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Fast Quantitation of Proteins and Antibodies Using the Octet® N1 System

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Abstract

Determining the concentration of a target proteins and antibodies using ELISA and HPLC can be labor-intensive and time-consuming. When the sample is in a complex matrix, these techniques are especially challenging.

The Octet® N1 system streamlines workflow and delivers rapid, direct quantitation of proteins, in a matter of seconds with high specificity and sensitivity, even in crude samples. The system provides a simple, rapid Dip and Read approach for protein quantitation. With the Octet® N1 system, proteins and antibodies can be quantitated, binding kinetics and affinity and concentration can be measured right at the bench from a drop of sample.

In this application note, assay design and best practices for the Octet® N1 system are provided, along with recommendations for biosensor preparation, data acquisition, curve fitting, and analysis of results.

Introduction

Traditional techniques for determining concentration of a target protein such as ELISA and HPLC are both elaborate and time-consuming, especially when analyzing complex matrices. For bioprocess development and production applications, the use of more rapid protein analysis techniques enables timely, informed process decisions. This application note describes the use of the Octet® N1 system to both streamline workflows and obtain rapid, direct quantitation of proteins in crude matrices.

About the Octet® N1 System

The Octet® N1 system provides a simple, rapid Dip and Read approach for protein quantitation in a small, affordably priced personal assay system. With the Octet® N1 system, proteins and antibodies can be quantitated in a matter of seconds with high specificity and sensitivity, even in crude samples. The system utilizes the same proprietary Bio-Layer Interferometry (BLI) technology as Sartorius' Octet® platform, enabling real-time analysis of interactions on the surface of disposable fiber optic biosensors. Affinity, concentration and binding kinetics can be measured right at the bench in a 4 µL drop of sample.

Quantitation of Proteins

The easy-to-learn Octet® N1 Software provides application modules for analysis of presence, quantity, activity and specificity of a protein of interest. Based on the specificity of the capture molecule immobilized on the biosensor, a protein of interest can be selectively measured even in complex sample matrices such as culture media, supernatant or cell lysate.

To evaluate protein concentration using the Octet® N1 system, the rate of protein binding to the surface of a disposable biosensor is measured in real time using a 4 µL drop of sample. The concentration of target protein in the sample is directly proportional to the binding rate. Using the Create Standard Curve application module, a standard curve can be generated from samples of known concentrations. The concentration of the protein in similar unknown samples can then be determined using the Quantitate Sample application module. Standard curves can be saved and utilized for subsequent quantitation experiments.

Dip and Read Biosensors for Protein Quantitation

Analyte-specific Dip and Read biosensors come pre-immobilized with capture molecules such as: anti-human IgG Fc, anti-murine IgG Fv, Protein A, Protein G, Protein L, anti-human Fab-CH1, anti-GST, Ni-NTA and anti-penta-HIS. These ready-to-use biosensors are suitable for quantitative analysis of IgGs or recombinant proteins that have affinity for these capture molecules. The Streptavidin Biosensor can be loaded with biotinylated capture molecules to customize an assay for other target proteins. Binding to the biosensor surface is highly specific, enabling differentiation between a target protein and other media components. Measurements can be made quickly and precisely even in unpurified samples, greatly simplifying analyses at all stages of research, process development and manufacturing. Rapid, easy and accurate quantitation of proteins can be achieved for bioprocess development applications such as hybridoma screening, clone selection, measurement of antibody titers, expression monitoring during or post-production, or optimization of growth conditions and expression systems.

Determining Antibody Titer in Hybridoma Samples

Monoclonal antibodies are essential tools in biomedical research and are of great commercial value as reagents and biotherapeutics. While technology for producing monoclonal antibodies from hybrid cell lines has undergone major advances in recent years, the methods available for screening individual hybridoma clones have varied very little over the same time period. Techniques such as ELISA for screening cell lines can be cumbersome, requiring significant amounts of material, multiple wash steps and excessive hands-on time (Figure 1). Label-free detection with the Octet® N1 system provides a simple, fast and accurate solution for characterization and monitoring of hybridoma cultures. Here we present how the Octet® N1 system was used to streamline quantitation in the monoclonal antibody production process for a tissue culture group at a Sartorius customer site.

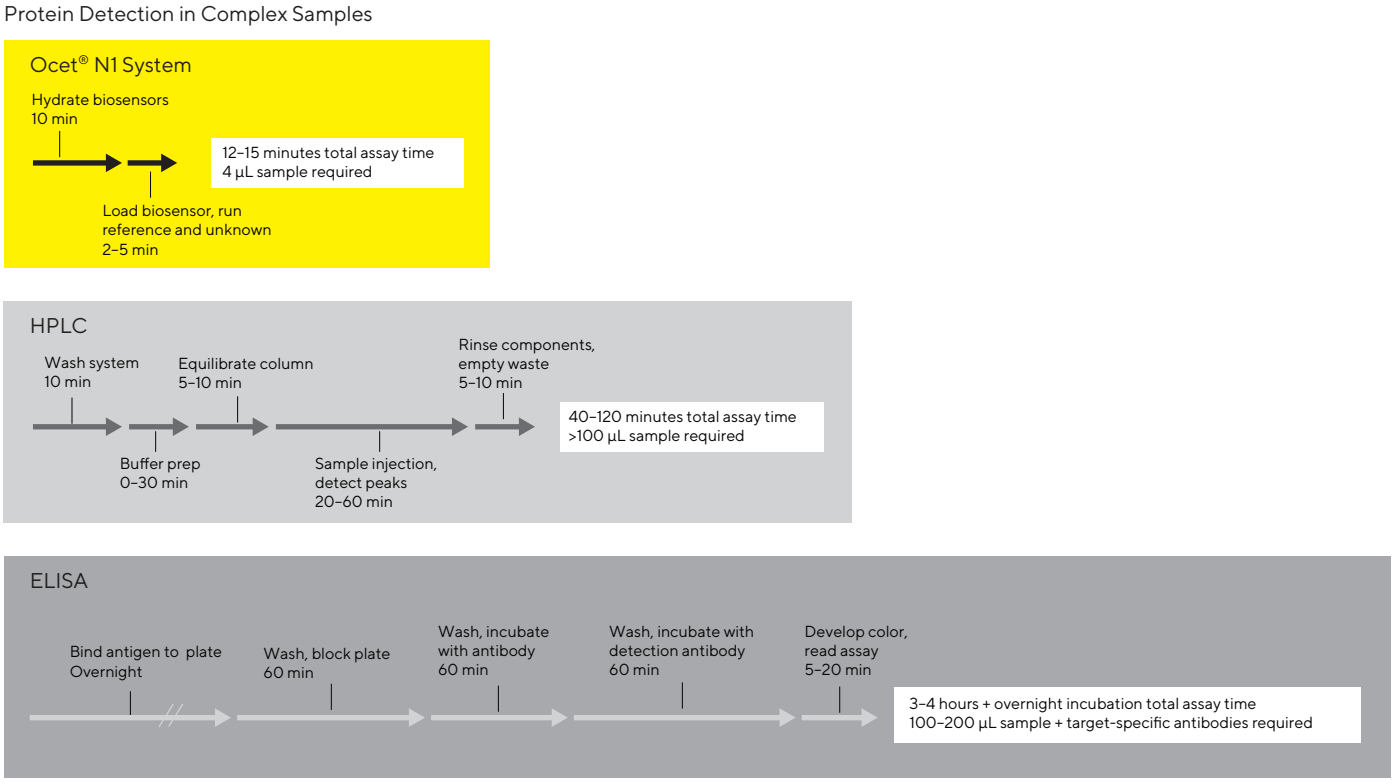


Figure 1: Comparison between protein detection using the Octet® N1 system and alternative techniques.

For this customer, a leader in production of antibodies, ELISA kits, dyes and reagents for the life science industry, it is critical to produce high-quality reagents in an efficient and timely manner. The group faced issues with variable protein titer and occasional low protein recovery. Without an efficient method to determine titer in complex hybridoma supernatants, the amount of antibody being produced in culture remained uncertain until harvesting and purification was performed. The group stood to save a great deal of time and materials by harvesting earlier in the production process. Quantitation using the Octet® N1 system and Protein G Biosensors provided a solution for easy and efficient assessment of antibody titer in complex hybridoma supernatants during production.

Generating a Standard Curve

Prior to running a quantitation assay on a hybridoma sample, isotype-matched standard curves were generated. For each standard curve, a purified monoclonal antibody of the same isotype as the unknowns was spiked into hybridoma culture medium at known concentrations. The matrix used for calibration samples was Iscove's Modified Dulbecco's Medium (IMDM) with nutritional supplements and 1% fetal bovine serum. Protein G Biosensors (Sartorius part no. 18-5082) were pre-hydrated with the same media.

Using the Create Standard Curve module in Octet® N1 Pro Data Analysis Software, sample data were entered and 4 µL of each concentration was run for 30 seconds with shaker enabled. A reference biosensor was also run in media with no target antibody in order to subtract background response caused by non-specific binding of media components and serum to the biosensor. Following data acquisition, the software calculated binding rates and automatically generated a standard curve which can be fitted by linear, linear point-to-point or 5PL equations. The standard curve was then saved for use in subsequent quantitation experiments.

Figure 2 shows real-time acquisition data from standard curves for three mouse antibody isotypes and three rat antibody isotypes. Note that even in the presence of complex media components and a low serum amount, binding rates for each concentration can be determined quickly with high sensitivity down to 1.56 µg/mL for some isotypes. The working dynamic range of the Octet® N1 system is broad, enabling measurement of antibodies from below 1 µg/mL up to 4 mg/mL (actual dynamic range depends on analyte and biosensor choice). In contrast, ELISA tends to have a narrower dynamic range and often requires dilution of samples.

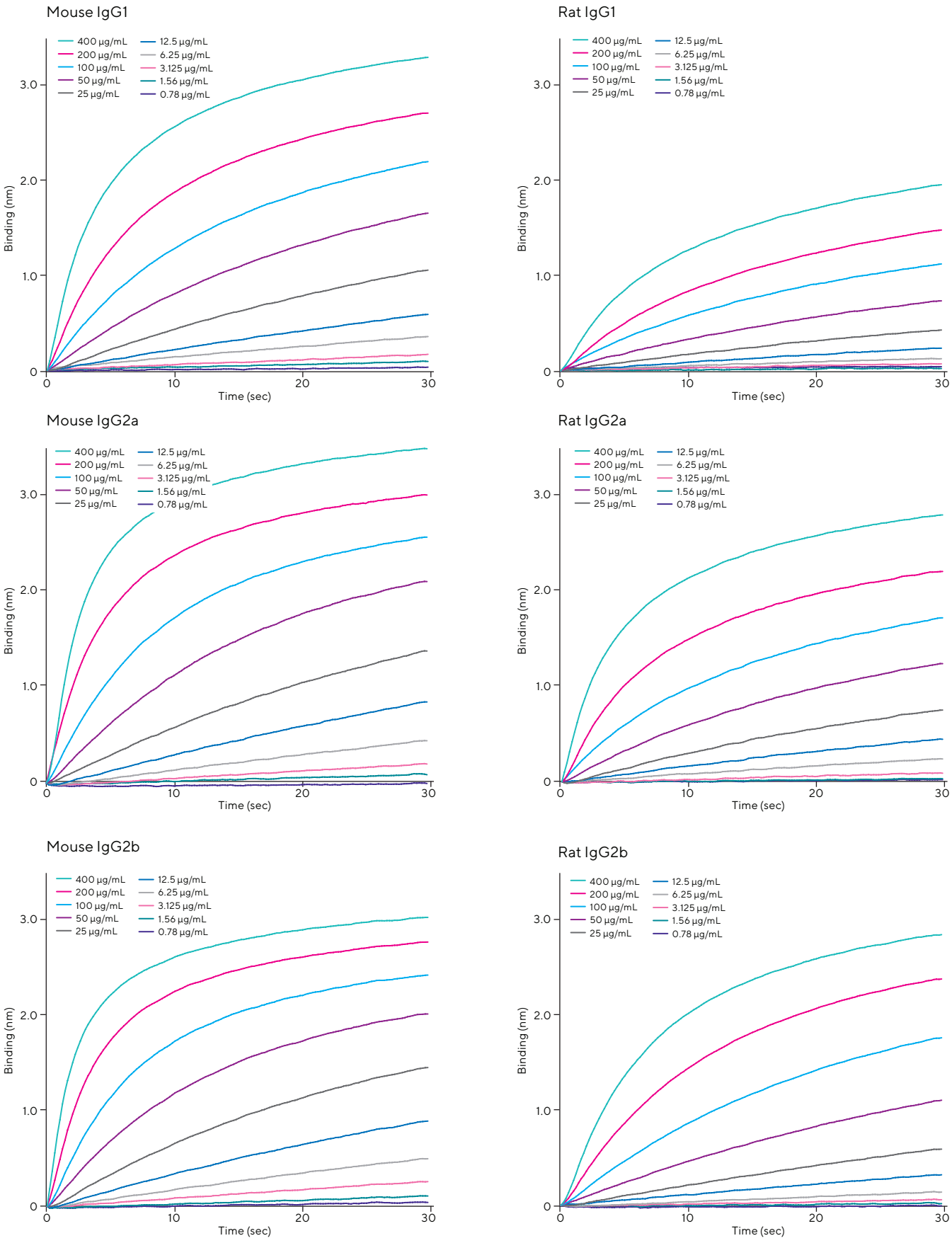


Figure 2: Acquisition data from standard curves for three mouse antibody isotypes and three rat antibody isotypes using Protein G Biosensors. Purified IgG was added to hybridoma culture medium containing 1% FBS to a concentration of 400 µg/mL and serially diluted. Background subtraction was performed using data obtained from a Protein G Biosensor in media without IgG.

Quantitating Unknown Samples

To determine antibody concentration in the unknown samples, supernatant was harvested and a 4 µL drop of sample was pipetted into the Octet® N1 system. Sample information was entered in the Quantitate Sample module in Octet® N1 Pro Data Analysis Software. A pre-hydrated Protein G Biosensor was loaded in the instrument. The assay was run for 30 seconds with shaking enabled, as with the standard samples. A reference sample consisting of media alone was also run. After data acquisition, unknowns were automatically fit to the standard curve and concentrations calculated.

Figure 3 shows data acquired from six hybridoma samples, along with standard curves calculated from the data in Figure 2. Each sample was matched to the standard curve with the same species/isotype for highest accuracy. All samples fell within the range covered by their respective standard curves, which is important for accurate quantitation. Calculated sample concentrations in this experiment ranged from 12.8 µg/mL to 230 µg/mL. The concentration data determined by this method has enabled this group to make predictions on yield from large scale monoclonal antibody production, and better estimate appropriate times for harvesting. Use of the Octet® N1 system can also be applied to optimization of culture conditions, screening and selection of the highest producing clones for production or cell banking, or determining the effect of supplements and growth factors on titer.

Comparison of Octet® N1 and HPLC Quantitation Data

Recombinant antibody production is the major focus in many drug discovery research and bioprocessing applications, and accurate concentration data is essential to evaluating the productivity of antibody producing clones. A number of techniques are available for the purpose of quantitating antibodies in cell culture supernatants, including ELISA, HPLC, or spectroscopy. In this section we show platform comparison data, courtesy of another Sartorius customer, which clearly demonstrate the accuracy of the Octet® N1 system when compared directly with HPLC.

A recombinant antibody of interest, Antibody A1, was transiently transfected into 293F cells (Invitrogen) and grown in serum-free media in two flasks over an 8-day expression cycle. To assess titer, suspension media was collected daily and monitored for yield. To quantify, 100 µL of supernatant was loaded onto a POROS® 20 micron Protein G column (0.1 mL, Applied Biosystems) and run on a Waters® e2695 HPLC system. At Days 5 and 6, the Octet® N1 system was utilized in parallel with HPLC. Protein A (Sartorius part no. 18-5010) or Protein G Biosensors (Sartorius part no. 18-5082) were pre-hydrated in culture media prior to use. Purified Antibody A1 protein was spiked into the same media at known concentrations and a standard curve was generated for each biosensor type using the Create Standard Curve module in Octet® N1 Pro Data Analysis Software. A 4 µL drop of culture supernatant from flasks 1 and 2 at Days 5 and 6 was analyzed on each biosensor for 30 seconds using the Quantitate Sample module as described above. A reference biosensor run in media alone was used in each experiment to subtract background response. Samples were analyzed in duplicate with one replicate neat and the other at a 2-fold dilution in culture media, with back-calculated original concentrations averaged to yield final concentration value. Note that in this experiment the exact protein to be quantitated was also used to generate the standard curve. Measuring sample concentration against a standard curve made with the same protein will give the most accurate results. When quantitating antibodies, if the identical antibody is not available, it is recommended to use one of the same isotype as the one being analyzed. It is also important to dilute the standards in the same buffer or media as the samples.

Table 1 and Figure 4 present a direct comparison of data for Antibody A1 transient transfection between the Octet® N1 system and HPLC. Calculated titer in µg/mL is shown for each flask versus collection day. Samples run using Protein A biosensors were matched to the calibration curve for Protein A, and Protein G samples matched to the Protein G curve. The results show that titers calculated from Octet® N1 system data using both Protein A and Protein G Biosensors are very similar, and also similar to the titer calculated from the HPLC analysis. The percent CVs between the three methods are 12% or lower for each flask and time point. The chart in Figure 4 clearly illustrates the consistency in data produced with the Octet® N1 system using different biosensor types in separate experiments, as well as the consistency in data between the Octet® N1 system and HPLC. While the results obtained from the two platforms are similar, HPLC systems require considerably more processing time, sample volume, instrument handling and maintenance. With the Octet® N1 system, accurate, real-time binding data can be acquired in a matter of seconds with minimal processing, providing a fast and convenient alternative for protein quantitation.

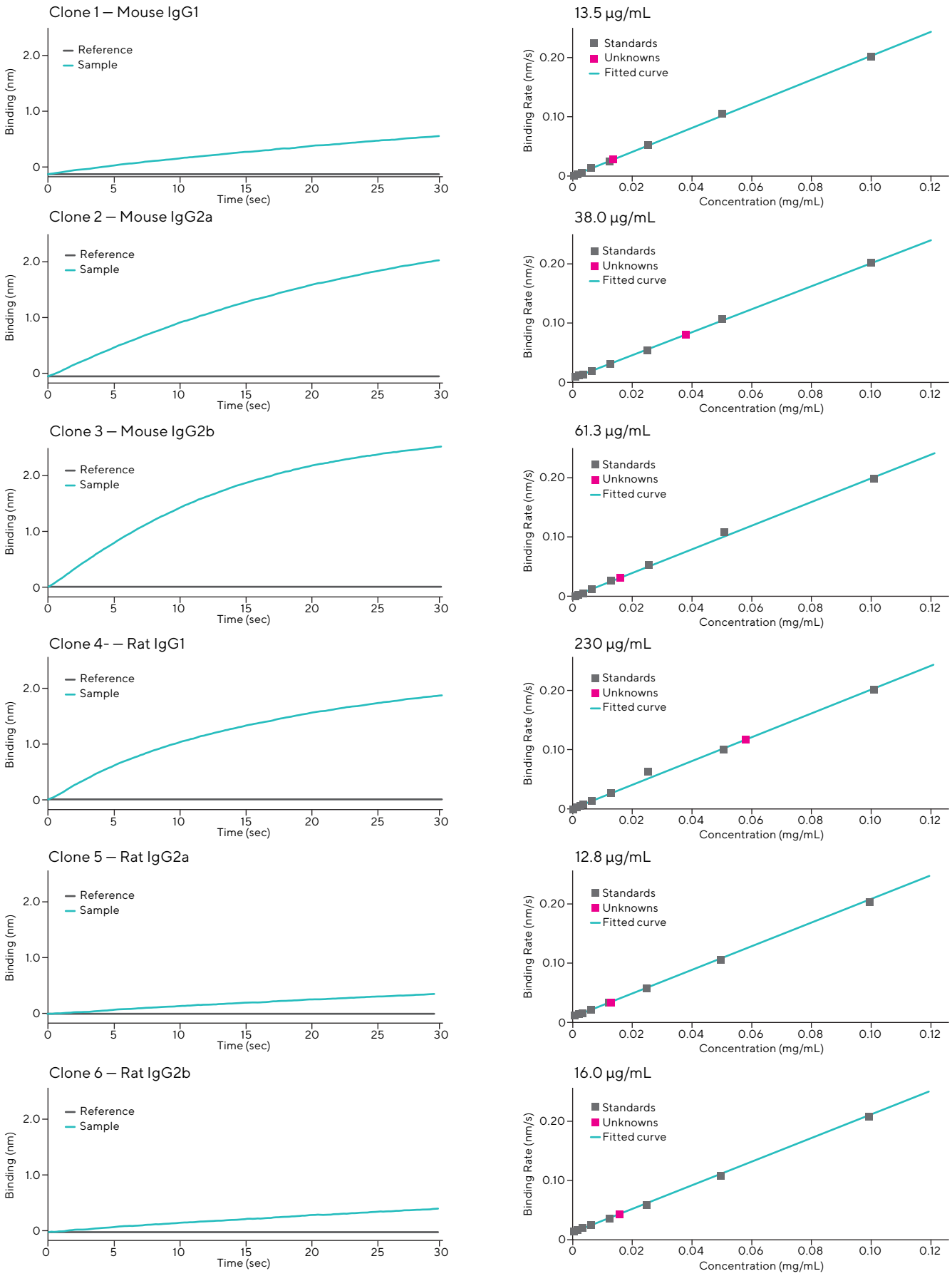


Figure 3: Quantitation of six hybridoma supernatant samples. Acquisition data is shown on the left, along with the reference biosensor data used for background subtraction for each sample. Isotype-matched standard curves from the acquisition data in Figure 2 are shown on the right, with sample binding rates fitted and calculated concentrations listed.

Table 1: Summary of quantitation data obtained on the Octet® N1 system and HPLC for Antibody A1 transient transfection supernatants. Calculated titer in µg/mL for replicate runs is listed for each flask versus collection day.

Day	Flask 1						Flask 2					
	Pro A - Octet® N1		Pro G - Octet® N1		Pro G - HPLC		Pro A - Octet® N1		Pro G - Octet® N1		Pro G - HPLC	
5	27.81	27.92	28.44	30.37	33	n/a	22.97	23.84	18.69	26.34	25	n/a
6	31.46	32.94	31.66	34.76	36	36	27.28	26.04	25.36	28.83	29	n/a

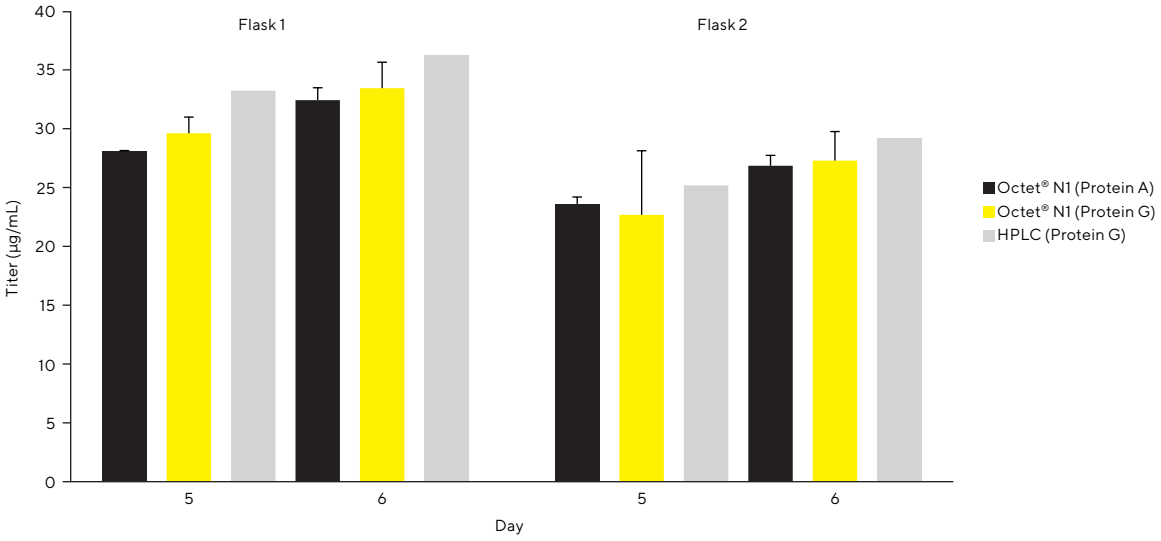


Figure 4: Octet® N1 system versus HPLC comparison for Antibody A1 transient transfection supernatants. Titers calculated from Octet® N1 system data using both Protein A and Protein G Biosensors are very similar to each other and to the titer calculated from HPLC data, demonstrating the consistency between platforms.

Specific Detection of Fab Fragments in the Presence of Free Light Chains

In the production of recombinant IgG and Fab fragments, over-expression of light chains can often be problematic. A majority of currently available methods for detection and quantitation of human Fab fragments utilize binding agents such as Protein L, which targets epitopes that reside on the antibody light chain. Using these methods, accurate determination of Fab concentration can be difficult due to ligand cross-binding to contaminating free light chains (Figure 5). Here we describe a method for selective analysis of intact Fab fragments and IgG using an affinity ligand that targets the CH1 domain on the heavy chain of the Fab fragment.

Sartorius' Anti-Human Fab-CH1 Biosensors allow highly specific binding to the CH1 region of human Fab, F(ab')2 and IgG. Anti-Human Fab-CH1 Biosensors exhibit no cross-binding to antibody light chains, and when used in conjunction with the Octet® N1 system offer a fast, simple method for selective quantitation of Fab/F(ab')2 fragments

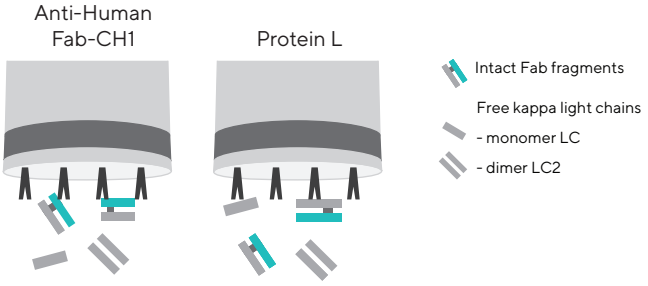


Figure 5: Selectivity of Anti-Human Fab-CH1 versus Protein L Biosensors for intact Fab fragments.

in the presence of contaminating free light chain species. To demonstrate the high level of specificity of Anti-Human Fab-CH1 Biosensors, the effect of free light chains on the analysis of a human Fab fragment was investigated. Samples were prepared in conditioned CHO cell culture media (Aragen Biosciences) containing approximately 17 µg/mL of a Fab fragment derived from whole human IgG (Jackson Immunoresearch) and spiked with free human kappa light chain from Bence Jones protein (Meridian Life

Science) at final concentrations of 0 µg/mL, 10 µg/mL or 50 µg/mL. The Fab fragment was also serially diluted in the same conditioned CHO cell culture media to generate a standard curve (data not shown). Conditioned media was also used to pre-wet Anti-Human Fab-CH1 Biosensors (Sartorius part no. 18-5104) and Protein L Biosensors (Sartorius part no. 18-5085) for 10 minutes. Standard curves and unknowns were run on both biosensor types using the Create Standard Curve and Quantitate Sample modules in Octet® N1 Pro Data Analysis Software, respectively. A 4 µL drop was used for each sample and data acquired for 30 seconds.

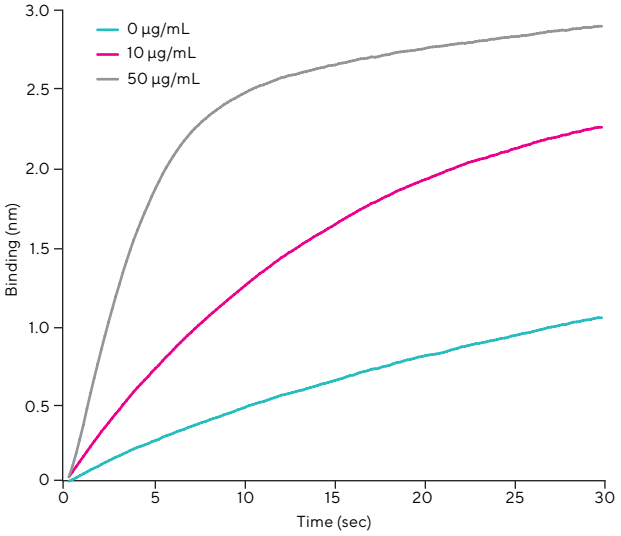
Data obtained on the Fab samples containing free kappa light chain for both biosensors are presented in Figure 6. The binding curves for Fab fragments analyzed on Anti-Human Fab-CH1 Biosensors were unaffected by the presence of free light chains, while Protein L Biosensors show increasing signal with higher concentrations of light chains. Since the concentration of Fab in the sample is constant, this increase is suggestive of co-binding of free light chains by Protein L. A chart comparing calculated concentration of Fab between the two biosensors is shown in Figure 7. These data clearly demonstrate that increased binding to Protein L Biosensors corresponds to higher concentration of free light chains, whereas binding to Anti-Human Fab-CH1 Biosensors remains constant. This high specificity of Fab binding makes the Anti-human Fab-CH1 Biosensor extremely useful for pre-purification analysis of Fab samples.

A range of biosensors are available for use on the Octet® N1 system with high levels of specificity for analysis in complex matrices. For example, Anti-Mouse IgG Fc (Sartorius part no. 18-5088) and Anti-Human IgG Fc (Sartorius part no. 18-5060) Biosensors offer selective capture of mouse or human IgGs, respectively. Streptavidin Biosensors can be loaded with a biotinylated capture molecule of choice to create custom biosensors that enable an unlimited range of assays.

Tips for Quantitation Assays

Pre-hydrate biosensors for at least 10 minutes in buffer matrix that exactly matches the sample to be analyzed. This will minimize background response from non-specific binding to the biosensor.

Protein L — Free light chain cross-reacts with Protein L Biosensor



Anti-hu Fab-CH1 — Free light chain does not influence signal from Fab binding to Anti-Fab Biosensor

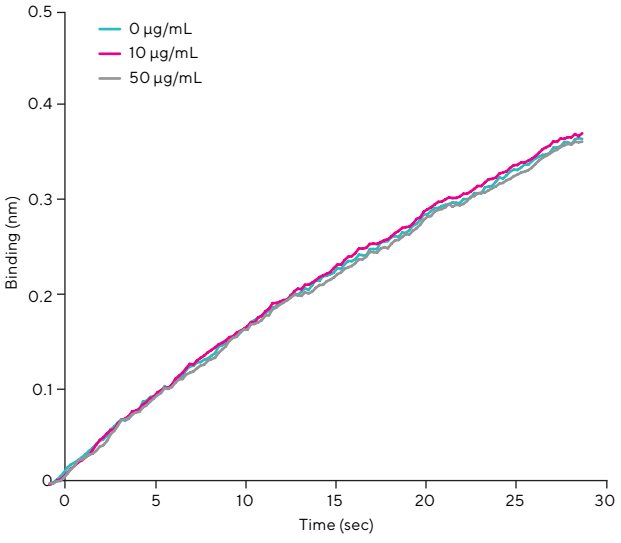


Figure 6: Effect of free kappa light chains on quantitation of human Fab fragment samples using Protein L Biosensors (left) compared to Anti-Human Fab-CH1 Biosensors (right). Varying levels of human kappa light chain were spiked into samples containing 17 ug/mL human Fab fragment and analyzed on both biosensor types. Real-time binding data is shown. Increased response on Protein L Biosensors indicates cross reactivity to free light chains.

When generating standard curves or running unknowns, always run a reference sample that matches the matrix of the sample(s) to be analyzed but does not contain the protein of interest. This will allow for subtraction of any response generated by non-specific binding to the biosensor.

For accurate results, a standard curve must be generated using the same protein as the sample(s) to be quantitated. The standards should be diluted in a buffer matrix that exactly matches that of the unknown sample(s).

Standard curves can be saved and utilized for subsequent experiments, as long as the biosensors are from the same manufacturing lot as those used to run the samples. The concentration of sample(s) being analyzed should fall within the concentration range of the standard curve for accurate quantitation.

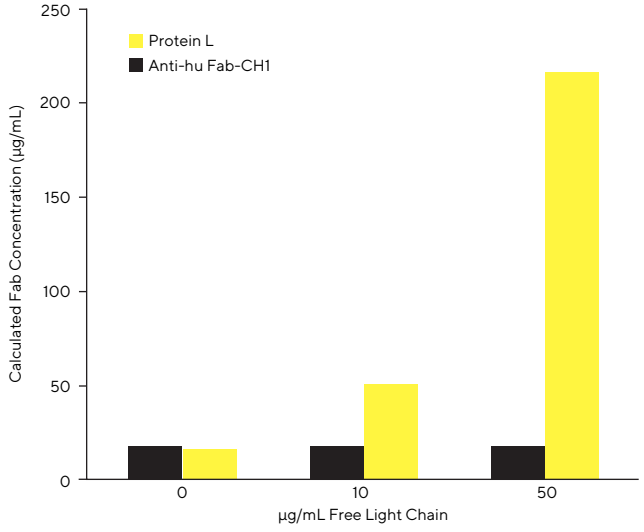


Figure 7: Increasing binding rates and calculated concentrations with Protein L Biosensors corresponds directly with increasing concentration of free light chains in the Fab samples, even though concentration of Fab remained constant. Anti-Human Fab-CH1 Biosensors display high specificity for intact Fab molecules.

Table 2: Calculated Fab concentrations

Free light chain (µg/mL)	Anti-Hu Fab-CH1	Protein L
0 µg/mL	16.17	15.52
10 µg/mL	16.88	50.57
50 µg/mL	16.02	214.6

Conclusion

We have presented methods and data demonstrating quantitation of proteins on the Octet® N1 system and how this capability can be utilized to improve workflows when paired with a wide selection of pre-immobilized biosensors. The ability to quantitatively measure target proteins directly in complex matrices using 4 µL of sample allows rapid, simple, powerful analyses on the Octet® N1 system that are not possible with other platforms.

References

1. Octet® N1 Quick Start Guide: Provided with all Octet® N1 systems.
2. Octet® N1 Demo and Starter Guide: Provides detailed step-by-step guidance on setting up assays using the Octet® N1 starter kit.
3. Sartorius Technical Note 28: Biotinylation of protein for immobilization onto Streptavidin biosensors.
4. Sartorius Application Note 9: CaptureSelect Affinity Ligands for Antibody Detection and Characterization.

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