Enabling a Healthier World

Lonza Biologics

Technical Note

Precision Pairing with bYlok® Bispecific Design Engineering Technology

How bYlok® technology drives accurate HC-LC pairing and provides a close-to-nature solution for bispecific antibodies

Introduction

Bispecific antibodies (bsAbs) can simultaneously bind two different antigens via distinct antigen binding sites. The dual binding properties of bsAbs enable a wide variety of applications and benefits. Since bsAbs can target two distinct disease-associated antigens, they can provide access to novel therapeutic targets not available to traditional monoclonal antibodies (mAbs). In addition, the presence of multiple antigen binding sites allows for increased targeting precision and increased therapeutic potency compared to traditional mAbs, whilst also contributing to a reduction in the development of clinical resistance to antibody-based therapeutics. In recent years, increasing numbers of bsAbs have entered clinical development. Indeed, as of June 2023, 316 bsAbs were in clinical trials according to the ClinicalTrials.gov trial registry [1], and an average of 75 bsAbs have entered trials every year for the past four years [2].

Despite their benefits, bsAbs can be challenging to generate. One of the most commonly reported issues with bsAb production concerns heavy chain (HC) and light chain (LC) mispairing. The formation of incorrect hetero- and/or homo-dimeric species creates challenges for downstream processing, due to the similarity of their physiochemical properties to those of the intended molecule. BsAbs derived from two independent parental antibodies typically require two HCs and two LCs. Consequently, in addition to the original homodimeric parental mAb species, there are eight potential heterodimeric pairing combinations, only one of which generates the desired bsAb format. Four incorrect heterodimeric pairings are due to HC mispairings, which was addressed by Genentech in 1996 [3] using "knobs-into-holes" (KIH) technology. This technology involves the mutation of four amino acids on one HC to form the "knob" and the mutation of three amino acids forming the "hole", aiding correct HC pairing. An additional disulphide bridge between the HCs is sometimes introduced in tandem with the KIH to stabilize the molecule. The technology is now widely used to express bsAbs with the correct HC pairing.

The remaining three incorrect pairings arise from HC-LC mispairing. In a typical dual binding bsAb, there are four possible HC-LC combinations, only one of which will generate the correct bsAb format. One strategy for solving the HC-LC mispairing problem is the 'common light chain' approach, which requires the parental mAbs to share an identical light chain. Whilst effective, this may limit therapeutic possibilities. Another strategy is to express each 'half-mAb' in a separate cell line. This approach is inefficient, however, as it requires twice the effort and resources. Only recently have alternative technologies for driving the formation of the desired HC-LC combination been developed. To solve this mispairing issue, Lonza offers bYlok® bispecific pairing technology (bYlok®), a novel, proprietary solution accessible via licensing. The technology is based on work by Vaks et al., 2018 [4], in which the native disulphide bond between the constant domain of the heavy chain (CH1) and the constant domain of the light chain

(CL) interface in one Fab arm is removed. An artificial disulphide bond between cysteines at the interface of the variable domain of the heavy chain (VH) and the variable domain of the light chain (VL) is then introduced. This minor structural modification, when used in combination with KIH technology, drives the correct pairing of heavy and light chains, to produce the desired bsAb format (Figure 1). Using bYlok® technology, it is possible to generate four bsAb formats. Two bsAbs contain the bYlok® modifications on the arm with the "knob" and two will contain the modifications on the arm with the "hole" (Figure 2).

In this technical note, we describe the construction and expression of bYlok® bsAbs using Lonza's GS Xceed® Gene Expression System and GSquad® vectors with GS piggyBac® technology. We also detail the evaluation of bYlok® bsAbs for HC-LC chain pairing, titer, binding affinity, stability and immunogenicity to demonstrate the effect that bYlok® modifications have on these critical quality attributes (CQAs). In addition, we demonstrate how improvements to bsAb HC-LC pairing enable standard downstream processes to be used for bsAb purification.

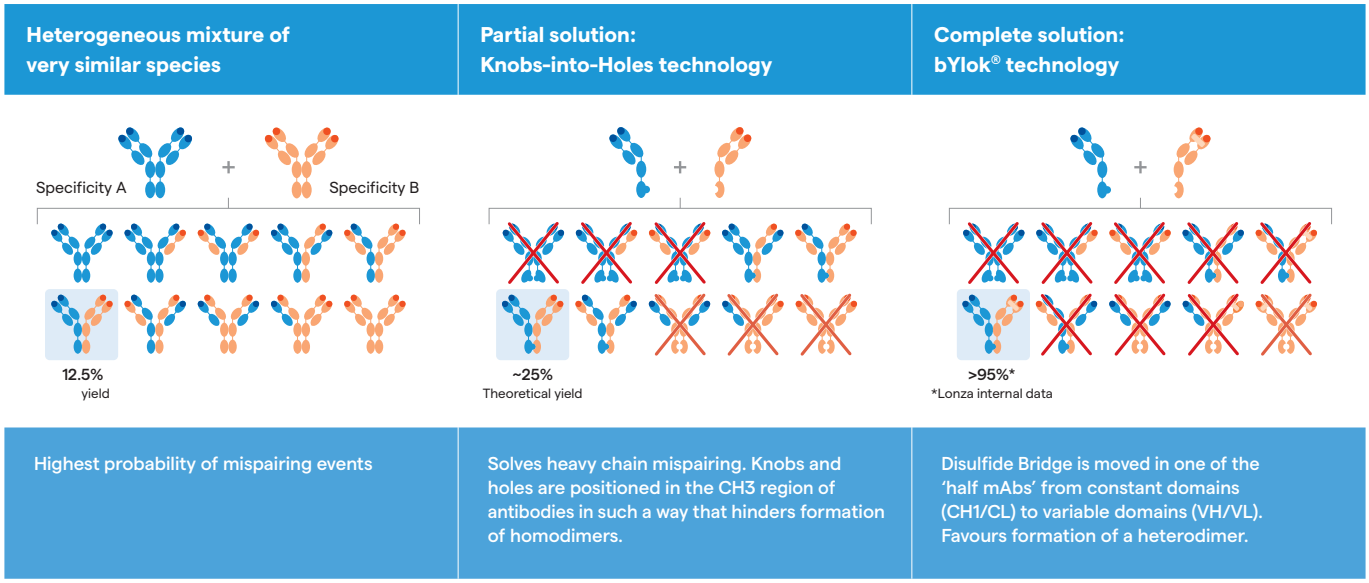

Figure 1
How bYlok® overcomes the limitations of existing bispecific pairing solutions.

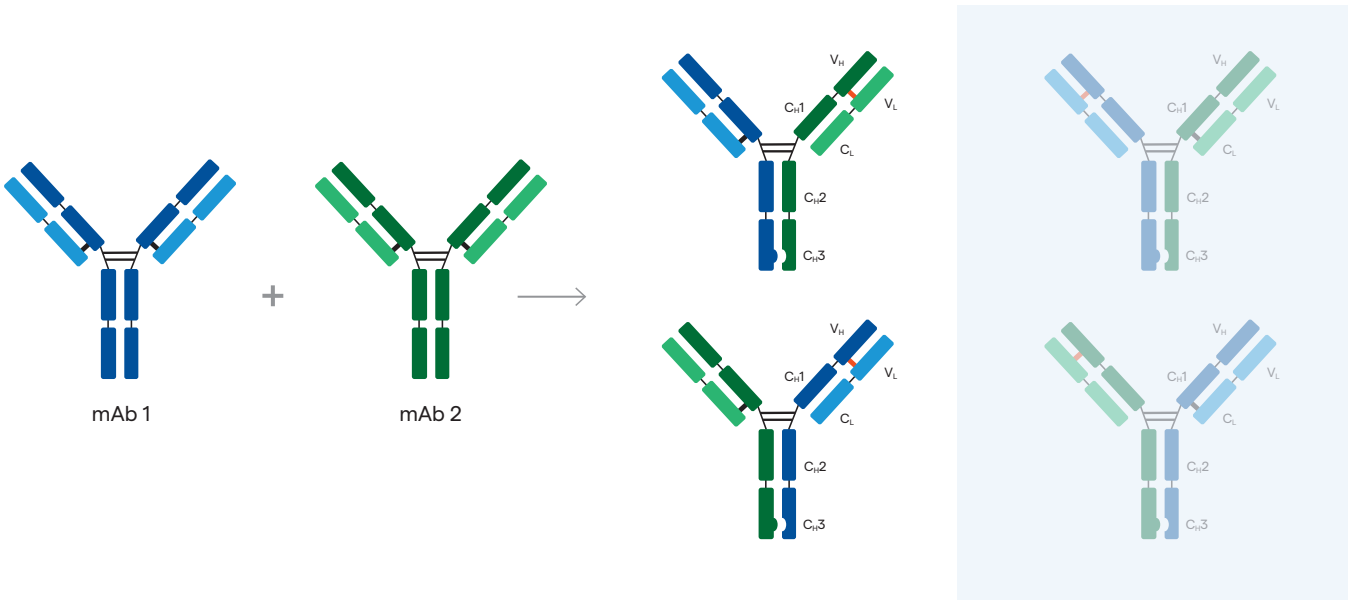

Figure 2
bsAb formats that can be generated using Lonza's bYlok® system in conjunction with KIH technology. The data presented in this technical note are generated from bYlok® molecules with the bYlok® modification on the Fab arm containing the KIH 'hole' (unshaded).

Case Study:
Generation and Evaluation of bsAbs

Materials and Methods

Design of bsAbs

Three parental therapeutic mAbs were selected to generate 8 bsAbs (Figure 3). Trastuzumab binds to human epidermal growth factor receptor 2 (HER2), panitumumab binds to epidermal growth factor receptor (EGFR) and alemtuzumab targets CD52, a glycoprotein on the surface of mature lymphocytes.

For this study, bYlok® modifications were introduced on the Fab arm containing the KIH 'hole', using Lonza's bYlok® bispecific pairing technology sequence design tool (Figure 2). In pre-clinical studies, it is recommended that bYlok® users assess all four possible bsAb formats.

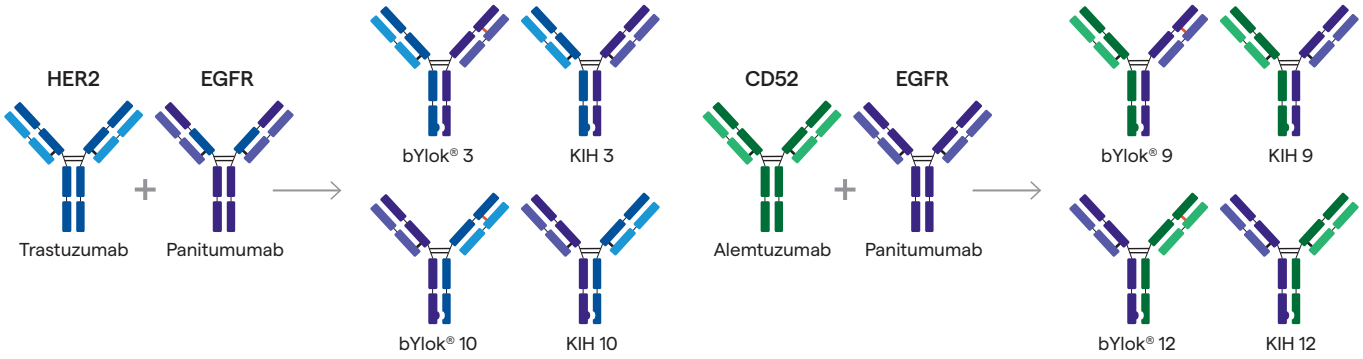

Figure 3
Different therapeutic mAbs used to produce 8 different bsAbs.
The bsAbs with a bYlok® prefix were generated using bYlok® and KIH technology and those with a KIH prefix are control bsAbs generated using KIH technology, without the bYlok® modification.

Construction and expression of bsAbs with GS Gene Expression System®

The bYlok® bsAbs and KIH bsAb controls were constructed and expressed using Lonza's GS Gene Expression System®. The GS Gene Expression System® offers an industry-leading toolbox of host cells, vectors, systems and know-how to address different protein expression challenges (Figure 4). The GSquad® vectors can be used to express up to four genes in the GS Xceed® CHOK1SV GS-KO® host cell line. The multigene vector format simplifies expression of complex proteins, such as bsAbs, allowing expression of all four antibody chains from a single vector in one cell line, streamlining development and reducing timelines.

Stable pools expressing bYlok® bsAbs, parental mAbs and KIH bsAb controls were constructed according to the GS Gene Expression System® Manual.

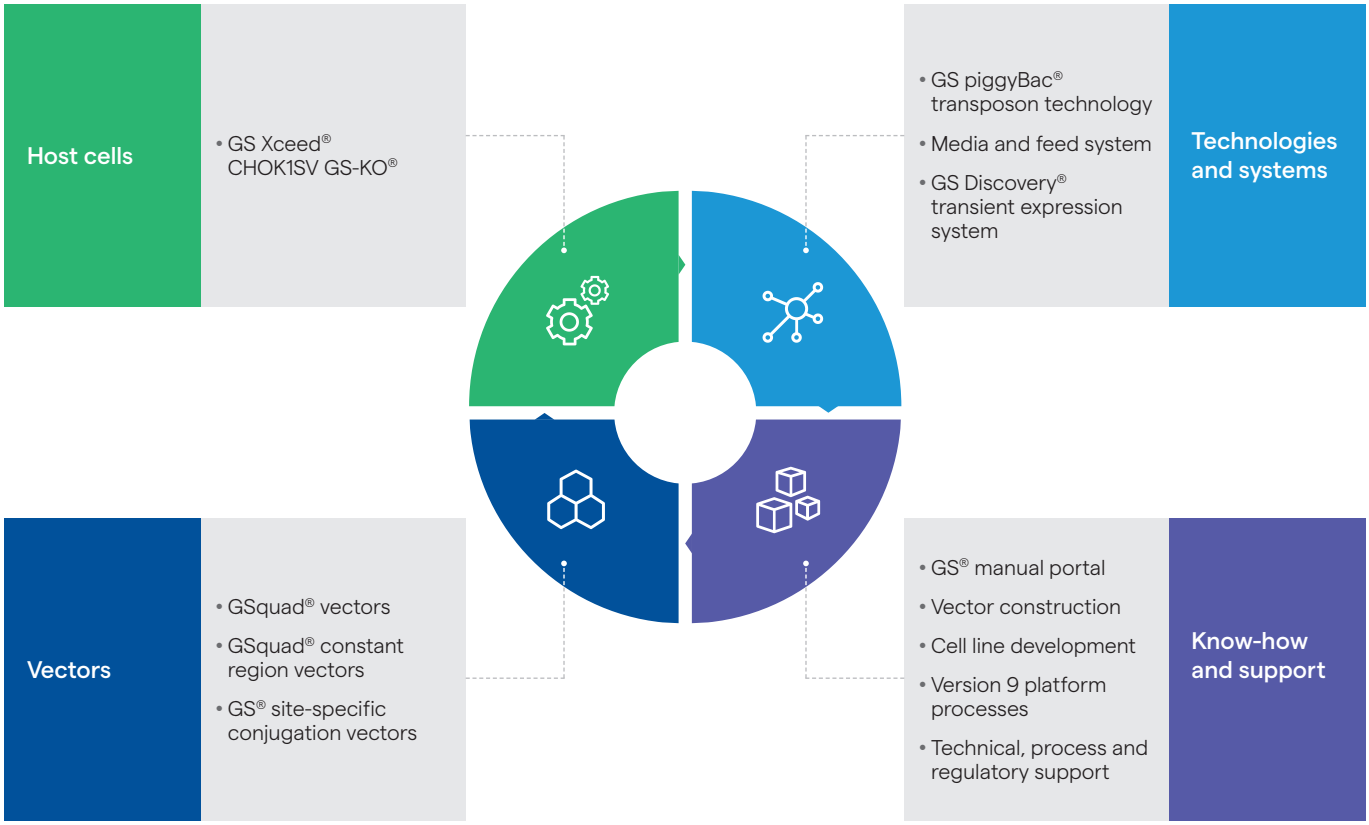

Figure 4
Lonza's GS Gene Expression System® – one toolbox, taking you from discovery to commercial production

Titer analysis

Productivity of pools expressing the bsAb molecules was assessed in 150 mL shake flasks using GSv9® media system. Pools expressing the bYlok® bsAbs and KIH bsAb controls derived from the trastuzumab/panitumumab (bYlok® 10; KIH 10) and alemtuzumab/panitumumab (bYlok® 12; KIH 12) families were also cultured in 5 L bioreactors using GSv9® media and feed regimes. Lonza's proprietary GSv9® media, feeds and know how are available to access via the GS Gene Expression System® research or commercial licenses. Cell culture supernatant was collected for titer analysis using using the Octet® platform (ForteBio).

Optimizing titer with GSquad® and GS piggyBac®

To determine if titer could be further improved, bYlok® 10, bYlok® 12 and their respective KIH bsAb controls were constructed and expressed using Lonza's GSquad® vector system with GS piggyBac® transposon technology. The GSquad® vector system uses a common workflow for the construction of expression vectors encoding for one (single gene vector; SGV), two (double gene vector; DGV), three (triple gene vector; TGV), or four (quadruple gene vector; QGV) product genes in two steps. In step one, product gene cassettes are cloned into GSquad® part vectors using standard cut-and-paste cloning methods. In step two, a one-pot assembly reaction allows formation of the final GS piggyBac® destination vector (SGV, DGV, TGV or QGV) using type II restriction enzymes (Figure 5). Sequence confirmation of the final vector is aided by the use of in-built sequencing primer binding sites.

Inverted terminal repeats (ITRs) contained within the GS piggyBac® destination vector, in combination with a hyperactive transposase, allow for insertion of product genes at transcriptionally active loci, enabling construction of high titer stable pools.

Stable pools expressing bYlok® 10, bYlok® 12 and a standard IgG2 mAb control were assessed for productivity in 50 mL shake flasks using GSv9® media and feeds. Titers were determined using the Octet® platform.

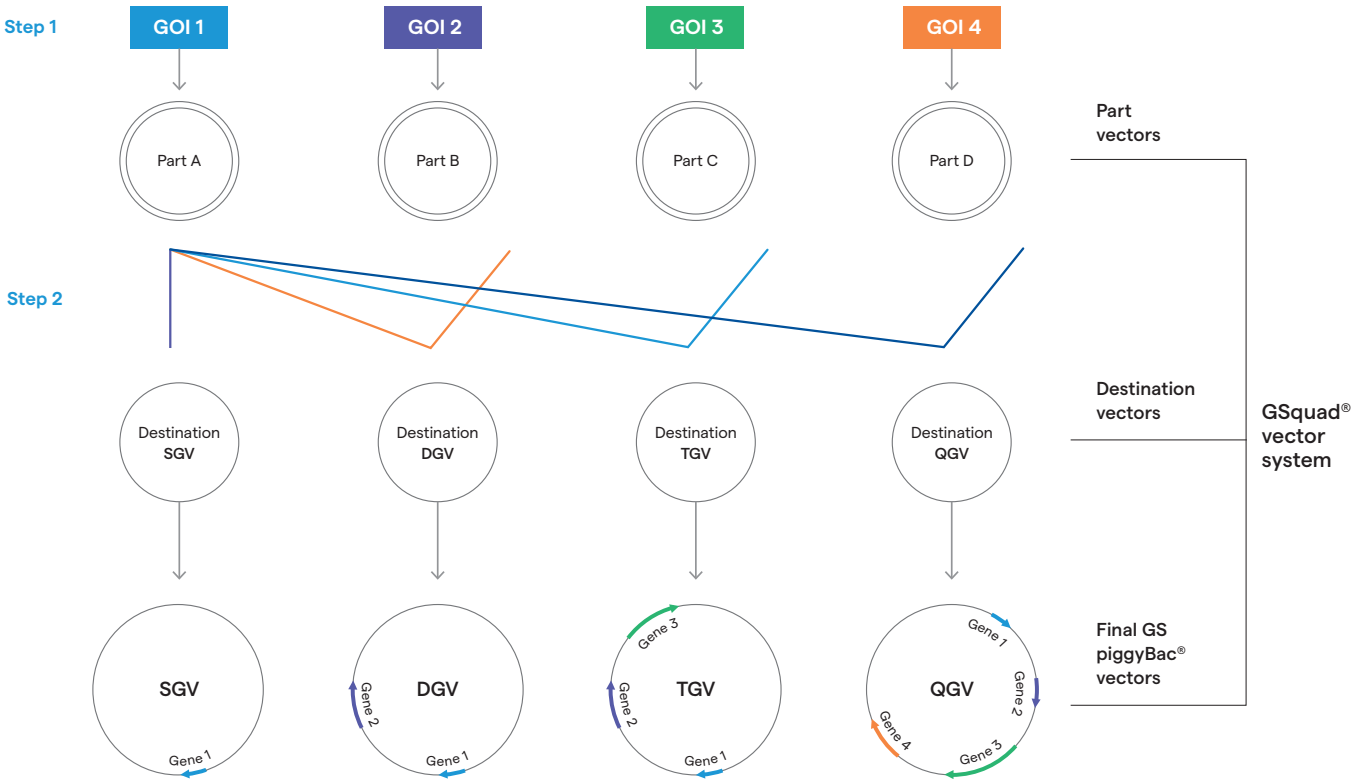

Figure 5
Lonza's GSquad® system for use with GS piggyBac® technology

Downstream processing

To determine the applicability of standard mAb purification methods for bYlok® bsAb purification, bsAbs were purified using Lonza's standard downstream processing workflow. Supernatants were clarified by centrifugation and bsAbs captured by Protein A chromatography. The bsAbs were then purified using Sartobind® Q 100 anion exchange resin (Sartorius) and POROS™ XS cation exchange resin (Thermo Fisher Scientific). Purified bsAbs were analysed by gel permeation chromatography (GPC) to determine the presence of aggregates and fragments. IgG purity was measured using the LabChip GXII System (Caliper Lifesciences) and the presence of host cell proteins (HCPs) was measured by ELISA.

bsAb characterization

Protein A-purified bsAb samples were frozen until characterization studies were performed. Liquid chromatography–mass spectrometry (LC-MS) was then used to determine correct bsAb heterodimerization.

Binding specificities and affinities of the bsAbs was assessed by several means. An ELISA was implemented whereby one antigen was immobilized and incubated with the bsAb. A second biotin-labeled antigen was added, followed by a streptavidin and horse radish peroxidase (HRP) substrate to produce a fluorescent signal upon binding of the bsAb. The fluorescent signal is generated only when bsAbs have dual binding capabilities. Surface plasmon resonance (SPR) was employed to further assess bsAb binding kinetics.

Nano differential scanning fluorimetry (Nano-DSF) was used to evaluate bsAb thermal stability.

The immunogenicity risk associated with the introduction of bYlok® modifications was assessed by Epibase® *in silico* HLA class II binding T-cell epitope profiling.

Results and Discussion

Analysis of bsAb heterodimerization

LC-MS data generated from two representative bYlok® modified bsAbs, bYlok® 10 (trastuzumab/panitumumab bYlok® bsAb and bYlok® 12, (alemtuzumab/panitumumab bYlok® bsAb), demonstrated >95% correct HC-LC pairing. These data were consistent for bYlok® bsAbs produced at both 150 mL shake flask and 5 L bioreactor scales (Figure 6). In contrast, KIH controls that did not contain the bYlok® modification KIH 10 (trastuzumab/panitumumab KIH bsAb) and KIH 12 (alemtuzumab/panitumumab KIH bsAb), demonstrated 40% – 70% correct pairing.

Further byproduct analyses using LC-MS indicated that the most represented byproducts generated from KIH controls comprise species in which LCs are swapped. For KIH 10 and KIH 12, LC swapping occurred at a rate of 35% and 30%, respectively. These observations are supported by data generated with additional alemtuzumab/panitumumab bsAbs and trastuzumab/panitumumab bsAbs (data not shown). Together, these data demonstrate that bYlok® modifications can significantly improve HC-LC pairing in bsAbs and offers a novel solution to the industry-wide HC-LC mispairing problem for IgG-like bsAbs.

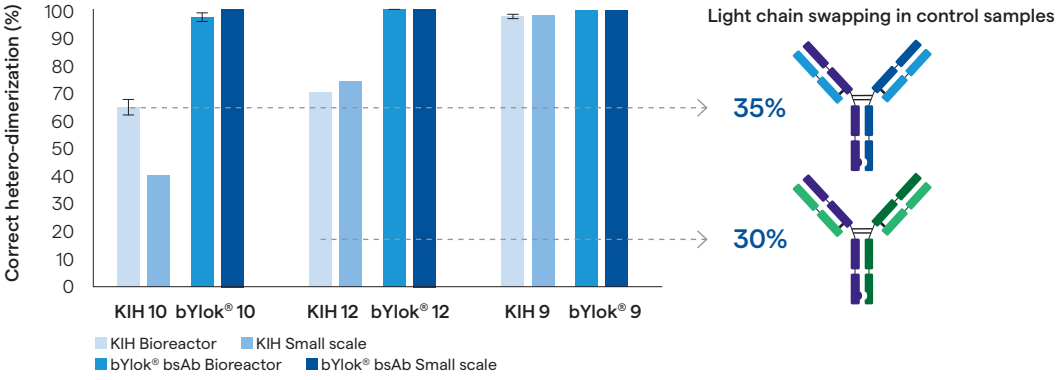

Figure 6
Correct heterodimerization rates in bsAbs generated with bYlok® and KIH technology, compared to control bsAbs produced using only KIH technology

Titer of bsAbs expressed using the GS Gene Expression System®

Titres of new mAb formats, such as bsAbs, are commonly reported to be lower than their parental mAb counterparts. Titer data generated at the 5 L bioreactor scale using the GS Gene Expression System® demonstrated that bYlok® 10 (a trastuzumab/panitumumab bsAb with bYlok® modification) achieved titers of 649 mg/L, which was between those of the parental mAbs. These data are comparable to the corresponding KIH control, KIH 10 (Figure 7). This observation is supported by analysis of titer data from all trastuzumab/panitumumab bsAb formats, at both shake flask and bioreactor scale (data not shown). These results indicate that the presence of bYlok® modifications does not have a negative impact on titers of these complex molecules, compared to KIH controls.

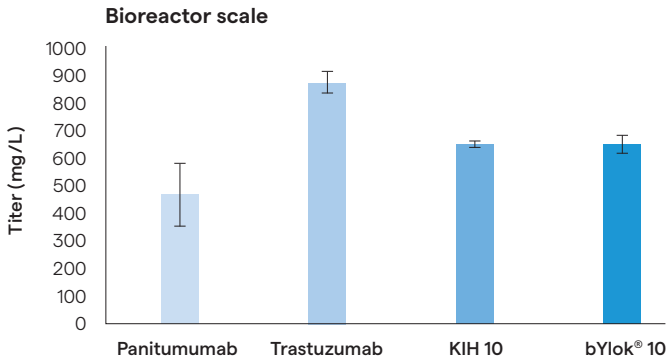

Figure 7
Titer of bsAbs generated using GS Gene Expression System®, with and without bYlok® modifications, compared to parental mAbs

Titer of bsAbs expressed using the GS piggyBac® System

Titer data generated using the GS piggyBac® system at 50 mL scale demonstrate that titers for bYlok® bsAbs bYlok® 10 and bYlok® 12 reached approximately 1200 mg/L and 930 mg/L, respectively (Figure 8). These values represent up to a four-fold increase in titer compared to that observed for the GS Xceed® controls without GS piggyBac® technology. These results indicate that use of the GS piggyBac® system may be preferred for high-titer expression of bYlok® bsAbs.

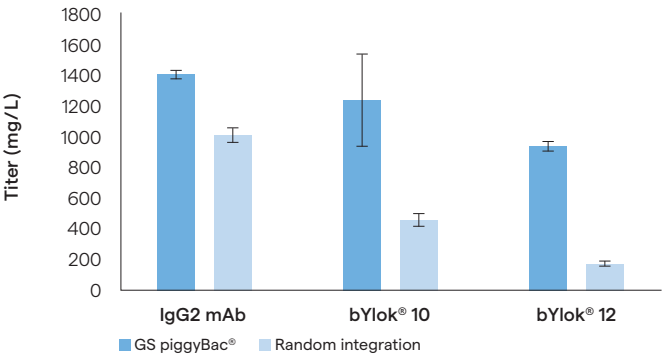

Figure 8
Titer comparison of bYlok® bsAbs expressed using GSquad® vectors with GS piggyBac® technology

Analysis of dual binding capabilities and binding kinetics

Dual binding affinities of bYlok® bsAb, bYlok® 10 (trastuzumab/panitumumab), were confirmed by ELISA assay. The binding affinities of bYlok® 10 to both EGFR and HER2 were comparable to the binding affinity of each parental mAb to their target antigen. These data indicated that bYlok® modifications do not adversely affect binding (Figure 9). Similar dual binding performance was observed for bYlok® bsAb bYlok® 12 (alemtuzumab/panitumumab) using a cell-based assay (data not shown).

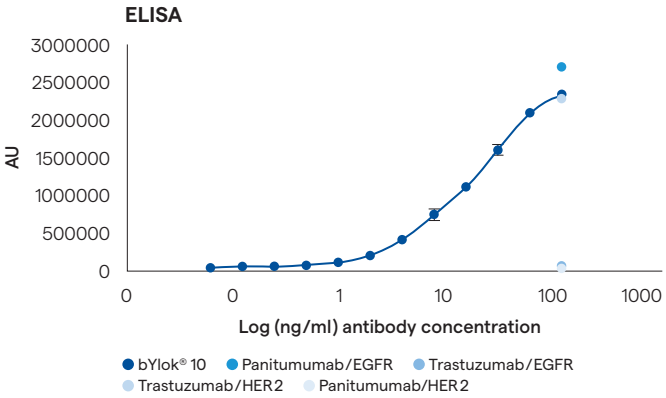

Figure 9
Binding affinities of trastuzumab/panitumumab bsAb bYlok® 10 as determined via quantitative ELISA

To further investigate the effect of bYlok® modifications on binding kinetics, SPR was used. Data generated demonstrated that bYlok® 10 has binding affinities to HER2 and EGFR antigens which are, as expected, between the binding affinities of the parental mAbs and are comparable to KIH 10 bsAb control (Table 1). These results further suggest that the bYlok® modification does not have an effect on the binding capabilities of bsAbs compared to their KIH controls. (Note that SPR data for bYlok® 12 could not be obtained due to CD52 immobilization).

Table 1
SPR binding affinities of a bsAb generated using bYlok® modifications and KIH technology, the comparative bsAb produced using only KIH technology and the two parental mAbs

	K_D (nM)	
	HER2	EGFR
bYlok® 10	373	47
KIH 10	569	54
Trastuzumab	542	0
Panitumumab	0	157

Stability

Protein engineering approaches such as the relocation of disulphide bonds has the potential to affect protein stability, which in turn can cause aggregation. The standard method for measuring protein stability is NanoDSF, which measures the change in intrinsic fluorescence of proteins as a function of temperature and time. To determine if the introduction of the bYlok® modification affected the stability of the molecules in this study, we compared the thermal stability profiles of bYlok® 10 and the corresponding KIH control. We demonstrated that the thermal stability of bYlok® 10 was not adversely affected compared to that of control bsAb KIH 10 or the two parental mAbs (Figure 10; Table 2).

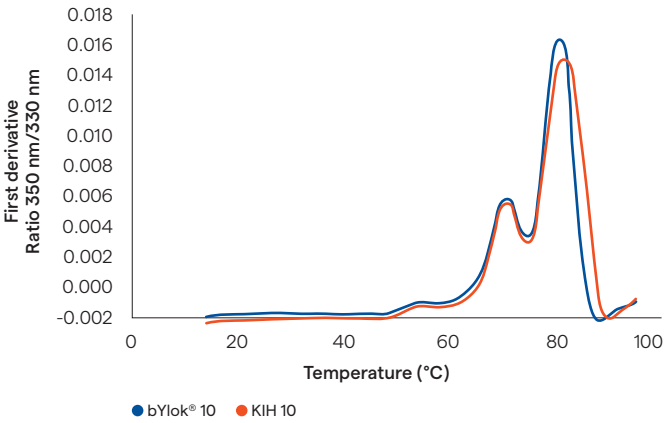

Figure 10
Comparison of the thermal stabilities of bYlok® bsAb bYlok® 10 and control bsAb KIH 10

Table 2
Thermal stabilities of bYlok® bsAb bYlok® 10, the control bsAb KIH 10 and their two parental mAbs

	Thermal stability by nanoDSF		
	T_{Agg} (°C)	T_{m1} (°C)	T_{m2} (°C)
bYlok® 10	78.0 ± 1.2	71.3 ± 0.2	80.8 ± 0.1
KIH 10	79.1 ± 0.1	70.8 ± 0.1	82.3 ± 0.01
Trastuzumab	79.0 ± 0.1	71.3 ± 0.01	80.5 ± 0.1
Panitumumab	80.5 ± 0.2	71.8 ± 0.4	81.6 ± 0.1

Immunogenicity

Immunogenicity of antibody-based therapeutics is a key factor when developing them for use in the clinic. Since bsAbs comprise heavy and light chains from two different parental antibodies, they tend to generate higher immunogenicity risk compared to each parental mAb alone. Indeed, the results from an Epibase® *in silico* HLA class II binding T-cell epitope profiling assay indicate that bYlok® bsAb, bYlok® 10, and its corresponding KIH control bsAb, KIH 10, both exhibited increased immunogenicity risks, as determined by the global DRB1 score, compared to the parental mAbs (Table 3). No change in the DRB1 score of the LCs of bYlok® 10, KIH 10 or the parental mAbs was observed; however, an increase in the DRB1 score was observed when KIH technology was utilized for HCs. When bYlok® technology was also employed, only a negligible increase in DRB1 score (<50) was observed. Similar data were obtained for bYlok® 12 (not shown). These results suggest that the main contributor to the increased immunogenicity risk in bYlok® bsAbs and KIH bsAb controls is the KIH technology.

Downstream processing

Downstream processing of bsAbs can be challenging due to the similarity in properties between incorrectly and correctly assembled heterodimeric species. In some cases, extensive adaptations and iterative purification steps, from more than one cell line, are required to purify bsAbs with optimum yields and purities. In an analysis of bYlok® bsAb, bYlok® 9 (alemtuzumab/panitumumab bsAb), it was demonstrated that standard downstream purification methods reduced aggregates and HCP levels to an acceptably low level, while increasing overall IgG purity to 97% (Table 4). These data suggest that bsAbs with bYlok® modifications can be purified using standard downstream protocols. We hypothesize that the recovery of high-purity bYlok® bsAbs using standard downstream protocols is possible because bYlok® technology confers a minor structural modification, driving the formation of bsAb formats that are closer to natural mAbs.

Table 3
Immunogenicity measured by global DRB1 score of bYlok® bsAb bYlok® 10, the control bsAb KIH 10 and their two parental mAbs

Epibase® *in silico* HLA Class II binding T Cell epitope profiling platform for the assessment of the immunogenicity risk					
	Global DRB1 Score				
	LC	LC	HC Knob	HC Hole	Full mAb/BsAb
bYlok® 10	49.3**	**533.3***	678.7**	**505.1***	1766.4
KIH 10	49.3**	533.3*	678.7**	491.9*	1753.2
Trastuzumab	533.3*		330.4*		863.7
Panitumumab	49.3**		535.2**		584.5

*Chains derived from trastuzumab **Chains derived from panitumumab **BOLD** – Chains containing bYlok® modification

Table 4
Purity analysis of a bsAb generated with bYlok® modifications and prepared using standard downstream processing methods

bYlok® 9	GPC		Caliper NR	HCP ELISA
	Aggregates (%)	Fragments (%)	IgG purity (%)	HCP (ng/mg)
PrA affinity	10.3	0.3	93.1	473.4
Purified product	0.8	Not detected	97.1	8.0

Conclusions

The data presented in this technical note demonstrate that bsAbs generated using Lonza's bYlok® modification display a high percentage (>95%) of correct HC-LC pairing compared to controls, significantly reducing HC-LC mispairing issues. Analytical characterization studies show that the bYlok® modification does not adversely affect CQAs such as titer, binding affinity, thermal stability, or predicted immunogenicity. Additionally, bYlok® bsAbs have a minor modification to the natural mAb design and can be purified to acceptable levels using standard downstream purifications protocols, indicating that bYlok® bsAbs can be processed using existing mAb manufacturing workflows.

In summary, Lonza's bYlok® bispecific pairing technology offers a novel solution for addressing the industry-wide HC-LC mispairing issue. This easy-to-implement technology facilitates the development of high-quality IgG-like bsAbs for use in pre-clinical and clinical studies.

References

[1] Clinicaltrials.gov Database Search for Clinical Trials with bispecific antibodies | Recruiting, Not yet recruiting, Active, not recruiting, Completed, Enrolling by invitation Studies https://clinicaltrials.gov/ct2/results?term=bispecific+antibodies&-Search=Apply&recrs=b&recrs=a&recrs=f&recrs=d&recrs=e&recrs=m&age_v=&gndr=&type=&rslt=

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Lonza Edition No. 08-2023