TECH NOTE

Characterization and Quantitation of AAVs on Alto Digital SPR using AAVX & AAV9 capture surfaces

Overview

Nicoya's AAVX & AAV9 Capture Kits use AAV-specific VHH molecules to capture AAVs on the sensor surface. This capture strategy offers an optimal orientation for analyte binding and enables users to capture AAVs from crude samples, which may be incompatible with direct coupling methods. The AAVX & AAV9 Capture Kits 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

Adeno-associated viruses (AAVs) are small, non-enveloped DNA viruses widely used as vectors for gene therapy due to their low immunogenicity, ability to infect dividing and non-dividing cells, and potential for long-term gene expression. As the development of AAV-based therapeutics accelerates, there is a growing demand for robust analytical techniques that can provide detailed insights into titer, vector quality, stability, and interactions with biological targets.

Surface plasmon resonance (SPR) is a label-free, real-time technique that enables precise measurement of molecular interactions, including those involving large, multivalent particles such as AAVs. By immobilizing AAVs, receptor fragments, or antibodies onto a sensor surface, researchers can assess the binding kinetics, specificity, and affinity of AAV capsids under near-physiological conditions.

Nicoya's Alto Digital SPR platform offers an accessible and automated approach to AAV characterization, enabling researchers to measure high-quality screening, kinetic, and quantitation data with minimal sample consumption. Full kinetic analysis of AAVs can be done with just 2 µL of sample and titer measurement can be done with only 3 µL resulting in 100x less sample consumption than a conventional SPR or BLI instrument. See Table 1 for a full summary of performance metrics.

In this technical note, we demonstrate protocols for using Alto to evaluate AAV binding interactions using Anti-AAV capture surfaces. These surfaces enable capture of AAV serotypes 1-8 & rh10 using an AAVX capture surface and the capture of AAV9 with an AAV9 capture surface (Figure 1). The immobilization and regeneration conditions are pre-optimized for these capture surfaces, reducing the time and effort needed to acquire high-quality binding data and accurate titers. With high specificity, ease of use, and broad serotype compatibility, these capture kits give researchers a powerful tool to accelerate AAV development and address key challenges in gene therapy.

Alto Performance	
Dynamic Range	Approximately 1.00 E+10 to 1.00 E+12 GC/mL
Limit of Detection (LOD)	≤8.89 E+09 GC/mL
Throughput	Titer up to 40 unique samples or kinetic analysis of up to 48 unique samples in a single assay
Sample Consumption	4 uL for standards, 3 uL for unknowns, 2 uL for kinetics

Table 1: *Performance metrics of Alto for AAV characterization*

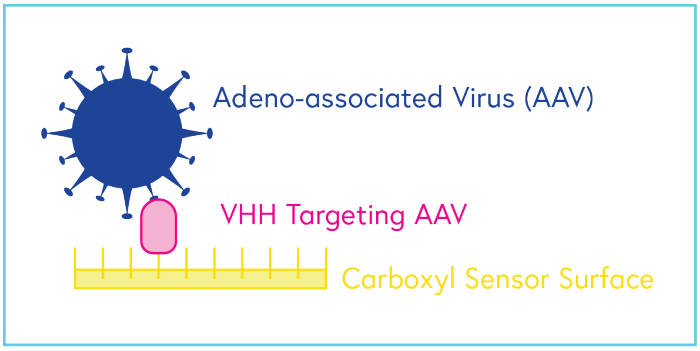

Figure 1: *Schematic representation of an assay using an AAV-specific capture surface*

Materials

- Alto 16-Channel Instrument with Nicosystem Pro Software (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4
- 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)
- Streptavidin Kit (ALTO-R-STV-KIT)
- AAVX Capture Kit: Anti-AAVX VHH and 10 mM Sodium Acetate, pH 4.5 (ALTO-R-AAVX-KIT)
- AAV9 Capture Kit: Anti-AAV9 VHH and 10 mM Sodium Acetate, pH 5.5 (ALTO-R-AAV9-KIT)
- AAV2 empty capsids: ProGen, CAT#: 66V020
- AAV8 empty capsids: ProGen, CAT#: 66V080
- AAV9 empty capsids: ProGen, CAT#: 66V090

Assay Optimization Tips

- For best performance, it is recommended (but not required) that samples are purified. Crude matrices are compatible with Alto.
- For quantitation assays, it is recommended to match the assay buffer/matrix of the standards as closely as possible to the unknowns.
- Quantitation standards require a volume of 4 µL and unknowns require a volume of 3 µL. It is recommended to use a high standard concentration in order to take advantage of the large dynamic range offered by the quantitation assay (ten 3-fold dilutions).
- For kinetics 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 any steric hindrance or mass transfer effects.
- The Anti-AAVX and Anti-AAV9 molecules are biotinylated and therefore compatible with streptavidin protocols. For kinetics applications, it is recommended for the Anti-AAVX or Anti-AAV9 to be immobilized by amine coupling to the sensor surface. For quantitation applications, either direct immobilization or a streptavidin surface may be used.
- AAV 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)

* *When using a TBS-T running buffer, use PBS-T or HBS-T in auxiliary buffer for capture molecule immobilization.*

Methods

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 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".

Assay protocols

The following assay protocols describe the optimal conditions used for capture of AAVs by their respective capture surfaces.

Dilution instructions for the capture reagents are shown in Figure 2.

Capture molecule and ligand concentrations with their associated immobilization levels are listed in Table 2.

AAV2 and AAV9 Capture with AAVX & AAV9 Capture Kits

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. 5 ug/mL Anti-AAVX VHH diluted in 10 mM Sodium Acetate pH 4.5 or 5 ug/mL Anti-AAV9 VHH diluted in 10 mM Sodium Acetate pH 5.5 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. AAV2 or AAV9 diluted in the running buffer was introduced to each even-numbered active sensor for 1800 s (see Table 2 for the full list of ligand concentrations used).

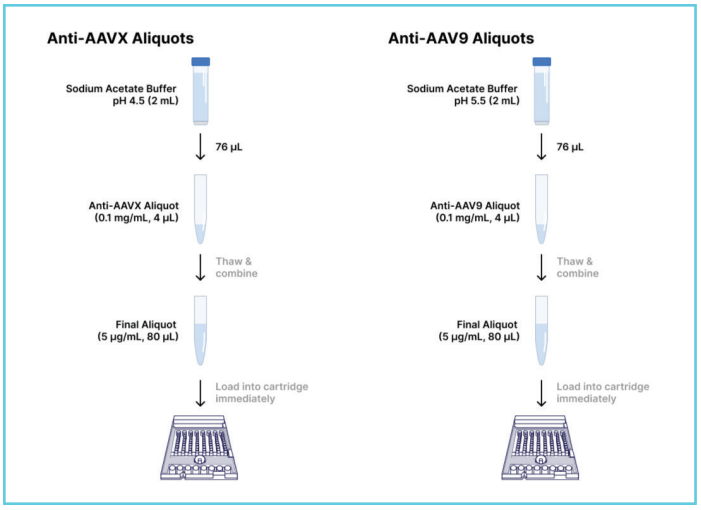

Figure 2: *Schematic representation of an assay using an AAV-specific capture surface*

AAV8 Quantitation

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. 10 ug/mL his-streptavidin diluted in 10 mM Sodium Acetate pH 5.5 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. 5 ug/mL Anti-AAVX VHH diluted in PBST was captured onto all even sensors for 900 s.
7. All sensors were conditioned for 60 s with 10 mM glycine-HCl, pH 1.5.

Capture surface	Immobilization of capture reagent (RU)	Serotype of ligand	AAV concentration (GC/mL)	AAV capture (RU)
AAVX-specific VHH	1983 ± 93	AAV2	*1.00 E+11*	*48 ± 14*
			4.00 E+11	*806 ± 51*
			8.00 E+11	*1508 ± 133*
AAV9-specific VHH	2147 ± 108	AAV9	*1.00 E+11*	*213 ± 39*
			4.00 E+11	*804 ± 73*
			8.00 E+11	*1325 ± 147*

Table 2: *Capture reagent immobilization and ligand capture response*

8. Alto automatically executed ten threefold serial dilutions of AAV8 on the cartridge, starting from a 7.12 E+12 GC/mL stock.
9. The lowest AAV8 standard concentration was exposed to all sensors for 1200 s and allowed to dissociate for 465 s. The sensor surface was regenerated using 10 mM glycine-HCl, pH 1.5 for 60 s.
10. Step 8 was repeated for the remaining nine AAV concentrations to generate a response curve for each of the ten serial dilutions.
11. 8.79 E+11 GC/mL AAV8 was allowed to associate with the sensor surface for 1200 s, followed by a dissociation period of 465 s. A 10 mM glycine-HCl, pH 1.5 was applied for 60 s to regenerate the sensor surface.
12. Step 11 was repeated with 8.79 E+10 GC/mL AAV8.

Data analysis for quantitation

1. The test was opened under the analysis tab in the Nicosystem user portal.
2. The 'Build streptavidin surface' tab was opened to assess the immobilization of streptavidin to ensure sufficient and/or optimal levels.
3. The 'Immobilize Biotin Ligand' tab was opened to assess the immobilization of the biotinylated Anti-AAVX to ensure sufficient and/or optimal levels.
4. The 'Quantitation - Standards' tab was opened to assess the quality of the response curves for the standards (sufficient signal, good regeneration, etc.)
5. The 'Quantitation - Unknowns' tab was opened to view the plotted standard curves and calculated unknown concentrations automatically determined by the Nicosystem.
6. Final images and .CSV files were downloaded.

Results & Discussion

The capability of Anti-AAVX and Anti-AAV9 VHHs to serve as capture surfaces in SPR assays on the Alto platform was evaluated. Figure 3A shows the immobilization of the Anti-AAVX VHH, with an average immobilization level of 1983 RU. Figure 3B shows the immobilization of the Anti-AAV9 VHH, with an average immobilization level of 2147 RU. Table 2 summarizes the average binding response and standard deviations for both capture molecule immobilization and subsequent AAV capture. AAVs were measured at concentrations of 1.00 E+11, 4.00 E+11, and 8.00 E+11 GC/mL. They were loaded onto a single cartridge with n=16 for each concentration. Sensorgrams overlaying all replicates from each test, showing the capture of AAV2 and AAV9 on the Anti-AAVX and Anti-AAV9 surfaces, respectively, are shown in Figure 4. Both capture molecules displayed reproducible and consistent capture responses of their respective AAVs as demonstrated from the ligand capture levels and respective standard deviations listed in Table 2.

Raw sensorgrams generated by Alto's Nicosystem showing the capture of AAV2 and AAV9 are displayed in Figure 5. The Anti-AAVX and Anti-AAV9 functionalized surfaces capture their ligands with very high affinity, which is evident from the stable baselines following the association phase. Minimal dissociation of the AAV2 and AAV9 from the capture surfaces is observed, indicating a robust capture surface for analyte analysis. Gly-HCl pH 1.5 serves as an effective regeneration solution to fully remove bound AAV from the capture surfaces.

The AAVX capture surface was also used to demonstrate titer measurement of AAV8 unknowns using Alto's quantitation module. In this assay, the biotinylated Anti-AAVX was immobilized on a streptavidin surface with an average immobilization level of 1146 ± 43 RU, highlighting its compatibility with this strategy (Figure 6). Nicosystem's analysis software automatically processes curves in each lane to generate an interactive standard curve consisting of 10 three-fold dilutions (Figure 7A). Average response values at each concentration are taken from an interval in the association phase using report point tools. The average response value of this interval is used to generate the standard curve where a 5PL logistic equation is used to fit the data as shown in Figure 7B. In this test, the calculated standard concentrations deviated a maximum of 3.2% from the actual concentration when the AAV titer was >1.00 E+11 GC/mL and averaged less than 10% across the entire concentration range. The standard of 8.89 E+09 GC/mL had a detectable response of >10 RU at the end of the association phase, indicating an LOD of ≤8.89 E+09 GC/mL. Full analysis is included in Table 3.

Using the calculated model, unknown concentrations of AAV8 are plotted against the standard curve to determine their concentration. The Nicosystem analysis software generates a summarized table of data, which includes the calculated AAV8 standard and unknown concentrations, shown here in Table 3. The calculated concentrations of the 'unknown' samples had a maximum difference of 11.2%

from the actual concentration at the lower end of the linear range.

This data demonstrates that AAVX capture surfaces can be used to accurately quantify AAV titers of < 1.00 E+11 GC/mL on Alto using only 3 µL of sample, up to 100x less sample than traditional SPR & BLI systems. Up to 40 unique AAV titers can be quantified in a single quantitation assay with minimal hands on time thanks to the automated dilutions performed by Alto.

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.

An ideal regeneration solution is strong enough to fully 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 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 AAVX and AAV9 capture surfaces.

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.

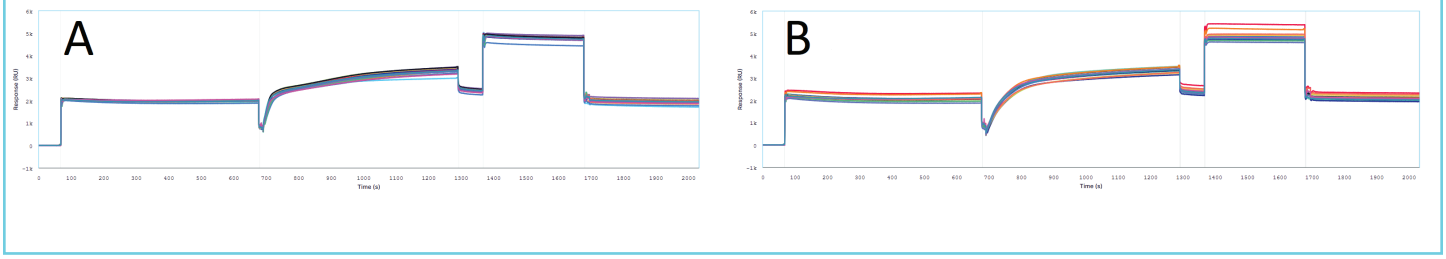

Figure 3: *Immobilization of AAVX and AAV9-specific VHHs. Capture reagent immobilization on sensor surface using A) 5 ug/mL of Anti-AAVX VHH in sodium acetate pH 4.5 and B) 5 ug/mL of Anti-AAV9 VHH in sodium acetate pH 5.5. Both sensor surfaces were exposed to 200 mM EDC/NHS from Nicoya's Carboxyl Surfacing Kit, followed by immobilization of capture reagent and blocking of sensors using 1 M ethanolamine. Image was generated in the Nicosystem software.*

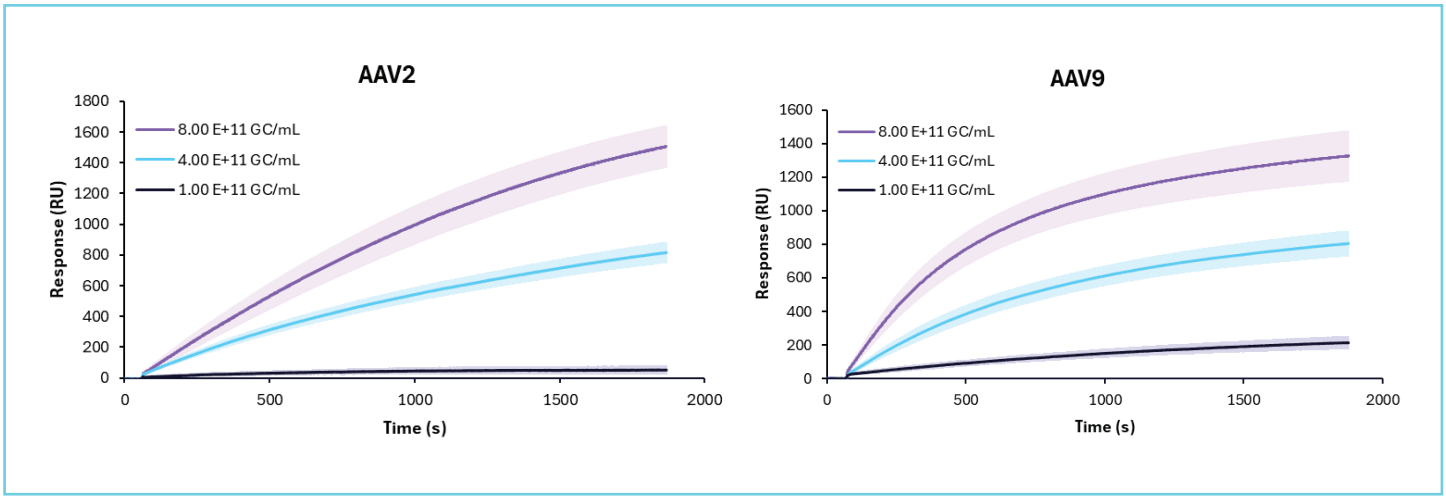

Figure 4: *AAV2 capture on AAVX capture surface (left) and AAV9 capture on AAV9 capture surface (right). Active sensor surface saturated with capture reagent and exposed to AAV diluted in PBST. Black, blue and purple sensorgrams correspond to the average binding responses of 1.00 E+11 GC/mL, 4.00 E+11 GC/mL, and 8.00 E+11 GC/mL of AAV2 & AAV9, respectively. The shadowed region around each curve represents the standard deviation. Data was collected from one cartridge run on Alto for each capture reagent, with 16 replicates for each concentration.*

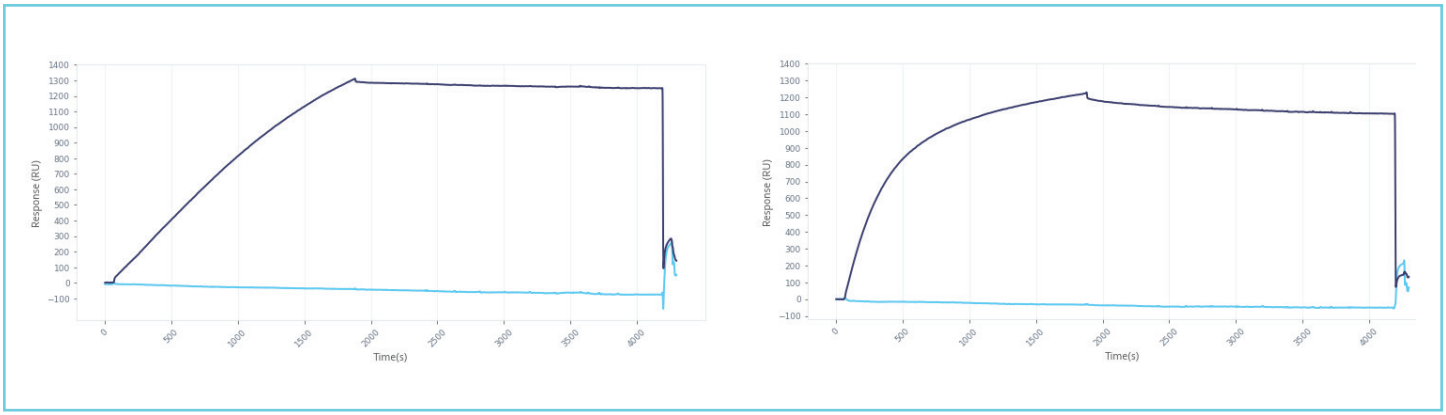

Figure 5: *Raw sensorgrams of AAV2 capture by AAVX capture surface (left) and AAV9 capture by AAV9 capture surface (right). Reference (light blue trace) and active sensor (dark blue trace) functionalized with Anti-AAVX VHH or Anti-AAV9 VHH are exposed to PBST and 8.00 E+11 GC/mL of AAV2 or AAV9, respectively, for 1800 s. This is followed by a long buffer rinse to demonstrate the rigidity of the capture surface. Finally, the sensors are exposed to glycine-HCl, pH 1.5 for 60 s, removing all captured ligand.*

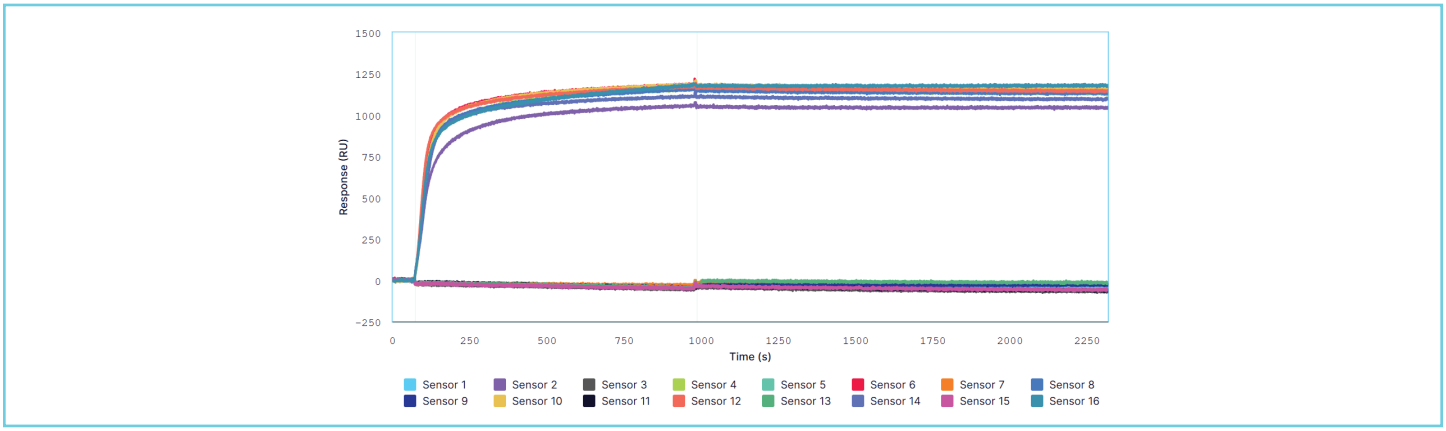

Figure 6: *Immobilization of 5 µg/mL biotinylated Anti-AAVX in PBS-T to the active (even) sensors of a streptavidin-functionalized surface.*

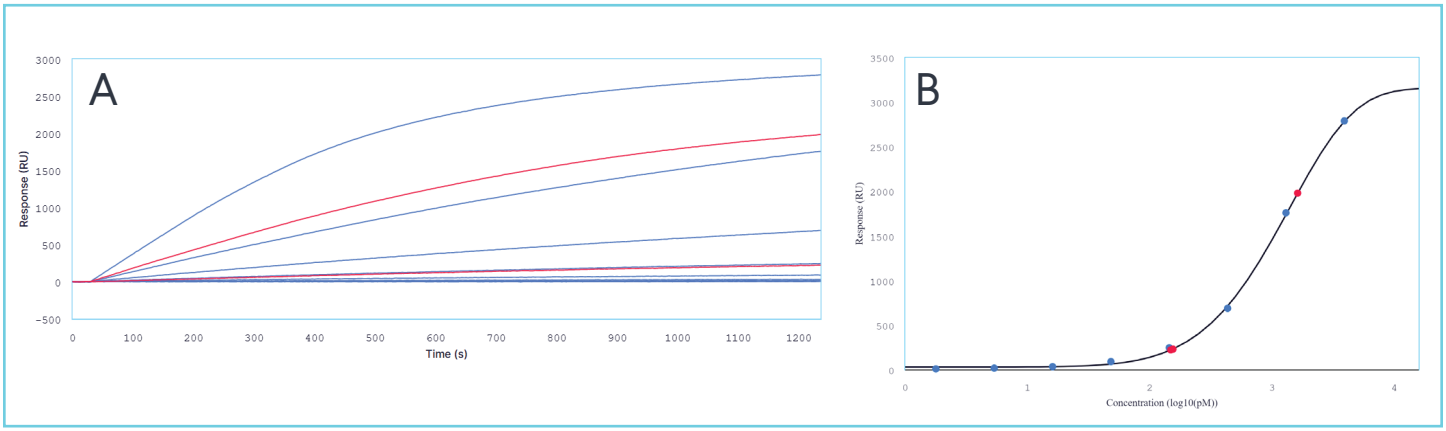

Figure 7: *Quantitation of AAV8 using an AAVX capture surface. A) Sensorgram view of corrected binding curves of AAV8 binding to the immobilized AAVX capture reagent, containing ten three-fold dilutions of the known standard analyte sample (blue curves) and two unknown analyte samples (red curves). B) 5-parameter logistic (5PL) calibration curve (black curve) created from plotting the log10 of the concentration for the ten standard analyte concentrations against their binding response (blue points). The unknown concentrations are plotted on the calibration curve (red points) and their concentration is solved from the 5PL model.*

Standard concentration (GC/mL)	Calculated standard concentration (GC/mL)	Residual %
2.16 E+12	2.15 E+12	0.3
7.20 E+11	7.09 E+11	1.5
2.40 E+11	2.48 E+11	3.2
8.00 E+10	7.39 E+10	8.2
2.67 E+10	2.10 E+10	27.2
8.89 E+09	7.66 E+09	16.0
Unknown concentration (GC/mL)	**Calculated unknown concentration (GC/mL)**	**Residual %**
8.79 E+11	9.77 E+11	11.2
8.79 E+10	8.97 E+10	2.0

Table 3: *Quantitation analysis of AAV8 using an AAVX capture surface*