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Octet kinetics assay: method development guideline

1. Purpose

1.1 The purpose of this document is to describe the procedure for developing a ligand binding kinetics assay on Octet® QKe, RED96, RED96e, RED384 and HTX instruments. The procedures described are for developing binding kinetics assays for release testing of product batches and related applications. The document is intended as a general guideline only. Actual method development procedures may be different as they may be product dependent.

1.2 The procedures are aimed at developing kinetics binding assays to establish specificity, accuracy, precision, linearity and range as stipulated by the USP 1032 requirements for ligand binding assays.

2. Materials required

- ForteBio biosensors: Any part number relevant to the capture molecule
- Ligand/receptor/capture molecule: Molecule to be immobilized onto the biosensor surface
- Product/analyte:As needed
- 10X Kinetics Buffer (KB) Part No. 18-1092
- Other assay buffers as needed
- Black 96 or 384-well plates:Greiner catalog numbers 655209 and 781209 for 96-well and 384-well plates respectively.

Note: 96 well plates require 200 µL of reagents or sample while 384-well plates require a minimum of 40 or 80 µL for the round and flat bottom plates, respectively

3. General assay development sequence

See Figure 1.

i Ligand (capture) and analyte molecules assignment

ii Biosensor hydration

iii Ligand optimal density scouting

iv Assay buffer optimization

v Analyte binding optimization

vi Assay robustness and specifications testing

4. Ligand and biosensor selection

Determine which of the two binding molecules should be assigned as the ligand (for loading/capture/immobilization onto the biosensor surface). Generally, the larger molecule should be selected as the analyte unless it is an antibody.

4.1 **Ligand selection.** It is recommended that the antibody be selected as the ligand for an antibody-antigen interaction to avoid antibody avidity effects.

4.2 **Biosensor selection.** When one binding partner has an intrinsic tag such as a HIS or GST tag, assign that molecule as the ligand. In this case, an anti-tag biosensor should be selected for use. However, in cases of high affinity binding, some anti-tag biosensors may not be appropriate. In such cases, it is recommended that one of the partner molecules be biotinylated and High Precision Streptavidin (SAX) biosensors used for the studies.

Note: refer to ForteBio Technical Note 28: Biotinylation of Protein for Immobilization onto Streptavidin Biosensors for biotinylation procedures.

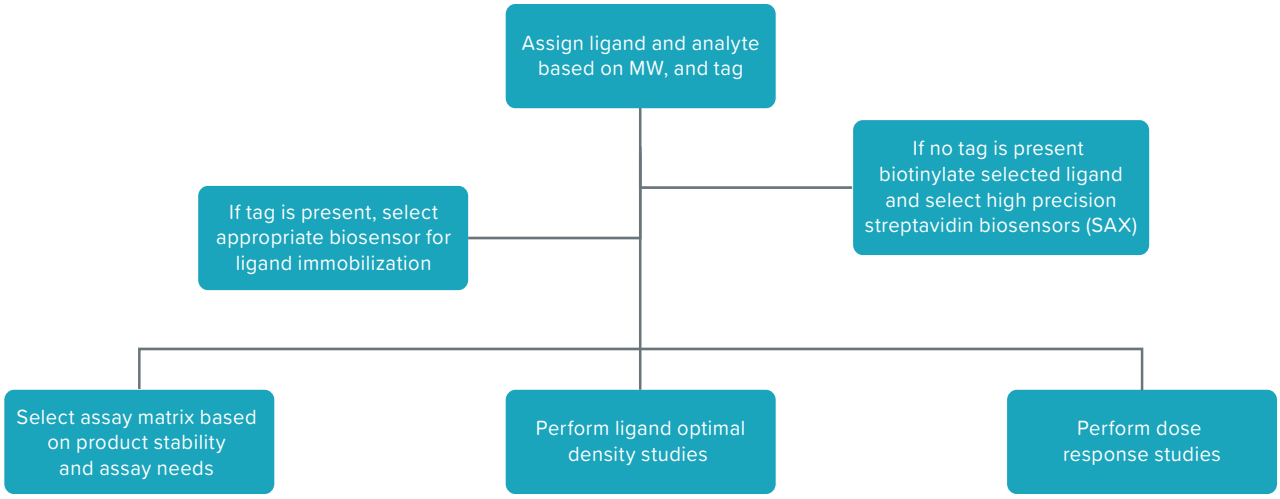

Figure 1: Overview of suggested ligand binding assay sequence on the Octet platform.

5. Biosensor hydration

5.1 Hydration plate preparation.

Note: While ForteBio recommends the use of 1X Kinetics Buffer, binding molecule stability and activity should dictate the selection of assay buffer.

Pipette buffer only into wells corresponding to the number of biosensors intended for immediate use. Hydrate the biosensors passively on the lab bench.

5.2 Hydration optimization. Biosensor hydration is typically recommended for a minimum of 10 minutes but may need to be optimized. Optimal hydration should result in minimal drift in the baseline step of the assay. To optimize hydration, select three different hydration times. Use at least two biosensors per condition to allow for replicates and stagger the hydration to enable simultaneous evaluation on the instrument. Recommended hydration times to evaluate are 5, 10 and 20 minutes.

Note: Actual total biosensor hydration time will depend on the instrument equilibration setting used prior to the start of the experiment. This is typically set at 10 minutes.

Pipette 200 µL of assay buffer into the biosensor hydration plate and an appropriate volume into corresponding wells of the sample plate. Perform a baseline step assay (recommended time is 2–5 minutes but could be longer). Select the hydration time and baseline step time conditions where the assay baseline drift is minimized and establish this as the optimal hydration time for the rest of the studies.

6. Ligand density scouting

The purpose of the load scouting experiment is to assess the optimal loading/capture/immobilization molecule concentration that results in adequate analyte binding without the introduction of assay artifacts. Assay artifacts could include an undesired drift in the baseline immediately following ligand capture, or an undesired biphasic behavior in the analyte dissociation step such as an initial fast off-rate (undesired) followed by a slower rate.

6.1 Hydrate the selected biosensors as established in Section 5.

6.2 Pipette at least three different ligand molecule concentrations in duplicate into a 96- or 384-well sample plate targeting low density, mid density and high density. It is recommended that the mid density concentration be approximately 150 nM of the ligand based on a 5-minute loading time. High- and low-density concentrations can be estimated from this value. Select a constant concentration of product/analyte to test for binding against each ligand concentration. As some estimate of the expected K_D is required, the analyte concentration should be estimated at above the expected K_D, typically 10–20X of the expected K_D. If the K_D is unknown, use a high analyte concentration.

Note: Analyte solubility in the chosen sample matrix may limit analyte concentration.

6.3 Perform the load scouting binding assay in this sequence: baseline, loading, baseline 2, association and dissociation. Assay step times should be developed and will depend on the expected K_D of the binding partners. The baseline 2 step time should be optimized to establish a step time with minimal drift, i.e. extend assay step time, to achieve minimal drift prior to the biosensor dipping into analyte for association time.

Note: The Octet Data Acquisition software allows the user to extend an assay step time or move the steps earlier than initially set based on real-time data observation. This feature is especially useful during assay development and should be used during these scouting experiments.

6.4 Select the lowest ligand concentration that a) results in an optimal analyte binding response and b) that does not introduce artifacts in the dissociation step to use for further method development. Optimal analyte response may be dependent on analyte molecular weight but should produce appreciable signals to allow for a dose response analysis during analyte titration.

7. Assay buffer optimization

It should be noted that assay buffer/matrix can affect assay performance. A mini design of experiment (DOE) to scout for optimal assay buffer should be performed keeping in mind the desired analyte stability. Ideally, the assay should be run using analyte sample matrix. However, sample dilution may be necessary if the sample matrix causes interference with the assay.

7.1 Select different buffers or sample matrix conditions to evaluate for assay performance. Conditions may include different pH, ionic strength, detergent types and amounts, etc. Selection of the conditions to evaluate should depend on the biophysical properties of the binding molecules, i.e. when the molecules are highly charged, pH and ionic strength of the buffer may be evaluated to determine optimal conditions. When the molecule is highly hydrophobic, detergents should be evaluated and spiked into the base buffer.

7.2 Design a mini DOE with the buffer conditions as the variables. The design space may be wider for initial DOE variables but should be tightened for optimization. Ligand molecule concentration should be as determined in Section 5, while analyte concentration should be high enough (typically 10–20X of the expected K_D) to result in optimal binding.

7.3 Spike the loading and analyte samples into each buffer or matrix condition.

7.4 Run a full binding experiment in the sequence: baseline (2–5 minutes), loading (5–10 minutes), baseline 2 (2–5 minutes), association (5–10 minutes) and dissociation (2–10 minutes). Actual binding assay step time will depend on the analyte. For each assay condition, include a negative control where all steps identical except the analyte concentration which should be 0 M (buffer or matrix without product/analyte).

8. Analyte binding

Kinetic binding assays should demonstrate binding curvature at the top concentrations evaluated, typically concentrations above the expected K_D. A dose response analysis should be performed with at least 3–5 data points. An ideal binding profile should have at least two data points below the expected K_D and two data points above the expected K_D. It should be noted that this may not be possible in some cases where the analyte is small but the binding affinity between the two molecules is high (pM). The analyte should be prepared in the same buffer or matrix as previously determined. Multiple lots of the analyte should be tested to demonstrate assay reproducibility.

8.1 Prepare serial dilutions of the analyte in replicates in the same assay buffer or matrix. The serial dilution could be either 2-fold or 3-fold but should be scouted to establish data with the binding curvature at the high concentration points and appreciable binding at concentrations lower than the reported K_D.

8.2 Establish assay shaking speed. The recommended shake speed for Octet binding assays is 1000 RPM. The shake speed may however need to be scouted and optimized depending on sample type. For example, for virus-like samples, the optimal shake speed may be lower (typically ~ 400 RPM). Shake speed evaluation should aim at achieving differentiation of analyte binding response as a function of concentration. To evaluate shake speed, keep all assay conditions constant while changing the shake speed. Evaluate three different shake speeds; 1000 RPM, 600 RPM, and 400 RPM.

8.3 Use the established assay conditions and parameters to establish analyte association and dissociation step times. An ideal top analyte concentration should result in binding curves exhibiting sufficient curvature. The top analyte concentration should be 10–20X of the expected K_D but can be adjusted to achieve binding curvature or response plateau. The dissociation step should result in sufficient response decay to allow for off-rate analysis, typically >5 % loss of response from the end of the association step.

8.4 Once the association and dissociation step times have been established, perform an analyte dilution series starting with the pre-determined top analyte concentration. Dilute samples 2–3-fold in the assay buffer or matrix. Prior to analyte dilution, prepare stock analyte sample in the pre-determined assay buffer/matrix.

9. Data analysis

Octet Data Analysis software allows the user to analyze data either through rate-based analysis or as an end-point analysis.

9.1 Select a model for data analysis ranging from a 1:1 binding model to a heterogenous binding model. For most binding pairs, the 1:1 model is appropriate, however, depending on the binding molecules in question, more complex models may be observed.

9.2 First fit the data using the local fit model.

9.3 Evaluate the kinetics output for each sample concentration to determine if outliers exist that may not satisfy acceptable statistical parameters. Ideally, the kinetics parameters should pass the coefficient correlation requirement of > 80- % and the chi-squared requirements of < 3.0. These loose specifications may be tightened as more data is collected, and then can be changed depending on assay requirements and behaviors.

9.4 If outlier analyte concentrations have been determined and eliminated, the assay should be repeated in replicates using the acceptable analyte concentrations. The new data should be fit to the global fit algorithm to determine the reportable dissociation constant (K_D).

10. Qualifying assay parameters

10.1 Testing for specificity

Specificity is established through the difference in response signals between a) the control (reference) sample and an irrelevant sample (negative control) and then b) the sample buffer or matrix.

10.1.1 Immobilize the capture molecule on six different biosensors.

10.1.2 Perform the binding of the control sample (reference), the irrelevant product and the sample buffer in duplicate.

10.1.3 In the analysis software, subtract the sample buffer binding data from that of the control and the irrelevant product data to establish specificity. Specificity is established when the irrelevant product exhibits no binding.

10.2 Testing for accuracy

Assay accuracy expresses the closeness of agreement between the value found and the value which is accepted either as a conventional true value or an accepted reference, and is calculated as a % recovery of the accepted or reference value. It is evaluated through the relationship between the nominal concentration and the measured sample concentration, and is confirmed using an acceptance criteria. A reference sample is therefore necessary for determining accuracy.

10.2.1 Immobilize the capture molecule on six different biosensors.

10.2.2 Perform a dose response binding assay of the reference product sample (3 concentrations in duplicates) Include duplicate samples of matrix alone (negative control). Repeat the assay to produce 3-replicates of the dose response assay.

10.2.3 Perform the same experiment using steps 10.2.1 and 10.2.2 with the test sample as the analyte.

10.2.4 In the analysis software, subtract the sample matrix (negative control) data from that of the reference and test samples.

Calculate and report the following:

- Mean K_D value (n=3) for each reference and test sample.
- Relative affinity for each assay.
- The %Recovery for the determined relative affinity calculated using the following formula:

$$\%Recovery = \frac{Determined\ Relative\ Affinity\ (n=3) \times 100\%}{Theoretical\ Relative\ Affinity}$$

10.3 Testing for linearity

The linearity of the procedure is its ability (within a given range) to obtain test results which are directly proportional to the amount of analyte in the sample.

10.3.1 Determine analyte concentrations for linearity assessment. This should be pre-diluted analyte samples to cover the full range of the affinity analysis as determined in Section 8.

10.3.2 Prepare the pre-determined analyte concentrations in assay buffer or matrix (enough for replicate analysis). Ensure accurate matching of matrix by using a stock analyte sample in an identical buffer or matrix.

10.3.3 Perform binding assays.

10.3.4 Fit the data to a local fit and compare the determined K_D numbers for each concentration with the average K_D obtained from the global fit.

10.3.5 Report the individual K_D, the mean K_D, % recovery and % CV of the replicates.

$$\%Recovery = \frac{Mean\ K_D\ Determined\ by\ Local\ Fitting\ (n=3) \times 100\%}{K_D\ Determined\ by\ Global\ Fitting}$$

10.3.6 Establish acceptance criteria.

10.4 Testing for repeatability/precision

Repeatability expresses the precision of the method under the same operating conditions and over a short interval of time. It is also referred to as intra-assay precision.

Repeatability should be assessed using n reportable values, 3 assays and 2 reportable values yielded from each assay. Sample should be assayed in replicate as per the method.

Precision should be established using replicate runs on different instruments or with different operators. For each variable (instrument, operator, etc.) record reportable parameters. Table 1 below shows an example of reportable parameters: K_D and % CV.

Assay	Mean K_D (M)	% CV
1		
2		
3		
Average		

Table 1: Precision testing results.

10.5 Robustness testing

The robustness of the analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. Key variables to test for robustness include:

a Biosensors ligand immobilization variability

b Biosensor lot to lot variability

c Plate well position variability

d Plate to plate variability

e Immobilization ligand lot to lot variability

f Analyte lot to lot variability

10.6 Stability indicating testing

To establish that the technique is stability indicating, accelerated stability studies where the analyte is degraded and tested for binding to the immobilized ligand should be performed. Determine the degradation conditions based on the biophysical properties of the analyte; examples of degradation conditions include high temperatures, low pH and enzymatic degradation among others. Perform the assays in triplicate.

10.6.1 Determine test sample conditions, for example, 3–4 degradation conditions.

10.6.2 Subject the samples to the forced degradation conditions, for example varying high temperature conditions.

10.6.3 Perform binding assays as previously determined. Include a control sample and a non-degraded sample.

10.6.4 Determine and record reportable parameters. Binding response at known concentrations can be used as a reportable parameter. Table 2 below shows an example of reportable parameters: response signals and % recovery which is the ratio of the degraded sample's response and the control sample response at equivalent analyte concentration.

Assay	Temp °C/pH	Maximum response (nm)	% CV	% Recovery
1				
2				
3				
Control				N/A

Table 2: Stability testing results.

Reference

USP guidelines chapters 111, 1032, 1033, 1034.

DISCLAIMER

This document does not supersede the need for the sponsor to consult with regulatory bodies on the appropriate method design and supporting data requirement for kinetics binding assays.

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