Validated quantitation and activity assay of antibody fragment molecule (Fab) for process development and quality control

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Background
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.

Octet platform solution
The group was able to develop 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. 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 of Octet systems in process development and QC studies.

Assay development and results

METHOD
An Octet RED 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.

BIOSENSORS REGENERATED AND RE-USED
Many ForteBio 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).

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 to 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
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.

Figure 2: Complete regeneration of the biosensor was achieved with 50 mM sodium hydroxide buffer supplemented with 1% SDS.
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 Data Analysis 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, calcu-
lated 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 experi-
ments to monitor the robustness of the assay.

OCTET FAB ACTIVITY DATA RELIABLE REGARDLESS OF THE TYPE OF CURVE-FITTING

Octet Data Analysis software provides several data-fitting
options including linear point-to-point, 4P (weighted or un-
weighted), 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.

<table>
<thead>
<tr>
<th>Theoretical (µg/mL)</th>
<th>Octet assay (µg/mL)</th>
<th>%CV</th>
<th>% Accuracy</th>
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<tbody>
<tr>
<td>2000</td>
<td>682</td>
<td>14.3</td>
<td>34%</td>
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<td>1000</td>
<td>702</td>
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<td>70%</td>
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<tr>
<td>500</td>
<td>526</td>
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<td>106%</td>
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<tr>
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<td>1.3</td>
<td>102%</td>
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<td>64</td>
<td>2.1</td>
<td>102%</td>
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<td>15.6</td>
<td>13</td>
<td>2.7</td>
<td>83%</td>
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<tr>
<th>Theoretical (µg/mL)</th>
<th>Octet assay (µg/mL)</th>
<th>%CV</th>
<th>% Accuracy</th>
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<td>3.8</td>
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<td>200</td>
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<td>100</td>
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<td>50</td>
<td>49.9</td>
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<td>102%</td>
</tr>
<tr>
<td>12.5</td>
<td>12.0</td>
<td>3.8</td>
<td>96%</td>
</tr>
<tr>
<td>6.25</td>
<td>5.8</td>
<td>5.9</td>
<td>93%</td>
</tr>
<tr>
<td>3.13</td>
<td>3.1</td>
<td>2.6</td>
<td>99%</td>
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Figure 4: Linearity of the Fab assay in the 3.13-400 µg/mL range.

<table>
<thead>
<tr>
<th>Control lot</th>
<th>Day</th>
<th>A280 (µg/mL)</th>
<th>Octet assay (µg/mL)</th>
<th>% Activity</th>
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<tbody>
<tr>
<td>1</td>
<td>Day 0</td>
<td>7680</td>
<td>7610</td>
<td>99</td>
</tr>
<tr>
<td>1</td>
<td>Day 1</td>
<td>7680</td>
<td>7745</td>
<td>101</td>
</tr>
<tr>
<td>1</td>
<td>Day 2</td>
<td>7680</td>
<td>7670</td>
<td>100</td>
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<td>2</td>
<td>Day 0</td>
<td>7680</td>
<td>7290</td>
<td>95</td>
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<td>2</td>
<td>Day 1</td>
<td>7680</td>
<td>7551</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>Day 2</td>
<td>7680</td>
<td>7528</td>
<td>98</td>
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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.

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).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Octet assay (µg/mL)</th>
<th>A280 (µg/mL)</th>
<th>%CV</th>
<th>% Activity</th>
<th>% CV</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>99% Fab</td>
<td>49100</td>
<td>50500</td>
<td>5.0</td>
<td>97</td>
<td>97</td>
<td>98</td>
</tr>
<tr>
<td>50% Fab</td>
<td>430</td>
<td>880</td>
<td>6.7</td>
<td>49</td>
<td>49</td>
<td>98</td>
</tr>
<tr>
<td>5% Fab</td>
<td>46</td>
<td>1100</td>
<td>3.3</td>
<td>4.2</td>
<td>4.2</td>
<td>84</td>
</tr>
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</table>

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

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

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

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.
**Means and standard deviations**

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std dev</th>
<th>Std error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<tbody>
<tr>
<td>1</td>
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<td>102.923</td>
<td>4.83258</td>
<td>0.9477</td>
<td>100.97</td>
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<tr>
<td>2</td>
<td>10</td>
<td>101.200</td>
<td>6.98888</td>
<td>2.2101</td>
<td>96.20</td>
<td>106.20</td>
</tr>
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</table>

**Repeatability (n = 8)**

97%
95%
101%
99%
100%
99%
90%
96%

Avg = 97%
%CV = 3.6

**Figure 10**: Fab activity assay demonstrated high intermediate precision, or repeatability, with CVs below 3.6%

**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.
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).

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.