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Application Note

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Validated Quantitation and Activity Assay of Antibody Fragment Molecule (Fab) for Process Development and Quality Control

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Abstract

The analytical group at Boehringer Ingelheim, Fremont, USA needed a robust assay to measure the biological activity of an antibody fragment (Fab) molecule for in-process testing as well as stability and lot release testing in their Quality Control (QC) department. The developed Fab activity assay is accurate and robust, with intermediate and intramediate precision less than 10%. Drug activity measurement using the Octet® system has become a critical parameter for their product evaluation and has resulted in increased Fab drug product consistency and quality. This document describes the various steps involved in development and initial validation of the Octet® assay and presents experimental results from Boehringer Ingelheim that demonstrate the value in process development and QC studies.

Octet® Platform Solution

Boehringer Ingelheim, Fremont, USA developed a working Fab activity assay on the Octet® RED system in less than a week. Relative to the overnight incubation and four-hour assay time of their ELISA protocol, the Octet® assay provided an analysis time of only one hour per 96-well microplate, including sample preparation time. This Octet® assay was used to monitor Fab activity for all process development studies. The assay was subsequently qualified for use in quality control in less than one month. Drug activity measurement using the Octet® system has become a critical parameter for their product evaluation, and has resulted in increased Fab drug product consistency and quality.

Assay Development and Results

Method

An Octet® instrument and Streptavidin Biosensors loaded with a biotinylated small molecule ligand (capture molecule) were used for all experiments. The Fab samples were prepared in a PBS formulation buffer containing BSA and were analyzed in 96-well, black, polypropylene, flat-bottom microplates from Greiner (Part No. 655209). The assay format ensured detection of active Fab molecules only; degraded, inactive or clipped variants of the molecule do not bind to the anti-Fab ligand on the biosensor. Results from the quantitation assay are expressed in terms of percent activity of the Fab molecule, calculated as the ratio of the Fab concentration determined by the Octet® assay to the concentration value determined by A280 absorption spectroscopy.

Low ligand consumption

The small molecule ligand for the Fab was synthesized with a 1:1 biotin:ligand molar ratio. Loading onto Streptavidin Biosensors was optimized for ligand concentration and loading time by incubating a range of concentrations for 800 seconds at 30°C with 1000 RPM shaking of the microplate (Figure 1). Real-time monitoring showed that 1.25 µg/mL of ligand incubated for 60 seconds at 1000 RPM resulted in optimal loading on the biosensor. These parameters were chosen as final conditions for all subsequent studies.

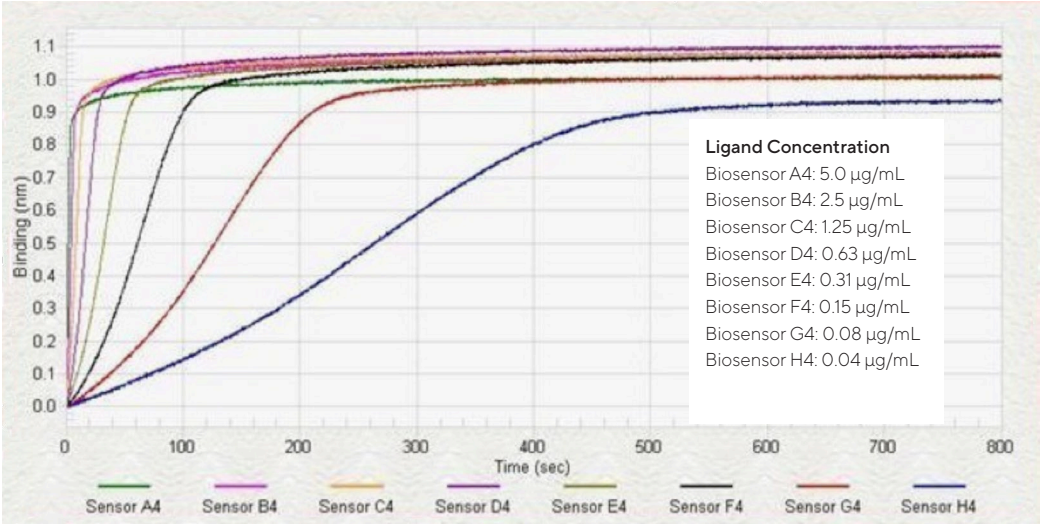


Figure 1: Ligand loading on Streptavidin Biosensor was optimized by testing several concentrations of biotinylated ligand. A 1.25 µg/mL solution of ligand incubated for 60 seconds at 1000 RPM demonstrated optimal ligand loading.

Biosensors regenerated and re-used

Many Sartorius biosensors can be regenerated and re-used for multiple tests. Effective re-use of a biosensor requires careful optimization of the regeneration protocol. For the Fab assay, phosphoric acid, citric acid, glycine and sodium hydroxide were screened as regeneration solutions. Sodium hydroxide treatment achieved partial regeneration. Complete regeneration for 10 cycles was seen when 50 mM sodium hydroxide buffer was supplemented with 1% sodium dodecyl sulfate (SDS) (Figure 2).

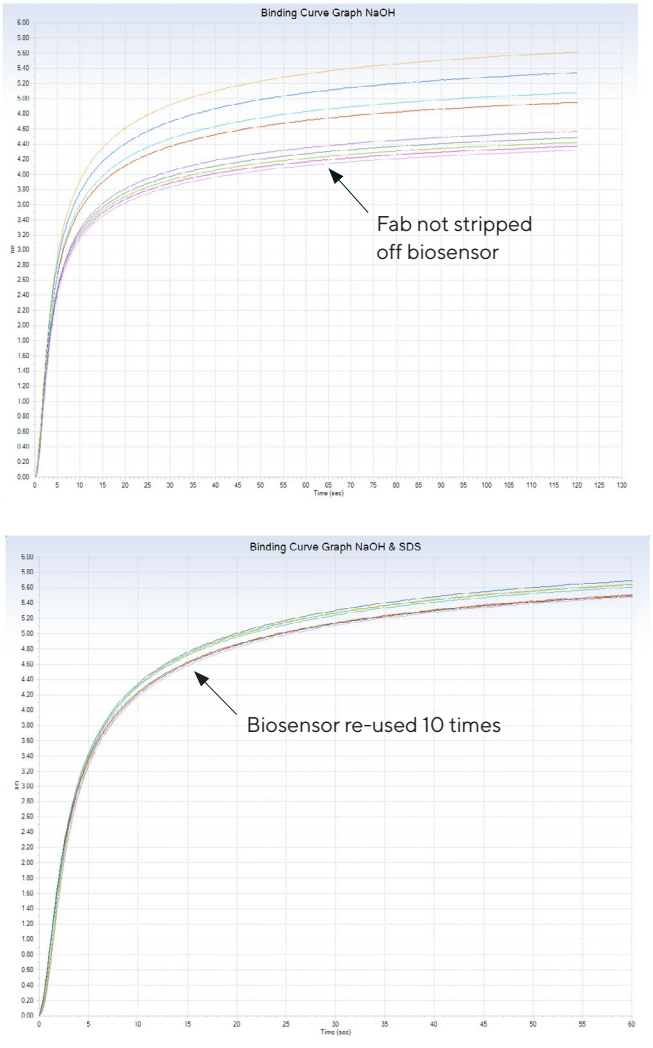


Figure 2: Complete regeneration of the biosensor was achieved with 50 mM sodium hydroxide buffer supplemented with 1% SDS.

Linear dynamic range greater than 2 logs

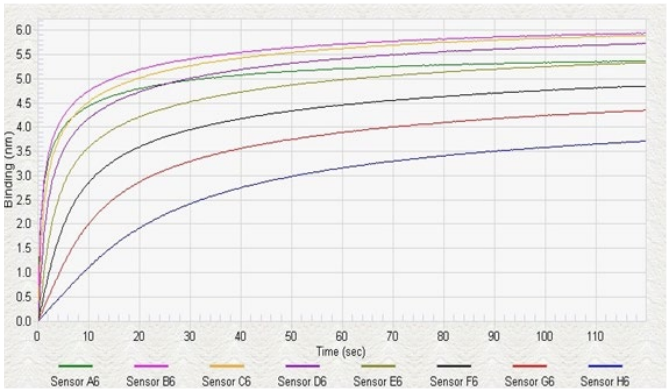
Purified bulk drug substance was diluted in PBS buffer containing 0.1% BSA. To determine the dynamic range for detection of the Fab molecule, concentrations from 15.6 µg/mL–2 mg/mL were tested in quadruplicate (Figure 3) using a microplate shake speed of 400 RPM and a 2-minute read time per sample. The two highest concentrations, 1 and 2 mg/mL, saturated the biosensor quickly, resulting in high CVs and unacceptable deviation from linearity. A lower range of Fab concentrations (3.13-400 µg/mL) was then tested in quadruplicate. This range provided acceptable results, with each concentration exhibiting a low CV and a calculated recovery within 10% of the theoretical values (Figure 3). The entire operating range of the standard curve was linear, with an R^2= 0.999 (Figure 4). As the linearity, accuracy and precision at each concentration in the dynamic range were optimal, 3.13–400 µg/mL was selected to be the operating range for the Fab activity assay.

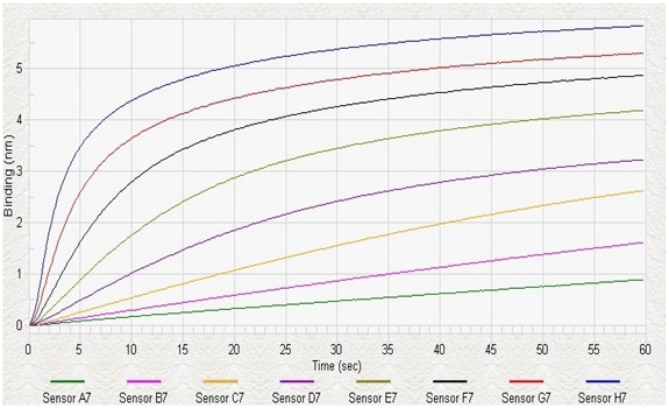
Saved standard curves are reliable

In Octet® Analysis Studio software, standard curves can be saved and applied to future experiments. To validate this approach, a standard curve was generated on Day 0 that was used to quantify the control sample prepared on Day 0 and on two subsequent days (Days 1 and 2). As shown in Figure 5, calculated percent activity values of the control sample for all three days were statistically identical. Control sample at four dilutions, in duplicate, were run with unknowns in all subsequent experiments to monitor the robustness of the assay.

Octet® Fab activity data reliable regardless of the type of curve-fitting

Octet® Analysis Studio software provides several data-fitting options including linear point-to-point, 4P (weighted or unweighted), and 5P (weighted or unweighted). Four Fab samples of known concentration and four other control samples were each assayed at four dilutions in duplicate, and the results were analyzed using linear point-to-point and four-parameter data fits (Figure 6). The known samples recovered within ±5% of the expected values and the eight replicates were measured with low CVs (<5%), regardless of the curve fitting model.





Theoretical (µg/mL)	Octet® assay (µg/mL)	%CV	% Accuracy
2000	**682**	**14.3**	**34%**
1000	**702**	**12.2**	**70%**
500	526	11.3	105%
250	265	2.3	106%
125	128	1.3	102%
62.5	64	2.1	102%
31.3	30	2.7	96%
15.6	13	2.7	83%

Theoretical (µg/mL)	Octet® assay (µg/mL)	%CV	% Accuracy
400	400.2	3.8	100%
200	200.1	4.2	100%
100	99.9	3.2	100%
50	49.9	3.7	100%
25	25.5	4.2	102%
12.5	12.0	3.8	96%
6.25	5.8	5.9	93%
3.13	3.1	2.6	99%

Figure 3: Testing of different dynamic ranges determined the 3.13–400 µg/mL range to be optimal with acceptable percent recoveries and CVs.

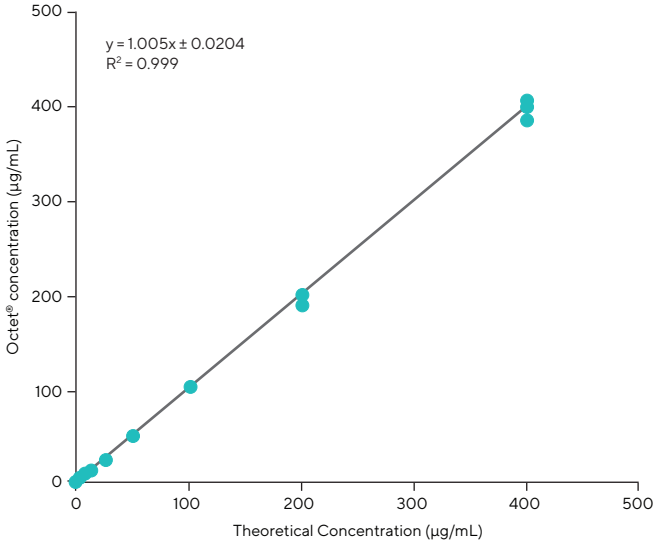


Figure 4: Linearity of the Fab assay in the 3.13–400 µg/mL range.

Control lot	Day	A280 (µg/mL)	Octet® assay (µg/mL)	% Activity
1	Day 0	7680	7610	99
1	Day 1	7680	7745	101
1	Day 2	7680	7670	100
2	Day 0	7680	7290	95
2	Day 1	7680	7551	98
2	Day 2	7680	7528	98

Figure 5: The performance of saved standard curves for Fab quantitation.

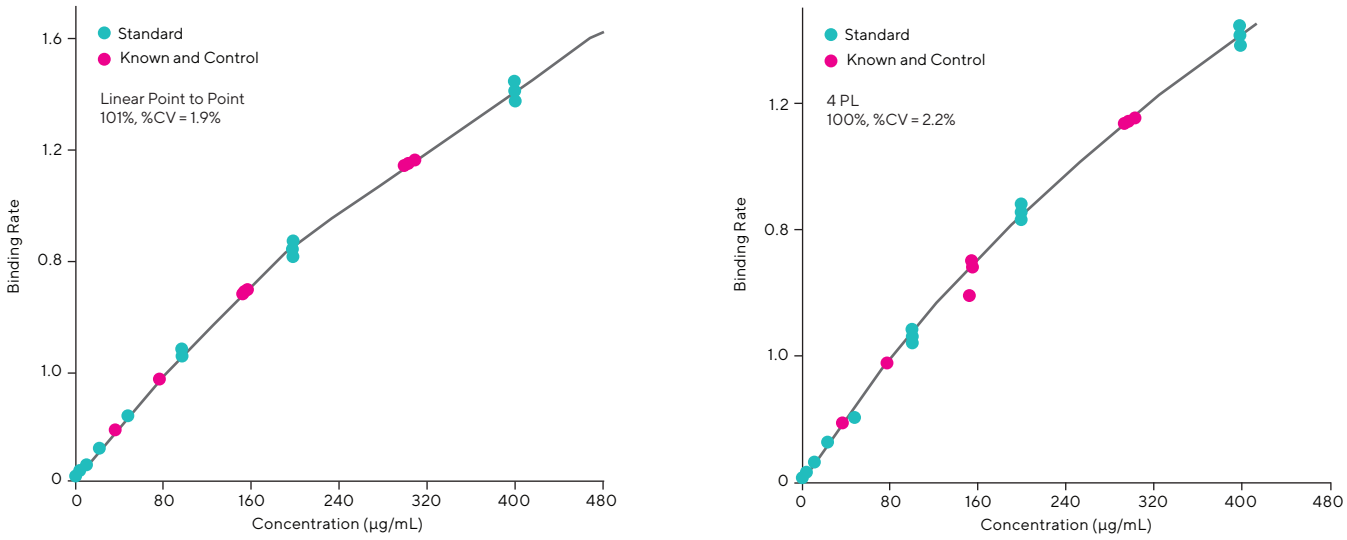


Figure 6: Known and control samples recovered within ±10% of expected values and the eight replicates produced very low CVs (<5%) for all curve fits evaluated.

Specific detection of Fab fragments in the presence of monoclonal antibodies

Six different monoclonal antibodies were titrated and tested with the Octet® assay. None of the six antibodies produced a binding signal, demonstrating the high specificity of the Octet® assay for the Fab molecule (Figure 7).

Excellent accuracy

During Fab production, the culture medium contains many host cell proteins in addition to the Fab. The assay must display high specificity for the intact Fab molecule in the presence of these impurities during the purification process. To test for assay specificity and accuracy, Fab samples were enriched for one of the major host cell contaminants. The purity of the samples was verified by size exclusion chromatography and capillary electrophoresis, and determined to be of 50% and 5% Fab content. The enriched impurity samples were run in the assay and the percent activity directly correlated with percent purity of the sample, *i.e.*, 50% purity yielded 49% activity and 5% purity yielded 4.2% activity (Figure 8). The Octet® assay demonstrated excellent accuracy for the Fab molecule over the entire range of impurity levels.

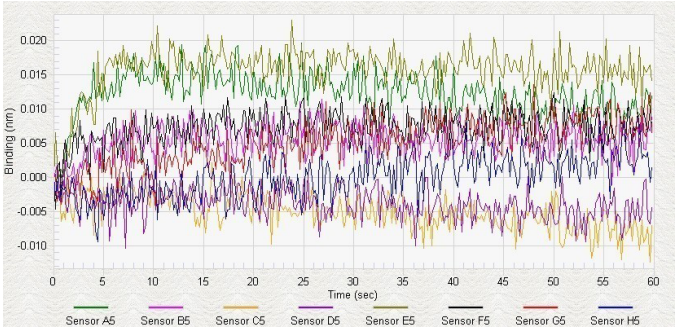


Figure 7: Absence of signal for six monoclonal antibodies tested.

Sample	Octet® assay (µg/mL)	A280 (µg/mL)	% CV	% Activity	% Accuracy
99% Fab	49100	50500	5.0	97	98
50% Fab	430	880	6.7	49	98
5% Fab	46	1100	3.3	4.2	84

Figure 8: Results indicate excellent selectivity and accuracy for Fab fragment in the presence of major host cell impurity.

Octet® assay complements A280 spectroscopy for in-process testing

A280 spectroscopy cannot differentiate between the Fab molecule and in-process impurities, instead measuring total protein concentration in the sample. The Octet® assay specifically detected the Fab molecule in the presence of impurities. The Fab concentration values reported by the two methods showed that the first purification step resulted in a partially purified sample containing approximately 15% of a major host cell impurity (Figure 9), and was confirmed by a secondary method.

Octet® values correlated very well (within 5%) with A280 spectroscopy values for the purified Fab molecule following the first purification step (Figure 9).

Sample	Octet® assay (µg/mL)	A280 (µg/mL)	% CV	% Activity	% Major impurity
First purification step	7680	9500	2.6	81	15%
First intermediate step	4220	4380	4.4	96	<1%
Second intermediate step	7570	7680	1.8	99	<1%
Final purification step	67880	65790	7.6	103	<1%
Bulk drug substance	50100	49500	5.0	101	<1%

Figure 9: Octet® assay correlated with A280 spectroscopy for the detection of purified Fab molecule, and showed specificity for Fab molecule in the presence of major host cell impurity.

Octet® Fab assay displayed good precision

Precision (repeatability) was evaluated by analyzing the assay control sample independently diluted eight times in a single analytical run (Figure 10). The Fab activity assay demonstrated acceptable repeatability with a 3.6% CV for the eight independently diluted control samples. Intermediate precision was determined by running the purified Fab control sample on 36 different occasions by two different operators from different laboratories. The average percent activity was 102% with a CV of only 5.3% for all runs (Figure 11).

Repeatability (n = 8)
97%
95%
101%
99%
100%
99%
90%
96%
Avg = 97%
%CV = 3.6

Figure 10: Fab activity assay demonstrated high intra-mediate precision, or repeatability, with CVs below 3.6%.

Means and standard deviations

Level	Number	Mean	Std dev	Std error mean	Lower 95%	Upper 95%
1	26	102.923	4.83258	0.9477	100.97	104.87
2	10	101.200	6.98888	2.2101	96.20	106.20

n = 36	% Activity	%CV
Avg	102	5.3

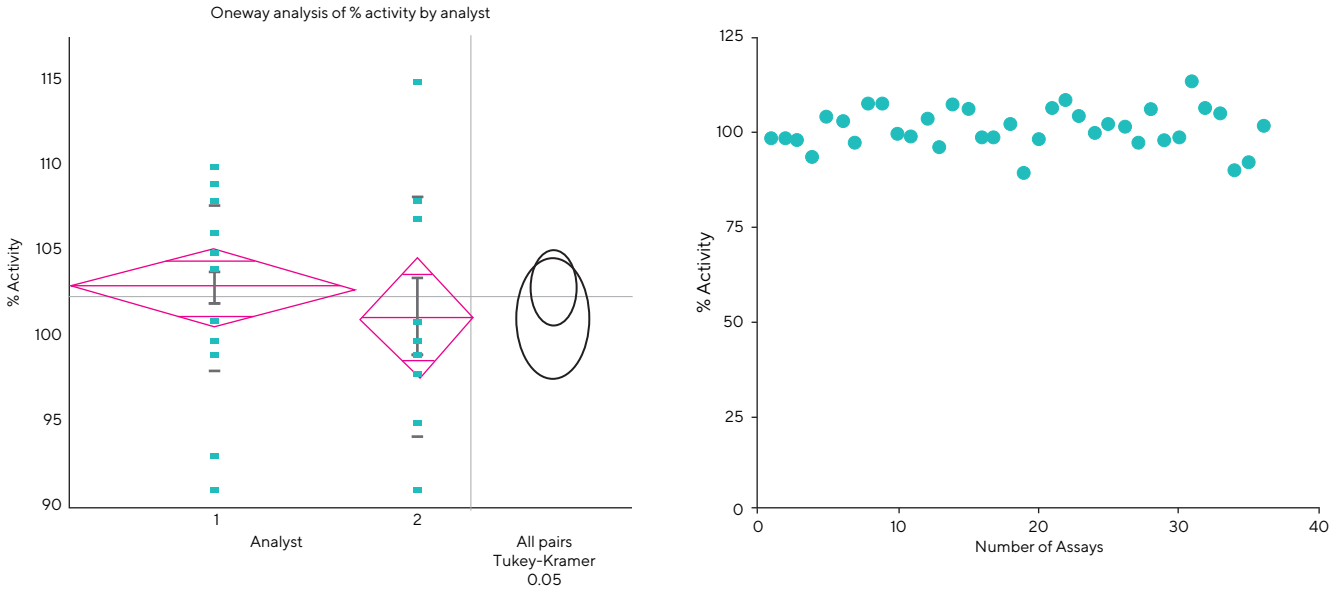


Figure 11: Good intermediate precision was observed. The average activity for the Fab control samples was 102% with a CV of only 5.3% for 36 runs.

Conclusion

The activity assay developed by Boehringer Ingelheim, Fremont, USA for their Fab molecule is currently being used for lot release and stability testing within the QC department. The assay has faster turn around times than ELISA and Biacore systems. The Fab activity assay is accurate and robust, with intermediate and intramediate precision less than 10%. Drug activity measurement using the Octet® system has become a critical parameter for their product evaluation, and has resulted in increased Fab drug product consistency and quality. The activity data generated on the Octet® system may be submitted to regulatory agencies for evaluation.

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