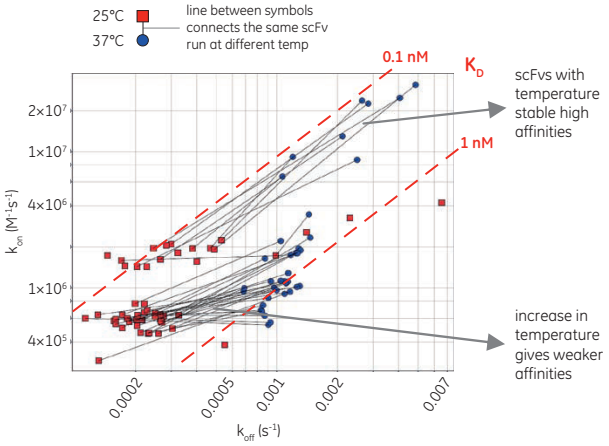


Fig 8. On/off-rate map for scFv antibodies specific to troponin I. The group with temperature-stable high affinities is most likely to be robust, and preferable for use in diagnostic analyses run at varying temperatures.

Off-rate ranking of chimeric Fab fragments binding to PSA

Aimed at developing another diagnostic test using Fab fragments against PSA, a panel of 192 Fab fragments was screened for binding to PSA with a capture approach using Human Fab Capture Kit. Although full kinetic characterization requires analysis of a series of antigen concentrations, fitting the dissociation phase of a single antigen concentration to a 1:1 model provides valuable ranking information based on the off-rate already at the screening stage. This off-rate ranking approach is an alternative to report point screening and 2-over-2 kinetics analysis for selection of the best candidates.

Analysis of Fab fragments was initially performed using capture onto an immobilized anti-HA antibody via a HA tag fused to the Fab fragments. However, the stability of the anti-HA capture was too low for accurate off-rate ranking. Also, low-expressing clones were not detected because the capturing ability of the anti-HA antibody was fairly low.

To address this, Human Fab Capture Kit was used. The stable ligand capture and low baseline drift obtained with the kit allowed single concentrations of analyte to be reliably analyzed and fitted to a 1:1 model. This enabled rapid off-rate ranking (Fig 9) and identification of clones with low-level expression that would have been missed with the initial HA tag capture. All 192 samples were assayed in 11 h, and the top 20 fragments were selected for sequencing and further evaluation on the diagnostic platform.

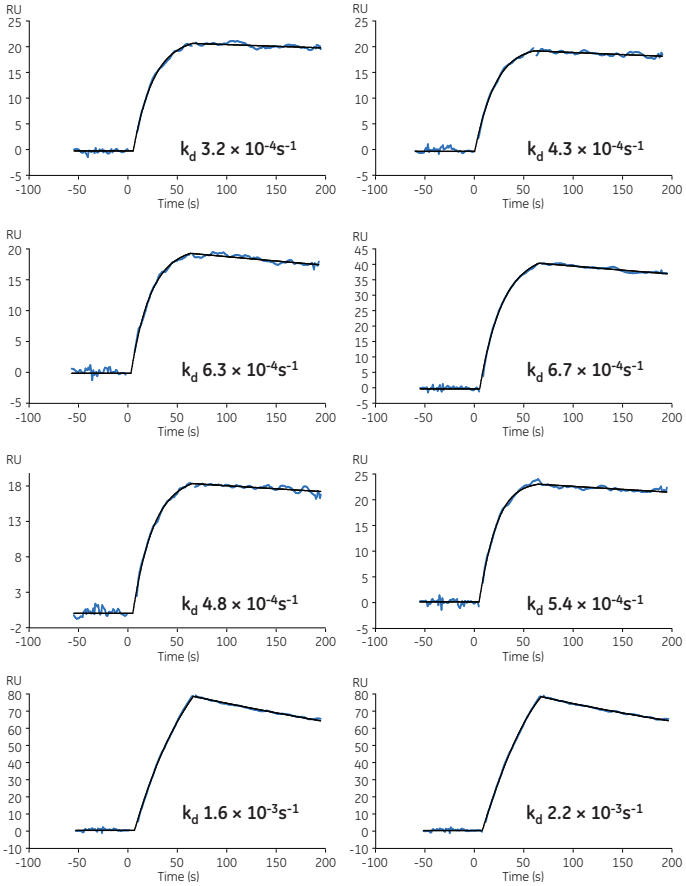


Fig 9. The fitted sensorgrams above represent the range of off-rates that were present among the analyzed Fabs.

Conclusions

The high capacity of Biacore 4000 allowed sampling of a large diversity pool from phage displayed combinatorial antibody libraries. In less than 20 h, 960 samples were screened and ranked based on binding stability. Moreover, 2-over-2 kinetics analysis provided on-rates, off-rates, and affinity for 96 scFvs in less than 11 h. Taken together, this demonstrates that Biacore 4000 provides the throughput and data quality needed to resolve the analysis bottleneck in the screening workflow.

Crude sample preparations were analyzed without the need for purification, saving both time and resources. The capture-based approach minimizes assay development time and allows the convenient analysis of multiple analytes or antibody batches in a single analysis.

The results show that kinetic data enables better-informed selection of antibody fragments. Off-rate based screening for initial selection and rapid on- and off-rate mapping for characterization are the keys to success in antibody development.

Acknowledgements

We thank our collaborators at the Biomedical Diagnostics Institute, Dublin City University, Ireland for their enthusiasm and excellent work in performing the experiments, as well as providing background material for Figure 1.

Ordering information

Product	Code no.
Biacore 4000	28-9643-21
Biacore 4000 Antibody Extension Package†	28-9664-57
Human Fab Capture Kit‡	28-9583-25
HBS-EP+ 10×	BR-1006-69

† Includes software CD containing Biacore 4000 Antibody Extension Software, license agreement with product key, and Certificate of Software Conformance.

‡ Includes Human Fab Binder and immobilization buffer for 10 immobilizations and regeneration solution for 1000 regeneration injections.

Related literature	Code no.
Biacore 4000, Data file	28-9694-94
Biacore 4000 Antibody Extension Package, Data file	28-9694-95
Human Fab Capture Kit, Data file	28-9623-69

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