

Customized quantitation of recombinant therapeutic proteins using High Precision Streptavidin biosensors (SAX)

Steve Turner¹, Scientist I, Weillei Ma², Senior Scientist, Nick Moss¹, Director Instrumentation Technology, John Welsh¹, Senior Manager, R&D and David O Apiyo², Marketing Applications Manager

¹ Pall Life Sciences, ² ForteBio

Introduction

The accurate determination of recombinant protein titer is critical to the selection of high-producing clones during cell line development and in optimization of bioreactor conditions during production of therapeutic proteins. Concentration measurements for these therapeutic proteins are often done using enzyme-linked immunosorbent assays (ELISAs), RP-HPLC or coomassie-stained SDS-PAGE gels. Although these techniques are prevalent, they are encumbered by drawbacks that include long assay times, extensive hands on and in some cases, low throughput. In addition, techniques such as ELISA tend to exhibit high variability, resulting in lower accuracy.

Bio-Layer Interferometry (BLI) is an optical analytical technique that utilizes the changing interference pattern of white light shown over a reflective biosensor surface immobilized with a ligand to detect interactions with an analyte in solution. The binding between the ligand and the analyte produces an increase in optical thickness on the tip of the biosensor that can be measured as a wavelength shift from the reference surface, and is a direct measure of the change in thickness of the biological layer upon binding events (Figure 1).

Octet® instruments utilize BLI technology and are a suitable replacement for both ELISA and HPLC techniques for quantitation of recombinant proteins. Octet systems are high-throughput (up to 96 samples can be analyzed at a time), accurate, fast and are relatively easy to use. Moreover, due to their sample plate-based format, Octet instruments can be used to accurately quantitate recombinant proteins in their crude form, allowing for rapid analysis of bioreactor samples during cell line development.

The measured wavelength shift is directly proportional to the concentration of the binding analyte.

This Application Note demonstrates the use of BLI in the quantitation of five biologically-relevant protein-based drug molecules: Insulin, Factor VIII, Factor IX, Erythropoietin (EPO) and Granulocyte Colony Stimulating Factor (GCSF). We specifically illustrate the use of BLI in monitoring concentration during protein expression and hence cell line development for these five proteins, however, the methods described here can, with proper assay development, be used for other recombinant proteins. Although only single-analyte step detection methods typically used for relatively high sample concentrations are discussed,

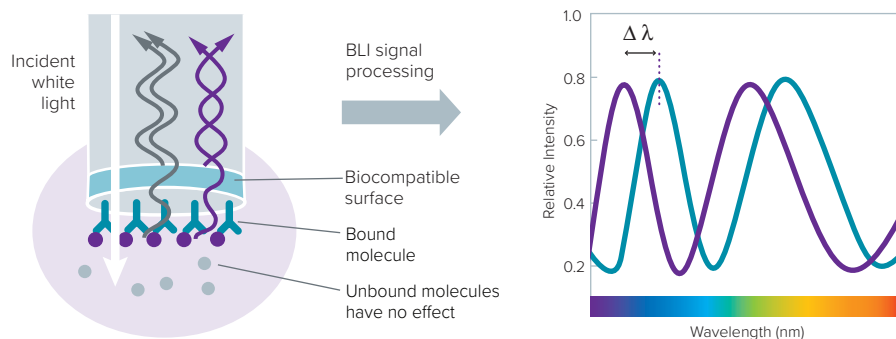


Figure 1: Relative intensity of light reflection pattern on a biosensor surface using BLI.

the technique can be modified to include signal amplification steps that can be used to increase assay sensitivity beyond what is reported.

Insulin, Factor VIII, Factor IX, GCSF and EPO

Human Insulin, produced by beta cells of the pancreas, is a relatively small 5.8 kDa protein. It plays a critical regulatory role in the control of glucose homeostasis and is involved in regulating carbohydrate and fat metabolism in the body. While Insulin plays a regulatory role in the body, Factor VIII and Factor IX are both blood coagulants that act as anti-hemophilic factors (AHF). A deficiency in these two proteins can lead to Hemophilia A and B for Factor VIII (280 kDa) and Factor IX (55 kDa) respectively. In addition to these three proteins, EPO and GCSF also play critical roles in the body. EPO is a 34 kDa glycoprotein produced in the adult kidney and in fetal liver. It controls erythropoiesis by stimulating maturation of erythroid precursor cells in bone marrow. Its interactions with receptors expressed on the cell surface results in increased numbers of red blood cells¹. GCSF (18.7 kDa) is also a glycoprotein but is produced by a variety of tissues and tumor cell lines that regulate hematopoiesis and is required for the clonal growth and maturation of normal bone marrow cell precursors. Commercial forms of GCSF such as filgrastim and biosimilars are clinically used to induce granulopoiesis in conditions such as myelosuppressive chemotherapy, acute myeloid leukemia and severe chronic neutropenia². Together these five proteins form part of an ever growing list of proteins used for therapeutic purposes.

In general, recombinant proteins intended for therapeutic use are often produced in mammalian host cells, mainly Chinese hamster ovary (CHO) cells. Recombinant human EPO is now available in sufficient amounts for clinical use in the treatment of anemia that results from chronic renal failure³. Accurate quantitation techniques for these proteins are therefore critical in enhancing their rapid development.

Materials and reagents

All biotinylated antibody reagents were obtained from Life Technologies and are 13 kDa camelid antibody fragments specific to each antigen and selective for both recombinant and plasma derived proteins: Part nos 7103362500 (insulin), 7103130500 (GCSF), 7103372500 (EPO), 7103002500 (Factor IX) and 7102862500 (Factor VIII).

Recombinant insulin (Part no. cyt-270) and Factor VIII (Part no. pro-318) were both obtained from ProSpec. EPO (Part no. 100-64) and GCSF (Part no. 300-23) were sourced from Pepro-Tech and recombinant Factor IX was sourced from Cambridge Bioscience.

EX-CELL® NS0 Serum-Free Medium for NS0 Cells, Dulbecco's Modified Eagle's Medium-high glucose, EX-CELL® CD CHO-3 Medium, Chemically Defined, and EX-CELL® 293 Serum-Free Medium for HEK 293 Cells were obtained from Sigma. SFX-CHO HyClone and B-PER Reagents were obtained from ThermoFisher Scientific and CD DG44 Medium was sourced from Life Technologies.

Instruments

ForteBio's Octet RED384 and Octet RED96 systems.

Agilent Zorbax 300SB-C18 RP-HPLC column; 5 µm, 4.6 × 250mm.

Method

The method discussed here can be applied to all Octet instruments. Data is acquired using the advanced quantitation mode in Octet Data Acquisition software. High-precision Streptavidin (SAX) biosensors are first hydrated in the relevant assay matrix prior to immobilization or loading of biotinylated antibody for each respective antigen. To achieve optimal assay conditions, antibody loading studies are first performed and are used to determine optimal antibody loading concentrations. Refer to ForteBio *Technical Note 40: High Precision Streptavidin Biosensor (SAX) Quantitation and Kinetic Assays* for more information. This process is applicable to any custom quantitation of recombinant proteins using the Octet platform. Once the optimal antibody loading concentration is determined, the assay is run as stipulated in Table 1.

ANTIBODY LOADING ON SAX BIOSENSORS

In these studies, commercially available biotinylated camelid VHH antibody fragments were used. However, antibody biotinylation can be done using reagents that target the antibody's primary amino groups. The most commonly used reagents are NHS-esters of biotin with spacer arms or linkers that allow for optimal orientation of the antibody once immobilized onto the biosensor surface. For an in-depth understanding of the protein biotinylation process, refer to ForteBio *Technical Note 6: Biotinylation of Protein for Immobilization onto Streptavidin Biosensors*. The antibody should be protein carrier-free and should be in an amine-free buffer. For optimal performance it is crucial to remove all free biotin after the biotinylation reaction, either by dialysis or through the use of a desalting column. To achieve consistent antibody biotinylation, the number of biotins per antibody molecule should be kept at a minimum. An initial antibody load scouting assay (Figure 2), where the antibody concentration is varied, typically around 100 nM or 15 µg/mL with concentrations above and below 15 µg/ml while the analyte

concentration is left constant should be performed. The goal is to saturate biosensor surface and select the lowest antibody concentration (devoid of assay artifacts) that yields maximum and reproducible analyte response signals.

RECOMBINANT PROTEIN CONCENTRATION ANALYSIS

Protein concentration determination on Octet systems requires the development of a standard reference curve. A standard reference curve is obtained using a purified standard sample of the respective protein. Known concentrations of the purified standard sample are spiked into the matrix of interest and are serially diluted (often two-fold) within the desired concentration range using an analyte depleted assay matrix.

A biosensor blocking step using biocytin that blocks non-reacted streptavidin molecules or using BSA in the binding matrix/buffer may be necessary post antibody capture to prevent non-specific binding (NSB). In addition, the use of detergents in the sample diluent to prevent potential non-specific hydrophobic interactions is recommended especially when dealing with glycoproteins. NSB refers to either the binding of the analyte to a biosensor surface not coated with the specific antibody or to observation of an appreciable binding of the analyte-depleted matrix to the antibody-coated biosensor surface and should be investigated and eliminated for each assay during development. If blocking is performed, a short baseline step should be established in the analyte sample matrix prior to sample detection.

DATA ANALYSIS

Octet Data Analysis software allows users to select between one of two binding rate analysis models: an initial slope of binding rate analysis and an end-point R-equilibrium analysis. The initial slope option should always be used when the analyte binding step is a simple single step post antibody loading. If the assay is modified to allow for a signal amplification step such as what would be used in a higher sensitivity assay, the detection step should be allowed to reach equilibrium, and the R-equilibrium binding rate model used to analyze the data. The analyzed data can be fit using one of several models; however, care should be taken to ensure that accurate analyte concentration is determined off the linear portion of the standard curve. The unknown analytes concentration is extrapolated off the generated standard curve.

Assay protocol

To start the Octet instrument, first turn on the computer followed by the instrument. Wait at least 1 hour for the lamp to warm up before running an experiment.

- 1 Hydrate SAX biosensors in assay matrix for at least 10 minutes.
- 2 Load samples onto the sample plate using 200 μ L per well for 96-well microplates and 80 μ L (Greiner microplates, Part no. 655209) or 40 μ L per well (ForteBio 384-well, black, tilted-bottom polypropylene microplates, Part no. 18-5076) when using 384-well microplates.
- 3 Open Octet Data Acquisition software to design the method.
- 4 In the Experiment Wizard window, select the **New Quantitation - Advanced Quantitation** method option. Next, click the green **forward** arrow.
- 5 In the plate definition window, click **Modify** to input assay parameters.
- 6 Under available assays, select **Standard Assay**.
- 7 Under assay parameters, insert cells using the **Insert** command. Use the pull down arrow to select assay steps in this order: Buffer, Capture antibody, 2nd buffer, Sample. Input the appropriate assay step times and the shaking speed per **Table 1**. Note that each buffer column can be used only once within the same assay when the method is developed in the advanced quantitation mode. Click **OK**.
- 8 Input sample plate information (example sample plate is shown in **Figure 3**).
- 9 The software will automatically assign the required number of biosensors.
- 10 Review the method and click **Go**.

Recombinant protein quantitation

The evaluation of recombinant therapeutic protein quantitation on the Octet system suggests varying limit of quantitation (LOQ) and a concentration dynamic range with varying media conditions (**Figures 4–8, Table 2**). All samples investigated exhibited better sensitivity in ForteBio's 1X Kinetics Buffer implying that for low sample concentrations, it is best to dilute off potential sample matrix effects. However, in cases where sensitivity is lost due to matrix effects, a wider concentration dynamic range can be detected.

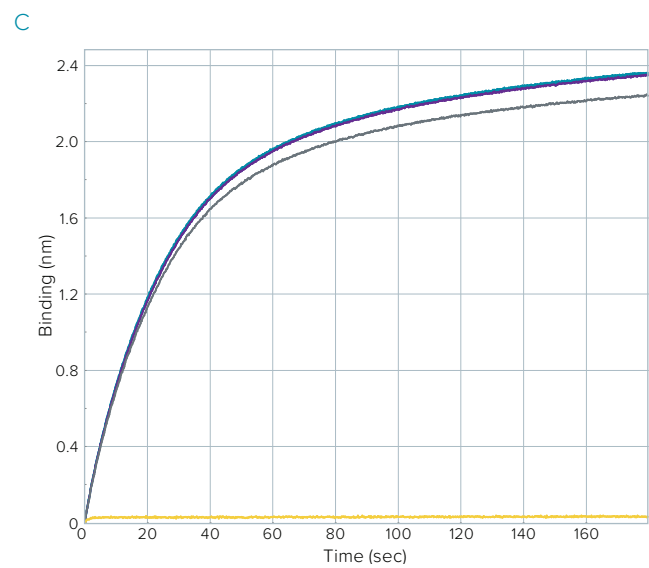
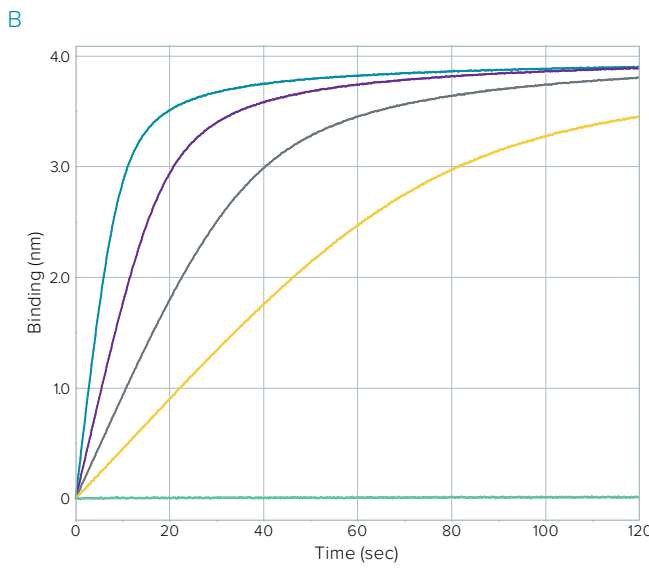
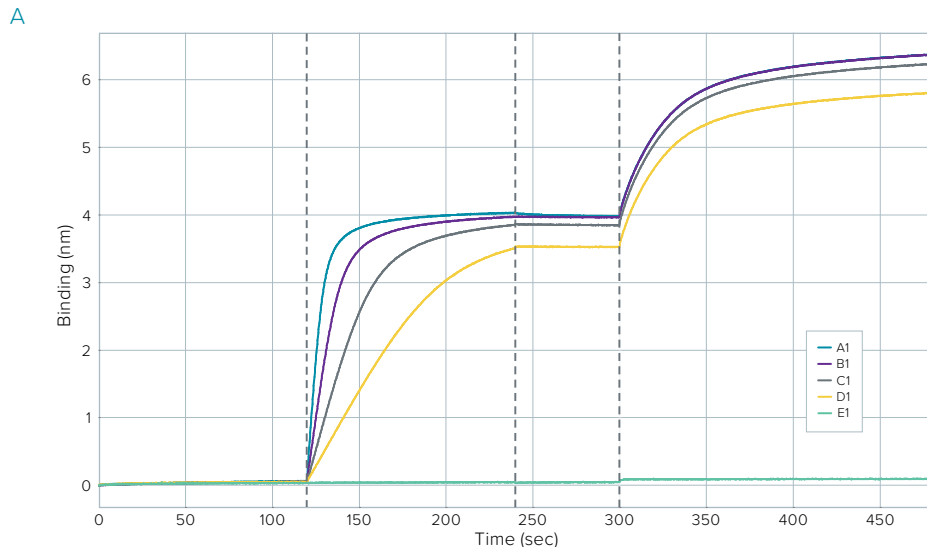


Figure 2: Antibody load scouting. Biotinylated anti-insulin loading was screened at 20, 10, 5 and 2.5 µg/mL. (A) shows raw data obtained when 5 µg/mL of recombinant insulin was used to probe for binding at the different antibody concentrations. Note: NSB was not observed when insulin was bound to biosensor surface with no antibody loaded (curve E1) (B) - antibody and (C) - antigen show the respective aligned response signals at 20 µg/mL (dark blue), 10 µg/mL (red), 5 µg/mL (blue) and 2.5 µg/mL (green). No discernible difference in signals was observed at the top three concentrations implying that 5 µg/mL is optimal for anti-insulin antibody loading. Similar studies were performed for the other recombinant proteins with 5 µg/mL deemed optimal in all cases.

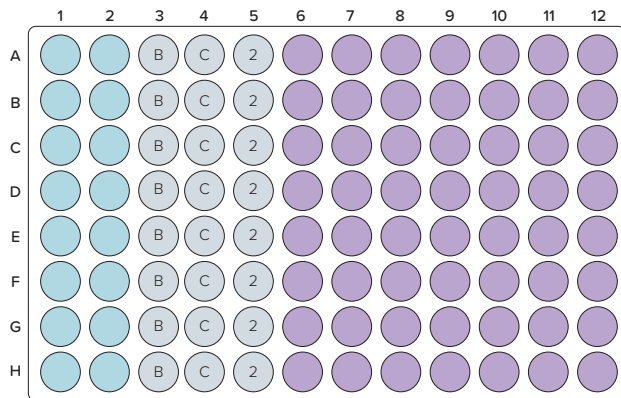


Figure 3: 96-well plate design for Advanced Quantitation on the Octet system showing standard samples (blue), buffer (B), capture antibody (C), 2nd buffer (2) and unknown concentration samples (purple). At least two replicates are required for the standard sample.

Step	Step type	Time (s)	Shaker (rpm)
1	Buffer	180	1000
2	Capture Antibody	300	1000
3	2nd Buffer	180	1000
4	Sample	300	1000

Table 1: Octet Advanced Quantitation assay method step setup for the customized quantitation of recombinant proteins

Insulin

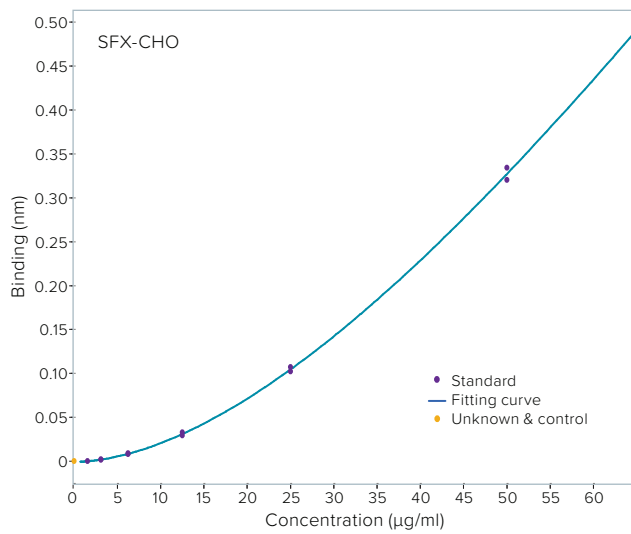
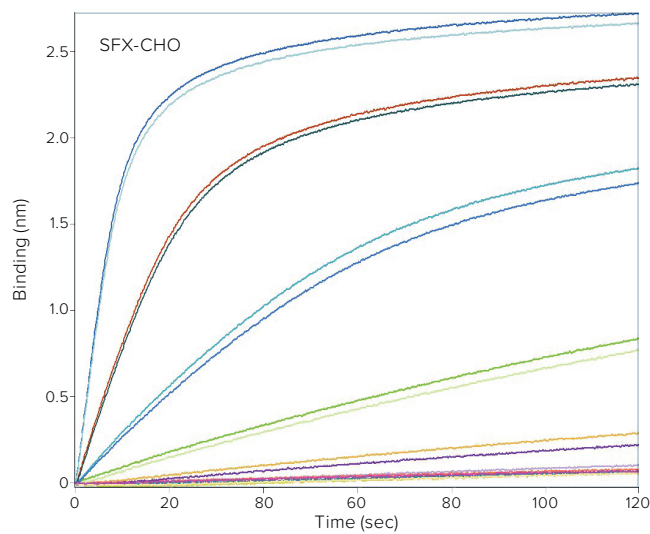
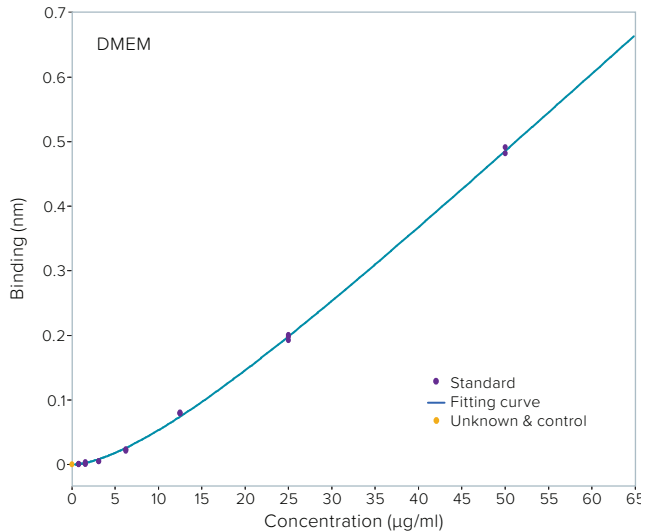
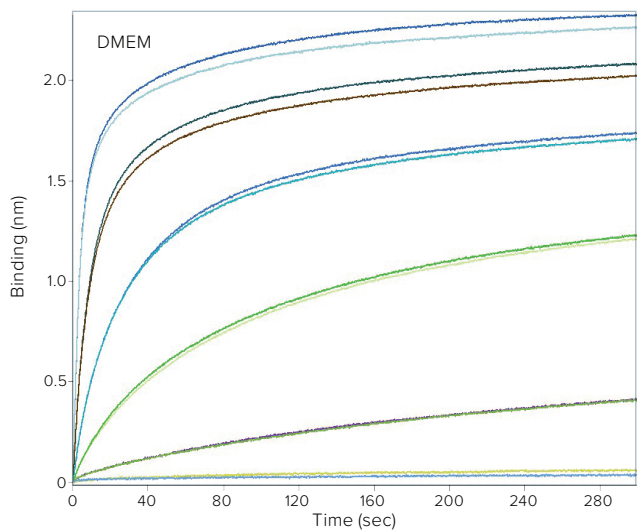
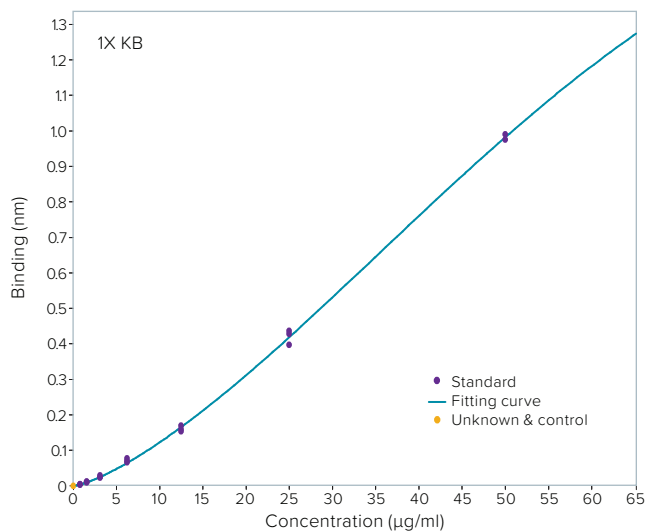
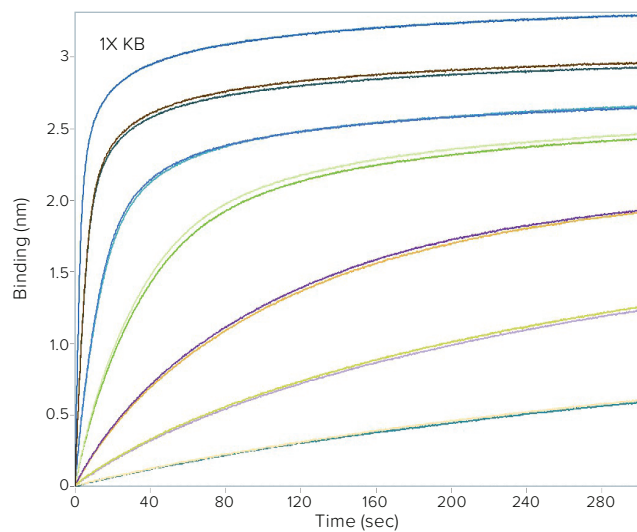


Figure 4: Binding curves (left) for the quantitation of recombinant insulin in three different media types with the corresponding standard curves (right). Standard curves were generated using initial slope binding rate analysis. Data was fit using a 5-PL Dose response curve fitting equation.

Factor IX

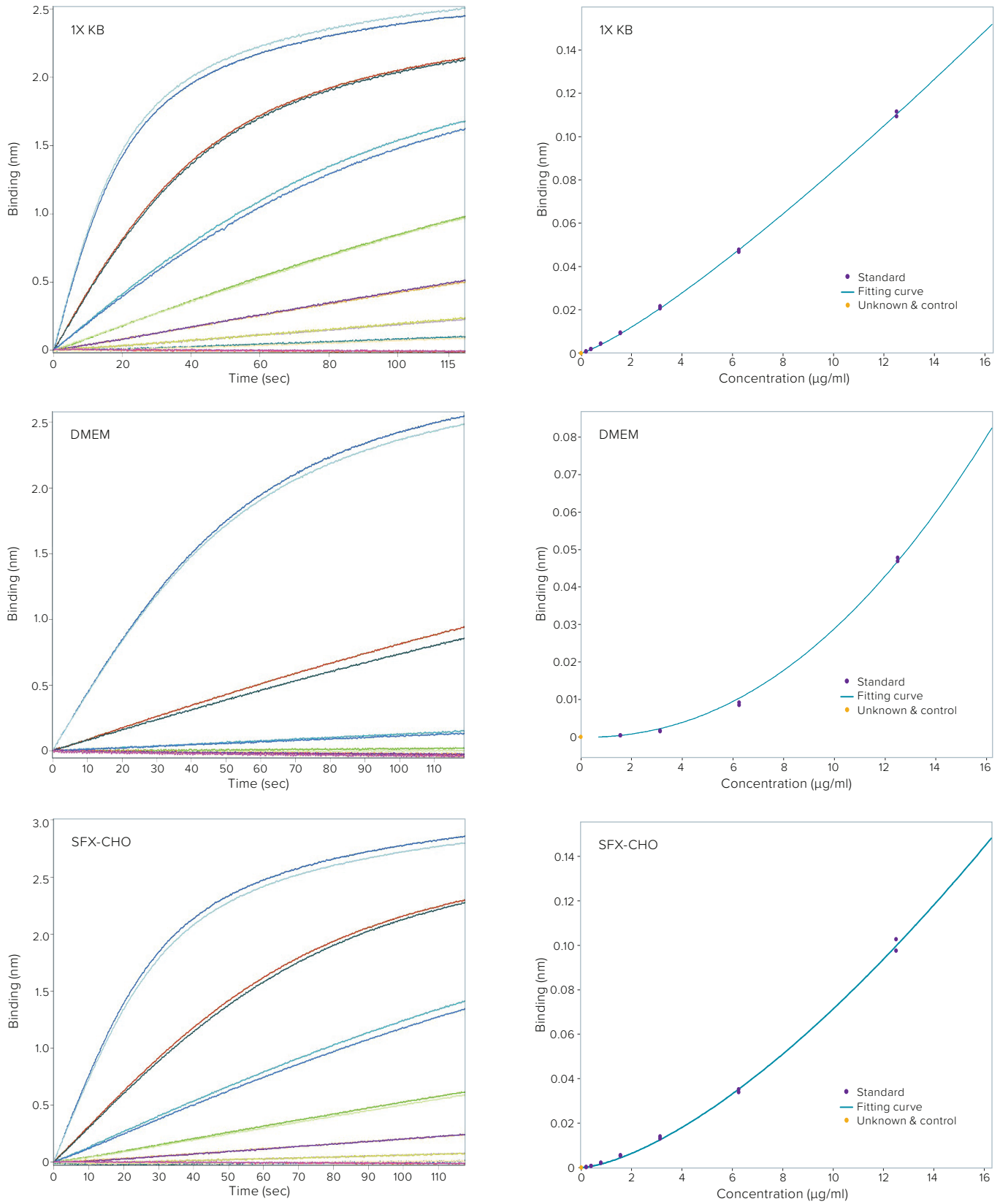


Figure 5: Binding curves (left) for the quantitation of recombinant Factor IX in three different media types with the corresponding standard curves (right). Standard curves were generated using initial slope binding rate analysis. Data was fit using a 5-PL Dose response curve fitting equation.

GCSF

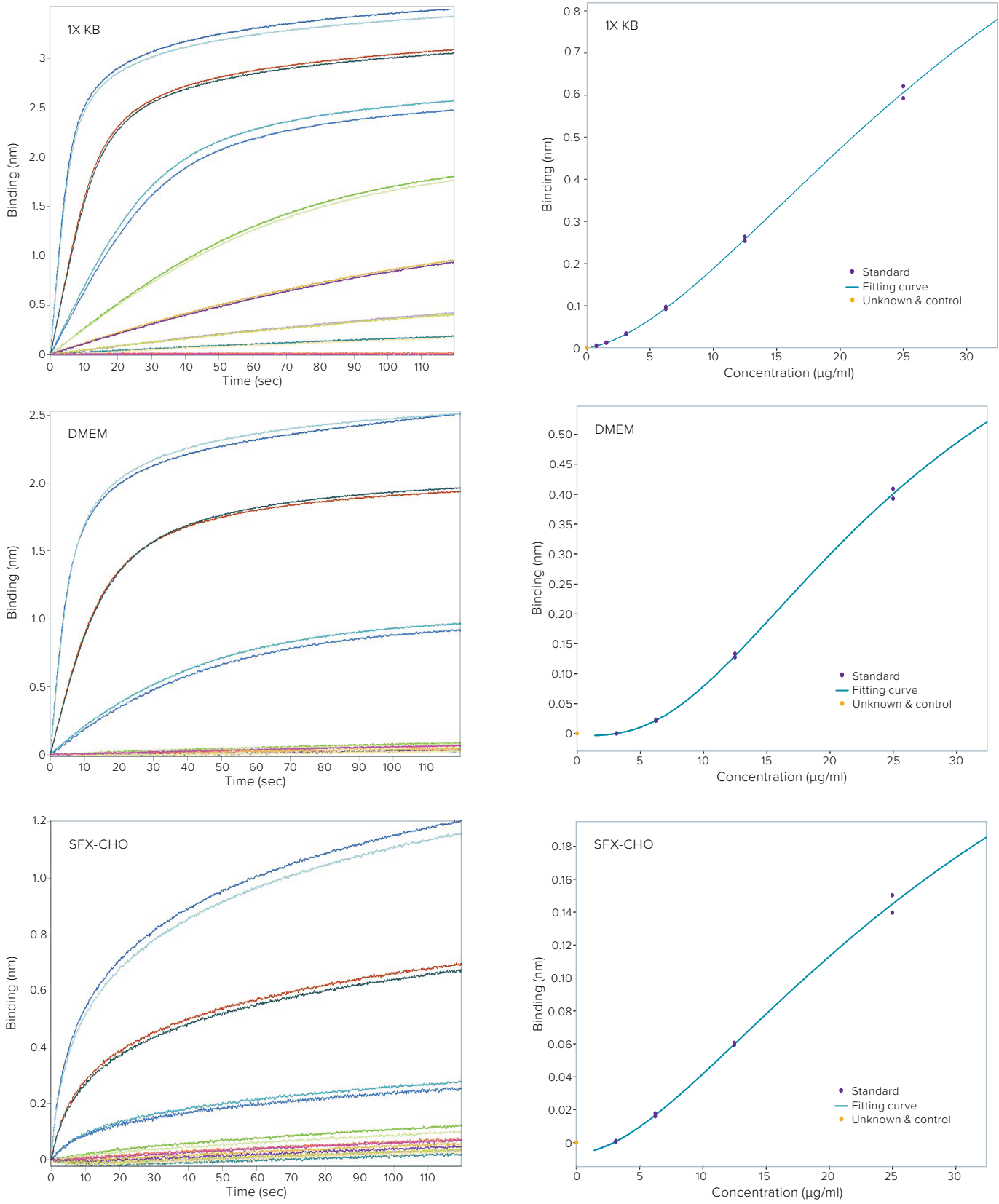


Figure 6: Binding curves for the quantitation of recombinant GCSF in three different media types with the corresponding standard curves (right). Standard curves were generated using initial slope binding rate analysis. Data was fit using a 5-PL Dose response curve fitting equation

EPO

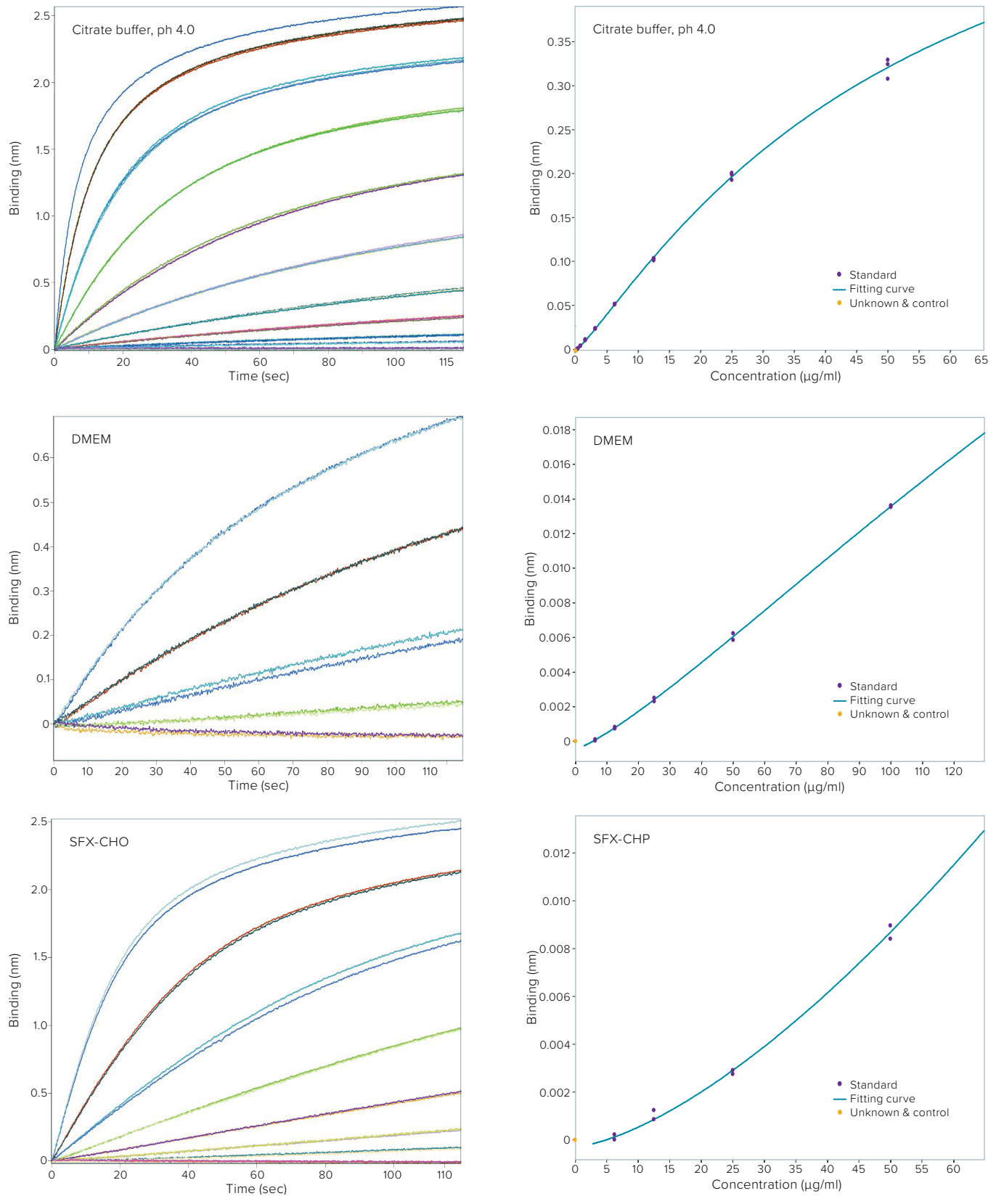


Figure 7: Binding curves (left) for the quantitation of recombinant EPO in three different media types with the corresponding standard curves (right). Standard curves were generated using initial slope binding rate analysis. EPO binding assay is pH-sensitive with the lowest LOQ obtained in 20mM Citrate buffer, 250mM NaCl, pH 4.0. Data was fit using a 5-PL Dose response curve fitting equation.

Factor VIII

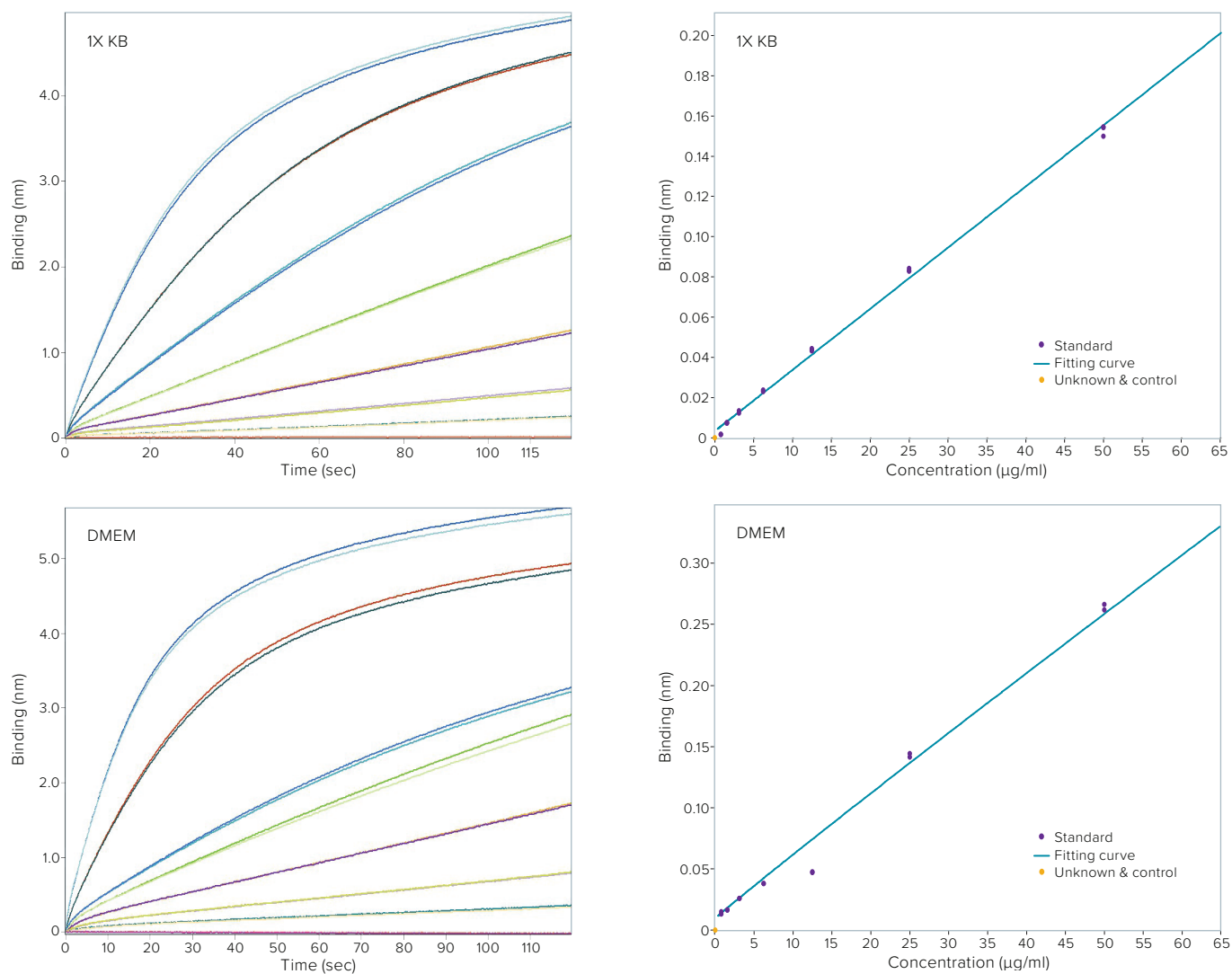


Figure 8: Binding curves (left) for the quantitation of recombinant Factor VIII in two different media types with the corresponding standard curves (right). Standard curves were generated using initial slope binding rate analysis. Data was fit using a 5-PL Dose response equation.

	Insulin	Factor VIII	Factor IX	GCSF	EPO
1X KB, µg/mL	0.78 – 50	0.78 – 50	0.19 – 12.5	0.39 – 25	n.d
DMEM Media, µg/mL	0.78 – 50	0.78 – 50	3.13 ≥ 12.5	3.13 – 25	n.d
SFX-CHO Media, µg/mL	0.78 – 50	n.d	0.19 – 12.5	3.13 – 25	12.5 ≥ 50
20mM Citrate buffer, 250mM NaCl, pH 4.0, µg/mL	n.a	n.a	n.a	n.a	1.56 – 50

Table 2: Dynamic range for recombinant therapeutic protein quantitation in different media types. n.a = not applicable; n.d = not determined

MATRIX EFFECT ANALYSIS

While non-specific interactions can result in false positives during a binding analysis, matrix interference by cell culture media components can have the opposite effect; quenched signal response despite positive binding between the immobilized antibody and the antigen. To investigate whether quantitation could be performed on upstream samples with

minimum pre-processing, and to determine the minimum dilution required for a successful quantitation analysis where cell culture media interference is observed, a matrix effect study was performed for each protein using several different common cell culture media. A binding rate probe at constant sample concentration for each sample (6.25, 6.25, 3.13, 1.56 and 1.56 µg/mL (for Insulin, Factor VIII, GCSF, Factor IX and EPO

respectively) was performed with the rate of binding used to predict the potential matrix effect. For all samples except EPO, biosensor equilibration, pre-load baseline and antibody loading were performed in 1X Kinetics Buffer. For EPO, these steps were performed in 10X Kinetics Buffer to minimize NSB. The subsequent post-antibody loading baseline and the antigen binding steps were performed in the respective media for all analyte samples. When the binding rate was deemed low enough to suggest potential signal quenching due to matrix effects, sample dilution in 1X Kinetics Buffer was performed. Figure 9 shows the binding rate data for the minimum dilution required for each media type for the respective recombinant proteins.

Octet system comparison to HPLC

The advantages of using HPLC for the quantitation of biomolecules include its applicability in diverse sample types including small organic molecules, large biomolecules and polymers amongst others. HPLC however has the disadvantage of lack of a “universal” detector. The fact that for proteins, the detection in HPLC is achieved at a wavelength of 280nm (not specific to any given protein) means that the detection has to be accompanied with good column separation resolution otherwise data accuracy is compromised. HPLC also lacks the through-put most critical in bioprocessing laboratories hence can be time consuming,



Figure 9: Binding rate analysis at constant protein concentration in common cell culture media. Low binding rates in undiluted samples suggest potential matrix effect hence the need for sample dilution.

especially for regulated analysis under good manufacturing practices (GMP). The BLI assays on Octet systems on the other hand can be optimized for specificity towards target biological molecules. The simple dip and read plate design method also allows for the rapid analysis of samples during protein expression and early on in the drug development process and can therefore facilitate necessary adjustments in the development of optimal conditions

To compare the performance of the Octet systems with HPLC, recombinant insulin was used as an example. Samples were analyzed side by side at Aragen Biosciences using the Octet RED96 instrument and a Zorbax 300SB-C18 RP-HPLC column.

Insulin samples were serially diluted 2-fold in either PBS or DMEM media at 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0 µg/mL. Half of the prepared samples were used for the RP-HPLC studies while the other half was used to run concurrent BLI assay quantitation studies. For the Octet system, the assay used was as per the assay protocol section while for RP-HPLC the following conditions were used:

Sample flow rate: 1 mL/min

Eluents:

A: 0.1% Trifluoroacetic acid (TFA) in water

B: 0.08% TFA in Acetonitrile

Elution Gradient: 30–50 % B in 10 minutes

Detection: 280 nm

Run time per sample: 10 minutes

The results suggest that HPLC exhibits excellent sample recovery at high insulin concentrations but significantly over estimates concentrations at the low end of the dynamic range. The Octet systems on the other hand have a more limited linear dynamic range of quantitation for insulin. However, the systems exhibit excellent recovery of low concentration insulin samples implying better sensitivity (Table 3). Moreover, it takes significantly less time to assay the same number of samples on an Octet system as it does via HPLC, providing significant time savings that can lower overall analysis costs.

GMP operations

The Octet platform is equipped with the necessary tools for use in GMP environments. The platform includes both IQOQ tools and 21 CFR Part 11 features to help meet GMP requirements including electronic security, audit trails, user access management and other compliance options.

In quantitation assays, precision, linearity and accuracy are key parameters that must be demonstrated to allow the assay to be validated for use in manufacturing. In addition, the limit of quantitation (LOQ) should be determined to increase confidence in the assay's performance. As an example, insulin was used to determine precision and accuracy, while the LOQ was determined for all samples as shown in Table 2.

Three samples (25, 6.25 and 1.56 µg/mL) of insulin were spiked in 1X Kinetics Buffer and treated as unknown samples. A standard curve, also in 1X Kinetics Buffer, was generated for the 0–50 µg/mL range. All samples were run in triplicate. Data was analyzed using the initial slope binding rate. The standard samples exhib-

Starting insulin, µg/mL	Insulin measured using RP-HPLC (PBS), µg/mL	Insulin measured using Octet (1X Kinetic Buffer), µg/mL	% Recovery	
			Insulin measured using RP-HPLC, µg/mL	Insulin measured using Octet, µg/mL
0.78	not determined	0.77		98.2
1.56	not determined	1.56		100.0
3.13	not determined	3.06		97.8
6.25	20.0	6.24	319.7	99.8
12.50	26.7	12.50	213.8	100.0
25.00	39.2	25.00	156.7	100.0
50.00	62.6	50.00	125.1	100.0
100.00	113.5		113.5	
200.00	212.8		106.4	
400.00	414.1		103.5	
800.00	813.6		101.7	

Table 3: A comparison of data accuracy for insulin quantification between Octet systems and HPLC.

ited concentration %CVs of < 3% (Table 4) while the unknown samples exhibited concentration %CVs well below 5% indicating excellent precision. Dose recovery for the unknown samples was found to be within 90–110 %, indicating excellent method accuracy (Table 5).

Best practices for custom recombinant protein quantitation assays

- 1 Always hydrate the biosensors for at least 10 minutes in the expected assay matrix. Note that in some cases, it may be necessary to hydrate the biosensors for longer than 10 minutes. A good rule of thumb is to hydrate the biosensors for approximately 20 minutes in cell culture spent media if the analyte is to be analyzed in its crude matrix during cell line expression monitoring.
- 2 An antibody-loading scouting experiment is recommended to ensure the optimal antibody is selected. Although the selected antibody concentration is 5 µg/mL for the five analytes mentioned in this application note, it is important to note that for some analytes the optimal concentration may be different. In principle, antibody loading should saturate the biosensor surface without a post-loading drift and should yield the highest analyte response signals. In addition, the rate of antibody loading should be gradual to ensure uniformity in loading. A rapid loading slope may indicate non-optimal biotinylation process that may affect antigen binding. A key advantage of the Octet platform is that the software allows the user to increase assay step time if necessary. This allows for flexibility in using low sample concentrations as the assay loading step can be increased accordingly during the scouting experiment.
- 3 During assay development, make sure to test for potential non-specific binding interactions (NSB) by running reference biosensors and samples. A reference analysis is two-fold: first test for NSB of the analyte to a biosensor blocked with an irrelevant antibody; second test for NSB between the assay media and the coated antibody. In the first case, the presence of NSB should suggest further assay development while in the second case the NSB can be subtracted using reference biosensors. Further assay development if necessary, is best done in the instrument's Kinetics.
- 4 For data accuracy, it is important to ensure matrix matching between the standard sample and the analyte. For example, if the cell culture media is SFX-CHO media, the standard samples should be spiked into SFX-CHO spent media to allow for comparable analyte environments, hence com-

Conc. µg/mL	Binding rate, nm/s	Conc. avg, µg/mL	Conc. % CV
50.00	1.0200	50.0	2.3900
25.00	0.5000	25.0	1.8800
12.50	0.1872	12.5	1.2400
6.25	0.0611	6.2	1.4800
3.13	0.0189	3.1	0.3476
1.56	0.0058	1.6	2.8300
0.78	0.0012	0.8	0.2831
0.00	0.0001	0.0	0.0000

Table 4: Insulin Quantification: Standard samples exhibit excellent precision, < 3% CV.

Conc. µg/mL	Binding rate, nm/s	Conc. avg, µg/mL	Conc. % CV	% Recovery
25.00	0.5182	25.9	1.4100	104
6.25	0.0640	6.4	0.1638	102
1.56	0.0066	1.7	1.4000	106

Table 5: Insulin Quantification: Unknown samples, n=3, exhibit excellent precision and accuracy.

parable analyte specificity. If the systems cannot be matrix matched, it is recommended that the analyte be diluted 1:10 in 1X Kinetics Buffer to allow for matrix consistency between the standard samples and the analyte.

- 5 Determine the useful standard sample concentration range and work in the linear portion of the standard curve. Standard samples should always be run in replicates to increase confidence in the assay.
- 6 Note that for recombinant protein quantitation, the recommended assay shaking speed is 1000 RPM. However, there may be analytes where a different shaking speed may be required. 1000 RPM is typically recommended in tandem with a longer assay time to improve assay sensitivity. In general, the higher the RPM, the better the sensitivity. In cases where the analyte is high in concentration, the assay dynamic range may be expanded by lowering the shaking speed to the default 400 RPM.
- 7 Although 1X KB is used in these studies, sample diluent buffer (higher % BSA) can also be used and is recommended for most proteins not included in this Application Note.

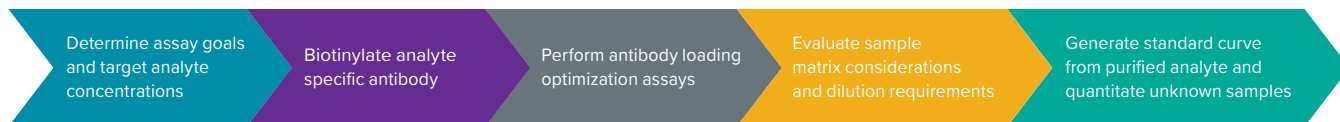


Figure 10: A summary of the work-flow for custom quantitation assays

Conclusion

The simple Dip and Read approach on Octet platforms in combination with High Precision Streptavidin biosensors (SAX) provide an easy to use, time-saving technique that enables high throughput, accurate quantitation of recombinant proteins. The LOQ for insulin concentration when determined on the Octet was 0.78 µg/mL in both DMEM and 1X Kinetics buffer while it was much higher (6.25 µg/mL) in both media when determined on RP-HPLC. Moreover, while it takes more than 10 minutes to assay one sample on RP-HPLC, the Octet systems take approximately the same time to assay as many as 8 samples (Octet RED96e), 16 samples (Octet RED384) and 96 samples (HTX). The technique can be a suitable replacement for HPLC

and ELISA as it is more robust to various cell culture media and can be used both upstream and downstream in the therapeutic protein drug development process. In addition, Octet platforms are equipped with GMP-compliant features that allow for the development of robust methods that can be quickly validated for assay transfer to QC laboratories.

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ForteBio
47661 Fremont Boulevard
Fremont, CA 94538
888.OCTET-75 or 650.322.1360
fortebio.info@moldev.com

ForteBio Analytics (Shanghai) Co., Ltd.
No. 88 Shang Ke Road
Zhangjiang Hi-tech Park
Shanghai, China 201210
salesops.china@moldev.com

Molecular Devices (UK) Ltd.
660-665 Eskdale
Winnersh Triangle
Wokingham, Berkshire
RG41 5TS, United Kingdom
+44 118 944 8000
uk@moldev.com

Molecular Devices (Germany) GmbH
Bismarckring 39
88400 Biberach an der Riss
Germany
+ 00800 665 32860