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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.

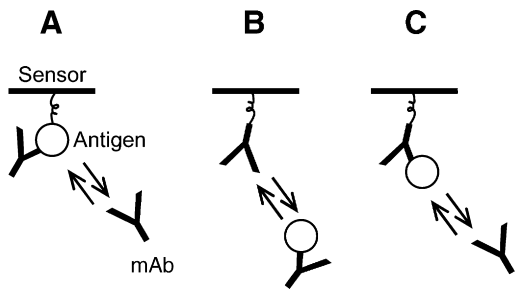


**Fig. 1.** Outline of three biosensor-based assay orientations that can be used to explore blocking, as applied to epitope binning mAbs in a pairwise manner: (A) in tandem blocking; (B) premix blocking; and (C) classical sandwich.

## 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-