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Application Note

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Instant Determination of Protein Presence Using the Octet® N1 System

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Abstract

Western blots and HPLC can be used during upstream processing for protein expression and bioreactor monitoring to check for the presence or absence of target protein. These techniques require multiple reagents and a significant amount of time to analyze crude samples. In contrast, the Octet® N1 system streamlines workflows and enables direct quantitation of proteins, in a matter of seconds with high specificity and sensitivity, even in crude samples.

When paired with pre-immobilized biosensors, the Quick Yes/No module on the Octet® N1 system provides access to real-time sample information during bioprocess development and production. A variety of applications are available, including quick checks for the presence of protein, relative expression ranking, construct screening, and detection of Fab fragments in the presence of free light chains. The ability to qualitatively measure target proteins directly in complex matrices using 4 µL of sample allows rapid, simple, and powerful analyses on the Octet® N1 system that is not possible with other platforms.

Introduction

Often, precious time is lost during protein expression and bioreactor monitoring when simply checking for the presence or absence of the target protein. Common techniques like Western blot and HPLC require multiple reagents and require significant amounts of time to analyze crude samples. This application note describes the use of the Octet® N1 system for instant, specific protein detection in crude matrices, enabling access to real-time sample information during bioprocess development and production.

About the Octet® N1 System

The Octet® N1 system provides a simple, rapid Dip and Read™ approach to protein analysis in an affordably priced personal assay system. Protein and antibody detection can be performed in a matter of seconds with high specificity and sensitivity, even in crude samples. The Octet® N1 system utilizes the same proprietary Bio-Layer Interferometry (BLI) technology as Sartorius Octet® platform, enabling real-time, label-free 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.

Quick Check for Protein Presence

The easy-to-learn Octet® N1 Software provides application modules for analysis of presence, quantity, activity and specificity of a protein of interest. Here we demonstrate examples of how the Quick Yes/No application module on the Octet® N1 system can be successfully utilized in a variety of assay formats. Rapid detection of the presence or absence of a target protein and relative ranking of concentration are simple yet important functionalities that can provide critical information at various stages in research, process development and manufacturing. More importantly, the ability to make these determinations in crude samples without the need for prior purification steps can streamline workflows, saving valuable time and resources.

Rapid, simple and direct methods for small scale qualitative evaluation of constructs, expression levels and protein activity can be performed using the Quick Yes/No module and disposable Dip and Read Biosensors. These methods complement or replace more elaborate and time-consuming techniques such as ELISA, SDS-PAGE and Western blot (Figure 1).

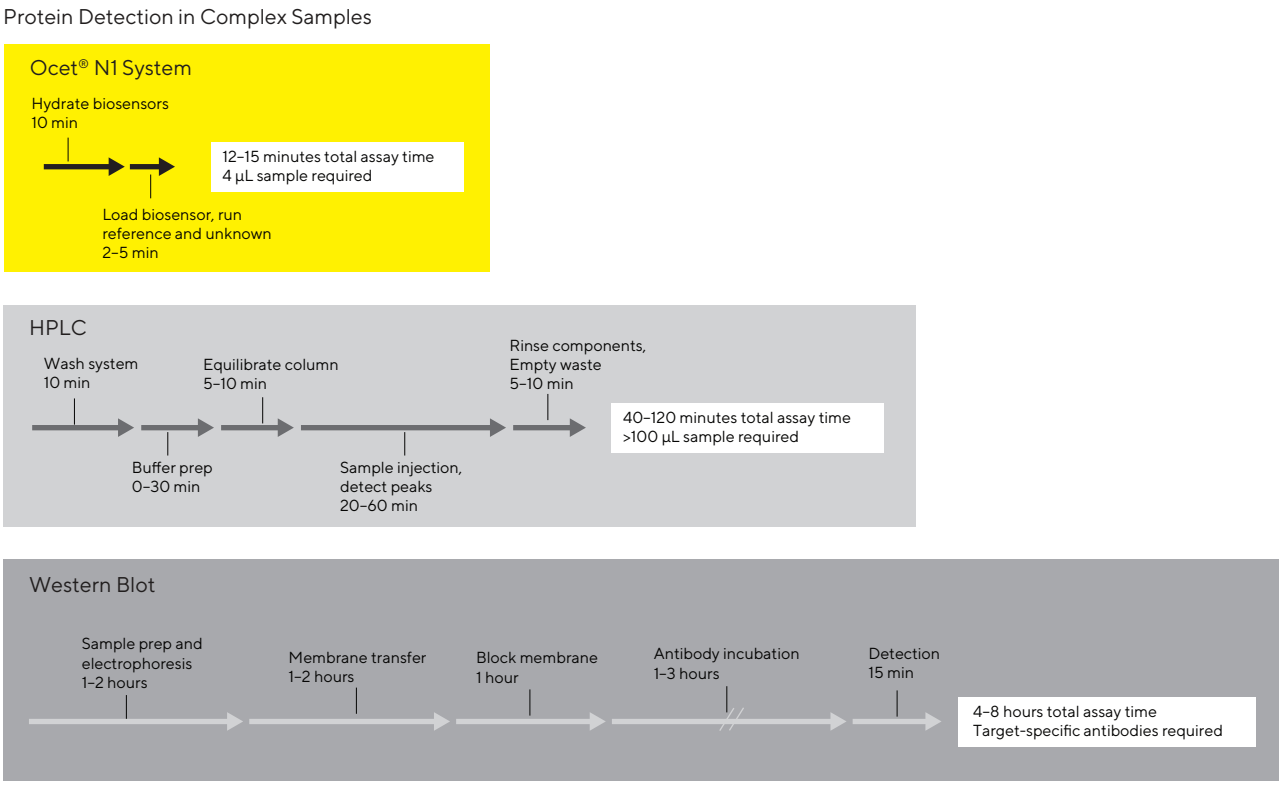


Figure 1: Comparison between protein detection using the Octet® N1 system and alternative methods.

Relative Expression Ranking

In all stages of biopharmaceutical discovery and development, there is a need for rapid qualitative analysis of samples. For example, identifying optimal secreting candidates from transfected CHO cells or selecting from numerous hybridoma cell clones for monoclonal antibody production can be challenging and labor intensive. Easy assessment of different growth conditions, expression systems and purification methods in bioprocess development can facilitate optimization for bioproduction. Similarly, manufacturing procedures require constant monitoring of expression levels in bioreactors.

The Quick Yes/No module on the Octet® N1 system provides a specific, fast and simple method for comparison of protein levels between samples. Relative amounts of antibody or target protein present in samples can be assessed in a matter of minutes based on binding to a pre-immobilized ligand on a biosensor, allowing for easy ranking of clones or conditions.

Here we use Anti-GST Biosensors for real-time detection and ranking of a GST-tagged protein spiked into conditioned CHO cell media at varying concentrations. The Anti-GST Biosensor (Sartorius part no. 18-5096) consists of a high affinity anti-GST antibody immobilized on the biosensor surface, allowing for specific label-free analysis of GST analytes, even in complex samples. For this experiment, GST-tagged Ubiquitin (EMD Millipore) was diluted in CD-CHO-DG44 culture media (Aragen Biosciences) to concentrations ranging from 2.5 µg/mL to 2000 µg/mL, each in triplicate. Anti-GST Biosensors were hydrated for a minimum of 10 minutes in the conditioned media prior to use. A 4 µL drop of each sample was analyzed using the Quick Yes/No module with shaker enabled. Octet® N1 Software was used to calculate binding rates from the real-time data.

The Anti-GST Biosensor binds to the GST tag on the target protein in a highly specific manner and differentiates between target protein and other media components, allowing for measurements to be made in unpurified samples. To minimize background response in a complex solution, biosensors must be pre-hydrated for at least 10 minutes in a matrix matched as closely as possible to that of the sample prior to use. Pre-hydration in the sample matrix minimizes signal from non-specific binding to the biosensor surface during an assay.

Figure 2 shows the real-time binding of GST-Ubiquitin to Anti-GST Biosensors in twelve samples. A greater concentration of target protein in the sample results in a faster binding rate. Binding rates are automatically calculated by Octet® N1 Software, making rapid ranking of expression levels possible. In Table 1, relative expression ranking and percent CVs are shown. Percent CVs for triplicate samples are low (< 11%) indicating excellent data reproducibility.

Protein Detection in Crude Samples for Construct Screening

The ability to pre-screen multiple vectors for protein expression on a small scale enables rapid identification of failures prior to scale-up, reducing time and expense as well as facilitating successful large-scale production of an increased number of proteins. To determine presence or absence of an expressed protein in cell culture, samples are typically analyzed using time-consuming procedures such as HPLC, multi-step ELISA or Western blot. Using the Octet® N1 system with BLI label-free technology, a target protein can be detected directly in crude and unpurified samples, such as *E. coli* cell lysate, in a matter of seconds with a high level of sensitivity.

A protein purification group at Novartis that studies histone demethylases (HDM) was interested in performing structural studies on a small HDM protein complex. Two HDM protein fragments were co-expressed in *E. coli* on separate vectors, with one of the constructs containing a HIS tag. The individual expressed fragments formed a tight protein complex. Visualization of soluble lysate on an SDS-PAGE gel, however, indicated little to no protein complex compared to the untransformed control (Figure 3). The available option for detecting protein from a poorly expressing construct was multiple purification runs over an HPLC column, which required substantial effort and material. As a simple solution for expression screening, the Octet® N1 system and Anti-Penta-HIS Biosensors were utilized.

Anti-Penta-HIS Biosensors (Sartorius part no. 18-5077) come with the highly specific Penta-HIS antibody from Qiagen pre-immobilized on the biosensor surface. The biosensor enables specific detection and quantitation of HIS-tagged proteins in purified or partially purified samples, cell culture supernatants or cell lysates. Binding of a HIS-tagged protein to the biosensor can be monitored in real time.

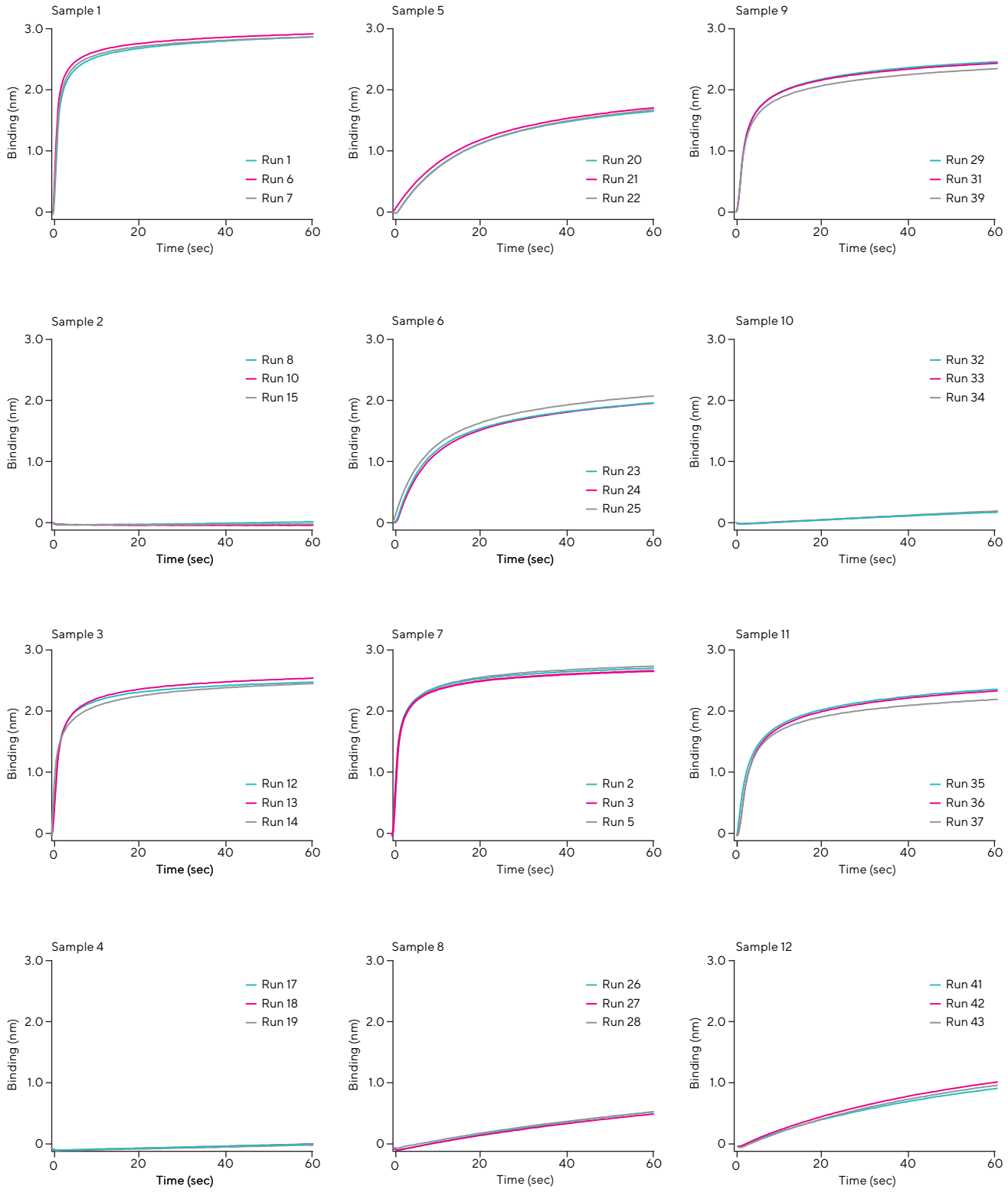


Figure 2: Real-time binding curves in triplicate showing detection of relative levels of GST-tagged Ubiquitin in twelve samples using Anti-GST Biosensors. Sample volume was 4 µL for each analysis.

E. coli cell cultures containing HDM constructs and untransformed negative control were harvested and lysed by resuspending in BugBuster™ reagent (Novagen) with benzonase and protease inhibitors. After clearing by centrifugation and filtration, total protein content was normalized via Bradford assay. Lysates were then diluted 1:10 with PBS buffer. Diluted negative control lysate was used to pre-hydrate biosensors. A 4 µL drop of each sample was loaded into the Octet® N1 system and detection performed using the Quick Yes/No module.

Typically, matrix effects can cause non-specific binding, increased drift, or interfere with the binding of a protein to the biosensor. When measuring proteins in crude samples such as complex media or cell lysate, it is recommended that the samples be diluted appropriately with Sartorius Sample Diluent (part no. 18-5028) to mitigate these effects. In this experiment, samples were diluted 1:10, however a dilution factor of 50 to 100-fold may be required depending on the nature of the samples and the type of biosensor being utilized.

Figure 4 shows real-time binding data for the HDM expressor and the negative control. Only the lysate sample containing the HDM protein constructs generates a positive signal when compared to the negative control, indicating the presence of the target protein complex in the co-expression line. Subsequent HPLC analysis showed the yield of HDM protein complex from this construct to be adequate for crystallography studies. In this example, the presence of target protein complex was detected in five minutes using a drop of sample, with no need for purification and with greater sensitivity than SDS-PAGE. Rapid detection of an expressed protein directly in crude samples demonstrates how the Octet® N1 system can save time and resources when evaluating constructs for scale-up.

Table 1: Expression ranking using the calculated binding rates. Percent CVs were calculated from the triplicate data shown in Figure 2.

Highest expression

Lowest expression

Rank	Binding rate	%CV
Sample 1	1.991	10.8%
Sample 7	1.646	2.4%
Sample 3	13.73	3.5%
Sample 9	0.864	3.2%
Sample 11	0.614	2.2%
Sample 6	0.230	3.7%
Sample 5	0.112	0.6%
Sample 12	0.031	4.3%
Sample 8	0.015	2.0%
Sample 10	0.004	4.3%
Sample 4	0.002	8.9%
Sample 2	0.000	NA

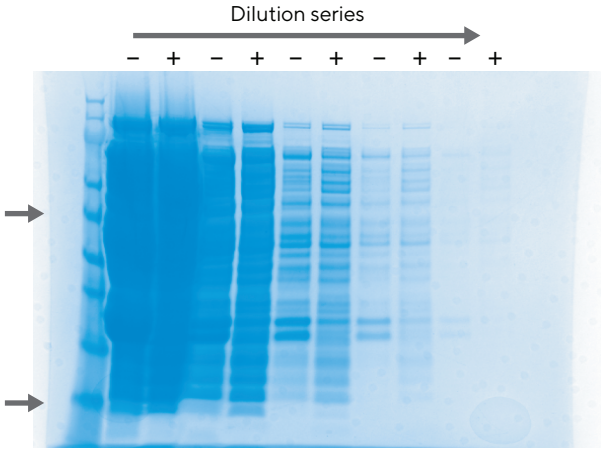


Figure 3: Coomassie stained SDS-PAGE gel analysis of HDM protein fragments co-expressed in *E. coli*. Soluble lysates from transformed (+) and negative control (–) cultures were normalized for protein content, then serially diluted and loaded onto the gel. Arrows indicate expected migration of the two fragments of the HDM complex. No obvious expressed bands can be visualized above background in transformed samples.

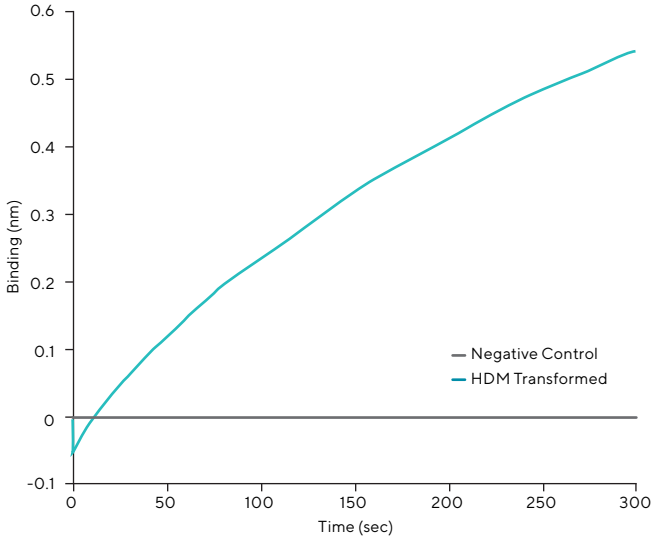


Figure 4: Real-time binding data from transformed and untransformed negative control *E. coli* lysates. Soluble lysates were normalized for protein content and diluted 1:10. A 4 µL drop per sample was loaded, then binding to Anti-Penta-HIS Biosensors was measured in real time for 300 seconds. Data was reference-subtracted using the negative control sample. The positive nm shift in the transformed lysate indicates the presence of the HIS-tagged HDM protein complex.

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

Often in production of recombinant IgG and Fab fragments, over-expression of light chains can become an issue. Because the majority of available Fab binding agents target epitopes that reside on the antibody light chain, accurate quantitation becomes problematic due to ligand cross-binding to contaminating free light chains (Figure 5). By using an affinity ligand that targets the CH1 domain on the heavy chain of a Fab fragment, this problem can be eliminated. Sartorius recently launched its Anti-Human Fab-CH1 Dip and Read Biosensor for 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 and simple method for differentiation of Fab/F(ab')2 fragments and contaminating light chain species.

To demonstrate the high level of specificity of the Anti-Human Fab-CH1 Biosensors, the effect of free light chains on the detection of a human Fab fragment was investigated. A purified Fab fragment derived from whole human IgG (Jackson ImmunoResearch) was diluted to 10 µg/mL in Sample Diluent (Sartorius part no. 18-5028). Sample Diluent 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 at least 10 minutes.

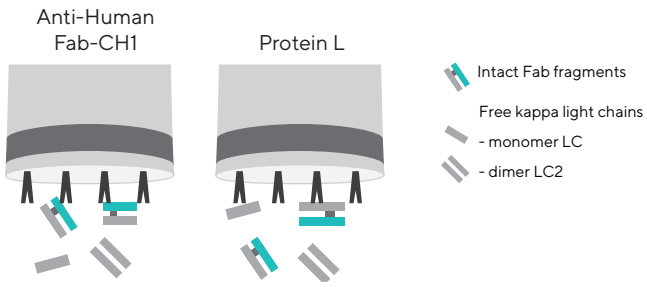


Figure 5: Selectivity of Anti-Human Fab-CH1 vs. Protein L Biosensors for intact Fab fragments.

Purified free human kappa light chain from Bence Jones protein (Meridian Life Science) was spiked into Fab fragment samples to final concentrations ranging from 0.5 µg/mL to 50 µg/mL. Binding analysis was performed on Protein L and Anti-Human Fab-CH1 Biosensors using the Quick Yes/No module for 60 seconds per sample with shaker enabled.

Results of the binding comparison experiment are displayed in Figures 6 and 7. Figure 6 shows real-time binding data for human Fab samples to each biosensor type. Note that the binding curves were unaffected on the Anti-Human Fab-CH1 Biosensors, as compared to the Protein L Biosensors where the binding response increased. This is suggestive of co-binding of free light chains by Protein L. A graph comparing binding rates between the two biosensors is shown in Figure 7. These data clearly show that increased binding rate to Protein L Biosensors corresponds to higher concentration of free light chain, whereas binding to Anti-Human Fab-CH1 remains constant.

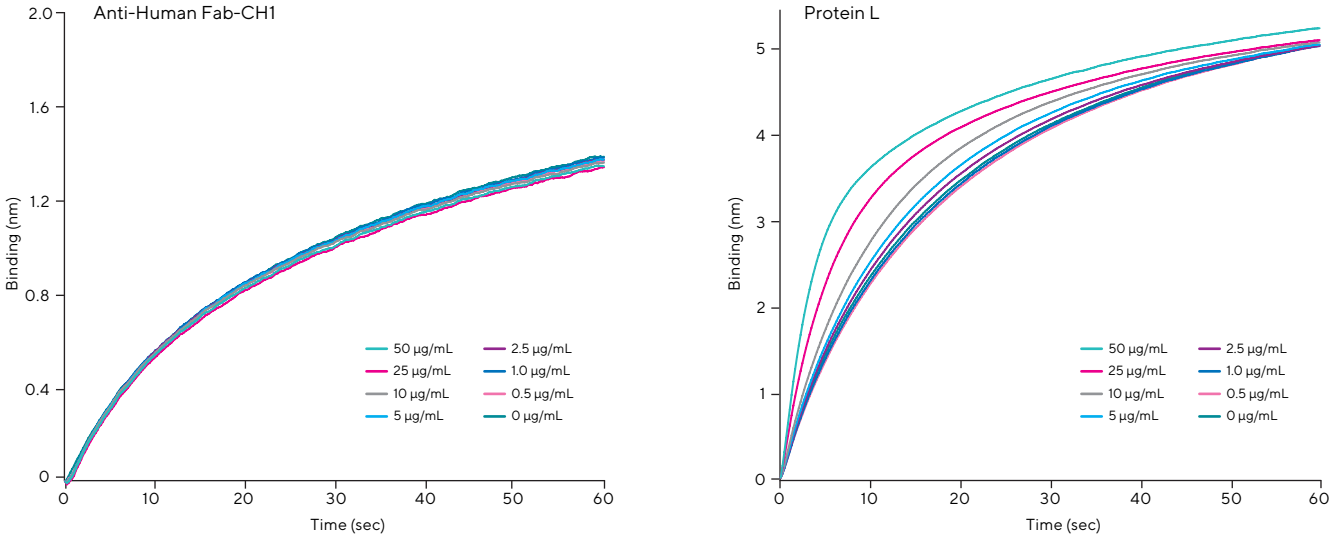


Figure 6: Effect of free kappa light chains on detection of human Fab fragments using Anti-Human Fab-CH1 Biosensors (left) compared to Protein L Biosensors (right). Varying levels of human kappa light chain were spiked into samples containing 10 µg/mL human Fab fragment and analyzed on both biosensor types. Real-time binding data is shown. Increased response on Protein L Biosensors indicates cross-reactivity to free light chains and consequently, unreliable results for specific measurement of Fab concentration.

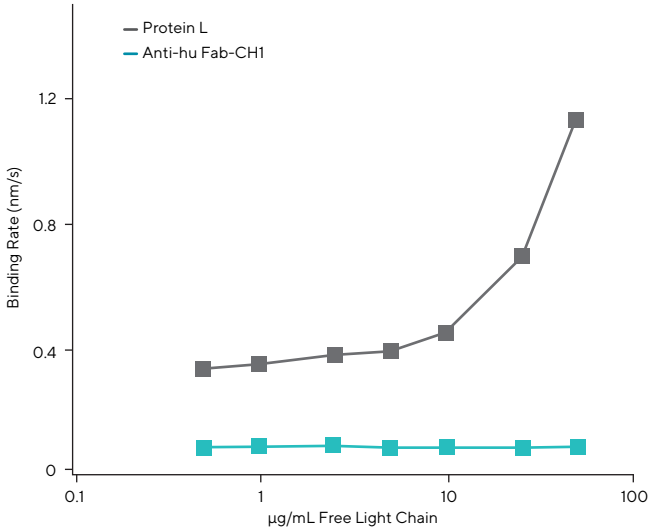


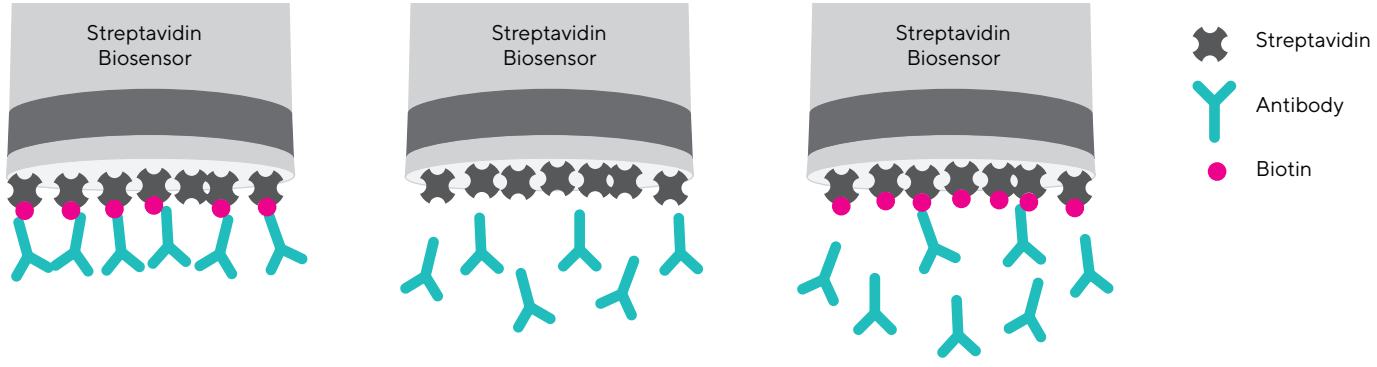
Figure 7: Effect of free kappa light chains on detection of human Fab fragments using Anti-Human Fab-CH1 Biosensors compared to Protein L Biosensors. Varying levels of human kappa light chain were spiked into samples containing 10 µg/mL of human Fab fragment and analyzed on both biosensor types. Increased response on Protein L Biosensors indicates cross-reactivity to free light chains.

This high specificity of Fab binding makes the Anti-Human Fab-CH1 Biosensor an extremely useful tool for pre-purification detection and analysis of Fab samples. A range of biosensors are available for use on the Octet® N1 system with high 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. In addition, Streptavidin Biosensors can be loaded with a biotinylated molecule of choice, for an unlimited range of assays.

Streptavidin Biosensors for Confirmation of Protein Biotinylation

Biotinylation of proteins is a common strategy in many protein research applications as a way to take advantage of the extremely high affinity interaction of biotin to avidin and streptavidin. Biotin and avidin/streptavidin have become standard reagents for a diverse array of detection and immobilization methods utilized in applications such as ELISA, Western blot, immunoprecipitation, affinity purification and flow cytometry. A variety of biotinylation reagents with different functional group specificities are available for biotin conjugation to proteins. However, issues can arise with biotinylation procedures including protein loss, inactivation and uncertainty as to whether the conjugation reaction was successful (Figure 8).

The Quick Yes/No module on the Octet® N1 system combined with Streptavidin Biosensors (Sartorius part no. 18-5019) provides a useful tool to quickly check for successful biotinylation. Confirmation of binding to Streptavidin Biosensors can be assessed in a matter of seconds, and only 4 µL of reaction mixture is required. To illustrate, a human/mouse IL-5 monoclonal antibody (R&D Systems) was biotinylated at a 1:1 molar coupling ratio (MCR) using EZ-Link® NHS-PEG4 biotin (Thermo Scientific). A sample with no biotin was also run in parallel as a negative control. Reactions were then desalted using Zeba desalting spin columns (Thermo Scientific). After



Successful biotinylation: Biotinylated antibodies bind to streptavidin, giving a strong positive binding response.

Unsuccessful biotinylation: No biotin present to attach antibodies to streptavidin. Zero binding response.

Successful biotinylation without desalting: Free biotin competes for binding to streptavidin. Zero to very low binding response.

Figure 8: Use of Streptavidin Biosensors and the Quick Yes/No module to confirm successful bioconjugation reaction. Biotinylation of an antibody is used to illustrate three possible scenarios: successful biotinylation and desalting, unsuccessful biotinylation or successful biotinylation without desalting. A strong binding response will only be observed upon successful biotinylation followed by efficient desalting to remove unincorporated biotin.

desalting, concentrations for each sample were determined spectrophotometrically and normalized with PBS. Streptavidin Biosensors were pre-wet for at least 10 minutes in Sample Diluent. Biotinylated IL-5 mAb samples were then diluted to 100 µg/mL using Sample Diluent. Loading on Streptavidin Biosensors was performed in 4 µL of each sample with shaking for 30 seconds.

Figure 9 shows the real time run data for loading biotinylated IL-5 mAb onto the Streptavidin Biosensors. No binding response was evident in the sample without biotin. In contrast, the biotinylated sample showed a very strong binding response, demonstrating that a successful conjugation reaction can be quickly confirmed.

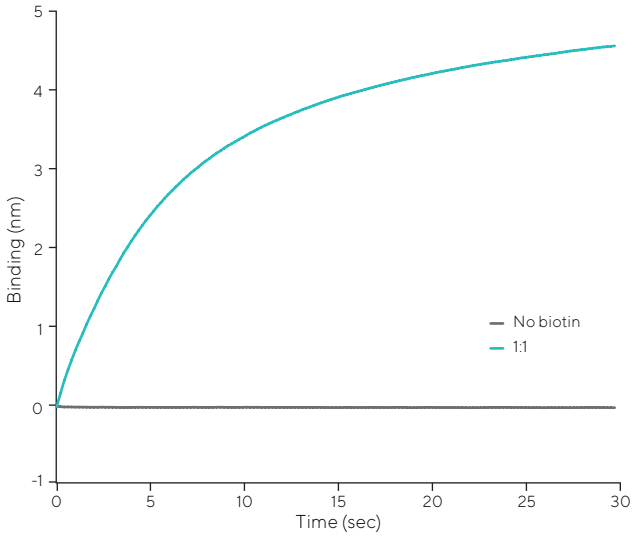


Figure 9: Effect of biotinylation on binding to Streptavidin Biosensors. IL-5 monoclonal antibody was biotinylated using a 1:1 molar coupling ratio. A sample with zero biotin gives no signal, however the conjugated sample shows a strong binding response.

Tips for Running Quick Yes/No Analysis for Protein Presence

- Cell lysates or supernatants must be pre-cleared of insoluble debris prior to analysis.
- Dilute crude samples such as cell lysates appropriately to mitigate matrix effects. Dilution recommendations are biosensor-dependent and can be found in corresponding biosensor technical notes
- 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 surface.
- Run a reference or negative control sample that matches the matrix of the sample to be analyzed but does not contain the protein of interest. This will allow for subtraction of any response signal generated by non-specific binding to the biosensor surface.

Conclusion

We have demonstrated several applications in which the Quick Yes/No module on the Octet® N1 system can be utilized to improve workflows when paired with a wide selection of pre-immobilized biosensors. The ability to qualitatively measure target proteins directly in complex matrices using 4 µL of sample allows rapid, simple and powerful analyses on the Octet® N1 system that is not possible with other platforms.

References

1. Octet® N1 System Quick Start Guide: Provided with all Octet® N1 systems.
2. Octet® N1 System Demo and Starter Guide: Provides detailed step-by-step guidance on setting up assays using the Octet® N1 system starter kit.
3. Sartorius Technical Note 28: Biotinylation of protein for immobilization onto Streptavidin Biosensors.
4. Sartorius Technical Note 10: Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors.
5. Sartorius Technical Note 11: Biotinylating in Antibody Stocks Containing Carrier Protein.

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