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Octet® Potency Assay: Development, Qualification and Validation Strategies

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Abstract

The determination of drug analyte characteristics can be affected by the test method used. In antibody drug candidate ligand binding potency assays for example, FcγRIIIa binding is a common characteristic tested to assess the potency of the drug. Factors such as the amount of FcγRIIIa ligand immobilized on the biosensor for assessing its affinity to the drug molecule, assay temperature and assay flow rate or shaking speed (for plate based platforms) among others can affect the binding behavior and in turn the accuracy of the method. These factors typically need to be evaluated in the pre-qualification stage of an analytical method development. While they can be evaluated as single variables, an ideal analytical platform is one that allows the user to evaluate multiple factors in-tandem in a design of experiment (DOE) to enable an understanding of the effect of their interactions to the output parameters. The Octet® platform is highly suited for a fast evaluation of the interactions between these potential key assay inputs and allows for relatively fast time to results due to its high-throughput and ease of use.

KBI BIOPHARMA

Introduction

Kinetic analysis of biomolecular interactions is critical during drug discovery and development. The affinity of an interaction directly affects the dose required for a biopharmaceutical to be effective. Real-time data on the kinetics and affinity of binding can provide useful information at every stage of biopharmaceutical reagent development. Moreover, understanding the mechanism of binding can provide insights into the desirability of a drug candidate during development, including implications for the drug's stability upon complex formation with its binding target. Binding kinetics assays and specifically affinity constant (K_D) analysis are increasingly being used for biological product lot release. Regulatory requirements necessitate that such products be QC tested using methods that have been appropriately developed, qualified and validated under GMP conditions.

In this application note, we discuss the strategies for the development and validation of a potency assay using Octet® systems. We have highlighted the Octet® system's ease-of-use and fast time to results by showcasing strategies for the development and validation of a method for evaluating the binding of an Fc gamma receptor III molecule to the widely characterized NISTmAb.

Fc receptors are widely distributed cell-surface proteins that act as communication points between effector antibodies and their biological implements. There are three classes of Fc receptors, which bind to antibodies through their Fc region and impart different activities including Fc-gamma receptor I (CD64) that is responsible for phagocytosis and the activation of monocytic cell lines, Fc-gamma receptor II (CD32) that is mainly responsible for antibody-dependent cellular phagocytosis and Fc-gamma receptor III (CD16), which is responsible for antibody-dependent cellular cytoxicity (ADCC). Glycosylation and other modifications to the Fc region of an antibody can affect Fc gamma receptor binding hence these receptor molecules are a good tool for evaluating antibody drug efficacy and for antibody product lot release assessment. In this application note, we use affinity constant (K_D) as the reportable parameter to determine Percent Relative Potency to a reference lot.

Bio-Layer Interferometry

The Octet® platform utilizes a Dip and Read format in combination with Bio-Layer Interferometry (BLI) to monitor the interactions between biological molecules. BLI is an optical technique where white light incident to a reflective biosensor surface immobilized with a ligand results in changing interference patterns of the reflected white light detected upon the interaction between the ligand and the analyte which is kept in solution. Binding events between the ligand and the analyte result in 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 proportional measure of the change in thickness of the biological layer (Figure 1).

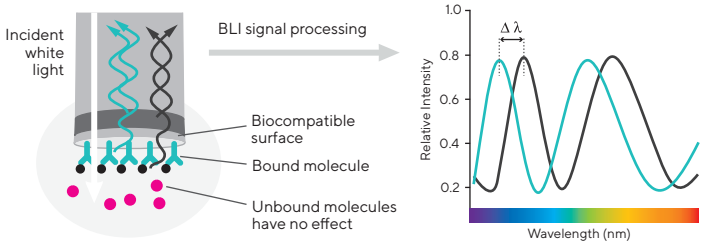


Figure 1: Relative intensity of the light reflection pattern from the two surfaces on the biosensor. Octet® BLI systems measure the difference in reflected light's wavelength (Δλ) between the two surfaces.

Materials and Reagents

Materials and reagents for the studies:

Material/reagent	Vendor	Catalog #
Ni-NTA Biosensors	Sartorius	18-5102
FcγRIIIa	R&D Systems	4325-FC-050
NISTmAb	National Institute of Standards and Technology (NIST)	RM8671
96-well plates	Griener Bio-One	655209
384-well plates	Griener Bio-One	781209
Octet® RH96 system	Sartorius	NA
Diluent (1X PBS, 0.1%Tween 20, 0.2% BSA)	KBI (prepared day of use)	NA

Method Development

Determination of analyte characteristics, including potency, can be affected by the test method used. Factors such as the amount of FcγRIIIa captured on the biosensor for the detection of the analyte, the temperature and shaking speed of the reaction, the sample matrix, and the equilibration time allowed for binding can affect the binding behavior. Each of these factors can also impact critical assay attributes such as accuracy. The Octet® platform is highly suited for a fast evaluation of the interactions between these potential key assay inputs and allows for relatively high-throughput method development. Three key input variables: temperature, shake speed and ligand loading density, were identified to be critical to the performance of the potency assay and were evaluated in a mini-design of experiment (DOE) prior to establishing the method. Each variable was examined at three or four levels. To establish assay performance at these conditions, the analyte concentration, sample matrix and assay step run times were maintained at constant values. A control condition was set at the Sartorius default conditions for ligand binding assay (kinetics) with the shaking speed at 1,000 RPM and temperature at 30°C for these studies.

Optimal assay behavior can be split into four distinct parts for kinetic determination assays:

1. The baseline(s) must be flat and absent of upward or downward drift (Figure 2, 0–60 seconds).
2. The loading density should be adequate to ensure sufficient protein is loaded on the biosensor, but not too high as to cause steric hindrance of the subsequent binding event. Multiple concentrations of the loading protein (Figure 2) are typically evaluated, often with a following association step although this is not mandatory. In general, low loading density is recommended when using Ni-NTA Biosensors.
3. The association step should show a concentration-dependent signal over at least 1.5 orders of magnitude (in concentration) as seen in Figure 2 (60 to 360 seconds). This step should also not show binding heterogeneity unless it is known that there is a 2:1 binding event occurring as indicated by a sigmoidal curve (similar to Figure 2) followed by a linear increase in signal rather than a plateau.
4. The dissociation step should show at least a 5% drop in binding signal (Figure 3, 300 to 900 seconds).

Data Acquisition Setup

For most of the kinetics experiments, a 384-well plate was used to enable high-throughput development (example shown in Figure 4). Additional buffer and loading wells were added as needed. Control points were performed at the beginning of a run prior to setting the shake speed or temperature to ensure no mechanical effects (*i.e.,* degradation due to increased temperature) on the control sample.

Biosensor Selection

Ni-NTA Biosensors were selected for this assay for robustness, ease of use, and to take advantage of the commercial availability of poly-histidine tagged (HIS-tag) receptors. FcγRIIIa is available commercially with many different purification conjugates. Using FcγRIIIa with a histidine tag at the C-terminus ensured the optimal orientation of the protein binding to the biosensor and provided the most distance between the biolayer of the biosensor and the binding site to prevent hindrance of FcγRIIIa binding to NISTmAb.

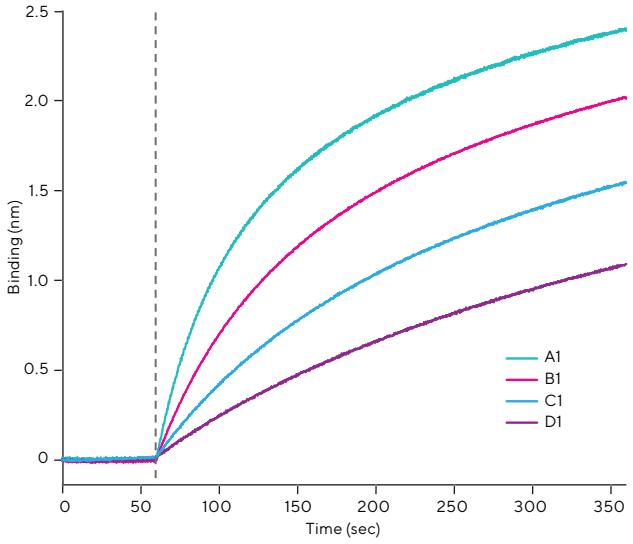


Figure 2: Four concentrations (10, 5, 2.5 and 1.25 µg/mL) were chosen for evaluation while targeting 1 nm response over 300 seconds. The 1.25 µg/mL concentration was chosen for further development. The baseline (first 60 seconds) and the loading step (60 to 360 seconds) for all four concentrations are shown.

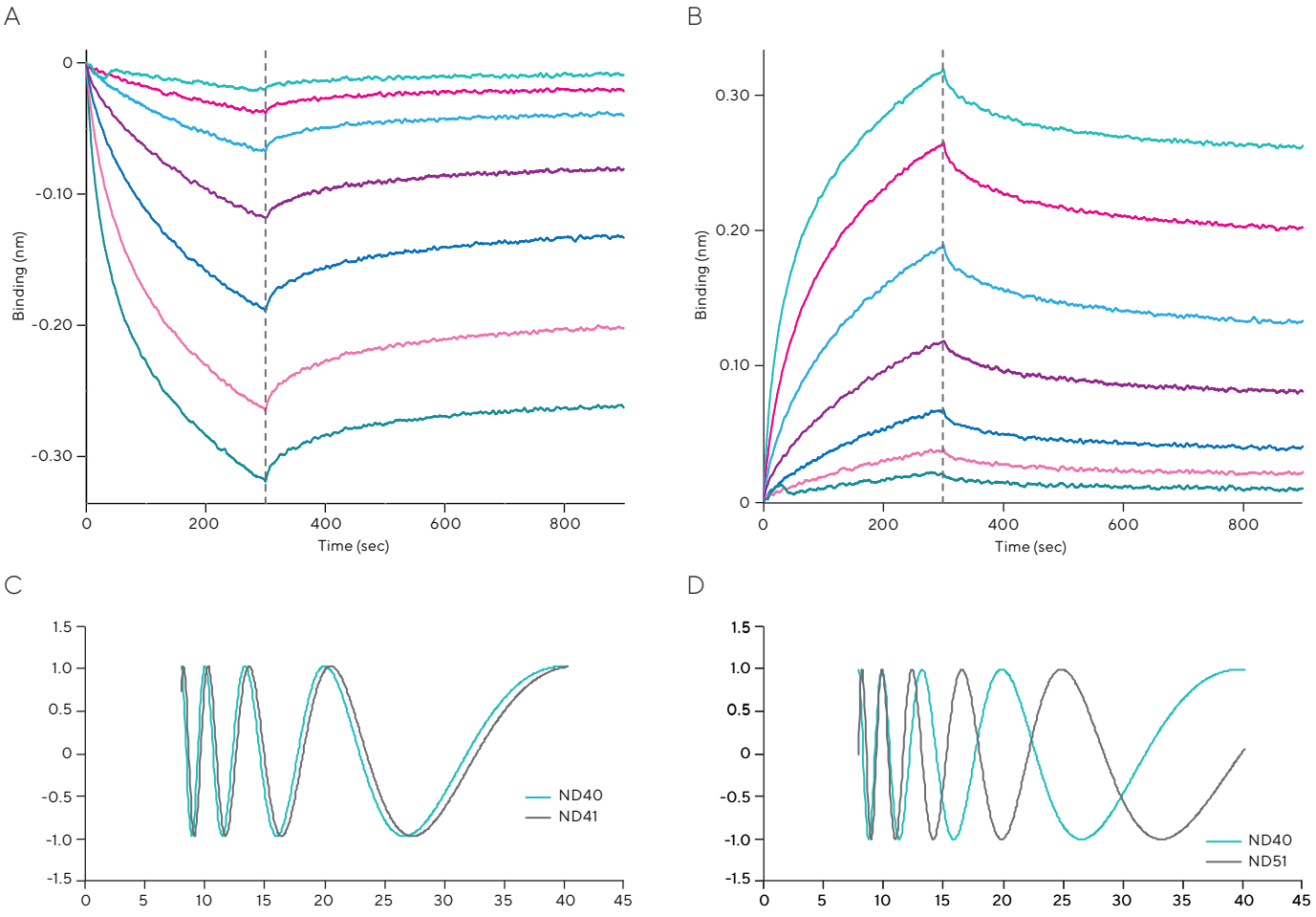


Figure 3: A) Inverted signal of NISTmAb associating to a FcγRIIIa bound to the bisosensor tip (loading of FcγRIIIa not shown). B) Flipped data from A using the "Flip Data" feature on Octet® Analysis Studio Software. C) Typical wavelength shift (left to right) from a small change in optical thickness (ND = Optical Thickness). D) Wavelength shift from a large change in optical thickness (right to left) resulting in an inverted signal.

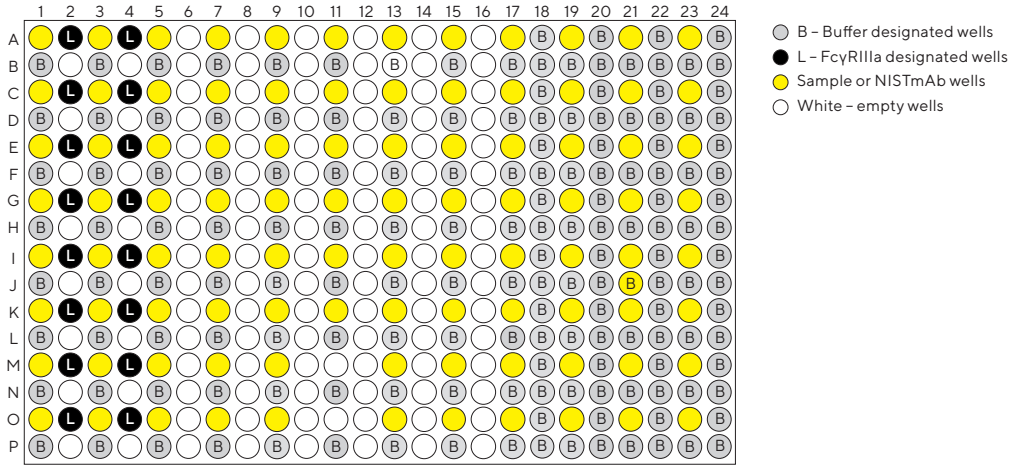


Figure 4: Sample Diluent was used in buffer wells and the zero point of all curves. 8-point curves (including zero point) were plated in a single column. Each baseline step had designated wells, and each curve used previously unused buffer wells for the dissociation step.

Ligand Density Assessment

Proteins such as Fc receptors typically provide optimal loading at concentrations <10 µg/mL. For this assessment, four concentrations were analyzed in duplicate for optimal signal and lack of saturation of the sensor (Figure 4). A FcγRIIIa concentration of 1.5 µg/mL was initially chosen as the ideal loading concentration based on a signal of 1 nm after 360 seconds, a typical initial benchmark.

Antibody Binding

NISTmAb concentration scouting was performed beginning with a range of 500 to 1.56 µg/mL. Curve shape, R_{Max}, Chi^2, R^2, Global Fit vs Local Fit, and Steady State were all considered when determining the optimal antibody binding. These attributes were also evaluated when establishing data processing parameters. The working range was determined to be 200 to 3.125 µg/mL based on acceptable assay performance. During the association step, signal inversion occurred. Signal inversion is a phenomena that arises when the optical thickness at the tip of the biosensor experiences a large change (Figure 4).[1] This is usually attributed to large molecules or complexes binding to a biosensor and is indicated as a decrease in signal. To verify that binding is occurring and not dissociation, the decrease in signal should be concentration-dependent and often a following step should be included, such as a dissociation step. The final assay steps are shown in Table 1 and were used for the theoretical pre-qualification/validation assessment.

Table 1: Octet® assay steps.

Step	Step type	Time (s)	Shaker speed (RPM)
1	Baseline	60	1,000
2	Loading	300	1,000
3	Baseline 2	120	1,000
4	Association	300	1,000
5	Dissociation	600	1,000

Pre-Qualification/Validation Assessment

For NISTmAb binding to FcγRIIIa, a short screening assay was performed as described above to determine the optimal FcγRIIIa loading concentration and NISTmAb concentration range. A DOE was then planned (Table 2) to determine the optimal loading concentration and if the platform conditions (30°C and 1,000 RPM) were suitable. A control preparation was performed at these platform conditions (including 1.5 µg/mL loading) to observe day-to-day repeatability and to calculate Percentage Relative Potency. The DOE approach, coupled with the fast assay time of the Octet® platform allows for the method parameters to be scouted in minimal time.

Table 2: Pre-qualification/validation development DOE.

Parameter	Range	Number of points
Temperature	28–35°C	4
Loading concentration	0.75–3.0 µg/mL	4
Shake speed	800–1,200 RPM	3

The results were analyzed using statistical analysis software which showed the optimal conditions for this assay were a 1.1 (±0.1) µg/mL loading concentration for FcγRIIIa, a 1,000 (±100) RPM plate shake speed, and a 30°C (29.5-31.5°C) assay temperature.

Critical Process Parameter Assessment

The pre-qualification DOE also provided the data required to assess specificity, precision/repeatability, and the working range of the assay. A diluent blank was performed as part of each NISTmAb curve. These blanks all demonstrated no matrix interference, indicating specificity of the assay. Due to the nature of the DOE, evaluating precision required assessing the data points from center points of the DOE. The average Percentage Relative Potency was 91% with a %RSD of 7%, suggesting good precision of the assay. Further, all points in the DOE showed R^2 ≥0.97, suggesting the working range of the assay (200 µg/mL to 3.125 µg/mL) is suitable for qualification.

Hydration of the biosensors was also evaluated. The baseline signal immediately after biosensor hydration of 10, 15, and 20 minutes was comparable, demonstrating that a 10 minute hydration time was suitable for the final method.

Method Qualification

Method Qualification, while not always required, can be a useful tool in early phases of drug development and provide critical data leading up to a validation. In general, the qualification of a potency method involves evaluating linearity, specificity, accuracy, precision, and range. Method Qualification also serves to set system suitability criteria for the assay as well as sample acceptance criteria for release testing and/or stability samples. For instance, the results from the accuracy calculations may allow for a criterion of 70% to 130% relative potency for test articles. When test samples meet this criterion, they are considered equivalent to reference. The results of an Octet® Percentage Relative Potency method qualification generally allow criteria to be set for: R^2, X^2, maximum response signal, minimum response signal, and a range of Percentage Relative Potency (potency comparison to reference). Typically, the results from running a qualified method (during development or stability experiments, etc.) in conjunction with the process (purification, culture, etc.) can provide the data to set criteria for a validation protocol. Validation of a method is performed after qualification and includes similar parameters to the qualification but with well-defined acceptance criteria in addition to validation specific parameters such as robustness.

Method Validation

Method Validations are completed to ensure an analytical method is suitable for its intended purpose. This provides an assurance of reliability for routine testing in GMP environment. Validation involves comprehensive protocol-driven experiments that evaluate and document the performance of an assay: As this method was being established as a potency assay, linearity, specificity, accuracy, precision, range, robustness, and ruggedness were evaluated as recommended by ICH Guideline Q2 (R1)[3] "Validation of Analytical Procedures: Text and Methodology."

Linearity is the expected relationship between known potencies of samples and their measured values using a range of 50% to 150% of the nominal relative potency, but treating their nominal concentration as 100%. Five levels were tested over the 50% to 150% range including 100%. The R^2 values of the resulting curves were all ≥0.95, indicating good linearity.

Accuracy is the degree of closeness to the expected value and was determined using results obtained from the linearity studies by calculating the percent recovery for each linearity level. For example; a Percentage Relative Potency of 46% at the 50% linearity level returns a 92% recovery. The average %recovery was calculated to be 97% with a range of 85% to 118% recovery. These results showed the method was accurate.

Precision is the variability in the data from replicate determinations under normal assay conditions. Repeatability of the method was assessed by testing multiple preparations at the nominal concentration. The average relative potency was 101% with a %RSD of 6%. Intermediate precision of the method was assessed using a second analyst to test multiple preparations at the nominal concentration. The average relative potency between two analysts was 101% with a %RSD of 8%. These results were within the expected limit.

The range of the method is demonstrated when precision, accuracy and linearity of the method show suitable performance. Suitable performance was demonstrated spanning the working range of 50% to 150% of the nominal potency. This corresponded to 100 µg/mL to 300 µg/mL for the highest concentration of the dose-response curve.

Specificity of the method was verified by testing a buffer blank and a generic non-human antibody, both diluted in the same scheme as NISTmAb. The Percentage Relative Potency of the blank and generic antibody were determined to be not-comparable to NISTmAb and specificity of the method was confirmed.

Robustness of this assay was evaluated by testing the working range of the parameters generated by the results of the development DOE. This involved making small but deliberate changes to the assay loading concentration, shake speed, and temperature. These changes in methodology returned results within 70% to 130% proving the method is robust.

Ruggedness of this assay was tested by evaluating normal test conditions that may vary over time. To test ruggedness of the assay, a DOE was performed on the parameters with the most risk for variance. This included biosensor lot, FcγRIIIa lot, and analyst to analyst variability (Tables 3 and 4).

The results of the DOE were analyzed by performing a Fit Least Squares analysis. The results of this analysis are shown in Figure 5. The Effect Summary Table showed no statistically significant interactions (*i.e.,* all ρ values were greater than 0.05). The Prediction Profile and Interaction Profiler showed no clear substantial trends between different variables. The effects of this DOE prove the method is rugged.

Table 3: Ruggedness DOE.

Parameter	# of Points
Biosensor lots:	5
FcγR(III)A lots:	2
Analysts:	3

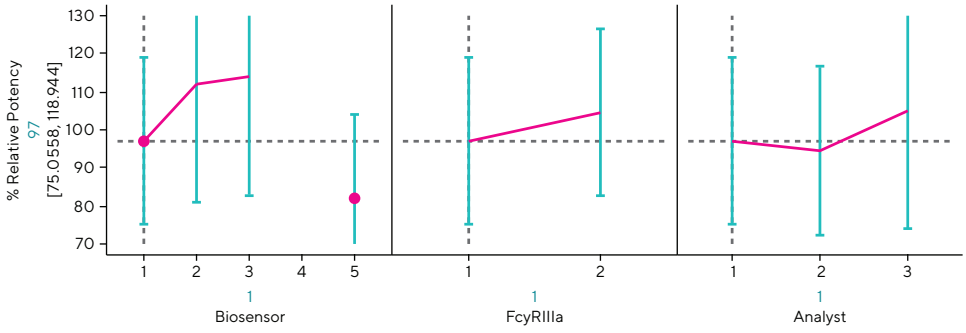
Table 4: A 30 run DOE showing the various combination of parameters tested.

Run	Biosensor	FcyR(III)A	Analyst	K_D (nM)	%Relative potency
1	1	2	1	25	100%
2	1	2	3	21	93%
3	5	1	1	31	82%
4	1	2	2	30	109%
5	1	1	2	28	103%
6	2	1	1	23	112%
7	3	2	2	25	91%
8	4	2	3	24	80%
9	5	2	1	21	119%
10	3	1	1	22	114%
11	5	1	2	30	110%
12	3	2	3	22	88%
13	5	2	2	26	96%
14	3	2	1	20	127%
15	5	1	2	28	113%
16	1	1	2	22	86%
17	4	2	1	23	108%
18	3	1	2	27	105%
19	5	2	1	24	104%
20	1	1	1	19	109%
21	1	2	2	25	98%
22	1	1	3	18	105%
23	4	2	3	18	109%
24	3	1	2	30	115%
25	3	2	2	22	86%
26	1	2	1	23	109%
27	5	1	1	31	82%
28	5	2	2	25	97%
29	1	1	1	25	85%
30	3	2	1	33	76%

A Effect summary table

Source	LogWorth	PValue
Biosensor*FcyRIIIa	0.677	0.21029
Biosensor*Analyst	0.629	0.23475
Biosensor	0.611	0.24497
Biosensor*FcyRIIIa*Analyst	0.551	0.28098
FcyRIIIa	0.216	0.60810
FcyRIIIa*Analyst	0.171	0.67448
Analyst	0.079	0.83416

B Prediction profiler



C Interaction profiler

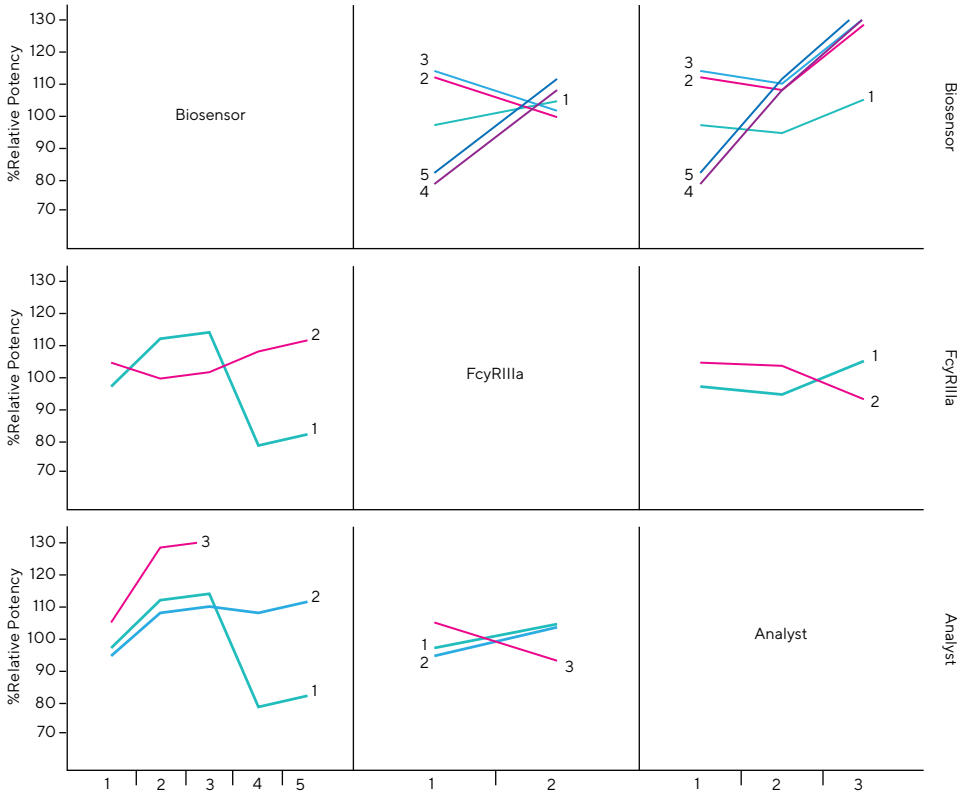


Figure 5: A) Effect screen of multiple parameters showing no significant interactions. LogWorth = -Log (p-Value). B) Prediction Profiler showing results are not able to predict future trends in data. C) Interaction Profiler showing the interactions between two variables have no predictable effect on %Relative Potency.

Results

A Percentage Relative Potency method for FcyRIIIa has been developed and analyzed in a representative method validation. For this validation exercise, the representative raw data can be seen in Figure 6 and analyzed results in Table 5. The results show that this method is linear, specific, accurate, precise and robust over a specific range in accordance with ICH Guidelines Q2 (R1).[3]

Table 5: Results of the validation exercise.

Parameter	Reportable result	Result
Linearity	R^2 of triplicate preps	$R^2 \geq 0.95$
Specificity	Diluent and non-specific mAb comparable to reference	Not comparable
Accuracy	%Recovery of linearity preparations	85% to 118% recovery
Repeatability	Average %relative potency and %RSD	Average = 101%, %RSD = 6%
Intermediate precision	Average %relative potency and %RSD of Analyst I and Analyst II	Average = 101%, %RSD = 8%
Range	Method range	50% to 150% for highest concentration
Robustness	%Relative potency at modified conditions	70% to 130% Relative Potency

Octet® Systems in GxP Laboratories

The use of Octet® systems in GxP laboratories is constantly expanding. KBI Biopharma has successfully developed 30+ methods on the Octet® platform used for titer, potency, kinetics, and identity testing. Many of these methods are being used to support Manufacturing, Drug Substance or Drug Product Release testing, and Long-term Stability testing in a GxP environment. While the assay and sample acceptance criteria are dependent on the method variability as well as the process variability, these methods generally exhibit ≤10 %RSD between replicates over long term testing.

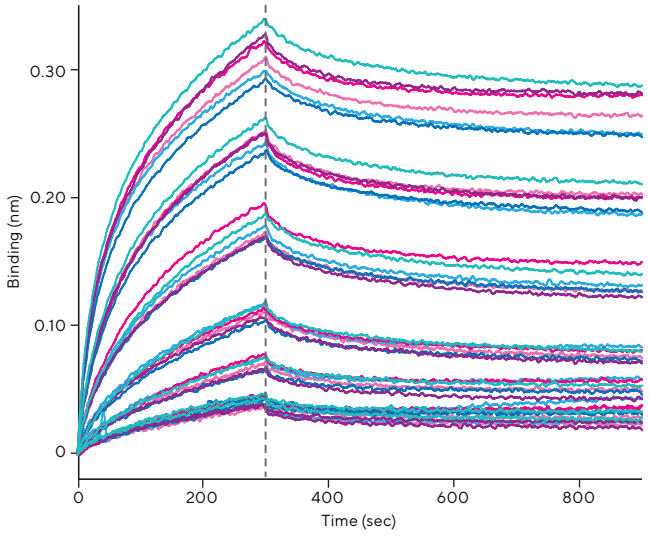


Figure 6: Replicate binding curves (n=6) of NISTmAb binding to FcγRIIIa.

Conclusion

Functional biological activity is a critical quality attribute (CQA) essential to verifying the potency of a drug molecule.[2] Potency assays can be used throughout the development process in comparability and formulation studies, and are required for release of every lot of therapeutic protein. The Octet® platform offers a fast, accurate, and robust solution for measuring potency of a drug molecule. Here we have described considerations for the development of a Percentage Relative Potency method capable of early-phase comparability studies and subsequent method validation for lot release. With the speed of the Octet® RH96 system, we could rapidly achieve Design of Experiment results which led to development, optimization, and potential validation practices.

References

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2. ICH Guideline Q6B, "Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products."
3. ICH Guideline Q2R1, "Validation of Analytical Procedures: Text and Methodology."

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