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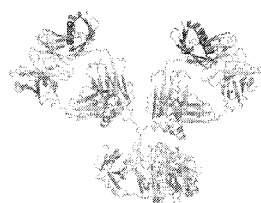
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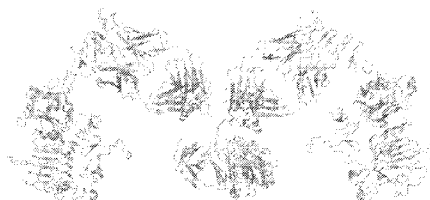
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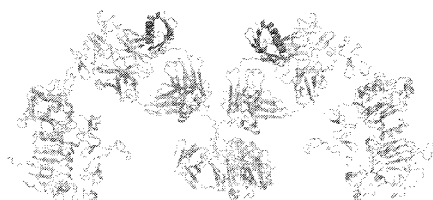
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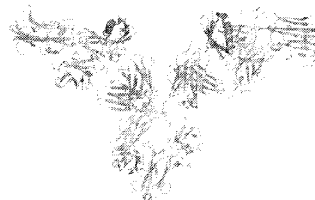
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FIGS. 1A-1D

(57) Abstract: The present disclosure provides compositions, methods, and systems related to a dual-affinity ratiometric quenching bioassay. In particular, the present disclosure provides novel compositions and methods that combine selective biorecognition and quenching of fluorescence signals for rapid and sensitive quantification of antibodies in complex samples.



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COMPOSITIONS, SYSTEMS, AND METHODS RELATED TO A DUAL-AFFINITY RATIOMETRIC QUENCHING BIOASSAY

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of U.S. Provisional Patent Application No. 63/089,806 filed October 9, 2020, which is incorporated herein by reference in its entirety for all purposes.

FIELD

[0002] The present disclosure provides compositions, methods, and systems related to a dual-affinity ratiometric quenching bioassay. In particular, the present disclosure provides novel compositions and methods that combine selective biorecognition and quenching of fluorescence signals for rapid and sensitive quantification of antibodies in complex samples.

BACKGROUND

[0003] Accurate quantification of monoclonal antibodies (mAbs) in complex media, such as the serum of patients undergoing immunotherapy, industrial cell culture harvests and streams, and fluids derived from transgenic plants and animals is paramount to ensure the health of patients and product quality in biopharmaceutical industry. To date, a myriad of assays have been developed for antibody detection and measurement, including lateral flow assays, ELISA, and immunoaffinity chromatography. These, however, differ widely in terms of duration (from minutes to hours), performance (sensitivity, limit of detection, and dynamic range), and cost. Likewise, the biorecognition moieties utilized to target the antibody analyte include protein-based affinity tags such as antigens, secondary antibodies and antibody-binding receptors (e.g., Protein A, Protein G, and Fc receptors FcγRs), as well as synthetic affinity tags such as aptamers and peptides. Finally, detection modalities vary significantly ranging from optical (e.g., UV/vis, fluorescence, and surface plasmon resonance) to electrochemical (e.g., impedance and amperometry) and acoustic (e.g., photoacoustic and quartz crystal microbalance). Fluorescence holds a preeminent place among detection modalities, owing to its high sensitivity, flexibility, and availability of fluorescence spectrophotometers. The generation of a fluorescence signal by the affinity tags can be accomplished either by chemical conjugation (e.g., by labeling them with synthetic fluorophores), or enzymatically by fusing them with enzymes (e.g., horseradish peroxidase or luciferase) that convert substrates into fluorescent products. Of major interest are combinations of fluorophores and labelling

strategies that can engage in phenomena such as static or dynamic quenching and energy transfer. Such combinations of fluorophores and labelling strategies enable continuous monitoring of the titer of analytes as their concentration evolves with time and provide information regarding the biomolecular structure of the analyte-tag complex.

SUMMARY

[0004] Embodiments of the present disclosure include a ratiometric bioassay method for detecting a target antibody in a sample. In accordance with these embodiments, the method includes combining an antigen coupled to a first fluorophore, an antibody-binding moiety coupled to a second fluorophore, and a sample comprising or suspected of comprising a target antibody, exposing the antigen coupled to the first fluorophore, the antibody-binding moiety coupled to the second fluorophore, and the sample to fluorescent light comprising an excitation wavelength of the first and/or the second fluorophore, and detecting fluorescence emission from the first and/or the second fluorophore. In some embodiments, the fluorescence emission from the first and the second fluorophore is reduced, and wherein the reduced fluorescence emission is proportional to the antibody concentration in the sample.

[0005] In some embodiments, the antigen is capable of being bound by the antibody in the sample.

[0006] In some embodiments, the antibody-binding moiety comprises a polypeptide capable of binding a region of the target antibody that does not comprise the antigen-binding site. In some embodiments, the antibody binding moiety comprises Protein L, Protein A, or Protein G. In some embodiments, the antibody binding moiety is Protein L.

[0007] In some embodiments, the first fluorophore is a high-energy fluorophore and the second fluorophore is a low-energy fluorophore. In some embodiments, the second fluorophore is a high-energy fluorophore and the first fluorophore is a low-energy fluorophore.

[0008] In some embodiments, the high-energy fluorophore is selected from the group consisting of Fluorescein, Oregon Green 488, Oregon Green 514, Rhodamine Green, Rhodamine Green-X, Eosin, 4', 6-diamidino-2-phenylindole, Alexafluor 405, Alexafluor 350, Alexafluor 500, Alexafluor 488, Alexafluor 430, Alexafluor 514, and Alexafluor 532.

[0009] In some embodiments, the low-energy fluorophore is selected from the group consisting of Rhodamine, Rhodamine B, Rhodamine Red-X, Tetramethylrhodamine, Lissamine, Texas Red and Texas Red-X, Naphthofluorescein, Carboxyrhodamine 6G, Alexafluor 555, Alexafluor 546, Alexafluor 568, Alexafluor 594, Alexafluor 610, Alexafluor

633, Alexafluor 635, Alexafluor 647, Alexafluor 660, Alexafluor 680, Alexafluor 700, and Alexafluor 750.

[0010] In some embodiments, the antigen is coupled to fluorescein or Alexafluor 350. In some embodiments, the antibody-binding moiety is coupled to Rhodamine.

[0011] In some embodiments, the method further includes determining the concentration of the target antibody in the sample based on the reduction in fluorescence emission of the first and/or the second fluorophore.

[0012] In some embodiments, the method further includes incubating the sample comprising or suspected of comprising the target antibody, the antigen coupled to the first fluorophore, and the antibody-binding moiety coupled to the second fluorophore for 30 minutes or less prior to measuring the fluorescent emission of the first and/or the second fluorophore.

[0013] In some embodiments, the method further includes incubating the sample comprising or suspected of comprising the target antibody, the antigen coupled to the first fluorophore, and the antibody-binding moiety coupled to the second fluorophore for 10 minutes or less prior to measuring the fluorescent emission of the first and/or the second fluorophore.

[0014] In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:20.

[0015] In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an moiety-to-fluorophore ratio of about 1:0.2 to about 1:20.

[0016] In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:1 to about 1:20.

[0017] In some embodiments, the sample is undiluted. In some embodiments, the sample is a whole blood sample, a plasma sample, a serum sample, a urine sample, a saliva sample, a tissue sample, a cell culture sample, a cell lysate sample, or a cell culture media sample.

[0018] In some embodiments, the antigen comprises a protein forming the capsid of a virus, or a fragment thereof.

[0019] In some embodiments, the antigen comprises the spike (S) protein of the SARS-CoV-2 virus, or a fragment thereof.

[0020] Embodiments of the present disclosure also include a composition for performing a ratiometric bioassay to detect an antibody in a sample. In accordance with these embodiments, the composition includes an antigen coupled to a first fluorophore, an antibody-binding moiety coupled to a second fluorophore, and a sample comprising or suspected of comprising a target antibody. In some embodiments, fluorescence emission from the first and the second fluorophore is reduced upon exposure to fluorescent light comprising an excitation wavelength

of the first and/or the second fluorophore. In some embodiments, the reduced fluorescence emission is proportional to the antibody concentration in the sample.

[0021] In some embodiments, the antigen is capable of being bound by the antibody in the sample.

[0022] In some embodiments, the antibody-binding moiety comprises a polypeptide capable of binding a region of the target antibody that does not comprise the antigen-binding site. In some embodiments, the antibody binding moiety comprises Protein L, Protein A, or Protein G.

[0023] In some embodiments, the first fluorophore is a high-energy fluorophore and the second fluorophore is a low-energy fluorophore. In some embodiments, the second fluorophore is a high-energy fluorophore and the first fluorophore is a low-energy fluorophore.

[0024] In some embodiments, the high-energy fluorophore is selected from the group consisting of Fluorescein, Oregon Green 488, Oregon Green 514, Rhodamine Green, Rhodamine Green-X, Eosin, 4', 6-diamidino-2-phenylindole, Alexafluor 405, Alexafluor 350, Alexafluor 500, Alexafluor 488, Alexafluor 430, Alexafluor 514, and Alexafluor 532.

[0025] In some embodiments, the low-energy fluorophore is selected from the group consisting of Rhodamine, Rhodamine B, Rhodamine Red-X, Tetramethylrhodamine, Lissamine, Texas Red and Texas Red-X, Naphthofluorescein, Carboxyrhodamine 6G, Alexafluor 555, Alexafluor 546, Alexafluor 568, Alexafluor 594, Alexafluor 610, Alexafluor 633, Alexafluor 635, Alexafluor 647, Alexafluor 660, Alexafluor 680, Alexafluor 700, and Alexafluor 750.

[0026] In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.5 to about 1:20.

[0027] In some embodiments, the antibody binding moiety is coupled to the second fluorophore at an moiety-to-fluorophore ratio of about 1:0.2 to about 1:20.

[0028] In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:1 to about 1:20.

[0029] In some embodiments, the sample is undiluted. In some embodiments, sample is a whole blood sample, a plasma sample, a serum sample, a urine sample, a saliva sample, a tissue sample, a cell culture sample, a cell lysate sample, or a cell culture media sample.

[0030] In some embodiments, the antigen comprises a protein forming the capsid of a virus, or a fragment thereof.

[0031] In some embodiments, the antigen comprises the spike (S) protein of the SARS-CoV-2 virus, or a fragment thereof.

[0032] Embodiments of the present disclosure also include a kit for performing a ratiometric bioassay to detect a target antibody in a sample. In accordance with these embodiments, the kit includes an antigen coupled to a first fluorophore, an antibody binding moiety coupled to a second fluorophore, and at least one container.

[0033] In some embodiments, the kit further comprises a buffer and/or instructions for performing the bioassay.

[0034] In some embodiments, the kit further comprises the target antibody or a fragment or derivative thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] FIGS. 1A-1D: Crystal structures of the complexes (A) Trastuzumab:^{Rh}PrL (derived from PDB IDs: 4HKZ and 1IGY), (B) Trastuzumab:^{Fl}Her2 (derived from PDB IDs: 6OGE and 1IGY), (C) Trastuzumab:2^{Rh}PrL:2^{Fl}Her2 (overlap of panels A and B), and (D) Adalimumab:^{Rh}PrL:^{Fl}TNF- α (derived from PDB IDs: 3WD5, 4HKZ, 1HEZ, and 1IGY). The heavy chain of the mAbs are in light blue, the light chain of the mAbs are in salmon, ^{Rh}PrL is in red, and the antigens ^{Fl}Her2 and ^{Fl}TNF- α are in green. The structures of the complexes were generated by overlapping the crystal structures published on the Protein Data Bank using the open source molecular visualization software PyMOL.

[0036] FIG. 2: DARQ assay: a target mAb in solution is incubated with rhodamine-labeled Protein L (^{Rh}PrL) and fluorescein-labeled antigen (^{Fl}AG). Upon formation of the mAb:^{Rh}PrL:^{Fl}AG complex, the fluorescent emission of ^{Rh}PrL and ^{Fl}AG is decreased in a mAb concentration-dependent fashion.

[0037] FIGS. 3A-3B: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry ($\lambda_{\text{ex}} = 480 \text{ nm}$; $\lambda_{\text{em}} = 525 \text{ nm}$) of the dual fluorescence affinity complex Trastuzumab:2^{Rh}PrL:2^{Fl}Her2 formed by mixing ^{Rh}PrL and ^{Fl}Her2 at constant stoichiometric amounts with solutions of varying concentrations of Trastuzumab in either (A) PBS (non-competitive conditions) or (B) CHO cell culture harvest (competitive conditions). The red line marks the region of linearity of the fluorescence signal vs. mAb concentration. The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0038] FIGS. 4A-4B: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometric analysis ($\lambda_{\text{ex}} = 480 \text{ nm}$; $\lambda_{\text{em}} = 525 \text{ nm}$) of the dual fluorescence affinity complex Adalimumab:2^{Rh}PrL:2^{Fl}TNF- α

formed by mixing ^{Rh}PrL and $^{Fl}TNF-\alpha$ at constant stoichiometric amounts with solutions of varying concentrations of Adalimumab in either (A) PBS (non-competitive conditions) or (B) CHO cell culture harvest (competitive conditions). The red line marks the region of linearity of the fluorescence signal vs. mAb concentration. The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0039] FIG. 5: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry ($\lambda_{ex} = 335$ nm; $\lambda_{em} = 445$ nm) of the dual fluorescence affinity complex Adalimumab: $2^{Rh}PrL$: $2^{AF350}TNF-\alpha$ formed by mixing ^{Rh}PrL and $^{AF350}TNF-\alpha$ at constant stoichiometric amounts with solutions of varying concentrations of Adalimumab in PBS. The red line marks the region of linearity of the fluorescence signal vs. mAb concentration. The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0040] FIGS. 6A-6B: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry ($\lambda_{ex} = 480$ nm; $\lambda_{em} = 525$ nm) of the dual fluorescence affinity complex Adalimumab: $2^{Rh}PrL$: $2^{Fl}TNF-\alpha$ formed by mixing ^{Rh}PrL and $^{Fl}TNF-\alpha$ at constant stoichiometric amounts with solutions of varying concentrations of Adalimumab at different incubation time (1, 5, 10, and 20 min) in either (A) PBS (non-competitive conditions) or (B) CHO cell culture harvest (competitive conditions). The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0041] FIGS. 7A-7B: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry ($\lambda_{ex} = 480$ nm; $\lambda_{em} = 573$ nm) of the dual fluorescence affinity complex Trastuzumab: $2^{Rh}PrL$: $2^{Fl}Her2$ formed by mixing ^{Rh}PrL and $^{Fl}Her2$ at constant stoichiometric amounts with solutions of varying concentration of Trastuzumab in either (A) PBS (non-competitive conditions) or (B) CHO cell culture harvest (competitive conditions). The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0042] FIGS. 8A-8B: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry ($\lambda_{ex} = 480$ nm; $\lambda_{em} = 573$ nm) of the dual fluorescence affinity complex Adalimumab: $2^{Rh}PrL$: $2^{Fl}2TNF-\alpha$ formed by mixing ^{Rh}PrL and $^{Fl}Her2$ at constant stoichiometric amounts with solutions of varying

concentrations of Trastuzumab in either (A) PBS (non-competitive conditions) or (B) CHO cell culture harvest (competitive conditions). The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0043] FIGS. 9A-9B: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry ($\lambda_{\text{ex}} = 480 \text{ nm}$; $\lambda_{\text{em}} = 525 \text{ nm}$) of the affinity complexes (A) Trastuzumab: 2^{Fl} Her2 and Adalimumab: $^{\text{Fl}}$ 2TNF- α and (B) Trastuzumab: 2^{Rh} PrL and Adalimumab: 2^{Rh} PrL formed in PBS by mixing either $^{\text{Rh}}$ PrL or fluorescein-labeled antigen ($^{\text{Fl}}$ Her2 or $^{\text{Fl}}$ TNF- α) with the corresponding mAb. The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0044] FIGS. 10A-10B: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry of the affinity complex Adalimumab: 2^{PrL} : 2_{AF350} TNF- α at (A) $\lambda_{\text{ex}} = 335 \text{ nm}$; $\lambda_{\text{em}} = 445 \text{ nm}$, and (B) $\lambda_{\text{ex}} = 335 \text{ nm}$; $\lambda_{\text{em}} = 573 \text{ nm}$. The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0045] FIGS. 11A-11C: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry of the affinity complex Adalimumab: 2^{Rh} PrL: $2^{\text{TNF-}\alpha}$ at the excitation and emission wavelengths of (A) $\lambda_{\text{ex}} = 480 \text{ nm}$; $\lambda_{\text{em}} = 573 \text{ nm}$, (B) $\lambda_{\text{ex}} = 480 \text{ nm}$; $\lambda_{\text{em}} = 525 \text{ nm}$, and (C) $\lambda_{\text{ex}} = 525 \text{ nm}$; $\lambda_{\text{em}} = 573 \text{ nm}$. The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0046] FIGS. 12A-12B: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometry of the affinity complex Adalimumab: 2^{Rh} PrL: 2^{AF350} TNF- α at the excitation and emission wavelengths of (A) $\lambda_{\text{ex}} = 335 \text{ nm}$; $\lambda_{\text{em}} = 573 \text{ nm}$, (B) $\lambda_{\text{ex}} = 525 \text{ nm}$; $\lambda_{\text{em}} = 573 \text{ nm}$. The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

[0047] FIGS. 13A-13C: Values of normalized fluorescence (relative fluorescence units, RFU) vs. antibody concentration measured by fluorescence spectrophotometric analysis of the affinity complex Adalimumab: 2^{Rh} PrL: 2^{Rh} TNF- α at the excitation and emission wavelengths of (A) 480 nm and 573 nm, (B) 480 nm and 525 nm, and (C) 525 nm and 573 nm. The vertical blue line marks the point of stoichiometric equivalence, where the concentration of each labeled protein is twice the concentration of antibody.

DETAILED DESCRIPTION

[0048] More than 100 monoclonal antibodies (mAbs) are in industrial and clinical development to treat myriad diseases. Accurate quantification of mAbs in complex media, derived from industrial and patient samples, is vital to determine production efficiency or pharmacokinetic properties. To date, mAb quantification requires time and labor intensive assays. As described further herein, embodiments of the present disclosure include a novel bioassay termed Dual-Affinity Ratiometric Quenching (“DARQ”), which combines selective biorecognition and quenching of fluorescence signals for rapid and sensitive quantification of therapeutic monoclonal antibodies (mAbs). The reported assay relies on the affinity complexation of the target mAb by the corresponding antigens and Protein L (PrL, which targets the Fab region of the antibody), respectively labeled with fluorescein and rhodamine. Within the affinity complex, the mAb acts as a scaffold framing the labeled affinity tags (PrL and antigen) in a molecular proximity that results in ratiometric quenching of their fluorescence emission. Notably, the decrease in fluorescence emission intensity is linearly dependent upon mAb concentration in solution. Control experiments conducted with one affinity tag only, two tags labeled with equal fluorophores, or two tags labeled with fluorophores of discrete absorbance and emission bands exhibited significantly reduced effect. The assay was evaluated in non-competitive (pure mAb) and competitive conditions (mAb in a Chinese Hamster Ovary (CHO) cell culture harvest). The “DARQ” assay is highly reproducible (coefficient of variation ~0.8-0.7%) and rapid (5 min), and its sensitivity ($\sim 0.2\text{-}0.5\text{ ng}\cdot\text{mL}^{-1}$), limit of detection ($75\text{-}119\text{ ng}\cdot\text{mL}^{-1}$), and dynamic range ($300\text{-}1600\text{ ng}\cdot\text{mL}^{-1}$) are independent of the presence of CHO host cell proteins.

[0049] In accordance with these embodiments, the bioassays of the present disclosure involve the formation of a 5-protein affinity complex comprising the target mAb, its two corresponding antigens, and two Protein L (PrL) molecules. PrL is a surface protein derived from *Peptostreptococcus magnus* and binds the light chain of the Fab region of antibodies, specifically those belonging to the κI , κIII , and κIV subtypes. As described further herein, Trastuzumab (brand name: Herceptin[®]) and Adalimumab (brand name: Humira[®]) were used as model κI -mAbs, owing to their demonstrated therapeutic value in fighting cancer and autoimmune diseases. Accordingly, Tumor Necrosis Factor- α (TNF- α) and human epidermal growth factor receptor 2 (Her2) were employed as model antigen (AG) molecules. The fluorophores used herein, namely fluorescein ($\lambda_{\text{ex}}/\lambda_{\text{em}}$: 494/512 nm) and rhodamine ($\lambda_{\text{ex}}/\lambda_{\text{em}}$: 552/575 nm), were chosen for their expected absorbance-emission spectral overlap and

extensive use in fluorescent labeling applications. Specifically, the antigens were labeled with fluorescein ($^{\text{Fl}}$ Her2 or $^{\text{Fl}}$ TNF- α); whereas PrL was labeled with rhodamine ($^{\text{Rh}}$ PrL). Within the affinity complex, the mAb acts as a molecular scaffold that maintains 2 $^{\text{Rh}}$ PrL and 2 fluorescein-labeled antigen molecules ($^{\text{Fl}}$ Her2 or $^{\text{Fl}}$ TNF- α) in a predefined orientation and distance (~ 3.10 nm between the centers of mass of PrL and TNF- α , and ~ 4.98 nm between the centers of mass of PrL and Her2), as illustrated in FIG. 1.

[0050] Embodiments of the present disclosure demonstrate that fluorescence emission of both fluorophores decreases linearly with the antibody concentration (FIG. 2). In some cases, the decrease of the fluorescein signal ($\lambda_{\text{em}} = 525$ nm) was more pronounced than that of the rhodamine signal. Without being bound by a particular mechanism, one possible explanation is that the fluorophores displayed on $^{\text{Rh}}$ PrL and $^{\text{Fl}}$ AG in complex with the mAb engage in a quenching mechanism. A set of control experiments was performed comprising different fluorophores and affinity complex schemes. To this end, Alexafluor 350 (AF350, $\lambda_{\text{ex}}/\lambda_{\text{em}}$: 335/445 nm) was adopted as a control fluorophore since it does not exhibit significant spectral overlap with rhodamine, which limits the potential for non-radiative energy transfer. In one embodiment, the control assays included (i) single-affinity labeling by complexing the target antibody with either $^{\text{Rh}}$ PrL or $^{\text{Fl}}$ AG alone; (ii) homogeneous dual-affinity labeling by complexing the Adalimumab using $^{\text{Rh}}$ PrL and $^{\text{Rh}}$ TNF- α ; (iii) dual fluorescence – dual affinity labeling by complexing the Adalimumab using $^{\text{Rh}}$ PrL and $^{\text{AF350}}$ TNF- α ; and (iv) single fluorescence – dual affinity labeling by complexing the Adalimumab using unlabeled PrL and $^{\text{AF350}}$ TNF- α .

[0051] Collectively, the results provided herein demonstrate that the mechanism underlying the bioassays of the present disclosure involve fluorescence quenching, reliant on the labelling of the antigen and PrL as an acceptor-donor pair. The decrease in fluorescein emission with mAb concentration is not due to a traditional FRET mechanism, since the rhodamine signal does not concurrently increase. In some cases, quenching arises predominantly in the high-energy fluorophores (e.g., AF350 and Fl), as observed in the initial assays and control test (iii). However, the exhibited quenching requires the presence of Rh, since control test (i) conducted with $^{\text{Fl}}$ AG alone and (iv) conducted PrL and $^{\text{AF350}}$ TNF- α did not show any decrease in fluorescence signal of either high-energy fluorophore. In some cases, no quenching of the low-energy dye (i.e., Rh) was observed (e.g., in control test (i) with $^{\text{Rh}}$ PrL alone and (iii) with $^{\text{Rh}}$ PrL and $^{\text{Rh}}$ TNF- α).

[0052] Additionally, the high affinity and selectivity of the interaction of mAb with PrL and AG molecules provides high sensitivity (~ 0.2 - 0.5 ng·mL $^{-1}$), low limit of detection (75-119

ng·mL⁻¹), and broad dynamic range (300-1600 ng·mL⁻¹), and eliminates any variability associated to the presence of other proteins in solution. When evaluated in buffered aqueous solution (non-competitive) or in a Chinese Hamster Ovary (CHO) cell culture harvest (competitive), the assay did not exhibit any appreciable variation of performance. Furthermore, the fast kinetics of formation of the mAb:2^{Rb}PrL:2^{Fl}AG complex makes DARQ a rapid (< 5 min) and reproducible (coefficient of variation ~0.8-0.7%) mix-and-read assay with considerably higher throughput compared to commercial mAb-specific ELISA kits (see, e.g., Table 5).

[0053] The current technologies utilized by analytical labs at both clinical and biomanufacturing sites for antibody quantification in complex media are laborious and involve expensive consumables or equipment. To overcome these issues, a novel “DARQ” (dual affinity ratiometric quenching) assay was developed for antibody quantification that is rapid, robust, and reproducible, and accurate. The assay relies on a highly selective affinity interaction between the target antibody, the fluorescein-labeled corresponding antigen, and rhodamine-labeled Protein L. The antibody captures the fluorescently labeled proteins, framing them in a supramolecular affinity complex, wherein the fluorophores are constrained in proximity within a dense proteinaceous structure. This translates into a decrease of their fluorescent emission that is linearly dependent upon the antibody concentration. Control assays performed using different combinations of fluorescent affinity tags, while not revealing the specific nature of the mechanism at hand, indicate that it is of the nature of energy transfer quenching. From a technology standpoint, the high binding strength and selectivity of both antigen and Protein L to the target antibody accelerates the formation of the affinity complex, leaving no residual free antibody in solution. The combination of biorecognition and fluorescence quenching makes the assay rapid (~ 5 min), highly sensitive (< 0.5 ng·mL⁻¹), and reproducible (CoV < 1.7%). Reliance on Protein L, which targets the antibody’s Fab region of κI, κIII, and κIV subtypes, does not limit the applicability of the assay, given that most therapeutic antibodies currently on the market belong to the κI group. This makes DARQ an ideal mix-and-read assay for at-line monitoring of antibody concentration in bioprocessing fluids (e.g., clarified harvest and chromatographic fractions) or a point-of-care test (POCT) for clinical laboratories.

[0054] Section headings as used in this section and the entire disclosure herein are merely for organizational purposes and are not intended to be limiting.

1. Definitions

[0055] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the present disclosure. The phrase “in one embodiment” as used herein does not necessarily refer to the same embodiment, though it may. Furthermore, the phrase “in another embodiment” as used herein does not necessarily refer to a different embodiment, although it may. Thus, as described below, various embodiments of the invention may be readily combined, without departing from the scope or spirit of the invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

[0056] The terms “comprise(s),” “include(s),” “having,” “has,” “can,” “contain(s),” and variants thereof, as used herein, are intended to be open-ended transitional phrases, terms, or words that do not preclude the possibility of additional acts or structures. The singular forms “a,” “and” and “the” include plural references unless the context clearly dictates otherwise. The present disclosure also contemplates other embodiments “comprising,” “consisting of” and “consisting essentially of,” the embodiments or elements presented herein, whether explicitly set forth or not.

[0057] For the recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range of 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the number 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, and 7.0 are explicitly contemplated.

[0058] “Correlated to” as used herein refers to compared to.

[0059] “Coefficient of variation” (CV), also known as “relative variability,” is equal to the standard deviation of a distribution divided by its mean.

[0060] “Component,” “components,” or “at least one component,” refer generally to an antigen, an antibody-binding moiety, a target antibody, a fluorophore, a calibrator, a control, a sensitivity panel, a container, a buffer, a diluent, a salt, an enzyme, a co-factor for an enzyme, a detection reagent, a pretreatment reagent/solution, a substrate (e.g., as a solution), a stop solution, and the like that can be included in a kit for assay of a test sample in accordance with

the methods described herein and other methods known in the art based on the present disclosure.

[0061] “Controls” as used herein generally refers to a reagent whose purpose is to evaluate the performance of a measurement system in order to assure that it continues to produce results within permissible boundaries (e.g., boundaries ranging from measures appropriate for a research use assay on one end to analytic boundaries established by quality specifications for a commercial assay on the other end). To accomplish this, a control should be indicative of patient results and optionally should somehow assess the impact of error on the measurement (e.g., error due to reagent stability, calibrator variability, instrument variability, and the like).

[0062] “Quality control reagents” in the context of the assays and kits described herein, include, but are not limited to, calibrators, controls, and sensitivity panels. A “calibrator” or “standard” typically is used (e.g., one or more, such as a plurality) in order to establish calibration (standard) curves for interpolation of the concentration of an analyte, such as an antibody or an analyte. Alternatively, a single calibrator, which is near a reference level or control level (e.g., “low”, “medium”, or “high” levels), can be used. Multiple calibrators (i.e., more than one calibrator or a varying amount of calibrator(s)) can be used in conjunction to comprise a “sensitivity panel.”

[0063] “Dynamic range” as used herein refers to range over which an assay readout is proportional to the amount of target molecule or analyte in the sample being analyzed. The dynamic range can be the range of linearity of the standard curve.

[0064] “Limit of Blank (LoB)” as used herein refers to the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested.

[0065] “Limit of Detection (LoD)” as used herein refers to the lowest concentration of the measurand (i.e., a quantity intended to be measured) that can be detected at a specified level of confidence. The level of confidence is typically 95%, with a 5% likelihood of a false negative measurement. LoD is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. LoD can be determined by utilizing both the measured LoB and test replicates of a sample known to contain a low concentration of analyte. The LoD term used herein is based on the definition from Clinical and Laboratory Standards Institute (CLSI) protocol EP17-A2 (“Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline - Second Edition,” EP17A2E, by James F. Pierson-Perry et al., Clinical and Laboratory Standards Institute, June 1, 2012).

[0066] “Limit of Quantitation (LoQ)” as used herein refers to the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LoQ may be equivalent to the LoD or it could be at a much higher concentration.

[0067] “Linearity” refers to how well the method or assay’s actual performance across a specified operating range approximates a straight line. Linearity can be measured in terms of a deviation, or non-linearity, from an ideal straight line. “Deviations from linearity” can be expressed in terms of percent of full scale. In some of the methods disclosed herein, less than 10% deviation from linearity (DL) is achieved over the dynamic range of the assay. “Linear” means that there is less than or equal to about 20%, about 19%, about 18%, about 17%, about 16%, about 15%, about 14%, about 13%, about 12%, about 11%, about 10%, about 9%, or about 8% variation for or over an exemplary range or value recited.

[0068] “Reference level” as used herein refers to an assay cutoff value that is used to assess diagnostic, prognostic, or therapeutic efficacy and that has been linked or is associated herein with various clinical parameters (e.g., presence of disease, stage of disease, severity of disease, progression, non-progression, or improvement of disease, etc.). It is well-known that reference levels may vary depending on the nature of the assay (e.g., antibodies employed, reaction conditions, sample purity, etc.) and that assays can be compared and standardized. Whereas the precise value of the reference level may vary between assays, the embodiments as described herein should be generally applicable and capable of being extrapolated to other assays.

[0069] “Antibody” and “antibodies” as used herein refers to monoclonal antibodies, monospecific antibodies (e.g., which can either be monoclonal, or may also be produced by other means than producing them from a common germ cell), multispecific antibodies, human antibodies, humanized antibodies (fully or partially humanized), animal antibodies such as, but not limited to, a bird (for example, a duck or a goose), a shark, a whale, and a mammal, including a non-primate (for example, a cow, a pig, a camel, a llama, a horse, a goat, a rabbit, a sheep, a hamster, a guinea pig, a cat, a dog, a rat, a mouse, etc.) or a non-human primate (for example, a monkey, a chimpanzee, etc.), recombinant antibodies, chimeric antibodies, single-chain Fvs (“scFv”), single chain antibodies, single domain antibodies, Fab fragments, F(ab’) fragments, F(ab’)₂ fragments, disulfide-linked Fvs (“sdFv”), and anti-idiotypic (“anti-Id”) antibodies, dual-domain antibodies, dual variable domain (DVD) or triple variable domain (TVD) antibodies (dual-variable domain immunoglobulins and methods for making them are described in Wu, C., et al., *Nature Biotechnology*, 25(11):1290-1297 (2007) and PCT International Application WO 2001/058956, the contents of each of which are herein

incorporated by reference), or domain antibodies (dAbs) (e.g., such as described in Holt et al. (2014) Trends in Biotechnology 21:484-490), and including single domain antibodies sdAbs that are naturally occurring, e.g., as in cartilaginous fishes and camelid, or which are synthetic, e.g., nanobodies, VHH, or other domain structure), and functionally active epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, namely, molecules that contain an analyte-binding site. Immunoglobulin molecules can be of any type (for example, IgG, IgE, IgM, IgD, IgA, and IgY), class (for example, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2).

[0070] “Antibody fragment” as used herein refers to a portion of an intact antibody comprising the antigen-binding site or variable region. The portion does not include the constant heavy chain domains (*i.e.* CH2, CH3, or CH4, depending on the antibody isotype) of the Fc region of the intact antibody. Examples of antibody fragments include, but are not limited to, Fab fragments, Fab’ fragments, Fab’-SH fragments, F(ab’)₂ fragments, Fd fragments, Fv fragments, diabodies, single-chain Fv (scFv) molecules, single-chain polypeptides containing only one light chain variable domain, single-chain polypeptides containing the three CDRs of the light-chain variable domain, single-chain polypeptides containing only one heavy chain variable region, and single-chain polypeptides containing the three CDRs of the heavy chain variable region.

[0071] As used herein, the term “antigen” refers to any substance that is capable of inducing an immune response. An antigen may be an organism (*e.g.*, a fungus), a whole cell (*e.g.*, bacterial cell) or a colony of cells, a virus, or a portion or component thereof. Examples of antigens include, but are not limited to, microbial pathogens, bacteria, viruses, proteins, glycoproteins, lipoproteins, peptides, glycopeptides, lipopeptides, lipopolysaccharides, oligosaccharides and polysaccharides, and portions or components thereof.

[0072] “Epitope,” or “epitopes,” or “epitopes of interest” refer to a site(s) on any molecule that is recognized and can bind to a complementary site(s) on its specific binding partner. The molecule and specific binding partner are part of a specific binding pair. For example, an epitope can be on a polypeptide, a protein, a hapten, a carbohydrate antigen (such as, but not limited to, glycolipids, glycoproteins or lipopolysaccharides), or a polysaccharide. Its specific binding partner can be, but is not limited to, an antibody.

[0073] The terms “specific binding partner,” “specific binding member,” and “binding member” are used interchangeably herein and refer to one of two or more different molecules

that specifically recognize the other molecule compared to substantially less recognition of other molecules.

[0074] As used herein, when an antibody or other entity (*e.g.*, antigen binding domain) “specifically recognizes” or “specifically binds” an antigen or epitope, it preferentially recognizes the antigen in a complex mixture of proteins and/or macromolecules, and binds the antigen or epitope with affinity which is substantially higher than to other entities not displaying the antigen or epitope. In this regard, “affinity which is substantially higher” means affinity that is high enough to enable selective detection of a target antigen or epitope which is distinguished from entities using a desired assay or measurement apparatus. Typically, it means binding affinity having a binding constant (K_a) of at least 10^7 M^{-1} (*e.g.*, $>10^7 \text{ M}^{-1}$, $>10^8 \text{ M}^{-1}$, $>10^9 \text{ M}^{-1}$, $>10^{10} \text{ M}^{-1}$, $>10^{11} \text{ M}^{-1}$, $>10^{12} \text{ M}^{-1}$, $>10^{13} \text{ M}^{-1}$, etc.). In certain such embodiments, an antibody is capable of binding different antigens so long as the different antigens comprise that particular epitope. In certain instances, for example, homologous proteins from different species may comprise the same epitope.

[0075] As used herein, the term “subject” refers to any human or animal (*e.g.*, non-human primate, rodent, feline, canine, bovine, porcine, equine, caprine, etc.).

[0076] As used herein, the term “sample” is used in its broadest sense and encompass materials obtained from any source. As used herein, the term “sample” is used to refer to materials obtained from a biological source, for example, obtained from animals (including humans), and encompasses any fluids, solids, and tissues. In particular embodiments of the present disclosure, biological samples include blood and blood products such as plasma, serum and the like. However, these examples are not to be construed as limiting the types of samples that find use with the present disclosure.

[0077] As used herein, the term “antibody sample” refers to an antibody-containing composition (*e.g.*, plasma, blood, purified antibodies, blood or plasma fractions, blood or plasma components etc.) taken from or provided by a donor (*e.g.*, natural source) or obtained from a synthetic, recombinant, other *in vitro* source, or from a commercial source. The antibody sample may exhibit elevated titer of a particular antibody or set of antibodies based on the pathogenic/antigenic exposures (*e.g.*, natural exposure or through vaccination) of the donor or the antibodies engineered to be produced in the synthetic, recombinant, or *in vitro* context.

[0078] As used herein, the term “an amount effective to induce an immune response” (*e.g.*, of a composition for inducing an immune response), refers to the dosage level required (*e.g.*, when administered to a subject) to stimulate, generate and/or elicit an immune response in the

subject. An effective amount can be administered in one or more administrations (e.g., via the same or different route), applications or dosages and is not intended to be limited to a particular formulation or administration route.

[0079] As used herein, the term “under conditions such that said subject generates an immune response” refers to any qualitative or quantitative induction, generation, and/or stimulation of an immune response (e.g., innate or acquired).

[0080] As used herein, the term “immune response” refers to a response by the immune system of a subject. For example, immune responses include, but are not limited to, a detectable alteration (e.g., increase) in Toll-like receptor (TLR) activation, lymphokine (e.g., cytokine (e.g., Th1 or Th2 type cytokines) or chemokine) expression and/or secretion, macrophage activation, dendritic cell activation, T cell activation (e.g., CD4+ or CD8+ T cells), NK cell activation, and/or B cell activation (e.g., antibody generation and/or secretion). Additional examples of immune responses include binding of an immunogen (e.g., antigen (e.g., immunogenic polypeptide)) to an MHC molecule and inducing a cytotoxic T lymphocyte (“CTL”) response, inducing a B cell response (e.g., antibody production), and/or T-helper lymphocyte response, and/or a delayed type hypersensitivity (DTH) response against the antigen from which the immunogenic polypeptide is derived, expansion (e.g., growth of a population of cells) of cells of the immune system (e.g., T cells, B cells (e.g., of any stage of development (e.g., plasma cells), and increased processing and presentation of antigen by antigen presenting cells. An immune response may be to immunogens that the subject's immune system recognizes as foreign (e.g., non-self antigens from microorganisms (e.g., pathogens), or self-antigens recognized as foreign). Thus, it is to be understood that, as used herein, “immune response” refers to any type of immune response, including, but not limited to, innate immune responses (e.g., activation of Toll receptor signaling cascade) cell-mediated immune responses (e.g., responses mediated by T cells (e.g., antigen-specific T cells) and non-specific cells of the immune system) and humoral immune responses (e.g., responses mediated by B cells (e.g., via generation and secretion of antibodies into the plasma, lymph, and/or tissue fluids). The term “immune response” is meant to encompass all aspects of the capability of a subject's immune system to respond to antigens and/or immunogens (e.g., both the initial response to an immunogen (e.g., a pathogen) as well as acquired (e.g., memory) responses that are a result of an adaptive immune response).

[0081] Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the present disclosure. All publications, patent applications, patents and other references

mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

2. Dual-Affinity Ratiometric Quenching (DARQ) Bioassay

[0082] Embodiments of the present disclosure include a ratiometric bioassay method for detecting a target antibody in a sample. In accordance with these embodiments, the method includes combining an antigen coupled to a first fluorophore, an antibody-binding moiety coupled to a second fluorophore, and a sample comprising or suspected of comprising a target antibody. The first fluorophore, the antibody-binding moiety coupled to the second fluorophore, and the sample can be combined in any suitable vessel, container, tube, and the like that allows for a binding complex to form (see, e.g., FIG. 2). The method includes exposing the antigen coupled to the first fluorophore, the antibody-binding moiety coupled to the second fluorophore, and the sample to fluorescent light comprising an excitation wavelength of the first and/or the second fluorophore, and detecting fluorescence emission from the first and/or the second fluorophore. In some embodiments, the fluorescence emission from the first and the second fluorophore is reduced, and wherein the reduced fluorescence emission is proportional to the antibody concentration in the sample. In some embodiments, and as described further herein, the ratiometric bioassays of the present disclosure involve fluorescence quenching of the first and/or the second fluorophore to determine antibody concentration, rather than a FRET-based mechanism. That is, the ratiometric bioassays of the present disclosure do not rely on emission from a first fluorophore to excite a second fluorophore.

[0083] In some embodiments, the antigen coupled to the first fluorophore is any antigen that is characterized as being capable of binding to the target antibody. The antigen can be the fully characterized antigen identified as being capable of binding to the target antibody, or it can be any fragment or derivative thereof, provided the portion of the antigen (e.g., epitope) recognized by the target antibody is functionally intact. As would be readily apparent to one of ordinary skill in the art based on the present disclosure, the compositions and methods provided herein can include the use of any antigen known to bind a target antibody, and any antigen subsequently developed or identified as being capable of binding a target antibody. Antigens can be obtained through any means known in the art, including but not limited to, chemical synthesis, protein purification, and genetic and cellular engineering.

[0084] As described further herein, the antigen is coupled to a first fluorophore that can be either a high-energy fluorophore or a low-energy fluorophore. In some embodiments, the antigen can be coupled to another agent in addition to the first fluorophore, provided that the

additional agent does not interfere with the ability of the fluorophore to emit fluorescence or the ability of the antigen to bind the target antibody (e.g., purification tag). As would be recognized by one of ordinary skill in the art based on the present disclosure (see, e.g., Materials and Methods), coupling a fluorophore to an antigen can be done by any means known in the art. In some embodiments, coupling an antigen to a fluorophore is referred to as functional coupling because both the fluorophore and the antigen maintain at least some degree of functionality as compared to their functionality prior to coupling.

[0085] In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.5 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:1 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:1.5 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:2 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:3 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:4 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:5 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:10 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:15 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:2.0 to about 1:20. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:15. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:10. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:5. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:4. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:3. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:2. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:1. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:1 to about 1:10. In some

embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:2 to about 1:8. In some embodiments, the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:3 to about 1:6.

[0086] In some embodiments, the antibody-binding moiety comprises a polypeptide capable of binding a region of the target antibody that does not comprise the antigen-binding site. In some embodiments, the antibody-binding moiety coupled to the second fluorophore includes any antibody-binding moiety that is characterized a being capable of binding to the target antibody. The antibody-binding moiety can be a fully characterized polypeptide or protein identified as being capable of binding to the target antibody, or it can be any fragment or derivative thereof, provided the portion of the antibody-binding moiety that can bind to the target antibody is functionally intact. As would be readily apparent to one of ordinary skill in the art based on the present disclosure, the compositions and methods provided herein can include the use of any antibody-binding moiety known to bind a target antibody, and any antibody-binding moiety subsequently developed or identified as being capable of binding a target antibody. For example, in some embodiments, the antibody binding moiety comprises Protein L, Protein A, or Protein G, including any fragments, variants, or derivatives thereof.

[0087] As described further herein, the antibody-binding moiety is coupled to a second fluorophore that can be either a high-energy fluorophore or a low-energy fluorophore. In some embodiments, the antibody-binding moiety can be coupled to another agent in addition to the second fluorophore, provided that the additional agent does not interfere with the ability of the fluorophore to emit fluorescence or the ability of the antibody-binding moiety to bind the target antibody (e.g., purification tag). As would be recognized by one of ordinary skill in the art based on the present disclosure (see, e.g., Materials and Methods), coupling a fluorophore to an antibody-binding moiety can be done by any means known in the art. In some embodiments, coupling an antibody-binding moiety to a fluorophore is referred to as functional coupling because both the fluorophore and the antibody-binding moiety maintain at least some degree of functionality as compared to their functionality prior to coupling.

[0088] In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.5 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:1 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-

to-fluorophore ratio of about 1:1.5 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:2 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:3 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:4 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:5 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:10 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:15 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:2.0 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:15. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:10. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:5. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:4. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:3. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:2. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:1. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:1 to about 1:10. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:2 to about 1:8. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:3 to about 1:6.

[0089] As described further herein, the method includes exposing the antigen coupled to the first fluorophore, the antibody-binding moiety coupled to the second fluorophore, and the

sample to fluorescent light comprising an excitation wavelength of the first and/or the second fluorophore, and detecting fluorescence emission from the first and/or the second fluorophore. In accordance with these embodiments, the first and second fluorophores can be either high-energy or low-energy fluorophores. In some embodiments, the antigen is coupled to a first fluorophore that is a high-energy fluorophore, and the antibody-binding moiety is coupled to a second fluorophore that is a low-energy fluorophore. In some embodiments, the antigen is coupled to a first fluorophore that is a low-energy fluorophore, and the antibody-binding moiety is coupled to a second fluorophore that is a high-energy fluorophore. As described further herein, a high-energy fluorophore (or higher-energy fluorophore) generally has an excitation spectra bathochromically shifted from the excitation spectra of a low-energy fluorophore (or lower-energy fluorophore). In addition, the high-energy fluorophore may have an emission spectra that is also bathochromically shifted from the excitation spectra of the low-energy fluorophore.

[0090] In some embodiments, the high-energy fluorophore and/or the low-energy fluorophore include any fluorophore(s) capable of binding N-hydroxysuccinimide (NHS). In some embodiments, the high-energy fluorophore is selected from the group consisting of Fluorescein, Oregon Green 488, Oregon Green 514, Rhodamine Green, Rhodamine Green-X, Eosin, 4', 6-diamidino-2-phenylindole, Alexafluor 405, Alexafluor 350, Alexafluor 500, Alexafluor 488, Alexafluor 430, Alexafluor 514, and Alexafluor 532. In some embodiments, the low-energy fluorophore is selected from the group consisting of Rhodamine, Rhodamine B, Rhodamine Red-X, Tetramethylrhodamine, Lissamine, Texas Red and Texas Red-X, Naphthofluorescein, Carboxyrhodamine 6G, Alexafluor 555, Alexafluor 546, Alexafluor 568, Alexafluor 594, Alexafluor 610, Alexafluor 633, Alexafluor 635, Alexafluor 647, Alexafluor 660, Alexafluor 680, Alexafluor 700, and Alexafluor 750. In some embodiments, the antigen is coupled to fluorescein or Alexafluor 350. In some embodiments, the antibody-binding moiety is coupled to Rhodamine.

[0091] In some embodiments, the method further includes determining the concentration of a target antibody in a sample based on the reduction in fluorescence emission of the first and/or the second fluorophore. In some embodiments, the concentration of a target antibody in a sample is based on the reduction in fluorescence emission of the first fluorophore. In some embodiments, the concentration of a target antibody in a sample is based on the reduction in fluorescence emission of the second fluorophore. In some embodiments, the concentration of a target antibody in a sample is based on the reduction in fluorescence emission of the first fluorophore and the second fluorophore.

[0092] In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:1 to about 1:20. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:1 to about 1:15. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:1 to about 1:10. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:1 to about 1:5. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:5 to about 1:20. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:10 to about 1:20. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:15 to about 1:20. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:5 to about 1:15. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:5 to about 1:10. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:10 to about 1:20. In some embodiments, the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:10 to about 1:15.

[0093] In some embodiments, the method further includes incubating the sample comprising or suspected of comprising the target antibody, the antigen coupled to the first fluorophore, and the antibody-binding moiety coupled to the second fluorophore for 30 minutes or less prior to measuring the fluorescent emission of the first and/or the second fluorophore. In some embodiments, the method further includes incubating the sample comprising or suspected of comprising the target antibody, the antigen coupled to the first fluorophore, and the antibody-binding moiety coupled to the second fluorophore for 20 minutes or less prior to measuring the fluorescent emission of the first and/or the second fluorophore. In some embodiments, the method further includes incubating the sample comprising or suspected of comprising the target antibody, the antigen coupled to the first fluorophore, and the antibody-binding moiety coupled to the second fluorophore for 10 minutes or less prior to measuring the fluorescent emission of the first and/or the second fluorophore. In some embodiments, the method includes incubating for about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, about 5 minutes, about 6 minutes, about 7 minutes, about 8 minutes, about 9 minutes,

about 10 minutes, about 11 minutes, about 12 minutes, about 13 minutes, about 14 minutes, about 15 minutes, about 16 minutes, about 17 minutes, about 18 minutes, about 19 minutes, about 20 minutes, about 21 minutes, about 22 minutes, about 23 minutes, about 24 minutes, about 25 minutes, about 26 minutes, about 27 minutes, about 28 minutes, about 29 minutes, or about 30 minutes. In some embodiments, the method includes incubating for over 30 minutes.

[0094] In accordance with the above embodiments, the bioassay methods and compositions of the present disclosure can be used with any sample that contains, or is suspected of containing, a target antibody. In some embodiments, sample is undiluted (e.g., sampled directly) prior to the bioassay being performed. In some embodiments, sample is diluted prior to the bioassay being performed, such as with a suitable buffer or other agent. In some embodiments, the sample is obtained from an organism or a portion of an organism. In some embodiments, the sample is obtained from a bodily fluid of a mammal (e.g., a human). In some embodiments, the sample is a whole blood sample, a plasma sample, a serum sample, a urine sample, a saliva sample, or a tissue sample. In some embodiments, the sample is obtained from an industrial biological, chemical, or biochemical process. For example, in some embodiments, the sample is a cell culture sample, a cell lysate sample, or a cell culture media sample. As would be recognized by one of ordinary skill in the art based on the present disclosure, other samples may also be used with the bioassays of the present disclosure.

3. Compositions and Kits

[0095] Embodiments of the present disclosure also include a composition for performing a ratiometric bioassay to detect an antibody in a sample, as described above. In accordance with these embodiments, the composition includes an antigen coupled to a first fluorophore, an antibody-binding moiety coupled to a second fluorophore, and a sample comprising or suspected of comprising a target antibody. The first fluorophore, the antibody-binding moiety coupled to the second fluorophore, and the sample can be combined in any suitable vessel, container, tube, and the like that allows for a binding complex to form (see, e.g., FIG. 2). In some embodiments, fluorescence emission from the first and the second fluorophore is reduced upon exposure to fluorescent light comprising an excitation wavelength of the first and/or the second fluorophore. In some embodiments, the reduced fluorescence emission is proportional to the antibody concentration in the sample.

[0096] In some embodiments, the antibody-binding moiety comprises a polypeptide capable of binding a region of the target antibody that does not comprise the antigen-binding site. portion of the antibody-binding moiety that can bind to the target antibody is functionally

intact. As would be readily apparent to one of ordinary skill in the art based on the present disclosure, the compositions and methods provided herein can include the use of any antibody-binding moiety known to bind a target antibody, and any antibody-binding moiety subsequently developed or identified as being capable of binding a target antibody. For example, in some embodiments, the antibody binding moiety comprises Protein L, Protein A, or Protein G, including any fragments, variants, or derivatives thereof.

[0097] In some embodiments, the antibody-binding moiety in the composition is coupled to a second fluorophore that can be either a high-energy fluorophore or a low-energy fluorophore. In some embodiments, the antibody-binding moiety can be coupled to another agent in addition to the second fluorophore, provided that the additional agent does not interfere with the ability of the fluorophore to emit fluorescence or the ability of the antibody-binding moiety to bind the target antibody (e.g., purification tag). As would be recognized by one of ordinary skill in the art based on the present disclosure (see, e.g., Materials and Methods), coupling a fluorophore to an antibody-binding moiety can be done by any means known in the art. In some embodiments, coupling an antibody-binding moiety to a fluorophore is referred to as functional coupling because both the fluorophore and the antibody-binding moiety maintain at least some degree of functionality as compared to their functionality prior to coupling.

[0098] In some embodiments, the antibody-binding moiety in the composition is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.5 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:1 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:1.5 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:2 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:3 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:4 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:5 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:10 to about 1:20. In some

embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:15 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:2.0 to about 1:20. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:15. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:10. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:5. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:4. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:3. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:2. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:0.2 to about 1:1. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:1 to about 1:10. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:2 to about 1:8. In some embodiments, the antibody-binding moiety is coupled to the second fluorophore at an antibody-binding moiety-to-fluorophore ratio of about 1:3 to about 1:6.

[0099] In some embodiments of the composition, the first and second fluorophores can be either high-energy or low-energy fluorophores. In some embodiments of the composition, the antigen is coupled to a first fluorophore that is a high-energy fluorophore, and the antibody-binding moiety is coupled to a second fluorophore that is a low-energy fluorophore. In some embodiments of the composition, the antigen is coupled to a first fluorophore that is a low-energy fluorophore, and the antibody-binding moiety is coupled to a second fluorophore that is a high-energy fluorophore. In some embodiments, a high-energy fluorophore (or higher-energy fluorophore) generally has an excitation spectra bathochromically shifted from the excitation spectra of a low-energy fluorophore (or lower-energy fluorophore). In addition, the high-energy fluorophore may have an emission spectra that is also bathochromically shifted from the excitation spectra of the low-energy fluorophore.

[0100] In some embodiments of the composition, the high-energy fluorophore is selected from the group consisting of Fluorescein, Oregon Green 488, Oregon Green 514, Rhodamine Green, Rhodamine Green-X, Eosin, 4', 6-diamidino-2-phenylindole, Alexafluor 405, Alexafluor 350, Alexafluor 500, Alexafluor 488, Alexafluor 430, Alexafluor 514, and Alexafluor 532. In some embodiments, the low-energy fluorophore is selected from the group consisting of Rhodamine, Rhodamine B, Rhodamine Red-X, Tetramethylrhodamine, Lissamine, Texas Red and Texas Red-X, Naphthofluorescein, Carboxyrhodamine 6G, Alexafluor 555, Alexafluor 546, Alexafluor 568, Alexafluor 594, Alexafluor 610, Alexafluor 633, Alexafluor 635, Alexafluor 647, Alexafluor 660, Alexafluor 680, Alexafluor 700, and Alexafluor 750. In some embodiments, the antigen is coupled to fluorescein or Alexafluor 350. In some embodiments, the antibody-binding moiety is coupled to Rhodamine.

[0101] In some embodiments, the bioassay methods and compositions of the present disclosure can be used with any sample that contains, or is suspected of containing, a target antibody. In some embodiments, sample is undiluted (e.g., sampled directly) prior to the bioassay being performed. In some embodiments, sample is diluted prior to the bioassay being performed, such as with a suitable buffer or other agent. In some embodiments, the sample is obtained from an organism or a portion of an organism. In some embodiments, the sample is obtained from a bodily fluid of a mammal (e.g., a human). In some embodiments, the sample is a whole blood sample, a plasma sample, a serum sample, a urine sample, a saliva sample, or a tissue sample. In some embodiments, the sample is obtained from an industrial biological, chemical, or biochemical process. For example, in some embodiments, the sample is a cell culture sample, a cell lysate sample, or a cell culture media sample. As would be recognized by one of ordinary skill in the art based on the present disclosure, other samples may also be used with the bioassays of the present disclosure.

[0102] In some embodiments, a sample refers to any fluid sample containing or suspected of containing a target antibody. The sample may be derived from any suitable source. In some cases, the sample may comprise a liquid, fluent particulate solid, or fluid suspension of solid particles. In some cases, the sample may be processed prior to the analysis described herein. For example, the sample may be separated or purified from its source prior to analysis; however, in certain embodiments, an unprocessed sample containing at least one target antibody may be assayed directly. In a particular example, the source of a target antibody is a mammalian (e.g., human) bodily substance (e.g., bodily fluid, blood such as whole blood (including, for example, capillary blood, venous blood, etc.), serum, plasma, urine, saliva, sweat, sputum, semen, mucus, lacrimal fluid, lymph fluid, amniotic fluid, interstitial fluid,

lower respiratory specimens such as, but not limited to, sputum, endotracheal aspirate or bronchoalveolar lavage, cerebrospinal fluid, feces, tissue, organ, one or more dried blood spots, or the like). Tissues may include, but are not limited to oropharyngeal specimens, nasopharyngeal specimens, skeletal muscle tissue, liver tissue, lung tissue, kidney tissue, myocardial tissue, brain tissue, bone marrow, cervix tissue, skin, etc. The sample may be a liquid sample or a liquid extract of a solid sample. In certain cases, the source of the sample may be an organ or tissue, such as a biopsy sample, which may be solubilized by tissue disintegration/cell lysis. Additionally, the sample can be a nasopharyngeal or oropharyngeal sample obtained using one or more swabs that, once obtained, is placed in a sterile tube containing a virus transport media (VTM) or universal transport media (UTM), for testing.

[0103] A wide range of volumes of the fluid sample may be analyzed. In a few exemplary embodiments, the sample volume may be about 0.5 nL, about 1 nL, about 3 nL, about 0.01 μ L, about 0.1 μ L, about 1 μ L, about 5 μ L, about 10 μ L, about 100 μ L, about 1 mL, about 5 mL, about 10 mL, or the like. In some cases, the volume of the fluid sample is between about 0.01 μ L and about 10 mL, between about 0.01 μ L and about 1 mL, between about 0.01 μ L and about 100 μ L, or between about 0.1 μ L and about 10 μ L. In some cases, the fluid sample may be diluted prior to use in an assay. For example, in embodiments where the source containing a target antibody is a human body fluid (e.g., blood, serum), the fluid may be diluted with an appropriate solvent (e.g., a buffer such as PBS buffer). A fluid sample may be diluted about 1-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 10-fold, about 100-fold, or greater, prior to use. In other cases, the fluid sample is not diluted prior to use in an assay.

[0104] Embodiments of the present disclosure also include a kit for performing a ratiometric bioassay to detect a target antibody in a sample. In accordance with these embodiments, the kit includes an antigen coupled to a first fluorophore, an antibody binding moiety coupled to a second fluorophore, and at least one container. In some embodiments, the kit further comprises a buffer and/or instructions for performing the bioassay. In some embodiments, the kit further comprises the target antibody or a fragment or derivative thereof.

[0105] In some embodiments, the kit comprises at least one component for assaying the test sample for a target antibody and corresponding instructions. Instructions included in kits can be affixed to packaging material or can be included as a package insert. While the instructions are typically written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this disclosure. Such media include, but are not limited to, electronic storage media (e.g., magnetic

discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. As used herein, the term “instructions” can include the address of an internet site that provides the instructions.

[0106] The kit can also comprise a calibrator or control (e.g., purified, and optionally lyophilized, target antibody or fragment thereof) and/or at least one container (e.g., tube, microtiter plates) for conducting the assay, and/or a buffer, such as an assay buffer or a wash buffer, either one of which can be provided as a concentrated solution, a substrate solution for the detectable label, or a stop solution. In some embodiments, the kit comprises all components that are necessary to perform the assay (e.g., reagents, standards, buffers, diluents, and the like). The instructions also can include instructions for generating a standard curve.

[0107] The kit may further comprise reference standards for quantifying a target antibody. The reference standards may be employed to establish standard curves for interpolation and/or extrapolation of antibody concentrations. Standards can include proteins or peptide fragments composed of amino acids residues or labeled proteins or peptide fragments for various analytes, as well as standards for sample processing, including standards involving spikes in proteins and quantitative peptides. Kits can also include quality control components (for example, sensitivity panels, calibrators, and positive controls). Preparation of quality control reagents is well-known in the art and is described on insert sheets for a variety of products. Sensitivity panel members can be used to establish assay performance characteristics, and can be useful indicators of the integrity of bioassay kit reagents, and the standardization of assays. The kit can also include other reagents required to conduct a bioassay or facilitate quality control evaluations, such as buffers, salts, enzymes, enzyme co-factors, substrates, detection reagents, and the like. Other components, such as buffers and solutions for the isolation and/or treatment of a test sample (e.g., pretreatment reagents), also can be included in the kit.

[0108] As would be recognized and understood by one of ordinary skill in the art based on the present disclosure, the various embodiments of the ratiometric bioassay methods and compositions described herein can be used to detect antibodies specific for any antigen, such as an antigen from a pathogenic organism. In some embodiments, the ratiometric bioassay methods and compositions of the present disclosure can be used to detect and/or quantify antibodies in a sample from a subject that has been infected with a pathogenic organism or from a recombinant system that has been engineered to express such antigen-targeting antibodies. Pathogenic organisms include, but are not limited to, fungi, bacteria, viruses, and parasites. In some embodiments, detecting and/or quantifying antibodies specific for one or more antigens of a pathogenic organism indicate that the pathogenic organism has elicited an immune response in the subject. In some embodiments, recombinant systems that have been

engineered to express antigen-targeting antibodies include mammalian cells (*e.g.*, CHO and HEK293 cells) or bacteria (*e.g.*, *E. coli* or *Pichia pastoris* or *Saccharomyces cerevisiae*).

[0109] In some embodiments, the ratiometric bioassay methods and compositions of the present disclosure can be used to detect antibodies in a sample from a patient (*e.g.*, whole blood sample, a plasma sample, a serum sample, a urine sample, a saliva sample, a tissue sample, and the like) that has been infected with, or is suspected of being infected with, a coronavirus (*e.g.*, coronavirus OC43, coronavirus 229E, coronavirus NL63, coronavirus HKU1, MERS-CoV, SARS-CoV, or SARS-CoV-2 (COVID-19)). Currently, most available serological tests that detect SARS-CoV-2 antibodies are lateral flow assays (LFA) that are based on simple positive or negative detection of antibodies, which is feasible for inexpensive and point-of-care (POC) use and large-scale surveillance but not informative regarding the amount, type, or function of the antibodies. An alternative for accurately detecting antibodies against SARS-CoV-2 is the enzyme-linked immunosorbent assay (ELISA), which can measure not only the presence but also the titer (amount) and type (IgG, IgM, monomeric or dimeric IgA) of antibody. ELISA assays allow for a better measure of the strength of the humoral response, but are complex and can only be performed in a laboratory setting. Additionally, ELISA is not ideal for virus neutralization/blocking tests, which is crucially important in studying the humoral response during vaccine development and vaccination but not widely available. Current neutralization assays usually involve propagation of viruses and require such assays to be conducted in a biosafety level 3 (BSL3) lab settings, which unfortunately is unavailable to many researchers or the public.

[0110] In some embodiments, the ratiometric bioassay methods and compositions of the present disclosure can be used to detect antibodies in a sample from a patient (*e.g.*, whole blood sample, a plasma sample, a serum sample, a urine sample, a saliva sample, a tissue sample, and the like) that has been infected with, or is suspected of being infected with, a coronavirus (*e.g.*, coronavirus OC43, coronavirus 229E, coronavirus NL63, coronavirus HKU1, MERS-CoV, SARS-CoV, or SARS-CoV-2 (COVID-19)). Currently, most available serological tests that detect SARS-CoV-2 antibodies are lateral flow assays (LFA) that are based on simple positive or negative detection of antibodies, which is feasible for inexpensive and point-of-care (POC) use and large-scale surveillance but not informative regarding the amount, type, or function of the antibodies. An alternative for accurately detecting antibodies against SARS-CoV-2 is the enzyme-linked immunosorbent assay (ELISA), which can measure not only the presence but also the titer (amount) and type (IgG, IgM, monomeric or dimeric IgA) of antibody. ELISA assays allow for a better measure of the strength of the humoral response, but are complex and

can only be performed in a laboratory setting. Additionally, ELISA is not ideal for virus neutralization/blocking tests, which is crucially important in studying the humoral response during vaccine development and vaccination but not widely available. Current neutralization assays usually involve propagation of viruses and require such assays to be conducted in a biosafety level 3 (BSL3) lab settings, which unfortunately is unavailable to many researchers or the public.

[0111] Further, in accordance with the ratiometric bioassay methods and compositions described herein, embodiments of the present disclosure include detecting and/or quantifying coronavirus-neutralizing monoclonal antibodies in a sample obtained from a bioreactor wherein cells recombinantly engineered to express that monoclonal antibody have been cultured and produced said antibody, either as an intracellular or extracellular product. Currently, analytical assays utilized in the biopharmaceutical and biomanufacturing industries for the quantification of recombinant monoclonal antibodies rely on ELISA tests. The slow kinetics of ELISAs make them inherently off-line assays, thereby reducing the ability of optimizing the process in real time.

[0112] Neutralizing antibodies identified using the disclosed methods and compositions can specifically bind to any known or as yet undiscovered coronavirus, such as, for example, coronavirus OC43, coronavirus 229E, coronavirus NL63, coronavirus HKU1, MERS-CoV, SARS-CoV, or SARS-CoV-2 (COVID-19). In some embodiments, the neutralizing antibodies are directed against SARS-CoV-2 (COVID-19). In the context of the present disclosure a “neutralizing antibody” is an antibody that binds to a virus (e.g., a coronavirus) and interferes with the virus’ ability to infect a host cell. Coronavirus spike proteins are known to elicit potent neutralizing-antibody and T-cell responses. The ability of a virus (e.g., coronavirus OC43, coronavirus 229E, coronavirus NL63, coronavirus HKU1, MERS-CoV, SARS-CoV, or SARS-CoV-2 (COVID-19)) to gain entry into cells and establish infection is mediated by the interactions of its Spike glycoproteins with human cell surface receptors. In the case of coronaviruses, Spike proteins are large type I transmembrane protein trimers that protrude from the surface of coronavirus virions. Each Spike protein comprises a large ectodomain (comprising S1 and S2), a transmembrane anchor, and a short intracellular tail. The S1 subunit of the ectodomain mediates binding of the virion to host cell-surface receptors through its receptor-binding domain (RBD). The S2 subunit fuses with both host and viral membranes, by undergoing structural changes.

[0113] SARS-CoV-2 utilizes the Spike glycoprotein to interact with cellular receptor ACE2. The amino acid sequence of the SARS-CoV-2 spike protein has been deposited with the

National Center for Biotechnology Information (NCBI) under Accession No. QHD43416. Binding with ACE2 triggers a cascade of cell membrane fusion events for viral entry. The high-resolution structure of SARSCoV-2 RBD bound to the N-terminal peptidase domain of ACE2 has recently been determined, and the overall ACE2-binding mechanism is virtually the same between SARS-CoV-2 and SARS-CoV RBDs, indicating convergent ACE2-binding evolution between these two viruses. This indicates that disruption of the RBD and ACE2 interaction (e.g., by neutralizing antibodies) acts to block SARS-CoV-2 entry into the target cell. In addition, neutralizing antibodies directed against coronaviruses have been identified and isolated (see, e.g., Liu et al., Potent neutralizing antibodies directed to multiple epitopes on SARS-CoV-2 spike. *Nature* (2020). doi.org/10.1038/s41586-020-2571-7; Rogers et al., *Science* 15 Jun 2020:eabc7520; DOI: 10.1126/science.abc7520; Alsoussi et al., *J Immunol* June 26, 2020, ji2000583; DOI: /doi.org/10.4049/jimmunol.2000583; Kreer et al., *Cell*, S0092-8674(20)30821-7. 13 Jul. 2020, doi:10.1016/j.cell.2020.06.044; Tai et al., *J Virol*. 2017 Jan 1; 91(1): e01651-16; and Niu et al., *J Infect Dis*. 2018 Oct 15; 218(8): 1249–1260).

[0114] In accordance with the above, the ratiometric bioassay methods and compositions described herein can include the use of a coronavirus antigen, such as an antigen from the RBD of the Spike protein of SARS-CoV-2, in combination with an antibody-binding moiety (e.g., Protein L, Protein A, or Protein G). In some embodiments, the coronavirus antigen is coupled to a first fluorophore, and the antibody-binding moiety is coupled to a second fluorophore, wherein the fluorescence emission from the first and the second fluorophore is reduced, and wherein the reduced fluorescence emission is proportional to the antibody concentration in the sample, as described further herein. In this manner, neutralizing antibodies directed against SARS-CoV-2 can be detected and/or quantified in a sample from a patient or from a recombinant source. In some embodiments, the coronavirus antigen (e.g., peptide comprising a rRBD of a coronavirus spike protein) may be prepared using routine molecular biology techniques, as would be recognized by one of ordinary skill in the art based on the present disclosure. The nucleic acid and amino acid sequences of RBDs of various coronavirus spike proteins are known in the art (see, e.g., Tai et al., *Cell Mol Immunol* 17, 613-620 (2020). doi.org/10.1038/s41423-020-0400-4; and Chakraborti et al., *Virology Journal* volume 2, Article number: 73 (2005); and Chen et al., *Biochemical and Biophysical Research Communications*, 525(1): 135-140 (2020)). An exemplary RBD domain of a SARS-CoV-2 spike protein can comprise the following amino acid sequence:

RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISNCVADYSVLVNSASFST
FKYGVSPTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCVI

AWNSNNLDSKVGGNYNLYRLFRKSNLKPFERDISTEIQAGSTPCNGVEGFNCYFP
 LQSYGFQPTNGVGYQPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLT
 GTGVLTESN KKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCS.

[0115] In some embodiments, the ratiometric bioassay methods and compositions described herein can be used to assess the coronavirus neutralization capacity or titer of a sample from an individual subject, or the coronavirus neutralization capacity or titer of a sample from multiple subjects (e.g., pooled plasma samples) or the coronavirus neutralization capacity or titer of a sample from a bioreactor (e.g., a cell culture fluid obtained from cells engineered to express the coronavirus neutralizing antibody). In some embodiments, the ratiometric bioassay methods and compositions described herein can be used to determine whether a subject has been infected with a coronavirus. In some embodiments, the ratiometric bioassay methods and compositions described herein can be used to determine whether a subject that has been vaccinated against a coronavirus has elicited a sufficient immune response to the vaccine (e.g., produced sufficiently neutralizing coronavirus antibody titers). In some embodiments, the ratiometric bioassay methods and compositions described herein can be used to determine whether the engineered cells have expressed (either intracellularly or extracellularly) the coronavirus-targeting antibody.

4. Materials and Methods

[0116] *Materials.* Pierce recombinant Protein L (PrL), NHS-Rhodamine, NHS-Fluorescein, and Coomassie (Bradford) protein assay were from Thermo Scientific (Waltham, MA, USA). Human TNF- α from Shenandoah Biotechnology Inc. (Warwick, PA, USA), while Her2 was sourced from ACROBiosystems (Newark, DE, USA). Anti-TNF- α hIgG₁ (Adalimumab) was sourced from InvivoGen (San Diego, CA, USA), while anti-Her2 hIgG₁ (Trastuzumab) was obtained from Selleckchem (Houston, TX, USA). Phosphate Buffer Saline at pH 7.4 (PBS), sodium bicarbonate, sodium hydroxide, aqueous HCl, dimethylsulfoxide (DMSO) and Amicon Ultra centrifugal filters (0.5 mL, 3KDa MWCO) from Millipore Sigma (St. Louis, MO, USA). 96 well non-binding microplates (F-Bottom/Chimney Well, Solid, Black) were from Greiner Bio-One (Monroe, NC, USA), while the 96 well non-treated microplates were from Thermo Fisher Scientific (Waltham, MA, USA). The null Chinese Hamster Ovary (CHO-S) cell culture fluid was donated by the Biomanufacturing Training and Education Center (BTEC) at NC State University.

[0117] *Protein labeling and quantification.* NHS-Fluorescein and NHS-Rhodamine were initially dissolved in DMSO at $5 \text{ mg} \cdot \text{mL}^{-1}$ and $10 \text{ mg} \cdot \text{mL}^{-1}$, respectively. PrL, Her2, and TNF- α were initially dissolved in 50 mM sodium bicarbonate at pH 8.5 at $10 \text{ mg} \cdot \text{mL}^{-1}$. A volume of 5.4 μL of NHS-Fluorescein solution in DMSO and 50 μL of antigen (either Her2 or TNF- α) solution were incubated at 0°C for 2 hours in dark. A volume of 18 μL of NHS-Rhodamine solution in DMSO and 50 μL of PrL solution were incubated at 0°C for 2 hours in dark. Following incubation, the fluorescent labels were removed by centrifugation with Amicon Ultra centrifugal filters (0.5 mL, 3KDa MWCO) at $14,000 \times g$ for 20 min, followed by diafiltration with PBS at pH 7.4. The concentration of $^{\text{Rh}}\text{PrL}$, $^{\text{Fl}}\text{Her2}$, and $^{\text{Fl}}\text{TNF-}\alpha$ in PBS was determined by Bradford assay, following manufacturer's specifications. The absorbance of the solutions of $^{\text{Rh}}\text{PrL}$ and fluorescein-labeled antigen ($^{\text{Fl}}\text{Her2}$ and $^{\text{Fl}}\text{TNF-}\alpha$) were measured by UV spectrophotometry at a wavelength of 494 nm and 552 nm, respectively, using a Synergy H1 plate reader (Biotek, Winooski, VT). From these values, the values of fluorophore-to-protein (F/P) ratio for the various labeled proteins were calculated using Equation 1.

[0118] Moles dye per mole protein = $\frac{A_{\text{max of labeled protein}}}{\epsilon' \times \text{protein concentration (M)}} \times \text{dilution factor}$ (1)

[0119] *Quantification of Trastuzumab and Adalimumab via DARQ assay in non-competitive conditions.* A volume of 500 μL of $57.4 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ fluorescein-labeled antigen ($^{\text{Fl}}\text{Her2}$ or $^{\text{Fl}}\text{TNF-}\alpha$) and 500 μL of $20 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ $^{\text{Rh}}\text{PrL}$ were mixed with 11 mL of PBS to reach a total volume of 12 mL at 25.2 nM of each protein. A volume of 230 μL of $^{\text{Rh}}\text{PrL}/^{\text{Fl}}\text{Her2}$ mixture was dispensed in 48 wells of a 96 well non-binding microplate, and 20 μL of solution of Trastuzumab at different concentrations was added to every well to achieve a final concentration of 23.2 nM for both $^{\text{Rh}}\text{PrL}$ and $^{\text{Fl}}\text{Her2}$, and a concentration of Trastuzumab varying between 0.92 and 18.4 nM. Additional sets of samples serving as negative control were prepared by replacing either $^{\text{Fl}}\text{Her2}$ or $^{\text{Rh}}\text{PrL}$ with a corresponding volume of PBS. Upon mixing the mAb solution with the fluorescently labeled affinity tags, the solutions were incubated for either 1, 5, 10, or 20 min at room temperature, and analyzed by fluorescence spectrophotometry using a Synergy H1 plate reader operated at the excitation wavelength of 480 nm and emission wavelengths of 525 nm and 573 nm. An identical set of experiments was repeated using Adalimumab, $^{\text{Rh}}\text{PrL}$, and $^{\text{Fl}}\text{TNF-}\alpha$.

[0120] *Quantification of Trastuzumab and Adalimumab via DARQ assay in competitive conditions.* The experiments described herein were repeated by dissolving the lyophilized mAb, $^{\text{Rh}}\text{PrL}$, and $^{\text{Fl}}\text{Her2}$ in clarified cell culture harvest produced with a null (non mAb-expressing) Chinese Hamster Ovary (CHO-S) cell line.

[0121] *Evaluation of the fluorescence quenching mechanism.* PrL, Her2, and TNF- α were initially dissolved in 50 mM sodium bicarbonate at pH 8.5 at 10 mg·mL⁻¹; in parallel, NHS-Fluorescein, NHS-Rhodamine, and NHS-AlexaFluor 350 (AF350) were dissolved in DMSO at 5 mg·mL⁻¹, 10 mg·mL⁻¹, and 10 mg·mL⁻¹ respectively. A volume of 2.26 μ L of NHS-Rhodamine solution in DMSO and 25 μ L of TNF- α solution were incubated at 0°C for 2 hours in dark; in parallel, 22.1 μ L of NHS-Rhodamine solution in DMSO with 140 μ L of PrL solution, and 1.80 μ L of NHS-AF350 solution in DMSO with 62 μ L of TNF- α solution were incubated at 0°C for 2 hours in dark. Following incubation, the fluorescent labels were removed by diafiltration as described above. The solutions of ^{Rh}PrL, ^{Rh}TNF- α , and ^{AF350}TNF- α in PBS were analyzed via Bradford assay and UV spectrophotometry at the wavelength of 552 nm to determine the values of fluorophore-to-protein (F/P) ratio using Equation 1. A volume of 500 μ L of ^{Rh}TNF- α solution at 57.4 μ g·mL⁻¹ and 500 μ L of ^{Rh}PrL solution at 20 μ g·mL⁻¹ were mixed with 11 mL of PBS to produce 12 mL of ^{Rh}TNF- α /^{Rh}PrL stock solution wherein the concentration of each protein is 25.2 nM. In parallel, a volume of 500 μ L of ^{AF350}TNF- α solution at 57.4 μ g·mL⁻¹ and 500 μ L of ^{Rh}PrL solution at 20 μ g·mL⁻¹ were mixed with 11 mL of PBS to produce 12 mL of ^{AF350}TNF- α /^{Rh}PrL stock solution wherein the concentration of each protein is 25.2 nM; additional stock solutions were made with identical composition, wherein unlabeled TNF- α was used in lieu of ^{Rh}TNF- α to produce a TNF- α /^{Rh}PrL stock solution, and unlabeled PrL was used in lieu of ^{Rh}PrL to produce a ^{AF350}TNF- α /PrL stock solution.

[0122] Aliquots of 230 μ L of ^{Rh}PrL/^{Rh}TNF- α stock solution were dispensed in 48 wells of a 96 well non-binding microplate, to which 20 μ L aliquots of Adalimumab solutions at different concentrations were added to achieve a final concentration in each well of 23.2 nM for both ^{Rh}PrL and ^{Rh}TNF- α , and varying between 0.92 and 18.4 nM for Adalimumab. Another 48 wells were filled with samples with identical composition, with the sole difference that ^{Rh}PrL/TNF- α was used as stock solution (unlabeled TNF- α). Upon mixing, the samples were incubated for 5 min at room temperature and analyzed by fluorescence spectrophotometry at the excitation wavelength of 480 nm and emission wavelengths of 525 nm and 573 nm, along with an excitation of 525 nm and emission at 573 nm.

[0123] Aliquots of 230 μ L of ^{AF350}TNF- α /^{Rh}PrL stock solution were dispensed in 48 wells of a 96 well non-binding microplate, to which 20 μ L aliquots of Adalimumab solutions at different concentrations were added to achieve a final concentration in each well of 23.2 nM for both ^{AF350}TNF- α and ^{Rh}PrL, and varying between 0.92 and 17.8 nM for Adalimumab. Another 48 wells were filled with samples with identical composition, with the sole difference

that AF350-TNF- α /PrL was used as stock solution (unlabeled PrL). Upon mixing, the samples were incubated for 25 min at room temperature and analyzed by fluorescence spectrophotometry at the excitation wavelength of 335 nm and emission wavelengths of 445 nm and 573 nm, along with an excitation of 525 nm and emission at 573 nm.

[0124] *Statistical data analysis.* The Student's t-test was performed to identify the Limit of Detection (i.e., statistically significantly different from a blank sample). The Wald-Wolfowitz runs test (departure from linearity) was performed to respectively determine dynamic range and sensitivity. The coefficient of variation (CoV) was calculated to evaluate reproducibility and repeatability of the tests.

[0125] It is understood that the foregoing detailed description and accompanying examples are merely illustrative and are not to be taken as limitations upon the scope of the disclosure, which is defined solely by the appended claims and their equivalents.

[0126] All publications and patents mentioned in the above specification are herein incorporated by reference as if expressly set forth herein. Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art and may be made without departing from the spirit and scope thereof.

5. Examples

[0127] It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods of the present disclosure described herein are readily applicable and appreciable, and may be made using suitable equivalents without departing from the scope of the present disclosure or the aspects and embodiments disclosed herein. Having now described the present disclosure in detail, the same will be more clearly understood by reference to the following examples, which are merely intended only to illustrate some aspects and embodiments of the disclosure, and should not be viewed as limiting to the scope of the disclosure. The disclosures of all journal references, U.S. patents, and publications referred to herein are hereby incorporated by reference in their entireties.

[0128] The present disclosure has multiple aspects, illustrated by the following non-limiting examples.

Example 1

[0129] *Protein labeling and quantification.* The fluorescent affinity tags were initially prepared by labeling PrL with NHS-rhodamine (^{Rh}PrL), Her2 with NHS-fluorescein (^{Fl}Her2), and TNF- α with NHS-AF350, NHS-fluorescein, and NHS-rhodamine (AF350-TNF- α , ^{Fl}TNF- α ,

^{Rh}TNF- α). The fluorophores were conjugated to the free amine groups of the lysine residues of the proteins via NHS chemistry. The resulting values of fluorophore-to-protein (F/P) ratios and rhodamine-to-fluorescein (Rh/FI) ratios, listed in Table 1, were determined via Bradford assay and UV spectrophotometry. The rhodamine:fluorescein ratio of 8-9 adopted in this study was inspired by the report of Lichlyter et al. on a FRET-based immunosensor, where a ratio <5 resulted in the fluorescence of the donor (fluorescein) dominating the signal; whereas the donor fluorescence becomes undetectable for ratios > 27. In designing the assay, it was hypothesized that by maintaining an optimized acceptor:donor ratio ~ 8-9, the fluorescent affinity tags framed in the affinity complex would exhibit a ratiometric fluorescence effect that is correlated to the concentration of the target mAb.

[0130] *Table 1:* Values of fluorophore to protein (F/P), and rhodamine-to-fluorescein (Rh/FI) and rhodamine-to-AF350 (Rh/AF350) ratios for ^{AF350}TNF- α , ^{FI}TNF- α , ^{Rh}TNF- α , ^{FI}Her2, and ^{Rh}PrL.

Protein	Fluorophore	F/P Ratio (mol)	Rhodamine:Fluorescein	Rh/AF-350 Ratio
PrL	NHS-Rhodamine	5.01	9.17	N/A
Her2	NHS-Fluorescein	0.55		
PrL	NHS-Rhodamine	11.9	7.99	N/A
TNF- α	NHS-Fluorescein	1.49		
PrL	NHS-Rhodamine	7.32	13.60	N/A
TNF- α	NHS-Rhodamine	0.54		
PrL	NHS-Rhodamine	7.32	N/A	20.9
TNF- α	NHS-AF350	0.35		

Example 2

[0131] *Quantification of Trastuzumab by DARQ assay.* Large-scale manufacturing of therapeutic mAbs relies on recombinant expression by CHO cells and purification using a series of chromatographic steps. The mAb titer along the production pipeline is currently monitored via off-line assays – typically, ELISA and analytical Protein A HPLC – that suffer from laborious sample preparation, long run time, and expensive equipment and consumables. To overcome the limitations of current assays a novel, affordable, and rapid (single-step) assay was developed that can be applied at-line for accurate mAb quantification in process fractions. The assay was first demonstrated using Trastuzumab/Her2 as model mAb/AG pair.

Trastuzumab, marketed as Herceptin by Genentech, is among the earliest mAbs utilized for human therapy and is currently utilized in the fight against breast and stomach cancer.

[0132] An ensemble of stock solutions of Trastuzumab were initially prepared either in PBS (non-competitive conditions) or CHO cell culture harvest (competitive conditions, CHO host cell protein titer $\sim 0.4 \text{ mg} \cdot \text{mL}^{-1}$, which is typical of industrial cell culture fluids, and contacted with a pre-formulated $^{\text{Rh}}\text{PrL}/^{\text{Fl}}\text{Her2}$ mix to achieve a final concentration of 23.2 nM for both $^{\text{Rh}}\text{PrL}$ and $^{\text{Fl}}\text{Her2}$, and a concentration of Trastuzumab varying between 0.92 and 18.4 nM. The molar excess of $^{\text{Rh}}\text{PrL}$ and $^{\text{Fl}}\text{Her2}$ enables the rapid formation of the $\text{mAb} \cdot ^{\text{Rh}}\text{PrL} \cdot ^{\text{Fl}}\text{Her2}$ affinity complex. After a short incubation time (25 min), the samples were analyzed by fluorescence spectrophotometry at $\lambda_{\text{ex}} = 480 \text{ nm}$ and $\lambda_{\text{em}} = 525 \text{ nm}$ (green) or 573 nm (yellow). The resulting value of normalized fluorescent emission intensity vs. mAb concentration in solution are reported in FIG. 3A and FIG. 3B for non-competitive and competitive conditions, respectively. In both cases, the normalized fluorescent signal was found to decrease linearly with the antibody concentration, indicating that the framing of $^{\text{Rh}}\text{Pr}$ and $^{\text{Fl}}\text{Her2}$ at a fixed molecular orientation and distance by Trastuzumab results in a ratiometric quenching of fluorescence emissions at the 525 nm wavelength. The values of limit of detection (LOD), dynamic range (DR), and sensitivity (S), collated in Table 2, were derived from statistical analysis of the raw data (Tables 7 and 8). Notably, no significant difference among the listed parameters was observed between competitive and non-competitive conditions – likely a result of the high affinity and selectivity of PrL and Her2 for Trastuzumab – indicating robustness of the assay. With $\lambda_{\text{ex}} = 480 \text{ nm}$ and $\lambda_{\text{em}} = 573 \text{ nm}$, the normalized rhodamine signal was found to decrease linearly with antibody concentration (FIG. 7), although with a smaller sensitivity when compared to the observed decrease at $\lambda_{\text{em}} = 525 \text{ nm}$. These results suggest that a FRET-like interaction between fluorescein and rhodamine is not realized, as it would have manifested in an increase in fluorescence emission at 573 nm concurrent with the decrease at 525 nm.

[0133] *Table 2:* Values of sensitivity (S), limit of detection (LoD), dynamic range (DR), and coefficient of variation (CoV) for the DARQ quantification of Trastuzumab using the dual fluorescence affinity complex Trastuzumab: $2^{\text{Rh}}\text{PrL}:2^{\text{Fl}}\text{Her2}$.

	S ($\text{ng} \cdot \text{mL}^{-1}$)	LoD ($\text{ng} \cdot \text{mL}^{-1}$)	DR ($\text{ng} \cdot \text{mL}^{-1}$)	CoV (%)
Her2 in PBS	0.54	160	531-1358	1.16
Her2 in CHO	0.36	75	250-1480	0.82

Example 3

[0134] Quantification of Adalimumab (Humira) by DARQ assay. The assay was repeated using Adalimumab/TNF- α as the mAb/antigen pair. Adalimumab, released in the US in 2002, is listed by the World Health Organization's among the safest and most effective therapeutics for treating a number of autoimmune disorders, including rheumatoid arthritis and Crohn's disease. Like Trastuzumab, Adalimumab is a κ I-IgG1 antibody and is mass manufactured via recombinant expression in engineered CHO cell lines.

[0135] The application of the assay to the Adalimumab/TNF- α pair returned once again a linear decrease of normalized fluorescent emission intensity vs. mAb concentration under both non-competitive (FIG. 4A) and competitive conditions (FIG. 4B). The resulting values of LOD, DR, and S (Table 3), derived from statistical analysis of the raw data (Tables 9 and 10) are also on par with those obtained with Trastuzumab. The normalized fluorescent signal generated with $\lambda_{\text{ex}} = 480$ nm and $\lambda_{\text{em}} = 573$ nm decreased linearly with antibody concentration (FIG. 8), but with a lesser sensitivity when compared to the $\lambda_{\text{em}} = 525$ nm.

[0136] Table 3: Values of sensitivity (S), limit of detection (LoD), dynamic range (DR), and coefficient of variation (CoV) for the DARQ quantification of Adalimumab using the dual fluorescence affinity complex Adalimumab:2^{Rh}PrL:2^{Fl}TNF- α .

	S (ng·mL ⁻¹)	LoD (ng·mL ⁻¹)	DR (ng·mL ⁻¹)	CoV (%)
TNF-α in PBS	0.24	51	369-1613	2.76
TNF-α in CHO	0.23	78	469-1672	1.28

Example 4

[0137] Fluorescence quenching mechanism. The findings reported in FIGS. 3 and 4 were somewhat unexpected: while the choice of fluorophores and the molecular distance between the fluorescent affinity tags are characteristic of Förster Resonance Energy Transfer phenomena (e.g., FRET), the outcome resembles that of fluorescence quenching.

[0138] It was initially hypothesized that the results could be ascribed to amino acid-driven fluorescence quenching, which has been observed in prior studies with several organic dyes including fluorescein and rhodamine. The molecular architecture of the Adalimumab:2^{Rh}PrL:2^{Fl}TNF- α complex exemplified in FIG. 1 indeed suggests that some fluorescent dyes can be “caged” by a proteinaceous shell that, owing to the aromatic residues (e.g., tryptophan) contained therein, quenches their fluorescence emission. To evaluate this hypothesis, the fluorescence emission of the single dye – single affinity complexes Adalimumab:2^{Fl}AG and Adalimumab:2^{Rh}PrL (FIG. 9) were measured, as well as the single

dye – dual affinity complexes Adalimumab:2PrL:2^{AF350}TNF- α (FIG. 10) and Adalimumab:2^{Rh}PrL:2TNF- α (FIG. 11). All four complexes, however, showed negligible fluorescence quenching with mAb concentration, indicating that protein “caging” of the fluorophores is not responsible for the observed ratiometric quenching.

[0139] Experiments were then conducted to investigate whether the mechanism underlying the DARQ assay is of the nature of energy transfer quenching or some other immobilization based quenching effect. To this end the fluorophore AF350 (blue) was utilized in lieu of Fl. Unlike the fluorescein-rhodamine pair ($J\text{-integral}_{\text{Fl-Rh}} \sim 5 \cdot 10^{15} \text{ m}^{-1} \cdot \text{cm}^{-1} \cdot \text{nm}^4$), in fact, AF350 and fluorescein do not engage in energy transfer ($J\text{-integral}_{\text{AF350-Fl}} \sim 0$). Accordingly, the fluorescence emission of the affinity complex Trastuzumab:2^{Rh}PrL:2^{AF350}Her2 was measured, and the same linear decrease in fluorescence signal was observed, as above (FIG. 5; Table 4, and FIG. 12), indicating that the DARQ assay does not operate upon energy transfer mechanisms.

[0140] *Table 4:* Values of sensitivity (S), limit of detection (LoD), dynamic range (DR), and coefficient of variation (CoV) for the DARQ quantification of Adalimumab using the dual fluorescence affinity complex Adalimumab:2^{Rh}PrL:2^{AF350}TNF- α .

	S (ng·mL⁻¹)	LoD (ng·mL⁻¹)	DR (ng·mL⁻¹)	CoV (%)
TNF-α in PBS	0.54	233	527-1761	11.4

[0141] Experiments were conducted to evaluate whether other quenching phenomena occur, such as self-quenching (homo-FRET), that drive the linear decrease in fluorescence emission. To this end, the fluorescence emission of the affinity complex Adalimumab:2^{Rh}PrL:2^{Rh}TNF- α formed by Rh-only labeled affinity tags was measured. Once again, however, no fluorescence quenching was observed with mAb concentration (FIG. 13), excluding the occurrence of self-quenching (homo-FRET).

[0142] Collectively, these results suggest that the mechanism underlying the DARQ assay is a dynamic quenching mechanism. The fluorescence quenching observed in these results consistently favors the quenching of the high-energy fluorophores (i.e., AF350 and Fl), yet it requires the presence of a low-energy fluorophore (Rh); in fact, no ratiometric quenching is observed when PrL is unlabeled, as shown in FIG. 10.

[0143] Further corroborating this hypothesis is the observation that the DARQ assay featured a 2-fold higher sensitivity for Adalimumab than for Trastuzumab. This can be attributed to the substantial difference in the size of the antigens. As shown in FIG. 1D, in fact,

TNF- α is a small protein (MW ~ 17.3 kDa, rhodamine ~ 3.2 nm), and the fluorescein moieties displayed on its surface are all located at short distance (~ 3 – 4 nm) to the rhodamine moieties conjugated on PrL. Conversely, as shown in FIG. 1C, Her2 has a much higher size (MW ~ 88.7 kDa, rhodamine ~ 5.5 nm), and can carry several fluorescein moieties at a distance from ^{Rh}PrL at which dye-dye quenching does not occur.

Example 5

[0144] *Effect of incubation time of the mAb:^{Rh}PrL:^{Fl}AG complex on DARQ.* Experiments were conducted to evaluate the performance of the assay with respect to the incubation time of the target mAb with the fluorescently labeled proteins using Adalimumab/TNF- α as model mAb/antigen pair. The assay was conducted as explained above, while reducing the incubation time from 20 min to 10, 5, and 1 min. As anticipated, a linear decrease in the normalized fluorescent emission intensity vs. mAb concentration was observed for all incubation time points under both non-competitive (FIG. 6A) and competitive conditions (FIG. 6B). The resulting values of S, LOD, and DR, obtained from statistical analysis of the raw data (Tables 9-10 and Tables 11-16), are reported in Table 5. Exception made for a slight increase in the dynamic range obtained with longer incubation times (10 and 20 min), the assay performance parameters did not vary with incubation time. This is coherent with the rapid binding kinetics characteristic of mAb:PrL and mAb:antigen complexes.

[0145] *Table 5: Values of sensitivity (S), limit of detection (LoD), dynamic range (DR), and coefficient of variation (CoV) for the quantification of Adalimumab via the dual fluorescent affinity tag assay conducted at different incubation times.*

	Incubation time (min)	S (ng·mL ⁻¹)	LoD (ng·mL ⁻¹)	DR (ng·mL ⁻¹)	CoV (%)
TNF-α in PBS	0	0.26	48	338-1318	2.16
	5	0.24	72	560-1443	2.57
	10	0.24	39	312-1443	2.54
	20	0.24	51	396-1613	2.36
TNF-α in CHO	0	0.24	67	349-1443	1.72
	5	0.21	22	125-1672	2.05
	10	0.21	56	0353-1443	1.77
	20	0.23	78	469-1672	1.28

Example 6

[0146] *Comparison of DARQ with established analytical methods for antibody quantification.* Table 6 compares the performance of the DARQ assay to that of state-of-the-art assays for the quantification of Adalimumab and Trastuzumab. DARQ performs comparably to the reference assays in terms of limit of detection and dynamic range but is considerably superior in terms of ease and speed of operation. Chromatographic assays require extensive sample preparation, a trained operator, and expensive equipment. Commercial ELISA kits are attractive owing to their low limit of detection and ample dynamic range; on the other hand, they suffer from high variability, entail a series of time-consuming steps (blocking, incubation of the sample and primary/secondary detection antibodies, intermediate washes, and enzymatic propagation of the signal), and require expensive automated liquid handlers to secure acceptable throughput. Other assays have recently been commercialized, which feature low limit of detection (~10 ng/mL for each mAb) and a 10-to-100-fold span of dynamic range. Cardinali et al. have developed a peptide-based ELISA that performs comparably to antibody-based ELISAs while improving on throughput. The Quantum Blue Adalimumab lateral flow assay (BÜHLMANN Laboratories) performs well and is rapid compared to the other testing methods; however, its applicability to industrial settings is limited by the need of a separate 15-minute test for each sample. The HMSA and UPLC methods, respectively developed by Wang et al. and Russo et al., are impressive in range and sensitivity, but require laborious sample preparation. The DARQ assay provides competitive assay parameters (sensitivity, limit of detection, and dynamic range), while dramatically reducing assay variability and time to measurement. This makes DARQ a practical choice for mAb quantification when time or equipment are limited.

[0147] *Table 6: Survey of reported analytical methods for the quantification of Adalimumab and Trastuzumab: limit of detection (LoD), dynamic range (DR), time to result, and dilution factor. Abbreviations: Homogenous Mobility Shift Assay (HMSA), Ultra-performance liquid chromatography (UPLC), Multiple Reaction Monitoring Mass Spectrometry (MRM-MS).*

Assay	Target	LoD (ng·mL ⁻¹)	DR (ng·mL ⁻¹)	Time to Run	Dilution Factor
DARQ	Adalimumab	141	370 - 1,600	~ 5 min	No sample dilution
DARQ	Trastuzumab	75	250 - 1,480	~ 25 min	No sample dilution

ELISA	Adalimumab	11	11 - 300	~ 6 hrs	Application based
ELISA	Trastuzumab	10	30 - 1,000	~ 6 hrs	Application based
Lateral Flow	Adalimumab	800	1300 - 35,000	~15 min (per sample)	20-fold
HMSA (Homogenous Mobility Shift Assay)	Adalimumab	18	120 - 1,600 ug/mL	~3 hrs (per sample)	Extensive sample preparation
Peptide ELISA	Trastuzumab	5000	10,000 - 180,000	~5 hours	500-fold
UPLC MRM-MS	Trastuzumab	0.74	0.03 - 42180	~4 hours (per sample)	Extensive sample preparation

[0148] Raw data corresponding to the embodiments described in the above Examples are provided in the Tables below.

[0149] *Table 7:* Raw emission data of the dual fluorescence affinity complex Trastuzumab:2PrL:2Her2 formed by mixing ^{Rh}PrL and ^{Fl}Her2 in PBS with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 20 minutes. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	9359	9544	9547	9852	10130	9949
0.92	9277	9190	9264	9725	9644	9622
1.15	9191	9031	9183	---	---	---
1.47	8843	8953	9087	---	---	---
1.84	8957	8765	8925	9199	9123	9011
2.3	8723	8742	8669	---	---	---
2.75	8535	8584	8543	---	---	---
3.67	8155	8088	8150	8516	8550	8262
4.59	7897	7806	7664	---	---	---

5.51	7736	7673	7580	---	---	---
5.97	7627	7411	7340	8039	7961	7954
6.7	7310	7045	7050	7662	7518	7487
7.34	7263	7261	7162	7781	7599	7610
9.18	7130	6845	6896	7124	7036	7026
10	7414	7233	7280	---	---	---
10.6	7429	7240	7281	---	---	---
11.2	7175	7079	7026	---	---	---
11.6	6903	6836	6783	---	---	---
12.3	6497	6417	6365	6947	6818	6890
13.6	6839	6940	6860	---	---	---
15.9	6747	6716	6856	---	---	---
18.4	6303	6348	6241	6793	6778	6440

[0150] *Table 8:* Raw emission data of the dual fluorescence affinity complex Trastuzumab:2PrL:2Her2 formed by mixing ^{Rh}PrL and ^{Fl}Her2 in CHO with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 20 minutes. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	18755	18347	18129	12337	12335	12279
0.92	18046	18154	18237	11983	12066	12213
1.15	17736	17779	17495	---	---	---
1.47	17666	17648	17067	---	---	---
1.84	18206	18013	16989	11457	11609	11414
2.3	16108	16116	16243	---	---	---
2.75	16635	16370	16082	---	---	---
3.67	15950	15826	15359	11078	10855	10811
4.59	16715	15579	15576	---	---	---
5.51	15473	14993	15415	---	---	---

5.97	14726	14716	14924	9843	9853	9930
6.7	15498	14849	14677	9861	9691	9683
7.34	15464	14784	14760	9661	9604	9468
9.18	14398	14299	14188	9063	9069	8983
10	9003	8829	8857	---	---	---
10.6	9028	8792	8876	---	---	---
11.2	9028	8893	8944	---	---	---
11.6	8790	8802	8702	---	---	---
12.3	13806	13700	13684	8629	8497	8642
13.6	8627	8643	8455	---	---	---
15.9	8684	8584	8677	---	---	---
18.4	12877	12866	12680	8832	8759	8714

[0151] *Table 9:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL:2TNF- α formed by mixing Rb PrL and Fl TNF- α in PBS with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 20 minutes. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	8847	8890	9029	8224	8140	8246
0.89	8391	8182	8350	7780	7606	7583
1.11	8231	8175	8328	---	---	---
1.43	8204	8087	8131	---	---	---
1.78	7849	7762	7748	7203	7127	7072
2.23	7477	7265	7322	---	---	---
2.67	7225	7014	7176	---	---	---
3.56	6576	6584	6565	6215	6064	6177
4.45	6158	6077	6054	---	---	---
5.34	5757	5590	5530	---	---	---
5.79	5167	5099	5076	4727	4506	4638

6.5	4856	4735	4917	4239	4107	4244
7.13	4526	4301	4198	3953	3791	3835
8.91	3442	3252	3246	3208	2994	2925
9.75	2592	2334	2480	---	---	---
10.3	2400	2164	2210	---	---	---
10.9	2296	2142	2126	---	---	---
11.3	2069	2018	1928	---	---	---
11.9	2508	2250	2160	1777	1697	1705
13.2	1691	1560	1634	---	---	---
15.4	1476	1522	1514	---	---	---
17.8	1570	1573	1504	1476	1511	1460

[0152] *Table 10:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL:2TNF- α formed by mixing ^{Rh}PrL and ^{Fl}TNF- α in CHO with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 20 minutes. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	12405	12553	12432	12310	12521	12647
0.89	11582	11659	11606	11511	11452	11570
1.11	11522	11624	11233	---	---	---
1.43	11275	10909	10953	---	---	---
1.78	10921	10910	10851	10873	10903	10740
2.23	10378	10418	10412	---	---	---
2.67	10158	10045	10107	---	---	---
3.56	9580	9305	9364	9432	9291	9253
4.45	8989	9324	8983	---	---	---
5.34	8777	8490	8438	---	---	---
5.79	7869	8186	7882	7940	7892	8006
6.5	7319	7332	7112	7483	7426	7445

7.13	7210	7241	7304	7196	7233	7233
8.91	6368	5953	6096	5928	5912	5858
9.75	5855	5624	5691	---	---	---
10.3	5283	5260	5182	---	---	---
10.9	4995	4863	4863	---	---	---
11.3	4665	4648	4623	---	---	---
11.9	4924	4581	4631	4529	4566	4497
13.2	4378	4286	4274	---	---	---
15.4	4010	4026	3957	---	---	---
17.8	3117	3023	3034	3656	3534	3524

[0153] *Table 11:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL:2TNF- α formed by mixing Rh PrL and Fl TNF- α in PBS with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 10 minutes. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	8943	8914	9057	8408	8320	8350
0.89	8449	8377	8467	7803	7716	7945
1.11	8262	8155	8399	---	---	---
1.43	8270	8035	8111	---	---	---
1.78	7909	7830	7812	7409	7253	7195
2.23	7393	7307	7393	---	---	---
2.67	7171	6970	6888	---	---	---
3.56	6440	6384	6517	6406	6163	6110
4.45	6065	6080	6021	---	---	---
5.34	5617	5614	5495	---	---	---
5.79	5204	4972	5047	4543	4260	4484
6.5	4928	4594	4732	4326	3873	4126
7.13	4505	4270	4243	3943	3821	3848

8.91	3404	3192	3312	3106	2925	3006
9.75	2730	2524	2739			
10.3	2681	2466	2516	---	---	---
10.9	2609	2408	2331	---	---	---
11.3	2201	2201	2025	---	---	---
11.9	2498	2352	2273	1907	1866	1822
13.2	1917	1774	1705	---	---	---
15.4	1646	1605	1612	---	---	---
17.8	1646	1548	1595	1527	1537	1489

[0154] *Table 12:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL:2TNF- α formed by mixing Rh PrL and Fl TNF- α in PBS with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 5 minutes. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	8905	9109	8893	8653	8806	8612
0.89	8381	8313	8305	8031	8019	8107
1.11	8285	8337	8386	---	---	---
1.43	8252	8310	8216	---	---	---
1.78	7824	7826	7746	7662	7540	7508
2.23	7444	7340	7350	---	---	---
2.67	7128	6953	7060	---	---	---
3.56	6532	6429	6453	6607	6653	6435
4.45	6042	5910	5864	---	---	---
5.34	5679	5381	5400	---	---	---
5.79	5177	5015	4977	4960	4863	4657
6.5	4767	4660	4760	4521	4571	4525
7.13	4437	4212	4211	4303	4051	4244
8.91	3486	3297	3310	3723	3200	3479

9.75	3152	2778	3107	---	---	---
10.3	2759	2786	2870	---	---	---
10.9	2973	2650	2682	---	---	---
11.3	2568	2508	2537	---	---	---
11.9	2600	2405	2294	2335	2132	2126
13.2	2194	1969	1985	---	---	---
15.4	1888	1720	1758	---	---	---
17.8	1704	1631	1676	1644	1642	1613

[0155] *Table 13:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL:2TNF- α formed by mixing R^h PrL and F^I TNF- α in PBS with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 1 minute. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	9305	9481	9388	9165	9068	9103
0.89	8940	8801	8974	8771	8615	8527
1.11	8626	8649	8594	---	---	---
1.43	8643	8629	8526	---	---	---
1.78	8107	8207	8293	8133	8059	8132
2.23	7899	7922	7788	---	---	---
2.67	7742	7345	7500	---	---	---
3.56	7022	6847	6817	7053	6894	6938
4.45	6347	6254	6174	---	---	---
5.34	6031	5853	5823	---	---	---
5.79	5575	5254	5277	6122	5884	5774
6.5	5139	5012	5128	5627	5552	5511
7.13	4863	4611	4621	5335	5110	5293
8.91	3909	3829	3814	4854	4394	4564
9.75	4447	4105	4265	---	---	---

10.3	4155	4073	4048	---	---	---
10.9	4076	3950	3910	---	---	---
11.3	4015	3842	3797	---	---	---
11.9	3134	2867	2825	3991	3769	3677
13.2	3783	3492	3513	---	---	---
15.4	3241	2984	2957	---	---	---
17.8	1969	1983	1986	2642	2582	2545

[0156] *Table 14:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL:2TNF- α formed by mixing Rh PrL and Fl TNFA- α in CHO with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 10 minutes. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	12620	12407	12646	12685	12688	12593
0.89	11739	11588	11572	11664	11625	11741
1.11	11681	11815	11399	---	---	---
1.43	11256	10965	10752	---	---	---
1.78	10940	10941	10894	10624	10885	10705
2.23	10343	10334	10405	---	---	---
2.67	10225	10056	9990	---	---	---
3.56	9565	9285	9462	9407	9309	9278
4.45	9121	9505	9119	---	---	---
5.34	8816	8433	8589	---	---	---
5.79	8017	8375	7930	7839	7869	7755
6.5	7492	7325	7350	7366	7317	7394
7.13	7263	7342	7251	7310	6979	6978
8.91	6502	5949	6296	5933	5904	5747
9.75	5790	5620	5700	---	---	---
10.3	5529	5524	5434	---	---	---

10.9	5133	5270	5048	---	---	---
11.3	5253	4832	4634	---	---	---
11.9	5081	4707	4912	4686	4694	4614
13.2	4560	4237	4446	---	---	---
15.4	4165	4146	4155	---	---	---
17.8	3325	3252	3084	3780	3684	3743

[0157] *Table 15:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL:2TNF- α formed by mixing ^{Rh}PrL and ^{Fl}TNF- α in CHO with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 5 minutes. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	12879	12912	12815	12964	12967	12938
0.89	11813	12157	12098	11945	12134	11924
1.11	11864	11885	11507	---	---	---
1.43	11672	11267	11044	---	---	---
1.78	11214	11392	11117	11191	11088	11080
2.23	10433	10566	10677	---	---	---
2.67	10204	10386	10362	---	---	---
3.56	9853	9600	9660	9796	9427	9403
4.45	9165	9454	9618	---	---	---
5.34	8978	8427	8655	---	---	---
5.79	8029	8514	8082	7943	7743	8019
6.5	7517	7612	7266	7424	7370	7489
7.13	7351	7523	7357	7276	7111	7307
8.91	6697	6220	6455	6216	6180	5991
9.75	6129	6235	6285	---	---	---
10.3	5962	6082	6077	---	---	---
10.9	5528	5618	5629	---	---	---

11.3	5722	5361	4938	---	---	---
11.9	5209	4819	5129	4755	4896	5077
13.2	4832	4803	4690	---	---	---
15.4	4458	4556	4488	---	---	---
17.8	3584	3286	3310	4138	3999	3963

[0158] *Table 16:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL:2TNF- α formed by mixing ^{Rh}PrL and ^{Fl}TNF- α in CHO with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 1 minute. Concentrations were done in triplicates (at minimum) to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity					
0	12808	12903	12912	13224	13496	13350
0.89	12084	12290	12045	12602	12510	12890
1.11	12147	12014	11892	---	---	---
1.43	11628	11416	11444	---	---	---
1.78	11486	11532	11462	12024	11755	12005
2.23	10863	10896	10954	---	---	---
2.67	10715	10752	10589	---	---	---
3.56	10241	9981	9941	10714	10246	10499
4.45	9356	9729	9414	---	---	---
5.34	9417	8761	9089	---	---	---
5.79	8424	8821	8395	9535	9504	9610
6.5	7727	7926	7449	9401	9339	9381
7.13	7619	7685	7598	9215	8809	8949
8.91	7256	6818	6766	8036	7943	7857
9.75	7995	7837	7780	---	---	---
10.3	7543	7613	7692	---	---	---
10.9	7465	7404	7302	---	---	---
11.3	7491	7084	7126	---	---	---

11.9	5701	5360	5496	7143	7166	7063
13.2	7044	6727	7033	---	---	---
15.4	6666	6512	6346	---	---	---
17.8	3964	3772	3735	5884	5544	5723

[0159] *Table 17:* Raw emission data of the dual fluorescence affinity complex Trastuzumab:2Her2 formed by mixing ^{F1}Her2 in PBS with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 20 minutes. Concentrations were done in triplicates to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity		
0	287	282	331
0.92	288	302	337
1.15	263	304	299
1.47	300	268	278
1.84	257	276	277
2.3	266	263	290
2.75	264	263	284
3.67	273	245	275
4.59	261	246	282
5.51	250	275	266
5.97	267	253	286
6.7	272	268	271
7.34	294	291	253
9.18	276	262	290
12.3	253	270	278
18.4	261	291	277

[0160] *Table 18:* Raw emission data of the dual fluorescence affinity complex Trastuzumab:2PrL formed by mixing ^{Rh}PrL in PBS with an excitation at 480 nm and reading at 573 nm for each concentration of antibody. Incubation time was 20 minutes. Concentrations

were done in triplicates to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity		
0	9807	9716	9904
0.92	9774	9865	9925
1.15	9935	10075	10049
1.47	9991	9837	9789
1.84	9281	9289	9190
2.3	9171	9120	9204
2.75	9346	9156	9299
3.67	9199	9207	9196
4.59	8846	8984	8936
5.51	8909	8905	8784
5.97	8734	8817	8893
6.7	8980	9031	8984
7.34	8908	8850	8980
9.18	8922	8842	8788
12.3	8966	8880	8819
18.4	8704	8733	8709

[0161] *Table 19:* Raw emission data of the fluorescence affinity complex Adalimumab:2TNF- α formed by mixing 125 I-TNF- α in PBS with an excitation at 480 nm and reading at 525 nm for each concentration of antibody. Incubation time was 20 minutes. Concentrations were done in triplicates to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity		
0	9540	9832	9871
0.891	9647	9663	9747
1.11	9802	9771	9795
1.43	9801	9450	9314
1.78	9653	9919	9904

2.23	9817	10000	9980
2.67	9893	9760	9663
3.56	9700	9611	9305
4.45	9319	9529	9682
5.34	9481	9559	9507
5.79	10640	9373	9440
6.5	9437	9211	9076
7.13	9058	9226	13273
8.91	9140	9141	8959
11.9	8806	8806	8670
17.8	8861	8630	8558

[0162] *Table 20:* Raw emission data of the dual fluorescence affinity complex Adalimumab:2PrL formed by mixing ^{Rh}PrL in PBS with an excitation at 480 nm and reading at 573 nm for each concentration of antibody. Incubation time was 20 minutes. Concentrations were done in triplicates to measure the error. This data was normalized and used to perform statistical analysis on the results.

Antibody Concentration (nM)	Emission Intensity		
0	634	550	537
0.891	565	499	567
1.11	518	523	508
1.43	557	540	507
1.78	523	576	545
2.23	551	546	556
2.67	563	578	537
3.56	549	533	531
4.45	558	543	557
5.34	554	596	561
5.79	562	552	546
6.5	585	578	554
7.13	489	535	557
8.91	548	528	574

11.9	539	553	562
17.8	535	569	543

CLAIMS

What is claimed is:

1. A ratiometric bioassay method for detecting a target antibody in a sample, the method comprising:

combining an antigen coupled to a first fluorophore, an antibody-binding moiety coupled to a second fluorophore, and a sample comprising or suspected of comprising a target antibody;

exposing the antigen coupled to the first fluorophore, the antibody-binding moiety coupled to the second fluorophore, and the sample to fluorescent light comprising an excitation wavelength of the first and/or the second fluorophore; and

detecting fluorescence emission from the first and/or the second fluorophore;

wherein the fluorescence emission from the first and the second fluorophore is reduced, and wherein the reduced fluorescence emission is proportional to the antibody concentration in the sample.

2. The bioassay method according to claim 1, wherein the antigen is capable of being bound by the antibody in the sample.

3. The bioassay method according to claim 1 or 2, wherein the antibody-binding moiety comprises a polypeptide capable of binding a region of the target antibody that does not comprise the antigen-binding site.

4. The bioassay method according to any of claims 1 to 3, wherein the antibody binding moiety comprises Protein L, Protein A, or Protein G.

5. The bioassay method according to any of claims 1 to 3, wherein the antibody binding moiety is Protein L.

6. The bioassay method according to any of claims 1 to 5, wherein the first fluorophore is a high-energy fluorophore and the second fluorophore is a low-energy fluorophore.
7. The bioassay method according to any of claims 1 to 5, wherein the second fluorophore is a high-energy fluorophore and the first fluorophore is a low-energy fluorophore.
8. The bioassay method according to any of claims 1 to 7, wherein the high-energy fluorophore is selected from the group consisting of Fluorescein, Oregon Green 488, Oregon Green 514, Rhodamine Green, Rhodamine Green-X, Eosin, 4', 6-diamidino-2-phenylindole, Alexafluor 405, Alexafluor 350, Alexafluor 500, Alexafluor 488, Alexafluor 430, Alexafluor 514, and Alexafluor 532.
9. The bioassay method according to any of claims 1 to 8, wherein the low-energy fluorophore is selected from the group consisting of Rhodamine, Rhodamine B, Rhodamine Red-X, Tetramethylrhodamine, Lissamine, Texas Red and Texas Red-X, Naphthofluorescein, Carboxyrhodamine 6G, Alexafluor 555, Alexafluor 546, Alexafluor 568, Alexafluor 594, Alexafluor 610, Alexafluor 633, Alexafluor 635, Alexafluor 647, Alexafluor 660, Alexafluor 680, Alexafluor 700, and Alexafluor 750.
10. The bioassay method according to any of claims 1 to 9, wherein the antigen is coupled to fluorescein or Alexafluor 350.
11. The bioassay method according to any of claims 1 to 10, wherein the antibody-binding moiety is coupled to Rhodamine.
12. The bioassay method according to any of claims 1 to 11, wherein the method further comprises determining the concentration of the target antibody in the sample based on the reduction in fluorescence emission of the first and/or the second fluorophore.

13. The bioassay method according to any of claims 1 to 12, wherein the method further comprises incubating the sample comprising or suspected of comprising the target antibody, the antigen coupled to the first fluorophore, and the antibody-binding moiety coupled to the second fluorophore for 30 minutes or less prior to measuring the fluorescent emission of the first and/or the second fluorophore.

14. The bioassay method according to any of claims 1 to 12, wherein the method further comprises incubating the sample comprising or suspected of comprising the target antibody, the antigen coupled to the first fluorophore, and the antibody-binding moiety coupled to the second fluorophore for 10 minutes or less prior to measuring the fluorescent emission of the first and/or the second fluorophore.

15. The bioassay method according to any of claims 1 to 14, wherein the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.2 to about 1:20.

16. The bioassay method according to any of claims 1 to 15, wherein the antibody-binding moiety is coupled to the second fluorophore at an moiety-to-fluorophore ratio of about 1:0.2 to about 1:20.

17. The bioassay method according to any of claims 1 to 16, wherein the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:1 to about 1:20.

18. The bioassay method according to any of claims 1 to 17, wherein the sample is undiluted.

19. The bioassay method according to any of claims 1 to 18, wherein the sample is a whole blood sample, a plasma sample, a serum sample, a urine sample, a saliva sample, a tissue sample, a cell culture sample, a cell lysate sample, or a cell culture media sample.

20. The bioassay method according to any of claims 1 to 19, wherein the antigen comprises the spike (S) protein of SARS-CoV-2, or a fragment thereof.
21. The bioassay method according to any of claims 1 to 19, wherein the antigen comprises the capsid protein of a viral vector for gene therapy, or a fragment thereof.
22. A composition for performing a ratiometric bioassay to detect an antibody in a sample, the composition comprising:
- an antigen coupled to a first fluorophore;
 - an antibody-binding moiety coupled to a second fluorophore; and
 - a sample comprising or suspected of comprising a target antibody;
- wherein fluorescence emission from the first and the second fluorophore is reduced upon exposure to fluorescent light comprising an excitation wavelength of the first and/or the second fluorophore, and wherein the reduced fluorescence emission is proportional to the antibody concentration in the sample.
23. The composition according to claim 22, wherein the antigen is capable of being bound by the antibody in the sample.
24. The composition according to claim 22 or claim 23, wherein the antibody-binding moiety comprises a polypeptide capable of binding a region of the target antibody that does not comprise the antigen-binding site.
25. The composition according to any of claims 22 to 24, wherein the antibody binding moiety comprises Protein L, Protein A, or Protein G.
26. The composition according to any of claims 22 to 25, wherein the first fluorophore is a high-energy fluorophore and the second fluorophore is a low-energy fluorophore.

27. The composition according to any of claims 22 to 26, wherein the second fluorophore is a high-energy fluorophore and the first fluorophore is a low-energy fluorophore.

28. The bioassay method according to any of claims 22 to 27, wherein the high-energy fluorophore is selected from the group consisting of Fluorescein, Oregon Green 488, Oregon Green 514, Rhodamine Green, Rhodamine Green-X, Eosin, 4', 6-diamidino-2-phenylindole, Alexafluor 405, Alexafluor 350, Alexafluor 500, Alexafluor 488, Alexafluor 430, Alexafluor 514, and Alexafluor 532.

29. The composition according to any of claims 22 to 28, wherein the low-energy fluorophore is selected from the group consisting of Rhodamine, Rhodamine B, Rhodamine Red-X, Tetramethylrhodamine, Lissamine, Texas Red and Texas Red-X, Naphthofluorescein, Carboxyrhodamine 6G, Alexafluor 555, Alexafluor 546, Alexafluor 568, Alexafluor 594, Alexafluor 610, Alexafluor 633, Alexafluor 635, Alexafluor 647, Alexafluor 660, Alexafluor 680, Alexafluor 700, and Alexafluor 750.

30. The composition according to any of claims 22 to 29, wherein the antigen is coupled to the first fluorophore at an antigen-to-fluorophore ratio of about 1:0.5 to about 1:20.

31. The composition according to any of claims 22 to 30, wherein the antibody binding moiety is coupled to the second fluorophore at an moiety-to-fluorophore ratio of about 1:0.2 to about 1:20.

32. The composition according to any of claims 22 to 31, wherein the first fluorophore and the second fluorophore are present in the composition at a first-to-second fluorophore ratio of about 1:1 to about 1:20.

33. The composition according to any of claims 22 to 32, wherein the sample is undiluted.

34. The composition according to any of claims 22 to 33, wherein sample is a whole blood sample, a plasma sample, a serum sample, a urine sample, a saliva sample, a tissue sample, a cell culture sample, a cell lysate sample, or a cell culture media sample.
35. The composition according to any of claims 22 to 34, wherein the antigen comprises the spike (S) protein of SARS-CoV-2, or a fragment thereof.
36. The composition according to any of claims 22 to 34, wherein the antigen comprises the capsid protein of viral vector for gene therapy, or a fragment thereof.
37. A kit for performing a ratiometric bioassay to detect a target antibody in a sample, the kit comprising:
- an antigen coupled to a first fluorophore;
 - an antibody binding moiety coupled to a second fluorophore; and
 - at least one container.
38. The kit according to claim 37, wherein the kit further comprises a buffer and/or instructions for performing the bioassay.
39. The kit according to claim 37 or claim 38, wherein the kit further comprises the target antibody or a fragment or derivative thereof.



FIGS. 1A-1D

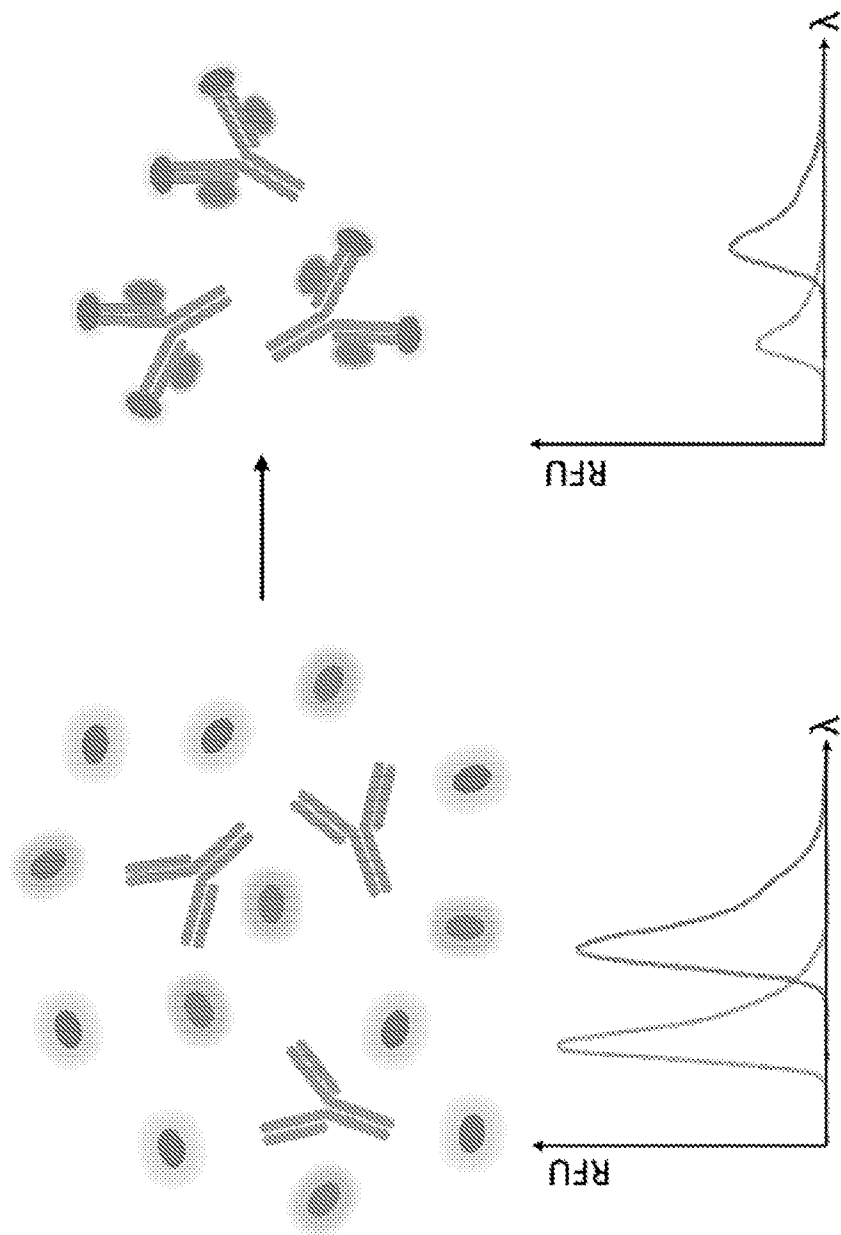
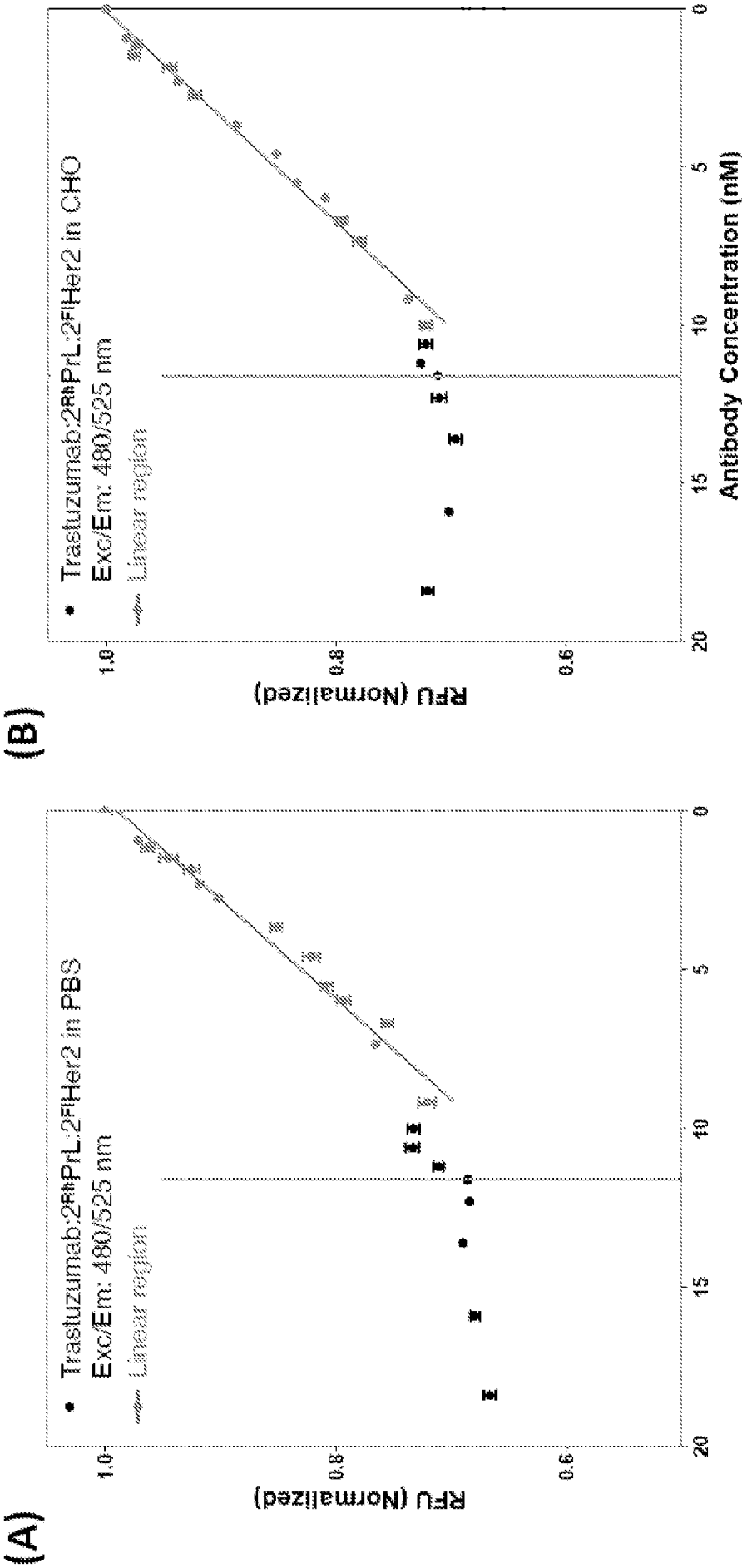
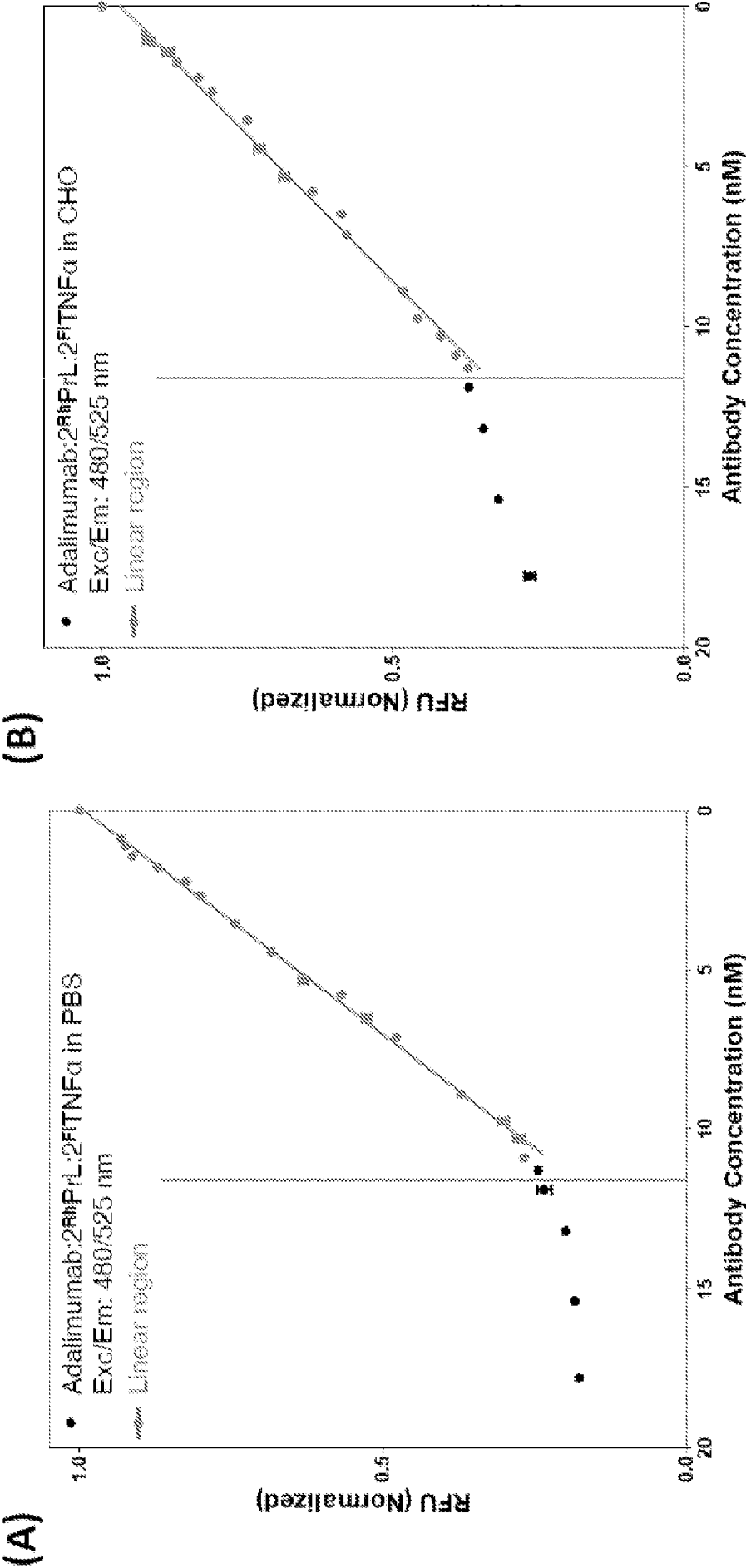


FIG. 2



FIGS. 3A-3B



FIGS. 4A-4B

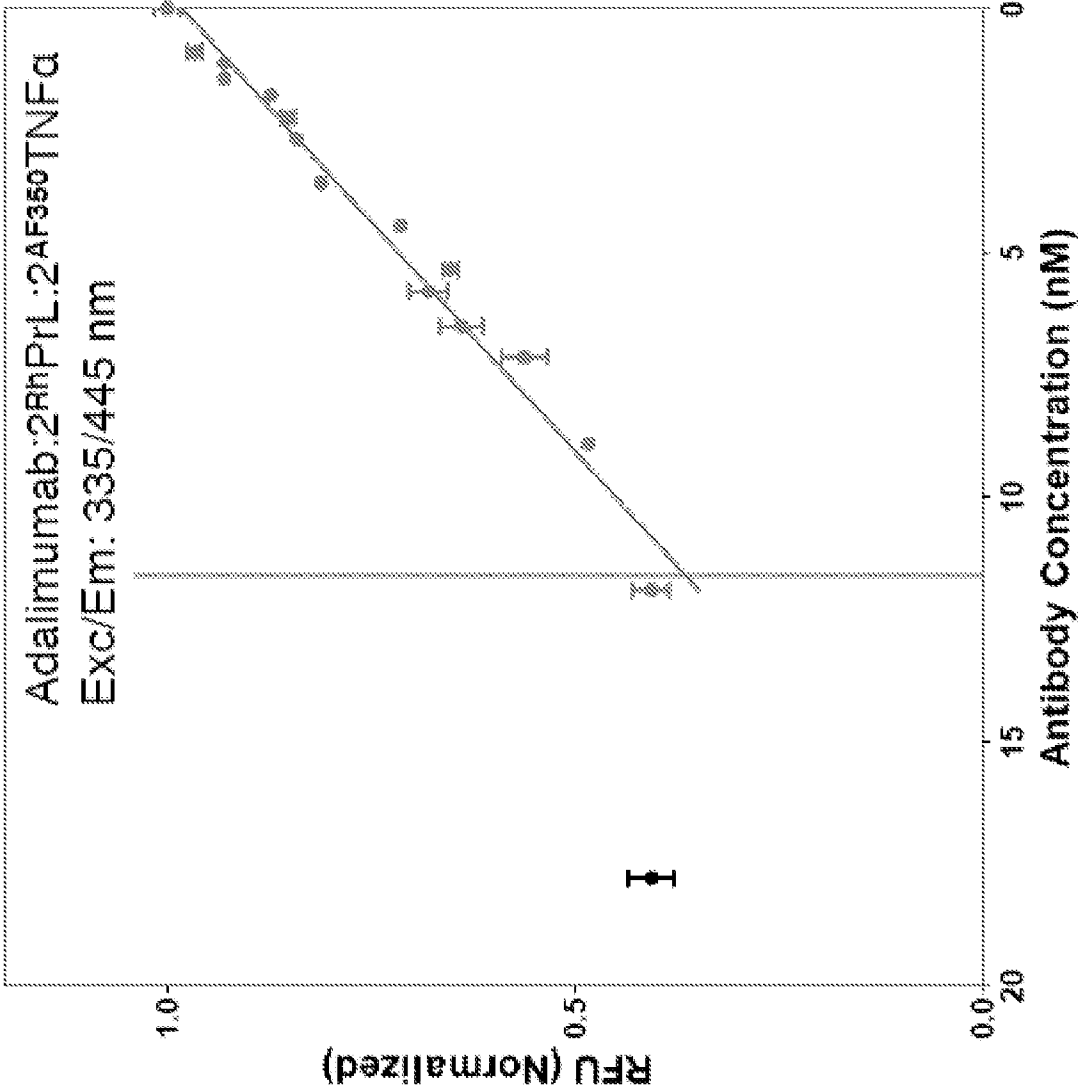
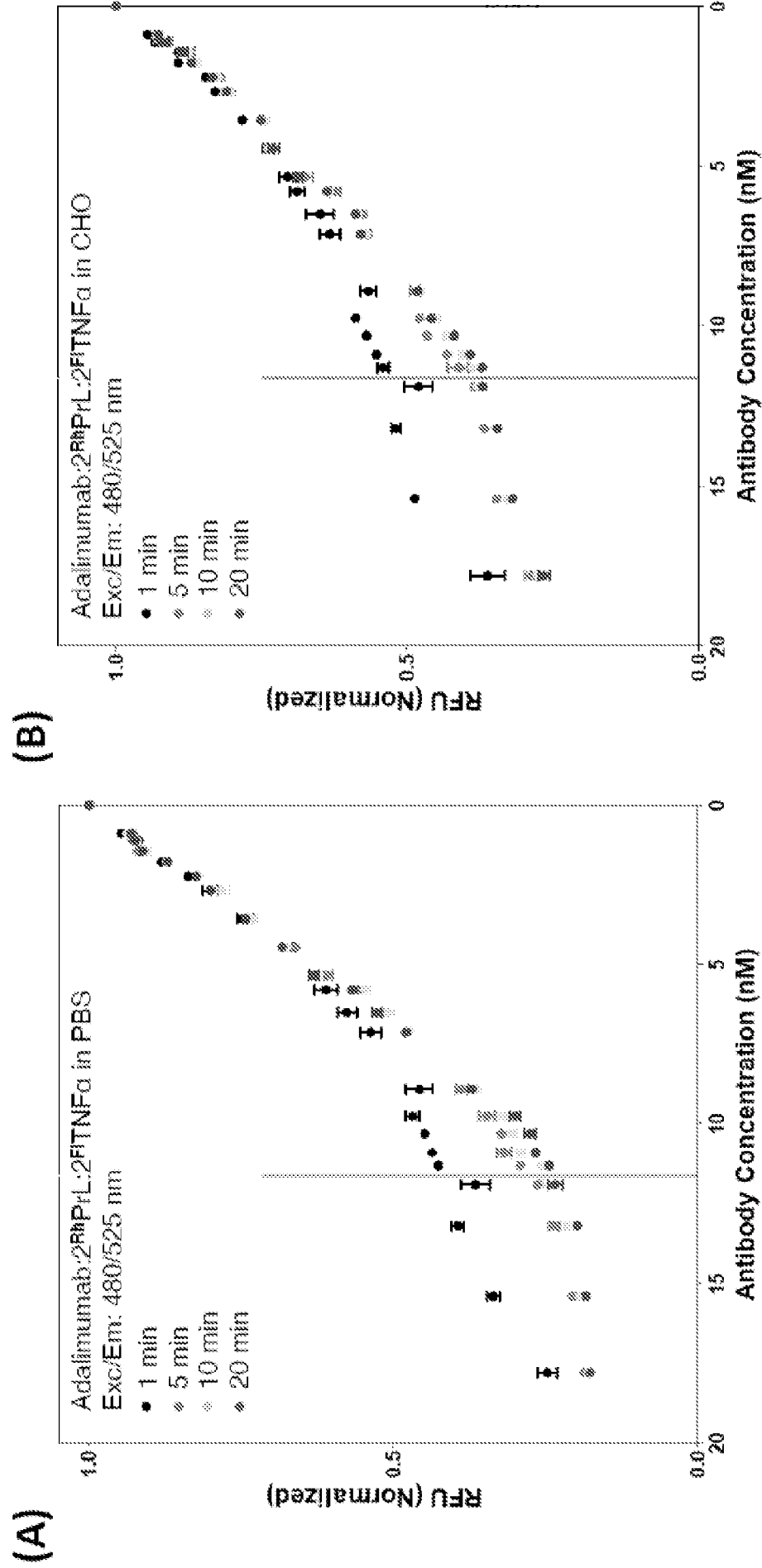
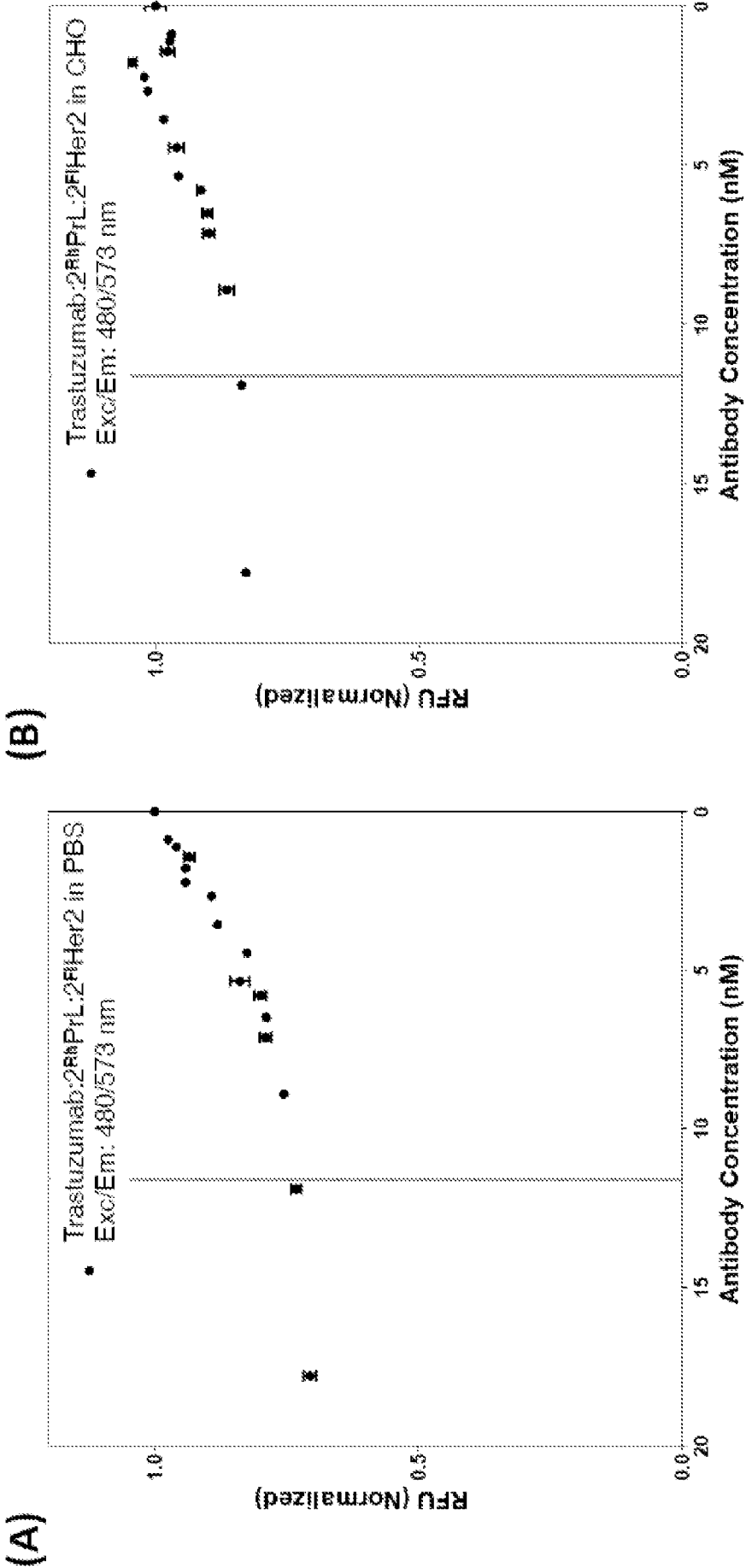


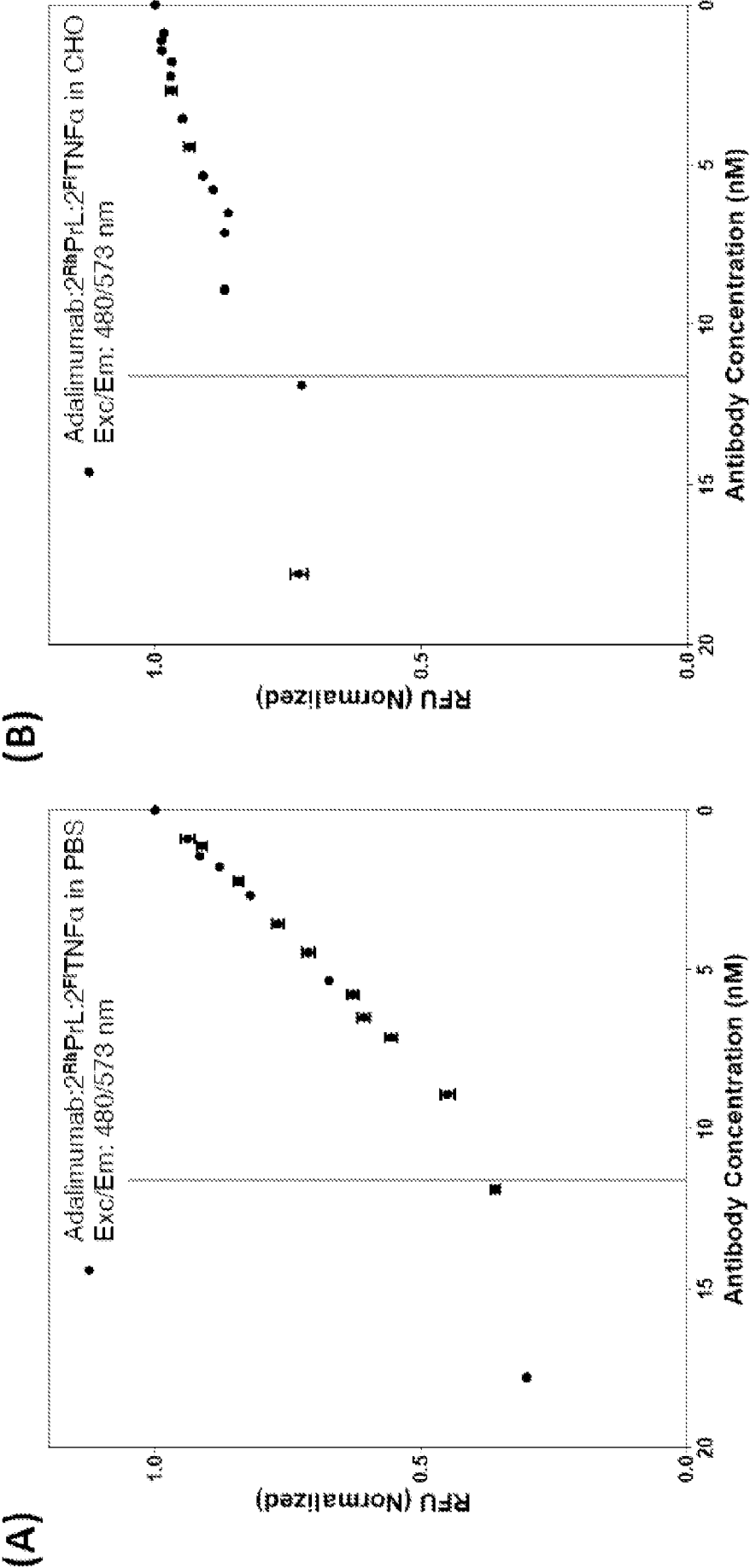
FIG. 5



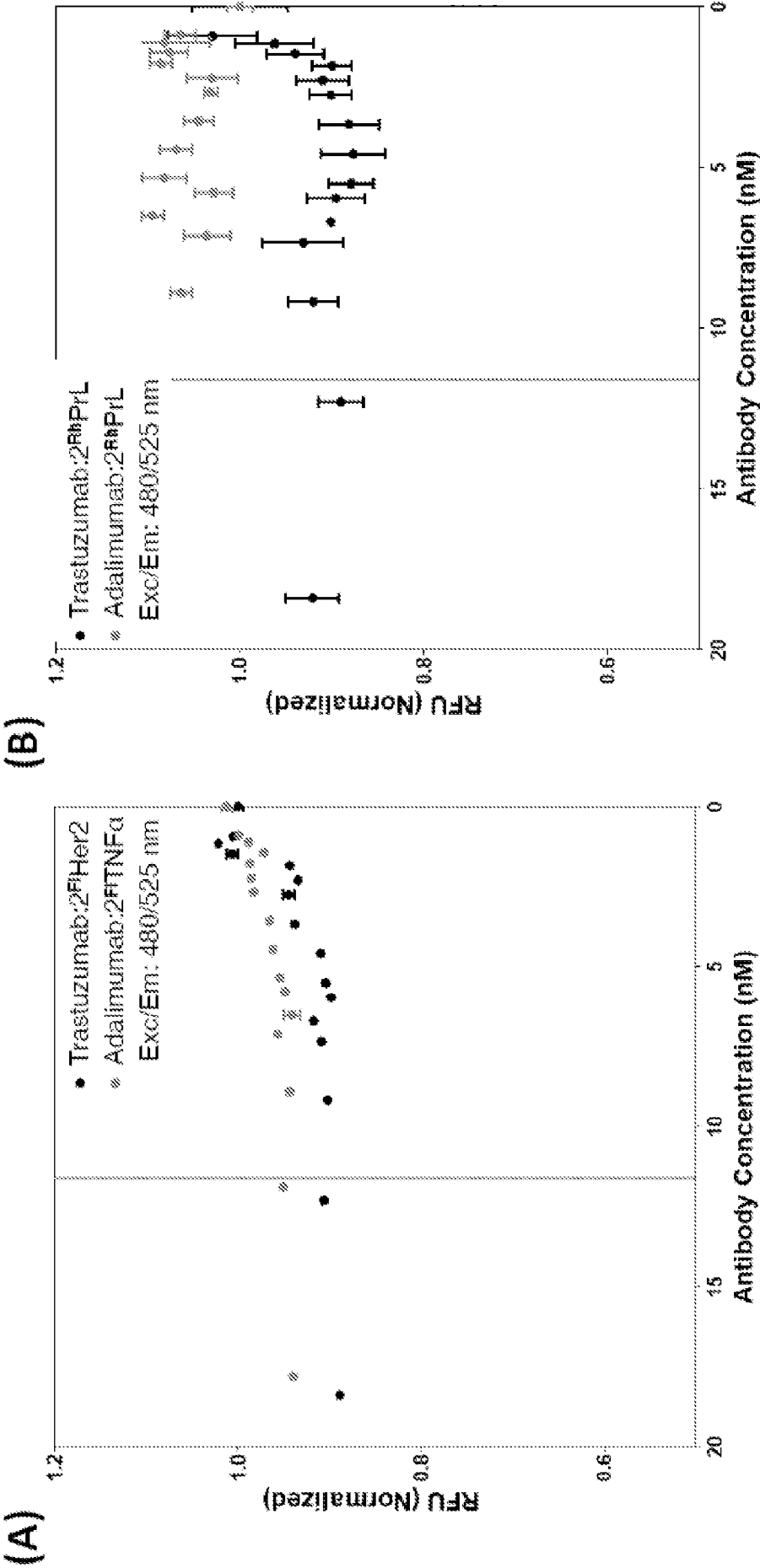
FIGS. 6A-6B



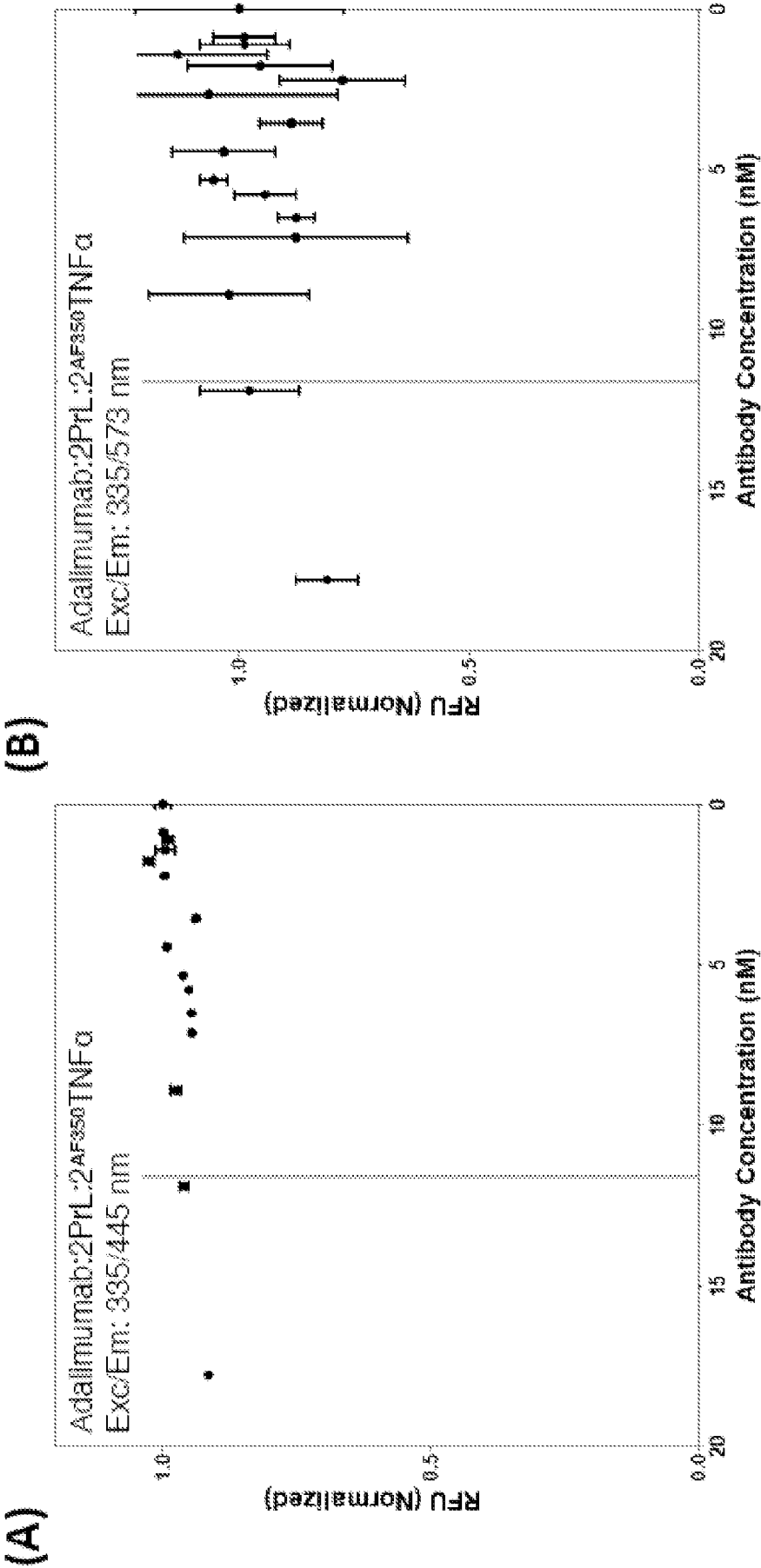
FIGS. 7A-7B



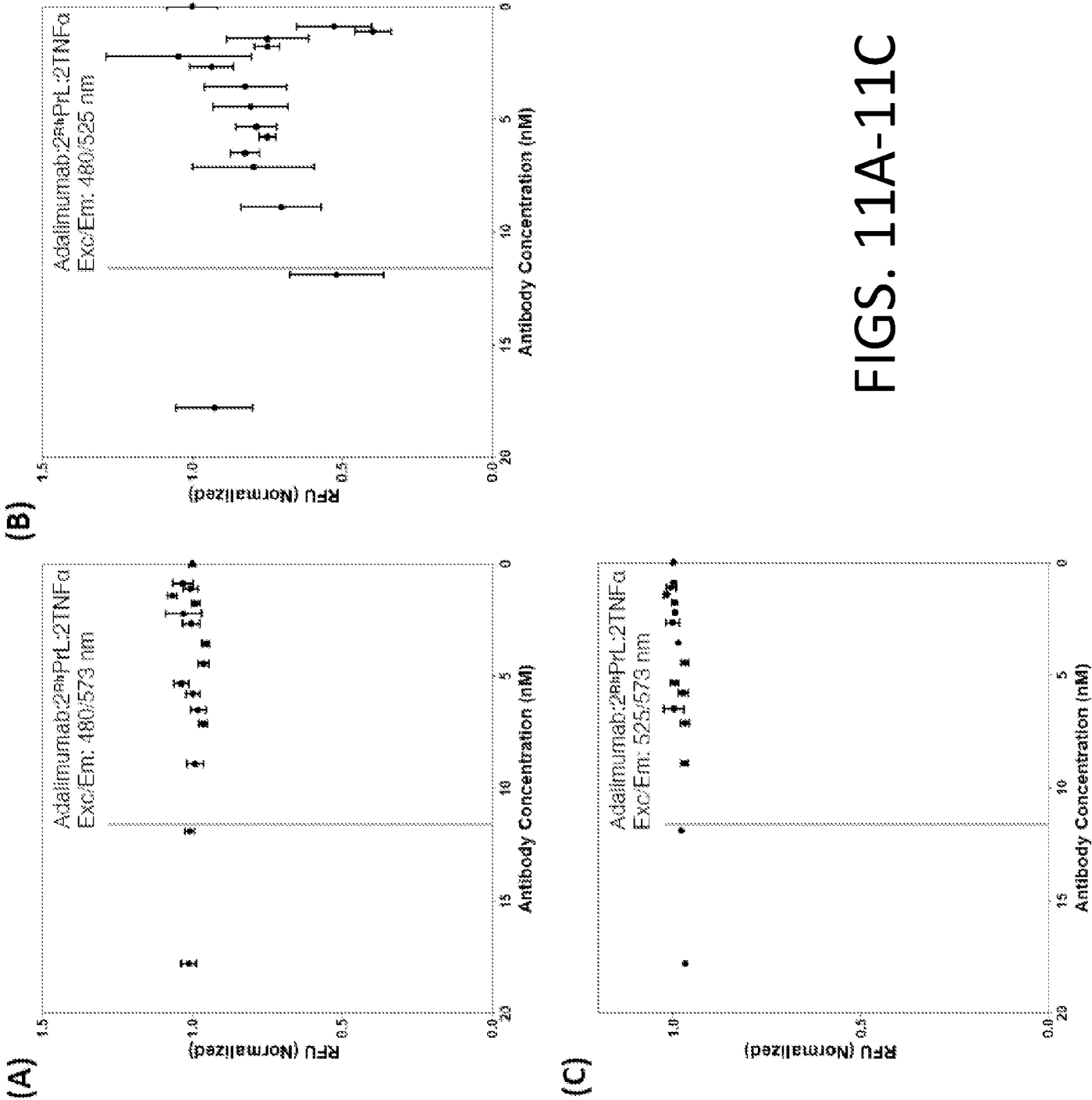
FIGS. 8A-8B



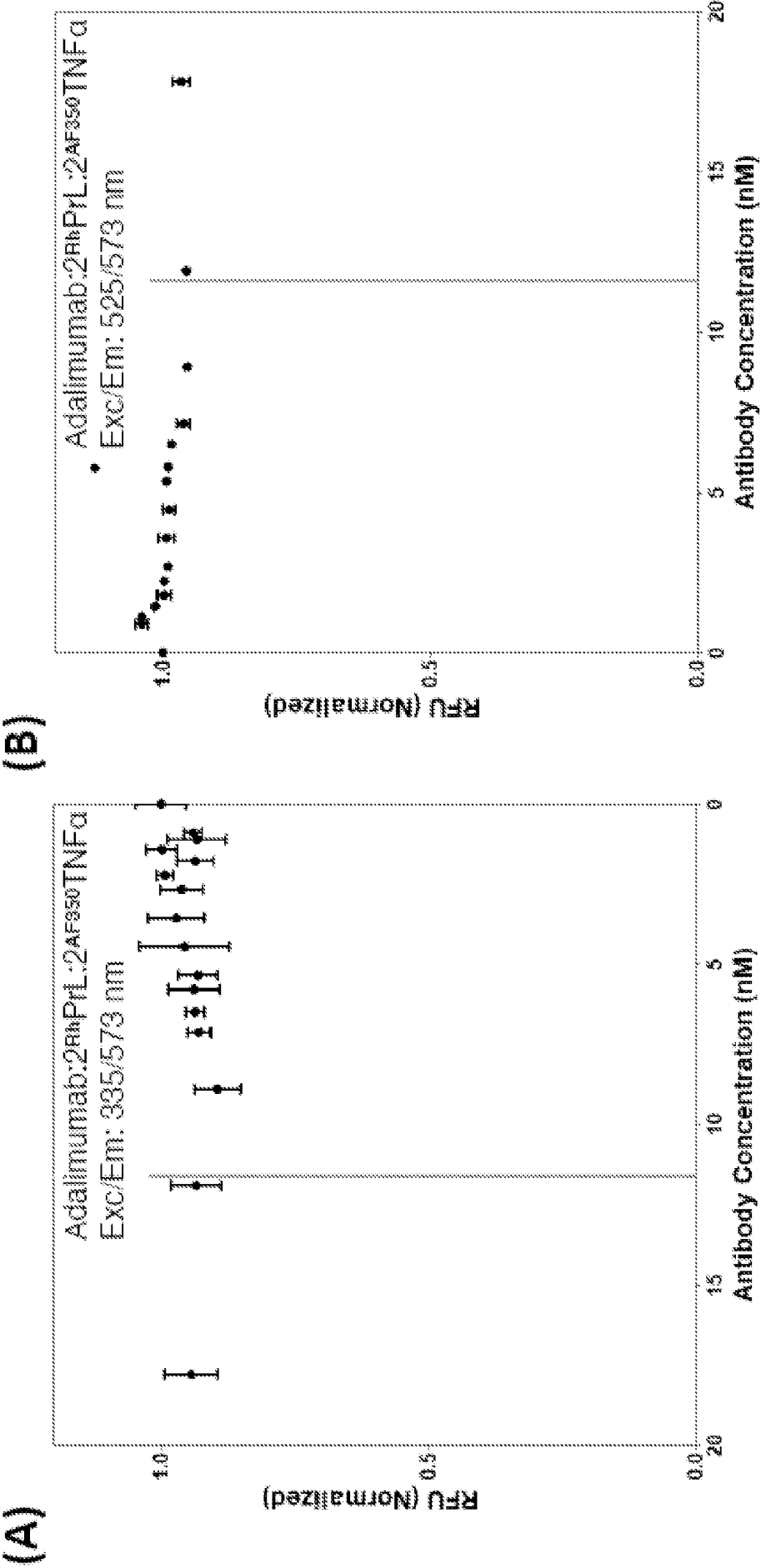
FIGS. 9A-9B



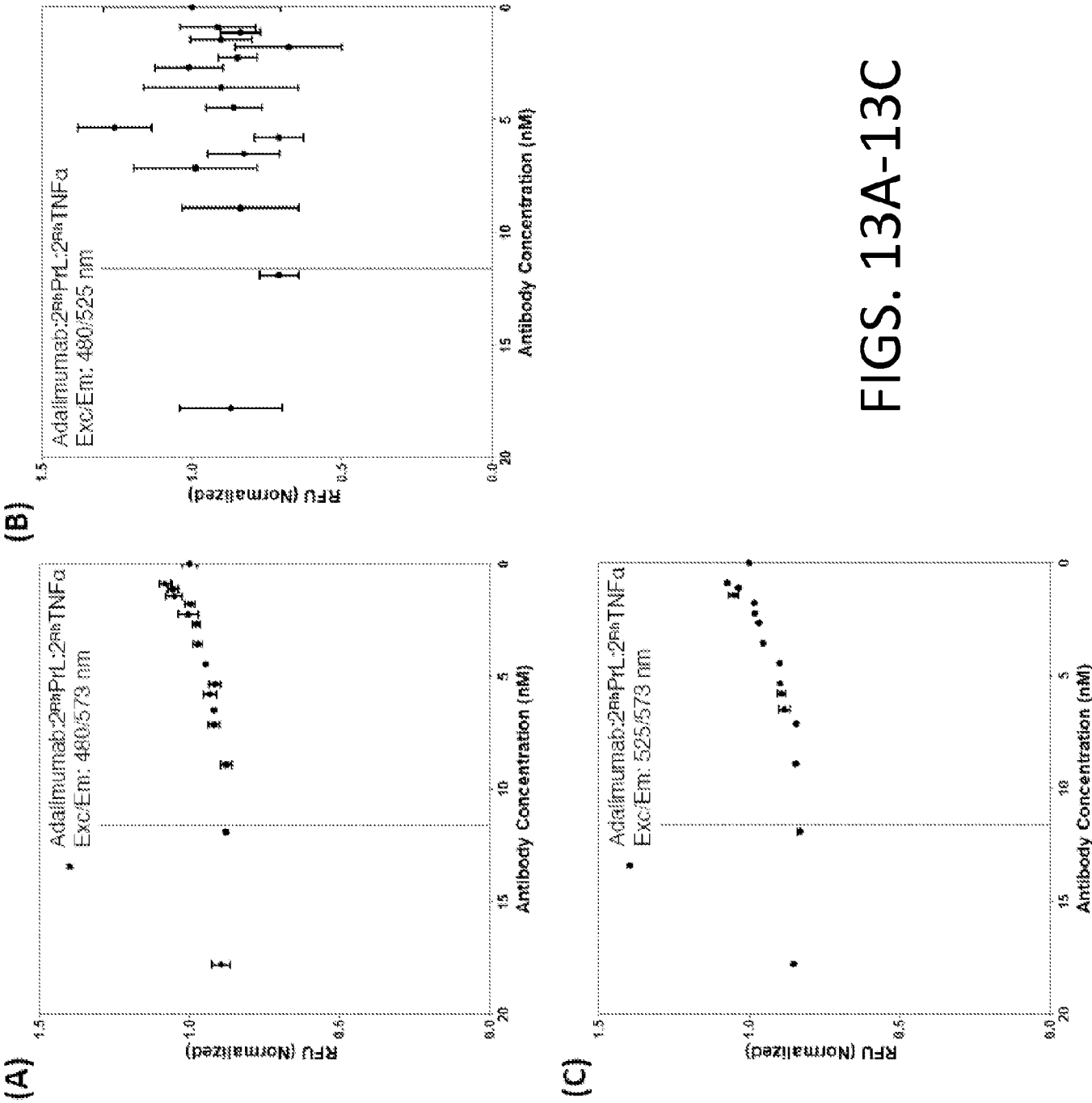
FIGS. 10A-10B



FIGS. 11A-11C



FIGS. 12A-12B



FIGS. 13A-13C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US21/54245

A. CLASSIFICATION OF SUBJECT MATTER

IPC - G01N 33/533; C12M 1/34 (2021.01)

CPC - G01N 33/533; G01N 21/6486; C12Q 2563/131; Y10S 436/80; C12M 1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---	CA 2818556 A1 (UNIV OF CALIFORNIA) 31 May 2012; pages 5, 14-18, 26, 30-35, and 44	1-2, 22-23, 37-39 ---
Y		3, 24
Y	BOGNÁR. "Aptamers against Immunoglobulins: Design, Selection and Bioanalytical Applications". International Journal of Molecular Sciences. 11 August 2020; page 2; DOI: doi:10.3390/ijms21165748	3, 24

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 January 2022 (11.01.2022)

Date of mailing of the international search report

FEB 17 2022

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US21/54245

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-21, 25-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.