TECH NOTE

Analysis of antibody-antigen binding kinetics on Alto Digital SPR using mouse Fc-specific capture reagents

Overview

Mouse Fc-specific capture reagents can be used to capture mouse antibodies or Fc-tagged proteins directionally. These capture reagents offer an optimal orientation for analyte binding and enable users to capture ligands from crude samples or matrix compositions that may be incompatible with direct coupling methods. Mouse capture protocols have been optimized for Alto™, Nicoya's Digital surface plasmon resonance™ (SPR) platform, allowing users to reduce experiment design time by offering pre-developed assay configurations. Protocols are included for capturing mouse IgG1, IgG2A, and IgG2B using Nano-CaptureLigands® from Proteintech and the AffiniPure® Goat Anti-Mouse IgG, Fcγ fragment specific from Jackson ImmunoResearch Laboratories, Inc.

Introduction

Mouse antibodies are widely used in studies involving mouse models, which are essential for understanding biological mechanisms and evaluating therapeutic targets in human diseases. SPR is a gold-standard technique for characterizing and validating antibody interactions. Whether screening crude hybridoma isolates for hits or measuring binding affinity of purified antibodies, a robust and consistent immobilization strategy is required for high-quality SPR analysis.

Commercially available reagents that specifically bind the Fc region of mouse antibodies—such as anti-mouse antibodies and VHH fragments—can be used for directional capture of mouse Fc-containing proteins in SPR assays. These mouse Fc-specific capture reagents are covalently attached to SPR carboxyl sensors via amine coupling, creating a surface coated with the desired capture reagent. Mouse Fc-containing proteins can then be reversibly captured on the sensor surface, enabling the collection of reproducible kinetic data.

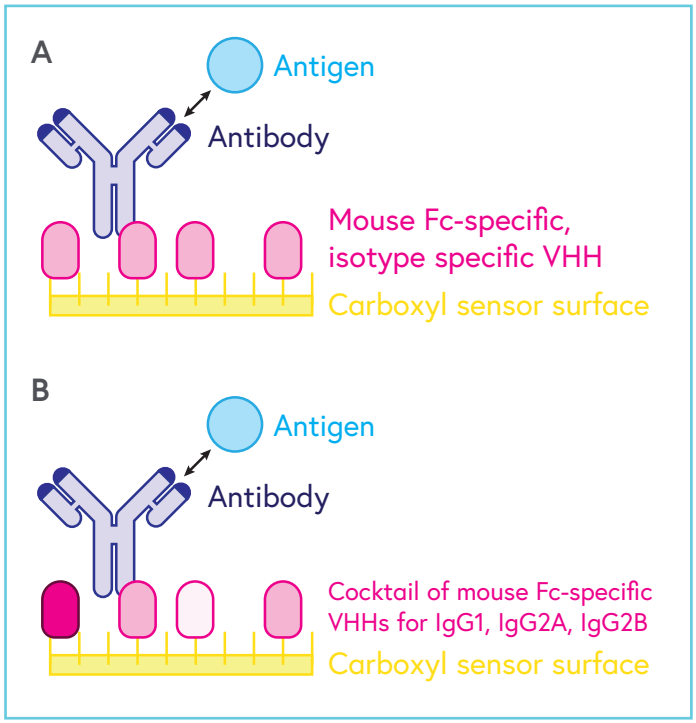

Figure 1: *Schematic representation of the VHH capture systems used in this study. A) A single mouse Fc-specific VHH from Proteintech is immobilized on the sensor to capture antibodies with a specific mouse isotype. B) A cocktail of three mouse Fc-specific VHHs is immobilized on the surface to capture antibodies of isotypes IgG1, IgG2A, or IgG2B.*

Developed in collaboration with Proteintech, a leading manufacturer of antibodies, proteins, nanobodies, and immunoassays, this technical note describes protocols for using mouse Fc-specific Nano-CaptureLigands from Proteintech on the Alto Digital SPR platform. Nano-CaptureLigands are VHHs (nanobodies) whose small size and single-chain structure make them ideal for SPR

applications, offering improved performance compared to conventional IgG capture reagents. Mouse IgG1-, IgG2A-, and IgG2B-specific Nano-CaptureLigands were tested individually (Figure 1A) and as a combined cocktail to support applications where the isotype is unknown (Figure 1B). As a model system, binding kinetics were measured for the interaction between the SARS-CoV2 spike protein and mouse antibodies of isotypes IgG1, IgG2A, and IgG2B.

Additionally, the AffiniPure® Goat Anti-Mouse IgG from Jackson ImmunoResearch Laboratories, Inc., an isotype agnostic capture reagent, was assessed for compatibility with the Alto system for applications involving mouse IgG2C and IgG3 isotypes (Figure 2). While binding data for these isotypes are not shown here, the reagents' performance is demonstrated for IgG1, IgG2A, and IgG2B. All capture reagents described are compatible with both kinetic analysis and high-throughput screening workflows on Alto.

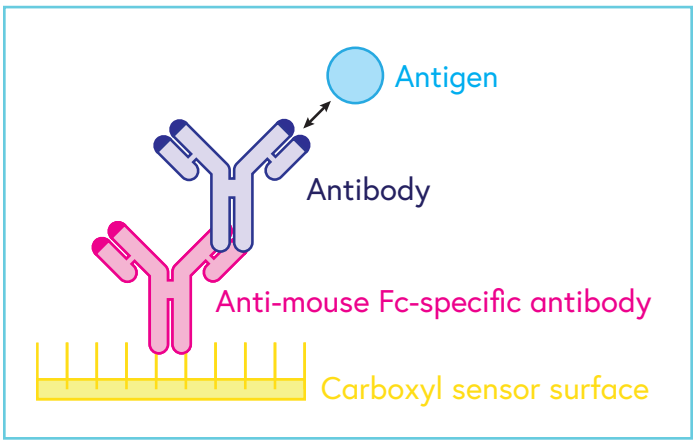

Figure 2: *Schematic representation of the anti-mouse IgG capture system used in this study. The anti-mouse IgG binds mouse antibody ligands via the Fc region of all isotypes.*

Materials & Equipment

- Alto 16-Channel Instrument with Nicosystem Pro Software (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)
- Running Buffer: PBS-TE + BSA (0.1% Tween 20, 5 mM EDTA, 0.5% BSA), pH 7.4
- Immobilization Buffer: 10 mM MES, pH 6.0 (ALTO-R-IMB-6.0)
- Regeneration Solution: 10 mM Glycine-HCl, pH 1.5 (ALTO-R-GLYHCL-1.5)
- Alto Carboxyl Surfacing Kit: Cleaning, normalization, activation solutions (ALTO-R-CBX-SURF)
- Capture reagent: ChromoTek Nano-CaptureLigand® mouse IgG1, Fc-specific VHH, biotinylated: Proteintech cat# smsG1B-1
- Capture reagent: ChromoTek Nano-CaptureLigand® mouse IgG2A, Fc-specific VHH, biotinylated: Proteintech cat# smsG2aB-1
- Capture reagent: ChromoTek Nano-CaptureLigand® mouse IgG2B, Fc-specific VHH, biotinylated: Proteintech cat# smsG2bB-1
- Capture reagent: AffiniPure® Goat Anti-Mouse IgG, Fcγ fragment specific, Jackson ImmunoResearch Laboratories, Inc: Cat # 115-005-071
- Mouse IgG1 ligand: SARS-CoV-2 (2019-nCoV) Spike Antibody, Mouse Mab, Sino Biological: Cat # 40591-MM42
- Mouse IgG2B ligand: SARS-CoV-2 (2019-nCoV) Spike Neutralizing Antibody, Mouse Mab, Sino Biological: Cat # 40592-MM57
- Mouse IgG2Aligand: SARS-CoV-2 Spike S1 Subunit Antibody, R&D Systems: Cat # MAB105805
- Analyte: SARS-CoV-2 (2019-nCoV) Spike Protein (S1 Subunit, Fc Tag), Sino Biological: Cat # 40591-V02H

Assay optimization tips

- Optimizing buffer conditions to capture the IgG ligand is not necessary; it is recommended (but not required) that the ligand be in the running buffer.
- For best performance, it is recommended (but not required) that samples are purified. However, crude matrices are compatible with Alto.
- For most applications, the user should choose the lowest ligand density that still provides an analyte binding signal. This prevents multi-phasic behavior and other artefacts that can come from over saturating the sensor surface. It is recommended that the user choose a ligand density that gives a maximum analyte response (R_{max}) between 50-150 RU to give a sufficient signal-to-noise ratio to resolve kinetics while avoiding steric hindrance or mass transfer effects.
- Mouse capture reagents are compatible with a wide range of running buffers and buffer additives. Recommended buffers:
  - PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
  - HBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-HBST)
  - TBS-T* (0.1% Tween 20), pH 7.4 (ALTO-R-TBST)

** TBS-T should not be used in auxiliary buffer well for capture molecule immobilization.*

Method

Experimental setup

The experimental setup was completed remotely on Alto's Nicosystem™ user portal, followed by run initiation on the instrument.

1. From a laptop, the experiment was designed and saved in the Nicosystem.
2. On the instrument, the designed method was selected to launch Alto's on-screen setup guide.
3. An Alto 16-Channel Carboxyl Cartridge was placed in the instrument, and samples were loaded into the cartridge following the experiment setup guide.
4. The experiment was initiated on the Alto device by selecting "Run Method".

Alto assay protocol

The assay protocol below describes conditions used for an assay with mouse IgG1, Fc-specific VHH as the capture molecule, anti-spike mouse IgG1 antibody as ligand and spike S1 as analyte. This protocol, including all steps and contact times, was identical for all interactions tested in this study. Capture molecule, ligand, and analyte concentrations were optimized independently for each interaction. Capture molecule and ligand concentrations are listed in Table 1 and analyte concentrations are listed in Table 2.

The following steps were completed automatically by Alto with no operator supervision:

1. Carboxyl sensors were normalized with normalization solutions.
2. Carboxyl sensors were cleaned with 10 mM HCl for 60 s.
3. Carboxyl sensors were activated with 200 mM EDC/NHS for 600 s.
4. The mouse IgG1 Fc-specific VHH capture reagent diluted in 10 mM MES, pH 6.0 was immobilized onto all sensors for 600 s (see Table 1 for full list of capture molecule concentrations used).
5. All sensors were blocked with the 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.
6. All sensors were conditioned for 60 s with 10 mM glycine-HCl, pH 1.5.
7. 1 µg/mL samples of anti-spike mouse IgG1 antibody in the running buffer were introduced to each even-numbered active sensor for 300 s (see Table 1 for full list of ligand concentrations used).
8. Alto executed five automated SARS-CoV-2 spike Protein, S1 Subunit (spike S1) serial dilutions on the cartridge. Each sample was diluted from 600 nM stock, producing 200 nM, 66.7 nM, 22.2 nM, 7.41 nM, and 2.47 nM solutions in the running buffer (see Table 2 for spike concentration for each interaction).
9. The lowest spike S1 concentration was exposed to each sensor for 180 s, followed in tandem by the four other analyte concentrations, lowest to highest. After the final analyte concentration, the analyte dissociated in the running buffer for 900 s, followed by a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5. This constitutes a full single-cycle kinetics (SCK) round.
10. After regeneration, steps 8 and 9 were repeated up to five more times for a maximum of 6 binding cycles.

Alto data analysis

1. The test was opened under the analysis tab in the Nicosystem user portal.
2. The "Build Capture Surface" tab was opened to assess VHH immobilization levels across 4 lanes of the cartridge to ensure sufficient and/or optimal levels.
3. The "Raw data" tab was opened to ensure that the ligand capture levels and regeneration are sufficient and/or optimal.
4. The "Capture Kinetics" tab was opened and a 1:1 Langmuir binding model was automatically applied to the data.
5. Processing tools were used as required.
6. Final images and .CSV files were downloaded.

Results & Discussion

Several mouse Fc-specific capture reagents were evaluated for use as a capture surface in SPR assays. For each experiment completed, the capture reagents were immobilized onto both the reference and active sensors of the cartridge. Figure 3 shows immobilization sensorgrams for each of the capture reagents tested and Table 1

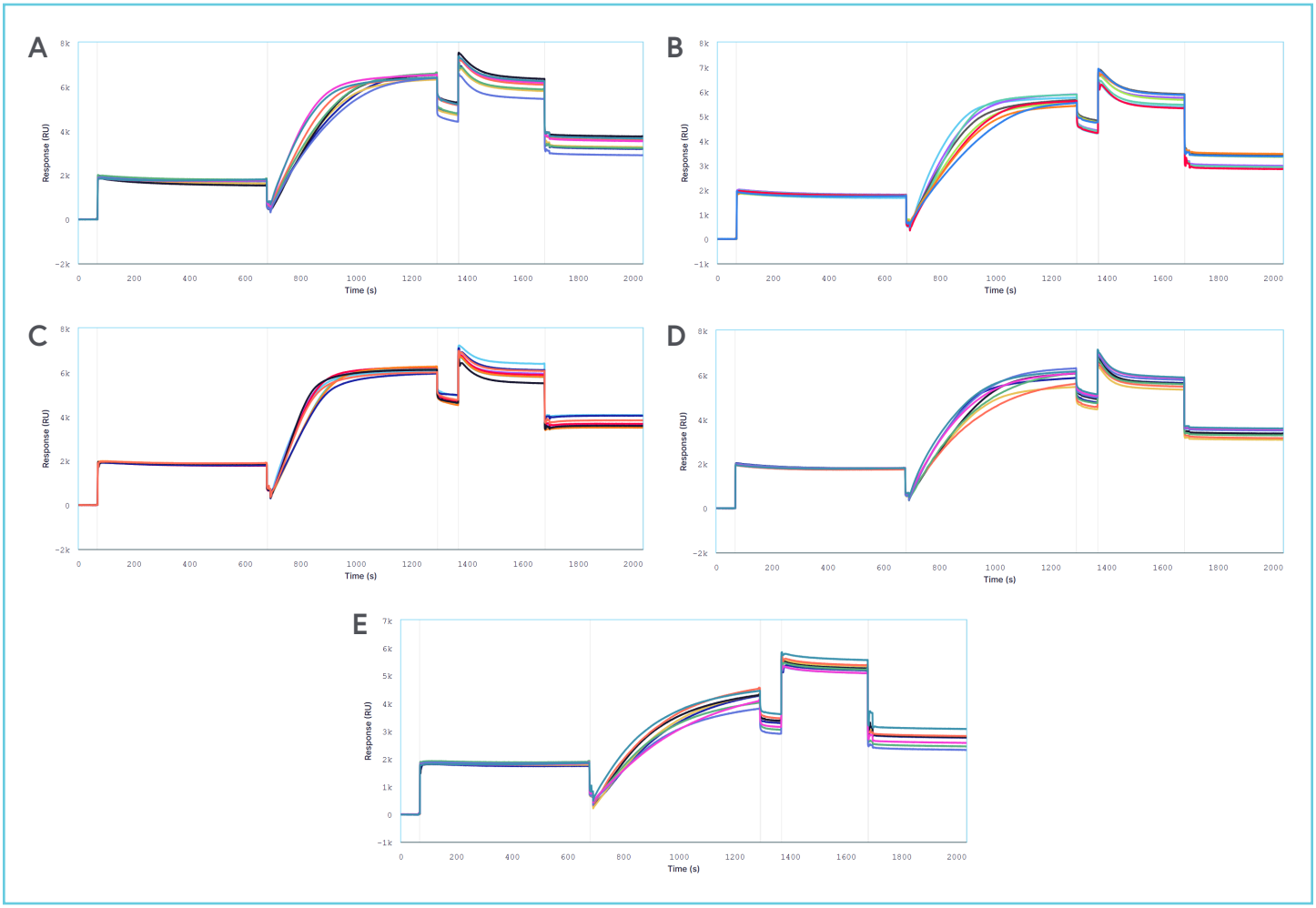

Figure 3: *Immobilization on the sensor surface of A) 5 µg/mL mouse IgG1 Fc-specific VHH, B) 5 µg/mL mouse IgG2A Fc-specific VHH, C) 5 µg/mL mouse IgG2B Fc-specific VHH, D) cocktail of 2.5 µg/mL each of mouse IgG1, IgG2A and IgG2B Fc-specific VHHs, and E) 5 µg/mL anti-mouse IgG antibody. The sensor surface is activated with 200 mM EDC/NHS from Nicoya's Carboxyl Surfacing Kit, followed by immobilization of the capture reagent and blocking of sensors with 1 M ethanolamine. Images were generated in the Nicosystem software.*

Capture reagent	Capture reagent concentration	Immobilization of capture reagent (RU)	Antibody ligand	Antibody concentration	Antibody capture level (RU)
Mouse IgG1 specific VHH	5 µg/mL	3203 ± 259	Mouse IgG1	1 µg/mL	45.5 ± 9.4
Mouse IgG2A specific VHH	5 µg/mL	3770 ± 184	Mouse IgG2A	0.5 µg/mL	61.2 ± 18.2
Mouse IgG2B specific VHH	5 µg/mL	3442 ± 292	Mouse IgG2B	1 µg/mL	40.1 ± 14.9
Mouse VHH cocktail	2.5 µg/mL of each VHH for IgG1, IgG2A, and IgG2B	3447 ± 165	Mouse IgG1	0.25 µg/mL	71.7 ± 18.5
			Mouse IgG2A	0.5 µg/mL	45.2 ± 13.5
			Mouse IgG2B	0.5 µg/mL	126.6 ± 14.2
Anti-mouse IgG antibody	5 µg/mL	2787 ± 231	Mouse IgG1	1 µg/mL	41.3 ± 9.8
			Mouse IgG2A	0.5 µg/mL	34.4 ± 4.6
			Mouse IgG2B	0.5 µg/mL	74.6 ± 10.7

Table 1: *Capture reagent immobilization and antibody ligand capture levels for tested anti-mouse capture reagents.*

summarizes the average capture reagent immobilization level and ligand capture for each interaction analyzed. Immobilization levels are approximately 3000 RU with %CV below 10% for all capture reagents, highlighting the reproducibility of Alto's sensor surfaces. Average ligand capture levels for each interaction ranged from 34.4-126.6 RU, which is relatively low for SPR data but produces a measurable analyte response between 50-150 RU. Low ligand density is beneficial for reducing steric hindrance and mass transport effects, resulting in the highest quality kinetics possible.

To test the ability of each mouse Fc-specific capture reagent to be used as a capture surface for kinetics determination, SCK assays using SARS-CoV2 spike S1 antigen binding to captured anti-spike antibodies were conducted. Figure 4A-I shows raw sensorgrams for active and reference sensors during ligand capture, analyte binding, and regeneration. Some bulk shift is observed for the spike S1 analyte due to high trehalose content of the stock. Complete regeneration of the ligands and bound analyte was achieved with 10 mM glycine-HCl, pH 1.5 demonstrating the reusability of the sensor surface for multiple rounds of binding. The antibody ligands show minimal dissociation following binding to the capture surfaces, highlighting the affinity of the capture reagents.

Average kinetic constants and binding affinity were calculated from a minimum of 8 replicates. A representative sensorgram for each interaction is shown in Figure 5A-I. The data were fit to a Langmuir 1:1 binding model analyzed in the Nicosystem software. Kinetic parameters for data obtained using all mouse Fc-specific capture reagents are reported in Table 2 and demonstrate excellent reproducibility across all replicates. As expected, the affinity measurements are consistent when the same ligand-analyte pair is tested across different mouse Fc-specific capture surfaces. Similarly, the k_a and k_d measurements are consistent across different mouse Fc-specific capture surfaces. The variability in k_a is slightly higher than the variability in k_d, which is likely due to subtle differences in capture molecule orientation and packing on the sensor surface.

Optimal SPR capture reagents are highly stable molecules that bind ligands with high affinity and specificity. While many antibodies and antibody fragments meet these criteria, selecting high-quality reagents remains a challenge for SPR users due to variability in performance. This technical note presents several capture reagents that bind

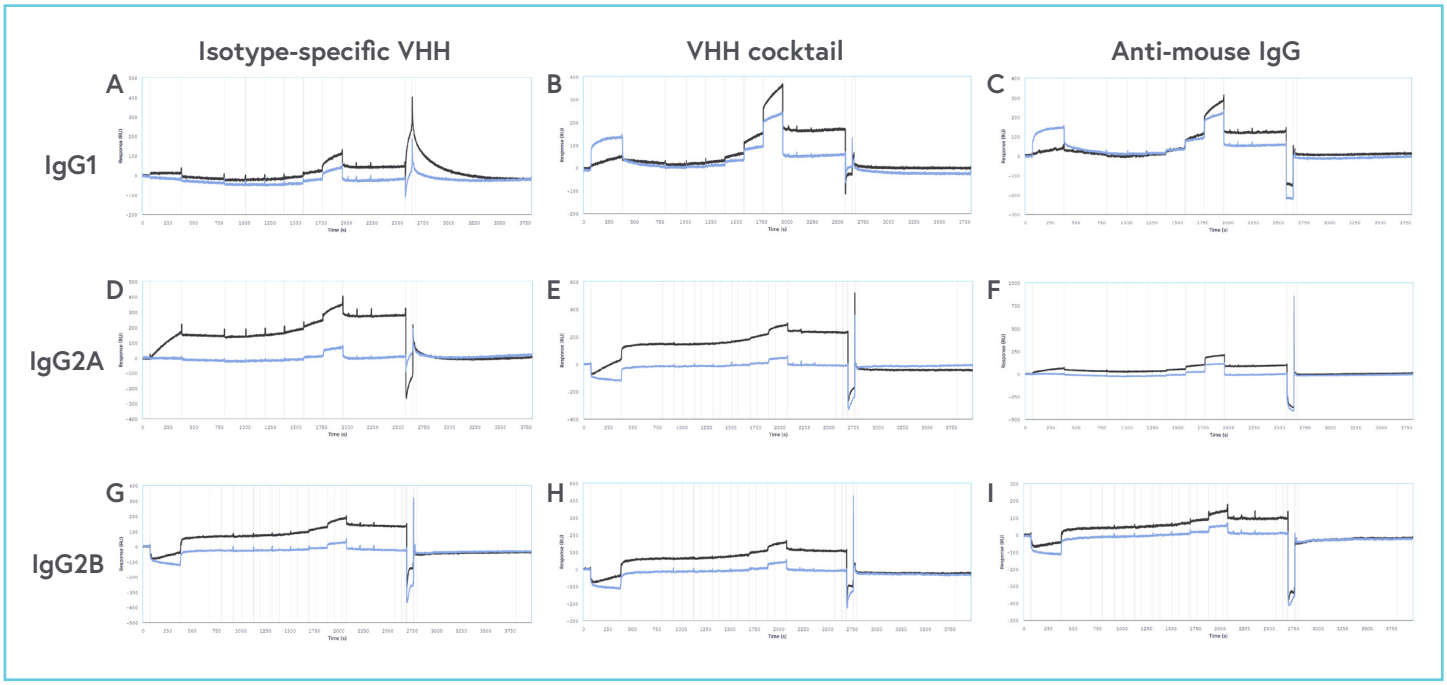

Figure 4: *Reference (light blue trace) and active sensor (black trace) binding data showing capture of mouse IgGs by the mouse Fc-specific capture reagents, binding of spike S1 to the capture reagents and regeneration with glycine-HCl, pH 1.5. Spike S1 was titrated with five 3x serial dilutions in tandem from low to high. The interactions analyzed were: A) mouse IgG1 captured by mouse IgG1 VHH, B) mouse IgG1 captured by VHH cocktail, C) mouse IgG1 captured by anti-mouse IgG antibody, D) mouse IgG2A captured by mouse IgG2A VHH, E) mouse IgG2A captured by VHH cocktail, F) mouse IgG2A captured by anti-mouse IgG antibody, G) mouse IgG2B captured by mouse IgG2B VHH, H) mouse IgG2A captured by VHH cocktail, I) mouse IgG2A captured by anti-mouse IgG antibody.*

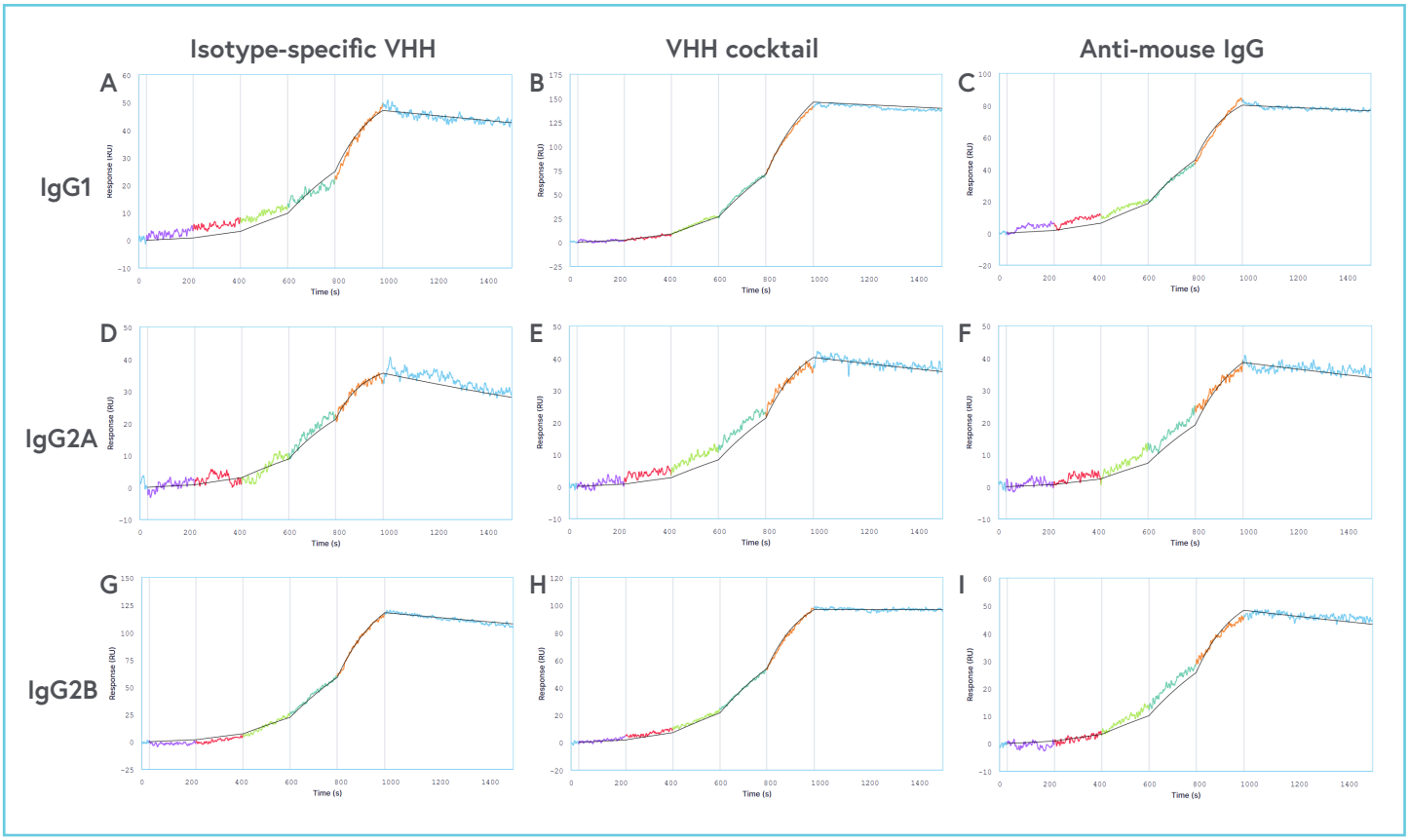

Figure 5: *Multi-cycle kinetics of spike S1 (analyte) binding to mouse IgGs (ligand) captured by mouse Fc-specific capture reagents on Alto. Spike S1 was titrated with five 3x serial dilutions in tandem from low to high. Spike S1 concentrations vary between interactions; stock concentrations are listed in Table 2. Black curves represent the Langmuir 1:1 binding fit model generated by the Nicosystem software. The interactions analyzed were: A) mouse IgG1 captured by mouse IgG1 VHH, B) mouse IgG1 captured by VHH cocktail, C) mouse IgG1 captured by anti-mouse IgG antibody, D) mouse IgG2A captured by mouse IgG2A VHH, E) mouse IgG2A captured by VHH cocktail, F) mouse IgG2A captured by anti-mouse IgG antibody, G) mouse IgG2B captured by mouse IgG2B VHH, H) mouse IgG2A captured by VHH cocktail, I) mouse IgG2A captured by anti-mouse IgG antibody.*

Spike antibody isotype	Capture reagent	Spike stock concentration	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
IgG1	Mouse IgG1 VHH	600 nM	$7.55 \times 10^4 \pm 1.65 \times 10^4$	$2.49 \times 10^{-4} \pm 5.52 \times 10^{-5}$	6.93 ± 1.34
	Mouse VHH cocktail	900 nM	$1.78 \times 10^4 \pm 2.33 \times 10^3$	$1.40 \times 10^{-4} \pm 7.33 \times 10^{-5}$	6.78 ± 2.62
	Anti-mouse IgG	900 nM	$1.76 \times 10^4 \pm 6.36 \times 10^3$	$1.39 \times 10^{-4} \pm 7.19 \times 10^{-5}$	8.55 ± 3.83
IgG2A	Mouse IgG2A VHH	200 nM	$1.41 \times 10^5 \pm 1.16 \times 10^4$	$2.65 \times 10^{-4} \pm 1.39 \times 10^{-5}$	1.94 ± 1.13
	Mouse VHH cocktail	200 nM	$1.29 \times 10^5 \pm 4.86 \times 10^4$	$1.98 \times 10^{-4} \pm 3.89 \times 10^{-5}$	1.71 ± 0.95
	Anti-mouse IgG	200 nM	$9.92 \times 10^4 \pm 1.45 \times 10^4$	$2.11 \times 10^{-4} \pm 7.67 \times 10^{-5}$	2.20 ± 0.94
IgG2B	Mouse IgG2B VHH	600 nM	$6.57 \times 10^4 \pm 1.26 \times 10^4$	$1.54 \times 10^{-4} \pm 3.31 \times 10^{-5}$	2.41 ± 0.66
	Mouse VHH cocktail	200 nM	$1.26 \times 10^5 \pm 2.97 \times 10^4$	$1.04 \times 10^{-4} \pm 2.34 \times 10^{-5}$	0.829 ± 0.099
	Anti-mouse IgG	600 nM	$7.55 \times 10^4 \pm 1.65 \times 10^4$	$2.49 \times 10^{-4} \pm 5.52 \times 10^{-5}$	6.19 ± 0.52

Table 2: *Kinetic parameters measured for spike S1 binding analyte binding to anti-spike mouse IgG1, IgG2A and IgG2b captured by mouse-Fc specific capture reagents.*

the Fc region of various mouse antibody isotypes. When the isotype of the antibody is known, the use of isotype-specific VHH capture reagents from Proteintech is recommended. VHH molecules offer several advantages: their small size enables efficient surface packing, improves the limit of detection (LOD) compared to full-length antibodies, and reduces the likelihood of steric interference with ligand-analyte interactions. VHHs also exhibit greater thermal and chemical stability than conventional antibodies[1], which is particularly beneficial for multi-cycle kinetic assays that require frequent sensor regeneration. These advantages may not be readily apparent in the current study due to the similar sizes of the ligand and analyte (150 kDa vs. 102 kDa) and the use of single-cycle kinetic protocols, which subject the surface to fewer regeneration cycles. When the antibody isotype is unknown, users can choose between a cocktail of isotype-specific VHHs or an isotype-agnostic anti-mouse antibody (from Jackson ImmunoResearch Laboratories, Inc.). The VHH cocktail retains the benefits of single-domain antibodies but cannot be used for capturing IgG2c and IgG3 isotypes. For Alto users seeking guidance on selecting the most suitable capture reagent for their experiments, Nicoya's Customer Success team is available to help.

Regeneration

Surface regeneration in SPR involves the removal of non-covalently bound reagents from the sensor and restoring it for subsequent binding events. The choice of regeneration solution must be optimized for each specific interaction.

Antibodies and VHH fragments are highly stable molecules and are typically compatible with many regeneration solutions. An ideal regeneration solution is strong enough to completely remove the ligand and analyte, but not harsh enough to damage the capture surface. As shown in Figure 4, each regeneration step results in a sharp change in signal that returns it to the same baseline position as before the ligand capture step. This is indicative of a successful regeneration. Glycine-HCl, pH 1.5 (ALTO-R-GLYHCl-1.5) is the recommended regeneration solution for all capture reagents used in this study.

Several regeneration solutions are available in Nicoya's Regeneration Optimization Kit (ALTO-R-REGEN-OPT), for those who wish to find the best reagent that suits their binding interaction.

References

1. Muyldermans S. Nanobodies: Natural Single-Domain Antibodies. Annu. Rev. Biochem. 2013; 82: 775-797.