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Exploring blocking assays using Octet, ProteOn, and Biacore biosensors

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ABSTRACT

We demonstrate the use of label-free real-time optical biosensors in competitive binding assays by epitope binning a panel of antibodies. We describe three assay orientations that we term *in tandem, premix,* and *classical sandwich* blocking, and we perform each of them on three platforms: ForteBio's Octet QK, Bio-Rad's ProteOn XPR36, and GE Healthcare's Biacore 3000. By testing whether antibodies block one another's binding to their antigen in a pairwise fashion, we establish a blocking profile for each antibody relative to the others in the panel. The blocking information is then used to create "bins" of antibodies with similar epitopes. The advantages and disadvantages of each biosensor, factors to consider when deciding on the most appropriate blocking assay orientation for a particular interaction system, and tips for dealing with ambiguous data are discussed. The data from our different assay orientations and biosensors agree very well, establishing these machines as valuable tools for characterizing antibody epitopes and multiprotein complexes of biological significance.

Label-free real-time biosensors, such as those commercialized by Biacore (GE Healthcare), typically employ optical phenomena to detect the association and dissociation of an interacting pair of proteins or other biomolecules, one of which is attached to the sensor. In an effort to meet the high-throughput demands of drug discovery, biosensors are multiplexing. Many commercial platforms have emerged that can handle large numbers of interactions simultaneously in an automated mode by redesigning the concept of a flow channel and the way in which samples are addressed and/or delivered [1]. This evolution inspired us to compare the performance of two relatively new parallel-processing biosensors, ForteBio's Octet QK and Bio-Rad's ProteOn XPR36 array system, with that of a traditional serial flow Biacore 3000 platform.

The Octet uses disposable fiber-optic sensors that detect biomolecular interactions via biolayer interferometry, whereas the ProteOn and Biacore are surface plasmon resonance (SPR)[1]-based detectors. The Octet is a nonflow dip-and-read system that addresses eight interactions at a time by immersing a column of ligand-coated sensor tips into the analyte-containing wells of a microplate. In contrast, the ProteOn and Biacore platforms use sophisticated microfluidics to flow analyte over ligand-coated sensors. The ProteOn creates a six-by-six crisscrossing interaction array via six parallel injections over a sensor chip that swivels 90° from the ligand to the analyte direction. Thus, six analytes flow over six strips where spots that contain ligand (reaction spots) alternate with those that do not (interspots) for a total of 36 interaction surfaces and 42 reference surfaces. The Biacore 3000 injects a single analyte over up to four serially addressed flow cells, which can be either coated with ligand or used as a reference surface.

We recently compared the above-named platforms head to head in determining the kinetic rate and affinity constants of antigen/antibody interactions [2]. In the current article, we focus on competitive binding (or "blocking") assays because the use of biosensors in this context is reported less frequently [3] despite it being of utmost importance in drug discovery. Blocking assays can reveal whether one molecule's binding a second molecule prevents the binding of a third molecule. Many drugs are designed to interfere with a biological interaction, such as that between a ligand and its receptor, as exemplified by the anticancer drugs bevacizumab [4] and tamoxifen [5] that target a natural ligand and a receptor partner, respectively.

During the past decade, there has been a trend toward using monoclonal antibodies (mAbs) as drugs to treat diverse diseases [6,7]. The quest for a mAb that targets a specific region (or "epitope") on an antigen (Ag) is often more important than the identification of a tight-binding mAb because affinity can be matured via standard protein engineering protocols [8]. Thus, epitope binning an array of mAbs is a practical application of blocking assays in a diagnostic or research setting for several reasons. First, binning a set of new mAbs against a previously characterized mAb can identify mAbs that bind similar epitopes and may share functional characteristics [9,10]. A mAb with a desirable function but undesir-

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[1] *Abbreviations used:* SPR, surface plasmon resonance; mAb, monoclonal antibody; Ag, antigen; ELISA, enzyme-linked immunosorbent assay; AR, amine-reactive; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SNHS, sulfo-*N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; IgG, immunoglobulin G; PBS, phosphate buffered saline; BSA, bovine serum albumin.

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able cross-reactivity, for example, can be used to discover a more ideal mAb. Second, binning can discriminate mAbs likely to exhibit distinct functional characteristics [11]. Once a set of mAbs has been epitope binned, a representative mAb from each bin can be tested in a low-throughput functional assay. This method of selection has a better chance of converging on a mAb with the desired biological activity than does choosing a set of mAbs based on their apparent affinity. Third, identifying pairs of mAbs that can bind Ag simultaneously can be useful in designing reagents for other assays that rely on sandwiching interactions [12]. Fourth, identifying multiple epitopes broadens the scope of a patent [13].

Using a biosensor to investigate blocking offers several advantages over other methods such as competitive enzyme-linked immunosorbent assay (ELISA), which requires labels and provides only an end-point analysis. Indeed, epitope binning via ELISA is often hindered by the inability to find a secondary reagent that can detect one mAb selectively over another when mAbs are competed against one another in pairs. Although biotinylating or reformatting a mAb may distinguish it from other mAbs in a test panel, this can make sample preparation tedious and unsuitable for performing high-throughput screening in a combinatorial-type format. Furthermore, ELISAs may be unfeasible if the plated reagent is precious and/or expensive. In contrast, biosensors reveal the entire binding profile between an interacting pair of molecules without labels, and the ligand-coated surfaces and/or solution binding partners can often be reused.

We draw on two biological interaction systems to explore biosensor blocking using three different assay orientations that we term *in tandem, premix,* and the *classical sandwich*. Fig. 1 illustrates how they can be used in the context of epitope binning. Because biosensors are essentially mass-based detectors, determining whether two mAbs block one another's Ag-binding activity is simply a yes/no readout regardless of the assay orientation used. Under conditions that favor complete blocking, no binding response will be detected at the sensor for any of the arrowed interactions illustrated in Fig. 1 if the two mAbs have overlapping epitopes. In contrast, a binding signal at each of these interactions identifies two mAbs that can bind Ag simultaneously at distinct nonoverlapping epitopes. Using this yes/no detection of the arrowed interactions, we assign mAbs to epitope bins according to their blocking profiles relative to one another. mAbs belong to the same bin if they satisfy two criteria. First, members of the same bin must block one another's ability to bind Ag. Second, they must exhibit a similar blocking profile to one another when each is paired against the other mAbs in the panel. Thus, the observation that two mAbs block one another is necessary, but not sufficient, to conclude that they belong in the same bin. This article deals with the specifics of each assay orientation in turn and discusses the strengths and weaknesses of each biosensor platform with an emphasis on throughput.

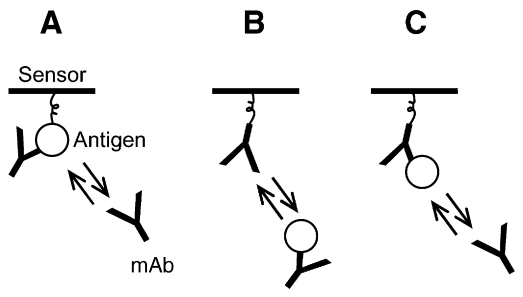

Fig. 1. Outline of three biosensor-based assay orientations that can be used to explore blocking, as applied to epitope binning mAbs in a pairwise manner: (A) in tandem blocking; (B) premix blocking; and (C) classical sandwich.

Materials and methods

Materials

Octet QK equipped with amine-reactive (AR) biosensor tips and coupling buffer, 100 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes, pH 5.0), was purchased from ForteBio (Menlo Park, CA, USA). ProteOn XPR36 equipped with GLC sensor chips and coupling reagents (10 mM sodium acetate [pH 4.5], sulfo-*N*-hydroxysuccinimide [SNHS], 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride [EDC], and 1 M ethanolamine–HCl [pH 8.5]) was purchased from Bio-Rad (Hercules, CA, USA). Biacore 3000 equipped with CM5 sensor chips and coupling reagents (10 mM sodium acetate [pH 5.0], EDC, *N*-hydroxysuccinimide [NHS], and ethanolamine) was purchased from GE Healthcare (Piscataway, NJ, USA). Ags 1 and 2 were human recombinant proteins purchased from R&D Systems (Minneapolis, MN, USA); Ag 1 was fused to a human Fc1 partner thus giving a dimer with a total molecular mass of approximately 100 kDa, whereas Ag 2 was a 30-kDa monomer. Eighteen murine mAbs were generated in-house using hybridoma technology (full-length immunoglobulin G [IgG] nos. 1–7 against Ag 1 and nos. 8–18 against Ag 2) and purified from ascites fluid using protein A. Polyclonal goat $F(ab')_2$ fragment against human IgG Fc (product no. 55053) was purchased from Cappel (MP Biomedicals, Solon, OH, USA) and used as a capture reagent. Immunopure Gentle IgG Elution Buffer (product no. 1851520) was purchased from Pierce (Rockford, IL, USA) and diluted 2:1 (v/v) with 4 M NaCl to provide a universal regeneration cocktail. All other reagents were purchased commercially and were of the highest grade available. All binding assays were performed at 25 °C.

General Octet assays

Sample plates were agitated at 1000 rpm. Immediately prior to analysis, AR sensors were prewet for 5 min in 0.1 M Mes (pH 5.0), which served as the running buffer for immobilizing ligands via a standard EDC/NHS-mediated chemistry. This involved activating a column of tips with a freshly mixed solution of 200 mM EDC in 50 mM NHS, coupling ligands at 10 to 100 μg/ml, and blocking excess reactive groups with 1 M ethanolamine, allowing 5 min for each step. We define the observed immobilization level as the increase in shift recorded at the end of the ethanolamine wash relative to the activated surface. Ligand-coated sensors were then immersed (150 s) in phosphate-buffered saline (PBS) + 5 mg/ml bovine serum albumin (BSA), which served as the running buffer for all binding assays. This buffer was used as a wash step (typically 2.5 min) after each binding and/or regeneration step.

Octet in tandem blocking assay

Ag 1 was amine coupled onto a single column of tips to a final mean level of 1.26 nm along the column with a standard deviation (0.05 nm) within the noise of the instrument (0.1 nm). Ag-coated tips were each dipped into a different "saturating" IgG (nos. 1–7, each at 300 nM, 15 min), using a buffer blank in the eighth well of the column, and then moved into a column of wells containing a fixed "competing" IgG (100 nM, 15 min). Ag-coated tips were regenerated for 50 s. In this way, the same array of saturating IgG was retested against five different competing IgGs (nos. 2–6), allowing each competing IgG its own column in the plate.

Octet premix blocking assay

Three IgGs (nos. 2, 3, and 5) were coupled onto their own columns of tips to mean levels of 2.93, 2.56, and 2.18 nm along a col-

umn, respectively, with a standard deviation within instrument noise. Ag 1 (final 30 nM) was premixed separately with seven different IgGs (nos. 1–7, each at final 300 nM) or buffer (the "Ag-only" control) and bound for 15 min. The same set of premixed reagents was tested over each column of IgG-coated tips, which were discarded after a single use.

Octet classic sandwich assay

Eleven different IgGs (nos. 8–18) were coupled onto their own columns of single-use tips, which were then immersed into Ag 2 (100 nM, 10 min) followed by an array of IgGs (each at 100 nM, 10 min). The same aliquots of reagents were reused to analyze all of the IgG-coated tips.

General ProteOn assays

ProteOn assays were performed in PBS + 0.005% (v/v) polysorbate 20 running buffer flowed at 30 μl/min except where noted otherwise. IgGs were coupled onto chips in the ligand direction in three steps. First, six flow cells were activated in parallel for 3 min with a freshly mixed solution of 200 mM EDC in 50 mM SNHS that was typically diluted 10-fold in water. Second, IgGs were coupled at a single concentration within the range of 45 to 80 μg/ml in 10 mM sodium acetate (pH 4.5) for varying contact times onto separate flow cells until the desired levels were reached. Finally, excess reactive groups on all six channels were blocked with ethanolamine for 3 min.

ProteOn in tandem blocking assay

A polyclonal anti-human IgG Fc antibody was coupled onto all channels of a chip to uniformly immobilize approximately 1820 RU with less than 1% variation along a strip. Ag 1 (100 nM) was then captured via its human IgG Fc fusion partner in the ligand direction for 2 min to saturating levels of approximately 200 RU. Six saturating IgGs (nos. 1–6, each at 200 nM) were injected for 200 s in the ligand direction and then again as competing IgGs in the analyte direction to create a six-by-six crisscrossing matrix. The Ag surfaces were regenerated with three 18-s pulses at 100 μl/min to allow another round of IgGs to be tested in tandem (nos. 1–5 and 7).

ProteOn premix blocking assay

Six different IgGs (nos. 1–6) were coupled onto separate flow channels of a chip to final mean levels of 703 to 15,567 RU with less than 2% variation within a strip. Ag 1 (final 30 nM) was premixed with one of five different IgGs (nos. 2–6, each at final 300 nM) or buffer (the Ag-only control) and injected for 3 min in the analyte direction. No regeneration was attempted. The entire analysis was duplicated on another chip.

ProteOn classical sandwich assay

Six different IgGs (nos. 8, 10, 11, 12, 17, and 18) were immobilized onto separate channels of a chip to final mean levels of 6610 to 12,320 RU with less than 3% variation along a strip. The running buffer was then switched to PBS + 5 mg/ml BSA. The chip was rotated to the analyte direction and 100 nM Ag 2 was injected for 2 min, followed by a 4-min injection of the same set of IgGs (each at 200 nM) that were immobilized on the chip.

General Biacore assays

All assays were performed at 5 μl/min using PBS as the running buffer for amine coupling and PBS + 5 mg/ml BSA for all other steps. Chips were preconditioned at 100 μl/min using multiple 30-s pulses of 75 mM phosphoric acid, a 1:1:1 (v/v/v) mixture of Pierce Gentle IgG Elution Buffer/4 M NaCl/150 mM phosphoric acid, and 50 mM NaOH. The flow rate was returned to 5 μl/min for the immobilization, which was performed in three steps as follows. First, individual flow cells were activated for 7 min using a freshly mixed solution of 200 mM EDC in 50 mM NHS. Second, ligands were diluted to a single concentration, typically 25 to 40 μg/ml in sodium acetate (pH 5.0), and injected for various contact times on separate flow cells until the desired immobilization levels were reached. Third, excess reactive esters were blocked with a 7-min pulse of 1 M ethanolamine. One flow cell per chip was left unmodified to provide a reference surface. After every binding cycle, the chip was regenerated at 10 μl/min with two 30-s pulses unless noted otherwise.

Biacore in tandem blocking assay

Ag 1 was immobilized at three different levels (520, 1730, and 4200 RU). Then 300 nM saturating mAb was injected for 4 min and followed by an identical injection of 300 nM competing mAb. The Ag-coated chip was then regenerated. This process was repeated for all 49 pairwise permutations of mAbs 1 to 7 using additional control cycles in which buffer injections replaced the saturating and/or competing mAbs.

Biacore premix blocking assay

IgGs 2, 3, and 5 were amine coupled to a chip at similar levels (6300, 6200, and 6400 RU, respectively). Then 50 nM Ag 1 was mixed with 250 nM of each of seven IgGs (nos. 1–7) in separate vials and allowed to incubate for 10 min at room temperature. These mixtures, as well as replicate aliquots of 50 nM Ag 1 alone, were injected for 3 min with regeneration after each injection.

Biacore classical sandwich assay

IgGs 11, 12, and 17 were amine coupled to a chip at levels of 6600, 7400, and 7100 RU, respectively. A 2-min injection of 100 nM Ag 2 was followed by a 2-min injection of 200 nM IgG (one of nos. 8–18), allowing each IgG its own binding cycle, by regenerating the surfaces after each IgG injection. After the standard regeneration, the IgG 12 surface required additional regeneration with three 18-s pulses of a 1:1:1 (v/v/v) mixture of Pierce Gentle IgG Elution Buffer/4 M NaCl/150 mM phosphoric acid.

Data processing

Octet and Biacore data were processed in Scrubber (version 2.0a, BioLogic Software, Campbell, Australia), and ProteOn data were processed in prototype software (also from BioLogic Software). Data were aligned to zero on both the *x* and *y* axes to establish an origin for all binding steps of interest. Octet data were not processed further, but ProteOn data were internally referenced along a ligand strip by subtracting the "interspots" from the "reaction spots" [14], and Biacore data were referenced across flow cells by subtracting the responses of an unmodified flow cell from the reaction flow cells.

Results

In tandem blocking

Of the three assay orientations sketched in Fig. 1, in tandem blocking (Fig. 1A) is probably the simplest conceptually. It involves coating the sensors with Ag and presenting two mAbs one after an-

other, namely, a saturating mAb followed by a competing one. The competing mAb should bind the mAb-saturated Ag only if its epitope does not overlap that of the saturating mAb.

Fig. 2 demonstrates how this assay orientation was used on the Octet to test whether a panel of seven mAbs blocked one another's binding to the dimeric Ag 1. Regenerating the Ag-coated tips after each binding cycle not only increased the unattended throughput of the assay but also made it more cost-effective in terms of both the number of tips used and the mass of Ag needed to coat them. Two controls were built into the assay. First, we verified that every mAb in the panel could bind free Ag on the tip by allowing each one its turn to saturate it (Fig. 2B). Second, we bound the same mAb in tandem to provide a "self-blocking" control. Four of the five mAbs that we tested in this way effectively blocked themselves, as shown by the baseline curves colored pink, green, light blue, and black in panels 2, 3, 4, and 5, respectively, of Fig. 2C. In contrast, mAb 6 was unable to fully block itself from binding in tandem (orange line in panel 6 of Fig. 2C) due to it dissociating notably from the Ag-coated tip (Fig. 2B).

Panel 1 of Fig. 2C shows that saturating the Ag with mAb 1 blocked mAbs 2, 3, and 4 (baseline curves) but not mAbs 5 and 6 (black and orange curves). A similar result was obtained when the Ag was saturated with mAb 3 (panel 3 in Fig. 2C), suggesting that mAbs 1 and 3 may belong to the same bin. In contrast, saturating the Ag with mAbs 2, 4, and 7 (see panels numbered accordingly in Fig. 2C) blocked all competing mAbs tested (nos. 2–6), suggesting that these three mAbs may bin together. For the most part, the response seen for the competing mAb was either unaffected or abolished by the saturating mAb, thereby providing a clear indication of which pairs of mAbs blocked one another.

Using this method to discern blocking profiles of each mAb relative to one another, we assigned them to three bins: bin A = mAbs 1 and 3; bin B = mAbs 5 and 6; and bin C = mAbs 2, 4, and 7. A pair of mAbs from this panel could bind Ag 1 simultaneously only when one mAb was from bin A and the other was from bin B because bin C blocked all mAbs tested. This suggested that bins A and B represented distinct nonoverlapping epitopes, whereas bin C's epitope(s) overlapped those of the other bins. These results are summarized in Table 1.

The same set of seven mAbs was analyzed on the ProteOn using a similar strategy except that Ag 1 was captured via its human IgG Fc fusion partner using a polyclonal anti-human IgG Fc antibody that was preimmobilized onto the chip (Fig. 3). This mode of immobilization was used as an alternative to amine coupling the Ag directly because it did not appear to preconcentrate on the ProteOn chip, likely reflecting the different chemistry of its alginate matrix compared with the proprietary matrix of AR Octet tips and the Biacore's CM5 sensor chip (discussed later). We took full advantage of the ProteOn's ability to address 36 interactions at a time by injecting six mAbs in tandem, rotating the chip between the saturating and competing IgGs. Regenerating the Ag surfaces enabled us to run a second binding cycle that incorporated the seventh member of the mAb panel and served to duplicate the binding of five of the others (data not shown). Using this method, we dis-

Table 1
Pairwise blocking results for the Octet data shown in Fig. 2

Saturating mAb	Competing mAb				
	2	3	4	5	6
1	Y	Y	Y	N	N
2	Y	Y	Y	Y	Y
3	Y	Y	Y	N	N
4	Y	Y	Y	Y	Y
5	Y	N	Y	Y	Y
6	Y	N	Y	Y	Y
7	Y	Y	Y	Y	Y

Note. Y, blocks; N, does not block. Three patterns of blocking activity are discerned whether the table is read from left to right or from top to bottom: bin A = mAbs 1 and 3; bin B = mAbs 5 and 6; and bin C = mAbs 2, 4, and 7.

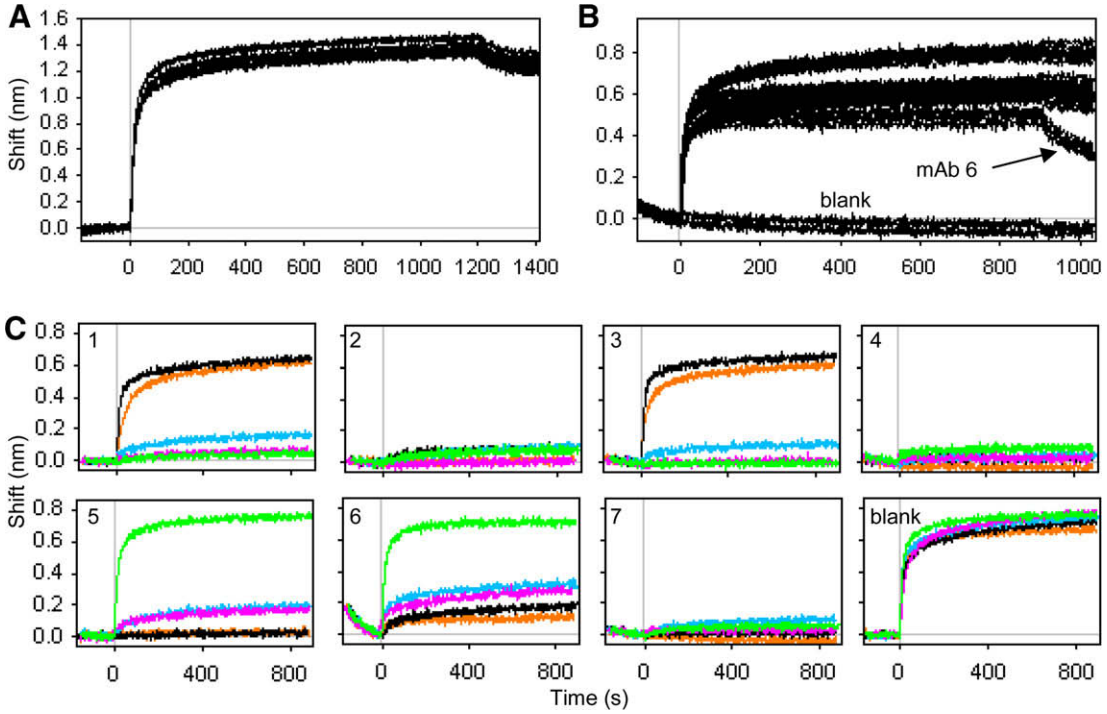

Fig. 2. In tandem blocking on the Octet. (A) Amine coupling of Ag 1 onto a single column of sensors. (B) Five repeat binding cycles of an array of saturating mAbs (nos. 1–7) and a buffer blank binding Ag 1 surfaces. (C) Overlay plots of five competing mAbs (nos. 2, 3, 4, 5, and 6) distinguished by color (pink, green, light blue, black, and orange, respectively) binding Ag 1/mAb complexes. Panels are grouped and numbered by the saturating mAb.

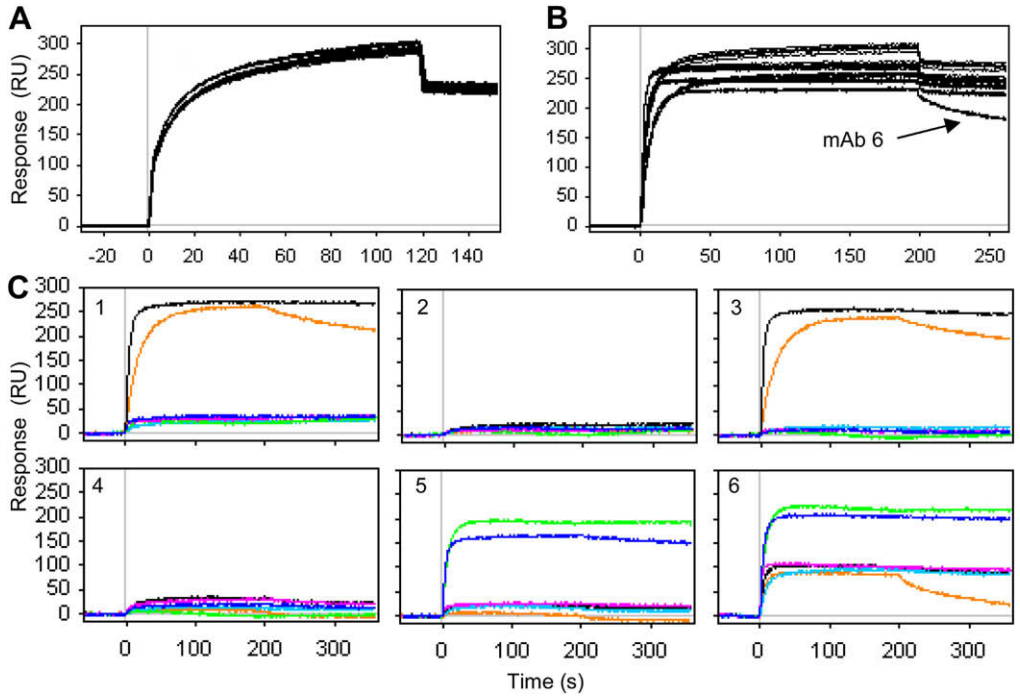

Fig. 3. In tandem blocking on the ProteOn. (A) Capture of Ag 1 via preimmobilized anti-human IgG Fc polyclonal on chip. (B) Overlay plot of six saturating mAbs (nos. 1, 2, 3, 4, 5, and 6) binding Ag 1 in the ligand direction. (C) Overlay plots of the same six competing mAbs binding Ag 1/mAb complexes in the analyte direction, distinguished by color (blue, pink, green, light blue, black, and orange, respectively). Panels are grouped and numbered by the saturating mAb.

cerned three bins with distinct patterns of blocking activity that agreed with the Octet data and were generated much faster.

A similar assay was conducted on the Biacore by testing every pairwise permutation of the seven mAbs (and buffer blanks) over amine-coupled Ag 1 (Fig. 4). The resulting bins agreed with those determined on the other sensors.

Premix blocking

We used the same set of reagents to illustrate the premix approach diagramed in Fig. 1B. This involved premixing the Ag separately with each member of an array of mAbs, in large molar excess over Ag, and then presenting these preformed complexes to mAb-coated sensors. An Ag/mAb mixture will bind to the immobilized mAb only if the two mAbs in question do not block one another.

Fig. 5 compares data collected in a premix mode on three different biosensors. A representative mAb from each of the three tentative bins deduced from the in tandem strategy (Table 1) was immobilized onto the sensors to probe for Ag that had been premixed with an array of mAbs. Ag not premixed with mAb established the Ag-only binding response (red curves) for the mAb-coated sensors. In each case, no binding was detected for a "self-sandwich" control, which used the same mAb in the premix and on the sensor, as shown by baseline curves colored green, black, and pink for the left, center, and right panels, respectively.

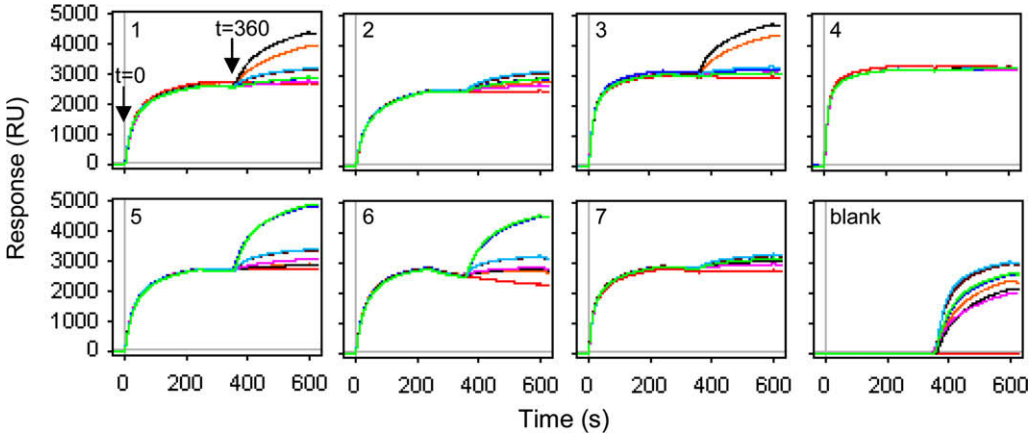

Fig. 4. In tandem blocking on the Biacore. A saturating mAb was injected at time zero, followed by a competing one at 360 s over Ag 1 on chip. Overlay plots are grouped and numbered by the saturating mAb. Buffer replaced the saturating mAb in the blank panel. Competing mAbs (nos. 1, 2, 3, 4, 5, 6, and 7) are distinguished by color (blue, pink, green, light blue, black, orange, and brown, respectively), with buffer (no competing mAb) shown in red. Although data were collected over three differing capacity surfaces, for clarity we show the results from only one of these surfaces with 4200 RU Ag immobilized.

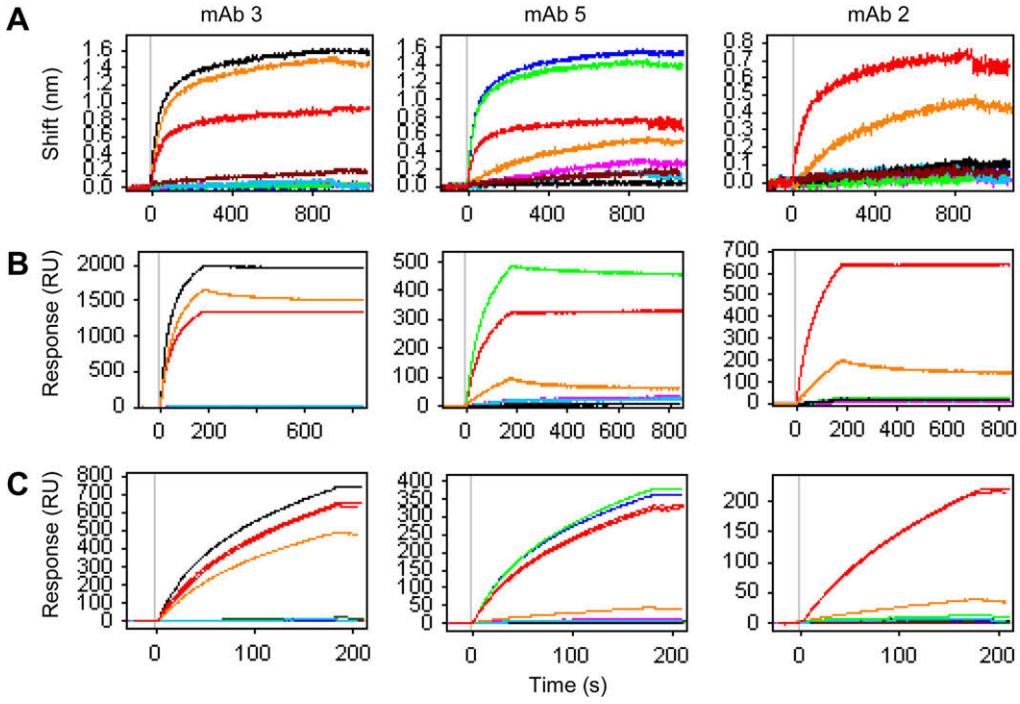

Fig. 5. Premix blocking on three biosensor platforms: (A) Octet; (B) ProteOn; (C) Biacore. The left, center, and middle panels show data from three different immobilized mAbs (nos. 3, 5, and 2, respectively), as numbered along the top. The immobilized mAbs were allowed to bind Ag 1 alone (red) or Ag 1 premixed with a mAb (nos. 1, 2, 3, 4, 5, 6, and 7, which are colored blue, pink, green, light blue, black, orange, and brown, respectively). Only five premixed mAbs (nos. 2–6) were analyzed on the ProteOn.

Fig. 5A shows premix data collected on the Octet. The left and center panels include examples of curves that were either below or above the red curve due to premixed mAbs that did or did not, respectively, block the Ag from binding to the mAb-coated sensors. In contrast, all but one of the premixed mAbs in the right panel abolished Ag binding. In one case, the binding was lowered but not completely abolished, perhaps indicating that the premixed mAb (no. 6) had such a weak affinity for the Ag that it could achieve only partial blocking at the concentration tested (see Discussion). Consistent with the in tandem approach, opposing sets of premixed mAbs generally blocked the mAbs immobilized in the left and center panels (nos. 3 and 5, respectively), whereas all premixed mAbs blocked the mAb immobilized in the right panel (no. 2).

Similar results were obtained on the ProteOn but in a more high-throughput manner. Ag premixed with buffer or one of five different mAbs was injected over six immobilized mAbs to address 30 pairs of mAbs and six Ag-only controls in a single binding cycle. Fig. 5B shows data from three of the six ligand channels that had distinct blocking patterns. Ag-binding responses were abolished or augmented relative to the red curve (Ag alone), diagnosing mAbs that did or did not block Ag from binding to the immobilized mAbs. Of the six mAbs tested, only mAb 6 did not fully block Ag binding at the concentration tested, even against itself, consistent with this mAb's weak affinity for the Ag. These data corroborated the Octet data.

The bins were further confirmed on Biacore by testing all seven premixed mAbs in a multicycle mode over three mAbs on the chip, which relied heavily on being able to regenerate all the immobilized mAbs (Fig. 5C).

Classical sandwich

As cartooned in Fig. 1C, the classical sandwich assay involves amine coupling a mAb onto the sensor, binding Ag, and then binding another mAb. The second mAb can bind the captured Ag only if its epitope does not overlap that of the immobilized mAb. To illustrate this strategy, we studied a set of mAbs (nos. 8–18) raised against a monomeric Ag (Ag 2). If a multimeric Ag was used, it could potentially bind the immobilized mAb with one subunit and the solution mAb with another subunit even if the two mAbs bound the same epitope [15].

A typical binding cycle on the Octet involved coating a column of sensor tips with a single mAb, allowing those tips to bind Ag 2, and then testing whether an array of mAbs could bind the mAb-tethered Ag 2 (Fig. 6). Some mAbs were pooled in the sandwich step to increase the assay's throughput if the accumulated data suggested that they belonged to the same epitope bin as the immobilized mAb.

Fig. 7 shows the same classical sandwich assay performed on the ProteOn in a higher throughput mode. Its two-dimensional format enabled us to analyze six competing mAbs over six immobilized mAbs at once, thereby addressing 36 interactions in a single

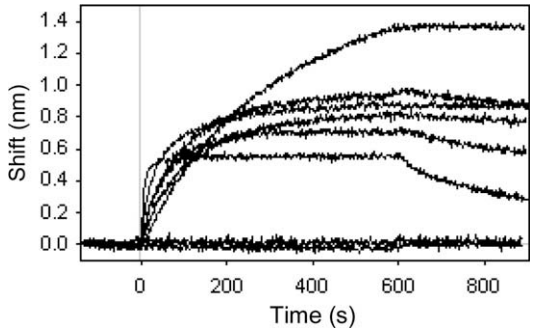

Fig. 6. Classical sandwich assay on the Octet. The overlay plot depicts an array of 11 mAbs (nos. 8–18) presented in a sandwich step to Ag 2 that was captured via tips coated with mAb 18. Each member of the array was allowed a separate well except mAbs 12 to 15, which were pooled. From this panel of 11 mAbs, 6 bound (nos. 8–11, 16, and 17) and 5 did not (nos. 12–15 and 18), indicating that the mAb on the sensor blocked the latter but not the former. As expected, no binding was detected for the self-sandwich control when mAb 18 was presented in solution.

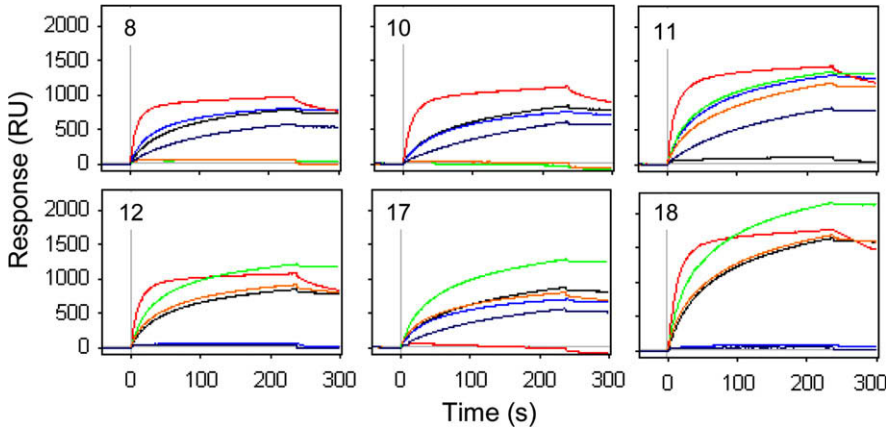

Fig. 7. Classical sandwich assay on the ProteOn. The panels represent the sandwich step for six mAbs (nos. 8, 10, 11, 12, 17, and 18, which are colored green, orange, black, blue, red, and dark blue, respectively) injected over Ag 2 that was captured via the same six mAbs on chip. The data are grouped and labeled by the immobilized mAb.

binding cycle. Six of these interactions provided self-sandwich or negative controls, confirming that none of the mAbs could bind Ag 2 that was captured by the same mAb on the chip. By regenerating the immobilized mAbs, additional binding cycles were run to test other competing mAbs (data not shown).

We conducted a similar assay on Biacore using a multicycle mode to test an array of competing mAbs over three mAbs on the chip, as depicted in Fig. 8A–C. We discerned four patterns of blocking activity; one example of each is shown in Fig. 8D–G.

Table 2 summarizes the epitope binning results for the classical sandwich assay deduced from Figs. 6–8. By conducting further experiments that addressed all additional pairwise permutations (data not shown), we assigned bins unambiguously to all 11 mAbs tested: bin D = mAb 8; bin E = mAbs 9 and 11; bin F = mAb 10; bin G = mAbs 12, 13, 14, 15, and 18; bin H = mAb 17; and bin I = mAb 16. For the most part, the bins represented groups of mAbs with mutually exclusive epitopes, such that any two mAbs from different bins could bind Ag simultaneously. Bin F was a notable exception because it also blocked bins D and I (full data not shown for bin I).

Discussion

The main result from our study was that the epitope bins agreed well across the three assay orientations regardless of the biosensor used. However, some assay orientations and biosensors provided clearer analyses than others.

Intermediate responses

Although it is obvious in most of our examples when mAbs did and did not bind, some mAbs showed intermediate binding responses such as in panel 6 of Figs. 2C, 3C, and 4. These data represent an in tandem assay on different platforms using a mAb (no. 6) that did not saturate the Ag-coated sensors (Figs. 2B and 3B and panel 6 of Fig. 4) prior to presenting an array of competing mAbs, which were thus able to bind free and bound Ag. However, when mAb 6 was used as the competing mAb (orange curves in Figs. 2–4), it clearly binned with mAb 5 (black curves). Similarly, mAb 6 gave an intermediate binding response when it was used in solution in the premix approach (orange curves in Fig. 5). The result was cleaner when it was coupled to the sensor (data not shown).

Taken together, these data illustrate that weak binders, such as mAb 6, can often give ambiguous results when studied in some assay orientations and that switching the roles of the two mAbs within an assay and/or exploring other orientations may improve confidence in the results. When an intermediate response is obtained in a premix approach, it is prudent to titrate in the competing mAb to confirm that blocking (or sandwiching) increases in a dose-dependent manner. Weak binders are best studied when they

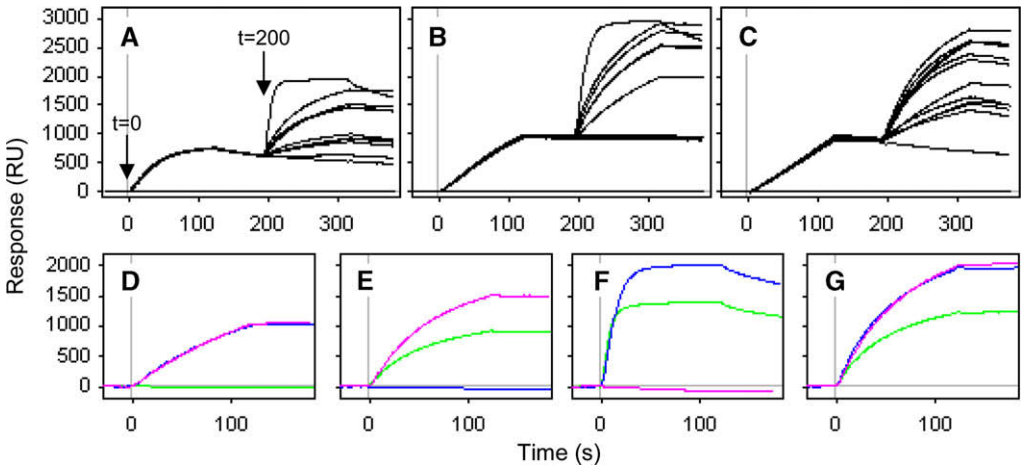

Fig. 8. Classical sandwich assay on the Biacore. Data were collected over three immobilized mAbs: mAb 11 (A), mAb 12 (B), and mAb 17 (C). Ag 2 was injected at time zero, and mAbs 8 to 18 were injected at 200 s. Panels D to G are grouped by competing mAb, where the colors (green, blue, and pink) identify the mAb surfaces (nos. 11, 12, and 17, respectively). Each panel shows an example of a competing IgG that showed a distinct blocking pattern: (D) IgGs 9 and 11; (E) IgGs 12, 13, 14, 15, and 18; (F) IgG 17; and (G) IgGs 8, 10, and 16.

Table 2
Blocking results from the classical sandwich assay deduced from Fig. 6 (Octet), Fig. 7 (ProteOn), and Fig. 8 (Biacore)

Biosensor	mAb on sensor	Competing mAb										
		8	9	10	11	12	13	14	15	16	17	18
Octet	18	N	N	N	N	Y	Y	Y	Y	N	N	Y
ProteOn	8	Y		Y	N	N					N	N
ProteOn	10	Y		Y	N	N					N	N
ProteOn	11	N		N	Y	N					N	N
ProteOn	12	N		N	N	Y					N	Y
ProteOn	17	N		N	N	N					Y	N
ProteOn	18	N		N	N	Y					N	Y
Biacore	11	N	Y	N	Y	N	N	N	N	N	N	N
Biacore	12	N	N	N	N	Y	Y	Y	Y	N	N	Y
Biacore	17	N	N	N	N	N	N	N	N	N	Y	N

Note. Y, blocks; N, does not block. Five patterns of blocking activity emerge whether the table is read from left to right or from top to bottom: bin D = mAbs 8, 10, and 16; bin E = mAbs 9 and 11; bin G = mAbs 12, 13, 14, 15, and 18; bin H = mAb 17. All mAbs were binned tentatively except mAbs 11, 12, 17, and 18, which were assigned unambiguously because they were competed against every member of the mAb panel.

are assigned the role of competing mAb in the in tandem approach or coupled to the sensor in the premix or classical sandwich approach. However, mAbs with very fast off-rates may be ill suited for coupling to the sensor in the classical sandwich approach because the Ag may have fully dissociated before presenting the sandwiching mAb. To verify that any change in binding response is truly Ag dependent in the premix and classical modes, and that there are no unwanted cross-reactions between the mAbs and the sensors, we routinely prescreen the mAbs alone in the absence of Ag. Sometimes, viewing the data in alternative ways, such as grouping tandem assay data by the saturating mAb rather than by the competing one, may be more intuitive.

In general, if the affinities (K_D values) for each Ag/mAb pair are known, then working at mAb concentrations well above the K_D (10×) should give a clean result (either full blocking or an obvious sandwich response) regardless of the assay orientation used. If, as is more often the case, the K_D values are unknown, then we would work at a mAb concentration presumed to be above the estimated K_D and rerun the assay at higher concentrations for those mAbs that showed intermediate responses if they were not too precious. We obtained intermediate responses for mAb 6 because we were unable to work at a high enough concentration of that mAb.

Comparison of assay orientations

Deciding on a suitable blocking strategy for epitope binning relies on the interplay of several factors (Table 3). A multivalent or aggregated Ag is best studied using either an in tandem method or a premix one, whereas the classical sandwich assay is amenable only to monomeric Ags. In general, one would couple the purest or most precious reagent, either the mAb or Ag, if it is regenerated easily and has a decent off-rate. If one binding partner cannot be coupled easily to the sensor and/or loses activity on coupling, it might be amenable to a capture method, as used to generate the ProteOn data in Fig. 3. Otherwise, the assay can be reoriented to allow that molecule to be in solution. The number of mAbs in the set may also dictate which strategy is chosen based on how many pairs of mAbs can be addressed in a single experiment. For example, the in tandem approach requires only one immobilized ligand, the Ag, to screen every permutation of an array of mAbs.

Of the three blocking strategies discussed here, the premix approach is probably the most reliable due to an internal Ag-only control that establishes the activity of the immobilized mAbs. Relative to this response, the Ag-binding response goes either up or down in the presence of the premixed mAb, indicating that the Ag/mAb complex can or cannot bind the immobilized mAb. Furthermore, the scope of the premix approach can be extended to quantitative applications such as determining active concentration [15] and solution affinity [2,16].

Comparison of biosensor platforms

Although SPR biosensors provide superior sensitivity compared with that currently available on the Octet QK, they generally support a limited number of immobilized ligands per experiment and rely on being able to regenerate them. Therefore, deciding on the most appropriate platform to use for blocking is a trade-off between the sensitivity offered by SPR and the convenience of the Octet's single-use sensors and ability to reuse

Table 3
Summary of the three assay orientations discussed here, as applied to epitope binning

Parameter	In tandem blocking	Premix	Classical sandwich
On sensor	Ag	mAb	mAb
Bind	Saturating mAb followed by competing mAb	Ag premixed with a large molar excess of mAb	Ag followed by mAb
Ag	Can be multivalent	Can be multivalent	Must be a monomer
mAb	All are native, but the first mAb must saturate the immobilized Ag and remain bound before presenting the competing mAb	One is immobilized, and the other is native; saturating the Ag with the premixed mAb gives the cleanest result, although it is not absolutely necessary	One is immobilized, and the other is native; no need to saturate binding sites
Theoretical outcome for last step	The competing mAb does or does not bind, indicating that it is not blocked by the saturating mAb in the former case and is blocked in the latter case	The Ag-binding signal is unaffected, abolished, or augmented, indicating that the premixed mAb does not bind Ag detectably, blocks it, or does not block it binding the immobilized mAb, respectively	The sandwiching mAb binds or does not bind, indicating that it is not blocked by the immobilized mAb in the former case and is blocked in the latter case
Self-blocking control	Apply the same mAb in the saturating and competing steps	Use the same mAb in solution and on the sensor	Same as for premix

samples. Although our model systems regenerate easily, scouting regeneration conditions can often be laborious on traditional biosensors. The throughput offered by the ProteOn, however, may make regeneration unnecessary if all of the desired permutations are covered within the 36 interactions that can be addressed in a single binding cycle. A unique feature of the Octet is that ligands can be loaded offline onto an entire tray of sensors as well as online onto a column at a time. Using precoated sensors increases the unattended throughput of the Octet by eliminating the need for online immobilization and, thus, freeing up wells in the sample microplate for additional analyte. However, batch immobilization requires reconstituting a large amount of ligand in a coupling buffer that might render the ligand inactive or unusable in other applications.

In the premix approach, we found that the sandwiching responses were more obvious on the Octet than on the other two platforms, particularly the Biacore. This may be due to the Octet's dip-and-read well-based format, which allows longer analyte-binding steps and favors rebinding of the analyte to the ligand-coated sensors. In contrast, not only is the contact time on flow-based SPR platforms limited by the capacity of the injection syringe and available flow rates, but longer association times consume more sample.

In our experiments, the total volume of sample needed for a given assay strategy on each of the three biosensors was roughly equivalent because the Biacore injected smaller volumes than the parallel biosensors but needed many more binding cycles due to its serial handling of samples. The Biacore's multicycle mode often meant that the total assay run time was longer than that on the other platforms.

Resolving epitope bins

Once two mAbs have been assigned to the same bin, it is possible that at some point in the future another mAb will be tested that blocks one and not the other, meaning that they would no longer be in the same bin. For example, mAbs 8 and 10 appeared to share a similar pattern of blocking activity toward other members of the panel until they were discriminated by mAb 16, which blocked mAb 10 but not mAb 8 (data not shown). Because a mAb's bin can be resolved only by the set of mAbs tested at that time, bins should be seen as a general description of a mAb's epitope rather than as a hard-and-fast definition of it. Rather, epitope binning should be considered as a complement to epitope mapping. For example, in the quest for a mAb that will interfere with the binding of the Ag's natural partner, defining the epitope precisely is often not necessary. The "gold standard" for defining an epitope in terms of the residues that form the Ag/mAb interface is to solve the X-ray crystal structure of the complex in question. However, this static view can often miss the importance of residues that contribute indirectly to the conformation of the overall complex. From a practical perspective, generating structures for every protein/protein complex of interest is very labor-intensive and rarely feasible. An alternative and more commonly used way of defining an epitope is to perform a mutational analysis on the Ag that elucidates the relative importance of each residue to the mAb-binding activity [17].

General blocking assays

The principles on which our assays are founded can be applied to any type of blocking experiment where a molecule (B) has two possible binding partners (A and C) that do not interact with one another directly. In the context of epitope binning, B is the Ag and both A and C are mAbs. On the other hand, in determining whether a drug (A) blocks a ligand (B) from binding its receptor (C), twice as many assay orientations can be explored as those described here because the drug and receptor can switch roles. For example, a premix assay may work better if the receptor, rather than the drug, is coupled to the sensor. With carefully designed experiments, biosensors can be used to identify blocking partners for diverse interacting systems and provide information about functional activity that drives drug discovery.

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