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Harmonizing Regulatory Guidelines for Assay Validation: A Focus on Kinetics-Based Assays Using Octet® BLI Systems

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Abstract

Validation of an assay is a key part in ensuring accurate and reliable data, which is crucial in scientific research, clinical diagnostics, and pharmaceutical industries decision making. To date, all regulatory validation guidelines cover end-point, potency-based assays and although they do offer a framework for assay design and subsequent validation they do not cover many of the parameters that are important in a kinetics-based assay.

This document represents an attempt to harmonize the current regulatory guidelines for assay validation and apply them to a kinetic-based assay in order to derive a common set of parameters. Although the focus of this document is the Octet® BLI systems the overarching recommendations and assay validation parameters are applicable to all label-free, real-time assay formats, such as surface plasmon resonance.

This document outlines the process of validating a ligand binding assay for Fcγ receptor binding analytes such as antibodies, or other relevant structural isoforms.

Introduction

Method development and subsequent validation of a ligand binding assay (LBA) is a critical part of drug production that is an integral constituent of safety and efficacy. Taking an assay from conception through to validation can often be a time-consuming process and therefore, this document aims to provide an overview of the current regulatory guidelines and combine them in such a way that Octet® BLI users can directly implement for validation of a kinetics-based ligand binding assay. Octet® BLI systems allow users to study label-free biomolecular interactions in real-time, which directly provides kinetics and affinity data for the interaction being assessed. Applicable throughout the drug discovery process, Octet® BLI's dip and read technology effectively shortens the time to results, which allows better decisions to be made more rapidly. The biologic's structure plays a critical role in determining the binding kinetics and affinity and therefore, label-free analysis ensures that the observed real-time results directly reflect the active binding concentration of the interactants.

As outlined later in the document, the coefficient of variation (CV) between assays is a critical parameter to control and Octet® BLI systems offer exceptional precision compared to standard end-point assays such as ELISA or cell-based assays. This precision in a validated assay ensures that any changes in structure or function over time are quickly identified and remedial action can be taken, substantially reducing time spent on quality investigations such as for an out of specification (OOS) acceptance criteria failure. In addition to real-time kinetics, Octet® BLI software allows users to assess their data via potency (EC_{50}) measurements.

Although frequently used in multiple areas of research and drug discovery, Octet® BLI systems face similar roadblocks to other technologies when being considered for validated assays. Oftentimes, these include a reluctance to change from traditional end-point assays that have been the mainstay for validated assays but as mentioned above, these often have a lower throughput and precision than label-free real-time assays. Reluctance to implement change can stem from a lack of awareness that current regulatory guidelines for LBAs are applicable to Octet® BLI assays and that regulators are broadly supportive of a controlled change to more precise technologies as shown by their inclusion in regulatory guidelines such as USP<1108> (Assays to Evaluate Fragment Crystallizable (FC)—Mediated Effector Function)[6].

As shown in this document, outside of preparing suitable documentation, the major factor in the adaptation of Octet® BLI assays for validation is a lack of understanding as to how current regulatory guidelines can be applied to a new or existing assays. No claim of regulatory compliance is made in this document.

Harmonizing Current Validation Guidelines

The Octet® BLI Validation Guidelines are an attempt to harmonize the current regulatory guidelines and create a series of recommendations for validating ligand binding assay(s) (LBA) performed on an Ocet® BLI system. The guidelines are based upon the most recent FDA[1], ICH[2,3,4] and USP[5] documents that contain recommendations for users looking to validate suitable assays. Although Analytical Procedure (AP) is the preferred term for a broad range of assays in ICH guidelines, the FDA terminology, which has been adopted in the harmonzing document ICH M10, of Ligand Binding Assay is used throughout this document.

Ligand binding assays (LBA) are used to measure the interaction between a surface bound ligand and a solution-based analyte and are commonly used in drug discovery, research, and manufacturing to understand the efficacy and potency of a particular drug. Validation of an assay ensures the assay is fit for its intended purpose and can produce specified parameters consistently. A non-validated assay may still provide useful information but may not be as consistent and may not be suitable for all intended purposes.

The recent ICH Q2(R2) supersedes ICH Q2(R1) and provides a complete revision of the original guidelines, with application of more recent analytical procedures (LBA) and aligns with the content of the Q14 guide (Analytical Procedure Development). ICH M10, although not directly relevant here due to the lack of biological matrix, provides an excellent overview of multiple parameters required for succesful validation of an LBA.

The FDA Bioanalytical Method Validation: Guidance for Industry (final version) was published in May 2018 and "provides recommendations for the development, validation, and in-study use of bioanalytical methods". Importantly the guide stresses that the "recommendations can be modified with justification, depending on the specific type of bioanalytical method"; which allows their application to technologies such as Octet® BLI.

The FDA guidance provides an excellent description of the purpose of assay design and development:
The purpose of bioanalytical method development is to define the design, operating conditions, limitations, and suitability of the method for its intended purpose and to ensure that the method is optimized for validation.

Submission of pivotal studies, such as IND (Investigational New Drug), NDA (New Drug Application) or BLA (Biologics License Application), require full validation of any bioanalytical methods used. Whereas, exploratory methods, such as candidate selection, that are used to support regulatory decision making may not require full validation. Users can decide on the level of assay qualification that supports their internal decision making for assays that are not intended to produce data that will be submitted for regulatory approval, or considered in regulatory decisions.

Prior to validation it is important to consider the primary purpose of the assay as this ensures that during the design and development phase any parameters that require acceptance criteria are well understood and controlled. The level of documentation varies with each stage of assay development and although LBA design and development does not require extensive documentation and record keeping, any procedural changes and issues (and their resolutions) encountered should be recorded appropriately (see Table 2 in Bioanalytical Method Validation Guidance for Industry)[1].

General considerations for assay validation are that the assay must be fit-for-purpose (FFP) and appropriate for the intended purpose of the study.
Key questions that should be addressed to ensure the LBA is validated correctly include:

- Does the method measure the intended analyte?
- What is the variability associated with these measurements?
- What is the range in measurements that provide reliable data?
- How do sample collection, handling, and storage affect the reliability of the data from the bioanalytical method?

Although not within the scope of this document it should be recognized that the assay parameters discussed can also be applied to design and development (and, where required, qualification) of a LBA too.

Here, an assay designed to test an antibody analyte binding to an Fcγ Receptor ligand protein will be discussed and the steps required to take the assay through to full validation proposed.

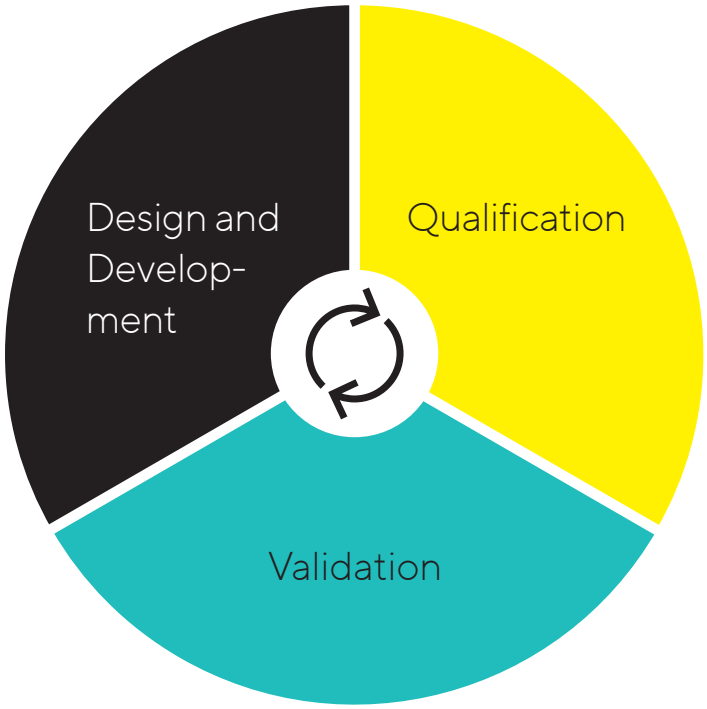
Assay Validation Lifecycle

A critical understanding in LBA validation is that the process should be considered as a lifecycle and not as a one-off event. Over time, changes in reagents (see **Critical Reagents**), instrumentation and other consumables can have an impact on a validated method's ability to meet its original acceptance criteria. Therefore, it is critical that each stage of the assay lifecycle is performed as completely as possible in order for the validation to build upon a stable foundation.

As discussed below (**General Assay Development Considerations**), creation of an assay control strategy (ACS) for the LBA should be defined before validation and confirmed after validation. This ensures that the assay performs as expected during routine use throughout its lifecycle. The ACS includes parameters that require control and a sufficiently detailed procedural description that would allow a skilled analyst to perform the method, analysis and interpret the results.

In general, the final validation should be thought of as the documented performance of the method that was developed during the previous two stages and that the assay is fit for its intended purpose.

Figure 1: *LBA Is Not a One-off Event and Should Be Viewed as a Continuous Process With a Lifecycle.*



Assay Design and Development

Although all projects are different and therefore, present different challenges, the intended use of the LBA is the first thing that must be defined every time a new assay is designed. It is important to have as much knowledge as possible about the analyte and ligand that will be used to perform the LBA. For example, does either the analyte or the ligand form dimers or higher order oligomers, is either molecule highly hydrophobic or charged at the pH the assay will be performed at or is anything already known about its stability at the concentrations that are expected to be used in the LBA? During assay design and development, it is essential that the analyte and ligand used to develop the assay are as similar, ideally identical to the samples expected to be used in the full validation (see **Reference Standards and QC Samples**). Octet® BLI offers several kits that can aid in rapid assay development.

Prior to starting assay development, it is highly recommended that the user is familiar with USP Guidelines<1032> (Design and Development of Biological Assays) and the **Octet® BLI Technical Note Octet® BLI Kinetics Assay: Method Development Guideline** and Application Note **Analysis of Fc-gamma Receptor-IgG Interactions on the Octet® Platform**.

An iterative approach is applied to designing and developing LBA, which includes:

1. Assay Orientation and Biosensor Selection
2. Biosensor hydration
3. Optimal Ligand Density—Ligand Scout
4. Assay Buffer Optimization and Non-specific Binding
5. Analyte Binding Optimization
6. Assay Robustness and Specifications Testing

Assay Orientation and Biosensor Selection

The first step of assay design is determining which member of a binding pair to immobilize or capture (ligand) and which member to leave in solution (analyte). Protein stability, size, and valency are big factors in this decision. More sensitive proteins may not tolerate the relatively harsh conditions imposed by immobilization on amine reactive surfaces. In this case, the less sensitive molecule must be immobilized or a different biosensor surface chemistry can be considered. Size is another factor as smaller molecules produce smaller signals upon binding.

Since certain Fcγ receptors-IgG interactions are relatively low affinity, concentrations of analyte for association may need to be quite high (up to µM). In contrast, the ligand molecule immobilization step is typically performed at lower concentrations.

Therefore, limitations on availability of reagents will need to be considered when choosing assay orientation. In general, binding partners that carry an intrinsic affinity tag should be assigned as the ligand and used with an appropriate anti-tag biosensor and several biosensor options are available for studying Fcγ receptors-IgG kinetics, depending on the desired format (Table 1 and Figure 2). Where the K_D of the binding interaction is known to be low, affinity capture may not always be appropriate and alternative capture or immobilization methods may be required.

When capturing HIS-tagged Fcγ receptors, Ni-NTA Biosensors (NTA) can be used, which binds to a HIS-tag attached to recombinant proteins. In this format, the Fcγ receptor protein is loaded onto the biosensor as the ligand, followed by association with IgG (Figure 2).

Since nickel (Ni2+) can also bind weakly to non-HIS-tagged proteins there is a potential for an increase in non-specific binding (NSB) to the IgG analyte when using Ni-NTA Biosensors and blocking and buffer conditions may need to be optimized to minimize NSB (see **Assay Buffer Optimization and Non-specific Binding**). Another method of reducing NSB when using HIS-tagged Fcγ receptors is to use an antibody specific for HIS-tagged proteins. QIAGEN's Penta-HIS antibody is highly sensitive and specific for HIS-tagged proteins. This antibody can easily be biotinylated for immobilization on streptavidin-based biosensors, or purchased pre-conjugated on Octet® HIS1K Biosensors, which, can then be used to capture HIS-tagged Fcγ receptors (Table 1 and Figure 2).

In general, amine-coupling of Fcγ receptors using AR2G Biosensors is not recommended as the resulting heterogenous orientation of receptor molecules on the biosensor surface can interfere with activity via steric restriction of access to binding sites or even conformational changes in binding sites. This interference is often reflected in low signal and sub-optimal kinetics[7]. Surface heterogeneity is especially important to consider when validating an Fcγ receptors assay where ligand activity and response (R_{max}) may be an assay acceptance criterion.

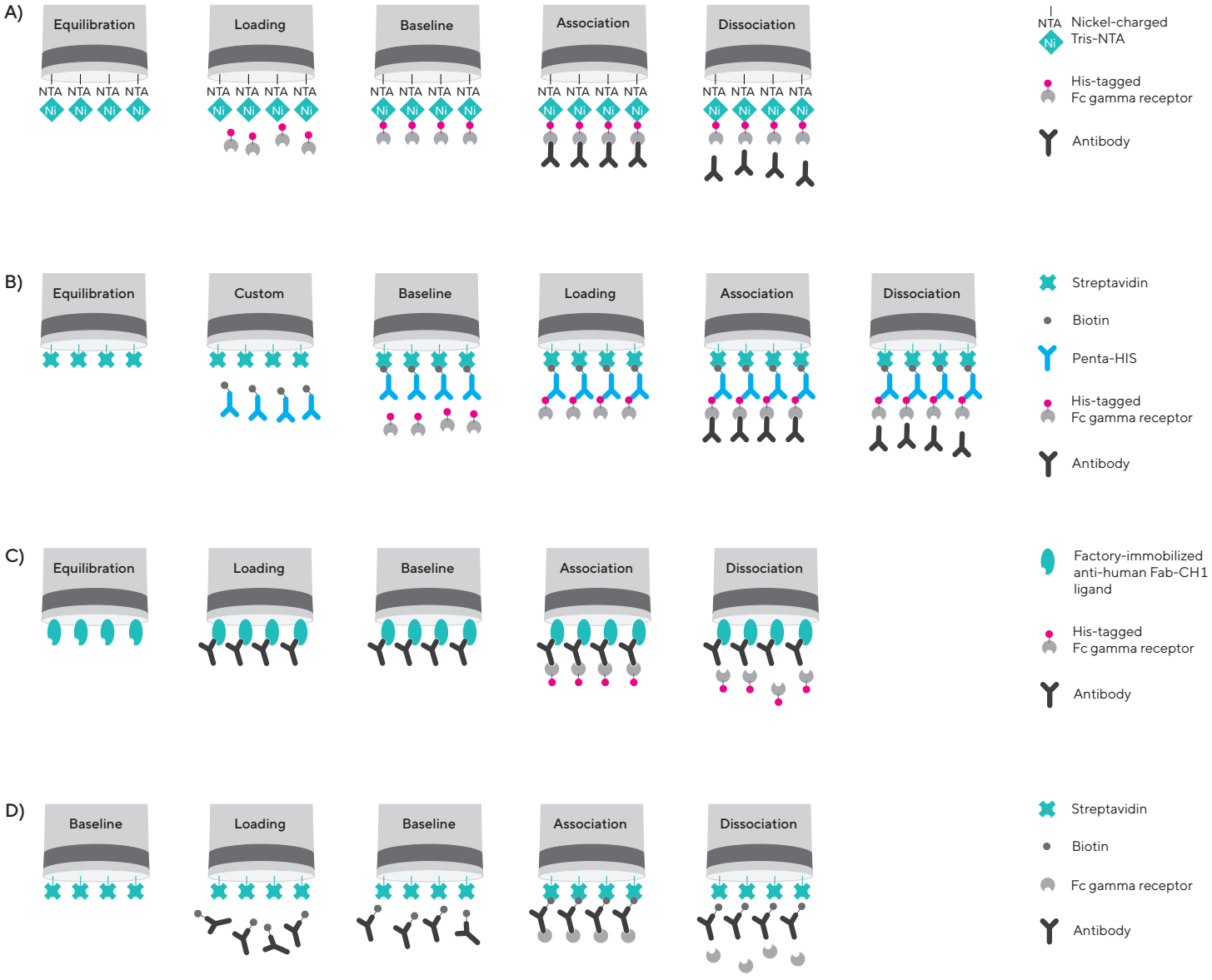
Therefore, use of streptavidin biosensors such as SA, SAX, SAX2 and SSA Biosensors is recommended over amine coupling when working with Fcγ receptors. Oriented capture via HIS-tag or a biotinylated AviTag™ offers a more robust and homogeneous binding kinetics assay. For the reverse assay orientation in which IgG is immobilized on the biosensor and Fcγ receptor protein is not limited in supply and remains in solution as analyte, the recommended biosensor selection is Anti-Human Fab-CH1 (FAB2G). The anti-human Fab-CH1 ligand molecule pre-immobilized on the surface has a high specificity towards the CH1 region of human IgG allowing a homogeneous surface to be created via oriented capture of IgG with minimal steric hindrance for Fcγ receptor binding (Figure 2).

This capture method is highly specific and reliable for analyzing Fcγ receptor-IgG kinetics but is more conducive to a platform-approach when testing multiple IgGs and receptors than a single analyte-ligand pair that would be used in assay validation.

Avidity is an important consideration in kinetics experiments as it can affect the overall calculation of K_D. While the affinity of a molecule to its binding partner is defined as the strength of the non-covalent association between one ligand binding site with one analyte binding site, the avidity of a molecule is determined by the total strength of all the binding associations possible between the two molecules (see **Application Note Optimizing Kinetics Assays to Avoid Avidity Effects**).

In general, the unmodified sensor surface for BLI is stable in extreme pH and temperature ranges, and also in relatively high concentrations of organic solvents. However, after immobilization or capture of a ligand, the stability of the active surface depends on the properties of the ligand. Choosing the correct sensor surface chemistry is a critical step in assay design and several key factors should be considered.

Figure 2: *Sample Workflow for Fcγ Receptor Assays on Octet® BLI Systems.*



Note: (A) Sample workflow for Fcγ receptor kinetic assay on Ni-NTA Biosensors. (B) Sample workflow for Fcγ receptor kinetic assay on High Precision Streptavidin (SAX and SAX2) Biosensors using the Penta-HIS antibody. (C) Sample workflow for Fcγ receptor kinetic assay on FAB2G Biosensors. (D) Sample workflow for Fcγ receptor kinetic assay on High Precision Streptavidin (SAX) Biosensors, with biotinylated IgG immobilized on the biosensor tip.

Optimal Ligand Density—Ligand Scout

The amount of ligand immobilized (loaded) onto the biosensor can have significant impact on the results of a kinetic assay. For either Fcγ receptor assay orientation—receptor immobilized or antibody immobilized —loading as much protein as possible onto the biosensors in order to maximize signal is not necessarily the best approach. An excess of ligand molecules bound to the biosensor can lead to data artifacts due to crowding, steric hindrance, avidity and mass transport effects. Weaker, non-specific interactions can also be favored, especially at higher analyte concentrations, when the biosensor is over-loaded. Assay artifacts can impact the observed binding kinetics and 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. If, however, not enough ligand is immobilized, the signal for the analyte association step may be too low, giving poor signal-to-noise ratio. Ideally, loading levels should be optimized using a ligand scout for every receptor and biosensor format used and the density of the ligand molecules on the biosensor tip depends on ligand concentration and loading step time.

The microplate format used on the Octet® BLI platform allows for rapid testing of several experimental parameters at once during a ligand scout, minimizing time spent on optimizing ligand density during assay development. When analyzing Fcγ receptor-IgG kinetics, ligand loading strategy will differ depending on the assay orientation and the biosensor used, though in general for kinetic measurements slower loading with a lower concentration of ligand is generally preferable to a higher concentration over a shorter time.

When capturing HIS-tagged or AviTag™ biotinylated Fcγ receptors, a low density of receptor ligand will yield better results. When loading biotinylated IgG or Fcγ receptors directly onto SAX Biosensors, loading conditions should be optimized for each experimental system. An assay development step or scouting step in which the biotinylated ligand is titrated on the biosensors is recommended.

When using FAB2G Biosensors to immobilize IgG, i.e. the reverse assay orientation, optimal results are achieved with a higher level of ligand loading. The kinetics and curve fitting results obtained using higher loading levels are similar to those using lower loading levels, with improved reproducibility at higher loading. Users should see Application Note **Analysis of Fc-gamma Receptor-IgG Interactions on the Octet® Platform** for specific recommendations on ligand concentration and loading times.

The optimal loading concentration to select for a detailed kinetic analysis is the lowest ligand concentration that yields an acceptable response in the analyte association step (see **Analyte Binding Optimization**). The selected loading concentration should not saturate the biosensor or cause changes in the association or dissociation kinetics compared to lower concentrations. Examples of such changes are the appearance of secondary binding in the association curve (caused by non-specific interactions) or decreased dissociation rate (caused by avidity). Generally, the less ligand loaded on the biosensor the better, as long as analyte signal is sufficient. It is recommended that the 'threshold' setting in Octet® BLI Discovery Software is used to ensure receptor loading does not progress above predetermined levels. Ligand loading strategies are listed according to biosensor format in Table 1.

To perform a loading optimization experiment, several concentrations of ligand are loaded onto the biosensor. A typical immobilization concentration for a ligand molecule, such as an IgG1 molecule (150 kDa), is ~0.47 – 30 µg/mL (3.125 – 200 nm) across seven biosensors with the last biosensor used for assay buffer. Initial loading times are based on a three to five-minute loading time. If the ligand concentration is low (e.g., < 50 nm), a longer loading time may be required for sufficient immobilization signal. Overnight incubation of biosensors in ligand solution may also be performed at 4 °C independently of the Octet® BLI system. Overnight incubation can greatly improve results in cases where capture biosensors are being used to capture a ligand molecule from a dilute supernatant or cell culture sample.

Following successful immobilization of the ligand, an analyte response test is performed for each ligand concentration using a high concentration of analyte (10X K_D). If the K_D is unknown, a high analyte concentration of 100 – 1000 nm may be assessed at first and then refined using an empirical process. Initial association and dissociation times of 300 – 600 seconds are recommended and can be made longer or shorter during the assessment using the 'Extend Current Step' and 'Go to Next Step' options in the runtime window, respectively. A zero-ligand biosensor should also be run as a control for determining whether the analyte binds non-specifically to the biosensor. The recommended sequence for the optimization assay is:

1. Baseline
2. Loading
3. Baseline 2
4. Association
5. Dissociation

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.

Assay step times should ultimately be developed and will depend on the expected K_D of the binding partners. The loading concentration to select for an assay should be the lowest concentration of immobilized ligand that yields an acceptable signal in the analyte association step. Other important parameters to consider are that the ligand should show no dissociation from the biosensor during Baseline 2 and that the analyte should reach equilibrium during the association phase. In general (unless expected), the data should fit a 1:1 model and heterogeneity should not be present in the association or dissociation phases (this is often caused by high ligand loads that promote analyte rebinding to the surface)[8].

The amount of ligand that is required is dependent on the assay format but in general for kinetic assays, the goal is to minimize the effects of mass transport limitation (MTL) of analyte to the surface and immobilization of small quantities of ligand minimizes the effects of MTL and improves measurement of kinetics-related observations. Therefore, we recommend a ligand load density between 0.5 – 1.5 nm shift (y-axis). For Octet® BLI assays, agitating the sample plate in a fluidic-free format creates a turbulent flow over the biosensor, which is not subject to laminar forces and is highly efficient at replacing the volume close to the surface of the biosensor. If mass transport effects are an issue, the supply of analyte to the surface must effectively be raised. This can be accomplished by reducing the level of immobilized ligand or increasing the shaking speed during the assay to increase flow rate.

Assay Buffer Optimization and Non-specific Binding

For Fcγ receptor kinetics assays, we recommend using Sartorius Kinetics Buffer as a sample buffer, which is available as a 10X solution (18 – 1105). This buffer contains the blocking agent bovine serum albumin (BSA), and a surfactant (Tween-20) to inhibit NSB to the biosensor tip and to other proteins. All samples (ligand and analyte) should be diluted in 1X Kinetics Buffer (prepared by diluting 10X KB in 1X PBS), and baseline and dissociation steps should be run in this buffer as well.

In general, non-ligand-loaded FAB2G Biosensors and ligand-loaded SAX Biosensors exhibit minimal NSB to IgG in 1X Kinetics Buffer. Where NSB is observed, adding additional BSA (up to 1%) or Tween-20 (up to 0.05%) to the 1X Kinetics Buffer can sometimes improve data quality.

Any histidine-containing protein can bind weakly to the Ni-NTA complex on NTA Biosensors therefore, it is important to check for NSB of IgG analyte.
A preliminary assay where IgG is associated to a NTA biosensor that is not loaded with Fcγ receptor ligand should be performed and a positive signal in the association step indicates the analyte is binding directly to the biosensor. Where background NSB signal is minimal, it can potentially be subtracted during data analysis by double reference subtraction though a better approach is to mitigate NSB by adjusting assay conditions by modifying the assay buffer and | or adding a blocking step after ligand loading. Adding a greater amount of Tween-20 (up to 0.05%) or increasing the salt concentration can increase the stringency of the binding and help decrease non-specific signal. However, these approaches may also decrease binding of antibody to the receptor in the association step, so must be tested prior to running the assay.

In general, mitigating NSB by increasing the BSA concentration or blocking with BSA is not recommended when the Fcγ receptor is immobilized or captured. This is due to the large molecular weight and size of BSA (~66 kDa) which could result in steric interference. After loading the biosensor with Fcγ receptor, use of a blocking agent such as casein (0.2%) or HIS-tagged ubiquitin (~8 kDa) at 0.5 µg/mL can be effective in reducing NSB. Since the Octet® instrument enables processing of up to 96 samples at a time, this entire optimization can be done in a single experimental run (approximately a 20-minute assay) and recommended buffer and blocking conditions are summarized by biosensor in Table 1.

Analyte Binding Optimization

In the association step, the rate of binding of the analyte to the ligand is measured. Kinetic binding assays should demonstrate binding curvature at (at the minimum) the top concentrations evaluated, typically concentrations above the expected K_D (users are recommended to refer to the **Label-Free-BLI-SPR-Biosensor-Analysis-Octet-Compendium** for further information about the role curvature plays in kinetics). A dose response analysis should be performed with at least 6 analyte concentrations. 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, which allows accurate global fitting of the data set and ensures accurate results. The analyte should be prepared in the same buffer as previously determined and any established assay conditions and parameters used in order to establish analyte association and dissociation step times.

The analyte concentration range used is ligand dependent for Fcγ receptor. For example, for a high affinity Fcγ receptor, such as CD64 (FcγRI), it may not be possible to observe signal at or below the expected K_D and an analyte concentration series of 2-fold or 3-fold starting 10 – 20X above the expected K_D down to the limit of detection can be used (see **What is the Range in Measurements that Provide Reliable Data? — Sensitivity**). The analyte concentration series should be scouted to establish data with binding curvature at high concentrations (where possible).

For low affinity Fcγ receptor, which have a global affinity in the range of 0.1 – 10 µM, a top analyte concentration of 10 – 20X above the expected K_D may create artifacts in the data it is best to start at 5X above K_D and work with 2-fold dilution down to the limit of detection.

Low affinity Fcγ receptor-IgG interactions can exhibit fast association rates ($>1 \times 10^5$ $M^{-1}s^{-1}$) and reach steady state equilibrium quickly. In these situations, the association step must not be run for too long as weaker, non-specific, interactions may begin to be favored over the primary interaction. This secondary binding can lead to heterogeneity in the association curves and impact the data fitting quality. Therefore, it is recommended to start with an association time of 30 – 60 seconds and as for the analyte concentration series establish data with sufficient binding curvature at high concentrations. In general, limiting the association time can improve fitting of the binding model.

As a general guideline, the association signal for the highest analyte concentration should be >0.4 nm. Where a low binding response is observed, optimization steps to improve the signal can be taken such as increasing the analyte concentration, optimizing buffer conditions or increasing the ligand load. In the case of increasing the ligand load, care must be taken to ensure that analyte rebinding does not occur[8,9].

Establish assay shaking speed. The recommended shake speed for Octet® BLI binding assays is 1000 RPM. The shake speed may however need to be scouted and optimized depending on sample type. 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.

Dissociation time is easier to optimize than association time as it is generally accepted in the kinetics community that determining accurate dissociation kinetics requires a > 5% decrease in response during dissociation in order to determine accurate K_D values. Therefore, an initial longer dissociation time depending on the expected affinity of the Fcγ receptor may be used and then a suitable dissociation time determined from the subsequent data fit. It is preferable that a visual drop in the response is also observed in addition to a mathematical decrease of 5% prior to performing any required regeneration.

Table 1: *Suitable Biosensors for Determination of Kinetic Parameters*

Biosensor	High Precision Streptavidin (SAX & SAX2)	HIS1K Biosensors	Ni–NTA (NTA)	Anti–Human Fab–CH1 2nd generation (FAB2G)	Anti-Human Fc-Capture (AHC & AHC2)	Anti-Mouse Fc-Capture (AMC & AMC2)	Amine Reactive Second-Generation (AR2G)
Catalog Number	SAX – 18-5117 SAX2 - 18-5136	18-5120	18-5101	18-5125	AHC - 18-5060 AHC2 - 18-5142	AMC – 18-5088 AMC2 - 18-5163	18-5092
Molecule immobilized on biosensor (ligand)	Biotinylated FcγR or biotinylated IgG	HIS–tagged FcγR	HIS–tagged FcγR	IgG	IgG	IgG	IgG
Recommend-ed for	High and low affinity receptors, including FcγRIIa	High and low affinity receptors, except FcγRIIa	High and low affinity receptors. Lower affinity receptors may give low signal in association step	High and low affinity receptors. Check for cross–reactivity with FcγRIIa and FcγRIIIb	Capturing human IgGs or human Fc-fusion proteins for antigen kinetic analysis	Capturing mouse IgGs or mouse Fc-fusion proteins for antigen kinetic analysis	Not Recommended
Ligand loading strategy	Depends on ligand. For biotinylated IgG immobilization start with 200 – 300 nm for 10 min. Use lower concentration for biotin-receptor immobilization.	Lower Loading 50 – 100 nm for 5 min	Lower Loading 50 – 100 nm for 5 min	Higher Loading e.g. 200 – 500 nm for 5–10 min	Lower Loading 50 – 150 nm for 5 min	Lower Loading 5 – 150 nm for 5 min	Not Recommended
Recom-mended loading response	Must be optimized	0.3 – 0.4 nm	0.3 – 0.4 nm	2.0 – 3.0 nm	2.0 – 4.0 nm	2.0 – 4.0 nm	Not Recommended
Recom-mended buffer	1X Kinetics Buffer, may need added BSA (up to 1%) and | or Tween-20 (up to 0.05%)	1X Kinetics Buffer	1X Kinetics Buffer, may need to be optimized by increasing salt and | or Tween-20 to reduce NSB	1X Kinetics Buffer	1X Kinetics Buffer	10X Kinetics Buffer	Not Recommended
Blocking	Typically not required	Typically not required	May be required, recommend dipping into 0.2% casein in assay buffer for 5 minutes after loading step.	Typically not required	Typically not required	Typically not required	Not Recommended
Special considerations	Biotinylate ligand protein using 1:1 molar coupling ratio. Loading conditions should be optimized	For low affinity receptors, use short association step time, 30 – 60 seconds	Check for non–specific binding. Optimize buffer and blocking con-ditions if needed. If signal is low in association step, increase loading concentration or step time. For low affinity receptors, use short associa-tion step, 30 – 60 seconds	May need to use High Concentra-tion Kinetics mode (10 Hz data acquisition rate) to capture initial data for fast on-rates	Pre-condition the biosensors before the first assay cycle for most consistent results when incorporating regeneration	Pre-condition the biosensors before the first assay cycle for most consistent results when incorporating regeneration	Not Recommended

Referencing

When performing a Fcγ receptor-IgG assay a reference sample must always be included. The reference sample contains no analyte and therefore, the assay buffer which the analyte is prepared in is suitable for use. This reference sample is than assessed in the same manner as the analyte containing samples (i.e., Fcγ receptor loaded).

Use of a reference sample allows for subtraction of any assay drift caused by dissociation of the captured IgG ligand from the pre-immobilized capture molecule. This background dissociation must be subtracted out using a buffer-only reference sample. Double referencing with both a reference sample and a reference biosensor can be performed when there is a small amount of non-specific binding. A reference biosensor is a biosensor dipped into buffer or irrelevant protein instead of ligand during the loading step (i.e., not Fcγ receptor loaded). It is then run through a replicate assay using the same analyte samples as the ligand-loaded biosensors. Reference biosensors enable subtraction of NSB of analyte to the biosensor. A separate reference biosensor should be included for every sample | analyte concentration when performing double referencing.

Data Acquisition Rate

As discussed in **Analyte Binding Optimization** Fcγ receptor-IgG interactions often exhibit fast association rates and the standard rate of data acquisition (5.0 Hz, averaging by 20) in Octet® BLI Discovery Software may not be the ideal setting for measuring kinetics. When data is acquired by the Octet® BLI system, there is a small delay from when the biosensor dips into the sample to when the first data points are reported to allow the software to average the collected data. When binding is very fast, this delay can cause the reported signal for the association step to initiate well above the baseline, leading to inaccuracies with curve fitting in data analysis. If this effect is observed and affects curve fitting, the data acquisition rate can be increased to enable more rapid reporting of binding data. The data acquisition rate refers to the number of binding signal data points reported by the Octet® BLI system per second and is reported in Hertz. A higher acquisition rate generates more data points per second with less averaging and monitors faster binding events better than a slower acquisition rate. The rate setting can be changed in the Advanced Settings box in the Run Experiment tab in Octet® BLI Discovery Software. Select the acquisition rate for High Concentration Kinetics (10.0 Hz, averaging by 5). Data collected at a higher acquisition rate may have lower signal-to-noise ratio and appear noisier than data collected at the standard rate. Acquisition rate should always be determined based on consideration of the binding rate, the amount of signal generated in the assay as well as experimentation with the settings.

Quality of Reagents

Reagent quality is a critical factor with any kinetics assay, especially for Fcγ receptor-IgG interactions and procurement of critical reagents, whether in-house or purchased commercially, should be considered early in method development. Aggregation of the antibody or receptor can impact kinetics due to increased avidity. Dimeric and multimeric IgG have been shown to dissociate much more slowly from Fcγ receptors and require lower concentration for the same level of binding than monomeric IgG[10,11].

Antibody samples should be fully evaluated for purity, activity and quality using analytical techniques before use in a kinetics experiment. Reagents that have been stored at 4 °C for long periods, especially at very high or very low concentrations, should not be used. Careful consideration should be given to storage conditions and handling of receptor proteins and multiple freeze-thaw cycles should be avoided.

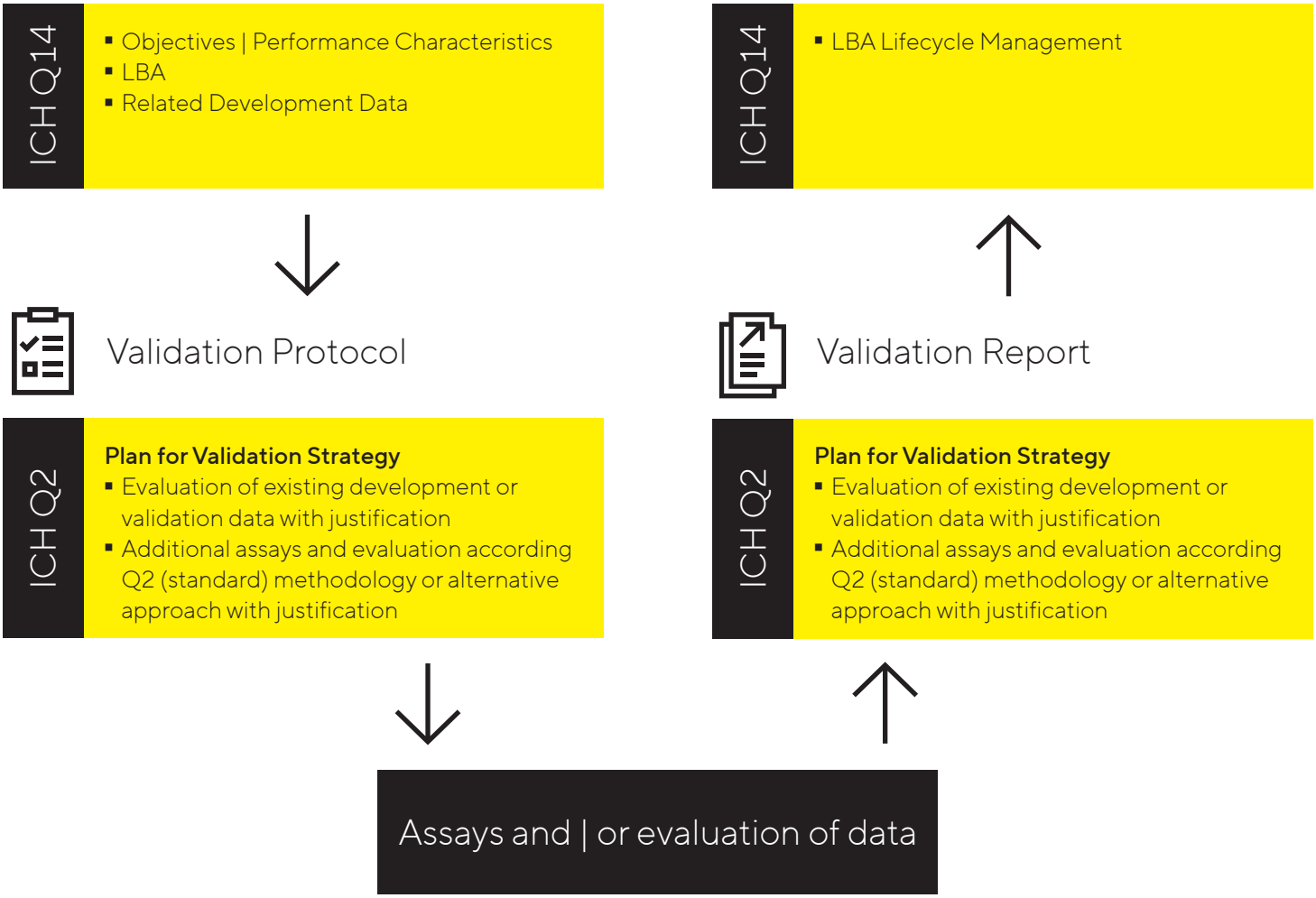
General Assay Development Considerations

Although validation of an LBA can be thought of as the final stage in a process it is important to remember that a validated assay is part of a lifecycle and as such, data derived during development studies can often be used in lieu of validation data and therefore, does not need to be repeated[3]. It is expected that prior knowledge (both internal and external) is used for informing the decision making process during assay development and therefore, additional time spent during design and development is critical to ensure that assay redundancy is not experienced during subsequent validation (Figure 3). It is also important to consider that where a previously established LBA is used for a new purpose, validation testing can be abbreviated where scientifically justified.

System suitability is an integral part of an LBA and is best assessed during assay development (see **System Suitability**). A key aspect of ensuring the designed LBA is fit for purpose is to ensure that the robustness of the assay is considered from the beginning.

Method development itself does not constitute assay robustness, therefore it is important to understand the validation principle during assay design and development and what should be assessed prior to assay validation. Robustness is assessed by establishing the effects of random events on the precision of the LBA and it is recommended that users refer to Section 5.1 of the ICH Q14 guidelines for a full understanding[3]. In general, typical variations include parameters such as different analyst, different days, and different equipment where applicable. With this is mind, Sartorius offer a Biosensor validation service where customers can assess the performance of multiple lots of biosensors prior to purchasing more trays from one (or more) of the evaluated lots (see **How do Sample Collection, Handling, and Storage Affect the Reliability of the Data from the Bioanalytical Method? — Robustness & Stability**).

Figure 3: *Validation Study Design and Evaluation. Adapted from ICH Q2 (R2) Guidelines[2].*



LBA Development

Once basic assay design has been performed and the optimal ligand and analyte concentrations determined, the assay is subsequently developed in order to optimize the parameters of the LBA such that the necessary bioanalytical parameters are satisfied and the assay is fit for validation. It is important to bear in mind that the assay development is an ideal opportunity to assess parameters that are to be performed during validation and therefore, development and subsequent validation should be relevant to the planned sample analysis workflow. As discussed above, assay developments can take two forms, a minimal (traditional) approach or an enhanced approach. Although outside of the scope of this application note, it is recommended that users review the requirements for a minimal or enhanced approach in ICH Q14[3]. Here, a minimal approach is presented that includes the elements:

1. Identifying which attributes of the drug substance or drug product need to be tested by the LBA.
2. Selecting an appropriate technology to perform the LBA.
3. Conducting appropriate development studies to evaluate LBA performance characteristics.
4. Defining an appropriate LBA description including the assay control strategy (e.g., parameter settings and system suitability).

Element 1 is best addressed by defining an intended purpose of the LBA, which for this application note can be defined as: *Measurement of kinetics and affinity of an anti- Fcγ Receptor I (CD64) monoclonal antibody for Drug Substrates.*

It is also best to clearly define the purpose of the LBA to the CQA for biological activity. For example, CD64 binding assessment could be defined as: *Binding of therapeutic antibodies to CD64 has a significant impact on the efficacy and safety of the drug and therefore, is recognized as a CQA. CD64 is expressed on the surface of immune cells and plays a crucial role in mediating antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). The LBA should be able to measure the kinetics and affinity of the antibody and detect whether there are significant changes in kinetics and affinity upon production of the Drug Substrate.*

Here, Element 2 can simply be defined as an Octet® BLI system for the purpose of this Application Note. Element 3 is best defined by regulatory validation guidelines for LBA and typically include these parameters:

- Specificity and selectivity – the ability of the LBA to measure the analyte of interest without interference from other substances
- Accuracy – closeness of the LBA value to the true value
- Precision – intra- and inter-assay variation of the LBA
- Linearity – ability of the assay to produce results that are proportional to the analyte concentration
- Range – the analyte concentration range over which the LBA can produce accuracy and precision within the determined criteria
- Sensitivity – the lowest concentration of analyte that can be reliably detected above assay buffer response
- Robustness – evaluate the LBA ability to remain unchanged despite small and deliberate variations

It is also important to remember that other critical parameters are present in the validation guidelines and must be addressed where required. As shown in the next section, when validating an assay based on kinetic parameters these parameters can become less applicable.

Assay Control Strategy

Element 4 is generally an afterthought for most assay development that does not lead to validation but it is critical in ensuring the lifecycle of a validated assay.

The ICH Q14 guideline on LBA development describes a minimal and enhanced approach to developing and refining LBA[3]. In both formats, the aim of development is to create a control strategy that ensures that the LBA performs as expected during routine use throughout its lifecycle and consists of a set of controls, derived from current understanding of the LBA including development data, risk assessment and robustness. It is important to note that prior knowledge can play a key role in the development of the assay control strategy. The assay control strategy should be defined before validation and then confirmed after validation is finalized. The use of design of experiments studies is encouraged. In its simplest form the control strategy can be broken down into:

- LBA parameters
- LBA description (including a system suitability test)

The LBA description describes the details necessary to perform the assay and is required at a level of detail that would allow a skilled analyst to perform the assay, analysis and interpret the results. For further information on data analysis and interpretation see **Analysis of Fc-gamma Receptor-IgG Interactions on the Octet® Platform**.

In general, the LBA description contains information about samples, reference materials and reagents and their preparation. Assay steps that pertain to the use of the Octet® BLI system and generation of the kinetics and affinity data and any other necessary steps should be covered too. While the minimal approach remains acceptable, some or all elements of the enhanced approach might be used to support development and lifecycle management of LBA. One such useful feature is the Analytical Target Profile (ATP), a prospective summary of performance characteristics that describes the intended purpose and anticipated performance criteria of the LBA. An example in shown in Figure 4.

Although not designed as a pro forma, the ATP can act as a useful template to capture information for LBA design, qualification and validation. The primary focus should be on the intended purpose of the assay. This should be clear enough as to capture the predicated analyte, ligand and assay format. The link to a biological function and | or CQA should then be stated. The form can then be used to capture any characteristics, their acceptance criteria and rationale that are required. Each element discussed above can be captured on the ATP for the assay.

Figure 4: *Example of Analytical Target Profile.*

Intended purpose		
Measurement of the kinetics and affinity of a monoclonal IgG1 antibody against Fcγ receptor I (CD64) in Drug Substance and in Drug Product at release and for stability testing.		
Molecule immobilized on biosensor (ligand)		
IgG antibodies play an important role in controlling inflammatory and anti-inflammatory responses of the innate immune system. CD64 binds to the Fc portion of IgG antibodies triggering immune cell phagocytosis and is also involved in the release of inflammatory cytokines, which help to coordinate the body's response to infection or injury. The assay should be able to measure the kinetics and affinity of the drug and detect if there are significant changes in biological activity.		
Characteristics of the reportable result		
Characteristic	**Acceptance criteria**	**Rationale**
Specificity	Method is specific for the intended mechanism of action of the active ingredient.	Critical characteristic of a bioassay to ensure specificity towards the targeted biological activity.
Selectivity	The response of blank samples spiked with a negative control or process contaminant, for example an Fc silenced antibody, should be below the detection limit	
Precision	Intra- and inter-assay variability of <20 % CV	Parameters assessed based on compendial guidance[1,2]. Selected performance characteristic ensures that the intended method delivers the quality reportable result.

System Suitability

System suitability ensures that the system used for the LBA is adequate for the intended analysis with acceptance criteria based on LBA performance criteria.

The test is used to verify that the measurement system and the analytical operations associated with the analytical procedure (LBA) are adequate during the intended time period of analysis and enable the detection of potential failures.

A well-designed system suitability test can represent an important aspect of risk mitigation and should be performed using samples that are independent of any reference samples, samples, or QC samples used for the validation. It is important to maintain records of the system suitability for any future audits.

System suitability can be easily performed on Octet® BLI systems through use of either the performance qualification – kinetics (PQ-K) kit (PN: 18-5134) or performance qualification – quantitation (PQ-Q) kit (PN: 18-1164). For the purposes of this Application Note the PQ-K kit is suitable to show system suitability.

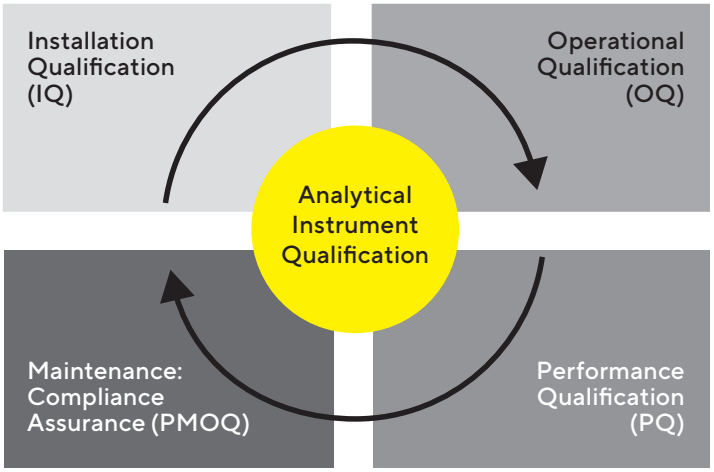
The PQ-Kinetics assay verifies that the signal levels, variability, and kinetic and affinity constants are within specification for a 1:1 binding interaction. And generates statistical data on system parameters:

- Baseline drift
- Association (k_a) rate constant
- Dissociation (k_d) rate constant
- Affinity (K_D) constant

All required reagents and buffer are provided prediluted for the PQ-Kinetics test, which takes approximately 2 hrs 30 minutes. Upon competition, a printable .pdf report file is automatically generated for documentation of assay specifications and results.

It is recommended that performance qualification is performed as part of an overarching system lifecycle plan that includes installation and operational qualification (IQOQ) to ensure the Octet® BLI system is performing within its defined specifications. It is also recommended that a maintenance compliance schedule (PMOQ) service is performed every six months to ensure the system is maintained in a calibrated state and to reduce the risk of using an out-of-compliance system (Figure 5) (see **Octet® System Qualification Products and Services**).

Figure 5: *Regulations and Global Standards, Including US FDA 21 CFR Part 11 Require Documented Verification That Your Instruments Are Delivered, Installed and Routinely Calibrated and Functioning According to Their Operational Specifications.*



Development of an LBA for Kinetic-based Validation

Although primarily aimed at end-point assays that produce calibration curves that can be assessed using a sigmoidal function and parallel line analysis (PLA), both ICH and FDA guidance state that modifications can be made with science based-justifications depending on the specific type of bioanalytical method where justified. ICH Q2(R2) also states that the applicant is responsible for designing validation studies and protocols that are most suitable for their product[2]. Prior to validation the method and validation procedure containing a specific and detailed written description should be established. This document may take many formats including a protocol, study plan, report, or Standard Operating Procedure (SOP).

The following sections offers guidance on how current guidance can be applied to validation of a kinetics-based assay using Octet® BLI.

The examples provided in this document are examples for illustrative purposes. They suggest how the concepts described in ICH Q14, ICH Q2(R2), ICH M10 and the FDA Bioanalytical Method Validation: Guidance for Industry could be applied and should not be used as a template or the sole basis for a regulatory submission. Appropriate regulatory consultation should be sought when required.

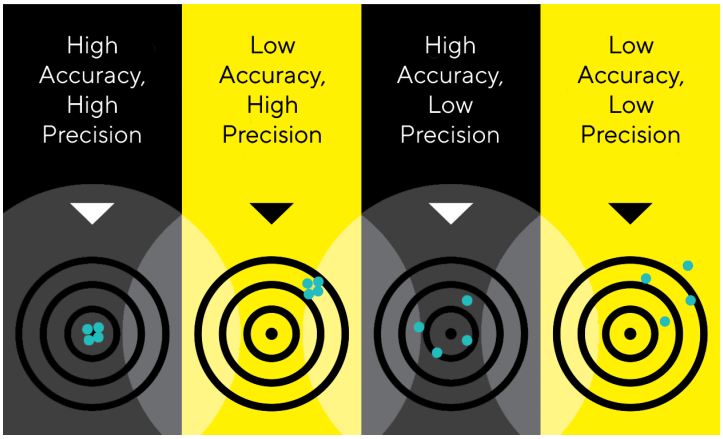
It is important to recognize that when designing and developing a kinetic-based assay rather than a standard quantitation potency-based assay that certain parameters and acceptance criteria are not applicable.

An iterative process is usually performed for developing and optimizing Octet® BLI assays. This often includes finding suitable conditions for ligand immobilization | capture, coating and conjugation, concentrations of analyte, association and dissociation times and regeneration conditions (see **Octet® BLI Technical Note Octet® BLI Kinetics Assay: Method Development Guideline**). Throughout development it is important to use samples that are as similar as possible (if not the same) to the samples expected to be used during validation.

Once successfully developed, the LBA analyte concentration series used in an assay is, in general, relatively insensitive to minor changes that would be used in a potency-based assay. For example, if a top analyte concentration of 10 nm was used to assess a high-affinity interaction with Fcγ RI, then assessing linearity (ability of the assay to produce results that are proportional to the analyte concentration) across a range of 80 – 125% would require top analyte concentrations of 8 nm and 12.5 nm, respectively. This would not be expected to produce a significant change in the observed kinetic parameters and therefore, the linearity parameter becomes void.

This process can also be applied to the 'Range' criteria in that the analyte concentration range over which the LBA can produce accuracy and precision within the determined criteria should not become a variable in a kinetic-based qualification or validation. Kinetic assays use an analyte concentration series that is composed of multiple concentrations, which remains constant during the assay and are subsequently fitted to a global mathematical model. Although accuracy is related to linearity | range, it is also intrinsically linked with precision (both intra- and inter-), which remains a critical parameter in kinetic assays. As per ICH Q2(R2), due to the removal of linearity | range, accuracy can be inferred once precision and specificity have been established (Figure 6)[2].

Figure 6: *Assay Development is Intrinsically Linked to the Data Results Where (A) High Accuracy, High Precision is the Ideal Scenario and the Assay is Both Accurate and Precise, Meaning the Assay Requires No Further Optimization. (B) Low Accuracy, High Precision Occurs When an Assay is Not a Good Indicator of the True Value but the Average Result is Precise. In This Scenario, Development Should Focus On Improving the Accuracy of the Result. (C) Where High Accuracy, Low Precision is Observed, Assay Development Should Focus On Decreasing Assay Variables Both Intra- And Inter-Assay. (D) The Worst Scenario is Low Accuracy, Low Precision Where neither Accuracy or Precision is Correct and Assay Development Should Focus on All Parameters Involved in Increasing Both Accuracy and Precision.*



The recent ICH Q2(R2) guidelines provide clear guidance on parameters that can be interpreted for kinetic based assays in a phase-appropriate manner during clinical development[2]. In these cases, where a non-linear response is observed, a model that describes the relationship between the response of the LBA and concentration of the analyte is necessary and should occur across a wide analyte concentration range. This subsequently allows the LBA to be assessed across a given working range to obtain values that are proportional to the true value. With the removal of certain classical acceptance criteria, kinetic assays require additional acceptance criteria to be added for the purpose of validation, which include k_a, k_d, K_D, and R_{max}.
By definition a critical quality attribute is a property or characteristic that should be within an appropriate limit, range or distribution to ensure the desired product quality so k_a, k_d, K_D, and R_{max} can be treated as such.

These acceptance criteria are investigated during assay development and qualification and are best thought of as replacement accuracy and precision parameters.

With this in mind, the updated validation parameters for a kinetics-based assay looks like:

- Specificity and selectivity – the ability of the LBA to measure the analyte of interest without interference from other substances.
- Precision – intra- and inter-assay variation of the LBA, with focus on the values determined from the kinetic model fit.
- Sensitivity – the lowest concentration of analyte that can be reliably detected above assay buffer response.
- Robustness – evaluate the LBA ability to remain unchanged despite small and deliberate variations.

As discussed above, during LBA design and development the following four questions from the FDA Guidelines form the basis of a successful development and can now be expanded out to form the basis for assay development, qualification, and subsequent validation[1]:

Does the Method Measure the Intended Analyte? — Specificity and Selectivity

Specificity and selectivity are the absence of interference in the LBA from potentially interfering substances and is best evaluated using known positive control samples (QC samples) and negative controls. It is also important to show that the analyte can be differentiated from potential interfering substances, which is production process specific. It is important at this stage that any material used as a positive control is as similar as possible, or identical, to the analyte to be used for validation i.e., concentration, purity, and stability. The stability of the analyte is important to know as early as possible in the development process as it will influence subsequent validation parameters.

How to Test Specificity and Selectivity

Specificity is related to cross-reactivity in that it's important to demonstrate that the ligand binds only to the analyte and does not cross-react with coexisting structurally related molecules. As per ICH Q2(R2) "...specificity or selectivity of an LBA can be demonstrated through absence of interference" and "can be shown by demonstrating that the identification and | or quantitation of an analyte is not impacted by the presence of other substances (e.g., impurities, degradation products, related substances, matrix, or other components present in the operating environment)."[2]

Therefore, for an Fcγ Receptor kinetics-based assay a suitable positive control would be either another antibody with the same isoform (for example an IgG1 which presents a different paratope than the one being used as the reference standard) or the reference standard itself. The negative control used can depend upon at what stage of production the validation is being performed but suitable components may include process contaminants such as Protein A or Host Cell Protein (HCP) spiking. Where the antibody being assessed is in a highly purified form then a suitable negative control may include an antibody with a different isoform that does not bind the Fcγ Receptor (e.g., IgG2 for Fcγ RI) or an Fc silenced antibody of the same isoform that has been shown by the manufacturer to not bind Fcγ Receptor. It is also possible to spike the positive control with the negative control antibody in order to show selectivity.

Acceptance Criteria

Specificity – The kinetic parameters (k_a, k_d, K_D, and R_{max}) of the QC samples are not impacted by the presence of another substance e.g., process contaminant or negative control. and should have a %CV <20 for the applicable parameters Selectivity - The response of blank samples spiked with a negative control or process contaminant, for example an Fc silenced antibody, should be below the detection limit (LLOD).

What is the Variability Associated with These Measurements? — Precision

Precision is a fundamental parameter in all assays and is defined as the closeness of agreement between measurements of the same homogenous sample (QC | Reference Standard). ICH Q2(R2) guidelines extend precision out further to an intra-assay parameter of repeatability and inter-assay parameter of intermediate precision[2]. A third form of precision, reproducibility, is also described in the ICH Q2 (R2) guidelines but not considered here[2].

Precision is intrinsically linked to accuracy and accuracy must be ensured before precision is considered. As accuracy is the degree of closeness to a known or nominal value, which is accepted as the reference value, so assay design must ensure that an accurate value can be obtained. Where a reference value is not known or available, users are advised to read the **Octet® BLI Technical Note Octet® BLI Kinetics Assay: Method Development Guideline** for advice on developing a suitable assay to provide a reference value.

Here, Fcγ receptor values are well-known across a range of binding species.

Precision results are usually expressed as the variance, standard deviation, or coefficient of variation. Here, the coefficient of variation (%CV) is used. Individual parameters for k_a, k_d, K_D, and R_{max} should be assessed and unique parameters set.

How to Test Precision

As mentioned above, precision can be classified as intra-assay (repeatability) and inter-assay (intermediate precision). Intra-assay (repeatability) is assessed by performing replicates of the assay using a QC | Reference Standard, ideally within a single assay. As only a single analyte concentration series is being assessed as the reportable range, it is recommended that not less than three independent replicates (of the concentration series) are performed when determining repeatability parameters.

Inter-assay (intermediate precision) is assessed in the same manner as repeatability but requires the use of different analysts over multiple assays. As with repeatability, it is important that independently prepared replicates are assessed and other normal laboratory variations performed (e.g., time of day, different assay buffer preparation, different pipettes…). Inter-assay (intermediate precision) should be assessed with not less than six independent assays (performed over ≥ 2 days) but unlike intra-assay (repeatability), not less than three replicates should be performed. Therefore, the minimum requirements for testing of intra- and inter-assay precision are triplicate measurements performed across six independent assays for a total of eighteen replicates.

It is recommended that intra-assay (repeatability) is performed using as many samples as are expected to be used in future assessment after validation, for example, if the assay will contain 12 samples in total (including any reference standards, then repeatability should be assessed with that number also to ensure the assay is fit for purpose and there are no effects from sample evaporation or other factors). It is important to note here that different lots of analyte and ligand should not be used, precision is not an ersatz robustness assay. As per ICH Q14, variations tested should be based on the LBA and understanding from development and risk assessment and studying these effects individually is not necessary[3].

Acceptance Criteria

Intra-assay (repeatability) – For an LBA kinetic assay, repeatability data should be calculated and presented for each assay and should have a %CV <20 for the applicable parameters.

As above, where multiple samples are assessed in a single assay then a cutoff point may be required in %CV <20%. For example, if 12 samples yield a %CV of 25% but 9 yields a %CV of 18%, the assay has a repeatability limit of 9 samples.

Inter-assay (intermediate precision) — For an LBA kinetic assay, data should be calculated by combining data from all assays and should have a %CV <20 for the applicable parameters.

What is the Range in Measurements that Provide Reliable Data? — Sensitivity

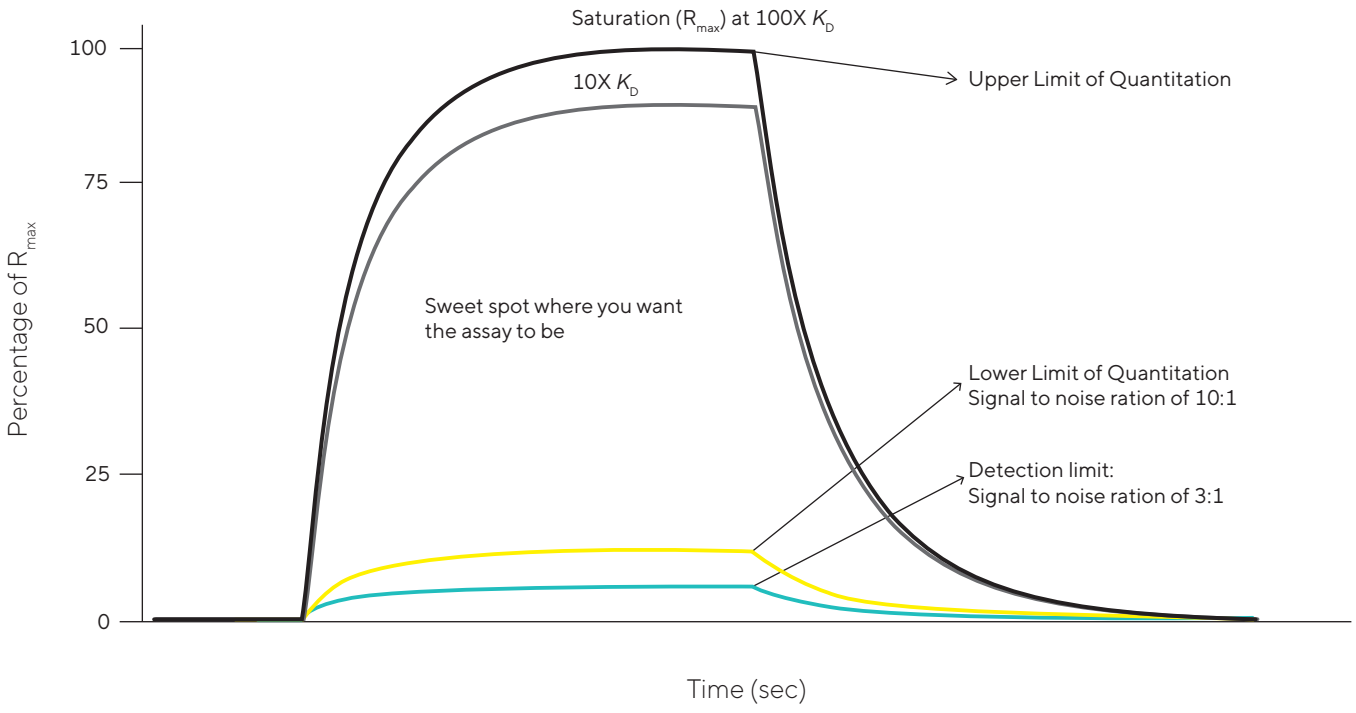
Sensitivity is defined as the lowest analyte concentration in the matrix that can be measured with acceptable accuracy and precision (i.e., quantitation limit). Although current guidance is based around calibration curves, the overall idea of understanding the sensitivity of an assay is important for a kinetic-based validation of approach. Although a single analyte concentration series is assessed during the assay validation, it is important to remember that samples of unknown status and or concentration will be assessed in the validated assay. Although these samples will be assessed against a well-characterized reference standard, it is important to understand the quantitation limits can provide useful information for not only determining whether data is acceptable and passes but also as useful indicator of sample properties including stability and aggregation.

The lower- and upper limits of quantitation of the assay, LLOQ and ULOQ, respectively describe the range across which measurements provide reliable data and should be determined during method development.

The LLOQ is an important parameter to determine as all analyte concentrations of the reference standard should fall within the assay sweet spot (Figure 7) and any subsequent sample testing that exhibits analyte responses below the LLOQ can be used to flag failing samples (e.g., samples that exhibit stability issues would show a lower response due to a lower active concentration of analyte than the well-characterized reference standard).

The ULOQ can be an important parameter in kinetic assays as the observed response in a 1:1 kinetic assay is dependent upon the amount of ligand present and the amount of analyte present, therefore, R_{max} should be well defined (with regards to precision). As above for LLOQ, the ULOQ allows the user to assess whether any samples contain any aggregate or higher order material as a corresponding increase in response above the validated R_{max} parameter will be observed. Therefore, determination of the ULOQ is desirable as any value above that can be an indicator that the sample should be assessed orthogonally. An additional lower range parameter, detection limit (DL) should also be determined and can be estimated using different approaches as shown below (Figure 7).

Figure 7: *All Curves, if Left for Long Enough, Will Reach Equilibrium. Equilibrium Level Relative to R_{max} Will Depend on Concentration Relative to K_D Only at Concentrations Around 100X K_D Will the Equilibrium Be a Saturating One.*



Note: Kinetic-based Sensitivity shows Multiple Important Parameters including the Detection Limit, Lower Limit of Quantitation and the Upper Limit of Quantitation (ULOQ). ULOQ shows where all the ligands are saturated, any response above that would indicate an issue with the analyte. Ideally the assay should be away from these limits in the assays 'sweet spot'

How to Test Sensitivity

An important distinction that should be made when assessing a kinetics-based assay is the difference between a detection limit and a quantitation limit. The detection limit is the minimum concentration of analyte that can be measured above the background noise of the instrument. The detection limit simply means that the analyte cannot be seen by the system being used but may be present at lower concentration.

As discussed below, there are multiple ways of determining the detection limit (DL) and is merely a measure of the system and assay ability to generate a positive response and is not linked to the accuracy of that response, especially when considering an analyte concentration series for a kinetic assay. A signal to noise ratio of 3:1 is considered acceptable for the detection limit.

Where accuracy is required at low levels, the quantitation limit (QL) represents the lowest amount of analyte that can be determined with accuracy and precision and is used for quantitative assays but as with the detection limit here, represents a useful value above which LBA assays should be performed.

Biosensor systems exhibit baseline noise and therefore, the DL and QL for a kinetics-based assay can be determined using two main methods:

- Signal to Noise
- Standard Deviation of a linear response and a slope

It is important to note that assays that are close to the detection limit may exhibit higher %CV results as small changes represent larger changes in percentage and are amplified in the resultant calculation.

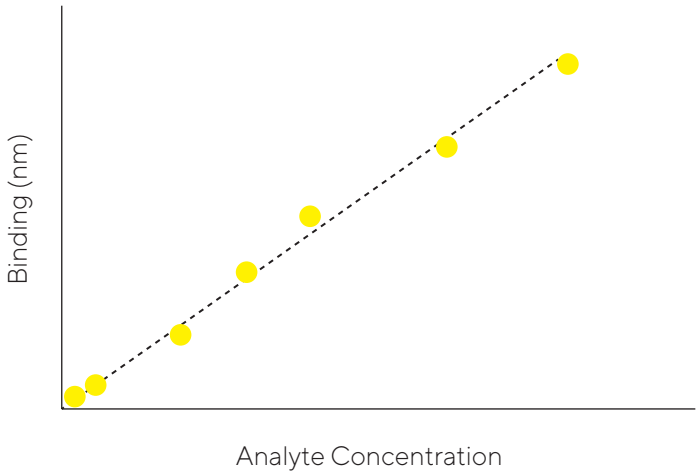
Signal to Noise

Sensitivity is determined by measuring the signal to noise ratio through the use of assay buffer blanks and samples with a known low analyte concentration. The DL or QL is the minimum concentration at which the analyte can be reliably detected or quantified. Here, for a kinetic assay, the lowest analyte concentration in the concentration series should be assessed and further dilutions of that sample performed until the signal to noise ratio calculation becomes unacceptable.

Standard Deviation of a Linear Response and a Slope

Similar to the signal to noise assay setup, the DL or QL is determined using the analyte concentration series and further dilutions of the lowest analyte concentration performed with the aim of generating a linear response to which a straight line can be fit (Figure 8).

Figure 8: *Example of Linear Response of Analyte With Linear Regression Performed.*



Two equations can then be used to determine DL and | or QL.

$$DL = \frac{3.3\sigma}{S} \quad \text{and} \quad QL = \frac{10\sigma}{S}$$

Where S is the slope of the calibration curve and can be determined from the linear regression shown above (Figure 8). σ is the standard deviation of response and can estimated using the following methods.

Standard Deviation of the Blank

This is the easiest way to determine the QL as multiple assay buffer blanks are included in assays for precision and this data can be analyzed and the standard deviation of the responses calculated and used here. This method is preferred compared to visual inspection as it is objective.

Visual Inspection

Visual inspection is subjective compared to the objective tests of signal to noise and standard deviation of buffer and therefore, the least preferable. In a visual inspection, the limit is determined by analyzing samples with a known concentration and establishing the minimum level at which the sample can reliably be resolved, detected, or quantified.

ULOQ

Determination of the ULOQ of quantitation for a kinetic assay is performed to determine the saturation Rmax above which no further analyte can bind. The simplest method of determining the ULOQ is through the use of a high analyte concentration (~100X K_D) for the same contact time as other analyte concentrations. Where the assay is of high enough affinity, use of the same highest analyte concentration as used in the LBA may be considered and an increased contact time used but limits in analyte availability may provide a false response value as analyte concentration becomes rate limiting as the assay progresses.

Acceptance Criteria

Signal to Noise
A signal to noise ratio of 3:1 is acceptable for DL and 10:1 for quantification based upon the ICH Q2(R2) guidelines[2]. Lower Limit of Quantitation (LLOQ) as defined by the FDA guidelines is ≥ 5X the response of the zero calibrator (assay buffer)[1].

Presentation of the relevant data is considered an acceptable justification for the use of signal to noise.

Standard Deviation of a Linear Response and a Slope
A signal to noise ratio of 3:1 is acceptable for DL and 10:1 for quantification based upon the ICH Q2(R2) guidelines[2]. Lower Limit of Quantitation (LLOQ) as defined by the FDA guidelines is ≥ 5X the response of the zero calibrator (assay buffer)[1].

Where DL and or QL is obtained by calculation as above, the value should be validated using a suitable number of samples prepared near or at the DL. It is worth noting that where the QL is ≥ 10X lower than the lowest analyte concentration response, the confirmatory validation can be omitted with justification.

ULOQ
There are no set acceptance criteria for the ULOQ but it is advised that users assess the ULOQ across multiple ligand preparations to ensure a wide distribution of results. This can be achieved by combining ULOQ with robustness testing.

How do Sample Collection, Handling, and Storage Affect the Reliability of the Data from the Bioanalytical Method? — Robustness & Stability

Determining robustness is crucial because it ensures the reliability and consistency of the assay results under normal usage conditions. It also helps to define the boundaries of the assay conditions, which is important for maintaining the quality control of the assay. This is crucial for assays involving biological samples, which may degrade or change over time. Method development is not robustness testing. Robustness is often thrown around as a term without experimental data to back it up and more often used as a description of a 'good' assay. Robustness is currently used as a wide-ranging term that would previously have been separated into robustness and ruggedness. For ease here, robustness as defined by ICH Q14 and Q2(R2) will be used[2,3]. The robustness of an analytical procedure (LBA) is a measure of its capacity to meet the expected performance requirements during normal use. Robustness is tested by deliberate variations of analytical procedure (LBA) parameters. Prior knowledge and risk assessment can inform the selection of parameters to investigate during the robustness study. Those parameters likely to influence procedure performance over the intended period of use should be studied.

For most LBA, robustness should be considered during assay development and would therefore not need to be repeated during validation. Robustness is tested by deliberate variations of the LBA, including such parameters as:

- Shake speed
- Assay temperature
- Concentration of assay buffer
- pH of assay buffer

An important part of LBAs are reference standards and critical reagents. Critical reagents are requisite components of an assay, and for an Octet® BLI assay would include the reference standard (analyte) and the Fcγ receptor (ligand). It is important during robustness testing to assess reference standards and ensure all critical reagents are defined.

Additional considerations for LBA are discussed below.

It is important to note that data from inter-assay precision (intermediate precision) can also be used to show robustness. Stability refers to the ability of reagents used in the assay to maintain their properties over time under specific conditions. Stability testing is important to understand how changes in storage conditions, such as temperature and time, might affect the analyte and the final assay results. Stability information is used to set storage conditions, retest periods or expiry dates for the analytes or reagents.

Unlike robustness, stability should be assessed during validation and is used to show that specific storage and use conditions do not cause degradation of the starting materials at relevant time intervals.

How to Test Robustness

Of the list above, multiple analytical procedures can be tested (and minimized) during assay development through the implementation of IQOQ and PQ (see **System Suitability**) such as shake speed which has an acceptance criterion of 400 ±20 RPM and 1000 ±50 RPM. Therefore, in order to test the effect of shake speed 950 and 1050 RPM would be assessed as a maximum range of 950–1050 RPM would be expected on an Octet® BLI system that had IQOQ certification.

Similar assay set up can be performed for assay temperature, where a system with IQOQ certification would have a maximum range of ± 1 °C for the heater plate temperature and due to thermal dissipation, a maximum range of ± 2 °C for the actual sample temperature. Therefore, in order to test the effect of temperature on an assay performed at 25 °C, 23–27 °C would be assessed as a maximum range of 23–27 °C would be expected on an Octet® BLI system that had IQOQ certification. For parameters such as assay buffer, it is expected that pipettes have a regular maintenance and calibration schedule and therefore, any variation in volume can be attributed to the systematic error of the device. For example, the Sartorius Picus® Nxt 50–1000 µL pipette has a systematic error of 0.45% at 1000 µL. Therefore, if a 1X Octet® Kinetics Buffer solution was prepared from a 10X stock, a maximum error of 4.5 µL would be expected from the pipette. Octet® make biosensor robustness simpler by offering a biosensor validation service for certain biosensors (18–9901), which allows users to assess up to three different lots of the biosensors whilst Octet® hold a required quantity of the biosensors from the specific lots during robustness testing. After testing, the user may then purchase more trays of the required lot to ensure sufficient biosensors for future testing.

In general, it is advisable to assess three different lots of the required biosensor to ensure that assay robustness is validated from a biosensor viewpoint. As per the ICH Q14 guidelines, LBA require biological reagents, for example here an Fcγ receptor (ligand), should be investigated during a robustness study. It is advisable here to use a reputable protein supplier who can provide multiple lots of the same protein for assessment and, where required, hold quantities of the required lot for future use in a call-off order. It is also advisable to assess other protein suppliers, though variation in protein quality should be expected and factored into acceptance criteria.

How to Test Stability

For both reference standards and critical reagents stability parameters should be determined during assay development. These include handling, storage (e.g., temperature, how long samples can be stored and storage container materials), and assessment of freeze-thaw stock solutions where applicable.

Storage and analytical conditions for stability tests should reflect those used for study samples for example bench-top (short-term) samples should be thawed (if applicable) in the same way as study samples and kept on the bench top at the same temperature for at least the same duration as expected for study samples.

Where samples are required to be frozen, stability should be tested on QC samples that have been frozen for at least 12 hours between thawing cycles. Recent guidelines (ICH M10) allow for bracketing approach to freezing biological drugs e.g., where stability is shown at –70 | –80 °C and at –20 °C, there is no requirement to assess temperatures between those points at which study samples may be stored[4]. Stability testing of critical reagents should be based upon the performance in the LBA and upon general guidance for reagent storage conditions. Meaning it is acceptable to extend the expiry date beyond that recommended by the supplier, where backed by relevant performance parameters. These should be documented in order to support the extension. It is important to note that reference to data published in the literature is not considered sufficient. It is recommended that the user refers to ICH M10 for information on stability of stock and working solutions[4].

Acceptance Criteria

Robustness
Defining acceptance criteria for robustness is not akin to other variables as: *The robustness of an analytical procedure (LBA) is a measure of its capacity to meet the expected performance requirements during normal use*.

Therefore, each robustness parameter will have its own acceptance criteria; although in general it is possible to apply general parameters as above for acceptable changes. For example, assessing different lots of the ligand could be given an acceptance criteria of a %CV <20, as this is in line with accuracy and precision values.

It is important at this stage to determine which reagents are critical to the LBA. A critical reagent is something that can be shown to have a direct impact of the results of the LBA method and is not limited to the analyte and ligand. Reagents that are determined to be critical should be stored under appropriate conditions throughout its use in LBA validation and subsequent testing. It is important to note that partial revalidation of the LBA is required when changing to a different lot of a critical reagent.

Stability
Stability samples should be treated as QC samples in a repeatability assay and kinetic parameters calculated. It is expected that %CV <20 for the applicable parameters is observed.

Reference Standards and QC Samples

Reference standards (RS) are a critical reagent and form an essential part of method development and validation. The reference standard is used to prepare quality control samples (QC) and three types of reference standards are normally used:

1. Certified (e.g., United States Pharmacopeia (USP) compendial Standard)
2. Commercially supplied
3. Custom synthesized (In-house)

For certified and commercially supplied reference standards, certificates of analyses (CoA), including the source, lot number, and expiration date (except for USP standards) should be supplied.

For custom synthesized reference standards that do not have a CoA, evidence should be provided to show the identity, purity, source and lot number.

It is recommended that the user refers to ICH M10 and the FDA guidelines for information on the use of expired reference standards, stock and working solutions[1, 4].

QC samples are intended to mimic samples that would subsequently be tested under the conditions of the validated assay and therefore, should be freshly prepared independently and under the conditions expected for sample testing.

For these validation guidelines, it is assumed that an antibody (biological drug) is being tested. Biological drugs have a highly complex structure and binding to reagents may be affected by a change in the manufacturing process of the antibody. Therefore, it is recommended that the batch of reference standard (RS) used for preparing QCs is from the same batch of drug that would be used in non-clinical and clinical studies. Once the assay has been validated, if the RS is changed then partial-validation should be performed with the original and new material to ensure the performance characteristics are within the specified acceptance criteria.

Critical Reagents

Critical reagents are requisite components of an assay that have a direct impact on the results of the assay and therefore, their quality needs to be assured. Critical reagents include binding reagents such as binding proteins, aptamers, antibodies or conjugated antibodies and by definition bind the analyte, which leads to an instrument signal corresponding to the analyte concentration.

As discussed above for quality of reagents reliable procurement of critical reagents both, in-house or purchased commercially, should be considered early in method development and should be identified and defined in the assay method. Correct documentation of critical reagents (at minimum identity, source, batch | lot number, purity (if applicable), concentration (if applicable) and stability | retest date | storage conditions) should be recorded.

As critical reagents are requisite components of an assay it is necessary to ensure consistency between new and original batches and this is achieved using a lifecycle management approach. Where minor changes to critical reagents are made (e.g., the source of one reagent) a single comparative accuracy and precision assessment is sufficient for characterization. Where major changes to critical reagents are made (e.g., change in production method or supplier of antibodies) additional validation assays are necessary. Ideally, this would consist of assessing the method with new and old reagents directly but at the minimum it is necessary to:

- Evaluate binding and reoptimize assays
- Verify performance with QCs
- Evaluate cross-reactivities

As critical reagents are well-defined any retest dates or validation parameters for extension or replacement of the critical reagent should be documented. See stability for more information on expiration dates.

Glossary of Terms Used in Document

Between (inter) assay: Between (inter) assay refers to the distinct period between or among several analytical or validation runs.

Bench-top stability: Bench-top stability is the stability of an analyte in a matrix under conditions of sample handling during sample processing.

Blank: A blank is a sample of a biological matrix to which no analytes have been added that is used to assess the selectivity of the bioanalytical method.

Critical reagents: Critical reagents are requisite components of an assay, which include antibodies, labeled analytes, matrices, etc.

Critical Quality Attribute: A biological property or characteristic that should be within an appropriate limit, range or distribution to ensure the desired product quality.

Detection Limit (DL) | Lower limit of quantification (LLOQ): The LLOQ is the lowest amount of an analyte that can be quantitatively determined with acceptable precision and accuracy.

Freeze-thaw stability: Freeze-thaw stability refers to the stability of the analyte in the matrix upon freezing and thawing.

Freshly prepared: Freshly prepared refers to QC sample preparation (i.e., spiked) on the day of the experiment; not frozen before use.

Full validation: Full validation refers to the establishment of all validation parameters that apply to sample analysis for the bioanalytical method for each analyte.

Interference: Interference refers to the action of sample components, including structurally similar analytes, metabolites, impurities, degradants, or matrix components that may impact quantitation of the analyte of interest.

LBA parameter: Any factor (including reagent quality) or analytical procedure operational step that can be varied continuously or specified at controllable, unique levels.

Long-term stability: Long-term stability assesses the degradation of an analyte in the matrix relative to the starting material after periods of frozen storage.

Nominal concentration: The nominal concentration is the actual or intended concentration of the calibrator or quality control samples.

Precision: Precision is the closeness of agreement (i.e., degree of scatter) among a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Quality control sample (QC): A QC is a biological matrix with a known quantity of analyte that is used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of study samples analyzed in an individual run.

Reference standard | sample: A reference standard is a chemical substance of known purity and identity which is used to prepare calibration standards and quality controls. Three types of reference standards are usually used: (1) certified (e.g., USP compendial standards), (2) commercially-supplied, and (3) custom-synthesized.

Sample: A sample is a generic term encompassing controls, blanks, unknowns, and processed samples.

Sensitivity: Sensitivity is defined as the lowest analyte concentration in the matrix that can be measured with acceptable accuracy and precision (i.e., QL).

Specificity: Specificity is the ability of the method to assess, unequivocally, the analyte in the presence of other components that are expected to be present (e.g., impurities, degradation products, matrix components, etc.).

Spiked samples: A spiked sample is a general term that refers to calibrators (calibration standards) and quality controls.

Stability: Stability is a measure of the intactness an analyte (lack of degradation) in a given matrix under specific storage and use conditions relative to the starting material for given time intervals.

Stock Solution: A stock solution refers to an analyte in a solvent or mixture of solvents at a known concentration, which is used to prepare calibrators or QCs.

Study samples: Study samples refer to samples from subjects or patients enrolled in a study.

System suitability: System suitability is a determination of instrument performance (e.g., sensitivity and chromatographic retention) by analyzing a set of reference standards before the analytical run.

Upper limit of quantification (ULOQ): The ULOQ is the highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy.

Within (intra)-run: Within-run refers to the time period during a single analytical or validation run.

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