A Tiered Approach for Characterization to Ensure Quality, Reproducibility, and Long-Term Stability of Critical Reagents in Regulated Bioanalysis to Support PK/ADA/NAb Assays for Biologics and Vaccines Programs

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ABSTRACT: The robustness of good laboratory practice and clinical data is reliant upon a clear understanding of the bioanalytical assays. One of the most important components of ligand-binding based assays is critical reagents used to directly or indirectly measure biologic markers or signals. High quality, reproducible, sustainable critical reagents through the development lifecycle could avoid unnecessary rework, multiple validations, cross-validations, and ensure consistency of the data. Numerous analytical methods (UPLC-size exclusion chromatography, cation exchange chromatography, biacore/octet, and high-resolution mass spectrometry) have been evaluated by using current critical reagents. A comprehensive analytical toolbox of biochemical and biophysical methods has been employed to evaluate the quality of critical reagents and explore potential issues if there are any. Moving forward, this “tiered approach” of critical reagents characterization will be used not only to establish critical quality attributes for new reagents but also to evaluate stability in support of reagents recertification.

KEYWORDS: regulated bioanalysis, PK/ADA/Nab, critical reagents, tiered approach, characterization, biologics and vaccines

Tier I: A280, SEC, IEX or intact mass

Tier II: Binding affinity

Tier III: Peptide mapping, PTMs monitoring

In 2019, 5 out of the top 10 selling drugs, based on revenue, were protein therapeutics. The clinical and commercial success of biotherapeutic drugs has led to an increased focus on their development within the industry.^{1,2} High quality bioanalytical assays are needed to support the development of these drugs. Such assays are needed to support preclinical toxicology studies, clinical drug development, and postmarketing studies.

Ligand-binding assays (LBA), on platforms such as MSD and ELISA, together with cell-based assays,^{3,4} are traditionally used to assess the pharmacokinetic (PK) and immunogenicity properties of biotherapeutic development candidates.^{5–7} For vaccine development, flow cytometry and real-time quantitative PCR (Q-PCR), in addition to LBA and cell based assays are used to assess vaccine efficacy and safety.⁸ Each of the assay types mentioned require specific reagents. In that these are often a key factor with respect to ensuring assay performance and quality, they are typically referred to as “critical reagents”. Examples of critical reagent classes include monoclonal antibodies (mAb), polyclonal antibodies (pAb), stable cell lines, recombinant proteins, peptides, polysaccharides, and nucleotides.^{3,9,10} To ensure consistent assay performance, the

identity and quality of critical reagents need to be well controlled during their production.

In this paper, we propose a tiered approach to characterize critical reagents used in regulated bioanalysis. A comprehensive analytical toolbox of biochemical, functional, and biophysical methods (ultrahigh performance liquid chromatography-size exclusion chromatography, ion exchange chromatography, biolayer interferometry, surface plasmon resonance, and high-resolution mass spectrometry) is utilized to ensure the quality, reproducibility, and long-term stability of critical reagents. Several case studies will be discussed in this paper, including an investigation of reagent batch consistency by IEX, the determination of the incorporation ratio of Biotin/Sulfo-Tag to mAbs by the intact mass method, the characterization of a pAb positive control for use in an antidrug antibody assay

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(ADA) by an assessment of direct binding, and the monitoring post-translational modifications (PTMs) by peptide mapping via high resolution mass spectrometry (HRMS). Implementation of the proposed approach is anticipated to minimize bioanalytical assay delays and improve assay consistency during drug development.

METHODS AND EXPERIMENTS

Pharmacokinetic Assay (PK) Procedure. Streptavidin-coated microplates were blocked with 1% BSA/PBST. Standards, controls, or samples were incubated with a mixture of Biotin and Sulfo-Tag-labeled anti-idiotypic mAbs. After incubation, the mixture was added to the blocked streptavidin-coated microplate. Unbound material was removed by plate washing after which MSD read buffer was added to the plate wells. The bound complexes were detected by ECL on an MSD Sector Imager 6000 Analyzer. Data regression was performed using Watson LIMS, and the concentration of a therapeutic monoclonal antibody drug in the samples was interpolated from a standard curve using a five-parameter Marquardt curve fit with 1/Y² weighting.

Antidrug Antibodies Assay (ADA) Procedure. Unknown serum samples and serum samples containing positive control were diluted 1:9 (final concentration = 10% human serum) with 300 mM acetic acid to enable antibody complex dissociation before analysis. Samples were then incubated with a conjugate/neutralization mixture consisting of Biotin-labeled therapeutic protein drug, Sulfo-Tag-labeled therapeutic protein drug, and 1 M Tris pH 9.5. During the incubation, antidrug antibodies formed a bridge between the labeled drugs used for capture and detection. The sample mixture was then added to a blocked MSD streptavidin-coated microtiter plate. The biotin-labeled therapeutic protein drug molecule bound to the streptavidin-coated surface of the well resulting in the immobilization of the bridged complex. The plate was washed to remove any unbound complexes and tripropylamine (TPA) MSD read buffer was added to each well. With the use of the MSD 6000 plate reader, an electrical current was introduced across the plate associated electrodes. The consequent electroluminescence was measured by photodiodes and was quantified as ECL units.

Size Exclusion Chromatography (SEC). All mAb samples were diluted with water to 1 mg/mL final concentration. A 10 μ L aliquot of mAb samples was injected onto a Waters BEH200 (1.7 μ M, 4.6 mm $\times$ 150 mm) column (Waters, Milford, MA) using 50 mM phosphate, 450 mM arginine mono HCl, pH 7.0 as mobile phase at a flow rate of 0.5 mL/min. The column temperature was maintained at 30 $^{\circ}$ C, while the autosampler was kept at 5 $^{\circ}$ C. The chromatographic run time was 8 min. The SEC experiments were performed on a Water Acquity H Class Bio system with detection based on UV absorbance at 280 nm. All data were imported and analyzed by Empower 3 software (Waters, Milford, MA).

Cation Exchange Chromatography (CEX). All mAb samples were diluted with water to 1 mg/mL final concentration. A 10 μ L aliquot of mAb samples was injected onto a YMC-BioPro SP-F (4.6 mm $\times$ 100 mm) column (YMC, Kyoto, Japan). Mobile phase A was 20 mM phosphate, 10 mM NaCl, pH 5.7, and mobile B was 20 mM phosphate, 250 mM NaCl, pH 5.7. The gradient started at 2% B for 2 min, increased gradually to 30% in 6 min followed by 30% B to 45% B in 12 min, and then washed with 100% B for 6 min followed

by equilibration at 2% B for 6 min. The flow rate throughout the run was 0.8 mL/min. The autosampler was kept at 5 $^{\circ}$ C. The CEX experiments were performed on an Agilent 1260 Infinity Quaternary LC system (Agilent, Santa Clara, CA) with wavelength detection at 280 nm. All data were imported and analyzed by Empower 3 software (Waters, Milford, MA).

Binding Kinetic Measurements by Octet. The binding of pAb or mAb positive controls to a therapeutic protein drug was measured by biolayer interferometry (BLI) using an Octet RED 96 (ForteBio, Menlo Park, CA). A total of eight streptavidin (SA) biosensors (ForteBio, Menlo Park, CA) were hydrated in 0.2 mL of PBS for 10 min, followed by equilibration with PBS for 60 s. Afterward, SA biosensors tips were loaded with a biotinylated therapeutic protein drug in a concentration of 111 nM in PBS, followed by an additional 60 s for blocking in 10 mg/mL Biocytin (ForteBio, Menlo Park, CA). The binding kinetics were measured by dipping the coated SA biosensors tips into the wells containing a mAb or pAb PC at varying concentrations from 0.27 nM to 200 nM with 2-fold serial dilutions. The binding interactions were monitored over a 300 s association period, followed by a 600 s dissociation period in new wells containing PBS. All samples were agitated at 1000 rpm over the entire course of the experiment. The assay generated a kinetic profile to characterize a therapeutic monoclonal antibody drug affinity to a pAb or mAb PC through determining both its on (k_a) and off (k_d) rates and the equilibrium dissociation constant (KD), calculated by dividing the value of k_a into k_d . The final results were reported as KD. The kinetics data were analyzed by ForteBio Data Analysis 7.0 software with a 1:1 model.

Antibody Conjugation by Biotin and Sulfo-Tag Procedures. The buffers of mAbs were exchanged to PBS, pH 7.8 by Amicon Ultra Centrifugal Filters with 50 kDa molecular weight cutoff (Millipore, Burlington, MA) before conjugation. Then material at 1 mg/mL was challenged at different molar ratios with EZ-Link Sulfo-NHS-LC-Biotin (challenge ratio: 10 and 30) (Thermo Scientific, Waltham, MA) and MSD Sulfo-Tag NHS-Ester (challenge ratio: 12 and 36) (Meso Scale Discovery, Rockville, Maryland). Reactions ran for 2 h at room temperature. The excess of unlabeled molecules was removed using Amicon Ultra Centrifugal Filters with 50 kDa molecular weight cutoff (Millipore, Burlington, MA). Labeled mAbs were diluted to 0.5 mg/mL in PBS, pH 7.4.

Intact Mass Method to Determine the Incorporation Ratio of Biotin/Sulfo-Tag to mAbs. Conjugated mAbs were analyzed by HRMS using a Waters Xevo G2 Q-TOF system (Waters, Milford, MA) after they were separated with a POROS R2/10 2.1 mm $D \times$ 30 mm L column (Thermo Scientific, Waltham, MA) on a Waters Acquity UPLC system. Mobile phases were 0.1% formic acid (FA) in water as mobile A and 0.1% FA in acetonitrile (ACN) as mobile B. The LC flow rate was 0.4 mL/min, and the column temperature was maintained at 75 $^{\circ}$ C. The antibody was eluted using a gradient of 0.2–2.4 min of 2%–99% B. MS spectra were acquired on a Waters Xevo G2 Q-TOF system which was scanned in a range of m/z 800–4000. The mean molar incorporation ratio was determined manually after spectra deconvolution using MaxEnt1 of MassLynx v4.1 (Waters, Milford, MA).

Reduced Peptide Mapping by LC–MS and Data Analysis. The antibody samples (100 μ g of antibody) were denatured and reduced in a solution of 50 mM Tris at pH 8.0 with 6.0 M guanidine HCl and 20 mM DTT at 56 $^{\circ}$ C for 30

min, followed by alkylation with 50 mM IAM for 30 min at room temperature in the dark. Then 11 mM DTT was added to quench the alkylation. A 20 μ L aliquot of the reduced and alkylated samples was diluted with 100 μ L of digestion buffer (50 mM Tris pH 8.0) before the Lys-C enzyme with an enzyme/substrate ratio of 1:40 (w:w) was added. The solution was incubated at 37 °C for 1 h. Then 480 μ L of digestion buffer (50 mM Tris pH 8.0) was added into the Lys-C enzyme digested samples, followed by the addition of trypsin at an enzyme/substrate ratio of 1:20 (w:w). The mixture was incubated at 37 °C for 3 h. After digestion, 10% TFA was added at a 1:10 ratio to quench the digestion. The digested samples were stored at –80 °C until analysis.

The peptides were separated by RP-UPLC on a Waters Acquity UPLC H-class (Waters, Milford, MA) using an ACQUITY UPLC BEH C18 (1.7 μ m, 2.1 mm $\times$ 150 mm) column. Mobile phase A was 0.1% FA in HPLC water, and mobile phase B was 0.1% FA in ACN. The peptides were eluted using a gradient started at 2% B for 2 min, increased gradually to 18% B in 38 min, followed by increasing from 18% B to 35% B in 47 min. Next, the gradient was increased from 35% B to 95% B in 5 min. The column was then washed with 95% B for 20 min, followed by equilibration at 2% B for 20 min. The LC flow rate was 0.2 mL/min, and the column temperature was maintained at 60 °C. The eluted peptides were monitored using a Thermo Fisher LTQ Orbitrap Velos mass spectrometer in positive ion mode (Thermo Scientific, Waltham, MA). MS spectra were acquired on a Thermo Fisher LTQ Orbitrap Velos MS system with a MS resolution of 60000. For the analysis of tryptic peptides, the mass spectrometer was operated in data-dependent acquisition mode with dynamic exclusion enabled. After the survey scan, the four most intense precursors were selected for subsequent fragmentation. The signal threshold for triggering an MS/MS event was set to 500 counts. The MS data were searched against the known protein sequences for confirmation and post-translational modifications (PTMs) using the Thermo BioPharma Finder 3.2 software (Thermo Scientific, Waltham, MA). The peptide peak areas were calculated from the extracted ion chromatograms of full scan MS data. For monitoring PTMs, the instrument was operated in MS1-only mode with a scan range from m/z 300 to 20000. MS/MS was performed to confirm peak identification. The relative percentage of the modified peptide was determined by the peak area of the modified peptide versus the sum of peak areas of the modified and unmodified species for each peptide.

RESULTS AND DISCUSSION

A Tiered Approach of Critical Reagents Characterization for Regulated Bioanalysis. In regulated bioanalysis there is an increasing need to assess the identity and quality of critical reagents, especially when a new batch of reagent is generated. In general, guidance from regulatory agencies related to protein analysis focuses on the characterization of the critical quality attributes (CQAs) of therapeutic proteins through the determination of their physicochemical and immunochemical properties, biological activity, purity, and impurities, etc. However, there is no clear guidance for the characterization of critical reagents. In this paper, we propose a tiered approach (Figure 1) for the evaluation of protein-based critical reagents with a goal of ensuring the consistency of the bioanalytical assays in which they are employed. This approach contains three tiers. The tier I assays, which include A280,

Figure 1. A tiered approach for critical reagents characterization

SEC, IEX, and intact mass assessment are executed following the generation of the initial batch of a protein based reagent. The results are used to establish critical quality attributes for the reagent. These assays establish baselines for protein concentrations, aggregation/purity level, charge profile, and protein identity, including unlabeled as well as Biotin/Sulfo tag labeled critical reagent. These Tier 1 characterizations are then executed following the production of subsequent reagent to assess batch to batch consistency. In some cases, tier 1 assay results have been found insufficient to identify lot variations. In such cases, further characterization via the Tier II and Tier III assays are employed. Tier II assays, employing Biacore or Octet are used to assess differences in the binding affinity of antibody reagents. Finally, tier III assays, which consist of peptide mapping using LC-HRMS, serve to identify chemical modifications, such as oxidation and demethylation etc. and any other forms of post-translational modifications (PTMs).

Case Studies and Discussion on Tier I Assays. SEC and IEX as Tier I assays to Support Reagents Development for Clinical PK Assay. In one case of PK assay development, after screening 31 anti-idiotypic mAbs (anti-ids), a pair of anti-ids were selected as capture and detection reagents. Anti-ids are highly specific for binding to their intended drug of measurement. The selection was based on human serum tolerance, soluble target tolerance, and maximizing the ratio of the signal-to-noise at the lower limit of quantitation (LLOQ). After the identification of the two anti-ids, large scale batches of the selected antibodies were produced to support assay development and future sample testing. Both small-scale and large-scale anti-ids were assessed upon their initial production using SDS-PAGE, analytical SEC, and binding ELISA. The quality attributes of these anti-ids showed comparable purity (>95%) and EC50 (Figure S1 in Supporting Information).

Method development based on a comprehensive design of experiments (DOE) approach was initiated using the large-scale anti-ids. The goal of this work was to optimize the PK assay in terms of minimum required dilution (MRD), *in vitro* matrix stability, impact of endogenous analyte, disease state matrix interference, accuracy, precision, curve-fit and calibration model, matrix stability, dilutional linearity, selectivity, and parallelism. However, an assessment on soluble target interference using the large-scale anti-ids production batch did not meet the clinical PK assay requirement; a large difference between small-scale anti-ids and large-scale anti-ids on assay performance was observed (Table 1).

Since the assessments using SDS-Page, SEC, and binding ELISA that were conducted following their generation failed to differentiate the new reagents from the original batch, a Tier I critical reagents characterization approach was triggered to investigate the assay performance difference by head-to-head comparison of the small and large scale batches. First, an assessment of potential degradation using analytical SEC was

Table 1. Significant Difference in Assay Performance between the Small-Scale and Large-Scale Anti-ids

	small scale anti-ids	large scale anti-ids
ECL count (a) of 10 ng/mL mAb drug in human serum without target	16004	66766
ECL count (b) of 10 ng/mL mAb drug in human serum with 25 ug/mL soluble target	16312	6148
% inhibition of soluble target ^a	−1.9	90.8

^aInhibition % = $[(a - b)/a] \times 100$.

performed on the initial and newly synthesized batches of mAbs. The purity of these four anti-IDs was found to be >95%, which is in agreement with the assessments conducted following their generation (Figure 2). However, when the

Figure 2. Overlay SEC chromatograms of small-scale and large-scale anti-ids. (A) Small-scale (in red) and large-scale capture anti-ids (in blue) showed different retention time. (B) Small-scale (in red) and large-scale detection anti-ids (in blue) showed similar retention time.

chromatograms were overlaid, a retention time shift of the capture anti-ids by about 10 s was identified when the small-scale and the large-scale batches were compared (Figure 2A). The retention time of the two batches of detection anti-ids were identical (Figure 2B). The changed retention time triggered further investigation using cation exchange chromatography (CEX) to characterize the charge profiles of these anti-ids (Figure 3). The small-scale and the large-scale capture anti-ids showed different CEX chromatograms (Figure 3A). In contrast, the two detection anti-ids gave similar charge profiles (Figure 3B). The results from SEC and CEX suggested that the two capture anti-id batches (the small-scale vs the large-scale) have different size and charge profiles. Further, LC–MS studies identified different molecular weights between the two capture anti-ids (Figure S2 in Supporting Information) but the same MWs of the two detection anti-ids, which further confirmed that the observed assay performance difference was largely because of the change on large scale capture anti-id.

On the basis of the above data, additional production of the capture anti-id was requested, and the new batch exhibited similar performances as compared to the initial batch.

Figure 3. Overlay CEX chromatograms of small-scale and large-scale anti-ids. (A) Small-scale (in red) and large-scale capture anti-ids (in blue) have different charge profiles. (B) Small-scale (in red) and large-scale detection anti-ids (in blue) have similar charge profiles.

This case study supports the Tier I analytical characterization approach, SEC and IEX provide a quick and economical first line approach for identifying differences between batches, while LC–MS is used in a confirmatory manner and is especially useful if differences are not readily apparent in SEC or IEX.

Intact Mass as a Tier I Assay for the Determination of Incorporation Ratios of Labeled Reagents in ADA Assay. Electrochemiluminescence (ECL) on the mesoscale development platform (MSD) has gained popularity in the past decade, mostly due to its better sensitivity and broader dynamic range as compared to enzyme linked immunosorbent assays (ELISA).³ The MSD-based assays require biotin and sulfo-tag labeled capture and detection reagents. In the preparation of these labeled reagents it is important to control the ratio of label-to-protein, known as the incorporation ratio (IR), as changes in IR from lot to lot of reagent may significantly impact assay performance.¹¹ A spectrophotometric assay using 4'-hydroxyazobenzene-2-carboxylic acid (HABA assay) has been used historically to characterize the IRs of labeled critical reagents. However, this technique suffers from high data variability as well as challenges with respect to the determination of unlabeled materials.

High resolution mass spectrometry (HRMS) may be used as an alternative to the HABA assay for the characterization of incorporation ratios. This technique has been employed to characterize antibody-drug conjugates (ADC).¹² The concept and techniques between characterization of ADC and labeled critical reagents are similar—the determination of the Biotin/Sulfo-Tag incorporation ratio for critical reagents is analogous to the determination of the drug/antibody ratio (DAR) for ADCs.

Given the advantages of HRMS with respect to data quality, it is proposed as a Tier I assay for IR determinations. Figure 4

Figure 4. Deconvoluted mass spectrum of intact mass for biotinylated mAb therapeutic drug with a 7 (A) and 17 (B) mean incorporation ratios after using 10:1 (A) and 30:1 (B) challenge ratios, respectively.

panels A and B show the HRMS results for an mAb therapeutic drug labeled at challenge ratios of 10:1 and 30:1 with NHS-LC-biotin. The biotin incorporation ratios were determined to be 7 and 17 by HRMS, for the samples prepared at 10:1 and 30:1 challenge ratios, respectively (Figure 4A,B and Table S1). In addition, HRMS can also provide information on the unlabeled drug in the reagent, which may impact ADA assay performance due to the potential binding of the unlabeled drug to the positive control.

Direct Binding by SPR/BLI as a Tier II Assay to Support Reagents Development for Clinical ADA Assay.

Assessment of the binding affinity between proteins may provide insight into assay performance issues. In that such assessment is not needed for all reagents, it is classified as a Tier II assay in our approach. Binding affinities may be determined using either ForteBio's Octet RED96 or GE Healthcare's Biacore T200 or other instruments. Octet and Biacore utilize different technologies. Octet is a bio-layer interferometry (BLI) platform that monitors the interaction of light waves and the changes that occur during the binding reaction. Biacore is a surface plasmon resonance (SPR) based platform, which measures small changes in the polarized light refractive index near the sensor surface where the binding interaction occurs.^{13–16}

Positive controls (PCs) for antidrug antibody (ADA) assays are an example of one reagent type where the tier II characterization of binding affinity may be useful. PCs are typically pAbs generated by the immunization of rabbits with a protein based drug followed by several steps of columns-based purifications, such as affinity purification against the

therapeutic protein drug and/or a gel filtration chromatography. The successful development and validation of ADA assays, especially with respect to assay sensitivity, highly depends on the performance of the PC. In 2019, the US Food and Drug Administration issued guidance indicating that screening and confirmatory ADA assays should achieve a minimum sensitivity of 100 nanograms PC per milliliter (ng/mL).^{17,18} Therefore, generation of a pAb with sufficient affinity as a PC and characterization of this pAb is essential for ADA assays. The case study presented below illustrates the value of the binding affinity assessment of PCs during ADA assay development. During ADA method development, the focus is on optimizing assay sensitivity, specificity, selectivity, drug tolerance, precision, reproducibility, and robustness. The sensitivity of the assay is evaluated by testing serial dilutions of a PC antibody at known concentrations in a pooled matrix from treatment-naïve subjects. However, after extensive assay development work, including assessment of different ADA assay formats, the sensitivity of the assay under development did not meet the US FDA guidance. As shown in Table 2, the

Table 2. Different ADA Sensitivities Using a pAb or mAb as a Positive Control

PC	mean ECL counts		
	negative control	PC at 100 ng/mL plus 10 μ g/mL drug	PC at 100 ng/mL plus 100 μ g/mL drug
pAb as a positive control	100	127	102
mAb as a positive control	100	641	254

mean electrochemiluminescence (ECL) counts were around 100 for the negative control, and 127 for a pAb PC at 100 ng/mL in the presence of 10 μ g/mL therapeutic protein drug; the ADA signal in the presence of PC was not significantly different from that of the negative control, indicating that the assay lacked sufficient sensitivity. To address the insufficient assay sensitivity, three mAbs with high affinities were evaluated to replace the pAb PC as a positive control for this ADA assay. The EC₅₀s of these three mAbs as measured by a direct binding ELISA were determined to be 0.042 nM, 0.056 nM, and 0.074 nM, respectively, which indicates these mAbs bind to this therapeutic drug tightly. By switching positive control from a pAb to a mAb, the sensitivity of this ADA assay increased five times (Table 2).

To understand why the sensitivity of the ADA assay with the pAb PC was insufficient, an investigation was initiated. Unlike mAbs or highly purified therapeutic proteins, there are limited reports on how to characterize a pAb, as a pAb is a mixture of antibodies from different B cells that recognize multiple epitopes of an antigen.¹⁹ An analytical size exclusion chromatography (SEC) assessment was used as a Tier I approach to evaluate if the observed ADA assay performance was due to the aggregation of the pAb. The purity of the pAb was determined by SEC to be 95%, thus ruling out that aggregation was a contributing factor to the low sensitivity. Next a Tier II assessment of the binding affinity between the pAb PC and the therapeutic protein drug was conducted using the Octet platform. Figure 5 panels A and B show the representative binding kinetics of the testing drug to the pAb and the mAb PCs. The K_D values were determined to be 0.894

Figure 5. Representative affinity sensorgrams for binding between a therapeutic monoclonal antibody drug and a pAb (A) and mAb (B) PCs.

nM and 0.012 nM for the pAb and mAb, respectively. As the result of this reagent characterization, it could be concluded that the low sensitivity of the clinical ADA assay was not due to issues with the purity of the pAb positive control, ADA assay format, or other reagents used in this ADA assay, but rather was due to a low binding affinity of the pAb PC to the therapeutic protein (Table 2).

Peptide Mapping by HRMS as Tier III Assays to Support Critical Reagents Characterization. In some situations, such as switching the positions of amino acids within a protein or post-translational modifications (PTM), the Tier I and II assays might not be able to identify these changes. It is reported that antibodies are prone to isomerization at aspartate in DG motifs, and deamidation at asparagine residues, in particular in NG motifs in CDRs, which may cause potential loss of antibody binding affinity and thus have a negative impact to LBA assay.^{15,20,21} In such cases, Tier III assays are used to identify protein modifications at the amino acid level. Such changes may occur, for example, during

antibody production, storage, or when subjected to multiple freeze and thaw cycles.

A tier III, peptide mapping approach with HRMS was used to identify the “hot spots” in a mAb reagent. About 100 μ g of mAb was reduced, alkylated, and digested by Trypsin and LysC enzymes. The digested peptides were subsequently injected into an Waters Acquity H Class UPLC coupled with a Thermo Orbitrap HRMS as detector. The raw mass data, including total ion scan and MS/MS spectra, were analyzed using Thermo BioPharma Finder 3.2 software based on the theoretical reagent mAb sequence. The sequence coverage of the sample was confirmed by comparing the experimentally determined amino acid sequences to the theoretical sequences. Peptide mapping analysis allowed the identification and relative quantification of PTMs. Many common PTMs cause a shift in reversed-phase LC retention relative to the native peptide. In combination with direct MS and MS/MS analysis the peptide mapping process can be used to interrogate modifications with relatively large mass shifts, such as C-terminal lysine (+128 Da) or glycation (+162 Da), as well as small mass differences, such as deamidation (+1 Da), oxidation (+16 Da), or pyroglutamination (+17 Da).^{22,23} Figure 6 shows a representative total ion chromatogram (TIC) of the reduced peptide mapping of a mAb. Figure 7 shows a representative MS/MS spectra and assignment of fragment ions of the nonoxidized and oxidized peptides from trypsin/Lys-C digested mAb, where there is a 16 Da difference of b4 and y5 ions between unmodified peptide and oxidized peptide. This example indicates that oxidation occurred at M254 of this mAb. A calculation of the percentage of modified protein in the sample can be done automatically by BioPharma Finder 3.2 software using the modification summary function, based on the MS1 peptide peak areas after confirming the peak identity with MS/MS. The relative percent of the modified peptide was determined by the peak area of the modified peptide versus the total peak area of the modified and unmodified species for each peptide, as shown in Table 3.

Although the oxidation of M254 was not within the CDR region of the tested mAb, this example demonstrates the utility of Tier III peptide mapping to conduct a characterization of critical reagents at the amino acid level and facilitate assay troubleshooting as required.

CONCLUSIONS

In summary, the ability to characterize critical reagents is necessary to ensure the consistency of bioanalytical assays used

Figure 6. Representative total ion chromatogram of LC-MS reduced peptide mapping of a monoclonal antibody.

Figure 7. MS/MS spectra and assignment of fragment ions of the tryptic peptide DTLMISR for the given mAb in Figure 6: (A) unmodified peptide, (B) oxidized peptide. M254 (corresponding to position 4 in the peptide above) oxidation was identified automatically by BioPharma Finder 3.2 software from Thermofisher.

Table 3. An Example of Observed Post-translational and Chemical Modifications Monitored for a Monoclonal Antibody

PTM	PTM% ^a
HC M254 (oxidation)	1.52
HC N317 (deamidation)	1.46
G448+Lys	21.85

^aModification % was calculated based on the extracted ion chromatographic (XIC) peak areas: (modified peptide)/(modified peptide + unmodified peptide) × 100.

to support good laboratory practice and clinical studies during drug development. An analytical tool box, including UPLC-SEC, IEX, Biacore/Octet, and HRMS methods was established to address the reagent related issues that may occur during assay development, validation, and use. A tiered approach to utilize these methods is proposed to characterize critical reagents and ensure the consistent performance of bioanalytical assays in which they are employed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspsci.0c00135>.

Materials; characterization of small-scale and large-scale capture anti-ids; intact mass spectrum of reduced mAbs; biotinylated mAb therapeutic drug mean incorporation ratios by HRMS (PDF)

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Notes

The authors declare no competing financial interest.

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