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Strategies for the Development of a High Throughput Octet® Bio-Layer Interferometry Method to Measure Pharmacokinetics of Monoclonal Antibodies in Preclinical Animal Models

Wahala M Wahala, Ph.D[1]; Lori Armstrong, M.S; Susan Buist, Ph.D.
Elanco US Inc., 2500 Innovation Way, Greenfield, IN, 46140

[1] **Corresponding Author:** wahala.wahala@elancoah.com
Email: octet@sartorius.com

Introduction

Therapeutic antibodies are developed to treat various diseases, including cancers, immunological disorders, and infectious diseases. The safety and efficacy of these therapeutic molecules may be influenced by the way they interact with the body. Therefore, it is vital to understand how a body reacts to a biopharmaceutical after administration. Pharmacokinetic studies (PK) measure the variations of drug levels in the body as a function of time and are an essential part of the drug development process. Properly designed PK studies facilitate the determination of the proper dosage, distribution, safety, and efficacy throughout the duration of drug treatment.

Reliable and sensitive bioanalytical methods are required to quantitate drug molecules in samples collected from PK studies. ELISA or other immunoassay formats are the commonly utilized methods to analyze samples from these studies. However, developing a conventional plate-based immunoassay requires a reasonable investment of time, and additionally these assays are not high throughput for analyzing clinical samples unless the process is automated. In this application note, we evaluated the feasibility of utilizing the Bio-Layer Interferometry (BLI) platform to rapidly design, establish, and qualify a method with which to quantitate therapeutic canine antibodies from rat PK study samples to support an urgent project request.

Table 1
Materials and Reagents Utilized for the Development of the PK Method

Material/Reagent	Vendor	Cat. No.
Octet® High Precision Streptavidin (SAX) Biosensor	Sartorius	18-5118
Rabbit Anti-Canine IgG Fc Fragment Secondary Antibody [Biotin] (polyclonal antibody)	Novus Biologicals	NBP1-73513
Rabbit Anti-Canine IgG F(ab)2 Secondary Antibody (polyclonal antibody)	Novus Biologicals	NBP1-73923
Normal Rat Serum (NRS)	abcam	ab7488
Black 96-well plates	Greiner Bio-one	655209
Octet® R8	Sartorius	
Octet®-AS Offline Biosensor Immobilization Station	Sartorius	
20x HBS-EP Buffer	Teknova	H8022
Diluent (1x HBS-EP, 0.1% BSA)	N/A	N/A
Glycine (10 mM) pH 1.5	GE Healthcare	BR-1003-54

Method and Assay Optimization

Biosensor and Assay Format Selection

A fit-for-purpose assay design concept was used to determine the assay format and biosensor selection for a non-GLP study, which does not require a fully validated assay. Generally, IgG can be quantitated using protein A and G biosensors. However, our therapeutic antibody of interest is in a rat serum matrix consisting off-target rat IgG and other serum components that also bind to these sensors. Therefore, to minimize off-target binding, a High Precision Streptavidin SAX biosensor-based capture assay format was selected. The basic assay format is shown in Figure 1. To summarize, the biotinylated capture antibody was immobilized onto the SAX biosensors. The therapeutic antibody (drug) molecule in the serum sample was then captured. To increase the assay specificity and sensitivity, capture of the drug was confirmed by use of a detection antibody specific to the target drug molecule.

Compared to the selection of commercially available anti-human antibodies, availability of anti-species specific (canine) antibodies are limited, and most of these antibodies are not well characterized. Based on previous experience and institutional knowledge, there is a possibility of cross reactivity of the detection antibody with the capture antibody which can be minimized by selecting an antibody pair generated from the same animal species. After an initial characterization of a selection of antibodies from several vendors, a biotinylated rabbit anti-canine Fc specific antibody (polyclonal) and a rabbit anti-canine F(ab)2 specific antibody (polyclonal) were selected as the antibody pair for developing an orientation-directed binding assay. Even though biotinylated target protein could have been used to capture the target protein specific canine antibodies in rat serum samples, we decided to use the aforementioned antibody capture assay format to establish a universal assay platform with which to analyze any canine antibodies in rat sera to support any future pre-clinical PK studies conducted in rats.

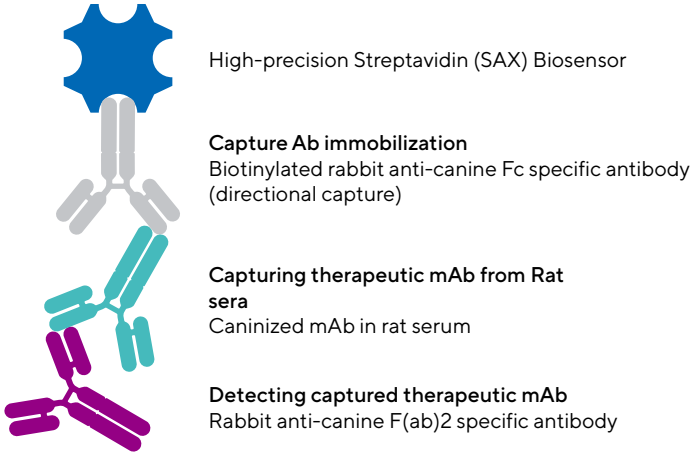

Figure 1
Assay Format
Biotinylated rabbit anti-canine Fc specific antibody was immobilized onto high precision streptavidin (SAX) biosensors. Utilizing an Fc-specific antibody allows directional capture of the Fc portion of therapeutic caninized mAb in the rat serum matrix. For additional specificity/sensitivity, the Fab portion of the therapeutic mAb was detected using a Rabbit anti-canine F(ab)2 specific antibody.

Assay Optimization

Assay optimization experiments were carried out using a standard curve spanning the expected range of the drug molecule in the serum. The standard curve was prepared by serially diluting the drug molecule in 1:10 diluted normal rat serum (matrix). All initial assay optimizations were performed in kinetic mode at a temperature of 30° C. Concentrations of spiked samples were calculated by fitting to a standard curve generated by plotting signal response (Req) levels for each standard concentration.

A. Buffer Optimization

The intended purpose of the assay is to quantitate the therapeutic antibody in a complex matrix such as serum or plasma. These complex matrices tend to produce higher background signals in an assay. So, optimization of buffer and blocking reagents are crucial steps in the assay development workflow.

Initial experiments were conducted in HBS-EP assay buffer containing 0.1% BSA. However, when testing standards diluted in rat serum, the assay produced an elevated amount of non-specific binding to the sensors. To minimize non-specific binding from rat serum samples, an additional blocking step consisting of normal rat serum diluted 1:10 in the HBS-EP assay buffer was introduced after the capture antibody immobilization step. To further aid in reducing the non-specific/off target binding, the amount of NaCl and Tween-20 was varied from 150 mM-500 mM and 0.05% or 0.09%, respectively to determine the best combination of NaCl and Tween 20 in the assay buffer.

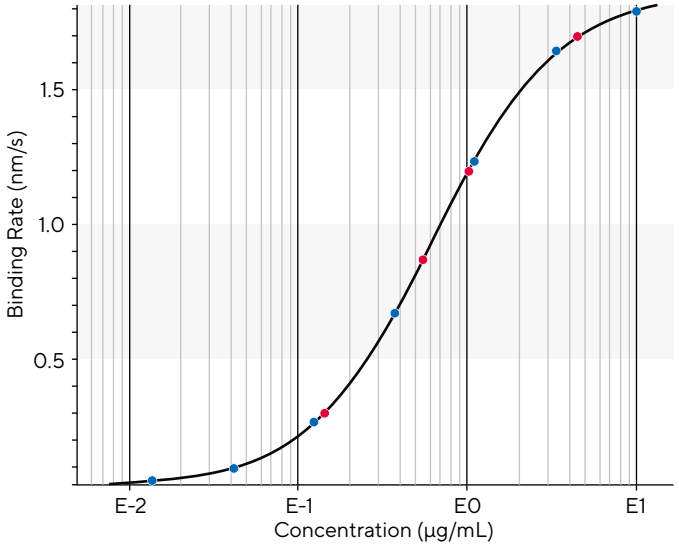

Figure 2

Octet Standard Curve

mAb standards were diluted 3-fold ranging from 10 µg/mL to 0.013 µg/mL in assay buffer. Known concentrations of mAb (4, 1, 0.5 and 0.125 µg/mL) were spiked into 1:10 diluted rat serum. Assay was performed as a kinetic assay and the data was analyzed using the quantitation module of the Octet data analysis software. Spiked samples are shown in red in the graph.

Increasing the concentrations of NaCl and Tween 20 decreased the background signal, however, the specific signal was also reduced (data not shown). Therefore, HBS-EP buffer containing NaCl (150 mM), Tween-20 (0.05%), 0.1% BSA was selected as the optimal assay buffer composition for future assays. After a few scouting experiments, 25 µg/mL and 5 µg/mL were selected as capture and detection antibody concentrations, respectively.

B. Shaking Speed Optimization

Since sensitivity is a critical parameter of a PK assay, different shaking speeds were tested to improve the assay sensitivity. The experiment was conducted in the assay buffer selected from the previous set of experiments (HBS-EP buffer containing 150 mM NaCl, 0.05% Tween-20, 0.1% BSA). SAX sensors were immobilized with 25 µg/mL of biotinylated rabbit anti-canine Fc specific antibody in the assay buffer. Rabbit anti-canine F(ab)2 specific antibody was diluted at 5 µg/mL in assay buffer and used as the final detection reagent. Octet® signals for the wells containing anti-canine F(ab)2 specific antibody were used for interpolation of the antibody concentrations. Shaking speeds of 800 and 1000 rpm were tested for the serum blocking, drug molecule capture in matrix, baseline, and detection antibody steps. Standards were diluted 3-fold ranging from 10 µg/mL to 0.013 µg/mL, and simulated spiked samples at varied concentrations were also included to calculate percent recovery at both speeds (Figure 2, standards in blue, spiked samples in red). Drug molecule at 0 µg/mL was used as a reference control. A shaking speed of 800 rpm provided the better recovery, with all samples within ±20% bias, and was selected as an assay parameter (Table 2A and 2B).

A. Shaking Speed—1000 RPM

Spiked mAb:	Spiked Conc. (µg/mL)	Calc Conc. (µg/mL)	% Bias
	4	5.73	43.25
	1	0.9944	-0.56
	0.5	0.5816	16.32
	0.125	0.1333	6.64

B. Shaking Speed—800 RPM

Spiked mAb:	Spiked Conc. (µg/mL)	Calc Conc. (µg/mL)	% Bias
	4	4.5	12.50
	1	1.02	2.00
	0.5	0.5472	9.44
	0.125	0.1425	14.00

Table 2

Shaking Speed Optimization and Percent Recoveries of Spiked mAb in Serum

Known concentrations of mAb at 4, 1, 0.5, and 0.125 µg/mL were spiked into 1:10 diluted normal rat serum. Shaking speeds of 1000 rpm (A) and 800 rpm (B) were tested. Spiked sample concentrations were interpolated, and percent bias was calculated for each shaking speed. With a shaking speed of 800 rpm, all samples were within ±20% bias. 800 rpm was selected as the optimal shaking speed parameter.

C. Assay Linearity

Using an experimental setup similar to the previous experiment, assay linearity was tested with simulated spiked drug molecule at 4, 1, 0.5, 0.125, and 0.0313 µg/mL against a standard concentration range from 10 µg/mL to 0.013 µg/mL by carrying out 3-fold dilutions. All dilutions were done using 1:10 diluted normal rat sera. Data was collected for 300 or 400 seconds for the final detection antibody binding step. Shaking speed was set at 800 rpm for serum block, drug capture, baseline, and final detection antibody steps. Shaking speed for all other steps was set at 1000 rpm. As shown below (Figure 3 and Table 3A and 3B, the quantitation assay had a very strong assay linearity (R2 > 0.99) in the range tested indicating that the Octet® based quantitation assay is suitable to test the PK study samples. Readings collected from 3 separate runs at 300 and 400 seconds both provided similar assay linearity. For both runs, recovery of all spiked samples was within ±20% bias.

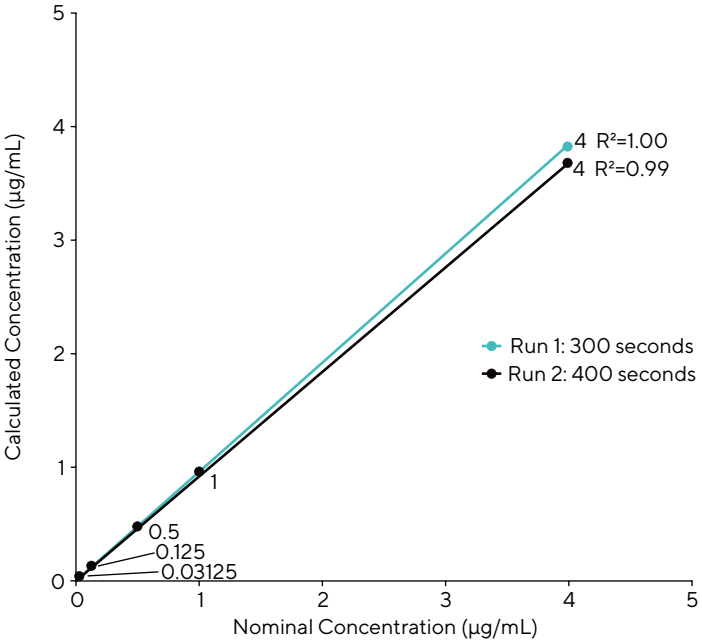

Figure 3

Assay Linearity

To test the linearity of the assay, standard mAb was spiked into 1:10 diluted serum matrix starting at 4, 1, 0.5, 0.125 and 0.03125 µg/mL. Calculated numbers were graphed against the nominal concentration of spiked amount of standard mAb to show the assay linearity.

A.300 Second Detection

Spiked mAb:	Spiked Conc. (µg/mL)	Calc Conc. (µg/mL)	% Bias
	4	3.83	-4.25
	1	0.9505	-4.95
	0.5	0.479	-4.20
	0.125	0.1273	1.84
	0.03125	0.0362	15.65

B.400 Second Detection

Spiked mAb:	Spiked Conc. (µg/mL)	Calc Conc. (µg/mL)	% Bias
	4	3.67	-8.25
	1	0.9494	-5.06
	0.5	0.4793	-4.14
	0.125	0.1268	1.44
	0.03125	0.0358	14.37

Table 3

Spike Recovery of mAb at 300 and 400 Second Detection Times

Sensor Regeneration and Initial Cycles of Regeneration Are Needed Before Analysis

Sensor regeneration is required to repeatedly use the sensors for replicate measurement of the samples. This will reduce the total assay time when testing the study materials along with decreasing the cost of consumables. Regeneration conditions at acidic pH were tested using the maximum standard concentration of 10 µg/ml with glycine-HCl solutions at pHs 1.5, 2.0, 2.5 and 3 to select the reagent that fully dissociates the analyte–ligand binding without drastically affecting the binding activity of the ligand. Sensors were neutralized in the assay buffer after the glycine-HCl regeneration step.

After each detection step in the assay, sensors underwent 3 cycles of regeneration, each consisting of a dip into the glycine solution for 5 seconds, then a dip into assay buffer for a 5 second neutralization. Signals from the rabbit anti-canine F(ab)2 specific detection step were used to calculate the binding response. The entire assay was run for 6 cycles with 5 regeneration steps total. The first run was performed with a fresh set of sensors without prior regeneration.

A. Binding Response Changes vs. Regeneration Cycle

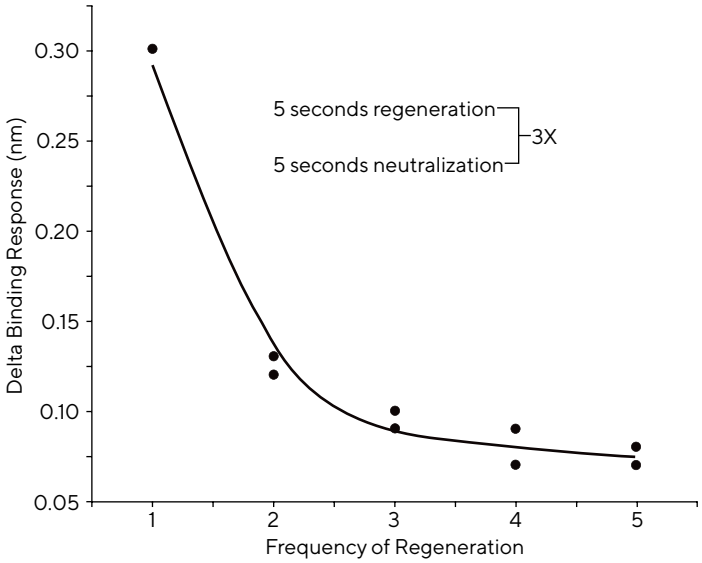

Figure 4A

Binding Response Changes Over Multiple Regeneration Cycles

Biosensors were regenerated a total of 5 times, and the change in binding response between cycles was calculated. Response decreased the greatest from regeneration 1 to 2, and response stabilized between cycles 3 and 5.

B. Glycine Biosensor Regeneration

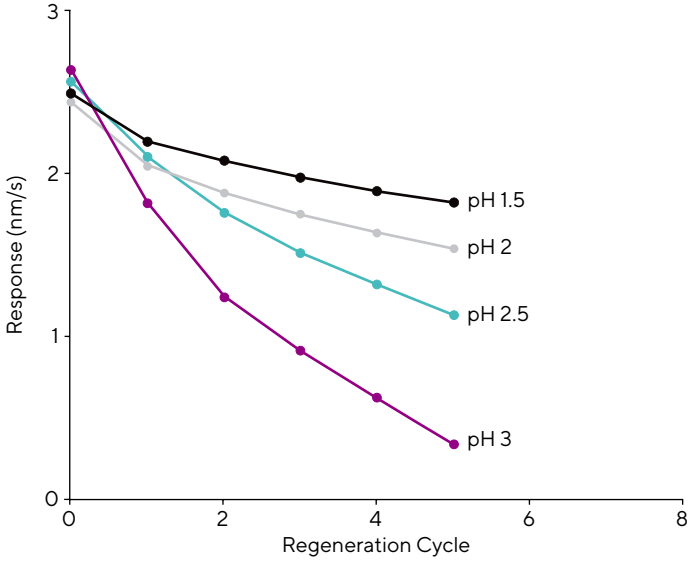

Figure 4B

Testing Biosensor Regeneration Buffers

Biosensors were regenerated for a total of 5 cycles in glycine-HCl buffer at pH 1.5, 2, 2.5, and 3, and binding response was monitored. Glycine buffer at pH 3 had the greatest binding change after regeneration, and Glycine buffer at pH 1.5 impacted binding the least.

Glycine Regeneration Buffer (pH)	1.5	2	2.5	3
Regeneration Cycle 0 Response (nm)	2.5	2.45	2.58	2.62
Regeneration Cycle 1 Response (nm)	2.2	2.05	2.1	1.81
Regeneration Cycle 2 Response (nm)	2.08	1.88	1.76	1.24
Regeneration Cycle 3 Response (nm)	1.98	1.75	1.52	0.9125
Regeneration Cycle 4 Response (nm)	1.89	1.64	1.32	0.6206
Regeneration Cycle 5 Response (nm)	1.82	1.54	1.13	0.331
Total Signal Loss from Regeneration	**0.68**	**0.91**	**1.45**	**2.29**

Table 4

Effects of Glycine Regeneration Buffer pH on mAb Binding Response (nm) Between Regeneration Cycles

Irrespective of the pH of the regeneration buffer, after each regeneration step binding rates decreased. The decrease was the most clear/ drastic between the first and second run where the first assay was run without prior sensor regeneration. This decrease was reduced between regeneration cycles 3 and 5 suggesting prior regeneration of sensors is a prerequisite before the actual sample analysis. (Table 4 and Figure 4)

As indicated by Table 4 and Figure 4A, the lowest binding response decrease (Δ binding response) after regeneration steps was observed with Glycine at pH 1.5, so this condition was selected for sensor regeneration. Data was also collected to determine the impact of acid regeneration steps on the assay dilution linearity. As depicted in Figure 5 dilution linearity was not affected even after multiple cycles of sensor regeneration. The graph below shows the assay linearity after three individual runs with regeneration steps.

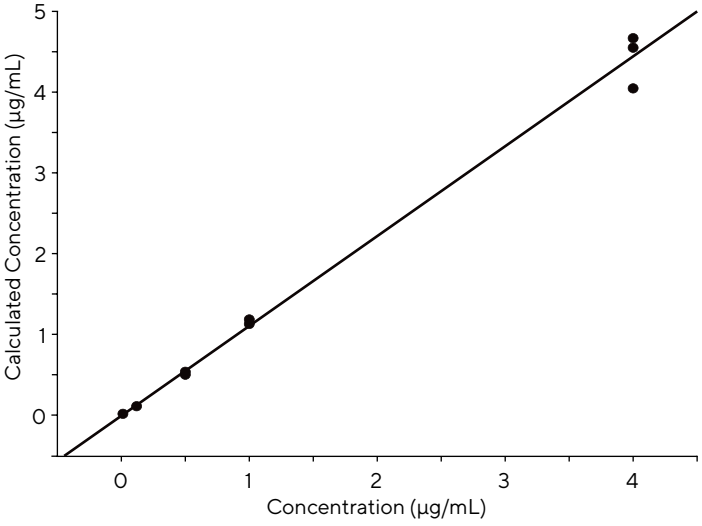

Figure 5

Assay Linearity After Three Individual Runs with Sensor Regenerations at pH 1.5: $R^2 = 0.99$

Introducing Octet® AS Offline Biosensor Immobilization Station to Improve Throughput

The final step of the assay was to introduce the Octet® AS offline biosensor immobilization station, which is capable of simultaneous and uniform reagent loading onto all 96 biosensors in a biosensor tray to increase the throughput and precision of the PK assay while also decreasing total assay handling time. Thus, the Octet® AS was used to simultaneously handle 96 biosensors to perform all offline assay steps that do not require online signal monitoring, such as initial biotinylated capture antibody loading, baseline, serum blocking, capture of the drug molecule from PK study samples, and sensor regeneration steps. Only the final detection step was monitored in real-time using the Octet® instrument.

SAX sensors were loaded with 25 µg/mL of biotinylated rabbit anti-canine Fc specific antibody in assay buffer. Drug molecule was diluted 3-fold ranging from 10 µg/mL to 0.013 µg/mL in 1:10 normal rat serum (NRS) in assay buffer to generate a standard curve. Simulated spiked samples were prepared by adding the drug molecule at 4, 2, 1, 0.5, 0.125, 0.0625, 0.031, and 0.0156, µg/mL in normal rat serum diluted 1:10 in assay buffer.

The rabbit anti-canine F(ab)2 specific antibody detection reagent concentration was increased from 5 µg/mL to 50 µg/mL to generate an equivalent signal with a decreased 60 second reaction time compared to the previous reaction time of 300 seconds. This allowed for the detection step to be completed faster for the entire plate reducing the chances for any low level capture drug dissociation from the biosensor while the detection steps are carried out one column at a time. On the other hand a Octet® RH96 system will be able to read the entire 96-well plate in a single read step.

Before the first quantitation run, biotinylated canine anti-Fc specific antibody was loaded onto the sensors and the sensors were serum blocked. After these two initial steps, sensors were regenerated with Glycine at pH 1.5. The serum blocking and regeneration steps were repeated once, for a total of 2 regeneration cycles, before the sensors were used in the quantitation assay.

Final Assay Format and Plate Map Showing Placement of Standards, Study Samples, and Quality Control Samples

Final assay setup, parameters and the plate layout is shown in Figure 6 and Tables 5A and 5B. Due to sample arrangement logistics, only four QC controls were setup at 4, 1, 0.5 and 0.125 µg/mL for samples analysis. Standard curve duplicates were setup at the beginning and the end of the plate to ensure that the sample loaded sensors remaining in the assay buffer does not impact the final quantitation due to possible reagent dissociation. All the samples collected from each animal were run using a single assay plate to maintain consistency of the data throughout the sample collection regimen. Each sample set underwent a duplicate run after sensor regeneration.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10	R0001 T0	R0001 T2	R0001 T4	R0001 T6	R0001 T8	R0001 T10	R0001 T12	10			
B	3.33333	R0002 T0	R0002 T2	R0002 T4	R0002 T6	R0002 T8	R0002 T10	R0002 T12	3.33333			
C	1.11111	R0003 T0	R0003 T2	R0003 T4	R0003 T6	R0003 T8	R0003 T10	R0003 T12	1.11111			
D	0.37037	R0004 T0	R0004 T2	R0004 T4	R0004 T6	R0004 T8	R0004 T10	R0004 T12	0.37037			
E	0.12346	R0001 T1	R0001 T3	R0001 T5	R0001 T7	R0001 T9	R0001 T11	4 µg/mL	0.12346			
F	0.04115	R0002 T1	R0002 T3	R0002 T5	R0002 T7	R0002 T9	R0002 T11	1 µg/mL	0.04115			
G	0.01372	R0003 T1	R0003 T3	R0003 T5	R0003 T7	R0003 T9	R0003 T11	0.5 µg/mL	0.01372			
H	0	R0004 T1	R0004 T3	R0004 T5	R0004 T7	R0004 T9	R0004 T11	0.125 µg/mL	0			

Figure 6

Final Assay Plate Map for Standards, QC Samples, and Clinical Rat Serum Study Samples

Standard curve duplicates were placed in the 1st and last columns of the plate. All timepoints collected from a single animal were analyzed on the same plate. Samples were analyzed in a duplicate run after biosensor regeneration.

A. Initial Biosensor Regeneration

Step	Step Type	Time (sec)	Shaking Speed (RPM)	
1	Equilibration	90	1000	Octet® AS Station
2	Loading Biotin Ab	60	1000	
3	Baseline	60	1000	
4	Serum Blocking (1:10 NRS)	120	800	
5	Regeneration (3x)	5	400	
6	Neutralization (3x)	5	400	

B. Sample Analysis and Quantitation

Step	Step Type	Time (sec)	Shaking speed (RPM)	
1	Baseline	60	1000	Octet® AS Station
2	Serum Blocking (1:10 NRS)	120	800	
3	mAb Drug in Matrix Capture	150	800	
4	Baseline	60	800	
5	Baseline	30	800	Octet® R8
6	Detection Ab Association	60	800	
7	Regeneration (3x)	5	400	Octet® AS Station
8	Neutralization (3x)	5	400	

Table 5

Finalized Assay Parameters for Analyzing Canine Antibodies From Rat PK Study Samples

Table 5A: Initial Biosensor Regeneration. Biotinylated antibody loading, baseline, 1:10 diluted normal rat serum (NRS) blocking, and initial biosensor regeneration steps were performed on the AS offline biosensor immobilization station.

Table 5B: Sample Analysis and Quantitation. The AS offline biosensor immobilization station was utilized for the baseline, 1:10 NRS blocking, and mAb drug in matrix capture steps. The remainder of the assay, baseline and detection antibody association steps were performed on the Octet R8 utilizing the quantitation assay method format. The assay plate was returned to the immobilization station to regenerate the biosensors offline for the duplicate assay run.

Spiked at (µg/mL)	Calc Conc. (µg/mL)	% Bias	Standard Deviation
4	4.493	12.33	0.393
1	0.959	-4.11	0.023
0.5	0.554	10.70	0.012
0.125	0.121	-2.81	0.008

Table 6

QC Sample Percent Bias Data From 8 Individual Assay Runs

Results and Summary of Sample Analysis

Samples collected at 13 different time intervals from four groups of animals were tested. As indicated in Table 6 and Figure 7, QC samples tested showed the expected recovery (within 20%) and assay linearity indicating the samples runs were successful. The quantity of the drug molecule in all the samples, with the exception of samples collected at time 0, were within the assay linearity (between 4 µg/mL–0.0313 µg/mL) range (Figure 7) indicating accurate quantitation of drug molecule in these samples.

The data generated from this study (Figure 8A and 8B) were used for PK profile analysis of the biotherapeutic molecules. A clear immunogenic response was observed with mAbs A and B by 120 hr after administration, an expected result of administering caninized mAbs to rats. Therefore, the first 5 days of exposure were used to characterize the four mAbs of interest. The highest C_{max} and greatest exposure, based on AUC over the first 120 hr, was achieved by mAb D. The other three mAbs were comparable based on C_{max} and AUC (Table 7). While rodent PK data cannot be directly correlated to canine PK, this data suggests mAb D may have the potential for greater absorption and overall exposure in dog compared with the other mAbs. This mAb also happened to have the greatest *in vitro* potency. The potency and rodent PK were combined to drive the decision to further characterize mAb D in dogs.

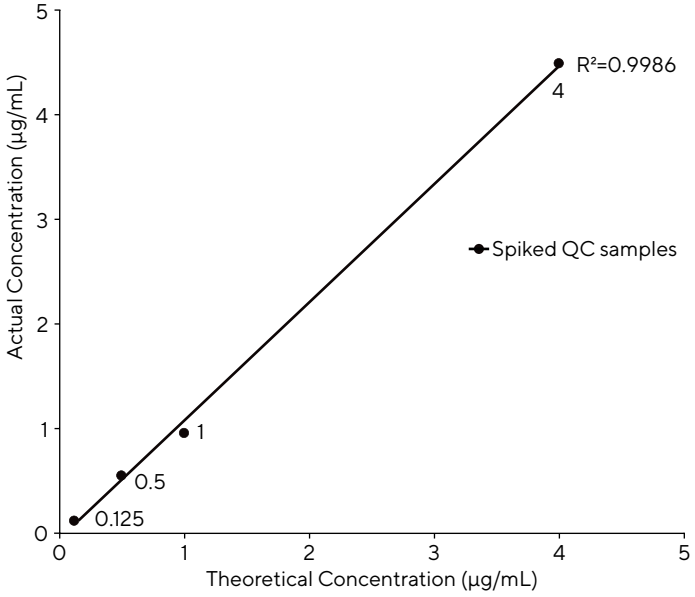

Figure 7

Assay Linearity for QC Samples From 8 Individual Assay Runs

A. Analysis of Rat Sera

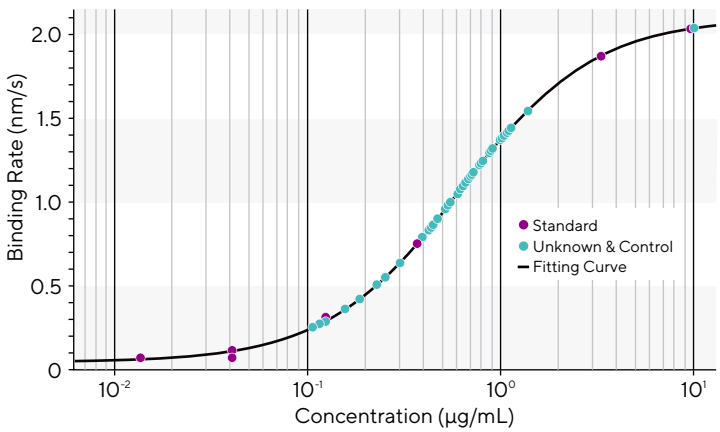

Figure 8A
Graph shows the standard curve and concentration of serum sample analyzed.

B. 336-hour Rat Exposure

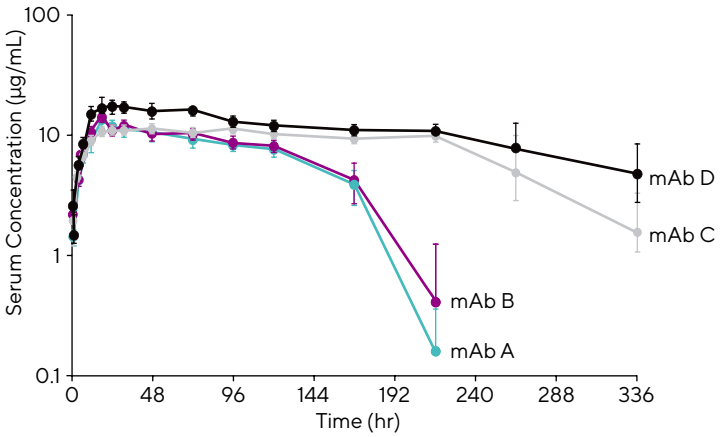

Figure 8B
Mean (±SD) serum concentration vs time profiles for mAb A (green), B (purple), C (gray), and D (black). Samples were serially collected from 4 subjects per dose group over 336 hr following subcutaneous administration of 5 mg mAb/kg body weight.

PK Parameter		mAb A	mAb B	mAb C	mAb D
C_{max}	µg/mL	13.4 (5)	14.9 (20)	12.1 (5)	19.0 (9)
T_{max}	hr	18 (0)	18 (0)	62 (45)	25 (23)
AUC_{0-120}	µg*hr/mL	1120 (12)	1190 (9)	1250 (1)	1740 (7)

Data presented as Mean (%CV)

Table 7
Mean Rat Serum PK Parameters Following Subcutaneous Administration of 5 mg mAb/kg Body Weight

Conclusions

We established an immune capture assay using the Octet® R8 platform to quantitate therapeutic molecules in serum samples collected from a pre-clinical PK study. With the combination of the off-line Octet® AS instrument, an Octet®-based PK assay was converted to a semi-automated high throughput assay. Compared to a conventional plate-based immunoassay, the assay was established in a very short time window to support an urgent PK assay need.

North America
Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
USA
Phone +1 888 OCTET 75
or +1 650 322 1360
Email: orders.US07@sartorius.com

Europe
Sartorius Lab Instruments GmbH & Co. KG
Otto-Brenner-Strasse 20
37079 Goettingen
Phone +49 551 308 0
Email: euorders.UK03@sartorius.com

Asia Pacific
Sartorius Japan K.K.
4th Floor, Daiwa Shinagawa North Bldg.
1-8-11, Kita-Shinagawa 1-chome
Shinagawa-Ku
Tokyo 140-0001
Japan
Phone +81 3 6478 5202
Email: orders.US07@sartorius.com

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For questions, email: AskAScientist@sartorius.com