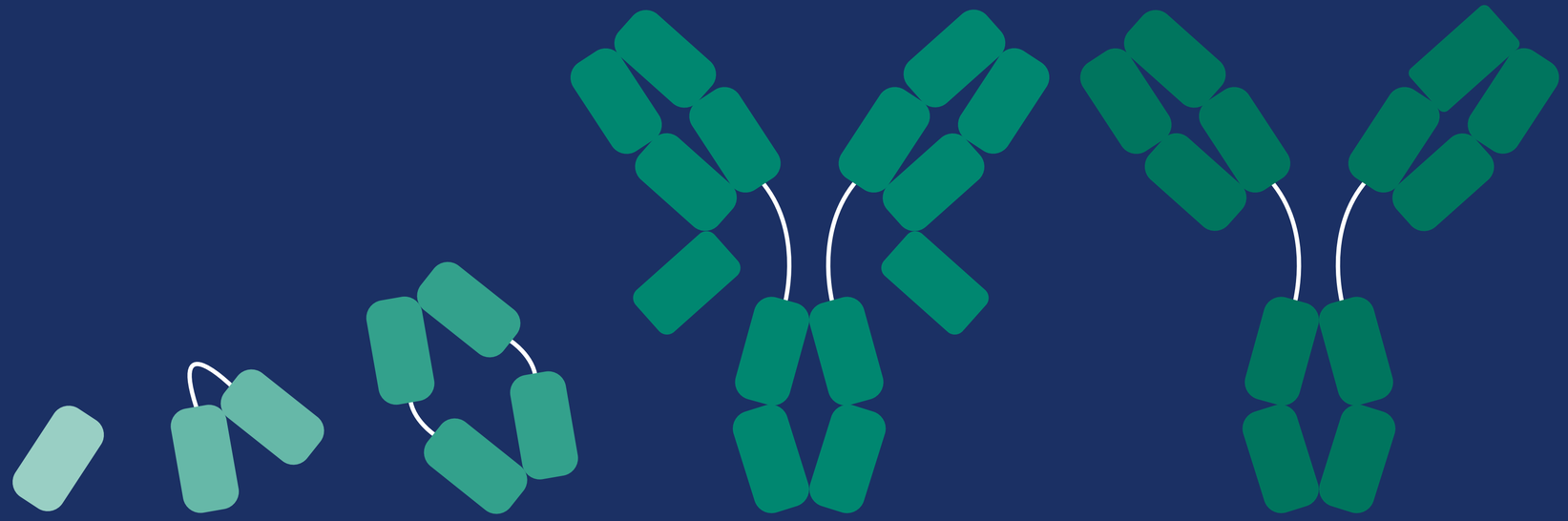
eBook

Purification of antibody therapeutics

Approach for diversified pipeline of multispecific antibodies and antibody fragments



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Introduction

Introduction

Antibody therapeutics are the largest class of biotherapeutics, and their development has been ongoing for decades. The first antibody therapeutic drug, which treated transplant rejection, was approved in 1986. Over the years, development beyond traditional monoclonal antibodies (mAb) has increased, and today, we see about a quarter of the antibody molecules in the development pipeline being antibody variants such as multispecific and bispecific antibodies, antibody fragments, and antibody drug conjugates.

A few treatments using bispecific antibodies and antibody fragments are approved drugs. These approvals in combination with the molecular pipeline and United States Food and Drug Administration (FDA) publishing development guidelines tells us that the antibody variants will continue to be an important biopharmaceutical along with traditional monoclonal antibodies (mAb).

Scientific progress and protein engineering capabilities have unleashed a large variation in different types of antibody variants and have enabled the development of new treatments for different indications. Addressing manufacturability already in development is key to allow for safe and efficient manufacturing over the complete workflow. Manufacturing platform approaches conveniently used for many traditional mAbs are being adopted or tweaked to fit the respective molecule. In the following articles, you will learn about considerations and tips for developing protocols for antibody variants.

Josefin Bolik
Global Product Manager, antibody capture
Cytiva

02

Cytiva Research and Development department supports antibody variant processes

Cytiva Research and Development department supports antibody variant processes

Antibody variants can, in some cases, be purified using conventional purification protocols, whereas other variants need new ligands or new base matrixes or have different regulatory requirements and application needs. Cytiva looks at the pipeline of molecules to get an understanding for the development requirements before our experienced development team starts to work on new innovations. We talked with Eva Heldin, section manager of antibody application in R&D at Cytiva, about how they support clients in developing and manufacturing antibody variant therapeutics.

Eva Heldin
Section Manager of antibody application in R&D
Cytiva

Q **How are chromatography protocols affected when working with antibody variants?**

A Production of monoclonal antibodies commonly includes platform processes to make the development and manufacturing standardized and process development quicker. With the diverse molecular pipeline, an existing platform may not work, and there is a need to develop new platforms. Depending on the domains of the target molecule and the impurity profile, protein A chromatography may be the technique of choice. However, there are also molecules where other affinities must be used for good separation and high purity.

Common mAb process-related impurities must be addressed in the polishing steps along with product-related impurities that are present for antibody variants. Therefore techniques like multimodal chromatography (MM) or hydrophobic interaction chromatography (HIC) may be needed instead of or in additional to conventional ion exchange chromatography (IEX). Multimodal (or mixed mode) resin interactions includes both ionic exchange and hydrophobic interactions and have been shown efficient for aggregate removal.

Q **Looking at the manufacturing aspects, what considerations do you see for antibody variants?**

A The demands for manufacturing are largely the same as for conventional mAbs. Impurities must be removed in the chromatography steps to specified levels, and the resins must withstand cleaning agents to avoid bioburden incidents. The target molecule needs to be stable over the process and in storage to reduce aggregation and degradation otherwise low yield and inefficient processes will result. Resins must be available and applied in research-scale formats to process development and manufacturing formats for predictable and smooth scale-up.

In addition, for antibody variants, molecules must be engineered for manufacturability to allow for efficient production. The design of the molecule can improve the ability to purify the target molecule from product-related impurities using available chromatographic techniques. The molecules are also designed to reduce formation of product-related impurities thereby making the process more efficient. Analytical methods must be considered already in protein engineering to avoid unpleasant surprises in late-stage process development.

Q **How is Cytiva adapting to support antibody variant processes?**

A We are looking into how we can support the molecular pipeline and listen to our clients to hear about their needs and challenges. Processes for antibody variants may benefit from new resins, formats, or protocols. As an example, Cytiva recently launched the MabSelect™ VL protein L resin with affinity for the variable kappa light chain and MabSelect™ VH3 protein A resin that interacts only with the variable heavy (VH) chain. This provides a great complement to traditional protein A and are additional tools in the antibody purification toolbox. Compared to its predecessor, MabSelect™ VL has improved alkaline stability and provides high dynamic binding capacity. MabSelect™ VH3 affinity resin uses an engineered protein A ligand that interacts only with the variable heavy chain of the VH3 sequence family of the human antibody. These resins allow for separation of product-related impurities and takes pressure off the subsequent polishing steps.

Our applications team is purifying different types of antibodies to develop useful insights and share knowledge. To improve process understanding further, mechanistic modeling methods can be considered. Mechanistic modeling simulates and predicts chromatographic behavior and experiments *in silico*. Using mechanistic modeling helps in the selection of resins and in the optimization of process parameters. It also speeds up process development.

Antibody purification by chromatography

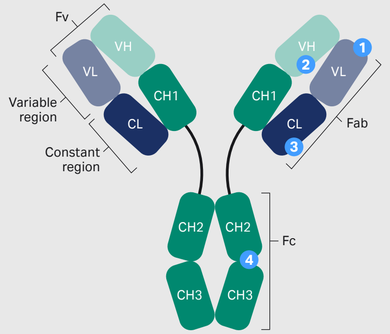
A typical antibody purification platform starts with an affinity capture step followed by one or two polishing steps.

Capture

Affinity chromatography

Select affinity resins for your monoclonal antibody, multispecific antibody, or antibody fragments based on the domains in the target molecule and the impurity profile.

* *Variable region of a human antibody's kappa light chain subclasses 1, 3, and 4 interacts with protein L.*
† *VH3 sequence interacts with protein A.*



Protein L ligand
1 MabSelect™ VL resin ▶

3 KappaSelect resin ▶
3 LambdaFabSelect resin ▶

Protein A ligand
2 4 MabSelect PrismA™ resin ▶
2 4 Fibro™ PrismA fiber units ▶
2 MabSelect™ VH3 resin ▶
4 MabSelect SuRe™ resin ▶
4 MabSelect SuRe™ LX resin ▶

Polishing 1

Cation exchange chromatography (CIEX) or multimodal CIEX

Select Capto™ resins ▶

Polishing 2

Anion exchange chromatography (AIEX) or multimodal AIEX

Select Capto™ resins ▶

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03

Optimization of a two-step purification method for bispecific antibodies

Optimization of a two-step purification method for bispecific antibodies

Collaborative study to develop a downstream process for a bispecific antibody (bsAb)

Alligator Bioscience AB develops antibody-based pharmaceuticals for cancer treatment, specializing in mono- and bispecific antibodies for tumor-directed immunotherapies. The company is active in the early stages of drug development, from early concepts to clinical phase II studies.

This article shows the results from our collaboration with Alligator to develop an efficient downstream purification process for a cancer immunotherapy bispecific antibody (bsAb). The main findings of the study were:

- Exchanging MabSelect SuRe™ resin to the more recently developed MabSelect PrismA™ resin increased the loading capacity by 53% (using a load of 70% of the Q_{b10} for MabSelect PrismA™ resin).
- The stability of the bsAb with respect to pH and salt limited the window of usable conditions for the polishing step, but conditions could be optimized for purification on Capto™ adhere resin in both bind/elute (B/E) and flow-through (FT) mode.
- The evaluated conditions for elution from Capto™ adhere resins were considered good conditions for the B/E application with respect to the stability of the bsAb.
- Using the same conditions for the polishing step—but in FT rather than for elution in B/E mode — improved yield significantly (> 90% yield) with acceptable HCP levels and protein A clearance.
- The improved process increases both product yield and process economy.

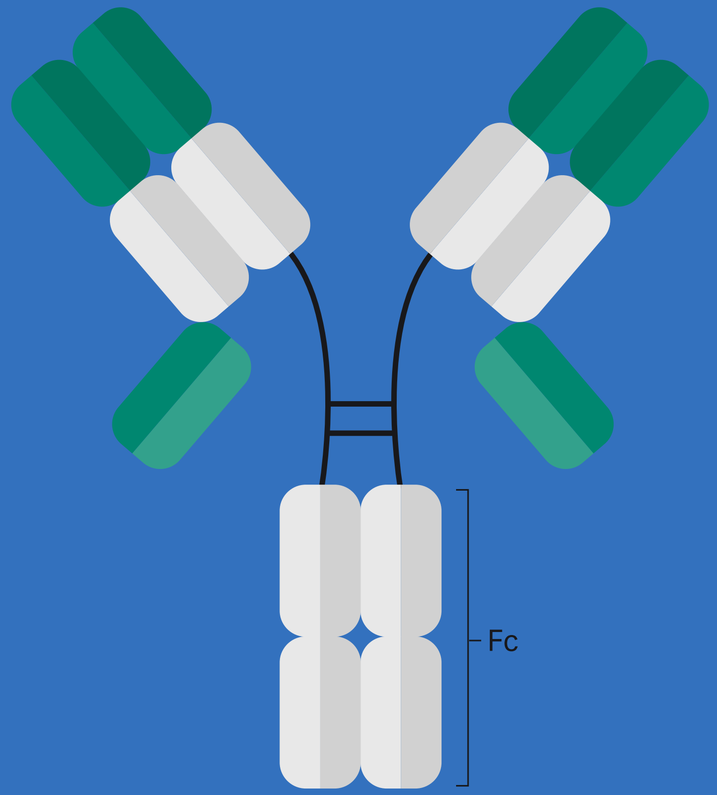


Fig 1. The bsAb format for the antibody used in this study. A recombinant protein is attached c terminally to the light chain (light green), thereby creating a second binding domain. The Fc part of the antibody is a fully human IgG1 without any further modifications.

Introduction

Bispecific antibodies (bsAbs) have gained increasing interest over recent years because they can bind two antigens, offering unique modes of action compared to monoclonal antibodies. New and improved antibody bioprocesses and recombination techniques have contributed to a surge in the numbers of bispecific antibody constructs currently in clinical trials. As of 2019, one bsAb has been approved and 57 bsAbs are in clinical trials (1).

There are many types of bsAb formats with two different sites to target different antigens. This dual specificity of bsAbs means that they can, for example, bind to target cells using one antigen-binding site and recruit other cells or molecules with the second antigen-binding site.

When it comes to purification of Fc-containing bsAbs, manufacturers typically use a platform approach based on protein A chromatography for capture of the antibody. The subsequent polishing steps are added to obtain a product of desired final product quality.

This article describes the further development of a two-step purification of a bsAb. The original capture step using MabSelect SuRe™ resin was changed to using MabSelect PrismA™ resin to increase the process economy. The bsAb used in this study is an anti-tumor, human IgG1 symmetric bispecific antibody, see Figure 1 for the bsAb format.

Current purification process based on MabSelect SuRe™ resin

The original downstream process for purification of the bsAb was based on a platform commonly used for regular monoclonal antibodies (mAb) except that the traditional low pH virus inactivation step was replaced by a solvent detergent step due to the pH sensitivity of the antibody. To immediately increase the pH after elution from MabSelect SuRe™ resin, the elution pool was collected in a vessel pre-prepared with 200 mM sodium phosphate buffer, pH 6.9. Figure 2 shows a schematic view of the original process.

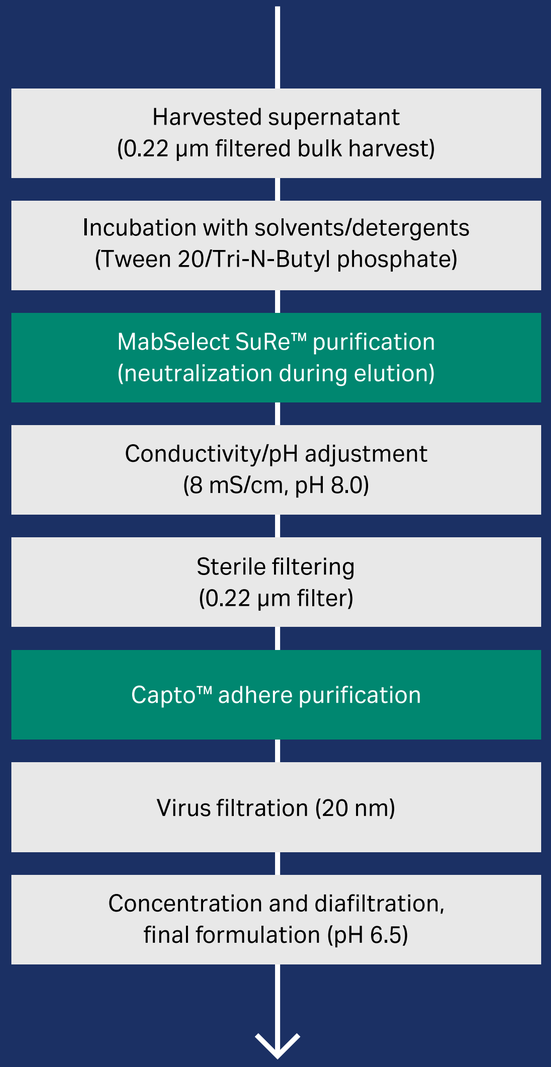


Fig 2. Original downstream process for the purification of the bsAb.

Evaluating capture of the bsAb with MabSelect PrismA™ resin

Despite the high yield and purity obtained using MabSelect SuRe™ resin in the original process, our more recent protein A resin — MabSelect PrismA™ — was evaluated as a potential replacement, with the aim of further improving the process economy.

As early research material with a non-optimized cultivation yield was used as input for the experiment, the titer of the clarified harvest was low (0.8 g/L). The residence time (RT) was therefore set to 4 min rather than 6 min as recommended for MabSelect PrismA™ resin to shorten the step during process development. Frontal analysis at 4 min RT showed that the dynamic binding capacity (DBC) at 10% breakthrough (Q_{b10}) for MabSelect SuRe™ and MabSelect PrismA™ resins was 42 g/L and 64 g/L of resin, respectively.

A modified capture step using MabSelect PrismA™ resin

Viral inactivation was performed with Tween 20 and tri-n-butyl phosphate at neutral pH. The supernatant provided by Alligator containing the target bsAb was treated for 90 min with the viral inactivation solution at room temperature. After inactivation, we adjusted the pH to 7.0 with 0.5 M NaOH and filtered the virus inactivated supernatant through a 0.22 µm filter.

Our goal for testing MabSelect PrismA™ resin was to investigate if we could increase the loading capacity compared to MabSelect SuRe™ resin.

Based on the breakthrough capacities, the load was set to 45 g of bsAb/L of MabSelect PrismA™ resin corresponding to 70% of Q_{b10}.

Columns:	Tricorn™ 5/100 (CV = 2 mL); Tricorn™ 10/100 (CV = 7.4 mL); HiScale™ 16/20 (CV = 24 mL) packed with MabSelect PrismA™ resin
Sample:	Cell culture supernatant containing bsAb and added viral inactivation solution
Column load:	70% of Q_{b10} (45 g/L)
Equilibration:	10 mM sodium phosphate, 150 mM NaCl, pH 7.0, 3 CV
Wash:	10 mM sodium phosphate, 150 mM NaCl, pH 7.0, 7 CV
Elution:	100 mM glycine-HCl, pH 3.2, 8 CV (eluate peak collected from 90 to 72 mAU)
Re-equilibration:	10 mM sodium phosphate, 150 mM NaCl, pH 7.0, 3 CV
Flow velocity:	4 min RT
Cleaning in place (CIP):	0.5 M NaOH, 3 CV, 5 min RT
Detection:	A_{280} nm
System:	ÄKTA pure™ 25

Runs were repeated at different scales, starting with a Tricorn™ 5/100 column (CV = 2 mL) packed with MabSelect PrismA™ resin, which resulted in a bsAb yield of 87%. Scale-up to a wider Tricorn™ 10/100 column (CV = 7.4 mL) gave a yield of 91%. Finally, as more material was needed for the polishing step, the capture step was performed in a larger HiScale™ 16/20 column (CV = 24 mL), which gave a yield of 95%. Increasing yield when increasing the size of the column is typically observed in downstream processing.

Eluted fractions were collected into a 50 mL tube prepared with 0.3 to 0.4 CV of a 200 mM sodium phosphate neutralization buffer, pH 6.9 to maintain bsAb integrity.

The conditions for the improved capture step are detailed on following pages.

Evaluation of Capto™ adhere resin polishing, bind/elute mode

We first performed the polishing step in B/E mode with Capto™ adhere resin using the conditions from the original downstream process. When using this approach, the target molecule binds to the ligand coupled to the resin through mixed-mode interactions. Subsequent changes in pH releases the molecule from the resin to allow collection of the eluate.

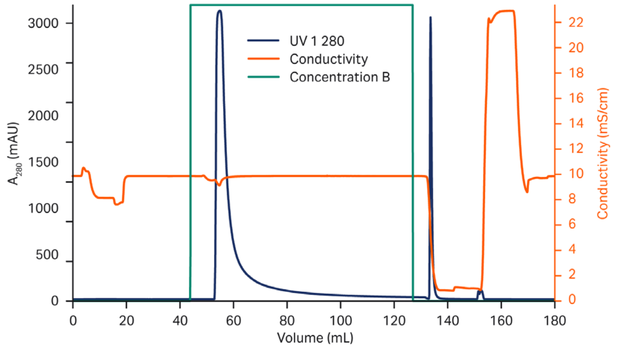
The resin used was Capto™ adhere packed in a Tricorn™ 5/200 column with a 20 cm bed height. MabSelect PrismA™ resin-purified bsAb was buffer exchanged to 10 mM sodium phosphate, 80 mM sodium chloride, pH 7.4. Sample was loaded at 27 mg of bsAb/mL resin. Eluate between 324 and 297 mAU was collected for analysis. Yield was calculated to 67% while levels of HCP (4 ppm), leached protein A (2 ppm), and aggregate (0.4%) were all well below the specifications set at the beginning of the study (Fig 3).

Stability test of bsAb in the Capto™ adhere resin polishing step

To improve the Capto™ adhere resin step, we evaluated the stability of the bsAb using the extreme buffer conditions in terms of pH and salt. From that study, we identified pH range between pH 5 and 7.1 in the presence of 40 to 200 mM NaCl where stability of the bsAb could be expected. The purified bsAb (20 mg/mL) was diluted on a 96-well plate to a concentration of 4 mg/mL with nine different buffer conditions within the range above. Samples were incubated for 2 h on a shaker.

The yield was estimated by comparing areas of the start sample to areas from samples analyzed by size exclusion chromatography (SEC). We observed no effect on aggregate level or yield at the selected conditions (Fig 4).

Column: Tricorn™ 5/200, 20 cm bed height (CV = 3.98 mL)
Resin: Capto™ adhere
Sample: MabSelect PrismA™ eluate containing bsAb
Sample load: 27 mg/mL resin
Elution buffer 20 mM sodium acetate, 80 mM sodium chloride, pH 5.5
Flow velocity and flow rate: 150 cm/h, 0.49 mL/min, RT 8 min
Detection: $A_{280\ nm}$
System: ÄKTA pure™ 25



	Bind elute mode
Yield	67%
HCP	4 ppm
Protein A	2 ppm
Aggregate	0.4%

Fig 3. Chromatogram of the Capto™ adhere resin polishing step in B/E mode. The table shows that 67% yield of the bsAb was achieved.

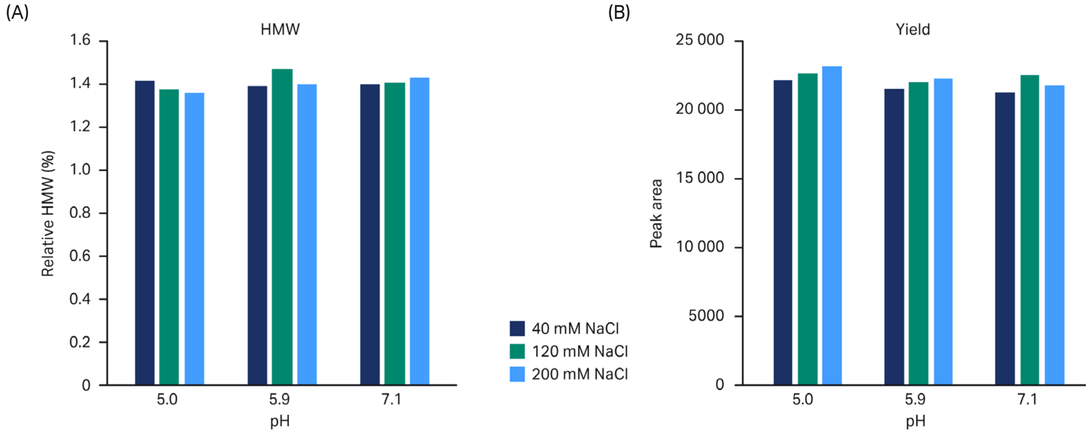


Fig 4. Comparison of (A) aggregate levels and (B) yield of bsAb treated for 2 h in phosphate buffers at: pH 5.0, 5.9, and 7.1 and with three salt concentrations: 40, 120, and 200 mM NaCl.

Polishing step: screening experiments

Capto™ adhere resin can also be used in flow-through (FT) mode where impurities bind to the resin while the target molecule is collected in the chromatography flowthrough. An FT process usually gives better process economy as the load can be significantly higher than for B/E mode.

To improve process yield and process economy, we evaluated a potential FT method using 96-well PreDictor™ plates filled with 6 µL of Capto™ adhere resin. The process development was designed to find optimal chromatographic conditions under non-binding conditions, with high product yield and HCP levels below target as high-molecular weight (HMW) content was already within specifications.

The equilibration buffers tested were composed of 100 mM sodium phosphate with pH from 5.0 to 7.1 and NaCl concentrations from 40 to 240 mM. The wells were equilibrated with 3 × 200 µL of equilibration buffer, and sample load was set to 157 mg/mL resin; a load that could also be applied in a FT step. The plate was then incubated on an orbital shaker for 60 min at 1100 rpm and then emptied into a UV plate with centrifugation at 500 × g for 1 min. The absorbance was read by using a UV reader.

Results for the binding capacity of the bsAb are shown in the heatmap in Figure 5A and HCP concentration is shown in Figure 5B.

From the plate experiments, we observed that the HCP clearance was improved in the lower pH/salt-range. However, in the earlier stability screening, we observed that below these conditions, the bsAb showed stability issues. Thus, we decided to compare the apparent best condition from the PreDictor™ plate study (40 mM NaCl, pH 5.0) with a more robust condition from a process perspective (80 mM NaCl, pH 5.5). The pH 5.5 condition is identical to the elution condition used in the original process where Capto™ adhere resin is operated in B/E mode.

Column studies of polishing step with Capto™ adhere resin in flow-through mode

The sample from the MabSelect PrismA™ resin capture step was buffer exchanged to loading buffer before start and Capto™ adhere resin chromatography at the two conditions stated above.

Yield from the polishing was over 90% for both FT methods. A significantly higher yield of 94% was observed for FT mode compared to the original B/E process (see Fig 3) with a yield of 67%.

96-well plate: PreDictor™ brand Capto™ adhere, 6 µL
Sample: MabSelect PrismA™ purified bsAb, 157 g/L resin
Equilibration buffers: 100 mM sodium phosphate, pH 5.0 to 7.1; NaCl 40 to 240 mM

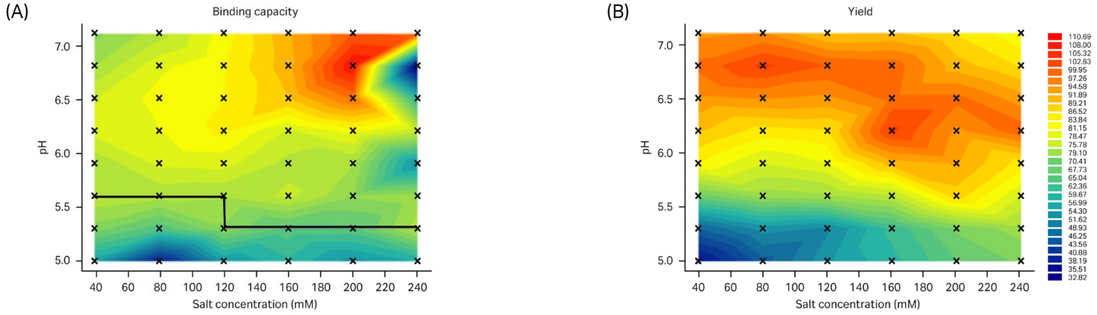


Fig 5. Binding capacity of bsAb (A) where blue indicates the lowest binding capacity (i.e., most suitable for an FT application) and red the highest. The marked area shows the conditions where HCP levels are < 100 ppm, which was the threshold value set for the study. The yield of HCP in percent is shown in (B) where blue gave the best clearance of HCP. From the plate experiment, two conditions were selected: 40 mM NaCl, pH 5.0 and 80 mM NaCl, pH 5.5 for FT in column, both having good clearance of HCP and low binding capacity for bsAb.

Column: Tricorn™ 5/20 (CV= 0.47 mL)
Resin: Capto™ adhere
Sample: MabSelect PrismA™ purified bsAb, 530 g/L resin
Equilibration buffer: (A) 20 mM sodium acetate, 80 mM sodium chloride, pH 5.5
(B) 100 mM sodium phosphate, 40 mM NaCl, pH 5.0
Flow rate: 0.12 mL/min, RT 4 min
System: ÄKTA pure™ 25

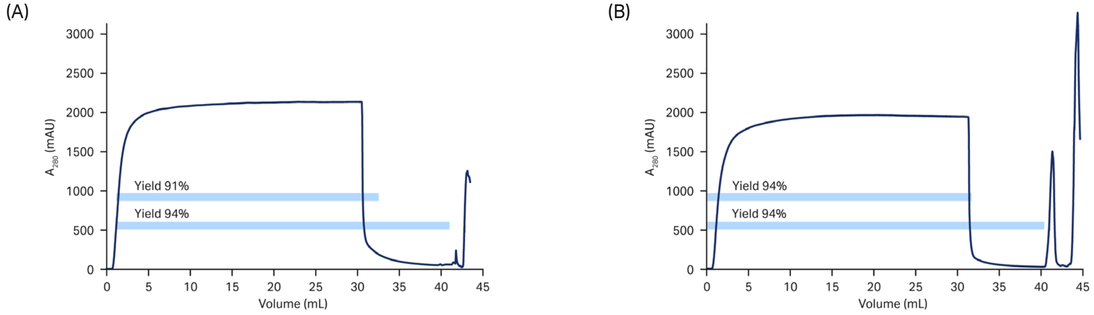


Fig 6. Polishing purification in FT mode of bsAb on Capto™ adhere resin using (A) 20 mM sodium acetate, 80 mM sodium chloride, pH 5.5 and (B) 100 mM sodium phosphate, 40 mM NaCl, pH 5.0. The lower bars show yields with the wash fraction included while the upper bars show yield without wash fractions.

Analysis of aggregates, HCP, and leached protein A

Size exclusion chromatography (SEC) using a Superdex™ 200 Increase 10/300 GL column (CV = 24 mL) was used to analyze aggregate levels.

The aggregate content in the start sample from the MabSelect PrismA™ resin capture was already low at about 1%. The Capto™ adhere resin steps succeeded in further reducing the aggregate content to < 1%. (Fig 7). The aggregate content for selected fractions in the respective chromatography runs are shown in the bars in each chromatogram and are within the specifications set for this study.

The concentration of HCP in start samples and the eluate from the B/E elution buffer run, in pooled fractions from the FT buffer run, and in selected fractions from the FT buffer run were analyzed using Cygnus technologies third generation CHO HCP ELISA reagents adapted to Gyrolab® workstation (Gyros Protein Technologies Group), (Fig 8).

Figure 8A clearly shows an increasing HCP concentration (ng HCP/mL) with loading the FT buffer run at pH 5.5 (11 to 110 ppm). In the load at pH 5.0 (Fig 8B), the HCP concentration is more evenly distributed through the sample loading step. The HCP amounts were about the same in the two pools collected — 48 and 53 ng HCP/mg bsAb for pH 5.5 and 5.0, respectively — which is below the original specification of 100 ng HCP/mg bsAb set for this study. Also, the wash fraction may be included in the pool as this fraction made only a minor contribution to the HCP level and has no significant influence on protein A or HMW content.

The purification results for the bsAb from Alligator are summarized in Table 1 and 2. Comparing mode of operation, (FT vs B/E) shows improved yield at the cost of lower HCP clearance. As similar results are obtained at both FT conditions, we conclude that the FT approach is robust. To avoid stability issues, the step should be operated at a pH above 5.0, that is the conditions used for elution in B/E mode
is preferred also in FT.

Column: Superdex™ 200 Increase 10/300 GL (CV = 24 mL)
Resin: Capto™ adhere
Buffer: 100 mM sodium phosphate, 300 mM NaCl, pH 6.8

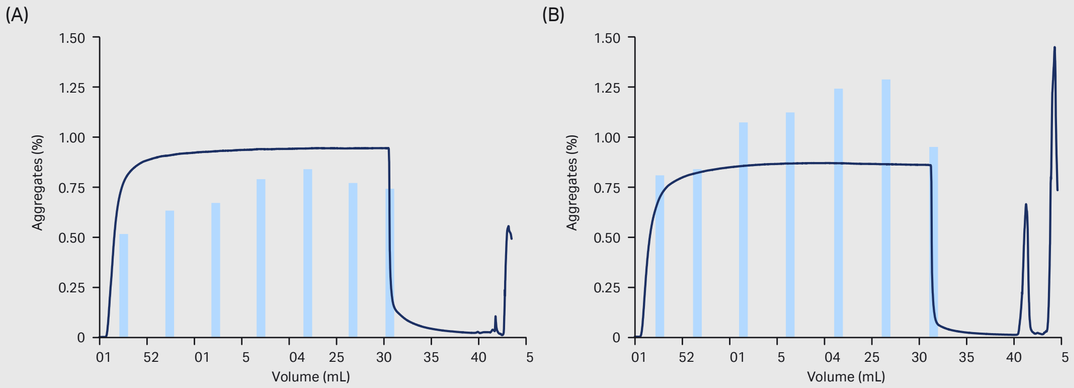


Fig 7. Aggregate analysis using size exclusion chromatography from (A) the Capto™ adhere resin FT run at 20 mM sodium acetate, 80 mM sodium chloride, pH 5.5 and (B) 100 mM sodium phosphate, 40 mM NaCl, pH 5.0.

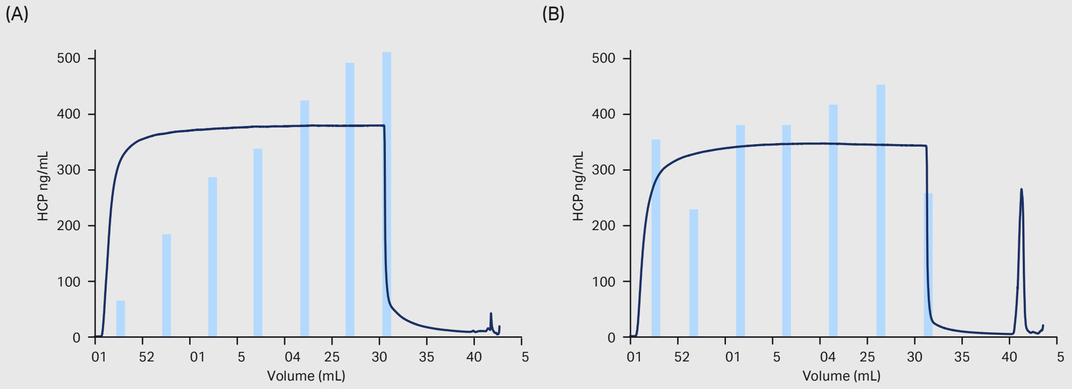


Fig 8. HCP concentrations in selected fractions from the (A) 20 mM sodium acetate, 80 mM sodium chloride, pH 5.5 (B) 100 mM sodium phosphate, 40 mM NaCl, pH 5.0 were analyzed by Gyrolab® workstation (Gyros Protein Technologies Group).

Table 1. Results from the capture step with MabSelect PrismA™ resin and polishing step with Capto™ adhere resin

Capture step with MabSelect PrismA™ resin	**Load 70% of Q_{b10} (45 g/L resin)**		
Yield	93%		
In elution pool:	HCP 131 ppm Protein A: 11 ppm Aggregate: 0.8%		
Polishing step with Capto™ adhere resin	**B/E**	**FT pH 5.5**	**FT pH 5.0**
Yield	67%	91%	94%
Purified/mL resin	18 mg	485 mg	495 mg
HCP	4 ppm	48 ppm	53 ppm
Protein A	2 ppm	3 ppm	3 ppm
Aggregate	0.4%	0.7%	1.1%

Table 2. Overall yield

Yield MabSelect PrismA™ resin	**93%**	**93%**	**93%**
Capto™ adhere resin	B/E	FT pH 5.5	FT pH 5.0
Yield	67%	91%	94%
Overall yield	62%	85%	87%

Conclusions

This study concluded the following:

- Switching the capture step from MabSelect SuRe™ resin to MabSelect PrismA™ resin allowed a 53% increase in sample load at 4 min RT. This could probably be further improved at longer RT as the breakthrough curve indicated faster kinetics on MabSelect PrismA™ resin compared to MabSelect SuRe™ resin.
- The optimized conditions for polishing were considered suitable for the FT application in respect to stability of the bsAb.
- The conditions applied in FT polishing mode also improved yield significantly (above 90% yield) but still with acceptable HCP and protein A clearance. This provides the potential for improved process economy.
- The two conditions compared for FT polishing mode resulted in similar yield and purity, and we have good reason to believe that the FT mode and conditions are robust.

References

1. Suurs FV, Lub-de Hooge MN, de Vries EGE, de Groot DJ. A review of bispecific antibodies and antibody constructs in oncology and clinical challenges. *Pharmacol. Ther.* 2019;201:103-119. doi:10.1016/j.pharmthera.2019.04.006

News

PrismA ELISA ligand leakage detection kit specifically designed for use with MabSelect PrismA™ resins

Web article

How to use MabSelect PrismA™ for antibody purification

04

Capture of bispecific antibodies and removal of product-related impurities

Capture of bispecific antibodies and removal of product-related impurities

Using MabSelect™ VL protein L affinity resin for the capture of bsAb and removal of challenging product-related impurities.

The biotherapeutics pipeline is becoming increasingly diverse as antibody variants such as bispecifics, conjugates, and fragments move through preclinical stages to commercial manufacturing. Many of these variants, especially asymmetric bispecific antibodies, are prone to aggregation or to forming product-related impurities such as homodimers and half antibodies during cell culture. The similarities between these impurities present extra challenges for downstream processes, especially post-capture. One potential solution is to initiate polishing at the capture step using differences in avidity. Here we present data on applications using MabSelect™ VL protein L resin with novel selectivity to purify challenging entities.

Introduction

MabSelect™ VL affinity resin uses a protein L ligand with strong affinity for the variable region of the human antibody kappa light chain (Fig 1). The resin offers high productivity for affinity capture of bispecific antibodies (bsAb) and antibody fragments containing the kappa light chain. It also offers a good capture alternative for antibody variants that do not bind to protein A. This resin has substantially improved dynamic binding capacity (DBC) and alkaline stability compared to its predecessor, making it well suited for cost-efficient capture of antibody variants. MabSelect™ VL resin allows for good resolution of product-related impurities in the capture of bispecific antibodies, and it provides a tool for efficient purification of antibody variants to high purity. Here we present a typical application for separation of bispecific antibodies from mispaired homodimers.

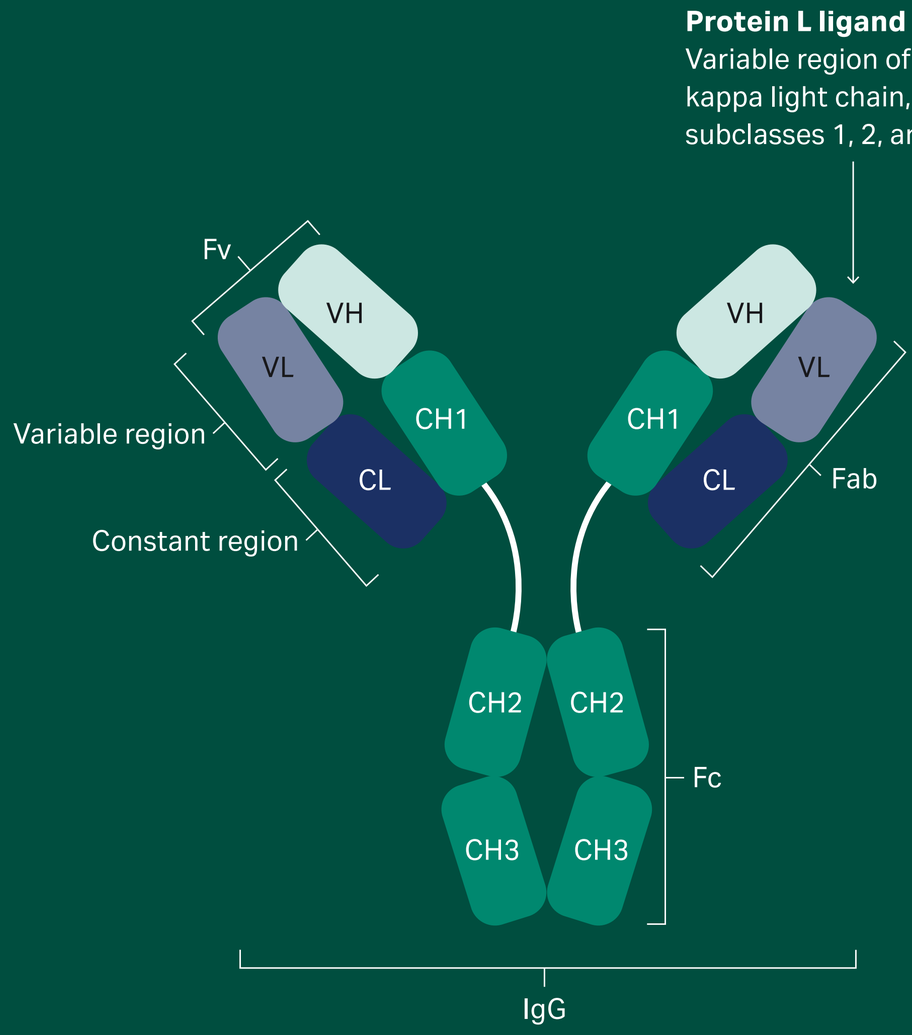


Fig 1. The structure of an antibody. The arrow indicates where protein L interacts with the antibody.

A bsAb with four chains can have various combinations of the heavy- and light-chain regions, as well as half antibodies that represent the desired bsAb and product-related impurities (Fig 2). The type and amount of product-related impurities depend on the assembly technique, such as Fc heterodimerization used to produce asymmetric molecules, knobs-into-holes technology, CrossMAb technology, or light-chain method used for correct pairing of light and heavy chains.

Separation of bsAb and mispaired homodimers by pH elution

MabSelect™ VL chromatography resin is designed to bind the kappa class light chains of IgG monoclonal antibody variants and can be used to separate the target molecule from mispaired versions in the capture step. We evaluated a separation based on kappa and lambda light chains and on differences in avidity using a feed composition comprised of a kappa light chain (Trastuzumab kappa class 1 Anti-HER2 light chain), lambda light chain (Avelumab lambda class 2 Anti PDL1 light chain), and a FC chain (Anti-HER2 heavy chain 1 and 2) (Thermo Fisher Scientific). The different chains were targeted to express in a ratio of 30:30:40 respectively.

Initially, the elution pH for the bispecific kappa-lambda heterodimer and the mispaired kappa homodimer was determined with a gradient elution from pH 5 to pH 2.5 by loading 5 mg of the bispecific cell culture on a Tricorn™ 5/100 column packed with MabSelect™ VL resin (Fig 3). The fractions were collected during the elution gradients and analyzed with liquid chromatography–mass spectrometry (LC-MS) to confirm the separation of the different entities. Mispaired lambda homodimers do not bind to the column as they do not contain any kappa light chains and can be found in the flowthrough. The gradient elution from pH 5 to pH 2.5 separates mispaired kappa homodimer and the kappa-lambda heterodimer bispecifics due to differences in avidity.

To further show the performance of MabSelect™ VL resin in separation of the bispecific kappa-lambda light chain mAb heterodimers from mispaired homodimers in cell culture harvest, the efficacy of a stepwise pH elution was tested. Step elutions were performed for different column formats packed with MabSelect™ VL resin. A 5 mg sample of bispecific cell culture harvest was applied to a Tricorn™ 5/100 column, a 2.5 mg sample was applied to a HiTrap™ 1 mL column, and a 12 mg sample was applied to a HiScreen™ column.

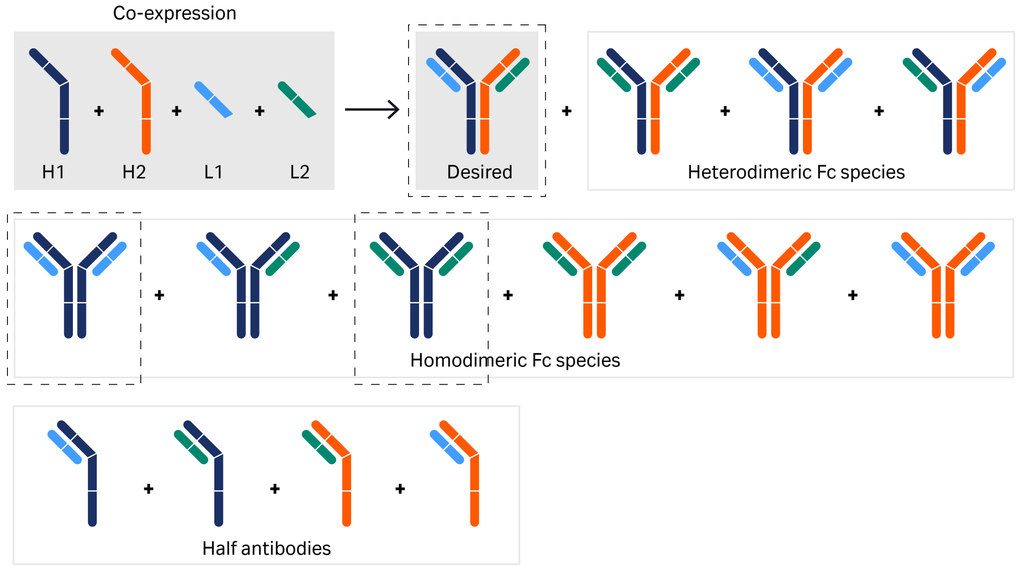


Fig 2. Possible combinations of the heavy- and light-chain regions in the expression of a bispecific mAb. The desired bispecific molecule and the mispaired homodimers are separated in the example described below.

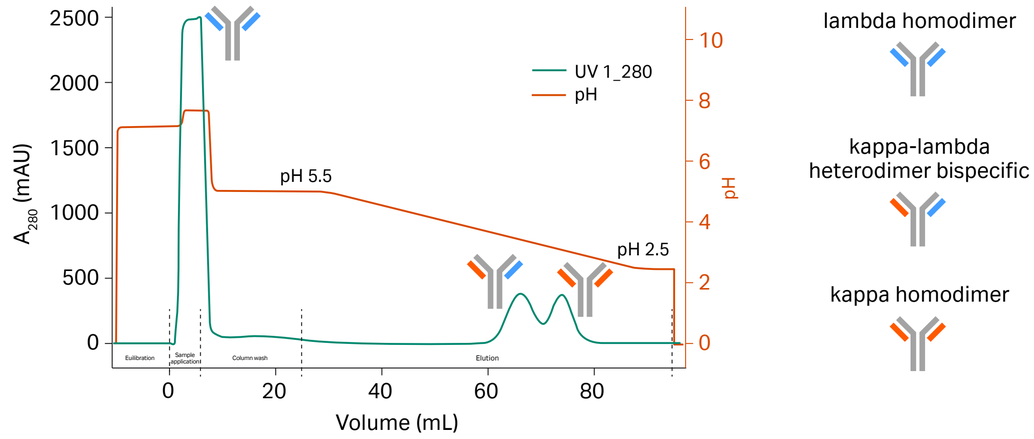


Fig 3. Gradient pH elution separates the desired bsAb from the mispaired homodimers.

Results

Gradient elution of bsAb sample

A linear gradient elution was performed using MabSelect™ VL resin packed in Tricorn™ 5/100 column to find the elution pH to be used in the step gradient runs (Fig 4). The results are presented in Table 1. The elution pH difference for MabSelect™ VL resin between the two peaks was 0.4.

Column: Tricorn™ 5/100
Resin: MabSelect™ VL
Sample: 5 mL of bsAb at a flow rate of 0.250 mL/min via a Superloop™ assembly
Equilibration: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2
Wash: 50 mM citrate pH 5.0, 10 CV at 1 mL/min
Gradient: 0 to 100% of 50 mM citrate, pH 2.5 for 20 CV at a flow rate of 1 mL/min per step, followed by 100% for 5 CV
CIP: 3 CV of 0.1M NaOH at 0.2 mL/min
System: ÄKTA pure™

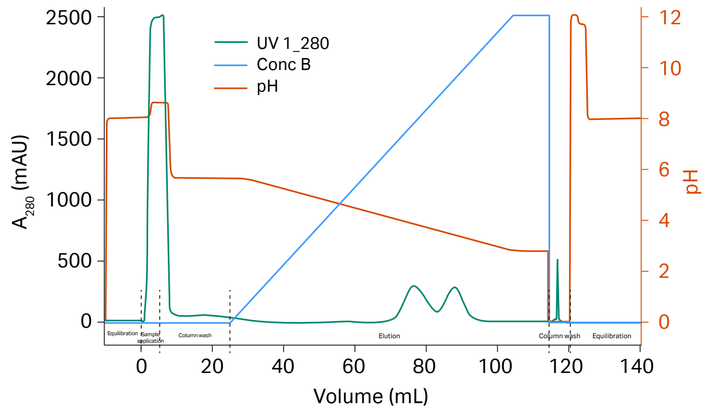


Fig 4. Chromatogram of linear gradient elution performed using MabSelect™ VL resin packed in Tricorn™ 5/100 column on ÄKTA pure™ chromatography system.

Table 1. Elution pH found in the gradient elution pH 5 to 2.5. These values will be used as step elution settings (%B) with elution buffer 50 mM citrate, pH 2.5.

Resin	Column	Elution pH peak1	%B peak 1	Elution pH peak 2	%B peak 2
MabSelect™ VL	Tricorn™ 5/100	3.5	65	3.1	79

Step elution of bsAb sample

A step elution was then performed according to the elution pH obtained from the gradient elution (Table 1). Solutions of 50 mM citrate with pH 3.5 and pH 3.1 were used for elution steps 1 and 2, respectively.

Figure 5 presents the MabSelect™ VL resin step elution with different column formats. The results show similar elution profiles for Tricorn™ 5/100 and HiScreen™ columns. The elution peaks for HiTrap™ 1 mL column were slightly delayed and broader compared to the other two columns. This is expected due to the smaller resin volume relative the void volume of the ÄKTA pure™ system compared to the other column formats. The relative larger void volume compared to the volume of HiTrap™ 1 mL column causes dilution of the sample. All three column formats resulted in separation of the bsAb and the kappa homodimer.

(A)
Column: Tricorn™ 5/100
Resin: MabSelect™ VL
Sample: 5 mg of bsAb cell culture harvest
Equilibration: 5 CV of 20 mM sodium phosphate, 150 mM NaCl, pH 7.2 at 1 mL/min
Wash: 10 CV of 50 mM citrate, pH 5.0 at a flow rate of 1 mL/min
Elution: step gradient with 50 mM citrate buffer, pH 3.5 and pH 3.1at 1 mL/min
CIP: 3 CV of 0.1 M NaOH at 0.2 mL/min
System: ÄKTA pure™

(B)
Column: HiTrap™ (1 mL)
Sample: 2.5 mg of bsAb cell culture harvest
Equilibration: 5 CV of 20 mM sodium phosphate, 150 mM NaCl, pH 7.2 at 0.5 mL/min
Wash: 10 CV of 50 mM citrate, pH 5.0 at 0.5 mL/min
Elution: step gradient with 50 mM citrate buffer, pH 3.5 and pH 3.1 at 0.5 mL/min
CIP: 3 CV of 0.1 M NaOH at 0.1 mL/min
System: ÄKTA pure™

(C)
Column: HiScreen™
Sample: 12 mg bsAb cell culture harvest
Equilibration: 5 CV of 20 mM sodium phosphate, 150 mM NaCl, pH 7.2 at 2.3 mL/min
Wash: 10 CV of 50 mM citrate, pH 5.0 at 2.3 mL/min
Elution: step gradient with 50 mM citrate buffer, pH 3.5 and pH 3.1 at 2.3 mL/min
CIP: 3 CV of 0.1 M NaOH at 0.47 mL/min
System: ÄKTA pure™

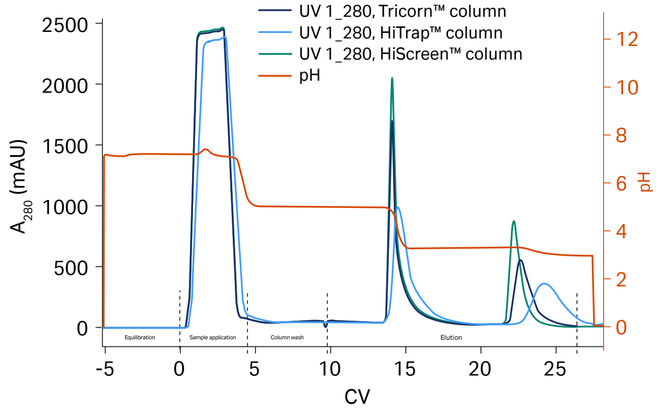


Fig 5. Chromatogram showing purification of bsAb sample using MabSelect™ VL resin packed in (A) Tricorn™ 5/100 (dark blue), (B) HiTrap™ 1 mL (light blue), and (C) HiScreen™ (green) columns and eluted in a step gradient with 50 mM citrate buffer, pH 3.5 and pH 3.1.

LC-MS analysis of elution fractions

To confirm the separation of product-related impurities such as homodimers and half-antibodies, the fractions were analyzed with LC-MS.

The samples used in the gradient and step elution runs were treated before LC-MS analysis with FabRICATOR (IdeS, Genovis AB). Fc/2 fragments were present in all fractions and corresponded to the peak molecular weight (M_r) of approximately 25 000 (Fig 6). Fraction A12 was composed of a main peak of approximately M_r 97 000, which matched the MW of the bsAb. Fraction B3 contained the bsAb (M_r 96 970) and the kappa homodimer (M_r 97 600) but also smaller fragments (M_r 23 600 and 46 200), which are likely variable light change (VL) and dimerized VL. The remaining fractions (B4, B5, and B6) contained only the kappa homodimer and no bsAb. Fragments, such as VL and dimerized VL, were present in fractions B4 and B6, respectively.

The results show that the mispaired kappa homodimer and the kappa-lambda heterodimer bispecific mAb are separated well which can be seen in the M_r of 97 000 and 97 600 separation in LC-MS from the different elution fractions. The data also shows that the bsAb can not only be purified from the kappa homodimer but also separated from the single and dimerized VL in the sample.

The LC-MS analysis performed on the fractions from the step elution shown in Figure 7 are presented in Figures 8 and 9. The step elution marks a baseline separation of the mispaired kappa homodimer and the kappa-lambda heterodimer bsAb. The LC-MS data from the gradient and step elutions show a clear separation of the bsAb from the impurities such as mispared kappa homodimer and single and dimerized VL.

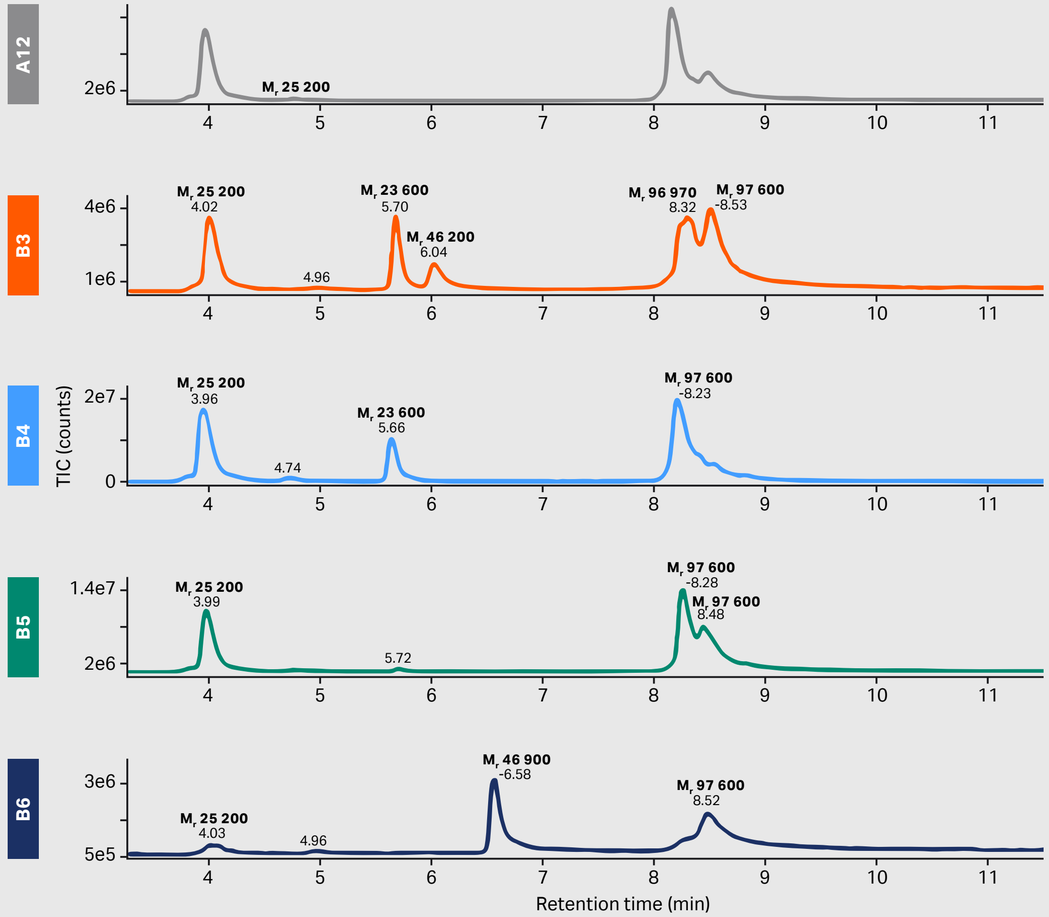


Fig 6. LC-MS data on fractions from gradient elution of bsAb sample using MabSelect™ VL resinchromatography system.

Column: Tricorn™ 5/100
Resin: MabSelect™ VL
Sample: bsAb
Sample load: 5 mg
Start buffer: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2
Wash: 50 mM citrate, pH 5.0
Elution buffer: 50 mM citrate buffer
Flow rate: 1 mL/min
Gradient: step gradient elution, pH 3.5 and pH 3.1
System: ÄKTA pure™
Detection: UV 280

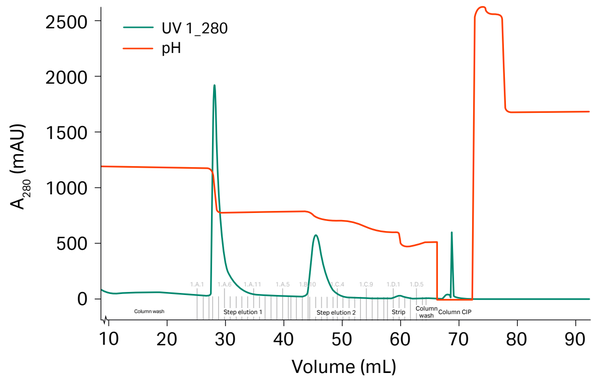


Fig 7. Chromatogram of step elution with fraction IDs of bsAb using MabSelect™ VL chromatography resin in Tricorn™ 5/100 column.

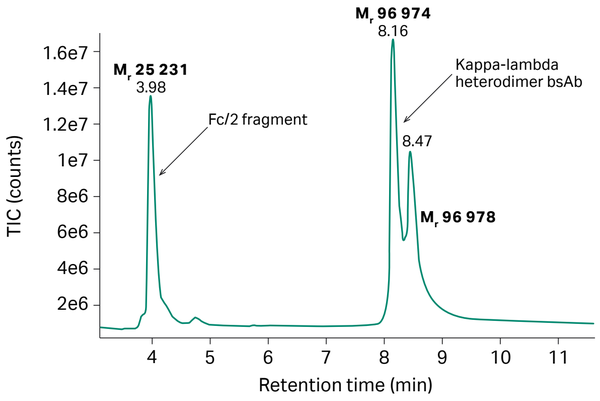


Fig 8. LC-MS data of fraction A5 from step elution showing MW of Fc/2 (M_r 25 000) and kappa-lambda heterodimer bsAb (M_r 97 000).

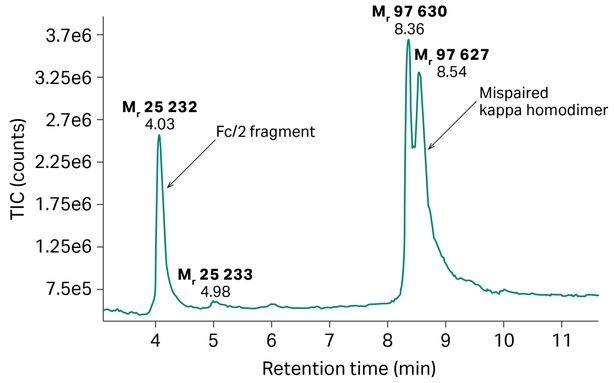


Fig 9. LC-MS data of fraction C2 from step elution showing MW of Fc/2 (M_r 25 000) and mispaired kappa homodimer (M_r 97 600).

Conclusions

We have shown that:

- MabSelect™ VL resin can separate kappa-lambda heterodimer bsAb from impurities (kappa homodimer and VL) due to differences in avidity. The desired bsAb was collected in the first elution peak over a gradient pH elution. Because the kappa homodimers and VL domain bind stronger to the resin, they eluted later in the second peak.
- A step elution with two static pHs resulted in a baseline separation of the kappa-lambda heterodimer bsAb and the impurities in the cell culture harvest.
- The resolution in a step pH elution with MabSelect™ VL resin is comparable for the different column formats (Tricorn™ 5/100, HiTrap™ 1 mL, and HiScreen™) investigated in this study. The elution peaks for HiTrap™ 1 mL column were slightly delayed and broader compared to the other two columns. This difference is expected due to the smaller resin volume relative the void volume of the ÄKTA pure™ system compared to the other column formats. All three column formats result in baseline separation of the bsAb and the kappa homodimer.

Material and methods

A linear gradient elution was performed using MabSelect™ VL resin packed in Tricorn™ 5/100 column to find the optimal elution pH. Next, step elutions were performed for different column formats packed with MabSelect™ VL resin to compare performance (Table 3). The buffers used in the gradient and step elution are listed in Table 2. ÄKTA pure™ chromatography system with preprogramed UNICORN™ software methods were used for both the linear gradient and step elution runs.

Table 2. Buffers used in the gradient and step elution

Buffer	Composition
A1 (equilibration buffer)	20 mM sodium phosphate, 150 mM NaCl, pH 7.2
A2 (wash buffer)	50 mM citrate, pH 5.0
B1 (elution buffer)	50 mM citrate, pH 2.5
A3 (CIP)	100 mM NaOH

Table 3. Elution setup for step elution based on the results from the gradient elution

Column	%B step 1	CV	%B step 2	CV
Tricorn™ 5/100 (2 mL)	65	8	79	5
HiTrap™ (1 mL)	65	8	79	8
HiScreen™ (4.7 mL)	65	8	79	8

LC-MS analysis of elution fraction

To further confirm the separation of product-related impurities such as homodimers and half-antibodies in a step elution, the fractions under peak 1 and peak 2 of the step elution using MabSelect™ VL resin were analyzed with LC-MS to determine the masses of the different entities in the elution pools. A 5 mg bsAb sample was applied to MabSelect™ VL resin in Tricorn™ 5/100 column, and a step gradient elution was performed including strip (100 mM citric acid, pH 2.1) and Milli-Q wash before CIP with 100 mM NaOH. Selected fractions were analyzed with LC-MS.

The samples were treated with FabRICATOR (IdeS, Genovis AB) prior to LC-MS analysis to digest bsAb samples. Samples were incubated for 3 h with the enzyme and analyzed using LC-MS. Reverse phase chromatography with gradient of 0.1% formic acid and 0.1% formic acid in acetonitrile solvents as buffers were used prior to MS as a separation technique. Using high accuracy mass spectrometry enabled the detection of homo- and heterodimers along with small fragment molecular weights.

Product

Next-generation protein L chromatography

Web cast

Chromatography process development going smarter

05

Optimize antibody fragment purification

Optimize antibody fragment purification

Downstream purification strategies to optimize bioprocessing of antibody fragments

Antibody fragments such as single domain antibodies (dAbs), antigen-binding fragments (Fab), and single chain variable fragments (scFvs) have unique properties that can make them better suited to certain therapeutic applications than full-sized monoclonal antibodies (mAbs). Fragments are smaller than mAbs, which can allow them to more effectively reach their targets, and they're also often easier and less expensive to produce. But purification of antibody fragments isn't as straightforward as for mAbs — fragments show wider variation in binding affinities and can be trickier to separate from impurities. Here we'll look at some purification approaches that can help you harness the potential of antibody fragments.

What types of antibody fragments are used in bioprocessing?

Antigen-binding fragments (Fabs) are antibody structures that bind antigens but lack an Fc region. Considered the first generation of antibody fragments, Fabs were first generated by enzymatic cleavage of an intact antibody to yield an Fc fragment and two Fab fragments, each composed of one variable heavy chain (VH) and one variable light chain (VL). Today, Fab fragments are produced using genetic engineering and can be generated by simpler expression systems, such as *E. coli*.

Single chain variable fragments (scFvs) are recombinant monovalent structures with affinity for a single antigen. With an approximate size of M_r 25 000, an scFv is a fusion of the VH and VL chains. An scFv comprises the complete antigen-binding site of its parent antibody molecule.

Single domain antibodies (dAbs) are some of the smallest functional antibody fragments that retain full antigen-binding specificity. They consist of the VH or VL domains and are around one-tenth of the molecular weight of a full-sized antibody. Single domain antibodies are stable under harsh conditions of temperature, pressure, and denaturing chemicals.

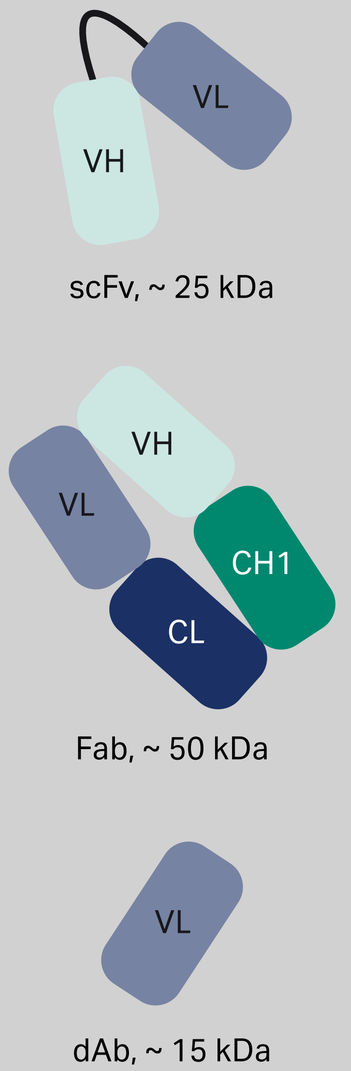


Fig 1. The structure of an antibody. The arrow indicates where protein L interacts with the antibody.

Production and purification of antibody fragments

Antibody production workflows involve expression of the protein in mammalian or bacterial cells, followed by downstream clarification of the culture to remove cellular material, then antibody capture and polishing. Here we'll look at options for each step.

Antibody fragment expression

Because antibody fragments are small and not glycosylated, you can use bacterial or yeast cell expression systems to produce them, making them simpler and less expensive to make than mAbs. Tangential flow filtration (TFF) with hollow fiber filters is a good option for clarifying viscous and high-solid feeds after microbial fermentation.

Antibody fragment purification

Typically, mAbs can be purified using a platform approach, where standard unit operations and operating conditions are constant.

The Fc region common to mAbs means that a near-generic approach is possible. Fab fragments, scFvs, and single domain antibodies require a broader strategy, but newer affinity resins open efficient capture alternatives. We offer a variety of chromatography resins suitable for antibody fragment purification, including Fabs, scFvs, and single domain antibody lambda and kappa light chains. Your approach for the capture chromatography step will depend on the structure and properties of your fragment.

Capturing fragments containing kappa light chains

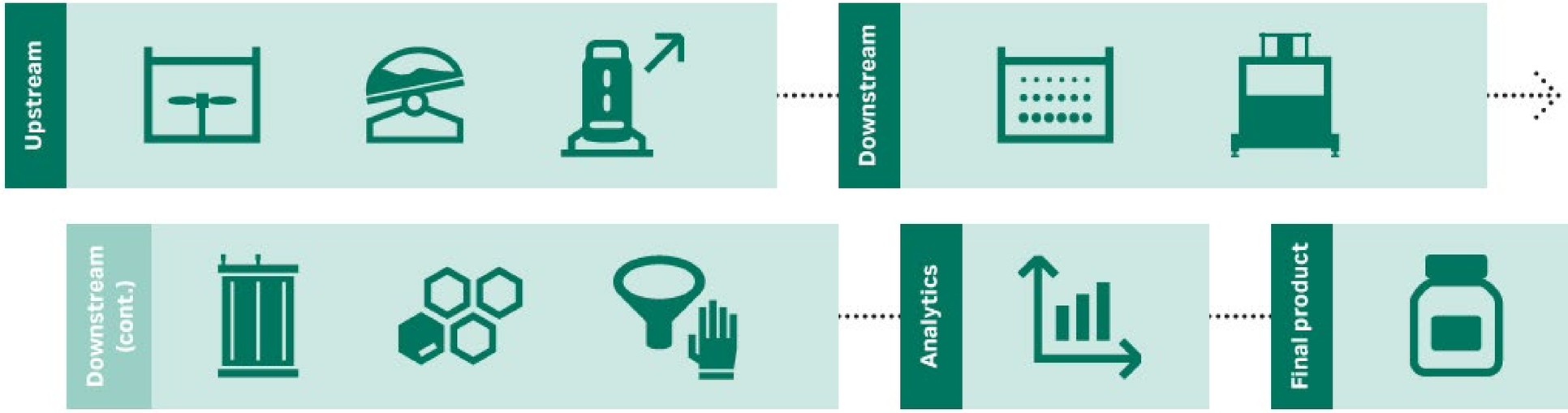
Because of its binding specificities, protein L has broad potential as an affinity ligand for antibody fragments. Native protein L interacts with immunoglobulin (Ig) kappa light chains and will bind to several antibody classes, including IgG, IgM, IgA, IgE, and IgD. It has no immunoglobulin class restrictions. Approximately 60% of mammalian IgG light chains are kappa chains with protein L binding activity (1).

Where previous generations of protein L ligands lacked the binding capacity and alkaline stability needed for industrial scale purifications, newer ligands, like the one in MabSelect™ VL resin, overcome those challenges. MabSelect™ VL resin includes a rigid base matrix, allowing for high flow rates and high productivity as well as low ligand leakage and suitability for large-scale manufacturing. Its broad affinity for a range of antibody fragments of different sizes that contain the variable region of the kappa light chains is useful for a wide range of purification applications for Fabs, scFv, and dAbs as well as for bispecific antibodies.

KappaSelect affinity resin binds to the constant region of the kappa light chain and can be used to capture relevant Fabs in conditions where MabSelect™ VL resin is not suitable.

Capturing fragments containing lambda light chains

For capture of antibody Fabs containing the lambda light chain, choose LambdaFabSelect.



Capturing fragments containing heavy chains

If you want to capture heavy chain fragments that contain the VH3 domain subtype, you might want to consider MabSelect PrismA™ resin or Fibro™ PrismA units. The PrismA protein A ligand binds to the VH3 domain subtype of human IgG Fabs in addition to binding in the Fc region, making it suitable for some antibody fragment purifications.

Intermediate purification and final polishing

For intermediate fragment purification and final polishing, separation based on different selectivity from the primary technique (orthogonal) can involve anion exchange (AIEX) or hydrophobic interaction chromatography (HIC). Your intermediate and polishing steps are based on impurity levels after capture. These steps reduce aggregates, HCP, and endotoxin and can be run in bind/elute or flowthrough mode.

Learn more about antibody fragments

Protein A and protein L chromatography for protein purification

References

1. De Château M, Nilson BH, Erntell M, et al. On the interaction between protein L and immunoglobulins of various mammalian species. *Scand J Immunol.* 1993;37(4):399-405. doi:10.1111/j.1365-3083.1993.tb03310.x

Web articles

Purifying monoclonal antibodies and fragments with protein A, protein L, or other ligands

Support for process development and manufacturing of mAbs, bispecific antibodies, fragments and more

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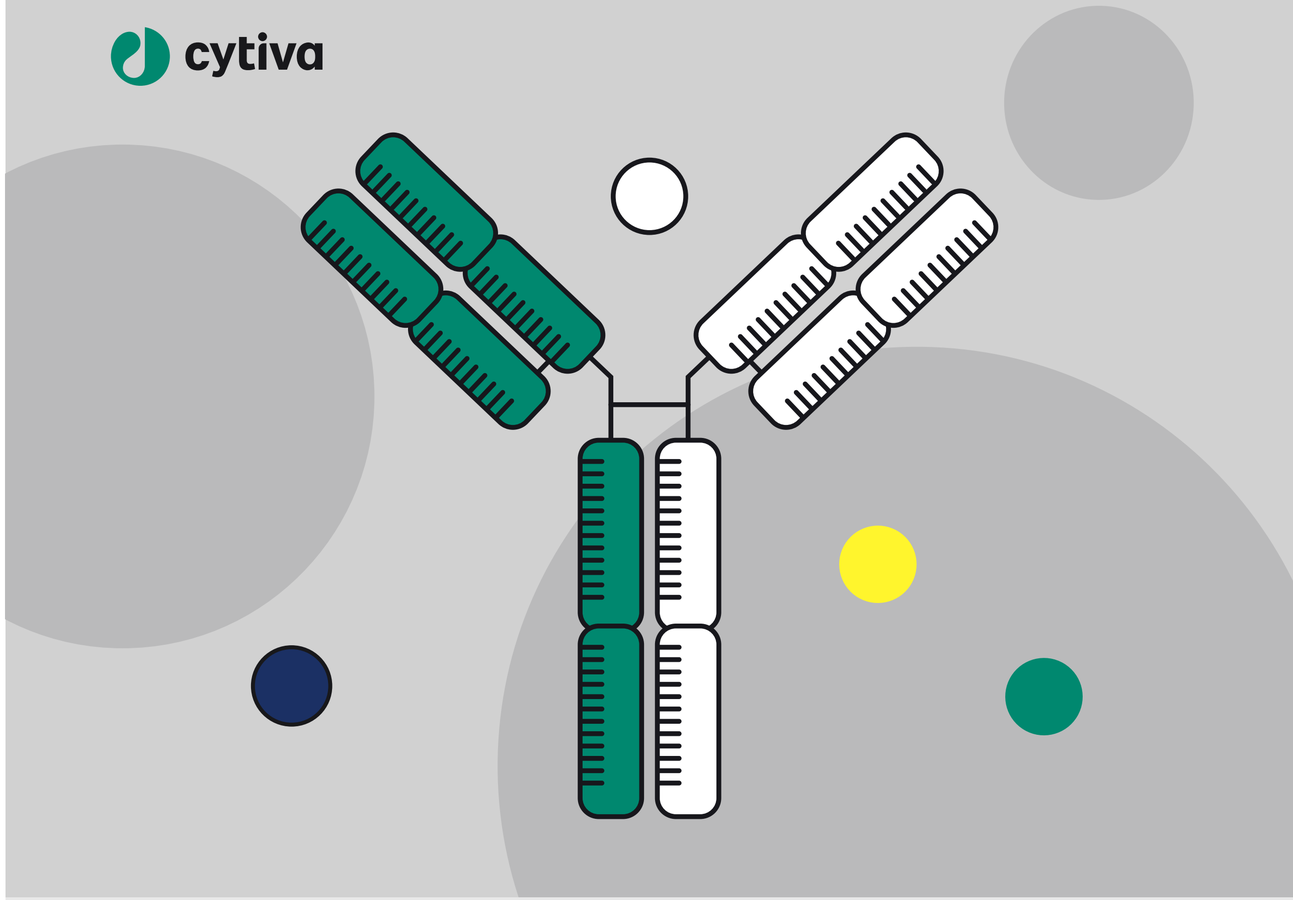

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