HuCAL® Antibodies Technical Manual
Affinity Determination

Affinity is the strength of binding between an antigen and an antibody binding site. This binding strength is based on the intrinsic affinity of the antibody and its valency. Monovalent antibody formats show only an intrinsic affinity while the binding strength of bi- or multivalent formats is affected by their avidity (intrinsic affinity plus valency). The equilibrium dissociation constant between the antibody and its antigen is designated K_D and is the ratio of the experimentally measured off- and on- rates, k_d and k_a, respectively. The lower the K_D value, the higher the affinity of the antibody. To get true affinity values, it is important to avoid avidity effects. Therefore, monovalent Fab fragments are recommended for affinity measurement.

Measuring antibody affinity is a useful tool for characterizing, evaluating, and ranking antibodies. Although different technologies are available, essentially there are two different principles applied to determine affinities:

- Kinetic data with association on and dissociation off rates (k_a and k_d), which is acquired by using methods measuring the interaction between an immobilized ligand and an analyte in solution, for instance, bio-layer interferometry (BLI) or surface plasmon resonance (SPR)
- Endpoint analysis, which measures concentrations after equilibrium is reached between antigen-bound and free antibody, for example by ELISA titration

Kinetic data are often measured in label-free technologies where none of the reagents are required to carry a label or dye, and no secondary reagents are necessary. For most kinetic measurements, one binding partner (the antigen or antibody) is immobilized on a solid support. Measured affinities for one antibody-antigen pair may vary depending on the method used due to the differences in set-up, antigen presentation or steric hindrance.

Whenever one of the two partners is bi- or multivalent, the set-up must be chosen with care to avoid avidity influencing the measurement. This is always the case when one molecule binds the immobilized partner with two binding sites simultaneously, for example when IgG binds with both arms to coated antigen.

There are many technologies available for K_D determination. This chapter describes the use of BLI for affinity determinations of HuCAL recombinant monoclonal antibodies. Bio-Rad offers services using BLI for affinity determination and affinity ranking of HuCAL antibodies generated as part of a custom project.

Bio-Layer Interferometry

Bio-Rad uses the Octet RED384 (Pall FortéBio) to carry out real time, label-free affinity determination and off-rate ranking services for HuCAL Fab antibodies. Bio-layer interferometry is based on the optical interference pattern of light reflected from a sensor surface. The antigen (ligand) is immobilized on the surface of an amine-reactive sensor while the Fab antibody (analyte) is in solution. Binding of the antibody to its immobilized antigen leads to an increase in optical thickness at the sensor surface, which results in a wavelength shift.

Affinity determination

By default, PBS containing 0.1% BSA and 0.02% Tween 20 is used as running buffer; the additives are used to reduce non-specific binding. To avoid avidity effects during affinity measurement, monovalent Fab fragments are recommended. The affinity determination must be performed with at least five different concentrations of the purified Fab antibody in running buffer, to ensure that a measurement with an optimal affinity fit can be obtained.

The ligand is immobilized on Amine Reactive 2nd Generation (AR2G) Biosensors (Pall FortéBio, catalog #18-5092), according to the manufacturer's instructions. The immobilization is achieved through EDC/Sulfo-NHS based amide bond formation to create a covalent bond between a reactive amine on the antigen and the carboxy-terminated sensor surface. For optimal immobilization, loading buffers with different pH values (typically pH 4, 5 and 6) are tested in the corresponding assay.

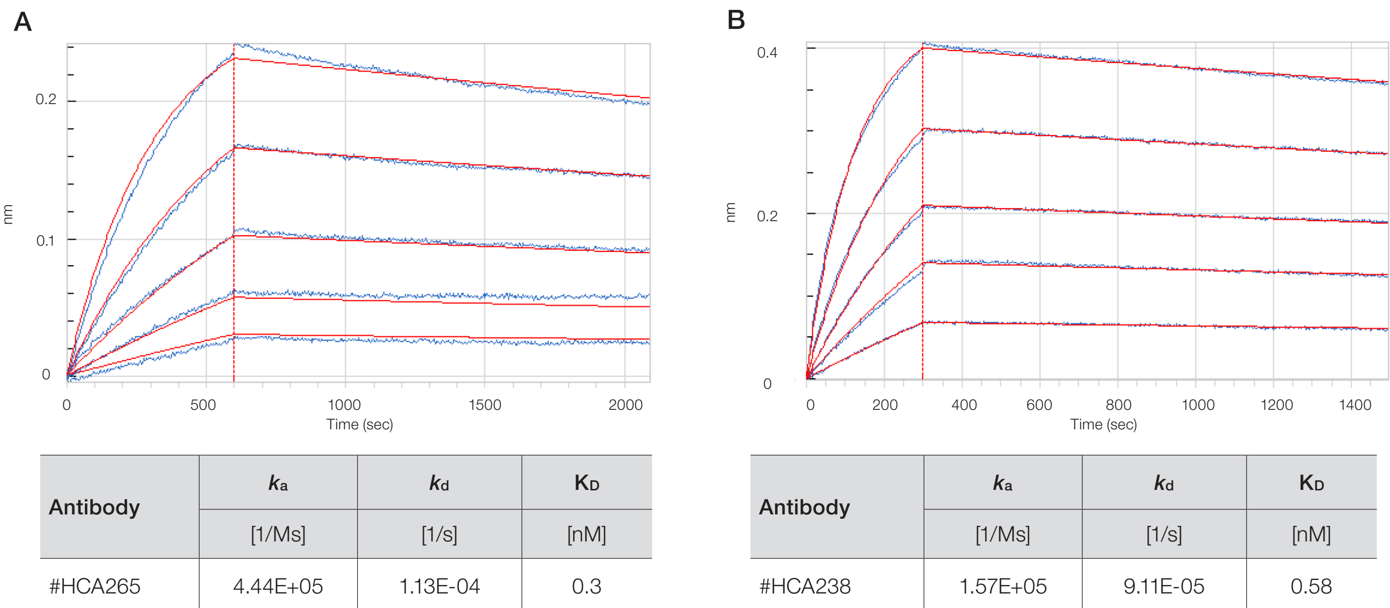
Protocol

1. Hydrate the sensors for 10 min in distilled water.
2. Set a baseline for 180 sec in distilled water.
3. Activate the sensors for 300 sec in 20 mM EDC/10 mM sulfo-NHS.
4. Load the ligand (usually 5-10 µg/ml) for 600 sec in 10 mM sodium acetate buffer with appropriate pH value.
5. Quench potential remaining binding sites for 300 sec with 1 M ethanolamine pH 8.5.

Affinity measurement

Use purified Fab antibody in running buffer. Include a reference sensor with immobilized ligand and run in running buffer without analyte for the association and dissociation steps.

1. Dilute the analyte (Fab) to eight different concentrations with running buffer, for example 1:2 dilutions, starting with 200 nM.
2. Set a baseline for 180 sec in running buffer.
3. Measure the association of the Fab antibody to the immobilized ligand for 600 sec and allow the Fab to dissociate for at least 1200 sec.
4. Regenerate the sensor by placing it into appropriate regeneration buffer, for instance 10 mM glycine, pH 1.5, or 50 mM HCl, depending on the antigen. Repeated regeneration steps may be necessary for complete regeneration.
5. After the regeneration, repeat steps 2-4 with the next concentration of the analyte.
6. Repeat steps 2-4 for all dilutions.
7. Analyze the data set using the FortéBio Data Analysis software by selecting the appropriate model to fit the data.



Antibody	k_a [1/Ms]	k_d [1/s]	K_D [nM]
#HCA265	4.44E+05	1.13E-04	0.3

Antibody	k_a [1/Ms]	k_d [1/s]	K_D [nM]
#HCA238	1.57E+05	9.11E-05	0.58

Fig. 1. Example affinity measurements. A, inhibitory, anti-idiotypic antibody, Human Anti-Panitumumab Antibody, clone AbD23897, cat. #HCA265, in monovalent Fab format. **B**, drug-target complex specific antibody, Human Anti-Omalizumab/hIgE Antibody, clone AbD20760, #HCA238.

Off-rate ranking

As well as affinity determination the Octet RED384 can be used for an off-rate ranking of antibodies as part of the antibody selection process. This secondary screening approach allows the antibodies to be ranked according to their dissociation rates k_d. The clones with the preferred k_d values can be selected for further characterization. Up to 95 antibodies can be analyzed in parallel. With this method, the antibodies can be used in crude *E. coli* lysate, therefore no purification step is needed before off-rate ranking is performed.

The immobilization of the ligand is carried out in accordance with the method for affinity determination described previously.

Protocol

Use Fab antibodies in crude *E. coli* lysate. Purification of the antibodies is not necessary. Include a reference sensor with immobilized ligand and run in control *E. coli* lysate containing a non-specific antibody for the association and dissociation steps.

1. Dilute the crude *E. coli* lysates 1:1 in running buffer.
2. Set a baseline for 180 sec in control lysate/running buffer mix.
3. Measure the association of the Fab antibody in crude lysate to the immobilized ligand for 450 sec.
4. Measure the dissociation of the Fab for 450 sec.
5. Analyze the data set using the FortéBio Data Analysis software by selecting the appropriate model to fit the data.
6. Rank the Fab antibodies according to their k_d values.

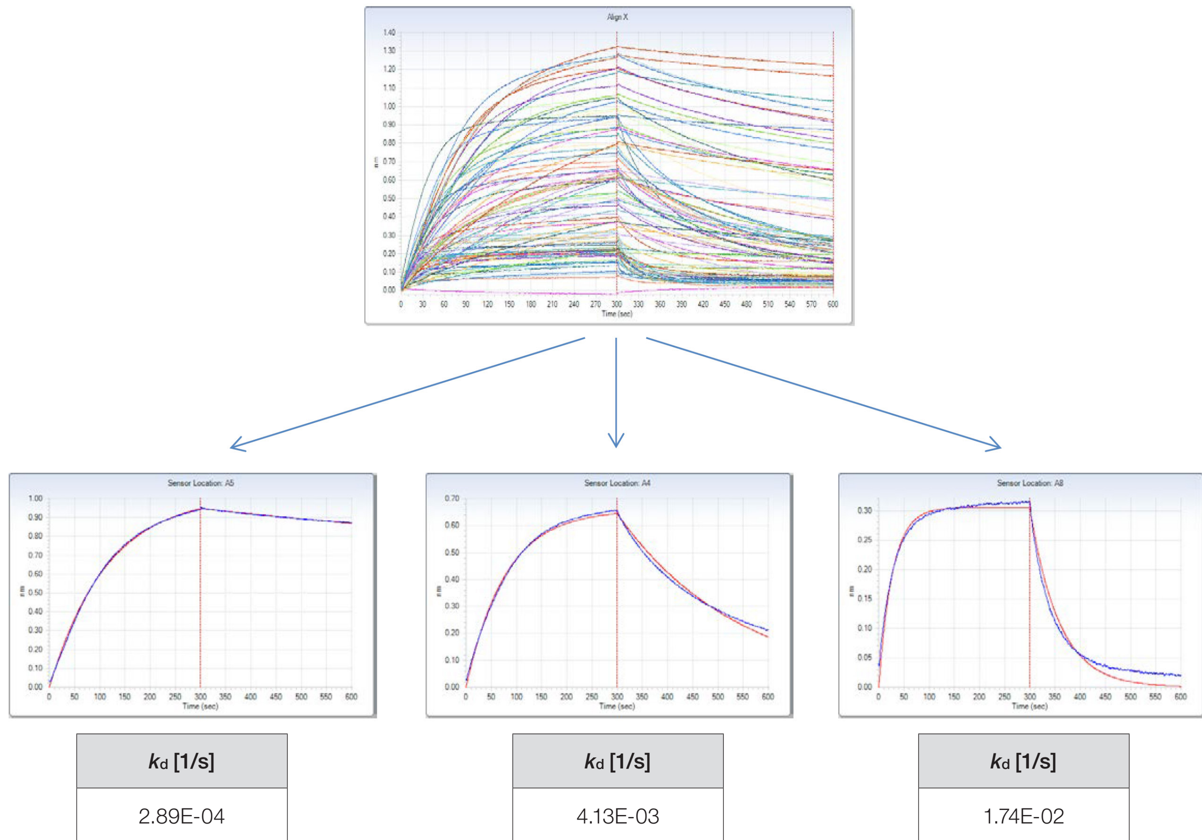


Fig. 2. Example data for off-rate ranking secondary screening experiment. The sensorgrams of 95 hits from an anti-cetuximab antibody generation are shown (above); individual sensorgrams for three of these 95 hits are presented (below), which show distinct dissociation behavior resulting in different k_d values.

For more information about off-rate ranking using the Octet RED384 see: Ylera F et al. (2013). Off-rate screening for selection of high-affinity anti-drug antibodies. Analytical Biochemistry. 441 (2):208-13.

Technical Assistance

Experienced technical advisors are available to help with any questions regarding HuCAL antibodies and their applications, both before project start and after antibody delivery. Since HuCAL technology provides many opportunities for optimizing the antibody generation process according to customer needs, we encourage the discussion of your antibody project with one of our scientists at an early stage. They can help with project design, antigen preparation, and provide information about the many options that are simply not available with typical animal based technologies. Once the antibodies are delivered, they can also provide valuable advice about protocols and detection systems.

Contact us at **antibody_sales_muc@bio-rad.com** or visit **bio-rad-antibodies.com/HuCAL** for more information.

General technical support requests related to catalog antibodies and accessory products can also be addressed to **antibody_tech_uk@bio-rad.com**.

Confidentiality

We treat all data and material provided to us by customers as completely confidential, including customer name and affiliation. Information from customers is used only as necessary for the successful and safe performance of the antibody generation project. We are pleased to provide a signed Confidential Disclosure Agreement (CDA) upon request.

Visit **bio-rad-antibodies.com** for more information.

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