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Facilitating high throughput bispecific antibody production and potential applications within biopharmaceutical discovery workflows

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ABSTRACT

A major driver for the recent investment surge in bispecific antibody (bsAb) platforms and products is the multitude of distinct mechanisms of action that bsAbs offer compared to a combination of two monoclonal antibodies. Four bsAb products were granted first regulatory approvals in the US or EU during 2023 and the biopharmaceutical industry pipeline is brimming with bsAb candidates across a broad range of therapeutic applications. In previously reported bsAb discovery campaigns, following a hypothesis-based choice of two specific target proteins, selections and screening activities have often been performed in mono-specific formats. The conversion to bispecific modalities has usually been positioned toward the end of the discovery process and has involved small numbers of lead molecules, largely due to challenges in expressing, purifying, and analyzing large numbers of bsAbs. In this review, we discuss emerging strategies to facilitate the production of expanded bsAb panels, focusing particularly upon combinatorial methods to generate bsAb matrices. Such technologies will enable screening in bispecific formats at earlier stages of discovery campaigns, not only widening the accessible protein space to maximize chances of success, but also advancing empirical bi-target validation activities to assess initial target selection hypotheses.

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Introduction

Bispecific antibodies (bsAbs) are molecules containing antibody-derived fragments able to bind two different antigen epitopes with high specificity. A myriad of bsAb formats have been reported, comprising different types and numbers of antibody-derived fragment, ranging from IgG-like molecules to linear strings of single domain (e.g., VHH) antibodies.^{1–3} Dual engagement (either simultaneous or sequential) of two antigen targets facilitates novel modes of action for ‘obligate’ bsAbs that are not possible using mono-specific antibodies, even in combination. For example, bsAbs may recruit T cells to diseased cells to initiate cell killing, bring two cell surface receptors into close proximity to regulate cell signaling or enable more specific targeting and depletion of a cell population uniquely expressing two antigens (see refs. 1–3 for detailed overviews of bsAb modes of action). BsAbs that do not offer a functional advantage over a matched mAb combination may still potentially offer practical advantages, for instance when mAb co-formulation is problematic or due to the reduced cost and complexity of developing manufacturing processes or clinical trial design for one versus two biologics.^{2,4–6} However, the expanded functionality offered relative to monospecific mAb therapeutics has been the major driver for bsAb development and the majority of marketed or late-stage (Phase 3 or pivotal Phase 2) bsAbs are obligate bsAbs.^{7,8}

The high-throughput (HTP) production of monoclonal antibodies is a vital component of an efficient mAb discovery process, as it enables large numbers (100–1000 molecules) of selection outputs to be generated and screened.⁹ This offers the potential for greater panel diversity, increasing the chance of discovering a mAb with desirable antigen binding, biological function and molecular properties.^{9–11} For a bsAb, especially an obligate bsAb, the desired molecule specifications are more complex than for a typical mAb; for example relative binding valencies and affinities to the two targets or the molecular geometries might also need to be explored to achieve the desired biological activity.^{12–18} Therefore, screening in bsAb format early in the discovery process is potentially highly advantageous, but requires a HTP method for bsAb production and also the development of HTP screening assays. Without these HTP capabilities, much smaller numbers of parental mAbs can be explored in bispecific format, greatly reducing the initial diversity of molecules tested (Figure 1, Option 1.1). Maximizing diversity is particularly important when bsAbs are assembled from parental molecules derived from new selections campaigns, rather than from a small number of clinically validated binding modules (e.g., anti-CD3 modules are often pre-defined on platforms generating T-cell engaging bsAbs.^{15,19–21} Without HTP capabilities, multiple rounds of engineering and screening are also likely to be required subsequently to optimize bsAb properties such as

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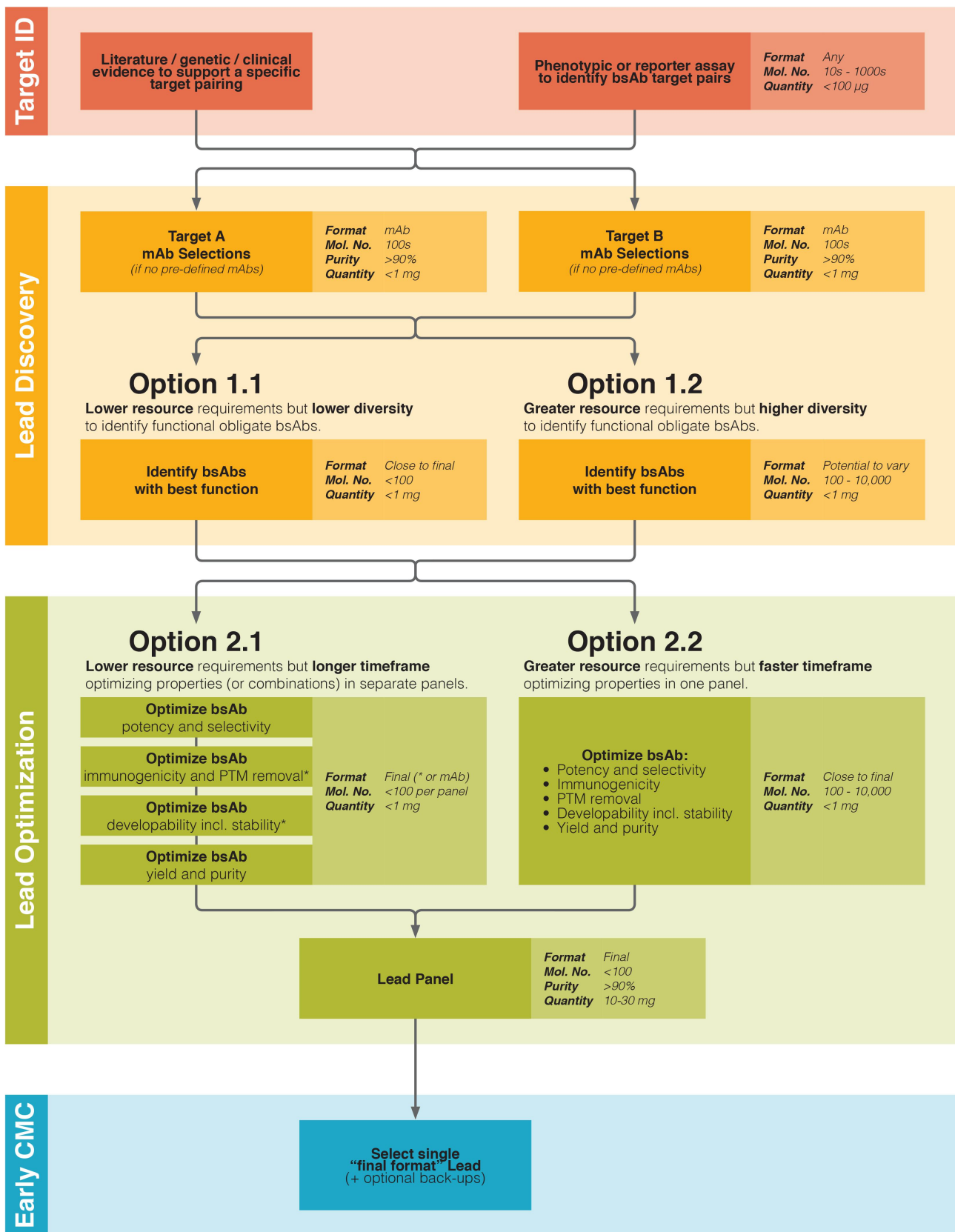


Figure 1. Schematic outlining the bsAb discovery process and highlighting applicable stages for HTP bsAb production and screening. The main bispecific antibody discovery process phases are here defined as: 1. 'Target ID', identify a target pairing hypothesized and/or demonstrated to enable the desired mode of action; 2. 'Lead Discovery', screen and select i. mAbs against these two targets and ii. Screen and select initial Hit bsAbs exhibiting the desired mode of action; 3. 'Lead Optimization', engineering and more in-depth screening of initial Hit bsAbs aiming to improve functional and biophysical properties; 4. 'early Chemistry, Manufacturing and Controls' (early CMC), includes cell line development for large scale bsAb manufacture, formulation optimization for the final bsAb drug and more detailed assessment of bsAb product quality. Estimated format type, maximum molecule numbers, minimum purity and material quantity requirements listed in the lighter shaded box, while the primary screening purpose is stated in the darker shaded box for each panel. Unless stated otherwise, sample purity requirements are dependent upon the sensitivity of the intended screening assay to bsAb related impurities and the control samples available. At the Target Identification stage, unbiased screening of very large bsAb

potency, selectivity, developability and immunogenicity (Figure 1, Option 2.1),²² extending overall project timelines. Efficient bsAb production is often limited by lower expression titer and more heterogeneous purity profiles relative to mAbs.^{1,3,23} Numerous technologies have been developed over the past decade to address these general challenges, including improvements in gene integration into host cell lines, cell line culture systems and new protein engineering solutions to drive correct chain pairing.^{1,24–28} An added challenge to developing a HTP bsAb production process is that acceptable sample yields and purities must routinely be obtained across all molecules in a panel, as bespoke extra steps to triage sample subsets are not feasible when handling large panels. As for HTP mAb production, to be resource and cost effective, an ideal HTP bsAb production process also involves minimal experimental steps and minimizes consumable requirements. In this review, we consider a range of established and potential future strategies to enable large bsAb panel production and discuss the applicability of each strategy for screening at different stages of a bsAb discovery pipeline.

Solutions to enable bispecific antibody panel production

A range of considerations are crucial in assessing the suitability of a bsAb panel production method, including protein yields, sample purity, speed, cost of consumables, compatibility with automation processes, and alignment with the intended downstream screening assays. Furthermore, the relative importance of these factors will vary depending upon the discovery process stage at which this method will be used (Figure 1). For example, early-stage biophysical screening assays to identify target binding responses across very large panels require relatively small sample quantities, while moderate levels of impurities can likely be tolerated (Figure 1, Target Identification and Lead Discovery). At this stage, transient expression in multi-well plates combined with a single protein capture/purification step may meet sample requirements, as well as being a process that with automation is scalable to very large panels (100–1000 molecules).²⁹ In contrast, later-stage developability assessments typically require highly concentrated and pure samples, but the screen size will likely be far smaller (Figure 1, Lead Panel).⁹ Here, lower throughput expression and purification methods likely need to be integrated into the process.

The bsAb format used by a given production method is also a key consideration (Figure 2), especially for obligate bsAbs where geometric requirements may be imposed by the intended mode of action. For instance, biparatopic antibodies designed to engage two epitopes on a single antigen molecule, which can increase binding avidity relative to mAbs unable to bridge across antigen molecules and binding monovalently, may favor specific molecular architectures, as demonstrated during the discovery of bsAbs targeting HIV and SARS-CoV-2 spike proteins.^{12,13,30,31} Meanwhile for T-cell engaging bsAbs, it has been demonstrated that valency of tumor-associated antigen (TAA) target binding, the relative position of the TAA and CD3 binding domains and the architecture of the bsAb binding arms are important design factors to explore to achieve potent target cell killing, while minimizing nonspecific T-cell activation.^{15–18} In such cases, it may prove essential to screen different formats initially to achieve the desired mode of action, favoring bsAb panel production methods incorporating format variety (Figure 1, Option 1.2). However, if the required ‘final format’ is already established prior to starting a bsAb discovery campaign, conducting all bsAb screening processes in this format is preferable so that early data is more predictive of the developability, manufacturability and *in vivo* activity of the molecules. If HTP production methods for this ‘final format’ are not available, a compromise approach is to produce bsAbs in an ‘intermediate format’ more amenable to HTP production for early screening activities (Figure 1, Target Identification and Lead Discovery) and then re-format later into the ‘final format’ once only smaller panels are required (Figure 1, Lead Panel).

IgG-like bispecific antibodies are the most commonly used ‘final format’, with 11 such bsAbs approved in the US or Europe (as of January 2024).^{8,41} In resembling the overall architecture and domain composition of a natural IgG antibody (Figure 2a, IgG-like), a bsAb retains key biological properties and functionalities. Firstly, the Fc domain confers a long serum half-life, reducing required dosing quantities and frequencies for many therapeutic applications.^{2,3,42} Secondly, high homology with a natural molecule minimizes the potential immunogenicity of the bsAb, as the probability of introducing novel T-cell epitopes is decreased.^{2,43,44} Thirdly, while immune cell re-directing bsAbs typically contain Fc domains engineered to ablate cytotoxic Fc-mediated effector functions (e.g., ADCC), a functional Fc may alternatively be integral to the intended bsAb mode of action and can be tuned using

panels in simplified *in vitro* functional assays provides expanded opportunity to discover novel bsAb target pairings, which is especially powerful for obligate bsAb modes of action. For a non-obligate bsAb campaign, screening in bsAb format at Lead Discovery stage is typically not required and smaller panels are required at Lead Optimization stage as properties such as bsAb potency and selectivity correlate well with those of the parental mAbs. For obligate bsAbs, the capability to produce and screen large panels incorporating more variables at Lead Discovery stage (Option 1.2) not only increases the overall chance of successfully identifying functional hits, but also reduces the number of parameters requiring screening during Lead Optimization, potentially allowing progression directly to a Lead Panel. When larger amounts of optimization work is necessary, the capacity to screen large panels in bsAb format (Option 2.2) provides the opportunity to shorten overall timelines by exploring many interdependent factors in one panel, rather than through multiple sequential and/or parallel optimization steps (Option 2.1). At Lead Panel stage, more in depth molecule profiling is typically performed,⁹ requiring greater amounts of ‘final format’ bsAb at higher purity, necessitating a bsAb production process incorporating increased protein expression scale and more thorough sample purification and quality control. The throughput of this process (e.g., 10 vs 100 bsAb panels) impacts how regularly projects can be accelerated directly to Lead Panel, without prior Option 2.1 or Option 2.2 screening stages. For bsAb modalities using well established bsAb targets (e.g., CD3 for T-cell engaging bsAbs^{15,19,20,21}) potential bsAb test panel sizes will be smaller during Target ID and the availability of clinically validated mAb(s) against these targets with demonstrated potency and immunogenicity may preclude a novel mAb selections campaign during the Lead Discovery phase. Limiting the number of variable domains options on one bsAb binding module will also reduce panel sizes later on, as further optimization is then only required on the second binding module. Four panels from top to bottom highlight the main phases of a bispecific antibody discovery process. Boxes representing individual bispecific antibody screening stages are overlaid and linked into a process workflow with connecting arrows.

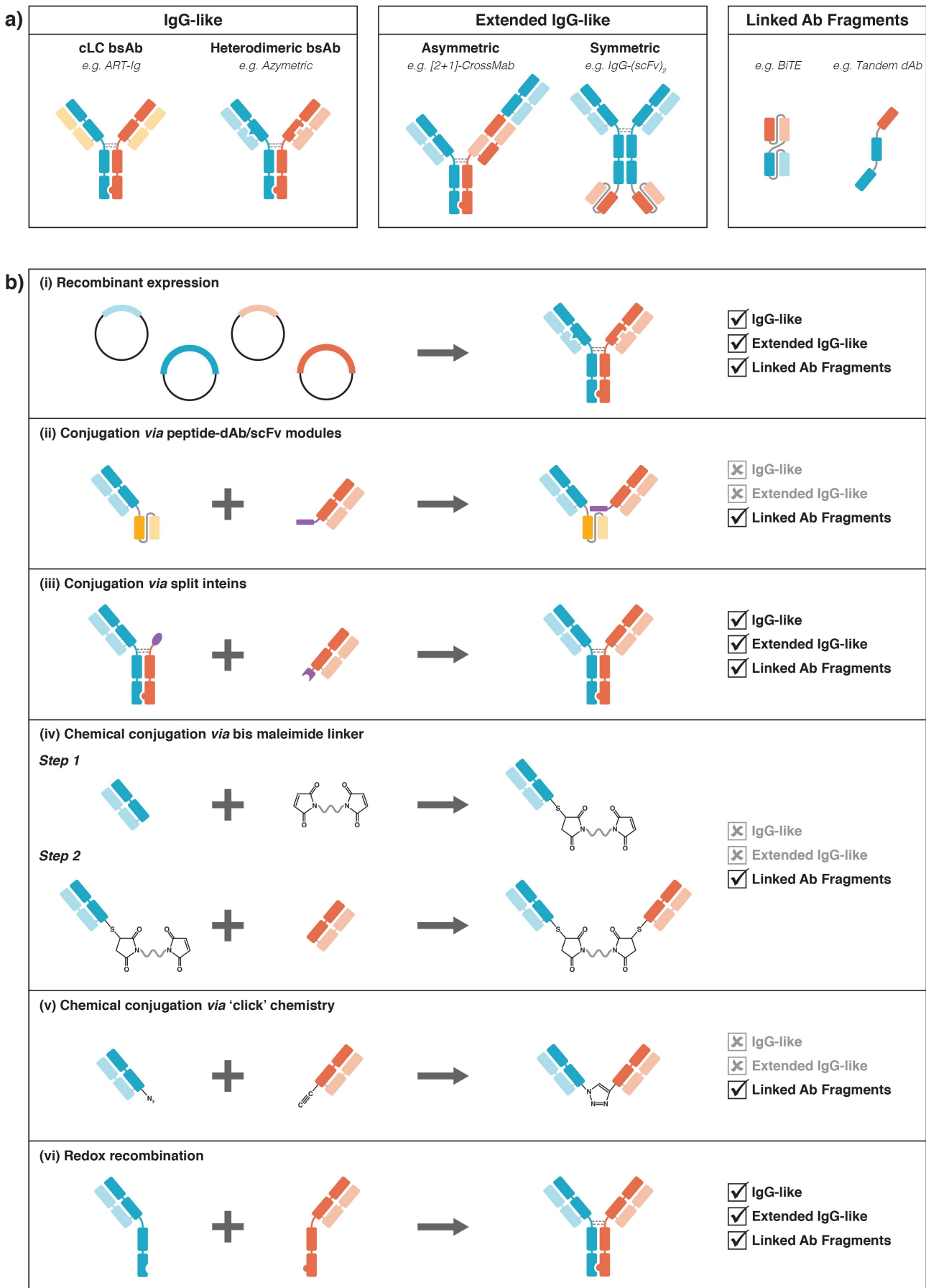


Figure 2. Overview of the major bsAb format classes and exemplar methods amenable to their HTP production. (a) Representative IgG-like (ART-Ig²² and Azymetric³²), extended IgG-like ([2+1]-CrossMab¹⁹ and IgG-(scFv)₂^{2,33}) and linked antibody fragment formats (BiTE³⁴ and tandem dAbs³⁵) depicted. Dashed grey lines represent disulfide bonds; full grey lines, peptide linkers; blue and red rectangular modules, immunoglobulin domains within protein chains binding

engineering solutions originally developed for monospecific mAbs.^{45,46} The developability profiles of IgG-like bsAbs are also typically more like standard mAbs than bsAbs composed of smaller antibody fragments in terms of both physical stability (level of aggregation and fragmentation propensity) and close compatibility with well-established mAb purification processes.^{1–3,47}

A fully end-to-end automated HTP bsAb production workflow (with >1000 bsAb throughput) requires process steps for expression vector generation, protein expression, protein purification, and biophysical validation that are compatible with automation processes. For instance, column-based purification steps requiring either buffer gradients or size-based separation are challenging to automate with this throughput. Meanwhile, automation of HTP affinity-based bsAb purification processes has been reported using commercially available Phynexus Phytip® columns or magnetic purification resin reagents alongside Hamilton Microlab® STAR or Tecan Freedom Evo® liquid-handlers.^{29,37,48} The integration of individual process steps into systems with increased functionality, such as sample concentration measurement and normalization, additionally requires plate-handling robotics plus software to link and control all hardware components within the system. Furtmann et al. describe the development of dedicated *E. coli*, DNA handling and Protein Science Stations within their end-to-end bsAb production workflow, which each use a PlateButler® PreciseFlex robotic arm for plate handling directed by PlateButler® software.²⁹ Furthermore, an integrated dataflow linking the software controlling each robotic station to a central database GDB® using custom Python®-based scripts is a crucial component of their overall process to ensure reliable tracking of samples throughout and robust data capture and storage.²⁹ Specific process challenges potentially preventing integration into fully end-to-end HTP bsAb workflows will be highlighted throughout this review.

Five different solutions enabling HTP bsAb production at varying stages of the discovery process are discussed below.

Bispecific formats containing HC-HC and HC-LC pairing technologies

The first solution to enable HTP IgG-like bsAb production considered here is the introduction of technologies to drive correct chain pairing when samples are generated in standard recombinant eukaryotic expression systems (see below for an alternative solution involving the redox recombination of half antibodies). An IgG-like bsAb comprises two heavy chains (HCs) and two light chains (LCs) and assembly into the correct molecule is dependent upon the specificity of interdomain interactions (Figure 2b (i)). Co-expression of four IgG chains without molecular engineering to drive correct chain pairing

would result in a heterogeneous mixture of species, from which the desired bsAb (on average only 12.5% of the sample assuming equal chain expression levels) is challenging to extract.⁵ The earliest engineering solutions focused on modification of the CH3-CH3' interface to promote heterodimeric versus homodimeric HC-HC interactions, such as the knob-in-hole (KiH) mutation set which introduces new steric interactions and remains commonly employed across several established bsAb platforms.^{1,3,24,25,49,50} Alternative solutions were subsequently developed aiming to further improve bsAb purity profiles and biophysical characteristics by introducing electrostatic charge pairs,^{51–53} promoting β -strand exchange⁵⁴ or optimizing multiple bonding parameters via computational and structural guided design.^{1,32,55–57}

IgG-like bispecific antibodies containing a common LC (cLC) can be assembled using these HC-HC pairing technologies and have a relatively simple purity profile. A range of purification strategies have been developed to remove remaining homodimer and/or half-antibody impurities following initial protein A affinity capture.⁵⁸ For example, by introducing mutations to ablate protein A binding into one HC, homodimer species containing either no or two HC protein A binding sites can be separated from the correct bsAb, containing one site, by an extra protein A affinity chromatography step across a pH gradient.⁵⁹ Alternatively, by engineering the two HCs to ensure an isoelectric point (pI) difference between the homodimer and half-antibody species relative to the correct bsAb, these impurities can be removed by an additional ion exchange chromatography (IEX) step following initial protein A affinity capture.^{22,53,60,61} However, these additional purification steps often involve using chromatography columns and are less amenable to HTP production than plate or magnetic bead based affinity matrices, with reported automation solutions comprising preparative scale chromatography typically capable of processing less than 24 samples per batch.^{62,63} Multi-step purification approaches are therefore only practical to use for bsAb production when sample numbers are small (<100) and once higher purity (>90%) samples are necessary, for example at lead panel stage, when more complete binding, functional and biophysical characterization work packages are standardly performed (Figure 1, Lead Panel).⁹

There is precedent for the production of large cLC bsAb panels for early-stage screening, when high sample purities and the associated needed for multi-step purifications are not required. 545 HER2-HER3 bsAbs (derived from 22 anti-HER2 and 32 anti-HER3 parental mAbs) were produced via transient HEK cell transfection and a single step protein-A purification, enabling phenotypic screening to identify molecules blocking ligand-driven signaling of the HER2/HER3 heterodimer.⁶⁴ Only five bsAbs demonstrated increased potency relative to

target 1 or target 2 respectively; yellow rectangular modules, immunoglobulin domains within a common light chain; circular module connectors, a mutation set to drive correct HC-HC pairing; triangular and square module connectors, a mutation set to drive correct HC-LC pairing. (b) Six exemplar methods amenable to HTP bsAb production: (i) Recombinant Expression, (ii) Conjugation via peptide-dAb/scFv modules³⁶ (iii) Conjugation via split inteins,³⁷ (iv) Chemical conjugation via bis maleimide linker,³⁸ (v) Chemical conjugation via 'click' chemistry³⁹ and (vi) Redox recombination.⁴⁰ The bsAb format classes that each method can generate are marked in tickboxes. Additional features specifically depicted in (b): (i) black circles, DNA plasmids; colored plasmid overlays, DNA sequences encoding individual bsAb chains (ii) purple module, connector peptide; yellow rectangular modules, scFv with specific, high affinity to connector peptide (note this differs to key in (a)) (iii) oval purple module; N-terminal intein fragment; concave purple fragment; C-term intein fragment (iv) solid grey line; chemical or peptide linker. Top panel contains cartoons of six representative bispecific antibody formats. Bottom panel contains schematics for six different bispecific antibody high throughput production methods.

a combination of trastuzumab and pertuzumab (benchmark anti-HER2 mAbs) in an MCF-7 cell proliferation assay, demonstrating the value of using both a large initial test panel to increase molecular diversity and an unbiased screening approach to maximize the chance of successfully identifying functional hits.

The major limitation to the use of cLC bsAb formats, however, is that at least one of the parental antibodies typically needs to be sourced via either *in vitro* display technology or transgenic animals with limited LC diversity. While a range of platforms have been established to accelerate cLC mAb discovery,^{49,60,65–68} introducing a HC-LC pairing technology into an IgG-like bsAb potentially allows the use of any two parental antibodies, such as existing therapeutics or mAbs derived from human vaccinees. A wide variety of HC-LC pairing technologies have been developed, including Roche's CrossMab format, in which the CH1 and CL domains are switched onto the LC and HC respectively on one bsAb arm^{19,24}; WuXi's WuxiBody format, in which CH1-CL domains are replaced altogether on one arm with the C α -C β constant domains from a T cell receptor^{50,69}; and AstraZeneca's Duetmab format, which moves the HC-LC disulfide bond position on one bsAb arm.⁷⁰ Additional HC-LC pairing technologies rely on modification of the CH1-CL interface, introducing distinct, opposite polarity mutation sets to the CH1-CL domains of each bsAb arm.^{71–74} Though offering maximum flexibility regarding possible parental mAbs, the purity profile of bsAbs containing both HC-HC and HC-LC pairing technologies is more complex than cLC bsAbs and efficiency of correct HC-LC pairing is crucial to minimize impurities containing mis-paired LCs, which are especially challenging to remove from the correct bsAb via standardized platform approaches (see "Solutions to enable biophysical validation of large bispecific antibody panels" section below). Therefore, when high purity (>90%) levels are required across a panel, the production of non-cLC IgG-like bsAbs via the co-expression of four chains is again typically limited to small number of molecules (<100), to allow bespoke purification steps (Figure 1, Lead Panel).

Developing screening assays with a higher tolerance to specific bsAb product impurities and including additional controls can facilitate early-stage screening of large, partially purified IgG-like bsAb panels for initial hit identification purposes (Figure 1 – Target Identification and Lead Discovery). This is simpler when engagement of both targets is required for measurable activity, in which case any sample impurities are unlikely to give false positive hits, but will lower the effective correct bsAb concentration in the sample. The first discovery stage for emicizumab, a bsAb mimicking Factor VIII via crosslinking factor IX and factor X, approved by the Food and Drug Administration (FDA) in 2017 to treat patients with hemophilia A, took this approach.²² Approximately 200 × 200 anti-Factor IX and anti-Factor X parental mAbs were combined to generate around 40,000 bsAbs using an IgG-like format containing only HC-HC chain pairing technology (theoretical purity approx. 20% correct bsAb). 94 bsAb positive hits were successfully identified from screening this very large, but relatively crude purity, panel in a HTP enzymatic assay and progressed for further engineering.²² The production of such

large bsAb numbers would have been unfeasible in terms of both resource and time costs if additional sample purification was required.

Technologies to drive correct chain pairing are also required to produce asymmetric extended IgG formats (Figure 2a, asymmetric extended IgG-like).^{19,69} While deviation from a standard IgG architecture carries potential risks of reduced expression levels, reduced molecular stability and reduced *in vivo* half-life (discussed in "Bispecific formats with only 1–2 different chains" section below), the inclusion of additional antigen binding domains can enable a wider range of bsAb modes of action. For example, Columvi®, approved by the FDA in June 2023 for the treatment of diffuse large B-cell lymphoma, is a Fab-IgG CD20-CD3 bsAb, with an additional N-terminal Fab domain appended to allow avid, bivalent binding to the TAA CD20.^{19,75} In summary, while chain pairing technologies enable the production of 'final format' IgG-like and extended IgG-like bsAb panels, sample throughput is limited by variable purity levels across a panel, often necessitating multiple purification steps. Therefore, co-expression of 3–4 chains containing pairing technologies in a recombinant eukaryotic expression system is a process more commonly used to generate bsAbs during late Lead Optimization stages of a biopharmaceutical discovery workflow when lower 'final format' molecule numbers (<100), at a higher purity level are required (Figure 1).

Bispecific formats with only 1–2 different chains

Reducing the number of different chains required to produce a bsAb is a second potential solution and is the simplest method to reduce the potential heterogeneity of the protein sample post-expression and therefore simplify the required downstream purification processes. A range of single polypeptide bispecific formats are very well established, including tandem-domain antibodies, tandem single chain variable fragments and single chain Diabodies (Figure 2a, linked Ab fragments).^{1,34,35,76–78} Further, linking these simple units to antibody CH2-CH3 domains facilitates homodimerization via Fc domain formation, yielding formats with increased valency while still requiring only a single DNA construct.^{1–3,79} Relatively simple formats such as tandem-domain antibodies also offer the advantage that soluble production in *E. coli* expression systems is often possible, greatly increasing the speed and reducing the cost of protein expression.⁸⁰ However, despite the convenience offered by bacterial expression, with increasing format complexity and the introduction of domains containing post-translational modifications (PTMs), mammalian expression systems typically become the most viable route for protein production.

BsAb formats encoded by a single HC and a single LC DNA construct offer molecular architectures more closely related to a standard IgG antibody, with additional antigen binding modules appended to either the Fab or Fc region (Figure 2a, symmetric extended IgG-like). Examples include tetravalent IgG-(scFv)₂ or IgG-(dAb)₂ formats.^{3,33,81,82} The mAb² format developed by F-star Therapeutics offers an alternative solution, whereby the Fab distal loops of the Fc CH3 domains are

engineered to provide the binding site for the second antigen.⁸³ The recently reported 2×VH format also maintains an extended IgG-like format, replacing the variable domain on each light chain with a variable heavy domain with a second binding specificity.⁸⁴ These formats all circumvent problems of incorrect chain pairing and are very compatible with existing, well-established expression and purification platforms for IgG monoclonal antibodies. In addition, bi-valent (or multi-valent) interactions with each target antigen may be favorable for specific mechanisms of action such as clustering of either cell surface receptors or soluble proteins.^{1–3} For example, a biparatopic antibody targeting the HER2 receptor with a format capable of bivalent binding to each epitope demonstrated enhanced receptor clustering, internalization and lysosomal degradation relative to a combination of the two parental mAbs.⁸⁵

Deviations from the standard IgG format can present both greater immunogenicity risks and additional molecular developability challenges though, requiring additional work packages at the later stages of the discovery and development process.^{42–44} For instance, flexible interdomain peptide linkers may be susceptible to cleavage and the addition of extra domains may offer alternative pathways to protein aggregation.⁴² Mitigation of these difficulties has been achieved and bispecific antibodies with appended domains have progressed to clinical studies.^{86–88} If a final format containing appended domains is intended, screening in format early in the discovery process is beneficial not only to flag developability challenges, but also to enable the selection of antibody domains or fragments most compatible with the ‘final format’ (Figure 1, Lead Discovery and Lead Optimization). For example, a series of anti-NGF domain antibodies (dAbs) displayed ~1000-fold greater NGF inhibition in mAb-dAb format compared to as individual dAbs, and may have been prematurely excluded from the discovery process if dAb naïve selection outputs had not been screened in format.⁸¹

For the bispecific formats described in this section, relatively simple sample mixtures are typically obtained directly following protein expression, enabling simplified downstream purification steps more compatible with process automation. Therefore, adopting these formats to facilitate material generation for intermediate-sized panels (100s of molecules), represents an attractive option, especially if the chosen format shares a similar architecture to the intended bispecific antibody ‘final format’ (Figure 1, Options 1.1, 1.2 and 2.2). However, to generate very large panels of bispecific antibodies (1000s–10000s of molecules) such as those that would be required to interrogate combinations from two *de novo* mAb selection campaigns, 1–2 unique expression vectors would need to be generated, and subsequently protein expression and purification performed, for every molecule in the screen. Recently, an automated end-to-end platform bsAb production process was reported based on the symmetric cross-over dual variable domain-Ig (CODV-Ig) format, which delivers exactly this expanded capability.²⁹ This platform was used to generate a panel of more than 25,000 bsAbs and mAb controls to enable the successful identification of variable domains, variable domain positions and interdomain linker lengths to improve

both potency and production titers.²⁹ The resource requirements to produce such large panels can potentially be streamlined, however, using the combinatorial approaches reviewed below.

Recombinant DNA design to enable linking of antibody fragments

The production of antibody fragments containing linkage units or handles and the subsequent assembly into bsAb molecules is a much more resource-efficient route to HTP bsAb production than expressing each bsAb individually. Via this combinatorial approach, very large bsAb panels can potentially be generated from a relatively small number of starting protein modules, greatly reducing the numbers of DNA plasmids, protein expressions and protein purifications required. Ideally the linkage reaction will be very efficient, both to reduce the starting protein module quantities required and so that an extra purification step is not required to remove unreacted modules from the final bsAbs. Also, the linkage formed will preferably be either a permanent covalent bond or very high affinity interaction (binding affinity sub picomolar), to maximize the stability of the bsAbs across a range of screening assay conditions. Here, methods developed using linkages fused to protein modules via recombinant DNA design (the third potential solution) will be considered, while methods using chemical conjugation (fourth solution) and those using native antibody features (fifth solution) are discussed in the following two sections.

As the primary purpose of combinatorial or ‘Plug and Play’ bsAb production methods is typically to generate very large panels for early stage screening (Figure 1, Target Identification and Lead Discovery), the presence of non-natural linkage groups or linkers and the use of antibody fragments can be tolerated, as the molecules will be converted to a final bsAb format with better developability profile at a later screening stage. Smaller antigen-binding antibody fragments, such as Fabs, scFv or dAbs can be reliably produced in standard expression systems and isolated with single step purification processes and so are convenient starting modules.^{36,89} An early ‘Dock and Lock’ bsAb production strategy relied on covalent disulfide bond formation to lock in binding between two fusion peptides derived from cAMP-dependent protein kinase and an A-kinase anchor protein.^{89,90} The Fab-K_D-Fab screening format developed by Bhatta et al. contains a much simpler linkage between a peptide from the yeast transcription factor GCN4 and an anti-GCN4 scFv and has proven very amenable to HTP bsAb production (Figure 2b (ii)).³⁶ Fab moieties recombinantly engineered with a decahistidine tag and either the GCN4 peptide or the anti-GCN4 scFv can be transiently expressed in mammalian cells and purified via nickel-affinity capture. Purified Fab pairs are then mixed at a 1:1 ratio to generate large bsAb matrices in a single step without the need for additional purification.³⁶ Three Fab-K_D-Fab format panels containing > 1000 molecules were screened in primary human cell phenotypic assays to successfully identify new obligate bispecific target pairings that: 1) inhibit B cell receptor activation, 2) inhibit extracellular matrix accumulation, and 3) modulate T cell subset function.³⁶ This empirical approach to

bispecific target pairing discovery, coupled with the development of HTP, disease-relevant phenotypic screening assays, offers greater opportunity to identify novel bsAb therapeutic opportunities (Figure 1, Target Identification).

Several protein bioconjugation technologies that employ covalent peptide or isopeptide linkages between tags and/or protein fragments have been applied to bispecific protein production, including SpyTag/SpyCatcher-related systems, split-inteins or SortaseA-mediated coupling (reviewed in greater detail).^{91,37,92–94} For example, the conjugation of single Fab-Fc antibody fragments bearing a hexahistidine-tagged split intein (C-term portion) in place of the second Fab arm, with Fab fragments fused to a hexahistidine-tagged split intein (N-term portion), is the basis of a process developed using post-translational trans-splicing (Figure 2b (iii)).³⁷ Upon mixing of Fab-Fc and Fab fragments under mild reducing conditions, the two split intein portions recombine and a bsAb product is released. The recombined intein molecule side product, together with non-reacted Fab-Fc or Fab fragments can be captured on a nickel-affinity reagent, and the remaining bsAb product oxidized to re-form interchain disulfide bonds. This bsAb generation process has been successfully miniaturized in both 96- and 384-well plate formats and an automated process line established, demonstrating the feasibility of this approach to produce very large (>1000 bsAb) panels for early stage screening.³⁷ The authors of this study highlight that varied bsAb formats could potentially be produced via this method, by either varying the input parental antibody fragments or using orthogonal-acting intein variants to assemble three fragments. The production of multi-format screening panels offers added value to obligate bsAb discovery, where the bsAb architecture may require optimization (Figure 1 – Target Identification and Lead Discovery).

Chemical conjugation of antibody fragments

The first reported bsAb was produced via a chemical approach, involving the reduction of two separate (Fab)₂ samples to break the interchain disulfide bonds, prior to sample mixing and re-oxidation.⁹⁵ Whilst bispecific product was successfully obtained, the sample purity was restricted by inefficient (Fab)₂ dissociation into individual chains and by uncontrolled chain association after chain mixing and re-oxidation.⁹⁵ Investment in the antibody-drug conjugate (ADC) field over the past decade has accelerated the development of chemical conjugation strategies, including those to specifically target single nucleophilic side chain residues. These advances have triggered research re-visiting the use of chemical conjugation to produce bsAbs.^{96,97} Linking antibody fragments via chemical conjugation provides a fourth potential solution to HTP bsAb production, and the feasibility of using current conjugation chemistries is considered in this section.

To provide an efficient route for HTP bispecific antibody production, a chemical conjugation strategy should fulfil the following five requirements: 1) high final bsAb yield; 2) high final bsAb purity; 3) stable final bsAb product; 4) no toxic reagents required; and 5) expedient synthesis. The heterogeneous product mixture obtained in the seminal Nisonoff and River report⁹⁵ was subsequently improved upon by capping

free thiols with an alternative covalent linkage after initial Fab reduction, prior to mixing with the second Fab.⁹⁸ However, this method required the use of a toxic arsenite reagent, which can be challenging to remove completely prior to bsAb use in downstream applications, such as cellular assays.⁹⁸ More recently, cysteine-reactive crosslinkers have been widely used to conjugate antibody fragments, including MDX-H210, an anti-HER2 × CD64 bsAb that was evaluated in clinical studies.^{38,97,99–101} The suitability of this bispecific format as an ‘intermediate’ format for the early stage screening of T-cell engager (TCE) bsAbs was validated by comparing a panel of maleimide cross-linked Fab bispecifics with matched full-length IgG-like bsAbs in a T-cell cytotoxicity assay.⁹⁷

However, sample heterogeneity can arise from cysteine crosslinking conjugation reactions when more than one cysteine is present in the input antibody fragments, via either disulfide scrambling or the conjugation of multiple cysteine residues.^{97,102,103} These complications can be limited by either engineering a single unpaired cysteine residue into the input antibody fragments or by using ‘re-bridging’ maleimides that form a new covalent connection between the reduced disulfide bonds (Figure 2b (iv)).^{38,99,100,104} Next-generation re-bridging maleimide conjugates also provide the added benefit of greater stability with respect to degradation via retro-Michael reactions.¹⁰⁵ Nevertheless, a major hurdle to producing large bsAb panels via cysteine crosslinking remains the requirement for low throughput purification steps, such as size exclusion chromatography (SEC) to remove impurities including unreacted antibody fragments and aggregate species.^{96,97}

Bio-orthogonal click chemistries potentially satisfy all the chemical conjugation requirements outlined above for developing an efficient HTP bsAb production method.¹⁰⁶ Pre-functionalized click handles can be introduced into antibody fragments using either modified next-generation re-bridging maleimide reagents or by incorporating unnatural amino acids at defined sites of the input antibody fragments.^{39,99,100} This facilitates the rapid and specific bio-orthogonal conjugation of two antibody fragments via a ‘click’ reaction to produce stable covalently linked and homogeneous bsAbs in high yields (Figure 2b (v)).^{96,107} The use of strained click handles enables copper-free click reactions, additionally negating the need for toxic metal catalysts and producing only nitrogen gas, if any by-product.^{108,109} Site-specific conjugation and click strategies have been successfully combined to provide modular, two-step methods to generate bispecifics.^{107,110–112} Using next-generation maleimide conjugation reagents to functionalize the input antibody fragments, and copper-free click reactions with rapid reaction kinetics, good conversion to ‘clicked’ bsAbs can be achieved.⁹⁶ For example, yields up to 85% were obtained for Fab-Fab bsAbs generated using a sequential conjugation-click methodology, followed by a single protein A or SEC purification step.⁹⁶ If affinity-based purification steps are sufficient, these can be readily miniaturized³⁷, therefore this workflow is potentially compatible with automation to enable HTP bsAb production.

The linker component of a chemically conjugated bsAb provides the opportunity to introduce additional variability or functionality into a bsAb panel. While recombinantly engineered hinge sequences can introduce extra flexibility into biparatopic bsAbs to enable binding to both antigen

epitopes,³¹ this flexibility could potentially be provided by chemical linkers of varied length and rigidity. Chemical linkers can also be further modified with additional small molecules, such as drug compounds to produce bispecific ADCs, fluorescent tags to enable bsAb use in imaging applications, or purification tags to aid in bsAb production.^{96,113} Although chemical conjugation production methods potentially provide more versatile applications for bsAb panels, deviation from the intended 'final format' needs to be carefully considered ahead of use in more complex cellular assays or *in vivo* studies.⁹⁷ Therefore, the production of large bsAb panels via chemical conjugation has clearest application during either Target Identification or Lead Discovery stages of a bsAb discovery campaign, to enable screening activities in an 'intermediate', exploratory format (Figure 1, Option 1.2).

Redox recombination of two monoclonal antibodies or their derivatives

As outlined above, there are multiple advantages to using an IgG-like bsAb 'final format' when monovalent engagement of each antigen target is sufficient to enable the desired mode of action. While production methods enabled by chain pairing technologies can feasibly generate Ig-like bsAb panels up to around 100 molecules, expansion beyond this number is limited by the low throughput purification steps typically required to achieve consistent sample purities. The combinatorial methods discussed above are far more resource efficient for HTP bsAb production, but are typically limited to non-IgG-like bsAb formats (with the exception of post-translational trans-splicing.³⁷) Redox recombination methods allow a balance to be struck, offering the advantages conferred by combinatorial approaches, while maintaining an Ig-like bsAb format.

Bispecific antibodies of the IgG4 immunoglobulin sub-class can be found naturally in human sera.¹¹⁴ These are derived from a dynamic process termed Fab-arm exchange (FAE), whereby the two half-antibodies comprising an IgG4 molecule, which have fewer intact intermolecular hinge disulfide bonds than in IgG1, are able to exchange to pair with half-antibodies derived from an IgG4 molecule with a different antigen selectivity. These bsAbs would not typically encounter both target antigens and function as bsAbs *in vivo*, but their monovalent antigen binding provides a mechanism to de-tune immune inflammation following chronic antigen exposure.^{115,116} Dynamic FAE was mimicked *in vitro* by adding mild reducing agent to a mixture of two IgG4 antibodies, yielding a mixture containing bsAbs.¹¹⁷ The potential application of this reaction to develop a process to routinely and reliably generate bsAbs from any two parental antibodies was quickly recognized.^{40,118–120} By reverting to the IgG1 hinge sequence, the bispecific antibodies produced via a controlled-FAE (cFAE) process were stable following reducing agent removal, while the introduction of complementary CH3 mutation sets into the two parental antibodies favored heterodimer rather than homodimer formation (Figure 2b (vi)).^{40,118–120} LC exchange was not observed during cFAE, as validated using stable isotope labeling using amino acids in cell culture (SILAC) mass spectrometry (MS).¹²¹ Further work

demonstrated that an anti-CD20 homodimer produced via cFAE containing the CH3 mutation sets used to drive heterodimerization retained comparable IgG1 Fc effector functions to an anti-CD20 mAb, while cFAE-derived bsAbs displayed similar pharmacokinetic properties to an IgG1 mAb.⁴⁰

Four FDA-approved bispecific antibody products, amivantamab, teclistamab, talquetamab and epcoritamab, have been developed to date using Genmab's Duobody® platform, which uses a cFAE-based bsAb production process from the discovery stage through to clinical manufacture.^{40,41,118,122} This process involves four main steps: 1) expression of two parental antibody panels (each panel containing one of two complementary CH3 mutation sets to drive correct HC-HC pairing) in a mammalian expression system, 2) purification of the parental antibody panels via protein A capture, 3) combination of parental antibodies in 1:1 ratio and addition of mild reducing agent to initiate cFAE reaction, and 4) removal of reducing agent to halt cFAE reaction. Amivantamab was selected following the unbiased screening of a cFAE-produced panel of 40 bispecific antibodies derived from five anti-MET mAbs combined with each of eight anti-EGFR mAbs, rather than the rational pairing of two mAbs based on their individual properties.¹²³ Blockade of both MET and EGFR signaling pathways were part of the bispecific antibody target product profile, which was especially challenging given that available parental anti-MET antibodies were agonists. Therefore, screening in bsAb format was crucial to not only ensure that monovalent antigen binding achieved the intended signaling reduction, but also that any bridging of the MET and EGFR antigens did not result in receptor agonism. Screening in final format from the outset de-risked that signaling may be differentially impacted by MET-EGFR bridging via a bsAb with a different geometry.¹²³

Further adaptations of the cFAE process have looked to bypass the requirement to purify the parental antibodies prior to mixing and reduction. An early variation involved mixing two *E. coli* cultures expressing the parental half-antibodies and then isolating the desired bsAb from the combined cell lysate via protein A capture.¹²⁰ However, use of this process for HTP bsAb production is limited by the extra steps introduced to maximize bsAb purity (measuring half-antibody expression levels and adjusting relative bacterial culture volumes), while the potency of bsAb panels derived from *E. coli* may be reduced relative to molecules produced from mammalian cells due to absent PTMs, particularly N-linked glycosylation (e.g., Asn-297 aglycosylation will reduce Fc interactions with FcγR receptors and C1q unless Fc engineering performed).^{45,124} Both these limitations can be avoided by using a mammalian expression system to produce cell supernatants containing the two parental antibodies, as standard mammalian cell culture media conditions have been shown to promote cFAE.^{28,125} Steinhardt et al. obtained high final bsAb purity levels by introducing amino acid mutations to ablate protein A binding into one parental antibody HC and adding excess of the cell supernatant containing this partner so that any excess parental antibody species remaining following cFAE should not bind protein A during the final bsAb capture step.¹²⁵ This modification ensures that the final bsAb purity is

less reliant on the accurate determination of parental antibody concentrations in the cell supernatants, increasing compatibility with automated processes. However, the volumes of cell supernatant required in each reaction mixture to obtain sufficient bsAb sample for screening are instead likely to limit process miniaturization and the production of panels > 100 bsAbs. Inclusion of the early purification step for a relatively small number of input parental antibodies, as in the original process described in the previous paragraph, enables more concentrated (and more accurately quantified) protein samples to be used for the cFAE step, increasing potential process throughput.

Format Chain Exchange (FORCE) is a further adaptation of the cFAE process developed to enable the combinational production of bsAb matrices with high material purity and expansion beyond an IgG-like format.⁴⁸ Dummy hexahistidine-tagged Fc chains containing either knob or hole mutations are initially expressed together with each parental HC (containing hole or knob mutations respectively) and LC, to generate monovalent heterodimers. cFAE reactions using complementary heterodimers (knob plus hole), result in correct bsAb production, while any remaining heterodimers or paired dummy Fc molecules can be removed via a single nickel-affinity purification step. Automation of both heterodimer expression and purification, and the cFAE process itself, was developed using liquid handling systems and utilized to produce an exemplar 4 mAb A × 4 mAb B × 9 format panel (total 144 HER1/2 × DR5 bsAbs). Subsequent screening via HTP bridging ELISA enabled bsAb formats preferable for dual antigen engagement to be identified.⁴⁸ Extended format panels produced by cFAE were also crucial to enable optimal positioning and antigen binding affinity of one CD3 and two different TAA binding moieties within a [2 + 1] T-cell engaging trispecific antibody to be screened.¹⁵ Screening in format was crucial in this example to assess selective CD8+ T cell-mediated killing of cells expressing both antigens versus those expressing only a single antigen.¹⁵

In summary, the use of 'final format' panels throughout the bsAb discovery process allows early functional screening for obligate bsAbs and prevents potential hit attrition after re-formatting steps, but may not be feasible for all modalities (Figure 1). Combinatorial production methods are the most resource-efficient route to HTP bsAb panel production during early stages, of which cFAE is well established and allows the production of both IgG-like and extended IgG format panels (Figure 2). BsAb formats without an Fc region, favorable for applications requiring a short bsAb serum half-life or a small bsAb size for increased tissue or tumor penetration, cannot be produced via cFAE. For these formats, the combination of antibody fragments using processes detailed under the third and fourth proposed solutions above provide alternative routes to generate large panels, though not necessarily in final format. Across all formats, bsAb production using an expression system similar to the clinical manufacturing process is important at later stages of the discovery workflow to test for process compatibility (Figure 1, Lead Panel).

Solutions to enable biophysical validation of large bispecific antibody panels

In addition to the considerations discussed above, the feasibility and complexity associated with bsAb analytics is an important factor in selecting a suitable HTP bsAb production method. FDA guidelines suggest bsAb characterization should be in line with standard mAb practices for final regulatory filings,¹²⁶ but molecular quality and stability profiles vary across bsAb formats and more complex analytical methods are typically required during a bsAb discovery campaign versus that for a standard mAb. At Lead Optimization and early Chemistry, Manufacturing and Controls (early CMC) stages, a comprehensive biophysical characterization of PTMs (e.g., Met/Trp oxidation, Asn deamidation, Asp isomerization, Asn-linked glycosylation), aggregation, fragmentation, hydrophobicity and thermostability, aided by forced degradation studies, can identify potential issues that may occur during late-stage clinical development (Figure 1).⁹ This can help to triage lead bsAbs and it provides an opportunity to begin defining critical quality attributes to be monitored through late-stage development and clinical manufacture. In contrast, a more pragmatic approach to biophysical analysis is required for bsAb panels generated to support early-stage Target Identification and Lead Discovery activities, due to both larger panel sizes and more limited material quantities (Figure 1). Like standard mAb campaigns, at these earlier stages bsAb characterization methods should be selected in line with sample requirements for downstream screening assays. As a minimum, bsAb identity should be confirmed by reduced MS, while endotoxin levels should be measured ahead of use in cellular screening assays with endotoxin-sensitive cells. Aggregate species can interfere with both binding and functional assays, and should therefore be quantified, typically via analytical SEC, particularly if using a bsAb format more prone to aggregation, such as those containing scFv modules or appended domains.^{1,42,127}

As we have discussed, combinatorial approaches for generating large bsAb panels can yield simpler purity profiles and more consistent purity levels across a panel than the recombinant co-expression of 3–4 chains. For instance, minimal amounts of species containing mis-paired LCs are produced via cFAE, though HC homodimers and half-antibody fragments still often persist.¹²¹ These impurities often retain antigen binding and therefore may complicate the interpretation of screening data. They also exhibit similar molecular properties to the correct bsAb, which makes their quantification difficult. Traditional chromatographic methods can often be optimized to characterize molecule-specific impurities, but it is not feasible to determine the identity of each species present in large, diverse bsAb panels. MS represents a solution to this analytical challenge by providing unambiguous, label-free identification, and to some degree, quantification, of the heterogeneous peptide mixtures. The application of MS to analyze bsAb panels is discussed in more detail below.

Table 1. List of liquid chromatography methods employed to characterize and/or purify IgG-like bispecific antibodies. Examples of bispecific antibody formats analyzed (or purified) using these standalone methods are provided under 'offline MS', while examples where these methods have been used in tandem with mass spectrometry are listed under 'online MS'.

| bsAb sample state | Liquid chromatography method | Physical property used for separation | Examples of IgG-like bispecific antibody formats analyzed/purified |
|-------------------|--|---|--|
| Denatured | Reversed phase chromatography | Hydrophobicity | Online MS Common LC ^{128,129} 4 Unique Chains ^{130–133} cFAE-derived ¹²⁵ |
| Native | Hydrophobic interaction chromatography | Hydrophobicity | Offline MS WuXi-Body ¹³⁴ DuetMabs ¹³⁵ 4 Unique Chains ^{133,136} |
| | Ion exchange chromatography | Charge | Online MS mAb combination ¹³⁷ Offline MS 4 Unique Chains ²² cFAE-derived ^{40,122} |
| | Size exclusion chromatography | Size (hydrodynamic volume) | Online MS 4 Unique Chains ¹³⁰ Offline MS cFAE-derived ¹³⁸ |
| | Mixed-mode size exclusion chromatography | Size (hydrodynamic volume) and hydrophobicity | Online MS Cross-mAb ^{139,140} Offline MS 4 Unique Chains ¹⁴¹ cFAE-derived ¹⁴² Online MS Common LC ¹⁴³ |

Mass spectrometry methods using chromatographic resolution to analyze bispecific antibody panels

By linking liquid chromatography methods to electrospray ionization MS (ESI-MS), it is possible to exploit differences in physicochemical properties between the desired product and related impurities to identify each species present and accurately determine sample purities in a relatively HTP manner. The most ubiquitous method used is reversed-phase liquid chromatography MS (LCMS), which uses an organic phase gradient to separate the sample constituents based on their respective hydrophobicity under denaturing conditions (Table 1). Gradients can be optimized to achieve chromatographic resolution between correctly and incorrectly paired species if they exhibit sufficiently different hydrophobic profiles, allowing absolute quantification of each species based on the UV absorbance profile.¹²⁸ When analyzing large bsAb panels or symmetric IgG formats in their intact format, however, it is unlikely that absolute chromatographic separation between all product species will be obtained for all samples using a platform method, as has been observed previously,¹²⁵ therefore orthogonal methods are likely required to achieve this.

Recent advancements in MS instrumentation allowing detection of a higher mass to charge ratio range have enabled precise mass determination of biologics in their native state.^{144,145} This in turn enables use of non-denaturing LC methods to separate heterogeneous bsAb mixtures, although limited examples of MS-hyphenation exist in the current literature (listed in Table 1). “Offline” (not coupled to MS analysis) hydrophobic interaction chromatography (HIC) and IEX have both proven effective for analyzing the proportion of HC homodimers, and allowing separation from the desired bispecific during purification.^{40,122,135,136} Both methods can also prove effective for the separation of impurities with mis-

paired or missing light chains,^{134,135} particularly when connected to “online” MS analysis by using compatible buffers as demonstrated for IEX.¹³⁰ Chromatographic separation of correct bsAb from impurity species using these methods can also be improved through protein engineering, such as maximizing the difference in pI between the two half-antibody bsAb components.²² SEC can resolve bsAb impurities of significantly different masses, such as half-antibodies.¹³⁸ However, by reducing the buffer salt content and promoting hydrophobic interactions with the column, chromatographic separation of species with more similar sizes can also be achieved. This mixed mode SEC (mmSEC) technique, has resolved half-antibodies, homodimers and LC mis-paired species,^{141,142} and can also be connected to “online” MS.¹⁴³ Although chromatographic resolution is often possible using mmSEC, run times are typically greater than 30 mins,^{141,142} making the analysis of large bsAb panels (>1000) impractical.

Mass spectrometry methods using m/z signal alone to analyze bispecific antibody panels

In lieu of chromatographic resolution between product-related impurities, absolute quantification of IgG-like bsAb species with mis-paired LCs can be achieved by MS signal intensity alone through the spiking of impurities to create calibration curves.^{131,146} This method allows for HTP impurity quantification by employing short (under five minute) desalting reversed-phase gradients. However, spiking of each impurity is required for accurate quantitation due to differing MS responses between species, particularly those very different in mass, which would be very labor intensive across diverse, heterogeneous panels. As a potential solution, relative quantitation between species of comparable masses without impurity-spiking has been shown

to provide reasonable estimates of impurity levels in a HTP manner.^{147,148} Without chromatographic separation, a sufficient mass difference (>25 Da) is required between the correct bsAb and all impurity species to achieve resolution in the deconvolved mass spectra.¹³² An additional, though much less commonly encountered, challenge is to identify and quantify impurities with both LCs mis-paired, as this species is identical to the correct bispecific in mass. An elegant, probability-based mathematical model has been derived to estimate the abundance of this species compared to species containing a single mis-paired LC, based on relative chain expression levels.¹³¹ Enzymatic digestion of a bsAb sample to generate Fabs for MS analysis allows experimental validation,¹⁴⁹ a step likely reserved for samples containing high mis-pairing levels in initial analyses, to conserve resources.

Preparing large bsAb panels through combinatorial approaches such as cFAE typically requires the use of robotic liquid and microplate handling to ensure accuracy and minimize hands-on sample preparation time. Waldenmaier et al.¹⁵⁰ have recently reported a fully automated mAb characterization workflow to support cell line selection, starting with mAb purification from cell-line supernatant, prior to intact, reduced, and peptide mapping LCMS analyses and data processing. The sample preparation stages of this procedure could potentially be modified to analyze panels of > 1000 bsAbs to support early screening campaigns, providing both time savings and improved data quality and consistency. However, instrument analysis time for liquid chromatography methods still limits the sample throughput, which can be addressed by using alternative sample introduction methods. Recent advancements in solid-phase extraction have enabled development of the first ultra-HTP method for analyzing IgG-like bsAb panels by using RapidFire coupled to an Orbitrap MS instrument.¹⁴⁸ Sawyer et al. were able to quantitatively analyze panels of IgG4 bsAbs at a rate of 15 seconds per sample and achieve baseline mass resolution of glycoforms with mass differences of 160 Da within 7 ppm accuracy. Although this method is still limited when impurity masses are very similar to the correct bsAb, it provided the first truly HTP purity screening method for very large bsAb panels, and has since been applied to a variety of bsAb formats.¹⁵¹ Pu et al. were able to demonstrate similar mAb glycoform resolution at a further increased rate of 1.5 seconds per sample via infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI), though throughput is dependent upon HTP sample desalting prior to analysis.¹⁵² More recently, Zacharias et al. have also achieved glycoform resolution and relative quantitation of mAbs at speeds of one second per sample without the requirement for additional sample preparation by utilizing Acoustic Ejection ESI-MS.¹⁵³ These results using a prototype high-resolution quadrupole time-of-flight instrument are highly encouraging, demonstrating high mass accuracy (<1.5 Da mass error), although this required a compromise on the mass range analyzed to achieve the reduced sample times. Simultaneously detecting half-antibody impurities and full bsAb species is therefore unlikely to be feasible at the same speed.

These exciting developments in HTP MS could potentially be combined with automated sample preparation and data analysis to enable cell supernatant to bsAb purity data to be generated for 1000 molecules within a day. They offer the

opportunity to increase the level of characterization conducted at the Lead Discovery stage and to provide more data to triage bsAb hits. However, methods that provide greater chromatographic resolution will still be required to determine sample purity levels with a higher accuracy at later stages of development to validate molecular quality (Figure 1, early CMC).

Conclusions and outlook

The capability to produce large bsAb panels (100s-1000s molecules), including across varied formats, offers potential advantages at multiple stages of the bsAb discovery process. Early on, target pair identification via unbiased phenotypic screening in a biologically relevant functional assay offers an alternative to hypothesis-driven selection, expanding the available target space and increasing the chance of finding novel pairings.³⁶ At the Lead Discovery stage, functional screening in bsAb format once mAb selection campaigns are completed is highly preferable and the ability to test larger, more diverse panels at this early stage increases the chance of identifying molecules with the desired mode of action. An alternative approach, a HTP single-cell-based bsAb functional screening pipeline, allowing approximately 22,300 CD19×CD3 scFv-scFv format bispecific T-cell engager antibodies (BiTEs) to be screened without the need for protein expression and purification has recently been reported.¹⁵⁴ This streamlined workflow will be more complex to adapt to IgG-like bsAb formats; nevertheless it offers a very efficient option to increase early bsAb screening throughput even if often requiring an 'intermediate format' rather than the intended 'final format'.

The design of a bsAb screening cascade during Lead Discovery and Lead Optimization phases is often a delicate balance between the extra resource required to produce, validate, and screen larger panels enabling multiple properties to be optimized simultaneously, versus the extended timelines if these properties are screened using multiple smaller panels (Figure 1). The development of HTP bsAb production methods, automated bsAb analytical workflows and miniaturized HTP screening assays together tip this balance, increasing the feasibility of screening larger bsAb panels and providing the opportunity to compress project timelines. Additionally, integrating computer-aided, rational bsAb design strategies can potentially help streamline screening panels contents; for example, structural models can be used to triage bsAb formats or antigen binding epitopes.^{13,18} In recent years, rapid progress has been made in developing machine learning strategies to firstly predict and secondly optimize biotherapeutic properties, aiming to reduce experimental screening requirements.¹⁵⁵⁻¹⁵⁷ To train accurate machine learning models, however, large and high-quality experimental datasets must initially be generated. Therefore, even as new machine learning strategies more regularly become integrated into the bsAb discovery process over the coming years, HTP bsAb production and screening will remain a crucial capability.

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Abbreviations

| | |
|----------------|---|
| ADC | antibody-drug conjugate |
| ADCC | antibody-dependent cellular cytotoxicity |
| bsAb | bispecific antibody |
| cAMP | cyclic adenosine monophosphate |
| CD20 | cluster of differentiation 20 cell surface protein |
| CD3 | cluster of differentiation 3 cell surface protein complex |
| CH1(2,3) | heavy chain constant region 1(2,3) |
| CL | light chain constant region |
| cLC | common light chain |
| CMC | Chemistry, Manufacturing and Controls |
| CODV-Ig | cross-over dual variable domain antibody |
| Da | Dalton |
| dAb | domain antibody |
| DNA | deoxyribonucleic acid |
| DR5 | death receptor 5 |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| EGFR | epidermal growth factor receptor |
| ESI | electrospray ionization |
| Fab | fragment antigen-binding |
| (c)FAE | (controlled) Fab-arm exchange |
| Fc | fragment crystallizable |
| FcRn | neonatal Fc receptor |
| FDA | U.S. Food and Drug Administration |
| FORCE | format chain exchange |
| HC | antibody heavy chain |
| HEK | human embryonic kidney |
| HER1(2,3) | human epidermal growth factor receptor 1(2,3) |
| HIC | hydrophobic interaction chromatography; |
| HIV | human immunodeficiency virus |
| HTP | high throughput |
| IEX | ion exchange chromatography |
| IgG | immunoglobulin G |
| KiH | knob-in-hole |
| LC | antibody light chain |
| LCMS | liquid chromatography mass spectrometry |
| mAb | monoclonal antibody |
| MALDI | matrix-assisted laser desorption/ionization |
| MET | hepatocyte growth factor receptor |
| mmSEC | mixed mode size exclusion chromatography |
| MS | mass spectrometry |
| NGF | nerve growth factor |
| ppm | parts per million |
| PTM | post-translational modification |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| scFv | single-chain variable fragment |
| SEC | size exclusion chromatography |
| SILAC | stable isotope labeling using amino acids in cell culture |
| TAA | tumor-associated antigen |
| TCE | T-cell engager |

| | |
|-----|--|
| UV | ultraviolet |
| VH | heavy chain variable region |
| VL | light chain variable region |
| VHH | camelid heavy chain variable domain antibody |

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