TECH NOTE

Analysis of antibody-antigen binding kinetics on Alto digital SPR using a Protein A capture reagent

Overview

Nicoya's Protein A Capture Kit allows users to capture antibodies or Fc-tagged proteins directionally, offering an optimal orientation for analyte binding. This method also enables users to capture ligands from crude samples or matrix compositions, which may be incompatible with direct coupling methods. This technical note describes protocols for use of the Protein A Capture Kit with Alto™, Nicoya's digital surface plasmon resonance™ (SPR) instrument, to measure the binding kinetics of Influenza A H5N1 hemagglutinin (HA) antigen and a H5N1 HA antibody. Alto's pre-optimized Protein A Capture Kit and protocols allow users to reduce experiment design time by offering a pre-developed assay configuration.

Introduction

Protein A is a surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*. It is used routinely in biochemical research for the capture and purification of antibodies and antibody-based molecules.[1] It is composed of five homologous immunoglobulin-binding domains that fold into a three-helix bundle. Each domain is able to bind one immunoglobulin molecule, specifically the heavy chain within the Fc region of most immunoglobulin molecules.[2] These properties are leveraged in SPR for capturing many species and isotypes of immunoglobulins, particularly IgGs (antibodies) and antibody-based molecules such as Fc-tagged proteins.

Alto's Protein A Capture Kit is used by amine coupling the Protein A to carboxyl sensors to create a Protein A coated surface. The Protein A Capture Kit is compatible with capture kinetics and capture screening assays. For example, when using Alto's capture kinetics protocol, users may capture their IgG molecules onto Protein A and measure binding between their analytes and the captured IgG (Figure 1).

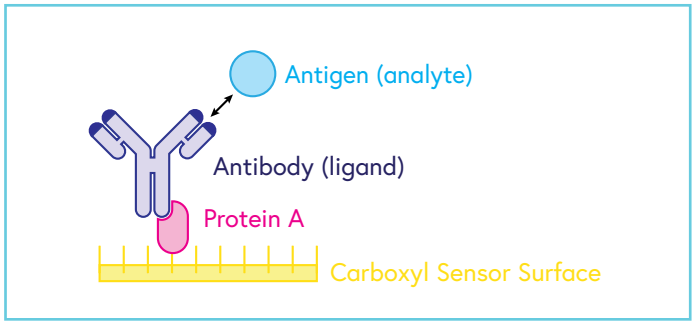

Figure 1: *Schematic representation of an assay using the Protein A Capture Kit.*

The affinity of Protein A varies for different classes & subclasses of immunoglobulins. Table 1 summarizes the affinity of Nicoya's Protein A for a few of the most common species and isotypes of IgG.

* *Please enquire about the binding strength of other species not listed in Table 1.*

Binding Strength	IgG species & isotypes
++++ (strong)	Human total IgG, IgG1, IgG2 & IgG4, Mouse IgG 2a, Rabbit total IgG
+++ (medium-strong)	Mouse IgG 2b
++ (medium)	Mouse total IgG & IgG3
+ (weak)	Mouse IgG1
- (non-binder)	Human IgG3

Table 1: *Binding strength of common IgG species & isotypes*

Materials

Materials included in the Protein A Capture Kit (ALTO-R-PROA-KIT):

- 10x Protein A Aliquots (ALTO-R-PROTEINA)
- 1.5 mL 10 mM Sodium Acetate pH 5.0 (ALTO-R-IMB-5.0)

Other equipment & materials used:

- Alto 16-Channel Instrument with Nicosystem Pro Software (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)
- Running Buffer: PBS-TE (0.1% Tween 20, 5mM EDTA), pH 7.4 (ALTO-R-PBST)
- Alto Carboxyl Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Regeneration Buffer: 10mM Glycine-HCl pH 1.5 (ALTO-R-GLYHCL-1.5)
- Influenza A H5N1 Hemagglutinin/HA rabbit antibody (Sino Biological Cat# 86001-RM02)
- Recombinant Influenza A H5N1 Hemagglutinin/HA Protein (SIno Biological Cat# 11048-V08H1)

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 the analyte be purified.
- For most applications, the user should choose the lowest ligand density that still provides an analyte binding signal to prevent multiphasic behavior and other artefacts from oversaturating the sensor surface. It is recommended that the user choose a ligand density that gives a maximum analyte response (Rmax) between 50-150 RU to give a sufficient signal-to-noise ratio to resolve kinetics while avoiding steric hindrance or mass transfer effects. As shown in Figure 2, the two sensorgrams with lower ligand concentrations have better kinetic fits and a more resolvable off-rate.
- The Protein A Capture Kit is 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 not to be used in auxiliary buffer for capture molecule immobilization.*

- Protein A aliquots are single-use. Do not freeze-thaw or combine freeze-thawed aliquots with fresh aliquots.

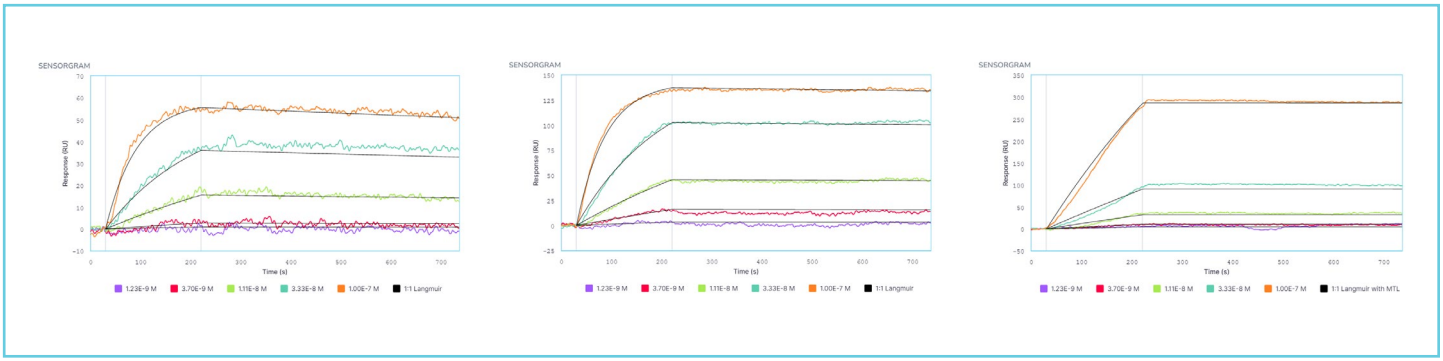

Figure 2: *Multi-cycle kinetics of H5N1 HA (analyte) binding to captured RM02 (ligand) on Alto. The analyte was titrated from 1.23 nM to 100 nM. Black curves are the Langmuir 1:1 binding fit model analyzed in the Nicosystem Software. The left figure has ligand concentration of 0.1 µg/mL, the middle figure has ligand concentration of 0.25 µg/mL and the right figure has ligand concentration of 1 µg/mL.*

Experiment Setup

The experimental setup was remotely completed on Alto's User Portal, followed by run initiation on the instrument:

1. From a laptop, a capture kinetics or capture screening experiment was designed and saved in the User Portal.
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".

Sample Preparation

Diagrammatic representation for the preparation of 5 µg/mL Protein A aliquots is shown in Figure 3.

1. Retrieve the Protein A aliquot (0.4 mg/mL, 4 µL) and allow it to come to room temperature prior to dilution.
2. Add 316 µL of 10 mM Sodium Acetate buffer pH 5.0 to the 4 µL Protein A aliquot to create the final aliquot solution (5 µg/mL, 320 µL).
3. Mix the solution by pipetting up and down.
4. Immediately load 3 µL of 5 µg/mL Protein A solution into Wells C1 -> C8 of the cartridge for capture kinetics or load 65 µL of 5 µg/mL Protein A solution into Well R6 for capture screening. Dispose of any leftover solution.

Assay Protocol

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

1. Carboxyl sensors were normalized with normalization solutions.
2. Carboxyl sensors were primed with 10 mM HCl for 60 s.
3. Carboxyl sensors were activated with 200 mM EDC/NHS for 600 s.
4. The Protein A from the Protein A Capture Kit diluted in 10 mM Sodium Acetate pH 5.0 was immobilized onto all sensors for 600 s.
5. All sensors were blocked with the 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.

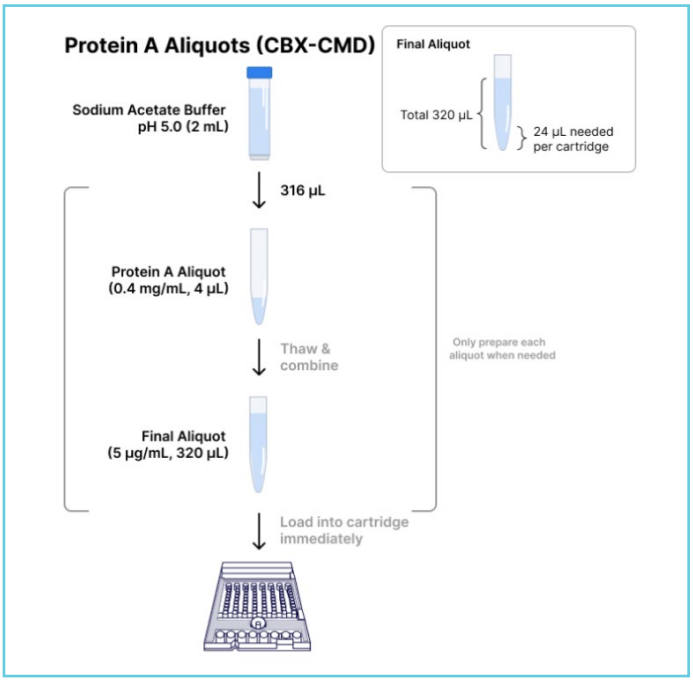

Figure 3: *Dilution instructions for the Protein A Capture Kit for CMD cartridges.*

6. All sensors were conditioned for 60 s with 10 mM Gly-HCl pH 1.5.
7. 0.1 µg/mL samples of Anti-H5N1 HA antibody RM02 in the running buffer were introduced to each even-numbered active sensor for 300 s.
8. Alto executed five automated H5N1 HA serial dilutions on the cartridge. Each sample was diluted from 300 nM stock, producing 1.23 nM, 3.70 nM, 11.1 nM, 33.3 nM, and 100 nM solutions in the running buffer.
9. The lowest H5N1 concentration was exposed to each sensor for 180 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 10 mM glycine-HCl pH 1.5.
10. Step 9 was repeated for the remaining four H5N1 HA analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round.

Data Analysis

1. Open the test under the analysis tab in the portal.
2. Check the build surface tab and assess Protein A immobilization levels across all 16 sensors in the cartridge to ensure sufficient and/or optimal levels.

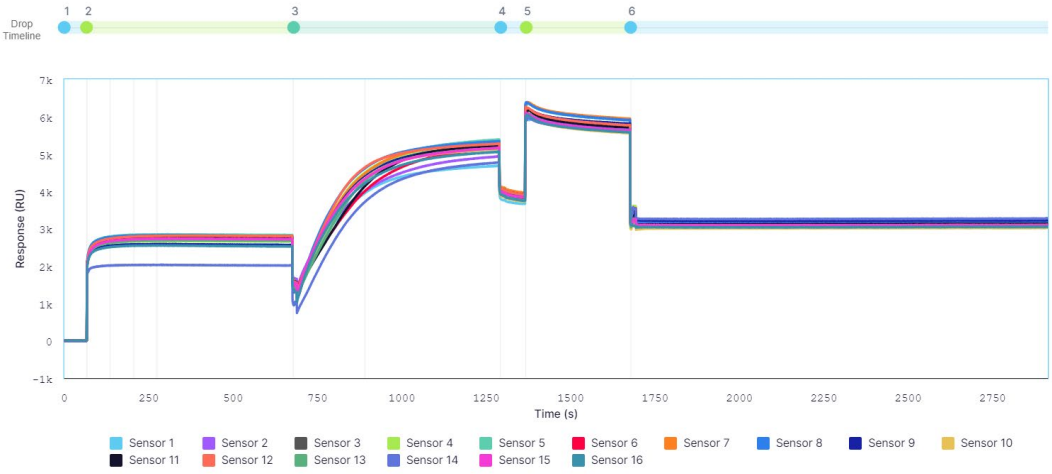

Figure 4: *Activation of the 16 channels with 200 mM EDC/NHS from Nicoya's Surfacing Kit, followed by immobilization of 5 µg/mL of Protein A in 10 mM sodium acetate pH 5.0 and blocking of sensors with 1 M Ethanolamine. The image was generated by the Nicosystem Software.*

3. Check the 'Round 1' tab under 'Raw data' to ensure that the ligand capture levels and regeneration are sufficient and/or optimal.
4. Click the Capture Kinetics tab. A 1:1 Langmuir binding model will be automatically applied to the data.
5. Use processing tools as required.
6. Download final images and/or .CSV files.

Results & Discussion

The capability of Protein A was evaluated for use as a capture surface in SPR assays. For each experiment completed, the Protein A was immobilized onto both the reference and response sensors of the cartridge. Figure 4 shows an immobilization overlay for one of the cartridges used as part of this study, with an average immobilization level of 3138 RU for the Protein A. Table 2 summarizes the average immobilization level and error for this step as well as the ligand capture step.

To test the ability of Protein A to be used as a capture surface for kinetics determination, MCK assays using H5N1 HA antigen binding to a captured human IgG (RM02) were performed. The recapture of the human IgG ligand was consistent across all five analyte concentrations and regenerations for a full round as shown in Figure 5. This figure also demonstrates the low susceptibility of NSB to the Protein A surface as evidenced in the lack of response in the reference channel for the H5N1 HA analyte. Complete 100% regeneration of the ligand and bound analyte was achieved with 10 mM Glycine-HCl, pH 1.5 demonstrating the reusability of the sensor surface. The ligand, RM02, shows minimal dissociation following binding to the Protein A surface, highlighting the rigidity of this capture system.

Kinetic values for MCK kinetics were calculated based on the sensorgrams obtained on one cartridge with Alto, tested across 2 lanes and 2 rounds. A representative example of a sensorgram is shown in Figure 6. Only lanes using 0.1 µg/mL RM02 ligand are used for these calculations. The data were fit to a Langmuir 1:1 binding model analyzed in the Nicosystem Software.

	5 µg/mL Protein A	0.1 µg/mL RM02	0.25 µg/mL RM02	1 µg/mL RM02
Average	3138 RU	49.7 RU	126.4 RU	597.9 RU
Std Dev	75.3 RU	12.3 RU	24.6 RU	132.0 RU

Table 2: *Immobilization level and Std. Dev. of Protein A and capture of RM02 across entire cartridge*

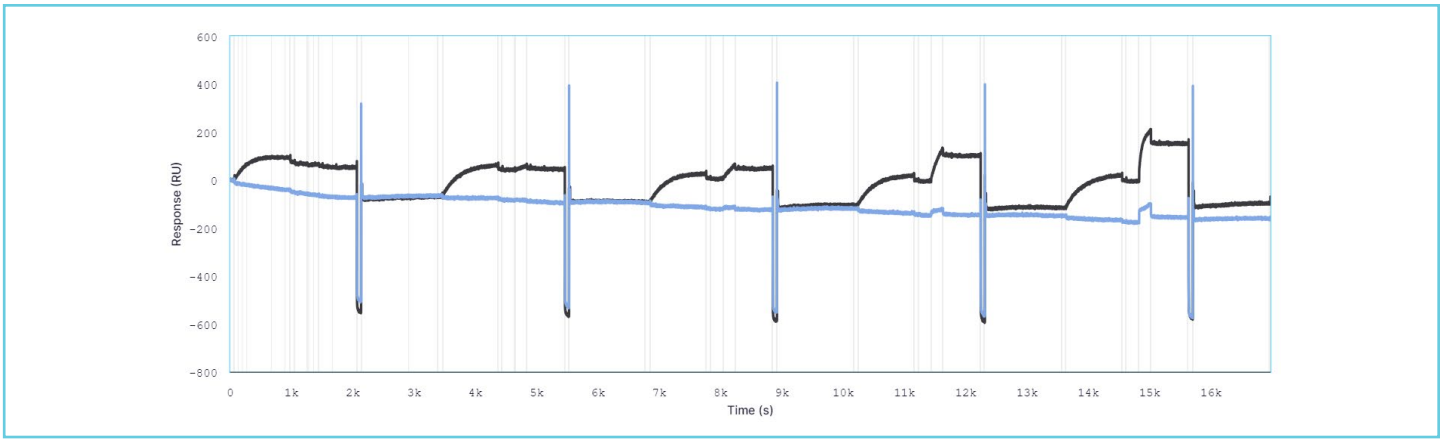

Figure 5: *Reference (blue trace) and active channel (black trace) binding data showing binding of H5N1 HA to captured RM02 for each analyte concentration. Following the dissociation of each analyte concentration, regeneration was achieved with glycine-HCl pH 1.5.*

Kinetic parameters for data obtained using the Anti-HIS Ab capture molecule are reported in Table 3 and demonstrated excellent reproducibility across all channels and rounds. From the kinetic analysis, association and dissociation rate constants when Anti-HIS Ab was used as the capture molecule in an MCK format were determined to be $1.27 \times 10^5 \pm 2.12 \times 10^3$ $M^{-1}s^{-1}$ and $1.18 \times 10^{-4} \pm 4.24 \times 10^{-6}$ s^{-1}, respectively, resulting in a KD of 929 ± 19.1 pM.

Regeneration

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

The Protein A Capture Kit is 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 5, each regeneration step results in a sharp change in signal that returns it to the same baseline position as before the analyte association step. This is indicative of a successful regeneration. Glycine-HCl, pH 1.5 (ALTO-R-GLYHCl-1.5) is the recommended regeneration solution for Protein A-IgG binding.

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

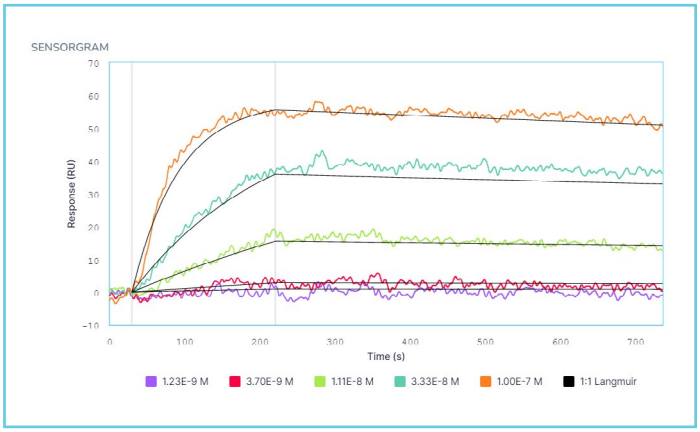

Figure 6: *Multi-cycle kinetics of H5N1 HA (analyte) binding to captured RM02 (ligand) on Alto. The analyte was titrated from 1.23 nM to 100 nM. Black curves are the Langmuir 1:1 binding fit model analyzed in the Nicosystem Software.*

	k_a	k_d	K_D
Average	1.27×10^5 M^{-1} s^{-1}	1.18×10^{-4} s^{-1}	929 pM
Std Dev	2.12×10^3 M^{-1} s^{-1}	4.24×10^{-6} s^{-1}	19.1 pM
% CV	1.68 %	3.60 %	2.04 %

Table 3: *Binding kinetic rate constants for H5N1 HA and antibody on Alto*

References

1. Goding, J.W. Use of staphylococcal protein A as an immunological reagent. Journal of Immunological Methods. April 1978. 20: 241-253.
2. Graille M., Stura E.A., Corper A.L., Sutton B.J., Taussig M.J., Charbonnier J.B., Silverman G.J. Crystal structure of a Staphylococcus aureus protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. Proceedings of the National Academy of Sciences of the United States of America. May 2000. 97 (10): 5399–404.