Chapter 11

Kinetic Screening in the Antibody Development Process

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Abstract

Kinetic screening is of paramount importance when it is to select custom-made antibodies, tailored for their respective scientific, diagnostic, or pharmaceutical application. Here a kinetic screening protocol is described, using a Biacore A100 surface plasmon resonance biosensor instrument. The assay is based on an Fc-specific antibody capture system. Antibodies from complex mixtures, like from mouse hybridoma supernatants are captured on the sensor surface in an oriented manner. The method uses a single injection of one antigen concentration for the determination of six relevant screening parameters, which comprehensively describe the antibody's kinetic rate profile and its valence mode. The method enables the scientist to rank and finally select rare and outstanding antibodies according to their kinetic signatures.

Key words: Kinetic screening, Molar Ratio, Valence, Dissociation half-life ($t_{1/2\ diss}$), Binding Late, Stability Late, Antibody Capture Level, k_d, Surface plasmon resonance

1. Introduction

The term kinetic antibody screening (1–4) means to select antibodies according to their antigen binding kinetic rate properties, rather than to their sole equilibrium dissociation constant K_D (M). Typically, kinetic screening is integrated in a workflow, where it refines the hit selection of a preceding ELISA-based screening, from which the outcome is initial information about an antigen binding event from sometimes hundreds of potentially suitable binders (see Note 1).

Based on an automated surface plasmon resonance (SPR) instrument, a method is described, which uses six easy-to-access parameters and two simple graphical depictions to quickly select best in class antibodies, even from large sample sets. The screening parameters are: the amount of antibody captured on the sensor surface (Capture Level, CL); the antigen-binding signal at the end

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of the antigen association phase (Binding Late, BL) and the antigen binding signal at the end of the antigen dissociation phase (Stability Late, SL). The Molar Ratio (MR) describing the antibody–antigen binding valence mode. The dissociation rate constant k_d, also called the "off rate" describes how fast the antigen dissociates from the antibody–antigen complex. Another way, to describe this is to calculate the antibody–antigen complex half-life $t_{1/2\ diss}$ in minutes. A high $t_{1/2\ diss}$ value indicates an antibody as binder with high antigen complex stability. The parameters are relevant for all binding molecules, no matter whether they will be used for a pharmaceutical, diagnostic, or research application. An example demonstrates how kinetic screening facilitates how to adapt the antibody selection process according to the latter antibody application specifications.

An antibody for its use in an in vitro diagnostic instrument, which is characterized by a short sample incubation at 37°C, followed by stringent washing steps prior to analysis is to be produced. The antibody is kinetically screened according to the following selection scheme: first, to adapt the instruments physical parameters, the screening takes place at 37°C. Second, an antibody is selected for rapid antigen complex formation (high BL value), so that enough binding signal can be generated in the limited period of the instrument's incubation time. Third, an antibody with sufficiently high antigen complex stability (BL = SL) is to be selected to withstand the stringent washing steps (small k_d means high $t_{1/2\ diss}$ value). The antibody's binding valence should indicate a functional antigen-binding mode. The Molar Ratio should be between MR = 1 and MR = 2. The exploration of these kinetic screening parameters facilitates the production of fit for purpose antibodies, kinetically tailored for their respective application. The method supports the scientist with a high degree of information, already at early project states, and is suitable for diagnostic as well as therapeutic antibodies.

2. Materials

2.1. Biacore Sensor Preparation

1. Biacore A100 (GE Healthcare, Piscataway, NJ, USA).
2. A100 antibody extension package software (GE Healthcare).
3. CM5 series S sensor (GE Healthcare).
4. 96-Well microtiter plates and deep well reagent plates (GE Healthcare).
5. At least 50 µl of hybridoma supernatant.
6. 0.2 µm Filtrated and degassed Bidest. water.
7. Biadesorb solution 1 (GE Healthcare).
8. Biadesorb solution 2 (GE Healthcare).
9. Bianormalization solution (GE Healthcare).

10. HBS-N buffer: 10 mM HEPES pH 7.4, 150 mM NaCl.
11. Activation buffer: 10 mM Sodium acetate, 150 mM NaCl pH 4.5, 0.05% Tween 20.
12. Polyclonal rabbit anti-mouse RAMFcy antibody (GE Healthcare).
13. EDC: 400 mM 3-(*N*,*N*,-dimethylamino)propyl-*N*-ethylcarbodiimide.
14. NHS: 100 mM *N*-hydroxysuccinimide.
15. 10 mM Sodium acetate pH 5 buffer (immobilization buffer).
16. 1 M Ethanolamine pH 8.

2.2. Kinetic Screening

1. Biacore A100 (GE Healthcare).
2. A100 antibody extension package software (GE Healthcare).
3. A100 Biaevaluation Software 1.1 (GE Healthcare).
4. CM5 series S sensor (GE Healthcare).
5. RAMFcy sensor.
6. 96-Well microtiter plates and deep well reagent plates (GE Healthcare).
7. At least 50 µl of hybridoma supernatant.
8. 1 mg/ml Antigen solution.
9. 0.2 µm Filtrated and degassed Bidest. water.
10. HBS-ET + CMD buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mg/ml CMD, 0.005% Tween 20.
11. HBS-ET buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20.
12. Carboxymethyl Dextran (CMD) 100 mg/ml stock solution in water.
13. 10 mM Glycine buffer pH 1.7 (regeneration buffer).

2.3. Data Processing

1. A100 antibody extension package software (GE Healthcare).
2. A100 Biaevaluation Software 1.1 (GE Healthcare).

3. Methods

3.1. Sensor Preparation

1. A Biacore CM5 sensor series S is mounted into a Biacore A100 system and is hydrodynamically addressed and normalized according to the manufacturer's instructions (see Note 2).
2. HBS-N buffer, activation buffer, immobilization buffer, and the Biadesorb solution 1 and 2 are used as buffers during the immobilization.
3. An antibody species specific Fc capture system (1) is prepared. In each flow cell, a polyclonal rabbit anti-mouse RAMFCy

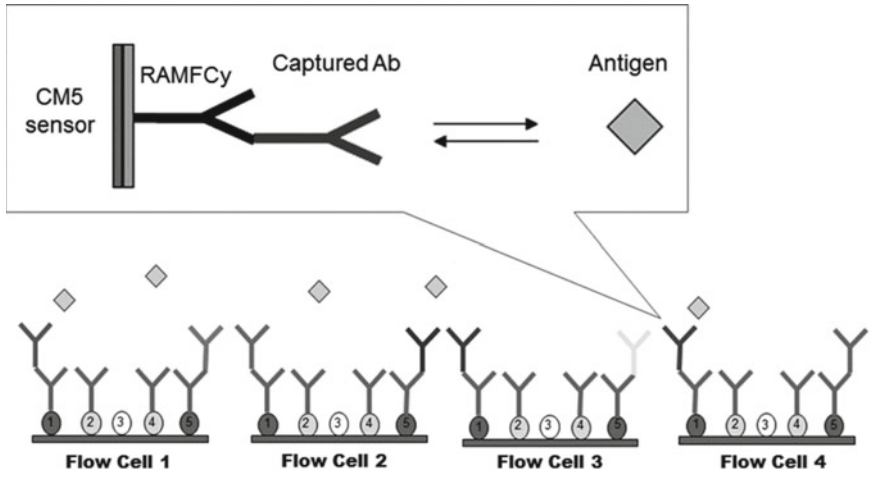

Fig. 1. Biacore A100 biosensor setup. A mouse Fc-specific antibody is covalently immobilized. In each flow cell, spot 1 and spot 5 are used to capture antibodies. Referenced binding signals are calculated from spot 1 minus 2 and spot 5 minus 4. Spot 3 is a blank spot, which serves as a control spot for unspecific binding to the blank sensor surface. Four flow cells can handle eight different antibodies in a single analyte injection. Acidic regeneration of the sensor elutes all complexes from the capture system.

antibody is immobilized on the spots 1, 2, 4, and 5 (Fig. 1). RAMFcy antibody is covalently immobilized using EDC/NHS coupling via primary amines (5).

4. After EDC/NHS activation of all spots on the sensor by a 10 min injection of EDC/NHS at 25°C, 30 µg/ml RAMFcy in 10 mM sodium acetate pH 5 buffer are injected for 10 min at 10 µl/min at 25°C. 10,000 Relative response units (RU) are immobilized on spots 1, 2 and 4, and 5.
5. The sensor's chemical binding capacity is saturated by a 2 min injection of 1 M ethanolamine at pH 8. The assay setup of the sensor references unspecific binding and corrects drifting baselines (spot 3).
6. For more details on the sensor preparation, immobilization procedure and instrument handling, please refer to the Biacore Methodology Handbook.

3.2. Kinetic Screening

At least 50 µl of hybridoma supernatant should be available for each antigen to be measured by kinetic screening. The hybridoma supernatants are diluted at least 1:2 with HBS-ET + CMD buffer (see Note 3). HBS-ET buffer is used as system running buffer during the kinetic screening. The screening temperature should be adjusted to the temperature of the antibody's latter field of application (see Note 4), like described in the introductory example.

Prior to each analysis run, the sensor is conditioned by 5 cycles capturing 25 nM of an arbitrary monoclonal mouse antibody.

Every 25th cycle, this antibody is repeatedly injected to control the stable antibody capture level performance of the surface.

Roughly calculated, one cycle takes 30 min to analyze eight antibodies. Depending on the regeneration stability of the sensor, a kinetic screening cycle can be repeated several hundred times. In this way, within 15 h, 120 hybridoma primary cultures can be analyzed for their kinetic properties, including double referencing (see Note 5).

One assay cycle comprises four steps:

(a) Antibody capturing.

(b) Antigen association phase monitoring.

(c) Antigen dissociation phase monitoring.

(d) Regeneration of the sensor.

1. Before the antibody is injected the baseline is defined by the setting of the Baseline Start (BS [RU]) report point (Fig. 5 and see Note 6).
2. The antibody capturing step is done at 10 µl/min for 2 min injection time on the measurement spots 1 and 5 (Fig. 1).
3. The amount of captured antibody is monitored as Capture Level (CL [RU]) at the end of the antibody association phase (see Note 7).
4. The antigen is injected at 150 nM for 2 min at 30 µl/min (see Notes 8 and 9) over all four flow cells.
5. At the end of the antigen's association phase, the Binding Late (BL [RU]) report point is taken. When the antigen injection is stopped, system buffer is floating over the sensor again and only the dissociation of the antigen from the captured antibody is monitored.
6. Dissociation is monitored for 3 min at 30 µl/min. At the end of the dissociation phase, the Stability Late (SL [RU]) report point is set.
7. The RAMFcy sensor is regenerated by a 1 min injection of 10 mM glycine buffer pH 1.7 at 30 µl/min over all flow cells (see Note 6).
8. After the regeneration procedure the Baseline End (BE [RU]) report point is set.

3.3. Data Processing and Visualization for Binding Late/Stability Late Report Points

Kinetic screening data can be quickly evaluated by plotting the Stability Late (SL) report points over the Binding Late (BL) report points (Fig. 2), which dramatically simplifies the visualization of the complex kinetic behavior of the molecular interactions. Antibodies populating on the BL = SL trendline show no measurable antigen complex dissociation within the monitored time frame and thus form tight antigen complexes.

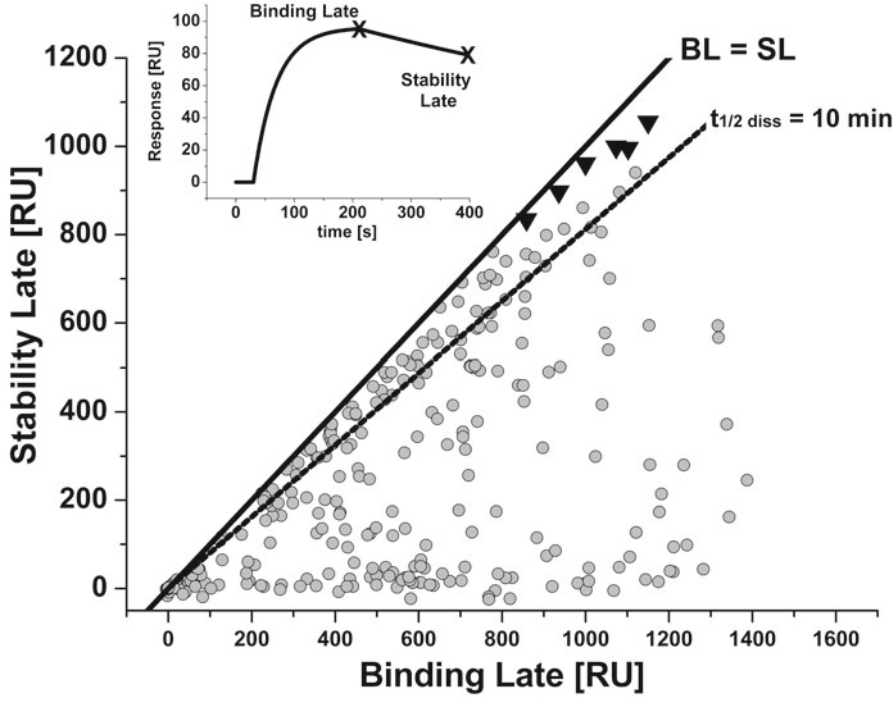

Fig. 2. 37°C kinetic screening of 296 mouse hybridoma clone culture supernatants versus a 72-kDa protein receptor analyte. Each data spot in the Stability Late (SL)/Binding Late (BL) plot represents an antigen–antibody interaction. The origin of the report points is shown in the enframed Biacore sensorgram (Response Units RU over time in seconds). The *black line* indicates values, where BL = SL. *Black triangles* mark antigen-antibody interactions of hybridoma supernatants, which are selected for further processing.

Best in class antibodies show highest BL values and at the same time populate as close as possible to the BL = SL trendline (see Note 10).

Figure 2 outlines the value of kinetic screening. From 225 ELISA preselected binders six antibodies were selected for being further developed into clone cultures. Kinetic screening is the ultimate filter for the selection of rarely occurring antibodies.

3.4. Data Processing and Visualization

The detection of an antibody forming a tight complex with its respective antigen is not sufficient to classify an antibody as a positive hit. The functionality and specificity of the interaction is to be proven, because e.g. unspecific binding hydrophobic artifacts can also produce highly stable complexes.

An important tool is the calculation of the Molar Ratio of the antibody–antigen interactions.

The Molar Ratio is calculated from the Binding Late value, the antibody Capture Level and the Molecular Weights (MW) of the interacting molecules:

MR = (BindingLate(RU)/CaptureLevel(RU)) × (MW(antibody)/MW (antigen)).

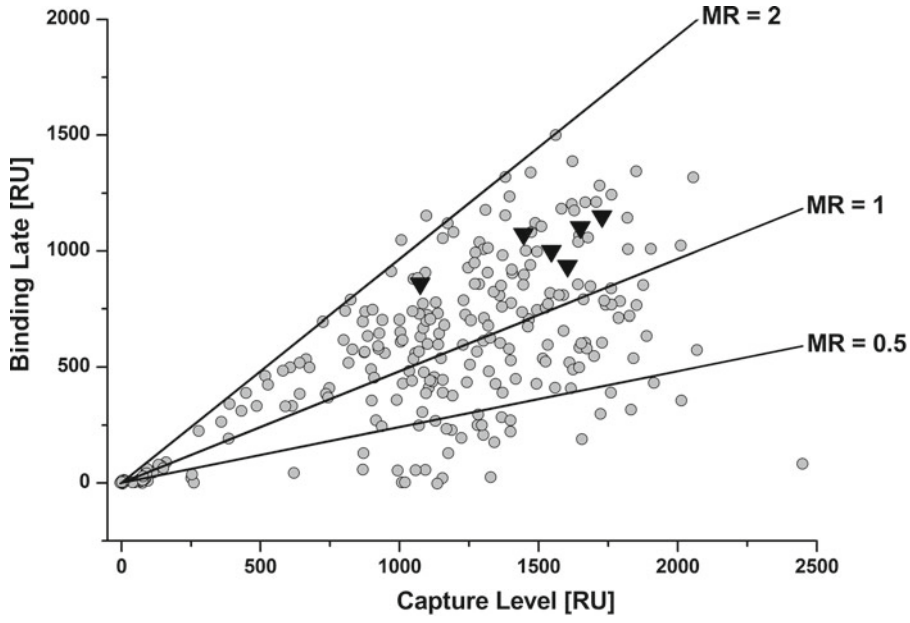

Fig. 3. Exemplary valence analysis of the data set from Fig. 2. Binding Late (RU) is plotted over the antibody Capture Level (RU). Corridors are formed by *lines*, indicating areas of the same Molar Ratios. The *lines* help to quickly classify antibodies according to their valences. The antibodies populate all corridors and therefore bind to their 72-kDa antigen with different valences. *Black triangles* indicate the selected clone cultures from Fig. 2.

To graphically visualize the valence analysis, Binding Late is plotted over the antibody Capture Level. Valence corridors, calculated using the MR formula, allocate the antibodies according to their virtual Binding Late values (Fig. 3), e.g., for the plotting of the MR = 2 trendline use the formula: BL at MR(2) = (MW(antigen) × 2 × Capture Level (RU)/MW (antibody)). Just replace the Molar Ratio values to calculate different trendlines.

When the antibody shows a MR = 1, it binds to an antigen with single valence. Obviously, there is some steric hindrance, which avoids full bivalent antibody binding.

When the antibody shows MR = 2, it is able to simultaneously bind to two antigens.

Here antibodies in the range of MR = 1 and MR = 2 were selected, when they showed a suitable BL/SL ratio (Fig. 3) at the same time. For further details about the valence analysis see Notes 9 and 11.

3.5. Data Analysis for k_d Ranking

If possible, calculate the "off rate", which means the antigen dissociation rate constant k_d (1/s) (6). The dissociation rate constant k_d describes the decay of complexes per second. k_d is calculated from a linear regression fitting of the antigen dissociation phase, which is a function of the Biaevaluation software. Using the dissociation rate constant, the complex half life $t_{1/2\ diss}$ in minutes

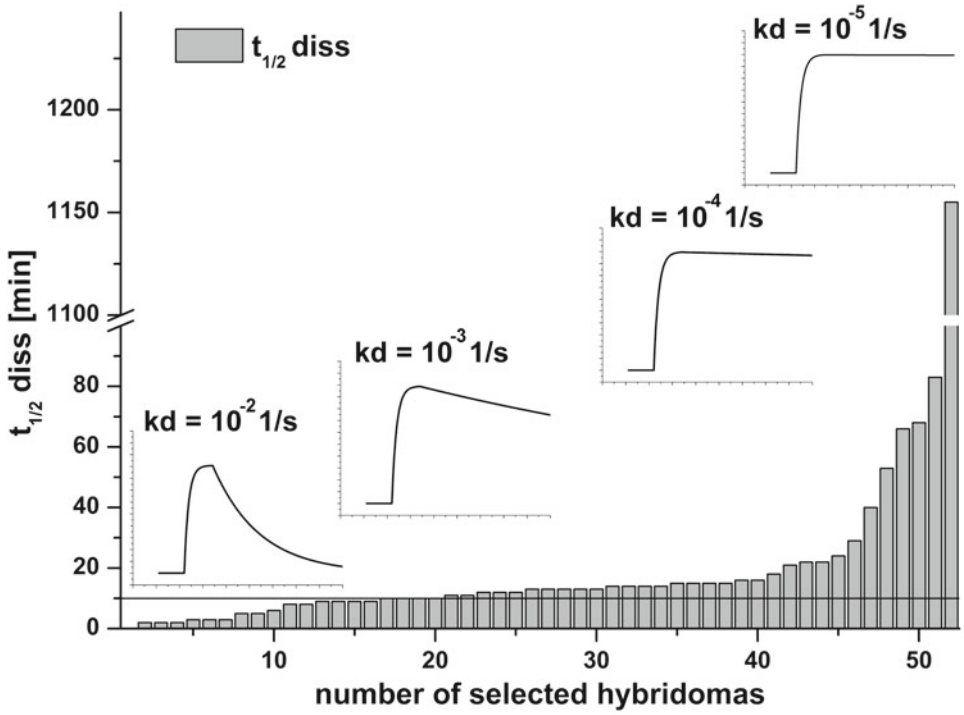

Fig. 4. From a set of 529 ELISA positive mouse hybridoma primary cultures, 51 cultures were selected at 37°C and ranked according to their $t_{1/2\ \mathrm{diss}}$ values (*filled bars*) versus a 6 kDa antigen. The sensorgrams reflect the $t_{1/2\ \mathrm{diss}}$ values. The *line* indicates the $t_{1/2\ \mathrm{diss}} = 10$ min value.

is calculated according to the first order kinetics half life law: $t_{1/2\ \mathrm{diss}} = \ln(2)/(60 \times k_{\mathrm{d}})$ (7). Finally the antibodies are ranked according to their antigen complex half-lives (Fig. 4).

In Fig. 4, a single antibody labeled as rare event and showing outstanding kinetic properties was identified among 529 ELISA hits. Antibodies with an antigen complex stability of $t_{1/2\ \mathrm{diss}} > 10$ min at 37°C are suitable binders for the use in an in vitro diagnostic assay as described in Subheading 1 (see Notes 11 and 12).

4. Notes

1. Kinetic screening succeeding after high throughput ELISA assays is a recommendable workflow. Most ELISA formats coat the antigen unspecific in microtiter plates, which denatures the antigen and blocks epitopes. The bivalent antibody recognizes the antigen in an avidity mode, which deteriorates the affinity information. The antibody concentration in primary hybridoma supernatants is mostly unknown in early process steps. A strong ELISA binding signal could originate from a high antibody

concentration, binding with low avidity. The protocol described here delivers kinetically resolved affinity information, as long as the analyte in solution is monomeric and efficiently selects binders from a pool of just potentially suitable ELISA-preselected antibodies.

2. The A100 performs hydrodynamic addressing, which separates four sensor flow cells each into five sensitive measurement spots, which then can be triggered singly or in certain combinations. 20 signals are simultaneously monitored. Applying Figs. 2 and 3 and the Subheading 3, the protocol can be effectively transferred to other Biacore instruments, such as 2000, 3000, T100/200, 4000, or any other commercially available biosensor-based kinetic systems.
3. 1 mg/ml CMD minimizes unspecific binding to the dextran layer on the CM5 sensor surface.
4. When enough antibody and antigen is available, try to screen at different temperatures, e.g., at 13, 25, and 37°C. This allows you to select antibodies according to their optimal temperature-dependent kinetic binding rate signatures.
5. Whenever possible, sample buffer is injected (0 nM antigen) to double reference. The antibody/antigen binding signals are referenced versus their control spots and additionally versus a 0 nM antigen injection to avoid baseline drifts or positively drifting signals during the dissociation phase. When a higher throughput is necessary you can omit the double referencing or reduce the association and dissociation time. But keep in mind that data quality is the top priority. When the throughput allows it, perform double referencing or add more antigen analytes, e.g., for off-target or cross-reactivity tests.
6. It is important that the captured antibodies are tightly captured without dissociation, but finally are completely regenerated from the surface. An important control is the setting of two additional report points before the antibody capturing (Baseline Start, BS) and after the regeneration step (Baseline End, BE). Both report points shouldn't deviate much. Sometimes, it is necessary to perform a regeneration buffer scouting to figure out suitable regeneration conditions, which do not harm the capture system and guarantee constant capture performance (Fig. 5).
7. Since SPR is a mass-sensitive system, the antibody capture level impacts the latter assay sensitivity. This is especially an issue when one needs to screen primary hybridoma supernatants with low antibody concentrations or antigens with low molecular weight. The smaller the analytes' molecular weight, the higher the antibody capture level is needed. For an antigen range of about 10 kDa, at least 500 RU antibody capture level

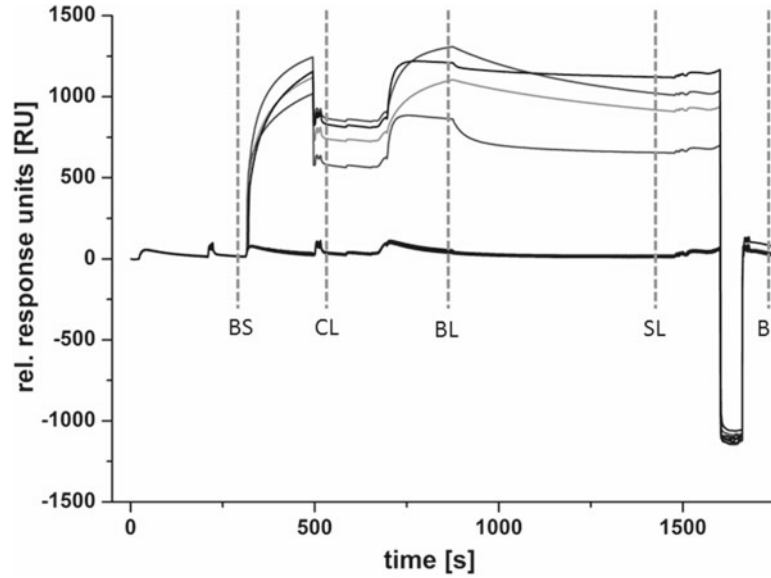

Fig. 5. A sensorgram (relative response units over time in seconds) overlay plot of four unreferenced screening cycles. *Dashed lines* indicate the time points of the report point settings. BS: Baseline Start defines the baseline signal prior to the antibody capturing. *CL* Antibody Capture Level is determined after the injection of the antibody containing solutions. *BL* Binding Late determined at the end of the antigen injection. *SL* Stability Late is determined at the end of the antigen dissociation phase. Clearly, different antigen association and dissociation phase signatures can be identified. *BE* Baseline End is determined after the regeneration of the capture system.

is recommended. To increase the antibody capture level increase the injection time, but do not use a more concentrated hybridoma supernatant due to the risk of unspecific binding and instrument clogging.

8. When the antigen is not available at sufficiently high concentration or generally slowly associates, increase the association time to up to 10 min. However, keep in mind that this increases the antigen consumption and the instrument's runtime.
9. The oriented presentation of the captured antibodies allows the selection of antibodies according to their valence by calculating their Molar Ratio. The correct calculation of the Molar Ratio requires saturating the antibody during the antigen association phase. In order to get information of low affinity antibodies it is useful to inject the antigen at higher concentration of at least 150 nM.
10. A high Binding Late (BL) value resembles a fast association rate constant k_a (1/Ms), even when k_a is not quantified. To select an antibody with highest k_a value is the most important screening parameter when it is to select diagnostic and pharmaceutical antibodies.

11. Primary hybridoma cultures mostly show low Molar Ratio values. In contrast, clone culture hybridoma supernatants should show reasonable Molar Ratios (Fig. 3). Usually, the whole data set is characterized by small MR values, when high molecular weight antigens (MW > 50 kDa) are used, because neighboring antibodies presented on the sensor surface are sterically blocked simply by the antigens' size. When the MR values statistically distribute, cultures with too low Molar Ratio (MR = 0.01) or overstoichiometric binding (MR > 2.5) should be deselected. If the complete run is characterized by generally understoichiometric or overstoichiometric Molar Ratio values, check the antigen's stability, buffer formulation, p*I*, molecular weight, and oligomeric status. In the worst case, the Molar Ratio indicates that the data obtained by the run is not meaningful. Always visually check the sensorgrams and do not just rely on k_d and $t_{1/2\ diss}$ values. A $k_d = 1.0E{-}05$ 1/s resulting in $t_{1/2\ diss} = 1{,}155$ min and a binding late signal of BL = 0.5 RU is an artifact, identifiable by low Molar Ratio values. Therefore, carefully consider all six parameters in a comprehensive way. It is not relevant to focus only on antibody–antigen complex stability. Fast association rates, reflected by considerable Binding Late values, are equally important. For an application in an equilibrium system (= without washing), a fast antibody–antigen association rate (= high BL value) is even of more of importance than just a high complex stability.
12. A comprehensive kinetic screening is shown in a table with Binding Late (BL), Stability Late (SL), k_d (1/s), $t_{1/2\ diss}$ (min), antibody Capture Level (CL) and the Molar Ratio (MR) at a specific temperature.

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