



Research paper

Label-free epitope binning assays of monoclonal antibodies enable the identification of antigen heterogeneity

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ABSTRACT

Label-free biosensors are often used in the discovery of therapeutic antibodies to characterize the epitope binding regions of a panel of monoclonal antibodies that target a specific antigen, thus facilitating their organization into epitope groups or “bins”. When tested in a pairwise combinatorial manner, two antibodies that compete with one another for binding to a specific antigen may be grouped into the same epitope bin – that is, they recognize similar or overlapping epitopes – whereas two antibodies that bind simultaneously to the antigen are placed into different epitope bins. However, depending on the assay format used, results from such experiments can sometimes contradict one another. Here, we provide two examples that illustrate how antigen heterogeneity, either inherent in an antigen sample, or induced by the assay conditions, can confound the interpretation of epitope binning results and, in some cases, lead to erroneous conclusions. We highlight why assays that employ solution antigen are often more reliable than those that employ immobilized antigen and, by corroborating our binning results with assays that utilize native antigen, we determine which subpopulations of our heterogeneous antigen samples are biologically relevant and thus improve the correlation between epitope bins and functional activity. Furthermore, we provide recommendations for performing definitive binning assays and a diagnostic assay procedure that can be followed when antigen heterogeneity is suspected.

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1. Introduction

The current study broadens the scope of our previous work wherein we described how label-free biosensors can be used to perform epitope binning assays on a panel of monoclonal antibodies (mAbs) (Abdiche et al., 2009). A common assumption in the interpretation of epitope binning assay results is that all

mAbs in the test panel bind a uniform population of the antigen (Ag). Therefore, when a pair of mAbs is tested for binding to a specific Ag, the mAbs either compete with one another for a similar or overlapping epitope on the same Ag molecule or are able to bind an Ag molecule simultaneously at distinct, non-overlapping epitopes. However, we have found that some Ag preparations may comprise subpopulations of molecules, e.g., a native form and a denatured form. Such a preparation may be problematic if employed as an immunogen or panning substrate in the discovery of therapeutic mAbs or if used as a reagent to characterize mAbs. Since only mAbs that bind native epitopes can be considered as therapeutic candidates, an Ag preparation that contains a contaminant or non-native form of the Ag may inadvertently lead to the selection of mAbs that bind biologically irrelevant epitopes and thus lack *in vivo* efficacy.

Determining whether a protein preparation contains an unacceptable level of heterogeneity can be difficult to assess because purifying a protein to homogeneity can be challenging and some proteins may be naturally heterogeneous. For

Abbreviations: mAb, monoclonal antibody; Ag, antigen; hPGRN, human progranulin; hPCSK9, human proprotein convertase subtilisin kexin type 9; hGFR α 3, human glial cell-derived neurotrophic factor family receptor alpha 3; hFABP4, human fatty acid binding protein 4; hART, human artemin; CREB, cyclic AMP-responsive element binding protein; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; NC, negative control.

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example, glycoproteins often comprise mixtures of glycoforms and this inherent heterogeneity may play an important role in the glycoprotein's biological function (Rudd and Dwek, 1997). Standard mass-based techniques such as SDS-PAGE, size exclusion chromatography (SEC), light scattering, and mass spectrometry may be employed to readily detect some forms of heterogeneity in an Ag preparation, including degradation products, aggregates, or variants due to glycosylation or phosphorylation. However, these techniques may be unable to discriminate between alternate conformations of an Ag that may arise due to misfolding or partial denaturation.

One way of detecting heterogeneity in a protein sample is to probe for Ag subpopulations using a panel of mAbs that target the protein of interest. We refer to an Ag as being "heterogeneous" if mAbs in a test panel bind to distinct subpopulations of molecules in a *single* Ag sample. When performing epitope binning experiments on large panels of mAbs, we have discovered cases where mAbs in the panel bind epitopes on separate subpopulations of molecules, *i.e.*, a subset of mAbs binds exclusively to one subpopulation, while another subset binds to another distinct subpopulation. The probability of encountering such instances increases with the diversity of epitopes that are targeted by the mAb panel, so one may expect that discovering "heterogeneity" in an Ag preparation is more likely when investigating a large number of mAbs. Some level of heterogeneity in an Ag sample may be undetectable in epitope binning assays if all mAbs bind the same Ag subpopulation; in this case, any contaminant can be considered to be "inert" because it does not affect the binning outcome. Some or all of these Ag subpopulations may be biologically relevant. When the results of epitope binning assays with recombinantly-expressed Ag are corroborated with the results from assays that employ a native form of the Ag, they can facilitate the selection of mAbs that bind native epitopes and the rejection of those that bind biologically irrelevant epitopes.

In this work we demonstrate how epitope binning assays can be applied to determine if an Ag is "heterogeneous" and we develop recommendations for conducting definitive binning assays with monovalent Ags. First, we use human progranulin (hPGRN) as a model Ag with a sandwiching pair of mAbs to demonstrate that the same binning result can often be obtained regardless of the assay format employed, if the mAbs in the test set bind to the same Ag population. Second, to introduce the concept of Ag heterogeneity and how it can confound the interpretation of epitope binning assays, we simulate a heterogeneous Ag by mixing hPGRN with an unrelated model Ag, human proprotein convertase subtilisin kexin type 9 (hPCSK9), and bin this mixture using an anti-hPGRN mAb and an anti-hPCSK9 mAb. This example demonstrates that binning with a heterogeneous Ag yields a different outcome depending upon the assay format employed. Third, we perform binning assays with human glial cell-derived neurotrophic factor family receptor alpha 3 (hGFR α 3), an Ag that appeared to be heterogeneous in one of our sample preparations. Finally, we use human fatty acid binding protein 4 (hFABP4) as a model Ag to demonstrate why investigators should be cautious when performing assays that employ immobilized Ag. In this example, direct immobilization of the Ag exposed additional denatured epitopes that led to the identification of mAbs that bound denatured epitopes (*i.e.*, false positives) and resulted in discordant results from different binning assay formats.

2. Material and methods

2.1. General methods

Biosensor experiments were performed on Biacore 2000 and 3000 platforms equipped with CM4 sensor chips (GE Healthcare Inc., Piscataway, NJ), a ProteOn XPR36 platform equipped with GLC and NLC sensor chips (BioRad Inc., Hercules, CA), and an Octet QK384 platform equipped with amine-reactive and streptavidin sensors (ForteBio Inc, Menlo Park, CA). All biosensor data were collected at 25 °C in HBST+ running buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 0.05% v/v Tween20), unless stated otherwise. Purified recombinant hPGRN with a 6-His tag at the C-terminus was purchased from R&D Systems (Minneapolis, MN, Catalog# 2420-PG). Recombinant hPCSK9 (mature form) with a 6-His tag at the C-terminus was prepared in-house by transient transfection of HEK293 cells and purified via a standard IMAC column followed by SEC. Two constructs of recombinant hGFR α 3 monomer (Gly31-Trp382, GenBank accession no. NP_001487) were expressed in-house with a 6-His tag at the N-terminus (preparations P1a and P1b represent two independent batches of this construct) or at the C-terminus (preparation P2). These proteins were expressed by transient transfection of CMV-based expression plasmids in 293-Freestyle cells using 293fectin according to the manufacturer's protocol (Life Technologies, Grand Island, NY), and purified from the conditioned media by nickel-NTA affinity chromatography (Qiagen, Valencia, CA). Purified recombinant hGFR α 3 monomer expressed in human cells was purchased from Creative BioMart (Shirley, NY, Catalog# GFRA3-3153H); we refer to this preparation as P3. Three preparations (referred to here as P4-Fc, P5-Fc, and P6-Fc) of purified recombinant hGFR α 3 fused to a human-Fc γ 1 were purchased commercially; P4-Fc (R&D Systems, Catalog# 670-FR) and P5-Fc (Creative BioMart, Catalog# GFRA3-1620H) were both expressed in insect cells, whereas preparation P6-Fc (Creative BioMart, Catalog# GFRQ3-3242H) was expressed in human cells. Purified recombinant hFABP4 with a C-terminal 6-His tag was prepared in-house in mammalian cells. Murine mAbs raised against hPGRN (mAbs 1 and 2), hPCSK9 (mAb 3), hGFR α 3 (mAbs 4 and 5), and hFABP4 (mAbs 6, 7, 8, and 9) were generated in-house via standard hybridoma technology and purified from ascites fluid via protein A affinity chromatography. Mouse anti-polyHistidine mAb (Catalog# MAB050) and purified recombinant human artemin (hART, Catalog# 2589-AR) were purchased from R&D Systems. An isotype-matched negative control (NC) mAb against an irrelevant Ag (bovine herpesvirus) was made in-house by PCR-based amplification of heavy and light variable domain cDNA reverse transcribed from hybridoma CRL-1853 (ATCC) RNA. The NC heavy and light chains were cloned into a CMV-based expression vector, and the mAb produced recombinantly as a mouse IgG1 isotype by transient transfection of 293F cells followed by protein A purification. Biotinylations were performed using EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce, Inc., Rockford, IL), per the manufacturer's instructions, using a linker:protein molar ratio of 2:1 for Ags and 15:1 for mAbs. Streptavidin-coupled Dynabeads M-280 were purchased from Life Technologies and used per the manufacturer's instructions. MAb used in direct immunofluorescence FACS experiments were labeled using a DyLight 649 microscale antibody labeling kit according to the manufacturer's protocol (Pierce, Inc.). Pooled human plasma from healthy donors was

purchased from Bioreclamation (Liverpool, NY). Buffer exchanges were performed using BioRad Micro Bio-Spin 6 Columns.

MAb concentrations are reported in molar binding sites (assuming an IgG has two binding sites and each binding site has a mass of 75 kDa) when the mAb's concentration relative to the anticipated affinity for its binding partner and/or the concentration of a premixed binding partner is relevant to the assay being performed. MAb concentrations are given in $\mu\text{g/ml}$ for immobilizations. Ag concentrations are reported as "nominal" to indicate the possibility of heterogeneity or partial activity with respect to binding a given mAb; in this case, the nominal total protein concentration in the Ag preparation was determined by light absorption at 280 nm and corrected using the theoretical extinction coefficient based upon the primary amino acid sequence. The active concentrations of individual subpopulations in a given Ag preparation were not determined.

2.2. Binning with hPGRN

An Octet equipped with streptavidin sensors was used to bin hPGRN with mAbs 1 and 2 using an in tandem binning format. Sensors were coated with 1 $\mu\text{g/ml}$ biotinylated mouse anti-6-His mAb for 15 min to provide a capture surface for hPGRN, which was then captured at 1 $\mu\text{g/ml}$ for 5 min to generate a sparse immobilization level. MAbs at a concentration of 270 nM binding sites (or buffer) were then bound consecutively for 10 min, varying the mAbs and the order in which they were bound, thereby addressing every pairwise permutation of buffer, mAb 1, and mAb 2. Each interaction was analyzed on duplicate or triplicate sensors. The anti-His surfaces were regenerated with two 30-s exposures of 75 mM phosphoric acid and the assay was repeated.

An Octet equipped with amine-reactive sensors was used to bin hPGRN with mAbs 1 and 2 in a classical sandwich format. Sensors were activated with a freshly prepared mixture of 20 mM EDC and 5 mM sulfo-NHS, coupled with 20 $\mu\text{g/ml}$ mAb 1 or mAb 2 in 0.1 M MES pH 5.0 buffer, and then excess reactive esters were blocked with 1 M ethanolamine pH 8.5, allowing 10 min per step. Amine-coupled mAbs were then used to capture 30 nM hPGRN (or buffer) followed by 270 nM mAb binding sites (or buffer). Surfaces were regenerated with 75 mM phosphoric acid and the assay was repeated. The same surfaces were used to perform a premix binning assay, which involved premixing 50 nM hPGRN with and without 270 nM mAb binding sites and then testing these equilibrated mixtures for binding to the amine-coupled mAbs. Additional samples of mAb alone (*i.e.*, without any premixed hPGRN) were used to monitor any nonspecific cross-reactions between the solution mAbs and the amine-coupled mAbs. Interactions were analyzed on triplicate sensors, allowing 15 min per mAb-binding step.

2.3. Binning with a mixture of hPGRN and hPCSK9 to simulate a heterogeneous antigen

An Octet equipped with streptavidin sensors was used to bin a mixture of hPGRN and hPCSK9 (*i.e.*, a simulated heterogeneous Ag) with an anti-hPGRN mAb (mAb 1) and an anti-hPCSK9 mAb (mAb 3). To prepare the reaction surfaces for an in tandem binning assay, sensors were coated

with 1 $\mu\text{g/ml}$ biotinylated mouse anti-6-His mAb for 15 min and used to capture 1 $\mu\text{g/ml}$ hPGRN for 5 min followed by 1 $\mu\text{g/ml}$ hPCSK9 for 3 min. Two mAbs were then bound, one after another, to the co-immobilized Ag surfaces. Reaction surfaces for classical sandwich and premix binning assays were prepared by saturating the streptavidin sensors with 1 $\mu\text{g/ml}$ biotinylated mAbs 1 or 3. To perform a classical sandwich assay, a mixture of 30 nM hPGRN and 40 nM hPCSK9 was captured *via* the mAb-coated sensors and then mAbs 1 or 3 were tested for sandwiching. To perform a premix binning assay, an Ag mixture was prepared at the concentrations stated above and premixed with or without mAbs 1 or 3. The equilibrated mixtures were then tested for binding to the immobilized mAbs. Additional control cycles were run in all three binning assay formats, where the Ag mixture or the mAbs were replaced with buffer. Across all three assay formats, mAbs were analyzed at a concentration of 270 nM binding sites, binding steps were allowed 15 min, and interactions were analyzed on quadruplicate sensors.

2.4. Binning with hGFR α 3

Interaction analysis of hGFR α 3 (preparation P1a) was conducted in HBST+ running buffer supplemented with 1 mg/ml, or in some experiments, 10 mg/ml, BSA.

In tandem binning was performed on a ProteOn equipped with a neutravidin-coated (NLC) sensor chip. Biotinylated hGFR α 3 was prepared at final concentrations of 0.3, 0.6, 1.1, 2.3, 4.5, and 9 $\mu\text{g/ml}$ and injected for 3 min along parallel ligand channels to give final capture levels ranging from 45 RU to 575 RU; in this way, we scouted for a sparse surface capacity appropriate for an in tandem binning analysis. In the analyte direction, mAb 4 or mAb 5 was injected for 3 min to saturate the immobilized Ag and then buffer, mAb 4, or mAb 5 was injected for another 3 min. A concentration of 500 nM mAb binding sites was used. Surfaces were regenerated with 2:1 v/v Pierce IgG elution buffer:4 M NaCl (referred to hereafter as the regeneration cocktail), and the binning step was repeated.

Classical sandwich binning was performed on a Biacore equipped with a CM5 sensor chip. Anti-hGFR α 3 mAbs 4 and 5 were immobilized using an aldehyde-coupling method. Before coupling, the mAbs were diluted to 1 mg/ml in 100 mM sodium acetate buffer pH 5.5, and then oxidized by adding 50 mM sodium metaperiodate to give a final concentration of 1 mM. This mixture was incubated for 20 min on ice and then buffer-exchanged into HBST+ buffer. The buffer-exchanged oxidized mAbs were stored on ice prior to coupling. The sensor surface was prepared by injecting a freshly prepared mixture of 200 mM EDC and 50 mM NHS for 3 min and then injecting 5 mM carbonylhydrazide for 7 min. The remaining reactive esters were blocked using a 7-min injection of 1 M ethanolamine pH 8.5. The oxidized mAb was diluted into 10 mM sodium acetate pH 4.0 buffer to 50 $\mu\text{g/ml}$ and injected over a single flow cell for 7 min. The 10 mM sodium acetate pH 4.0 buffer was injected for 7 min on another flow cell to prepare a reference surface. After coupling the mAb, 100 mM sodium cyanoborohydride in 100 mM sodium acetate pH 4.0 buffer was injected for 4 min to stabilize the linkage. This procedure resulted in final immobilization levels of 13,000 RU for mAb 4 and 8,000 RU for mAb 5. Having prepared the

reaction and reference surfaces as described above, a classical sandwich binning was performed by capturing hGFR α 3 at a nominal concentration of 20 nM for 2 min and then injecting 1 μ M mAb (4 or 5) binding sites or buffer for 2 min. Surfaces were regenerated with three consecutive 18-s injections of the regeneration cocktail.

Premix binning was performed on a ProteOn equipped with a GLC chip. MABs 4 and 5 were amine-coupled onto separate ligand channels by activating the surfaces with a 2-min injection of a freshly prepared mixture of 4 mM EDC and 1 mM sulfo-NHS, coupling 15 μ g/ml mAbs in 10 mM sodium acetate buffer pH 4.5 using a 3-min injection, and blocking excess reactive esters with a 3-min injection of 1 M ethanolamine pH 8.5. This resulted in immobilization levels of approximately 4000 RU along a channel. Premixes of nominal 20 nM hGFR α 3 with and without 100 nM mAb binding sites were injected for 3 min in the analyte direction and surfaces were regenerated with the regeneration cocktail.

Magnetic separation using Dynabeads was performed to isolate distinct subpopulations of our heterogeneous hGFR α 3 preparation (P1a). Thus, 1 mg streptavidin-coated beads was washed three times in HBST+ and then coated with 10 μ g biotinylated mAb (4 or 5) – or buffer, to prepare blank beads – in a typical volume of 0.3 ml. The bead samples were then incubated for 30 min at room temperature with gentle rotation. MAb-coated (and blank) beads were separated with a magnet for 2 min, and washed four times with HBST+. Each bead type was then resuspended with 0.5 ml Ag at a nominal concentration of 500 nM in HBST+ with 10 mg/ml BSA and incubated overnight at 4 °C with gentle rotation. MAb-bound Ag was separated with a magnet and the resultant supernatants were centrifuged at 16,000 $\times$ g for 5 min to remove any remaining beads, diluted twofold in the ProteOn's running buffer (HBST+ with 10 mg/ml BSA), and injected for 3 min over the same chip as that used for the premix binning experiments described above.

2.5. Kinetic analysis of hGFR α 3

A surface for capturing murine mAbs was prepared using the Biacore Mouse Antibody Capture Kit (GE Lifesciences, Piscataway, NJ). The polyclonal rabbit anti-mouse Ig antibody from the kit was amine-coupled to a Biacore CM5 sensor chip in HBST+ running buffer using the instructions provided from the manufacturer, which resulted in immobilization levels of approximately 12,000 RU on all channels. The rest of the assay was performed in running buffer supplemented with 1 mg/ml BSA. Anti-hGFR α 3 mAbs 4 or 5 were each prepared at 1 μ g/ml and injected for 30, 60, and 120 s on separate flow cells, which gave typical capture levels ranging from 50 RU to 200 RU. In this way, we scouted for a low surface capacity that was appropriate for kinetic analysis. One surface was left blank (representing the bare polyclonal antibody) to serve as a reference surface. Monovalent hGFR α 3 (P1a, P1b, P2, or P3) was prepared as a threefold serial dilution with a top nominal concentration of 100, 600, or 1000 nM, depending upon the experiment, and samples were injected for 2 min, allowing a variable dissociation time, i.e., 15 min for the buffer and top concentration cycles and 1 min for all other cycles. All samples were analyzed in duplicate cycles over

freshly captured mAbs using a 3-min injection of 10 mM glycine pH 1.7 to regenerate the capture surfaces.

Using an Octet equipped with streptavidin sensors, a panel of hGFR α 3 preparations (P1b, P2, P3, P4-Fc, P5-Fc, and P6-Fc) was screened for binding to immobilized mAbs. Thus, 1 μ g/ml biotinylated mAbs 4 and 5 was captured for 15 min to saturate the sensors and then the Ag preparations were bound at a nominal concentration of 200 nM binding sites, allowing a 15-min association time and a 15-min dissociation time. All interactions were monitored on duplicate sensors.

2.6. Cell-based proliferation assays and Western blot

NBL-S neuroblastoma cells were maintained in NBL-S media (RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 100 U/ml penicillin/streptomycin) at 37 °C in an atmosphere of 5% CO₂. Cells were trypsinized, passed through a 70- μ m filter to remove clumps and plated in laminin/fibronectin-coated 96-well tissue culture plates (BD Biosciences) at 20,000 cells per well in NBL-S media with 0.5% FBS overnight. The following day, media was removed and replaced with 100 μ l OptiMEM (Gibco) with or without mAb (30 μ g/ml final concentration). After 10 min, a second 100 μ l aliquot of OptiMEM was added to each well, containing 100 ng/ml (2 $\times$ final concentration) recombinant hART, and plates were incubated at 37 °C for 72 h. Cell number was assessed using the CyQuant Direct Cell Proliferation Assay (Life Technologies) according to the manufacturer's protocol, and fluorescence was read with a Victor3V Multilabel Counter (Perkin Elmer). To determine GFR α 3-induced phosphorylation of cyclic AMP-responsive element binding protein (CREB), NBL-S cells were seeded at 3 $\times$ 10⁵ cells in 35-mm dishes coated with poly-D-ornithine/laminin (BD Biosciences), and left overnight in NBL-S media. The following day, media was removed and replaced with OptiMEM containing mAbs (400 nM binding sites). Following a 10-min incubation at room temperature, hART was added to the cells at 100 ng/ml, cells were incubated for 20 min at 37 °C, and lysed with 0.4 ml ice-cold lysis buffer of 62.5 mM Tris, pH 7.5, containing 2% SDS and protease inhibitors (Halt protease inhibitor cocktail, Thermo Scientific). The lysate was sonicated and centrifuged at 13,000 rpm for 10 min, and 15 μ l of supernatant loaded per lane on a 4–12% NuPAGE reducing gel (Life Technologies). After electrophoretic separation, proteins were transferred to nitrocellulose membranes and blotted with mAbs to CREB or S133 phospho-CREB (48H2 or 87G3, Cell Signaling). Goat anti-rabbit IRDye 800 (Odyssey) was used as the secondary and bound antibodies visualized on a LI-COR Odyssey Infrared Imager. For detection of hGFR α 3 by Western blot, 50 ng of recombinant hGFR α 3-hFc fusion protein was run on a denaturing 4–12% NuPAGE reducing gel (Life Technologies) and transferred to nitrocellulose. The membrane was subsequently blocked with 5% milk in phosphate-buffered saline (Mediatech Catalog# 21-040) and probed with mAb 4 or 5 (10 μ g/ml). Goat anti-mouse IRDye 800 (Odyssey) was used as detection antibody.

2.7. Flow cytometry (FACS) analysis

NBL-S cells were minimally trypsinized, filtered to remove clumps, and aliquoted into tubes at 1 $\times$ 10⁶ cells per tube. Cells

were suspended in a block buffer of 0.5% FBS in Dulbecco's phosphate buffered saline, containing Ca^{2+} and Mg^{2+} (Mediatech, Manassas, VA), for 30 min at 4 °C, centrifuged at 2000 rpm for 2 min, resuspended in block buffer containing primary mAb, i.e., mAb 4, mAb 5 or an isotype-matched negative control (NC) mAb at concentrations ranging from 0.005 µg/ml to 50 µg/ml and incubated for 1 h at 4 °C. Cells were washed four times with block buffer, then resuspended in block buffer containing phycoerythrin-conjugated goat anti-mouse IgG (1/500 dilution, Jackson ImmunoResearch) and incubated an additional 30 min at 4 °C. After repeating the washing steps, cells were resuspended in 150 µl block buffer and samples were run on a 5-laser BD Biosciences LSR II Flow Cytometer. Ten thousand events per sample were collected and the data were analyzed using Flow jo 9.2 software (Treestar, OR). U2O-S osteosarcoma cells (ATCC) at a density of 90% confluence were transiently-transfected with a CMV-based expression plasmid encoding full-length hGFR α 3 including the glycosphosphatidylinositol linkage sequence (Gly31-Trp400) or empty vector (negative control) in 75-cm² flasks, using 15 µg DNA and 30 µl lipofectamine2000 (Life Technologies), according to the manufacturer's protocol. Forty-eight hours after transfection, cells were collected and processed for flow cytometry as described above.

A panel of eleven human cancer cell lines with unknown but presumed low or absent expression of hGFR α 3 (Daudi cells – B lymphoblast, MCF-7 – breast cancer, A431 – skin cancer, BXPC3 – pancreatic cancer, CAL27 – tongue squamous, cFPAC – pancreatic cancer, HT-29 – colorectal cancer, H1975 – lung cancer, G361 – skin melanoma, MDA – epithelial, and SK Mel-5 – skin melanoma) and NBL-S cells (that express native hGFR α 3) were screened for binding to Dylight-labeled mAbs (4, 5, and NC) using 2 µg mAb per 100 µl volume (0.5×10^5 cells).

The number of hGFR α 3 receptors expressed on the surface of NBL-S and transiently-transfected U2O-S cells (collected 72 hours after transfection) was estimated by determining the mAb binding capacity using pre-calibrated anti-mouse-Fc-coated microspheres and Dylight-labeled mAbs (5 µg of mAbs 4, 5 or NC per 100 µl volume containing 0.5×10^5 cells), according to the manufacturer's protocol (Quantum Simply Cellular kit #815, Bangs Laboratories). All samples to be compared were run on the same day on a SORP LSR II (BD) cytometer following standard rainbow bead QC. Data was collected for the singlet cell population in the desired fluorescent channel (log APC), 10,000 events per sample. The number of mAb binding sites per sample was calculated using the Quickcal v2.3 data analysis program and the lot-specific Quickcal template included with the kit.

2.8. Binning with hFABP4

In tandem, classical sandwich, and premix binning assays with hFABP4 and mAbs 6, 7, 8, and 9 were performed on an Octet equipped with amine-reactive sensors.

To prepare the surfaces for an in tandem binning assay, hFABP4 was either amine-coupled or captured via its His-tag using a pre-immobilized mouse anti-6-His mAb. Amine-coupling was performed by activating the sensors with a freshly prepared mixture of 20 mM EDC and 5 nM sulfo-NHS, coupling 10 µg/ml hFABP4 or 30 µg/ml mouse anti-6-His mAb

in 0.1 M MES pH 5.0 buffer, and then blocking excess reactive esters with 1 M ethanolamine pH 8.5. The activation, coupling, and blocking steps were each allowed 10 min. The anti-His surfaces were then used to capture 2 µg/ml hFABP4 for 10 min. Having immobilized the hFABP4 (either by amine-coupling or by anti-His capture) anti-FABP4 mAbs at a binding site concentration of 270 nM to 400 nM were bound consecutively for 10 min each, varying the mAbs analyzed per sensor to address every pairwise permutation of mAbs. The surfaces were regenerated with two 30-s exposures of 75 mM phosphoric acid and all binning cycles were performed in quadruplicate.

To prepare the surfaces for classical sandwich and premix binning assays, anti-hFABP4 mAbs were each prepared at 30 µg/ml in 100 mM MES pH 5.0 buffer and amine-coupled onto replicate sensors, as described above. To perform a classical sandwich binning, recombinant hFABP4 (or buffer) was bound at a typical concentration of 200 nM followed by 20 µg/ml (i.e., 270 nM binding sites) anti-hFABP4 mAb or buffer, allowing 3 min per step. To perform a premix binning, 200 nM recombinant hFABP4 was prepared with and without 400 nM binding sites of each mAb (final concentrations) and these samples were bound for 15 min. All interactions were analyzed on freshly prepared surfaces and most interactions were analyzed on duplicate sensors.

A classical sandwich binning using native FABP4 extracted directly from human plasma was performed on a Biacore equipped with a CM5 sensor chip. Anti-hFABP4 mAbs (7, 8, and 9) and a negative control (i.e., an irrelevant) mAb were each prepared at 40 µg/ml in 10 mM sodium acetate pH 5.0 and amine-coupled onto separate flow cells using a standard EDC/NHS coupling chemistry; final immobilization levels were approximately 15,000 RU per channel. Human plasma was diluted four-fold in running buffer (HBST+) and injected for 10 min over all flow cells. Then, various anti-FABP4 mAbs were each injected for 2 min to test for sandwich pairing with the immobilized mAbs. The surfaces were regenerated with three 15-s injections of 75 mM phosphoric acid.

2.9. Biosensor data analysis

Octet and Biacore data were processed and analyzed in BiaEvaluation v. 4.1. Octet data were Y-averaged to zero prior to the start of the binding step of interest; no referencing was applied. Biacore data were double-referenced before data analysis and interpretation. To obtain double-referenced data, the responses of a reference surface were subtracted from those of an active surface to yield “reference-subtracted” responses. The “reference-subtracted” responses of a buffer analyte were then subtracted from those of the sample analytes to yield “double-referenced” responses. Kinetic data were analyzed globally using a 1:1 Langmuir model with mass transport (Myszka et al., 1998). ProteOn data were processed and analyzed in ProteOn Manager v. 2.1. ProteOn data were referenced by subtracting the responses of the interspots from those of the neighboring reaction spots, yielding “reference-subtracted” responses. The “reference-subtracted” responses of a buffer analyte were then subtracted from those of the sample analytes to yield “double-referenced” responses.

3. Results

3.1. Binning with a homogeneous monovalent antigen often results in the same outcome independent of the assay format employed

Using label-free biosensors, we routinely perform epitope binning assays with monovalent Ags in three different assay formats that we termed “in tandem”, “classical sandwich” and “premix” methods (Abdiche et al., 2009). Similar epitope binning assay formats have been employed widely in the ELISA and biosensor literature without a universally accepted terminology to describe them; our definitions merely provide a convenient way to distinguish them. In an in tandem binning format, two mAbs are presented one after another to an immobilized Ag to determine if a saturating level of the first mAb blocks the binding of the second mAb. A classical sandwich binning format uses an immobilized mAb to capture an Ag after which a second mAb is presented in solution to determine whether both mAbs can bind the Ag simultaneously and thus form a “sandwich pair”. In a premix binning format, an Ag is equilibrated in solution with the first mAb, and then the mixture is presented to a second, immobilized mAb. A comparison of the binding response of the mixture to that of Ag alone is used to determine whether both mAbs can bind the Ag simultaneously. The same surface preparation is used in classical sandwich and premix methods, but the order and content of the flowed analyte are different. For a monovalent Ag that is homogeneous with respect to the panel of mAbs being tested, the ability of mAbs to block one another or form sandwiching pairs is often independent of the assay format used and the order in which the mAbs are presented to the Ag.

Fig. 1A shows a schematic representation of the three assay formats described above for a simple binning experiment that uses a monovalent Ag and two mAbs. Fig. 1B shows the biosensor-based binning results obtained in each of these assay formats using hPGRN as a model Ag and two anti-hPGRN mAbs (mAb 1 and mAb 2). In this example, the in tandem data shows that the mAbs do not block one another's binding to the immobilized Ag, suggesting that they bind non-overlapping epitopes. They would thus be assigned to different epitope “bins”. This result is corroborated by the results from both classical sandwich and premix binning assay formats, which confirm that the two mAbs can bind the Ag simultaneously. Fig. 1C summarizes these results in terms of a sandwiching binning matrix, i.e., all three assay formats yield the same binning result irrespective of the order in which the mAbs are presented to the Ag.

3.2. Binning with a heterogeneous antigen can result in conflicting outcomes from different assay formats

In a simple case of Ag heterogeneity, two mAbs bind different epitopes that reside on separate subpopulations of an Ag sample, i.e., the epitopes targeted by the two mAbs are not available on the same Ag molecule. Fig. 2A shows schematically how a heterogeneous Ag affects the outcome from each assay format. To illustrate this concept, we simulated a heterogeneous Ag by mixing two unrelated Ags (i.e., hPGRN and hPCSK9) and performed binning assays on this mixture with an anti-hPGRN mAb (mAb 1, from the

example described above) and an anti-PCSK9 mAb (mAb 3). Fig. 2B shows the biosensor results obtained from these experiments. A heterogeneous Ag confounds the interpretation of these epitope binning assays because the results from different assay formats appear to contradict one another as follows: (1) the mAbs do not block one another when binned in an in tandem assay, (2) the mAbs do not sandwich pair with one another when binned in a classical sandwich assay, and (3) the mAbs have “no detectable effect” on one another when binned in a premix assay, i.e., for a given immobilized mAb, a sensorgram for an Ag/mAb premixed sample is indistinguishable from replicate sensorgrams for the Ag alone, where the total Ag concentration is held constant in the Ag alone and Ag/mAb premixed samples. We refer to this conflicting binning pattern across the three assay formats as the “signature heterogeneous binning profile” (Fig. 2C). In the context of a premix assay, we use the term “full block” to describe the situation where there is an undetectably low concentration of free Ag in a premixed sample under the given assay conditions. If this is achieved when the solution and immobilized mAbs are the same mAb, we refer to this special case as a “full self-block”. We also use the term “full block” or “full self-block” in the context of classical sandwich assays and in tandem assays when no binding of the competing mAb is detected.

3.3. Binning with hGFR α 3 exhibited the “signature heterogeneous binning profile”

When analyzing a large panel of hybridoma-derived mAbs raised against recombinant hGFR α 3, we observed binning results for some mAbs that conformed to the “signature heterogeneous binning profile” described above; we use two representatives from this set (mAbs 4 and 5) to illustrate our findings (Fig. 3). First, when binned using an in tandem assay, mAbs 4 and 5 were each able to block themselves but did not block one another. (The poor self-block for mAb 4 can be explained due to its rapid decay from the immobilized Ag, which exposed free Ag binding sites that mAb 4 subsequently reoccupied to the same level when injected in the second step — see dotted line in the top left panel of Fig. 3A.) Second, in a classical sandwich assay, mAbs 4 and 5 did not form a sandwich pair regardless of which one was coupled. Third, a hGFR α 3/mAb 4 premixed sample did not bind to coupled mAb 4, which demonstrated a “full self-block”, but when presented to coupled mAb 5, the same premixed sample showed binding that was identical to the binding of hGFR α 3 alone, thereby demonstrating “no detectable effect”. A similar result was obtained in the reverse orientation. Thus, a hGFR α 3/mAb 5 premixed sample barely bound to coupled mAb 5, consistent with a self-block (Fig. 3A), but when presented to coupled mAb 4, the same premixed sample demonstrated “no detectable effect”. As in the previous example with our simulated heterogeneous Ag, the apparent conflict in the binning results obtained across the different assay formats can be reasoned by presuming that the hGFR α 3 sample is a mixture of (at least) two distinct subpopulations, each selectively targeted by a different mAb.

To prove that mAb 4 and mAb 5 detected mutually exclusive subpopulations in our hGFR α 3 preparation, we depleted each subpopulation *via* treatment with mAb-coated Dynabeads and then tested the Dynabead-depleted hGFR α 3 samples for binding to coupled mAbs using a biosensor assay (Fig. 3 — far

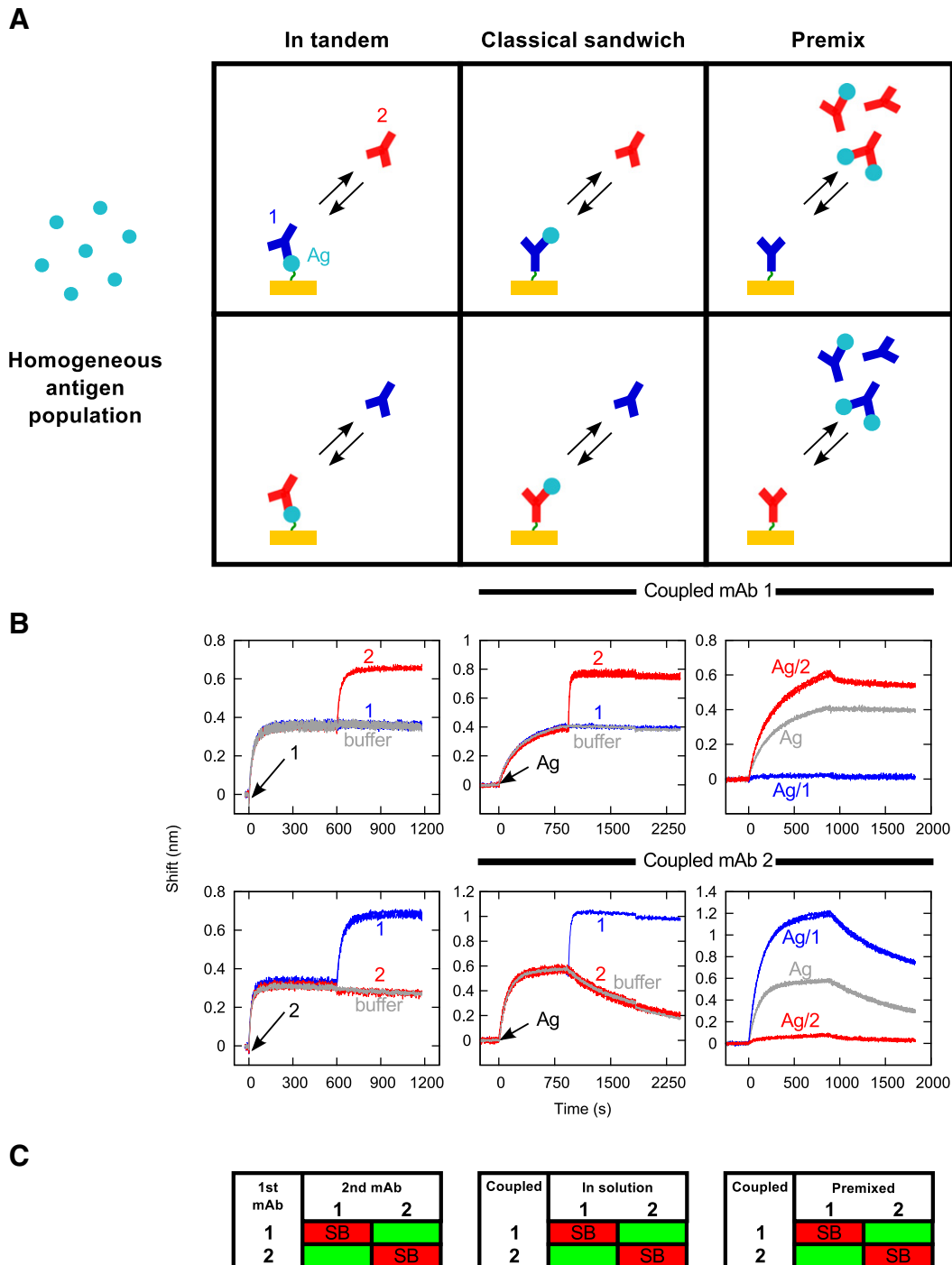


Fig. 1. Binning two mAbs (1 and 2) with hPGRN. (A) Schematic view of three different assay formats. (B) Biosensor data collected on an Octet – see [Material and methods](#) for experimental details. (C) Binning matrices: sandwiching pair – green; blocking pair – red; and self-block – SB (red) control.

right panel). Thus, samples that had been pre-depleted with mAb 4-coated beads showed no detectable binding to coupled mAb 4, demonstrating that the beads had fully depleted the Ag subpopulation targeted by mAb 4. However, when presented to coupled mAb 5, the same mAb 4-depleted samples bound with

an identical sensorgram as that for blank bead-depleted Ag. These observations suggested that the Ag subpopulation recognized by mAb 5 was not altered by pre-depletion with mAb 4, and that the two mAbs targeted different Ag subpopulations. This hypothesis was corroborated by running the

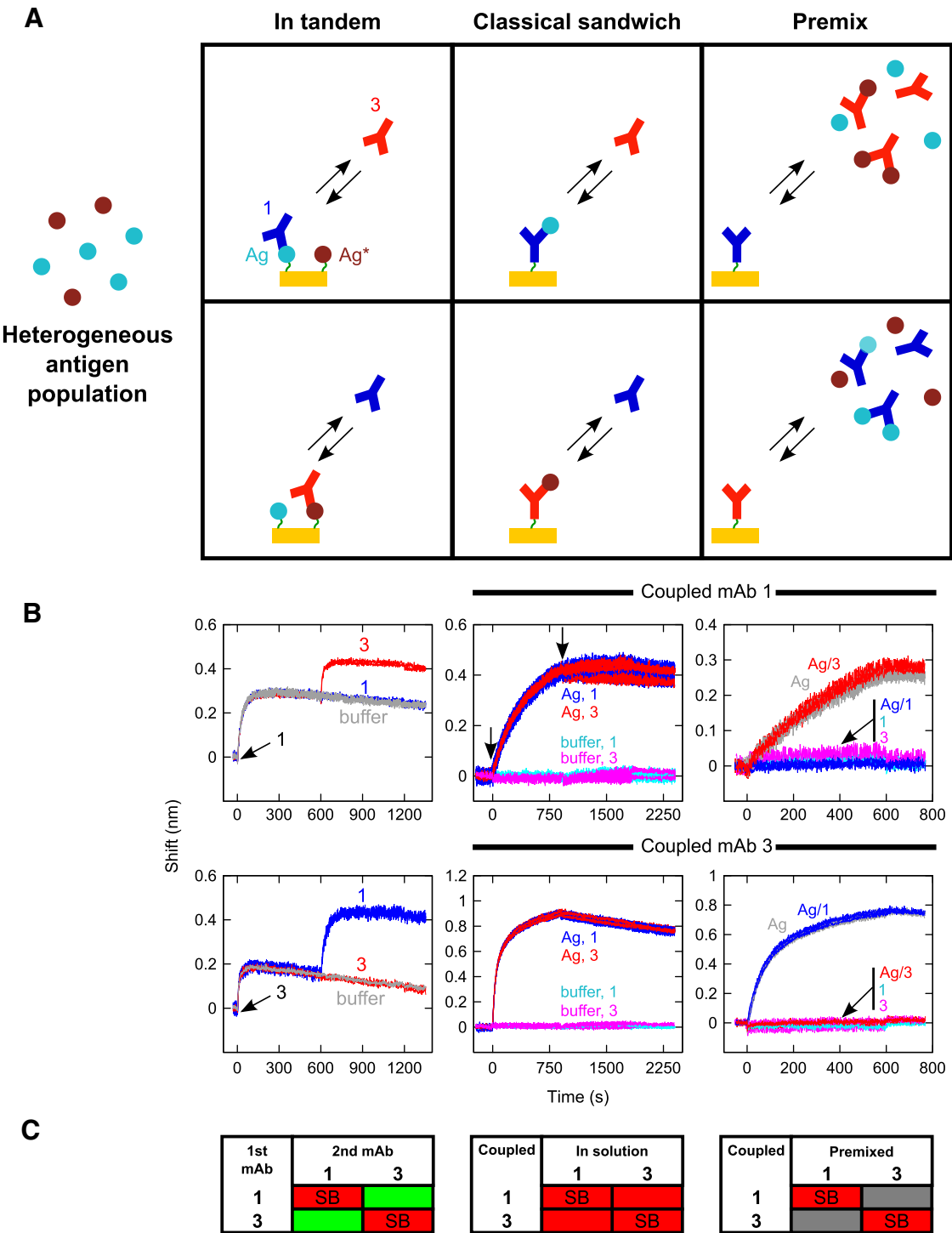


Fig. 2. Binning two mAbs (1 and 3) with a “heterogeneous antigen” that was simulated by mixing hPGRN and hPCSK9. (A) Schematic view of three different assay formats. (B) Biosensor data collected on an Octet — see [Material and methods](#) for experimental details. Vertical arrows in the center panel denote the start points for the first (Ag or buffer) and second injections (mAb or buffer). (C) Binning matrices: no blocking (or apparent sandwiching pair) — green; no sandwiching (or apparent blocking pair) — red; no detectable effect — grey; and self-block — SB (red). This example defines the “signature heterogeneous binning profile” for an Ag that exists as two subpopulations as identified by two mAbs whose epitopes reside on different Ag subpopulations.

experiment in a reverse manner, *i.e.*, Ag was pre-depleted with mAb 5-coated beads, and then presented to coupled mAb 4 or coupled mAb 5. The Dynabead results also suggested that mAb

5 was able to bind with low affinity to mAb 4’s subpopulation, explaining the small amount of depletion observed for the mAb 5-depleted sample on coupled mAb 4.

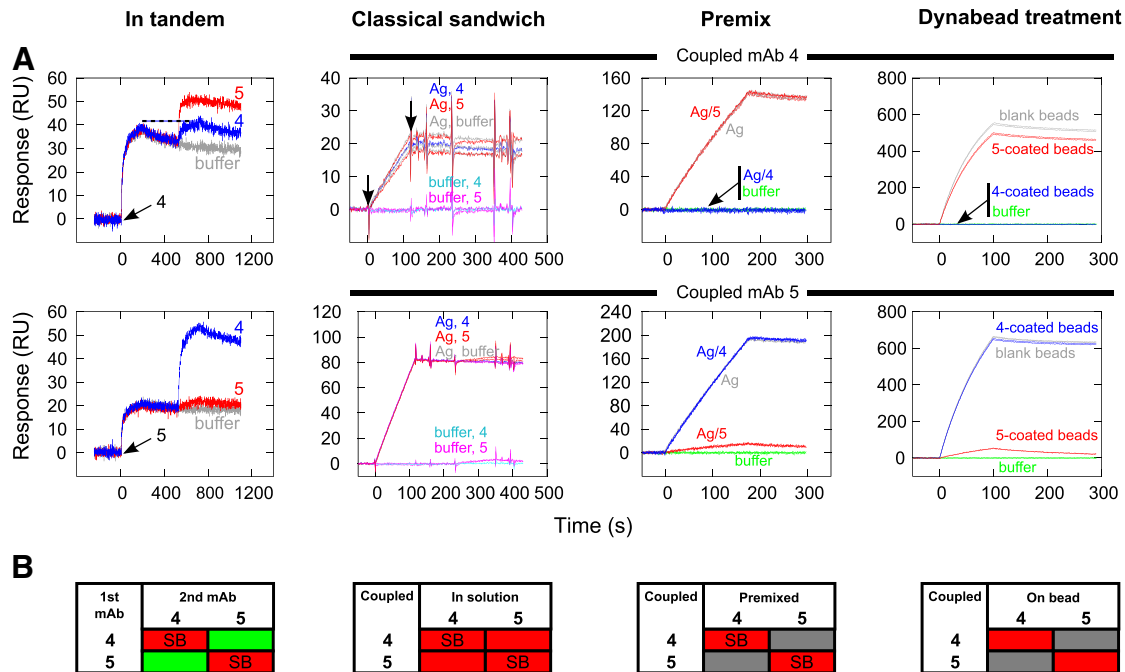


Fig. 3. Binning two anti-hGFR α 3 mAbs (4 and 5) with monovalent hGFR α 3 (preparation P1a) via different assay formats on SPR biosensors. (A) Biosensor data generated on a ProteOn XPR36 (except the classical sandwich data, which were generated on a Biacore 2000). Vertical arrows in the classical sandwich panel denote the start points for the first (Ag or buffer) and second injections (mAb or buffer). The dotted line in the top left panel indicates that the binding response for mAb 4 in the second injection reaches the same level as in the first injection, consistent with a self-block. (B) Binning matrices using the same color key as described in Fig. 2. The color scheme for the Dynabead treatment matrix is as follows: low or undetectable depletion – grey; significant or complete depletion – red. See [Material and methods](#) for experimental details.

3.4. Alternative sources of hGFR α 3 were more homogeneous with respect to our panel of mAbs

To examine whether the heterogeneity we described above was unique to our in-house batch of hGFR α 3 (preparation P1a), we explored a panel of additional preparations from different sources (see [Material and methods](#)). Preparations P1, P2, and P3 were monomeric constructs that encoded mature hGFR α 3; P1a and P1b were two independent batches of the same construct. Preparations P4-Fc, P5-Fc, and P6-Fc were bivalent constructs that encoded the mature hGFR α 3 fused via a flexible linker to human Fc γ 1. A biosensor-based kinetic analysis of our in-house heterogeneous preparation (P1a) gave apparent K_D values of 0.4 nM and 1.8 nM to immobilized mAbs 4 and 5, respectively (Fig. 4A); these affinities are described as apparent because they were determined to a mixed population of Ag. In contrast, the other monomeric preparations (P1b, P2, and P3) bound significantly to immobilized mAb 4 and with negligible signal to immobilized mAb 5, as exemplified by preparation P2 (Fig. 4B), which bound with a K_D of 0.2 nM to immobilized mAb 4 and bound with a very low response to immobilized mAb 5 at a nominal concentration of 1 μ M hGFR α 3, consistent with a very weak affinity. Similarly, all three of the hGFR α 3-hFc fusion constructs bound significantly to immobilized mAb 4 and barely to immobilized mAb 5. Fig. 4C shows that all six additional preparations bound significantly to immobilized mAb 4 and barely to immobilized mAb 5,

suggesting that they were all more homogeneous than our original preparation (P1a). Thus, mAb 4 bound an epitope that was common to all preparations, while mAb 5 bound an epitope that was unique to the P1a preparation. Heterogeneity, in this case, was sample dependent.

3.5. Cell-based assays discriminated the epitopes of mAb 4 and mAb 5

To determine whether mAb 4 or mAb 5 bound a native, functional epitope, we tested their ability to block the hART/GFR α 3/Ret-mediated signaling pathway with a cell proliferation assay in a human neuroblastoma cell line, namely NBL-S, which expresses both endogenous GFR α 3 and its co-receptor Ret (Baloh et al., 1998). The results of this assay confirmed that mAb 4 blocked hART-induced cell proliferation (indicated by the arrow in Fig. 5A), whereas mAb 5 did not. In addition, mAb 4, but not mAb 5, partially blocked the hART-induced phosphorylation of intracellular signaling protein CREB as determined by Western blot of NBL-S cell lysates (indicated by the arrow in Fig. 5B). Consistent with these observations, a FACS analysis showed that mAb 4 bound in a dose-dependent manner to native hGFR α 3 expressed on the cell surface of NBL-S neuroblastoma cells, while mAb 5 did not bind significantly above the background autofluorescence of the cells (Fig. 5C – left panel). This result was corroborated by a similar FACS analysis on another human neuroblastoma cell line that

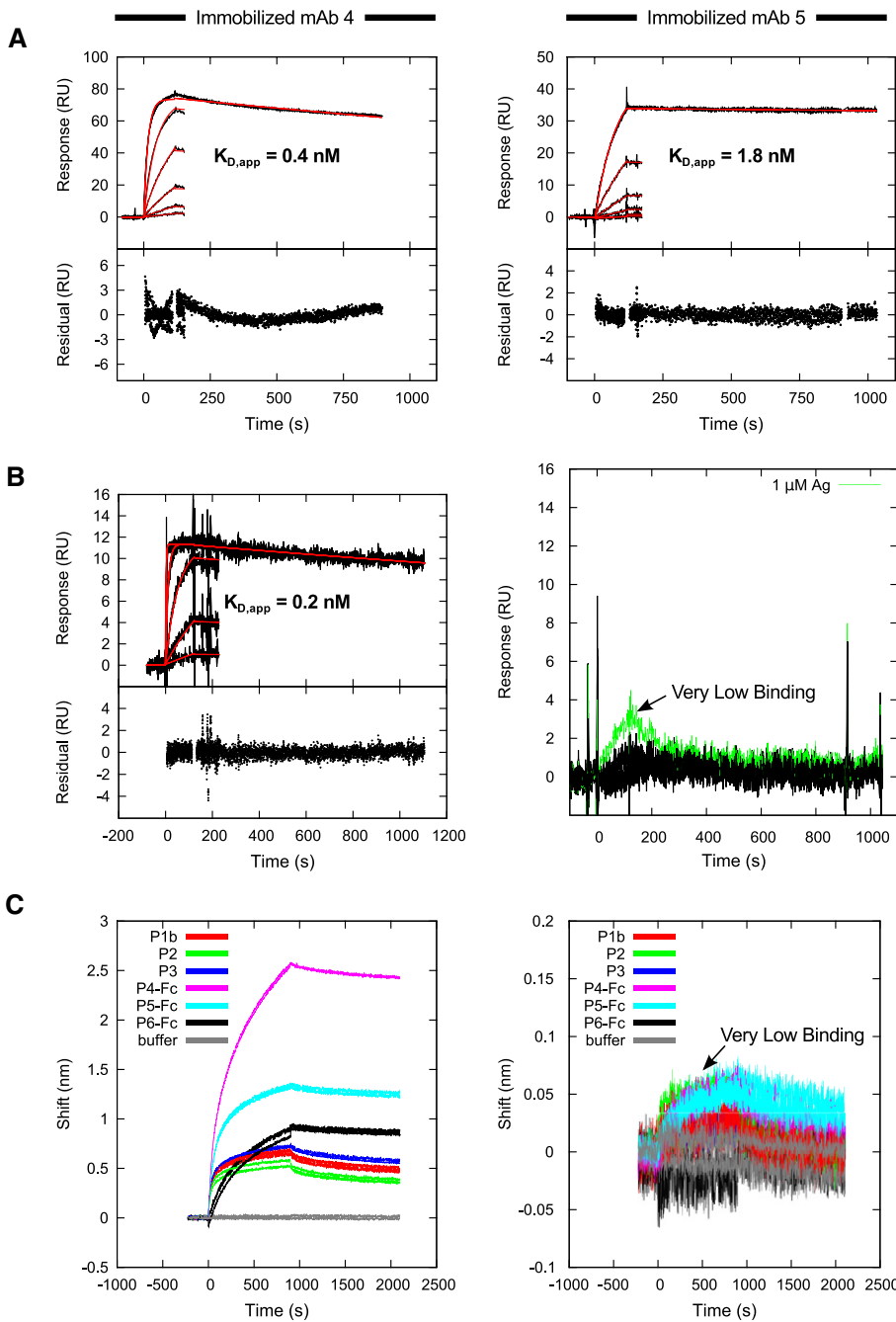


Fig. 4. Biosensor analysis of various preparations of hGFRα3 binding to immobilized mAb 4 (left) and mAb 5 (right). (A) Global kinetic analysis of the monovalent preparation P1a injected as a three-fold dilution series with a top nominal concentration of 100 nM (left) or 1000 nM (right) over anti-murine captured mAbs on a Biacore chip. (B) Similar analysis as that shown in panel A, using the monovalent preparation P2 as a five-fold dilution series with a top nominal concentration of 600 nM (left) or 1000 nM (right). Each panel shows an overlay plot of the measured data (black lines) and the global fit (red lines). The apparent kinetic rate constants for the data shown in panels A and B are shown below (ND – not determined).

hGFRα3	mAb	Apparent k_a ($M^{-1} s^{-1}$)	Apparent k_d (s^{-1})	Apparent K_D (nM)
P1a	4	6.4×10^5	2.3×10^{-4}	0.4
P2	4	7.8×10^5	1.6×10^{-4}	0.2
P1a	5	1.3×10^4	2.3×10^{-5}	1.8
P2	5	ND	ND	ND

(C) Octet data for various preparations of hGFRα3 (P1b – red, P2 – green, P3 – blue, P4-Fc – pink, P5-Fc – cyan, P6-Fc – black and buffer – grey) at a nominal binding site concentration of 200 nM interacting with streptavidin-captured biotinylated mAbs.

expresses native GFR α 3, namely SH-SY5Y (data not shown). However, by FACS and immunohistochemical analysis, both mAbs 4 and 5 bound to full-length hGFR α 3 when over-expressed on the surface of transiently-transfected U2O-S cells (Fig. 5C – center panel) or CHO cells (data not shown), suggesting that the epitopes of both mAbs could be recapitulated in an *in vitro* cell-based expression system. We determined empirically that the transiently-transfected cells expressed several hundred-fold more GFR α 3 receptors than the native cells, which may explain why mAb 5 could bind the former cells and not the latter (*i.e.*, avidity would have enhanced mAb binding to the transiently-transfected cells). No binding was detected for mAb 4 to empty vector-transfected U2O-S cells (Fig. 5C – right panel) or CHO cells (data not shown), confirming that the interaction was specific to GFR α 3; in contrast, mAb 5 bound slightly (*i.e.*, nonspecifically) to these cells.

To test the specificity of mAb 4 and mAb 5, we screened them for binding to a panel of eleven human carcinoma cell lines with presumed low or absent expression of GFR α 3 (see [Material and methods](#)); cells known to express native GFR α 3 (NBL-S neuroblastoma) were analyzed in the same experiment. Whereas the binding of mAb 4 was similar to or less than that of an isotype-matched negative control (NC) mAb to all cancer cell lines tested, mAb 5 showed significant binding to ten of the cell lines (all but Daudi cells, Fig. 5D and data not shown). Only mAb 4 bound specifically to the NBL-S cells. These results suggest that mAb 4 likely binds to a native GFR α 3 epitope, whereas mAb 5 is more promiscuous and may recognize a commonly-expressed extracellular epitope, not necessarily specific to GFR α 3.

Furthermore, by Western blot analysis, mAb 5 but not mAb 4, recognized denatured hGFR α 3 (Fig. 5E), suggesting that the high apparent affinity of $K_D = 1.8$ nM (Fig. 4A) determined for the interaction of mAb 5 with the P1a preparation likely represented binding to a denatured form of hGFR α 3 contaminating that particular sample.

3.6. Common treatments of antigens may promote heterogeneity that causes artifacts in the results of binning assays

Some Ags become heterogeneous (*e.g.*, may partially denature) upon exposure to conditions commonly used in analytical assays. We encountered this problem when binning with human fatty acid binding protein 4 (hFABP4). Four positive binders (mAbs 6, 7, 8, and 9) were identified using an ELISA format that employed recombinant hFABP4 adsorbed onto a standard polystyrene microtiter plate. Using a similar assay format on a biosensor, we showed that all four mAbs bound freshly amine-coupled hFABP4 to varying degrees. An in tandem binning assay revealed that none of the mAbs blocked one another, suggesting that the four mAbs bound non-overlapping epitopes and thus belonged to different “bins” (Fig. 6 – left panel). However, acid-regeneration of the amine-coupled hFABP4 augmented the binding responses of mAbs 6 and 9, decreased the binding response of mAb 8, and abolished the binding of mAb 7 (Fig. 6 – center panel). Thus, acid-treatment of the immobilized hFABP4 appeared to expose the epitopes of mAbs 6 and 9, make mAb 8's epitope less available, and destroy mAb 7's epitope. A different binning pattern was observed when we employed a milder immobilization method, namely capture of the hFABP4 *via* a pre-immobilized anti-His mAb. In this experiment, mAbs 7 and

8 bound with significant responses and did not block one another, mAb 9 barely bound, and no binding was detected for mAb 6 (Fig. 6 – right panel). While Fig. 6A shows a representative subset of the binning data, Fig. 6B shows the binning matrix for all sixteen interactions tested. Some interactions gave no (or low) binding responses due to no (or barely) detectable binding of the mAbs to the presented Ag population; these interactions are marked with an X. When solution-based hFABP4 was presented to the immobilized mAbs, it bound significantly to mAbs 7 and 8 but not to mAbs 6 or 9. Therefore, only mAbs 7 and 8 were amenable to classical sandwich and premix binning assays (Fig. 7).

We verified that mAbs 7 and 8 bound native epitopes by performing a classical sandwich binning assay with the native Ag. This was feasible because hFABP4 is a secreted soluble Ag that is abundant in human plasma (Stejskal et al., 2008) and mAb 8 bound it with high affinity, which rendered it an efficient capture mAb. The inset in Fig. 7 shows that solution-based mAb 7 was able to bind native FABP4 that was first captured from human plasma *via* immobilized mAb 8.

4. Discussion

4.1. When antigen heterogeneity is identified, assays that utilize native antigen can help discriminate which antigen subpopulations are relevant to drug discovery

Our results demonstrate that when biosensor-based epitope binning assays on monovalent Ags are performed rigorously, they can reveal whether an Ag is heterogeneous by identifying mAb pairs that give the “signature heterogeneous binning profile”, as described in Figs. 2 and 3. Ag heterogeneity does not necessarily imply that some subpopulations are irrelevant or non-native; it is possible that all distinct subpopulations within a heterogeneous Ag are functional. Thus, if Ag heterogeneity is suspected, other assays should be conducted to verify which subpopulations represent native epitopes. For example, when studying mAbs raised against hGFR α 3, we complemented our biosensor assays with FACS analysis on two different human neuroblastoma cell lines that express native GFR α 3. These results clearly identified that one of the subpopulations in our “heterogeneous” recombinant preparation of hGFR α 3 resembled the native Ag as expressed on neuroblastoma cells (the subpopulation targeted with high affinity by mAb 4), whereas the second subpopulation was not detectable on the surface of these cells (the subpopulation targeted with high affinity by mAb 5). While we did not identify the root cause of the heterogeneity present in one of our in-house preparations of recombinant hGFR α 3, the biosensor binning results prompted us to seek a more homogeneous source of the Ag to improve the quality and reliability of our screening, kinetics, and binning assays and thus aid in the selection of mAbs that bound native epitopes and the rejection of those that did not.

In our work with hFABP4, the use of plasma-derived Ag helped us to identify anti-FABP4 mAbs in our test panel that bound native epitopes. Furthermore, since we recapitulated these results with purified recombinant hFABP4, it increased our confidence that our recombinant preparation was in a native conformation (when analyzed in solution) and thus

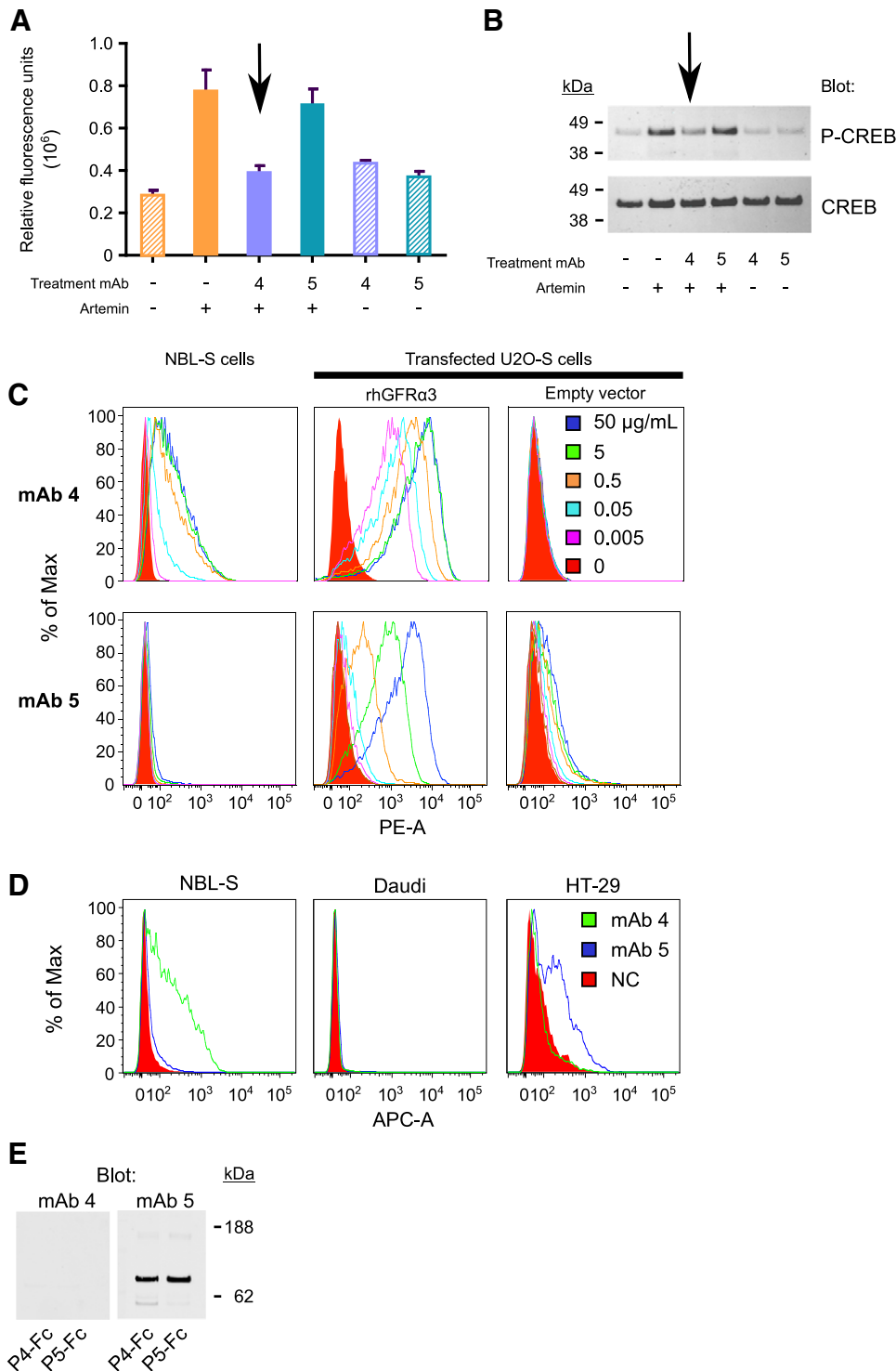


Fig. 5. Discriminating the epitopes of mAbs 4 and 5 using non-biosensor-based methods and various sources of hGFR α 3. (A) NBL-S cell proliferation assay. Data shown are the means $\pm$ SEM of six replicate samples per group. (B) Effect of mAbs on hART-induced CREB phosphorylation in NBL-S cells. Two identical Western blots were prepared from cell lysates and probed with antibodies to either phospho-CREB or CREB, as indicated. (C) FACS analysis of dose-dependent binding of mAbs 4 and 5 (at the concentrations shown in upper right panel) to untransfected NBL-S cells or U2O-S cells transfected with indicated plasmids. The solid red graph shows the binding of an isotype-matched negative control mAb (50 μ g/ml) or, in some experiments, no primary mAb. (D) FACS analysis of Dylight-labeled mAbs binding to a panel of human cell lines. NC, negative control mAb (E) Western blot analysis of mAbs binding to denatured hGFR α 3-Fc fusion proteins.

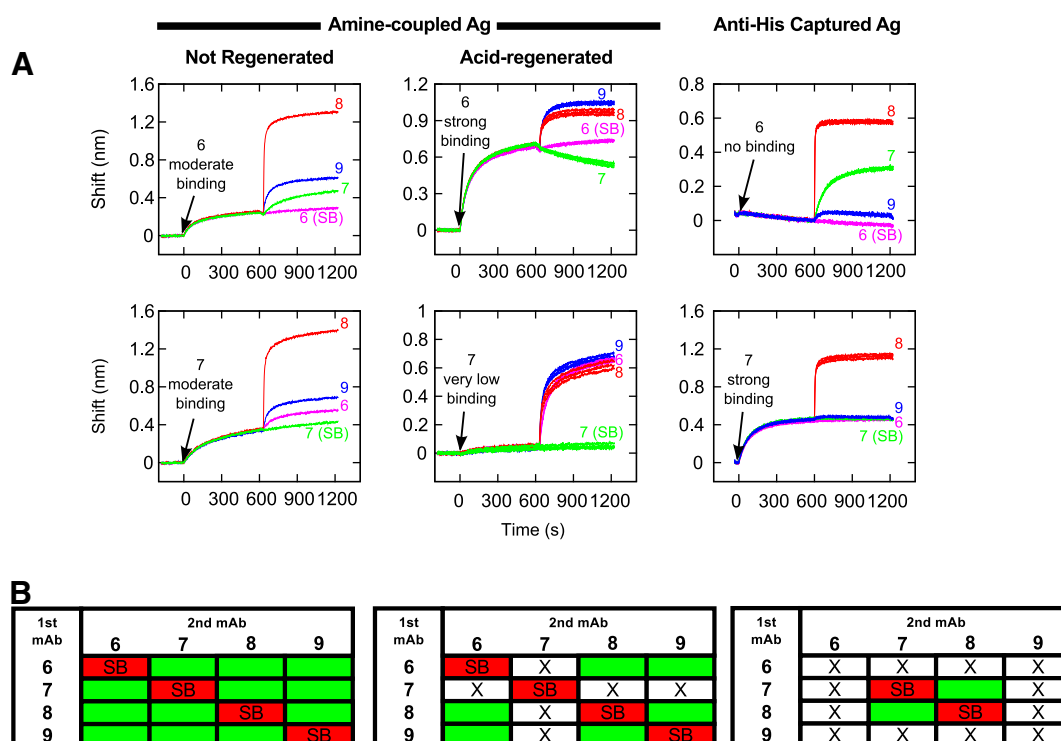


Fig. 6. Effect of immobilization and acid-regeneration on the outcome of in tandem binning assays with recombinant hFABP4. (A) Octet data for an array of mAbs binding to Ag that was immobilized in different ways (as indicated in the plot titles). A single data set is shown in the left panel, while the center and right panels each show triplicate analyses. Data are shown for a subset of the interactions tested. (B) Binning outcomes for the entire 4×4 competition matrix from each immobilization method; non-blocking pair – green, self block – SB (red) and barely binds or no binding – X.

was reliable for use in screening and binning assays that employed solution-based Ag.

4.2. An unidentified case of antigen heterogeneity may confound the interpretation of epitope binning assays and result in inaccurate affinity determinations

One of the primary uses of binning data is to combine them with functional data from cell-based or *in vivo* assays. Often, only a subset of epitope bins will contain functionally active mAbs. Typically, the results of a binning campaign on a large panel of mAbs are used to select a smaller panel for testing in more costly and time consuming cell-based and *in vivo* assays. In our work with hGFR α 3, if only the “classical sandwich” results were available, mAbs 4 and 5 would appear to belong to the same bin. If mAb 5 was chosen as a representative of this epitope bin for further characterization in a cell-based proliferation assay, the bin may be erroneously designated as “non-functional”. Since only mAb 4 was shown to be “functional” in this cell-based assay, the classical binning assay would be unable to predict functional activity. Therefore, not only can a heterogeneous Ag lead to a mAb being assigned to the wrong epitope bin, but it may lead to the rejection of functionally interesting bins. Moreover, a heterogeneous Ag can mislead an affinity ranking of mAbs in a kinetic analysis because the affinities are determined to a mixed Ag population. In our example with the “heterogeneous” hGFR α 3 preparation

P1a, if the Ag subpopulation targeted by mAb 4 comprised 50% of the total protein, then the k_a (and thus K_D) value would need to be corrected twofold. If the subpopulation targeted by mAb 5 comprised only 5% of the total protein, then the k_a (and thus K_D) value would need to be corrected twenty-fold. Thus, if the Ag was assumed to be homogeneous, this assay orientation would be unreliable for comparing the affinities of hGFR α 3 towards mAb 4 and mAb 5.

4.3. Diagnosing antigen heterogeneity using epitope binning assays can expedite target validation and the identification of therapeutic mAbs

If not identified early in drug discovery, pursuing mAbs that bind to non-native subpopulations of a heterogeneous Ag that are destined to fail in cell-based or *in vivo* functional assays will invalidate interesting targets and waste resources by investing them in the wrong mAbs. Several researchers have discovered mAbs that bound to surface-adsorbed Ag but not to solution-based Ag across a broad range of targets, including enzymes such as α -mannosidase (Mierendorf and Dimond, 1983), creatine kinase (Vaidya et al., 1985), lactate dehydrogenase (Vaidya et al., 1985), and tryptophan synthase (Friguet et al., 1984), toxins such as abrin (Zhou et al., 2007) and botulinum neurotoxin (Zhou et al., 2008) and other soluble proteins such as cytochrome C (Schwab and Bosshard, 1992), follicle stimulating hormone (Miller et al., 1983), and ovalbumin

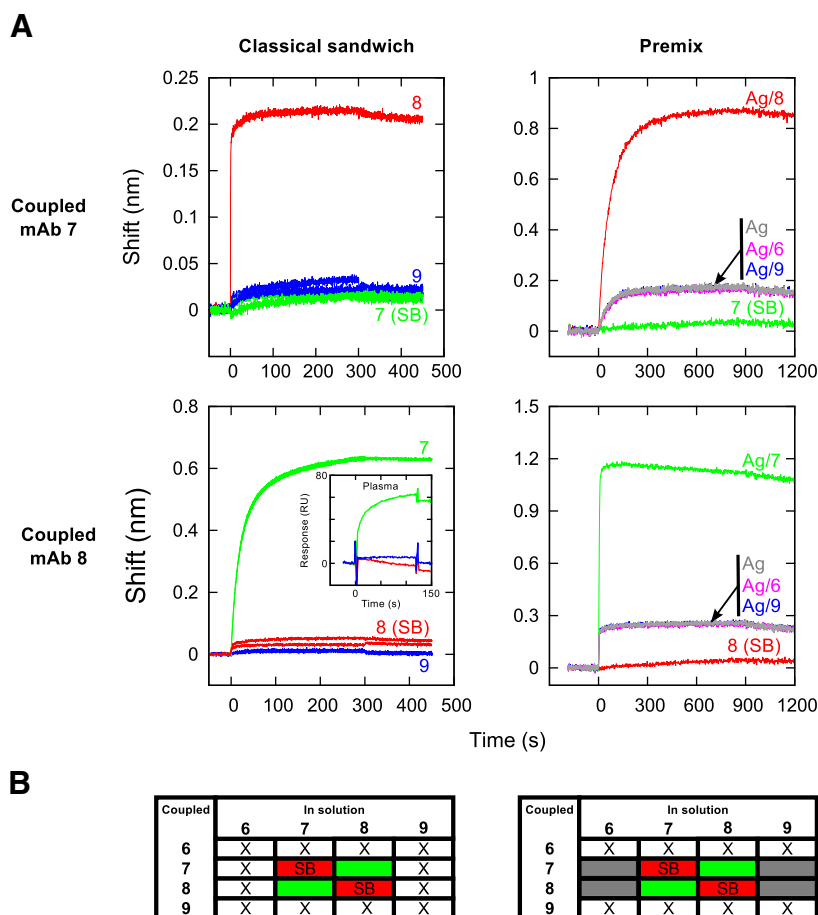


Fig. 7. Epitope binning with solution-based hFABP4. (A) Octet data using purified recombinant hFABP4 (main panels) and Biacore data using unpurified native FABP4 in human plasma (inset). (B) Binning matrix, where sandwiching pair – green, blocking pair – red, no detectable effect – grey, self block – SB (red), and barely binds or no binding – X.

(Houen and Koch, 1997). The discovery of such mAbs using hybridoma technology may be enhanced by adjuvant-induced Ag denaturation during the immunization protocol (Houen and Koch, 1997; Scibienski, 1973). Despite these results, ELISA employing plate-adsorbed Ag continues to be a commonly used assay format in the preliminary screening of hybridoma supernatants. Indeed, this method was used to identify the mAbs raised against hGFR α 3 and hFABP4 that we describe in this work, and in both cases it led to the discovery of false positives, i.e., mAbs that did not bind native epitopes. Also worrisome are the false negatives that one may encounter when preliminary screening is conducted with immobilized Ag, if conformational epitopes of interest have been distorted or masked upon immobilization of the Ag.

Some Ag preparations may contain a mixture of properly folded and denatured Ag prior to adsorption onto a plate; therefore, mAbs that target a denatured subpopulation of Ag have the potential to bind Ag even when the Ag is presented in solution to an immobilized mAb (as we demonstrated with the P1a preparation of hGFR α 3 in Figs. 3 and 4A). In this case, biosensor-based epitope binning assays can help identify whether mAbs in a test panel appear to bind distinct Ag subpopulations.

4.4. Recommendations for detailed binning studies

Our results lead us to a set of recommendations for conducting detailed binning assays with monovalent Ags in a way that yields the clearest possible result, maximizes the chances of accurately identifying Ag heterogeneity and thus ameliorates the correlation between epitope bins and functional activity.

4.4.1. Recommendation 1: use multiple binning assay formats

Whenever possible, bin in more than one assay format because the in tandem, classical sandwich, and premix methods are complementary, with each having its advantages and limitations (Abdiche et al., 2009). The premix method is the most informative because the “no detectable effect” outcome may indicate that the Ag is heterogeneous with respect to the panel of mAbs being tested. However, it requires *a priori* knowledge of the K_D values of each premixed mAb for the Ag to ensure that the concentration of premixed mAb used in the assay is high enough to saturate the Ag (discussed further in Recommendation 2: use a premix assay format whenever possible). The classical sandwich is the least informative format in the case of a heterogeneous Ag,

because mAbs that bind different subpopulations of a heterogeneous Ag will appear to block one another and therefore may be erroneously assigned to the same bin. Another drawback of the classical sandwich format is that, since it is amenable only to monovalent Ags, a false sandwiching pair can arise if a presumed monovalent Ag forms multimers or aggregates. Since both the classical sandwich and pre-mix methods employ solution Ag, they are preferable to an in tandem assay that relies upon immobilizing the Ag in a way that preserves its native conformation and allows all epitopes to be accessible. To minimize the chance of introducing heterogeneity when conducting in-tandem binning assays, the Ag should be immobilized via the mildest possible method, such as a reversible capture if the Ag is suitably tagged. However, one must rule out the possibility that some epitopes may be occluded upon site-specific capture, by complementing an in-tandem assay with an assay that employs solution Ag. In tandem assays should be conducted on sparse capacities of the immobilized Ag to minimize crowding artifacts that can complicate the interpretation of the results.

If Ag heterogeneity is suspected, employ all three binning assay formats to determine whether the results conform to the “signature heterogeneous binning profile”, as summarized in Fig. 2.

4.4.2. Recommendation 2: use a pre-mix assay format whenever possible

To obtain definitive results across an entire panel of mAbs in a pre-mix assay, whether the outcome is sandwiching, blocking, or “no detectable effect” (in the case of an inactive mAb, an irrelevant mAb, or a heterogeneous Ag) it is important to empirically verify that each Ag/mAb pre-mixed sample contains a very low concentration of free Ag; in the special case of a heterogeneous Ag, “free Ag” refers to the distinct subpopulation targeted by the pre-mixed mAb. Therefore, we employ experimental conditions that are likely to saturate the Ag with each pre-mixed mAb being tested by, (1) fixing the Ag concentration to be higher than the anticipated K_D of the solution-based interaction, (2) using a concentration of pre-mixed mAb that is in molar excess compared to the concentration of the Ag, and (3) using a pre-mixed mAb concentration that is sufficient to give a “full self-block”. The concentration at which the Ag is screened should be considered carefully to avoid introducing non-specific binding artifacts, i.e., it should be high enough to facilitate detection by the entire panel of immobilized mAbs, but not too high, which may promote aggregation of the Ag and nonspecific binding between the solution-based and immobilized mAbs (since a high Ag concentration necessitates the use of an even higher pre-mixed mAb concentration to saturate the Ag). Various factors affect the “limit of detection” of free Ag in the pre-mixed sample, such as the binding kinetics of the Ag for each of the immobilized mAbs (i.e., a high affinity mAb may be able to detect a lower free concentration of the Ag than a lower affinity mAb) and the sensitivity (i.e., signal-to-noise ratio) of the biosensor platform employed.

Three outcomes are possible when the binding responses of “Ag alone” and the “Ag/mAb pre-mixed sample” are compared for a given immobilized mAb. First, the Ag/mAb pre-mixed sample shows significantly diminished (or ideally,

no) binding to the immobilized mAb, indicating that the pre-mixed mAb and immobilized mAb compete for an overlapping epitope. Second, the Ag/mAb pre-mixed sample shows kinetically-altered binding compared to that for Ag alone, suggesting that the pre-mixed and immobilized mAbs bind the Ag simultaneously at non-overlapping epitopes. While a sandwiching response is often larger than the binding response of Ag alone (see pre-mix examples in Figs. 1 and 2), in some cases, the Ag/pre-mixed mAb complex may bind with significantly altered kinetics than Ag alone, resulting in a binding response that is smaller than that of Ag alone (discussed in Recommendation 3: when pre-mix assays give “intermediate responses”, disambiguate the result with follow-up assays). Third, the Ag/mAb pre-mixed sample shows an identical sensorgram (within the normal variation of replicate measurements) as that observed for Ag alone, suggesting that the pre-mixed and immobilized mAbs bind distinct epitopes that reside on separate subpopulations of a heterogeneous Ag; this result is compelling when the same Ag/mAb pre-mixed sample gives a “full self-block”.

4.4.3. Recommendation 3: when pre-mix assays give “intermediate responses”, disambiguate the result with follow-up assays

When the Ag/mAb pre-mixed sample shows a lower binding response than that for Ag alone, i.e. an “intermediate” response, we recommend a follow-up assay to determine if, (1) the binding response is due to Ag alone, which would imply that the pre-mixed and immobilized mAbs compete for an overlapping epitope and that a higher concentration of the pre-mixed mAb may be needed to fully block binding to the immobilized mAb or (2) the binding response is kinetically-altered because the detected solution species is the Ag/pre-mixed mAb complex, which would imply that the pre-mixed and immobilized mAbs do not compete for Ag and thus form a sandwich pair. One way to discriminate between these two scenarios is to use a secondary detection reagent that is specific for the pre-mixed mAb; in this case, binding of the secondary reagent confirms that the “intermediate” signal is due to a sandwiching interaction between the pre-mixed and immobilized mAbs. However, this approach is only feasible when the immobilized and pre-mixed mAbs can be differentiated from one another, e.g., based upon their species, subclass, tag or label.

4.4.4. Recommendation 4: perform antigen depletion experiments to confirm antigen heterogeneity

To confirm Ag heterogeneity, one can perform depletion experiments with mAb-coated magnetic beads (e.g., Dynabeads) to selectively remove each subpopulation from the Ag preparation, and then use biosensor experiments to test if subpopulations have been depleted (as we demonstrated in Fig. 3).

4.5. Other forms of antigen heterogeneity

This study has demonstrated how to diagnose a simple case of Ag heterogeneity, where an Ag exists as two distinct subpopulations as identified by the mAbs in the test panel, i.e. a given mAb binds to one of the two Ag subpopulations, but not to both. Other forms of Ag heterogeneity can occur, for example a mAb that binds more than one Ag subpopulation. Such a scenario would have its own “signature heterogeneous

binning profile". For example, in the thought experiment where an Ag exists as two subpopulations as distinguished by mAb X and mAb Y, where mAb X binds an epitope that is presented by both Ag subpopulations and mAb Y binds a distinct non-overlapping epitope that is presented on only one Ag subpopulation, binning in all three assay formats would give the same result of "non-blocking", as shown in Fig. 1B. In this case, the binning outcome depends only on the Ag subpopulation that is common to both mAbs. However, Dynabead depletion experiments would diagnose the presence of two Ag subpopulations, because an Ag sample depleted by mAb X would show no binding to immobilized mAb X or immobilized mAb Y, whereas an Ag sample depleted with mAb Y would show no binding to immobilized mAb Y but a partial binding response (compared to non-depleted Ag) to immobilized mAb X. Thus, a Dynabead depletion experiment can be considered a fourth binning assay format because it provides complementary information that may be overlooked in a standard binning experiment. Other – more complex – forms of heterogeneity can be postulated and the combined use of binning and depletion experiments can support or eliminate the possibility of such heterogeneity existing.

5. Conclusions

Our work has illustrated how to identify Ag heterogeneity through the use of epitope binning assays on label-free biosensors. While our examples have drawn from our work on monovalent Ags, multivalent Ags can be analyzed using similar techniques – although they present a more complex situation. Therefore, assays with multivalent Ags should be designed with particular care to avoid misleading results. When applied in a rigorous manner and on a diverse panel of mAbs, which is possible to accomplish in high-throughput on various commercially available label-free biosensor platforms (Abdiche et al., 2011), binning assays can be highly informative to the discovery of therapeutic mAbs and can be predictive of functional activity.

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