GEN Genetic Engineering & Biotechnology News eBook

Streamlining "Library-to-Lead" for your Antibody Therapeutics

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High Throughput mAb Characterization

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- Identify unique epitopes
- Build your IP portfolio

The LSA™ Array SPR instrument is a fully integrated antibody characterization platform that can analyze up to 384 binding interactions simultaneously, delivering up to **100x the data** in **10% of the time** with **1% of the sample requirements** of other systems.

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Welcome

The number of therapeutic antibodies entering the market continues to grow annually, with 2017 seeing ten approvals in either the European Union or United States supported by a robust late-stage clinical pipeline that will likely yield even more approved drugs in 2018 and 2019. Making a drug is an expensive and time-consuming endeavor requiring an incredible level of detail and rigor to ensure that drugs are safe, efficacious, manufacturable and convenient to administer to the patient. While antibody generation is highly commoditized, with modern *in vivo* and *in vitro* libraries typically producing vast numbers of clones, the analytical tools used to characterize them at the molecular level to understand their mechanism of action often fall orders of magnitude behind in throughput. This series of articles reviews some technological advances that can help triage library-to-leads and accelerate drug discovery.

The Carterra® LSA™ is a high throughput array-based surface plasmon resonance (SPR) instrument that can streamline the characterization of antibody libraries in terms of their binding kinetics, affinity, and epitope specificity, which are key parameters used to assess clones and select leads. The LSA analyzes up to 384 binding interactions in parallel with minimal sample consumption, making it significantly faster and more efficient than other methods.

To learn more about Carterra's high throughput Array SPR capabilities, visit us online at: **www.carterra-bio.com**

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Streamlining "Library-to-Lead" for your Antibody Therapeutics

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Epitope Binning for Next-Generation Biotherapeutic Discovery

High-Throughput Antibody Characterization

Yasmina Abdiche, Ph.D.

Monoclonal antibodies (mAbs) are one of the most successful therapeutic drug classes, generating lucrative sales for the pharmaceutical industry. In 2016, more than 20 therapeutic antibodies gained blockbuster status, as defined by their annual sales exceeding $1 billion, with the top-tier drugs—adalimumab (AbbVie), infliximab (Johnson & Johnson and Merck), rituximab, bevacizumab, and trastuzumab (Roche/Genentech)—each generating annual sales between $6 and 16 billion. Significant investment in antibodies to treat cancer, autoimmune disease, infectious diseases, rheumatoid arthritis, pain, heart disease, and many more therapeutic areas has resulted in a clinical pipeline of about 500 investigational drugs, of which more than 50 are in late-stage clinical evaluation with nine under review for first market approval as of 2017. Additionally, antibody-based assays are important in the diagnostic reagents industry.

The innate ability of antibodies to bind their targets with exquisite specificity and high affinity has been leveraged in the discovery of

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therapeutic and diagnostic antibodies, so selecting antibodies with appropriate binding characteristics is an important step in early-stage research programs. While an antibody's epitope largely dictates its biological function, this property can neither be predicted by *in silico* methods nor shifted rationally by engineering, so it must be selected empirically by screening a myriad of clones routinely produced by modern antibody libraries. Arguably, the epitope is a more relevant screening parameter than affinity, since the latter can be optimized by standard protein-engineering methods, whereas the former cannot. Indeed, it is highly desirable to discover antibodies with unique epitopes that may offer mechanistically differentiated modes of action and intellectual property opportunities. To meet the demand for evaluating these enormous antibody libraries, analytical tools are evolving to accelerate screening while minimizing sample consumption.

This article introduces how array-based surface plasmon resonance imaging (Array SPRi) methods can be used to perform high-throughput "epitope binning" assays that characterize the "epitope landscape" of an antibody library and facilitate the triaging of hits to leads, ultimately driving R&D costs down.

Biophysical Methods for Epitope Characterization

The gold standard for defining an antibody's epitope is with structural data, available from biophysical tools such as X-ray crystallography, cryo-electron microscopy, nuclear magnetic resonance, or mass spectrometry. However, while these techniques give a precise, albeit static, description of an epitope with atomic-level resolution, they are low-throughput, resource-intensive, and often require highly specialized staff to operate them, limiting their use to confirming leads at late-stage research, rather than screening for potential leads in early-stage research. Functional (or loss of function) methods for epitope characterization include antigen mutagenesis and peptide-based epitope mapping approaches, but they require the production of specialized reagents, which may not be readily available. Epitope binning is a competitive immunoassay that is used to assess the epitope diversity of an antibody library, in a relative sense, by clustering antibodies into epitope families or "bins" based on their ability to block one another's binding to their specific antigen, in a pairwise and combinatorial manner. Since "bin buddies" likely share similar (or near-identical) functional characteristics, bin representatives can be chosen to distill a large panel of clones to a small subset that retains the epitope diversity of the whole panel. By merging epitope-binning data with the results from orthogonal assays, such as functional cell-based data, binding affinity for the specific target, cross-reactivity to orthologs, and sequence data, a more comprehensive picture of the antibody library can emerge to inform the selection of leads worthy of further characterization. Identifying sandwich pairs by choosing antibodies from discrete, non-overlapping "bins" is fundamental to the design of diagnostic reagents that are used to detect biomarkers and support clinical programs.

Label-free biosensors, such as those employing surface plasmon resonance (SPR) or biolayer interferometry (BLI) detection, are biophysical

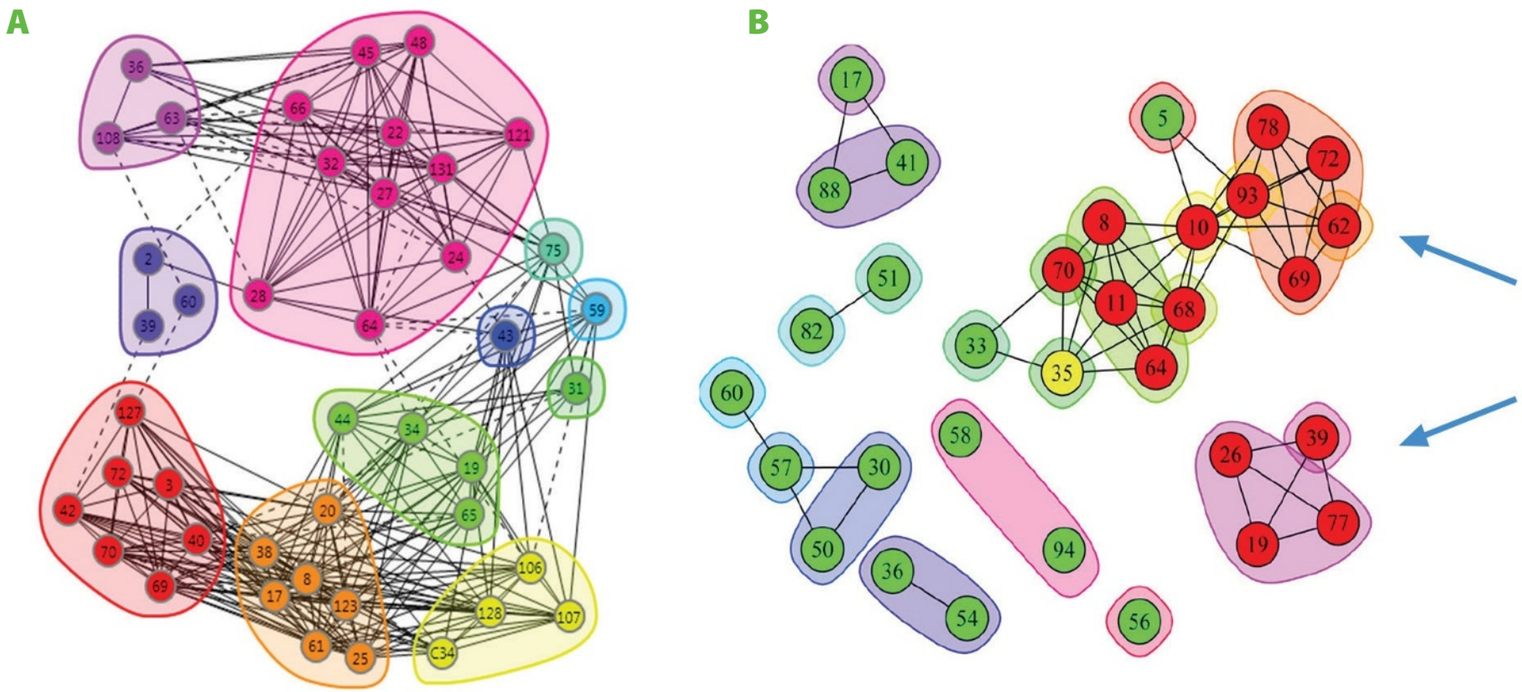

Figure. Examples of network plots used to visualize blocking relationships between antibodies and their deduced epitope clusters or "bins." A) Antibodies are colored by bin; the dotted chords represent a unidirectional (or asymmetric) blocking relationship (reproduced from Y.N. Abdiche et al., 2017, *PLOS ONE*). B) Antibodies colored by functional activity where red, yellow, and green indicate a block, partial block, or no block in a cell-based assay, respectively (reproduced from Y.N. Abdiche et al., 2014, *PLOS ONE*). The arrows indicate two discrete subsets of germline-encoded function-blocking antibodies.

tools well suited for performing epitope-binning analyses, and the results from these studies help elucidate an antibody's mechanism of action and identify clones with unique epitopes for therapeutic or reagent purposes. However, the limited throughput, high sample requirements, and costly consumables of traditional biosensor platforms make it impractical to perform epitope-binning assays on panels of antibodies larger than about 20 or so because the size of the experiment scales geometrically with the antibody panel.

Introducing Carterra's Approach

In contrast, Array SPRi technology by Carterra (formerly Wasatch Microfluidics) allows epitope-binning assays to be performed on much larger panels of antibodies in a facile manner. By employing a one-on-many configuration, samples are analyzed in a highly parallel style, which significantly accelerates throughput while conserving precious samples, allowing routine binning on 96-antibody arrays and expansion to 384-antibody arrays. Using specifically designed software, the results of a binning experiment are represented graphically in various ways, such as via proprietary network plots, in which blocking relationships between antibodies are indicated with chords, and bins are inscribed by envelopes (*Figure A*).

Furthermore, by merging data from independent assays, networks can be colored by various parameters, providing an intuitive visualization

tool for organizing multi-parameter information and facilitating the discrimination of clones with unique behaviors. For example, merging epitope-binning results with cell-based function-blocking data and sequence data on a panel of human antibodies produced from four healthy donors revealed two subsets of clones (*Figures A* and *B*). These clones displayed unique modes of neutralization against a *Staphylococcus aureus* virulence factor, exhibiting a strongly biased germline usage. This remarkable finding underscored an elegant example of convergent evolution, as the antibodies populating each bin cluster shared strikingly similar sequences that resulted in their nearly identical binding mechanism, despite their isolation from the naïve B cells of different individuals. The resolution afforded by the binning assay, therefore, enabled a deeper appreciation of the evolution of the human immune repertoire, expanding the concept of germline-restricted usage of antibodies to bacterial pathogenic proteins.[1]

Recently, Carterra announced the production of their LSA platform that integrates printing and imaging with automated switching between single-channel (large flow cell) and multichannel (96-printhead) modes, allowing routine binning of a panel of 384 antibodies in a single, unattended run, using less than 2 µg per antibody (1 µg for printing and 1 µg for sandwich pairing). This simplified workflow will not only enable higher-order epitope-binning assays but will also accelerate kinetic screening by enabling capture-based kinetics against multiple targets on 1,152 crude antibodies per unattended run. Considering the cost of development for a therapeutic antibody (from the bench to commercial release) is estimated to be $1 billion, the pharmaceutical industry is demanding higher-throughput analytical methods to facilitate the identification of product candidate leads with mechanistically differentiated modes of action as early as possible. ■

Recently, Carterra announced the production of their LSA platform that integrates printing and imaging with automated switching between single-channel (large flow cell) and multichannel (96-printhead) modes.

Yasmina Noubia Abdiche, Ph.D.
is chief scientific officer at Carterra.
Website: www.carterra-bio.com.

Reference

1. Y.A. Yeung *et al.*, 2016, *Nat Commun*

APPLICATION NOTE

High-Throughput Kinetics with Array SPR

Introduction

Throughput, speed, resolution, and sample consumption are typically key limiting factors for detailed kinetic characterization early in monoclonal antibody (mAb) discovery campaigns. Here, we show that Array SPR can facilitate the generation of high quality kinetic data from a large panel of clones rapidly and with minimal sample consumption. In this example of a single day's run, 384 independent kinetic measurements were made on an array comprised of 43 unique mAbs, each immobilized at 8–16 capacities, using a capture approach which does not require purified antibodies. This method required <1 µg per mAb and only 2 µg of the recombinant monomeric antigen. The array format provided well-described and highly reproducible kinetic measurements for clones spanning a 10,000-fold affinity range for their target antigen. These data clearly demonstrate the efficiency and quality of kinetic analysis that is possible using Array SPR.

Method

A capture kinetic analysis of a large panel of mAbs binding their specific monomeric antigen (as analyte) was performed using Array SPR. A moderate density (~1,000 Response Units) anti-human IgG-Fc capture "lawn" on a XanTec

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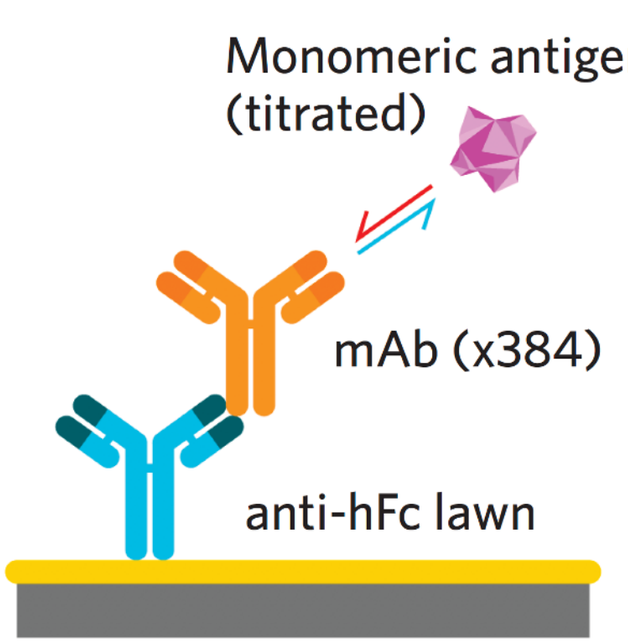
HC30M chip (30nm polycarboxylate) was prepared via amine coupling. To prepare the lawn, the chip was activated with 133mM EDC and 33.3mM S-NHS in 100mM MES pH 5.5 and goat anti-Human IgG Fc (Southern Biotech) was coupled for 10 minutes at 25µg/mL in 10mM sodium acetate pH 4.5 and quenched using 1M ethanolamine HCl pH 8.5. Carterra's continuous flow microspotting (CFM) technology was used to capture a panel of mAbs for 5 minutes at 2 µg/mL to create a 384-mAb array with 8–16 individual spots per clone. A purified recombinant monomeric form of the specific antigen targeted by the mAbs was injected over the entire printed array at 7 concentrations in a 3-fold dilution series ranging from 0.4–300nM using a 5-minute association phase and a 25-minute dissociation phase per analyte concentration. Running buffer was 10mM HEPES pH 7.4, 150mM NaCl, 3mM EDTA, 0.05% Tween 20 with 0.5 mg/mL BSA. Binding data were double referenced by subtracting the responses from an interspot (local reference) surface and the responses from a buffer analyte injection and globally fit to a 1:1 Langmuir binding model for estimation of k_a (association rate constant), k_d (dissociation rate constant), and K_D (affinity) using the Carterra™ Kinetics software.



Results

The results of a capture kinetic experiment performed on a 384-mAb array using 0.4–300nM monomeric antigen as analyte, are shown as a "tile view" in *Figure 1*, where each panel represents the binding responses (colored by analyte concentration, with a blue/green palette) and global fit (in red) obtained for the antigen interacting with a single mAb-coated spot. Since we had fewer than 384 unique mAbs to study, we arrayed each mAb onto 8–16 individual spots within the array to highlight the spot-to-spot reproducibility of the array format, which shows that the results are invariant of a spot's address within the array. The use of multiple spots per clone also meant that the apparent kinetic rate and affinity constants of each antigen/mAb interaction could be reported with statistical confidence (*Figure 2*). The use of a wide analyte concentration range enabled us to characterize clones across a broad affinity range, with apparent K_D values from <39pM to >222nM. The analysis discerned clones varying ~73-fold in their apparent association rate constant (k_a) and >7,000-fold in their dissociation rate constant (k_d), as shown by the histoplots in *Figure 2*. Examples of three clones showing diverse binding kinetics and affinities are shown in *Figure 3*. *Figure 4* summarizes the kinetic diversity of the studied panel, in terms of an iso affinity plot, where the three clones highlighted in *Figure 3* are shown as blue symbols.

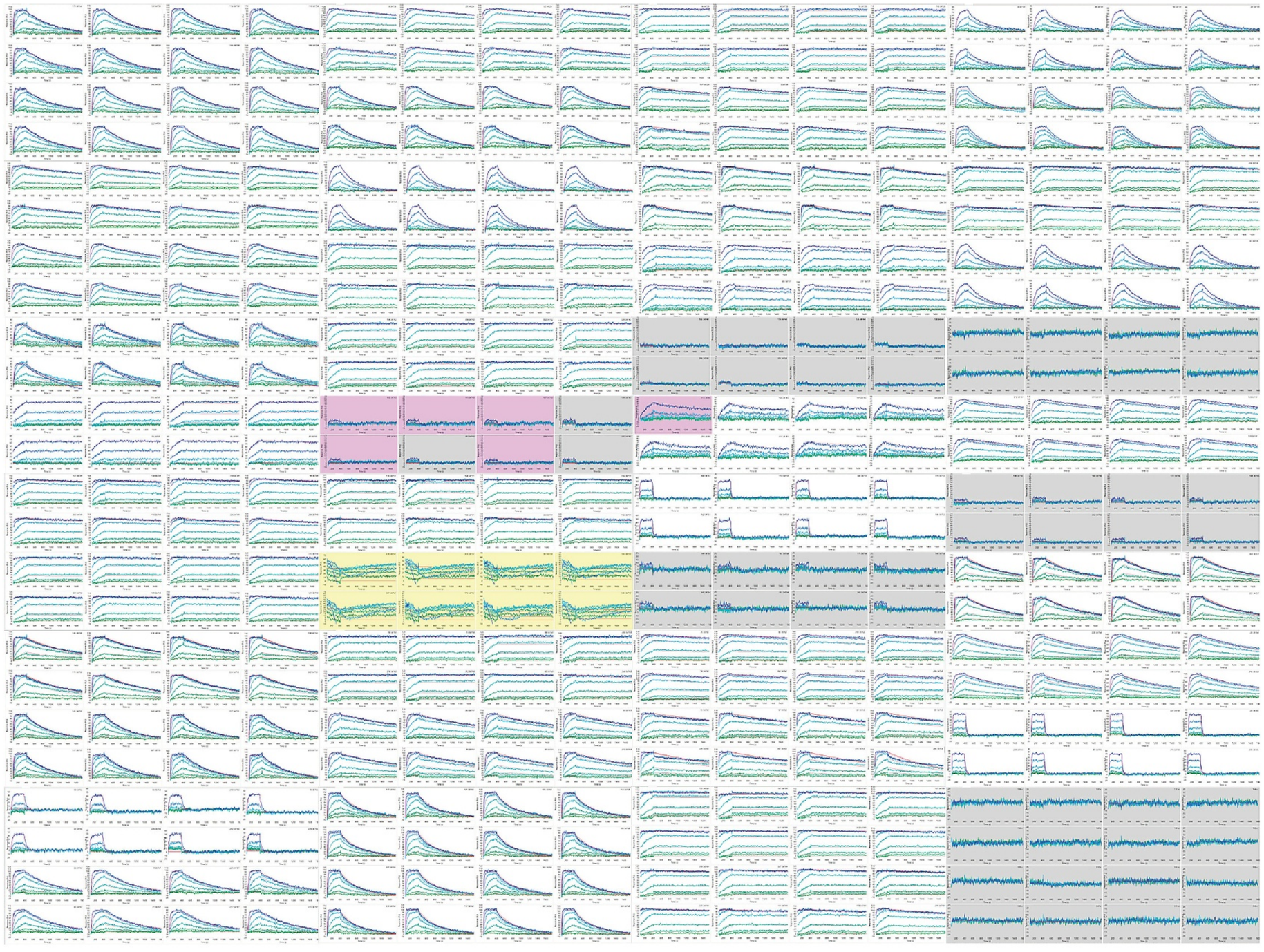


Figure 1. Capture kinetics was performed on 384 arrayed mAbs simultaneously using Array SPR. The array comprised 43 unique clones each captured onto 8–16 individual spots of an anti-human-IgG Fc-coated chip and their specific monomeric antigen was injected as analyte from 0.4–300nM over the entire array. Data with potential quality issues was automatically flagged by the software. Samples where the standard deviation of the residuals was >10% of the calculated R_{max}, which indicates a poor fit, are highlighted in yellow. Samples where the observed binding level was < 50% calculated R_{max} are highlighted in purple. Grey highlighting indicates barely binding or non-binder clones.

Figure 2. Affinity (K_D) values and kinetic rate constants (k_a and k_d) from *Figure 1* are reported as mean values from 8–16 replicates per clone and error bars reflect the standard deviation.

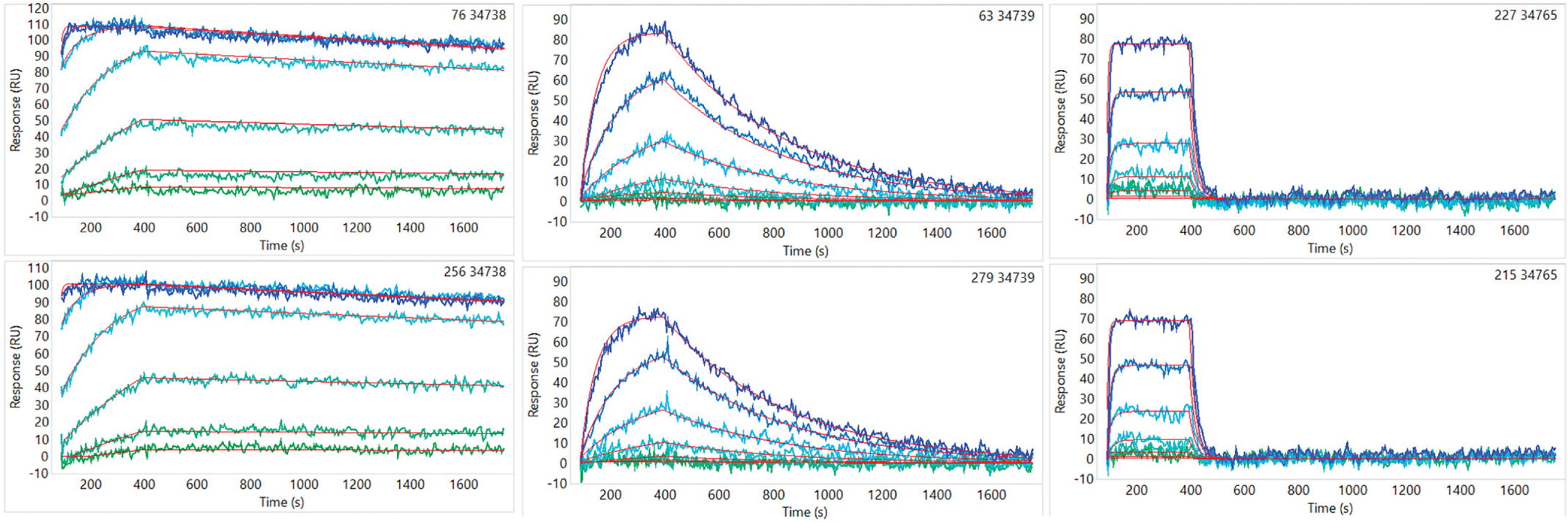


Figure 3. Example of duplicate spots for three clones with diverse kinetics (slow, medium, and fast dissociation rates, from left to right).

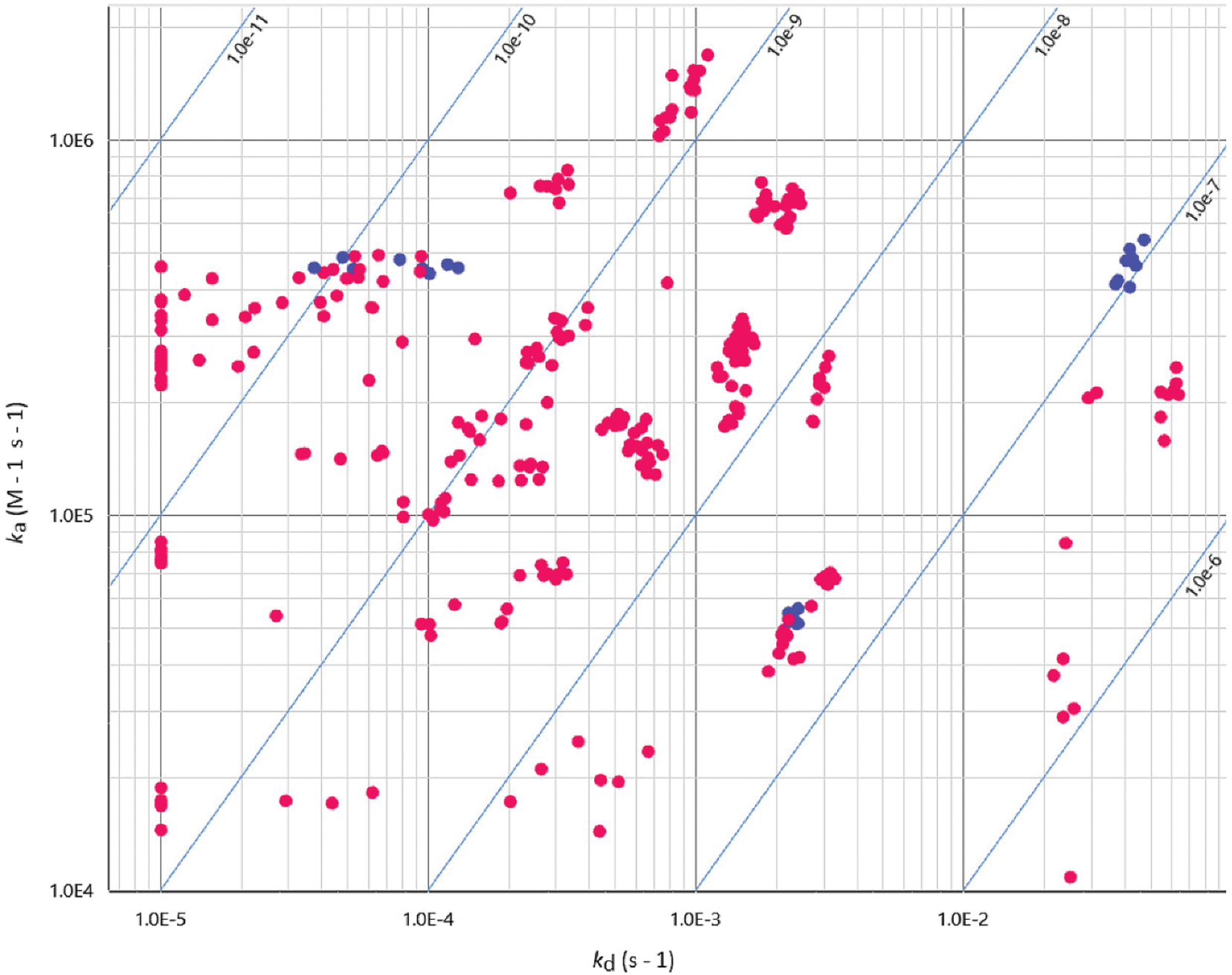


Figure 4. Iso-affinity plot, depicting the relationship between the association rate constant (k_a, y-axis) and the dissociation rate constant (k_d, x-axis) for all replicate mAb measurements (non-binders and clones flagged as poor fits in *Figure 1* are excluded). The diagonal lines represent lines of equal or "iso" affinity (K_D). The blue dots represent the replicate measurements for the three clones shown in *Figure 3*. Note that a k_d value of $1x10^{-5}$ (1/s) was used as a limit in the analysis, for clones showing barely any detectable dissociation within the allowed dissociation phase (of 25 min), as much longer dissociation phases would need to have been monitored to provide sufficient signal decay to accurately estimate slower dissociation rates.

Summary

Array SPR facilitates the rapid generation of kinetic screening data from up to 384 samples in parallel. The use of Carterra's CFM technology to print the 384-spot array allows for the efficient immobilization of mAbs to a capture surface from low concentration (and unpurified) samples. These reloadable arrays yield high-quality and reproducible binding kinetics of clones with diverse affinities and binding rate constants. Capture kinetic experiments performed by Array SPR take significantly less time and consume significantly less antigen than would be required for any other method to complete an analysis of this scale. Additionally, analyzing these large data sets is quick and easy using Carterra's powerful and intuitive Kinetics software, with a typical analysis taking only a few minutes.

This is part of collaborative work with Adimab, whom we thank for supplying the mAb panel. ■

Retooling Protein Characterization

If the Biotherapeutics Workshop Is to Progress beyond Rough-Hewn Products, It Will Need a Range of Finely Calibrated Analytical Platforms

Lisa Heiden, Ph.D.

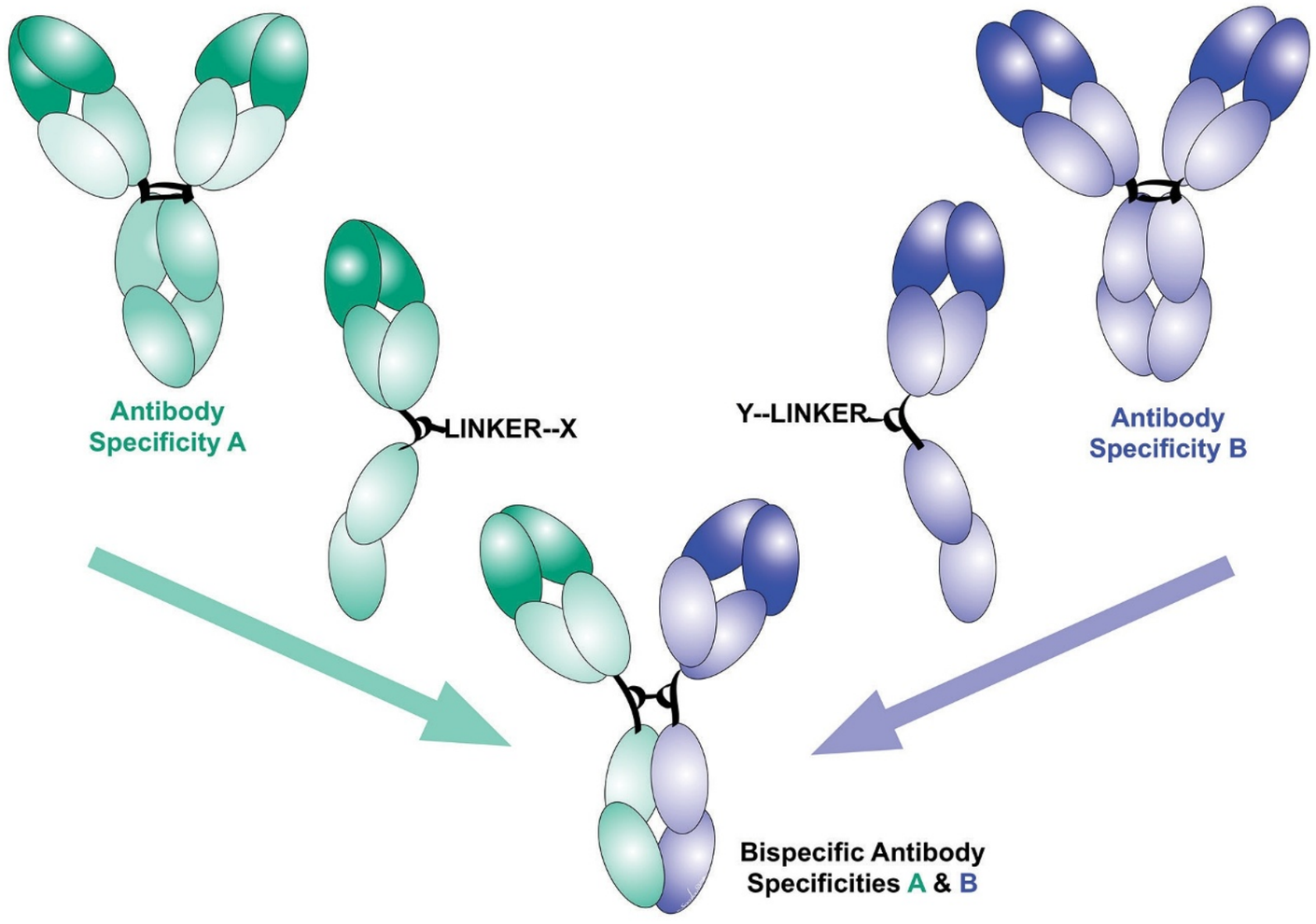
Andrew Brookes / Getty Images

Protein characterization approaches go hand in hand with analytical instrumentation platforms. This point was expressed in various ways by the presenters at Peptalk: The Protein Science Week, a Cambridge HealthTech Institute event recently held in San Diego.

The event's speakers and presenters also emphasized how protein characterization could advance specific research areas.

"Biotherapeutics, ranging from insulin to antibodies to viruses to siRNA, are interesting molecular tools to address a wide range of diseases," said Wafa Hassouneh, Ph.D., applications scientist, Wyatt Technology. "In the quest to develop effective and robust biotherapies, novel molecules have to be characterized to determine their properties and behavior."

Our increasing knowledge of cell-signaling pathways and mechanisms is opening up a plethora of opportunities for novel target-based biotherapeutic drugs. "I think everyone agrees



Schematic illustration of Sorrento Therapeutics' approach to generating chemically linked bispecific antibodies. Two immunoglobulin G antibodies with distinct specificities are chemically dissociated and modified and then linked together by means of a nonreducible, covalent bond that is introduced at the hinge region. Chemically modified antibody "A" can react only with chemically modified antibody "B," leading to the exclusive formation of the bispecific heterodimers.

harnessing the patient's immune system is powerful," declared Gunnar F. Kaufmann, Ph.D., senior vice president, Sorrento Therapeutics. "We are now getting to the point where we can make therapeutics targeting immune pathways."

The complexity of targets is increasing with the shift toward more complicated therapeutic intervention, such as bispecific antibodies (BsAbs). Emerging technologies offer significant functional advantages, such as synergistic mechanism of action and increased selectivity. They are also "putting an increasing emphasis on more detailed biophysical characterization studies," suggested Paul Belcher, Ph.D., functional leader, Biacore, GE Healthcare.

Stabilizing Membrane Proteins

Integral membrane proteins (IMPs), central players in cell-signaling pathways, are therapeutic targets for a significant percentage of approved protein drugs. Yet targeted IMPs are often not fully characterized due in part to challenges in preserving native, functional

conformations. High concentrations of detergents required for maintaining IMP solubility in aqueous solutions typically interfere with protein-characterization methods.

Nanodisc technology is a synthetic model membrane system increasingly being employed for overcoming associated IMP challenges. Han Xu, Ph.D., principal scientist, Amgen, discussed nanodisc as a reliable and enabling method for solubilizing and stabilizing IMPs into detergent-free lipid-bilayer environments.

The IMP is stabilized within the nanodisc structure "by a pair of membrane scaffold proteins and the lipid bilayer, which mimics the endogenous cellular membrane without detergent interference," explained Dr. Xu. "Thus, IMPs are likely to be presented in their native form or in a form very close to their native form in nanodisc."

"This approach," he continued, "enables a wide range of detection methods such as SPR (surface plasmon resonance), BSI (back scattering interferometry), and AUC (analytical ultracentrifugation) to be employed for detailed biophysical and biochemical characterization of membranes."

Dr. Xu noted that he had determined binding properties of peptide and small molecule inhibitors of Kv.1.3. He reported that he had used a chimeric ion channel, Kcs-Kv.1.3 nanodisc, and direct binding technologies. His results, he asserted, would have been impossible using micelles or liposomes.

Kv.1.3, an important therapeutic target, is a potassium voltage-gated ion channel with key roles in regulating activation of immune response signaling pathways. Although Kv.1.3 function remains to be fully elucidated, dysregulation is implicated in chronic inflammatory syndromes. There is considerable interest in developing more specific Kv1.3 inhibitors for attenuating immune responses.

Retooling Monoclonals into Bispecifics

Most BsAb technologies require genetic/protein engineering to fuse antibody (Ab) specificities together. Sorrento's approach, however, involves "an off-the-shelf approach," said Dr. Kaufmann. "We make IgG-like BsAbs utilizing proteins we already have."

Any two homodimeric Abs can be cut in half and joined together. Many companies already have plenty of antibodies, so there is no need to reinvent the wheel, said Dr. Kaufmann. "Just tweak already developed antibodies," he suggested.

Chemically modified Ab halves can't react with each other but can react with another specificity's half, forming BsAbs having properties the two parental Abs had as a mix, but not individually. "BsAbs binding to both targets at the same time have much stronger binding than either parental Ab," Dr. Kaufman points out.

Yanwen Fu, Ph.D., associate director of antibody technologies and chemical biology at Sorrento, described how easily research tools could be developed: "You can quickly combine any two specificities together and see what they do." Comprehensive protein characterization ensures the BsAb is suitable "not only for discovery, but also for future process development," Dr. Fu emphasized.

Cancer cells evolve mechanisms to undermine immune system networks and trigger shutdowns, like PD-L1 upregulation, which downregulates cytotoxic T cell activity and dampens immune responses. The c-Met /PD-L1 BsAb is designed to block aberrant c-Met phosphorylation/activation signaling while activating immune responses.

"It is critical to show that BsAbs keep the integrity and natural properties of parental antibodies," Dr. Fu insisted. Flow cytometry of surface-bound c-Met/PD-L1 bispecific-IgG on breast cancer cells verified strong binding affinity, and ELISAs showed that this BsAB suppressed c-Met phosphorylation induced by hepatocyte growth factor, validating c-Met blocking activity. PD-L1 blocking activity was verified in immunomodulatory assays with T and antigen-presenting cells wherein the BsAb increased IL-2 release, a T cell activity marker.

In vivo animal models will aim to show that the BsAb induces synergistic anticancer activity by inhibiting c-Met signaling while inducing immune-mediated cell killing. "We are always happy to partner with others who have Abs they want to turn into BsAbs," informed Dr. Kaufmann.

Applying New Tools and Techniques

Applied scientific knowledge and analytical instrument platform capabilities are inextricably interwoven. Philip Chapman, product manager at Bio-Rad Laboratories, polled customers, asking, "What was it that swung you to buy your chromatography system from us?"

"The number one answer we got was the software," Mr. Chapman said. The software, he asserted, is intuitive, easy to use, contains method/application templates, and is integral to the modular design of the company's next-generation chromatography (NGC™) system.

Dr. Hassouneh discussed Wyatt Technology's suite of analytical tools, which are also detailed in the company's webinars. "These tools help answer various questions," she maintained. "What biotherapeutic is synthesized? What post-translational modifications, impurities, or degradants are present? How does the biotherapeutic behave in different conditions? How robust it is during scale up? What interactions does it have with targets?"

Weighing in on techniques, Dr. Belcher pointed out that "over 60% of approved antibody therapeutics have used Biacore SPR either in research and development, R&D filing (investigational new drugs), or BLA (biological licensing agreement)." After stating that Biacore's DiPIA (Developments in Protein Interaction Analysis) online community and biennial conferences are available as venues for exchanging scientific expertise, he highlighted one particular upcoming event: "SPR in the drug discovery workflow will be covered at DiPIA 2016, an event for SPR practitioners that will be held in June in Berlin."

"No one biophysical technique can answer all the questions for a given CQA (critical quality attribute)," Dr. Belcher added. "Protein characterization requires a whole suite of techniques."

Seeing the (Scattered) Light

DLS (dynamic light scattering) assesses size distribution, and MALS (multiangle light scattering)

measures molar mass. Both are utilized throughout drug discovery, beginning with quality assessments of proteins to identify the best candidate molecules.

"Binding studies are no better than the quality of the solutions and molecules used in the studies," Dr. Hassouneh warned. She proposed using Wyatt's DynaPro® DLS Plate Reader II, which is, she said, capable of checking for aggregates in seconds: "You can run all your solutions through it to identify aggregated solutions before they are inadvertently loaded onto delicate instruments."

"Promising biotherapeutic candidates are tested for stability and robustness as they are pushed further down the pipeline," noted Dr. Hassouneh, who added that thousands of conditions could be screened per day with DynaPro to determine aggregation and stability of the biotherapeutic.

Wyatt's MALS detectors (Dawn® Heleos® II, Mini-Dawn™ Treos®, and uDawn™) are used in conjunction with chromatography to determine the absolute molar mass, size, conformation, and/or conjugation of macromolecules such as proteins without reference to molecular standards.

MALS is combined with their Calypso® composition gradient to characterize biomolecular interactions. "Calypso is a great label-free, immobilization-free tool to characterize simple or complex biomolecular interactions," Dr. Hassouneh exclaimed.

The Astra® software package leverages instruments' capabilities, providing powerful analysis tools including the protein conjugate analysis tool, which enables determination of molecular weight distributions of PEGylated proteins, antibody glycosylation status, or drug-to-antibody ratio.

Real-Time Reporting

Biacore uses the biophysical phenomenon of SPR to detect and characterize biomolecular interactions in a real-time, label-free, and contact-free environment.

Real-time allows one to see the entire event as it occurs, not just a snapshot or endpoint, as in ELISA. Real-time measures the kinetics of interactions such as how quickly drugs recognize their protein targets or how long they occupy receptors.

"Kinetics has therapeutic consequences affecting multiple aspects of drug function such as dosing and pharmacokinetics," says Dr. Belcher. "Kinetics allows the linking of structure to function, allowing a greater understanding of biological mechanisms."

"Contact-free" means the detection system is, on principle, outside of where protein interactions take place. This enables one to work with opaque samples or complex mixtures such as crude antibody hybridoma supernatant or even blood, making it highly suitable to process-development workflows.

The FDA requires biosimilars to be highly similar to the innovator product, with no clinically meaningful differences in terms of safety, purity, and potency. "Contact-free" enables crude protein products to be analyzed early on against the innovator molecule, allowing the identification of problems much earlier in the production process.

Biacore added a comparability function to its

platform in 2015, allowing direct statistical comparisons of one sample to another. The comparability software enables drug companies to assess how similar their fledging products are to the innovator partner they are hoping to compete against.

"It overcomes the challenge of characterizing kinetic interactions that are not a simple 1:1," concluded Dr. Belcher. "Our comparison tool enables that because it provides a direct statistical comparison. It doesn't require a mathematical model, meaning it's always applicable."

Streamlining Separation

Moving from a single column—bind, elute, and analyze your fraction—to more multicolumn applications or automated batch purifications is part of NGC.

Chromatography, based on size separation, is an essential protein purification tool. For downstream characterization processes, it's critical "to ensure that you're looking at what you intended to look at, and that you haven't co-purified something affecting protein integrity," said Jeff Habel, Ph.D., applications manager, Bio-Rad Laboratories.

Traditional chromatography was labor intensive. Skilled, experienced people were essential because it was so difficult to learn. Previously, even a simple His-tag purification took days. To accelerate such separations, Bio-Rad developed NGC.

NGC is designed to automate processes and effectively reduce days to hours. The idea is to let users load samples, press a button, and walk away to do other work while the entire purification process happens by itself.

According to Bio-Rad, NGC automates processes for empirically determining optimal purification conditions. For example, NGC can prepare a range of buffers. "Just specify the salts and pH you want, and the automation scouts through a range of different conditions during the run," explained Mr. Chapman. Then, he continued, the user may overlay the different chromatograms to identify the separation's optimal conditions. The modularity of the system enables columns and pumps to be switched out as the user goes from pilot studies, which involve purifying small amounts of protein, to scale up efforts, which require larger columns.

The NGC "open the box and start to use it" approach is meant to make chromatography accessible to more people. "Smaller research groups or for-profit labs can now do their own purification work as part of their work flow," asserts Mr. Chapman.

NGC is designed to be customizable. For example, instrument modules can be matched to customer requirements. As requirements expand, "just drop in new modules," Mr. Chapman advised. "The software recognizes them, and the functionality is immediately there." ■

Antibody Validation Group Urges Multipronged Approach

Although poorly characterized antibodies are blamed for the loss of hundreds of millions of dollars—the bitter fruit of failed or unreliable experiments—dissatisfaction with antibody validation has yet to be adequately addressed. A comprehensive framework for antibody validation across research applications is still lacking.

In hopes of stimulating the research community to achieve a higher standard of antibody reproducibility, the International Working Group on Antibody Validation (IWGAV), an independent group of international scientists, issued a set of guidelines. These guidelines, which appeared in *Nature Methods*, emphasize that antibody validation is best pursued in an application—and context-specific manner.

In *Nature Methods*, representatives of the IWGAV, including the group's chair, Mathias Uhlén, professor of microbiology at Sweden's Royal Institute of Technology, published a paper entitled, "A Proposal for Validation of Antibodies." The paper outlined five "conceptual pillars" to guide antibody validation in specific research applications:

Hero Images / Getty Images

- Genetic strategies: Measure the relevant signal in control cells or tissues in which the target gene has been knocked out or knocked down using techniques such as CRISPR-Cas or RNA interference (RNAi).

- Orthogonal strategies: Use an antibody-independent method for quantification across multitudes of samples and then examine the correlation between the antibody-based and antibody-independent quantifications.

- Independent antibody strategies: Use two or more independent antibodies that recognize different epitopes on the target protein and confirm specificity via comparative and quantitative analyses.

- Expression of tagged proteins: Modify the endogenous target gene to add sequences for an affinity tag or a fluorescent protein. The signal from the tagged protein can be correlated with detection through antibody-based methods.

- Immunocapture followed by mass spectrometry (MS): Couple immunocapture, the technique of isolating a protein from a solution through binding with a target-specific antibody, with MS analysis to identify proteins that interact directly with the purified antibody as well as proteins that may form a complex with the target protein.

"We suggest that at least one of these pillars should be used as a minimum criterion for claiming that a particular antibody has been adequately validated for a specific application," the authors of the *Nature Methods* article wrote. "The use of multiple strategies would further strengthen this conclusion."

The article also included recommendations for producers and users to ensure antibody reproducibility over time. For example, besides recommending that users of antibodies carry out at least one of the validation strategies described here in their own particular application or sample context, the article's authors also suggested that users adhere to appropriate reporting guidelines and, at a minimum, include catalog number, lot number, and perhaps RRIDs (Research Resource Identifiers) to ensure that any antibodies used in their research can be identified unambiguously.

Similar advice—to adopt at least one of the five pillars, and then go further—was offered to producers, which were encouraged to provide as much additional information

> We recognize that input from all stakeholders, including funders, publishers, antibody providers, and the research community, will facilitate widespread adoption of our recommendations.

regarding the antibody as possible, such as antibody concentration in characterization assays, concentration of cellular extract or of the immunogen, details of the immunogen used for antibody production, antibody–epitope affinity, antibody isotype, buffer formulation, and the material data sheet. Among the producers that may be interested in these suggestions is Thermo Fisher Scientific, which extended financial support to the IWGAV in 2015 to encourage the development of standards and help overcome the challenges associated with antibody specificity and reproducibility.

"This publication is an important first step toward the development of widely accepted standards for validating antibodies and ensuring high quality and consistent antibodies for biomedical research," said Dr. Uhlén. "We look forward to receiving feedback from the broader community of antibody users, publishers, funding agencies, and producers to help strengthen this initial proposal and ensure the reliability of these essential tools of biomedical research."

"We recognize that input from all stakeholders, including funders, publishers, antibody providers, and the research community, will facilitate widespread adoption of our recommendations," the authors of the *Nature Methods* article concluded. "Specifically, this wider community can provide critical insight into the timing for adoption of new proposals, the mechanisms used to enforce recommendations, and the specific responsibilities of each stakeholder as recommendations are implemented." ■

Antibody Characterization Balances Rigor and Reason

Emphasizes a Combination of Antibody-Dependent and -Independent Testing Methods

Angelo DePalma, Ph.D.

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Authentication is becoming a hot-button topic among researchers. For example, researchers who use cells that are meant to possess distinct characteristics have been making an issue of cell-line authentication.[1] With increasing frequency, researchers who use antibodies are raising authentication concerns of their own. These researchers are insistent that antibodies be validated for function and purpose.

Antibodies are critical research reagents, says Nicolas Schrantz, Ph.D., senior manager for antibody development at Thermo Fisher Scientific. They can lead to research success, but only if they perform as desired—which is to say, as advertised. "Developing an antibody that recognizes the correct target using a specific application is an art that requires scientific knowledge and technical expertise," notes Dr. Schrantz. "Unfortunately, many antibodies on the market that claim to have been validated for a specific application simply do not recognize the target they are

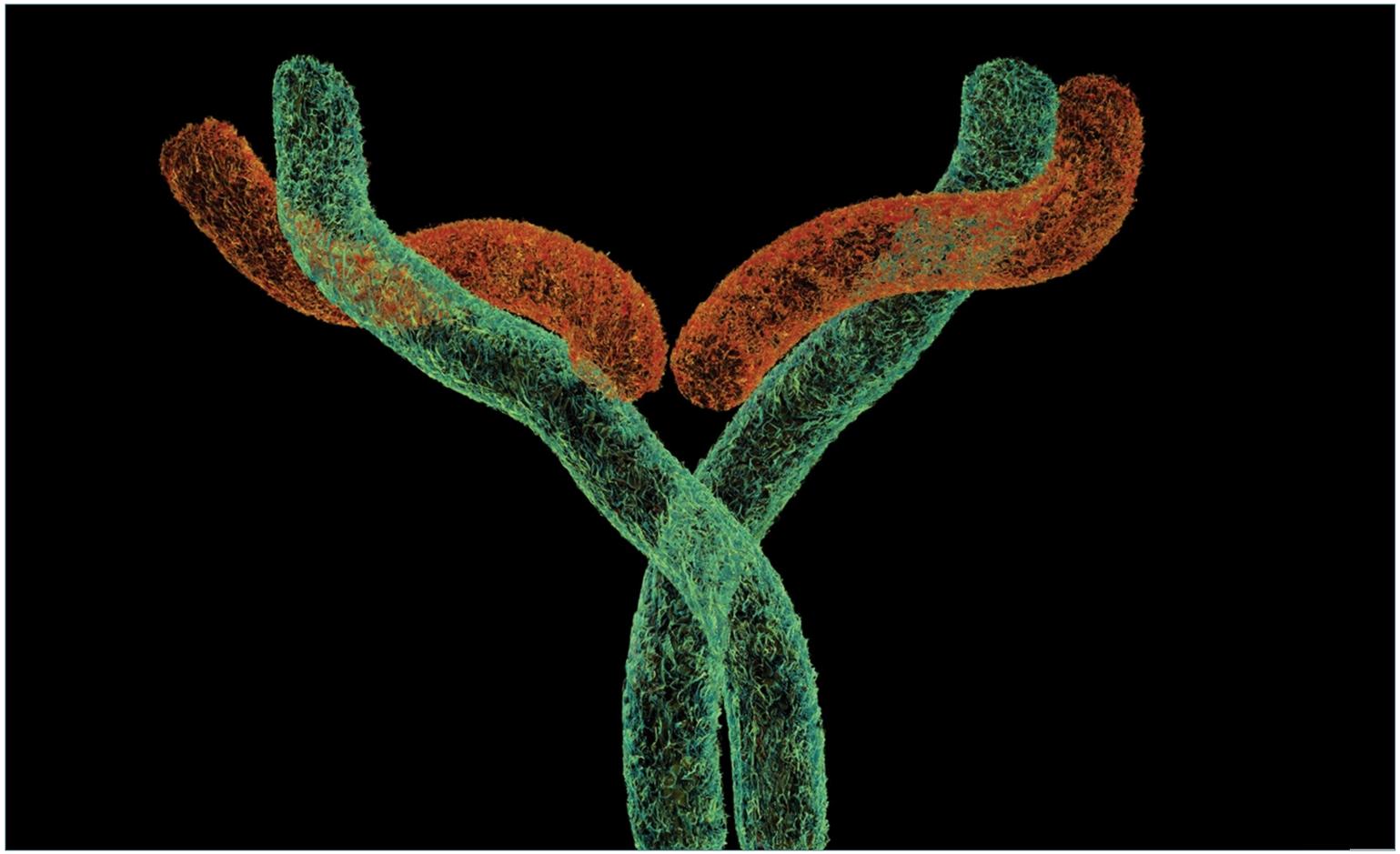
supposed to, or worse, recognize the wrong target. The existence of unreliable antibodies wastes researchers' time and money."

Two-Step Validation

Thermo Fisher Scientific has been working with individual researchers and the International Working Group for Antibody Validation (IWGAV) to define and implement standards for antibody testing. The company has already adopted validation standards for its Invitrogen antibody portfolio that adhere to IWGAV recommendations.

Thermo Fisher Scientific uses a two-step validation approach. The first step is target specificity verification. The second step is functional application verification.

Target specificity verification assures that antibodies bind to the correct target. Validating an antibody's specificity also ensures the absence of nonspecific binding.



The structure of antibodies is well known, but understanding their true function is where things get more nebulous. knorre/Getty Images

To accomplish target specificity validation, Thermo Fisher uses at least one standard method from a collection of standard methods. These methods span several categories: immunoprecipitation/mass spectrometry; genetic modification (knockout and knockdown testing); independent antibody verification (a testing approach that uses two differentially raised antibodies that recognize the same protein target); and biological verification (cell treatment, relative expression, neutralization, peptide array, and orthogonal approaches).

Thermo Fisher employs several knockdown and knockout methods to test antibody performance against genetically modified samples. They include mouse knockout models, dominant negative mutants, morpholinos, short interfering RNA, and most recently, gene editing. CRISPR-Cas9 gene editing allows creation of knockout cell models for use as controls for validating antibody specificity.

In independent antibody verification, antibodies should target non-overlapping epitopes on an antigen. "Obtaining comparable [affinity] results increases confidence that these antibodies are specific and suitable for the detection of their intended target," Dr. Schrantz tells *GEN*.

With the orthogonal approach, the idea is to correlate two methods, an antibody-dependent method and an antibody-independent method. For example, Western blot could be correlated with quantitative RT-PCR, or flow cytometry could be correlated with Thermo Fisher's own PrimeFlow™ RNA assay kit.

The second level of validation involves determining how well antibodies work in applications such as Western blotting, immunofluorescent imaging, flow cytometery, chromatin immunoprecipitation, and immunohistochemistry. "We test our antibodies using at least one of these methods," states Dr. Schrantz." And yes, we even use antibodies or kits from other vendors for this purpose."

Middle-Up Approach

Monoclonal antibodies (mAbs) are inherently heterogeneous because they are produced in living cells and can undergo unanticipated modifications during the biomanufacturing process. Living cells are capable of variability at every stage of protein expression, from the generation of amino acid chains during the formation of protein backbones, to the introduction of post-translational modifications (PTMs), some of which occur enzymatically (glycosylation), and some of which occur non-enzymatically (oxidation and deamidation). Additional modifications may occur during purification and storage.

GEN readers are familiar with chromatographic and compound analytical methods, as well as the importance of using multiple, sometimes orthogonal methods, for example, size-exclusion or ion-exchange chromatography and peptide mapping. Peptide mapping followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS)—the method of choice for

understanding site-specific PTMs—involves serious sample preparation and lengthy chromatography runs. Researchers refer to proteolysis-based mapping as bottom-up proteomics, and those based on intact proteins as top-down methods.

Peptide mapping is a multistep process generally involving antibody denaturing, reduction, and alkylation; digestion with a protease (usually trypsin); high-performance liquid chromatography (HPLC) on a octadecyl carbon chain (C18)-bonded silica column; and finally, mass spectrometry "online" with HPLC to identify the separated peptides. Together, the steps in peptide mapping take about one full day, including overnight digestion.

Peptide map data analysis is complex by virtue of the sheer number of peptides generated, all of which require identification and quantitation. Analysis gets even trickier when full digestion does not occur, or when products of nonspecific digestion are present.

Investigators involved in mAb characterization are therefore interested in alternatives to full peptide mapping. Many of these methods, including middle-up or middle-down, employ proteolytic enzymes that are somewhat more selective than trypsin.

Among these is the immunoglobulin-degrading enzyme from *Streptococcus pyrogenes* (IdeS), which was first reported by Swedish researchers in 2002,[2] and is now provided in commercialized in kit form by several vendors. IdeS reagents and kits are sold by Genovis, MilliporeSigma, Creative Enzymes, and Promega. "IdeS is effective due to its high cleavage specificity and simple operation," explains Chris Hosfield, Ph.D., a senior research scientist at Promega. A related enzyme, IdeZ (from *S. equi* subspecies *zooepidemicus*), has identical specificity but cleaves mouse IgG2a more efficiently than IdeS.

IdeS cleaves the IgG heavy chain below the hinge region. Post-digestion addition of a reducing agent yields a sample containing three fragments of about 25 kDa in size. This is the starting point for subsequent analysis—hence the "middle-up" designation. The approach identifies domain-specific oxidation, charge profiling, and N-glycan profiles. In a study published in *MAbs*,[3] domain separation was achieved with a 30-minute HPLC gradient, and oxidations were quantified through ultraviolet detection.

IdeS is faster than peptide mapping, taking up to about one hour instead overnight. The separation stage is faster because only three fragments are involved. "HPLC gradients are in the range of 30 minutes instead of three hours," asserts Dr. Hosfield. Data analysis is simpler for the same reasons. "For these reasons, many companies use IdeS-based analysis as a platform method, for example, in quality control lot-release settings."

The downside is that information on modifications is not site-specific. For example, one could detect an oxidation occurring in the Fc region, but not determine definitively which methionine within that region was affected.

A more recent study by a group at Genentech described an improved IdeS in which separation of

the IgG domains and variants took just 10 minutes.[4] Measured oxidation levels were comparable to those achieved by more complex and time-consuming peptide mapping.

Putting Validation to Use

The level at which manufacturers validate antibodies depends on the antibodies' intended use. An example of how validation level may complement antibody use is provided by Abbiotec, which manufactures both polyclonal antibodies (pAbs) and mAbs for research purposes. The "company characterizes its products for target reactivity, but according to CEO Hervé Le Calvez, Ph.D., it doesn't assay products for physicochemical properties, outside of purity determinations by ELISA or SDS-PAGE, which specifically test for contamination by other antibodies.

"Our approach is similar between pAbs and mAbs, especially when we use peptides as antigens," Dr. Le Calvez says. "We mainly produce GLP-grade antibodies, so we make sure screening, titering, and isotyping, if applicable, are done correctly before moving on with purification and testing." Purification methods for pAbs vary from standard Protein A or Protein G to antigen-affinity chromatography and fractionated precipitation.

"Because of the difficulties in characterizing pAbs from each bleed or animal," explains Dr. Le Calvez, "we offer the option to clone the genes coding for the antibodies and produce recombinant antibodies when the end goal is the diagnostics or therapeutics market."

One issue entering the validation/characterization equation is the antibody target. "It's one thing to validate an antibody against a well-known target such as TNFα [tumor necrosis factor alpha] or NF-κB [nuclear factor kappa B], but quite another if the target is the latest new protein identified by sequencing," insists Dr. Le Calvez. "Our products lean towards the latter."

There is much to learn, he adds, about the validation of antibodies that target new molecules that have not themselves been fully characterized. Abbiotec releases such products as a service to research groups that do not fully disclose their intentions. "In this respect," reveals Dr. Le Calvez, "we provide numerous antibodies for niches that are still undeveloped."

Lest We Forget...

In the characterization of intact, therapeutic-grade mAbs, conventional analysis tools are being deployed more systematically. As mass spectrometry (MS) becomes more user-friendly and generally accessible, mAb developers

Accurate mass determination, separation of protein isoforms, and detection of major and minor heterogeneities provides reliable answers about the protein and growth conditions directly from intact protein analysis.

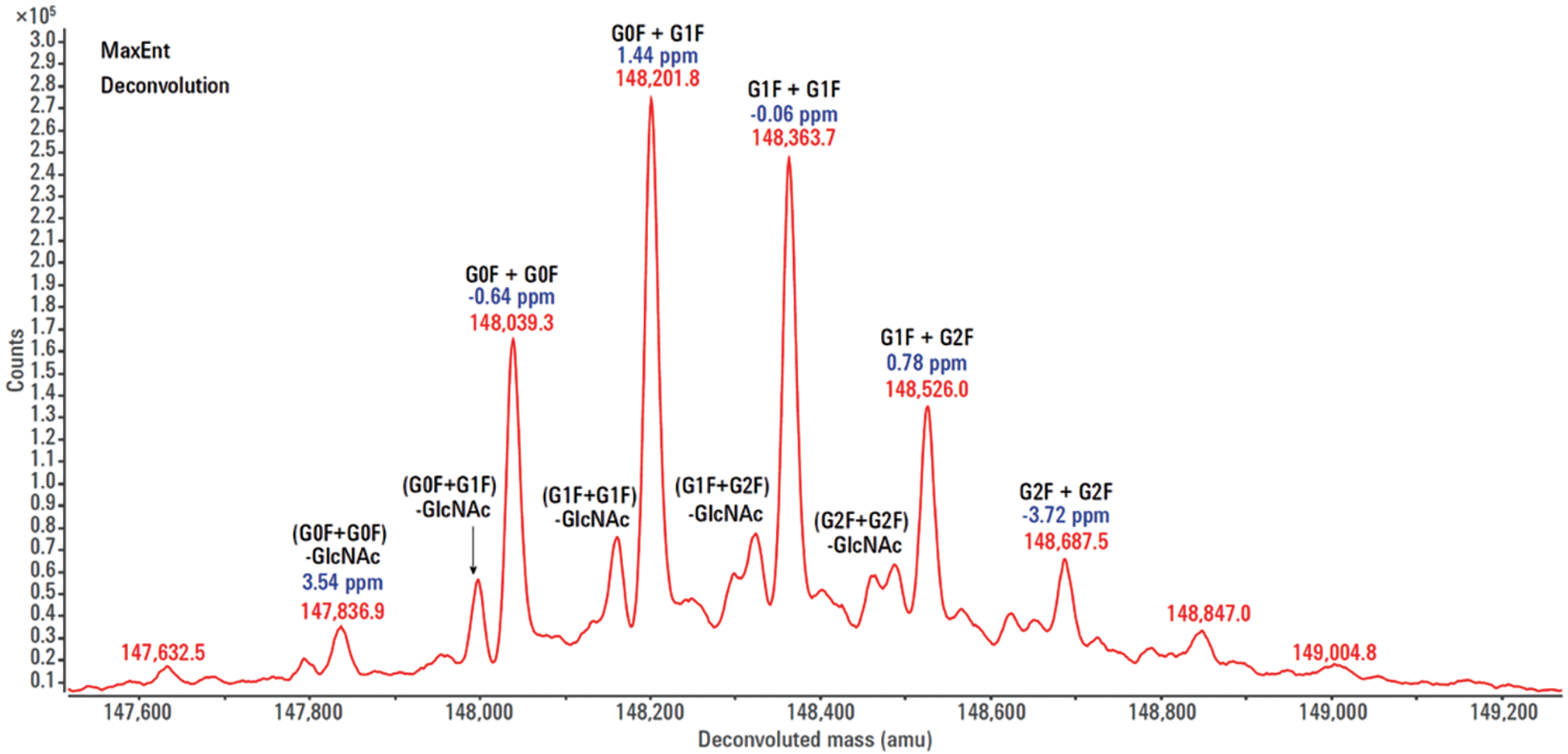


Figure. Major glycoforms and minor isoforms alike are clearly seen with the Agilent 6545XT AdvanceBio LC/Q-TOF system. The data was deconvoluted using the maximum entropy algorithm in the Agilent MassHunter BioConfirm software. This algorithm carefully preserves low-level peaks so that the heterogeneity of a molecules may be fully characterized.

routinely adopt this method to obtain a product's accurate molecular weight and heterogeneity (for example, with respect to PTMs). For development-stage antibody-drug conjugates, MS provides further insights into conjugation number, mAb sequence variations, and degradation products.

"Confidence in the information obtained from analyzing intact mAbs depends on measurement reliability," explains David Wong, Ph.D., a senior applications scientist at Agilent Technologies. "Accurate mass determination, separation of protein iso-forms, and detection of major and minor heterogeneities provides reliable answers about the protein and growth conditions directly from intact protein analysis."

Due to its high resolution in high mass ranges, quadrupole time-of-flight (Q-TOF) LC–MS is the mode of choice for analyzing intact proteins. Top vendors have "systematized"

these instruments toward specific purposes. For example, the Agilent 6545XT AdvanceBio LC/Q-TOF system includes hardware and software features for characterization of biomolecules up to 30,000 m/z.

"Q-TOFs have the flexibility to analyze not just intact proteins, but also to perform peptide sequence mapping and PTM identification and localization at the peptide level," Dr. Wong adds.

In a recent application note,[5] Agilent described a typical workflow involving the Agilent 1290 Infinity II UHPLC system (at the front end of separation), the AdvanceBio LC/Q-TOF, and the company's MassHunter BioConfirm software (for automatic data processing). The analyte was a NIST (National Institute of Standards and Technology) mAb standard.

LC–MS analysis showed mass resolution of all species falling between 2,000 and 5,000 m/z. Moreover, zoom-in spectra of each charge state showed the six major glycoforms of the NIST mAb. In addition to these major features, the analysis identified minor glycosylation heterogenicities, such as loss of N-acetylglucosamine (*Figure*).

Agilent analysis software includes a maximum entropy deconvolution algorithm, which preserves fine details of the intact protein. Typically, a mAb can have 30–70 positive charges under LC–MS conditions. Consequently, a mAb with a nominal mass close to 150,000 amu is generally detected in the range of 2,000–5,000 m/z.

According to Dr. Wong, the maximum entropy algorithm has been widely used for deconvolving the multiple charge state envelope that occurs when a protein is analyzed by LC–MS: "The method transforms the raw m/z spectrum of one or more intact proteins into the actual molecular weight of the protein, making it easier to directly compare [it] to theoretical sequence information or potential mass shifts caused by PTMs." ■

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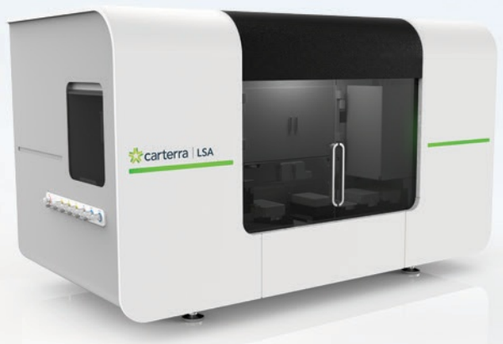

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