TECH NOTE

Analysis of antibody-antigen binding kinetics on Alto Digital SPR using a Human/rabbit IgG VHH capture reagent

Overview

Nicoya's Human/rabbit IgG VHH Capture Kit uses the Nano-CaptureLigand® human IgG/rabbit IgG, Fc-specific VHH (nanobody) from Proteintech to capture human and rabbit antibodies or hu/rb-Fc tagged proteins directionally. This capture strategy offers an optimal orientation for analyte binding and enables users to capture ligands from crude samples or matrix compositions, which may be incompatible with direct coupling methods. The Human/rabbit IgG VHH Capture Kit and protocols are pre-optimized for Alto™, Nicoya's Digital surface plasmon resonance™ (SPR) platform, allowing users to reduce experiment design time by offering a pre-developed assay configuration.

Introduction

A VHH (or nanobody) is a 12-15 kDa antibody fragment consisting of only a single variable antibody domain. The VHH fragment stands for Variable domain of Heavy-chain-only antibodies which are derived from the unique structure of antibodies found in camelid species[1]. The small size and single-chain structure of VHH fragments provide an SPR capture surface with higher overall performance than conventional IgG antibodies.

Nicoya's Human/rabbit IgG VHH Capture Kit is used by amine coupling the Fc-specific VHH (Nano-CaptureLigand® human IgG/rabbit IgG) to carboxyl sensors to create a human/rabbit IgG VHH coated surface. The Human/rabbit IgG VHH Capture Kit is compatible with capture kinetics and capture screening assays on Alto. For example, when using Alto's capture kinetics protocol, users may immobilize their human or rabbit IgG via Proteintech's anti-human/rabbit IgG VHH (Nano-CaptureLigand®) and measure binding between their analytes and the captured IgG molecule (Figure 1).

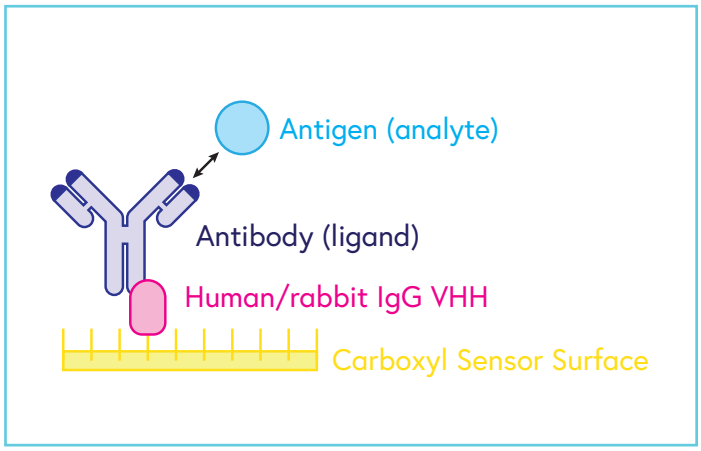

Figure 1: *Schematic representation of an assay using the Human/rabbit IgG VHH Capture Kit.*

In collaboration with Proteintech, a leading manufacturer of antibodies, proteins, nanobodies, and immunoassays, this technical note describes protocols for use of the Human/rabbit IgG Capture Kit with Alto, to measure the binding kinetics of Influenza A H5N1 hemagglutinin (HA) antigen and a H5N1 HA antibody.

Materials

Materials included in the Human/rabbit IgG VHH Capture Kit (ALTO-R-VHH-HR-KIT):

- ChromoTek Nano-CaptureLigand® human IgG/rabbit IgG, Fc-specific VHH, biotinylated: CAT# shurbGB
- 10 mM MES, pH 6.0

Other materials:

- 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, 5 mM EDTA), pH 7.4 (ALTO-R-PBST)
- Regeneration Solution: 10 mM Glycine-HCl, pH 1.5 (ALTO-R-GLYHCL-1.5)
- Alto Carboxyl Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Recombinant Influenza A H5N1 Hemagglutinin/HA Protein: Sino Biological, CAT#: 11048-V08H1
- Influenza A Virus Hemagglutinin/HA Antibody, Rabbit mAb: Sino Biological, CAT#: 86001-RM02

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 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.
- The Human/rabbit IgG VHH 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.*

- Human/rabbit IgG VHH aliquots are single-use. Do not freeze-thaw or combine freeze-thawed aliquots with fresh aliquots.

Experiment 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 Alto, 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 2.5 µg/mL Human/rabbit IgG VHH aliquots is shown in Figure 2.

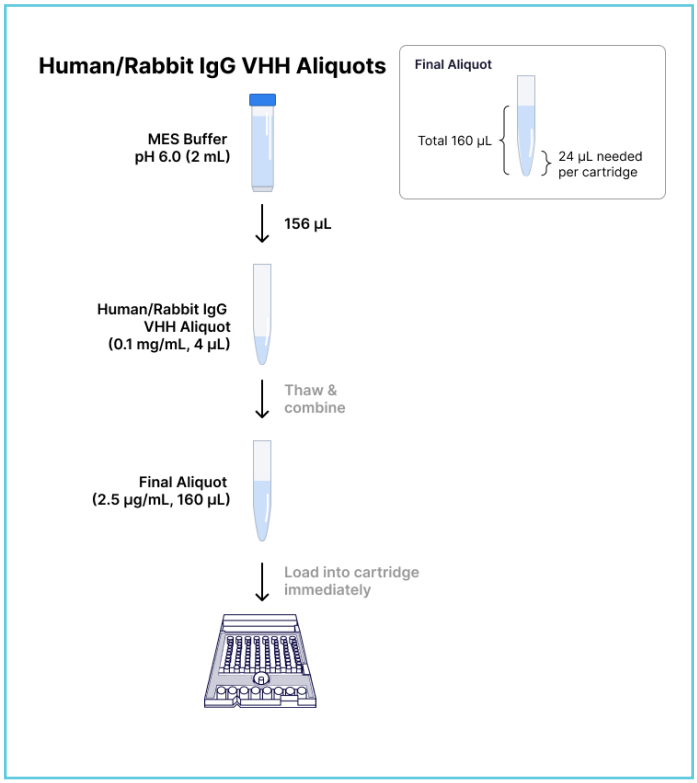

Figure 2: *Dilution instructions for the Human/rabbit IgG VHH Capture Kit for CMD cartridges.*

Preparation of 2.5 µg/mL Human/rabbit IgG VHH aliquots:

1. Retrieve the Human/rabbit IgG VHH aliquot (0.1 mg/mL, 4 µL) and allow it to come to room temperature prior to dilution.
2. Add 156 µL of 10 mM MES buffer pH 6.0 to the 4 µL Human/rabbit IgG VHH aliquot to create the final aliquot solution (2.5 µg/mL, 160 µL).
3. Mix the solution by pipetting up and down.
4. Immediately load 3 µL of 2.5 µg/mL Human/rabbit IgG VHH solution into Wells C1 to C8 of the cartridge for capture kinetics or load 65 µL of 2.5 µg/mL Human/rabbit IgG VHH 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 Human/rabbit IgG VHH from the Human/rabbit IgG VHH Capture Kit diluted in 10 mM MES, pH 6.0 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.
6. All sensors were conditioned for 60 s with 10 mM glycine-HCl, pH 1.5.
7. 0.2 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 150 nM stock, producing 0.617 nM, 1.85 nM, 5.55 nM, 16.7 nM, and 50 nM solutions in the running buffer.
9. The lowest H5N1 HA concentration was exposed to each sensor for 300 s, followed by dissociation in the running buffer for 900 s, and a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
10. Steps 7 and 9 were repeated for the remaining four H5N1 HA analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round.

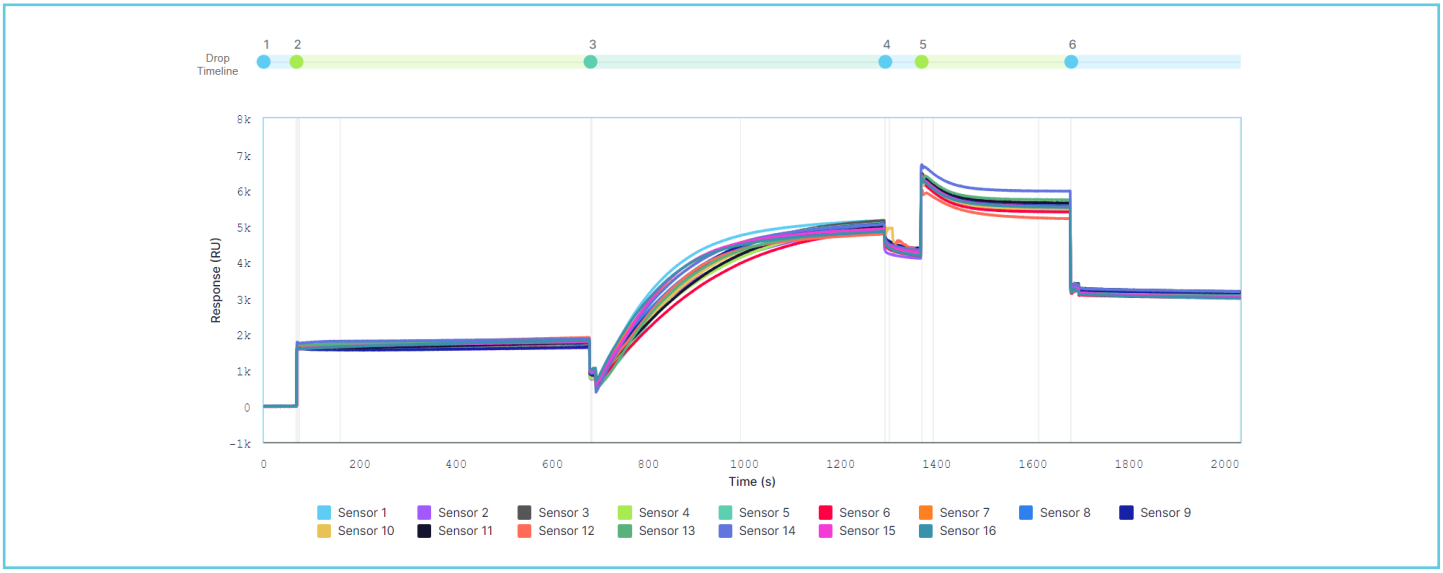

Figure 3: *Activation of the 16 channels with 200 mM EDC/NHS from Nicoya's Carboxyl Surfacing Kit, followed by immobilization of 2.5 µg/mL of Human/rabbit IgG VHH in 10 mM MES, pH 6.0 and blocking of sensors with 1 M ethanolamine. The image was generated in the Nicosystem software.*

Data analysis

1. The test was opened under the analysis tab in the Nicosystem User Portal.
2. The build surface tab was opened to assess VHH immobilization levels across all 8 lanes in the cartridge to ensure sufficient and/or optimal levels.
3. The 'Round 1' tab under 'Raw data' 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

The capability of Human/rabbit IgG VHH was evaluated for use as a capture surface in SPR assays. For each experiment completed, the Human/rabbit IgG VHH was immobilized onto both the reference and active sensors of the cartridge. Figure 3 shows an immobilization overlay for one of the cartridges used as part of this study, with an average immobilization level of 3185 RU for the Human/rabbit IgG VHH. Table 1 summarizes the average immobilization level and standard deviation for this step as well as the ligand capture step, which averaged 141.3 RU.

	2.5 µg/mL Human/ rabbit IgG VHH (RU)	0.2 µg/mL RM02 capture (RU)
Average	3185	141.3
Std Dev	63.8	14.0

Table 1: *Human/rabbit IgG VHH immobilization and capture of RM02 across entire cartridge.*

To test the ability of Human/rabbit IgG VHH to be used as a capture surface for kinetics determination, MCK assays using H5N1 HA antigen binding to a captured rabbit IgG (RM02) were performed. The recapture of the human IgG ligand was consistent across all five analyte concentrations and regenerations, with a sample cycle shown in Figure 4. This figure also demonstrates the low susceptibility of non-specific binding (NSB) to the Human/rabbit IgG VHH surface as evidenced by the minimal response in the reference channel for the H5N1 HA analyte. Complete 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 Human/rabbit IgG VHH 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 rounds. A representative example of a sensorgram is shown in Figure 5. The data were fit to a Langmuir 1:1 binding model analyzed in the Nicosystem software.

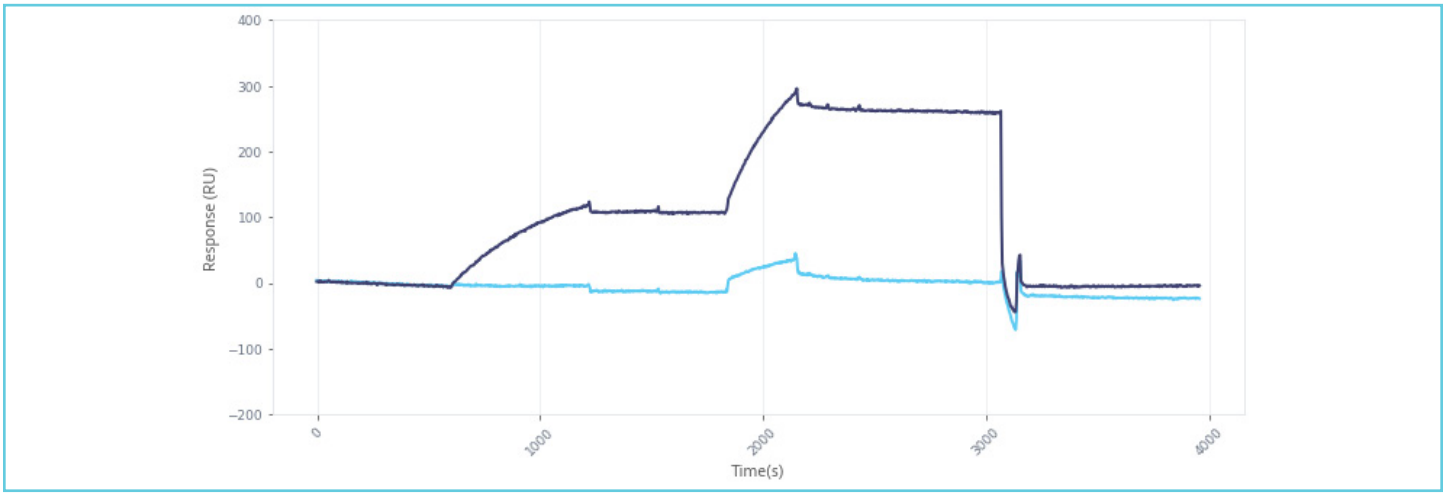

Figure 4: *Reference (light blue trace) and active channel (dark blue trace) binding data showing binding of 50 nM H5N1 HA to captured RM02. Following the dissociation of the analyte concentration, regeneration was achieved with glycine-HCl, pH 1.5.*

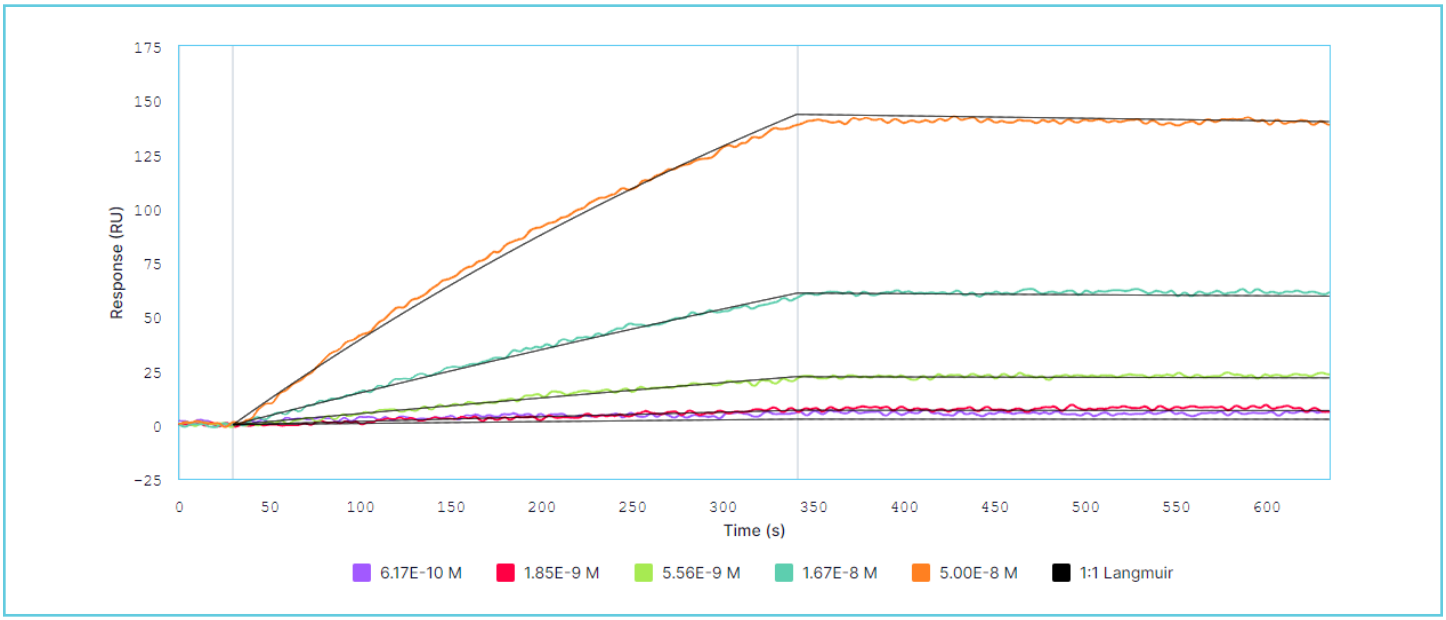

Figure 5: *Multi-cycle kinetics of H5N1 HA (analyte) binding to captured RM02 (ligand) on Alto. The analyte was titrated from 0.617 nM to 50 nM. Black curves represent the Langmuir 1:1 binding fit model generated by the Nicosystem software.*

k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (pM)
$3.15 \times 10^4 \pm 6.68 \times 10^3$	$3.10 \times 10^{-5} \pm 8.02 \times 10^{-6}$	984 ± 155

Table 2: *Kinetic parameters measured for H5N1 HA binding to antibody RM02 captured by the Human/rabbit IgG VHH capture molecule.*

Kinetic parameters for data obtained using the Human/rabbit IgG VHH capture molecule are reported in Table 2 and demonstrated excellent reproducibility across all channels and rounds. From the kinetic analysis, when Human/rabbit IgG VHH Ab was used as the capture molecule in an MCK format, association and dissociation rate constants (k_a and k_d) were determined to be $3.15 \times 10^4 \pm 6.63 \times 10^3$ $M^{-1}s^{-1}$ and $3.10 \times 10^{-5} \pm 8.02 \times 10^{-6}$ s^{-1}, respectively, resulting in a K_D of 984 ± 155 pM.

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.

The Human/rabbit IgG VHH 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 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 Human/rabbit IgG VHH - IgG binding.

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. Harmsen, M.M., De Haard, H.J. Properties, production, and applications of camelid single-domain antibody fragments. November 2007. Applied Microbiology and Biotechnology. 77 (1): 13–22.