Chapter 8
Bioanalytical Parameters in Immunoassays and Their Determination

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8.1 Introduction

The implementation of high-quality IAs is important in response to growing health concerns and increasingly stringent requirements for clinical testing. Essential bioanalytical parameters encompass precision, accuracy, selectivity, sensitivity, reproducibility, and stability. Therefore, the IVD manufacturers need to adopt an appropriate design and developmental plan with stringent quality control procedures to renovate and manufacture commercial IVD and POCT kits [1–4]. During the last decade, many commercial IAs for clinical testing have shown conflicting results, thereby signifying the need for even more stringent bioanalytical guidelines and regulatory requirements [5, 6]. Thus, all aspects of bioanalytical testing must be addressed while drafting appropriate regulatory guidelines for IVD. The continuously increasing number of IA formats and technologies [7], the emergence of novel technologies [8], improved healthcare monitoring and management procedures, and

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new healthcare delivery concepts [9, 10] are posing a great challenge to the development of globally harmonized IVD guidelines.

The increased bioanalytical requirements for high-quality IAs are evident from the evolving IVD guidelines, normally associated with a high cost. In addition, significant improvements in IAs can be achieved by considering the feedback from the end-users and analysts. This chapter provides a comprehensive view of the bioanalytical parameters and performance of IAs together with the trends in bioanalytical testing/validation and technical challenges.

8.2 Bioanalytical Parameters of an Immunoassay

8.2.1 Precision and Accuracy

The IA precision indicates the nearness of individual test results for repeated analysis of an analyte concentration in multiple aliquots of a single homogeneous volume of the biological matrix. The IA precision for various analyte concentrations within its detection range should be <15% of the coefficient of variation (CV). In addition, its precision for the analyte concentration at the lower limit of quantification (LLOQ) should be within 20% of the CV. The "within-run (intra-batch)" precision is derived from a single analytical run, whereas the "between-run (inter-batch)" precision is obtained from multiple analytical runs over time.

The IA accuracy shows how close its mean test results are w.r.t. the actual target concentration, i.e., the nominal value. The mean value is within 15% of the nominal value for the entire detection range, whereas the mean LLOQ is within 20% of the nominal value. The IA accuracy is determined by replicate analysis of quality controls (QCs) using known analyte concentrations.

8.2.2 Sensitivity and Specificity

The IA sensitivity*is* the lowest detectable analyte concentration (LLOQ), with acceptable accuracy and precision. The IA selectivity is its ability to differentiate and quantify a target analyte from native samples in the presence of various endogenous interfering substances including physiological and pharmacological substances. The selectivity should be ensured at LLOQ and evaluated for each analyte in case of multiplex IA.

8.2.3 Calibration Curve

A calibration curve or plot is required for the determination of analyte concentration in an IA from the signal response. The signal response-analyte concentration relationship must be highly reproducible, and the calibration plot must be generated for each analyte in the sample in case of multiplex IA. The calibration standards are prepared by spiking known analyte concentrations in the native biological sample matrix. They cover the wide concentration range of an IA. However, substitute matrices could be used in case of special biological matrices, e.g., cerebrospinal fluid, which is difficult to obtain. The calibration curve is generated by employing a blank sample (no analyte or internal standard), a zero sample (no analyte but contains internal standard), and at least six analyte samples at varied analyte concentrations to cover the range of IA including LLOQ (with the analyte and the internal standard). The calibration standards are within 15% of nominal analyte concentrations for all concentrations above the LLOQ. At LLOQ, it should be within 20% of the nominal concentration.

8.2.4 Stability

Stability of IA reagents reflects their ability to retain the original performance and properties when stored under the defined conditions for a specified duration. The stability of an analyte should be determined for the specified duration in a matrix and container system. The real-time stability is determined by storing the IA reagents at 4 °C, while the accelerated stability is determined by storing them at 25 °C or 37 °C. The accelerated stability at such temperatures provides an estimate of the stability of IA reagents at 4 °C (Table 8.1). These determined values are based on the temperature coefficient Q_{10}, the increased reaction rate at a temperature increase of 10 °C. The freezing/thawing stability of the samples must be analyzed for up to three cycles of freezing/thawing, and this step should be performed in

Table 8.1 Evaluating the stability of IA kit at 4 °C from the determined accelerated stability at 37 or 25 °C

	Estimate of days of stability of an IA kit at 4 °C based on testing at	
No. of days of testing	37 °C	25 °C
7	68	30
14	137	60
21	207	90
28	276	120
35	345	150
42	414	180
49	483	210
56	552	240

similar experimental conditions as prevalent at the analytical lab. Another consideration is the stability of IA reagents during sample collection, handling, and transport. For automated IAs, the stability of IA reagents once they are opened and stored onboard the instrument must also be evaluated. Similarly, the "in-use" stability of IA reagents after opening and storage as per the instructions for use (IFU) must be addressed. Another issue is the sample stability during multiple freezing and thawing cycles. The stability analysis should employ samples prepared from a freshly prepared stock solution of analyte in a suitable biological matrix that does not contain any specific analytes and interferences.

8.2.5 Reproducibility and Recovery

The reproducibility is obtained by the replicate determinations of analyte concentrations in an IA using the desired QCs and samples. The recovery of an analyte in an IA is a measure of its analyte extraction efficiency. It is calculated using the detection signal obtained from the analyte concentration added to the biological matrix in comparison to the detection signal obtained for the same analyte concentration in a solvent. The desired percentage of recovery should be in the range of 90–110, although the values between 80 and 100 are still acceptable. The sample recovery experiments are conducted for each sample matrix if the IA is intended for multiple matrices. Such experiments determine the presence and extent of the matrix effect for a sample type.

8.2.6 Bioanalytical Performance Parameters

8.2.6.1 Limit of Blank (LOB), Limit of Detection (LOD), and Limit of Quantification (LOQ)

LOB is the highest measurement result, which is likely to be observed with a stated probability, usually at 95% certainty, for a blank native sample that does not contain any analyte (Fig. 8.1) [11]. The blank native samples must be real sample matrix instead of a clean buffer matrix. LOB is calculated as

$$\text{LOB} = \text{Mean of blank samples} + 1.645\ \text{standard deviation (SD) of blank samples} \tag{8.1}$$

LOD is the lowest analyte concentration in a sample that can be consistently detected with a stated probability, usually at 95% certainty [11]. The LOD, determined by Eq. (8.2), is based on the replicate analysis of real samples with very low analyte concentrations.

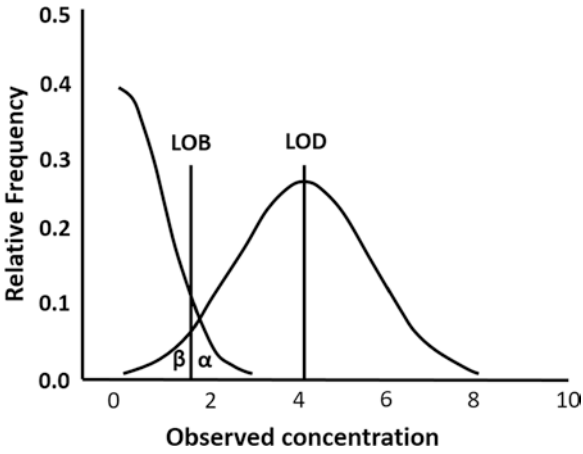

Fig. 8.1 The results for the blank sample having no analyte (left curve) should have 95% of the measurement results below the LOB (taking $\alpha = 0.05$). The results for the positive analyte sample having low analyte concentration at the LOD should have 95% of the results above the LOB (taking $\beta = 0.05$)

$$\text{LOD} = \text{LOB} + 1.645 \text{ SD of samples with low analyte concentration} \quad (8.2)$$

LOQ is the lowest analyte concentration that can be quantitatively detected with a stated accuracy and precision [11], which are set by the IA developers based on the predefined acceptance criteria, performance requirements, and end-user application. The lower LOQ (LLOQ) is the lowest calibration standard on the calibration curve where the detection signal for the analyte should be at least five times more than that of the blank sample. The precision of the determined analyte concentration should be within 20% of the CV, while its accuracy should be within 20% of the nominal analyte concentration. The upper LOQ (ULOQ) is the highest calibration standard on the calibration curve, where the analyte response is reproducible, the precision is within 15% of the CV, and the accuracy is within 15% of the nominal concentration.

The calibration curve should only be used between the LLOQ and the ULOQ for determining the analyte concentration in unknown samples. The practice of extrapolation below the LLOQ or above the ULOQ for the quantification of an analyte in samples is not recommended. The samples with analyte concentrations higher than the ULOQ must be diluted using the same matrix as the "real-world" sample. The samples with analyte concentrations below the LLOQ are reported as the zero concentration.

8.2.6.2 Lower Limit of the Linear Interval (LLLI) and Lower Limit of the Measuring Interval (LLMI)

LLLI is the lowest analyte concentration at which the detection signal shows a linear relationship with the analyte concentration. LLMI is the lowest analyte concentration at which all the specified performance characteristics of an IA, such as linearity, bias, and imprecision, are all met.

8.2.6.3 Linear Range and Analytical Measurement Range

The IA linear range is the analyte concentration range on the calibration curve, where the detection signal shows linearity with the analyte concentration in the sample. The linearity studies should be performed for each sample matrix of the analyte based on the intended use of the IA. The analytical measurement range of an IA is the analyte concentration range that an IA can determine in the sample without any sample pretreatment including dilution or pre-concentration.

8.2.6.4 Carryover and Errors

The carryover is defined as the error in the determination of analyte concentration in a sample when very concentrated samples are run before very low analyte concentration samples. The amount of analyte carried by the measuring system from a particular IA into subsequent IAs often introduces a significant error in the analyte determination [12]. It is calculated as the mean of the High-Low results minus the mean of the Low-Low results. The High-Low result is the result of low analyte concentration determination that immediately follows a high analyte concentration determination, whereas the Low-Low result is the result of a low analyte concentration determination that immediately follows a low analyte concentration determination.

The accuracy error is the deviation of the determined analyte concentration from the actual analyte concentration, defined as the sum of the random and systematic errors. The systematic error is the difference between the actual analyte concentration and the mean of an infinite number of analyte measurements carried out under repeatable conditions. The total analytical error is defined by Eq. (8.3) below, consisting of bias and imprecision based on a particular error model [13].

$$\text{Total analytical error} = \text{Bias} + 1.65\ \text{Imprecision} \tag{8.3}$$

8.2.6.5 Interference and Cross-Reactivity

The interference in an IA is the significant bias in the determined analyte concentration that may lead to imprecise results [14, 15]. It is due to the presence of nonspecific substances in a sample but could be tackled at the assay development stage by changing the formulation of IA reagents and/or the assay format [16–18]. However, if the interference from a nonspecific substance could not be obviated, this issue should be reported clearly in the instructions for use (IFU). The experimental procedure for the determination of such interferences in an IA involves the calculation of bias resulting from the addition of interfering substance. If the bias in an IA exceeds 10% after the addition of an interfering substance, it is important to determine the concentration of the interfering substances at which less than 10% bias is

achieved to establish the interference threshold. The interference thresholds of interfering substances should be mentioned clearly in the IFU.

The cross-reactivity, defined as the percentage of the measured concentration of cross-reactant over its absolute concentration, is an indicator of the nonspecific reaction between an antibody and a nonspecific structural analog like the specific analyte of an IA. The IA should not have any cross-reactivity to the substances that are mentioned in the product design specifications (PDS) of an IA. But if there is cross-reactivity against a substance that cannot be obviated, it should be reported clearly in the IFU.

8.2.6.6 Bias and Method Comparison

The bias is the difference between the actual analyte concentration determined in a sample (accepted reference value) and the expected analyte concentration. It is measured by determining the analyte concentration in a sample at the beginning and the end of a long series of IAs when the same IA components are used under the same ambient conditions.

The method comparison is an essential requirement of all regulatory submissions to demonstrate the alignment of the developed IA with the predicate IA. It is determined by the measurement of correlation and bias between the developed IA and the established IA based on the testing of real samples. Most IA developers employ the method comparison results for their product flyers, marketing literature, and technology claims. The method comparison guidelines recommend taking real samples covering the reference range of an IA along with samples that are lower and higher than the reference range. Only pristine samples, not subjected to heat, pH, stripping, or filter treatment, should be used with about half of the samples above the reference range, while others should be within and below the reference range. The use of a large number of samples improves the statistical confidence of method comparison, while samples stored for more than 12 months should not be used.

8.2.6.7 The Hook Effect and Quality Controls (QCs)

The hook effect is observed in case of sandwich IA at very high analyte concentrations [19], when the increased number of analyte molecules could bind to both the capture and the detection Ab and prevent them from forming the sandwich immune complexes. This could result in the decrease in detection signal at extremely high concentrations, which could fall to the detection signal corresponding to lower analyte concentration within the calibration curve range. It could lead to a misleading lower analyte concentration, while the actual analyte concentration would be much higher. Therefore, the Hook effect must be critically evaluated in case of sandwich IAs, and the highest analyte detection range of such assays should be restricted to a high analyte concentration where there is no hook effect.

The QC samples play an important role in IAs as they are responsible for the acceptance or rejection of an IA run. At least three concentrations of QCs should be taken in duplicate for an IA. The low QC should be within three times the LLOQ of an IA, while the middle QC should be in the mid concentration range of an IA, and the high QC should be in the high concentration range of an IA. If at least 67% of QCs and 50% of QCs at each level are within 15% of their nominal concentrations, the IA run is bioanalytically successful. The calibration standards and QCs are prepared from separate stock solutions, but they can be prepared from same spiking stock solutions if the stock solution is verified as stable and accurate.

8.3 Critiques and Outlook

The developed IAs must be precise, accurate, sensitive, selective, and reproducible for the reliable detection of clinical analytes in healthcare and bioanalytical settings. The variability in IA results is one of the critical issues that is often observed with the end-users. Consequently, the IVD developers must consider all the factors that could lead to variability in IAs during the developmental stage. All desired measures must be taken to obviate the variability, including the testing of IAs with several potential end-users via alpha and beta site clinical trials during development. The various factors that are usually responsible for IA variability are instrument, reagent lot, calibration lot, calibration cycle, operator, consumables, laboratory, and environment.

LOD as the basis of IA improvements has been often exaggerated. Indeed, there is a need to critically assess the bioanalytical performance of an IA based on all the parameters and the intended use. The improved LOD is not an indicator of improved analyte detection; despite a low LOD, the IA linear range could remain the same. The IA linear range should cover the entire analytically relevant analyte concentration range, where the cutoff value (minimum analyte concentration that could be present in the patient's sample) is much above the LLOQ of the IA to enable reliable analyte detection.

Sandwich IAs are prone to the hook effect [19], resulting in a falsely low analyte concentration, while the actual analyte concentration is very high. Therefore, the Hook effect must be investigated and set IA linearity accordingly. For automated IAs, "the carryover" by the instrument should be obviated as it could lead to erroneous results, i.e., falsely elevated analyte concentrations for patient samples with low analyte concentrations. The intended use of an IA must be mentioned clearly in the IFU so that the end-users only use the IA for analyte detection in the specified sample matrix. The IVD manufacturers must provide to the end-users the detailed instructions, the IA protocol, and the application note to avoid any errors in analysis. The calibration of an IA plays a prominent role in automated IAs, which makes it obligatory for the users to follow the calibration procedure provided by the manufacturer at the start of the measurement and periodically thereafter at the specified frequency. Additionally, the manufacturers have to critically evaluate the quality of

raw materials as their variability can affect the IA performance, leading to imprecision [20, 21]. Such materials must be specified to ensure their high quality and consistency.

The end-user scenario and conditions also play an important role in the development and validation of IA. As most IAs are developed in the standard bioanalytical labs under controlled ambient conditions, there is a lack of understanding of their performance in the end-user setting. The automated IAs must have random access capability as the running of a particular IA on an instrument should not impact the performance of subsequent IAs [22, 23]. All possible interferences for a particular IA must be scrutinized [14, 15, 24] and evaluated. The relevant and updated CLSI guidelines must be monitored as the list and concentrations of interfering substances may vary from time to time. A regulatory pre-submission at an early stage is needed to confirm the effects of all plausible interferences and their concentrations. If an IA has confirmed interference with some interfering substances, these must be clearly specified on the IFU. The selection of high concentrations of cross-reacting substances to be used for IA is often problematical as the regulatory authorities only recommend the use of sufficiently high concentrations of cross-reacting substances but not their exact concentrations. Thus, the use of varying high concentrations of cross-reacting substances by different IVD developers is often encountered. In general, there is a significant decrease in the error cases of IA during the last two decades, but the pre- and post-analytical steps still account for some significant errors [25, 26] that needs to be effectively tackled [27].

There are growing concerns about the conflicting results obtained from various IAs developed by different companies for the same analyte. Most IAs align well with the predicate IA that is specified by the regulatory authorities or selected by the developer based on the market intelligence. However, the developers are not considering if their developed IAs provide the same results as other most widely used commercial IAs. Therefore, to avoid discrepancy in results, the developers should evaluate the correlation of results obtained by their developed IA with those obtained by the predicate IA and the widely used IAs in healthcare. The developers should participate in external quality assessment and assurance schemes [28–30] and perform the validation of IA stringently as per the bioanalytical guidelines provided by the regulatory authorities [31].

The IA developers should know the end-user's scenario, i.e., analysis procedures, decision-making, sampling, etc. so that they could develop an IA that is well-suited to the intended bioanalytical application. They should monitor the performance of the developed IA using many real samples at the various clinical and bioanalytical settings available to the end-users. Moreover, they must keep themselves abreast of the bioanalytical guidelines and requirements, international standards, and trends in the field. If the international standard for an analyte is changed, the IA should employ the recent international standard only to avoid any discrepancy in results [32, 33]. The instruments must be well maintained, calibrated, and operating in stable conditions with stringent quality control during the entire course of IA development. Further, it is essential to check the integrity of data obtained by the instrument periodically. The persons involved in IA development

and its use should be fully trained in all aspects of IA, instrumentation, and data analysis. In case of automated IA, the IA system must be verified periodically for any defect that adversely impacts the analysis. Moreover, it is important for IVD manufacturers to continuously monitor the performance of their IAs in the market. The manufacturing lots of IA that demonstrate inadequate bioanalytical performances should be recalled immediately to prevent any adverse events and detrimental consequences. The new IA systems as the automated IAs developed on the old IA system may not show the same analytical performance on the new IA system. Thus, it is obligatory for the manufacturer to validate the IAs on the new IA system before launch. There have been several instances where the same IA from the same manufacturer demonstrated significant variations on several IA systems developed by the manufacturer [34].

8.4 Conclusion

There is a constant and increasing need for critically improved IAs to have the desired optimal bioanalytical performance for clinical analytes. To date, some conflicting results still exist among various commercial IAs for the same analyte in interrogation. The IA developers need to follow a design and developmental plan to conform to the most updated regulatory bioanalytical guidelines. The IA bioanalytical performance must be established by determining all analytical parameters and performing statistical analysis in accordance with the established CLSI guidelines. The intended use of the IA must also be evaluated critically via independent end-user trials during the development. IVDmanufacturers must have a complete control over the development of IA, including the lot-to-lot consistency of manufacturing lots and raw materials. The regulatory authorities should keep track of the evolving technologies to update the bioanalytical guidelines.

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