Instant determination of protein presence using the BLItz system

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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 BLItz™ system for instant, specific protein detection in crude matrices, enabling access to real-time sample information during bioprocess development and production.

About the BLItz system

The BLItz 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 BLItz system utilizes the same proprietary Bio-Layer Interferometry (BLI) technology as ForteBio’s 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 BLItz Pro™ Data Analysis 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 BLItz 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).

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 BLItz 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 (ForteBio 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. BLItz Pro 1.1 Data Analysis 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. A 4 µL drop of each sample was analyzed using the Quick Yes/No module with shaker enabled. BLItz Pro 1.1 Data Analysis software was used to calculate binding rates from the real-time data.

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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.
Anti-Penta-HIS biosensors (ForteBio 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.

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 BLItz 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 ForteBio 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.

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

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

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. ForteBio 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 BLItz 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 (ForteBio part no. 18-5028). Sample Diluent was also used to pre-wet Anti-Human Fab-CH1 biosensors (ForteBio part no. 18-5104) and Protein L biosensors (ForteBio part no. 18-5085) for at least 10 minutes.
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. 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 BLItz system with high specificity for analysis in complex matrices. For example, Anti-Mouse IgG Fc (ForteBio part no. 18-5088) and Anti-Human IgG Fc (ForteBio 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 BLItz system combined with Streptavidin biosensors (ForteBio 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 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.

**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 (fortebio.com/literature.html).
- 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 BLItz 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 BLItz system that is not possible with other platforms.

References

2. BLItz Demo and Starter Guide: Provides detailed step-by-step guidance on setting up assays using the BLItz starter kit.

BLItz system ordering and contact information

- To request a quote, visit www.fortebio.com/blitz.html and click Get Quote.
- For a list of available biosensor types, visit www.fortebio.com/biosensor-types.html.
- For technical support, go to www.fortebio.com/support.

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.

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.