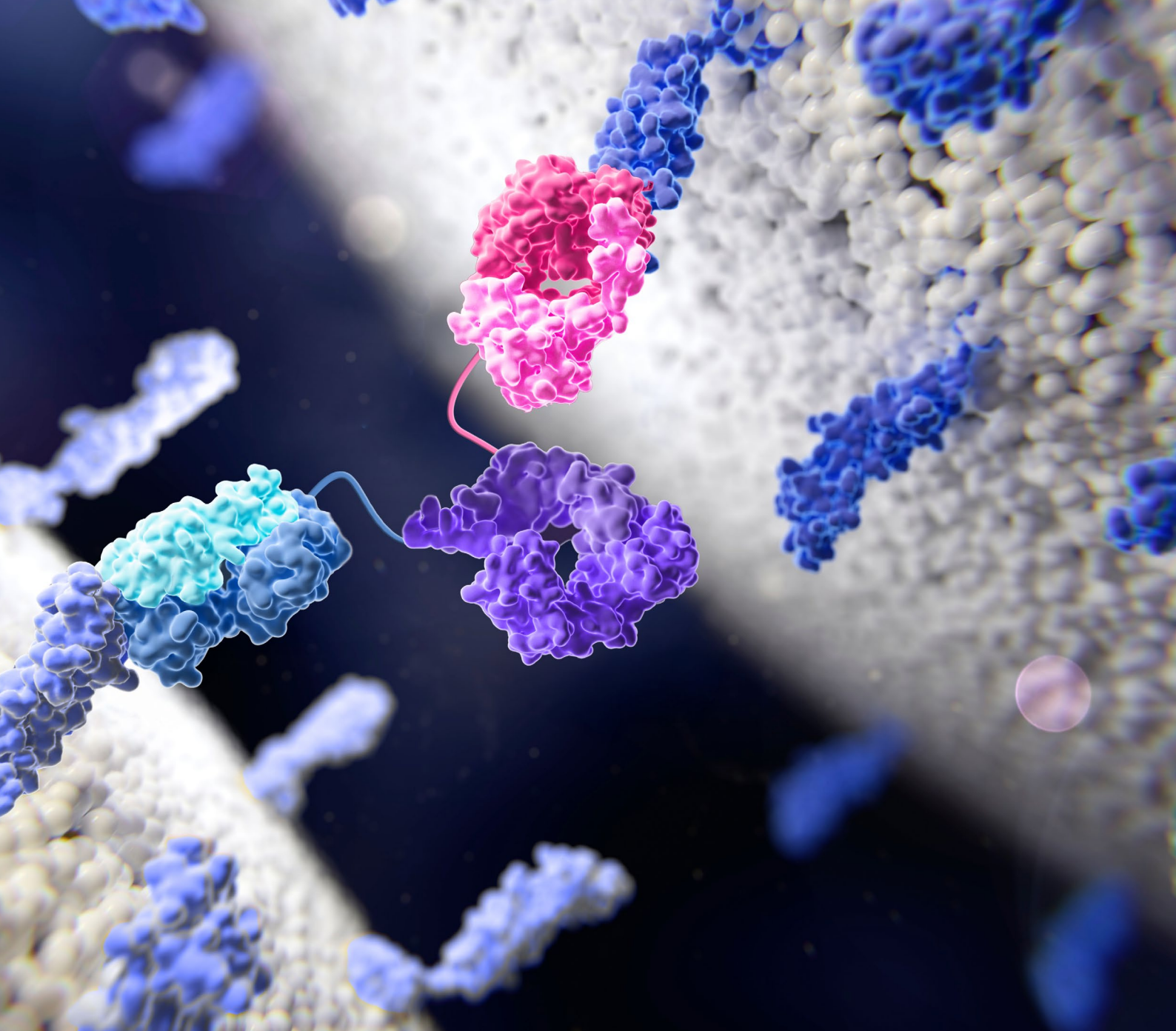




Dual-Targeting Breakthroughs:

Advancements in Bispecific
Antibody Development

Expert Insights

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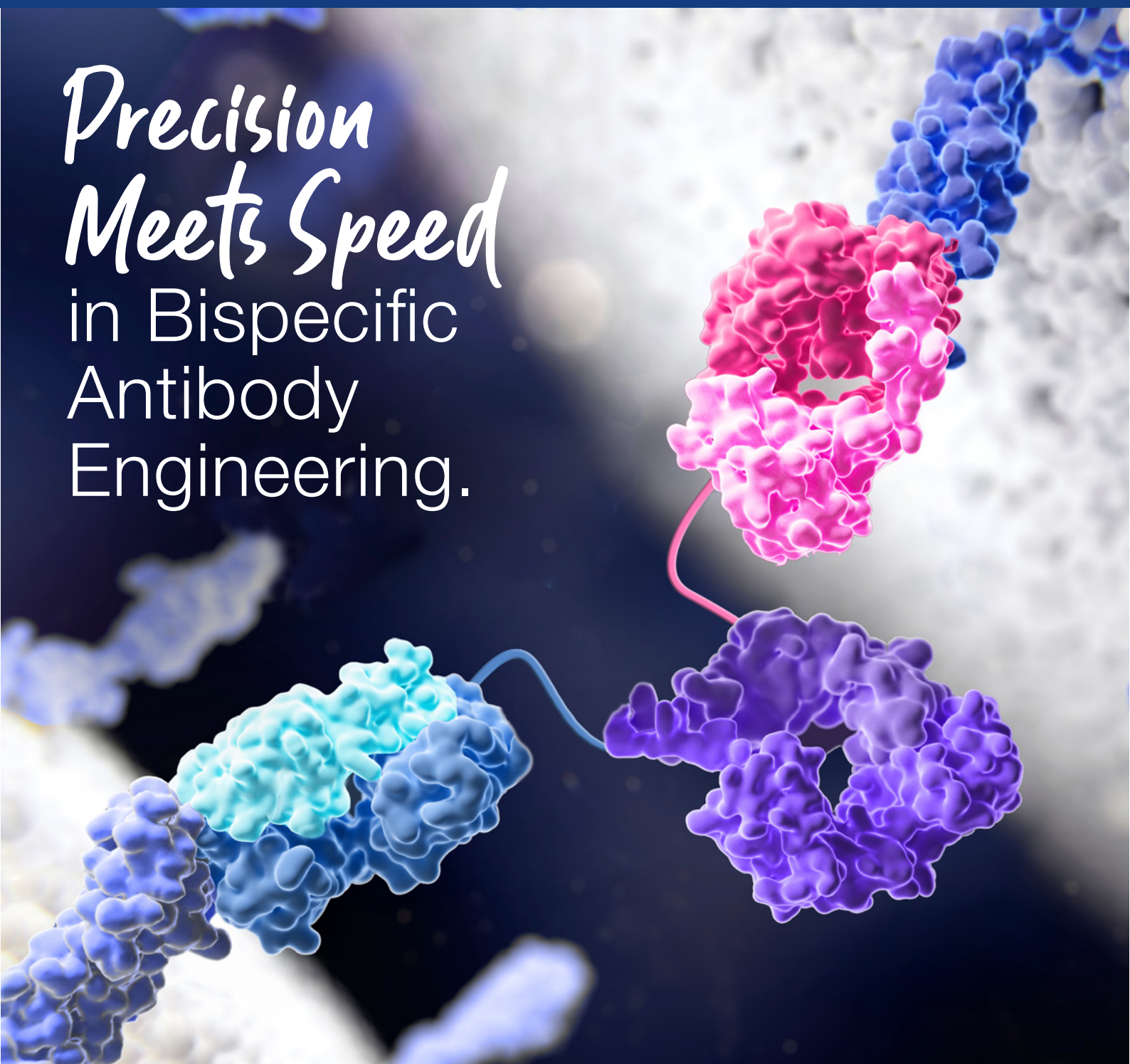
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Precision Meets Speed in Bispecific Antibody Engineering.



Accelerate bispecific success — from discovery to an IND

Bispecific antibodies are new modalities that offer both promise and complexity, from development to manufacturing. To overcome these challenges, you need solutions that deliver speed, precision, and efficiency at every stage. From optimizing antibody formats and characterizing critical quality attributes to biomanufacturing, we provide the solutions and expertise to drive your success.

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Introduction

Bispecific antibodies (bsAbs) represent a groundbreaking advancement in immunotherapy, offering the potential to revolutionize treatment for various diseases, including cancer, autoimmune, and inflammatory conditions. Their unique ability to engage two different antigens simultaneously enhances therapeutic efficacy and reduces dosage requirements. The development and optimization of bsAbs involve complex processes, from target identification to bioprocessing and manufacturing, which are crucial for improving productivity, selectivity, and overall therapeutic outcomes. This Expert Insights into bsAb development provides valuable knowledge for overcoming challenges and advancing the field, ultimately leading to more effective and accessible treatments for patients.

First, the feature by Taylor covers the advancements in bsAb development, highlighting their significant impact on immunotherapy. It discusses the entire process from target identification to bioprocessing and manufacturing, emphasizing the importance of efficient biomanufacturing. The feature also explores the therapeutic applications of bsAbs in cancer, autoimmune, and inflammatory diseases.

Carver *et al.* then covers the optimization of antibody production in Chinese hamster ovary (CHO) cells using targeted integration (TI) methodology [1]. It investigates the effects of antibody heavy-chain and light-chain gene dosage and positioning, demonstrating that increasing gene copy numbers enhances productivity. The study also explores the impact of gene positioning within plasmids on expression levels and antibody production, with implications for extending these findings to bsAbs.

Next, Parasnavis *et al.* covers the systematic workflow for studying domain contributions of bsAbs to selectivity in multimodal chromatography [2]. It integrates chromatographic screening, surface property mapping, and protein footprinting using covalent labeling followed by LC-MS analysis. The study reveals enhanced selectivity of multimodal resins compared to single-mode systems and identifies key binding patches in bsAbs.

Then, the interview with Prof. Steven Cramer covers innovations in bsAbs. Prof. Cramer discusses his journey into the field, contributions to chromatographic bioprocessing, and protein-surface interactions. It then

delves into bsAbs' structure, function, potential in cancer immunotherapy, and development challenges. It also explores future directions for the field and offers advice for young researchers.

An interview with Dr. Paul Royle explores the dynamic field of bispecific antibodies. Dr. Royle shares his experience partnering with researchers worldwide and Bio-Rad's role in advancing bsAb discovery. The conversation covers the unique mechanisms of bispecific antibodies, their promise in cancer immunotherapy and autoimmune disease, and strategies to improve their specificity and safety. It also highlights the impact of collaboration and innovation on the field's progress and offers Dr. Royle's perspective on future directions for bsAb research.

Next, an infographic covers the journey of bsAbs from concept to clinic, explaining their dual-targeting capability, applications in oncology and autoimmune diseases, and the discovery and development process. There are three bulletins from Bio-Rad included covering topics from cell line development for biopharmaceutical production, emphasizing the importance of optimizing productivity through host cell engineering; overcoming bispecific antibody purification challenges with CHT™ Ceramic Hydroxyapatite Media, discussing the benefits of using this multimodal media for high purity and reduced aggregation, and a note covering SpyLock technology, which enables rapid and covalent assembly of bsAbs.

Overall, bsAbs are crucial in advancing immunotherapy, offering innovative solutions for treating cancer, autoimmune, and inflammatory diseases. Understanding bsAb development, optimization, and applications provides valuable insights into cutting-edge therapeutic strategies, making it essential for anyone interested in the future of medical treatments and bioprocessing advancements.

Through the methods and applications presented in this Expert Insights, we hope to educate researchers on new technologies and techniques about bsAb development

and production. To gain a deeper understanding of available options for improving your research, we encourage you to visit [Bio-Rad's website](#).

Dr. Christene A. Smith

Editor at Wiley

References

- [1] Carver, J. *et al.* (2020). Maximizing antibody production in a targeted integration host by optimization of subunit gene dosage and position. *Biotechnology Progress*. <https://doi.org/10.1002/btpr.2967>.
- [2] Parasnavis, S.S. *et al.* (2022). Systematic workflow for studying domain contributions of bispecific antibodies to selectivity in multimodal chromatography. *Biotechnology and Bioengineering*. <https://doi.org/10.1002/bit.27967>.

Advancing Bispecific Antibody Development: From Concept to Clinic

Gwen Taylor, PhD

Introduction

The landscape of immunotherapy has been dramatically transformed by the emergence of bispecific antibodies (bsAbs), representing a significant leap forward in targeted therapeutic approaches. These innovative molecules, capable of simultaneously engaging two different antigens, have opened new frontiers in the treatment of various diseases, particularly in oncology and immunology [1–3]. The unique ability of bsAbs to bridge immune cells with target-cells has revolutionized cancer treatment strategies, while their application in autoimmune diseases and inflammatory conditions continues to expand [4,5]. Currently, the most common therapeutic application of bsAbs is in cancer immunotherapy, where bsAbs are engineered to simultaneously bind a cytotoxic T-cell and a target tumor cell that is to be destroyed.

bsAbs offer several advantages compared to conventional monoclonal antibodies (mAbs), including higher cytotoxic potential, ability to bind antigens expressed at relatively low levels, and lower effective dosage. As of early 2025, the FDA has approved 15 bsAbs, and while most bsAbs in development aim to treat cancer, others are focused on chronic inflammatory, autoimmune, and neurodegenerative diseases; vascular, ocular, and hematologic disorders; and infections.

This feature provides an overview of the steps involved in bsAb development, emphasizing the importance of efficient bioprocessing and biomanufacturing in bringing these breakthrough therapies from concept to clinical research.

Discovery and Development: Building the Foundation

bsAb development begins with target identification, followed by lead discovery, characterization, validation, and optimization [6]. The crucial initial phases of lead discovery require sophisticated tools and technologies that can efficiently screen and validate potential candidates while maintaining the delicate balance of dual specificity and manufacturability [7,8]. Success in this stage sets the foundation for all subsequent development steps and ultimately determines the therapeutic potential of the final product.

Target Identification

The first step of bsAb development is target identification, which involves identifying target pairings hypothesized or demonstrated to enable a desired mode of action. Two examples of known target pairings for bsAbs are: (1) CD19 and CD3, used in blinatumomab (Blinicyto), the first FDA-approved bsAb. It targets CD19 on B-cells and CD3 on T-cells, leading to T-cell activation and B-cell destruction. (2) EGFR and cMet, used in amivantamab (Rybrentav), which targets epidermal growth factor receptor (EGFR) and mesenchymal-epithelial transition factor (cMet) for the treatment of non-small cell lung cancer.

Lead Discovery

For bsAbs, lead discovery involves screening and selecting mAbs against two target proteins, typically employing phage display technology, which enables the creation and screening of large libraries of antibodies displayed on bacteriophage surfaces through multiple rounds of selection against target antigens. At this stage, unbiased screening of large panels of bsAbs in simplified *in vitro* functional assays provides an opportunity to discover novel bsAb target pairings. This approach allows for the exploration of a wide range of potential target combinations, maximizing the chances of identifying effective and innovative bsAb candidates early in the discovery process.

The [Pioneer™ Antibody Discovery Platform with SpyLock technology](#) (Bio-Rad) offers unprecedented flexibility and efficiency in the early stages of bsAb therapeutic development. This large and innovative phage display library system, combined with SpyLock technology, enables researchers to rapidly generate and evaluate bispecific candidates with high-throughput. The platform's efficiency in identifying optimal molecular formats significantly accelerates the early development timeline while reducing resource requirements.

The customization potential of lead discovery is further enhanced through the use of phage libraries such as the [Human Combinatorial Antibody Libraries](#) (HuCAL; Bio-Rad), which enable creation of precise custom antibody reagents for bioanalysis. This capability is particularly valuable when developing novel bispecific formats or targeting unique antigen combinations.

The versatility of the HuCAL service enables researchers to generate highly specific reagents for characterization and validation studies, ensuring comprehensive evaluation of potential therapeutic candidates throughout the development process.

Engineering and Expression

Once candidate antibodies are identified, engineering and assembly methods come into play, including knobs-into-holes technology for creating heterodimeric Fc regions and various DNA assembly techniques such as Gibson Assembly and Golden Gate Assembly for constructing modular antibody formats.

Expression systems, predominantly mammalian cell lines like HEK293 for screening and Chinese hamster ovary (CHO) cells for larger-scale production, are crucial for producing the engineered constructs. The expressed bsAbs undergo purification through multiple chromatographic steps, beginning with Protein A chromatography for initial capture, followed by size exclusion chromatography to isolate correctly assembled molecules and remove aggregates or fragments.

Functional Screening, Optimization, and Quality Control

Functional screening is a critical phase that incorporates various cell-based assays to evaluate cytotoxicity, T-cell engagement, and target cell killing, alongside stability assessments including thermal and accelerated stability testing. Optimization methods encompass affinity maturation through techniques like site-directed mutagenesis and CDR walking, as well as format optimization studies examining different linker lengths and variable domain orientations. Quality control measures utilize advanced analytical methods such as mass spectrometry for intact mass analysis and peptide mapping, along with various chromatographic techniques including ion exchange and hydrophobic interaction chromatography. These methods are typically employed in an iterative manner, with results from each stage informing subsequent optimization rounds, requiring careful integration of multiple techniques and thorough documentation of structure-function relationships to guide development decisions.

Developability profiling is another crucial aspect, evaluating and optimizing properties like stability, solubility, and potential immunogenicity. Increasingly, computational design leveraging artificial intelligence is being used to predict optimal variants based on antibody sequence and structural data. The process typically involves iterative optimization through rapid design-build-test cycles, refining bsAb properties through multiple rounds of *in silico* design and experimental

testing. Early-stage format screening is also vital to identify the most suitable configuration for the intended therapeutic application.

Characterization, Validation, and Optimization

Comprehensive characterization of bsAbs requires multiple analytical approaches to ensure safety and efficacy. Analytical characterization employs sophisticated techniques including surface plasmon resonance for binding kinetics and epitope mapping, flow cytometry for cell surface binding analysis, and analytical size-exclusion chromatography with multi-angle light scattering (SEC-MALS) for molecular weight determination and quality assessment.

Flow cytometry, e.g., using the [ZE5 Cell Analyzer](#) (Bio-Rad), along with [StarBright™ Antibodies](#), enables detailed analysis of cell-based assays, providing crucial data on binding specificity, functional activity, and potential off-target effects. The advanced capabilities of the ZE5 System allow sophisticated analyses of cellular interactions, immune response patterns, and therapeutic mechanisms. The integration of StarBright Antibodies enhances detection sensitivity and multiplexing capabilities, enabling more comprehensive characterization of cellular responses to bsAb treatment.

Multiplexed immunoassays, e.g., [Bio-Plex](#) (Bio-Rad), complement these analyses by enabling biomarker identification of up to 500 proteins, peptides, or nucleic acid targets in a single sample. This technology provides valuable insights into cytokine release profiles, immune response signatures, target engagement markers, and safety parameters. The ability to simultaneously analyze multiple biomarkers provides a more complete picture of the therapeutic's biological effects and potential safety considerations.

Biomarker monitoring is a crucial aspect in the development of bsAbs. The [QX600 Droplet Digital™ PCR \(ddPCR™\) System](#) (Bio-Rad) offers advanced six-color multiplexing and absolute quantification of nucleic acids, providing unprecedented precision in quantifying genetic markers and expression levels. This capability has proven particularly valuable for oncology research by facilitating structural variant analysis, rare variant detection, copy number variation, methylation detection, and determining loss of heterozygosity. It has also been used in gene expression studies, next generation sequencing (NGS) orthogonal testing, cell and gene therapy, and food and wastewater testing. The system's exceptional sensitivity and precision enable researchers to detect subtle changes in gene expression and molecular markers, providing crucial insights into therapeutic effectiveness and potential mechanisms of action.

Safety and Toxicology Considerations in bsAb Development

Safety and toxicology studies for bsAbs involve a comprehensive approach to evaluate their potential risks and efficacy. These studies typically include preclinical assessments through *in vitro* assays and animal studies, followed by clinical trials. *In vitro* assays focus on cytokine release, T-cell activation, and binding specificity, while animal studies assess toxicity and immunogenicity. Clinical studies, particularly Phase I trials, evaluate dose escalation, pharmacokinetics, and monitor for adverse events.

While bsAbs offer promising therapeutic potential, they can cause toxicities such as cytopenias, infections, and tumor lysis syndrome. Unique side effects include cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), which are generally less severe than those observed with chimeric antigen receptor (CAR) T-cell therapy. As bsAbs become more widely used, there is a growing need for consensus-based guidelines to help clinicians manage these immune activation toxicities effectively. Ongoing research continues to explore new targets and optimize the safety and efficacy profiles of bsAbs for various clinical applications.

Process Development and Manufacturing: Ensuring Quality and Consistency

The transition from discovery to manufacturing presents unique challenges in bsAb development. The complexity of these molecules demands sophisticated purification strategies and robust analytical methods to ensure product quality and regulatory compliance. The successful scale-up of bsAb production requires careful optimization of multiple parameters and comprehensive quality control measures.

Expression and Purification

bsAbs present complex challenges throughout the scale-up of expression and purification processes. For example, during expression, heavy and light chain mispairing can occur, which can result in undesired antibody variants, lower production yields, aggregation, and potential stability complications.

Large-scale purification of bsAbs is equally demanding, characterized by the presence of multiple product-related impurities including mispaired products, fragmented molecules, and higher aggregate levels [9]. Separation challenges can also include effective isolation of heterodimers from homodimers, removal of half-antibodies, and elimination of excess light chains. The molecular similarity between these impurities and the target bsAb makes their separation exceptionally difficult.

Several Bio-Rad [mixed-mode chromatography resins](#), including Nuvia™ aPrime 4A Media, Nuvia wPrime 2A Media, and CHT Ceramic Hydroxyapatite Media, offer exceptional selectivity for purification of target molecules, high-binding capacity, robust performance across different molecular formats, and consistent batch-to-batch reproducibility. Each media's unique properties make it particularly suitable for challenging separations often encountered with bispecific antibodies, including format-specific impurity removal, aggregate separation, host-cell protein clearance, and endotoxin reduction. The versatility of mixed-mode resins enables process developers to establish robust purification strategies that can be effectively scaled from laboratory to commercial production.

Quality Control and Regulatory Compliance

Regulatory compliance in the production of bsAbs requires a comprehensive set of analytical methods to ensure quality, safety, and efficacy. These methods are essential for characterizing the complex structure and properties of bsAbs, as well as for monitoring critical quality attributes throughout the development and manufacturing process. Key analytical methods required for regulatory compliance include:

1. Mass spectrometry (MS) to identify and quantitate product variants, especially during early development stages.
2. Chromatography for the purification and isolation of bsAbs, including ion exchange, Protein A affinity, and mixed-mode chromatography, provides enhanced selectivity of closely related variants and difficult-to-purify bsAbs, in addition to the removal of host cell proteins, DNA, and product-related impurities.
3. Binding assays to assess the specificity, affinity, and avidity of bsAbs to their target antigens.
4. Potency assays, such as functional assays to evaluate the biological activity and efficacy of the bsAb by measuring target-cell engagement, immune cell activation, cytotoxicity profiles, and functionality maintenance. The comprehensive analysis capabilities of the Bio-Rad [ZE5 Cell Analyzer](#) system enable the development and validation of robust potency assays that meet regulatory requirements while providing meaningful insights into product quality and consistency.
5. Immunogenicity assays to measure immune responses, including anti-drug antibody (ADA) detection.

6. Stability studies of the bsAb under various conditions to ensure product quality over time.
7. Methods to detect and quantify process-related impurities, chain mismatching, fragments, and homodimers. The [QX ONE Droplet Digital PCR System](#) or [QX200 Droplet Digital PCR System](#) provides precise quantification of process-related impurities, residual DNA monitoring, viral clearance validation, and batch release testing. The system's ability to provide absolute quantification without the need for standard curves makes it particularly valuable for regulatory compliance and batch release testing. The high precision and reproducibility of ddPCR technology ensures consistent quality control throughout the manufacturing process.
8. Structural analysis to confirm the correct pairing of heavy and light chains and overall structural integrity.
9. Post-translational modification analysis, especially when using mammalian cell expression systems.
10. Comparator studies: Clinical trials comparing the bsAb to approved monospecific products targeting the same antigens may be required for risk-benefit assessment

These analytical methods must be developed and validated in accordance with ICH guidelines and regulatory requirements. The FDA and other regulatory bodies may request specific assays, such as those for ADA detection, as part of the approval process for bispecific antibodies.

Patient Treatment Monitoring: Optimizing Therapeutic Outcomes

Early Detection and Response Monitoring

The success of bsAb therapy extends beyond manufacturing to patient monitoring where early detection and response tracking play crucial roles in improving patient outcomes. The ability to accurately monitor patient response and disease progression is vitally important for optimizing therapeutic outcomes and personalizing treatment strategies.

Biomarker detection can serve as a bsAb therapy qualifier by helping to identify patients who are most likely to benefit from the treatment approach [10]. In addition, the presence and level of specific biomarkers can indicate the potential effectiveness of a bsAb treatment over time and can enable molecular residual disease quantification as well as long-term recurrence surveillance. The exceptional sensitivity and precision of ddPCR technology, such as Bio-Rad's QX600 Droplet Digital PCR System equipped

with research use only assays like the [ESR1](#) mutation detection kit, supports research in the area of earlier intervention and more personalized treatment selections. The system's ability to provide absolute quantification of molecular markers makes it particularly valuable for research into monitoring treatment response and disease progression.

Long-Term Patient Management

Infection prevention and management are paramount issues for patients receiving long-term bsAb therapies, given the increased risk due to immunosuppression and hypogammaglobulinemia [11]. This involves keeping vaccinations up-to-date, providing prophylaxis against certain infections, considering immunoglobulin replacement therapy, and promptly addressing respiratory symptoms.

Monitoring and managing side effects, particularly CRS and ICANS, is essential, especially during initial doses. There is growing interest in optimizing treatment schedules to minimize toxicity while maintaining effectiveness, potentially through extended dosing intervals or fixed-duration treatment courses.

Continuous monitoring using ddPCR technology supports research for the early identification of resistance development, timely therapeutic adjustments, improved patient outcomes, and reduced treatment costs. The ability to detect minimal residual disease with high sensitivity has revolutionized patient care strategies, enabling more informed treatment decisions and optimal timing of therapy modification. The long-term success of bsAb therapy relies heavily on accurate and sensitive monitoring tools that can guide treatment decisions throughout the patient's journey.

Future Directions

bsAb Bioprocessing Advances

Looking to the future of bsAb bioprocessing, advanced protein engineering is expected to create more precise and effective bispecifics, including multispecific antibodies and next-generation designs to overcome resistance mechanisms. The integration of AI and machine learning in design and optimization processes is likely to accelerate the development of innovative bsAb therapies. Technological innovations are driving the creation of novel formats and engineered Fc regions, optimizing performance, and reducing immunogenicity. Advancements in bioprocessing and analytical testing technologies are crucial for meeting increasing demand, offering benefits such as reduced cost and time-to-market. The combination of next-generation cell lines with enhanced productivity

and AI technologies is anticipated to improve yields and reduce production costs. More sophisticated analytical methods and in-line testing technologies will ensure better quality and consistency in bsAb development. Additionally, techniques such as continuous processing and intensified downstream purification are expected to streamline manufacturing and reduce overall costs. These anticipated breakthroughs aim to address current challenges in bsAb bioprocessing, including complex design, immunogenicity, limited half-life, and off-target effects, while improving manufacturability, scalability, and overall therapeutic efficacy.

Ongoing bsAb Innovations

Today, researchers are focusing on enhancing bsAb targeting precision to selectively attack cancer cells while minimizing damage to healthy tissue, which is particularly crucial for solid tumor treatment. Simultaneously, efforts are underway to expand immune cell engagement beyond T-cells, potentially boosting therapeutic efficacy. A promising trend is the integration of bsAbs with antibody-drug conjugates, forming bsAb-ADCs, which could offer superior tumor selectivity and treatment effectiveness [12]. Novel delivery methods are being developed to enable long-acting delivery of bsAbs, potentially improving T-cell infiltration at tumor sites. While oncology remains a primary focus, the potential applications of bsAbs are expanding to include autoimmune diseases, ophthalmic conditions, hemophilia, and neurodegenerative disorders. Ongoing advancements in antibody engineering and protein design are expected to yield bsAbs with improved stability, reduced immunogenicity, and optimized pharmacokinetic profiles. These innovations collectively aim to develop more effective and versatile bsAbs, potentially leading to improved patient outcomes across a broader spectrum of diseases.

Future Perspectives

The field of bsAb development continues to evolve, driven by technological advances and growing clinical experience. Bio-Rad's comprehensive toolkit, from the Pioneer Antibody Discovery Platform with SpyLock technology to the QX600 Droplet Digital PCR System, positions researchers and manufacturers to meet current challenges while preparing for future innovations. The integration of these technologies throughout the development pipeline enables faster development timelines, more robust manufacturing processes, better patient monitoring strategies, and improved therapeutic outcomes.

Conclusion

This feature presents an overview of the current landscapes of bsAb research and development, bioprocessing/biomanufacturing, and impact on patient care. It provides a guide through the various stages of bsAb development, from initial discovery to final manufacturing to patient treatment monitoring, discussing the challenges and solutions at each step.

The journey from concept to clinic in bsAb development represents a complex interplay of scientific innovation, technological advancement, and clinical expertise. As we continue to push the boundaries of what's possible in immunotherapy, the integration of advanced technologies and robust analytical tools will remain essential for bringing these revolutionary treatments to patients in need. Bio-Rad's commitment to innovation and quality ensures that researchers and manufacturers will have access to the tools and technologies needed to bring these revolutionary treatments from the laboratory to the patient.

References

- [1] Falchi, L. *et al.* (2023). Bispecific antibodies for the treatment of B-cell lymphoma: promises, unknowns, and opportunities. *Blood*. <https://doi.org/10.1182/blood.2021011994>.
- [2] Devasia, A.J. *et al.* (2024). Bispecific antibodies in the treatment of multiple myeloma. *Blood Cancer Journal*. <https://doi.org/10.1038/s41408-024-01139-y>.
- [3] Wei, J. *et al.* (2022). Current landscape and future directions of bispecific antibodies in cancer immunotherapy. *Frontiers in Immunology*. <https://doi.org/10.3389/fimmu.2022.1035276>.
- [4] Zhao, Q. (2020). Bispecific Antibodies for Autoimmune and Inflammatory Diseases: Clinical Progress to Date. *BioDrugs*. <https://doi.org/10.1007/s40259-019-00400-2>.
- [5] Felten, R. *et al.* (2023). Novel therapeutic strategies for autoimmune and inflammatory rheumatic diseases. *Drug Discovery Today*. <https://doi.org/10.1016/j.drudis.2023.103612>.
- [6] Fawcett, C. *et al.* (2024). Facilitating high throughput bispecific antibody production and potential applications within biopharmaceutical discovery workflows. *mAbs*. <https://doi.org/10.1080/19420862.2024.2311992>.
- [7] Kareva, I. *et al.* (2021). Bispecific antibodies: A guide to model informed drug discovery and development. *Heliyon*. <https://doi.org/10.1016/j.heliyon.2021.e07649>.
- [8] Segaliny, A.I. *et al.* (2023). A high throughput bispecific antibody discovery pipeline. *Communications Biology*. <https://doi.org/10.1038/s42003-023-04746-w>.
- [9] Chen, S.W. and Zhang, W. (2021). Current trends and challenges in the downstream purification of bispecific antibodies. *Antibody Therapeutics*. <https://doi.org/10.1093/abt/tbab007>.
- [10] Sun, Y. *et al.* (2023). Bispecific antibodies in cancer therapy: Target selection and regulatory requirements. *Acta Pharmaceutica Sinica B*. <https://doi.org/10.1016/j.apsb.2023.05.023>.
- [11] Raje, N. *et al.* (2023). Monitoring, prophylaxis, and treatment of infections in patients with MM receiving bispecific antibody therapy: consensus recommendations from an expert panel. *Blood Cancer Journal*. <https://doi.org/10.1038/s41408-023-00879-7>.
- [12] Shim, H. (2020). Bispecific antibodies and antibody-drug conjugates for cancer therapy: Technological considerations. *Biomolecules*. <https://doi.org/10.3390/biom10030360>.

Maximizing Antibody Production in a Targeted Integration Host by Optimization of Subunit Gene Dosage and Position

Adapted from Carver, J., *et. al*

This study investigated the optimization of antibody production in Chinese hamster ovary (CHO) cells using targeted integration (TI) methodology, specifically examining the effects of antibody heavy-chain and light-chain gene dosage and positioning. A novel TI host capable of simultaneously integrating two plasmids at the same genomic site was used. Results demonstrated that increasing antibody gene copy numbers enhanced specific productivity, although with diminishing returns as more genes were added to the same TI locus. Additional random integration of antibody DNA copies into a TI cell line showed further productivity improvements. In addition, the positioning of antibody genes within the two plasmids was shown to significantly influence expression levels.

Introduction

Traditional CHO cell protein production has relied on random integration (RI) of expression plasmids into the host genome. While effective, this approach introduces variability between clones. The development of TI technology allows for precise recombination of plasmid DNA into predetermined genomic loci, potentially reducing clone-to-clone variation [1]. However, TI systems have historically produced lower antibody titers compared to RI methods, primarily due to limited plasmid copy number integration and challenges in identifying optimal transcriptional “hotspots” for recombinase-mediated cassette exchange (RMCE) [2–5].

The authors recently developed a TI host containing a landing pad with three incompatible LoxP sites (L3, LoxFAS, and 2L) at a transcriptional hotspot [6]. While conventional directional RMCE involves crossovers at two incompatible recombinase recognition sequences, addition of a third incompatible LoxP site enables this host to undergo RMCE with two donor plasmids simultaneously. This allows for integration of up to twice as many copies of antibody heavy-chain and light-chain DNA in a single locus compared to the conventional single-plasmid-based RMCE. In this study, this two-plasmid TI system was used to explore the effect of antibody gene copy number and position on antibody expression level.

Materials and Methods

For antibody expression constructs, both single-plasmid versions with L3 and 2L sequences, and two-plasmid versions with L3/LoxFAS and LoxFAS/2L sequences were created. All constructs included a puromycin resistance marker and antibody genes controlled by CMV promoters.

CHO cells were cultured in DMEM/F12-based medium using shake flasks at 37 °C. For generating stable cell lines, plasmids were transfected via electroporation, followed by puromycin and FIAU selection. Single-cell cloning was performed to isolate high-producing clones, which were identified through HTRF assay screening. Production assessment used 14-day fed-batch cultures with temperature shifts from 37 °C to 35 °C on day 3, and feeding on days 3, 7, and 10.

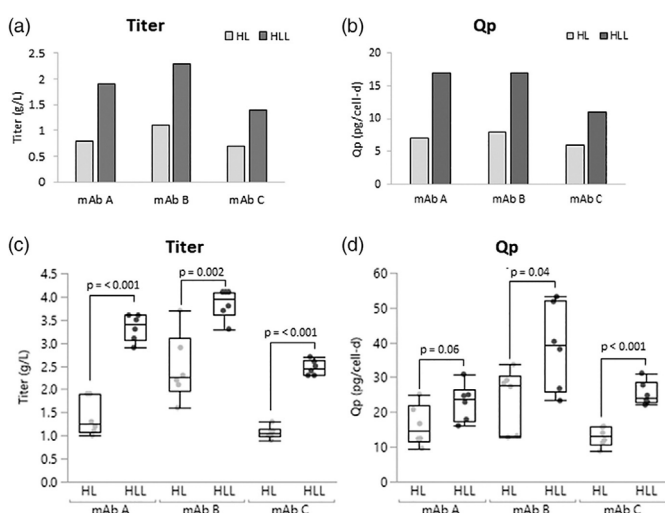
To evaluate gene copy numbers and expression, the team employed Droplet Digital PCR (using a ddPCR™ Supermix Kit from Bio-Rad) for DNA quantification and real-time PCR for mRNA analysis, normalizing results against reference genes. Protein levels were assessed through western blot analysis using specific antibodies against heavy and light chains. For supertransfection experiments, additional antibody genes were introduced through random integration into existing TI cell lines using a GS selection system with MSX selection. Throughout the study, cell growth, viability, metabolites, and antibody titers were monitored using standard bioanalytical methods.

Results

Addition of an extra light chain gene improves antibody titer and specific productivity

The effect of adding an extra light chain gene was investigated by comparing one heavy chain and one light chain (HL) configuration to one heavy chain and two light chains (HLL). For three different antibodies (mAbs A, B, and C), HLL pools showed approximately two-fold higher titer compared to HL pools ($p < 0.05$) (Figure 1). This improvement was primarily attributed to a 1.5- to 2.5-fold increase in specific productivity.

Figure 1



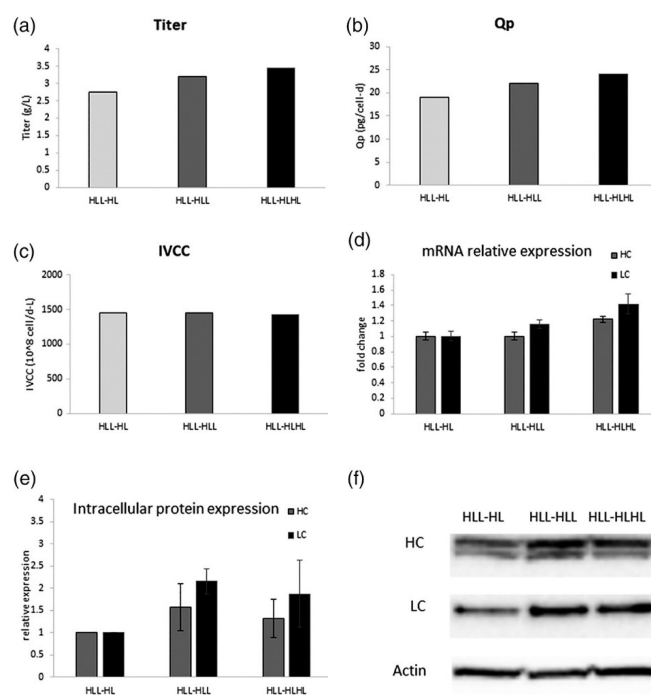
(a) Day 14 titer for RMCE pools of three mAbs comparing the HL and HLL configurations. (b) Day 14 specific productivity (Qp) for RMCE pools of three mAbs comparing the HL and HLL configurations. (c) Day 14 titer for clones generated from the pools in (a). The six best clones per configuration were tested. (d) Day 14 Qp for clones generated from the pools in (a). p value for (c) and (d) based on two-tailed student's t test and denoted if $p < 0.05$.

Transfection of up to seven antibody chain genes increases antibody specific productivity and titer in RMCE pools

The effects of increasing antibody chain numbers were investigated by comparing single-plasmid (HLL) to dual-plasmid configurations (HLL-HL and HLL-HLL) across five antibodies. Dual-plasmid configurations consistently showed higher titer and specific productivity (Qp) in 14-day fed batch production, with maintained stability for 120 days post-thaw.

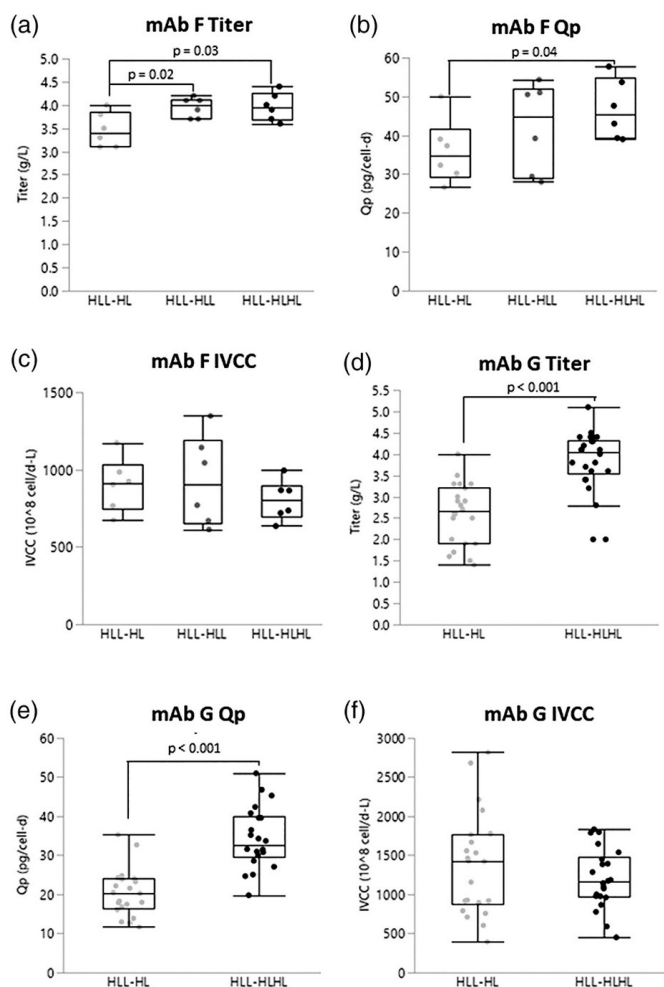
Further studies with mAb F compared five-chain (HLL-HL), six-chain (HLL-HLL), and seven-chain (HLL-HLHL) configurations. Both titer and Qp increased with additional chains while cell growth remained constant across configurations (Figure 2). Analysis revealed increased light-chain mRNA expression in six- and seven-chain configurations, with the seven-chain configuration also showing elevated heavy-chain mRNA levels. Western blot analysis showed similar light-chain protein levels in six- and seven-chain configurations, both higher than the five-chain version. Unexpectedly, heavy-chain protein levels increased with additional light chains despite unchanged mRNA levels, possibly due to improved protein folding stability from light-chain assistance.

Figure 2



(a–c) Day 14 titer, Qp, and growth (as expressed by the integral of viable cell concentration, IVCC) for RMCE pools of mAb F comparing three different plasmid configurations. (d) Seed train mRNA expression from the pools in (a). mRNA expression for heavy and light chain was normalized to the HLL-HL configuration; each bar is the average of four technical replicates (error bars are standard deviation). (e) Intracellular heavy and light chain protein expression from all pools was quantified from western blots and normalized to the HLL-HL configuration; each bar is the average of three technical replicates (error bars are standard deviation). (f) Representative western blot image of the data shown in (e). The two bands observed for heavy chain protein are due to variable glycosylation of this subunit.

Figure 3



(a) Day 14 titer, Qp, and integral of viable cell count (IVCC) for mAb F-expressing clones generated from RMCE pools with three different configurations. The best six clones per pool were evaluated. (b) Day 14 Qp from the clones in (a). (c) D14 IVCC from the clones in (a). (d) Day 14 titer for mAb G clones generated from pools with the HLL-HL and HLL-HLHL configurations. The best 22 clones per pool were evaluated. (e) Day 14 Qp from the clones in (d). (f) Day 14 IVCC from the clones in 3D. p value for (a–f) based on two-tailed student's t test and denoted if $p < .05$.

Clones with seven-chain HLL-HLHL configuration have high titer and specific productivity in two tested antibodies

To evaluate whether the seven-chain (HLL-HLHL) configuration's enhanced performance persisted after single cell cloning, clones were compared from HLL-HL, HLL-HLL, and HLL-HLHL configurations of mAb F. Analysis of the top six clones showed modest increases in titer and specific productivity (Qp) with additional chains, while growth remained consistent (Figure 3a,b,c). Despite expected gene copy numbers, significant clone-to-clone variability was observed in mRNA and protein expression, potentially due to epigenetic modifications or CHO cell genomic plasticity.

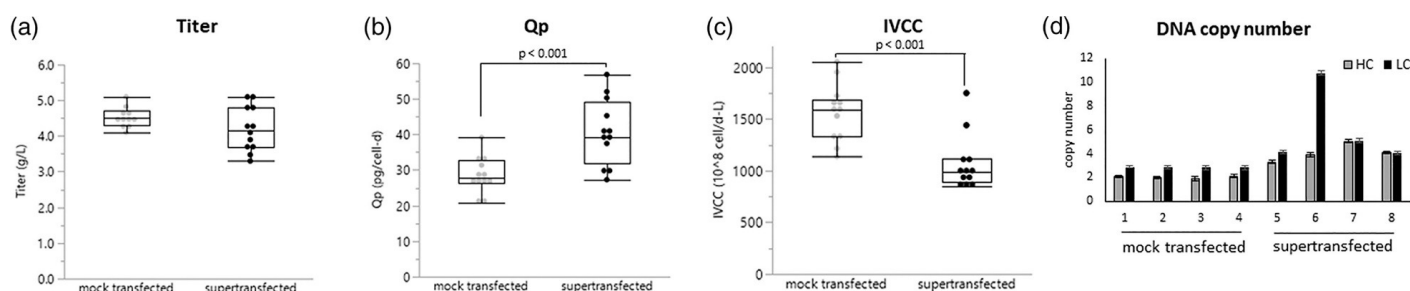
To validate these findings, mAb G was tested using HLL-HL and HLL-HLHL configurations. The seven-chain configuration demonstrated clearer superiority with mAb G than with mAb F, showing significantly higher titer primarily due to increased Qp, while maintaining similar growth rates (Figure 3d,e,f). These results confirmed that higher antibody copy numbers at the integration site generally improve specific productivity.

Random integration of antibody plasmid into a targeted integration cell line further increases specific productivity by 50%

Varying effects of the seven-chain (HLL-HLHL) configuration were observed across different antibodies, with mAb G showing a 52% titer increase compared to just 14% for mAb F. Further testing with eight total chain copies showed no additional productivity gains, suggesting diminishing returns from increasing gene copies at a single integration site.

To explore alternative approaches, genes outside the TI site were added through supertransfection of HL plasmid (mAb H) into an existing HLL-HL cell line, using an antibody-free GS plasmid as control.

Figure 4



(a) Day 14 titer, Qp, and IVCC for supertransfected and mock transfected clones generated from a mAb H expressing HLL-HL TI clone. The best six clones per condition were evaluated. (b) Day 14 Qp from the clones in (a). (c) Day 14 IVCC from the clones in (a). (d) Heavy and light chain gene DNA copy number from four supertransfected and four mock transfected clones. Bars represent the average of three technical replicates (error bars are standard deviation). p value for (a–c) based on two-tailed student's t test and denoted if $p < .05$.

While supertransfected clones showed a 50% increase in specific productivity (Qp) compared to controls, a corresponding decrease in cell growth (IVCC) resulted in minimal overall titer improvement (Figure 4). DNA analysis confirmed increased heavy- and light-chain copy numbers in supertransfected clones, while mock-transfected clones maintained original copy numbers.

Antibody gene position in a transfected plasmid affects its productivity

To examine how gene positioning affects antibody expression in the dual-plasmid targeted integration system used in this study, various arrangements of heavy- and light-chain genes from mAb H were compared. Two configurations were tested: one where the genes were on the same plasmid and one where they were separated across front and back plasmids (Figure 5a).

The results revealed that gene positioning significantly impacted expression levels (Figure 5b). Heavy-chain expression was consistently lower when placed after the light chain (LH configuration) compared to before it (HL configuration). Light-chain expression showed more complex patterns, with higher expression in the LH configuration in the front plasmid but lower expression in the back plasmid. The front plasmid generally supported stronger expression than the back plasmid for both chains.

These positional effects directly influenced antibody production, with titer and specific productivity correlating with mRNA expression levels (Figure 5c,d). Digital Droplet PCR using a ddPCR™ Supermix Kit (Bio-Rad) confirmed that these differences were due to gene positioning rather than copy number variation, as all configurations maintained similar gene copy numbers (Figure 5e).

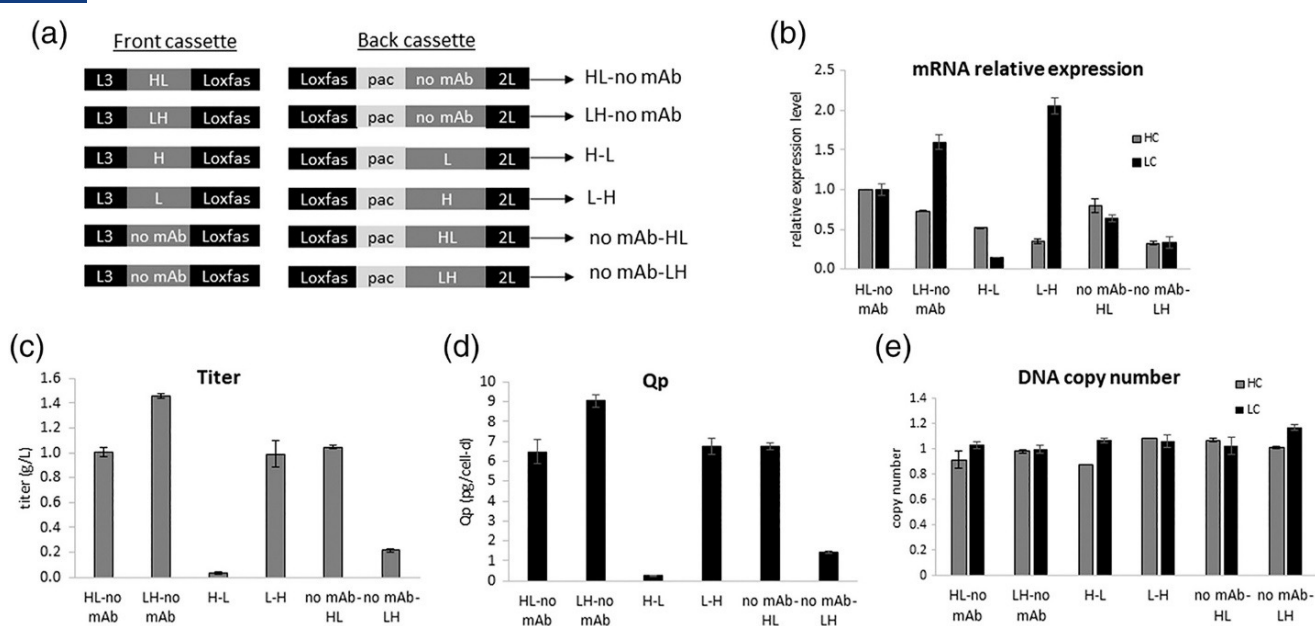
Discussion

In this study, antibody gene copy number correlated positively with productivity in TI hosts, although the relationship was not linear. The plateau effect observed with increasing copy numbers may be attributed to:

1. The ratio of heavy- to light-chain genes, with previous research showing optimal production typically requires higher light-chain expression.
2. Potential transcriptional interference when multiple genes are closely positioned at a single genomic site.

The successful enhancement of specific productivity through supertransfection suggests that utilizing multiple, dispersed genomic sites may be advantageous for maximizing transcriptional output. This finding indicates that incorporating additional TI sites could further improve antibody production.

Figure 5



(a) Schematic of the one heavy chain and one light chain plasmids transfected to assess the effect of plasmid position on chain expression. Antibody heavy chain gene (abbreviated as H) and light chain gene (abbreviated as L) were either expressed on the same plasmid or split between the front and back plasmids. L3, Loxfas, and 2L sites are noted, as is the pac puromycin resistance gene in the back plasmid. (b) Seed train mRNA expression of heavy and light chain from pools with DNA configurations outlined in (a). Expression for heavy and light chain was normalized to the mean of HL-no mAb configuration. Each bar represents the average of two pools per configuration (error bars are standard deviation). (c) Day 14 titer for the RMCE pools of the various configurations. Each bar represents the average of two pools per configuration (error bars are standard deviation). (d) Day 14 Qp for the pools in (c). (e) Heavy and light chain DNA copy number for the pools. Each bar represents the average of two pools per configuration (error bars are standard deviation).

The strong positional effects observed upon gene expression highlight the importance of careful vector design in TI systems. This becomes particularly relevant for complex proteins like bispecific antibodies, where precise control of subunit ratios is crucial for proper assembly.

These findings provide valuable insights for optimizing antibody production in TI systems, whether for conventional antibodies or more complex therapeutic proteins. The results suggest that a combination of targeted integration and strategic gene positioning, potentially across multiple genomic sites, may offer the best approach for maximizing antibody production while maintaining the benefits of reduced clone-to-clone variability. In the case of bispecific antibodies, the assembly of the correct product may be strongly dependent on the exact expression ratio of its different subunits.

References

- [1] Ho, S.C. *et al.* (2013). Generation of monoclonal antibody-producing mammalian cell lines. *Pharmaceutical Bioprocessing*. <https://doi.org/10.4155/pbp.13.8>.
- [2] Zhang, L. *et al.* (2015). Recombinase-mediated cassette exchange (RMCE) for monoclonal antibody expression in the commercially relevant CHO K1SV cell line. *Biotechnology Progress*. <https://doi.org/10.1002/btpr.2175>.
- [3] Nehlsen, K. *et al.* (2011). Towards rational engineering of cells: Recombinant gene expression in defined chromosomal loci. *BMC Proceedings*. <https://doi.org/10.1186/1753-6561-5-s8-o6>.
- [4] Nehlsen, K. *et al.* (2009). Recombinant protein expression by targeting pre-selected chromosomal loci. *BMC Biotechnology*. <https://doi.org/10.1186/1472-6750-9-100>.
- [5] Crawford, Y. *et al.* (2013). Fast identification of reliable hosts for targeted cell line development from a limited-genome screening using combined ϕ C31 integrase and CRE-Lox technologies. *Biotechnology Progress*. <https://doi.org/10.1002/btpr.1783>.
- [6] Ng, C.K.D. *et al.* (2019). *Targeted integration of nucleic acids*. WO 2019/126634 A2, issued Jun. 27, 2019.

Systematic Workflow for Studying Domain Contributions of Bispecific Antibodies to Selectivity in Multimodal Chromatography

Adapted from Parasnavis, S.S., *et. al*

This study established a systematic workflow to analyze selectivity differences and preferred binding regions in bispecific monoclonal antibodies (bsAbs) and their parental monoclonal antibodies (mAbs) across three multimodal cation-exchange resin systems. The methodology integrated chromatographic screening of parent mAbs and their fragments at various pH levels, combined with surface property mapping and protein footprinting using covalent labeling followed by LC-MS analysis. The investigation revealed that multimodal resins provided enhanced selectivity compared to single-mode systems. A notable finding was that while the bsAb typically eluted between its two parental mAbs, its retention behavior on Capto MMC showed pH-dependent transitions between the parental mAbs. Analysis of domain contributions indicated Fab-dominated interactions at higher pH, and protein footprinting successfully identified key binding patches, with the light chain demonstrating a particularly significant role in Parent A's enhanced binding compared to Parent B.

Introduction

Bispecific antibodies (bsAbs) have emerged as a significant therapeutic modality due to their unique ability to simultaneously engage two different antigens. However, their production and purification present distinct challenges due to the potential formation of various product-related variants, including parental mAbs, mispaired species, and fragments, which can pose a significant challenge for downstream processing. While several engineering strategies have been developed to minimize mispairing, such as “knobs-into-holes” technology and electrostatic steering at the CH₃ interface [1,2], downstream processing remains challenging.

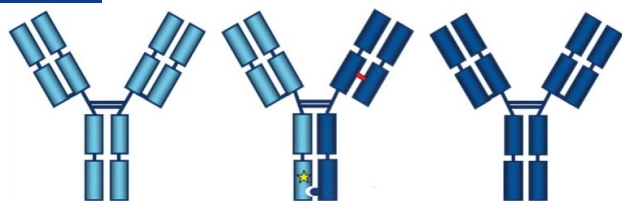
Recent developments have included mutations to modulate Protein A affinity and the use of different light chain isotypes to facilitate downstream processing [3–5]. However, product-related variants inevitably emerge during production. Multimodal resins have shown promise in providing increased chromatographic selectivity over single-mode resins for similar biomolecules, but a fundamental understanding of how these large multidomain biomolecules interact with chromatographic resins at the molecular level has been lacking.

In this study, a comprehensive framework was developed to evaluate how complex multidomain molecules like mAbs and bsAbs interact with chromatographic media, incorporating techniques such as chromatographic screening, protein surface mapping, and covalent labeling LC/MS. This framework was applied to analyze a bsAb and its two parent antibodies to understand how their binding patterns relate to chromatographic selectivity across several multimodal cation-exchange resin systems.

Materials and Methods

The parent mAbs (A and B with pI values of 8.62 and 8.46, respectively) and their bsAb (pI 8.49) were all IgG1s (AstraZeneca PLC.). The two parent mAbs had different Fab domains while the bsAb included Fabs from each of the parent molecules (Figure 1). Chromatographic resins used included MabSelect SuRe, LambdaFabSelect, KappaSelect, Nuvia cPrime and different multimodal cation-exchange resins from Cytiva and Bio-Rad Laboratories. Antibody fragments were generated using immobilized papain and pepsin enzymes, with fragments purified via protein A chromatography followed by size exclusion chromatography.

Figure 1



Relative structures of parent A (left), the bsAb (center), and parent B (right).

For surface property mapping, homology models of antibody fragments were generated using MOE software and electrostatic potential calculations were conducted using the APBS package. An in-house algorithm based on spatial aggregation propensity calculations was employed to generate hydrophobicity maps.

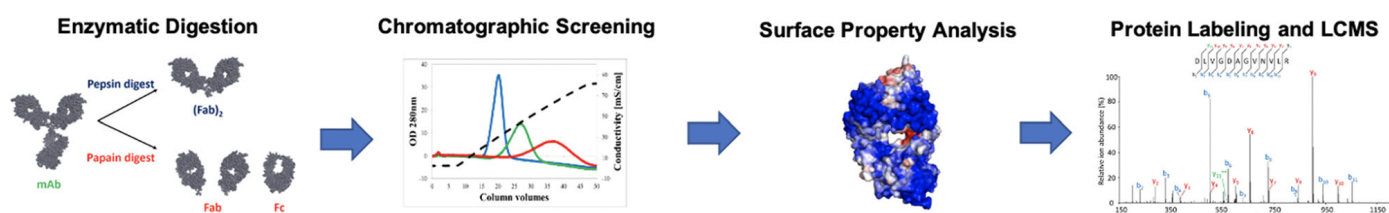
For protein labeling experiments, sulfo-NHS-acetate was used to label antibody lysine residues at pH 7.5 in both unbound and resin-bound states. The labeled antibodies were analyzed using LC-MS at both the intact protein and peptide levels. Subunit analysis was performed using FabRICATOR enzyme digestion followed by LC-MS on a Q Exactive HF-X mass spectrometer. For peptide mapping, the samples were reduced, alkylated, and digested with trypsin before LC-MS/MS analysis. Byos software was used for database searching and quantification of modification levels.

Results and Discussion

Workflow for a systematic study of mAb and bsAb chromatographic behavior

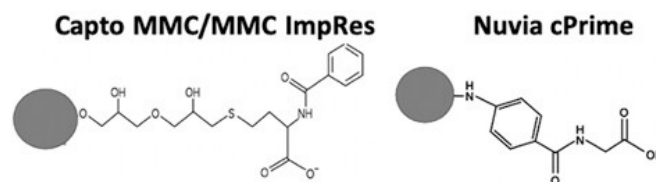
This study describes a comprehensive workflow for analyzing chromatographic selectivity behavior of parent mAbs and their bsAb (Figure 2). The strength of this approach lies in its integration of multiple analytical techniques to build a detailed understanding of binding mechanisms at both the molecular and macroscopic levels.

Figure 2



Workflow depicting the experimental and computational techniques utilized to probe the selectivity trends for the parent mAbs and their bsAb.

Figure 3

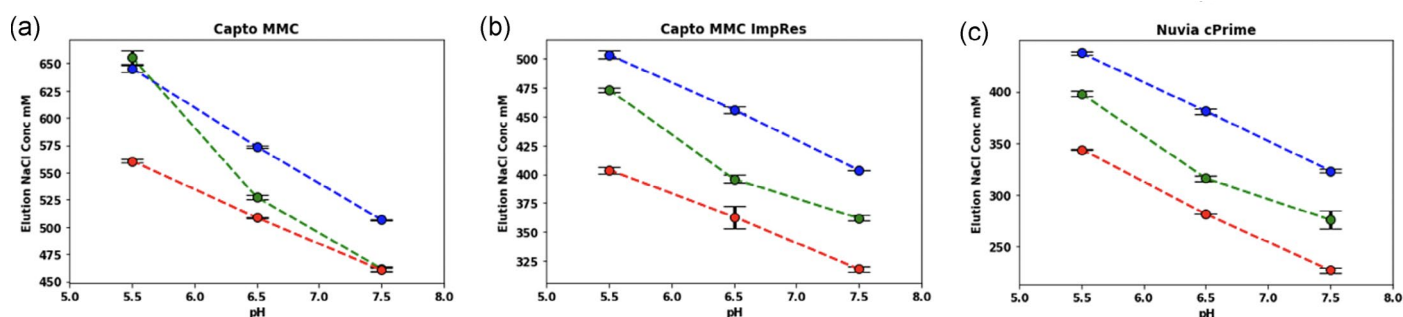


Capto multimodal cation exchange (MMC) and Capto MMC ImpRes resins have the same ligand head group but the Capto MMC resin system has a higher ligand density as well as a larger particle size as compared to the Capto MMC ImpRes resin system. The phenyl moiety on the Nuvia cPrime is located towards the base of the ligand and is less solvent-exposed as compared with the MMC series.

Linear gradient screening results for parental mAbs and bsAbs on MM CEX systems

Multimodal (also called mixed-mode) resins are specialized chromatography materials that combine multiple types of interactions to separate proteins, rather than relying on a single interaction mode. In this study, multimodal resins provided enhanced selectivity compared to single-mode systems, with the elution order following the molecules' respective pI values. The mAb and bsAb proteins had an elevated elution salt concentration on Capto MMC as compared with MMC ImpRes (Figure 3), likely due to the higher ligand density on the MMC system. In addition, while the bsAb eluted between Parents A and B on MMC ImpRes for all pH conditions, it exhibited a transitory behavior on the MMC resin (Figure 4). At pH 5.5, the bsAb coeluted with Parent A but then transitioned with pH to eventually behaving like Parent B at pH 7.5. This pH-dependent behavior observed with the Capto MMC resin suggests that specific domains may be involved in preferential binding at different pHs, a finding that could be particularly valuable for process development.

Figure 4



Linear gradient elution salt concentrations for Parent A, Parent B and bsAb at pH 5.5, 6.5, and 7.5 on (a) Capto MMC, (b) Capto MMC ImpRes, (c) Nuvia cPrime.

Domain contributions to parental mAb retention on multimodal resin systems

The chromatographic behavior of antibody fragments (Fab, Fc, and (Fab)₂) compared to intact antibodies was studied on resin systems Capto MMC and Nuvia cPrime (Figure 3) at pH 5.5 and 6.5. For Parent A, the (Fab)₂ fragment showed similar elution behavior to the intact antibody across all conditions, with a consistent elution order ((Fab)₂, then Fab, then Fc), though the separation between Fab and Fc fragments increased at higher pH. Parent B showed similar behavior to Parent A at pH 6.5, but displayed notable differences at pH 5.5. This result suggested that the (Fab)₂ domain plays a crucial role in binding to these multimodal resin surfaces, likely due to avidity effects.

Surface property mapping for parental mAbs

Surface property mapping revealed distinct differences in both the positive iso-surfaces and the surface hydrophobicity maps between Parent A and B Fab fragments (Figure 5). Even though the protein surface maps were different for the Fabs A and B, both have overlapping electrostatic potential (EP) and spatial aggregation propensity (SAP) regions that likely play a role in their interactions with these multimodal resins.

While the Fab fragments showed minimal pH dependence in their EP maps across pH 5.5–7.5 (due to pK_a values of residues being outside this range), the Fc fragment exhibited significant pH-dependent changes, with positive patches becoming less prominent and

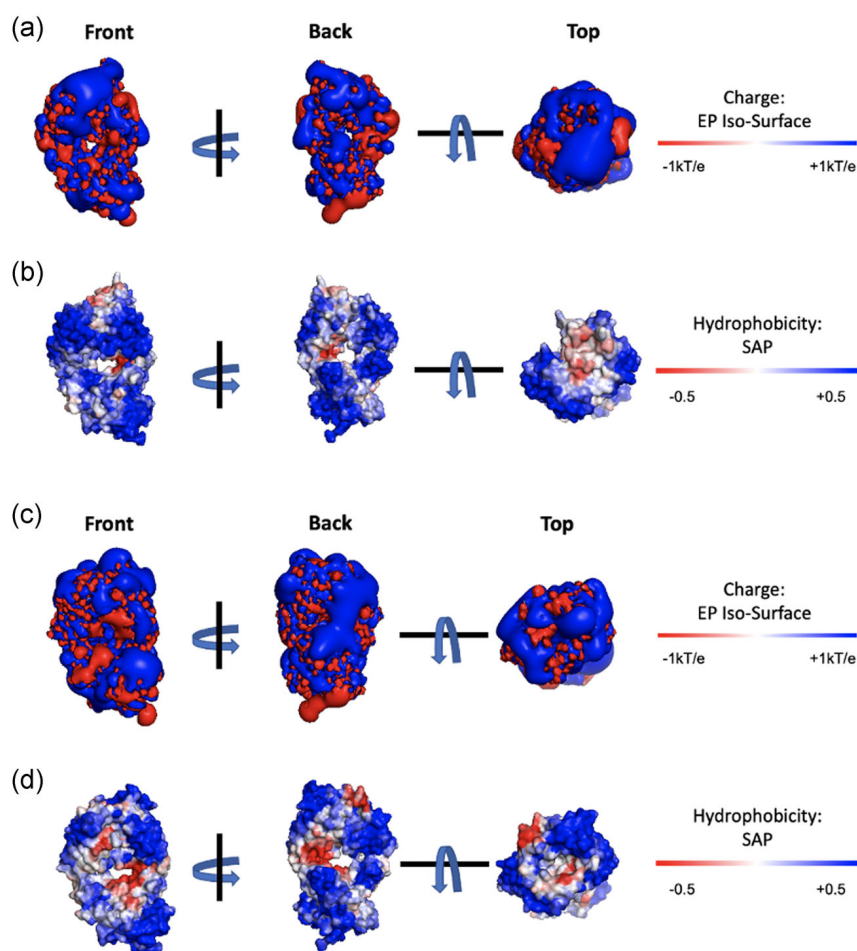
negative patches emerging as pH increased (Figure 6). Although the Fc fragment contained a large overlapping positive and hydrophobic patch in its donut loop region, this was likely less important for binding due to steric hindrance and proximity to a negative patch.

Covalent labeling and intact-mAb mass spectrometry results for parental mAbs and the bsAb

Based on domain contribution experiments and surface property analyses, it was hypothesized that Fab fragments were crucial for antibody binding to multimodal resins at high pH. To test this, lysine residues were covalently labeled in both unbound and resin-bound states. The molecules were further processed (before LCMS) to generate three fragments: Fd' (VH, CH1 and hinge), Fc (CH₂ and CH₃), and light chain (VL and CL). The difference in the number of labeled lysine residues between the unbound and bound conditions was then used to identify the domains of the molecules involved in resin binding.

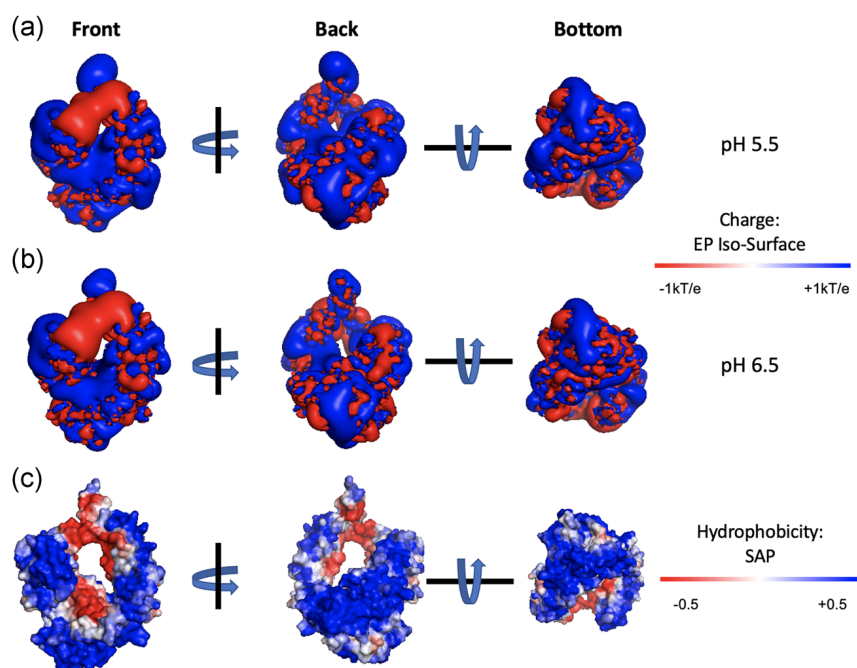
Results showed minimal differences in Fc fragment labeling across resins but significant differences in light chain and Fd' fragments, with Parent A showing more protected lysine residues (7.5 available, 3 protected) than Parent B (5.5 available, 2 protected). These findings indicated that the light chain fragment played a key role in the stronger binding of Parent A, while the bsAb showed labeling patterns reflecting both parent antibodies, suggesting both halves contributed to resin binding.

Figure 5



(a) Electrostatic iso-surface map for Parent A Fab at pH 6.5. (b) Spatial aggregation propensity (SAP) map for Parent A Fab. (c) Electrostatic iso-surface map for Parent B Fab at pH 6.5. (d) SAP map for Parent B Fab.

Figure 6



Surface property maps for the IgG1 Fc. (a) Electrostatic iso-surface map at pH 5.5 (b) and at pH 6.5. (c) SAP map showing regions of hydrophobicity on the surface.

Identification of the preferred binding regions using peptide mapping

Unbound and resin-bound parental mAbs and their bsAb were covalently labeled and then digested using trypsin followed by LC/MS to identify lysine residues involved in binding to the MM CEX resins. Results confirmed that the variable regions of the antibodies' light and heavy chains were the primary binding sites, while the constant regions showed minimal involvement. Four lysine residues in Parent A showed major differences between unbound and resin-bound states. These lysines were mapped onto wireframe structures and formed a concentrated binding patch in the complementarity-determining region. This indicated that these residues were likely the most shielded upon binding and thus played an important role at the interacting interface with the resins. Parent B had only one primary binding lysine residue and showed a more diffuse binding pattern. The bsAb incorporated binding characteristics from both parents, with key residues from both Parent A and Parent B contributing to resin interactions, explaining its intermediate elution behavior on most resins while showing parent B-like behavior on MMC resin. Interestingly, no significant differences in labeling were observed between the three resins indicating that the same preferred binding patches were likely important for all of the resins.

Conclusions

This study describes a systematic workflow to study how bsAbs and their parent mAbs interact with multimodal cation exchange resins, combining chromatographic screening, surface property mapping, and protein labeling techniques. The workflow provides a foundation for systematically studying chromatographic selectivity of large multidomain molecules, which could lead to improved biomanufacturability and expedited downstream process development.

References

- [1] Ridgway, J.B.B. *et al.* (1996). "Knobs-into-holes" engineering of antibody C(H)3 domains for heavy chain heterodimerization. *Protein Engineering*. <https://doi.org/10.1093/protein/9.7.617>.
- [2] Gunasekaran, K. *et al.* (2010). Enhancing antibody Fc heterodimer formation through electrostatic steering effects: Applications to bispecific molecules and monovalent IgG. *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.M110.117382>.
- [3] Tustian, A.D. *et al.* (2016). Development of purification processes for fully human bispecific antibodies based upon modification of protein A binding avidity. *mAbs*. <https://doi.org/10.1080/19420862.2016.1160192>.
- [4] Zwolak, A. *et al.* (2017). Rapid Purification of Human Bispecific Antibodies via Selective Modulation of Protein A Binding. *Scientific Reports*. <https://doi.org/10.1038/s41598-017-15748-0>.
- [5] Wang, C. *et al.* (2018). A systematic approach for analysis and characterization of mispairing in bispecific antibodies with asymmetric architecture. *mAbs*. <https://doi.org/10.1080/19420862.2018.1511198>.

Innovations in Bispecific Antibodies

Interview with Prof. Steven Cramer on Antibody Innovations



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In this insightful interview, we sit down with Prof. Steven Cramer to discuss his latest research on bispecific antibodies. Prof. Cramer shares his journey into the field, highlighting his significant contributions to chromatographic bioprocessing and protein-surface interactions. He delves into the unique structure and function of bispecific antibodies, their potential in cancer immunotherapy, and the current challenges in their development and manufacturing.

Can you tell us a bit about your background and how you became interested in the field of chemical and biological engineering, particularly in protein-surface interactions and molecular bioprocessing?

My background is in biomedical engineering, which I studied at Brown University. After that, I worked for a couple of years at a biotech manufacturer. Then, I went back to school for my PhD at Yale. Once I completed my PhD, I started as a professor at RPI in the mid-80s.

The reason I got into this field was personal. My dad had multiple sclerosis, and I was very interested in helping to create new medicines. Additionally, in the early 80s, the biotech industry was just starting to take off. I realized there weren't many people trained in biomanufacturing for these new kinds of drugs, and I wanted to make an impact in that area.

I became fascinated with the properties of biological molecules and how they interact with other molecules, ligands, and surfaces. As new drugs emerged, I saw a real need for both experimental and *in silico* tools to create new separation materials and better processes for purifying these drugs.

Your lab has made significant contributions to chromatographic bioprocessing. What has been the most rewarding aspect of your career so far?

Well, those are two separate questions in a way, right? I would say the main contributions we've made are developing mathematical models for predictive tools, the use of high throughput screening, and the integration of biophysics and separations technology. But what's been the most rewarding is probably the students.

I've really enjoyed mentoring students. Behind me is an academic tree showing my PhD advisor, Chaba Horvath, and his advisors, going back to some very famous physical chemists in Europe. I have all of my PhD students, 60 to date. My lab is still very full with 12 PhDs and postdocs. My former PhD students have made a dramatic impact both in academia and the biotech industry. They've been responsible for bringing a large percentage of the current biopharmaceuticals to the market. I'm extremely proud of my former students, both in academia and industry.

How do bispecific antibodies differ from traditional monoclonal antibodies in terms of their structure and function?

Disclaimer upfront, I'm a chemical engineer, not a molecular biologist. But I'm happy to answer that question. Regular monoclonal antibodies, or mAbs, are one of the most successful biopharmaceuticals right now. Bispecific antibodies, on the other hand, have a Y shape where one side binds to one target and the other side binds to another target. This opens up an incredible opportunity to both bind to targets and attract immune system components. Essentially, bispecifics have a double capability and can accomplish some of the same goals as cell therapies. Bispecifics have tremendous promise.

Your recent article '[Systematic workflow for studying domain contributions of bispecific antibodies to selectivity in multimodal chromatography](#)' discusses the systematic workflow for studying domain contributions of bispecific antibodies. Could you elaborate on the key findings and their implications for the field?

This is part of an ongoing effort in my lab to understand the preferred binding domains of

biological molecules in multimodal chromatography. Multimodal chromatography combines hydrophobic and electrostatic interactions, using ligands with both charge and hydrophobic groups. These combinations are useful for difficult separations, such as separating the desired bispecific antibody product from similar impurities.

We first look for interesting selectivity patterns between the product and its impurities. Then, we use biophysics, specifically covalent labeling mass spectrometry, to study these patterns. We compare the molecule in solution and bound to the surface, reacting them with chemical reactants. This helps us identify preferred binding patches and understand why we have selectivity.

This research is fundamental for understanding why certain ligands are useful for different problems. We're also developing *in silico* tools to enable fully computer-driven design of separations.

What are the top current challenges in the development and manufacturing of bispecific antibodies?

Since I focus primarily on the separation side, the main challenge is separating the product from very similar molecules. There's also the upstream side of biomanufacturing, where you make the molecules, and the downstream side, where you purify them. The formulation side prepares the product for the patient. The challenge is how to make these molecules, whether in different parts or as one whole thing, and the impact on product concentration and impurities. But I'm not an expert on the upstream side, so I'll leave my comments there.

How do you see the future of bispecific antibodies evolving, particularly in the context of cancer immunotherapy?

Bispecific antibodies are extremely powerful and have great potential. The jury is still out on how bispecifics or even trispecifics will evolve. If you can have two ligands binding to different important parts of the disease process, what about three? Is there an advantage to having three? The added complexity of these molecules compared to traditional monoclonal antibodies and cell therapies is significant.

Currently, cell therapy is unbelievably exciting but also very expensive. I have several students who are CTOs of cell therapy companies, and while it's a thrilling field, the cost is a major barrier. If we can achieve similar outcomes with protein-based biologics instead of cell-based biologics, it could have a dramatic economic impact on who can access these treatments. Hopefully, cell therapies will also become much cheaper soon.

What advice would you give to young researchers who are just starting out in the field of bioprocessing and biomanufacturing?

A good bioprocess engineer needs a combination of engineering and molecular knowledge. You must understand traditional engineering aspects, mass transport phenomena, thermodynamics, and have a strong background in these areas. But you also need to know your molecules, biology, and biochemistry. You must understand your product, impurities, and their properties to develop drugs quickly. Flexibility and creativity are crucial because drugs are changing all the time. You need a strong tool set and the ability to adapt and upgrade it. My advice is to have a balance of strong foundational understanding and a developed sense of intuition. Be an engineer but also a jazz musician, able to make intellectual jumps to new ideas.

Are there any upcoming projects or research areas that you are particularly excited about?

One of the projects I'm particularly excited about is using biophysics to define preferred binding domains of molecules with ligands but doing it entirely on the computer. We're trying to combine *in silico* predictions with high-throughput robotic screening to expedite process development. This requires a lot of innovation and is a major focus for us.

We're also deeply involved in exploring alternatives to chromatography, such as affinity precipitation, affinity coacervation, and phase separations. Many of these projects are still under wraps, but they hold great promise.

Additionally, we're doing significant work in mRNA and gene therapy, particularly with lentiviral vectors and AAV. What excites me the most is integrating the latest developments in simulations and biological understanding into these areas.

Unlocking Bispecific Antibodies

Interview with Dr. Paul Royle on Partnering for Discovery



Email: paul_royle@bio-rad.com Watch the video [here](#)

In this compelling conversation, we explore the rapidly evolving field of bispecific antibodies with insights from Dr. Paul Royle, a Bio-Rad expert who works closely with researchers worldwide. The discussion covers the unique mechanisms of bispecific antibodies, their expanding role in cancer immunotherapy and autoimmune disease, and the innovative strategies being used to enhance their specificity, safety, and therapeutic potential. Gain insight into how scientific breakthroughs and collaborative efforts are shaping the future of bispecific antibody research.

Can you tell us about your background and how your path led you to working with custom antibodies, especially in the context of emerging formats like bispecifics?

I am the Market Development Manager for Custom Antibody Services at Bio-Rad, and I've been with the company for almost 12 years, focusing almost exclusively on custom antibody services from both a sales and technical sales perspective. Over the years, I've worked as a technical sales specialist and also managed a global team of custom technical sales specialists. My role has given me the opportunity to engage closely with scientists to understand their specific requirements for custom antibodies and to collaborate with our laboratory teams to deliver tailored solutions. One of the most rewarding aspects of my job is gaining insights from a diverse range of customers—many of whom are developing cutting-edge biotherapeutic technologies, including bispecific and multi-specific antibodies and binding molecules. Whether they need bioanalytical tool antibodies to support their studies or antibodies to be incorporated into final bispecific formats for new drug development, these interactions have given me a broad perspective on the technologies and innovations shaping the field.

Before joining Bio-Rad, I spent seven years working at an immunology diagnostics company, where I developed a strong technical foundation and had the chance to interact with many interesting companies with different platforms and requirements. Prior to that, my background was in academic and postdoctoral research. While I'm not directly involved in developing bispecific drugs—since Bio-Rad is a life science company rather than a drug development company—I've had extensive

technical interactions and supported many organizations working in this space. I hope these experiences give me a valuable perspective on the market, and I'm always happy to share my insights with others in the field.

Where do you see the next breakthrough in bispecific applications—beyond oncology—for conditions like autoimmune or infectious diseases?

That's a really good question. It's such a broad field of research, and there are so many exciting avenues to explore. When most people think of bispecific antibodies, they usually picture bispecific T cell engagers, since these were the first approved drugs in this category. Essentially, a bispecific T cell engager is a molecule with one specificity directed at a tumor target and the other at a receptor or marker on an immune cell, typically a T cell. Many of the approved bispecifics target blood cancers, especially B cell malignancies, with one binding arm recognizing markers like CD19, CD20, or BCMA on tumor cells and the other targeting CD3 on T cells. The antibody brings the T cell into close proximity with the tumor cell, forming an immune synapse that enables T cell-mediated tumor killing. While this is the classic example, the field is much broader—even within immuno-oncology, it's not just T cells that can be recruited; natural killer cells can also be engaged if the antibody targets markers like NKG2D or CD16. Beyond oncology, there's significant interest in using bispecifics to deplete B cells responsible for autoimmune conditions, as many autoimmune diseases are driven by autoantibodies from rogue B cells. Some bispecifics are being developed to target inflammatory cytokine pathways associated with autoimmunity, which could lead to new treatments for serious diseases like lupus.



Jumping back to immuno-oncology, there are also innovative drugs that bring together different elements of immune checkpoint inhibition, rather than just connecting CD3 and tumor targets. Immune checkpoints are mechanisms cancer cells use to switch off immune cells; by targeting both the checkpoint ligand on the tumor and the receptor on the immune cell, bispecifics can not only form an immune synapse but also release the brakes on the immune system, potentially leading to more effective tumor killing. There's also fascinating work on conditionally activated bispecific antibodies that are only active in the unique environment of the tumor, such as areas with high ATP, specific proteases, or low pH. This helps ensure the antibodies are only active where needed, reducing off-target effects elsewhere in the body. For example, some antibodies are designed so their binding sites are masked until cleaved by tumor-associated proteases, or they only bind at lower pH, or require ATP to engage their target.

There are also innovations in conditionally deactivating bispecific antibodies, which address the risk of cytokine release syndrome—a potentially severe side effect of immune cell recruitment—by incorporating protease cleavage sites that allow the drug to be degraded once T cells are activated, acting as a form of autoregulation. While much of this work is in immuno-oncology, the field is not limited to it. For instance, there's an approved bispecific drug for hemophilia that operates completely differently: instead of targeting cancer, it mimics the function of clotting factor VIII by bringing together factors IXa and X, restoring clot formation in patients who are factor VIII-deficient and may not respond to traditional supplementation. Another exciting area is using bispecifics to cross the blood-brain barrier. Therapeutic antibodies are typically too large to cross, but by targeting receptors like transferrin or CD98 at the barrier, one arm of the bispecific can facilitate transport into the brain, allowing the other arm to act on its intended target.

Overall, it's an incredibly diverse and innovative field, with so many promising directions that it's hard to

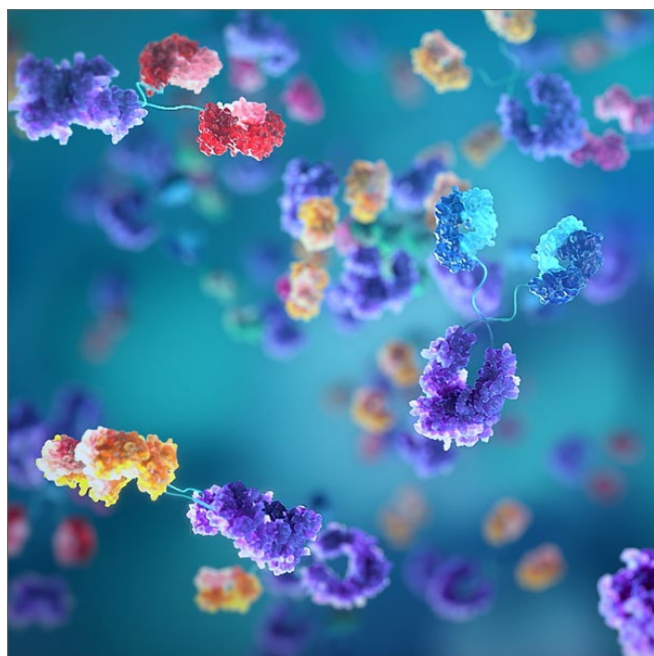
predict where the next big breakthrough will come. There's a lot of clever science happening, and the range of applications for bispecific antibodies continues to expand.

What are the critical gaps in current bispecific screening tools?

Much of what we do revolves around supporting bioanalytical studies, and when you take a step back and consider these studies more broadly, there are a few critical differences between antibodies and biologics compared to small molecules when it comes to analysis. For small molecule drugs, you'd typically use techniques like LCMS to measure drug levels in patient samples. However, bispecific antibodies often resemble normal antibodies in their biophysical characteristics, and since a patient's blood is already full of their own antibodies, you end up searching for an antibody in a background of antibodies. This makes it challenging to isolate the analyte using standard physical chemistry methods because of the high background of similar molecules. In pharmacokinetic studies, which look at how the body affects the drug—covering absorption, distribution, metabolism, and excretion—it's important to determine whether the drug concentration in circulation matches what you'd expect based on the dosage. For these kinds of studies, traditional biophysical methods can struggle to pull out the drug, so customers typically need specific reagents. We can generate antibodies that recognize a customer's drug antibody without cross-reacting with the patient's own antibodies by targeting unique features of that drug, and this need is very much case by case—each drug requires its own unique reagents for accurate measurement.

Another key aspect of biologics like bispecific antibodies is that, unlike small molecules such as aspirin or paracetamol, they're large enough to be detected by the immune system. So, during clinical trials, it's crucial to check whether the patient's immune system is recognizing the drug and mounting a response. If the immune system clears the drug, you'll see lower pharmacokinetic readings simply because the drug is no longer present. A common challenge our clients face is the lack of positive control material for immune responses to a newly developed molecule. When we generate antibodies for pharmacokinetic studies, they can also serve as positive controls in anti-drug antibody assays, which typically involve a workflow of screening, confirmatory, and neutralizing antibody assays. From a screening perspective, these are areas we're very actively engaged in.

The antibodies we generate to other antibodies or bispecifics are called anti-idiotypes. When working with a customer's bispecific antibody, it's important to understand the structure of the molecule, as bispecifics can be more complex than the classic Y-shaped antibody with one binding arm for each target. Some have additional binding sites—such as single-chain variable fragments, nanobodies, Fabs, or other fragments—grafted onto different parts of the molecule, giving them more than one binding site. Ideally, customers can provide individual antibodies or fragments for each binding site, allowing us to generate antibodies to each arm in separate campaigns and then test them on the final bispecific. Sometimes, though, customers only have the final format, so we generate antibodies to the whole molecule and hope to cover both binding arms. We then screen the resulting anti-idiotypes by forming complexes with the drug target and seeing if our antibody can bind that complex; if it can't, it's likely directed to the binding arm involved in the complex, and if it can, it's probably targeting the other arm. This approach allows us to sort the anti-idiotypes generated in a campaign, though using only the final molecule doesn't always yield the best possible antibodies to both arms, whereas separate campaigns for each arm give a better chance.



After generating these antibodies, we also have to carefully design sandwich pair screens to find the best pairs for pharmacokinetic assays. For example, if the bispecific has two copies of a binding arm, you might capture and detect using the same arm, but if it only has one of each, you'd need to use antibodies against one arm for capture and the other for detection.

There's quite a bit of nuance in designing these bioanalytical tools, but that's where our specialized antibody sales team at Bio-Rad excels in collaborating with customers to find the right solutions. It's a very interesting and dynamic area to work in.

What criteria do you believe researchers should prioritize when searching for therapeutic candidates, and how do Bio-Rad's tools simplify this process?

It's very difficult to say in terms of what researchers should prioritize, as it really depends on their specific drug target and how they envision their drug working. People often assume that generating antibodies with very high affinity is always best, since high affinity can allow for lower drug concentrations. However, there are scenarios where you might actually want a low-affinity antibody. For instance, if you're targeting a tumor antigen that's highly expressed on tumor cells but also present at lower concentrations on normal cells, using a bispecific with two low-affinity binding arms can be beneficial. In the tumor, where the target is abundant, both weak binding arms can engage, resulting in strong overall binding. But in normal tissues, where the target is scarce, only one binding arm may attach at a time, leading to much weaker binding and allowing the antibody to dissociate more easily—minimizing off-target effects. This relates to the concept of valency and avidity: affinity describes the strength of a single binding interaction, while avidity reflects the combined strength of multiple interactions. Avidity isn't simply additive; it can be much higher than the individual affinities, especially in high-density environments like tumors. So, you can engineer a weak antibody that binds strongly in tumors but not elsewhere. There are many considerations, and the best approach is highly customer-dependent. One of the advantages of the platforms we use for generating antibodies is the ability to manipulate screening conditions to select antibodies that meet specific requirements, whether that's affinity, binding under certain conditions, or other criteria. By adjusting the screening or enrichment conditions, we can tailor the antibodies to fit the project's needs. Ultimately, the best advice for researchers is to work closely with companies like Bio-Rad from the beginning, rather than waiting until later in the process. By sharing their requirements early and openly, researchers can benefit from our experience and potentially discover innovative solutions they might not have considered. Sometimes customers come in with preconceived ideas about how things should be done, but by telling us

what they're trying to achieve, we can often suggest alternative strategies or screening approaches that better fit their needs, thanks to the flexibility and innovation built into our platforms.

How does Bio-Rad's services enable bispecific antibody development?

Bio-Rad offers custom antibody generation through our Pioneer Antibody Discovery Platform, which includes a fully synthetic, human Fab library containing approximately 220 billion antibodies. This diverse library is optimized for rapid screening, allowing us to efficiently identify antibodies that meet specific criteria for therapeutic development. The platform integrates SpyTag/SpyCatcher technology—a protein ligation system derived from a *Streptococcus pyogenes* collagen adhesin domain—that enables irreversible covalent bonding between separately produced fragments. This technology is utilized across multiple workflows: displaying antibody fragments on phages, converting antibody formats without subcloning, and prototyping bispecific antibodies at the protein level.

A key innovation within the Pioneer Platform is SpyLock, a modified SpyCatcher that allows controlled sequential assembly of bispecific antibodies. By engineering

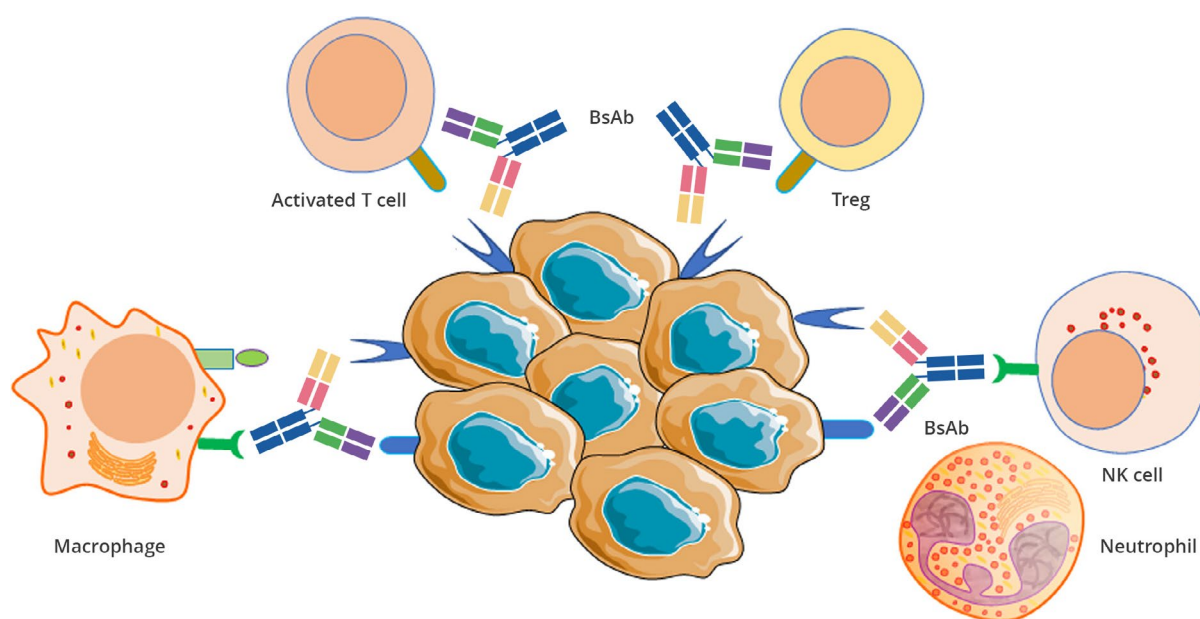
a cysteine residue into SpyLock and using bulky molecules to temporarily block its activity, we can first ligate one binding arm to the wild-type SpyCatcher component. Reducing agents then “unlock” SpyLock, enabling the addition of a second binding arm to form a 1:1 heterodimer. This approach eliminates the need for extensive subcloning or test productions, allowing customers to generate and screen hundreds of bispecific combinations in as little as 90 minutes. For example, we've applied this to immune checkpoint targets like PD-1/PD-L1, where bispecifics both bridge immune and tumor cells and release inhibitory signals.

The platform's flexibility extends to post-screening workflows. Using technologies like CrossMab, we convert high-performing bispecific candidates into mammalian-expressed IgG formats, ensuring functional consistency between prototypes and final therapeutics. This rapid prototyping capability, combined with the Pioneer Library's scale and diversity, empowers researchers to explore optimal affinity balances for their targets and accelerate lead candidate selection. Whether supporting immuno-oncology, autoimmune disease research, or other therapeutic areas, Bio-Rad's tools and expertise help customers navigate complex bispecific development with speed and precision. We're always eager to collaborate and tailor our services to meet specific project needs.

Paul Royle is the Market Development Manager for Custom Antibody Services at Bio-Rad Laboratories. With a background in marketing and life sciences, he specializes in driving growth and supporting clients in the biotech sector. Paul combines technical expertise with strategic insight to help researchers and industry partners access advanced antibody solutions.

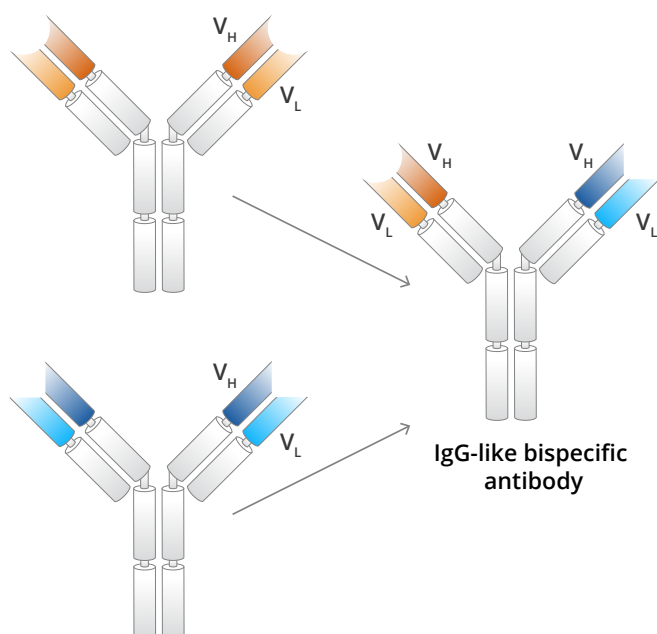
Charting the Course: Bispecific Antibody Development Pipeline

The Bispecific Antibody (bsAb) Journey



What are Bispecific Antibodies?

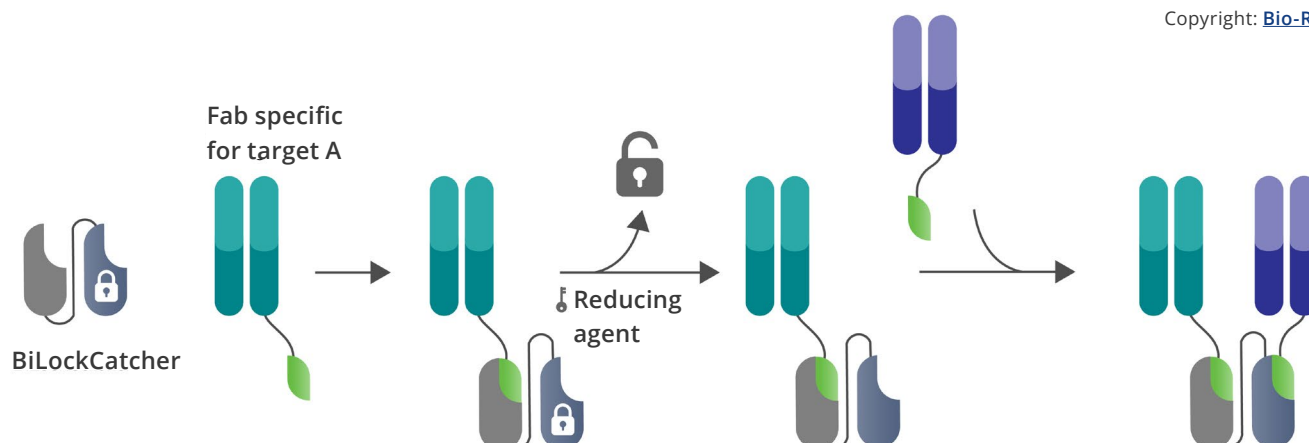
Bispecific antibodies (bsAbs) are innovative molecules that have the unique ability to simultaneously engage two different antigens. This dual-targeting capability revolutionizes cancer treatment by allowing for more precise and effective targeting of cancer cells while also engaging immune effector cells. Beyond oncology, bsAbs are expanding their applications into autoimmune diseases and inflammatory conditions, offering new therapeutic possibilities and improving patient outcomes.



Discovery and Development: Building the Foundation

Target Identification

Identifying the right target pairings is essential for achieving desired therapeutic effects with bsAbs. By engaging two different antigens simultaneously, bsAbs can precisely modulate biological pathways, enhance immune responses, and inhibit specific molecular interactions. This strategic selection of target pairings allows bsAbs to offer increased efficacy, reduced resistance, and broader therapeutic applications compared to traditional monoclonal antibodies.

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Engineering, Expression, and Functional Screening

SpyLock is a cutting-edge approach to expediting bispecific screening. It takes advantage of the autocatalytic reaction between SpyTag and SpyCatcher. This groundbreaking innovation with a 'lockable' SpyCatcher named SpyLock, which can be reversibly inhibited to prevent unwanted ligation with a SpyTag. The simplicity of SpyLock technology is reflected by its sequential antibody reactions for the generation of a bispecific prototype.

Characterization, Optimization, and Quality Control



Analytical Approaches

Flow Cytometry:

ZE5 Cell Analyzer is an essential tool used in cell analysis and immunoprofiling.

Multiplexed Immunoassays:

Multiplex Immunoassays allows simultaneous measurement of multiple potential biomarkers from a single sample.



Optimization Techniques

Cell-Based Assays:

Evaluate the functionality of bispecific antibodies. Ensure that the antibodies effectively engage their targets and initiate cell death.

Optimization:

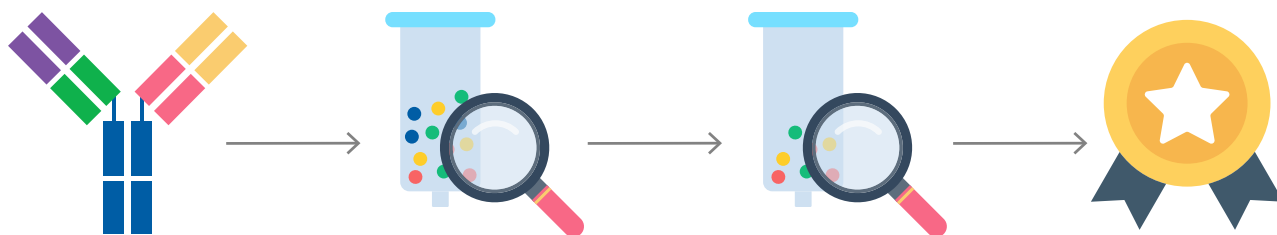
SpyLock capabilities in combination with our TrailBlazer technology allows you to prototype multiple antibody formats for optimization.



Quality Control Practices

Analytical Methods:

Ensure that bispecific antibodies meet QA/QC standards. QX200 or QX ONE Droplet Digital PCR System is used for impurity testing and quality control.

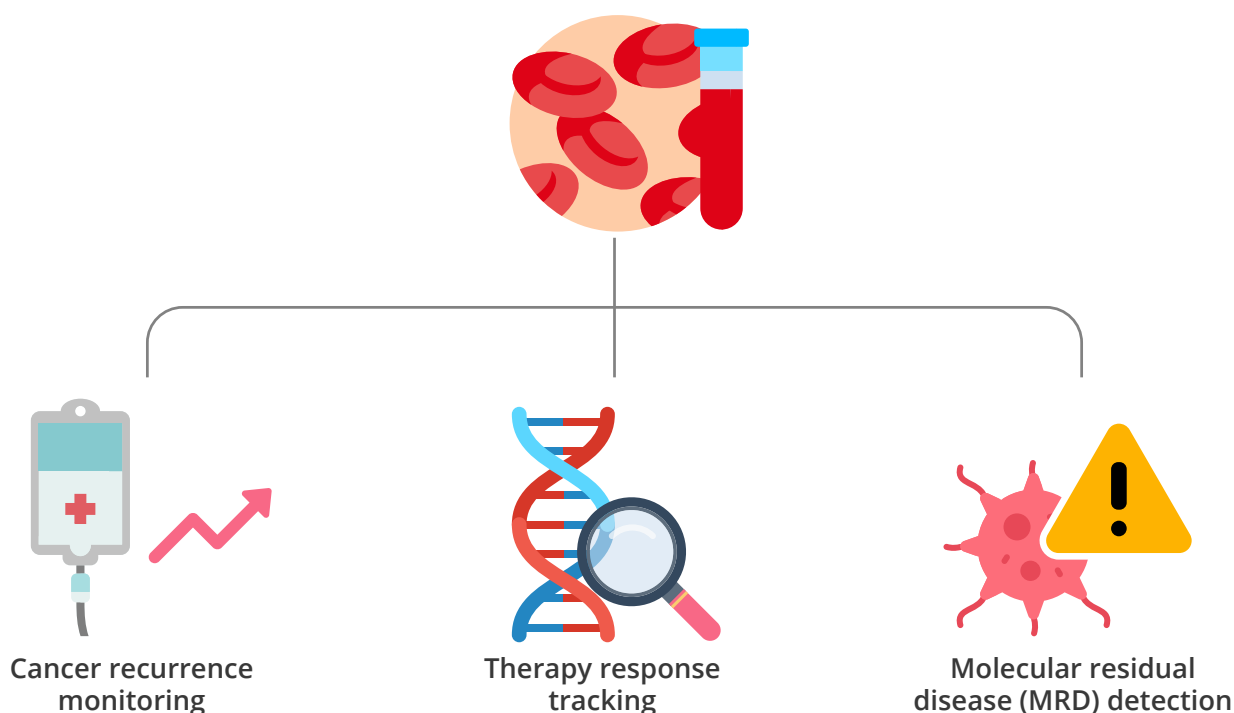


Process Development and Manufacturing: Ensuring Quality and Consistency

Challenges in bsAb production and purification can be addressed through the use of Bio-Rad's CHT Ceramic Hydroxyapatite Media or mixed-mode resins (Nuvia aPrime 4A Media or Nuvia wPrime 2A Media) for purification and enrichment of the correct bsAb format with removal of incorrect bsAb biproducts. Analytical methods to test for purity across for ensuring regulatory compliance and monitoring quality. Bio-Rad's QX ONE or QX200 Droplet Digital PCR System is used for impurity testing, contaminants, and QC, and the ZE5 Cell Analyzer in combination with StarBright Antibodies for cell potency assays.

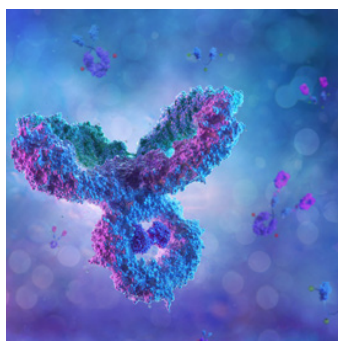
Clinical Research to Advance Patient Treatment and Monitoring

Powerful Research Tools for Clinical Research in Treatment Response Analysis, Molecular Residual Disease Detection, and Recurrence Monitoring



Bio-Rad's QX600 Droplet Digital PCR System, equipped with specialized assays like ESR1 mutation detection and MSI, plays a crucial role in clinical research by enabling precise therapy qualification. Circulating tumor DNA (ctDNA) analysis offers multiple applications, including research into therapy response tracking, molecular residual disease (MRD) detection, and cancer recurrence monitoring. These research tools ensure accurate and reliable results for advancing scientific knowledge.

Antibody Therapeutics



Advancing Cell Line Development to Streamline Biopharmaceutical Production

Biologics, including monoclonal antibodies, clotting factors, hormones, and the like, have been trending in the biotechnology pipeline for years, reaching 50% of U.S. Food and Drug Administration (FDA) approvals in 2022 (Senior 2023). Driving this trend are the clinical advantages of biologics over small molecules — namely, greater target specificity and fewer side effects — as well as technical advances in cell-based bioprocessing that have made biologics easier to produce. However, continual innovation in cell line development is required to continue this trend.

From DNA to Clone: The Process of Cell Line Development

Biologics originate from engineered cell lines. The process involves four basic steps. First, the recombinant DNA encoding the biopharmaceutical must be designed and produced. Typically, this involves constructing a DNA plasmid containing the gene of interest, a strong promoter, and a selectable marker that can be used later to screen for gene-bearing cells.

The next step is perhaps the most challenging: gene delivery. Cell lines vary considerably in their amenability to transfection. Additionally, there are many transfection methods from which to choose. The suitability of a transfection method often depends on the cell line selected for biopharmaceutical production: cationic lipids or calcium phosphate may be suitable for an easy-to-transfect cell line such as human embryonic kidney (HEK) 293 cells, whereas electroporation may be necessary for those less amenable to transfection (for example, Chinese hamster ovary [CHO] cells). Retroviral vectors are an option for cell lines that are particularly resistant to transfection. Ultimately, the goal is to integrate enough copies of the transgene into the cell line's genome so that a stable and high-expressing clone can be selected and enriched for bioproduction. Copy numbers assayed with techniques such as quantitative PCR or Droplet Digital™ PCR can serve as early indicators of cell line productivity.

After gene delivery comes cell selection and cloning: the process of enriching cultures with gene-bearing cells and selecting individual cells from this population for characterization. Cell selection typically depends on a selectable marker that was introduced alongside the gene of interest in the previous step. Common examples include antibiotic resistance markers and fluorescent proteins, which enable selection by antibiotic treatment and fluorescence-activated cell sorting (FACS), respectively.

Once a pool of gene-bearing cells is enriched, they must be cloned, as regulatory agencies expect biologics to be derived from monoclonal sources (Castan et al. 2018). Historically, cloning was done by limiting dilution, a process by which cells are heavily diluted such that single cells are deposited into individual wells in a microtiter plate. Alternatively, clones can be isolated by automated colony picking systems or, more commonly, FACS (if fluorescent markers are used for selection).

Once single cell clones are generated, they are scaled up and assayed for productivity, growth, and quality. Productivity is important to establish early during scale-up and can be achieved with high throughput using bio-layer interferometry systems and verified by enzyme-linked immunosorbent assay (ELISA) (Castan et al. 2018). At the end of the cloning process, several dozen clones are selected to create safety cell banks, and the best clone is selected for the master cell bank, which represents the endpoint of cell line development. The master cell bank serves as the source of the working cell banks used for biopharmaceutical production. These banks are subjected to extensive testing, including mycoplasma and viral testing, to ensure there is not contamination in the starting source material.

Starting Strong: Choosing a Cell Line

Although any cell line amenable to genetic manipulation can produce recombinant proteins, only a select few are suitable for biopharmaceutical production. The ideal cell line should be able to grow in serum-free media (animal components — serums — carry potential contamination risks), in suspension, and at a replication rate to enable commercial-scale production (Castan et al. 2018). Moreover, they should be able to produce recombinant proteins with the right posttranslational modifications and secrete them efficiently and with as few by-products as possible. Therefore,

choosing a suitable cell line at the beginning of the development process is key to maximizing productivity and minimizing risk in biopharmaceutical production.

The CHO cell line is a popular choice with a notable pedigree in biopharmaceutical production. This pedigree stems from the derivation of CHO auxotrophs — mutant CHO cell lines lacking the ability to synthesize critical amino acids — in the 1960s and 70s (Jayapal et al. 2007). Because these mutant CHO cell lines lacked the ability to synthesize critical amino acids, clonal selection after gene transfer could be carried out by simply cotransfecting the cells with the missing biosynthetic enzymes and growing them in nutrient-deficient medium, obviating the need for antibiotic or fluorescence-based selection while encouraging the selection of cells expressing high copy numbers of the gene of interest.

Once CHO cell lines had become established in the pharmaceutical industry, cell culture technology for CHO cells continued to evolve. For example, tools for site-specific integration of transgenes, such as Cre- or Flp-based gene targeting and, more recently, clustered regularly interspaced short palindromic repeats (CRISPR), made gene transfer more predictable and less labor intensive (Kim et al. 2012). The widespread use of CHO cells also streamlined production because all the industry players — cell culture suppliers, manufacturers, and regulatory bodies — had become familiar with the technology (Dumont et al. 2016).

The industry has recently begun turning to human cell lines, such as HEK 293 cells and HT-1080 cells, to produce more human-like (less immunogenic) recombinant proteins (Dumont et al. 2016). However bacteria, yeast, and insect cells do not replicate the posttranslational modifications produced by human cells, and CHO cells can only approximate them, missing some and adding others. As an example of the latter, CHO cells modify glycosylated proteins with N-glycans that are immunogenic to humans (Dumont et al. 2016). However, while human cells can produce less immunogenic proteins, they can also harbor and transfer pathogens that infect humans. Thus, choosing a cell line for biopharmaceutical production requires carefully considering its benefits and risks.

Optimizing Productivity: Host Cell Engineering

Improving the productivity of mammalian cell lines requires looking beyond the biotherapeutic gene of interest to the host cell's biology. A wide variety of cellular processes can bottleneck recombinant protein production and impact biopharmaceutical quality, including cell death, proliferation, metabolism, and protein processing and secretion. These processes are prime targets for host cell engineering efforts. Apoptotic pathways, autophagy, cell cycle, protein folding, the unfolded protein response pathway, metabolic pathways, and vesicle trafficking have all been successfully manipulated by overexpressing or suppressing the relevant endogenous players in recombinant CHO cells (Kim et al. 2012).

Host cell engineering has also been applied to suppress host cell protein production. Occasionally, during biopharmaceutical production, a recombinant cell line will secrete host cell proteins

that copurify with the biopharmaceutical. Suppressing the expression of such proteins can reduce the complexity of the downstream purification while freeing up cellular resources for biopharmaceutical production.

Technological advances have improved the engineering of recombinant cell lines. In particular, CRISPR has dramatically simplified the process of creating gene knockouts, especially multiple knockouts. microRNAs have also emerged as an alternative capable of tuning entire cellular pathways without burdening cellular transcription machinery. Finally, various omics technologies have unraveled the complexity of cellular pathways, enabling new targets to be identified and leveraged for engineering.

Ensuring Consistency: Genetic Clonality and Stability

Cloning is performed during cell line development to minimize heterogeneity in the final cell bank and, consequently, during production. Regulatory bodies generally agree and expect monoclonal cell lines while acknowledging that monoclonality is impossible to prove. Some in the pharmaceutical industry have pushed back on the requirement for monoclonality, arguing that monoclonal cell lines are heterogeneous due to the inherent genomic instability of immortalized cell lines and the requirement of 33 population doublings needed to achieve the 10^{10} cells needed for banking. They further argue that a rigorous control strategy is more important than clonality (Castan et al. 2018; Welch and Arden 2019). Even so, monoclonal cell lines are thought to experience less variability during the manufacturing lifetime than polyclonal lines, and regulatory agencies still expect to prove clonality.

The ease with which clonality can be proven depends on the method used for cloning (Castan et al. 2018). For limiting dilution, the probability of clonality depends on the dilution factor; the more dilute the cells are, the higher the probability is that a monoclonal line will be established. A second round of limiting dilution can also be performed to increase this probability. However, higher dilutions add to screening time, and performing the procedure twice adds to the number of cell doublings needed to derive the line. The tendency of a cell line to form aggregates — CHO cells do — can also complicate cloning by limiting dilution.

FACS of single cells into microtiter plates represents a less risky method, given the ability of flow cytometry systems to discriminate singlets from doublets. However, flow cytometry can stress cells and potentially impact outgrowth. Therefore, regardless of which method is chosen, it should be experimentally validated to achieve the highest possible probability of clonality.

The Future of Cell Line Development

Despite decades of research, cell culture and cell line development remain incompletely understood. Cell line development is advancing on several technological fronts, including the maturation of recombinant cell line systems beyond the CHO system, the development of streamlined genetic manipulation strategies, the application of systems biology approaches to process improvement, and the development of more sound control

strategies. Moreover, basic questions about clonality and cell line stability still need to be empirically addressed, namely, does polyclonality lead to lot-to-lot variability during manufacturing? And can tests be developed to detect potential manufacturing risks early in the cloning process?

As the technology and conceptual understanding of cell line development move forward, biologics can be expected to become more prevalent among drug approvals. In support of this trend, Bio-Rad™ Laboratories, Inc. offers an array of solutions that streamlines cell line development, ranging from highly sensitive copy number analysis by Droplet Digital PCR to robust assays for biopharmaceutical quality assurance and quality control.

References

- Castan A et al. (2018). Cell line development. In *Biopharmaceutical Processing*, G. Jagschies, E. Lindskog, K. Łacki, P. Galliher, eds. (Elsevier), pp. 131–146.
- Dumont J et al. (2016). Human cell lines for biopharmaceutical manufacturing: History, status, and future perspectives. *Crit Rev Biotechnol* 36, 1,110–1,122.
- Jayapal KP et al. (2007). Recombinant protein therapeutics from CHO cells — 20 years and counting. *Chem Eng Prog* 103, 40–47.
- Kim JY et al. (2012). CHO cells in biotechnology for production of recombinant proteins: Current state and further potential. *Appl Microbiol Biotechnol* 93, 917–930.
- Senior M (2023). Fresh from the biotech pipeline: Fewer approvals, but biologics gain share. *Nat Biotechnol* 41, 174–182.
- Welch JT and Arden NS (2019). Considering “clonality”: A regulatory perspective on the importance of the clonal derivation of mammalian cell banks in biopharmaceutical development. *Biologicals* 62, 16–21.

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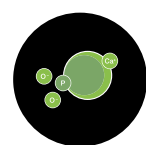
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Bispecific Antibody Purification

Overcoming Bispecific Antibody Purification Challenges with CHT™ Ceramic Hydroxyapatite Media



CHT CERAMIC
HYDROXYAPATITE

Abstract

The purification of bispecific antibodies (bsAbs) can be challenging due to the aggregation of the target molecules and chromatographic purification approaches. In Ingavat et al. (2023), researchers investigated the benefits of CHT Ceramic Hydroxyapatite Media in the downstream processing of this essential class of therapeutics in comparison to traditional cation exchange (CEX) resin purification strategies using three model molecules. For both asymmetric and symmetric IgG-like bsAbs, purity of over 97% was achieved using CHT Media with low levels of high molecular weight impurities (HMW). Using CHT Media resulted in an eightfold decrease in chromatography-induced aggregation compared to CEX-based workflows. Additionally, CHT Media effectively eliminated low molecular weight impurities (LMW) through post-load wash (PLW) optimization.

Introduction

BsAbs belong to a class of next-generation therapeutics that have been engineered to contain more than one antigen or epitope recognition site, in contrast with traditional monoclonal antibodies (mAbs). This additional recognition site enables dual targeting: two different biological targets are held in proximity, potentiating novel treatment options that could not be achieved by the application of a single mAb. The first bsAb was approved by the U.S. Food and Drug Administration (FDA) in 2014 and since then most applications have been in the field of oncology (FDA 2023).

BsAbs have been broadly classified into three categories based on their structure, namely fragment-based bsAbs (no fusion construct [Fc] region), asymmetric bsAbs, and symmetric bsAbs (Figure 1).

Asymmetric bsAbs are derived from different parental mAbs and to prevent random chain pairing, technologies such as knobs-into-holes (Figure 1, molecules A and B) and CrossMAb technology have been developed, in which each half of an asymmetric bsAb can contain one or two antigen recognition sites. Symmetric bsAbs (Figure 1, molecule C) have two different antigen recognition sites on each half of the molecule, and therefore are generally tetravalent (2+2).

As seen in Figure 1, antigen recognition sites can be in the traditional form with the CH1 domain present (molecule A, left arm) or single-chain variable fragments (molecule A, right arm), to give a single valency on each half of the molecule. Double valency can be introduced through a combination of both, as seen in molecules B and C.

Downstream processing of bsAbs involves numerous challenges, including a propensity for aggregation, which is considered a major hurdle. BsAbs tend to be less stable than their parental mAbs, with chromatography-induced aggregation observed in chromatography processes (Chen et al. 2021). CEX is a commonly employed strategy for aggregate removal in mAb purification. Here, the authors compared bsAb purity and aggregation following CEX and CHT chromatography polishing steps.

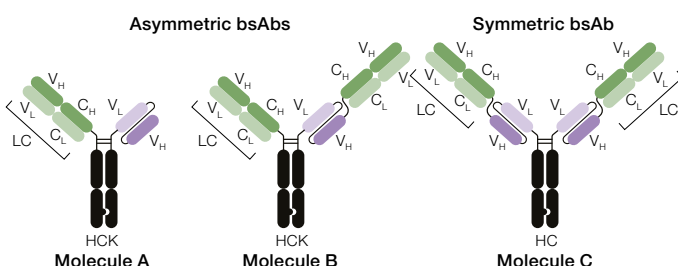


Fig. 1. Asymmetric and symmetric bispecific antibodies (bsAbs). Molecules A and B are asymmetric bsAbs representing 1+1 valency and 1+2 valency, respectively. Molecule C is a symmetric bsAb representing 2+2 valency. LC, light chain; HC, heavy chain; HCK, heavy chain knob.

Methods

Ingavat et al. (2023) produced molecules A, B, and C (Figure 1) by cell culture in stably transfected Chinese hamster ovary (CHO) K1 cells. Following harvest, the culture supernatant was centrifuged and filtered to remove cells and cellular debris. BsAbs in the harvested fluids were captured by protein A chromatography and, following elution, were adjusted to pH 6.5. Samples were aliquoted, frozen at -20°C , and used for the subsequent studies. For CEX/CHT purification, the frozen bsAb samples were thawed at room temperature, then adjusted to pH 5.5 for CEX load and pH 6.8 for CHT load. All conductivities were kept ≤ 5 mS/cm. Capto SP ImpRes ion exchange chromatography resin (Cytiva) was used as the CEX resin, and CHT ceramic hydroxyapatite multimodal chromatography media, Type II (40 μm) (Bio-Rad Laboratories, Inc.) was utilized as the CHT mixed-mode chromatography medium. Conditions used for chromatography are shown in Table 1, and experiments were performed at a flow rate of 150 cm/hr.

Table 1. Experimental details.

Chromatographic Approach	CEX Resin	CHT Mixed-Mode Resin
Resin	Capto SP ImpRes (Cytiva)	CHT, Type II, 40 μm (Bio-Rad)
NaP Gradient	—	10–400 mM sodium phosphate, pH 6.8
NaCl Gradient	50 mM sodium acetate, pH 5.5, 0–400 mM sodium chloride	10 mM sodium phosphate, pH 6.8, 0–400 mM sodium chloride

CEX, cation exchange.

BsAb concentration and purity were determined using size exclusion high performance liquid chromatography (HPLC-SEC). To ensure accurate bsAb monomeric quantification, the authors subtracted the integration of the main peak from the cell culture supernatant from that of the protein A capture chromatography, which effectively eliminated background interference from the calculation of bsAb concentration. The quantities of HMW and LMW components were determined by analyzing the peak areas eluting before and after the main peak, respectively. Host cell protein (HCP) contents were determined with the CHO HCP ELISA Kit, 3rd Generation (Cygnus Technologies). Host cell DNA (HCDNA) contents were determined using a qPCR assay.

Results

Aggregate Removal

In this study, bsAb aggregate removal by CHT Media was compared with a typical 40 μm CEX resin, Capto SP ImpRes (Cytiva). Elution of bsAbs loaded onto CHT Media was carried out through a sodium chloride gradient (mixed-mode elution profile) or a sodium phosphate gradient (similar to CEX mode), whereas elution of the CEX resin used a sodium chloride gradient (Figure 2).

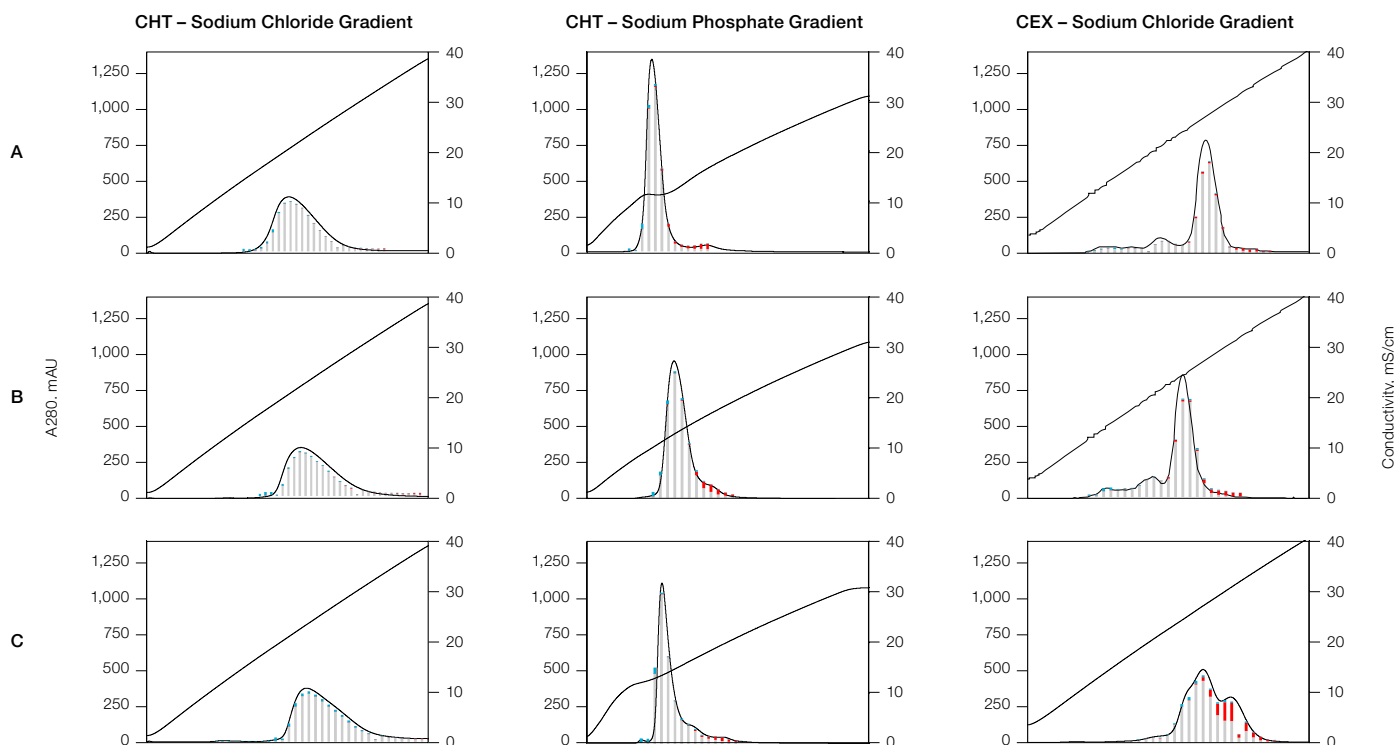


Fig. 2. Elution profiles of molecules A, B, and C eluted from CHT Media using CHT sodium chloride gradient, CHT sodium phosphate gradient, and eluted from Capto SP ImpRes using a CEX sodium chloride gradient. Each plot is an overlay of the UV280 chromatogram (mAU) with each elution fraction's HMW (■), monomer (■), and LMW (■) distribution as a bar graph and conductivity (mS/cm), during the elution phase (40 CV). CHT, calcium hydroxyapatite; CEX, cation exchange; HMW, high molecular weight impurities; LMW, low molecular weight impurities.

The eluate was fractionated (1 CV/fraction) and fractions with a higher purity than the load material were pooled. Yields were calculated based on total pooled material and based on HPLC-SEC analysis of these pools. It was observed that both types of CHT gradients had a better HMW reduction than CEX for all molecules (Table 3). It was also observed that the occurrence of chromatography-induced aggregation was mitigated when CHT Media was used, possibly due to the presence of calcium ions at calcium sites (C-sites) (Figure 3).

Removal of Other Impurities

The study also observed that CHT Media with a sodium chloride gradient achieved the best HCP clearance, whereas LMW and HCDNA removal was comparable to CEX. Increased phosphate concentration was required for large LMW removal in the sodium chloride gradient for CHT (Table 2).

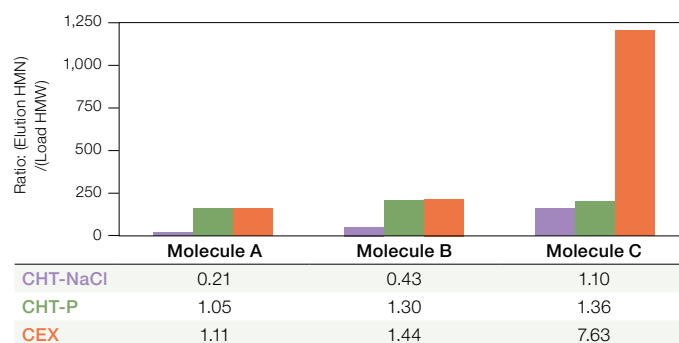


Fig. 3. Observed chromatography-induced aggregation. Chromatography-induced aggregation is the ratio of HMW content in all elution fractions:HMW content in load, based on HPLC-SEC analysis. HMW, high molecular weight impurities; CHT-NaCl, ceramic hydroxyapatite resin with sodium gradient; CHT-P, ceramic hydroxyapatite resin with phosphorous gradient; CEX, cation exchange resin.

Table 2. Yield, Purity, HMW, HMW reduction, LMW, HCP, and HCDNA of post-protein A eluate and pooled fractions obtained from CHT and CEX gradient elution.

	Yield	Purity	HMW	HMW Reduction*	LMW	HCP, ppm	HCDNA, ppm
Post-Protein A Eluate							
Molecule A	N.A.	93.6%	4.7%	N.A.	1.7%	5,839	39
Molecule B	N.A.	91.7%	4.1%	N.A.	4.2%	4,578	17
Molecule C	N.A.	91.5%	2.3%	N.A.	6.3%	1,304	7
CHT Sodium Chloride Gradient**							
Molecule A	76.4%	97.5%	0.5%	90.1%	1.0%	62	0.009
Molecule B	73.9%	96.5%	0.2%	96.3%	2.5%	12	n.d.
Molecule C	67.8%	97.7%	0.3%	85.5%	4.3%	35	0.012
CHT Sodium Phosphate Gradient							
Molecule A	82.2%	96.5%	1.8%	61.5%	1.7%	378	n.d.
Molecule B	80.4%	96.1%	0.9%	78.5%	3.0%	155	n.d.
Molecule C	53.6%	97.7%	0.4%	82.9%	1.9%	70	n.d.
CEX Sodium Chloride Gradient							
Molecule A	80.3%	95.7%	3.2%	31.7%	1.0%	301	0.008
Molecule B	69.4%	94.8%	2.9%	30.5%	2.3%	43	0.006
Molecule C	31.4%	92.9%	2.0%	10.5%	5.1%	243	0.026

HMW, high molecular weight impurities; LMW, low molecular weight impurities; HCP, host cell protein; HCDNA, host cell DNA; CHT, calcium hydroxyapatite; CEX, cation exchange

* HMW reduction = $(\Delta \text{HMW})/(\text{Post-protein A eluate HMW})$

** Molecules A and B were obtained with a modified post-load wash to remove low molecular weight (LMW)

Discussion

CHT Media is a bioceramic, spherical, and macroporous form of hydroxyapatite known for its physical and chemical robustness. Functioning as both the ligand and support matrix, its multimodal resin features metal affinity interactions with C-sites and CEX interactions with phosphate sites (P-sites). These sites allow for unique and precise separation of an extremely broad range of therapeutic modalities. CHT Type II (40 μm) Media was utilized for this work and is exclusively available from Bio-Rad Laboratories (Table 3).

Table 3. CHT Type II Media characteristics.

Functional groups	Ca^{2+} , PO_4^{3-} , OH^-
Available mean particle sizes	$40 \pm 4 \mu\text{m}$ and $80 \pm 8 \mu\text{m}$
Typical linear flow rate range	50–400 cm/hr
Operating pH range	6.5–14
Sanitization	1–2 N NaOH
Autoclavability	121°C, 20 min, in phosphate buffered saline, pH 7
Tap-settled density, g/ml packed bed	0.63 g/ml

Ingavat et al. (2023) have shown that CHT Media is effective in purifying both asymmetric and symmetric bsAbs, which resulted in products with at least 97% purity that consistently achieve high aggregate removal with both sodium chloride and sodium phosphate elution. Notably, chromatography-induced aggregation was mitigated in post-CHT products but occurred in post-CEX products. CHT Media with NaCl elution achieved the best HCP clearance, and LMW and HCDNA impurity removal was comparable to CEX.

References

- Ingavat N et al. (2023). Harnessing ceramic hydroxyapatite as an effective polishing strategy to remove product- and process-related impurities in bispecific antibody purification. *Bioresour Bioprocess* 10, 1.
- FDA (2023). Bispecific Antibodies: An Area of Research and Clinical Applications. Published online: <https://www.fda.gov/drugs/news-events-human-drugs/bispecific-antibodies-area-research-and-clinical-applications>
- Chen S et al. (2021). Current trends and challenges in the downstream purification of bispecific antibodies. *Antib Ther* 4, 73–88.

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SpyLock Technology

SpyLock Technology: A Cutting-Edge Solution for Rapid Bispecific Antibody Screening

Identifying the best antibody pairs for a bispecific antibody (BsAb) requires extensive subcloning, expression, and purification, along with experimental screening. The SpyLock service offers fast bispecific generation and preliminary screening in an alternative format that significantly reduces workload and accelerates candidate selection. SpyLock is based on the SpyTag/SpyCatcher protein ligation system and is an engineered SpyCatcher with functional control of its SpyTag reactivity. The fusion of SpyCatcher and SpyLock technologies allows for the sequential incorporation of two different fragment antigen-binding (Fab) regions containing SpyTags, resulting in bispecific prototypes. Fabs derived from nine anti-programmed cell death ligand 1 (PD-L1) and four anti-programmed cell death protein 1 (PD-1) antibodies were generated and used to construct 36 unique BsAbs with SpyLock technology. A bioluminescent assay displayed distinct efficacies in the checkpoint inhibition of 36 bispecifics. When paired with the Pioneer™ Antibody Discovery Platform, SpyLock technology emerges as an ideal solution for swiftly generating BsAbs tailored for high-throughput screening.

Introduction

BsAbs are a class of biologics gaining significant interest in the field of biotherapeutics. As of December 2024, 12 FDA-approved bispecific drugs are on the market in the U.S. and more than 450 are being investigated in clinical trials (the Antibody Society 2024). Despite some interest in using BsAbs in clinical diagnostics and medical imaging, most bispecifics are used as cancer immunotherapies and, to a lesser extent, are being investigated for autoimmune or other diseases.

Antibody drug development is a process that consists of hit-generation, selection, and characterization during which the number of candidates is narrowed down until one lead candidate remains. For monoclonal antibodies (mAbs), this often involves looking at the properties of hundreds of antibodies. The pairing of two antibody specificities can lead to a significantly higher number of BsAb combinations, requiring the production and screening of hundreds or thousands of candidates. For example, emicizumab, a clinically approved BsAb to treat hemophilia A, was identified after screening and optimizing 40,000 candidates (Kitazawa et al. 2012). Generating a large number of bispecific antibodies requires cloning,

expression, and purification steps, which can be labor-intensive, time-consuming, and expensive. A first screening step in an alternative format to validate general functionality can massively reduce the workload and accelerate BsAb candidate selection. A significantly reduced number of positively tested candidates can then be converted into the final format.

SpyLock Technology: Mechanism and Advantages

SpyTag, a short peptide of 13 amino acids (aa), reacts spontaneously and specifically with a SpyCatcher protein (113 aa) to form a covalent isopeptide bond (Figure 1) (Hatlem et al. 2019). This reaction is high yielding in diverse conditions of pH, temperature, and buffer.

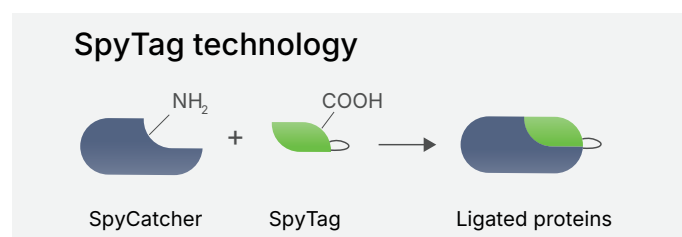


Fig. 1. SpyTag/SpyCatcher reaction.

Bio-Rad Laboratories, Inc. has developed a groundbreaking innovation called SpyLock, a lockable SpyCatcher that can be reversibly inhibited to control ligation with a SpyTag (Hentrich et al. 2024). By treating the SpyLock with a reducing agent it can be precisely unlocked, enabling controlled and efficient coupling with a SpyTag (Figure 2).

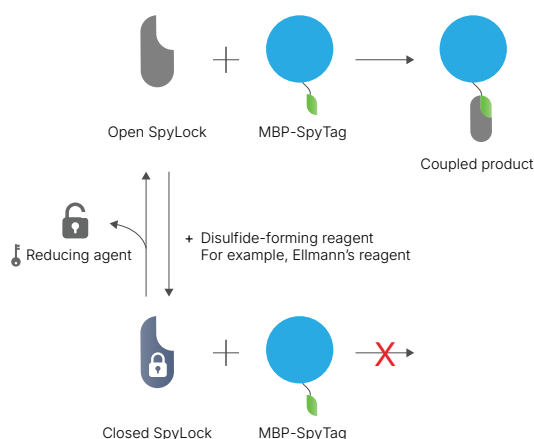


Fig. 2. **SpyLock: A lockable SpyCatcher.** MBP, maltose binding protein.

The simplicity of SpyLock technology is reflected in its sequential antibody coupling reactions for the generation of a bispecific prototype (Figure 3). A SpyCatcher–SpyLock fusion protein, the BiLockCatcher, reacts with the first SpyTagged antibody fragment (Fab specific to target A in Figure 3). After unlocking the SpyLock site using a reducing agent, the second SpyTagged antibody fragment (Fab specific to target B) is added to produce a bispecific antibody. Bio-Rad has developed a protocol that can be performed in 90 min, as well as a high-purity protocol, which usually yields >95% BsAb candidates.

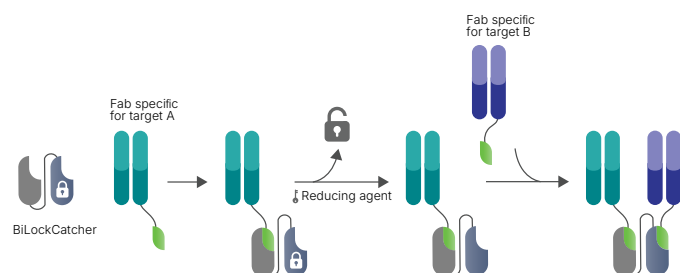


Fig. 3. **SpyLock's sequential reaction for bispecific antibody generation.**

Application: PD-1/PD-L1 Bispecific Screening

Programmed cell death protein 1 (PD-1) and its ligand, PD-L1, are common targets for cancer immunotherapy (Magnez et al. 2017). Bispecific targeting of PD-1 and PD-L1 has shown enhanced antitumor activity in mouse models when compared to combined antibody treatment or individual monotherapies (Kotaniides et al. 2020). We used a PD-1/PD-L1 immune checkpoint inhibitor screening assay based on reporter Jurkat-Lucia TCR-hPD-1 cells and Raji-APC-hPD-L1 antigen-presenting cells (InvivoGen, catalog #rajkt-hpd1). In this assay, a blockade of the PD-1/PD-L1 interaction results in luciferase expression (Figure 4).

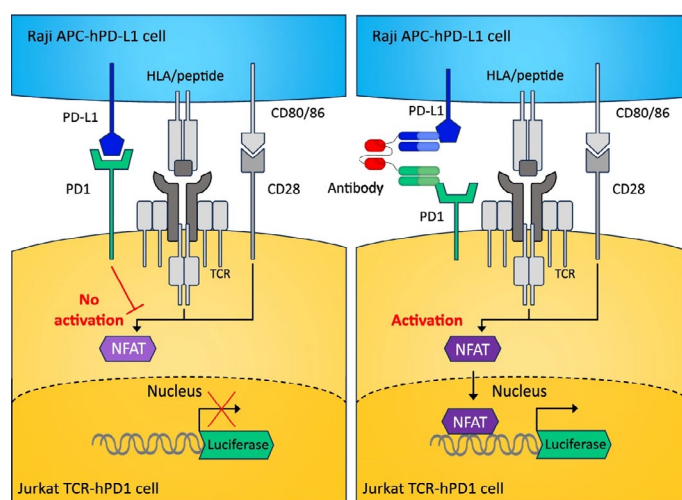


Fig. 4. **PD-1/PD-L1 assay schematic.** APC, antigen-presenting cells; CD, cluster of differentiation; HLA, human leukocyte antigen; hPD, human programmed cell death; NFAT, nuclear factor of activated T cells; TCR, T-cell receptor.

We generated 36 bispecific antibodies from four anti-PD-1 (Fab 1-4) and nine anti-PD-L1 (Fab A-I) antibodies originating from the Pioneer phage display library. All 36 bispecific antibodies exhibited a dose-dependent response, displaying distinct efficacies of the checkpoint inhibition (Figure 5A). The assay performance of one anti-PD-1/Anti-PD-L1 bispecific, comprising Fab 3 and Fab G (3-G), was characterized in greater detail (Figure 5B). The bispecific anti-PD-1/PD-L1 (3-G) was markedly more potent in the assay compared to the monospecific parental Fabs (Fab G and Fab 3), their equimolar mixture (Fab G + Fab 3), and a SpyLock control (aGFP-aGFP).

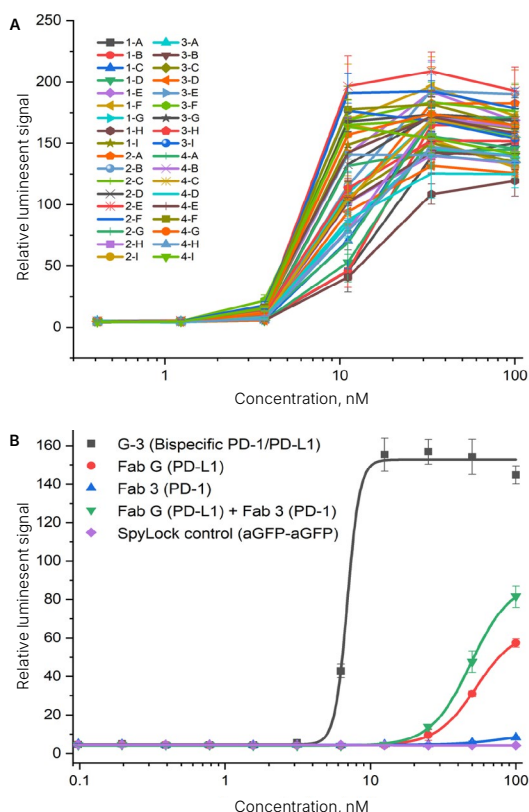


Fig. 5. Bioluminescent PD-1/PD-L1 bispecific antibody blockade assay. **A**, screening of 36 bispecific PD-1/PD-L1 antibodies, 1–4 are PD-1 Fabs, A–I are PD-L1 Fabs. **B**, a detailed test of one bispecific PD-1/PD-L1 in comparison to the monospecific antibodies alone or a mixture of the two. PD, programmed death; aGFP, anti-green fluorescent protein.

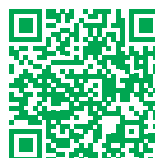
Conclusion and Future Directions

SpyLock technology seamlessly integrates into the Pioneer Antibody Discovery Platform, offering a powerful solution for high-throughput bispecific antibody generation. The BiLockCatcher, a SpyCatcher-SpyLock dimer, enables controlled and efficient bispecific assembly. This unique mechanism eliminates the need for laborious expression and purification of massive antibody combinations, relying instead on a single SpyTag-associated antibody fragment, significantly simplifying the workflow.

Through the PD-1/PD-L1 case study, SpyLock technology showcases its effectiveness in accelerating bispecific antibody generation, making it an ideal solution for high-throughput prototyping and screening of BsAb candidates.

References

- The Antibody Society (2024). Antibody Therapeutics Product. www.antibodysociety.org/antibody-therapeutics-product-data, accessed March 20, 2025.
- Hatlem D et al. (2019). Catching a SPY: Using the SpyCatcher-SpyTag and related systems for labeling and localizing bacterial proteins. *Int J Mol Sci* 20, 2129.
- Hentrich C et al. (2024). Engineered reversible inhibition of SpyCatcher reactivity enables rapid generation of bispecific antibodies. *Nat Commun* 15, 5939.
- Magnez R et al. (2017). PD-1/PD-L1 binding studies using microscale thermophoresis. *Sci Rep* 7, 17623.
- Kitazawa T et al. (2012). A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model. *Nat Med* 18, 1570–1574.
- Kotanides H et al. (2020). Bispecific Targeting of PD-1 and PD-L1 enhances T-cell activation and antitumor immunity. *Cancer Immunol Res* 8, 1300–1310.



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