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Rapid Determination of Optimal Concentration Series for Kinetic Binding Assays Using Parallel Multiparameter Assessment

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Abstract

Accurate determination of binding kinetics and affinity depends on selecting an analyte concentration series that appropriately samples the dynamic range of an interaction. However, identifying an optimal concentration range is often an iterative and time-consuming process that can require multiple experimental rounds. Parallel multiparameter assessment (PMA) provides an optimized assay development approach by evaluating multiple overlapping analyte concentration series within a single kinetic experiment. By assessing how kinetic and affinity parameters behave across different concentration subsets, PMA enables direct sampling of the kinetic landscape and identification of the concentration range that provides the most robust and informative data.

PMA allows users to determine whether an analyte concentration series contains sufficient information to support accurate kinetic analysis, identify concentration ranges that are under- or over-saturating, and establish conditions that maximize confidence in the derived kinetic parameters.

By reducing the need for iterative assay optimization, PMA accelerates assay development, increases confidence in kinetic characterization, and supports more efficient reagent utilization through selection of the most informative analyte concentration series. Furthermore, the approach can be extended to evaluate additional assay variables, providing a flexible framework for the rapid optimization of kinetic binding assays.

Introduction

Determination of accurate kinetics and affinity plays a critical role in drug discovery and development. An essential parameter in determining accurate kinetics and affinity is the use of an optimized analyte concentration series as the quality of the data generated is dependent on the ability of the concentration series to span the dynamic range of the assay.

Traditionally determination of an optimal analyte concentration series has followed the standard advice of: *"...when reliable, accurate kinetic constants are required, a dilution series of at least five analyte concentrations should be measured during the association step."*

An ideal binding profile should include at least two data points below the expected K_D and two data points above the expected K_D . Where the K_D is known, either from previous experimentation or from literature values, the analyte dilution series should ideally range from approximately $0.1 \times K_D$ up to $10\text{--}20 \times K_D$, using 2-fold or 3-fold dilutions. Since K_D is defined as the concentration of analyte at equilibrium that occupies 50% of the available binding sites on the biosensor surface, this concentration range helps ensure that the assay spans from approximately 90% R_{max} down to the limit of detection. Running several concentrations also demonstrates how well the fitted binding model applies across a concentration range surrounding the K_D ."

Although excellent advice, determining the optimal analyte concentration series using only a single analyte concentration per assay can be time-consuming and not provide the desired robustness in the kinetic rate constants. Therefore, an alternative, higher throughput approach is desirable and parallel multiparameter assessment (PMA) can be used to determine the optimal analyte concentration series.

When using PMA, it is important to ensure a wide range of analyte concentrations are assessed (Figure 1). For example, in Figure 1A, the analyte concentration range produces a binding signal that spans the dynamic range of the assay from near the limit of detection to just below saturation. The even spacing between the association curves indicates that the ligand on the biosensor has not been saturated with analyte. If the analyte concentrations tested in a kinetic assay are too high, ligand binding sites can become saturated, increasing the potential for artifacts and non-ideal performance related to non-specific binding or aggregation (Figure 1B). Conversely, if analyte concentrations are too low, signal may be weak and/or the resulting binding rates may be diffusion limited (Figure 1C).

PMA follows the traditional advice discussed above but is flexible enough to deal with known or unknown K_D .

If the K_D of the interaction is not known, a broad concentration PMA with 5 – 10-fold dilutions can be used to approximate the K_D before performing a more refined PMA.

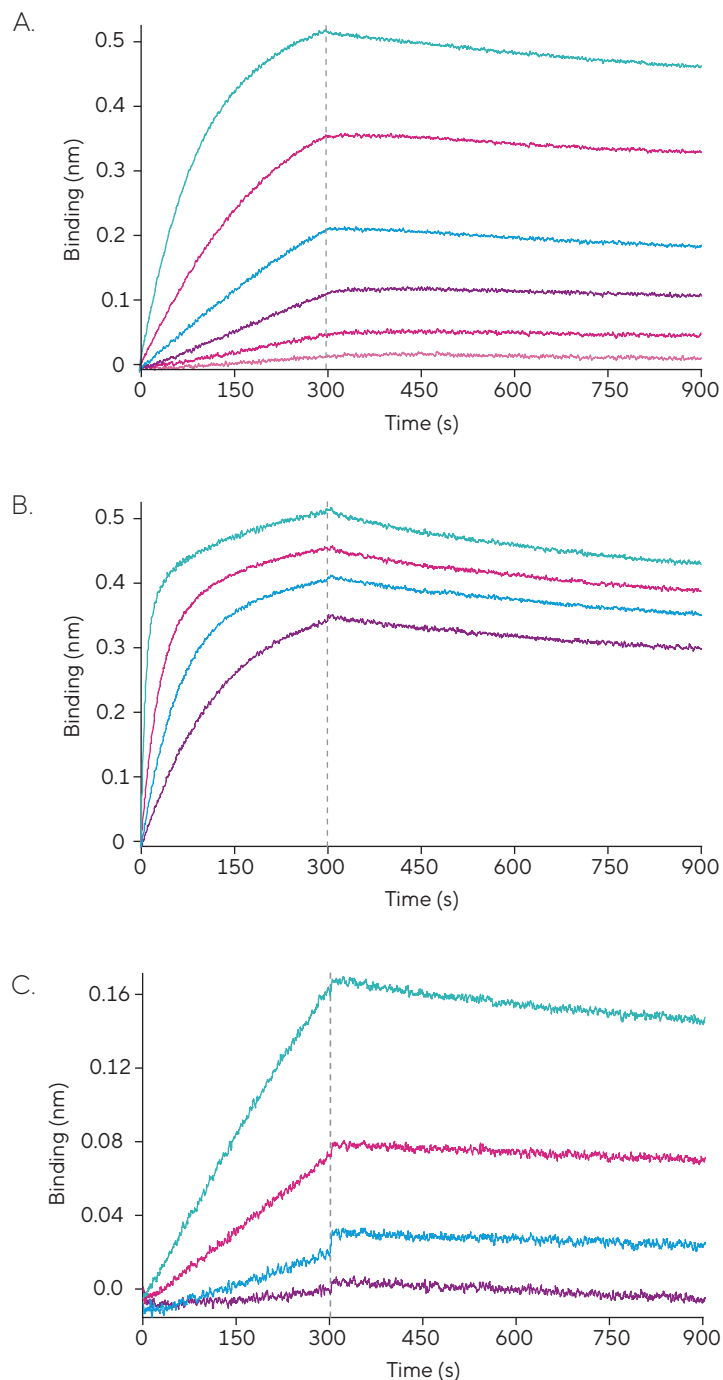


Figure 1: (A) Ideal range of analyte concentrations for a kinetics experiment, with signals spanning the dynamic range of the assay. Curve fits generated using such data will likely give reliable kinetic constants. (B) The range of analyte concentrations utilized is too high. Note that the curves are close together and exhibit heterogeneity most likely due to non-specific binding. The lower range of concentrations is not represented, which tends to give the most accurate data. (C) The range of analyte concentrations here is too low and data is limited by mass transport.

Sampling the Kinetic Landscape

As shown above, traditional kinetic analysis can be viewed as:

- Running an analyte concentration series
- Fitting the resultant data
- Obtaining k_a , k_d and K_D

PMA assays aim to improve the robustness of the determined kinetic parameters by evaluating data across the kinetic landscape through the use of multiple analyte concentration series in a single assay. PMA allows multiple different combinations of analyte concentrations to be assessed and identify where the kinetic parameters are most consistent. The underlying principle is that the most reliable kinetic constants should remain relatively consistent regardless of which valid subset of data is used. PMA allows sampling of the kinetic landscape and identification of the analyte concentration series where k_a , k_d and K_D converge.

Determining the optimal concentration series of analyte for an assay can be a laborious process that can require multiple iterations. In this technical note, a simple method to determine the optimal analyte concentration series of programmed cell death protein (PD-1) to analyze binding to Opdivo® (nivolumab) is shown.

Materials and Methods

Materials

Material	Supplier	Product Number
Octet® AHC2 Biosensors	Sartorius	18-5142
Octet® 96FW Microplate	Sartorius	18-5173
Octet® 384TW Microplates	Sartorius	18-5166
Octet® AE Evaporation Cover	Sartorius	18-5152
20X HBS-EP+	Teknova	H8022
Opdivo® (nivolumab)	Bristol-Myers Squibb	Supplied by WEP Clinical
Human PD-1 (His Tag)	SinoBiological	10377-H08H
GLY 1.7 (Octet® Regeneration Buffer Kit)	Sartorius	18-5171

Table 1: Materials required for performing binding assays.

Methods

All assays were performed in 1X HBS-EBT, which was prepared from 20X HBS-EP+ supplemented with 1 mg/mL BSA.

Octet® AHC2 Biosensors were used for assessment of binding of PD-1 to Opdivo®. All biosensors were hydrated for at least 10 minutes at room temperature prior to use in 1X HBS-EBT in either a 96- or 384-well, black, tilted bottom microplate. All assays were performed at 25 °C.

Each analyte concentration series was assigned to a replicate group. Octet® AHC2 Biosensors were regenerated between assessment of different analyte concentration series using three rounds of 5 second regeneration in glycine pH 1.7 and 5 second neutralization in 1X HBS-EBT.

The Octet® BLI PMA assay followed a general assay flow of:

Assay Step	Step Name	Time (s)	Shake Speed (RPM)*
1	Baseline	60	1,000
2	Loading*	600	1,000
3	Baseline 2	60	1,000
4	Association	180	1,000
5	Dissociation	300	1,000

Table 2: General Octet® PMA assay flow. * Opdivo® was captured to a threshold of 1.00 nm.

Analyte Preparation

Human PD-1 (His Tag) was prepared to a final concentration of 450 nM in 1X HBS-EBT and subsequent analyte concentration series prepared from this stock solution. Opdivo® was buffer exchanged into 1X HBS-EBT using a Vivaspin® 500 centrifugal concentrator and a final concentration of 1 µg/mL was used in all analyte concentration assessments. A reference sample of 1X HBS-EBT was used in all assessments in order to correct for baseline drift.

Data Analysis

Analysis was performed using an Octet® R8e BLI system using Octet® BLI Discovery and Analysis Studio Software version 13.1.0.25. Data was fitted using Octet® Analysis Studio software 13.1.0.38 to a global 1:1 model grouped by Replicate Group.

Results

The interaction of PD-1 with Opdivo® is well studied and published K_D values provide a useful starting point for assay design. Reported values include 3.06 nM in the European Public Assessment Report¹ (EPAR), 4.2 nM in the application note **How Low Can You Go? A New Era of Sensitivity in Octet® BLI**, and a general spread of single digit nanomolar K_D values have been reported across multiple surface plasmon resonance systems².

As discussed in the previous section, these literature values can be used to guide the selection of analyte concentrations for kinetic characterization experiments. The eight-channel Octet® R8 and Octet® R8e systems allow up to eight analyte concentrations to be assessed simultaneously (although it is recommended that a buffer blank is also included to control for excipient effects). As shown in Figure 2, a top concentration of 450 nM of human PD-1 (approximately 100-fold greater than the published K_D value) was chosen in order to ensure as much sampling of the kinetic landscape as possible. A 3-fold dilution series was performed both vertically and horizontally, which created six different analyte concentration series.

	Human PD-1 Analyte Concentration (nM)
1	1.85 - 450
2	0.617 - 150
3	0.206 - 50
4	0.069 - 16.7
5	0.023 - 5.56
6	0.008 - 1.85

Table 3: Human PD-1 analyte concentration series generated from a parallel multiparameter assessment (PMA) design. A 3-fold dilution scheme from a maximum concentration of 450 nM was used to create six overlapping concentration series for evaluation of the PD-1/Opdivo® interaction.

Note: The association time should be long enough to see curvature in the data traces, but not so long that the curves reach saturation for an extended period as this leaves opportunity for weaker, non-specific interactions to occur. Therefore, PMA assays can contain extended association periods to assess this parameter. The association time can then be curtailed in subsequent assays.

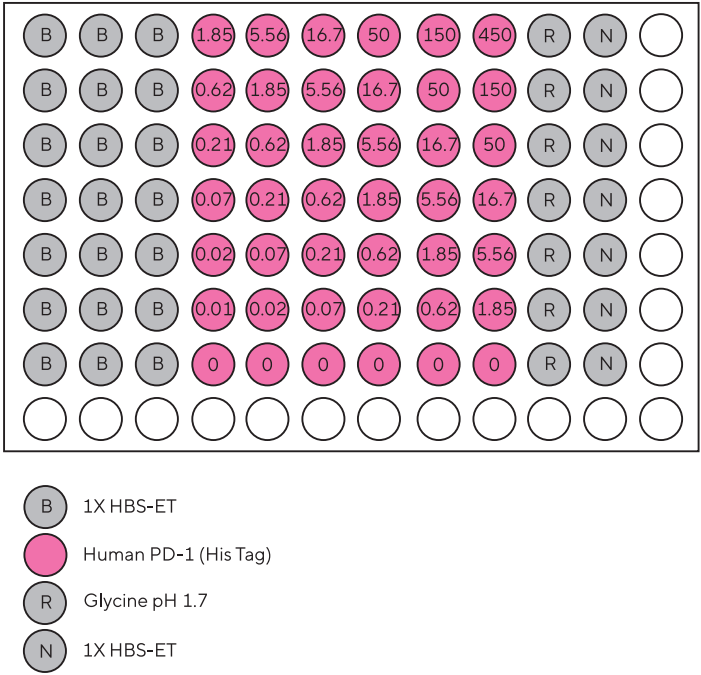
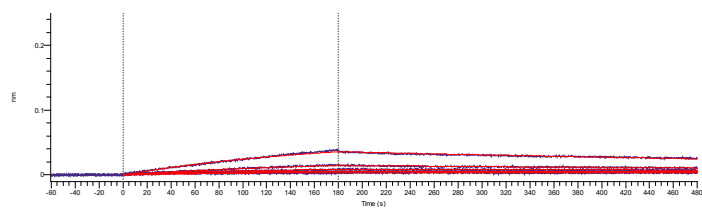
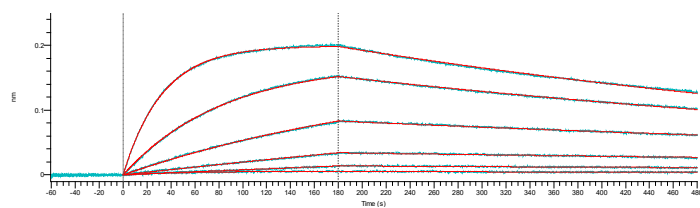


Figure 2: 96FW Microplate design of the parallel multiparameter assessment (PMA) concentration matrix. Human PD-1 was prepared as a 3-fold dilution series from 450 nM, creating six overlapping analyte concentration series.

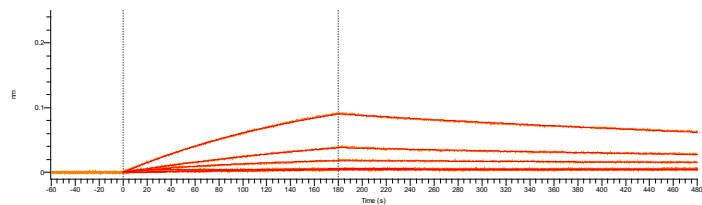
0.01 – 1.85 nM



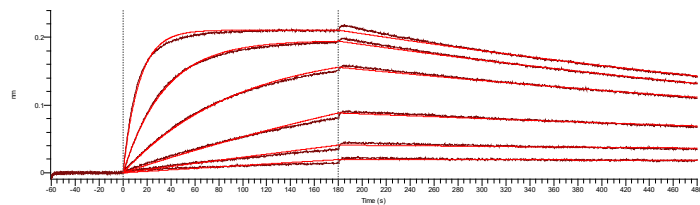
0.21 – 50 nM



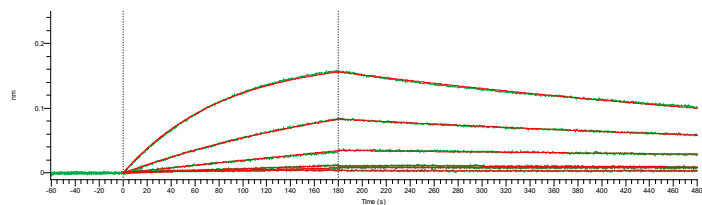
0.02 – 5.56 nM



0.62 – 150 nM



0.07 – 16.7 nM



1.85 – 450 nM

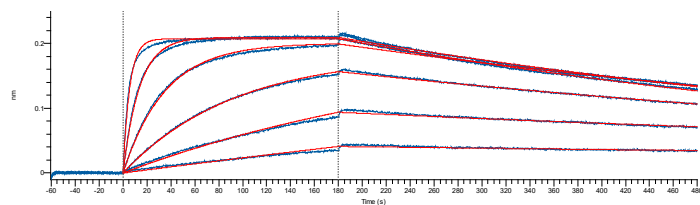


Figure 3: PMA enables rapid visualization of the kinetic landscape across multiple analyte concentration series in a single experiment. Comparison of sensorgrams reveals how concentration range influences kinetic information content, model fitting, and confidence in derived binding parameters, facilitating rapid identification of the optimal concentration series for assay development.

Human PD-1 Analyte Concentration (nM)	k_a ($M^{-1}s^{-1}$)	k_a Error	k_d (s^{-1})	k_d Error	K_D (nM)	K_D Error	R^2
0.01 – 1.85	3.00×10^3	9.25×10^4	2.47×10^{-4}	1.79×10^{-5}	82.5	2.54×10^{-6}	0.9479
0.02 – 5.56	3.63×10^3	1.44×10^4	8.62×10^{-4}	8.82×10^{-6}	237.3	9.39×10^{-7}	0.9878
0.07 – 16.7	5.24×10^5	2.95×10^3	1.16×10^{-3}	4.39×10^{-6}	2.22	1.50×10^{-11}	0.9966
0.21 – 50	5.20×10^5	1.07×10^3	1.25×10^{-3}	2.33×10^{-6}	2.39	6.67×10^{-12}	0.9988
0.62 – 150	5.28×10^5	8.26×10^3	1.39×10^{-3}	1.68×10^{-6}	2.65	5.23×10^{-12}	0.9991
1.85 – 450	5.39×10^5	1.12×10^3	1.47×10^{-3}	1.99×10^{-6}	2.72	6.74×10^{-12}	0.998

Table 4: Kinetic and affinity parameters determined from global fitting of each PMA concentration series to a 1:1 Langmuir binding model. Association rate constants (k_a), dissociation rate constants (k_d), affinity constants (K_D), associated fitting errors, and goodness-of-fit values (R^2) are shown for each analyte concentration range.

Discussion

When performing a full kinetic profile with several analyte concentrations, data are analyzed globally by fitting both the association and dissociation phases for all analyte concentrations simultaneously using a common set of rate constants. Global analysis across a broad analyte concentration range provides robust fitting and accurate estimation of binding constants. Therefore, accurate knowledge of analyte concentration is critical, as this value has a direct impact on the calculated kinetic and affinity constants.

0.01 – 1.85 nM

This concentration series exhibits very low linear responses, which lack curvature and therefore yield very little kinetic information. As shown in Table 4, the kinetic rate constants yield an affinity that is approximately 20-fold greater than the expected value. The lack of observable curvature during the dissociation phase – coupled with a binding decrease of less than 5% – makes determination of an accurate k_d impossible.

0.02 – 5.56 nM

Although showing a larger response, 0.62 – 5.56 nM shares a lot of the same issues as 0.01 – 1.85 nM in that the responses are linear and yield very poor kinetic information. This is highlighted by the large K_D and corresponding error that is close to the predicted value due to the 1:1 model attempting to fit unsuitable data. As above, the lack of observable curvature during the dissociation phase – coupled with a binding decrease of less than 5% – makes determination of an accurate k_d impossible.

0.07 – 16.7 nM

As the top concentration increases, curvature can start to be observed in the top analyte concentration (16.7 nM) which allows the model to fit the data more precisely and extrapolate more accurate kinetic rate constants. This is also shown for the dissociation phase where a more accurate kinetic rate parameter can be determined due to a visible decrease and binding decrease of greater than 5%. Although this concentration series exhibits an affinity close to the published values, the top concentration does not reach saturation and therefore, further kinetic information could be obtained by further increasing the top analyte concentration. This could also be achieved through a longer association period. It is also important to note that as only the top concentration exhibits any significant curvature and therefore, any change in activity of the human PD-1 analyte activity could decrease the observed curvature and affect the accuracy of the assay (as could be experienced due to multiple freeze-thaw cycles). It is also important to note here that the second highest concentration of analyte is 5.6 nM, and as discussed above, it is important to have more than two analyte concentrations above the K_D in order to determine accurate kinetics and affinity.

0.21 – 50 nM

In this concentration series, the top two concentrations exhibit good curvature and the top concentration (50 nM) reaches saturation, which helps in the determination of more accurate association kinetic parameters. A clear decrease in the signal during dissociation is also observed, aiding more accurate determination of the dissociation rate constant. Although the determined association and dissociation kinetic parameters are similar to the previous concentration series, the K_D error is decreased, indicating more confidence in the fit of the model to the data set. The top two concentrations (50 and 16.7 nM) are both above the predicted K_D along with the bottom two concentrations (0.21 and 0.63 nM) being below the predicted K_D .

Although the model provides a good fit for the data, it is important to assess all the concentration series to further sample the kinetic landscape and determine whether there is agreement in the determined kinetic parameters and affinity values.

0.62 – 150 nM

As shown in Figure 3, the top concentration in this series (150 nM) saturates for an extended period of time (~120 seconds), which may favor the opportunity for weaker, non-specific interactions to occur. As previously, the 50 nM concentration reaches saturation. The spread of the signal responses in this concentration series is well defined between the highest and lowest concentrations, with both the lowest concentrations still below the predicted K_D . As shown in Table 4, the association and dissociation rate constants are similar to those determined using the 0.21 – 50 nM concentration series, with no further decrease in the associated error values.

1.85 – 450 nM

As expected, this concentration series becomes 'top heavy' with the three highest concentrations (450, 150 and 50 nM) reaching saturation. This clustering is especially apparent when reviewing the dissociation phase for the three analyte concentrations. It is also apparent from the sensorgram (Figure 3) that there is space below the lowest concentration (1.85 nM) for a further analyte concentration, indicating that this concentration series may not sample the kinetic landscape effectively.

As with the previous two concentration series, there is no further decrease in the association or dissociation rate constants and their associated errors. This is also shown for the determined affinity and associated error.

From the data shown in Figure 3 and Table 4 it is clear that the assessed concentration series are sufficient to cover the kinetic landscape and help determine the optimal analyte concentration series.

Across the concentration series evaluated, the lowest ranges (0.01 - 1.85 nM and 0.02 - 5.56 nM) produced predominantly linear binding responses with minimal observable dissociation (<5% signal decrease). As a result, insufficient association kinetic information was obtained, preventing reliable determination of the dissociation rate constant (k_d), resulting in inaccurate affinity estimates. Kinetic parameter quality improved as higher analyte concentrations were included, with the 0.07 - 16.7 nM series showing observable curvature and dissociation, yielding affinity values close to published data. However, only the highest concentration provided meaningful kinetic information and saturation was not reached. The 0.21 - 50 nM series represented the best balance, with strong curvature, transient saturation at the highest concentration, clear dissociation, analyte concentrations distributed above and below the predicted K_D , and reduced fitting error, leading to greater confidence in the derived kinetic parameters. Further increases in concentration (0.62 - 150 nM and 1.85 - 450 nM) did not significantly improve parameter precision; instead, prolonged or excessive saturation caused the datasets to become increasingly 'top heavy', potentially introducing non-specific interactions and reducing effective sampling of the kinetic landscape without providing additional gains in confidence or accuracy.

As shown, the 0.21 - 50 nM series produced a precise K_D value for the interaction between PD-1 and Opdivo® and therefore would be the most suitable analyte concentration series for future kinetic analysis. As the association and dissociation rate constants stabilize above this concentration series there is little perceivable benefit in using three to nine times more analyte in order to determine the same parameters.

Note: Although surface density was not investigated here, a simple modification to the assay setup would allow the same analyte concentrations to be assessed at multiple ligand densities. This could further streamline assay development into a single experiment, especially when using the Octet® AE Evaporation Cover.

Conclusions

Parallel multiparameter assessment (PMA) provides a powerful and efficient approach for kinetic assay development by enabling multiple analyte concentration series to be assessed simultaneously within a single experiment. Rather than relying on iterative optimization, PMA allows direct sampling of the kinetic landscape and identification of the concentration range that yields the most reliable and reproducible kinetic parameters.

By identifying the optimal concentration series in a single experiment, PMA reduces assay development time and provides a data-driven framework for establishing robust kinetic assays. The approach increases confidence in reported kinetic and affinity parameters by demonstrating convergence across multiple valid concentration series and by identifying those datasets that contain the most informative kinetic content. This enables users to develop more reliable assays, make better-informed structure-kinetic and affinity comparisons, and reduce downstream reagent consumption through the use of a concentration series that is both scientifically justified and experimentally efficient. Furthermore, the PMA workflow can be readily extended to evaluate additional assay variables, such as ligand density and association time, making it a broadly applicable strategy for accelerating assay optimization and improving confidence in kinetic characterization.

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