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# Extending the throughput of Biacore 4000 biosensor to accelerate kinetic analysis of antibody-antigen interaction

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ABSTRACT

The surface plasmon resonance (SPR) biosensors are being routinely used in different stages of drug discovery and development. However, the lack of high throughput SPR biosensors continues to be a primary bottleneck for the rapid kinetic screening of large panels of monoclonal antibodies (mAbs). To further increase the throughput of the Biacore 4000 biosensor, we have developed three kinetic screening assays to characterize mAb-antigen interactions – (i) 16-mAb capture kinetic, (ii) single cycle kinetic (SCK), and (iii) parallel kinetic (PK). The performance of all three kinetic assays was evaluated by characterizing the binding of kinetically diverse human mAbs to four antigens with molecular weights of 14kD, 29kD, 38kD, and 48kD and binding affinities ranging from 130pM to 200 nM. The binding rate constants measured using all three kinetic assays were reproducible across multiple experiments and correlated with the values generated using the conventional 8-mAb capture kinetic assay on the Biacore 4000 ($R^2 > 0.94$). Moreover, the 16-mAb capture assay decreased experiment time and analyte consumption by 35% and 50%, respectively. This work illustrates the significance of the 16-mAb capture kinetic, SCK, and PK assays to increase the throughput of Biacore 4000 and to support rapid kinetic screening of mAbs.



## Introduction

The research and development of therapeutic monoclonal antibodies (mAbs) has become a key focus for the treatment of different diseases. Diverse biophysical, biochemical, and functional assays are being developed to meet the current throughput needs of the biopharmaceutical industry. Kinetic screening of mAbs has become an integral screening technique of today's mAb discovery workflow [1]. Among the different biosensor technologies capable of measuring mAb binding affinities, the surface plasmon resonance (SPR) and the surface plasmon resonance imaging (SPRi) biosensors are the preferred platforms to measure the kinetics of a mAb binding to its target antigen [2,3]. However, the lack of high throughput SPR/SPRi biosensors capable of reliably characterizing mAb-antigen interactions continues to be a critical bottleneck in the discovery and development of biotherapeutics. The traditional serial flow SPR biosensors, such as Biacore 3000 and Biacore T200, have limited throughput and can only screen approximately 100 mAbs per day from crude conditioned media (CM) [4], whereas the parallel flow biosensors such as Biacore 4000 (formerly Biacore A100) and MASS-1 assist in the kinetic screening of approximately 400 mAbs per day [5,6]. Array based SPRi biosensors such as IBIS MX96, Horiba XelPleX, Horiba OpenPlex, Plexera PlexArray enable the design of multiplex assays and allow simultaneous characterization of several hundred molecular interactions, thus enabling rapid kinetic screening of mAbs [7–9]. Newer technology platforms such as Biacore 8k and MASS-2 are currently emerging to meet the increasing demand for higher throughput SPR/SPRi biosensors.

Apart from the development of new biosensor technologies, researchers are also developing new methods to enhance the throughput of existing biosensors for kinetic screening of mAbs [10–12]. Taking advantage of the crisscrossing flow path design of the Biorad's ProteOn XPR36 biosensor, Bravman et al. developed the “one-shot” kinetic assay which enables the measurement of six different ligands simultaneously binding to six analyte

*Abbreviations:* RT-LF, real time label free; SPR, surface plasmon resonance; SPRi, surface plasmon resonance imaging; mAbs, monoclonal antibodies; SCK, single cycle kinetic; PK, parallel kinetic; CM, conditioned media; $k_a$, association rate; $k_d$, dissociation rate; $K_D$, equilibrium dissociation constant; SICK, single injection cycle kinetic; HA, hydrodynamic addressing; CHO, Chinese Hamster Ovary.

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concentrations [10]. The 36-ligand array kinetic assay developed by Abdiche et al. allows simultaneous characterization of up to 36 analyte-antigen interactions; further expanding the throughput of ProteOn XPR36 [11]. The use of ELISA detection reagents resulted in 1000-fold increase in the sensitivity of bio-layer interferometry (BLI) biosensor and the BLI-ELISA assay developed by combining ELISA detection and BLI biosensor allowed high throughput and accurate measurement of solution based binding affinity of mAb [12]. In this study, we focused on increasing the throughput of the Biacore 4000 biosensor and the three new kinetic assays that were developed in this study were used to characterize of mAb-antigen interactions.

The Biacore 4000 is an array-based biosensor with four independent flow cells (labeled as Flow Cell-1 through Flow Cell-4). Each flow cell contains five detections spots (labeled Spot-1 through Spot-5) that can simultaneously measure binding events [6]. The hydrodynamic addressing (HA) technology of the Biacore 4000 allows sample injection over either the outside spots (Spot-1 or Spot-5) or both outside spot and its neighboring inside spot (Spots-1 and Spot-2 or Spots-4 and Spot-5), but fails to deliver samples over just the inside spots (Spot-2 or Spot-4). Therefore, even though the Biacore 4000 has 16 detection spots, it only allows the design of kinetic assays by simultaneously capturing up to eight mAbs per cycle (two mAbs per flow cell) [5,6]. Moreover, the Biacore 4000 restricts users to design newer and more advanced assays single cycle kinetic (SCK) and parallel kinetic (PK) assays that were developed to eliminate the need to regenerate the sensor surface. To address all the aforementioned challenges, we have developed three kinetic assays using different combinations of pre-existing injection commands to accelerate the kinetic analysis of mAb-antigen interactions on the Biacore 4000 biosensor: i) 16-mAb capture kinetic, (ii) SCK, and (iii) PK assays.

The 16-mAb capture kinetic assay enables simultaneous characterization of up to 16 unique mAb-antigen interactions (four mAbs per flow cell). Fig. 1 provides a detailed schematic of different steps required to design the 16-mAb capture kinetic assay on the Biacore 4000. Depending on the mAb under investigation, the entire sensor surface can be immobilized with either an anti-Fc or an anti-Fab capture molecule (Fig. 1; Step-1). First, mAb is captured on Spot-1 and Spot-2 and later on Spot-4 and Spot-5 (Fig. 1; Step-2 and Step-3), followed by the regeneration of mAb captured on the outside spots; Spot-1 and Spot-5 (Fig. 1; Step-4 and Step-5). Finally, mAbs are captured on Spot-1 and Spot-5 (Fig. 1; Step-6 and Step-7) to achieve a total of 16 mAb capture surface. In this assay, Spot-3 is used as an internal reference to correct for changes in bulk refractive index.

To test the throughput and performance of the 16-mAb capture, SCK, and PK assays, we selected a panel of 16 mAbs that bound to 14kD, 29kD, 38kD, and 48kD antigens (four mAbs per antigen) with varying binding kinetic profiles. All three assays were designed as capture kinetic assay, where the mAb was first captured using an anti-human Fc immobilized sensor surface followed by the injection of antigen titration series. The reliability and reproducibility of the association rate ($k_a$), dissociation rate ($k_d$), and equilibrium dissociation constant ($K_D$) generated using the 16-mAb capture, SCK, and PK assays were assessed. All three kinetic assays developed in this study have the potential to significantly increase the throughput of the Biacore 4000 biosensor for the characterization of mAb-antigen interactions, and in turn will be able to meet the throughput requirement for rapid selection of lead therapeutic mAbs.

## Materials & methods

### *Reagents*

Coupling reagents, N-(3-dimethylaminopropyl)-N-ethyl-carbodiimide (EDC) and N-hydroxysuccinimide (NHS), ethanolamine—HCl (Catalog# BR100633), anti-human Fc specific capture mAb (Catalog# BR-1008-39) and CM5 sensor chips (Catalog# BR-1005-30) were purchased from GE Healthcare. Sodium acetate, pH 5.0 was purchased from Ricca Chemical (Catalog# 61-1). HEPES (Teknova, Catalog# H1035), NaCl (Gibco, Catalog# 24740-011), EDTA (BDH, Catalog# BDH8730-1) and Tween-20® (Bio-Rad, Catalog# 161-0781) were used to prepare running buffer.

All recombinant proteins were expressed using stable Chinese Hamster Ovary (CHO) cell lines and purified in-house. The mAbs used in this study were fully human mAbs expressed with either human IgG1 or IgG4. The antigens used in the study are monomeric ecto-domain of the single transmembrane receptor expressed with a C-terminal myc-myc-6xHis tag. The antigens from CM were purified using the HisTalon superflow cartridge (Clontech) following the manufacture's protocol. Briefly, the CM containing antigen was passed through the HisTalon superflow cartridge to allow antigen binding to the resin. After washing the cartridge with 20 mM sodium phosphate buffer containing 5 mM Imidazole, 0.5 M NaCl, pH7.2, the column bound antigen was eluted using 20 mM sodium phosphate buffer containing 200 mM Imidazole, 0.5 M NaCl, pH7.2. The mAbs were purified using the Protein A Fast Flow HiTrap cartridge (GE Healthcare) following the manufacture's protocol. The resin bound mAb was later eluted using Pierce IgG elution buffer, pH2.8 and the elute was immediately neutralized to pH7.0 using 1 M Tris-HCl pH8.0. The purified mAbs and antigens were later dialyzed into PBS containing 5% Glycerol, pH7.2 and further purified using size exclusion chromatography in PBS containing 5% Glycerol, pH7.2 buffer. Finally, all the purified mAbs and antigens were aliquoted and stored at −80 °C until used for kinetic assay.

### *Immobilization of anti-human Fc mAb on Biacore 4000*

The CM5 sensor surface was first immobilized with an anti-human Fc mAb using a standard protocol, reported previously [3]. Briefly, the entire sensor surface was first activated by injecting a 1:1 (v/v) mixture of 400 mM EDC and 100 mM NHS at a flow rate of 10 μL/min for 10 min. Monoclonal mouse anti-human Fc (10 μg/mL) prepared in 10 mM sodium acetate, pH5.0 was then injected at a flow rate of 10 μL/min for 10 min followed by the injection of 1 M ethanolamine, pH8.5 for 7 min at a flow rate of 10 μL/min. During the entire immobilization procedure, 10 mM sodium acetate, pH5.0 was used as the running buffer. Finally, the uncoupled residual protein was removed by treating the sensor surface with 10—12 s injection of 20 mM phosphoric acid, pH2.0, 8—10 times before performing kinetic assays.

### *Binding studies performed on the Biacore 4000*

All of the binding studies were performed in freshly prepared, filtered and degassed running buffer containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.05% Tween-20® (HBS-ET) at 25 °C. Before performing the binding studies, the CM5 sensor surface immobilized with anti-human Fc mAb was equilibrated with HBS-ET by priming the instrument at least three to four times. For the 8-mAb capture kinetic, PK and SCK assays, mAbs were first captured on both outside spot and its neighboring inside spot (Spot-1, Spot-

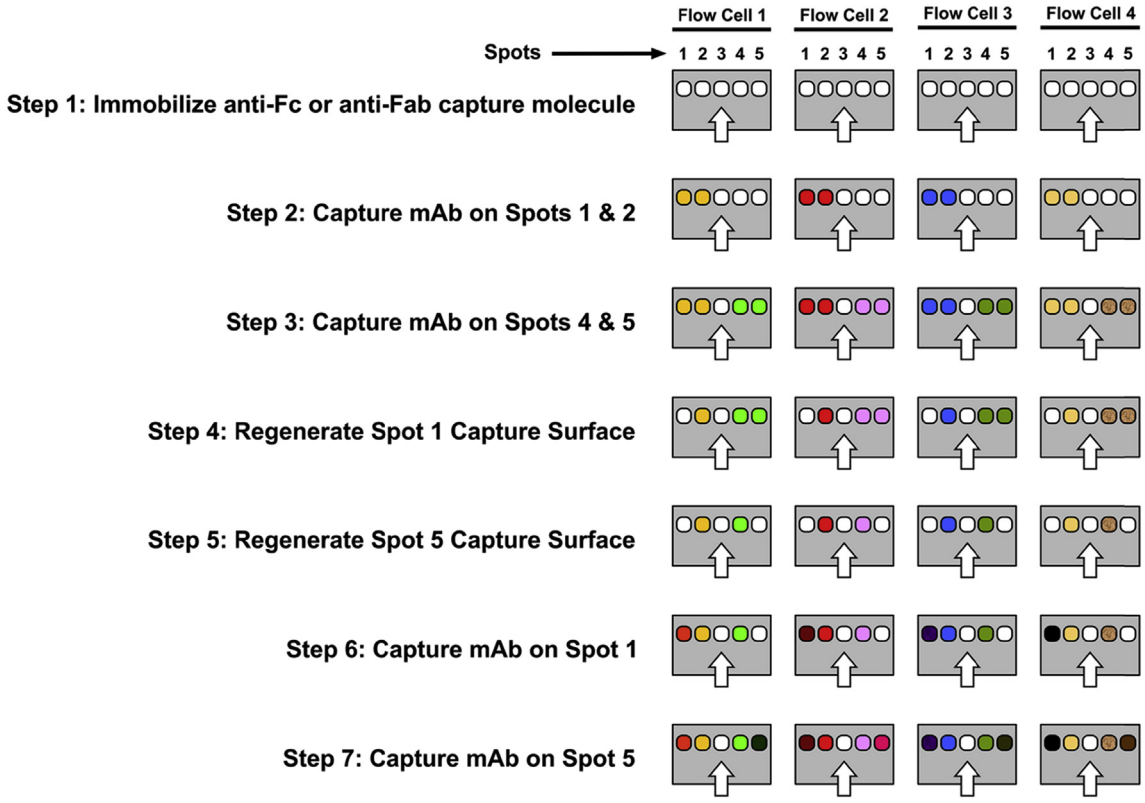


**Fig. 1.** Schematic representation of the experimental design for simultaneously capturing 16 mAbs on Biacore 4000.

2, Spot-4 and Spot-5) using the "Capture" injection command. However, the binding data generated only from Spot-2 and Spot-4 was used during the analysis. In the case of the 16-mAb capture kinetic assay, mAbs were captured on Spot-1, Spot-2, Spot-4, and Spot-5 as shown in Fig. 1. The "Capture" injection command was used to capture mAbs on Spot-2 and Spot-4 (Step-2 and Step-3 of Fig. 1) and the "General" injection command was used for the subsequent mAb capture steps (Step-6 and Step-7 of Fig. 1). Spot-3 was used as the reference surface for all of the kinetic assays performed in this study. Following the mAb capture, varying antigen concentrations (serially diluted by three-fold in HBS-ET buffer) were injected over the mAb capture surface for four minutes at a flow rate of 30 μL/min followed by a dissociation phase of 15 min.

For the SCK assay, a total of four antigen concentrations were injected over the mAb capture surface. The "Sample" injection command was used to inject the lowest antigen concentration followed by three "General" injection commands for the subsequent sequential injection of increasing antigen concentrations. The antigens were injected for 150 s followed by a dissociation phase of 120 s for the lowest three antigen concentrations. The dissociation time was added to the "General" injections by selecting the "Stabilization period after injection" which is available in the advanced settings of the "General" injection command. After the third "General" injection command, the dissociation time was extended to 600 s. Additional dissociation time of 300 s was added to the assay by injecting buffer using the "Enhancement" injection command. The analyte injection cycles were preceded by three to four buffer injection cycles and buffer cycles were later added after three replicate analyte injection cycles. At the end of each cycle, capture surfaces were regenerated and fresh mAb was captured at the beginning of each cycle. An example of a typical SCK binding cycle is provided in Supplementary File 1.

We observed that if the antigen is labeled "Buffer Supply", the four-needle system of the Biacore 4000 biosensor aspirates the running buffer from the four buffer tubing and injects the running buffer over the sensor surface. This circumvents the need for a dedicated well in the sample plate and thus assists in saving significant space in the sample and/or reagent plate. Therefore all of the buffer injections required for the second reference subtraction were performed by labeling the antigen as "Buffer Supply".

The "Regeneration" injection command fails to inject sample over just the outside spots (Spot-1 and Spot-5) and hence was found to be incompatible with the 16-mAb capture kinetic assay. The "General" injection command allowed sample injection over just the outside spots and hence was used to regenerate mAb captured sensor surface for all the binding studied presented in this embodiment. Another advantage of the "General" injection command is that it consumes lower sample volume and thus allows designing longer binding kinetic assays. In this study, the mAb capture surfaces were regenerated by injecting 20 mM phosphoric acid, pH2.0 for 10–12 s using the "General" injection command followed by an extra wash of the flow cell ("Extra wash after injection with" option available in the advanced settings) with the running buffer (labeled as "Buffer Supply").

### *Data processing and analysis*

The binding data were initially processed using the Biacore 4000 BIAevaluation software by aligning the raw data to the start of the analyte injection followed by the subtraction of the reference flow cell. The single reference subtracted data were exported as text files and a second reference subtraction was performed using the Scrubber fitting software (version 2.0c, BioLogic Software, Campbell, Australia) using the control buffer injections. The double

reference subtracted data generated using the 8-mAb/16-mAb capture kinetic and PK assays was globally fitted to a 1:1 Langmuir binding model with mass transport limitation using Scrubber software. The double reference subtracted SCK data was fitted using the SCK binding model with mass transport limitation using the Biacore 3000 BIAevaluation software (version 4.1.1).

## Results

### *A total of 16 monoclonal antibodies can be simultaneously captured for kinetic analysis on the Biacore 4000 without any bleeding between the capture spots*

The 16-mAb capture assay requires multiple regeneration steps (Fig. 1), and inefficient regeneration of the sensor surface can leave traces of the previously captured mAb. This can result in the bleeding of mAb between different capture spots. In order to determine the efficiency of the regeneration steps, we assessed mAb bleeding by measuring binding specificity following multiple regeneration steps included in the 16-mAb capture assay. Four mAbs binding to 28kD, 48kD, 71kD, and 97kD antigens were captured over different spots using an anti-human Fc immobilized sensor surface in different patterns as shown in the top panel of Fig. 2. Different antigens (50 nM) were later injected over the entire sensor surface and the observed binding response was normalized with respect to the maximum binding signal (bottom panel of Fig. 2). Irrespective of the location of the mAb on the capture surface, antigen only bound to the mAb specific capture spots. This demonstrated that the multiple regeneration steps involved in the 16-mAb capture assay did not leave any residual mAb. We also observed less than 1% deviation in the mAb capture levels during this 28 cycle experiment (data not shown), suggesting that multiple surface regenerations did not affect the activity of the anti-human Fc immobilized sensor surface.

### *The 16-mAb capture kinetic assay format generated reliable and reproducible binding kinetic parameters*

To assess the reliability and reproducibility of the 16-mAb capture kinetic assay, at least three independent experiments were performed on 16 kinetically diverse human mAbs using the 8-mAb and 16-mAb capture kinetic assay formats and the $k_a$, $k_d$, and $K_D$ values generated using both the assays were compared. Fig. 3 provides representative real time binding sensorgrams (black lines) along with the global fits (red lines) for data generated using the 8-mAb and 16-mAb capture kinetic assay formats, while the results of the kinetic analysis (±standard deviation) are summarized in Table 1. The binding affinities of the mAbs ranged from 130 pM to 200 nM and the binding kinetic values generated using the 16-mAb capture kinetic format correlated with those measured using the 8-mAb capture kinetic format, as indicated by the linear regression fits, which yielded $R^2$ values close to 1 (Fig. 4). The slope of the trend line for $k_a$, $k_d$, and $K_D$ correlation plots was also approximately 1, indicating that comparable kinetic values were measured using the 16-mAb capture kinetic assay. The $k_a$ and $k_d$ values measured for all 16 mAb-antigen interactions were within two-fold of each other for both capture kinetic assay formats. In addition, $K_D$ values for 15 out of 16 mAbs were also within two-fold of each other. The $K_D$ values for mAb-4 binding to 14kD antigen was found to be within three-fold of each other (133 pM measured using the 8-mAb capture format and 303 pM measured using the 16-mAb capture format).

### *Single cycle kinetic and parallel kinetic assays measured reliable and reproducible binding rate constants*

#### *Single cycle kinetic (SCK) assay*

The conventional kinetic assays are designed by serially injecting different analyte concentrations over multiple binding cycles, and the sensor surface is typically regenerated between each binding cycle to remove the analyte bound to the ligand surface. However, optimization of the regeneration conditions can be challenging, time consuming, and often detrimental to the activity of the ligand surface. To address these challenges, the SCK assay was developed, which eliminates the regeneration step during the characterization of protein-protein interactions and thus reduces the time required to generate the kinetic data [13]. Moreover, instead of running multiple binding cycles as is the case for the conventional titration kinetic assay, kinetic data can be generated using just two cycles (buffer injection followed by sequential injection of analyte titration series) which in turn reduces the experiment time required to characterize biomolecular interactions. The Biacore T200, Biacore X100, Biacore S200 and Biacore 8k biosensors have a pre-defined injection command to perform SCK assay, but is currently unavailable for the Biacore 4000 biosensor. This provided us the rationale to develop the SCK assay on the Biacore 4000. Experiments were initially performed using different combinations of injection commands and, based on our

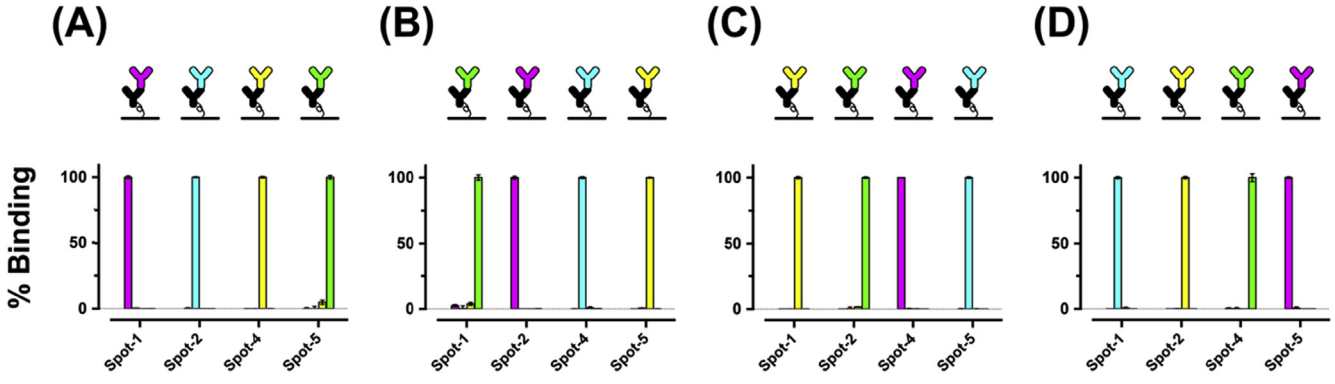


**Fig. 2. Antigen binding to monoclonal antibody captured on different spots.**
mAb to 28kD (magenta), 48kD (cyan), 71kD (yellow), and 97kD (green) was first captured on different spots immobilized with anti-human Fc mAb (black) followed by the injection of 50 nM antigen over the entire flow cell. The top panel provides the schematic of different patterns of mAb capture surfaces (**A-D**), while the bottom panel shows bar graph of the average of percent maximum binding from three independent injections of 28kD (magenta), 48kD (cyan), 71kD (yellow), and 97kD (green) antigen and the error bars represent standard deviation. Irrespective of the mAb capture surface, all the four antigens bound to their specific mAb.

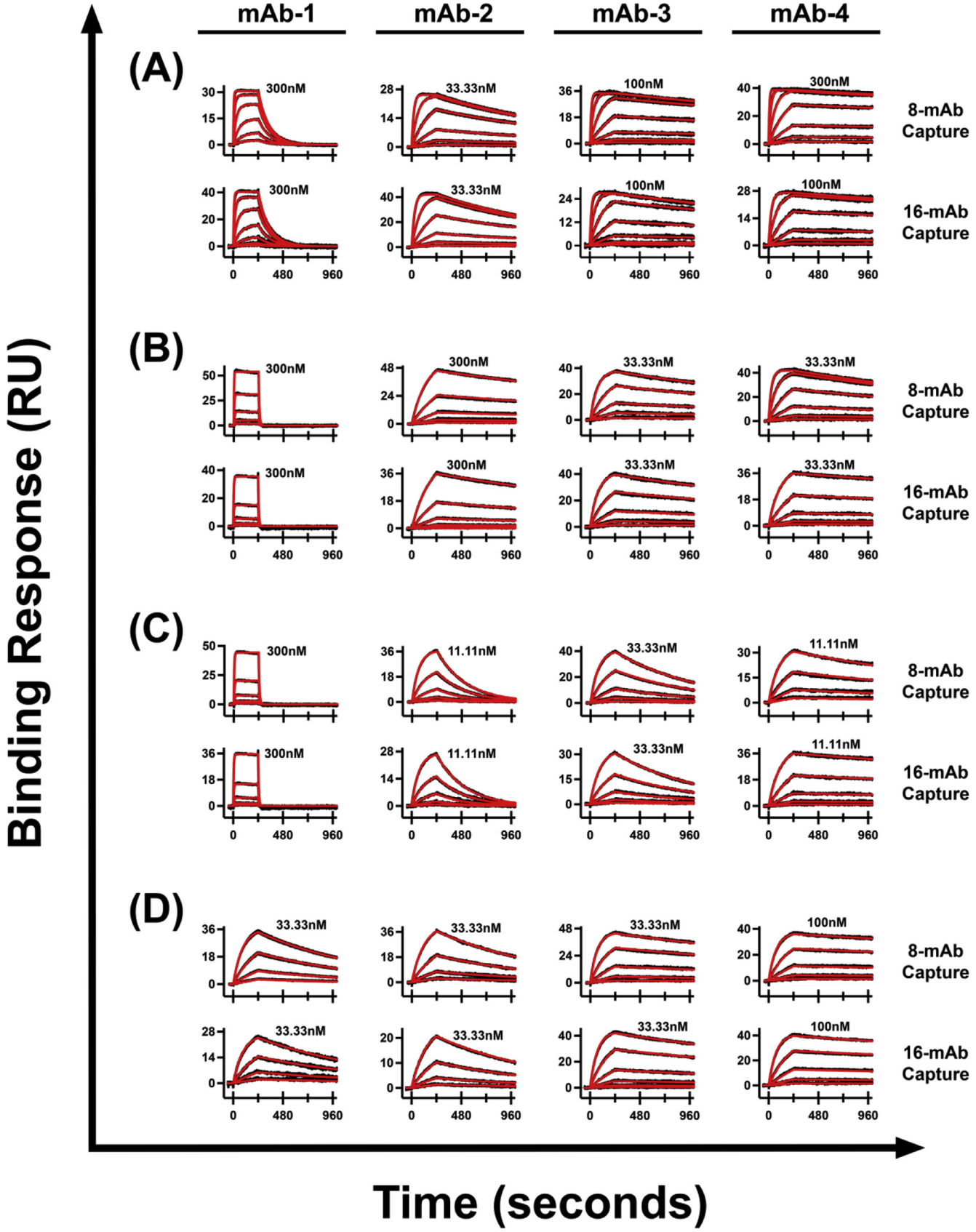


**Fig. 3. Representative binding sensorgrams for mAb-antigen interactions using the 8-mAb and 16-mAb capture kinetic assays.**
mAb was first captured using either the 8-mAb capture (top panel) or 16-mAb capture (bottom panel) kinetic assay followed by the injection of different concentrations of 14kD (**A**), 29kD (**B**), 38kD (**C**), and 48kD (**D**) antigen. Real time binding data for at least triplicate injections of same antigen concentration, serially diluted by three-fold in HBS-ET buffer is shown in black while red lines indicate global fits obtained using 1:1 binding model with mass transport limitation. The highest antigen concentration used to fit the binding data is also provided. Average binding kinetic parameters generated from at least three independent experiments (±standard deviation) using the 8-mAb capture and 16-mAb capture kinetic assay formats are tabulated in Table 1.

**Table 1**
Comparison of the binding kinetic parameters for mAb-antigen interactions using 8-mAb and 16-mAb capture kinetic assays.

| Antigen | mAb Captured | 8-mAb Capture Kinetic Assay (n = 5) | | | 16-mAb Capture Kinetic Assay (n = 3) | | |
|---|---|---|---|---|---|---|---|
| | | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) |
| 14kD | mAb-1 | $(7.48 \pm 0.20)\ 10^5$ | $(8.22 \pm 1.73)\ 10^{-3}$ | $(1.10 \pm 0.22)\ 10^{-8}$ | $(6.13 \pm 1.19)\ 10^5$ | $(9.78 \pm 0.41)\ 10^{-3}$ | $(1.63 \pm 0.26)\ 10^{-8}$ |
| | mAb-2 | $(1.60 \pm 0.10)\ 10^6$ | $(6.36 \pm 0.20)\ 10^{-4}$ | $(3.97 \pm 0.14)\ 10^{-10}$ | $(1.40 \pm 0.20)\ 10^6$ | $(6.66 \pm 0.31)\ 10^{-4}$ | $(4.84 \pm 0.85)\ 10^{-10}$ |
| | mAb-3 | $(9.96 \pm 0.40)\ 10^5$ | $(2.10 \pm 0.15)\ 10^{-4}$ | $(2.12 \pm 0.21)\ 10^{-10}$ | $(8.22 \pm 1.33)\ 10^5$ | $(2.51 \pm 0.41)\ 10^{-4}$ | $(3.15 \pm 0.91)\ 10^{-10}$ |
| | mAb-4 | $(5.71 \pm 0.57)\ 10^5$ | $(8.17 \pm 2.22)\ 10^{-5}$ | $(1.33 \pm 0.76)\ 10^{-10}$ | $(4.42 \pm 0.64)\ 10^5$ | $(1.30 \pm 0.31)\ 10^{-4}$ | $(3.03 \pm 1.05)\ 10^{-10}$ |
| 29kD | mAb-1 | $(1.14 \pm 0.34)\ 10^6$ | $(1.46 \pm 0.38)\ 10^{-1}$ | $(1.35 \pm 0.45)\ 10^{-7}$ | $(1.02 \pm 0.47)\ 10^6$ | $(1.51 \pm 0.35)\ 10^{-1}$ | $(1.57 \pm 0.30)\ 10^{-7}$ |
| | mAb-2 | $(3.28 \pm 0.90)\ 10^4$ | $(3.21 \pm 0.43)\ 10^{-4}$ | $(1.01 \pm 0.13)\ 10^{-8}$ | $(1.83 \pm 0.38)\ 10^4$ | $(3.34 \pm 0.15)\ 10^{-4}$ | $(1.86 \pm 0.31)\ 10^{-8}$ |
| | mAb-3 | $(3.98 \pm 0.95)\ 10^5$ | $(4.12 \pm 0.46)\ 10^{-4}$ | $(1.12 \pm 0.46)\ 10^{-9}$ | $(4.09 \pm 0.34)\ 10^5$ | $(3.22 \pm 0.21)\ 10^{-4}$ | $(7.89 \pm 0.28)\ 10^{-10}$ |
| | mAb-4 | $(1.06 \pm 0.19)\ 10^6$ | $(3.45 \pm 0.13)\ 10^{-4}$ | $(3.39 \pm 0.92)\ 10^{-10}$ | $(1.11 \pm 0.17)\ 10^6$ | $(3.79 \pm 0.18)\ 10^{-4}$ | $(3.45 \pm 0.40)\ 10^{-10}$ |
| 38kD | mAb-1 | $(1.06 \pm 0.40)\ 10^6$ | $(2.33 \pm 1.11)\ 10^{-1}$ | $(2.32 \pm 1.23)\ 10^{-7}$ | $(6.41 \pm 5.17)\ 10^5$ | $(3.18 \pm 1.83)\ 10^{-1}$ | $(5.59 \pm 1.19)\ 10^{-7}$ |
| | mAb-2 | $(1.03 \pm 0.09)\ 10^6$ | $(3.71 \pm 0.19)\ 10^{-3}$ | $(3.64 \pm 0.40)\ 10^{-9}$ | $(1.02 \pm 0.06)\ 10^6$ | $(3.70 \pm 0.05)\ 10^{-3}$ | $(3.63 \pm 0.19)\ 10^{-9}$ |
| | mAb-3 | $(3.62 \pm 0.48)\ 10^5$ | $(1.21 \pm 0.04)\ 10^{-3}$ | $(3.40 \pm 0.54)\ 10^{-9}$ | $(3.23 \pm 0.17)\ 10^5$ | $(1.20 \pm 0.03)\ 10^{-3}$ | $(3.73 \pm 0.11)\ 10^{-9}$ |
| | mAb-4 | $(8.20 \pm 0.57)\ 10^5$ | $(4.06 \pm 1.18)\ 10^{-4}$ | $(4.99 \pm 1.63)\ 10^{-10}$ | $(8.70 \pm 0.39)\ 10^5$ | $(2.30 \pm 1.31)\ 10^{-4}$ | $(2.62 \pm 1.40)\ 10^{-10}$ |
| 48kD | mAb-1 | $(2.70 \pm 0.32)\ 10^5$ | $(1.07 \pm 0.25)\ 10^{-3}$ | $(4.01 \pm 1.11)\ 10^{-9}$ | $(2.83 \pm 0.22)\ 10^5$ | $(8.84 \pm 0.25)\ 10^{-4}$ | $(3.13 \pm 0.24)\ 10^{-9}$ |
| | mAb-2 | $(1.98 \pm 0.28)\ 10^5$ | $(9.41 \pm 0.52)\ 10^{-4}$ | $(4.85 \pm 0.79)\ 10^{-9}$ | $(1.96 \pm 0.11)\ 10^5$ | $(9.36 \pm 0.22)\ 10^{-4}$ | $(4.78 \pm 0.16)\ 10^{-9}$ |
| | mAb-3 | $(3.96 \pm 0.52)\ 10^5$ | $(3.21 \pm 0.49)\ 10^{-4}$ | $(8.32 \pm 2.28)\ 10^{-10}$ | $(4.04 \pm 0.26)\ 10^5$ | $(2.85 \pm 0.11)\ 10^{-4}$ | $(7.06 \pm 0.26)\ 10^{-10}$ |
| | mAb-4 | $(1.39 \pm 0.12)\ 10^5$ | $(1.75 \pm 0.61)\ 10^{-4}$ | $(1.26 \pm 0.37)\ 10^{-9}$ | $(1.32 \pm 0.07)\ 10^5$ | $(1.60 \pm 0.08)\ 10^{-4}$ | $(1.21 \pm 0.12)\ 10^{-9}$ |

Binding kinetic values represent an average of at least three independent experiments (±standard deviation) performed using 8-mAb and 16-mAb capture kinetic assay formats.

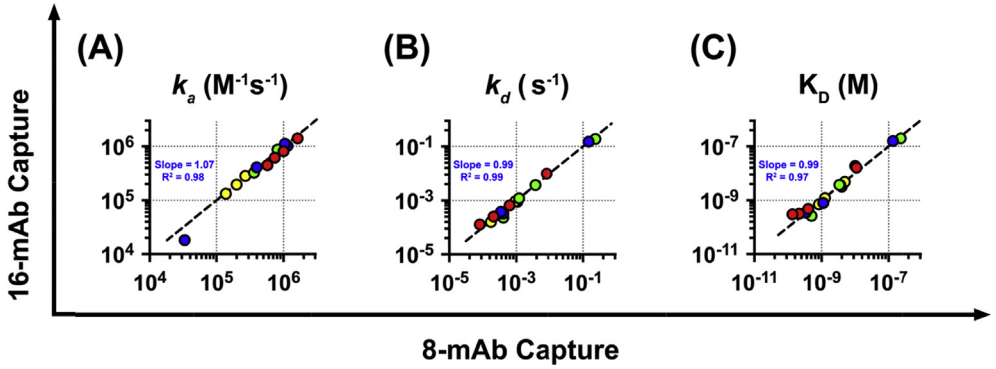


**Fig. 4. Correlation plot of binding kinetic values generated using the 8-mAb and 16-mAb capture kinetic assay formats.**
Graphical representation showing the correlation of binding kinetic values measured for mAb binding to 14kD (●), 29kD (●), 38kD (●), and 48kD (●) antigen. Scatterplots of average values of $k_a$ (**A**), $k_d$ (**B**) and $K_D$ (**C**) measured from at least three independent experiments using 8-mAb capture and 16-mAb capture kinetic assays are shown. The correlation plots were fit using a linear regression fit and the slope of the trend line along with the correlation coefficients ($R^2$) are shown in the plot. The trend line is represented as black dotted line. The slope of the trend line is approximately 1 illustrating that similar binding kinetic parameters were measured using the 16-mAb capture kinetic assay.

experience, the SCK method provided in the materials and methods section generated the best quality SCK binding data (data not shown).

The mAbs were first captured over an anti-human Fc immobilized sensor surface, and increasing concentrations of analyte were later injected. At least eight independent experiments were performed and Fig. 5 provides representative binding sensorgrams for at least five replicate injections of antigen binding to the mAb capture surface. The binding data generated using the SCK assay on the Biacore 4000 were reproducible, as the replicate antigen injections for all 16 mAbs included in this study exhibited good overlap. Moreover, the fitted data (red lines) overlapped with the real time binding sensorgrams for all 16 mAbs. The average $k_a$, $k_d$, and $K_D$ values for the 16 mAb-antigen interactions characterized using SCK assay are provided in Table 2.

*Parallel kinetic (PK) assay*

Similar to the SCK assay, the PK assay allows the characterization of mAb-antigen interactions without the regeneration of the ligand surface. The PK assay is a characterization technique currently available with the Biacore 8k biosensor and is similar to the "single injection cycle kinetic" (SICK) assay format of MASS-1 [3]. In the PK assay, mAb is first immobilized or captured across different flow cells followed by the simultaneous injection of an analyte concentration series across different flow cells of the sensor surface. Kinetic values can thus be generated using just two binding cycles – (i) buffer injection required for double reference subtraction, and (ii) injection of analyte concentration series.

To assess the performance of the PK assay on the Biacore 4000, we selected eight mAbs from the panel of 16 mAbs (two mAbs per antigen). At least four independent experiments were performed and representative binding sensorgrams for all eight mAbs are shown in Fig. 6. Four replicate injections of the same antigen concentration (black line) showed excellent overlap, indicating good reproducibility of the binding data using the PK assay. The global fits (red lines) also overlaid with the real time binding curves. Average values of the binding kinetic parameters from four independent experiments (±standard deviation) are provided in Table 3.

The binding kinetic parameters performed using both SCK and PK assays correlated with the values generated using the conventional 8-mAb capture kinetic format and the linear fits generated $R^2$

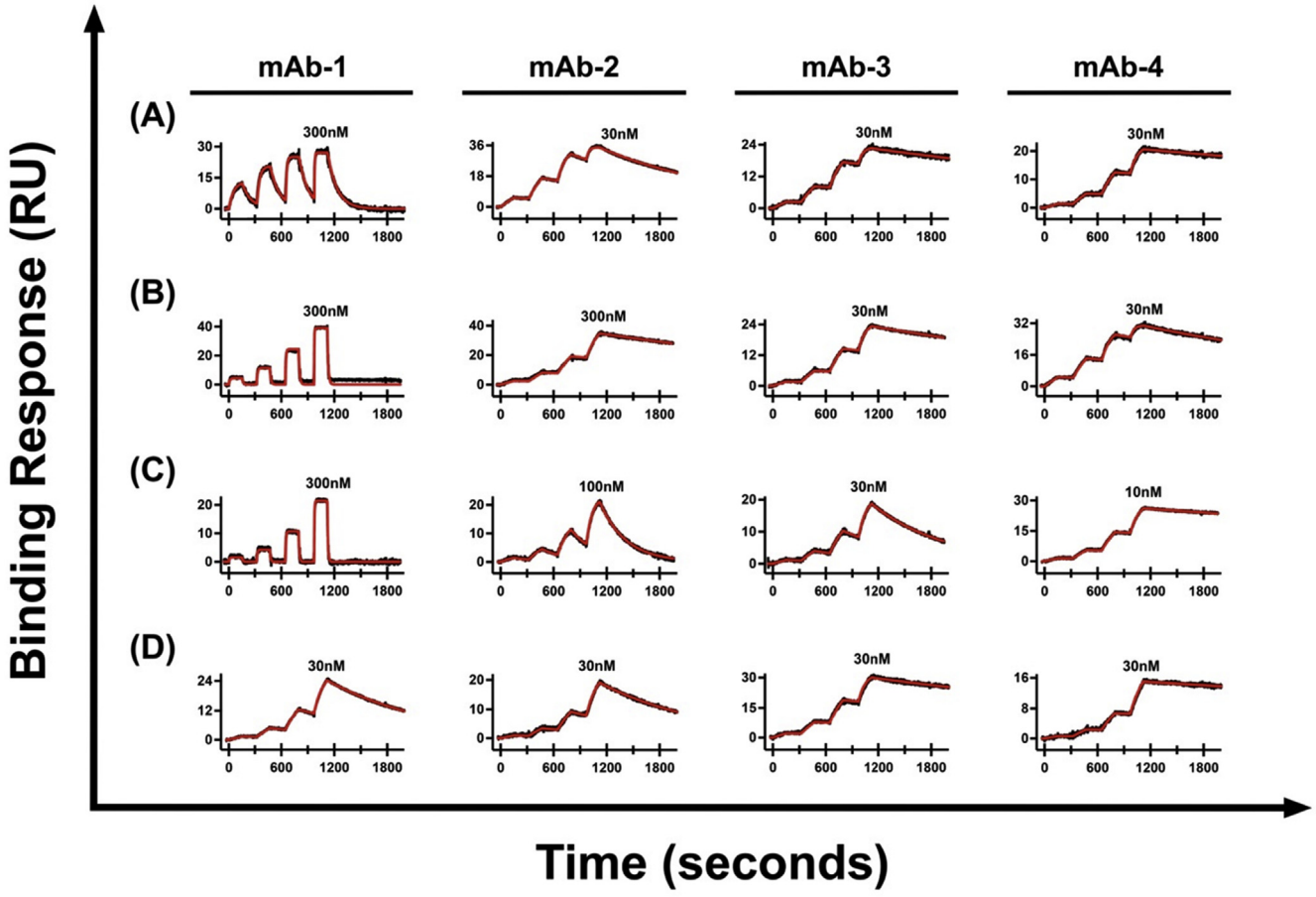


**Fig. 5. Representative binding sensorgrams for mAb-antigen interactions using the single cycle kinetic assay.**
mAb was first captured using the conventional 8-mAb capture assay format followed by the sequential injection of increasing concentrations of 14kD (**A**), 29kD (**B**), 38kD (**C**), and 48kD (**D**) antigen. Real time binding sensorgrams generated from at least five independent injections of antigen are shown as black lines and red lines represent modeled data generated by globally fitting real time data using single cycle kinetic model with mass transport limitation in the BIAevaluation software of Biacore 3000. Antigens were serially diluted by three-fold in HBS-ET running buffer and the highest antigen concentration used in the assay is reported as text in the figure. Results of the kinetic analysis from at least eight independent experiments are provided in Table 2.

**Table 2**
Binding kinetic analysis performed on 16 monoclonal antibodies captured using SCK assay format.

| Antigen | mAb Captured | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) |
|---|---|---|---|---|
| 14kD | mAb-1 | $(7.01 \pm 1.0)\ 10^5$ | $(8.22 \pm 1.17)\ 10^{-3}$ | $(1.19 \pm 0.19)\ 10^{-8}$ |
| | mAb-2 | $(1.17 \pm 0.09)\ 10^6$ | $(6.98 \pm 0.45)\ 10^{-4}$ | $(6.03 \pm 0.78)\ 10^{-10}$ |
| | mAb-3 | $(7.47 \pm 0.71)\ 10^5$ | $(2.33 \pm 0.18)\ 10^{-4}$ | $(3.13 \pm 0.28)\ 10^{-10}$ |
| | mAb-4 | $(4.11 \pm 0.3)\ 10^5$ | $(1.30 \pm 0.14)\ 10^{-4}$ | $(3.20 \pm 0.52)\ 10^{-10}$ |
| 29kD | mAb-1 | $(9.37 \pm 4.57)\ 10^5$ | $(1.13 \pm 0.44)\ 10^{-1}$ | $(1.27 \pm 0.26)\ 10^{-7}$ |
| | mAb-2 | $(2.20 \pm 0.43)\ 10^4$ | $(2.75 \pm 0.07)\ 10^{-4}$ | $(1.29 \pm 0.24)\ 10^{-8}$ |
| | mAb-3 | $(4.21 \pm 0.22)\ 10^5$ | $(2.68 \pm 0.16)\ 10^{-4}$ | $(6.40 \pm 0.54)\ 10^{-10}$ |
| | mAb-4 | $(8.79 \pm 0.27)\ 10^5$ | $(3.00 \pm 0.08)\ 10^{-4}$ | $(3.41 \pm 0.18)\ 10^{-10}$ |
| 38kD | mAb-1 | $(5.68 \pm 2.95)\ 10^5$ | $(9.98 \pm 3.45)\ 10^{-2}$ | $(1.93 \pm 0.55)\ 10^{-7}$ |
| | mAb-2 | $(1.17 \pm 0.05)\ 10^6$ | $(3.55 \pm 0.10)\ 10^{-3}$ | $(3.05 \pm 0.12)\ 10^{-9}$ |
| | mAb-3 | $(3.19 \pm 0.30)\ 10^5$ | $(1.06 \pm 0.16)\ 10^{-3}$ | $(3.33 \pm 0.50)\ 10^{-9}$ |
| | mAb-4 | $(8.77 \pm 0.53)\ 10^5$ | $(1.27 \pm 0.14)\ 10^{-4}$ | $(1.46 \pm 0.25)\ 10^{-10}$ |
| 48kD | mAb-1 | $(2.78 \pm 0.13)\ 10^5$ | $(8.19 \pm 0.15)\ 10^{-4}$ | $(2.95 \pm 0.10)\ 10^{-9}$ |
| | mAb-2 | $(2.26 \pm 0.35)\ 10^5$ | $(8.35 \pm 0.21)\ 10^{-4}$ | $(3.77 \pm 0.56)\ 10^{-9}$ |
| | mAb-3 | $(4.32 \pm 0.27)\ 10^5$ | $(1.96 \pm 0.08)\ 10^{-4}$ | $(4.53 \pm 0.17)\ 10^{-10}$ |
| | mAb-4 | $(1.76 \pm 0.24)\ 10^5$ | $(1.09 \pm 0.09)\ 10^{-4}$ | $(6.31 \pm 0.91)\ 10^{-10}$ |

Average of binding kinetic parameters generated from at least eight independent experiments (±standard deviation) is provided.

values greater than 0.94 (Fig. 7). Moreover, the slope of the trend line was found to be close to 1, which supports the reliability of the measured kinetic values. The affinity isotherm plots ($k_a$ versus $k_d$) shown in Fig. 8 provide the results of individual experiments performed using the 8-mAb capture, 16-mAb capture, SCK, and PK assay formats presented in this embodiment. The affinity isotherms

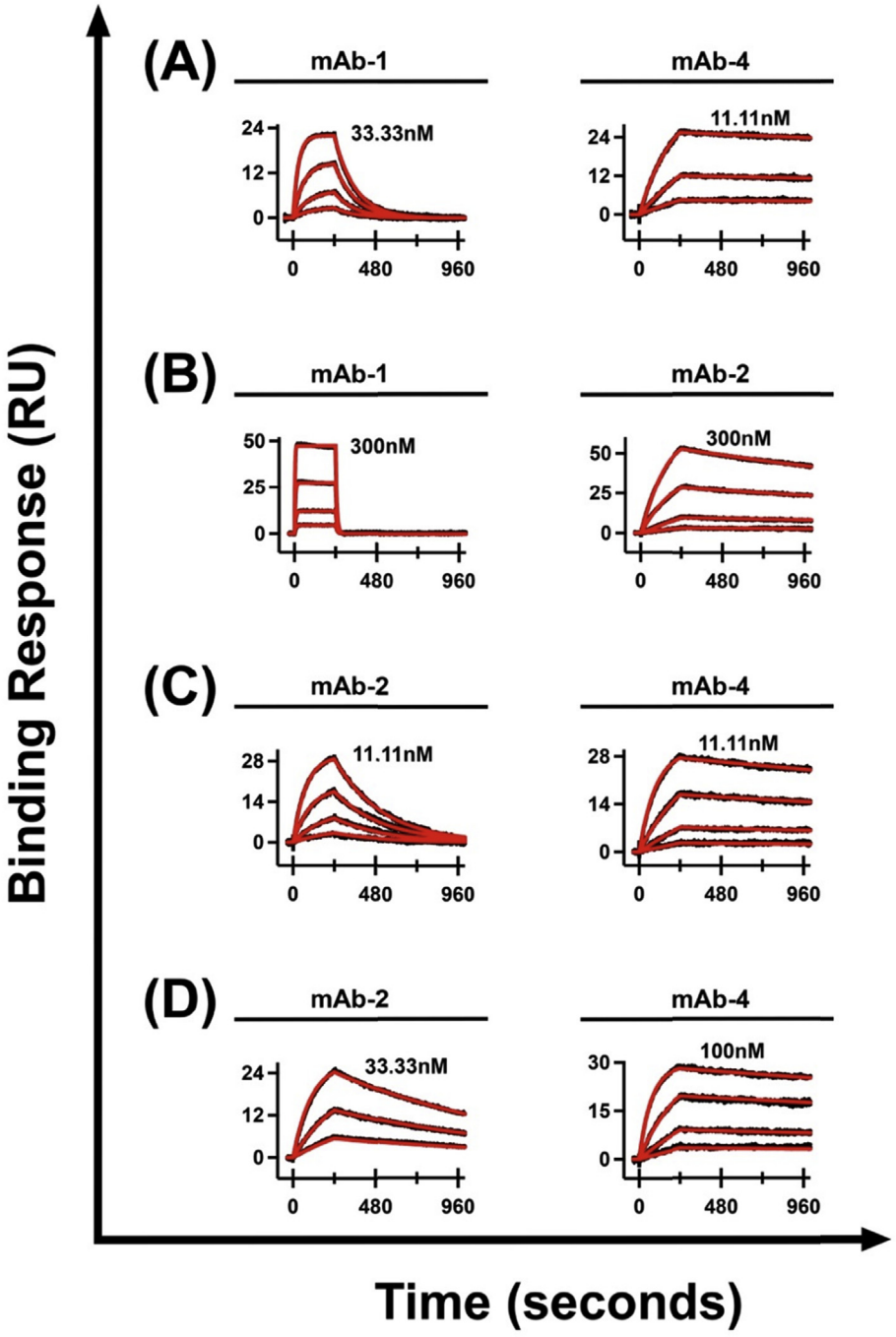


**Fig. 6. Representative binding sensorgrams for mAb-antigen interactions using the parallel kinetic assay format**.
A comparable level of mAb was captured on different flow cells followed by the simultaneous injection of varying antigen concentrations over different flow cells. Black lines represent real time binding data generated from at least four replicate injections of 14kD (**A**), 29kD (**B**), 38kD (**C**), and 48kD (**D**) antigen while modeled fits generated by globally fitting the real time binding data are shown as red lines. Highest antigen concentration used to fit the binding data is also provided and average kinetic values (±standard deviation) measured from four independent experiments are listed in Table III.

also showcase the experimental spread in the measured rate constants for each mAb determined from the different kinetic assay formats. We did not observe any significant experiment-to-experiment variability and the standard errors in the binding rate constants ($k_a$ and $k_d$) and $K_D$ averaged less than 18% and 26%, respectively. These results further support the reproducibility and reliability of the kinetic values measured using all three kinetic assay formats developed on the Biacore 4000 biosensor.

## Discussion

The Biacore 4000 biosensor has been utilized for more than a decade to support the discovery and development of therapeutic mAbs. However, the inability to inject samples over only the inside

**Table 3**
Binding kinetic parameters of eight mAbs generated using parallel kinetic assay format.

| Antigen | mAb Captured | $k_a$ (1/Ms) | $k_d$ (1/s) | $K_D$ (M) |
|---|---|---|---|---|
| 14kD | mAb-1 | $(8.76 \pm 1.06)\ 10^5$ | $(1.14 \pm 0.37)\ 10^{-2}$ | $(1.27 \pm 0.27)\ 10^{-8}$ |
| | mAb-4 | $(4.61 \pm 1.35)\ 10^5$ | $(9.3 \pm 0.21)\ 10^{-5}$ | $(2.17 \pm 0.68)\ 10^{-10}$ |
| 29kD | mAb-1 | $(10.04 \pm 1.76)\ 10^5$ | $(1.58 \pm 0.53)\ 10^{-1}$ | $(1.54 \pm 0.3)\ 10^{-7}$ |
| | mAb-2 | $(3.24 \pm 0.85)\ 10^4$ | $(3.43 \pm 0.4)\ 10^{-4}$ | $(1.1 \pm 0.21)\ 10^{-8}$ |
| 38kD | mAb-2 | $(10.96 \pm 2.7)\ 10^5$ | $(3.58 \pm 0.16)\ 10^{-3}$ | $(3.41 \pm 0.72)\ 10^{-9}$ |
| | mAb-4 | $(8.68 \pm 1.33)\ 10^5$ | $(1.94 \pm 0.67)\ 10^{-4}$ | $(2.36 \pm 1.22)\ 10^{-10}$ |
| 48kD | mAb-2 | $(2.33 \pm 0.31)\ 10^5$ | $(9.29 \pm 0.27)\ 10^{-4}$ | $(4.04 \pm 0.56)\ 10^{-9}$ |
| | mAb-4 | $(1.38 \pm 0.07)\ 10^5$ | $(1.54 \pm 0.19)\ 10^{-4}$ | $(1.12 \pm 0.16)\ 10^{-9}$ |

Values represent average of at least four independent experiments performed (±standard deviation).

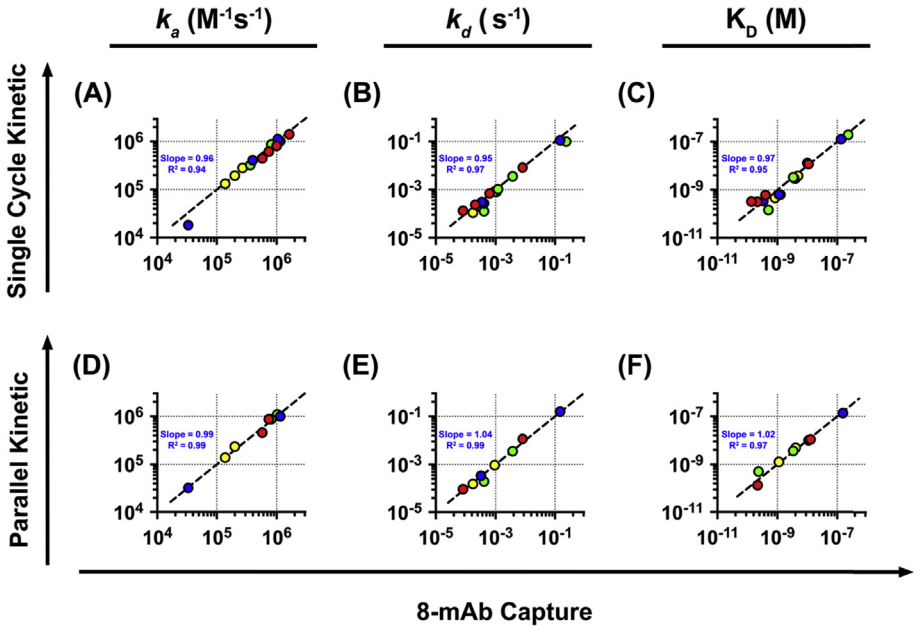


**Fig. 7. Correlation of binding kinetic parameters measured using the conventional 8-mAb capture kinetic with the single cycle kinetic or parallel kinetic assay formats.** Graphical representation showing the correlation of $k_a$ (**A**, **D**), $k_d$ (**B**, **E**), and $K_D$ (**C**, **F**) values measured for mAb binding to 14kD (●), 29kD (●), 38kD (●), and 48kD (●) antigen. The scatterplot represents average kinetic values measured from at least eight independent experiments performed using the SCK assay (**A**, **B**, **C**) or from four independent experiments performed using the PK assay (**D**, **E**, **F**). The correlation plots were fit using a linear regression fit in Excel and the slope of the trend line and the correlation coefficients ($R^2$) are shown in the plot. The trend line is represented as black dotted line. The slope of the trend line is approximately 1 illustrating that similar binding kinetic parameters were measured using the 16-mAb capture kinetic assay.

spots (Spot-2 or Spot-4) has restricted its application to characterize only up to eight mAbs per cycle using the capture kinetic assay format. This study focuses on the development of three binding kinetic assays to further increase the throughput of the Biacore 4000 biosensor for kinetic analysis of mAb-antigen interactions. All three assay formats developed in this study (16-mAb capture kinetic, SCK, and PK) are currently unavailable as a predefined injection command on the Biacore 4000. The results presented in Table 1 through Table 3, and Fig. 8 demonstrate that the $k_a$, $k_d$ and $K_D$ values measured using all three kinetic assays are reproducible, with minimal deviation in the measured binding rate constants in comparison to those calculated using the traditional 8-mAb capture kinetic assay format. The high reliability in the measurement of binding kinetic parameters using all three kinetic assays is also illustrated in Figs. 4 and 7, as $R^2$ values were observed to be greater than 0.94.

The 16-mAb capture kinetic assay format presented in this study provides a new approach to perform kinetic analysis on the Biacore 4000. Apart from the reliability and reproducibility of the measured binding rate constants, the 16-mAb capture kinetic assay provides two additional advantages in comparison to the conventional 8-mAb capture kinetic assay. First, since 16 mAbs are simultaneously captured over the sensor surface, analyte consumption is reduced by 50%. This is an important feature, especially when the analyte is expensive and/or its availability is limited. Secondly, the experiment time for performing kinetic analysis using the 16-mAb capture kinetic assay format is significantly reduced. A detailed comparison of the experiment time required to characterize mAb-antigen interaction using the 8-mAb capture and 16-mAb capture kinetic assay is provided in Table 4. Approximately 35% reduction in the experiment run time is observed for 16-mAb capture kinetic assay (Table 4), which translates to around 34 h of time savings for characterizing 48 mAbs binding to three antigens.

The BIAevaluation software of the Biacore 4000 biosensor only allows analysis of the data generated using the pre-defined assays provided by the manufacturer (GE Healthcare) and cannot be utilized to analyze data generated using novel assays. This limitation is another challenge associated with the Biacore 4000 biosensor. Any

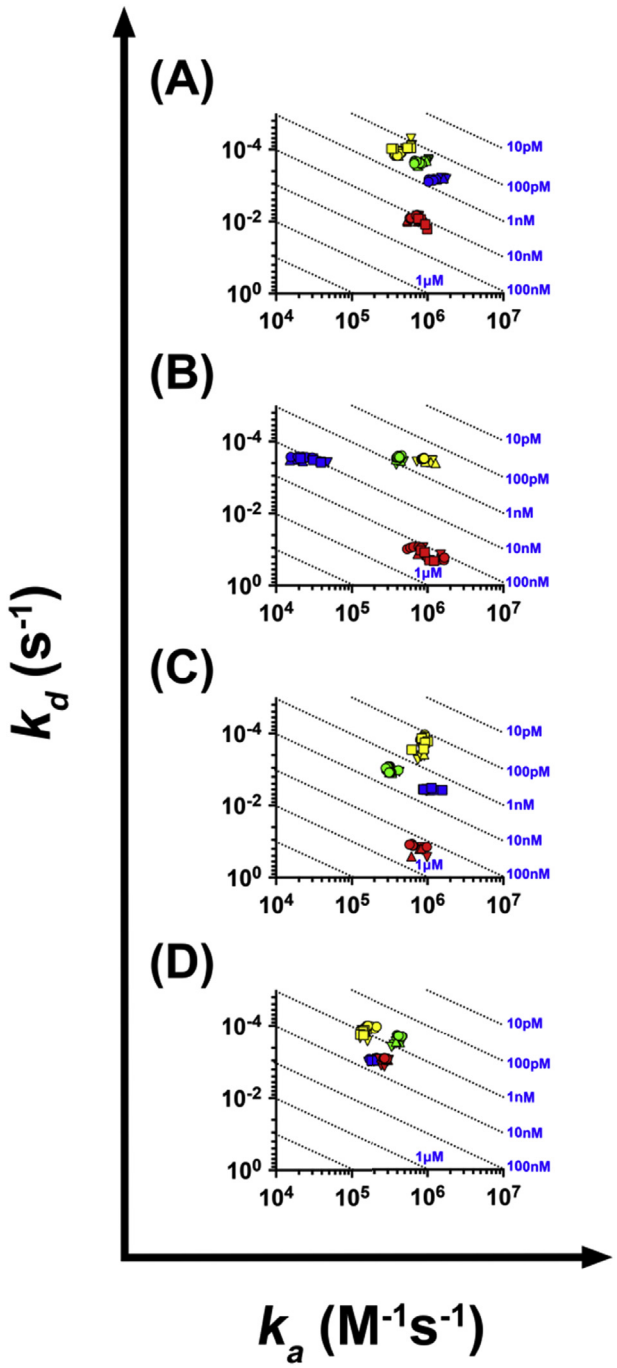


**Fig. 8. Affinity isotherm plot showing results of individual experiments performed using diverse assay formats.**
The scatter plot of $k_a$ versus $k_d$ values generated from individual experiments for mAb-1 (red), mAb-2 (blue), mAb-3 (green), and mAb-4 (yellow) binding to 14kD (**A**), 29kD (**B**), 38kD (**C**), and 48kD (**D**) antigen. The affinity isotherm plots also provide experimental spread in the binding rate constants generated using the 8-mAb capture kinetic (inverted triangle), 16-mAb capture kinetic (triangle), SCK (circle), and PK (square) assay formats.

new assay developed on the Biacore 4000, including the three kinetic assays described herein, typically necessitates the use of a third party curve fitting software to analyze the binding data. In this study, Scrubber software was used for fitting the double reference subtracted binding curves generated using the 16-mAb capture kinetic and PK assays while BIAevaluation software (version 4.1.1) was required to analyze SCK data. Moreover, the single reference subtracted data exported by Biacore 4000 BIAevaluation software lacks the information about the flow cell and injection cycle number, and these details had to be manually entered in the Scrubber software before analyzing the kinetic data; another caveat of the Biacore 4000 BIAevaluation software. Supplementary Fig. 1 provides a schematic of the steps required to import the single reference subtracted data generated using the 8-mAb capture and 16-mAb capture kinetic assays in the Scrubber software.

Since all 16 mAbs characterized in this study were human mAbs, binding studies were performed using an anti-human Fc immobilized sensor surface. The same method can be easily adopted for the characterization of murine IgG (hybridoma) or Fab fragments by immobilizing anti-mouse Fc or anti-Fab capture molecule on the sensor surface, respectively. The 16-mAb capture method can be further tailored to concurrently characterize antibodies with different Fc regions (human vs. mouse) or with different antibody formats (IgG vs Fab/scFv) by immobilizing different capture molecules on the inside spots (Spot-2 and Spot-4) and outside spots (Spot-1 and Spot-5). We classify this assay format as a 2x8-mAb capture kinetic assay. However, selection of the appropriate spot for the immobilization of the capture molecule will depend on the regeneration condition of the capture surface. Since the Biacore 4000 fails to deliver sample over just the inside spots (Spot-2 and Spot-4), it is imperative that the regeneration condition for the capture molecule immobilized on the inside spots should not affect the activity of the capture molecule immobilized on the outside spots (Spot-1 and Spot-5). Also, depending on the regeneration conditions, additional regeneration cycles might be required following the end of the dissociation time. Moreover, we need to ensure that the capture molecule selected for designing kinetic assay should be very specific to the antibody under investigation with no cross-reactivity.

The 2x8-mAb capture kinetic assay is routinely performed in our laboratory for simultaneous characterization of mAb panel comprising of human and murine mAbs binding to the same antigen. We typically use human antibody capture and mouse antibody capture kits from GE Healthcare for the kinetic analysis of human and murine mAbs, respectively. The anti-mouse Fc capture molecule requires 50–60 s injection of 10 mM Glycine, HCl, pH1.5 compared to a short 10–12 s injection of 20 mM phosphoric acid to regenerate anti-human Fc capture molecule. Immobilization of anti-mouse Fc capture molecule on the outside spots (Spot-1 and Spot-5) enables longer injection of 10 mM Glycine, HCl, pH 1.5 over the outside spots alone; thus retaining the activity of anti-human Fc capture surface, which we immobilize on the inside spots (Spot-2 and Spot-4). The EDC-NHS activated and ethanolamine blocked Spot-3 surface is used as a reference surface in the 2x8-mAb capture kinetic assay which allows the compensation for the bulk refractive index changes. Injecting the mAb sample containing a mixture of human and murine mAbs over both inside and outside spots enables mAb capture over the specific immobilized capture surface. Based on our experience, the kinetic values measured using the 2x8-mAb capture kinetic assay are similar to those generated using the traditional capture kinetic assay format (data not shown).

During the development of 16-mAb kinetic assay, we observed that injection of 3 M magnesium chloride (GE Healthcare recommended regeneration buffer for anti-human Fc capture molecule) over the outside spot resulted in the bleeding over to the inside spots; leading to the regeneration of the mAb captured over both the inside and outside spots. Therefore, regeneration of anti-hFc surface using 3 M magnesium chloride was incompatible with the 16-mAb capture assay and we had to identify different regeneration condition. After screening different regeneration conditions, we found that a 10–12 s injection of 20 mM phosphoric acid,

**Table 4**
Comparison of the throughput for the 8-mAb capture and 16-mAb capture kinetic assay formats to characterize mAb-antigen interactions.

| Number of Antigens | 16 mAb Panel | | 32 mAb Panel | | 48 mAb Panel | |
|---|---|---|---|---|---|---|
| | 8-mAb Capture | 16-mAb Capture | 8-mAb Capture | 16-mAb Capture | 8-mAb Capture | 16-mAb Capture |
| 1 | 12hr, 14min | 8hr | 24hr, 8min | 15hr, 36min | 36hr, 1min | 23hr, 18min |
| 2 | 21hr, 45min | 14hr, 6min | 43hr, 10min | 27hr, 54min | 64hr, 34min | 41hr, 36min |
| 3 | 32hr, 4min | 20hr, 40min | 63hr, 46min | 41hr, 6min | 95hr, 29min | 61hr, 30min |

The total run time for the kinetic assays performed by capturing the mAb for 30 s, 4 min of association time, and 15 min of dissociation time. The representative examples of 8-mAb capture and 16-mAb capture assays are provided in Supplementary File 2 through Supplementary File 5. A total of five antigen concentrations (duplicate injections) were used in the method. Each mAb-antigen binding study was initiated by two buffer injection cycles and then buffer cycles were added after every four antigen injection cycles.

pH2.0 was able to specifically regenerate mAb captured on the outside spot without affecting the mAb that was captured on its neighboring inside spot. Moreover, less than 1% deviation in the mAb capture level was observed during the 28 binding cycles performed to generate the data presented in Fig. 2. This provided us enough evidence that injection of 20 mM phosphoric acid, pH2.0 for 10–12 s is an appropriate regeneration condition and retained the binding activity of the anti-human Fc capture molecule. Hence all of the kinetic assays presented in this study were performed using this regeneration condition.

Due to the multiple regeneration steps involved in the 16-mAb capture kinetic assay format, the success of this assay entirely relies on the robustness of the regeneration condition. As optimization of the regeneration condition can be complex and requires extended assay development time, we also developed the SCK and PK assays to address such challenges, as both of these assays eliminate the need to regenerate the sensor surface. Fig. 8 illustrates that both SCK and PK assays generate reproducible $k_a$ and $k_d$ values from multiple experiments and the values correlate with those generated using the 8-mAb capture kinetic assay format (Fig. 7).

During the classical multiple cycle titration kinetic assays, different analyte concentrations are serially injected over the ligand surface over multiple binding cycles. Therefore, any atypical binding curve generated during a particular cycle can be discarded during the analysis and data can be analyzed using the remaining analyte titration curves. However, in the case of the SCK assay, every binding curve comprises of the entire analyte titration series and exclusion of any abnormal binding curve generated during the SCK assay results in the removal of the entire antigen titration curve. Therefore generation of reproducible binding sensorgrams is extremely vital for the successful implementation of the SCK assay.

The SCK data presented in this study was generated using a total of five individual injection commands (details provided in materials and methods section) as opposed to a single injection command for a pre-defined SCK injection. It is known that irrespective of the SPR/SPRi based microfluidic biosensor platform, binding artifacts are typically generated in between injections, usually caused due to changes in the microfluidics and buffer pumps along with the opening and/or closing of the pneumatic valves connected to the integrated fluidic cartridge (IFC). This typically introduces a delay in the start of the injection and alignment of multiple injections over numerous binding cycles can be challenging. Such misaligned data is difficult to analyze and even after the double reference subtraction procedure yields large spikes at the beginning and end of each injection. Although the SCK data presented in Fig. 5 was generated using five individual injections, we did not observe any significant disturbance in the binding curves, especially at the beginning and end of each injection. Moreover, it was encouraging to observe reproducible and overlapping SCK binding curves without any spikes in between each injection, which is a striking feature of the SCK data presented in Fig. 5. These findings further validate the SCK method developed on Biacore 4000 for the kinetic analysis of mAb-antigen interactions. Designing the SCK assay on the Biacore 4000 however, was found to be more complex than anticipated. Multiple experiments were performed by selecting different combinations of currently available injection commands and the method provided in the materials and methods generated the best quality SCK data.

As opposed to the five antigen concentrations utilized in the pre-defined injection command on different Biacore biosensors, our SCK method uses only four antigen concentrations. We have also generated the SCK data using the traditional five antigen concentrations for representative mAb-antigen interactions characterized in the study and the measured binding kinetic values were comparable to those reported in Table 2 (data not shown). However, we found that a two-fold serial dilution of antigen was required to generate SCK data using five antigen concentrations. No noticeable antigen binding was observed at the lowest concentration for the SCK data using five antigen concentrations, serially diluted by three-fold, and hence could not be included for data analysis. Since the 8-mAb/16-mAb capture kinetic and PK data were generated by diluting antigen by three-fold, we wanted to be consistent with the dilution factor while generating the SCK data. The use of four antigen concentrations; serially diluted by three-fold was found to be sufficient to provide adequate spread of antigen concentrations required to reliably measure binding rate constants and hence all the SCK assays were performed using just four antigen concentrations.

Similar to the SICK and one-shot kinetic assay formats [3,10], performing binding assays using a similar density of the mAb across different flow cells is a pre-requisite of the PK assay. This can be accomplished by either capturing the mAb using the covalently immobilized capture molecule or by directly immobilizing the mAb onto the sensor surface. We observed that the microfluidics of the Biacore 4000 uniformly delivers samples across all four flow cells and less than 4% variability in the immobilization density of the anti-human Fc or anti-mouse Fc capture antibody is typically observed across different flow cells. This enabled capturing similar levels of mAb across four flow cells using the same mAb sample. The PK assay developed in this work takes advantage of this uniform sample delivery and thus allowed global fitting of the binding curves generated across different flow cells; further reducing assay development time for the PK assay.

Although our data demonstrate the reliability of the PK assay designed on the Biacore 4000, its success will depend on the kinetic diversity of the mAbs under investigation. As opposed to the ProteOn XPR36, and MASS-1 biosensors, which provide simultaneous injection of up to six and eight antigen concentrations, respectively [3,10], the four flow cells of Biacore 4000 allows the use of only four antigen concentrations and hence can be challenging to characterize mAb-antigen interactions for a mAb panel with widely varying $k_a$, $k_d$ and $K_D$ values. This restricted use of antigen concentration series should be considered as a limitation before

designing PK assay on the Biacore 4000.

The results presented in this study demonstrate that the 16-mAb capture, SCK, and PK assays designed to characterize mAb-antigen interactions provide multiple advantages over the traditional 8-mAb capture kinetic assay format. All three kinetic assays generated reproducible and reliable binding kinetic values and further increased the throughput of the Biacore 4000 biosensor. We believe that these kinetic assays can be easily implemented in the mAb characterization workflow and will assist in accelerating the kinetic analysis of mAb-antigen interactions for rapid selection of lead therapeutic mAbs.

## Disclosure summary

All the authors are employees and shareholders of Regeneron Pharmaceuticals.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.04.020.

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