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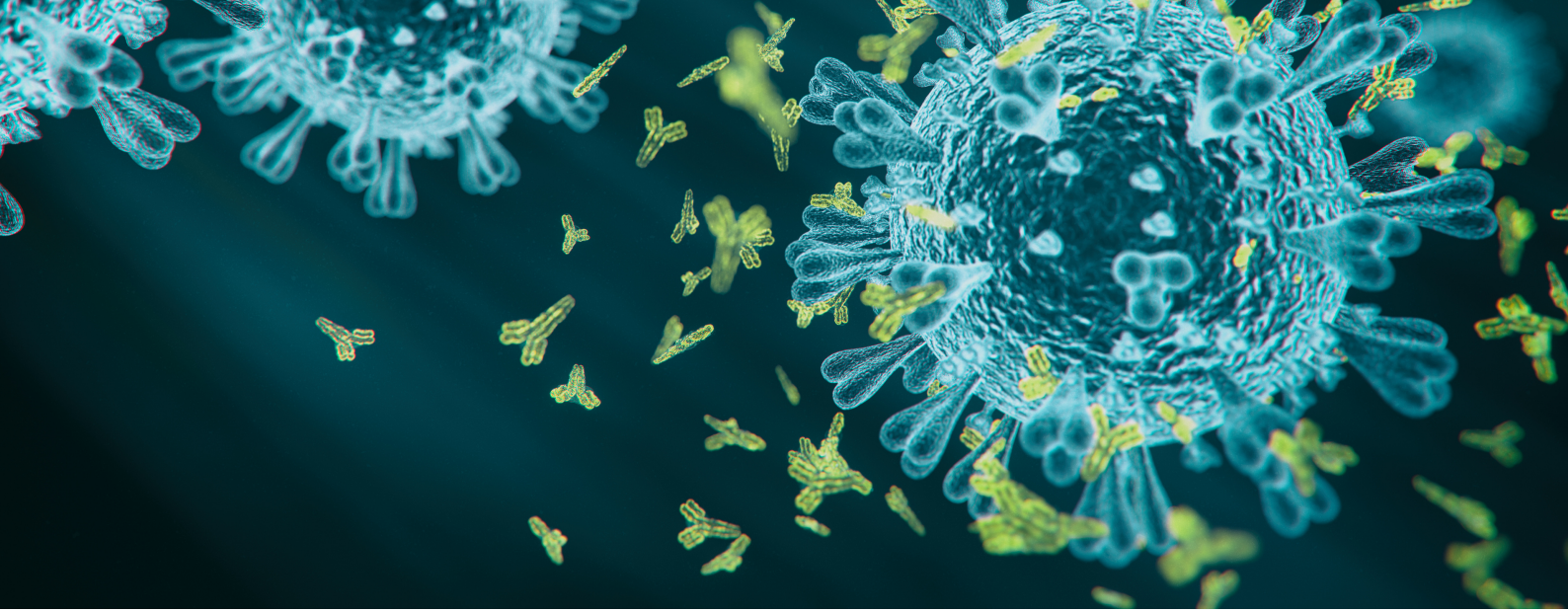

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Technology digest: why is it important to develop a critical reagent management strategy?

Written by Naamah Maundrell (Editor-in-Chief, Bioanalysis Zone)

The importance of critical reagents

When making pivotal decisions in support of drug development, data from ligand-binding assays (LBAs) can be difficult to manage. Ultimately, the robustness and reliability of the assay is directly influenced by the long-term availability and quality of the critical reagents used by the bioanalyst [1]. Critical reagents have been defined as the key components used in LBAs and include antibodies, biologics, peptides, as well as solid supports and matrices. These reagents have unique characteristics that are the backbone of assay performance in relation to sensitivity, specificity and robustness [2]. Critical ligand-binding reagents are predominantly produced via biological systems and are prone to lot-to-lot variability, which is importantly managed and monitored by life-cycle management [3]. The data generated by LBAs are dependent on the biological interactions of these analytes to the capture and detection reagents. Therefore, it is important to develop a critical reagent management strategy as even the smallest change in production could significantly alter the performance of the assay.

The maturation of drug-development programs to commercialization can span decades, so the production, quality, characterization, stability and sustainability of the critical reagents used are vital to the success of the bioanalytical laboratory [4]. Hence, why critical reagents are the crux of assay success. With this longevity and sustainability in mind, how can the continuing success of the LBAs be ensured? Replacing or extending the reagent based solely upon the assigned expiry date could miss changes in assay performance; and what happens when reagents change?

Key challenges of critical reagents

A crucial challenge of life-cycle management is when reagents change. Changing any reagent can also change the method, which in most cases means bridging is required to ensure comparable results [5]. LBAs should be managed through continuous monitoring where each run is performed with the method being recorded and monitored for shifts in signal, background and overall performance. Through monitoring and interpreting characterization data over time, insights can be provided into reagent stability and overall condition. This helps to maintain 'best practices' of the critical reagent program [2].

At Eurofins Bioanalytical Services (MO, USA), they employ a 'Test Method Summary Sheet', where every run from the method, regardless of study or data usage, is recorded with the lots used of critical and non-critical reagents, and the signal for the standards and/or control samples are recorded. The 'pass' or 'fail' status is also recorded and control samples' performance are assessed via use of Bland-Altman charts by tracking how they are performing either by signal, calculated concentration, or both.

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Ashley Brant, Senior Director of Scientific Affairs at Eurofins, commented on how the 'Test Method Summary Sheet' recording system works:

"At Eurofins we believe in monitoring assay performance across the board. Similar to how biomarker or serological assays are analyzed, we continually analzye the performance of the assay through time regardless of what study it is being run on. We monitor it by method and all the data goes into one huge spreadsheet so we can do statistics and monitor how the assay performs in real-time, in real-life. This way we can see how each of the critical reagents are impacting the assay. Even sometimes, the non-critical reagents, which we assume are non-critical, but these can end up being pivotal to the performance of the assay as well. We can analyze in real-time, make adjustments and ensure our assay is running as consistently as possible, the correct reagents are included as critical and therefore providing the most consistent data."

LBA development is dependent on sufficient high-quality critical reagents, where the resupply inventory needs to be monitored and characterization of new critical reagent lots is essential [3]. Results from a recent LinkedIn survey, investigating the key challenges of critical reagent life-cycle management, highlight that reagent characterization was the greatest challenge with 53% of respondents in agreement. This challenge was followed by reagent design (18%) and reagent production and supply (18%). This demonstrates the inherent variability of critical reagents, which means that a well-characterized and defined management system is essential.

Documentation of critical reagents

When generating critical reagents it is important to consider the documentation. With the everchanging work environment and staff turnover, having SOPs – documented analytical procedures – improves reproducibility and consistency of the bioanalytical laboratory. Documenting the history of each critical reagent can become invaluable in troubleshooting investigations especially when a reagent must be re-created [3,4].

Details on handling and storage are also important as changes in these can affect the quality of the reagents and therefore the reproducibility of the data. The stability of reagents can be affected by freeze–thaw cycles, storage times and temperatures. Thus, it is important to implement a systematic monitoring process for the attributes of critical reagents, as this can accelerate assay troubleshooting, identify detrimental trends and minimize delays in drug development [2].

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Critical reagent expiry/re-test dates

When critical reagents reach expiry or re-test dates, based on LBA data, a decision is made whether to extend and reassign an expiry/re-test date [5]. As long as LBA performance is monitored correctly and the LBA shows no decline, expiry/re-test dates for rare reagents are typically extended as they can be difficult to procure or generate [3]. At Eurofins Bioanalytical Services, they evaluate reagents and assign re-test dates by assay performance rather than relying solely on SOPs or manufacturer expiry, as there are examples when a reagent may appear stable based on the limited characterization performed by the manufacturer, but assay performance is not maintained.

Summary

Complete life-cycle management of critical reagents requires the bioanalytical laboratory to understand reagent characterization, supply chain sustainability, inventory management and database systems. Ineffective planning can interrupt the supply chain of critical reagents and cause delays to assay development and results, ultimately impacting cost and time. Therefore, efficient life-cycle management will ensure smooth operations in the bioanalytical laboratory [3]. Looking towards the future, as automated workflows accelerate drug-development timelines and the popularity of biologics increases, more emphasise will be put into critical reagent life-cycle management, rather than seeing it in a snapshot and switching the reagent out.

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Perspective

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Bioanalysis

Critical reagent screening and characterization: benefits and approaches for protein biomarker assays by hybrid LC–MS

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In recent years, hybrid ligand-binding assays (LBAs)/LC–MS assays have been increasingly used for quantitation of protein biomarkers in biological matrices. However, unlike in LBAs where the importance of critical reagent screening and characterization is well understood and widely reported, benefits of well-characterized hybrid LC–MS assay reagents are frequently underestimated. Two groups of analyte-specific reagents, binding reagents and assay calibrators, are considered the critical reagents for biomarker assays. In this article, we summarize the similarities and differences of critical reagents used in LBAs and hybrid LC–MS assays, overview the benefits and approaches of critical reagent screening, characterization, antibody conjugation and discuss bioanalytical considerations in hybrid LC–MS assay development for robust measurements of protein biomarkers in biological matrices.

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Keywords: critical reagents • endogenous biomarker • hybrid LC–MS assay • immunocapture (IC) • ligand-binding assay (LBA) • surrogate peptide

Hybrid ligand-binding assay (LBA)/LC–MS technology, often referred as 'hybrid LC–MS', has been increasingly used in drug discovery and development to overcome bioanalytical assay challenges that cannot be resolved using LBA alone [1–6]. Hybrid LC–MS biomarker assays are frequently required to differentiate between total and selected proteoforms, and provide consistent quantification results using different antibodies [6–10]. Furthermore, some immunoassays have limited utility for biomarker quantitation due to poor analytical specificity and potential interferences when using two antibodies [6]. One of the solutions to resolve discrepancies of LBA assay results is to use an independent method for protein quantitation. LC–MS technology is able to provide answers about LBA performance and has been used as an alternative platform for biomarker assays [6,8,11,12]. In addition, it is often difficult to generate and/or select two antibodies that recognize nonoverlapping epitopes on the target protein that are required for an LBA. Hybrid LC–MS strategy is commonly used when the target protein is small and/or available binding reagents bind to the same or overlapping epitopes.

Biomarker data quality is highly dependent on the quality of critical reagents [4,13]. Several industry recommendations and case studies describing the importance of LBA critical reagent characterization have been published up to date [14–20]. However, recommendations for critical reagents for hybrid LC–MS have not been extensively discussed in the literature. Critical reagents in LBAs are defined as biological materials that directly impact the readout of the assay [18]. It is well understood that critical reagents are essential components of LBAs and their performance needs to be monitored throughout the process of drug discovery, development and postmarketing surveillance. Since critical reagents are generated mostly in biological systems, lifecycle management of critical reagents helps to minimize assay performance issues caused by declining reagent activity or by lot-to-lot variation of commercial or in-house generated reagents. Proactive reagent characterization is essential in monitoring the quality of assays and enables quick troubleshooting. Some of the biophysical and physicochemical properties of LBA binding reagents that are measured include: binding characteristics, molecular identity, clonality, purity and

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concentration. Biomarker assay calibrator characterization and selection is another essential process to ensure that the calibrator material represents the endogenous form of the biomarker of interest [13].

In biomarker assays, the poor definition of the analyte has been identified as a bottleneck, and a roadblock, for the development of reference methods and globally accepted reference materials [3]. LC–MS methods are gaining acceptance in reference measurement procedures for protein standards, have been successfully used as complementary methods for immunoassays [3,21–23], and play a well-established role in characterizing therapeutic monoclonal antibodies (mAbs) [24]. LC–MS technology can also be successfully employed for characterization of biomarker assay binding reagents and calibrator materials. Well-characterized critical reagents are essential in the development of robust bioanalytical methods. In this article, we overview approaches and benefits of critical reagent screening, characterization, antibody conjugation for hybrid LC–MS assays and discuss the bioanalytical considerations of critical reagent screening and characterization in hybrid LC–MS assay development. These considerations are essential in ensuring assay reproducibility, selectivity, analytical sensitivity and detectability for robust measurements of protein biomarkers, especially low abundance biomarkers in biofluids or tissues. The main focus will be on bioanalytical considerations in critical reagents used for protein biomarker assays by hybrid LC–MS, since reagent screening, characterization and selection for pharmacokinetic (PK) and immunogenicity (IMG) assays have been extensively described in the literature [14–18,20,25–29].

Definition of critical reagents & benefits of reagent screening & characterization for protein biomarker assays by hybrid LC–MS

In general, analyte-specific biological reagents for biomarker assays, such as antibodies, antibody conjugates, peptides, proteins, protein conjugates, drug molecule and conjugated drug molecule are most often considered as critical reagents in LBA [20]. Analyte-specific reagents used in biomarker assays can be divided into two groups: binding reagents (generic and analyte-specific monoclonal and polyclonal antibodies (pAbs), engineered antibody fragments, other analyte binding proteins and aptamers) and assay calibrators (usually a recombinant or synthetic protein that closely represents endogenous analyte).

Biomarker assay binding reagents are usually screened, characterized and selected by following processes similar to that of PK assays. Yet, two caveats need to be taken into account when screening for binding reagents (usually antibodies) to be used in biomarker assays: these reagents are typically generated by immunizing animals with a recombinant protein that may not be structurally identical to the endogenous analyte; the calibrator used for reagent antibody screening may not have the same binding properties as the endogenous protein. Unsuitable capture reagents cause delays in assay development, regardless of whether a hybrid LC–MS assay or an immunoassay is used [30]. The reliability of immunocapture step is a key issue: several case studies were discussed in our bioanalytical group when selected antibodies were binding recombinant calibrators with good affinities, but could not recover any endogenous analytes [Unpublished Data]. For recombinant calibrators, it is recommended that selected calibrators perform similarly to the mammalian cell line produced material [13].

The major benefits of reagent screening and characterization may include but are not limited to the selection of the best binding reagents for a particular protein biomarker before and during assay development and validation. For example, for long-term biomarker projects, reagent screening, characterization and selection activities are frequently an iterative process throughout the different stages of assay development and validation. During this process, knowledge about the biology of an analyte, its binding properties and precise proteoforms that need to be measured is accumulated. As a result, assay performance is continuously improved by reselecting the most suitable critical reagents based on the collected knowledge. In these processes, reagent screening and characterization will be the key to ensure assay's precision, accuracy and reproducibility for robust measurement of protein biomarkers in biological matrices.

Assay calibrators are another group of critical reagents in biomarker bioanalysis, which require screening and characterization. They should represent the endogenous analytes well. Human protein biomarkers can be overexpressed in cell lines *ex vivo*; however, overexpression may cause improper folding of the protein of interest. Moreover, for long-term biomarker projects purified recombinant protein is preferred due to the availability and better stability. In some cases, it is possible to obtain a clinical sample with high levels of the target analyte and dilute it in pooled samples with low concentrations of the target; however, this approach can only be employed in small-scale studies. Since it is very difficult to obtain calibrators that are identical to their endogenous counterparts, their corresponding recombinant proteins that can be obtained from several international reference standard sources are usually used. In general, these proteins may have been screened, characterized and selected by numerous assay users [13]. However,

if a well characterized reference standard exists, it usually means there is a clinical utility for the use of that material, so its use would have to be verified for the intended use of the biomarker assay being developed.

Extensive characterization of assay reagents through biophysical and physicochemical measurements is not only beneficial but also essential for a better understanding of assay readouts and greater confidence in assay data. The design of reagent screening and characterization assays is usually dependent on the intended use of the assay and study purpose. Each reagent should have a certificate of analysis (CoA) or technical datasheet that provides a characterization profile, expiration date and storage temperature. Some of the data can be generated by the reagent manufacturer; however, the desired analytical data are usually incomplete. Therefore, the bioanalytical group would perform the required tests and generate a new certificate of analysis for both commercial and in-house generated reagents.

LBA & hybrid LC–MS biomarker assay reagents: similarities & differences

When developing biomarker assays using a LBA or a hybrid LC–MS approach, there are a number of concerns that need to be addressed including sensitivity, specificity and selectivity. It is well understood that sensitivity, selectivity and specificity of LBAs depend on the critical reagents [15,17,19]. The specificity of LC–MS detection is frequently highlighted in hybrid assays without emphasizing the importance of the quality of the immunocapture reagents. In fact, binding reagents play a key role in both assays; however, hybrid LC–MS assays require only one analyte-specific capture reagent. The requirement of only one binding reagent simplifies reagent generation, screening and the selection process. Generic workflows for protein quantitation by LBAs and hybrid LC–MS assays are presented in Figure 1: (A) LBA approach; (B) Hybrid LC–MS approach employing anti-protein capture; (C) Hybrid LC–MS approach employing anti-peptide capture.

For LBAs, selectivity is generally described as the ability of a binding reagent, which is usually a mAb or pAb, to bind to the measured analyte in the presence of unrelated matrix compounds [31,32]. The concept of specificity in a LBA is usually related to cross-reactivity (binding of non-analyte epitopes in the presence of structurally related molecules) [32,33]. Selectivity/specificity in LC–MS assays used for small molecule quantitation has similar definitions as for LBAs. Stable isotope labeled (SIL) internal standards (IS) that closely represent the endogenous analytes correct for any variations in extraction efficiency (recovery) and normalize matrix effects. Multiple reaction monitoring is commonly used for LC–MS assays in which high specificity is achieved by selecting unique masses (m/z ratios) corresponding to parent and product ions of the analyte. However, when using hybrid LC–MS assays with a protein capture step, we need to keep in mind that spiked IS cannot correct variations in extraction recovery and normalize matrix effects. The first step of a hybrid LC–MS assay is an immunocapture step to enrich the analyte of interest from the matrix, which is prone to extraction recovery variation. In the majority of cases it is not feasible to synthesize a SIL IS, which closely represents an endogenous protein and binds to the binding reagent with the same affinity. Therefore, it is critical to characterize the binding reagents using the same approaches as for LBAs, to ensure that all of the requisite proteoforms are captured. The major similarity between a LBA and a hybrid LC–MS assay is that both assays only measure what the capture reagent binds; therefore, it is critical to screen, characterize and select binding reagents that would capture the analyte of interest with a good specificity and selectivity. Some of the drawbacks of antiprotein antibodies and issues with poor extraction recovery can be avoided if 'stable isotype standards and capture by anti-peptide antibodies' (SISCAPA) approach is employed [34–43]. In the SISCAPA approach, summarized in Figure 1C, stable isotype labeled standards are added to a biological sample and the sample is digested before immunocapture. Sequence-specific anti-peptide antibodies are used to capture nonlabeled (analyte) and labeled (SIL-IS) peptides. The anti-peptide capture is an enrichment method that helps to avoid variations in extraction recovery by means of capturing the same peptide representing all protein forms. The major disadvantage of antipeptide immunocapture is that it will only detect the total analyte concentrations and this approach cannot differentiate protein–protein binding events such as the analyte bound to a target protein versus a free analyte.

As discussed above, one of the most important characteristics of capture reagents is a high binding affinity. The equilibrium constant for dissociation, K_d is calculated from a ratio of the binding kinetic parameters, $K_d = k_d/k_a$. The association constant (k_a) determines how fast the two molecules bind and form a complex. The dissociation constant (k_d) determines how fast the complex dissociates. High affinity (low K_d) is desired for both LBAs and hybrid LC–MS assays. Multiple washing steps are typically used in LBAs and hybrid LC–MS assay procedures. Consequently, the binding reagents with slow dissociation rates are preferred to minimize reagent/analyte dissociation during the washing steps. A fast association rate is usually preferred to reduce the time required for the binding reaction

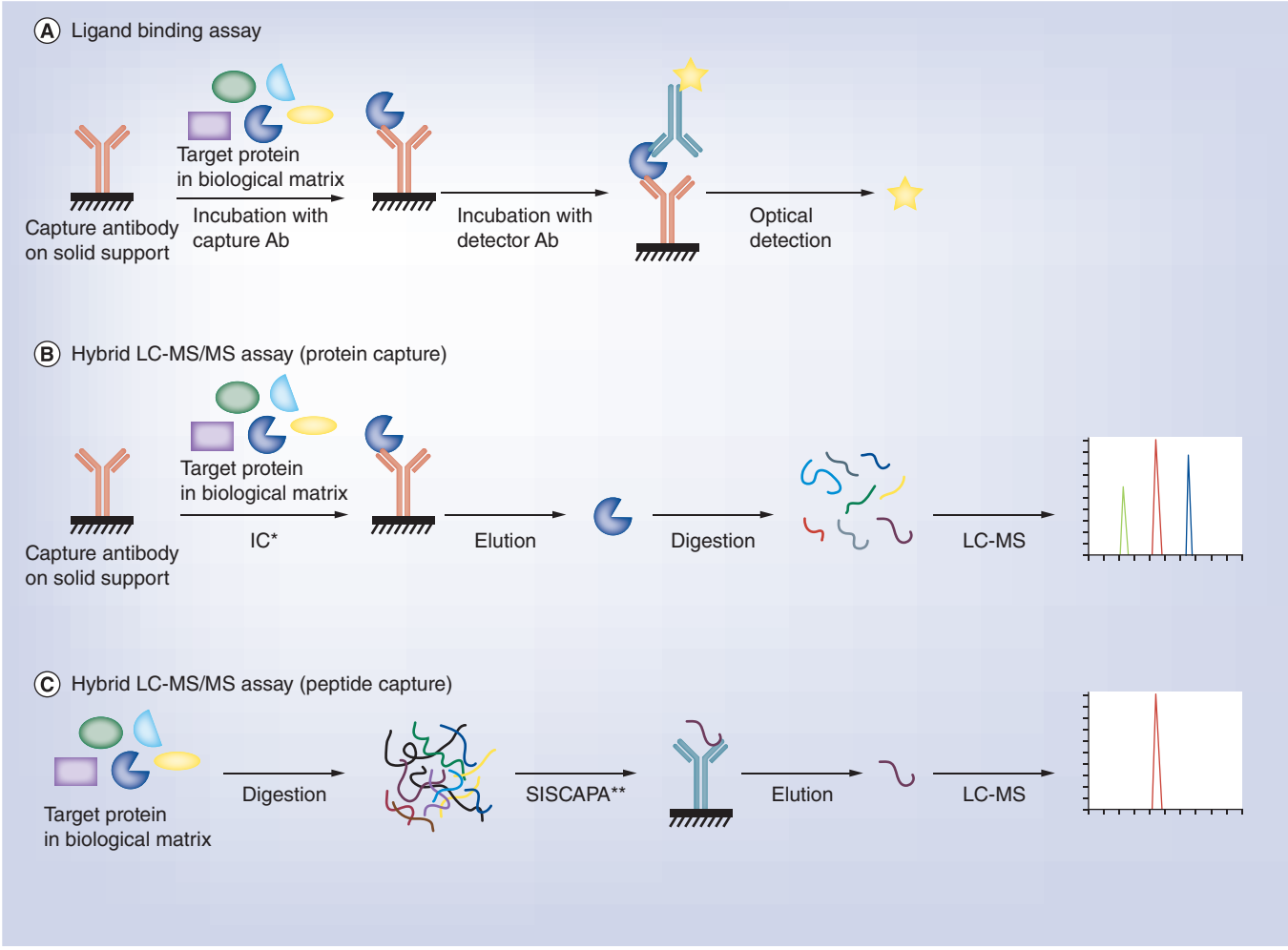

Figure 1. A general workflow. (A) Ligand-binding assay; **(B)** hybrid LC–MS assay using anti-protein capture; **(C)** hybrid LC–MS assay using anti-peptide capture.
Ab: Antibodies; IC: ImmunoCapture; SISCAPA: Stable isotope standards and capture by anti-peptide antibodies.

to reach equilibrium, though longer incubation times might be used if binding reagents with high association constants are not available.

Other physicochemical characteristics that need to be taken into consideration before assay development are the ability of a capture reagent to bind: 1) all of the relevant proteoforms; 2) the complexed analyte, if a total assay is desired (which usually requires a noncompeting antibody); and 3) only the free analyte, if a free assay is required: this assay requires a competing antibody with lower affinity to the target than that of the drug's affinity to avoid dissociation of drug–target complex. For the bioanalysis of complex endogenous analytes that exist in multiple proteoforms, hybrid LC–MS assays might be preferred if all of the relevant proteoforms share a common digestible peptide. In this particular case, anti-peptide capture can be selected to ensure that all protein forms are captured with similar immunocapture recoveries in the assay since the peptide of interest, obtained after digestion, binds to the capture reagent with the same affinity. On the contrary, similar immunocapture recoveries for different proteoforms of the same analyte might not be achievable when the proteins are captured using one antiprotein antibody. With an antipeptide antibody capture followed by LC–MS detection, all proteoforms can be quantified without a binding affinity bias, assuming tryptic digestion efficiency is the same for all forms.

Use of nonspecific and/or uncharacterized antibodies could result in nonreproducible results or lead to unfounded conclusions [44]. If an antibody does not have sufficient specificity for the intended analyte, issues will arise in assay development and validation. Moreover, if antibodies were not properly characterized and the original lot has been exhausted, lot-to-lot reagent comparability cannot be established and the original results may not be reproducible [44].

Many commercial antibodies fail tests for specificity, meaning that they often bind to more than one target or, in the extreme cases, the wrong target [45]. These nonspecific antibodies have been shown to introduce assay bias and skewed assay results [46]. Although it is still critical to select specific capture reagents for both LBA and hybrid LC–MS assays, antibody nonspecificity issues are alleviated by the use of highly selective LC–MS platforms. The specificity of immunocapture reagents is desired for hybrid LC–MS assays since it might increase the signal-to-noise ratio; however, it may or may not be as critical as for LBAs since LC–MS analysis adds another layer of specificity. In cases where new lots of the capture reagent need be qualified, a fit-for-purpose approach may be applied. For example, the acceptance criteria of the reagent may be solely based on its functionality in the assay. One of the big differences between LBA and hybrid LC–MS assays is the requirement of only one antibody for hybrid assays. As a result, hybrid LC–MS assays could save time in reagent generation and selection and are frequently preferred in drug discovery if reagents are limited and timelines are tight. Another difference between the two technologies is criticality of proteases in hybrid LC–MS assays that use enzyme digestion steps. Fortunately, for trypsin, the most popular protease used in LC–MS assays, quality control tests are usually performed by the reagent providers. Although other reagents such as internal standards (which are mostly SIL-peptides) used in LC–MS assays are considered as critical reagents, they are reagents for which stability and storage conditions are normally clearly established and routinely validated [33].

As mentioned previously, well-characterized reference standards are usually not available for endogenous protein bioanalysis; therefore, characterization of the protein calibrator is an extremely important step for both LBAs and hybrid LC–MS assays. It is well known from LBA results that assay calibrators may produce bias [13]. Ideally, the composition of a calibrator should be very close to that of the analyte found in subject specimens [46]. In all assays that include an immunocapture step, structurally heterogeneous analytes should bind capture reagents in a similar fashion as calibrators. It is not critical in relative quantitation for a recombinant calibrator and an endogenous compound to have similar affinities to the binding reagent; nevertheless, if affinities are significantly different, the amounts of the endogenous material can be significantly overestimated or underestimated. It has been reported in literature that using two commercially available soluble PD-1 (sPD-1) calibrators resulted in a 50-fold difference in back-calculated concentration of endogenous sPD-1 [47]. The authors concluded that the monomeric version of sPD-1 recombinant protein most closely resembled the structure of the endogenous material; therefore, the monomer was used as the calibrator material.

For hybrid LC–MS assays, it is also of a great importance to carry out some *in silico* work to make sure that the chosen surrogate peptide is not subjected to post-translational modifications (PTMs) and is the same in all the requisite proteoforms, unless differentiation of several proteoforms is desired. Surrogate peptides generated from calibrators via enzymatic digestion should have the exact same amino acid sequences (or masses) as those generated from the endogenous proteins. Issues could arise in cases where the calibrator peptides do not match the endogenous peptides due to: 1) PTMs; 2) isoforms resulting from splice variants; 3) sequence variants resulting from single nucleotide polymorphisms. Therefore, for hybrid LC–MS assays, carrying out peptide mapping experiments to characterize assay calibrators as well as endogenous proteins might be necessary [2,3]. On the other hand, for LBAs, splice variants, PTMs and single nucleotide polymorphisms can cause serious issues only if capture and detection antibodies do not bind to all of the requisite proteoforms.

For both LBAs and hybrid LC–MS assays, critical reagents should be defined in the assay method and monitored through the life cycle of the assay. Variations in reagent physicochemical properties due to prolonged storage, or change of the reagent lot may or may not impact the assay performance. Nevertheless, the reagent baseline characteristics should always be compared with the characteristics of the stored, or newly generated, reagent in order to ensure fast assay troubleshooting.

Analytical techniques for critical reagent screening & characterization

Characterization of assay antibodies and calibrators may require substantial efforts because it requires development of standardized methods for reagent screening and characterization. In addition, selection of the most suitable reagents before starting assay development might be a time consuming process. However, long term, a more specific and more robust assay could be developed as a result of these screening and characterization efforts.

Some of the physicochemical and biophysical properties of LBA and hybrid LC–MS critical reagents monitored include concentration, purity, aggregation, kinetic binding characteristics and molecular identity of calibrators and monoclonal capture reagents. It is usually too challenging to establish an identity for pAbs. For monoclonal capture reagents that serve as an endless resource, clonality needs to be established as well. Regardless of assumption that

a mAb is monoclonal as its name implies; it has been reported in the literature that more than 30% of individual hybridomas contained one or more additional productive heavy or light chains [48].

Chemical modifications of capture reagents are frequently required before they can be used in the sandwich and hybrid LC–MS assays. In peptide capture methods used for tissue assays, antibodies can be adsorbed or chemically cross-linked to protein A and/or protein G beads to avoid quality control issues related to biotinylation. However, protein A/G beads are not recommended for blood-based matrices because they are rich in endogenous IgGs. Moreover, protein A/G cannot be used with binding reagents lacking the Fc region. Therefore, capture antibodies are frequently biotinylated to enable binding to avidin/streptavidin surfaces, while LBA detector antibodies are conjugated to chemical moieties that allow detection of the sandwich formed. Characterization of the reagents used for biomarker assays goes hand in hand with the assay calibrator selection and are critical steps during assay development.

Reagent screening techniques

Kinetic screening and binding affinity measurement is the first step in selection of binding reagents. Several real-time, label-free (RT-LF) biosensor technologies have been developed with the capability of providing information on the kinetics and affinity of molecular binding interactions [49,50]. The two most popular technologies include, but are not limited to, surface plasmon resonance (SPR) and bilayer interferometry (BLI). SPR technology, such as Biacore systems from GE Healthcare or MASS 1/2 from Sierra Sensors, measures small changes in polarized light refractive index near the sensor surface where the binding interaction occurs, while BLI technology, such as Octet® from Pall Life Sciences, is based on the principles of optical interferometry, namely the interaction of light waves and the changes that occur during the binding reaction. BLI and SPR technologies do not require antibody conjugation for binding detection, they allow for real time monitoring of binding kinetics, and provide a superior way to quickly screen a large number of antibodies and select the best binders with the desired characteristics. SPR and BLI technologies are widely used in drug development, in the diagnostic industry, and in academic research [51–54]. If the assay requires the measurement of a particular form of an analyte (free, total, bound, cleaved, etc.), these technologies can be used to assess the binding epitopes and select the suitable reagents for the analysis.

Likewise, screening for anti-peptide antibodies with the highest affinities can be performed using a high throughput SISCAPA assay format. The technique was reported to allow several hundred supernatants to be screened per week per operator: a throughput that was comparable to the SPR technique [40]. MALDI immunoscreening (MiSCREEN) is another MS technique used for screening of anti-peptide antibodies [55]. MiSCREEN was compared with kinetic analysis of antibody–peptide binding SPR and it was shown that the selected antibodies had high affinities and low dissociation constants. Both mass spectrometric methods enable rapid screening and selection of high affinity anti-peptide antibodies.

Chemical conjugation techniques

As mentioned earlier, chemical conjugations are essential for capture and detection reagents used for sandwich LBA and hybrid LC–MS assays. Modification of capture reagents may result in altered reactivity with the target ligand [15]. Significant changes in binding kinetics and affinity might occur if too many chemical moieties are conjugated to the reagent causing the steric hindrance of binding epitopes. If possible, characterization of the binding reagent conjugates, especially with respect to the binding characteristics needs to be performed. After chemical conjugation, reagents are characterized to determine label incorporation ratio and binding properties. In hybrid LC–MS assays, the capture antibody is either biotinylated or conjugated to magnetic beads with pre-activated surfaces (tosyl- or epoxy-). The main advantage of using beads with activated surfaces is that coating the beads covalently with the capture reagent is more straightforward than with the beads with modified surfaces (carboxyl, amino, hydroxyl or sulfates). These surfaces need to be activated by adding appropriate crosslinkers. After conjugation, biotinylated reagents can be evaluated using SPR or BLI techniques to check their binding kinetics and affinity. Biotin conjugates can also be evaluated for label incorporation ratio using MALDI-TOF or high-resolution mass spectrometry technologies. Currently, binding reagents conjugated to magnetic beads, have to be tested in the bioanalytical assays therefore, it is important to document conjugation procedure carefully to ensure that assay performance is not affected, and performance is bridged when a new lot of beads is prepared. Several methods for thorough characterization of conjugated beads have been described in the literature [56,57], including a simple colorimetric bicinchoninic acid (BCA) assay that can be used to estimate the number of antibodies on the magnetic beads surface. Total antibody concentration in the bead suspension is obtained from subtracting the absorbance of

Table 1. Biophysical and biochemical characterization tests for capture reagents.

Purpose of analysis	Tests for capture reagents	Test for conjugated capture reagents
Concentration	Absorbance at 280 nm, BCA or Bradford assay	Absorbance at 280 nm (if there is no interference from the label), BCA or Bradford assay
Binding kinetics and affinity	SPR or BLI (k_a, k_d, K_d)	SPR or BLI (k_a, k_d, K_d) for soluble conjugates
Purity	SEC, SDS-PAGE, LC–HRMS	SEC, SDS-PAGE, LC–HRMS
Dimerization, multimerization, aggregation	SEC, DLS	SEC, DLS
Molecular weight	SEC, SDS-PAGE, LC–HRMS	SEC, SDS-PAGE, LC–HRMS
Isoelectric point, charge variants (optional)	cIEF methods (protein simple, other platforms, other IEF platforms)	N/A usually
Conjugate incorporation ratio	N/A	For soluble conjugates: MALDI, LC–HRMS For conjugated beads: BCA assay
Stability	Evaluate purity, aggregation, degradation (LC–HRMS), changes in binding properties (SPR or BLI)	Evaluate purity, aggregation, degradation (LC–HRMS), changes in binding properties (SPR or BLI)

BCA: Bicinchoninic acid; BLI: Bilayer interferometry; cIEF: Capillary isoelectric focusing; DLS: Dynamic light scattering; HRMS: High-resolution mass spectrometry; k_a: Association constant; k_d: Dissociation constant; K_d: Equilibrium dissociation constant; SEC: Size exclusion chromatography; SPR: Surface plasmon resonance.

the magnetic beads from that of the magnetic beads containing the antibody. The number of antibodies per bead is calculated by dividing the number of antibodies in the suspension by the number of particles in the suspension (number of beads/volume of bead suspension) [56]. It is important to ensure that the immobilized antibody density on the surface is high enough to capture sufficient target protein, but not too high in order to avoid steric hindrance and interference with binding epitopes [40,50,58–60]. To achieve higher sensitivities, the concentrations of antibodies should be optimized on the magnetic bead bioconjugates and the same should be true for any assays with an immunocapture step. Recommended characterization tests for nonconjugated and conjugated capture reagent are summarized in Table 1.

Reagent characterization techniques

A basic characterization recommended for all biological reagents include concentration, purity, identity, binding activity, dimerization, multimerization and aggregation. Total protein concentration is measured by traditional methods such as the measurement of UV absorbance at 280 nm, bicinchoninic acid (BCA) and Bradford assays. Biological reagent purity and identity are determined using analytical methods, such as size exclusion chromatography (SEC) with UV detection, SDS-PAGE and mass spectrometric methods. SEC provides information about reagent purity and aggregation state, and it is a useful tool to find out if the calibrator material in solution is a monomer, dimer or multimer. Aggregation levels can also be determined by dynamic light scattering technique. SDS-PAGE provides semiquantitative information on purity and some information on identity (approximate molecular weight). MS-based platforms (MALDI, high-resolution mass spectrometry) provide more precise information about the protein identity and primary structure [61,62]. These analytical tools can be used to monitor any physicochemical changes that may occur during the biological production process including, but not limited to, nonenzymatic modifications (oxidation and deamidation), enzymatically produced PTM as well as any changes to the primary amino acid sequences or changes in the charge-state or aggregations. A careful monitoring of these properties could help minimize reagent lot-to-lot variation and provide insight into reagent storage stability and help maintain the reagent quality during its lifecycle and ensure the long-term success of the established assays. Biochemical and biophysical characterization tests used for protein biomarker calibrators are summarized in Table 2.

Conclusion & future perspective

Hybrid LC–MS is rapidly growing as a complementary or alternative technique to conventional LBAs, and there is a clear need to address assay development issues by taking into consideration all the knowledge accumulated by LBA and LC–MS experts. In this article, we define critical reagents, discuss the benefits of reagent screening and characterization, and overview the processes and techniques used for screening, characterization and selection of these reagents as it pertains to hybrid LC/MS biomarker assays. Some additional thoughts and opinions on biological reagent selection for LBA versus hybrid LC–MS assays are also shared. These discussions are intended to simplify some of the areas where no clear guidelines exist. Reagent screening, characterization and selection for bioanalytical assays is a time-consuming process; however, well-planned reagent selection could speed up LBA and hybrid LC–MS assay development, shorten method development times and improve quality of the bioanalytical assays. Moreover,

Table 2. Biophysical and biochemical characterization tests for protein biomarker calibrators.

Purpose of analysis	Tests for calibrator material
Concentration	Absorbance at 280 nm, BCA or Bradford assay
Activity/functionality in the assay	SPR or BLI (k_a, k_d, K_d)
Purity	SEC, SDS-PAGE, LC–HRMS
Dimerization, multimerization, aggregation	SEC, DLS
Molecular weight	SEC, SDS-PAGE, LC–HRMS
Identity (desired)	LC–HRMS for intact analysis, peptide mapping, identification of sequence variants and post-translational modifications
Isoelectric point, charge variants (optional)	cIEF methods (ProteinSimple or other platforms)
Stability	Evaluate purity, aggregation (SEC, DLS), degradation (LC–HRMS), changes in binding properties (SPR or BLI)

BCA: Bicinchoninic Acid; BLI: Bilayer interferometry; cIEF: Capillary isoelectric focusing; DLS: Dynamic light scattering; HRMS: High-resolution mass spectrometry; k_a: Association constant; k_d: Dissociation constant; K_d: Equilibrium dissociation constant; SEC: Size exclusion chromatography; SPR: Surface plasmon resonance.

extensive characterization of biologic reagents through biophysical and physicochemical measurements could add valuable information to the existing knowledge about the measured analyte and provide greater confidence in the data generated from the assays. Assay reagents selected during the early assay development stage may or may not be adequate for later stages of assay development and used throughout the assay life cycle. As the project progresses, additional information may be gained about the analyte indicating the need for improving the quality of the assay reagents. Therefore, for many projects, critical reagent selection activities are an iterative process throughout the life cycle of the assay and the drug-development process.

Executive summary

Definition of critical reagents & rationale for reagent screening & characterization

- 'Critical reagents' are defined as essential components in biomarker assays whose characteristics could impact assay performance. These reagents should include both analyte binding reagents and assay calibrators.
- Binding reagents should be carefully screened and characterized to ensure optimal assay performance.
- Well-characterized reference standards are usually not available for endogenous protein bioanalysis, unless clinically validated assays are available on the market.
- Biomarker assay calibrators are not identical to the endogenous analytes; therefore, calibrator materials should be carefully screened, selected and characterized.

Similarities & differences between ligand-binding assays & hybrid LC–MS assay reagents

- Binding reagent characteristics are equally important for both ligand-binding assays (LBAs) and hybrid LC–MS assays.
- Extensive characterization of biomarker calibrators as well as endogenous analytes might be needed. *In silico* work as well as peptide mapping experiments has been suggested for hybrid LC–MS analysis of challenging analytes.
- Splice variants, post-translational modifications and single nucleotide polymorphisms can be a big challenge in biomarker hybrid LC–MS assay development. On the other hand, for LBAs, splice variants, post-translational modifications and single nucleotide polymorphisms cause serious issues only if the requisite proteoforms do not bind to the capture and detection reagents.

Value of analytical techniques for reagent screening & characterization

- Physicochemical and biophysical properties of LBA and hybrid LC–MS critical reagents monitored include concentration, purity, aggregation, kinetic binding characteristics and molecular identity of calibrators and monoclonal binding reagents.
- Broad characterization of biologic reagents through biophysical and physicochemical measurements could provide a greater confidence in assay data.

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Author contributions

R Santockyte drafted this manuscript. N Zheng and J Zeng contributed to the conception of this work and revision for critical content.

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Editorial

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Bioanalysis

Critical reagents: evolution of WRIB recommendations on critical reagents

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"Poorly characterized or monitored reagents can lead to poor assay performance, extensive time spent on troubleshooting, wasted resources, delayed timelines. Ultimately this can adversely impact drug development by leading to erroneous pharmacokinetics (PK), immunogenicity or biomarker data without appropriate controls and monitoring in place."

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Keywords: antibody reagents • commercial reagent characterization • critical reagents • immunogenicity and biomarker assays • reagents for PK • selection and characterization of assay reagents

Critical reagents are a key component in most assays utilized during drug development to generate essential data. For the purpose of this article, critical reagents are defined as 'key antibody reagents that impact key parameters such as specificity and sensitivity of the assays'. Antibodies serve as invaluable tools in both academic research and pharmaceutical R&D. They aid in characterizing protein expression, localization, quantitation in addition to serving as tools in measuring genomic, cellular biomarkers and in some cases for imaging biomarkers. BCC research, an organization that reviews antibodies market estimated that worldwide research spending alone is approximately US$2.5 billion on reagent antibodies, and this is an underestimate as it does not include pharmaceutical R&D spending [1]. Bradbury & Pluckthun along with 110 cosignatories highlight the importance of antibody generation, selection and characterization and that millions of dollars have been wasted on irreproducible research using ill-characterized antibodies [2]. Furthermore, a systematic analysis called for the attention to antibody selection and validation due to a high number of irreproducible research studies in 2020 [3,4].

This article primarily focuses on the use of antibodies in drug development setting as critical reagents in ligand binding (LBA), flow cytometry, and in some cases, LC–MS-based assays to measure drug concentration, immunogenicity and biomarkers. Over the last several years (2015–2019), the WRIB conference has addressed several aspects of critical reagents including: generation and characterization of reagents; application of fit-for-purpose approach to their use and characterization; long-term maintenance and impact; and regulatory guidance. Please refer to the WRIB white papers for more detailed recommendations [5–7]. For the purpose of this article, critical reagents are those used to capture and detect the analyte of interest. Some commonly used critical reagents include antibodies to capture a biotherapeutic, a biomarker of interest whether they are unlabeled or labeled with tags such as biotin.

What is the fuss about critical reagents? The answer – your assay is as good as your critical reagents are. Critical reagents fundamentally impact assay parameters, and therefore, impact the assay performance. At the assay level, critical reagents can impact specificity, selectivity and sensitivity of the assay. At the reagent level, the concentration, purity, stability and other physical and functional features can impact assay performance.

In a pharmaceutical drug development setting, there is relatively more awareness and efforts that go into selecting and characterizing critical reagents for development, and validation of bioanalytical, immunogenicity and biomarker assays. Poorly characterized or monitored reagents can lead to poor assay performance, extensive time spent on troubleshooting, wasted resources, delayed timelines. Ultimately this can adversely impact drug development by leading to erroneous pharmacokinetics (PK), immunogenicity or biomarker data without appropriate controls and

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monitoring in place. Regulatory guidelines related to bioanalytical, immunogenicity and biomarker assays only included high level discussion of critical reagents instead of detailed specifications [8–12].

While it is not necessary to characterize a key reagent to the same extent as a drug product, it is important to understand the impact of the reagent on assay performance and then implement the appropriate characterization and maintenance as fit-for-purpose. For this reason, numerous discussions took place and recommendations regarding critical reagents were developed at WRIB [7].

Critical reagent antibodies in drug development setting are used in a variety of assays including: PK assays using LBA format; anti-drug antibody (ADA) assays; cell-based or noncell-based Nab assays; flow cytometry-based assays; immunoaffinity (IA)-LC–MS/MS assays; the extensive array of LBA-based and non-LBA-based biomarker assays including genomic assays; and tissue-based assays such as immunohistochemistry (IHC). Labeled antibodies are also used (e.g., biotinylated or fluorescently labeled antibodies) in addition to naked antibodies (which require additional characterization to ensure batch-to-batch consistency) to ensure consistent performance in the assay.

In addition to reagent antibodies, the reference standards used in bioanalytical assays and calibration standards used in protein biomarker assays should be considered as critical reagents that require appropriate characterization and monitoring during clinical development of a drug program. While drug substances undergo extensive analytical characterization, use of a reference standard tends to be less of an issue with assays to measure drug concentration (PK assays); however, researchers need to be aware of its use and impact on assay performance when there are lot changes. Protein calibrators and their characterization in biomarker assays is a crucial element that may have a potentially significant impact on assay performance. This topic is well studied with illustrations using case studies by Cowan *et al.* [13]. Another unique reagent that can significantly impact assay performance of immunogenicity assays is the negative control matrix because of its relationship to the study population impacts the cut point of the assay [11].

A robust critical reagent program includes:

- Generation;
- Characterization;
- Qualification;
- Stability assessment;
- Long-term maintenance.

Use of monoclonal or polyclonal antibodies generated using newer technologies should be determined by the researcher to ensure consistent supply for the long term with intended use in mind. It is recommended to screen and select the antibodies with appropriate specificity, affinity and functional activity in the reagent generation phase as early as possible.

There are a variety of characterization parameters that may be applicable to critical antibody reagents based on the assay and intended use that include, identity, concentration, purity, binding and/or functional activity, labeling efficiency, freeze–thaw stability etc. During the discussion at the WRIB 2019 meeting, the expert team arrived at a recommendation for critical reagent characterization to minimally include: Concentration, Purity and Functionality by an orthogonal method (abbreviated as CPF) [7].

In addition to these three primary factors, additional critical quality attributes may be assigned, for example, conjugation ratio for labeling and aggregate assessments for aggregation prone-labeled reagents. Design of experiments or quality by design approaches may be utilized as needed to establish meaningful ranges for key quality attributes. Documentation of key quality attributes in the form of certificate of analysis or technical data sheet is recommended. A comprehensive and robust program to handle critical reagents should include standard operating procedures (SOPs) and instructions for preparation, qualification, labeling and storage.

Commercial reagents are often used in biomarker assays. These reagents are typically specified for research-use only. When using these reagents in drug development, it is important to understand the reagent quality. Additional in-house characterization may be needed to ensure consistent, sustained performance across multiple clinical trials [13]. When bridging different lots of commercial kits/reagents extra attention should be paid to ensure that the assay is performing consistently and that the data across studies can be bridged/pooled and so on. The Global Bioanalytical Consortium Group developed a robust reagent management process for drug development setting [14] and principles described here could also be helpful in developing such a fit-for-purpose reagent management program. An electronic inventory system may be helpful tool for such a reagent management system.

The complexities related to reagents may increase significantly when utilizing multiplex assays that require multiple reagents. In addition, as we look to the future of drug development, the complexity of classes of therapeutics is ever increasing, for example: development of new modalities such as bi- and tri-specific antibodies, multidomain therapeutics, gene and cell therapies including viral vectors and chimeric antigen receptor containing-Tcells (CAR-Ts), nanoparticles. In addition, the nature of biomarker assays is increasingly complex, for example: free and total ligand and drug measurements. Implementation of such assays will add even more demand on characterization of critical reagent antibodies used in these assays. The datasets generated from these assays ultimately inform our understanding of biological processes, and the impact of specific therapeutic on patient populations under evaluation. Therefore, it is crucial that the scientific community to truly appreciate the importance of characterizing critical reagents used in assays that generate the data we rely on.

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Critical reagents: an interview with Ashley Brant

Ashley works closely to drive new technology and overall strategy for growth providing biologics-focused bioanalytical solutions for clients. She serves as a single point of contact for client development programs with a focus on expanding large molecule capabilities as well as provides strategic scientific oversight of study designs. She is the scientific and technical advisor for clients and internal teams for development, validation, and application of assays to ensure compliance with regulatory requirements. During Ashley's distinguished scientific career, she has held positions of increasing responsibility within the biopharmaceutical industry. After receiving her MS from the University of Michigan, Ashley became a bioanalytical consultant assisting clients with project set-up and clinical study management and design. Subsequently a Principle Investigator and Associate Scientific Director with small to mid-size bioanalytical CRO's where she managed the ligand binding method development and validation workflows. An accomplished leader in the field of bioanalysis and large molecule drug development, he has been involved in various industry organizations, including AAPS and WRIB and is an invited speaker at multiple industry events.

Ashley Brant
Eurofins Bioanalytical Services (MO, USA)

Q) Can you explain why critical reagents are important in relation to assay performance?

In a ligand-binding assay, the critical reagents are really the backbone of the assay. If you were talking about a small molecule, you would think about the platform that you are working on (i.e the instrumentation used). The instrumentation doesn't make the big difference in how we will detect our analyte but our critical reagents do. That is how we select our analyte of interest and how we detect it.

Q) What are the challenges associated with changing reagents?

There are a lot of different challenges that come along with changing critical reagents. One is that if we have created that critical reagent differently then it essentially is a different molecule. Anything in the primary, secondary, tertiary structure of that reagent will impact how it interacts with the analyte of interest.

These large molecules have three dimensional interactions with each part interacting in a specific way. The better you get that lock and key interaction, the more specificity and selectivity we get. Therefore, it is critical that when you change a lot, even if it was slightly purified differently, then you are going to get something different as you will have different amounts of that critical reagent that is interacting with your analyte and changing the outcome.

Q) At Eurofins you utilize a Test Method Summary Sheet, could you explain how this recording system works?

Our opinion at Eurofins really comes back to the Eurofins multipronged approach throughout the industry (because we touch so many different industries whether it's food or pharmaceutical related, we take to monitoring the assay performance across the board).

Critical reagents: an interview with Ashley Brant

We continually analyze the performance of the assay through time. So regardless of what study it is being run on, we monitor it by method. If that method is performing on one study or we are doing a reagent qualification, all of that goes into one giant spreadsheet so that we can do statistics and then monitor how the assay actually performs in real-time, in real-life. That way we can see how each of those critical reagents is impacting that or even sometimes the non-critical reagents that we assume were 'non' but then ended up being pivotal to the performance of the assay. Then we can see that in real-time, make adjustments, and therefore, make sure that the assay is performing as consistently as possible and provide the most consistent data.

Q) How do you evaluate the expiry date or a re-test date of a critical reagent?

For a critical reagent expiration or re-test date that is assigned, either by a manufacturer, or something that was assigned by SOP, we aliquot it out and store it at minus 80 (not at refrigerated or room temperature) so that stability may be different than what they were assuming those conditions were. Then additionally, so many of our reagents we have conjugated and assigned that, so we like to assign a re-test date where we are continuing to monitor that performance. We then look at those statistics every three or six months and then make sure that we are within a performance and we are not seeing a downward or upward trend.

Q) Lastly, how do you see the management of critical reagents evolving over the next 5 years?

I think that the continuous management of critical reagents will be critical, I think it needs to happen across the board, as well as all the way down to the manufacturing side. I know that there is a big push for our CRO industry to currently update the COFAs. There was a White Paper that was put out recently, and I think that will continue to be evident as we also push that out into critical reagents. As the popularity of biologics increases, and now as we expand into gene and cell therapy, we will see the emphasis of watching the critical reagent life cycle rather than just seeing it in a snapshot and then swapping it out.

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Bioanalysis

EBF recommendation on practical management of critical reagents for PK ligand-binding assays

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Critical reagents play a crucial role in ligand-binding assays; the robustness and reliability of an assay is defined by the quality and long-term availability of these reagents. However, neither regulatory guidelines nor relevant scientific papers provide clear directions for set-up, life cycle management and, more importantly, the acceptance criteria required for the testing of the critical reagents for pharmacokinetic, biomarker and immunogenicity assays. The ambiguity from current guidelines can be a challenge for the bioanalytical community. Members of the European Bioanalysis Forum community undertook a more pragmatic approach on how to assess the impact of critical reagents. In this paper, a review and corresponding gap analysis of the current guidelines and relevant papers will be provided as well as decision trees proposed for lot-to-lot changes of critical reagents for pharmacokinetic assays.

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Keywords: antibodies • critical reagents • ligand-binding assays • major change • minor change • PK assay

Introduction & scope

Critical reagents play an important, but often overlooked role, in ligand-binding assays (LBAs). Unlike chromatographic methods, immunoassays rely on binding properties of the reagents to quantify the analyte of interest. The reagents used for LBAs are often produced using biological systems and may be prone to production variability. The reagents can be proteins, antibodies and conjugated antibodies. The unique binding characteristics define the capability of the assay to reproducibly and consistently quantify the analyte. Therefore, it is important to understand whether any change in the reagents significantly impacts the performance of the assay (functional assay) and as a result the reagent is deemed as critical.

Current the European Medicines Agency (EMA), US FDA and the Japanese Ministry of Health, Labour and Welfare (MHLW) guidelines [1–3] do not provide clear strategies for assessing critical reagents for LBAs. The guidelines simply state that "critical reagents have direct impact on the results of the assay and therefore their quality must be assured" [1], "The sponsor should appropriately characterize and document (i.e., determine the identity, purity and stability) all reference standards and the critical reagents" [2] and "the quality of critical reagent should be appropriately maintained throughout the period of use in analytical method validation and study sample analysis" [3]. As the process behind these statements is not defined in regulatory guidance, it remains a challenge for the bioanalytical community to agree on the best practices [4–11] for defining, qualifying and monitoring critical reagents.

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The Global Bioanalysis Consortium (GBC) released a recommendation on the life cycle management of critical reagents [10]. To further expand on this topic, the European Bioanalysis Forum (EBF) herein provides a gap analysis of current publications and in addition provides a practical recommendation on the assessment of critical reagents in LBAs used in support of pharmacokinetic (PK) studies. However, some scientific assessments proposed in the manuscript may be project specific and should not be required for all projects. Immunogenicity assay reagents (LBA or cell based), and biomarker assay reagents are out of scope for this recommendation.

Review of the current recommendations & definitions

There is agreement in the literature for the need to verify assay performance when critical reagents are changed and that the efforts to qualify reagents need to be documented. This documentation includes, but is not limited to, information regarding reagent identity, concentration, storage conditions and stability testing. A comprehensive list with recommendations regarding documentation is provided by the aforementioned GBC paper [10].

Current guidelines and scientific papers listed in Table 1 offer a valuable insight into the bioanalytical community perspective on critical reagents, but there are clear gaps that would benefit from more in-depth discussion. The major gaps are as follows:

- No formal definition of a critical reagent is provided. Most published papers offer high level definitions.
- No practical recommendations on reagent monitoring processes. Many papers list reagent monitoring and trend analysis as an effective life cycle management tool, however the actual process and execution is not presented in detail.
- Limited recommendations on life cycle management of reagent stability and expiry/re-testing, particularly in relation to commercial expiry dates versus in-house established dates. Although this area is partially addressed in a few publications [5,10], expiry and re-test date management is not clearly outlined.
- No practical recommendation on process control or execution for reagent lot-to-lot changes. This is a critical aspect of reagent handling and companies have different approaches. Specifically details on the design and evaluation of change control experiments would be of value.

Identifying critical & noncritical reagents

LBA involves a choice of several platforms, detection systems, single or multiplex and/or plate or beads formats. Each of these approaches use reagents that require careful monitoring.

Assay reagents are considered critical if their quality, nature, structure or specificity has a direct impact on the assay performance. These reagents define the assay readout and are most often, but not exclusively, capture/detection antibodies or peptides/proteins. In contrast, noncritical reagents can easily be replaced in an LBA without the need for lot-to-lot assessment. Some examples of critical and non-critical reagents are given in Table 2. In summary, the EBF defines critical reagents as reagents that require specific testing of newly introduced lots, where a loss of performance can lead to suboptimal results in routine bioanalysis and assay validation. In contrast, noncritical reagents changes can be monitored within study, and therefore no special testing runs are required.

In principle there are two approaches for obtaining critical reagents. The first is to obtain a large batch of the critical reagent to avoid any changes during a drug development program or a study that requires analysis over a long period of time. This approach will require the determination of a re-test date or monitoring of long-term stability of the critical reagents. The second approach is to have smaller batches of critical reagents, avoiding the requirement of long-term stability. However, this approach may inevitably lead to (multiple) lot-to-lot changes to confirm performance of the new lot. The approach chosen depends on the strategy and capability of the company. The careful identification of critical reagents must be performed in balance with scientific considerations taking the availability of the reagents into consideration.

It should be acknowledged that not all reagents can be tested for criticality during method development and validation. This can also be performed during sample analysis or transfer of the method to another facility. Reagents which are non-critical in some (if not most) LBAs, can indeed prove to be critical in other cases. This is often identified when the assay is transferred between laboratories and different providers are used to supply what are previously considered as noncritical reagents. In cases where the recipient laboratory employs reagents from other sources than the originator laboratory, method transfer between laboratories offers the opportunity to assess the impact that individual reagents may have on assay performance. If an impact on assay performance is discovered, alternative reagent lot(s) or preferred suppliers used in the recipient laboratory should be tested in at least one

Table 1. Overview of current guidelines and white papers.

Study (year)	Definition of critical reagents	Process recommendation for lot-to-lot changes	Recommendation for reagent stability	Ref.
EMA (2012)	Binding proteins Conjugates Antibodies Aptamers	Verification of method performance	Storage conditions and maintenance should be documented	[1]
FDA (2018)	Reference standards Antibodies Labeled analytes Matrices	Evaluate binding and re-optimize assays Verify performance with a standard curve and QCs Evaluate cross-reactivities	Storage conditions should be defined	[2]
MHLW (2014)	Conjugates Antibodies Components with performance impact	Perform partial validation when minor changes are made to an analytical method that has already been fully validated	Storage conditions should be used that ensure consistent quality	[3]
Nowatzke and Woolf (2007)	Conjugates Antibodies Enzymatic moieties Matrix	Verification of method performance Similarity assessment using a combination of calibrators and QCs	Not discussed	[4]
Rup and O'Hara (2007)	Conjugates Antibodies Cell lines	Verification of method performance Method re-optimization Test old and new lots in parallel Trend analysis	Default expiry dates for commercial reagents Trend analysis may extend reagent expiration date	[5]
Viswanathan *et al.* (2007)	Conjugates Antibodies	Not discussed	Storage conditions and maintenance should be documented Default expiry dates for commercial reagents Stability data required for newly established storage conditions	[6]
Staack *et al.* (2011)	Conjugates Antibodies Peptides Receptors or ligands (including fragments)	Not discussed	Monitor aggregation of reagents Characterize with functional and biophysical methods	[7]
O'Hara *et al.* (2012)	Components with performance impact Conjugates Antibodies Complex drugs/biologics Solid supports Matrix	Depends on extent of change and expected impact Verification of method performance Method acceptance criteria established *a priori*	Storage conditions and maintenance should be documented Trend analysis may extend reagent expiry date	[8]
O'Hara and Theobald (2013)	Components with performance impact	Verification of method performance Method acceptance criteria established *a priori*	Expiry dates should be based on formulation, storage conditions and available biophysical and functional data	[9]
King *et al.* (2014)	Drugs/biologics Conjugates Antibodies Proteins Peptides Matrix	Verification of method performance Similarity assessment i.e., test old and new lots in parallel (if available) Minor lot changes require 1 run with minimum 3 control levels (biomarker, PK, immunogenicity) Major lot changes require 3 runs with minimum 3 control levels (biomarker, PK, immunogenicity)	Default expiry dates for commercial reagents. If such information does not exist, dates can be assigned based on experience with similar classes of reagents Stability data required for newly established storage conditions Trend analysis may extend reagent expiry date	[10]
Bradford (2015)	Reagents that are specific to the analyte Drugs/biologics Conjugates Antibodies Matrix Receptors or ligands	Verification of method performance Similarity assessment, i.e., test old and new lots in parallel (if available)	Investigate conjugate stability Trend analysis	[11]

bridging run, head-to-head with the original assay reagents, to ensure the new lot(s) meet *a priori* acceptance criteria.

The most practical strategy for identifying critical reagents is to run direct comparisons of alternative lots of reagent against an original lot (head-to-head comparison). This is ideally done at the end of method development when the majority of performance characteristics have been defined and the assay format is fixed. Nevertheless, even when assays are developed from scratch, this may not be feasible due to limited reagent availability, or it is not possible to secure large quantities for a longer period. In this case, it is recommended that sufficient amounts of the original reagent are retained for bridging experiments between different reagent lots.

Table 2. A European Bioanalysis Forum overview of critical and noncritical reagents of ligand-binding assays.

Reagent	Considered as critical	Examples
Antibodies (capture/detection reagents)	Always	Monoclonal or polyclonal antibody used as capture reagent in a PK assay
Commercial or in-house conjugated reagents, including in-house conjugated beads	Always	In-house labeled reagents, e.g., Biotin/Sulfo-TAG labeled capture and detection antibodies
Commercial kits	Always in case of change of critical reagent lot	Change of capture reagents in the kit lot
Solid phase	Potentially	Plates (ELISA, MSD, Aushon etc.), paramagnetic beads, Bioaffy CDs (Gyrolab) etc.
Biological matrix	Potentially	If endogenous counterpart is present
Standard reagents	Rarely	Blocking reagents (BSA, Superblock, etc.), enzymatic substrates for detection (TMB, etc.), buffer components (PBS, Tween-20 etc.)

Solid-phase reagents such as plates or beads from vendors with a good history of supply, and lot-to-lot reproducibility, may be considered noncritical. Evaluation of multiple lot numbers during method development (assay prevalidation) may be advisable. Further assessment during validation may be required if the solid phase is considered variable, before proceeding to sample analysis. Alternatively, if variability is found during method development, it is advisable to source a single lot number of solid phase reagents for completion of the validation and sample analysis. However, this approach is not always possible and poses some restrictions on future analysis by the same method. Low binding and uncoated plates, such as those used for transfer to assay plates, electrochemiluminescence (MSD) or colorimetric assays (like ELISA) can be considered noncritical. Coated plates, such as streptavidin-coated MSD or ELISA plates, Ni2+ NTA, and Gyrolab CDs may be considered critical reagents.

For commercial kits particular consideration should be paid when using these for the purpose of a pharmacokinetics (PK) assay. If possible, evaluate the supplier history by considering their reliability to supply the kit for the duration of the study, and the frequency of kit changes made historically. It is recommended to secure sufficient commercial kits of the same lot number for the duration of the validation and for at least the sample analysis for the first study. If this is not possible, multiple lots should ideally be evaluated during method development, although this may not always be possible since kits are often produced in 'on-demand' batches. Therefore, the impact of lot changes should be closely monitored.

Most biological matrices may be considered noncritical for PK. However, in some applications, when an endogenous counterpart of the drug is present in the matrix, it may be necessary to prescreen lots or individual sources of matrix especially where endogenous components may cause assay problems such as high background or baseline levels.

Lot-to-lot changes of critical reagent: recommendations & considerations: a pragmatic approach

The above section has described a practical strategy for defining which reagents can be considered critical and noncritical during assay development and validation. However, challenges also exist when changing a critical reagent lot mid-study or when re-establishing an assay after a considerable period of time. The purpose of lot-to-lot changes testing is to verify that assay performance is not altered by switching to the new reagent lot, compared with the original reagent. A new critical reagent lot needs to be tested prior to its use in a study. The practices of accepting (or not accepting) a new critical reagent lot varies within the bioanalytical community.

It is the recommendation of the EBF that all critical reagents are tested in the PK assay using the assay run acceptance criteria and compared with expected assay performance, thus keeping unexpected results to a minimum. This EBF recommendation is also in line with previous recommendations provided by King (2014) [10], O´Hara (2012) [8]. The EBF suggests following the GBC recommendation on critical reagents [10] regarding the definition of minor and major critical reagents: "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." Conversely a major change requires "the most extensive reagent qualification level and is directed primarily towards the replacement of a critical reagent where the original source of a reagent is no longer available." The GBC advocates three qualification runs for major changes and one run for minor changes [10]. Furthermore, this paper recommends acceptable re-test dates based on the reagent type and the storage conditions.

In addition to the GBC recommendation, the EBF recommendations for both major and minor changes are to review the Certificate of Analysis (CoA)/technical datasheet and assess whether there is a change in lot, clone or

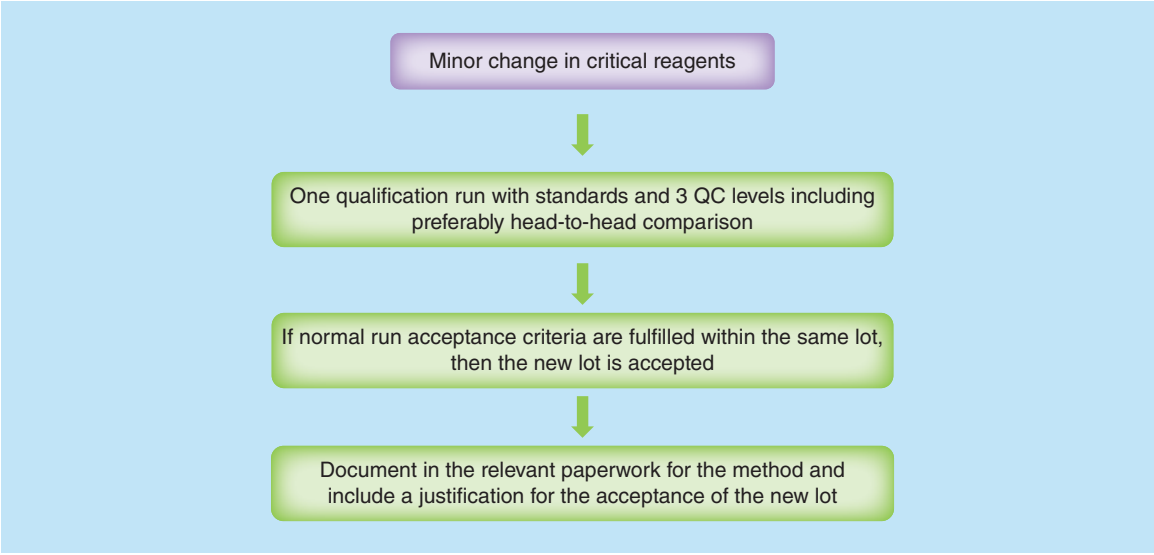

Figure 1. European Bioanalysis Forum recommendation for minor changes.

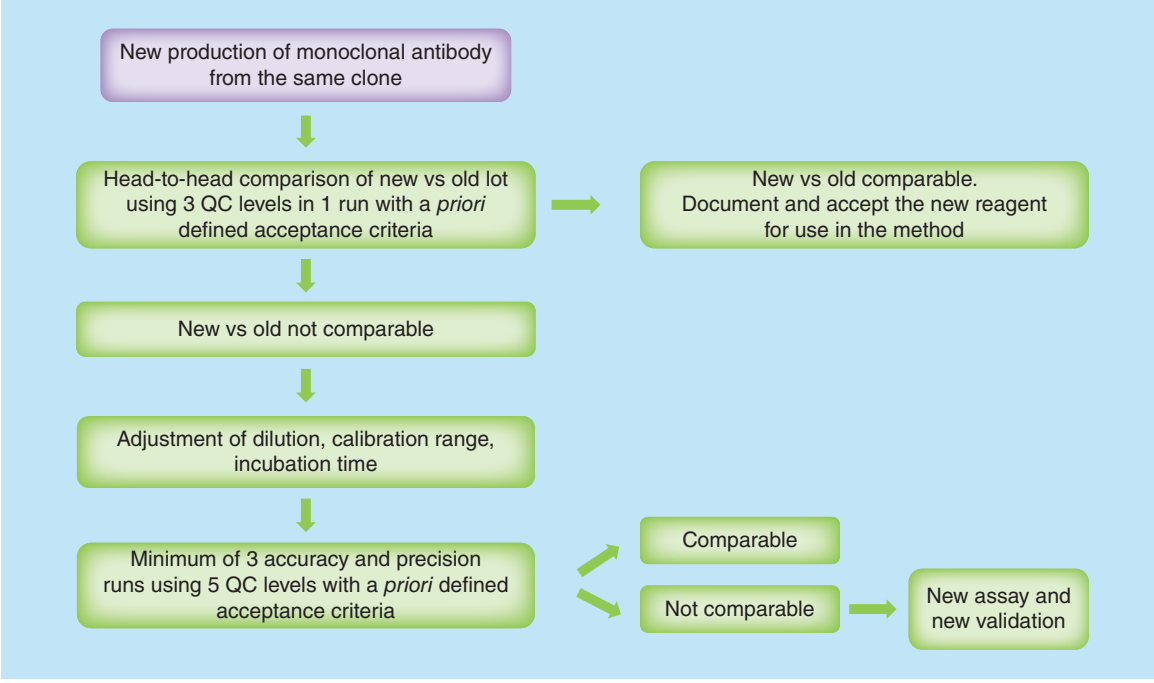

Figure 2. European Bioanalysis Forum recommendation for a new production of monoclonal antibody using the same clone.

concentration. In such cases it is recommended to perform a head-to-head comparison between the new and old lot in the PK assay. The evaluation may result in no further testing is required to an optimization or re-development of the assay. However, it is important to understand what impact the change in critical reagent may have on the assay and to evaluate which tests should be included in order to implement the new lot.

For evaluation of possible scenarios of critical reagent changes, EBF recommends the following decision trees (Figures 1–4), however it is important to keep in mind that these are the minimum requirements and it may be required to include additional experiments, based on scientific rationale or company policy.

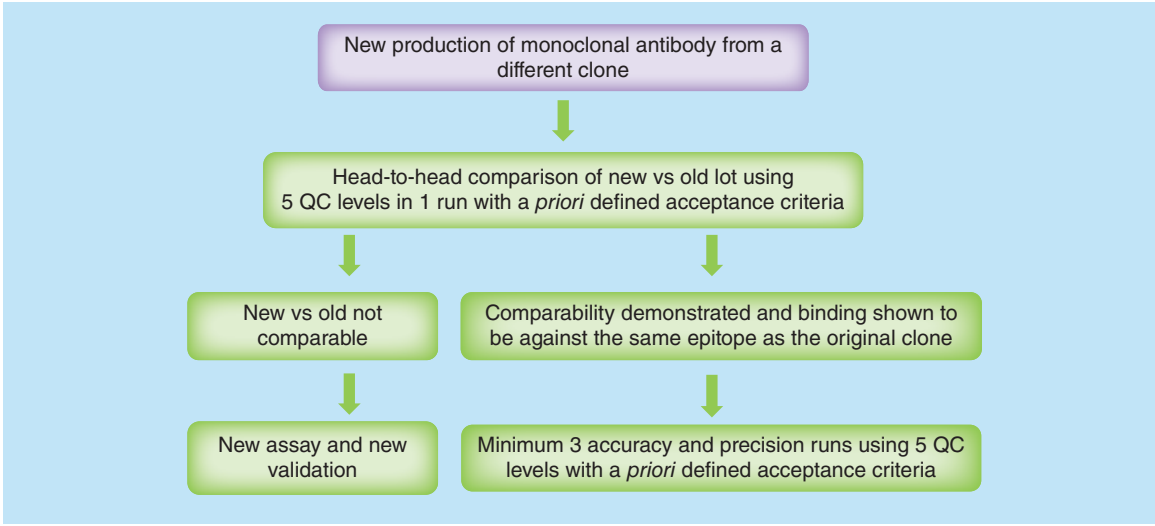

Figure 3. European Bioanalysis Forum recommendation for a new production of monoclonal antibody using a different clone

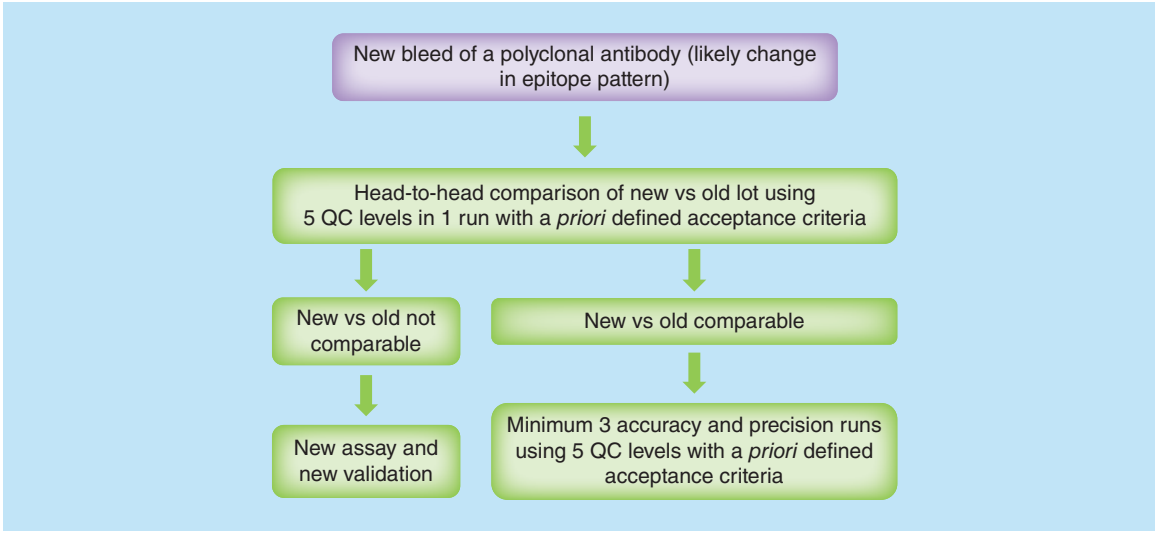

Figure 4. European Bioanalysis Forum recommendation for new production of a polyclonal antibody (new bleed from same or new animal).

Preferably, qualification and evaluation of a reagent change are performed well in advance of the introduction of the new reagent in bioanalysis. There are several options when replacing a critical reagent depending on the availability and stability:

1) Both the old and new lots of the critical reagent are available and are within re-test date;
2) Both the old and new lots of the critical reagent are available but the old lot is outside the re-test date;
3) Only the new lot of critical reagent is available (old lot is depleted).

Scenario 1 is always preferable as it offers a way to bridge the reagents. Hence it is recommended to retain some of the old material to allow a head-to-head experiment prior to a critical reagent change. Having the old lot as comparator in the bridging run helps to assess the critical impact of the change and serves as an internal assay control even if the old lot is used outside the re-test date as mentioned in scenario 2. However, this should be taken into account during the evaluation of results from the head-to-head comparison in the PK assay. Scenario 3 is not optimal but nevertheless quite common if reagent consumption is not effectively monitored or if the reagent is not

available at a certain time. For this reason, bioanalytical laboratories should consider having active reagent logging systems plus effective planning and communication between responsible parties to mitigate the risk of running out of a comparator reagent prior to qualification of new material.

The timing of the qualification of a new lot is also dependent on the reliability of the reagent supplier and communication with these vendors; suppliers might not always inform customers regarding a change in the production of the critical reagent and, as such, production alterations might have a significant impact on the assay performance. This should be kept in mind even when ordering the same catalog number but different lots of the critical reagent.

In summary, the bioanalyst should decide upfront, if the introduction of a new lot of (critical) reagent is a minor or a major change and what impact the possible changes may have on the assay functionality. Thereafter, performing a functional test in the PK assay by comparing the new reagent against the old reagent and then evaluating the results is needed. Based on this evaluation, a justification to either perform additional qualification/validation experiments, or to proceed with bioanalysis is required. The outcome of these experiments, in other words, the results, the evaluation and conclusions/justifications should be documented.

Stability

Reagent stability and expiry/re-test date management can be a challenge. Reagent stability, characterization and life cycle management is discussed in Rup (2007), O'Hara (2012) and King (2014) [5,8,10]. Many companies use commercial reagents in their assays. These usually come with vendor-specified expiry dates. Rarely can the vendor supply details on stability assessments and not uncommonly the expiry date is specified as, for example, 12 months from delivery date. There is an overall consensus in the industry that assigning a re-test date over an expiry date is more practical. EBF recommends that stability testing should be based on functional performance in the PK assay by confirming that the *a priori* assay acceptance criteria are still met. Re-test dates should be set from experience or procedures and generic stability tables and used for recommendation of suggested re-test periods, rather than strictly applying the expiry dates provided by the supplier.

It is recommended to monitor relevant assay data such as quality control (QC) level data or instrument responses, which yields valuable supportive information to monitor stability of the critical reagent, but is also useful to evaluate lot-to-lot testing of critical reagents during the life cycle of an assay. Monitoring of data throughout assay use/during bioanalysis offer a way to early identify issues (e.g., assay drift) and thereby provide an opportunity to take corrective action.

Documentation

Documentation is an important, but often neglected, part of reagent handling. Survey results from the GBC identified large inconsistencies in reagent-related documentation practices between companies [10]. A likely reason for this is that current EMA, FDA and MHLW guidelines do not provide a clear direction. An outline of best practices for documenting reagents can be found in King (2014) [10].

It is recommended to have a formal procedure for reagent handling, qualification and documentation practices. Such information will help support life cycle management and lot-change decisions. The testing and evaluation of lot-to-lot changes and reagent stability testing should be documented in the relevant method documentation.

Critical reagent information can be captured in CoA, a technical datasheet or in other relevant documentation such as a reagent book.

The EBF recommendation for the minimum information for a critical reagent is as follows:

- Name of reagent;
- Lot number;
- Source (e.g., cell line, method of expression), if applicable;
- Origin source for modified reagents (e.g., labeled reagents);
- Catalog number (for commercial reagents);
- Concentration, if applicable;
- Re-test date/expiry date;
- Manufacture date;
- Storage condition recommendation.

Conclusion & recommendation

Definition, qualification and monitoring of critical reagents play an important, but often overlooked, role in LBA. For LBA, critical reagents are (often biological) reagents/molecules that are involved in binding reactions that can alter the outcome of the assay. Consequently, these reagents influence the validity of an LBA assay and of the resulting data. However, to date there is no clear definition for critical reagents in regulatory guidelines or how to manage changes.

Whilst some scientific assessments proposed in this manuscript may be project specific and should not be required for all projects, the EBF recommendations for critical reagents in PK assays are:

- Identify the critical reagent per PK assay and clearly document in the method documentation (Section 3);
- Identify the noncritical reagents and monitor during routine sample analysis (Section 3);
- Ideally, ensure enough material is available to support an entire study and/or drug development program (Section 3);
- Use GBC definitions to evaluate what constitutes a minor or major change (Section 4);
- Where one single lot cannot be sourced for the entire period of use, retain enough material of the old lot for head-to-head comparisons of new lots (Section 4);
- Evaluate lot-to-lot changes of critical reagents using the CoA and test in the PK assay using *a priori* criteria (Section 4);
- Application of re-test dates instead of expiry dates to monitor and evaluate reagent stability (Section 5);
- Monitor QC and assay data during the assay life cycle (Section 5);
- Document reagent identity, lot-to-lot changes and evaluation/extension of re-test dates in the relevant paperwork for the method (Section 6);
- For commercial kits that are used for PK purposes, secure sufficient kits of the same lot number. If this is not possible then test new batches when available (Section 4).

Decision trees have been provided for evaluation of lot-to-lot changes depending on the degree of change (minor and major) and type of reagent (standard reagents/relabeling, monoclonal antibodies produced from the same or different clone and polyclonal antibodies from a new animal bleed). However, it is important to keep in mind that this is a recommendation for the minimum requirements and that additional testing might be required based on the scientific knowledge of the impact of a critical reagent on an assay.

The EBF recommends performing a functional evaluation of lot-to-lot change and stability testing in the PK assay, but also acknowledges that further characterization of the reagents may be implemented to minimize the chance of surprises during testing of new reagent lots. Whether additional reagent characterization is performed is a business decision and needs to be balanced against the additional time and cost of the characterization. This is especially important in early phases of drug development when attrition is high. It should also be noted that assay data robustness is independent of the level of reagent characterization applied and a highly characterized assay reagent does not automatically generate more robust assays.

Future perspective

It is important that the industry and regulatory authorities agree on the appropriate level of testing for critical reagents, and ensure that the difference between critical reagents (which are a component in the assay) and a reference standard, which is used directly for concentration determination, is recognized. Going forward, the bioanalytical community would also welcome a more open communication with the commercial suppliers to ensure more consistency across different reagent lots and potentially longer stability/re-test dates. It would be beneficial for the industry if commercial vendors provide more information regarding reagent characteristics including a fully comprehensive CoA or technical datasheet. Furthermore, commercial vendors should ensure timely communication to the end users when changes or a cease in production occurs to allow for timely assessment of the impact in the assay and provide adequate time to take any required mitigation steps. The EBF is also discussing the requirements for critical reagents for immunogenicity assays and biomarkers and expect to provide recommendations for immunogenicity assays in these areas as the next step.

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