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Quantitation of IgG and His-tagged proteins using the Carterra® LSA®

Key Highlights

- Quantitate 1,152 samples in a single run using ready-to-use capture surfaces
- Broad dynamic range across three orders of magnitude
- Single digit ng/ml limit of detection
- Requires as little as a few microliters of supernatant

Introduction

High-throughput quantitation of protein samples is desirable, particularly in early-stage antibody discovery workflows and cell line development. The Carterra LSA offers an HT-SPR protein quantitation workflow which can be performed as a standalone experiment or in conjunction with capture kinetics to obtain both detailed quantitation and kinetics. This workflow has an uninterrupted throughput of 1,152 samples per experiment, owing the sample plate capacity of up to three 384-well plates. As a capture-based approach, it works equally well for purified and crude protein samples, such as cell culture supernatants.

The Quant application requires use of a capture surface, and several ready-to-use sensor surfaces are available from Carterra, including Protein A, Proteins AG, and NiNTA. For these surfaces, capture sensitivities range from 10s ng/mL to 10's of µg/mL depending on molecule type and construct size. Custom capture surfaces can also be created for characterization of protein concentration based on other affinity tags or interactions (anti-V5, anti-Fab, etc.). The Quant feature in the Kinetics software utilizes a set of standard dilutions to be included for each set of 96-sample array captures. The choice of standard is important, as the similarity and behavior of the standard relative to the unknowns determine the accuracy of the measurement. A good standard should meet several criteria, including being as identical as possible in terms of sequence of the affinity tag, species, isotype (Fc capture) and molecular weight as the unknown proteins. Additionally, standard dilutions should extend above and below the anticipated unknown concentrations.

Experimental parameters, such as capture contact time, also depend on the expected unknown concentration range. If unknown samples are expected to be in the sub- to mid-µg/mL range, shorter contact times (5 min) will be sufficient, and quantitation can be performed using capture levels or initial capture slopes. For low-to-mid ng/mL samples, longer contact times of 20-40 minutes are suggested to allow more time for association and enhance detection sensitivity. The unique bidirectional injection fluidics of the LSA allow for extended injection times without additional consumption of sample in these cases where enhanced sensitivity is required.

Methods and Materials

Quantitation was performed using several sensor chips offered by Carterra: PAGHC30M (4292), PAGHC200M (4293), PAHC200M (4291), and NiHC200M (4299). To demonstrate the utility of the Quant app, the following commercially available proteins were used: Human IgG (Jackson Immuno 009-000-003) and mouse IgG (Jackson Immuno 015-000-003) were used in combination with Protein A/AG surfaces; His-tagged proteins hGRN (Sino Biological 10826-H08H) and hPD-1 (R&D Systems 8986-PD) were used for capture onto the NiNTA sensor.

Capture quantitation was performed in 1X HBSTE running buffer for Protein A and AG surfaces. 1X HBST was used in combination with NiNTA surface (EDTA is a chelator and will disrupt Ni-NTA complex). Each protein was used to set up 12 standard points and 8 "unknown" dilutions for quantitation. Each standard curve was prepared in triplicate, while the unknowns were prepared

using six replicates. Mouse and human IgG standard curve solutions ranged from 100 µg/mL down to 6 ng/mL, with unknowns ranging from 90 µg/mL down to 41 ng/mL. For His-tagged constructs, the standards ranged from 10 ug/mL down to 0.6 ng/mL, while the unknowns ranged from 9 µg/mL down to 4 ng/mL. Samples were prepared in deep 96-well plates (Greiner 780201) using the running buffer. The volume prepared in each well of the plate for the standard and unknowns was 250 ul, with 200 ul being injected for the measurement. The samples were returned to the well plate at the conclusion of the measurement. Each set of standards and unknowns for a single protein (i.e., human IgG) was located on the same plate, and thus would be captured in the same set of 96 prints. The quantitation experiment was set up using the Navigator control software Quant wizard, with each plate undergoing two consecutive prints. Contact times were set at 20 minutes per print. Sensor surface conditioning, which optimizes reproducibility of signals, was as follows: Protein A and AG surfaces were conditioned using standard conditioning sequence of 1-minute-long consecutive injections of 50 mM NaOH, 1 M NaCl, and 10 mM glycine pH 2.0. A NiHC200M sensor was conditioned and charged as described in the NiHC200M Biosensor Kinetic Assays on the Carterra LSA application note. Briefly, the sensor was conditioned using 1-minute pulses with 50 mM NaOH and 1 M NaCl. Initial charge and consecutive recharge procedure included two 2-minute pulses with 250 mM imidazole, followed by two 2-minute pulses with 250 mM EDTA, followed by one 2-minute pulse with 10 mM NiCl2.

Data were processed using Kinetics analysis software. The print data were filtered with settings of 7 RU height and 4 RU width, and Y-aligned to the baseline in overlay view. Under the Report Point tab, report points were created for early, late, and post-association phases. Referencing of a control sample can also be performed, but is often not necessary, as in this example. Standard concentrations were entered under the Report Point > Quant tab and 5PL fit was performed using appropriate selection of slope or RU for the early, late, or post-association phases report points. Unknown sample concentrations were automatically extrapolated from the standard curve fit.

Results

Four types of ready-to-use surfaces were investigated in this study, each was analyzed with a set of two proteins. Protein A and AG sensors were tested using human and mouse IgG preparations in concentrations ranging between 100 µg/mL and 6 ng/mL, **Figure 1** and **2A**, respectively. The NiHC200M sensor was tested using two His-tagged proteins, hPGRN with MW of 63 kDa and His-hu PD-1, MW 17 kDa at concentrations ranging between 10 µg/mL (158 nM hPRGN or 588 nM PD-1) and 0.6 ng/mL (9.5 pM hPRGN or 35 pM PD-1) (**Figure 2B**). Each protein was used to prepare a set of standards and unknown solutions for capture.

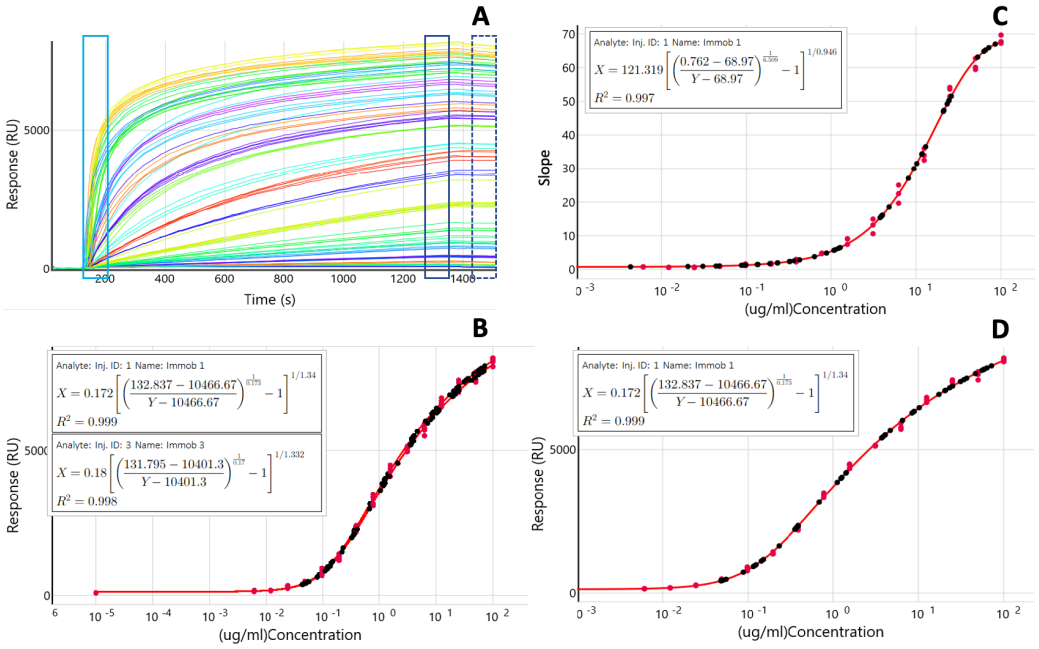

Figure 1: Human IgG capture on PAGHC200M surface. (A) Sensograms showing human IgG standards capture on PAGHC200M sensor. Each concentration is shown in a different color. Segment outlined in light blue was used to define the report point for initial slope analysis. Solid dark blue defines the region of the sensogram used for capture-level analysis. Dashed dark blue defines alternative report point location for capture-level analysis in the post-injection phase. B-D Response v. concentration plots showing the standards (red circles), 5PL standard curve fits (red line), and extrapolated unknowns (black circles). (B) Overlay plot of two sets of standards and unknowns. Replicates were performed by capturing from the same sample plate twice. (C) Standard curve generated using slope from early association phase report point. (D) Standard curve generated using response at late association report point.

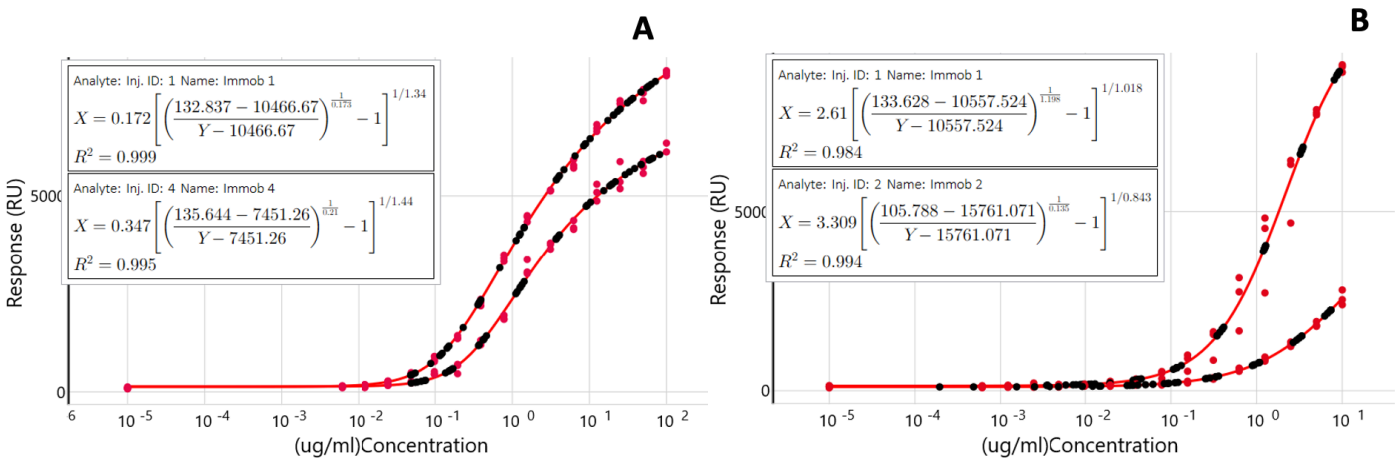

Figure 2: IgG sample capture onto PAGHC200M sensor. (A) Human IgG (top curve) vs mouse IgG (bottom curve) capture. (B) His-tagged hPGRN (top curve) and hPD-1 (bottom curve) capture onto NiHC200M sensor.

Quantitation on the LSA can be performed using two different modes: slope- or response-based. For this study two report points were created, slope analysis was used for the early association phase report point and RU analysis was selected for the late association phase. Report points for response-based quantitation can also be created in the post-injection window (**Figure 1A**, region shown in dashed line), particularly for crude samples, which can produce large bulk shifts during injection. As can be seen in **Figure 1C**, the standard curve produced from initial slope analysis works quite well at addressing the high and intermediate concentration ranges of 5-100 µg/mL IgG, while concentrations below 5 µg/mL are collapsed into the lower plateau. The lower concentration range benefits more from the capture level analysis with allowance for a longer contact time window (**Figure 1D**). This approach allows quantitation of samples in 10's ng/mL to 10's of µg/mL IgG. Thus, capture level analysis with extended contact times is recommended for characterization of a set of unknowns with a wide concentration range or at lower concentrations. The high sensitivity of this detection can allow for very low sample consumption as only a few microliters of higher expressing supernatants will be required. It is common to quantitate supernatants at a 10-to-100-fold dilution and using diluted supernatants reduces any potential refractive index artifacts in the signal as well.

Replicate measurements can be performed by including several samples per plate. If sample volume is limiting, performing multiple prints from the same sample wells is an option for analytical replicates, since only a small additional volume is required for consecutive prints. **Figure 1B** shows excellent agreement for sets of data collected for two prints from the same set of standards and unknowns.

Figure 2A and **B** demonstrate the prerequisite for choosing a standard, which closely resembles the unknown samples. **Figure 2B** shows two proteins of different molecular weight producing very different magnitude of capture response on a NiHC200M sensor. Capture level dependence on the construct size is somewhat intuitive, but there are other more subtle considerations for the standards beyond matching MW. **Figure 2A** shows standard curves for human (top) and mouse (bottom) IgG sample capture using PAGHC200M sensor chip. While these proteins are nearly identical in size, the difference in affinities of Proteins A and AG for the human vs mouse IgG isotypes translates into different capture levels from the same concentration range. This can be seen with other capture entity/affinity tag combinations, like a 6-His tag vs 10-His tag constructs on the NiHC200M or an N-terminal vs C-terminal location of FLAG-tagged construct on a custom anti-FLAG capture surface.

To determine the robustness of quantitation analysis, extrapolated values were compared with the actual concentrations of the "unknown" samples. Linear regression analyses for actual vs calculated concentrations are shown in **Figure 3**. Overall, proteins across all surfaces tested produced good agreement between actual and calculated concentrations in low 10's of ng- to sub 100 µg/mL range. Higher concentrations of unknowns tend to diverge slightly, likely due to being very close to the upper limit of the standard curve. Proteins of various molecular weight were represented in this study, from 150 kDa full length IgG to smaller 17 kDa human PD-1, and the quantitation experiment performed well throughout this range.

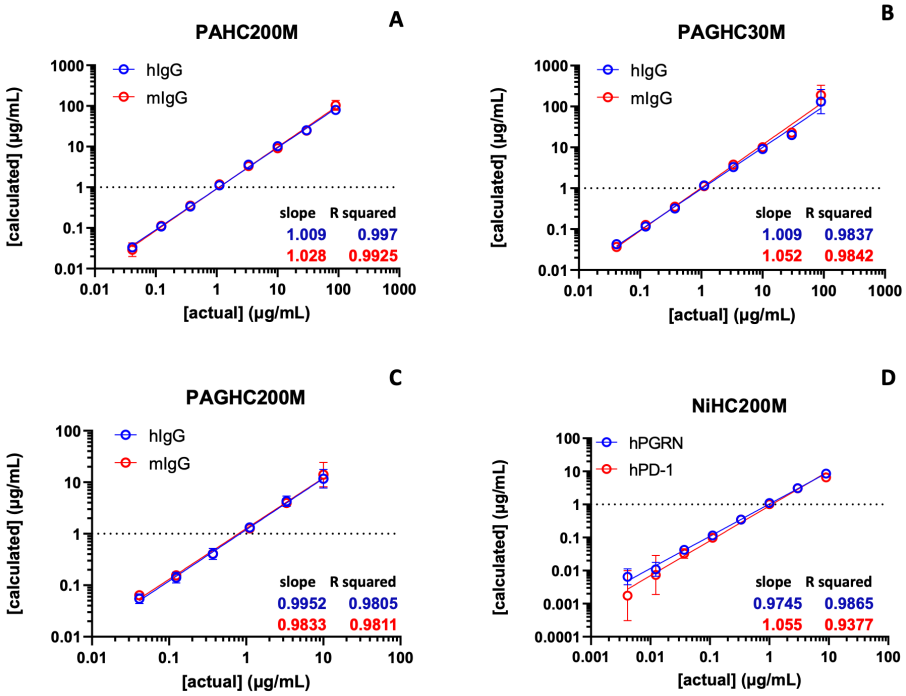

Figure 3: Correlation between actual protein concentrations and concentrations extrapolated from 5PL fit for (A) PAGHC200M with human and mouse IgG, (B) PAGHC30M with human and mouse IgG, (C) PAHC200M with human and mouse IgG, and (D) NiHC200M with hPGRN and human hPD-1. Plots were generated using GraphPad Prism 8. Slope and R-squared value are shown in corresponding color for each protein sample.

Table 1 summarizes performance characteristics found using four different surface types offered by Carterra using commercially available IgG and His-tagged proteins. Limit of detection for IgGs was approximately 40 ng/ml for IgG molecules on Protein A and AG and as low as 5 ng/ml for His-tagged proteins using NiNTA. Each of these surfaces demonstrates quantitation capabilities spanning more than 3 orders of magnitude, enabling a broad range of concentrations to be accurately assessed in a single run.

		Limits of detection	
Sensor type	**Protein**	**Low**	**High**
PAHC200M PAGHC30M PAGHC200M	hIgG	40 ng/mL or 300 pM	90 µg/mL or 600 nM
	mIgG	40 ng/mL or 300 pM	90 µg/mL or 600 nM
NiHC200M	hPGRN	5 ng/mL or 80 pM	10 µg/mL or 160 nM
	hPD-1	10 ng/mL or 600 pM	>10 µg/mL or >600 nM

Table 1: Summary of limits of detection for IgG and His-tagged proteins using select Carterra surfaces.

Summary

As a standalone feature, or in conjunction with detailed kinetic analysis, the Quant workflow is well suited for sensitive quantitation of proteins. Representative studies detailed here demonstrated IgGs being quantitated down to 40 ng/mL (300 pM) and for His-tagged proteins, quantitation was achievable down to 5 ng/ml (80 pM). In all examples, dynamic range spanned more than three orders of magnitude. It is important to note that variance with "similar" molecules was observed in binding responses on the same surface, presumably based on tag accessibility, protein size, and possibly other factors involved in binding. This illustrates the need to develop highly matched standards that can be used to accurately estimate unknown concentrations.

Also highlighted here are different approaches to analyzing quantitation results based on distinct aspects of the binding signals. The initial slopes of the responses are good for describing higher concentration samples, while the final bound level analysis is best for achieving sensitivity with regard to lower concentrations. Taking the responses immediately after the injection completes is helpful when working with high refractive index crude samples to avoid confounding from the supernatant matrix. Since these different approaches to data analysis can be done using the same data set, they provide flexibility in the analysis strategy depending on binding responses and assay conditions.

The ready-to-use chip surfaces described in these studies are ideal for turnkey quantitation. They allow for a consistent workflow and depending on assay criteria, can be reused in multiple assays. In addition, Carterra offers surfaces such as CMD200M (4280), CMD500M (4296), and HC200M (4287), which are ready for amine coupling of custom capture molecules. These would be advantageous for capture strategies such as anti-V5 or anti-HA.

In terms of assay metrics, the injected volume used to measure concentration on the LSA is 200 ul. This volume is returned to the source plate after the measurement and can be used to perform replicate injections in the same experiment or for other purposes. Assuming reasonable expression levels, this volume translates to only requiring a few microliters of supernatant in the conditions described here. Conducting a kinetics assay on material captured in the quantitation step further stretches the utility of the samples. On a per sample basis, the sensor chip cost for screening 1152 samples in a single run works out to be less than $1.50 per sample. If the assay criteria allow for reuse of sensor chips, this cost can be even further reduced. Overall runtime to quantitate 1152 samples is typically between 6 to 10 hrs. This is dependent on the capture times specified in the method, with lower expressing samples taking longer and higher expressing samples taking less time. Since the well plates are sealed, evaporation is not a concern and the LSA can cool the sample decks down to 15˚C, further stabilizing the samples.

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