Characterization of Critical Reagents for Ligand Binding Assays

Ligand binding assays are utilized throughout the large molecule drug development process. However their robustness, accuracy and reproducibility depend on the quality of critical reagents. The unique characteristics of these reagents are crucial to assay performance, and require careful characterization. To ensure their consistent characterization in bioanalytical laboratories, a team of pharmaceutical scientists have outlined recommendations and best practices for this process, particularly during the early stages of drug development.

Drug development relies on ligand binding assays (LBAs), which are bioanalytical procedures widely used to measure the immunogenicity of biotherapeutic molecules, and determine drug concentrations for pharmacokinetic analyses. The accuracy and performance of LBAs, however, depends on the quality of key reagents, called critical reagents. The Global Bioanalysis Consortium (GBC) and the European Medicines Agency classify critical reagents as analyte specific or binding reagents, specifically antibodies; peptides; engineered proteins; antibody, protein and peptide conjugates; reagent drugs; aptamers and anti-drug antibody (ADA) reagents including positive and negative controls (King et al. 2014). In 2014, the GBC expanded this definition to include biological matrices, in some instances (King et al. 2014). Utilizing poorly characterized critical reagents in LBAs could lead to inaccurate conclusions and delays in the drug development process. It is therefore necessary to properly characterize them early in the drug development program. However, their characterization and management varies between bioanalytical laboratories.

Best Practices and Recommendations for Critical Reagent Characterization

The initiative to generate a consensus within the pharmaceutical industry regarding the expectations and operations of a bioanalytical laboratory originated from a 2009 American Association of Pharmaceutical Scientists workshop on the Twenty-First Century Bioanalytical Laboratory. Following up on this initiative, O'Hara et al. (2012) developed and published a set of recommendations and best practices for the life cycle management, characterization and supply of critical reagents used in LBAs.

This article summarizes the guidelines for characterizing critical reagents, particularly during the early stages of drug development. In this context, characterization refers to the process of identifying the physicochemical attributes of these reagents (O'Hara et al. 2012). In addition, examples are shown to provide insight into the analytic methods applied at Bio-Rad for quality control and analytical antibody batch characterization as part of the manufacturing and product development of the anti-biotherapeutic antibody portfolio.

Characterizing critical reagents is essential for ensuring optimal assay performance over time as these attributes can serve as a guide for: 1) identification of the root causes of assay performance problems, 2) determination of when a reagent is deteriorating and 3) the generation of new reagents and screening of different lots.

Categories of Critical Reagents used in Ligand Binding Assays

- Antibodies
- Peptides
- Engineered proteins
- Chemically synthesized molecules
- Antibody, protein and peptide conjugates
- Complex biologics
- Solid supports and matrices

BIO-RAD

To enhance consistency in the industry and guide decision making, O'Hara et al. (2012) propose the following recommendations and best practices for characterizing critical reagents in LBAs.

1. It is important to generate a basic characterization profile for each critical reagent that includes assessment of concentration (antibody titer), binding activity, aggregation, purity level and molecular weight.
2. Include purification assays for protein reagents during early stages of drug development to reduce the impact of impurities, which could affect stability during long term storage.
3. Verify purified reagents using methods such as SDS-PAGE and SEC to confirm purity and monodispersity prior to use in the desired LBA.
4. As drug development progresses, express monoclonal antibody reagents in characterized mammalian cell lines to ensure their genetic stability and consistency across reagent lots.
5. Where appropriate, consider assessing optional characterization parameters such as determining protein A levels in a protein purified using a protein A column, bovine IgG levels from tissue culture expansion, and residual host cell protein levels.

Recommended Critical Reagent Characteristics to Measure

Sensitivity

- Concentration (antibody titer)
- Binding activity
- Potency
- Binding kinetics and affinity determination
- Neutralization activity
- Aggregation level

Specificity

- Cross-reactivity

Reproducibility

- Molecular weight
- Purity
- Isotype (for monoclonal antibodies)
- Formulation buffer selection
- Isoelectric point
- Conjugate incorporation ratio
- Endotoxin level
- Protein A level
- Host cell protein level
- Stability
- Bovine IgG level
- Functional assays

Antibody Critical Reagents Developed by Bio-Rad

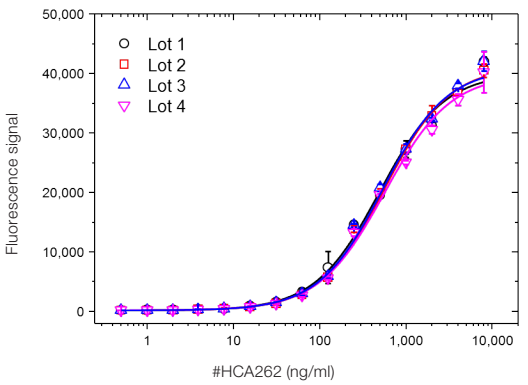
Bio-Rad develops highly specific, high affinity anti-biotherapeutic antibodies to support preclinical research, clinical trials and patient monitoring for innovator and biosimilar products. Because reproducibility and accuracy are essential for successful bioanalytical assay development, the antibodies for these assays must be made to a high quality and supplied to a consistent standard in order to minimize assay variability, so that the assay developer can have confidence in the results.

Bio-Rad's anti-biotherapeutic antibody reagents for ADA and PK assay development are generated using the Human Combinatorial Antibody Library (HuCAL®) and an improved, proprietary method of antibody phage display, resulting in fully human, monoclonal recombinant antibodies in Fab and full length immunoglobulin (Ig) formats. Integral to the process is in vitro guided selection, which enables the generation of highly specific inhibitory and non-inhibitory anti-idiotypic antibodies and specialized drug-target complex binders. The antibody sequence is always known and stored for long term security, and the methods used are well established, proven and highly reproducible. Fab antibodies are expressed in *E. coli* and full length Ig formats are expressed in a characterized human cell line using ultra-low bovine IgG FCS in the production method. Antibodies are then purified using affinity chromatography. The quality control and characterization process is thorough, consistent and incorporates several of the relevant best practices outlined by O'Hara et al. (2012). Every new antibody and subsequent lots are subject to a strict quality control procedure to ensure a high quality product every time, helping the assay developer with critical reagent life cycle management, characterization and supply, and the maintenance of optimal assay performance over time.

Anti-Biotherapeutic Antibody Quality Control

Batch-to-batch consistency

For reagents in full length Ig format, the antibody batches are tested and compared using an ADA bridging ELISA set-up. Figure 1 shows batch consistency with an anti-palivizumab antibody as an example of such an assay.



	EC50 (ng/ml)
Lot 1	494.1
Lot 2	564.3
Lot 3	538.4
Lot 4	566.9

Fig. 1. Batch-to-batch consistency. A microtiter plate was coated overnight with palivizumab at a concentration of 1 µg/ml. After washing and blocking with PBST + 5% BSA, PBST with 10% human serum, spiked with increasing concentrations of Human Anti-Palivizumab Antibody (cat. #HCA262, Lot 1-4) was added. Detection was performed using HRP conjugated palivizumab at a concentration of 2 µg/ml in HISPEC Assay Diluent (#BUF049A) and QuantaBlu Fluorogenic Peroxidase Substrate. HRP conjugation of palivizumab was performed using a LYNX Rapid HRP Antibody Conjugation Kit®. Data are shown as the mean of three measurements for each data point. EC50 values (table above) were calculated from the non-linear curve fits.

Quality Control for Batch Release

Every new product is first produced in three independent batches. The activity of the batches is compared in a LBA: ELISA titration for Fab antibodies (Figure 6) and ADA bridging ELISA for Ig antibodies (Figures 1 and 7). The batch closest to the average curve is chosen as the future reference batch. Every new batch is compared to the reference batch using the same assay protocol.

Before release, new batches of all antibodies are also routinely tested for specificity, purity (Figures 2 and 3), and in addition, full length Ig antibodies are tested by SEC for monodispersity (Figure 4).

In addition, for each new batch production, the antibody gene is re-sequenced to ensure product identity.

SDS PAGE

Purity of Fab format antibodies is assessed using SDS PAGE with subsequent Coomassie staining (Figure 2).

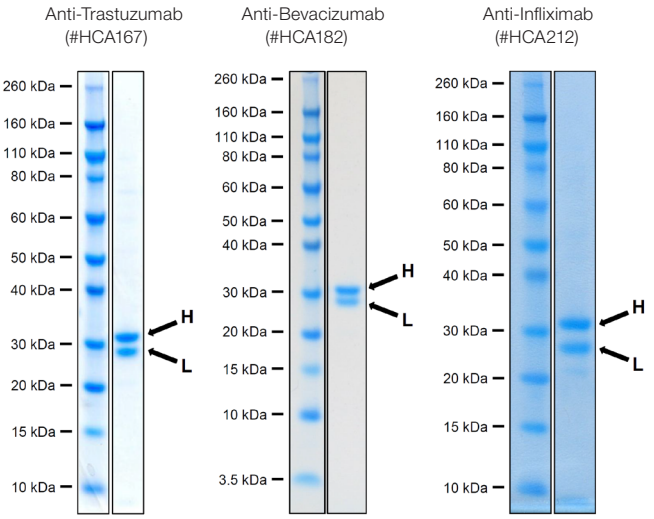
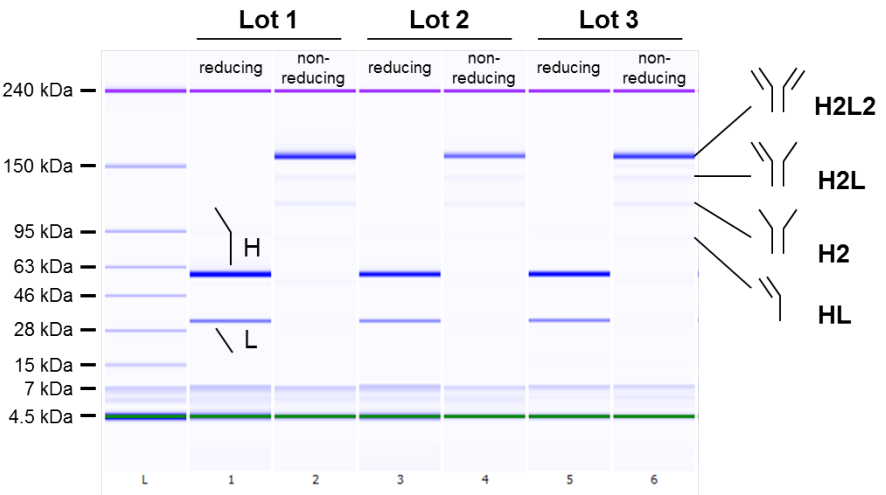


Fig. 2. Purity assessment of Fab format antibodies. Human Anti-Trastuzumab (#HCA167), Anti-Bevacizumab (#HCA182) and Anti-Infliximab (#HCA212) Fab Antibodies were analyzed by SDS-PAGE (2 µg per lane). Heavy chains (H) and light chains (L) are visible at ~32 kDa and ~28 kDa, respectively.

Capillary Electrophoresis (CE)

Purity of full Ig format antibodies is assessed using capillary electrophoresis (CE); the output is a 'virtual' gel, as shown in Figure 3.

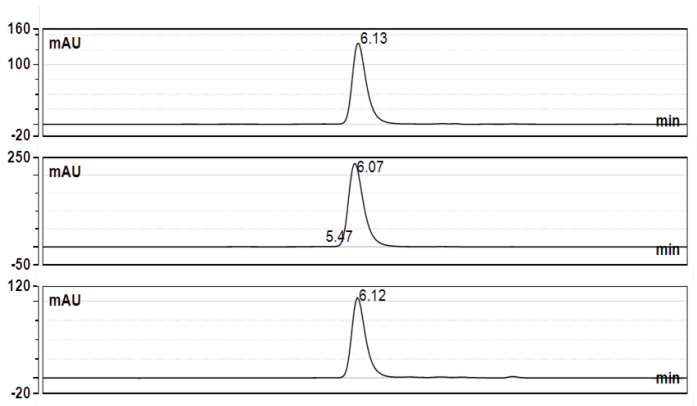


#	Anti-Palivizumab	Purity %
L	Ladder	
1	Lot 1 reducing	99
2	Lot 1 non-reducing	85
3	Lot 2 reducing	99
4	Lot 2 non-reducing	82
5	Lot 3 reducing	99
6	Lot 3 non-reducing	82

Fig. 3. Purity assessment of Ig format antibodies. Three different batches of Human Anti-Palivizumab hIgG Antibody (#HCA262, Lot 1-3) were analyzed by CE. Samples were denatured and run under reducing and non-reducing conditions. H and L chains under reducing denaturing conditions are visible at ~50 kDa and ~30 kDa, respectively. Intact IgG molecules (H2L2) are visible at ~150 kDa under non-reducing denaturing conditions.

Size Exclusion Chromatography (SEC)

SEC is used to look for the presence or absence of aggregates (Figure 4).

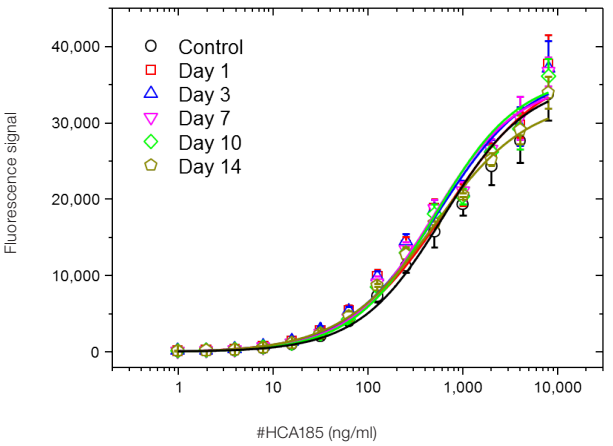


	#	Peak Name	Retention Time [min]	Rel. Area [%]
Lot 1	-	Aggregates	-	-
	1	hIgG	6.132	100.00
Lot 2	1	Aggregates	5.465	0.59
	2	hIgG	6.065	99.41
Lot 3	-	Aggregates	-	-
	1	hIgG	6.115	100.00

Fig.4. SEC analysis. Three different batches of Human Anti-Palivizumab hIgG Antibody (#HCA262, Lot 1-3) were analyzed by SEC on an analytical HPLC instrument.

General Characterization of Anti-Biotherapeutic Antibodies

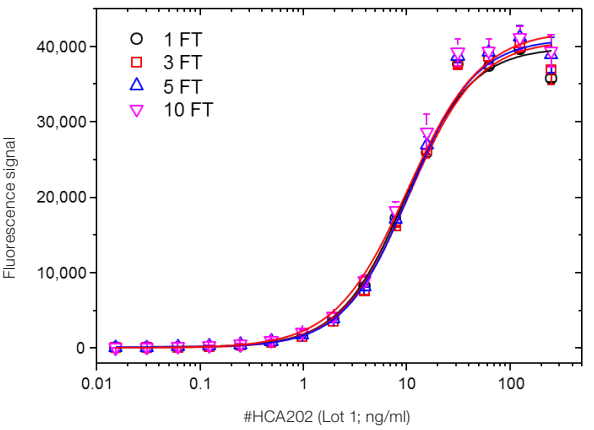
Antibody shelf life stability can be tested by using an accelerated stability testing protocol or by monitoring activity after several freeze-thaw cycles. In the example below, the Anti-Bevacizumab Antibody #HCA185 (human IgG1) was tested by measuring activity after up to 14 days incubation at 37˚C.



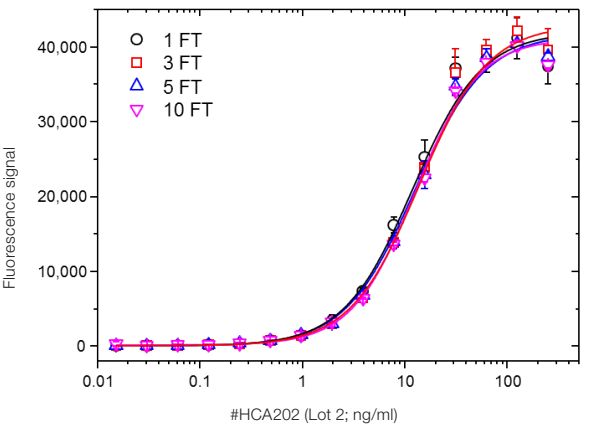
	EC50 (ng/ml)
Control	624.0
Day 1	667.2
Day 3	556.4
Day 7	482.4
Day 10	518.7
Day 14	496.4

Fig. 5. Accelerated stability test. Freshly thawed Human Anti-Bevacizumab hIgG Antibody (#HCA185) was diluted to 1 mg/ml, aliquoted and placed at 37˚C. Aliquots were removed from 37˚C at every time point and placed at 4˚C along with the control. For the assay a microtiter plate was coated overnight with bevacizumab at a concentration of 1 µg/ml. After washing and blocking with PBST + 5% BSA, PBST with 10% human serum, spiked with increasing concentrations of Human Anti-Bevacizumab hIgG Antibody (#HCA185) was added. Detection was performed using HRP conjugated bevacizumab at a concentration of 2 µg/ml in HISPEC Assay Diluent (#BUF049A) and QuantaBlu Fluorogenic Peroxidase Substrate. HRP conjugation of bevacizumab was performed using a LYNX Rapid HRP Antibody Conjugation Kit. Data are shown as the mean of three measurements. EC50 values (table above) were calculated from the non-linear curve fits.

In the example below, two independent batches of the Anti-Adalimumab Antibody #HCA202 (Fab format) were tested for activity after several freeze-thaw cycles using indirect ELISA (Figure 6).



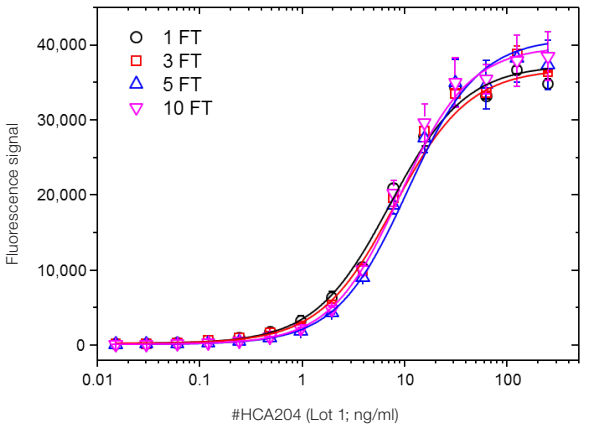
EC50 (ng/ml)			
1 FT	**3FT**	**5FT**	**10FT**
9.70	10.58	10.56	10.24



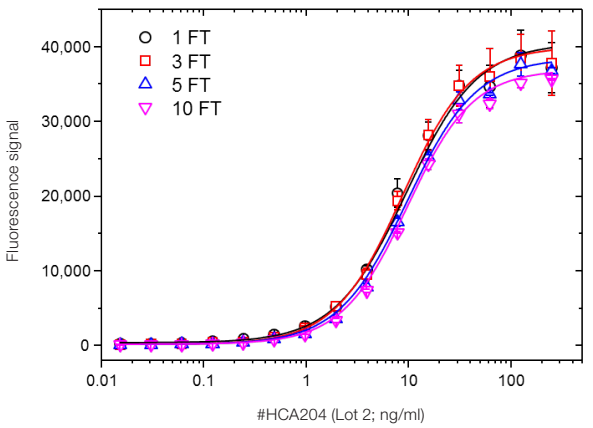
EC50 (ng/ml)			
1 FT	**3FT**	**5FT**	**10FT**
11.98	13.84	12.62	12.69

Fig. 6. Freeze-thaw test for Fab format. Human Anti-Adalimumab Fab-FH Antibody (#HCA202) lot 1 (top) and lot 2 (bottom), were subjected to the indicated number of freeze-thaw cycles (FT). For the assay, a microtiter plate was coated overnight with adalimumab at a concentration of 5 µg/ml. After washing and blocking with PBST + 5% BSA, PBST with 10% human serum spiked with increasing concentrations of Human Anti-Adalimumab Fab-FH Antibody was added. Detection was performed using HRP conjugated Mouse Anti-Penta Histidine-Tag Antibody (#MCA5995P) at a dilution of 1:2000 in HISPEC Assay Diluent (#BUF049A) and QuantaBlu Fluorogenic Peroxidase Substrate. Data are shown as the mean of three measurements. EC50 values (tables under graphs) were calculated from the non-linear curve fits.

Similarly, two independent batches of the Anti-Adalimumab Antibody #HCA204 (human IgG1) were also tested for activity after FT cycles, this time using an ADA bridging ELISA set-up (Figure 7).



EC50 (ng/ml)			
1 FT	**3FT**	**5FT**	**10FT**
7.25	7.94	10.67	9.04

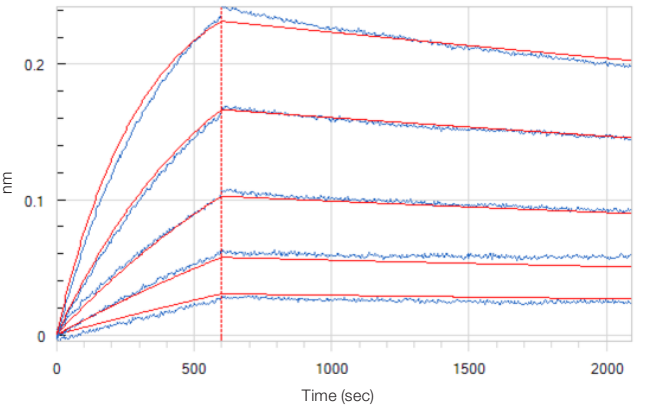


EC50 (ng/ml)			
1 FT	**3FT**	**5FT**	**10FT**
9.49	8.71	9.69	9.70

Fig. 7. Freeze-thaw test for Ig format. Human Anti-Adalimumab hIgG1 Antibody (#HCA204) lot 1 (top) and lot 2 (bottom) were subjected to the indicated number of freeze-thaw cycles (FT). For the assay, a microtiter plate was coated overnight with adalimumab at a concentration of 1 µg/ml. After washing and blocking with PBST + 5% BSA, PBST with 10% human serum, spiked with increasing concentrations of Human Anti-Adalimumab hIgG Antibody was added. Detection was performed using HRP conjugated adalimumab at a concentration of 2 µg/ml in HISPEC Assay Diluent (#BUF049A) and QuantaBlu Fluorogenic Peroxidase Substrate. HRP conjugation of adalimumab was performed using a LYNX Rapid HRP Antibody Conjugation Kit. Data are shown as the mean of three measurements. EC50 values (tables under graphs) were calculated from the non-linear curve fits.

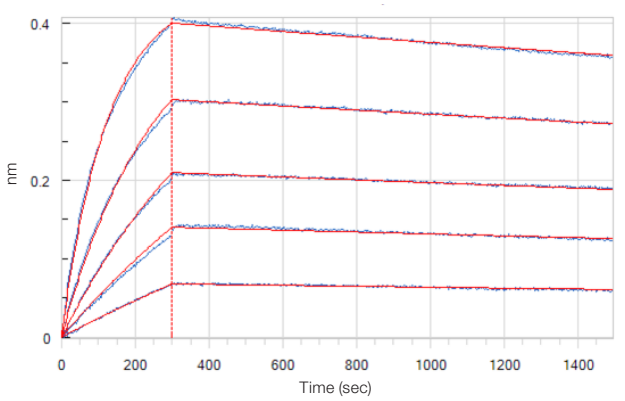
Antibody Affinity Measurement

Affinity is the strength of binding between an antigen and an antibody binding site. 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. We determine the affinity of all anti-biotherapeutic antibodies in their monovalent format using biolayer inferometry. Examples are shown below for an inhibitory anti-idiotypic antibody (Figure 8) and a specialized drug-target complex specific antibody (Figure 9). We use the monovalent Fab format for affinity determination, as the measured affinity truly reflects the intrinsic antigen-binding affinity due to the monovalent interaction with the antigen. Knowing the affinity of an antibody can give the assay developer an indication of the sensitivity of an assay that might be achieved.



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

Fig. 8. Affinity determination for an inhibitory (Type 1) anti-idiotypic antibody. 10 µg/ml panitumumab in 10 mM sodium acetate pH 4 was immobilized on Octet RED384 (Pall FortéBio) AR2G sensors with a coating density of 1.4 ± 0.1 nm. The Human Anti-Panitumumab Antibody (#HCA265), in a monovalent Fab format was then perfused over the sensor surface using a 1:2 dilution series with a starting concentration of 6.25 nM. An additional sensor perfused with experimental buffer only was used as reference for compensating any baseline drifts. Sensors were regenerated after each cycle with 10 mM glycine pH 2.0. Experimental buffer was PBS pH 7.4, 0.1% (w/v) BSA and 0.02% (v/v) Tween 20 Reagent. The experiment was performed at 30°C with a shake speed of 1000 rpm. A 1:1 interaction model was used to fit the data using the FortéBio Data Analysis software 8.2.0.7. Measured data are shown in blue; interaction fit is shown in red. Calculated association and dissociation rates and the K_D value are given in the table.



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

Fig. 9. Affinity determination for a drug-target complex specific (Type 3) antibody. 10 µg/ml omalizumab (anti-human IgE) in 10 mM sodium acetate pH 6 was immobilized on Octet RED384 (Pall FortéBio) AR2G sensors with a coating density of 2.8 ± 0.1 nm. The complex with human IgE (hIgE) was formed by adding 10 µg/ml of hIgE in experimental buffer. The additional signal of hIgE was 1.3 ± 0.1 nM. The complex-specific Human Anti-Omalizumab/hIgE Antibody (#HCA238) in a monovalent Fab format was then perfused over the sensor surface using a 1:2 dilution series with a starting concentration of 48 nM. An additional sensor with immobilized omalizumab/hIgE complex and perfused with experimental buffer only was used as reference for compensating any baseline drifts. Experimental buffer was PBS pH 7.4, 0.1% (w/v) BSA and 0.02% (v/v) Tween 20 Reagent. The experiment was performed at 30°C with a shake speed of 1000 rpm. A 1:1 interaction model was used to fit the data using the FortéBio Data Analysis software 8.2.0.7. Measured data are shown in blue; interaction fit is shown in red. Calculated association and dissociation rates and the K_D value are given in the table.

Summary

LBAs are instrumental in the drug development process. They utilize a set of reagents of which some are deemed critical to the performance of the assay. These critical reagents directly impact the quality and reproducibility of LBAs and consequently can delay the drug development process if they are of poor quality. Because they are so important to the success of LBAs, careful characterization of critical reagents should be performed, whether purchased or produced in-house. Best practices and recommendations for characterizing critical reagents are not fully standardized within the pharmaceutical industry. A team of scientists has addressed this concern by providing the best practices for critical reagents characterization. These guidelines focus on generating a characterization profile for each critical reagent that will ultimately be useful for ensuring the long-term robustness of LBAs.

Bio-Rad incorporates best practices for quality control and analytical antibody batch characterization as part of the product development and manufacturing of the anti-biotherapeutic antibody portfolio. These antibodies are being adopted as critical reagents in assays carried out as part of bioanalytical testing services provided by global contract research organizations, and in preclinical and clinical studies in pharmaceutical companies.

References

King LE et al. (2014). Ligand binding assay critical reagents and their stability: Recommendations and best practices from the global bioanalysis consortium harmonization team. AAPS J 16, 504-515. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4012044/

O'Hara DM et al. (2012). Ligand binding assays in the 21st century laboratory: Recommendations for characterization and supply of critical reagents. AAPS J 14, 316-328. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3326169/

Antibodies to Biotherapeutics

Bio-Rad has developed highly specific, high affinity anti-biotherapeutic antibodies to support preclinical research, clinical trials and patient monitoring for innovator and biosimilar products.

Select antibodies to your target from our industry-leading range of antibodies against marketed biotherapeutic monoclonal antibody drugs, or commission a custom made antibody against your proprietary biotherapeutic lead.

Special features of our ready made antibodies include:

- Application data and protocols to support pharmacokinetic (PK), anti-drug antibody (ADA) and inhibition assays
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- Recommended ELISA detection antibodies available directly conjugated to HRP
- Fully human immunoglobulins as controls and calibrators for ADA assays
- Unlimited and consistent supply throughout preclinical and clinical studies

Anti-Biotherapeutic Antibody Range

Antibody Target INN/Trade Name	Number of Products Available		Binding Modes Available			Affinities Available[1] (K_D, nM)	Supporting Data		
	Fab	Ig	Inhibitory	Non-Inhibitory	Complex Binder		PK/PD	ADA	Inhibition
Adalimumab/HUMIRA	3	5 IgG1 1 IgE	•		•	67.0-0.06	•	•	•
Alemtuzumab/LEMTRADA	2	2 IgG1	•			2.4-0.2	•	•	•
Bevacizumab/Avastin	1	2 IgG1	•			2.0-0.4	•	•	•
Cetuximab/ERBITUX	1	4 IgG1	•	•		13.5-0.5	•	•	•
Denosumab/Prolia	1	3 IgG1	•			7.1-0.8	•	•	•
Etanercept/Enbrel	1	1 IgG1 1 IgG4	•	•[2]		14.8-2.4	•	•	•
Golimumab/Simponi	5	5 IgG1	•	•	•	53-0.1	•	•	•
Infliximab/REMICADE	3	4 IgG1	•	•		3.9-0.12	•	•	•
Natalizumab/Tysabri	1	2 IgG1	•			12.2-2.1	•	•	•
Omalizumab/XOLAIR	2	2 IgG1	•		•	1.1-0.58	•	•	•
Palivizumab/Synagis	1	1 IgG1	•			1.5	•	•	
Panitumumab/Vectibix	1	2 IgG1	•			0.6-0.3	•	•	•
Rituximab/RITUXAN	3	1 IgG1	•			10.0-0.13	•	•	
Tocilizumab/Actemra	1	5 IgG1	•	•		31.0-0.1	•	•	•
Trastuzumab/Herceptin	5	2 IgG1 1 IgG4	•		•	2.5-0.02	•	•	•
Ustekinumab/STELARA	2	2 IgG1	•			2.8-0.2	•	•	•

[1] Given as a range; Lowest affinity antibody available - highest affinity antibody available. NB. Affinities are measured in the monovalent Fab format.
[2] One non-inhibitory antibody recognizes the TNFR2 domain; a second non-inhibitory antibody is specific for the TNFR2-Fc fusion region of etanercept.

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