Label-free Analysis from Microliter Amounts of Sample

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Abstract:

Bio-layer interferometry (BLI) has gained popularity in recent years as a reliable method for analyzing protein-protein and other biomolecular interactions. Like other label-free techniques, BLI has the advantage of probing unmodified proteins, thus eliminating potential perturbation by labels of the interactions being studied. We have developed new technology using BLI that enables rapid identification, quantitation and characterization of expressed protein from as little as 4 microliters of sample. New methods and data will be presented to illustrate the ease of incorporating BLI into a laboratory workflow.

Introduction:

BLItz instrument – A need was identified for an easy to use, compact, bench-top instrument for performing protein characterization in microliter amounts of sample. Utilizing label-free detection is attractive due to the low sample preparation time, the ability to work in a crude sample matrix and the real-time information obtained on amount, activity and specificity of the unknown. The BLItz instrument (**Figure 1**) is the result of a technology development project to satisfy all of these desires. The instrument is small (approximately 15 X 22 cm) and will fit most bench scientist's available space. The instrument is run manually, without complicated robotics or microfluidics, providing an easy to learn and easy to use platform.

Most importantly, the instrument allows for the analysis of presence, quantity, activity and specificity of a protein of interest from as little as 4 µL of sample. Data from each of these applications is outlined below.

Figure 1: The BLItz instrument

Figure 2: Biosensor testing a 4 µL sample

Biosensors – The BLItz instrument was designed to use the same biosensors (example in **Figure 2**) as the other instruments in ForteBio's product line. This allows for maximum flexibility in assay design taking advantage of the 12 factory immobilized biosensors available.

- Streptavidin
- Protein A
- Protein L
- Protein G
- Anti-hIgG Fc
- Amine reactive 2G
- Anti-mIgG Fv
- Anti-GST
- Anti-Penta-His
- Aminopropylsilane
- Anti-mIgG Fc Capture
- Anti-hIgG Fc Capture

BLI technology – Bio-layer interferometry (BLI) is an analytical technique that monitors the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer (**Figure 3**). Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern. This change, measured in nanometers (nm), is reported in real-time.

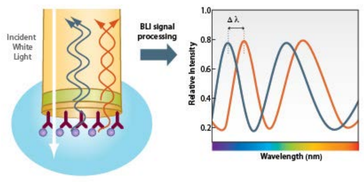

Figure 3: Bio-layer interferometry

Kinetics:

The ability to characterize the interaction between proteins can provide valuable information for further research decisions. Simply by inspecting real-time binding curves, information on binding and dissociation can be determined. Shown in **Figure 6** are the association and dissociation curves for 5 antibodies to a single antigen. All five antibodies were analyzed directly from crude cell culture supernatants. From this data, Culture 5 (red) clearly shows the highest binding intensity during association (step 2) and one of the tightest binding interactions with minimal loss during dissociation (step 3).

A follow-up experiment was performed using the clone from Culture 5 to determine kinetic parameters (**Figure 7**). By performing both of the assays on BLItz, both screening and kinetic analysis can be obtained from less than 12 µL of sample.

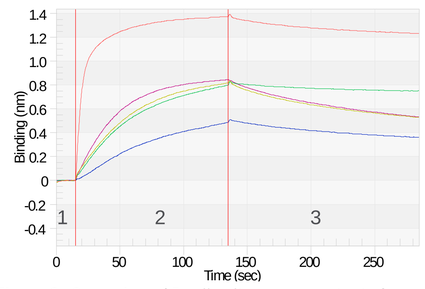

Figure 6: Screening of 5 cell culture supernatants for expression of antibodies specific for the immobilized antigen A21. Culture 5 (red) shows higher activity and a slow off-rate.

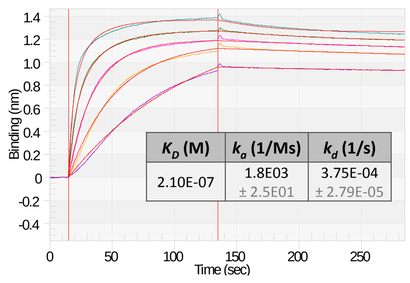

Figure 7: Full kinetic analysis of clone showing highest binding and slow off-rate (Culture 5).

Materials and Methods: Cell culture supernatants and antigen were a generous donation from Oncomed Inc, CA. The antigen was biotinylated using a standard protocol. Biotinylated antigen was immobilized onto Streptavidin biosensors (ForteBio PN 18-5019) off-line. Baseline, Association and Dissociation steps were performed in the BLItz instrument with the shaker enabled using the following protocol: Baseline (15 seconds, in tube of blank media), Association (120 seconds in 4 µL drop of sample), Dissociation (150 seconds in tube of blank media). Kinetic analysis was performed using a 2 fold dilution of clone from culture 5. Analysis was performed in BLItz Pro 1.0 software using a blank media run as a baseline reference.

Quick Yes/No Application:

In the laboratory, there is often a need for qualitative or semi-quantitative analysis of samples mid-process. The data presented in **Figure 4** show the real-time detection of a GST-tagged protein (Ubiquitin) in CD-CHO expression media using Anti-GST biosensors. By determining the binding rate of the unknown GST-Ubiquitin to the anti-GST biosensor in each of the 12 samples, one can rapidly rank the levels of expression in each sample (**Table I**). The specificity provided by the Anti-GST biosensor allows for the specific detection of GST-Ubiquitin in the presence of complex media in 4 µL of sample.

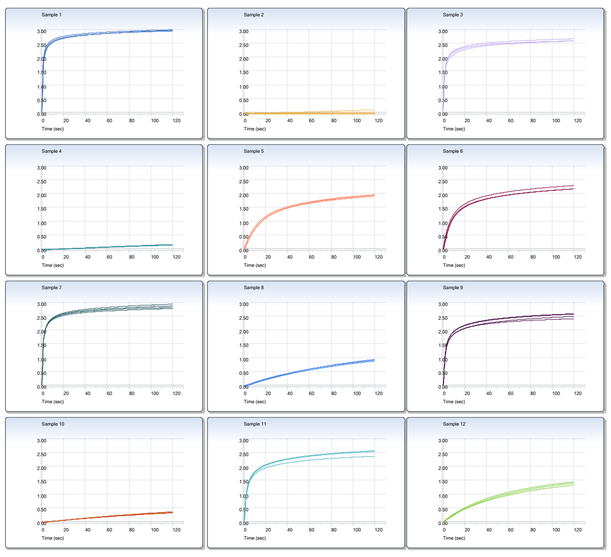

Figure 4: Triplicate analysis for levels of GST-tagged Ubiquitin in 12 samples using the GST biosensor on BLItz. Sample volume was 4 µL for each analysis.

Table I: Expression ranking using the calculated binding rates. Percent CVs shown were calculated from the triplicate data shown in Figure 4.

Highest expression

Lowest expression

Rank	Binding Rate	%CV
Sample 1	1.991	10.8%
Sample 7	1.646	2.4%
Sample 3	1.373	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

Materials and Methods: Anti-GST biosensors (ForteBio PN 18-5096) were hydrated in a control media sample CD-CHO-DG44 media (Aragen Biosciences, CA) for a minimum of 5 minutes prior to use. All samples were in CD-CHO-DG44 media and were analyzed with the shaker enabled for 120 seconds. BLItz Pro 1.0 software was used to calculate binding rates from the real-time data. Coefficients of variation (%CV) were calculated based on the triplicate assays for each sample.

Assay Development:

Screening antibodies for viable binding pairs and kinetic analysis of these interactions is a regular part of laboratory assay development.

The data presented in **Figure 8** show the screening of two IL-5 capture antibodies against two detection antibodies using Streptavidin biosensors. With the real-time binding analysis, it is simple to determine which antibodies form viable pairs by observing positive binding of the detection antibody in step 6. In addition, the ability of each capture to bind IL-5 can be determined in step 4 and the relative strength of the complete interaction can be assessed from step 7.

Other valuable information, such as the kinetic parameters of the capture antibody to analyte interaction, can be determined as shown in **Figure 9**.

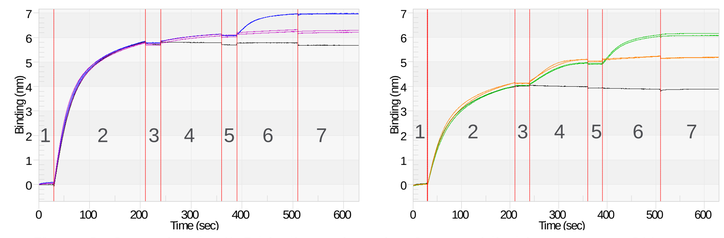

Figure 8: Screening for IL-5 binding pairs using Streptavidin biosensors on BLItz. MAB205 (left) and MAB405 (right) used as capture (step 2), followed by IL-5 (step 4) and detection antibody binding (step 6).

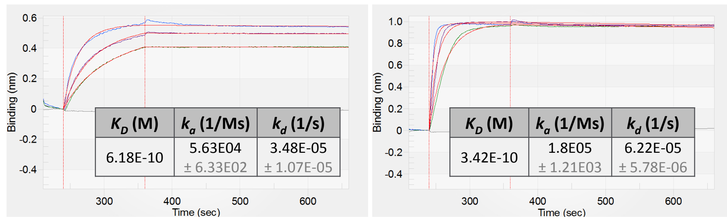

Figure 9: Kinetic analysis of MAB205 (left) and MAB405 (right) antibodies binding to IL-5.

Materials and Methods: Antibodies and Interleukin 5 (IL-5) were from R&D Systems, MN (capture antibodies PN MAB205, MAB405; detection antibodies PN BAM6051, AF205; IL-5 PN 205-IL). Capture antibodies were biotinylated using a standard protocol. Sample Diluent (ForteBio PN 18-1000) was used for all dilutions, baselines and hydrations. A typical antibody pair analysis was performed using Streptavidin biosensors (ForteBio PN 18-5019) on the BLItz instrument for the following steps: Baseline (30 seconds), loading of biotinylated capture (180 seconds) baseline 2 (30 seconds), analyte binding (120 seconds), dissociation check for analyte (30 seconds), binding of detection antibody (120 seconds), complex dissociation (120 seconds). Kinetic analysis performed using Streptavidin biosensors immobilized with the two capture antibodies. A two fold dilution series of IL-5 Sample Diluent starting at 888 nM was assayed using a baseline (30 seconds), association (120 seconds) and dissociation (300 seconds). A reference of Sample Diluent was used for baseline correction. Kinetic parameters calculated using BLItz Pro 1.0.

Quantitation:

Quantitation of a specific protein in the presence of a complex or high protein background is a challenge for A280 or other general laboratory methods. By taking advantage of the specificity inherent in the capture molecules pre-immobilized on the biosensor, the protein of interest can be specifically detected and quantified.

Figure 5 shows the real-time data and resulting dose response for detection and quantitation of human IgG (hIgG) in the presence of 1 mg/mL bovine serum albumin (BSA). Real-time data is shown in triplicate and the dose response is shown for the average of the triplicate measurements. Examples of other proteins assayed with various biosensor types is shown in **Table II**. Dynamic range and precision is shown for triplicate measurements.

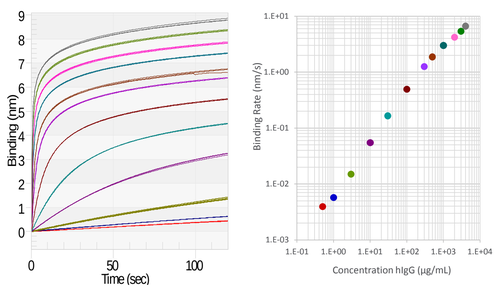

Figure 5: Triplicate analysis of 4 µL of human IgG using Protein A biosensors on the BLItz instrument. Analysis was performed in the presence of 1 mg/mL BSA at 4000, 2000, 1000, 500, 300, 100, 30, 10, 3, 1 and 0.5 µg/mL hIgG. Real-time binding curves (left) and resulting dose response curve (right) are shown.

Table II: Dynamic range and precision data from six biosensor types.

	Biosensor used	Anti-GST		Protein A		Protein G		Protein L		Anti-Human Fc		Anti-Murine Fv	
	Analyte detected	*GST-Ubiquitin*		*human IgG*		*mouse IgG*		*human IgG*		*human IgG*		*mouse IgG*	
		BR†(nm/s)	%CV	BR†(nm/s)	%CV	BR†(nm/s)	%CV	BR†(nm/s)	%CV	BR†(nm/s)	%CV	BR†(nm/s)	%CV
Dynamic range	0.25 µg/mL	---	---	---	---	---	---	---	---	0.001	4%	---	---
	0.5 µg/mL	0.004	4%	0.004	5%	0.001	1%	0.001	1%	0.002	1%	0.002	3%
	1 µg/mL	0.015	2%	0.006	4%	0.002	1%	0.002	4%	0.005	5%	0.004	1%
	3 µg/mL	0.031	4%	0.015	3%	0.007	7%	0.007	3%	0.011	2%	0.012	4%
	10 µg/mL	0.112	1%	0.055	1%	0.024	2%	0.023	3%	0.038	2%	0.045	2%
	30 µg/mL	0.229	4%	0.165	4%	0.070	3%	0.070	2%	0.099	4%	0.124	3%
	100 µg/mL	0.614	2%	0.496	3%	0.238	2%	0.273	1%	0.269	1%	0.324	1%
	300 µg/mL	0.864	3%	1.256	2%	0.642	2%	0.678	2%	0.546	1%	0.702	2%
	500 µg/mL	1.373	4%	1.869	2%	0.945	0%	1.040	1%	0.697	3%	0.841	1%
	1000 µg/mL	1.646	2%	2.997	2%	1.520	2%	1.838	2%	1.220	3%	1.160	1%
	2000 µg/mL	---	---	4.189	1%	2.618	3%	2.930	3%	---	---	1.763	5%
	4000 µg/mL	---	---	6.639	2%	3.701	6%	---	---	---	---	2.154	3%

†Binding rate (BR) calculated by BLItz Pro 1.0

Materials and Methods: All biosensors were from ForteBio, Inc. Human IgG and mouse IgG from Lampire Biological Laboratories, PA. Typical analysis was performed by diluting analyte into Sample Diluent (ForteBio PN 18-1000) at specified concentrations. Each 4 µL sample was analyzed in triplicate with the shaker enabled and a read time of 120 seconds on the BLItz instrument. BLItz Pro 1.0 software was used to calculate binding rates (BR). Coefficients of variation (%CV) were calculated based on the triplicate assays. Dynamic range was defined as a %CV of less than 10% and a separation of at least 3 times standard deviation from neighboring concentrations.

Conclusions:

- The results shown demonstrate the **flexibility of the compact BLItz instrument** when paired with the factory immobilized biosensors.
- Data from **Anti-GST, Protein A, Protein G, Protein L, Anti-mIgG Fv, Anti-hIgG Fc** and **Streptavidin biosensors** have been presented.
- Applications showing use of the BLItz instrument for **presence, quantity, binding kinetics** and **assay development** have all been presented to illustrate the utility of the instrument as a general purpose characterization platform.
- We envision this platform could be used in many other applications in a normal laboratory workflow. Some examples are:
  - Identification of high expressing clones for antibodies, antibody fragments or fusion proteins.
  - Confirming presence of a specific protein after purification or concentration.
  - Testing for presence and activity after biotinylation or other modification
  - Kinetic characterization of tagged, biotinylated or PEGylated proteins
  - Quantitating antibodies or antibody fragments in complex samples.
  - Optimizing buffer, salt and wash conditions for immunoassays.

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