mAbs 5:2, 270–278; March/April 2013; © 2013 Landes Bioscience

High throughput solution-based measurement of antibody-antigen affinity and epitope binning

Patricia Estep, Felicia Reid, Claire Nauman, Yuqi Liu, Tingwan Sun, Joanne Sun and Yingda Xu*

Protein Analytics; Adimab, LLC; Lebanon, NH USA

Keywords: high throughput, affinity, antibody, epitope, KD, MSD, kinetic exclusion, BLI

Advances in human antibody discovery have allowed for the selection of hundreds of high affinity antibodies against many therapeutically relevant targets. This has necessitated the development of reproducible, high throughput analytical techniques to characterize the output from these selections. Among these characterizations, epitopic coverage and affinity are among the most critical properties for lead identification. Biolayer interferometry (BLI) is an attractive technique for epitope binning due to its speed and low antigen consumption. While surface-based methods such as BLI and surface plasmon resonance (SPR) are commonly used for affinity determinations, sensor chemistry and surface related artifacts can limit the accuracy of high affinity measurements. When comparing BLI and solution equilibrium based kinetic exclusion assays, significant differences in measured affinity (10-fold and above) were observed. KinExA direct association (k_a) rate constant measurements suggest that this is mainly caused by inaccurate k_a measurements associated with BLI related surface phenomena. Based on the kinetic exclusion assay principle used for KinExA, we developed a high throughput 96-well plate format assay, using a Meso Scale Discovery (MSD) instrument, to measure solution equilibrium affinity. This improved method combines the accuracy of solution-based methods with the throughput formerly only achievable with surface-based methods.

Introduction

Binding affinity and epitopic coverage are critical biophysical characteristics for identification of therapeutically relevant antibodies.[1] Generation of 1000–3000 unique lead antibodies per week necessitates a high throughput methodology to measure affinity and determine epitopic diversity. When measuring affinities based on currently available methods, one must compromise either on throughput or accuracy.

Surface-based methods such as biolayer interferometry (BLI) and surface plasmon resonance (SPR) are widely used for affinity measurements by monitoring dissociation and association events in real time, with affinity (K_D) determined by the ratio of dissociation to association rate constants (k_d/k_a).[2,3] BLI based instruments, such as ForteBio's Octet RED384, allow quick evaluation of binding kinetics in a 96- or 384-well format. Antigen solutions can be re-used multiple times, resulting in a negligible amount of antigen consumption.[4] In addition, epitopic diversity can be assessed by interrogating the simultaneous binding of specific antibody pairs to the same target.[5] In contrast, while advancements in SPR instrumentation allow for high throughput analysis, antigen consumption remains a challenge when limited material is available.[6] For accurate surface-based K_D measurements, it is often recommended to use the lowest practical sensor loading density and multiple concentrations of analyte to minimize mass transport related effects and rebinding.[7-11] For high affinity interactions, even the most optimized conditions may not yield an accurate K_D, due to limitations in instrumentation sensitivity for slow dissociation kinetics. Within these limitations, BLI methodology is useful for ranking the affinity of antibodies down to the single digit nM range. Because of our need to assess hundreds of antibodies per week beyond the practical affinity limitation of BLI-based methods in high throughput mode, we developed a solution-based method for the rapid determination of K_Ds for hundreds of antibodies in the sub-nanomolar affinity range.

The kinetic exclusion assay (KinExA, Sapidyne) provides an assessment of free ligand at equilibrium, rather than measuring real-time association and dissociation rates to determine affinity. In this assay, we prefer to hold the ligand concentration constant while varying the receptor concentration (reverse orientation); following the establishment of an equilibrium, the percent of free ligand is determined to calculate an equilibrium K_D.[12,13] Kinetic exclusion can also directly measure association rate constants using the same principle. The sensitivity of the KinExA instrument allows for accurate and reproducible measurements. Affinities in the several hundred femtomolar range and on-rates as high as 10^7 $s^{-1}M^{-1}$ have been measured,[14,15] but one of the main limitations is that only a handful of K_D measurements can be made per day without accounting for time for samples to come to equilibrium. To overcome this speed limitaton, a magnetic beads-based method compatible with high throughput solution

*Correspondence to: Yingda Xu; Email: yingda.xu@adimab.com
Submitted: 11/16/12; Revised: 11/27/12; Accepted: 11/28/12
http://dx.doi.org/10.4161/mabs.23049

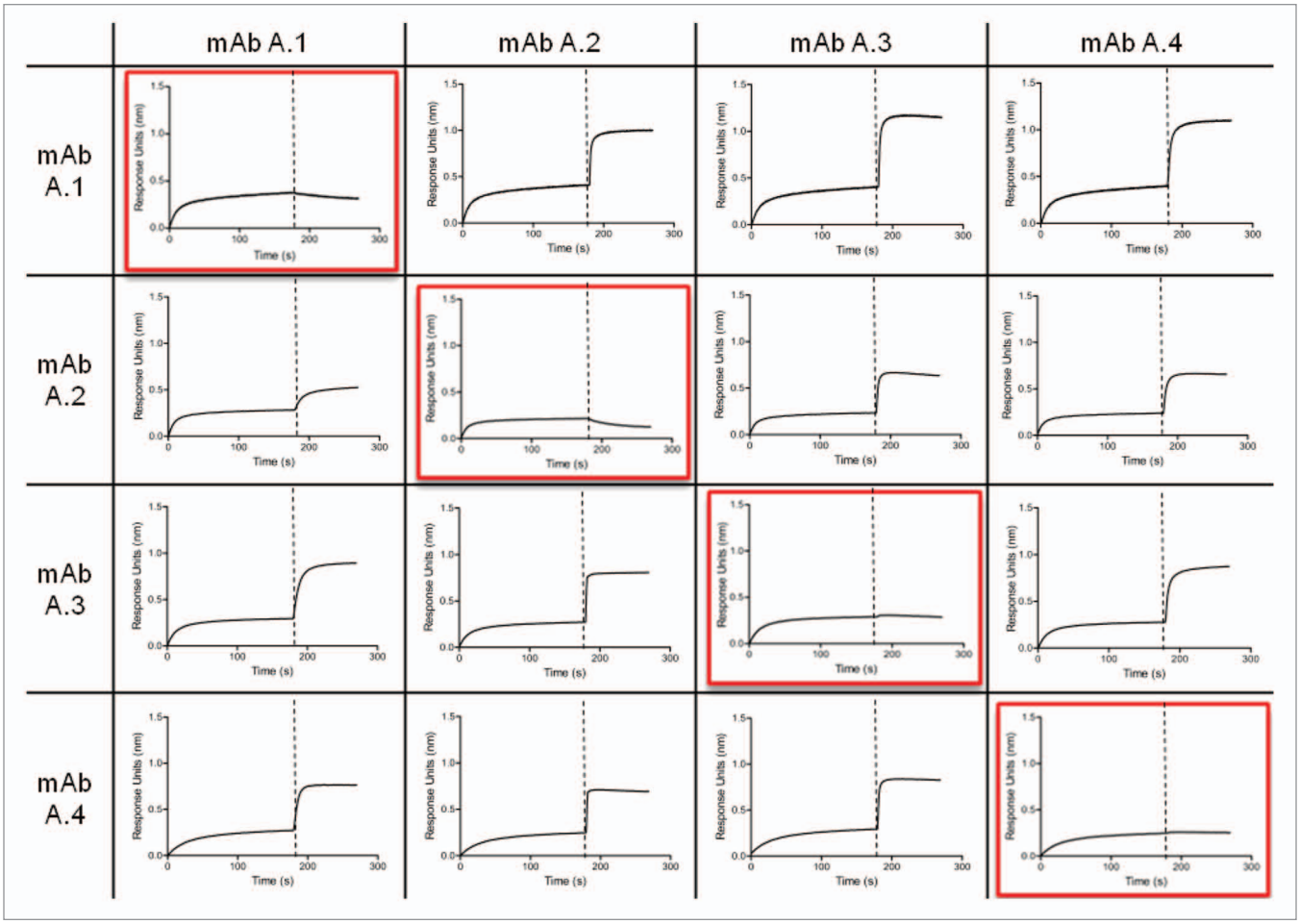

Figure 1. Octet RED384 Binning. Four antibodies were probed for competitive and non-competitive binding against the same antigen. In a typical binning assay, control antibodies were loaded onto AHQ sensors, blocked with a human IgG1 antibody, and the sensors were exposed to Antigen A (step 1) followed by an antibody of interest (step 2). Additional binding of the antibody of interest indicates an unoccupied epitope (non-competitor), while no binding indicates epitope blocking (self-competing antibodies shown by borders around graphs).

phase equilibrium K_D measurement was first reported by Haenel et al.[26] A newer technique employing the kinetic exclusion principle on Gyrolab discs allows up to 20 affinity measurements to be made in duplicate within 4 h.[16]

In this paper, we describe the development of a high throughput solution-equilibrium K_D based method that allows for the accurate determination of affinities into the femtomolar range. Combining solution-based K_D measurements with a BLI based approach for epitope binning allows for rapid identification of therapeutically relevant leads.

Results

Epitope binning by BLI. Octet RED384 offers the ability to quickly evaluate epitope distribution among a panel of discovered antibodies.[5] A bin is related to the epitope of the antigen and can be viewed as an antibody's footprint on an antigen's surface. In a typical epitope binning assay, the control antibody is first loaded to the sensor through its Fc-region, followed by blocking of remaining Fc-binding sites on the sensor. Next, the sensor is exposed to antigen, shown by the response increase in the first binding step of **Figure 1**, followed by exposure to a second antibody. When the antibody on the sensor competes with itself in solution, no additional binding to the antigen is observed. On the other hand, a binding signal is observed when an antibody binds to the antigen in a non-competitive manner with respect to the sensor-loaded antibody. For this 34-kDa protein, four mutually exclusive bins were identified. Furthermore, this assay mapped the epitopic distribution of seven sets of antibodies against their respective antigens and discovered multiple bins for each panel, finding up to seven bins for a 61kDa protein (**Table 1**).

Affinity screening by BLI. To limit antigen consumption, the Octet RED384 was chosen as the primary affinity screening tool for discovered antibodies. **Figure 2A** shows the apparent binding affinities for three samples measured at a single concentration of Fab with maximum antigen loading density: Fab 1 ranks the highest at 2.6 nM and Fab 3 ranks the lowest at 78 nM. Loading density optimization for the immobilized binding partner is necessary, however, to minimize mass transport limitation and rebinding events to obtain a more accurate K_D.[9,10] In **Figure 2B**,

the loading density of a Fc-fusion antigen was varied from 1.0 nm to 0.2 nm and exposed to seven Fab solutions at 50 nM. As the loading density decreased, the fitted k_a increased 30–50%. The fitted k_a further increased when the loading density of the antigen was fixed at 0.2 nm and the Fab concentration was titrated from 50 to 12.5 nM. These two optimized conditions combined to change the measured affinities by ~70%, and global fitting of the data supports that the lowest Fab concentration provided the most accurate k_a. The results of K_D measurements under optimal conditions for 48 antibodies are shown in **Figure 2D**. Even with optimized conditions and a 30 min dissociation step with background subtraction, there is an observed K_D detection limit at ~100 pM. Further optimization of experimental conditions is limited by instrument sensitivity.

K_D and k_a measurements by kinetic exclusion. An orthogonal K_D measurement tool was necessary to evaluate high affinity antibodies that could not be differentiated by Octet measurements. **Table 2** shows a side-by-side comparison for K_D measurements by KinExA 3200 and Octet RED384 for seven antibodies against four different antigens. A very significant K_D difference was observed ranging from 3 to 60-fold between the two measurements. KinExA direct k_a measurements for these interactions revealed much faster association rates compared with corresponding Octet measurements (**Table 2**). A comparison of 80 Octet vs. KinExA K_D measurements is shown in **Figure 3A**. While there is a rough correlation ($R^2 = 0.7$) between Octet and KinExA determined K_D values, the affinities measured by Octet are generally an order of magnitude lower.

MSD affinity measurements. To improve throughput, MSD technology was used to develop a solution equilibrium kinetic exclusion K_D measurement (MSD-SET) (**Fig. 4**). To maintain the accuracy of KinExA's measurements on MSD-SET, we found it necessary to optimize the contact time of the SET samples on the antibody-coated plate. During this step, as free antigen is depleted from solution, equilibrium shifts to favor the dissociation of pre-formed complexes. A longer contact time will thus potentially affect the accuracy of any ELISA based K_D measurement by artificially inflating the K_D value. This is illustrated by **Figure 3B**, where the same set of equilibrated samples was allowed to incubate on a coated MSD plate from 2 min up to 1 h. As the contact time increased, the measured K_D increased 9-fold. This effect would be more dramatic for low affinity antibodies with fast dissociation rates. MSD's high sensitivity, however, allows a distinct advantage over traditional ELISAs in that even very short contact times result in sufficiently strong signals. For most K_D measurements, we find 150 sec provides ample signal without perturbing equilibrium. The contact time can be extended for high affinity interactions where antigen concentration may be low, or conversely the contact time can be decreased for low affinity interactions and high antigen concentrations.

Upon further optimization of coating density, detection reagents and washing buffer composition, we measured three antibodies with affinities ranging from 5 nM to sub-pM to benchmark against KinExA. MSD-SET measurements showed good precision between duplicate measurements with an average of 20% error (**Fig. 3D**), while KinExA measurements had 13% error on average (**Fig. 3C**). Additionally, MSD-SET measurements for these antibodies provided K_D values within 2-fold of respective KinExA values. Further comparing measurements of over one-hundred antibodies against six antigens by both MSD-SET and KinExA showed that all measurements, including lower affinity antibodies (K_D > 10 nM), were within 2-fold.

Using a programmable liquid handler and an automated MSD plate reader, several hundred K_Ds can be measured in a matter of days. **Figure 5B** shows MSD-SET measurements of over a thousand solution-phase K_Ds ranging in affinity from femtomolar to near micromolar against 12 different antigens. By combining the epitope binning data from Octet and the MSD-SET affinity data, a more complete biophysical profile can be established for any given panel of antibodies (**Fig. 5B**, inset).

Table 1. Number of bins discovered per antigen

Antigen A, 59 kDa	4
Antigen B, 61 kDa	7
Antigen C, 51 kDa	4
Antigen D, 32 kDa	2
Antigen F, 4 kDa	3
Antigen G, 60 kDa	4
Antigen H, 18 kDa	4

Seven sets of antibodies were probed for epitopic coverage against their respective antigens of different sizes and types (cell-surface receptors, cytokines, peptides and enzymes).

Discussion

Discovering antibodies that target biologically relevant epitopes, whether they are active sites of enzymes or ligand-activated domains of cell-surface receptors, is critical for therapeutic lead identification. Broad epitopic coverage of a given antigen provides several advantages, including (1) the increased likelihood of identifying biologically relevant binding sites and (2) enabling the discovery of multiple antibodies that may act synergistically to enhance therapeutic efficacy.[17] Panels of lead antibodies that bind to peptides, cytokines, cell-surface receptors and enzymes ranging in MW from 4 kDa to 61 kDa were evaluated for epitopic coverage and affinity. Epitopic coverage was determined using the BLI based method, and the number of cross-blocked epitopes ranged from two to seven (with a mean of four) per target.

BLI methodology is suitable for rapid epitope binning. As shown in **Figure 1**, binning assessment of four different antibodies was performed in a single concurrent assay in a 96-well format. Real-time monitoring of sequential binding of two antibodies (or ligands) to the same target in two orientations provides the basis for asserting that two mutually exclusive bins are occupied.[5] Verifying the absence of non-specific binding of the second antibody to the control antibody or sensor is critical to eliminate false positives. A self-competition assay is routinely included to ensure the validity of the binning data; for example, antibodies that do not compete with themselves can indicate either antigen aggregation or non-specific binding. The Octet RED384 instrument allows direct assignment of bins, even for low affinity

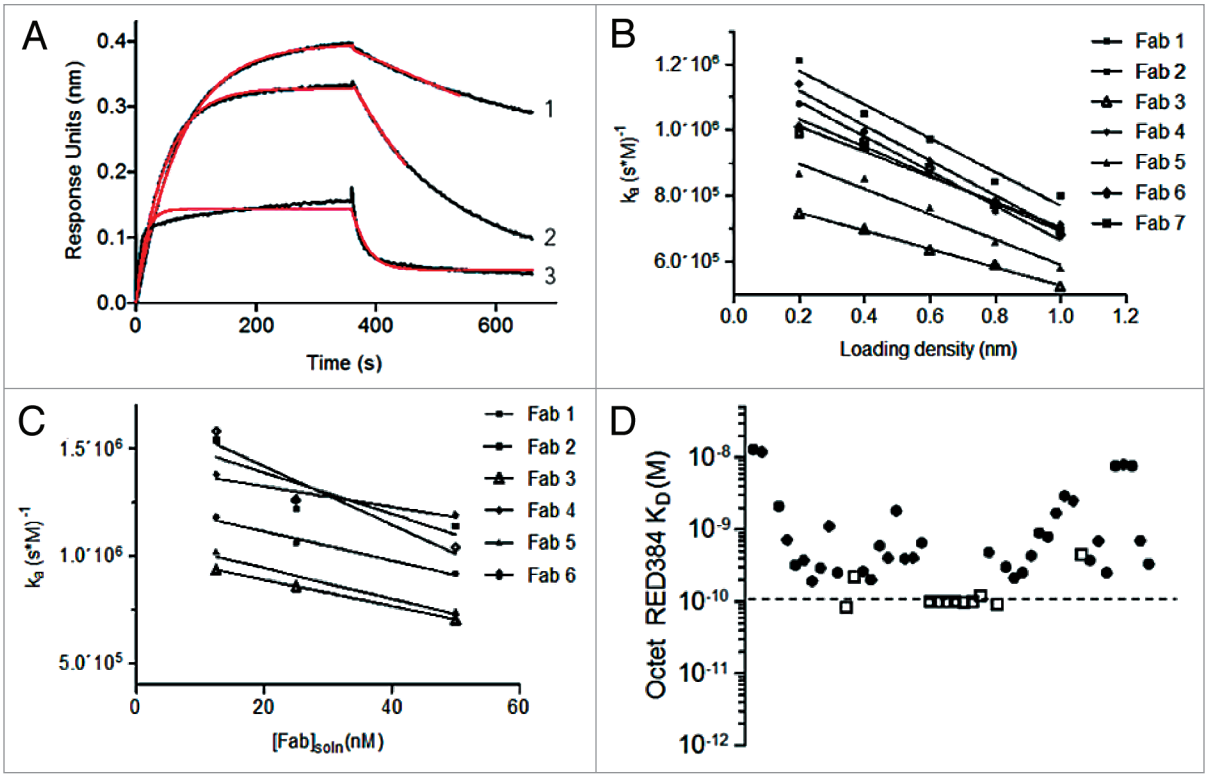

Figure 2. Octet RED384 Kinetic Measurements. (**A**) Octet RED384 K_D profile screening. Antigen A, with a human Fc, was loaded to 0.2 nm on AHQ sensors and exposed to solutions of 200 nM Fab in PBSF. Traces for Fabs 1 and 2 were fit using a truncated dissociation step; trace 3 was fit using the "Partial" fit function in the Octet Data Analysis software v. 7.0. (**B**) Octet RED384 loading density titration. The Fc-antigen was loaded onto AHQ sensors at densities ranging from 1.0 to 0.2 nm and exposed to 50 nM Fab solutions in PBSF. (**C**) Octet RED384 analyte titration. The Fc-antigen was loaded onto AHQ sensors at 0.2 nm and exposed to different concentrations of Fab solutions in PBSF (50–12.5 nM). (**D**) Optimized Octet RED384 K_D measurements of 48 antibodies. 48 antibodies were loaded onto AHQ sensors and then exposed to a solution of 25 nM monomeric antigen. The (❑) symbols indicate that a 5E-05 sec^{-1} limit has been reached for a 30 min dissociation step. The dotted line represents a 100 pM threshold.

clones, whereas these interactions may be missed in a traditional ELISA when only a single endpoint is determined.[5]

In addition to epitopic coverage, affinity is a critical parameter for antibody characterization, but obtaining rapid yet accurate affinity measurements can be quite challenging using existing surface-based technologies.[18] Exhaustive studies on SPR instruments have been performed to investigate the effects of sensor chemistry and other surface-related artifacts.[19-21] Since BLI based technologies rely on a similar principle, one can surmise that measurements based on BLI are subject to similar artifacts. For any surface-based measurement, parameters such as loading density, and analyte concentration must be optimized to allow accurate K_D measurements.[10] For example, as ligand density on the sensor is increased, the calculated association rate decreases, resulting in an underestimation of the affinity as seen in **Figures 2B and C**. This is most likely a mass transport related phenomenon.[18,19] Overloading of ligand on the sensor can also cause artifacts; biphasic binding profiles are often observed when all binding sites are not equally accessible to the analyte.[18,22] Current BLI instrument sensitivity poses a limit on loading densities and K_D determinations even under optimized conditions (**Table 2**). These factors likely contributed to the apparent 100 pM affinity limit shown in **Figure 2D**. To overcome these surface-related artifacts, we investigated several alternative techniques.

An obvious discrepancy emerges when comparing BLI to kinetic exclusion assay K_D measurements (**Fig. 3A**). BLI consistently underestimates the affinity of low-picomolar antibodies, reaching an apparent affinity limit at 100 pM (**Figs. 2D and 3A**). The data in **Table 2** shows that the association rate constants are consistently underestimated. Generally, a greater effect is observed on the k_a for high affinity antibodies. This is likely due to the sensitivity limitation of BLI since a certain amount of sensor-loading is required to observe a measurable signal; however, mass transport-related effects may not be avoidable at this loading density. In addition, high affinity measurements necessitate a low concentration of analyte, which further challenges the instrument's sensitivity limits. In addition to loading density, surface chemistry can further obscure the accurate determination of affinities. For example, mAbC.1 and mAbC.2 show a 26- and 85-fold difference in k_a (Octet vs. KinExA) that is likely caused by adverse interactions between the antigen and the Octet RED384 biosensors. This sensor-related artifact is not unique to the Octet RED384 but has also been reported for SPR based instruments.[19] Taken in concert with the data shown in **Figure 2B and C**, we believe that sensor-related artifacts and mass transport play a significant role in limiting the accuracy of K_D measurements by Octet RED384.

Solution phase kinetic exclusion assay by KinExA is an attractive alternative because it requires minimal antigen amounts if

Table 2. KinExA, Octet RED384 and MSD kinetic parameters

Antibody	KinExA kinetic parameters		Octet RED384 kinetic parameters				MSD-SET K_D	
	k_a (s*M)$^{-1}$ (95% Confidence Interval)	KinExA K_D (M)	k_a (s*M)$^{-1}$	Octet RED384 K_D (M)	k_a Fold difference (KinExA/ Octet RED384)	K_D Fold difference (Octet RED384/ KinExA)	MSD K_D	K_D Fold difference (KinExA/ MSD)
mAb B.1	6.30E06 (*9.7E06–3.9E06*)	1.80E-11	3.28E05 ± 8.31E02	1.88E-10	19	10	1.40E-11	1.3
mAb C.1	9.20E06 (*1.3E07–5.9E06*)	1.10E-11	1.08E05 ± 1.10E02	9.09E-11	85	8	1.20E-11	0.9
mAb C.2	2.00E06 (*2.7E06–1.4E06*)	3.40E-12	7.64E04 ± 4.14E02	2.05E-10	26	60	NA	NA
mAb D.1	6.10E05 (*7.7E05–5.2E05*)	2.10E-09	1.37E05 ± 4.63E02	1.20E-08	4	6	2.06E-09	1.0
mAb D.2	2.30E05 (*2.6E05–2.0E05*)	4.95E-09	6.30E04 ± 5.10E02	2.35E-08	4	5	1.3	1.3
mAb A.1	1.80E06 (*2.3E06–1.4E06*)	1.30E-11	2.33E05 ± 9.51E02	4.3E-11 *	8	3	8.70E-12	1.5

*Indicates samples that have reached a dissociation limit for a 2 h dissociation step. On-rate measurements were made by KinExA and Octet RED384 under optimized conditions. Italicized numbers in parenthesis denote 95% confidence intervals for KinExA on-rate measurements. Octet RED384 K_D measurements were taken from real-time measurement of association and dissociation phases, while KinExA and MSD K_D values were determined using solution equilibrium titrations.

the antigen is held constant and the antibody concentration is varied to obtain solution-based equilibria.[13] Additionally, large sample volumes and fluorescence-based detection allow the use of very low antigen concentrations, which is critical for accurate K_D determination of high affinity antibodies.[14] Solution-based equilibrium titrations also eliminate surface based artifacts, allowing free rotation of molecules in solution and binding kinetics are limited only by intrinsic affinity and the rate of diffusion. In some cases, this may provide a more relevant assessment of the actual biological interaction that is being targeted. Unfortunately, KinExA sacrifices throughput for sensitivity and accuracy, which limits this assay for applications where hundreds of high affinity antibodies have to be assessed on a weekly basis.

To overcome these limitations, we developed an electrochemiluminescence-based method for high throughput solution-phase equilibrium K_D measurements. Traditional sandwich ELISA assays have been used to measure K_Ds for decades, but low sensitivity and a narrow linear range make them unsuitable for screening antibodies with diverse affinities.[23,24] MSD-based ELISAs exhibit high sensitivity and broad linear range. We evaluated its application for quantification of free antigen in a kinetic exclusion assay. In an MSD experiment, the ECL-labeled reagents are activated locally by electric current and the resulting luminescent signal is detected by a CCD camera. The high signal-to-noise ratio and the broad dynamic range make MSD suitable to determine free antigen in a kinetic exclusion assay.[25] **Figure 4** shows the general design of a typical MSD-based solution equilibrium titration (MSD-SET).

In conclusion, we note that BLI is an effective tool for epitope binning and qualitative affinity assessment down to the single digit nM range, but, until improvements in sensitivity can be made to overcome sensor-related artifacts, solution-phase K_D measurements will be preferred for high throughput accurate K_D measurements for these high affinity antibodies. When only a small number of antibodies have to be analyzed, KinExA is a useful instrument. To meet the demand for measuring hundreds of accurate solution-based affinities on a weekly basis, MSD-SET is a suitable alternative. In a given assay, MSD-SET is quite capable of measuring affinities into the femtomolar range. Based on a five-year instrument depreciation model, each sample costs $0.60 on MSD-SET vs. $40 on KinExA. The integration of Octet RED384 binning and MSD-SET affinity measurements provides powerful decision support for the development of therapeutically relevant antibodies.

Materials and Methods

Instrumentation. All biolayer interferometry experiments were performed on a ForteBio Octet RED384 instrument using anti-human Fc capture (AHQ, 18–5005), streptavidin (SA, 18–5021), or anti-murine Fc capture (AMC, 18–5090) sensors. Kinetic exclusion assays were analyzed either by a KinExA 3200 with autosampler or by Meso Scale Discovery Sector Imager 2400 with standard-bind ECL-Multi-Array Plate (L15XA-3).

Reagents. *Octet RED384.* Human antibodies and Fabs were discovered and produced in-house using our proprietary antibody discovery platform. Antigen concentrations were determined using absorbance at 280 nm and the active binding site concentration was calculated from KinExA or MSD-SET experiments.[14] Antibodies were quantified using AHQ sensors, Fab were quantified using streptavidin sensors loaded with biotinylated

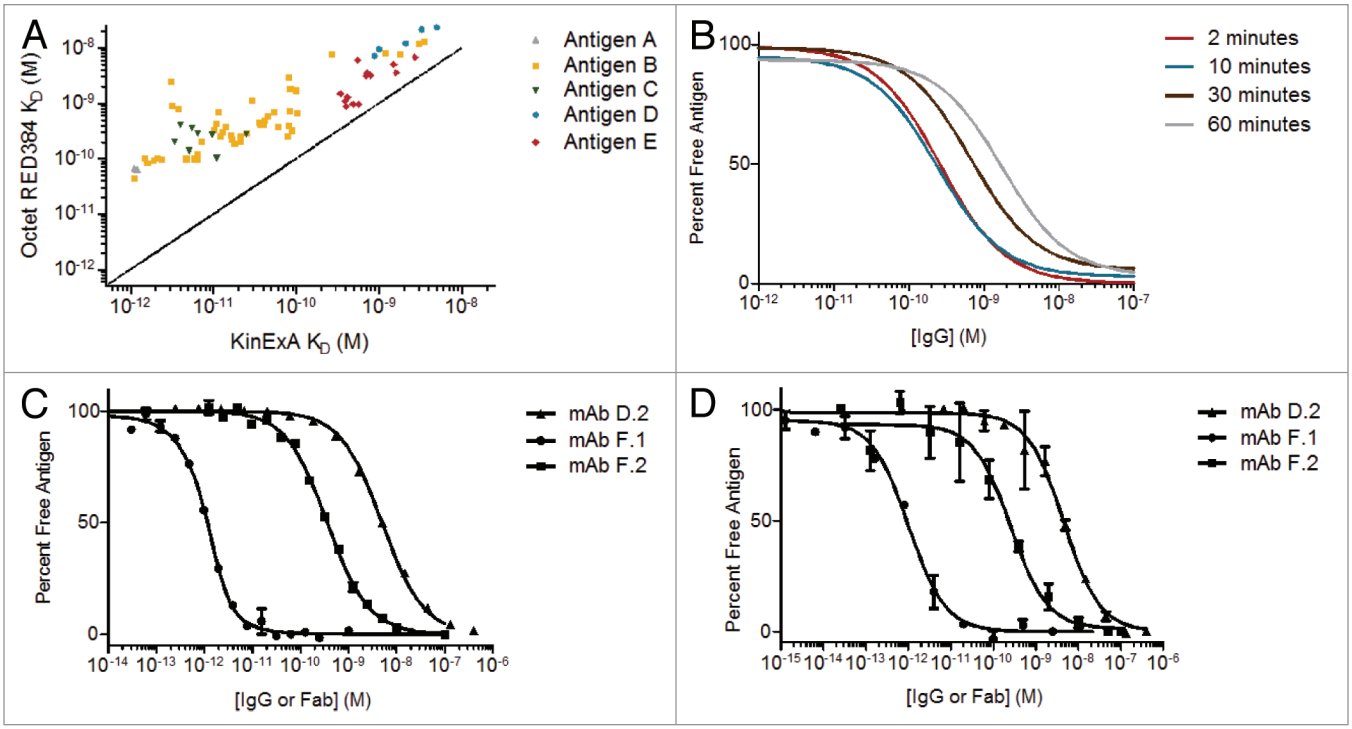

Figure 3. Octet RED384, KinExA and MSD-SET K_D Measurements. (**A**) Octet RED384 K_D values plotted against KinExA K_D values. Octet RED384 experiments were performed using AHQ sensors. KinExA experiments were performed in the reverse orientation, and both KinExA and MSD experiments were designed to obtain K_D-controlled curves. (**B**) MSD-SET K_D measurements with varying contact times. MSD-SET experiments were performed by incubating equilibrated solutions on an antibody-coated MSD plate for 2, 10, 30 and 60 min. As plate contact times increased, the fitted K_D values also increased. Only fits are shown for clarity. (**C**) Reproducibility studies of 3 antibodies by KinExA. Each of the 16 points on the curve represents duplicate measurements from a 3-fold dilution series, with error bars representing standard deviation. The K_Ds of mAb F.1, mAb F.2 and mAb D.2 were 680 fM, 360 pM and 5 nM, respectively. (**D**) Reproducibility studies of 3 antibodies by MSD-SET. MSD-SET experiments were performed as outlined in **Figure 4**. Plate incubation times were fixed at 150 sec to minimize disruption of equilibrium. Each of the 12 points on the curve represents duplicate measurements from a 5-fold dilution series, with error bars representing standard deviation. The K_Ds of mAb F.1, mAb F.2 and mAb D.2 were 1 pM, 260 pM and 4.4 nM, respectively.

anti-human-LC kappa protein (710.0833.100, CaptureSelect). All samples and assays were prepared in an assay buffer of PBS pH 7.3 + 0.1% BSA (PBSF).

KinExA. All experiments used a running buffer composed of PBSF with 0.01% Tween and 0.02% sodium azide. Conjugation of antibody to Azlactone beads (Sapidyne Instruments) was performed as recommended by the manufacturer. For detection of native antigen, control antibodies labeled with Dylight-633-NHS Ester dye (46417, ThermoPierce) were used. For detection of biotinylated antigen, Streptavidin-633 (S21375, AlexaFluor) at 250 ng/mL was used.

MSD-SET. 20 nM of antibody in PBS buffer was coated onto standard bind plates (L15XA-3). The plates were blocked with Blocker A (R93AA-1) and PBSF containing 0.05% Tween was used for plate washing. Streptavidin (R32AD-1) or control antibody labeled with sulfotag (R91AN-1) was used for detection of biotinylated or native antigen. Read buffer T with surfactant (R92TC-1) was used during plate reading.

Octet RED384 epitope binning. In a typical epitope binning assay, control antibodies were loaded onto AHQ sensors and remaining Fc-binding sites on the sensor were blocked with a human IgG1 antibody. The sensors were then exposed to the second antibody to check for non-specific binding to the sensor. Finally, the sensors were exposed to antigen followed by the second antibody. Raw data was processed using ForteBio's Data Analysis Software 7.0 and the antibody pairs were assessed for competitive binding. Additional binding by the second antibody indicates an unoccupied epitope (non-competitor), while no binding indicates epitope blocking (competitor).

Octet RED384 Surface based K_D measurements. For high throughput K_D screening, ligand (antibody or antigen) was loaded to the sensor with maximum loading. Following a short baseline in PBSF, the sensors were exposed to analyte at a single concentration in PBSF for an association step. Dissociation was monitored in PBSF. For optimized K_D measurements, the ligand was loaded onto AHQ, AHC, or SA sensors using a pre-determined loading threshold. The loaded sensors were exposed to a series of analyte concentrations (12.5–50 nM) and background subtraction was used to correct for sensor drifting. All experiments were performed with shaking at 1,000 rpm. ForteBio's data analysis software was used to fit the data to a 1:1 binding model to extract an association rate and dissociation rate. The K_D was calculated using the ratio k_d/k_a.

KinExA K_D and k_a measurements. Antibodies were coupled to Azlactone beads to perform the reverse orientation of the equilibrium K_D assay, described in detail by Rathanaswami et al.[12]

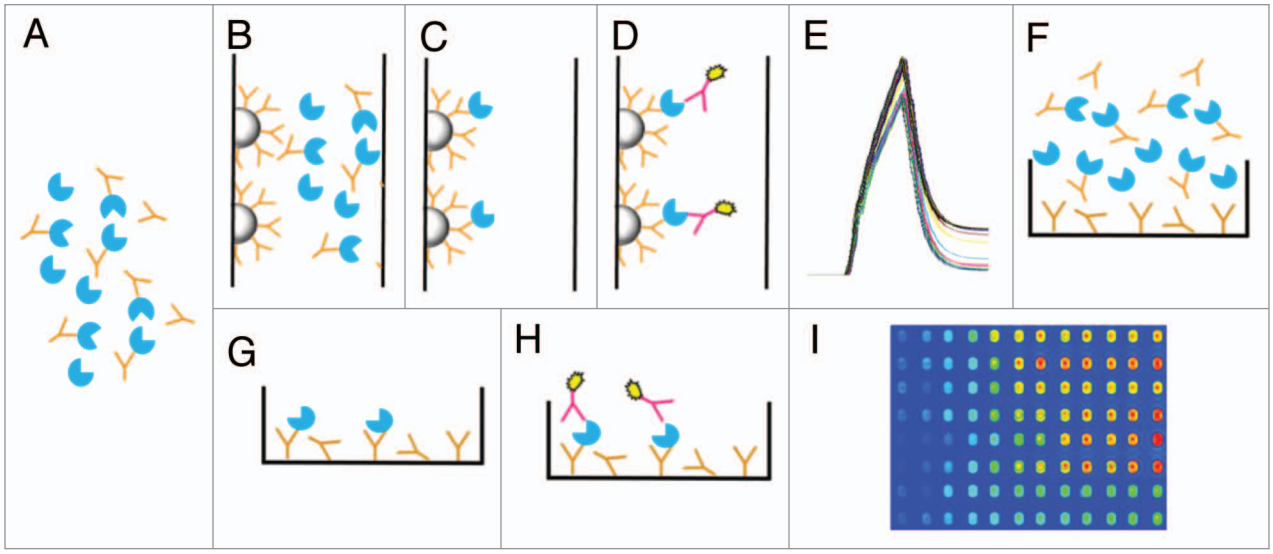

Figure 4. Solution Equilibrium Titrations (SET), KinExA and MSD experimental design. (**A**) SET. A solution equilibrium titration (SET) is prepared with antigen (blue) held constant and antibody (orange) titrated. The solution is allowed to come to equilibrium. (**B**) KinExA. A column is packed with antibody-conjugated beads. The SET sample is passed over the column at a fixed flow rate (typically 0.25 mL/minute). (**C**) Only free antigen is captured on the antibody-conjugated beads. (**D**) The captured antigen is detected with a fluorophore-labeled antibody (pink) that binds in a non-competitive manner on the antigen. (**E**) The fluorophore's signal results in the trace shown, where the end points of a raw signal trace can be used to plot signal as a function of antibody concentration and fit to a quadratic equation to extract a K_D. (**F**) MSD. Antibody is coated to a standard-bind MSD plate. The SET sample is added by a liquid handling robot to the antibody-coated plate. (**G**) After 150 sec, free antigen is captured on the plate, while the remaining SET solution is washed off the plate. (**H**) The captured antigen is then detected by a sulfotag-conjugated antibody that binds in a non-competitive manner on the antigen. (**I**) The sulfotag is electrochemically activated to provide signal that is measured by a CCD camera. The raw signal can then be plotted as a function of antibody concentration and fit to a quadratic equation to extract a K_D.

Briefly, the antigen concentration was held constant as the antibody or Fab was titrated in a 2 or 3-fold serial dilution. Solutions were allowed to come to equilibrium at room temperature for a minimum of 24 h and injected via KinExA's autosampler over a column packed with antibody-conjugated azlactone beads. The antigen captured on the beads was detected by a fluorescently-labeled antibody or streptavidin. Percent free antigen was plotted as a function of titrated antibody or Fab and fit to a quadratic equation. In a K_D- and antigen-controlled curve, where the antigen concentration is within 10-fold of the K_D, both K_D and active binding site concentration of the antigen can be determined. Ninety-five percent confidence intervals refer to results obtained from duplicate titration curves. Direct k_a experiments were performed based upon the equilibrium K_D measurements in that antibody concentrations where the antigen was 20–50% free were targeted for the experiment. The antigen and antibody were mixed and solution withdrawn at regular time intervals to quantify the amount of remaining free antigen. Percent free antigen was plotted as a function of time and fit directly using KinExA's data fitting software to extract the k_a.

MSD-SET K_D measurements. Solution equilibrium titrations were performed similar to KinExA, except sample volumes were between 100 and 200 μL and 3-to 5-fold serial dilutions were used. Antibodies (20 nM in PBS) were coated onto standard bind MSD-ECL plates overnight at 4°C or at room temperature for 30 min. Plates were then blocked with Blocker A for 30 min with shaking at 700 rpm, followed by three washes with wash buffer. Samples were applied and incubated on the plates for 150 s with shaking at 700 rpm followed by one wash. Antigen captured on a plate was detected with a sulfotag-labeled antibody or streptavidin by incubation on the plate for 3 min. The plates were washed three times with wash buffer and then read on the MSD Sector Imager 2400 instrument using 1x Read Buffer T with surfactant. The percent free antigen was plotted as a function of titrated antibody in Prism and fit to a quadratic equation. To improve throughput, liquid handling robots were used throughout MSD-SET experiments, including SET sample preparation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

All the antibodies characterized in this paper were discovered and produced by antibody discovery and high throughput expression departments at Adimab, LLC. We appreciate the critical advice and insight provided by Kristin Rookey, Dane Wittrup and Tillman Gerngross. We are also grateful for the instrument training and technical support provided by Wendy VanScyoc from Meso Scale Discovery.

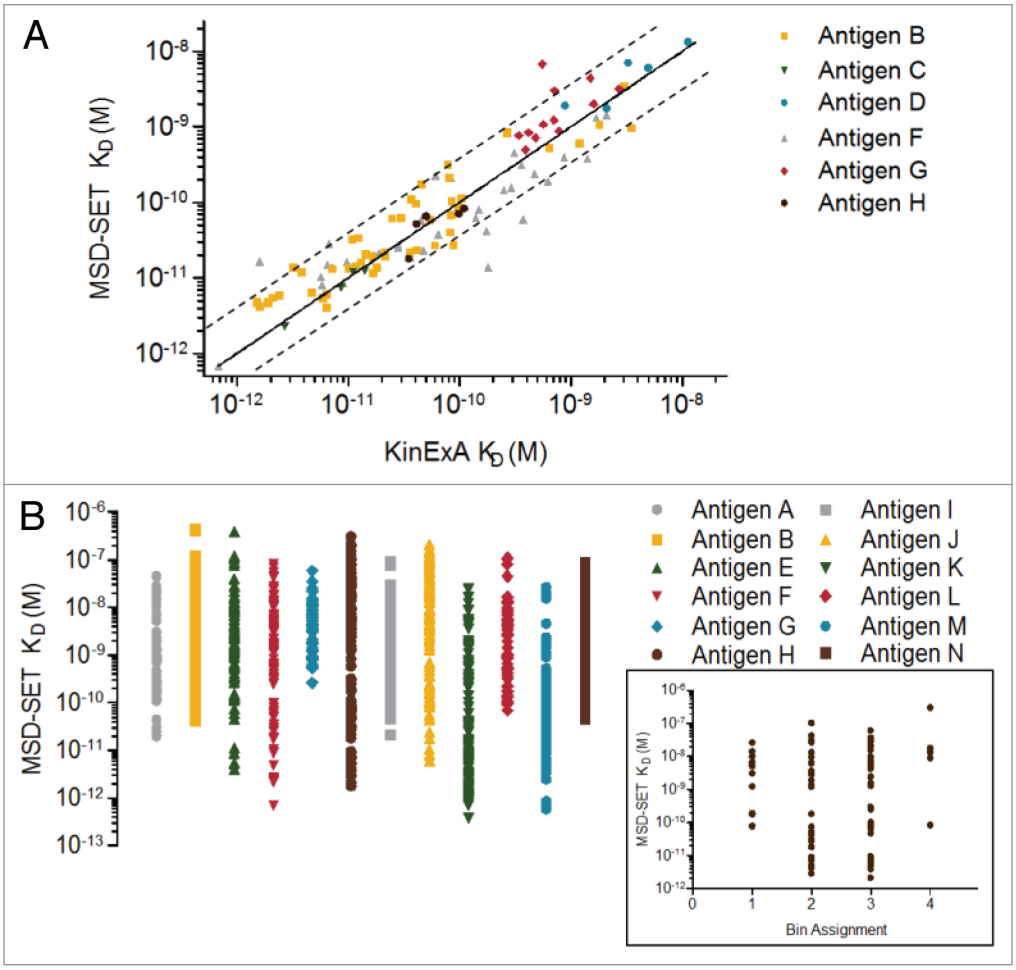

Figure 5. MSD-SET K_D measurements. (**A**) KinExA K_D values plotted against MSD-SET K_D values. KinExA and MSD experiments were performed to provide K_D-controlled curves. (**B**) MSD-SET K_D of 12 different panels of antibodies. MSD-SET was performed to provide K_D-controlled curves. Antigens used included cytokines, cell-surface receptors, enzymes and peptides. The inset shows affinities of antibodies binding to Antigen H across four separate bins.

References

1. Jostock T, Vanhove M, Brepoels E, Van Gool R, Daukandt M, Wehnert A, et al. Rapid generation of functional human IgG antibodies derived from Fab-on-phage display libraries. J Immunol Methods 2004; 289:65-80; PMID:15251413; http://dx.doi.org/10.1016/j.jim.2004.03.014
2. Rich RL, Cannon MJ, Jenkins J, Pandian P, Sundaram S, Magyar R, et al. Extracting kinetic rate constants from surface plasmon resonance array systems. Anal Biochem 2008; 373:112-20; PMID:17889820; http://dx.doi.org/10.1016/j.ab.2007.08.017
3. Bravman T, Bronner V, Nahshol O, Schreiber G. The ProteOn XPR36TM Array System—High Throughput Kinetic Binding Analysis of Biomolecular Interactions. Cellular and Molecular Bioengineering 2008; 1:216-28; http://dx.doi.org/10.1007/s12195-008-0036-4
4. Concepcion J, Witte K, Wartchow C, Choo S, Yao D, Persson H, et al. Label-free detection of biomolecular interactions using BioLayer interferometry for kinetic characterization. Comb Chem High Throughput Screen 2009; 12:791-800; PMID:19758119; http://dx.doi.org/10.2174/138620709789104915
5. Abdiche YN, Malashock DS, Pinkerton A, Pons J. Exploring blocking assays using Octet, ProteOn, and Biacore biosensors. Anal Biochem 2009; 386:172-80; PMID:19111520; http://dx.doi.org/10.1016/j.ab.2008.11.038
6. Abdiche YN, Lindquist KC, Pinkerton A, Pons J, Rajpal A. Expanding the ProteOn XPR36 biosensor into a 36-ligand array expedites protein interaction analysis. Anal Biochem 2011; 411:139-51; PMID:21168382; http://dx.doi.org/10.1016/j.ab.2010.12.020
7. Myszka DG, He X, Dembo M, Morton TA, Goldstein B. Extending the range of rate constants available from BIACORE: interpreting mass transport-influenced binding data. Biophys J 1998; 75:583-94; PMID:9675161; http://dx.doi.org/10.1016/S0006-3495(98)77549-6
8. Rich RL, Myszka DG. Survey of the 2009 commercial optical biosensor literature. J Mol Recognit 2011; 24:892-914; PMID:22038797; http://dx.doi.org/10.1002/jmr.1138
9. Figuera-Losada M, LoGrasso PV. Enzyme kinetics and interaction studies for human JNK1β1 and substrates activating transcription factor 2 (ATF2) and c-Jun N-terminal kinase (c-Jun). J Biol Chem 2012; 287:13291-302; PMID:22351776; http://dx.doi.org/10.1074/jbc.M111.323766
10. Karlsson R, Katsamba PS, Nordin H, Pol E, Myszka DG. Analyzing a kinetic titration series using affinity biosensors. Anal Biochem 2006; 349:136-47; PMID:16337141; http://dx.doi.org/10.1016/j.ab.2005.09.034
11. Abdiche Y, Malashock D, Pinkerton A, Pons J. Determining kinetics and affinities of protein interactions using a parallel real-time label-free biosensor, the Octet. Anal Biochem 2008; 377:209-17; PMID:18405656; http://dx.doi.org/10.1016/j.ab.2008.03.035
12. Darling RJ, Brault P-A. Kinetic exclusion assay technology: characterization of molecular interactions. Assay Drug Dev Technol 2004; 2:647-57; PMID:15674023; http://dx.doi.org/10.1089/adt.2004.2.647
13. Rathanaswami P, Richmond K, Manchulenko K, Foltz IN. Kinetic analysis of unpurified native antigens available in very low quantities and concentrations. Anal Biochem 2011; 414:7-13; PMID:21371417; http://dx.doi.org/10.1016/j.ab.2011.02.034
14. Bee C, Abdiche YN, Stone DM, Collier S, Lindquist KC, Pinkerton AC, et al. Exploring the dynamic range of the kinetic exclusion assay in characterizing antigen-antibody interactions. PLoS One 2012; 7:e36261; PMID:22558410; http://dx.doi.org/10.1371/journal.pone.0036261
15. Canziani GA, Melero JA, Lacy ER. Characterization of neutralizing affinity-matured human respiratory syncytial virus F binding antibodies in the sub-picomolar affinity range. J Mol Recognit 2012; 25:136-46; PMID:22407977; http://dx.doi.org/10.1002/jmr.2149

16. Salimi-Moosavi H, Rathanaswami P, Rajendran S, Toupikov M, Hill J. Rapid affinity measurement of protein-protein interactions in a microfluidic platform. Anal Biochem 2012; 426:134-41; PMID:22542978; http://dx.doi.org/10.1016/j.ab.2012.04.023
17. Nowakowski A, Wang C, Powers DB, Amersdorfer P, Smith TJ, Montgomery VA, et al. Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. Proc Natl Acad Sci USA 2002; 99:11346-50; PMID:12177434; http://dx.doi.org/10.1073/pnas.172229899
18. Svitel J, Boukari H, Van Ryk D, Willson RC, Schuck P. Probing the functional heterogeneity of surface binding sites by analysis of experimental binding traces and the effect of mass transport limitation. Biophys J 2007; 92:1742-58; PMID:17158569; http://dx.doi.org/10.1529/biophysj.106.094615
19. Drake AW, Tang ML, Papalia GA, Landes G, Haak-Frendscho M, Klakamp SL. Biacore surface matrix effects on the binding kinetics and affinity of an antigen/antibody complex. Anal Biochem 2012; 429:58-69; PMID:22766435; http://dx.doi.org/10.1016/j.ab.2012.06.024
20. Abdiche YN, Malashock DS, Pons J. Probing the binding mechanism and affinity of tanezumab, a recombinant humanized anti-NGF monoclonal antibody, using a repertoire of biosensors. Protein Sci 2008; 17:1326-35; PMID:18505735; http://dx.doi.org/10.1110/ps.035402.108
21. Myszka DG, Morton TA, Doyle ML, Chaiken IM. Kinetic analysis of a protein antigen-antibody interaction limited by mass transport on an optical biosensor. Biophys Chem 1997; 64:127-37; PMID:9127943; http://dx.doi.org/10.1016/S0301-4622(96)02230-2
22. Svitel J, Balbo A, Mariuzza RA, Gonzales NR, Schuck P. Combined affinity and rate constant distributions of ligand populations from experimental surface binding kinetics and equilibria. Biophys J 2003; 84:4062-77; PMID:12770910; http://dx.doi.org/10.1016/S0006-3495(03)75132-7
23. Hardy F, Djavadi-Ohaniance L, Goldberg ME. Measurement of antibody/antigen association rate constants in solution by a method based on the enzyme-linked immunosorbent assay. J Immunol Methods 1997; 200:155-9; PMID:9005954; http://dx.doi.org/10.1016/S0022-1759(96)00201-3
24. Nielsen UB, Geierstanger BH. Multiplexed sandwich assays in microarray format. J Immunol Methods 2004; 290:107-20; PMID:15261575; http://dx.doi.org/10.1016/j.jim.2004.04.012
25. Marchese RD, Puchalski D, Miller P, Antonello J, Hammond O, Green T, et al. Optimization and validation of a multiplex, electrochemiluminescence-based detection assay for the quantitation of immunoglobulin G serotype-specific antipneumococcal antibodies in human serum. Clin Vaccine Immunol 2009; 16:387-96; PMID:19158284; http://dx.doi.org/10.1128/CVI.00415-08
26. Haenel C, Satzger M, Ducata DD, Ostendorp R, Brocks B. Characterization of high-affinity antibodies by electrochemiluminescence-based equilibrium titration. Anal Biochem 2005; 339:182-4; PMID:15766727; http://dx.doi.org/10.1016/j.ab.2004.12.032