White Paper

Theme: Best Practices for Bioanalytical Methods: Recommendations from the Global Bioanalysis Consortium

Guest Editors: Binodh DeSilva and Philip Timmerman

Ligand Binding Assay Critical Reagents and Their Stability: Recommendations and Best Practices from the Global Bioanalysis Consortium Harmonization Team

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Abstract. The L4 Global Harmonization Team on reagents and their stability focused on the management of critical reagents for pharmacokinetic, immunogenicity, and biomarker ligand binding assays. Regulatory guidance recognizes that reagents are important for ligand binding assays but do not address numerous aspects of critical reagent life cycle management. Reagents can be obtained from external vendors or developed internally, but regardless of their source, there are numerous considerations for their reliable long-term use. The authors have identified current best practices and provided recommendations for critical reagent lot changes, stability management, and documentation.

KEY WORDS: critical reagents; ligand binding assays; lot changes; recommendations; stability.

INTRODUCTION

The Large Molecule L4 Harmonization Team was formed as part of the Global Bioanalysis Consortium (GBC) with the goal of reviewing the guidances, white papers, and relevant literature pertaining to the use of ligand binding assay (LBA) reagents in bioanalysis in the context of their stability in order to identify clearly defined best practices as well as potential gaps. LBAs are widely used to measure drug concentrations for pharmacokinetic evaluations, immunogenicity of biotherapeutic drugs, and biomarkers. Reagents are the foundation of these assays; specificity, selectivity, and sensitivity of LBAs are dependent on reagents.

The diversity of LBA reagents and their usage has resulted in the evolution of a broad range of management approaches often influenced by business constraints and

resource limitations. How reagents are used depends also on the application and the stage of drug development. For example, the analytical issues for non-clinically regulated toxicokinetics (TKs) and clinical pharmacokinetics (PKs) may be similar, but for clinical assays, there may exist a need to manage multiple reagent lot changes and their long-term storage stability as the program progresses through different phases of clinical development. While there is clearly no one size fits all approach, efforts to manage reagent life cycle often fit into either the (1) use of a large lot where stability and storage logistics would be a major concern or the (2) use of a small lot where the management of lot changes would be the main concern.

It has been recognized that some reagents are critical, and their changes significantly alter or curtail the performance of the assay that relies on them (1–6). Control of critical reagents is fundamental to the quality and long-term performance of LBAs. Although current regulatory documents have provided very specific guidance on the analytical validation of PK and immunogenicity assays, they have generally not adequately provided guidance regarding numerous aspects of critical reagents life cycle management including stability. The US FDA and European Medicines Agency (EMA) guidance for bioanalytical assays (7,8) do provide some general points to consider for critical reagents. In Japan, the draft guidance for ligand binding assay is now in public review. Brazil, China, and India do not have any guidance that address critical assay reagents for ligand binding assays.

The published guidance documents, and white papers have provided broad expectations, but there is a lack of commonality in practices in how to manage changing critical reagents, including major and minor lot changes,

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documentation, and criteria for performance testing of reagent stability and lot-to-lot continuity. Thus, industry needs a harmonized approach in order to enhance consistency and guide decision making based on sound scientific and regulatory principles.

Leading Principles

The authors reviewed and discussed LBA critical reagent global guidances and literature to identify gaps as well as consensus best practices through regular teleconferences over a period of many months and defined the scope and key topics for this effort.

It was recognized based on the experience and literature that reagents need to be considered and managed in the context of the assay life cycle (1–3). In particular, characterization of a critical reagent is important both in terms of rationale for initial selection and effective supply of the critical reagent throughout the life of the assay.

With this in mind, the team focused on four general areas:

- Critical reagent documentation
- In-house critical reagent *versus* commercial source reagents
- Changing critical reagents
- Stability of critical reagents.

Topics deemed out of scope included reference and internal standards, cell-based PK assays, and matrix other than that needed for antidrug antibody (ADA) assays. Commercial kits, which are widely used for biomarker analysis, were also considered out of scope, although some of the recommendations for reagents may also be applicable.

After this review, the authors formulated a set of draft recommendations which were first released on the GBC webpage in June 2012 for feedback from worldwide bioanalytical community. In addition, the team requested a direct comment through the use of a survey which covered both draft recommendations and general questions about relevant reagent practices. Feedback and comment on these draft recommendations were also obtained from conference presentations and round tables as well as responses from European Bioanalysis Forum (EBF), Japan Bioanalysis Forum (JBF), and many individual companies. The consolidated responses were used to refine the final recommendations and best practices which are outlined in this paper and focus on the four areas described above. Additionally, the authors identified areas where further discussions are needed. Overall, there was a wide range of practices along with some areas with a high degree of consensus. Nevertheless, the survey results reflect the inconsistencies in regulatory guidance regarding documentation, specifically for lot changes, with about 50% having a procedure to address lot changes and 50% without such a procedure. Most surveyed indicate having a procedure for critical reagent production, but no procedure for expiry extension. However, there is consensus that expiry extension is a data-driven decision.

RECOMMENDATIONS AND BEST PRACTICES

Critical Reagent Definition

While any component of an assay may be critical to its performance under certain circumstances, critical reagents for any specific assay need to be defined as such. Reagents that are difficult to make, replace, acquire, or substitute may be considered as rare critical reagents. Conversely, reagents that are more generic such as anti-light or heavy chain antibodies may also be critical in some assays. The use of immunocapture in combination with LC-MS could require the capture antibody to be considered as a critical reagent. Even assay buffers or blocking reagents may be critical to the performance of ADA assays. The need to define a reagent as critical demands active management of reagent availability and reproducibility. Documented procedures should be in place to define reagents as critical and the way these reagents are managed throughout the life cycle of the assay.

While most regulatory agencies have not defined critical reagents, they do discuss the key and critical nature of LBA reagents. The 2001 FDA Bioanalytical Method Validation Guidance for Industry (7) defines some reagents as a key (critical) and outlines the management of aspects of these reagents throughout the life of the assay. Specific reference to the appropriate characterization of antibody including cross-reactivity, definition of storage conditions, assessment of stability, and the need to assess performance of antibody and labeled analyte (tracer) when lots are changed supports the critical nature of these reagents and assessments. This need can easily be extended to other LBA reagents. The 2011 EMA guidelines for bioanalytical method validation (8) support and broaden the concept of key/critical reagents from the 2001 FDA guidance. These guidelines recognized that assay specificity was determined by the reagents used and that control of critical reagents is fundamental to the quality and long-term performance.

Identifying a reagent as critical has been raised by both regulatory agencies and in a number of publications (1–6). The authors recommend, within the scope of the GBC initiative, the following critical reagent definition: LBA reagents that are analyte specific are most often considered as critical reagents (antibodies, peptides, proteins, conjugates (label)), drug as reagent, and ADA reagents including positive and negative control. The EMA guidelines define critical reagents as "...binding reagents (e.g., binding proteins, aptamers, antibodies or conjugated antibodies) and those containing enzymatic moieties have(ing) direct impact on the results of the assay..." (8). The bioanalytical community, as evidenced in the literature and the survey results from this team's efforts, agreed and expanded on this definition to include peptides, such as receptors or ligands and fragments thereof; enzyme conjugates; and in some instances, biological matrices (3–6, GBC L4 team survey).

PK reference standards (drug) are not defined as critical reagents *per se*. Regulatory guidances are very clear on how PK reference standards need to be controlled and assessed as reference standards (7). However, PK reference standards can also become a critical reagent, both in immunogenicity and in target biomarker assays, typically after conjugation. Biomarker calibrators are generally far less well characterized

than drug reference standards, and thus, lot changes are more difficult to control, and often, an accredited source (e.g., US Pharmacopeial Convention) for a reference standard is unavailable. While biomarker calibrators were not in scope, specific guidance regarding biomarker calibrators is generally lacking, creating a large gap regarding an expected practice.

CHARACTERIZATION AND QUALIFICATION OF CRITICAL REAGENTS

The quality of critical reagents is a fundamental component for robust assay development. Reagents are developed and selected based on their specificity to the analyte of interest and cross-reactivity to matrix or other reagents in the assay. They can be tested and characterized in a variety of ways before and during method development. Knowledge of how and why a reagent was selected and its performance within the assay and in other tests is important for the selection and qualification of new lots of material, if needed. Early FDA guidance highlighted the need for reagents such as antibody and tracers to be characterized appropriately as well as the need to test for binding and key cross-reactivity when new lots are tested (7). Similarly, the Clinical and Laboratory Standards Institute guidelines emphasize the need to characterize reagents and provide guidance on key characteristics (9). Once assay method development is complete and the critical reagents are identified, it is then possible to define risks of resupplying critical reagents. This could include, for example, consideration for a complex immunization strategy. For commercial reagents, rarity may be a concern (e.g., single vendor/supplier, cell line availability), and for in house reagent, purification and conjugation complexity or challenges may be important to assess.

Guidance (7,8) indicates that critical reagent characterization and qualification shall be done for the intended use, but the degree of required characterization of reagents for any given assay varies considerably. Thus, the authors have limited the scope of review of reagent characterization and initial qualification and recommend simply that sufficient characterization is conducted to enable some form of consistency and process control in the generation of a new lot and that this qualification is documented. Reagent characteristics can include but are not limited to the following: identity, source, purity, concentration (or titer), binding affinity, isotype (monoclonal/polyclonal antibody), molecular weight, specificity, incorporation ratio, and aggregation level. Several recent publications discuss in detail critical reagent characterization and testing methods (5,6,10).

The stage of the drug is also a consideration for a scope of investment in reagent characterization.

PROCUREMENT OF CRITICAL REAGENTS

It is important to consider the procurement of reliable critical reagents prepared internally or obtained from commercial sources in sufficient quantities preferably for the life span of a clinical study. Reagents can be produced in-house or obtained from commercial sources as *custom-produced* or *off-*

the-shelf reagents. However, the apparently simple task of the selection of reliable source of the critical reagents is not trivial. It may require careful planning, considerations of pros and cons, and due diligence.

In-house Produced Reagents

In-house custom production of the reagents can be tailored to suit the assay design; they can be extensively characterized for their intended use, a certificate of analysis (CofA) can be customized, stability data can be generated, and more importantly, the lot-to-lot variability (especially in protein labeling) can be monitored and controlled. Furthermore, internal control of reagent production may allow better life cycle management. However, in-house production of reagents requires significant investment in skilled personnel and capital to establish an infrastructure for the production of the reagents and advanced planning. Obviously, the availability of in-house resources could be a major challenge and an impediment to this approach.

Commercially Produced Reagents

Commercial reagents are very widely used for the development of LBAs in a variety of formats. Major advantages of commercial reagents include the off-the-shelf availability of a wide variety of the reagents from multiple vendors, usually characterized for the intended use and with CofAs of some sort. Moreover, a wide variety of detection reagents (labeled proteins) can be obtained from multiple sources and tested for superior performance in the assay.

In addition to off-the-shelf reagents, many vendors offer the custom production and characterization of specific reagents. The main benefit of this approach is reputable manufacturers of assay reagent that generally have well-established manufacturing facilities, technical skills, and knowledge base to support the production and application of the reagents (11). Moreover, they can provide an excellent support to the end users both in terms of generation and conjugation of custom reagents and resolution of application issues. However, the commercial reagents are not devoid of challenges. There are several issues related to commercial reagents that may need careful consideration. Some of these are listed in Table I and includes lack of reagent stability data, lack of available reagent specifications/lot release acceptance criteria (though some vendors may release it upon request), and lack of other basic information about the reagent.

The major challenge is lot-to-lot variability of unlabeled and labeled reagents which may make it difficult to bridge the data on a long-term study where multiple lots of reagents are used. It is not uncommon for a manufacturer to change the lot of critical reagents, such as the polyclonal antibody pool used to make conjugates that may meet the lot release specifications but could be a significant risk to the end user. Such changes may not be communicated to the end users in a timely manner. Additionally, the availability of a particular reagent may be discontinued without prior notice.

Table I. Considerations for Reagents Obtained In-house and Commercially

	Pros	Cons
In-house produced reagents		
Availability and cost-effectiveness	Custom in house reagents can be tailored to suit the assay design	Needs special planning and infrastructure for in-house reagent production In-house production of reagents (e.g., recombinant proteins/antibodies) requires significant investment in personnel (expertise) and capital Cost-effectiveness and availability of in-house resources could be a major challenge
Characterization	Reagents can be extensively characterized for the intended use, and certificate of analysis can be customized Lot-to-lot variability (especially in protein labeling) can be monitored and controlled Stability data can be generated in-house	Reagent characterization can be time-consuming and expensive. Time is of essence in most developmental projects In-house expertise may be limited
Ease of use	Better control on life cycle of reagents. Availability of a sufficient amount of the reagents can be exerted by better planning	
Commercially produced reagent		
Availability and cost-effectiveness	Readily available off-the-shelf reagents from a variety of vendors Custom reagent production is not limited to production of critical reagents (e.g., recombinant proteins, antibody, and antibody conjugates) in-house. These reagents can be produced by commercial companies according to customer needs Specific reagents (e.g., recombinant proteins, antibody, and antibody conjugates) can be custom prepared Relatively inexpensive	Availability of a particular reagent may be discontinued without prior notice Lot-to-lot variability. Lot changes may meet vendor release specifications but may be a significant risk to consumer Lot change may reflect both conjugation and starting materials Custom reagents are generally more expensive than off-the-shelf reagents
Characterization	Generally characterized for intended use, and certificate of analysis is provided Specific reagents can be characterized as per customer specification	Certificate of analysis may not have all the characteristics that one would like to see Stability data are generally missing. It should be done in-house
Ease of use	A wide variety of conjugated proteins can be obtained and tested for superior performance in the assay Calibrators available in preweighed quantities Premade buffers and assay diluents tested to work with critical reagents are readily available Several vendors provide excellent technical support	May require considerable efforts to select a reliable reagent and a reliable vendor Concentration assignments for preweighed calibrators vary widely varied, especially if the concentration is expressed in international units Proprietary composition of specialty buffers (e.g., wash buffer) may create logistic issues Many vendors may not have competent technical support

Recommendations Regarding Procurement of Critical Reagents

Where in-house resources and facilities permit, one may opt for the in-house production of reagents; otherwise, commercial source of reagent is a viable option. However, in view of the availability of a commercial reagent from multiple vendors of variable capabilities (strength of technical support documentation and ability to provide multiple lots), it is prudent to invest significant efforts in due diligence and feasibility evaluation to select a reliable reagent and vendor. The need to understand specific characteristic of a reagent is the same whether it is generated internally or acquired from a commercial source.

It is beneficial to establish close relationships with vendors to improve the scope of technical support for trouble shooting and resolving reagent-related issues, obtaining timely information about reagent lot changes, and securing

the supply of specific lots of reagents for a longer period of time. Moreover, if there is only a single supplier of a critical reagent, then it is advisable to consider some contractual relationship that warns the user about product changes or cessation of production (2). Some helpful points to consider are listed below:

- Recombinant proteins and peptide with identical concentration unit assignments from different vendors may behave differently in an assay system. Therefore, it may be useful to select and retain sufficient quantities of this material for long-term needs. In some cases, a correction factor may be considered to compensate for content and proportional errors if this has been established during validation, or experimental data have been generated and are available to support it (12).

- Vendors typically have quality control/quality assurance and lot release processes. Although these processes may not be generally available for review, it may be possible to perform a QA audit of the vendor facility under certain circumstances.
- Often the documents that are not typically provided with the reagents (e.g., related to reagent characterization, stability, lot release specifications, etc.) can be obtained by making a specific request.
- Commercially prepared pre-coated solid phase supports, such as electrochemiluminescence and ELISA plates as well as beads, may need to be considered as critical reagents. One should establish and document internal processes (and lot acceptance test) to assure uniform coating of plates, especially where strips are used (13). Where practical for in-house biomarker assays built with commercial reagents, one should use pooled biological matrix (e.g., serum/plasma) containing endogenous analyte as QCs to monitor lot-to-lot performance of a commercial reagent (see 14).
- Interpret manufacturer expiry data, as a test/retest in that reagent may still be functional for intended purpose beyond “expiry date.” See “[Reagent Stability](#)” section for guidance.

CHANGING CRITICAL REAGENTS

Reagents for ligand binding assays are typically generated by a biological production process, rather than a fully controlled synthetic procedure. This results in higher variability and heterogeneity from one batch to the next. The production process is often followed by purification and labeling steps that add to the complexity. Since these assays are often needed over long periods of time to support the life cycle of the drug, multiple lots of reagents may be needed to support the assay over time. To minimize lot-to-lot variability, it is important to have well-defined and consistent reagent generation processes that are fully documented using manufacturing protocols and labeling procedures (4,5). Often, however, the levels of detail in protocols and procedures are less than adequate (see 15 for a recent example), and it is recognized that the better the procedures is documented, the greater the reproducibility of reagent production. This also facilitates the production of a replacement if a critical reagent must be changed.

Changing critical reagents represent perhaps the most significant gap and challenge the team identified. In the FDA 2001 guidance on bioanalytical method validation, it is stated that “...assay re-optimization or validation may be required when there are changes to key reagents.” Additionally, the EMA guidance (8) states that when reagent batches are changed, the analytical performance of the method must be verified “to ensure that it is not altered compared with the original or previous batch.”

Any manipulation of a reagent represents a new lot or batch of that reagent, including dilutions. O’Hara *et al.* (6) classify changes to reagents or reagent lots as major and minor changes that require varying levels of evaluation. The evaluation needs to be scientifically driven. It is recommended that a clear set of assay performance and acceptance

criteria are established *a priori*, and the criteria and experimental evaluations are well documented (4,6). This documentation could be either as a standard operating procedure (SOP) governing reagent changes or as part of the final method report (1).

Based on responses to our draft recommendation, we found that not all bioanalytical laboratories have some procedures, if not formal SOPs, governing their reagent lot changes. In some cases, a qualification may consist of a single run using the new reagent lot or a single run using both the old and new lots in the same run. This comparison to the old lot was considered useful, but not an absolute requirement as, in many instances, the old (or original) lot may not be available. Some labs have a more well-defined partial validation procedure described for lot changes. There was some variation in definition of new reagents under some circumstances such as the production of new lots from the same parent material. The authors considered the traceability for lots maintained and used over time, finding that it is important for a lot to be traceable to the parent lot and to be able to differentiate between the different manipulations of that lot.

The authors originally considered that the acceptance criteria for both new lots of reagents and for stability testing should include maximum instrument response. However, concerns were raised about the utility of signal control charts based on instrument response. Considerations need to be paid to the platform used to generate the signal and ability to compare signal obtained over time from the same instruments and between different instruments. The influence of variables such as ambient temperature should also not be overlooked. Thus, the authors decided to only include this as a tool for consideration in the final recommendations.

Recommendations on Changing Critical Reagents

The authors recommend testing of new reagents in a functional assay based on *a priori* assay acceptance criteria. A two-tiered approach for the qualification of these critical reagents is proposed, with the level of testing being driven by the extent of change to that reagent or the level of possible impact of those changes. Typically spiked QCs are used in the assay to define performance, but for biomarker assays, it may be important to consider also including sample-based QCs that contain the endogenous analyte to ensure commutability (14).

Minor Changes

Minor reagent changes are defined as those that are expected to have minimal effects on assay performance and may therefore be implemented without any deleterious effect on data production. Examples of such minor changes would be a new reagent lot derived from a previously qualified stock such as a new affinity purification of polyclonal sera from the same animals or a new conjugation using the same protein lot when the conjugation process has been demonstrated to be well controlled. The process used to prepare the new lot should be the same as that used to produce the predicate (or

original) lot. The first level encompasses activities that would be common to all reagent qualification programs (Fig. 1).

- Recommend testing of one run including three levels of QC samples but prefer also to include the lower and upper limits of quantification for a total of five QCs, as the extremes of the quantitative range are where the assay is most fragile for PK and biomarker assays. For immunogenicity assays, three levels including the negative and low positive control close to the cut-point should be included.
- If multiple reagents were to be changed at the same time, the reagents may be tested together in the same assay and qualified together. It is recommended that more than one assay run be performed in order to increase confidence in the result and to minimize the risk of accepting data from a false-positive or negative outcome. However, one run may be sufficient if only a single reagent is changed at a time.
- Recommend where possible testing in parallel with the current *versus* the original reagent. This comparison to the original lot is useful in monitoring or controlling assay performance drift. However, in the absence of comparator lots, the monitoring of the maximum and/or QC signal becomes more important.
- Once acceptable data have been generated from the appropriate qualification tests, results should be documented as acceptance for the change of reagent, and no further qualification is required.
- In cases where acceptance criteria are not met, further individual tests may be required to identify the cause of failure. A single failure might be resolved by a single reparation of standards and QCs and reanalysis. However, repeated failures would indicate, at the least, the need to prepare a new reagent lot and possibly reoptimize the assay. Failure at this level could also be indicative of the need to produce a new lot of parent reagent. This would be considered as a major change to the assay.
- Suggest monitoring maximum instrument response from QC samples as a tool for gauging assay performance only and not as an acceptance criterion. The value of trending the QC maximum instrument response increases as data are collated over longer duration or in larger studies (16).

Fig. 1. Summary of critical reagent management process

Major Changes

This is the most extensive reagent qualification level and is directed primarily towards the replacement of critical reagent where the original source of a reagent is no longer available. In such cases, a new supplier and/or reagent will be identified and characterized, and the assay performance will be demonstrated. Examples of major changes would include antibody lots obtained from new animals, new clones for monoclonal antibody production, or new cell lines for the generation of recombinant material, and each of these would be expected to trigger more extensive requalification. Less obvious but at times also essential to the maintenance of assay performance could be the adoption of a new lot of negative control, which is particularly relevant for immunogenicity assays. In addition to the recommendations for minor changes, the following considerations should also be taken into account for major changes to assay reagents (Fig. 1):

- A minimum of three runs should be performed to characterize assay performance with the new reagent.
- Test the new reagent in parallel with the current or original reagent over multiple runs. If assay acceptance criteria are not met by the new reagent and/or the assay performance is altered but the assay still remains fit for purpose, the new reagent may be acceptable but would require more extensive qualification work, such as a partial validation.
- Other data may also be required in order to define and properly document the full impact on assay parameters.

REAGENT STABILITY

Recent EMA guidance states that “Conditions guaranteeing the maintenance of the stability of both non critical reagents (e.g. buffers, diluents or acidification reagents) and more importantly of the critical reagents should be documented in order to ensure that the performance of the method is not affected over time” (8). This concisely summarizes the expectation that stability should be assured, but the guidelines do not specify how reagent stability should be assigned, monitored, and/or tested.

The term expiry date and “retest date” have been used interchangeably. However, retest date seems to be more accurate from a practical point of view. The initial retest dates are often assigned based on experience with similar reagents. However, the stability of critical reagents can also be verified and tested directly either through real-time or accelerated stability testing. Accelerated stability data predict reagents’ stability based on the Arrhenius equation (17,18). This may be particularly useful if the reagent is unique. Mathematical extrapolations are used to calculate the predicted shelf life. For example, 24 h at 37°C may be equivalent to 1 month at 4°C (17,18). However, not all reagents have linear degradation rates, as some may be more stable at elevated temperatures, and some may fail precipitously.

Stability testing often involves performing the assay and determining whether the control values conform to specifications; this is essentially immunoanalytical stability. According

to the European Bioanalysis Forum and the GBC reagent surveys, the verification of the reagent’s stability in the method is typically done using QCs and the same acceptance criteria used for method acceptance. The number of QCs ranged from 2 to 5 (for quantitative assays) and 2 (negative control and low positive control) or 3 (negative control, low positive control, and high positive control) (for ADA assays). Most companies surveyed did not perform additional investigations for stability; instead, they rely on assay performance and in-study acceptance criteria using three levels of QC. Most responders to the survey questioned the utility of multiple qualification runs.

Method validation qualifies the reagent used during validation for study support and may include limited stability testing if needed (e.g., freeze/thaw). After validation, additional reagent stability testing can be conducted as needed (e.g., long-term reagent stability at a specific storage temperature, 1 month at 2–8°C). Separate assay runs may be employed if needed for stability testing to cover gaps where samples are not run (e.g., between clinical trials/non-clinical studies) or after an assay has not been used for some time. Such runs should be documented, with the purpose of the run clearly stated, and the outcome recorded.

Considerations for Storage of Critical Reagents

The preparation of critical reagents often entails multiple steps and dilutions as well as preparation in buffer with multiple components. When assigning stability to the final product, all of these components should be taken into consideration. It is important to differentiate between the critical reagent itself and the buffer formulation prepared. The buffer used to prepare or dilute the critical or rare reagent is often composed of multiple components and has its own expiry date. Typically, however, the container, the storage conditions (temperature), and form (liquid/solid) will be altered after the addition of the reagent, and stabilizers, antimicrobial agents, or other excipients may also be added. The primary role of the buffer formulation is to maximize the stability of the reagent, and the expiry/retest date is not defined by the expiration of individual buffer components. In addition, critical reagents are often prepared by conjugating two molecules. For example, a biotinylated antibody prepared from a lot of antibody and a lot of biotin linker is a new reagent, with a new lot number and a new test/retest/expiry date, independent of the starting materials. The stability of conjugates may differ from the parent molecules. Though it is important to recognize that conjugation can inactivate a reagent to varying degrees this may or may not be detected in a functional assay.

There is a significant body of literature describing protein, peptide, antibody purification, and conjugations thereof and formulation to minimize degradation (for review, see 19) and maximize stability (20,21). Generally, these approaches are not applied to in-house reagents, although commercial reagents may include them. Formulation can improve reagent stability and prevent denaturation, aggregation, surface adsorption, deamidation, oxidation, isomerization, fragmentation, etc. They are widely used in the diagnostic industry to prepare reagents for use in both kits

and automated analyzers and for biotherapeutic drugs (22,23).

Bacterial contamination can rapidly degrade reagents. Preservatives to prevent bacterial/fungal contamination are widely used and include sodium azide, ProClin300, and traditionally, thimerosal (see 24 for review). These preservatives may be included for long-term storage at 4°C and/or when a stock vial may be used repeatedly, thus increasing the risk of contamination. Alternatively, individual aliquots can be prepared, stored frozen until use, and then discarded to minimize this and other risks to reagents if long-term frozen storage conditions are feasible.

Looking at reagents from an assay life cycle management perspective is fundamental to managing critical reagents (see 6). Generation of large amounts of a single lot of critical reagent reduces a risk of lot changes but requires that large amounts are stored in small aliquots, often at very low temperature to cover the anticipated life of the assay but would require stability to be demonstrated. Each aliquot may be used only once, and this reduces a risk from 4°C storage for weeks or months, minimizing damage due to freezing and thawing, and a risk of contamination introduced by pipetting from a single vial in multiple times as mentioned above.

Commercial reagents should be stored as recommended by the provider/vendor. If this is not possible or preferred, then appropriate storage stability data need to be generated in-house. Generally, critical reagents should be stored in long-term storage at -60°C or below unless there are specific known stability issues or evidence that adequate stability can be expected at other temperatures (see Table II and reference 6). Storage containers, both materials, and headspace volume can also be a factor to consider (25,26). Some suppliers suggest that enzyme-conjugated antibodies should not be frozen at all and should instead be kept at 2 to 8°C or at -10 to 30°C in 50% glycerol, which lowers the freezing point. Since freeze thawing (FT) generally reduces enzymatic activity and can affect the protein stability, multiple FT cycles should be avoided. However, often stability after one FT cycle may be adequate and could be tested and documented.

Recommendations Regarding Reagent Stability

If reagent stability testing and/or monitoring will be performed, the authors recommend defining how to monitor and/or test reagent stability, including responsibility for testing, where the data will be stored, what criteria will be used, and where to document this work. Reagent stability evaluation can include but is not limited to freeze-thaw cycles, storage times, and temperatures. In addition to the bioanalytical assay (functional test) to assess reagent stability and extension, other methods may be employed. These methods may include reagent-specific testing (e.g., HRP functional test), accelerated stability evaluation (within the assay or independently), as well as physical characteristics that are indicative of instability risk such as aggregate formation. For example, Staack *et al.* (5) describes in detail the testing scheme used in their lab to assess storage (retest/expiration date) and handling stability of bioanalytical assay reagents during assay development which includes both functional and biophysical testing under a variety of storage

conditions. However, the scope of testing should match the needs of the assay life cycle and the instability risk of the reagents.

The authors recommend that reagent stability testing minimum acceptance criteria include back-calculated concentrations of a minimum of three QC levels. However, the use of five QCs, as described in the [Changing Critical Reagents](#) section, is preferred and remains as a more robust approach.

Acceptance criteria based solely on back-calculated QC concentrations do not address the potential for significant changes in instrument response. A decrease in instrument response may be indicative of a reagent instability and thus issue. LBA methods are commonly designed with excess reagent degradation may result in a slow decline in performance that can be best detected through control charts (e.g., Levey-Jennings plots; 27) that include instrument response from all acceptable runs with the assay (validation and sample analysis). Sample analysis can provide assay performance data to inform trend analysis of maximum instrument, and QC instrument response which may provide the best initial indication that one or more reagents are failing.

The authors recommend defining a process for addressing stability-related failures to enable a scientifically defensible rationale for either repeating the stability test or concluding that the reagent is no longer stable. Failure of a reagent within the original assigned stability window can inform the assignment of dates for future lots, but reagent handling must also be considered.

Assigning Retest Date (Expiry Date)

The authors recommend assigning retest rather than expiry dates to define ongoing critical reagent stability initially. In the rest of this article, we will use retest date to be consistent. The retest dates for critical reagents can be assigned according to the reagent expiration date provided by the vendor or historical data. If no prior stability information is available, then the retest date could be assigned according to Table II. Initial dates can be assigned based on experience with similar reagents. It is also possible to assign very long retest dates and to monitor performance and then revise when changes in reagent performance are identified. This approach may be most useful with a well-understood class of reagent such as polyclonal antibodies. For novel reagent, an alternative is to start conservatively and set short time periods and then extend as you get experience with this reagents. In either case, how retest dates are assigned should be documented. Some reagents may be shared by multiple assays, but reagent stability remains assay specific. For consistency, the following general principles are proposed: reagents may be used on the date of retest, and if a reagent retest date is assigned to a month, the reagent can be considered stable on the last day of that month.

Extension of Reagent Stability

For long-term study support, extension of the expiry date of a reagent is often required. The reagent expiry or retest date can be extended based on performance evaluation from one or more analytical runs and/or from a trend analysis

Table II. General Guidance for Reagent Storage Conditions

Reagent type	Storage condition	Retesting ^a	Extension ^b
Purified proteins/antibody ^c	≤−60°C	8 years from preparation date	2 years
	2°C to 8°C	6 months from thaw date	6 months
Labeled proteins/antibody ^c	≤−60°C	4 years from preparation date	1 year
	2°C to 8°C	6 months from thaw date	NA
Lyophilized reagents	≤−60°C	8 years from receipt date without reconstitution	2 years
	2°C to 8°C	4 years from receipt date without reconstitution	NA ^b
Commercial biological matrix	≤−60°C	3 years from receipt date	1 year
	2°C to 8°C	1 week from thaw or receipt date	NA

NA not applicable

^aFor specific reagents, scientific judgment may need to be applied based on the nature of the individual components; when appropriate, document the basis for the retest date in the analytical or reagent preparation procedure

^bRetest date can be further extended upon additional supporting data

^cFor reagents in non-lyophilized form

during assay use. It is the best practice to conduct the test for extending reagent stability prior to the expiry date. For performance evaluation in order to extend the reagent stability window, we recommend that extension be based on data, and there are a number of potential ways to obtain this. The first step would be to test the reagent in the functional assay to confirm acceptable performance.

The following considerations should be taken into account for the reagents still within the retest date:

1. For reagents that are in regular/continuous use, the retest date can be reassigned based on acceptable performance of the reagent during routine use and trend analysis, without requiring a separate stability run. The trend analysis needs to be documented.
2. For reagents that are not in continuous use, the retest extension can be based on an independent stability evaluation run (single run). The new retest date is assigned based on predefined documented criteria for this class of reagents (e.g., monoclonal antibody stored below −60°C, extended for 6 months).

It is understood that expired reagents should not be used for regulated work. However, if reagent stability data had not been generated prior to the initial retest date or the reagent is theoretically expired, bioanalytical scientists may face a significant challenge in replacing it, as has been discussed. In order for this to be accomplished for regulated work, it is critical that the process and acceptance criteria used to allow the use of a reagent beyond its original retest and/or manufacturer's expiry date be defined *a priori*. For an expired reagent, the data generated prior to expiration date can be used to define the new stability duration. However, this approach has to be well defined and documented.

The following considerations should be taken into account for reagents already beyond the retest date (see also Fig. 1):

1. For a reagent in continuous use, retest extension could be based on a single, independent run demonstrating acceptable assay performance and the trending data. The appropriate new retest date would be based on predefined criteria for that class of reagents (e.g., monoclonal antibody stored at less than −60°C, extended up to 6 months) plus the statistical trend

analysis from accumulated data (e.g., no more than 1 year of extension could be given based on 1 year of past data) (see 28). This recommendation applies to the practice in statistical trend analysis with the consideration of avoiding over-optimistic conclusions drawn from the statistical analysis using data from short duration.

2. For a reagent not in continuous use, retest extension should be based on independent runs (demonstrating acceptable assay performance). In this case, the reagent should be put onto a more rigorous monitoring program with shorter retest extensions and/or multiple runs (to obtain acceptable performance trending data prior to being reclassified as a reagent in continuous use). Assay performance monitoring would also be very important during the extension period.

While reagents may be stable beyond the originally assigned retest date, they can fail, and this is their true expiry date.

CRITICAL REAGENT DOCUMENTATION

Regulations have provided limited guidance for the documentation of assay procedures specifically relating to reagents and stability. Generally, the regulations require documentation in the form of an SOP (EMA and FDA), documented analytical procedure (Organisation for Economic Co-operation and Development), or work instruction (Medicines and Healthcare Products Regulatory Agency) to ensure the reproducibility and consistency of the method. They have similar definitions, requiring documentation which will allow operators "...to carry out the operations correctly and always in the same manner." (7,8,29,30), and while there is agreement regarding the requirement for a procedure, there are inconsistencies and gaps in what and how to document.

These regulations indicate some of the areas to be documented in procedures: the type and use of apparatus, listing of materials and reagents and their preparation, and storage conditions (8). They also require specific information for reagent labeling to include identity, titer or concentration, storage, and expiry (29,30). The method validation guidance

provides additional information in the area of a study report. Here, we find specific indications of what the regulatory agencies want to be documented: assay procedure including details of analysis, reagent preparation, reagent storage conditions, and stability (8). However, comprehensive information regarding required documentation is lacking.

The bioanalytical community, as evidenced in the literature and from the GBC reagent survey, supports the need for documentation which includes the use of an SOP or similar document for procedures and the requirement for a CofA or related document for critical reagents (5). Their efforts in recent years to fill the regulatory gaps demonstrate the need for more comprehensive direction relating to documentation. The community recognizes the need to document all of the information related to a lot of reagent; the source of the raw material(s), method(s) of expression, purification, final formulation buffer, and characterization in “batch records” (4); and the need for well-documented procedures to support consistent and reproducible processes such as antibody purification and conjugation, which includes the degree and site of labeling (5). This would also include the qualification of reagents fully described by a protocol or general SOP with *a priori* acceptance criteria (3) and where to document specific information for critical reagents, such as reagent stability, reagent storage, identification, and preparation (31). One of the largest gaps in documentation was related to the evaluation of reagent stability, how to evaluate stability and change lots when reagents have exceeded stability, criteria to use for acceptance, and where to document stability. The community also recognizes the importance of capturing the early assay development and reagent characterization information and maintaining documentation to support life cycle management efforts (1,5,6).

Recommendations Regarding Documentation

The authors recommend an SOP or similar document as determined by your business practices that defines the requirements of reagent stability tests and/or monitoring including how, what, where, and when, recognizing that documentation may take many forms and would include items as appropriate to a specific reagent. Depending on the business practice, documentation for well-characterized critical reagent may take the form of a CoA (5) or some other form of internal documentation, such as record of analysis, when the term CoA is preferred for drug substance.

Best practice recommendations include documentation of the following:

- Identify critical reagents for a specific method, i.e., in the method or test procedure.
- Provide identity of each critical reagent, e.g., CoA, satisfying regulatory requirements for labeling of critical reagents (29).
- Describe the production of critical reagent. Early in the reagent life cycle, this might be a laboratory notebook and, later, a procedure/SOP including
- Reagent characterization sufficient to enable a consistent process (see “[CHARACTERIZATION AND QUALIFICATION OF CRITICAL REAGENTS](#)” section)

including performance in the method. The authors acknowledge that broad characterization data starting with assay development may become invaluable during life cycle management but also recognize the limitations in obtaining some of this information.

- Documentation of the reagent source (e.g., cell line, method of expression), which may reside with the provider, if applicable.
- Purification and/or conjugation methods used to produce critical reagents, which may reside with the provider, if applicable.
- Reagent formulation.
- Describe assay acceptance criteria for reagent lot changes and stability evaluations. A procedure that includes how to manage retest of failed runs is preferred.
- Describe reagent qualification, how reagent was qualified (procedure), location of qualification data, and assessment of performance, e.g., pass/fail.
- Describe how assay performance will be tracked throughout the method life cycle, e.g., control charting, and location for this information, e.g., validation report amendment.
- Procedure for determination of reagent stability including
 - How retest (expiry) dates are assigned
 - How stability will be evaluated including *a priori* acceptance criteria, procedure to be used, who will evaluate (if applicable), frequency, storage conditions, and retesting criteria.
 - How to document stability evaluations, e.g., separate stability batch; reagents stability-specific runs can be documented separately from the method validation, and this can be linked to method(s)
 - Where to document stability data, e.g., stability report
 - How stability will be extended
 - Define expiry/retest date, a procedure for extending expiry/retest date with *a priori* acceptance criteria.
- Track the storage conditions and history of critical reagents, the location of this information, and the retention period for this information.
- Procedure and *a priori* acceptance criteria for new reagent lots and location of this information.

Critical reagents obtained from commercial sources may have limited documentation regarding purification and labeling as described in the “[Commercially Produced Reagents](#)” section. This represents a risk that needs to be managed by the user.

OVERALL CONCLUSIONS

All LBAs utilize a set of reagents, and some of these are critical to the performance of assays. Given the diversity of assays, formats, reagent types, and applications, it is difficult to anticipate all of the potential issues that bioanalytical scientists may need to address in reagent selection and initial characterization. However, given the potential need for assays to support therapeutic programs that can span many years and perhaps even decades if one includes potential post-marketing support, critical reagents must be considered in the context of assay and project life cycle. As global

regulatory guidance continues to develop, it is expected that the foundational nature of reagents for LBA will also evolve. The GBC L4 team has focused on defining scientifically defensible best practices and recommendations drawing from existing guidances, relevant white papers, and the literature in both bioanalytical and diagnostic fields and from a survey of pharmaceutical companies to address what we believe are some key areas of focus.

In simple terms, we recommend that bioanalytical groups have an *a priori* plan or framework for LBA critical reagent management. This plan should

- Identify critical reagents and risks to resupply and lot changes, whether provisioned internally or from a commercial source,
- Have a scientifically justified plan to address lot changes and stability of critical reagents, and
- Define critical reagent-related documentation.

We have summarized the critical reagent management process we describe in Fig. 1. In considering the development of a practical and efficient critical reagent management plan, a tiered approach may be useful since as programs mature, an increased investment may be needed. Additionally, critical reagents that are difficult to make and obtain or that are more unstable may require additional investment.

Stability testing of reagents is not well defined in guidances and is the area with the most gaps and range of practices. The guidelines do not specify how reagent stability should be documented, assigned, and/or tested, nor are there standardized acceptance criteria. For regulated study support, reagent stability testing needs to be well defined, and the practice should be guided using an SOP.

Perhaps not surprisingly, given the broad scope of critical reagents, there remained a number of unresolved issues and areas for future work. Some areas, such as the scope and process of new reagent characterization, both biophysical and immunological specificity, are complex and reagent specific. Obtaining sufficient reagent characterization documentation from commercial vendors is particularly challenging and may require internal investigation. Ideally, adequate data pertaining to the stability and characterization of critical reagents used in bioanalytical methods would be generated prior to employment of these reagents, during the assay validation, and in the reagent life cycle, but this will not always be practical or possible. In these situations, the critical reagent management plan for such assays should acknowledge these risks.

For biomarker assays, the calibrators are typically not as well defined as PK assay reference standards. While there have been some international reference standards developed for clinical diagnostics (e.g., human troponin I, 32) until such standards are established for any given analyte, it may be useful to treat them in the same way as critical reagents. Companion diagnostics (biomarker) and post-marketing patient monitoring assays (PK and ADA) are increasingly being developed to support therapeutic programs and typically evolve from the assays used during development. To optimally support a premarket approval of a novel diagnostic test using phase III studies, the management of critical reagents with these potential utilities in mind must start early in the development of these reagents.

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