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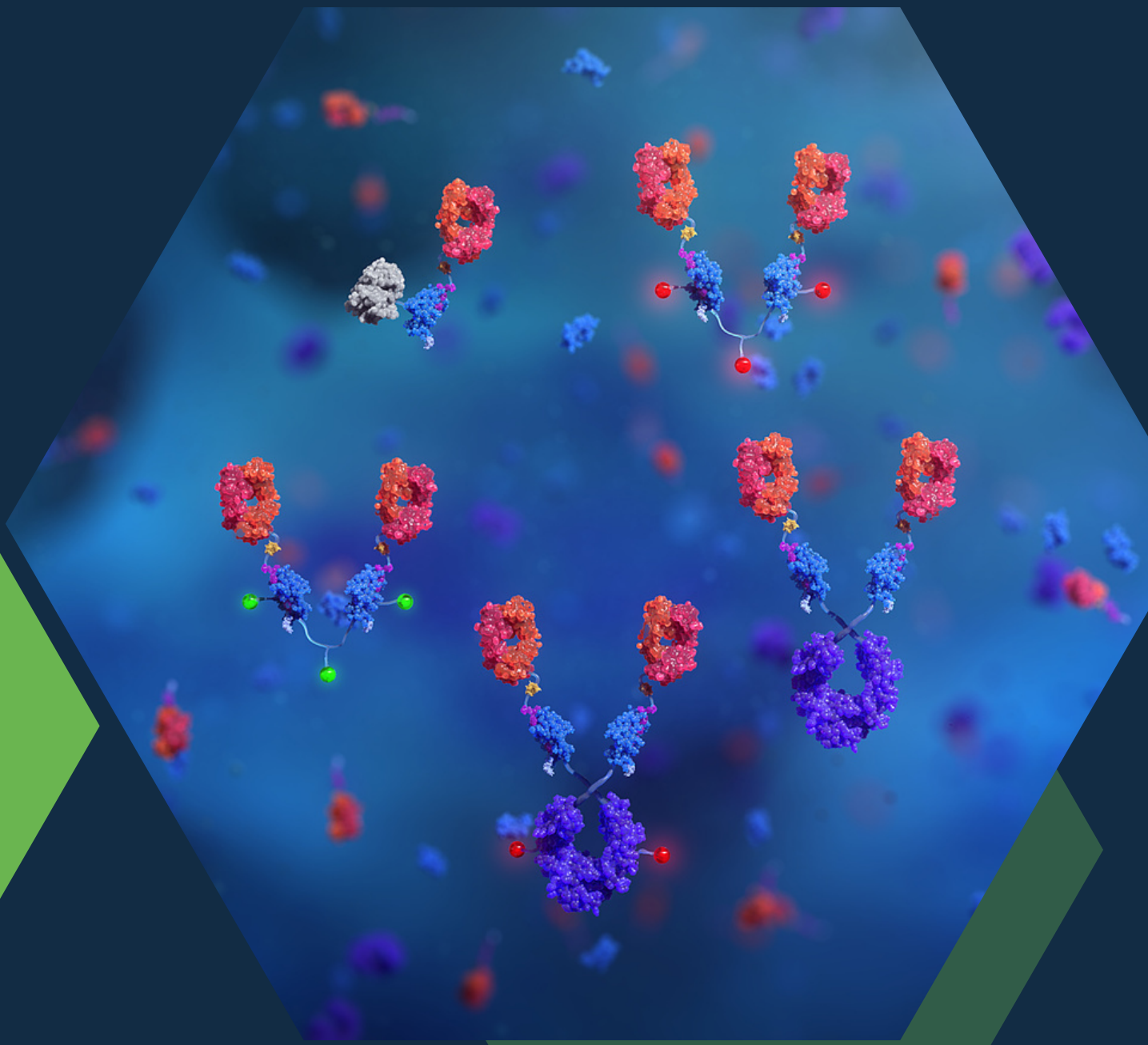

Antibody engineering and SpyTag-SpyCatcher technology

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Technology Digest: Antibody engineering and SpyTag-SpyCatcher technology

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Antibody engineering and the importance of high-quality antibody formats

Antibodies are among the most frequently used research tools in clinical assays and are incredibly valuable for basic scientific research. Over the last few years, there has been a lot of development in the methods and techniques used for antibody engineering, resulting in recombinant antibody formats, such as Fab (fragment antigen binding) fragments, becoming more widely available e.g. for use in immunoassays [1,2].

During drug development it is important to analyze and quantitate protein biotherapeutics to support pharmacokinetics, pharmacodynamics, and safety assessments. Large biological analytes, such as antibodies, are typically measured using ligand-binding assays in immunoassay formats, for example ELISA. Immunoassays use the specificity of an antigen-antibody interaction to quantify and detect target molecules within a sample and immunoassay platforms use at least one antibody or antigen, as well as binding reagents that possess specificity for the analyte [3].

When performing immunoassays, the use and control of high-quality reagents is critical to produce reliable and reproducible data, as well as to ensure consistent assay performance. Antibodies are essential components that directly impact the bioanalytical assay results and each immunoassay has different requirements, which is why it is beneficial to use an antibody format optimized for the relevant application [4]. In the past, full immunoglobulin G (IgG) antibodies were most commonly used for immunoassays, with the Fc portion of IgG often required solely for detection with a secondary reagent. To reduce production costs, IgG can be replaced with bacterially-expressed antibody fragments, like Fabs. However, it can be time-consuming to convert an antibody from one format to another, or to label it without affecting the antigen binding site. Therefore, protein ligation methods, based on the formation of a spontaneous bond, have been employed to generate antibodies from tagged antibody fragments and a catalog of standardized parts [1].

What is SpyTag-SpyCatcher technology?

Target-specific antibody fragments isolated through phage display can be quickly produced in bacteria. However, the modification of antibodies with probes or the generation of full-length antibodies can be labor-intensive, costly, and time-consuming [1]. SpyTag technology is a protein ligation method that irreversibly locks biological molecules together. This technology has been licensed by Bio-Rad (CA, USA) to covalently attach domains and labels to antibody fragments equipped with a SpyTag and offers a smart way to generate modular antibodies from SpyTagged antibody fragments and a catalog of standardized parts [1,5].

> *"The innovative SpyTag and SpyCatcher technology, also termed 'molecular superglue', simplifies both modular antibody assembly and site-specific labeling of antibodies. Through Bio-Rad's TrailBlazer Antibody Platform, recombinant antibodies contain a SpyTag and can be combined with a set of prefabricated SpyCatcher modules, leading to unprecedented flexibility in assay design. The reaction between SpyTag and SpyCatcher occurs autocatalytically and quantitatively in minutes. Depending on the SpyCatcher used, the antibodies are site-specifically labeled with, for instance, HRP, biotin, or fluorescent dyes, or they are multimerized or converted to immunoglobulin-like constructs with different isotypes or with Fc domains from various species," commented John Cardone, PhD, Marketing Manager at Bio-Rad Laboratories, Inc. (Oxford, UK).*

The SpyTag peptide — a 13 amino acid tag— and SpyCatcher protein are derived from the second immunoglobulin-like collagen adhesin domain from the fibronectin-binding protein of *Streptococcus pyogenes*. The collagen adhesin domain naturally contains an intrachain isopeptide bond between the side chains of a lysine (Lys) and an aspartic acid (Asp) [5,6]. By splitting this domain and conducting rational engineering of the fragments, a peptide, i.e., the SpyTag containing the reactive Asp residue, and a small protein, i.e., the SpyCatcher containing the reactive Lys residue and a glutamic acid (Glu) residue necessary for forming the catalytic triad, were obtained, which form an amide bond spontaneously when mixed [7,8]. The SpyTag reaction occurs with high yield in diverse conditions of pH, temperature, and buffer and since its conception, the two components have subsequently been optimized, creating versions 2 and 3 (SpyTag2-SpyCatcher2, SpyTag3-SpyCatcher3) where the reaction time was shortened from hours to minutes [5].

Reaping the benefits of SpyTag-SpyCatcher technology

The SpyTag-SpyCatcher technology can be used to build antibodies with new properties, helping to address the challenges of detecting proteins [9]. This technology enables the rapid change and production of different antibody formats and has provided a simple, specific, and genetically encodable method to create a diverse range of biomaterials [10]. By coupling a SpyTagged antibody to different SpyCatcher proteins the possibilities of assay design are increased, enabling the rapid assembly of multiple, stable products. These include bivalent Fabs and Ig-like antibodies with different isotypes. Importantly, all derivatives of SpyTag and SpyCatcher are compatible with each other and it is possible to engineer any recombinant antibody to contain a C-terminal SpyTag for use with SpyCatcher [5,9].

A key advantage of pre-conjugated SpyCatchers is that they are simple, fast, flexible, and site-directed. Conventional antibody conjugations can involve a random process, resulting in each antibody carrying a different number of labels at different positions. Additionally, the performance of the reagent in the application could decrease if some labels attach to the antibody-antigen binding site. Labeled SpyCatchers are coupled to the antibody C-terminus containing the SpyTag with defined stoichiometry, leaving the antibody binding site unmodified [5].

Meeting the demands of assay design with TrailBlazer™ Antibodies

The design and optimization of immunoassays can be time-consuming and complex. A key challenge arises in determining the optimal number of antibody formats needed for a project. Opting for a single antibody format is cost-effective but can limit the results gained from the assay. Recognizing this challenge, Bio-Rad has incorporated the SpyTag-SpyCatcher technology into their custom recombinant antibody generation service to form the TrailBlazer Antibody Platform [11]. By incorporating SpyTag-SpyCatcher technology with custom recombinant antibodies, site-directed conjugation or assembly into different stable antibody formats is enabled, providing an antibody toolbox to facilitate the rapid development of multiple assays [9]. Antibodies can be produced in a variety of configurations including full-length IgG and monovalent or bivalent Fabs, with a choice of isotypes and labels. This in turn means that the most beneficial antibody format and label can be explored, determining which format works best for the desired application and on different technology platforms [12].

Summary

Antibodies are among the most frequently used tools in clinical assays and are typically measured using ligand-binding assays in immunoassay formats. Immunoassays are key bioanalytical methods employed by scientists to detect and quantify target molecules within a sample using the specificity of an antigen-antibody interaction. However, a critical challenge of assay design can include only using an antibody in one format, which can limit results [1–3].

SpyTag-SpyCatcher technology, provided by Bio-Rad, is a protein ligation method where the SpyTag peptide forms a spontaneous amide bond with its protein partner SpyCatcher [5,8]. This 'molecular superglue' is a significant tool for molecular assembly, offering a smart way to generate modular antibodies from SpyTagged antibody fragments and a repertoire of standardized SpyCatchers [1,6]. The SpyTag-SpyCatcher technology (as applied to antibodies and known as TrailBlazer Antibodies) can be used to build antibodies with new properties to help address the challenges of detecting proteins [9]. By incorporating this technology with Bio-Rad's custom recombinant antibody generation service, robust assays can be quickly developed. TrailBlazer Antibodies can increase assay flexibility, ensuring that reliable and reproducible data is produced, which is critical for all stages of drug development [4,11].

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Learn more about the antibody toolkit for accelerated clinical monitoring studies in this webinar now available on demand: Antibody toolkit for accelerated clinical monitoring studies (workcast.com)

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Perspective

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Critical reagent generation, characterization, handling and storage workflows: impact on ligand binding assays

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The foundation of pharmacokinetics and antidrug antibodies assay robustness relies on the use of high-quality reagents. Over the past decade, there has been increasing interest within the pharmaceutical industry, as well as regulators, on defining best practices and scientific approaches for generation, characterization and handling of critical reagents. In this review, we will discuss current knowledge and practices on critical reagent workflows and state-of-the-art approaches for characterization, generation, stability and storage and how each of these steps can impact ligand-binding assay robustness.

Lay abstract: A critical part of clinical development for new biologic drugs is the use of tests known as ligand-binding assay. These assays must be able to accurately measure drug levels and to assess if the biologic drug interacts with the immune system in patients. In order to support patient efficacy and safety, scientists must use state-of-the-art approaches to develop and identify specific reagents for each new biologic drug. This review aims to cover all key steps that are needed to support the quality and performance of the unique components of ligand-binding assays from the beginning of assay development and throughout the entire life-cycle of the biologic drug.

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Background

Reliable and reproducible ligand-binding assay (LBA) assay performance and data interpretation rely on the quality and control of critical reagents. These reagents include unique antibodies specific for monoclonal antibody (mAb)-based drugs, recombinant proteins and replacement proteins, or labeled versions of the drug itself. Moreover, there have been many advances made in the design of novel biotherapeutic modalities. This has led to an increase in molecular diversity of scaffolds, and thus, the need for more complex assays that measure multiple analytes and/or domains. According to ICH M10, critical reagents are defined as assay components that have direct impact on the results of the bioanalytical assays [1]. LBA critical reagents are predominantly biological in nature, and therefore, can be difficult to acquire, generate, resupply or substitute [2]. Thus, the workflow for critical reagent life-cycle management encompassing characterization, handling and storage best practices is of key importance for LBA assay ruggedness and robustness [3]. Furthermore, early characterization of critical reagents should be viewed as a risk-mitigation strategy that will minimize future troubleshooting efforts, decreasing failures and resource investments.

Commonly used reagents for pharmacokinetics & antidrug antibodies assays

Critical reagents commonly employed in LBAs include labeled versions of the biotherapeutic drug, mAb and polyclonal antibody (pAb) anti-idiotypic antibodies (anti-Ids), recombinant proteins and in some cases, synthetically derived molecules designed to bind specifically proteins (Table 1). Most LBA platforms often require that one or more of these reagents be labeled with either biotin, SULFO-TAG, digoxin and/or fluorescent dyes (for capture

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Table 1. Typical critical reagents and reagent generation platforms.

Reagent type	Platforms	Species/expression system
Monoclonal antibodies	Hybridoma Phage display Yeast display B-cell cloning Single chain variable domain (V_{HH})	Mouse/rabbit/rat Bacteria Yeast Human, mouse rabbit Llamas/alpacas
Polyclonal antibodies	Not applicable	Rabbit Goat Llamas/alpacas
Proteins	Recombinant expression Purified natural proteins	Mammalian cell lines (Hek 293/CHO), bacterial (*Escherichia coli*), yeast and insect Serum/plasma
Aptamers	DNA RNA	Synthetic

n/a: Not applicable; V_{HH}: Single heavy chain variable domain.

and/or detection). mAb and pAb anti-Ids are the most common critical reagents for pharmacokinetics (PK) and antidrug antibodies (ADA) assays as capture and detection reagents for PK assays and as positive controls (PCs) for ADA assays, respectively. Table 1 shows the types of *de novo* generated critical reagents. Other types of critical reagents include human matrices, assay calibration reference standards preparations and in some cases, unique blocking reagents. However, for the purposes of this review, the critical reagents that will be discussed include mAb and pAb anti-Ids, recombinant proteins and conjugated versions of the drug itself.

Design & generation of mAbs as critical reagents

Anti-Ids can be generated by multiple technologies such as standard mouse/rat/rabbit hybridoma technology, phage/yeast display or single B-cell recombinant technologies. Each of these technologies has been reviewed elsewhere [4–6]. mAb anti-Ids are typically generated to ensure long-term and reliable supply of reagents to support bioanalysis during clinical development. Each anti-Id campaign should be carefully planned to ensure that the selection of species, immunogens or antigens, adjuvants (if needed) and screening strategy are aligned with the needs of the LBA.

The goal for most anti-Id campaigns is to produce a broad toolbox of both blocking and nonblocking reagents to test in early methods development. Antibody campaigns designed to target recombinant protein biotherapeutics or peptides will require a more in-depth understanding of the bioanalytical strategy to determine whether it is important to focus the antibody tools to the functional sites of the therapeutic. Selection of candidates should be based not only on optimal functional screening testing, but also on production robustness, determined by measurement of antibody IgG titers and by confirmation of clonality via intact LC–MS analysis and V-gene sequencing.

Overview of critical reagent workflow

Multiple paradigms for reagent life-cycle management have been published with the goal of providing a framework process to ensure quality, management of re-test dates and resupply, as well as support LBA maintenance [2,7–9]. All these approaches start with the early conception of a reagent generation strategy aligned with the bioanalytical strategy. Figure 1 shows a proposed workflow schematic of reagent generation, characterization, handling and storage practices. The generation and selection of analyte-specific reagents should be performed with the assay needs and bioanalytical objectives in mind. Understanding the biotherapeutic's molecular and biophysical properties, mode of action and the therapeutic target are important aspects to align the reagent generation strategy with the bioanalytical strategy.

Based on the selected reagent-generation strategy, specific tools are generated for a given biotherapeutic agent. Once the novel reagents are in hand, the first step is to perform quality control (QC) to establish the concentration, purity and identity prior to use in method development activities [10]. Geist *et al.*, reported that purity and identity impact assay performance and the information learned during early QC can help provide context to method development and/or data bridging [11]. Once this baseline is established, critical reagents should be catalogued ideally into a computerized inventory system for life-cycle management [8]. Additional characterization such as reagent pairing, determination of affinity and categorization as blocking or nonblocking anti-Ids, should be

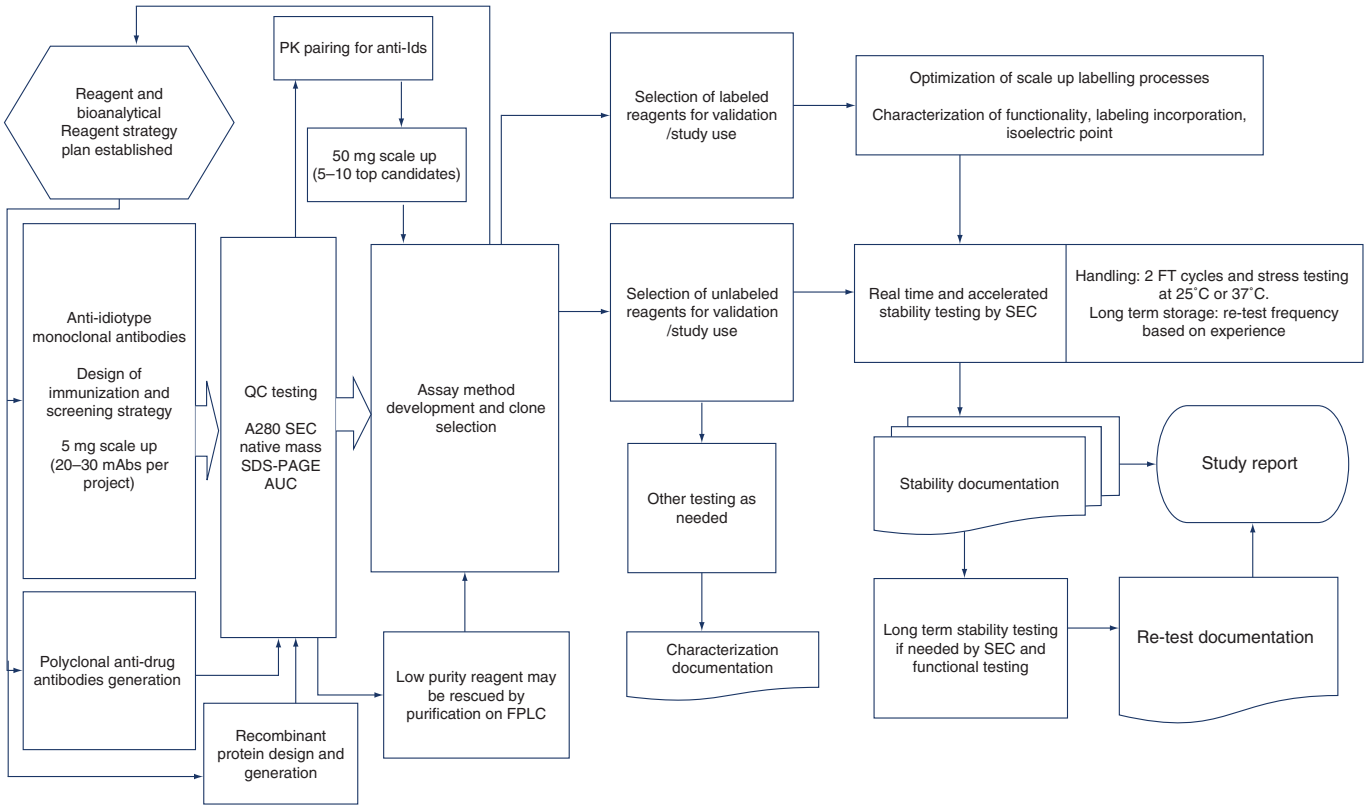

Figure 1. Reagent generation & characterization workflow to support the life cycle of ligand binding assays.
FPLC: Fast protein LC.

implemented to support method development activities. More in-depth biophysical characterization, particularly on conjugated critical reagents, may be considered to help in the selection of reagents that can support improved LBA performance and robustness [3,10]. Following reagent selection, it is important to gain an early understanding of stability. A program of accelerated and real-time stability testing using a subset of the same methods as those used during the initial QC process can be employed [10]. Long-term stability testing of reagents will help ensure that the quality of reagents remains through the life-cycle of the program.

QC of critical reagents

Concentration

Accurate measurement of reagent concentration is necessary for all subsequent steps of reagent characterization. There are multiple methods to determine concentration and the final method selection should be based on the type of reagents used (labeled or unlabeled), the storage buffer, typical concentrations expected and the throughput required in the bioanalytical lab. One of the most common methods is the spectrophometric absorbance measurement at 280 nm (A280). The available instruments that measure A280 differ in the volume requirements, LOD (may be as low as 60 µg/ml), dynamic range (may be as high as 200 mg/ml), throughput (1–96 samples) and ease of use. The addition of full spectrum scanning and advance analytics to the A280 measurement, allows the differentiation between A280 absorbance from tryptophan or tyrosine residues from interfering buffer components (NP-40 or Triton –X 100), contaminants substances or conjugates (SULFO-TAG) affecting the final calculation.

In addition, chromogenic methods can be used to determine concentration, such as the bicinchoninic acid method, the Lowry method or the Bradford method [12,13]. While these methods will quantify both unconjugated and conjugated molecules, they rely on the use of a reference standard curve for concentration determination, require larger amounts of sample and may be susceptible to interfering buffer components. The reference standard included in commercially purchased kits is typically bovine serum albumin (BSA), which may not be a suitable standard for all reagents. After addition of the reaction mixture, BSA can develop at a different rate from the test

material (due to different amino acid composition) and may not provide an accurate measurement if insufficient development time is allowed prior to readout. Alternatively, IgG reference standards may be used in chromogenic kits to quantify with accuracy the concentration of IgG-based reagents.

Characterization of purity & size estimation

Reagent purity is a key parameter to evaluate during any critical reagent characterization program [14,15]. Although industry and regulatory agencies recommend characterizing and documenting purity of critical reagents, none of them has defined the term of 'purity' for critical reagents [1,2,7,8,11,16]. While each sponsor may define purity for critical reagents, it is recommended to evaluate minimally the levels of reagent aggregates. Aggregation is the most common form of protein alteration for reagents and the increase of aggregate content may lead to nonspecific matrix interferences in LBA assays as well as altered assay signals [8].

There are multiple methods to evaluate purity and size of biological molecules. One of the most commonly used is size exclusion chromatography (SEC) [7,8]. SEC, either through HPLC or ultra-HPLC, respectively, can detect and quantify soluble biological aggregates with a size of 100 nm or lower [17]. These soluble aggregates are eluted earlier in comparison to the monomeric biotherapeutics. The SEC method must be optimized to the expected size and hydrophobicity properties of the biotherapeutic. Selection of the appropriate column size and mobile phase composition (salt, amino acid content and pH) is critical for accurate separation of peaks. Columns with a MW range of 10–500 kDa, an ethylene bridged hybrid particle of 1.7 µm and a pore size of 200 Å should provide appropriate resolution between monomers and aggregates for most IgG and IgG-like formats. SEC may also provide an estimation of fragments. However, the presence of low-MW species should be confirmed by orthogonal methods for appropriate sizing to discern whether they are fragments from the biological entity or buffer contaminants.

Another common tool in the evaluation of purity of critical reagents is the use of gel electrophoresis or capillary electrophoresis (CE) chromatography. Both the SDS-PAGE and CE-SDS are suitable analytical tools to estimate the presence and size of low-MW protein fragments in IgG-like molecules and recombinant proteins.

Analytical ultracentrifugation (AUC) is an orthogonal tool that may be used in instances where SEC data show possible chromatographic bias. Although, chromatography bias may be resolved with the addition of low concentration organic solvents into the mobile phase, this poses the potential risk of masking contaminant peaks in the chromatogram by forcing multiple peaks into one. AUC on the other hand, relies on the use of gravity or a centrifugal field to gauge information on the mass (MW), size and shape of biomolecules. AUC measures radial concentration distribution, by scanning at intervals ranging from seconds (for velocity sedimentation in a centrifugal field) to hours (for equilibrium sedimentation under gravity). Although AUC requires practically no sample preparation, this procedure needs an analytical ultracentrifuge, specialized software tools and scientific expertise to interpret data [18]. Therefore, AUC should be used as an orthogonal method to troubleshoot and resolve the presence of contaminant peaks on the SEC chromatogram. It should not, however, be used as the method of choice to determine purity.

Small differences in aggregate content may be observed between aliquots of the same reagent as well as between instruments and time points. Therefore, the changes in aggregation should only be considered a concern when there is a substantial change in purity that could put at risk the performance of the assay. In our laboratory, we have observed that as little as a 10% change in purity can have an impact in ADA assays. While there are approaches to remove large amounts of aggregates in a critical reagent (e.g., purification by fast protein LC), the recovery yield can be severely impacted. Generally, if the critical reagent of similar quality can be readily resupplied, it may be reasonable to purify and accept the recovery loss as future lots can be generated. However, if the reagent is commercially sourced or contracted it may be best to discuss minimum requirements of purity during the contract negotiation with the supplier [7].

Characterization of critical reagent identity

Understanding the robustness and reproducibility of the reagent generation process requires the use of multiple tools that can confirm the identity of the reagent [14,15]. Antibody variable (V)-gene sequencing and LC–MS evaluation of intact mass are methods that can determine identity and help ensure that subsequent lots can be generated in a reproducible manner.

When mAb anti-Ids are generated from hybridomas, it is essential to confirm clonality. Hybridomas that do not undergo at a minimum two rounds of subcloning can potentially express more than one heavy or light chain [19,20]. Uncloned hybridomas can also lose expression of either the heavy- or light-chain gene over time due

to genetic instability. Therefore, it is important to evaluate the heavy- and light-chain nucleotide sequence to help confirm clonality and uniqueness of the antibody. It also serves as a backup strategy in situations where the original hybridoma cell line loses genomic stability and fails to generate a new antibody lot. In these situations, a new lot may be generated with recombinant expression technology.

Evaluation of intact mass of both reduced and nonreduced deglycosylated anti-Ids can aid in the confirmation of clonality and appropriate pairing of heavy and light chains. Time-of-flight MS (MS-TOF) has become the more common technology for qualitative assessment of biotherapeutics and proteins by LC–MS; however, analyzers such as triple quadrupoles and ion traps have also be used [11,21]. Assessment of intact mass by LC–MS is not quantitative in nature. Therefore, orthogonal techniques such as SDS-PAGE or sodium dodecyl sulfate-CE should be used to provide a relative quantification of the levels of additional heavy or light chains, as well as mispaired dimers observed via LC–MS.

Multiple lots of the same anti-Id mAb should provide, in most cases, identical intact masses via LC–MS if generated either through transient recombinant expression or through a hybridoma cell line after at least two rounds of subcloning. In situations where bridging of anti-Ids in a validated PK assay fails, understanding the identity will help focus resources on troubleshooting activities to unveil the cause of bridging failure and obtain solutions for a path forward. The importance of LC–MS use for the characterization of critical reagents, has been reviewed in depth elsewhere [11].

Reagent pairing evaluations

Most PK assays utilize a pair of drug specific anti-Id mAbs as capture and detection reagents. A successful anti-Id campaign can generate 20–30 unique mAbs for testing in PK assay method development. Optimal anti-Id pairs may be selected in advance of method development through either epitope binning evaluation or on ligand-binding immunoassay platforms (ELISA, Meso Scale Discovery [MSD] or GyroLab). Epitope binning, typically used in drug discovery to identify antigen heterogeneity, has been proposed as a predictive tool for selection of optimal antibody pairs for immunoassays [22–26]. Regardless of the platform used, the goal should be to select multiple suitable candidate pairs rather than to identify a single pair for early methods development. This is because performance of anti-Id pairs can differ substantially depending on the platform used. For instance an antibody pair that shows optimal performance on epitope binning evaluation or ELISA may not show the same assay profile on GyroLab [27].

Other considerations in the choice of reagent pairing platform are: the ability of having simplified workflows, the platform's dynamic range and the availability of robotic systems that can offset the time that is required for testing manually hundreds of possible pair combinations. Although ELISA can offer a simplified workflow due to the end-point read out, a typically narrow dynamic range (e.g., 1 log) may prevent differentiation in the performance between pairs with oversaturated signals. In contrast, technologies with broader dynamic ranges (MSD or GyroLab) may allow better differentiation between pairs. Epitope binning is an attractive tool, as it does not require conjugated antibodies like most ELISAs, MSD or GyroLab assays do. However, method optimization and pre-emptive affinity ranking of anti-Ids is recommended to facilitate data interpretation. It is not recommended, however, to combine in the evaluation of epitope binning high with low-affinity antibodies. This is because the high-affinity antibody may displace the low-affinity counterpart during the epitope binning assay and provide a false positive result [24,28].

The importance of reagent conjugation design in PK & ADA assay robustness

Conjugation of critical reagents should be carefully designed with the goal of creating the most stable conjugate with the highest level of purity that will provide optimal assay performance [29]. Two main variables should be considered during the design: the conjugation chemistry selection and the degree of labeling (DoL) control [10,15]. Both variables require a good understanding of the amino acid composition as well as an empirical approach to achieve the desired LBA performance.

Conjugation chemistry selection

Critical reagents used in PK, ADA or neutralizing antibody (nAb) assays are commonly conjugated to either biotin, horseradish peroxidase, SULFO-TAG, fluorescent dyes or digoxin [11]. A common chemical approach used to attach labels is the cross-linking of conjugates to primary amines or thiol groups via an amide coupling or a sulfhydryl reaction, respectively.

The decision on the form of biotin to use should be based on their unique characteristics and properties such as spacer-arm length, reactive site, cleavability and solubility (hydrophobicity). For example, nonsulfonated biotinylation reagents such as iodoacetyl-LC-biotin or biotin-BMCC (1-biotinamido-4-[4′-(maleimidomethyl)-cyclohexane-carboxamido] butane) generate a nonreversible thioether bond with a long spacer arm to prevent binding interferences. However, nonsulfonated biotins are hydrophobic and need to be solubilized in organic solvents prior to be added to an aqueous solution reaction. In contrast, sulfonated biotinylated reagents such as sulfo-NHS-LC-biotin (sulfo-succinimidyl-6-[biotinamido] hexanoate) or sulfo-NHS-SS-biotin (sulfosuccinimidyl 3-[[2-(biotinamido) ethyl] dithio] propionate), are water soluble allowing for direct incorporation into aqueous solutions. These sulfonated reagents, nevertheless, bind covalently to primary amines by incorporating a nonpolar hydrophobic C6 alkyl chain spacer. The incorporation may lead to slow aggregation through time and loss of activity due to increase reagent hydrophobicity [30].

Additional forms of biotin such as the NHS-PEG_n-biotins offer increase water solubility without the incorporation of nonpolar spacers. They are commercially available in a variety of chain lengths from 4 to 12 unit polyethylene glycol (PEG) groups. The hydrophilicity of the PEG spacer reduces the risk of slow aggregation during long-term storage. Longer PEG spacers also allow greater distance between the biotin and other interacting molecules minimizing steric hindrance in the bioassay [31].

Although amine and thiol conjugations are highly effective in generating most conjugated reagents, the potential random nature of this labeling reaction may lead to insufficient or excessive incorporation or loss of functionality in some bioassays. The level of incorporation is associated with the number of exposed residues, which will differ between molecules with different amino acid content or tertiary structure characteristics. The decrease in LBA activity may be caused by under or overconjugation of the molecule or by labeling of residues key to the functionality of the anti-Id, recombinant protein or drug substance.

If optimization of standard conjugation through empirical testing is unsuccessful, alternative techniques such as enzyme-mediated protein ligation or N-glycan azide modification ('click' chemistry) may be used for a more directed approach. Enzyme-mediated protein ligation can be accomplished with the use of Sortase A (*Staphylococcus aureus*, transpeptidase) or BirA (*Escherichia coli* ligase). It requires, however, a specific reactive peptide tag motif to be engineered into the protein sequence to allow the enzyme cleavage prior to nucleophilic attack and conjugation to the desire label [32]. Conversely, click chemistry does not require protein engineering but rather the enzymatic modification of antibody N-glycans present on the heavy chain. The antibody N-glycan G1/G1F and G2/G2F structures are converted to G0/G0F glycans with the use of β-galactosidase. The G0/G0F glycan is then modified with the addition of azide using galactose-1-phosphate uridyltransferase enzyme. A (2 + 3) cycloaddition between the azide intermediate and dibenzocyclooctyne biotin generates the final pyrazole biotin conjugate. The drawback of this chemistry is its dependence on a commercially supplied kit, which can limit the scalability of the labeled material [33]. Conjugates generated through a targeted approach do not necessarily have improved stability over conjugates generated through lysine or thiol chemistry approaches. The stability will ultimately depend on the type of label selected, the extent of label incorporation and the selection of storage buffer.

Controlling the DoL

The optimal DoL will vary from one molecule to another, and thus, should be empirically determined. Controlling the level of conjugate incorporation is particularly important for hydrophobic labels with a relatively large MW such as SULFO-TAG or fluorescent dyes (>1000 g/mol). Excessive incorporation of these labels may impact the tertiary structure, biophysical properties and binding kinetics of the molecule.

There are multiple laboratory-controlled parameters that can impact DoL. An optimal DoL can only be achieved if multiple conditions are tested empirically. A recommended strategy is to use a checkerboard approach to test desalting conditions, several molar ratios of protein:label, incubation temperatures and incubation times to achieve the desired DoL needed for optimal assay performance [3]. In particular, addition of several molar ratios helps in the understanding of both nonoptimal and optimal incorporations and their correlation with assay performance.

Within the checkerboard, an important parameter to focus on is the selection of desalting method prior to conjugation. This first step is critical to ensure all nucleophilic buffer components in the storage/formulation buffer are completely removed. A nucleophile, in this context, is defined as a chemical component in standard formulation buffers (i.e., Arg) that has the ability to donate an electron to the label ester compound. When these components are present during the conjugation reaction, they can consume the active label available in the reaction

lowering the effective challenge ratio. Therefore, inefficient removal of nucleophilic factors can lead to lot to lot inconsistency in the labeling process [3].

Buffer exchange methods range from well-known technologies such as dialysis, centrifugation techniques and tangential flow filtration (TFF) systems to newer robotic pressure-based filtration technologies. Dead-end filtration methods such as the Amicon® or Zeba™ spin columns, are cost- and time-effective ways of conjugating small quantities of biological material. However, the unidirectional force of conventional centrifugation may cause the filter membranes to clog, due to the collapse of biological molecules on the membrane, increasing the risk of aggregate formation. Aggregation can be prevented with dialysis; however, it is low throughput due to the long buffer exchange time it requires. TFF, on the other hand, requires a large volume of antibody solution and a single streamline process. Therefore, TFF is more appropriate for the generation of antibody–drug conjugates in a manufacturing facility and less versatile for the standard bioanalytical lab. New robotic pressured-based filtration systems perform buffer exchange by applying uniform pressurization to a chamber while mixing multiple parallel samples. This technique, therefore, prevents the aggregation and loss of material observed in dead-end filtration methods while maintaining the throughput that cannot be achieved with dialysis. The aforementioned buffer exchange methods should also be used as a purification step after the conjugation reaction is completed, to remove unreacted excess label. Insufficient removal of unreacted label can lead to increase background in many bioanalytical assays.

Evaluation of DoL

Evaluation of DoL may be performed with the use of commercially available kits such as biotin quantitation kits (HABA) or spectrophotometric methods like 455-nm measurement for SULFO-TAG conjugates. These methods, provide an average estimation of label incorporation. Although these kits offer a convenient way to quickly assess whether the molecule was successfully tagged or not, they can fall short when trying to understand the impact of different lot conjugates in LBA performance. This is because the conjugation process not always leads to labeling of 100% of the molecules [10]. The percentage of unlabeled molecule can be observed by estimation of DoL on a MS-TOF instrument. The MS-TOF evaluates the intact mass DoL by measuring the mass shift of the intact deglycosylated nonreduced conjugate in comparison to its parental counterpart. Therefore, the intact mass DoL can provide the relative abundance or biodistribution of different species of conjugated molecules with various degrees of incorporation, from no incorporation (+0 labels) to very high incorporation (+10 or higher). The relative abundance is estimated from the deconvoluted spectra of integrated peaks, providing an estimation of the percentages of label distribution in the conjugate. A typical successful conjugation should lead to a Gaussian distribution of the labeled material. If intact mass DoL data are available, it is important to ascertain the amount of labeled and unlabeled reagent that the assay can tolerate to ensure assay performance, long-term stability and robustness. The DoL biodistribution observed by MS-TOF, may also provide a fingerprint that can serve as a QC parameter for the generation of subsequent batches to ensure lot-to-lot consistency. Although the MS-TOF instrument is not essential in the measurement of DoL, it is a helpful tool in troubleshooting the impact of conjugates in LBA performance.

The importance of in-depth reagent characterization in PK & ADA assay robustness beyond standard QC testing

Determination of purity by SEC during the QC process, provides information on soluble aggregate content within a narrow size (~100 nm), and therefore, it could potentially miss the presence of large soluble or insoluble aggregates [17]. Furthermore, although maintaining a high level of reagent monomer content is important for assay robustness, other molecular changes not detectable by SEC may also contribute to changes in assay performance [3]. For instance, purity does not provide information on the potential for reagent unfolding that could also influence functionality. Methods such as light scattering (LS; static LS [SLS] or dynamic LS [DLS]), full spectrum fluorescence and isoelectrofocusing may be useful techniques in understanding biophysical changes in critical reagents and their impact on performance [3].

Regardless of the methodologies used for in-depth reagent characterization, the main objective should be to establish a biophysical profile that enables consistency in lot preparations [7]. Additionally, reagent characterization should help in the understanding of LBA performance and data interpretation. The reagents' biophysical characteristics and the LBA data should be integrated together so that the reagent characterization efforts can assist in the interpretation of specific assay issues [7].

Full spectrum fluorescence

Full spectrum fluorescence evaluates the intrinsic fluorescence properties of a molecule by measuring the natural fluorescence emission of tryptophan and tyrosine amino acid residues. When a protein unfolds, tryptophan and tyrosine residues will be exposed leading to a change in the intrinsic fluorescence signal that correlates to conformational changes [34]. The full fluorescence spectrum allows the measurement of low-intensity peak shifts as the protein unfolds. When the data are obtained during a defined temperature range that is set to change at a defined rate (thermal ramp), the information can be used to generate a melting temperature point. Melting temperature point could be used to rank the robustness between molecules with the same amino acid composition (i.e., comparison between unlabeled and labeled reagents at multiple challenge ratios). This approach could help in the selection of conjugation conditions that will lead to optimal assay performance and robustness.

Light scattering

LS is a technique that uses the concept of 'elastic scattering'. Elastic scattering occurs when a laser hits a particle's electrons, which in turn re-emit the same radiation in all directions. LS measures molar mass and particle size of biological macromolecules, and therefore, it can measure soluble protein aggregates from approximately 1 nm to 5 µm in range [17]. There are two types of LS, SLS and DLS. SLS (measured at 266 and 473 nm) can measure both small and large aggregates at low and high protein concentrations. In SLS, the scattering intensity increases as the protein aggregate size increases (mean solute mass in kDa). DLS, on the other hand, measures the size distribution within a solution (polydispersity or sample uniformity) by calculating the nonrandomness from the random diffusion that occurs during Brownian movement. When LS is coupled to a thermal ramp, the aggregation temperature can be calculated to determine the tendency of a molecule to aggregate under stressed conditions [35]. Both SLS and DLS provide additional information that may help understand the impact of conjugation in the robustness of critical reagents.

Isoelectric point

Isoelectric point (pI) is another technique that may be used to evaluate the biophysical property changes after the introduction of the label. The pI is the pH at which a molecule has a neutral electrical charge. The pI can be determined by isoelectric focusing, where a current separates the molecules according to their pI on a pH scale [11]. The pI of a labeled molecule should be compared with the parental unlabeled molecule to determine whether a shift in pI has occurred as a result of the conjugation procedure. These data will inform the selection of optimal formulation buffer conditions to prevent aggregation of the reagent during storage. Large changes in pI may also lead to impact in assay performance, and therefore, this tool may serve as an additional variable to select optimal labeling conditions [3].

Critical reagent stability testing & characterization to inform buffer formulation, handling & storage

Clinical PK and ADA assays may likely need to be maintained for almost a decade or longer in most successful programs. This creates the challenge for the sponsor to choose to supply assay reagents with multiple small lots or a single large lot. Supply of a single large lot is an attractive idea to avoid the need for bridging of multiple new lots in the assay. However, it poses the additional challenge of ensuring reagent long-term stability to prevent assay drift through time [10]. Thus, careful consideration is needed to support formulation and long-term stability of critical reagents.

Buffer formulation

One potential factor that can improve the long-term stability of reagents is the careful selection of the formulation buffer [15]. It is not practical, however, to perform a formulation buffer study for each reagent tool in support of regulated bioanalysis. Since each drug program could have multiple tool reagent molecules, using standard buffers has both scientific and practical advantages. From a scientific perspective, many biophysical properties of molecules are buffer dependent, thus making comparisons in the robustness of tools across data sets more feasible. From a practical perspective, the use of standard buffers streamlines lab protocols and simplifies the preparation and storage of a few buffers sets.

In order to determine the best set of formulation buffers to support a reagent life-cycle program, several factors should be taken into account. These considerations include but are not limited to the pIs of the molecules, preferred

storage temperature, the need for stabilizers and antimicrobial agents [2]. The pH of the formulation buffer may induce changes to the tertiary and quaternary structure of a reagent. It may also impact its solubility and tendency to aggregate [7]. A standard buffer should have sufficient buffering capacity at the selected pH, be appropriate for protein stability and be compatible with multiple analytical techniques. The buffering capacity will be dependent on the dissociation constant of the selected weak acid or base and the corresponding salt [36]. As an example, a buffer containing acetate should be adjusted to a pH close to 5.0. This is because the buffering capacity of acetate is around the pKa of acetic acid (3.7–5.6). Selecting an appropriate pH should also be based on the pI of the molecule. Ensuring that the pH of the buffer does not overlap with the pI of the molecule, allows the molecule to be electrically charged, improves solubility and prevents aggregation and precipitation through long-term storage. Recommended buffer components that could be used in critical reagents with a pI >7.0 (i.e., mouse mAbs or recombinant proteins with a human Fc tag) are sodium acetate/NaCl at approximately pH 5 or histidine/NaCl at approximately pH 6. Molecules with pI <7.0 (i.e., rabbit antibodies) may need alternative salts with a higher pH buffering capacity such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or phosphate-buffered saline. If HEPES is selected, HEPES+ NaCl at neutral pH should provide optimal buffering capacity.

Additionally, a cryoprotectant may be added to the formulation buffer to minimize the effects of freeze–thaw cycles. Cryoprotectants prevent the formation of ice crystals, which destroy protein structure. This effect is believed to occur either through vitrification or by water replacement [37]. Recommended cryoprotectants are sucrose or trehalose due to their compatibility with downstream analytical techniques such as SEC, allowing for on-going long-term stability testing. Other common cryoprotectants such as glycerol also have the ability to prevent ice crystals by minimizing degradation through vitrification. However, glycerol may lead to downstream analytical challenges making it less compatible with a long-term reagent life-cycle management program. A tailored approach should be developed to empirically test stability of reagents under a predetermine set of standard cryoprotectants and formulation buffers to maximize long-term reagent ruggedness and robustness. If desired, antimicrobial agents such as sodium azide or ProClin™ 300 may be used to prevent contamination. This is particularly important when buffers containing sucrose, trehalose or acetate are used in reagents stored at 4°C to avoid bacterial contamination and degradation of critical reagents [2,8].

Long-term stability testing

For critical reagents, the term retest date is preferred to expiration date because more often than not the date can be extended if reagent quality can be demonstrated after a retest date [2]. By assigning a retest date instead of an expiration date, a continuous monitoring of critical reagents can be achieved. Stability testing of each critical reagent lot should be performed to inform on the physical intactness of the molecule as well on its functionality during prolonged storage. Special consideration should be taken to aliquot size and headspace during reagent stability studies, to mimic as close as possible or practical the actual inventory vials.

The long-term monitoring of critical reagents should be performed with the same methods utilized during the original characterization and should be supported by LBA performance (system suitability) [7]. A simplified way of performing real-time long-term stability testing is using SEC, surface plasmon resonance (SPR) and/or assay system suitability. The advantage of using SEC and SPR as complementary tools to assess system suitability is that these techniques may identify physical and functional changes in critical reagents before there is an actual impact in the assay. Changes observed by SEC and SPR may occur before assay system suitability is compromised. In the case where changes are observed, the retest date may be extended based on system suitability for a short period, while a new lot of the reagent is generated and bridged successfully in the method. This information, therefore, mitigates the risk of assay failures and delays due to an unanticipated decrease in the quality of critical reagents. A retest date scheduled from 2–5 years for antibody molecules and 6–12 months for recombinant proteins and peptides stored at -70°C or below is an acceptable practice, and would provide confidence in the reagent without overwhelming internal testing capacity [3,7].

Stability prediction & reagent handling

Stability testing of critical reagents should also inform on best practices for handling the reagent during laboratory activities. Once a reagent is thawed, it is important to understand whether maintaining the remaining aliquot at 4°C or subjecting it to a freeze–thaw cycle are likely to impact the stability and performance of the reagent in future assay runs. Appropriate handling may be predicted by freeze–thaw stress testing and accelerated stability testing studies. A critical parameter that plays an important role in freeze–thaw stress testing of a critical reagent is the

freezing rate. This parameter is challenging to predict, due to the lack of a specific instruments that can monitor the exact freezing temperature and freezing rate of each reagent. A stress freezing condition test that simulates a slow rate of freezing or mishandling conditions may be used to unveil vulnerabilities in the robustness of reagents. One approach is to place a test tube in the center of a prefilled 81-sample box containing 500-µl screw top vials prefilled with water at room temperature. In our experience, we have observed that this test allows us to uncover nonrobust reagents for which alternative buffer components can be tested to stabilize further the reagent.

Stability at 4°C after thawing a reagent may be predicted with the use of accelerated stability testing, utilizing the Arrhenius equation and the Q rule [2,38–40]. The Arrhenius equation describes mathematically the relationship between storage temperature and degradation rate, based on the activation energy required to exceed a molecule's energy barrier for degradation to occur. It is not realistic to measure the activation energy for each molecule tested, and therefore, this variable is typically assumed through the Q rule. The Q rule is a mathematical short cut that assumes that product shelf life decreases by a constant factor (Q10), when the storage temperature is lowered by 10°C. The Q10 value may be set to 2, 3 or 4 based on how much energy is assumed to be needed in order to exceed the energy barrier (the higher the number, the higher the energy needed for degradation, and therefore, the less conservative the assumption). According to this rule, a large temperature shift will lead to an exponential change on the assumed activation energy [39]. Therefore, the Arrhenius equation and Q rule may be used together to correlate the impact of extreme temperatures to a biological molecule for a short period of time to the impact at lower temperatures for a longer period of time (e.g., 1 week at 37°C may be equivalent to ~3 months at 4°C). The following formula serves to predict the number of days at which a reagent may be stored at 4°C after testing purity and functionality for at 37°C for 8 days.

Calculation of exponent to Q10:

$$n = \frac{Testing\ \ temperature - Pedicated\ \ Temperature}{10} \quad n = \frac{37 - 4}{10} = 3.3$$

Therefore, if a reagent is stressed at 37°C for 8 days, the number of days predicted to be stable at 4°C may be calculated with the following formula, assuming conservatively that a low activation energy will be needed to exceed the energy barrier:

$$number\ \ of\ \ days\ \ at\ \ predicted\ \ temperature = number\ \ of\ \ days\ \ at\ \ testing\ \ temperature \times (Q10)^{n}$$

$$Days\ \ at\ \ 4C = 8 \times (2)^{3.3} = 79$$

Although these mathematical models may predict stability, the assumptions applied may be incorrect and should not be considered as 'bullet proof' methods. Prediction of stability through stress testing should be confirmed through real-time stability. It is also widely accepted in industry that retest dates of critical reagents should be set from experience based on functional assay performance [41,42]. The acceptance criteria for functional performance should be set prior to stability testing [41]. In our experience, we have observed that a decrease in monomeric content of 10% or greater leads to substantial changes in the performance of drug bridging assays.

Documentation

While formulation buffers and stability predictions are important, the documentation of critical reagents is equally important. The first step to practicing appropriate documentation is to have a formal procedure described in a standard operating procedure [2,14,41]. The standard operating procedure should include the naming convention to allow for easier inventory management [8]. Additionally, the minimum information that should be included in the documentation is the name of the reagent, lot number, source, modifications, catalog number (for commercial reagents), concentration, retest date, manufacture date and storage condition recommendation [2,41]. Additional information includes purification method, buffer composition and a summary of purity, identity and functionality tests performed to date. Once all the information is documented, the characterization/stability data should be integrated appropriately into an inventory system for ease of use and retrieval [7,8].

Case studies

Below are several case studies to highlight important aspects of critical reagents. These case studies focus on impact on ADA assays and were presented at the 2019 Workshop on Recent Issues in Bioanalysis [3]. While critical reagent practices can impact PK assays, it is important to note that in general it may be easier to troubleshoot problems in PK assays due to their quantitative nature. On the other hand, ADA assays are qualitative, so monitoring of assay performance and troubleshooting can be more challenging.

Case study 1: impact of PC aggregation on ADA assay performance

In support of a Phase I clinical trial of an IgG1 biotherapeutic mAb, a mouse anti-Id mAb was developed as a PC for a validated ADA assay. In order to prepare for the additional trials, a second lot of the same anti-Id ('lot B') was generated. Attempts to bridge lot A and B of PC were unsuccessful in a titration experiment as demonstrated by substantially lower relative light units by lot B. An investigation revealed that lot A contained higher amounts of aggregate compared with lot B (13.2 vs 2.4%, respectively). The higher aggregate content in lot A corresponded to the lower percentage of monomer content in this lot. It was concluded that the higher aggregate content in lot A contained multimers of the PC that resulted in an artificially higher assay signal, which could not be replicated with material with a lower aggregate content. In addition, it was also discovered through a separate investigation that this PC displayed human-anti-mouse antibodies (HAMA) binding activity. The HAMA reactivity made this reagent not ideal for use as a PC in an ADA assay, due to the background issues that can be caused by HAMA. Even though this method had already been validated and used to analyze clinical samples, it was determined that the best course of action was to replace this suboptimal mouse anti-Id PC with another anti-Id that lacked the biophysical potential to aggregate and was devoid of HAMA activity. This case study highlights the importance of understanding the binding and biophysical properties early in the critical reagents workflow to avoid a method failure during clinical sample analysis.

Case study 2: impact of desalting procedure on nAb assay development

As part of the procedure to increase drug tolerance for a cell-based nAb assay for an IgG1 biotheraputic mAb, a bead extraction method was employed to remove excess drug from serum samples. The extraction step required biotinylated drug (sulfo-NHS-LC-biotin) for binding to streptavidin magnetic particles. In order to biotinylate the drug, buffer exchange and desalting was needed. Two commercially available desalting products were tested: one using a spin desalting column, the other using a centrifugal filtration device. It was determined that the biotin material desalted with the centrifugal filtration device yielded a fourfold higher signal at the low PC signal compared with the material prepared with the desalting spin column. Analysis by MS-TOF revealed that the centrifugal filtration device yielded a broad distribution of biotins, with 1–7 biotins per molecule and 5% of molecules containing no biotins. On the other hand, the desalting spin column yielded a narrower distribution of biotins, with 1–4 biotins per molecule and 30% of molecules containing 0+ biotins. The cause for differences in biotin incorporation and assay performance between the two desalting procedures was not fully elucidated. However, it was postulated that the desalting spin column did not optimally remove the original formulation components, which in this case included arginine, a nucleophile factor. This case study underscores the importance of identifying the appropriate labeling procedure to optimize assay performance.

Case study 3: impact of handling & storage on critical reagent function

Following freeze–thaw testing of a SULFO-TAG IgG1 biotherapeutic molecule used during validation of a clinical ADA method, the signal in the negative pool increased threefold. Assessment of purity by SEC (UPLC) did not reveal a change in aggregate content, and increase in aggregates was ruled out as a cause for the increase in the background signal. In an attempt to purify further the labeled material, a late eluting peak containing unreacted SULFO-TAG was removed. This purification step, however, did not reduce the signal back to the original background levels and suggested that other changes to the molecule not detectable by SEC were increasing the signal in the negative pool. A new lot of the conjugate was prepared using two desalting steps instead of one, the storage volume was increased to reduce the amount of headspace in the tube and the labeled material was stored in a more favorable buffer. These changes restored the stability and performance of the labeled material following freeze–thaw. Although the exact cause of the increase in background was not determined, this case study highlights the need for determining optimal labeling, formulation and storage conditions. This example also demonstrates molecules with comparable

chromatographic profiles can have other molecular differences not easily detected based on size alone that can impact assay performance.

Conclusion & future perspective

While there are no specific guidelines for the management of critical reagents, there is increasing awareness in the need for careful selection, characterization and handling [3,14,15,43]. It is incumbent on the pharmaceutical/biotechnology industries and CROs to adopt appropriate practices and quality standards to ensure critical reagents achieve reliable and reproducible performance during the life span of a product's clinical development. With the increasing number of more complex mAb-based drugs, it should be anticipated that the PK and ADA assays themselves would need more reagents to measure multiple analytes. The implementation of technologies commonly used for biophysical characterization of biotherapeutic drugs is becoming of increasing value for understanding critical reagent robustness and performance. This knowledge will be essential as new bioanalytical assay platforms, with the potential to improve sensitivity, may require the use of well-characterized conjugated reagents to ensure lot-to-lot consistency and assay performance. Biophysical characterization of reagents is at present out of reach for many bioanalytical laboratories. However, the advent of new user-friendly, compact and versatile instruments as well as vendor capability growth in reagent life-cycle management, should help bridge the gap in the next 5-10 years.

Executive summary

- Laboratories should develop a comprehensive workflow starting from a reagent generation design through life-cycle management plan. This will ensure that quality of critical reagents is implemented at every step and is maintained throughout clinical development.
- The selection of critical reagents in ligand-binding assays requires a strong understanding of the bioanalytical objectives and needs to be tailored for each biotherapeutic agent.
- Purity, concentration and activity need to be established early and maintained throughout the life-cycle management of critical reagents.
- Approaches to characterize biophysical properties such as size exclusion chromatography, biolayer interferometry, SPR, AUC, LS and pI can be helpful in identifying suitable reagents for methods development and for troubleshooting.
- Appropriate selection of formulation buffer, storage and handling should be established before assay validation and sample analysis.
- Careful consideration for selection, labeling and handling of critical reagents, particularly for antidrug antibodies assays, is especially important. This is because improper selection or mishandling of positive controls, or selection of binding or biophysical issues, may lead to changes in assay performance that cannot be easily monitored in a qualitative assay.

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Methodology

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Bioanalysis

Characterization of robust immune responses to a bispecific antibody: a novel class of antibody therapeutics

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Background: Anti-A/B is a bispecific monoclonal antibody that blocks activities of soluble targets A and B. Robust immune responses were observed in a multiple-dose cynomolgus monkey toxicology study, negatively impacting the toxicokinetics/pharmacodynamics profile of anti-A/B in some animals. This was unexpected as similar findings were not observed in the two previously studied parental molecules. **Methodology & Results:** This paper discusses our characterization strategy for evaluating the immunogenic domain(s) of anti-A/B and our mitigation plan to monitor immunogenicity in the first-in-human clinical study. The characterization results from the cynomolgus monkey and Phase I studies are discussed. **Conclusion:** The characterization strategy discussed informed understanding of immunogenicity results and clinical impact, which can be broadly applied to other molecules with multiple-binding domains.

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Keywords: bispecific monoclonal antibody • bridging ELISA • immunogenicity • pharmacodynamics • toxicokinetics

In recent years, advancements in bioengineering technology have expanded the diversity of antibody therapeutic modalities. Bispecific monoclonal antibodies (bsmAbs) are a novel class of monoclonal antibodies (mAbs) that have gained recent attention with the aim of improving drug efficacy by simultaneously binding to two targets in an effort to increase efficiency for treatment of complex diseases [1–3]. Bispecific molecules provide exciting opportunities for novel drug design and development; however, they also pose unique development challenges. Additional studies are needed to evaluate this unique class of therapeutics as there is currently relatively limited experience in developing bsmAbs compared with conventional bivalent mAbs. The recently published US FDA draft guidance for bsmAb development [4] provides general considerations and recommendations for bsmAb development programs, as well as regulatory, quality, nonclinical and clinical considerations in the context of those programs. This is the first FDA guidance on this topic and is informative to guide industry in the development of bsmAbs.

All protein therapeutics have the potential to induce anti-drug antibody (ADA) responses in patients. It is essential to monitor and understand the clinical relevance of this response to ensure the safety and efficacy of the potential therapeutics during drug development [5]. There are different types of ADAs: ADAs with no clinical impact, ADAs with impact on drug exposure and safety. The impact of ADAs should be monitored and managed during clinical trials. For example, presence of drug clearing as well as neutralizing ADAs can lead to reduced drug exposure; therefore impacting the pharmacokinetics/pharmacodynamics (PK/PD) and efficacy. In addition, ADAs that lead to clinical safety need to be monitored, necessitating the need for sensitive ADA assays that are drug tolerant [6–8].

Anti-A/B is a bsmAb therapeutic that binds to two targets A and B, which are implicated in the pathogenesis of inflammatory diseases. A and B are presented as the soluble forms only *in vivo*. The mechanism of action (MOA) for anti-A/B does not require simultaneous binding of the two targets. Anti-A/B was produced in *E. coli* through hetero-dimerization of the two half antibodies anti-A and anti-B, which is driven by the knob-into-hole (KIH) modifications made in the CH3 domain of the Fc region [9]. In a multiple-dose cynomolgus monkey toxicity/toxicokinetic (TK) study of anti-A/B, a high incidence (97%) of ADA response was observed. The observed ADA response negatively impacted the TK/PD profile of anti-A/B in some animals. In addition,

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a correlation between higher ADA titer levels and reduction of serum drug concentrations as well as PD effect was observed. Anti-A/B was generated from two parental mono-target and bivalent mAbs anti-A and anti-B. Both parental molecules were evaluated in nonclinical and clinical studies previously; however, neither molecule had the levels of ADA response that were observed with the anti-A/B. Therefore, the high ADA incidence and response of anti-A/B observed in the cynomolgus monkey TK study was unexpected. Characterization work was conducted to understand the immunogenic epitope(s) of anti-A/B. Because of lack of experience with this type of novel modality and the observation of the robust immune response in cynomolgus monkey studies, we revised our immunogenicity monitoring plan for the first in human clinical studies, including more frequent sampling and building in multiple interim analysis for earlier readouts. This paper discusses our considerations and strategy for assessing the ADA response of a bsmAb anti-A/B and summarizes the characterization results from the cynomolgus monkey toxicity/TK as well as Phase I study. Characterization of immunogenicity response to multiple domain biotherapeutics has been discussed elsewhere [10]. The strategy utilized in this study is another example of how this approach can be applied to characterize ADA response to bsmAbs.

Materials & methods

Materials

Anti-A/B, a humanized IgG bsmAb therapeutic with a KIH structure in the CH3 domain of the Fc region was produced in *E. coli* at Genentech (CA, USA). The surrogate positive control for the cynomolgus monkey ADA assay, a sheep anti-human IgG polyclonal antibody, was purchased from Binding Site (Cat# AU003CUS01, Birmingham, UK). Individual and pooled cynomolgus monkey serum samples were purchased from Bioreclamation (MD, USA). Three surrogate positive controls were used in the human ADA assay, including an affinity purified complementarity-determining region (CDR) specific goat polyclonal antibody to anti-A/B and two monoclonal anti-idiotypic antibodies to the anti-A and anti-B arms, respectively. All three surrogate positive control antibodies were generated in-house. Individual and pooled serum samples from healthy volunteers were purchased from Bioreclamation.

Conjugation of biotin and digoxigenin (DIG) to anti-A/B was carried out using EZ-Link SulfoNHS-LC-Biotin (Cat# 21335, Thermo Fisher Scientific, MA, USA) and 3-amino-3-deoxydigoxigenin hemisuccinamide, succinimidyl ester (Cat# A2952, Thermo Fisher Scientific), respectively, according to manufacturer instructions at a nominal 10:1 molar ratio of biotin or DIG to anti-A/B.

Anti-A/B cynomolgus monkey ADA assays

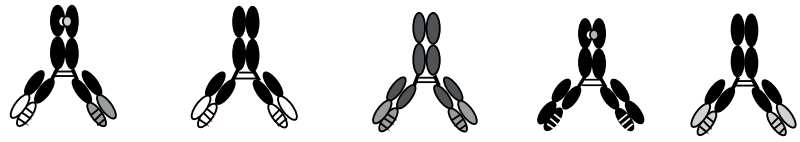
Screening assay

A homogenous bridging ELISA was developed and validated to measure anti-A/B ADAs in cynomolgus monkey serum. The assay format details were similar as described in our previous publication [11] however differed in a few assay conditions. In the anti-A/B cynomolgus monkey ADA assay, study samples were prepared at a minimum dilution of 1:20 in assay diluent (1x phosphate-buffered saline, 0.5% bovine serum albumin, 0.05% Tween 20 and 0.05% ProClin 300, pH 7.4); biotin-conjugated and DIG-conjugated anti-A/B at 2 µg/ml were co-incubated with prediluted controls or samples in a round-bottom polypropylene Costar plate (Cat#3365, Corning, AZ, USA) overnight at room temperature (RT) with agitation; horseradish peroxidase-conjugated IgG fraction mouse anti-DIG mAb (Cat# 200-032-156, Jackson ImmunoResearch, PA, USA) at 40 ng/ml were applied for detection.

The assay was validated at a contract research organization (CRO). A screening cut point factor (CPF) was established using a panel of serum samples from 100 drug naive cynomolgus monkeys. The screening assay threshold was then calculated to achieve a 5% untreated positive rate (UTPR). The assay relative sensitivity and drug tolerance were assessed using positive control sheep anti-human IgG polyclonal antibodies. Other assay parameters, such as matrix effect, hook effect, and specificity, were evaluated during assay validation according to recommendations described elsewhere [12,13] and will not be discussed in this paper.

Characterization assay

A competitive binding assay was developed by spiking five antibodies (each containing a specific domain of interest) into the sample using the same homogenous bridging ELISA described above to characterize samples that tested positive in the screening assay. Samples were incubated in the presence or absence of five characterization antibodies: unconjugated anti-A/B, anti-framework mAb (non-KIH isotype control), anti-KIH mAb (KIH isotype control) and anti-A and anti-B (variable domain control of each arm) (Figure 1). Samples were spiked with 0 or 25 µg/ml of



	Drug anti-A/B	Variable control (mAb or Fab) of arm A	Variable control (mAb or Fab) of arm B	Isotype control KIH	Isotype control w/o KIH
ADAs to framework	+	-	-	+	+
ADAs to KIH	+	-	-	+	-
ADAs to arm A variable	+	+	-	-	-
ADAs to arm B variable	+	-	+	-	-
ADAs to arm both A and B variable	+	+/-	+/-	-	-

Figure 1. Anti-drug antibody characterization strategy to understand the immunogenic epitope(s). + denotes significant percent signal reduction by competition; - denotes limited or minimum signal reduction by competition.

each characterization antibody individually and incubated at RT for an hour prior to analysis. The percentage of signal reduction from unspiked samples was calculated as follows: (signals of unspiked samples - signals of spiked samples) / (signals of unspiked samples) x 100. The confirmatory threshold for each characterization antibody was determined based on the percentage of signal reduction observed in serum samples from 20 drug-naive cynomolgus monkeys with a target UTPR of 1%. A sample with a signal reduction equal or above the confirmatory threshold of each characterization antibody was considered positive for that antibody.

Anti-A/B cynomolgus monkey toxicology study

Study design

An anti-A/B cynomolgus monkey toxicology study was conducted to determine the toxicity and TK of anti-A/B when administered at 14-day intervals (7 doses) via intravenous (iv.) or subcutaneous (sc.) injection to cynomolgus monkeys for at least 13 weeks. Anti-A/B was administered at dose levels of 0 (Group 1, n = 10) or 10 and 30 mg/kg/day to three animals/sex/group (Groups 2 and 3, respectively) via iv. route and 100 mg/kg/day via iv. or sc. (Groups 4 and 5, respectively) to five animals/sex/group. ADA samples were collected on days 0, 14, 28, 56, 83 and 143 post drug administration as part of a drug toxicity evaluation. Additional samples at the ADA sampling time points were also collected for TK and PD biomarker measurements.

ADA sample analysis

The above-described cynomolgus monkey ELISA was validated and used for analysis of ADA samples from an anti-A/B multiple dose cynomolgus monkey toxicology study. Samples were first tested in the screening assay and screened positive were titered by performing a minimum dilution of 1/20 in assay diluent followed by two-fold serial dilutions with titer diluent. Titer values were calculated and reported as the log10 of the dilution at which the obtained signal would equal the assay cutpoint. Titer values were used to determine the magnitude of ADA positive signals. Samples that screened positive were analyzed in the characterization assay.

Anti-A/B human ADA assays

Screening assay

The clinical anti-A/B ADA screening assay used a similar homogenous bridging ELISA format as the cynomolgus monkey ADA assay described above with a few exceptions in assay conditions. The clinical ADA assay had a minimum sample dilution of 1:50 in assay diluent and a master solution containing 3 µg/ml each of biotin- and DIG-conjugated anti-A/B.

The assay was validated at a CRO. A screening CPF was established using a panel of serum samples from 50 drug naive healthy volunteers. The screening assay threshold was calculated to achieve a 5% UTPR. The assay relative sensitivity and drug tolerance were assessed using an affinity purified CDR specific goat polyclonal antibody to anti-A/B. Other key assay parameters, such as matrix effect, hook effect and specificity, were evaluated during assay validation according to recommendations described elsewhere [12,13] and will not be discussed in this paper.

Confirmatory assay

A competitive binding step was added to the same homogenous bridging ELISA described above to confirm samples that tested positive in the screening assay. Diluted samples were incubated in the presence or absence of unconjugated anti-A/B prior to analysis. The percentage of signal reduction from unspiked samples was calculated as following: (signals of unspiked samples - signals of spiked samples) / (signals of unspiked samples) x 100. The confirmatory threshold for anti-A/B was determined based on the percentage of signal reduction of serum samples from 50 drug-naive healthy volunteers, with a target UTPR of 1%. A sample with a signal reduction equal or above the confirmatory threshold of each characterization antibody is considered positive for anti-A/B.

Characterization assay

The characterization assay is similar to the confirmatory assay described above, with the exception of using anti-A Fab and anti-B Fab as the competing reagents to incubate with samples prior to analysis. The two monoclonal anti-idiotypic antibodies to the anti-A and anti-B arms were used as the surrogate positive controls, respectively. The confirmatory threshold was obtained the same way as the confirmatory assay, using serum samples from 50 drug-naive healthy volunteers with a target UTPR of 1%. A sample with a signal reduction equal to or above the confirmatory threshold of each characterization antibody was considered positive for anti-A Fab and/or anti-B Fab.

Anti-A/B human PK assay

Anti-A/B concentrations in human sera were measured using a validated ELISA. In the assay, streptavidin-coated microtiter plates were incubated overnight with 100 µl of 0.5 µg/ml biotin-conjugated recombinant target A in phosphate-buffered saline for capture. An anti-A/B standard curve, ranging from 0.781 to 50 ng/ml (in-well concentrations) at 1:2 serial dilutions, was prepared in the standard diluent (1% serum in assay diluent). The quality controls and unknown samples were prepared at a minimum required dilution of 1:100 in sample diluent, and applied to the biotin-conjugated recombinant target A coated plates for a 2 hour incubation in RT. The plates were washed three times after the incubation. A detection mAb, raised against the CDR of the anti-B arm and conjugated to digoxigenin, was added to the plate for a one-hour incubation. After three washes, mouse anti-digoxin conjugated to horseradish peroxidase was then added and incubated for 1 hour. TMB substrate was added for color development. The plate was read on a plate reader at 450 nm for detection absorbance and at 630 nm for reference absorbance.

Anti-A/B Phase I study

The anti-A/B Phase I was a randomized and placebo-controlled study conducted in healthy volunteers with two parts. There were five single ascending-dose (SAD) cohorts in Part A and subjects received administrations of anti-A/B at 30 mg sc., 90 mg sc., 300 mg sc., 300 mg iv. and 750 mg iv. in cohorts A to E, respectively. Part B composed of three multiple ascending-dose (MAD) cohorts and each cohort received 3 administrations of anti-A/B at 150 mg sc., 300 mg sc., or 600 mg sc. once every 4 weeks (on Days 1, 29, and 57) in cohorts F to H. In each cohort, subjects were randomized at 6:2 active to placebo to receive treatment(s). The anti-A/B Phase I study had a planned enrollment of 40 subjects and 24 subjects in Parts A and B, respectively. The initiation of Part B was gated upon safety data review of up to the cohort C (300-mg sc.) in Part A. The validated human ADA ELISA was used for analysis of samples from Phase I study.

Results

Immunogenicity assessment of anti-A/B in cynomolgus monkeys

Anti-A/B cynomolgus monkey ADA assay

A homogenous bridging assay for anti-A/B ADA assay was validated at a CRO. A screening CPF of 1.45 was determined based on a panel of serum samples from 100 drug naive cynomolgus monkeys. The assay relative sensitivity was determined to be 32 ng/ml based on the surrogate positive control (sheep anti-human IgG pAb).

Table 1. Summary of anti-A/B anti-drug antibody results in cynomolgus monkey toxicology study and Phase I clinical study.

Study	Group/cohort	Treatment	ADA positive incidences	Titer range
Cynomolgus monkey study ADA results†				
	1	0 mg/kg, iv. & sc.	10% (1 of 10)	1.40
	2	10 mg/kg, iv.	100% (6 of 6)	1.54–5.78
	3	30 mg/kg, iv.	100% (6 of 6)	1.64–6.02
	4	100 mg/kg, iv.	90% (9 of 10)	1.49–6.75
	5	100 mg/kg, sc.	100% (10 of 10)	2.05–6.96
	All anti-A/B treated animals		97% (31 of 32)	1.40–6.96
Phase I study ADA results‡				
Part A (single ascending dosing)	A	30 mg, sc.	0 % (0 of 6)	n/a
	B	90 mg, sc.	67% (4 of 6)	1.81–3.42
	C	300 mg, sc.	50% (3 of 6)	1.87–3.95
	D	300 mg, iv.	83% (5 of 6)	1.77–3.97
	E	750 mg, iv.	67% (4 of 6)	1.86–3.35
	All anti-A/B treated subjects		53% (16 of 30)	1.77–3.97
Part B (multiple ascending dosing)	F	150 mg, sc.	80% (4 of 5)	1.70–3.13
	G	300 mg, sc.	100% (6 of 6)	1.70–2.70
	H	600 mg, sc.	100% (6 of 6)	1.70–2.89
	All anti-A/B treated subjects		94% (16 of 17)	1.70–3.13

†Minimum assay titer is 1.30.
‡Minimum assay titer is 1.70.
ADA: Anti-drug antibody; n/a: Not applicable.

The assay was able to tolerate up to 100 μg/ml of anti-A/B while detecting the surrogate positive control at 1 μg/ml. The minimum titer for the assay is 1.30.

High positive ADA responses in the anti-A/B cynomolgus monkey toxicology study

As described in the study design section, 32 cynomolgus monkeys received anti-A/B treatments at three levels via iv. or sc. and ADAs were detected in 31 of 32 (97%) animals (Table 1). Among the 31 ADA positive animals, 30 tested negative at baseline and developed treatment-induced ADAs; one animal in Group 3 (30 mg/kg iv.) was positive at baseline and had increased titers after treatment, which indicated treatment-enhanced ADAs. In addition, there was also a trend of increase of titer values with longer treatment duration (Figure 2A). For example, in the 31 ADA positive animals, the titer values ranged from 1.49 to 3.33 following the first dose on Day 14 versus 2.70 to 6.87 after the last treatment on Day 84. As the titer number was reported in log scale, the titer range represents a significant level of ADA production. There was no notable difference of the ADA positive incidence and titer range between the treatment groups (different dose levels and iv. vs sc.), suggesting that the ADA responses in the study were not treatment or dosing route dependent (Table 1). It is not uncommon to observe ADA responses in animal studies against humanized mAb therapeutics [14,15]. However, both the high incidence of ADA responses and high titer values observed in the anti-A/B cynomolgus monkey toxicology study were unexpected based on observations with the parental molecules (anti-A and anti-B), which had <51% ADA incidence for both parental molecules.

Impact of ADA on TK & PD in anti-A/B cynomolgus monkey toxicology study

In addition to ADA, TK and PD biomarker samples were collected throughout the study, including but not limited to the time points for ADA sampling. TK was measured using a validated LC–MS assay, which detects total anti-A/B levels in cynomolgus monkey serum. The assay principle was described elsewhere [16]. The validated cynomolgus monkey PK assay has a standard curve ranging from 0.100 to 25 μg/ml (neat). The LLOQ of the assay is 100 ng/ml (neat). The PD biomarker assay was an ELISA developed in-house to detect total soluble target B. The assay was qualified in cynomolgus monkey serum. It was a stepwise ELISA using mAbs to the biomarker

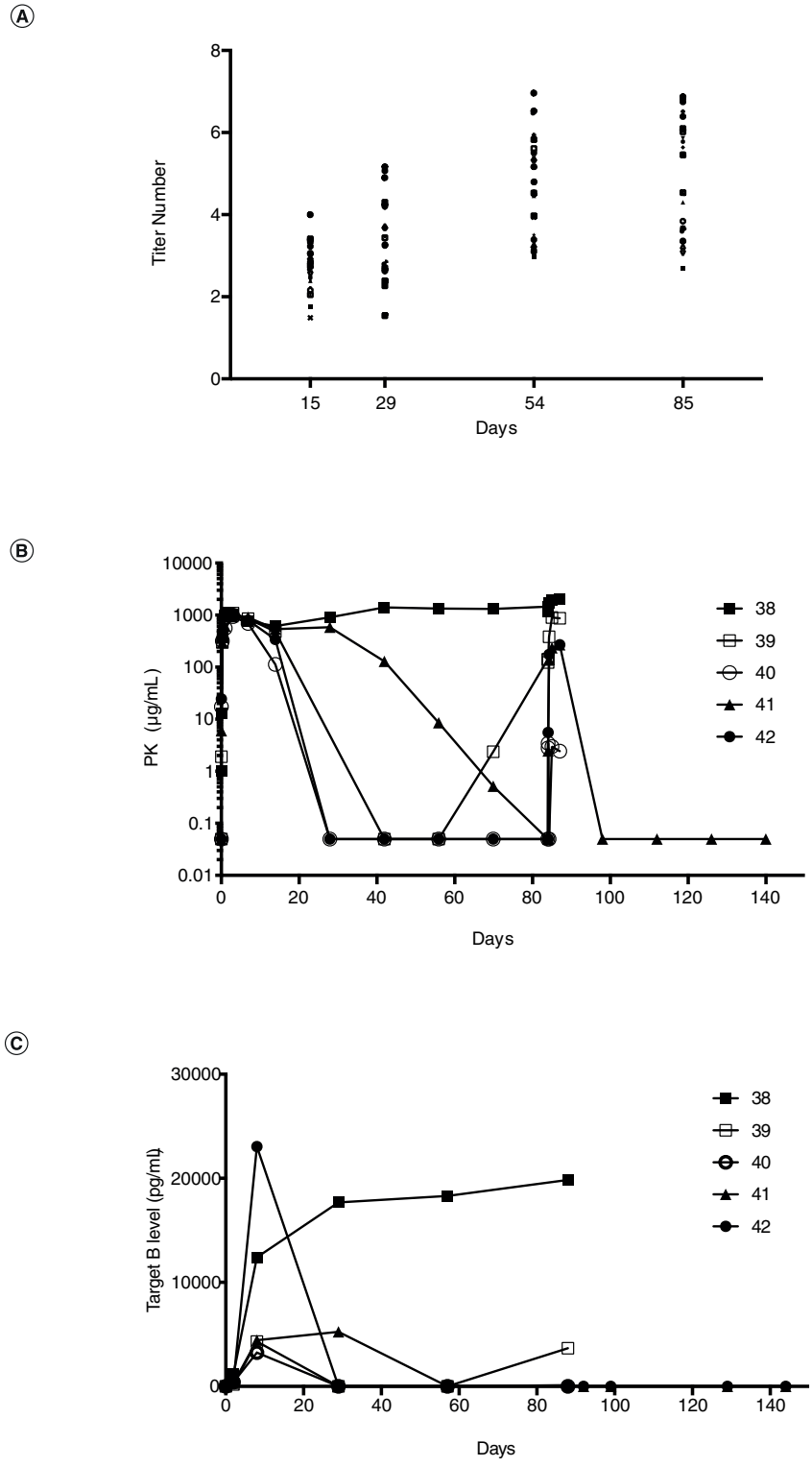


Figure 2. High titer values of anti-drug antibodies correlated with loss of pharmacokinetics and pharmacodynamics of anti-A/B in the toxicology cynomolgus monkey study. (A) A trend toward an increase of titer values with longer treatment duration (days). **(B)** Impact of high ADA titer values on the PK profile of anti-A/B, as measured by the change in its serum concentration over time (days). **(C)** Impact of high ADA titer values on the PD profile of anti-A/B, as measured by the change in the serum concentration of the target molecule B over time (days).
ADA: Anti-drug antibody; PD: Pharmacodynamics; PK: Pharmacokinetics.

Table 2. Anti-drug antibody titer results of five female animals in Group 5 of anti-A/B cynomolgus monkey toxicology study.

Study day	Animal ID #				
	#38	#39	#40	#41	#42
Baseline	Negative	Negative	Negative	Negative	Negative
D15	2.66	3.06	3.39	2.84	4.00
D29	Negative	4.25	5.16	3.26	4.90
D57	Negative	5.17	6.52	4.80	6.96
D85	Negative	3.36	6.87	6.08	6.83
Recovery	n/a	n/a	n/a	5.36	6.39

n/a – Not applicable for ADA samples not collected.
ADA: Anti-drug antibody.

for capture and detection. Details of assays are not discussed in this paper. It is expected that target B signals will increase with drug exposure as the clearance rate of drug-complexed target is reduced compared with uncomplexed target [17].

Overall, the observed high ADA response negatively impacted the TK/PD profile of anti-A/B in the cynomolgus monkey study. Data from female animals in Group 5 were used as an example (Table 2 & Figure 2B & C), as the observations from those animals were representative and consistent across the groups that received anti-A/B treatments. All five female cynomolgus monkeys in Group 5 receiving the highest dose of anti-A/B (100 mg/kg, sc.) tested ADA-positive (Table 2), however the ADA signals and patterns varied among the five animals. Animal #38 tested ADA negative at predose but had a single positive ADA timepoint on Day 15 (Day 14 after first treatment) only with a moderate titer value of 2.66. The other four animals tested positive at all post-treatment time points with a trend of increasing titer values (Table 2). Correspondingly, anti-A/B exposure was maintained in Animal #38 (Figure 2B) and an increase of target B levels was observed (Figure 2C). However, with the other four animals that were ADA positive, various levels of decreases in serum concentrations of anti-A/B and target B levels were observed, in comparison with Animal#38 (Figure 2B & C). The observed negative impact of ADAs on the TK/PD profile of anti-A/B in these five female animals from Group 5 is representative of other treatment groups as well. There was a good correlation between high ADA responses and decreased TK and PD readouts.

Characterization of the immunogenic epitope(s) of anti-A/B in cynomolgus monkeys

Further characterization was conducted to understand the immunogenic epitope(s) of anti-A/B, using five characterization mAbs: anti-A/B, anti-framework mAb without KIH, anti-framework mAb with KIH (anti-KIH mAb) and the variable domain control of the individual arms (anti-A mAb, anti-B mAb). As demonstrated in Figure 1, the totality of testing results from the five characterization mAbs was utilized to determine the immunogenic epitopes of anti-A/B. For example, if the ADA signals were specific to the variable domain of anti-B, a considerable signal reduction in the presence of anti-A/B and anti-B mAb, but not in the presence of the other three characterization antibodies would be expected. However, if signals are specific to the variable domain of both arms, the results may depend on the composition of the positive signals. Since the assay detects ADAs to both anti-A and anti-B (Figure 3A), ADA signal reduction in the presence of either anti-A or anti-B alone may not be effectively depleting all ADA signals. Consequently, considerable signal reduction may happen in the presence of anti-A/B however not necessarily with anti-A or anti-B mAb individually.

A proof of concept experiment was conducted to demonstrate these scenarios. Two anti-idiotypic antibodies, mAb1 for anti-A and mAb2 for anti-B, were spiked in at different proportions. When ADAs were composed of 50% mAb1 plus 50% mAb2, the addition of either unconjugated anti-A/B or anti-A plus anti-B led to complete signal reduction (Figure 3B). In contrast, the addition of only anti-A resulted in partial (~50%) signal reduction, which reflected the reductions of the signals from mAb1 (Figure 3B). A similar outcome was observed using anti-B as a completer (Figure 3B). When ADAs were composed of 90% mAb2 plus 10% mAb1, notable signal reduction was observed in the presence of unconjugated anti-A/B, anti-B and anti-A plus anti-B but minimum signal reduction was only observed in the presence of anti-A (Figure 3C). This is because ADAs to anti-A contributed to a small portion of the signals and depletion of this portion of signals was not very visible in the overall assay

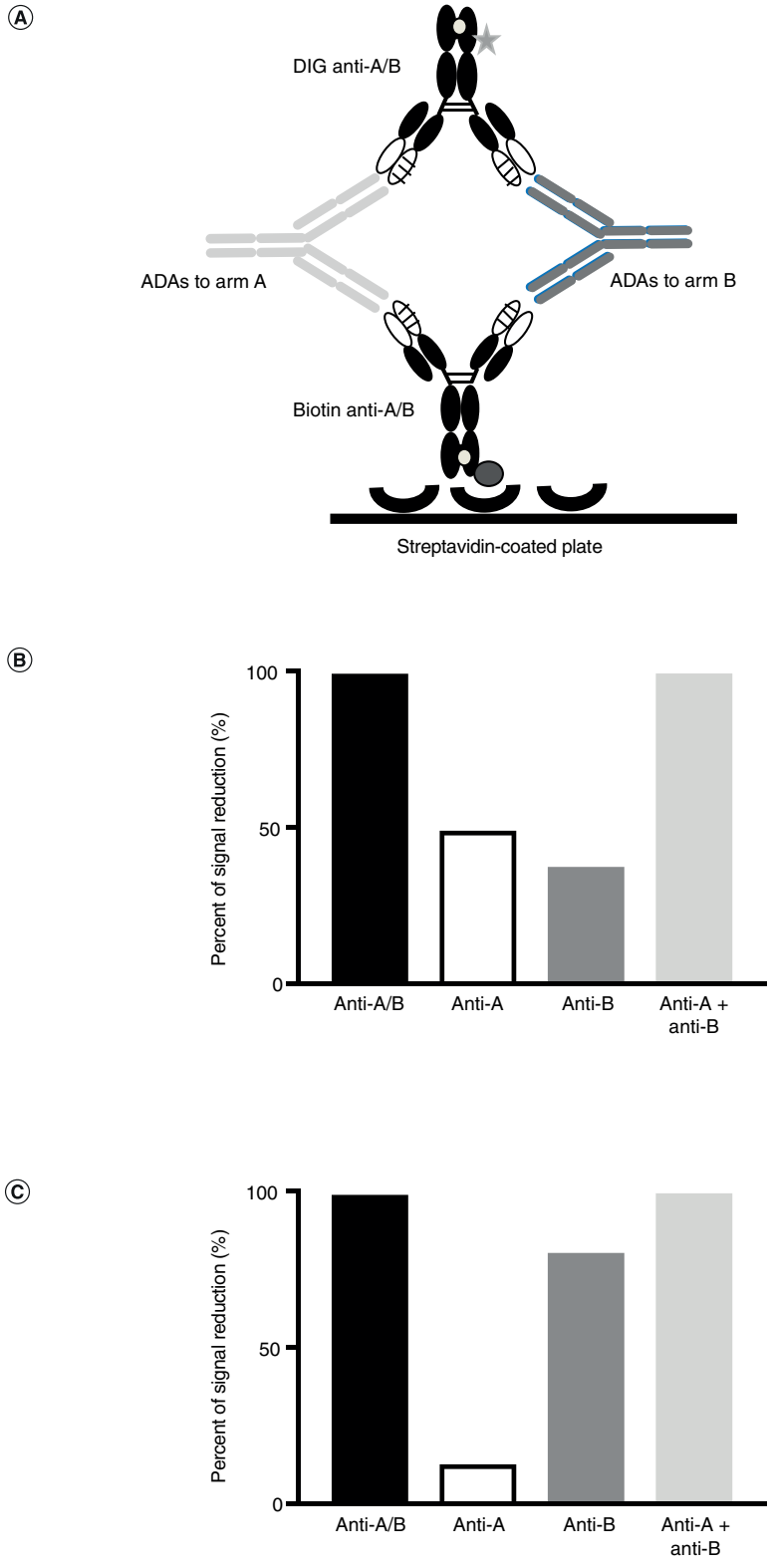


Figure 3. Domain mapping result interpretation depends on composition of anti-drug antibody species. (A) Assay detects ADAs to anti-A and anti-B. **(B)** Limited signal reduction using either anti-A or anti-B for depletion when ADA signals to anti-A or anti-B are similar. **(C)** Significant signal reduction using anti-A/B, anti-B and combination of anti-A and anti-B suggesting ADA signals are predominantly to anti-B.
ADA: Anti-drug antibody.

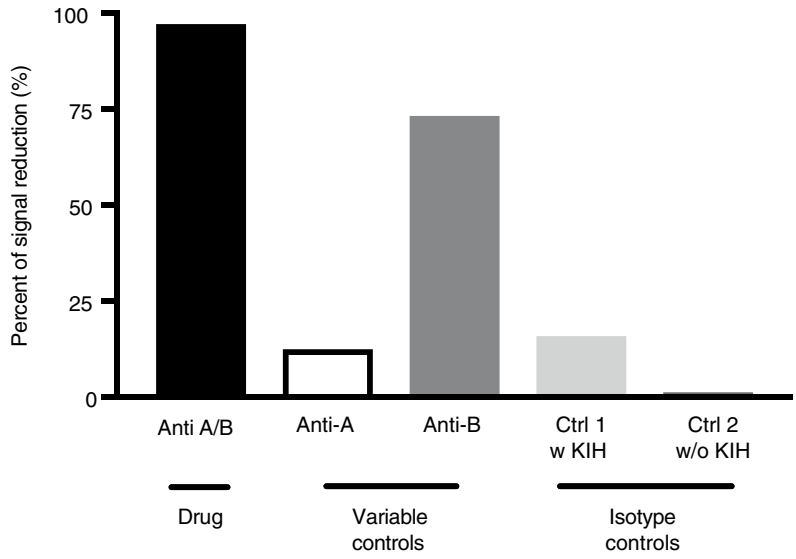


Figure 4. Anti-drug antibody signals predominantly to the variable domain of the anti-B arm of anti-A/B in cynomolgus monkeys.

signals. Therefore, the characterization results will inform the immunogenic domains that are responsible for the predominant ADA signals.

Determination of the immunogenic epitope(s) of study samples

In all tested samples from ADA positive animals, notable signal reduction was observed with the addition of unconjugated anti-A/B and anti-B only (Figure 4). Signal reduction was limited in the presence of the other three characterization antibodies (Figure 4). Data shown in Figure 4 was from one study sample; however, similar results were observed in all tested study samples. Characterization data of the study samples was similar to the proof of concept experiment results shown in Figure 3C. This suggested that the immunogenic signal seemed to be predominantly against the variable region of the anti-B arm of anti-A/B.

Both parental molecules anti-A and anti-B have been evaluated previously as individual bivalent mAbs in multiple animal studies. Up to 43% and 51% ADA positive incidence was observed in multiple-dose cynomolgus monkey studies for anti-A and anti-B, respectively. The observation of ADA signals being predominantly to the anti-B Fab of anti-A/B raises a question: why was anti-A immunogenic as a bivalent mAb but not as part of the anti-A/B bsmAb? We hypothesized that there could still be ADAs to anti-A Fab of the anti-A/B bsmAb, however they only account for a small fraction of the overall ADAs, therefore signal reduction from anti-A competition was insignificant compared with the response observed with anti-B mAb. It is a similar scenario as demonstrated in Figure 3C.

We tested a representative subset of samples from the cynomolgus monkeys treated with anti-A/B in the two homogenous bridging ELISA ADA assays that were designed to detect ADA for the two parental molecules. The CPFs were 1.87 and 1.45 for anti-A and anti-B ADA assays, respectively. We calculated sample signal to negative control ratio (signal to background ratio [S/B]) to allow comparison of the relative signals detected in the two assays. We observed that ADA-positive samples tested positive in both assays (Figure 5). More importantly, the S/B generated in the anti-B ADA assay was about ten-fold higher than the corresponding S/B in the anti-A ADA assay across all tested samples. These results confirmed that the ADA responses in the anti-A/B cynomolgus monkey study included ADAs to both anti-A and anti-B variable regions; however, ADAs were predominantly directed against the anti-B variable region.

Immunogenicity assessment of anti-A/B in clinic

Anti-A/B human ADA assay

A homogenous bridging human anti-A/B ADA assay was validated at a CRO. A screening CPF of 1.85 was determined based on a panel of serum samples from 50 drug naive healthy volunteers. The assay relative sensitivity

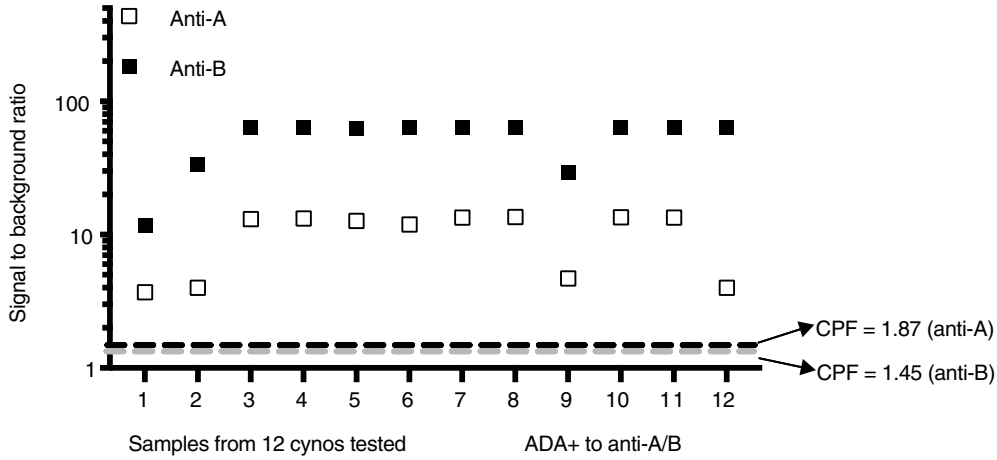


Figure 5. Anti-drug antibody responses against both anti-A and anti-B arms of anti-A/B in cynomolgus monkeys however predominantly toward anti-B arm.
ADA: Anti-drug antibody; CPF: Cut point factor.

was determined to be 22 ng/ml based on a surrogate positive control (affinity purified CDR specific goat pAb to anti-A/B). The assay was able to tolerate up to 100 µg/ml of anti-A/B in the presence of 40 ng/ml surrogate positive control. The minimum titer for the assay is 1.70. The confirmatory threshold was 45% for anti-A/B, 13% for anti-A Fab and 33% for anti-B Fab.

Anti-A/B human PK assay

The validated human PK assay has a standard curve ranging from 0.78 to 50 ng/ml (in-well concentrations) at 1:2 serial dilutions. The LLOQ of the assay is 140 ng/ml (neat concentration). Accuracy of the assay was assessed using controls prepared by spiking anti-A/B in pooled normal human serum at five levels representing the low, mid, and high portions of the assay standard curve. Accuracy of the assay was deemed acceptable with percent difference ranging from 2.8 to 7.0%. The coefficient of variation for the intra-assay precision ranged from 1.2 to 3.1%, and the coefficient of variation for the inter-assay precision ranged from 1.5% to 3.6%.

ADA incidence & characterization data in anti-A/B Phase I study

The Phase I Part A SAD study had the baseline prevalence of ADAs of 8% (3 of 39 subjects) and the post-dose incidence of ADAs of 53% (16 of 30 subjects) in subjects treated with anti-A/B (Table 1). ADAs were detected in all Cohorts except Cohort A. One of the 16 ADA-positive subjects was positive at baseline and developed transient, treatment-enhanced ADAs at one-time point post-treatment. The other 15 ADA-positive subjects were positive at post-dose and developed treatment-induced ADAs. The Phase I Part B MAD study had the baseline prevalence of ADAs of 5% (1 of 22 subjects) and the post-dose incidence of ADAs of 94% (16 of 17) in anti-A/B-treated subjects (Table 1). One of the 16 ADA-positive subjects was positive at baseline and developed persistent treatment-enhanced ADAs. The other 15 ADA-positive subjects developed treatment-induced ADAs.

Similar to the data from the cynomolgus monkey toxicology study, this Phase I study showed a trend toward an increase in titer values in most ADA-positive subjects throughout the treatment period. In Phase I Part A (SAD), except for Cohort A there was no notable differences of the ADA positive incidence and titer range between other treatment groups (Table 1), which suggested that the ADA responses did not discriminate between route of delivery or dose in the study. Phase I Part B (MAD) had a higher ADA incidence than Part A (Table 1), indicating that repetitive dosing increased ADA generation. ADAs in all tested positive samples were characterized for domain specificity, data shown in Figure 6 was from one study sample; however, the data is representative and similar results observed in all ADA positive study samples. The domain characterization data indicated that clinical ADAs were also predominantly directed against the anti-B (Figure 6), similar to the nonclinical data. Furthermore, the titer values in Phase I were significantly less than in the cynomolgus monkey study (Table 1).

The clinical impact of ADAs on safety and PK was evaluated. The presence of ADAs did not have apparent impact on safety in the study. The assessment of ADA impact on PK was restricted because of the limited sample

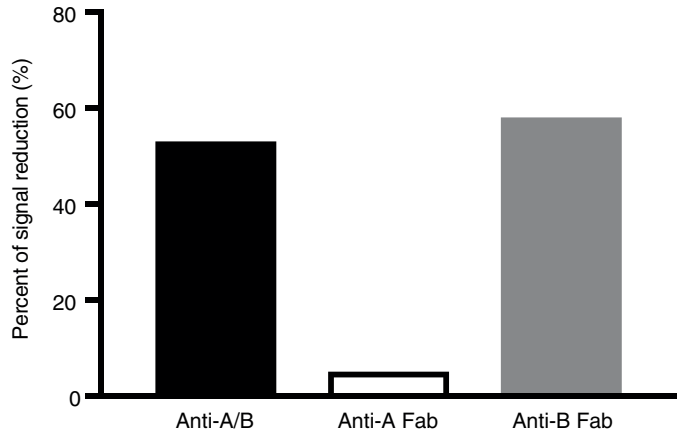


Figure 6. Anti-drug antibody signals predominantly to the anti-B arm of anti-A/B in humans.

sizes for the ADA-negative PK population due to the high incidences of ADAs, especially in Part B of the study. In Part A of the study, similar PK was observed between ADA-negative subjects and the overall population. There were three individuals with relatively higher ADA responses at the end of Part A, which correlated with lower serum concentrations of anti-A/B (data not shown). Since there was only 1 ADA-negative subject in Part B of the study, the impact of ADAs on PK could not be evaluated.

Discussion

An unexpected high incidence (97%) and robust ADA response were observed in a multiple dose cynomolgus monkey toxicology study of a bsmAb anti-A/B, these findings negatively affected the TK and PD readouts. Toxicity findings in some animals were consistent with ADA-related effects with administration of a humanized therapeutic antibody in nonhuman primates [18] rather than a direct pharmacologic or toxicologic effect of anti-A/B (data not shown). Further characterization was conducted to identify the immunogenic epitope(s) of anti-A/B (variable regions of the two arms, framework or KIH structure) by examining ADA responses to each domain. Implementation of this strategy helped us identify the variable domain of arm anti-B as the predominant immunogenic component of anti-A/B in animals. ADAs to the variable domain of the anti-A arm were also present; however, ADAs to the variable domain of arm B were considerably higher than those observed with ADAs to arm anti-A. This ADA assessment strategy was adapted in the Phase I study and similar results were observed in humans with high ADA incidence and ADAs being predominant to anti-B arm of the antibody.

It is not unexpected to observe ADAs in animals after receiving antibody therapeutic treatments. Both in-house and published data suggested that the ADA responses observed in nonclinical studies do not necessarily translate to the clinic [19–21]. BsmAbs, on the other hand, represent a novel class of mAbs with more engineered features and relatively limited development history. While a high ADA-positive incidence to anti-A/B in a nonclinical study is not unexpected, such high levels of ADA signals (up to titer number of 6.96) in an anti-A/B cynomolgus monkey study is not typically observed. There was concern that the high incidence and robust response in animals would translate to the clinic. Therefore, we proactively planned two interim analyses, in addition to the end of study analysis in the Phase I studies, in order to closely monitor anti-A/B immunogenicity in the clinic. The first interim analysis was planned up to Day 28 of Cohort C in Part A, which was right before starting Part B. The second interim analysis was planned after completion of Part A plus up to day 28 of Cohort G in Part B, so that the data could inform Cohort H dosing. Data from the first interim analysis indicated a preliminary ADA incidence of 22% (4 of 18) in subjects that received anti-A/B treatments, which included one ADA-positive subject from Cohort B and three from Cohort C. However, by the second interim analysis, we had the complete ADA incidence of 53% (16 of 30) in Part A and preliminary ADA incidence of 91% (10 of 11) in Part B. Given the high incidence of ADAs observed in both cynomolgus monkeys and healthy volunteers, we decided to end the development of this molecule.

While bsmAbs promise greater therapeutic efficacy and cost efficiency in comparison to combination therapies, the complexity of their design nonetheless presents unique analytical challenges for drug development. As illustrated with our experience of anti-A/B development, it is unclear why similar robust immune responses for anti-A/B

were observed in cynomolgus monkeys and in healthy subjects. Immunogenicity is complex and could be caused by multiple factors. Potential effect caused by the MOA of the drug was considered but was ruled out. The MOA does not require simultaneous binding of both targets. Our assay was designed to detect ADAs binding to the uncomplexed drug, since conjugated drug was used for capture and detection of ADAs in the study samples. Both targets are soluble proteins, it is unlikely that the ADA responses were due to soluble target B tethering to cells expressing target A (or *vice versa*) or target engagement is required for the ADA responses. *in silico* analysis was conducted and the results predicted greater immunogenicity in the anti-B variable region of anti-A/B, which is consistent with our observations in both cynomolgus monkey and human studies. We hypothesized that the high immunogenicity of anti-A/B is due to the presence of immunodominant neoepitopes in anti-A/B, which are not present in the parental molecule. A follow-up cynomolgus monkey immunogenicity study is conducted to investigate if a different format of anti-A/B have the same outcome. The study results will be evaluated to present in a future publication in order to contribute to our understanding of this relatively newer class of biologic therapies.

Conclusion

BsAbs are designed to bind to two different target antigens or two different epitopes on the same target antigen. They may have potential clinical benefits that traditional biotherapeutics do not offer. It takes more engineering modifications to generate a bsmAb, compared with a conventional mAb therapeutic. These engineering works can lead to various modifications of molecule structure and sequence; therefore, bsmAbs potentially may contain more domains with immunogenic potential. Production of bsmAbs takes additional manufacturing processes and may introduce unwanted variants, such as mismatched pairs and homodimers, which are additional risk factors for immunogenicity.

BsmAbs belong to a relatively newer class of modalities and ADA domain specificity characterization has been situational, which is not consistent across industry depending on the immunogenicity risk level and development phase of a program. Because bsmAbs target two antigens or two sites of an antigen, ADA domain specificity assessment would better inform the clinical impact of the ADAs to each target or each binding site of a target in patients. If domain specificity assessment could not be established in time for the first in human study, it should be considered for later phases. While the case study description in this paper is a bsmAb, the strategy could also apply to other multi-domain biotherapeutics.

Future perspective

New modality approaches can potentially improve drug development by enabling the capability of tackling challenging therapeutic targets and difficult disease indications. However, there are also unforeseen challenges in the development of new modalities, including bioanalytical work. Overcoming these challenges will continue to be an exciting and evolving area in the future. Customized bioanalytical strategies and methodologies should be considered for each program based on the target biology, MOA, development timeline and key reagent availability. It would also be informative for the industry to keep sharing their experiences and lessons learned to benefit the bioanalytical community.

Financial & competing interests disclosure

All authors are Genentech employees and stockholders of the Roche Group. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The cynomolgus monkey study described in the manuscript was conducted in compliance with the FDA Nonclinical Laboratory Studies Good Laboratory Practice Regulations. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Data sharing statement

The authors certify that this manuscript reports the secondary analysis of clinical trial data (NCT02748642) that have been shared with them, and that the use of this shared data is in accordance with the terms agreed upon their receipt.

Executive summary

Background

- Anti-A/B is a bispecific monoclonal antibody that blocks activities of soluble targets A and B.
- Robust immune responses were observed in a multiple-dose cynomolgus monkey toxicology study, negatively impacting the toxicokinetics/pharmacodynamic profile of anti-A/B in some animals.
- This was unexpected as similar findings were not observed in the two parental molecules.

Methodology & results

- Immunoassays for evaluation of anti-drug antibody (ADA) and pharmacokinetics measurements of anti-A/B in cynomolgus monkey and clinical studies were developed.
- Strategy for Anti-A/B ADA immunogenic domain characterization is discussed.
- The anti-A/B ADA immunogenic domain characterization data demonstrated that the ADA responses were predominantly toward the anti-B Fab of anti-A/B in both cynomolgus monkey and clinical study samples.

Conclusion

- Anti-A/B is a bispecific monoclonal antibody, a relatively newer class of modalities. The ADA domain characterization discussed helps to understand the nature of the ADAs and to inform a better assessment of the clinical impact of ADAs.
- The anti-A/B ADA domain characterization strategy discussed in this paper could also be applied broadly to other multidomain biotherapeutics.

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Guide to SpyCatcher Products Supporting TrailBlazer Antibodies

The innovative SpyTag and SpyCatcher technology, also termed “molecular superglue”, has been employed by the scientific community in many ways to build proteins with novel properties. We have created the brand TrailBlazer Antibodies, where recombinant antibodies containing a SpyTag are combined with a set of prefabricated SpyCatcher modules, leading to unprecedented flexibility in assay design. The reaction between SpyTag and SpyCatcher occurs autocatalytically and quantitatively in minutes. Depending on the SpyCatcher used, the antibodies are site-specifically labeled with, for instance HRP, biotin, or fluorescent dyes, or they are multimerized or converted to immunoglobulin (Ig)-like constructs with different isotypes or with Fc domains from various species.

Introduction to SpyTag Technology

SpyTag technology is a protein ligation method based on the SpyTag peptide and SpyCatcher protein (Zakeri et al. 2012), which are derived from the second immunoglobulin-like collagen adhesin domain (CnaB2) from the fibronectin-binding protein (FbaB) of *Streptococcus pyogenes* (Spy). The CnaB2 domain naturally contains an intrachain isopeptide bond between the sidechains of a lysine and an aspartic acid. By splitting this domain and rational engineering of the fragments, a peptide (the SpyTag containing the reactive Asp residue) and a small protein (SpyCatcher containing the reactive Lys residue and a Glu residue necessary for forming the catalytic triad) were obtained that form an amide bond spontaneously when mixed. This reaction occurs with high yield in diverse conditions of pH, temperature, and buffer. Following optimization of the two components (SpyTag2 and SpyCatcher2) (Keeble et al. 2017), the reaction time was shortened from hours to minutes (Figure 1), and has subsequently been improved to increase the reaction speed even further (SpyTag3 and SpyCatcher3) (Keeble et al. 2019). All versions of SpyTag and SpyCatcher are compatible with each other.

SpyCatcher2
113 aa
12.08 kD

SpyTag2
14 aa

Ligated proteins

- Spontaneous (autocatalytic) reaction
- Covalent isopeptide bond formation, irreversible
- Fast, quantitative reaction
- pH 5 to 8, temperature +4 to +37°C
- Robust to buffer conditions, Ca^{2+}/Mg^{2+} not needed
- Robust to detergents
- Reaction occurs also inside cells (in vivo)

Fig. 1. The reaction between SpyTag2 and SpyCatcher2 requires only mixing, is rapid, high yielding, and shows good specificity.

BIO-RAD

Recombinant Antibodies with SpyTag Technology

Bio-Rad has combined SpyTag technology with recombinant antibody expression to introduce completely new versatility to antibodies (Hentrich et al. 2021). The SpyTag2 is genetically fused to the C-terminus of recombinant antibodies (Figure 2; here the SpyTag is genetically fused to the heavy chain C-terminus of a Fab antibody fragment). A series of SpyCatcher-derived adapters have been created that can be coupled to the SpyTagged antibodies by mixing and incubating. SpyCatchers are available with labels such as HRP and biotin. Several variants have been designed, including the BiCatcher, where two SpyCatchers are genetically linked to allow formation of bivalent Fab2, and the FcCatcher, where SpyCatcher is fused to an Ig Fc domain to make a synthetic Ig-like molecule after the reaction with two SpyTag Fabs (Figure 3). A Fab with a SpyTag forms a covalent isopeptide bond to the chosen Catcher, enabling conjugation or conversion to multiple formats within one hour (Figure 4).

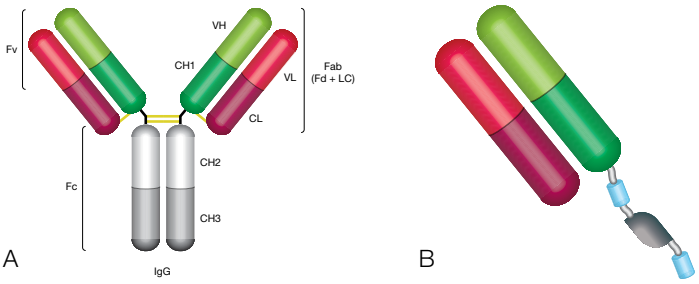

Fig. 2. Schematic images of A, full length immunoglobulin; B, monovalent Fab format with SpyTag (gray) and purification and detection tags (blue).

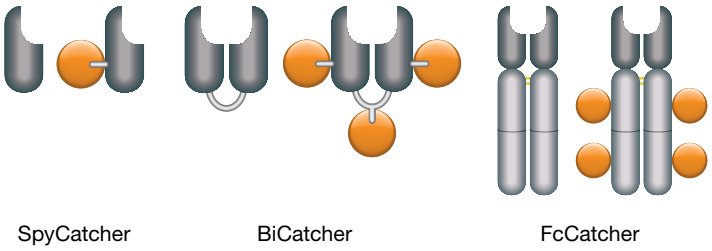

Fig. 3. Schematic images of different Catchers (gray) unlabeled (left) and conjugated (orange circle, right). SpyCatcher and BiCatcher have been modified to include a cysteine residue that enables site-specific conjugation with a known degree of labeling (DOL); FcCatcher is conjugated via primary amines and the DOL measured.

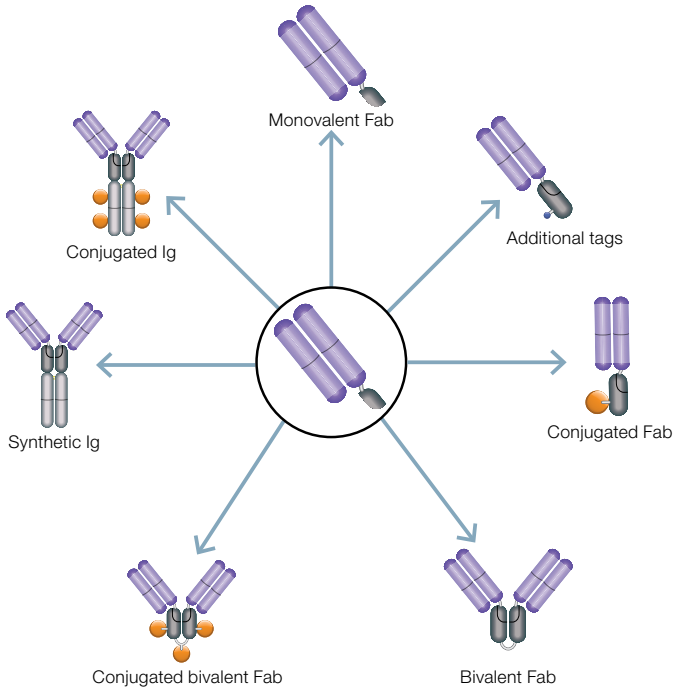

Fig. 4. A monovalent Fab with SpyTag (center) can be converted to multiple different formats via a formation of a covalent isopeptide bond with the chosen Catcher.

SpyCatcher Products and Services

All SpyCatchers and their derivatives (Table 1) are compatible with our recombinant antibodies that incorporate a SpyTag2 at the C-terminus of the heavy chain, for instance format Fab-F-Spy2-H, which also contains a FLAG- and a His-tag. SpyCatchers can be coupled to recombinant Fab antibodies as part of our TrailBlazer Antibody Custom Service. SpyCatchers can also be purchased from our catalog for coupling by the user to an antibody or protein with a reactive SpyTag1, SpyTag2, or SpyTag3.

Table 1. SpyCatcher products.

Product	Description	Availability	
Monovalent Format		**Custom Service**	**Catalog Number**
SpyCatcher2	SpyCatcher2 protein	Yes	TZC001
SpyCatcher2-CYS	SpyCatcher2 with an engineered cysteine residue. Can be used for site-specific chemical conjugation to a label of choice	Yes	TZC001CYS
SpyCatcher2: Biotin	SpyCatcher2 conjugated to biotin	Yes	Inquire
SpyCatcher2: HRP	SpyCatcher2 conjugated to HRP	Yes	Inquire
SpyCatcher2:PE	SpyCatcher2 conjugated to RPE	Inquire	Inquire
SpyCatcher3	SpyCatcher3 protein	Yes	TZC025
SpyCatcher3-CYS	SpyCatcher3 with an engineered cysteine residue. Can be used for site-specific chemical conjugation to a label of choice	Yes	TZC025CYS
Bivalent Format		**Custom Service**	**Catalog Number**
BiSpyCatcher2	BiSpyCatcher2 protein	Yes	TZC002
BiSpyCatcher2-CYS	BiSpyCatcher2 with one engineered cysteine residue. Can be used for site-specific conjugation to a label of choice	Yes	TZC002CYS
BiSpyCatcher2-CYS3	BiSpyCatcher2 with three engineered cysteine residues. Can be used for site-specific conjugation to a label of choice	Yes	TZC002CYS3
BiSpyCatcher2: Biotin	BiSpyCatcher2 conjugated to biotin	Yes	Inquire
BiSpyCatcher2: HRP	BiSpyCatcher2 conjugated to HRP	Yes	TZC002P
BiSpyCatcher2: PE	BiSpyCatcher2 conjugated to RPE	Yes	Inquire
Ig-Like Format		**Custom Service**	**Catalog Number**
hIgG1-FcSpyCatcher3	SpyCatcher3 fused to the hinge region, CH2 and CH3 of human IgG1	Yes	TZC009
hIgG1-FcSpyCatcher3: Biotin	Human IgG1-FcSpyCatcher3 conjugated to biotin	Yes	Inquire
hIgG1-FcSpyCatcher3: HRP	Human IgG1-FcSpyCatcher3 conjugated to HRP	Yes	Inquire
hIgG2-FcSpyCatcher3	SpyCatcher3 fused to the hinge region, CH2 and CH3 of human IgG2	Yes	TZC016
hIgG3-FcSpyCatcher3	SpyCatcher3 fused to the hinge region, CH2 and CH3 of human IgG3	Yes	TZC017
hIgG4-FcSpyCatcher3	SpyCatcher3 fused to the hinge region, CH2 and CH3 of human IgG4	Yes	TZC018
hIgG4-Pro-FcSpyCatcher3	SpyCatcher3 fused to the hinge region, CH2 and CH3 of human IgG4-Pro (S228P)	Yes	TZC019
hIgA-FcSpyCatcher3	SpyCatcher3 fused to the hinge region, CH2 and CH3 of human IgA1	Yes	TZC020
mIgG2a-FcSpyCatcher3	SpyCatcher3 fused to the hinge region, CH2 and CH3 of mouse IgG2a	Yes	TZC012
rIgG-FcSpyCatcher3	SpyCatcher3 fused to the hinge region, CH2 and CH3 of rabbit IgG	Yes	TZC013

Protocol for Fab Antibody Coupling to SpyCatchers, BiCatchers, and FcCatchers

The SpyTag-SpyCatcher coupling reaction is straightforward and fast.

1. Calculate the required volumes of Fab and SpyCatcher product starting with the amount of Fab you want to couple.
2. Mix Fab and SpyCatcher product.
3. Incubate for 1 hr at RT.

Detailed protocols are available on our website. The guidelines can also be applied to couple a protein with a reactive SpyTag to a SpyCatcher product.

Visit **bio-rad-antibodies.com/spycatcher** to download the protocols.

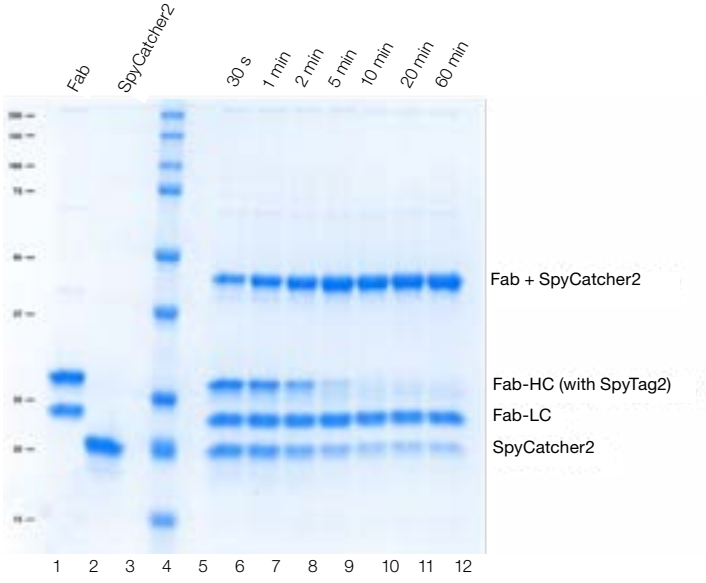

Fig. 5. Example of coupling kinetics for SpyTag-SpyCatcher reaction. His-SpyCatcher2 (#TZC001) coupling kinetic was analyzed on an AnyKD Criterion TGX Stain-Free Gel (#5678125). A SpyTagged Fab (AbD35759, Fab-F-Spy2-H format) was loaded in lane 1 and His-SpyCatcher2 in lane 2. Precision Plus Protein Unstained Protein Standard (#1610363) was run in lane 4. In lanes 6-12, the coupling reaction of the Fab with a 25% molar excess of His-SpyCatcher2 at RT was loaded. The reaction was stopped at the indicated time by addition of 4x Laemmli Sample Buffer (#1610747). All lanes were loaded with 3 µg protein.

Frequently Asked Questions

1 What are the biophysical parameters of SpyTag and SpyCatcher?

Table 2. Biophysical parameters for the original SpyTag and SpyCatcher proteins.

Element	Size (in Amino Acids)	Calculated Isoelectric Point, pI	Calculated Molecular Weight* (kD)
SpyTag1	13	8.95	1.47
SpyTag2	14	9.87	1.68
SpyTag3	16	10.37	1.93
SpyCatcher1	113	4.17	12.14
SpyCatcher2	113	4.12	12.08
SpyCatcher3	113	3.91	12.13

*Refer to the individual product datasheets for molecular weights of the various Bio-Rad SpyCatcher proteins and derivatives, which will be different due to the addition of tags and linkers.

2 What is the difference between SpyTag, SpyTag2, and SpyTag3, and SpyCatcher, SpyCatcher2, and SpyCatcher3?

The original SpyTag and SpyCatcher were developed based on the fibronectin-binding protein from *S. pyogenes*. This pair was optimized for reaction kinetics by phage display to create SpyTag2 and SpyCatcher2, which react together to form the isopeptide bond ten times faster than the original pair. SpyTag2 and SpyCatcher2 were further optimized by rational design to create SpyTag3 and SpyCatcher3, with a reaction speed improved by a further factor of ten. All SpyTag and SpyCatcher versions are compatible with each other. Our products contain SpyTag2. The coupling speed of SpyTag2 is comparable to that of SpyTag3 at the concentrations recommended in our antibody coupling protocols.

3 Why is SpyCatcher3 used for the FcCatchers and SpyCatcher2 for all other Catchers?

Our SpyCatchers and BiCatchers were originally developed with SpyCatcher2, before SpyCatcher3 was available. Our FcCatchers were developed later, after SpyCatcher3 had become available. We tested SpyCatcher3 for use in the SpyCatcher and BiCatcher products but did not observe significant performance benefits for version 3 over version 2, so these products continue to be made using SpyCatcher2.

4 Is the SpyTag-SpyCatcher bond stable?

The isopeptide bond formed when the proteins ligate is considered to be as stable as other peptide bonds between amino acids in a protein. The bond formation is considered irreversible.

5 Can SpyTag or SpyCatcher react with other proteins in my cells or lysates?

The SpyTag-SpyCatcher protein ligation reaction is very specific. Neither of the two elements has been shown to react with any other protein in cells or lysates.

6 Why is it better to conjugate via the SpyCatcher rather than to the antibody directly?

The use of pre-conjugated Catchers is simple, fast, flexible, and site directed. Conventional antibody conjugation is a random process resulting in each antibody carrying a different number of labels at different positions. Some labels might be attached at the antibody-antigen binding site leading to a decrease in performance of the reagent in the application. Labeled Catchers are coupled to the antibody C-terminus with defined stoichiometry, leaving the antibody binding site unmodified. Furthermore, we engineered SpyCatcher and BiCatcher versions with defined free thiol groups that can be conjugated site-specifically with labels such as biotin, resulting in a fixed degree of labeling (DOL). A SpyTagged antibody can be coupled to any of the different Catchers by a simple mixing step. This offers completely new flexibility for assay design.

#	Question	Answer
7	How are labels site-specifically attached to SpyCatcher?	We have genetically engineered SpyCatcher2 and SpyCatcher3 to contain one cysteine (Cys), and BiCatcher2 to contain either one or three cysteines. These Cys residues can be used for site-specific conjugation via maleimide chemistry for instance, enabling a fixed DOL for these Catchers. We have not introduced any additional Cys residues into the FcCatcher products to avoid unnatural folding. FcCatchers are conjugated via primary amines, and therefore are not site-specifically labeled. However, the conjugation protocol is controlled, ensuring a consistent DOL between batches. As the FcCatchers are conjugated with a label before coupling to the SpyTagged antibody fragment, the antibody-antigen binding site is never compromised.
8	Can a Catcher protein be conjugated to any probe or label?	Unconjugated SpyCatcher and BiCatchers are available for conjugation by the user to a label of choice. SpyCatcher and BiCatcher proteins are available with free cysteine residues to enable site-specific conjugation via thiol reactive groups, using for instance, maleimide chemistry. Random conjugation via primary amines is also possible using NHS ester reaction chemistry.
9	Can SpyTag-SpyCatcher technology be used with any antibodies?	It is possible to engineer any recombinant antibody to contain a C-terminal SpyTag for use with SpyCatchers. Contact us to discuss feasibility of a custom service project. During our research work to develop recombinant Fabs with the SpyTag, we found that the functional periplasmic expression of such antibody fragments in *Escherichia coli* led to rapid cleavage of the SpyTag by *E. coli* proteases. We developed a system to prevent this cleavage, making SpyTag technology accessible for *E. coli* produced antibodies (Hentrich et al. 2021). The production of an antibody with a SpyTag in *E. coli* requires a proprietary strain, please inquire for further information.
10	What is the composition of a synthetic Ig-like antibody?	SpyCatcher3 is genetically fused at its C-terminus to the N-terminus of the Ig hinge region followed by the constant domains of the antibody Fc domain (CH2 and CH3 in case of IgG1). This construct is named FcCatcher. Antibodies containing a SpyTag at the C-terminus will covalently bind to FcCatcher resulting in an Ig-like molecule. Our constructs typically use fully human Fab fragments (containing a full light chain and a heavy chain containing VH and CH1 domains) obtained via phage display. A series of tags, usually FLAG-tag, SpyTag2, and His-tag are genetically attached at the C-terminus of the heavy chain.
11	What is the ratio of SpyTag to SpyCatcher for the coupling reaction, and is there any uncoupled Fab or Catcher leftover in my product after coupling?	To drive the ligation reaction to completion, we recommend using an excess of one ligation partner. ▪ Monovalent SpyCatchers: use a 25% excess of monovalent SpyCatcher to make sure all Fab antibodies have reacted and are coupled to the SpyCatcher; excess SpyCatcher will be washed off when carrying out nonhomogeneous assays ▪ Bivalent SpyCatchers (e.g., BiSpyCatcher2 or FcSpyCatcher3): for bivalent SpyCatchers, use 25% excess of SpyTagged Fab to make sure that all Catchers carry two Fabs. In our experience, small amounts of unreacted Fabs do not interfere with the assay nor reduce sensitivity, as the bivalent coupled product usually binds with much higher avidity and therefore replaces any monovalent uncoupled Fab It is possible to set up coupling reactions with a 1:1 ratio, however, inevitable inaccuracies in protein concentration determination might lead to deviations from this ratio and unpredictable coupling stoichiometries.

#	Question	Answer
12	How long does the protein ligation reaction take?	Our protocol recommends one hour for the ligation reaction to complete. SpyTag2 ligation to the monovalent SpyCatcher2 is completed within about 10 min. Coupling to BiSpyCatcher2 and FcSpyCatcher3 takes several minutes longer since two SpyTagged Fab antibodies must couple to each bivalent Catcher molecule. For simplicity, our protocols recommend one coupling time for all Catchers. The reaction can also be left overnight. For SpyTag3, we recommend the same reaction times as for SpyTag2. SpyTag1 needs significantly longer coupling times, and these should be determined experimentally as the coupling kinetics is concentration dependent.
13	How can high background signal be reduced, if observed?	A Fab antibody coupled to a Catcher would be expected to show the same background as the same antibody clone in a different format. If a high background is observed, it is recommended to optimize the antibody concentration by testing various dilutions first. If working with an uncharacterized antibody, the coupled and uncoupled antibodies can be tested in parallel with the same secondary detection antibody for both. In western blot experiments, we have observed a faint additional band around 50 kD with some mammalian cell lysates, for instance, with HeLa cells, which is caused by unreacted SpyCatcher in the preparation. This band can be completely removed by addition of free SpyTag3 peptide to the sample after coupling followed by incubation for 5 min, whereby SpyTag3 peptide binds to uncoupled Catcher and completely blocks binding to the 50 kD protein. If a Fab coupled to an FcCatcher is to be used in western blotting, it is recommended to use nitrocellulose membranes rather than PVDF to avoid membrane background staining. In sandwich or bridging ELISA with a SpyTagged capture antibody and Catcher-coupled detection antibody, an increased background can appear due to unreacted SpyCatcher sites, which can react with the SpyTag of the capture antibody. This can be prevented by the addition of SpyTag3 peptide to the coupled detection antibody. We recommend using a 2.5-fold molar excess of peptide over SpyCatcher sites, i.e. 2.5-fold excess for SpyCatcher- and 5-fold excess for BiCatcher- and FcCatcher-coupled antibodies, and incubate for at least 5 min. Please inquire about the availability of SpyTag3 peptide.
14	Is there any difference in performance of SpyTag-SpyCatcher coupled antibodies to conventional antibodies?	SpyTag-SpyCatcher coupled antibodies are expected to perform in the same way as any other monovalent or bivalent Fab antibody or full-length Ig in the same application. Coupling a SpyTagged Fab antibody to a pre-conjugated Catcher protein avoids the potential for the label being attached to the antibody-antigen binding site, as can happen with random labeling of a conventional antibody. This is likely to result in a better performance of the conjugated SpyTag-SpyCatcher antibody, and consequently a lower concentration is typically needed.
15	Do synthetic Ig-like antibodies perform exactly like conventional full-length Igs?	The antibody-antigen binding sites in these two formats are identical and therefore the binding specificity is the same. Both molecules are bivalent and have the same avidity. Our FcCatcher proteins are produced in a mammalian cell line using the same procedure as for our full-length Igs, resulting in an identical Fc domain, including glycosylation. As the structure of the Ig-like molecule and the full-length Ig are not the same, their conformations will not be completely identical, and these differences in the geometry may affect certain assays. As the Fab is produced in *E. coli*, there will be lipopolysaccharides (endotoxin) from bacterial membranes present in the preparation. If necessary, endotoxin levels can be decreased prior to coupling to Catchers.

#	Question	Answer
16	Can antibodies be immobilized on beads and resins using SpyTag technology?	SpyTagged Fab antibodies coupled to a biotinylated Catcher protein can be captured on streptavidin-coated beads. If coupling the Fab to a biotinylated Catcher (SpyCatcher2:Bio or BiSpyCatcher2:Bio) for immobilization on beads, it is advisable to use an excess of Fab, to avoid the solution containing unreacted SpyCatcher sites. Uncoupled Fab will be removed during the washing step after the reaction with streptavidin beads. Alternatively, SpyCatchers can be immobilized directly on beads or column resin either site-specifically for example via cysteines, using SpyCatcher3-CYS, or via primary amines using SpyCatcher3. Antibodies can subsequently be immobilized and oriented on SpyCatcher resin by a simple 1 hr incubation of the SpyTagged antibody with SpyCatcher resin.
17	Can a SpyTagged antibody be coupled to other proteins using SpyTag-SpyCatcher technology?	If a protein contains the SpyCatcher domain, it can be coupled to a SpyTagged antibody. We recommend using genetic engineering to create a fusion protein whereby the SpyCatcher is either fused to the N- or C-terminus of the protein of interest.
18	Can common anti-Fab and anti-Ig secondary antibodies be used with SpyCatcher coupled antibodies?	Yes, we have successfully used anti-FLAG-tag, anti-His-tag, and anti-human Fab secondary antibodies, with SpyTagged Fab antibodies, both with and without a SpyCatcher protein coupled. In addition, anti-human, mouse, and rabbit Fc, and anti-IgG (H+L) specific secondary antibodies can be used for the respective FcCatcher coupled antibodies. SpyCatchers and BiCatchers carry a 6-His tag that can be used for detection. FcCatchers do not have additional tags.
19	In ELISA, can a SpyTagged Fab coupled to an HRP conjugated Catcher be used as a detection antibody in the same way as a conventional antibody conjugated to HRP?	Yes, when using as a detection antibody in ELISA, we recommend coupling the chosen Fab to an HRP conjugated BiCatcher (BiSpyCatcher2:HRP) or FcCatcher (hIgG1-FcSpyCather3:HRP). In our experience, the SpyTag-SpyCatcher coupled antibody can be used at a 4 to 10-fold lower concentration than the same clone in full-length IgG format directly conjugated to HRP, and result in the same assay sensitivity. If you are using a SpyTagged capture antibody for your sandwich or bridging assay in combination with an HRP conjugated Catcher coupled detection antibody, you might observe an increased background through partly uncoupled Catcher sites of the detection antibody that can react with the SpyTag of the capture antibody. The addition of SpyTag3 peptide to the coupled detection antibody will remove the background. We recommend using a 2.5-fold molar excess of peptide over SpyCatcher sites, i.e. 2.5-fold excess for SpyCatcher-, and 5-fold excess for BiCatcher- and FcCatcher-coupled antibodies, and incubate for at least 5 min. Please inquire about the availability of SpyTag3 peptide.
20	Can the SpyTag be used to immobilize antibodies on surfaces such as ELISA plates?	SpyCatcher can be directly coated to ELISA plates, or biotinylated Catchers can be immobilized on streptavidin-coated plates. The coupling reaction at the surface is slower compared to in solution, and the antibody concentration used is usually lower than for standard coupling reactions, hence the reaction might not be complete after one hour. Coupling the antibody to SpyCatcher surfaces leads to more active antibodies compared to immobilization by passive adsorption. Therefore, lower antibody concentrations are required to give the same signal. Alternatively, the Fab can be coupled to biotinylated SpyCatcher (SpyCatcher:Bio) followed by immobilization of the coupled biotinylated antibody on Streptavidin-coated plates.

Inquiries and Orders

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SpyCatcher Catalog Products

Inquiries
Contact your local Bio-Rad office or distributor.

How to order: bio-rad-antibodies.com/SpyCatcher

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TrailBlazer

TrailBlazer Antibody Custom Services

We understand that the design and optimization of immunoassays is complex and time consuming. There's never a 'one size fits all' solution. Having one antibody in one format limits your options, and the resulting assay may not answer all your questions. Producing several different conjugates or alternative formats can be tedious and costly, can delay your project, and may still result in a dead end.

TrailBlazer™ Antibodies open up more pathways to successful assay development.

We have incorporated SpyTag technology into our custom recombinant antibodies, enabling site-directed conjugation or assembly into different stable antibody formats. This provides you with an extensive antibody toolbox that enables rapid development of multiple assays, providing the results you need to make progress.

Your custom-made antibodies can be delivered in a variety of configurations at the outset. Test in monovalent or bivalent Fab, and full length immunoglobulin formats, with a choice of isotypes and labels.

You can explore which antibodies work best in your desired applications and on your different technology platforms.

What Is SpyTag Technology?

SpyTag technology is based on the SpyTag peptide and SpyCatcher protein (Zakeri et al. 2012), which are derived from the fibronectin-binding protein (FbaB) of *Streptococcus pyogenes* (Spy). The FbaB protein contains an intrachain isopeptide bond between the sidechains of a lysine and an aspartic acid within the Ig-like collagen adhesion domain. The SpyTag peptide incorporates the aspartic acid residue, and the SpyCatcher has the lysine residue; when the SpyTag and SpyCatcher are mixed, the isopeptide bond is formed between these amino acids. This reaction occurs with high yield in diverse conditions of pH, temperature, and buffer, and following optimization of the two components (SpyTag2 and SpyCatcher2, Keeble et al. 2017) the reaction time was shortened from hours to minutes (Figure 1) and has subsequently been improved to increase the reaction speed even further (SpyTag3 and SpyCatcher3) (Keeble et al. 2019). All versions of SpyTag and SpyCatcher are compatible with each other.

SpyCatcher2
116 aa
15.2 kDa

SpyTag2
13 aa

Ligated proteins

- Spontaneous (autocatalytic) reaction
- Covalent isopeptide bond formation, irreversible
- Rate constant 2.0 × 10^4 M-1 s-1 ($t_{½}$ = 74 sec; 10 µM)
- pH 5 to 8, temperature +4 to +37°C
- Robust to buffer conditions, Ca^{2+}/Mg^{2+} not needed
- Robust to detergents
- Reaction occurs also inside cells (in vivo)

Fig. 1. The reaction between SpyTag2 and SpyCatcher2 requires only mixing, is rapid, high yielding, and shows good specificity.

BIO-RAD

About HuCAL® Technology

HuCAL technology is proven and well published, and has been used by the Bio-Rad Custom Antibody Team to generate antibodies for research and diagnostic applications since 2004. The structural diversity of the human antibody repertoire is represented in the HuCAL PLATINUM® library by seven heavy chain and six light chain variable region genes, which give rise to 42 master framework combinations. Highly diverse genetic cassettes, encoding the complementarity determining regions (CDRs) of the antibody binding sites, are combined with these frameworks to create antibody genes that code for some 45 billion unique antibodies in Fab format (Knappik et al. 2000, Prassler et al. 2011).

Screening of the HuCAL library is performed in vitro enabling the successful selection of antibodies that it is impossible to generate by in vivo immunization of animals; such challenging targets include highly conserved or self-antigens, low immunogenic antigens, and conformational variants. As the sequence of any selected antibody is known, it is possible to reproduce the genes synthetically if needed. This sequence back up secures the future supply of the antibody, and recombinant production methods ensure a high level of consistency between batches.

How Is SpyTag Technology Used with Bio-Rad's Custom Antibodies?

Bio-Rad has incorporated SpyTag technology into the Human Combinatorial Antibody Libraries (HuCAL) antibody phage display platform, introducing completely new versatility. The SpyTag2 is genetically fused to the C-terminus of the recombinant Fab heavy chain (Figure 2b). SpyCatchers have been created with site-specific conjugated labels such as HRP or biotin. Further variants have been designed, including the BiCatcher, where two SpyCatchers are genetically linked to allow formation of bivalent Fab2, and the FcCatcher, where SpyCatcher is fused to an immunoglobulin Fc domain to make a full length Ig-like molecule after the reaction with two SpyTag Fabs (Figure 3). A Fab antibody with a SpyTag forms a covalent isopeptide bond to the chosen Catcher, enabling conjugation or fast conversion to bivalent Fab or a full length immunoglobulin-like molecule within just one hour (Figures 4 and 5).

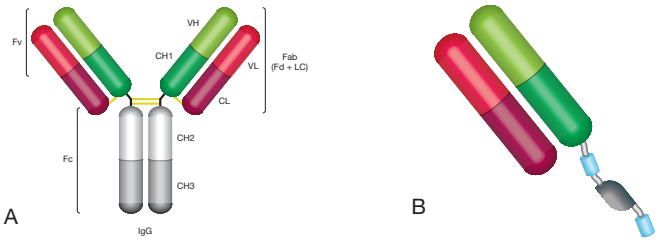


Fig. 2. Schematic images of A, full length immunoglobulin; B, monovalent Fab format with SpyTag (gray) and purification and detection tags (blue).

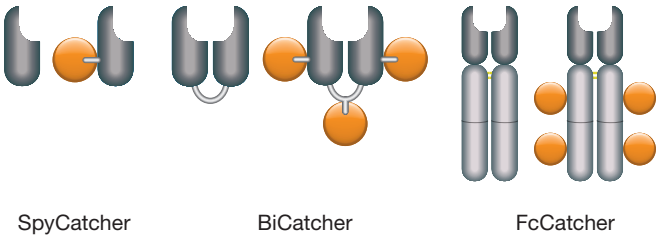


Fig. 3. Schematic images of different Catchers (gray) unlabeled (left) and conjugated (orange circle, right). SpyCatcher and BiCatcher have been modified to include a cysteine residue that enables site-specific conjugation with a known degree of labeling (DOL); FcCatcher is conjugated via primary amines and the DOL measured.

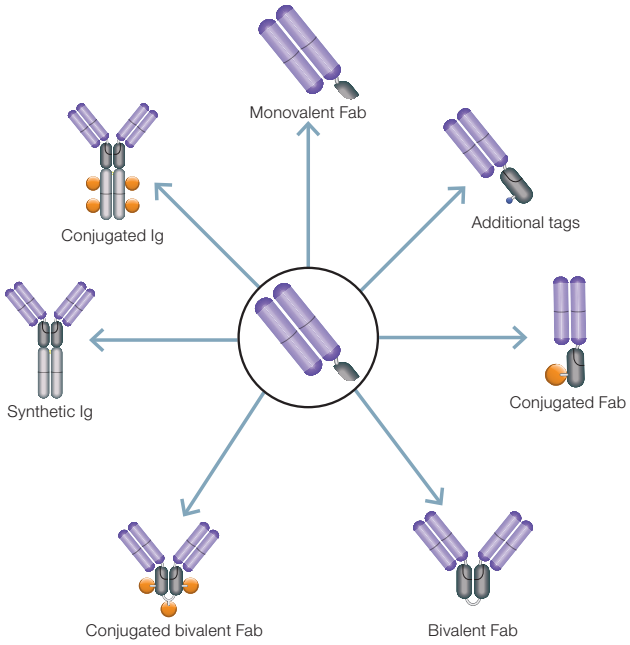


Fig. 4. A monovalent Fab with SpyTag (center) can be converted to multiple different formats via a formation of a covalent isopeptide bond with the chosen Catcher.

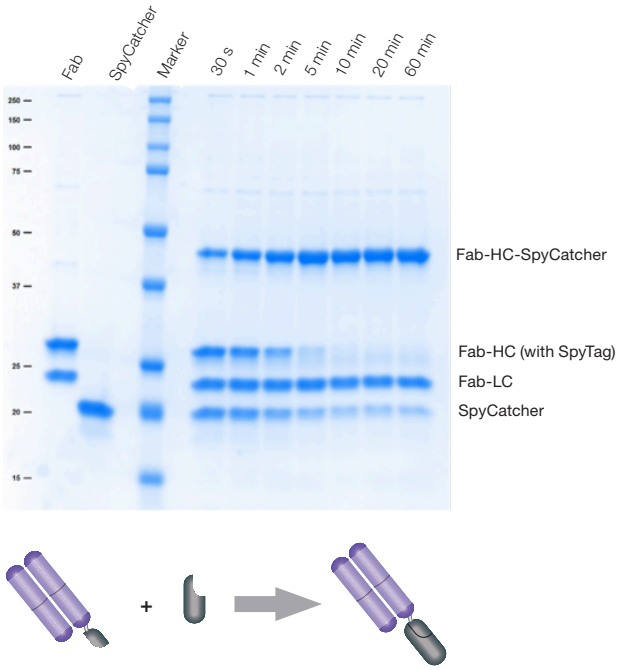


Fig. 5. Fab-SpyTag2 and SpyCatcher2 coupling reaction. In lane 1, both the Fab heavy chain (HC) and light chain (LC) are visible as they are not covalently linked; 30 sec after mixing the Fab-SpyTag2 and SpyCatcher2 the formation of the coupled Fab-HC-SpyCatcher2 can be visualized, and is seen to increase over the time course of one hr; the amount of individual Fab-HC-SpyTag2 and SpyCatcher2 is seen to decrease over the same time period. Coupling at room temperature, ratio Fab:Catcher = 1:1.25, 4 µM Fab-SpyTag2, 5 µM SpyCatcher2, Bio-Rad AnyKD gel, non-reducing conditions.

The Benefits of Site-Directed Conjugation

Several applications require or benefit from directly conjugated antibodies, such as a biotinylated or HRP conjugated detection antibody for a sandwich ELISA. Antibody conjugation protocols can be complex and time consuming, and conjugation is highly antibody specific. The conventional method is based on the random reaction of a label with the primary amines of the antibody. This process can result in some of the antibodies carrying a label at the antibody-antigen binding site, preventing them from binding to the antigen, decreasing the overall functionality of the antibody, and resulting in a sub-optimal assay. The degree of labeling follows a distribution curve that not only means each antibody carries a varying number of labels at different positions, but also that a new batch is likely to vary in the degree of labeling from the last. Site-directed conjugation through SpyTag-SpyCatcher coupling avoids these drawbacks as the antibody is labeled by coupling the already-conjugated SpyCatcher to the SpyTag located at the C-terminus of the heavy chain.

We have modified the SpyCatcher2 and BiCatcher2 by adding a cysteine residue, which facilitates site-specific conjugation, and results in a fixed degree of labeling (DOL). The result is an antibody with a fixed number of labels at a defined position. The labeling of an antibody simply requires mixing the antibody and conjugated Catcher together and leaving them at room temperature for one hour. Table 1 shows a list of currently available Spy- and BiCatchers.

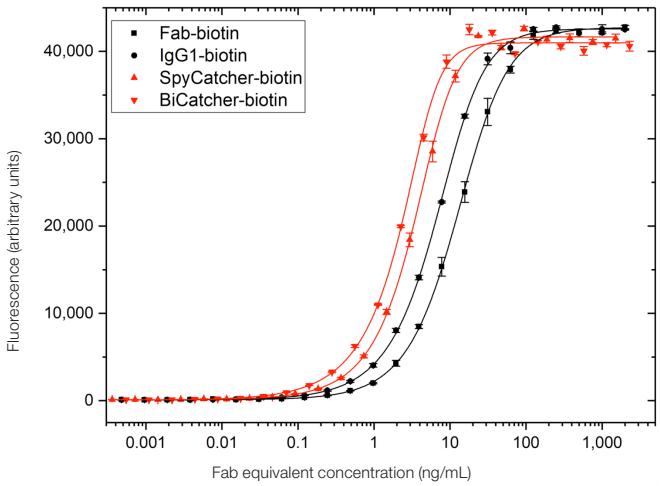


Fig. 6. Site-specific conjugation to biotin leads to better sensitivity. Fab antibody coupled to BiCatcher2-Biotin or SpyCatcher2-Biotin (red) perform better than the same antibody clone in Fab or IgG format (black) when chemically conjugated to biotin. A microtiter plate was coated overnight with adalimumab at a concentration of 1 µg/ml. Four different antibody variants were biotinylated: human anti-adalimumab (clone AbD18655) was biotinylated as Fab and as IgG via the Sulfo-NHS-LC-Biotin reagent; the same antibody with a SpyTag was coupled to biotinylated SpyCatcher2 or biotinylated BiCatcher2. After washing and blocking with PBST + 5% milk, the biotinylated antibodies were titrated to the given concentrations in PBST + 0.5% milk. Detection was performed using Neutravidin-HRP in HISPEC Assay Diluent, and QuantaBlu Fluorogenic Peroxidase Substrate. Data points are shown as the mean of three measurements.

Table 1. Catchers for coupling to recombinant Fab antibodies with a SpyTag. SpyCatcher and BiCatcher are expressed in *E. coli*, FcCatcher is expressed in a mammalian cell line; additional variants are in development, please inquire.

Monovalent	**Ig-like**
SpyCatcher2-HRP	Human
SpyCatcher2-Biotin	IgG1-FcSpyCatcher3
SpyCatcher2-Cys	IgG1-FcSpyCatcher3-HRP
SpyCatcher2-Flag3	IgG1-FcSpyCatcher3-Biotin
	IgG2-FcSpyCatcher3
Bivalent	IgG3-FcSpyCatcher3
BiCatcher2	IgG4-FcSpyCatcher3
BiCatcher2-HRP	IgG4-Pro-FcSpyCatcher3
BiCatcher2-Biotin	
BiCatcher2-Cys	Mouse IgG2a-FcSpyCatcher3
BiCatcher2-Flag3-His	Rabbit IgG-FcSpyCatcher3
BiCatcher2-PE	

Rapid Antibody Assembly into Different Formats

Recombinant antibodies can be converted to different formats by sub-cloning the gene fragments encoding the antibody variable domains into expression vectors containing the desired elements such as IgG constant domains, followed by expression and purification. This method is well established but can take several weeks to achieve. Using the simple SpyTag-SpyCatcher coupling reaction, monovalent Fab fragments can be converted into different antibody formats using BiCatchers and FcCatchers in less than an hour (Figures 3 and 5).

One antibody can be used in alternative formats based on suitability for the application, for instance a monovalent format for intrinsic affinity determination, and a bivalent format in a western blot to take advantage of avidity effects. The FcCatcher can be coupled to the antibody for those assays that require an Fc domain (Table 1). Mouse IgG2a-FcCatcher and rabbit IgG-FcCatcher are suitable for use with species-specific anti-Fc secondary reagents, and can also be used for multiplexing purposes by coupling different antibodies to different FcCatchers (Figure 7).

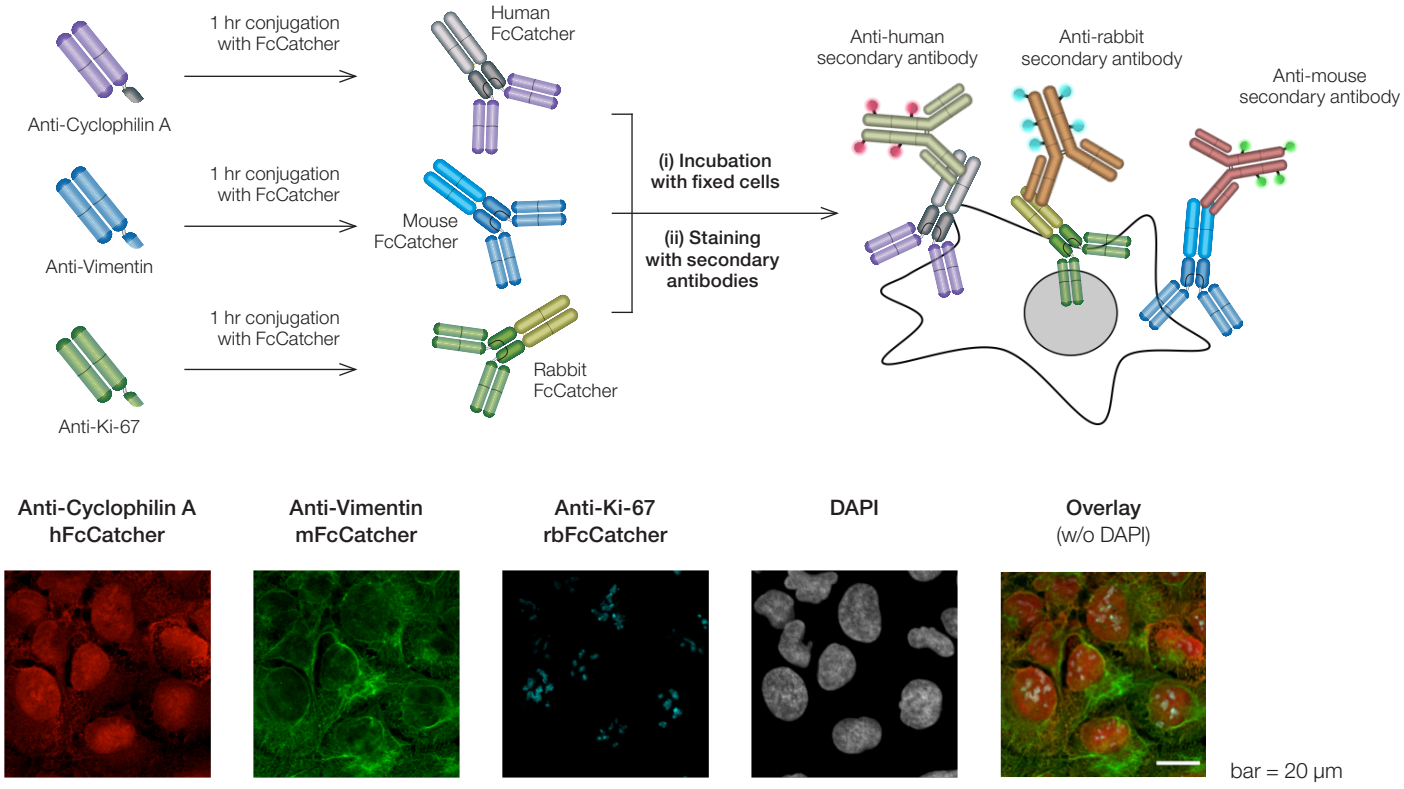


Fig. 7. Multiplexing immunofluorescence experiment. Three different Fab antibodies were coupled with three FcCatchers, simultaneously incubated with the fixed cells (U2OS) and after washing three different fluorescently labeled secondary antibodies were incubated simultaneously with the sample.

SpyTag Antibodies for Bioanalytical Method Development

Optimization of pharmacokinetic bridging ELISA

A robust pharmacokinetic (PK) assay requires a labeled detection antibody that can be produced with batch-to-batch consistency throughout the lifetime of a clinical study. To demonstrate suitability in this application, the performance of SpyTag coupled antibodies was compared with that of a full length immunoglobulin, IgG1 (Figure 8). The results were identical for the three different types of detection antibody.

Moreover, the concentration of the detection antibody coupled to BiCatcher and FcCatcher was reduced by a factor of 4 compared with the full IgG1 detection antibody, adding a reagent cost efficiency to the method.

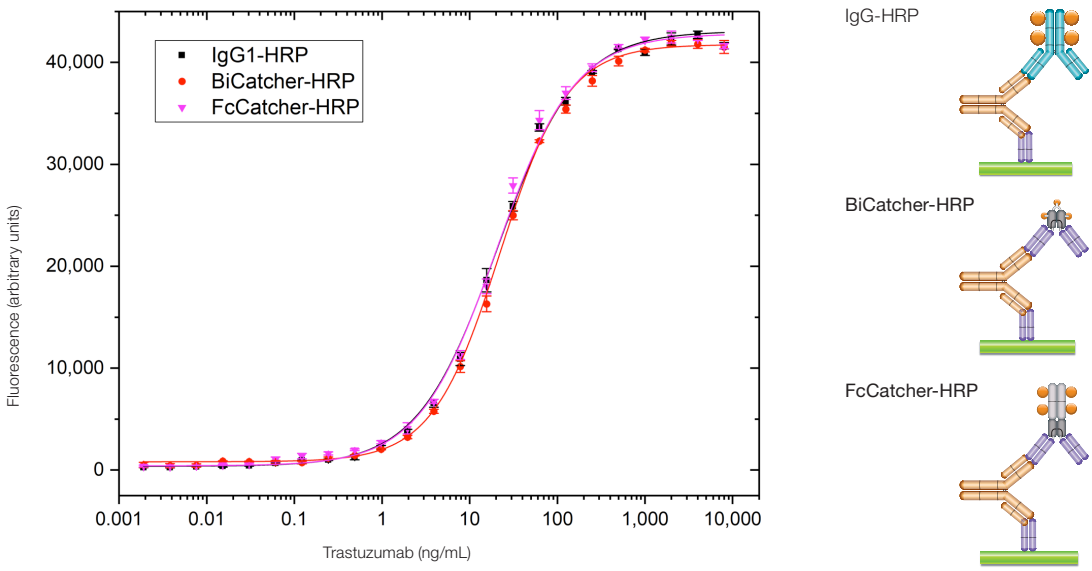
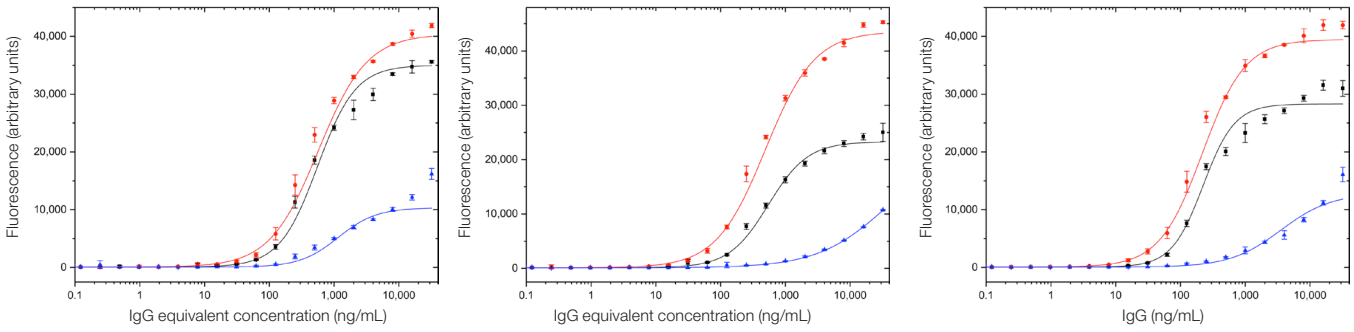


Fig. 8. Performance of SpyTag antibody formats compared with full immunoglobulin in a bridging ELISA. The three curves were generated each with the same capture antibody and the same detection antibody, the latter being prepared in different formats. A microtiter plate was coated overnight with human anti-trastuzumab antibody (clone AbD35758) at a concentration of 1 µg/ml in PBS. After washing and blocking with PBST + 5% milk, 10% human serum was added spiked with increasing concentrations of trastuzumab. Detection was performed using HRP conjugated human anti-trastuzumab, either clone AbD18018_hIgG1 (black square) or AbD18018-FSpy2H coupled to FcCatcher3-HRP (pink triangle) or BiCatcher2-HRP (red circle) at a concentration of 2 µg/ml IgG1, or 0.5 µg/ml FcCatcher and BiCatcher, in HISPEC Assay Diluent and QuantaBlu Fluorogenic Peroxidase Substrate. Data are shown as the mean of three measurements.

Quick Comparison of Performance in ADA Assay with Ig-Like Format

A set of antibodies in monovalent Fab-SpyTag format can be rapidly converted to Ig-like format using the FcCatcher for comparison as a reference standard in an anti-drug antibody (ADA) assay. This rapid modelling can help the user select the appropriate candidates for conversion to fully human IgG for the clinical assays (Figure 9). In instances where ADA isotyping is required, the FcCatchers with different human IgG isotypes offer a quick and cost effective alternative to full conversion to multiple antibody products.



A. Antibody + BiCatcher B. Antibody + FcCatcher C. Antibody in full IgG1 format

Fig. 9. Performance of high, medium, and low affinity anti-adalimumab antibodies compares well across BiCatcher, FcCatcher, and IgG formats. Antibody1, high affinity (red circle) KD 0.06 nM, antibody 2, medium affinity (black square) KD 0.2 nM, antibody 3, low affinity (blue triangle) KD 11 nM; the three different antibodies were compared in an ADA assay, coupled to **A,** BiCatcher, **B,** FcCatcher, and **C,** in full length IgG1 format.

Additional Benefits of Using Non-Animal-Derived Antibodies

Our recombinant monoclonal antibodies are selected from a synthetic, naïve antibody phage display library (HuCAL), and therefore benefit from the all the advantages of non-animal-derived antibodies, including precision, reproducibility, and flexibility.

The recently published recommendations of the European Commission Joint Research Centre (JRC) shine a spotlight on the versatility and scientific benefits of universal antibody libraries in combination with selection technologies, such as phage display, in generating precision antibodies for biomedical and scientific use (Barroso et al. 2020).

Antibodies generated through phage display libraries are developmentally, functionally, and structurally equivalent to those produced by animals, and improvements can be made to elevate quality further by refining or adapting the resulting gene sequences (Gray et al. 2016).

What Difference Could SpyTag Technology Make to Your Research Projects?

We have incorporated the option of SpyTag technology into our custom HuCAL antibody generation services. Monovalent Fabs generated from a new antibody project can be produced with a SpyTag from the outset, and existing HuCAL antibody clones can be easily converted to SpyTag format on request. A variety of Catchers is available for fast conversion from monovalent to bivalent Fab format and synthetic Ig-like construct, with and without labels. These products and services offer the user an enormous degree of flexibility to test different assay designs on different platforms, to achieve optimum results.

The degree of labeling of Catchers is measured and controlled between batches, and coupling Fab to Catcher is site-specific at a 1:1 ratio using SpyTag-SpyCatcher technology. This results in consistency of labeling between batches and avoids conjugation occurring at the active site.

Take Advantage of TrailBlazer Antibody Custom Services

- Rapid generation of highly specific Fab antibodies in as little as 8 weeks
- Isolation of antibodies against virtually any type of antigen, for instance proteins, peptides, biologics, low immunogenic antigens, and toxins
- Fast, flexible format conversion using SpyTag technology
- Site-directed conjugation with consistent DOL using SpyTag technology
- Improvement of binding affinity by molecular evolution, if needed
- Sequenced — antibody identity known, long-term secure supply guaranteed

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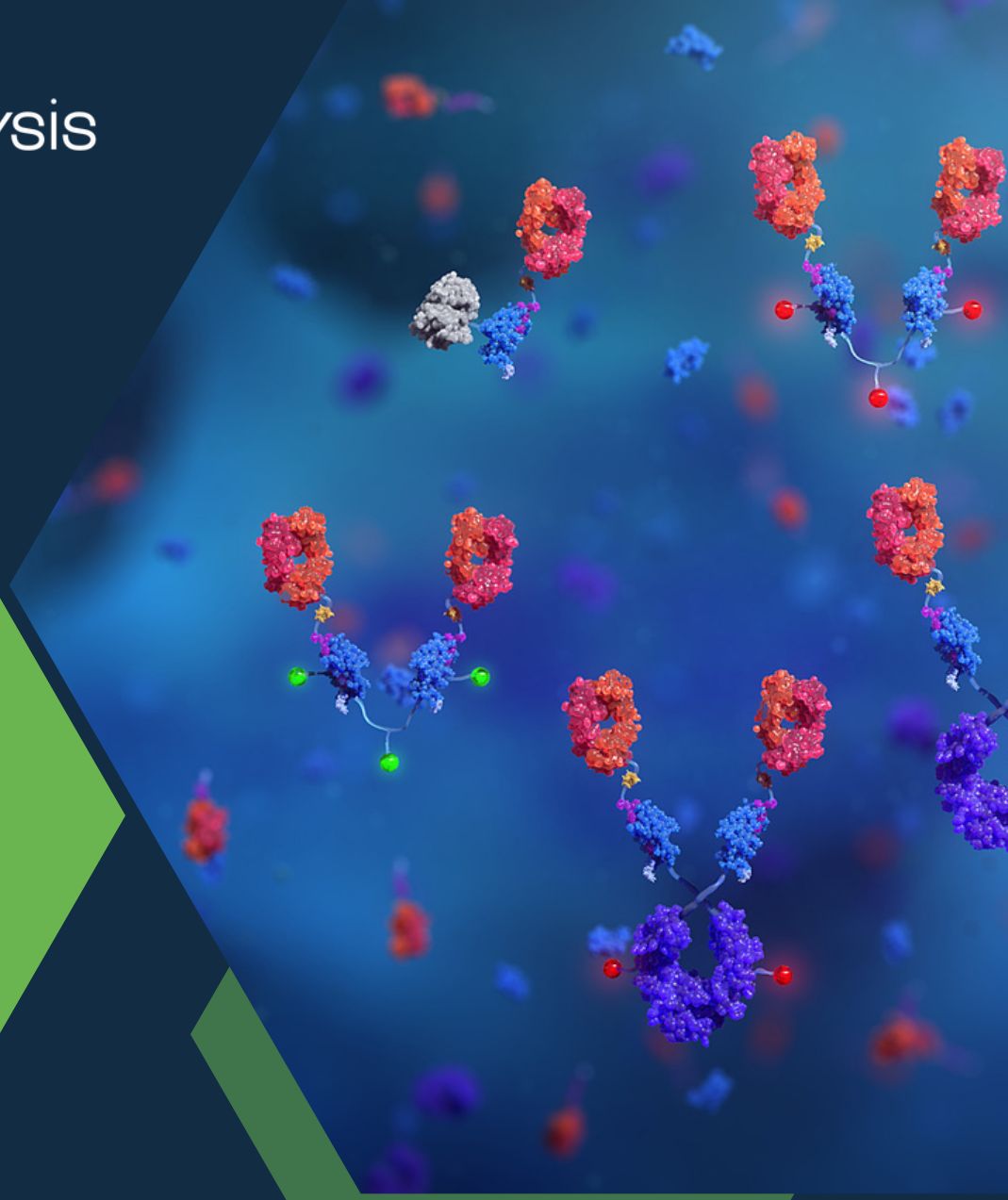

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