Development of High Throughput Screening Tools for Purification of Bispecifics

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Abstract

Antibodies have been used as therapeutics for over 30 years but can have a limited modality. In recent years advances in protein engineering have allowed us to build antibody structures with two (or more) specificities - bispecific antibodies. Despite the varied molecular engineering strategies that have been developed to ensure correct bispecific assembly, there remains the potential for incorrect pairing i.e. formation of monospecific homodimers, which often require significant and time-consuming USP and DSP optimization. Here we demonstrate the development of high throughput screening for purification of different bispecific formats.

Three bispecific molecules against the same two targets, were expressed in different formats that incorporated different molecular engineering strategies. These were expressed in the AbZelect™ CHO platform at small scale and purified. For two constructs, purification using platform affinity capture and polishing steps were effective however, for one format, heterodimeric Fab/scFv-Fc, these standard approaches failed to give acceptable purity or yields. We demonstrate how high throughput resin screening was performed using the Cytiva PreDictor plates to rapidly develop a method for effective purification of this construct and discuss an approach for rapidly screening different bispecific formats to identify the optimal arrangement for any two specificities, taking developability and function into account.

Background

Sponsor was looking to generate bispecific mAbs against two targets (A and B). Sponsor had specified three designs allowing for different valencies of the two specificities using different heterodimerization and purification approaches.

Bispecifics Process Flow

Bispecific and Vector Design followed by DNA synthesis
↓
Generate stable pools using different DNA ratios
↓
Small scale purification and analysis to determine optimal pools for progression
↓
Optimize purification processes
↓
Perform PQA to ensure that quality of bispecific

	Format 1 IgG-scFv	Format 2 Heterodimeric Fab/scFv-Fc	Format 3 Hetero-IgG-scFv
Valency	2A x 2B	1A x 1B	2A x 1B
Symmetry	Symmetric	Asymmetric	Asymmetric
Heterodimerization	N/A	Electrostatic	Knob into Hole
Purification	Protein A	Protein A & differential pI	Differential PrA binding
	Binding Site A / Binding Site B	Binding Site A / Binding Site B	Binding Site A / Binding Site B
	IgG-scFv	**Heterodimeric Fab/scFv-Fc**	**Hetero IgG-scFv**

Overview of bispecifics generated

Vector Construction and Expression

Vectors were constructed for use in Abzena's AbZelect™ CHO platform.

Format 1 (IgG-scFv) used a single vector, dual expression cassette system (containing both heavy and light chains on the same vector).

For both Format 2 (Heterodimeric Fab/scFv-Fc) and Format 3 (Hetero IgG-scFv) a two-vector approach was used with each vector containing half of the molecule. The two vectors were constructed separately so that ratios of each half of the asymmetrical molecule could be varied during transfection to obtain optimal heterodimer expression.

Triplicate stable pools were transfected using different vector DNA ratios and, following recovery, fed-batch productivities were set up in TubeSpins™ for cell growth and viability (ViCell) and titre assessment (Octet). All pools recovered and grew as expected in the AbZelect™ platform selection process and differing DNA ratios did not impact the growth of cells.

Pools were initially purified by Protein A only and characterized by analytical SEC and CE-SDS.

For format 1, no ratio optimization was performed.

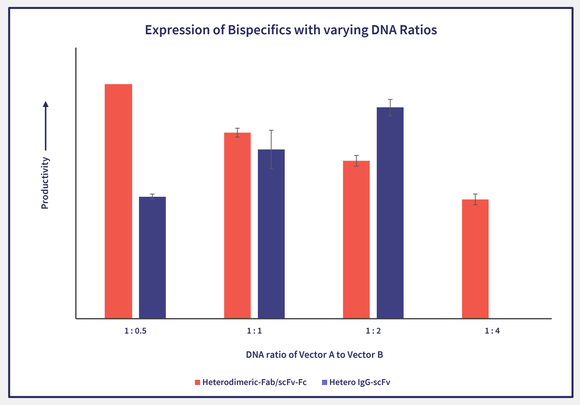
For the other two bispecifics, titres varied depending upon ratio.

For format 2 (heterodimeric Fab/scFv-Fc), the overall titre decreased as the DNA ratio of chain B (scFv-Fc) to chain A (heterodimeric Fab) increased, however this was counterbalanced by an increase in the product quality (as determined by the proportion of bispecific observed).

For format 3 (hetero IgG-scFv) the reverse trend was observed with titre improving as the DNA ratio of chain B (heterodimeric Fab) to chain A (Hetero IgG-scFv) increased but the product quality decreasing.

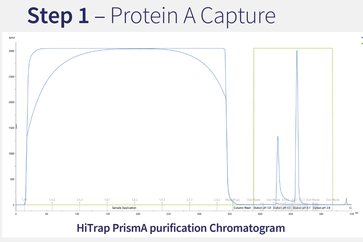
This highlights the importance of optimizing vector ratios and assessing the product quality obtained from stable pools

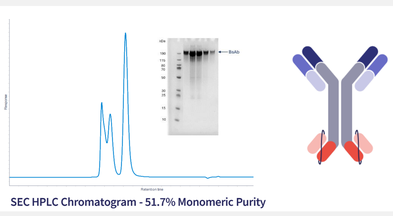
Pools containing the greatest proportion of each correctly paired bispecific were progressed to purification optimization



Purification of IgG-scFv, Heterodimeric Fab/scFv-Fc and Hetero IgG-scFv

Step 1 – Protein A Capture


HiTrap PrismA purification Chromatogram

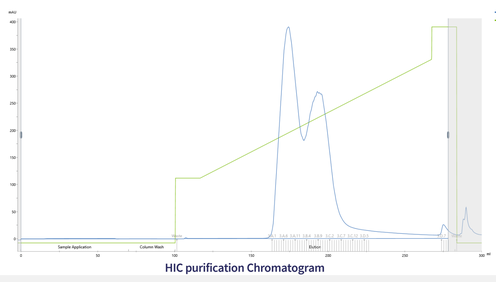


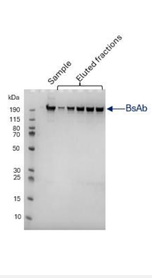
SEC HPLC Chromatogram - 51.7% Monomeric Purity

Standard Protein A affinity chromatography capture with step pH elution led to low product purity with high levels of HMWS, possibly a consequence of the scFv.

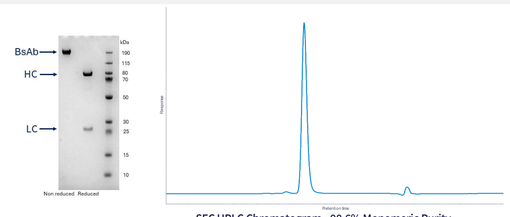
Introducing an additional purification step was necessary to remove aggregates. Based on several purification method screening runs, Hydrophobic Interaction Chromatography (HIC) was chosen as the successful technique to separate monomers from aggregates and achieve > 95% monomeric purity of final material.

Step 2 – Hydrophobic Interaction Chromatography

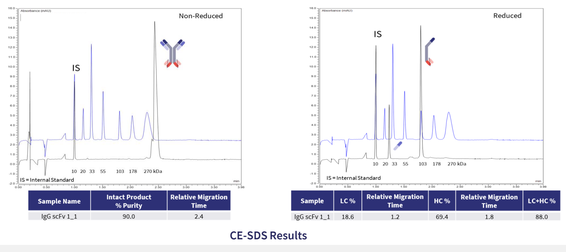

HIC purification Chromatogram



Final sample



SEC HPLC Chromatogram - 98.6% Monomeric Purity



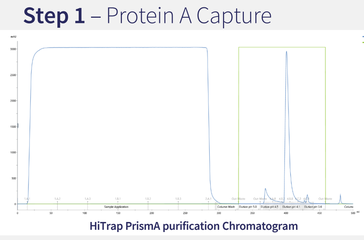
Sample Name	Intact Product % Purity	Relative Migration Time
IgG scFv 1_1	90.0	2.4

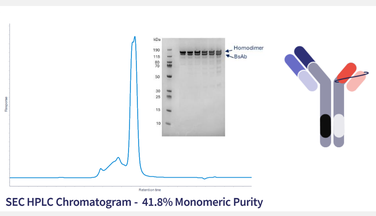
Sample	LC %	Relative Migration Time	HC %	Relative Migration Time	LC+HC %
IgG scFv 1_1	18.6	1.2	69.4	1.8	88.0

CE-SDS Results

An additional HIC purification step led to an increase in **monomeric purity from 51.7% to 98.6%**

Step 1 – Protein A Capture


HiTrap PrismA purification Chromatogram



SEC HPLC Chromatogram - 41.8% Monomeric Purity

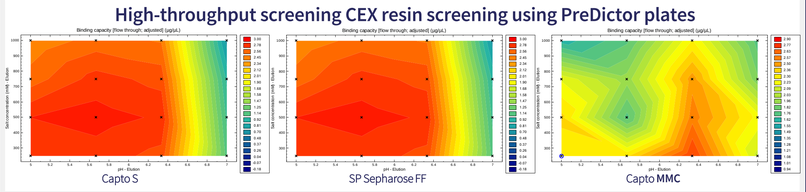
Standard Protein A affinity capture using step pH elutions resulted in high homodimer content but low product purity. An additional purification step was necessary to achieve bispecific purity >95%.

In this case, the Fc regions contained amino acid substitutions resulting in a pI differential between the two heavy chains. As a result, the heterodimeric molecule would be expected to have a different pI compared to either of the potentialunwanted monospecific homodimers.

High-throughput screening of different resins and chromatographic conditions for all stages in the chromatographic cycle (binding, wash, and elution) was performed using PreDictor Plates (Cytiva). Three different Cation Exchange resins (CEX) were tested & Assist software was used as a DoE tool. High-throughput PreDictor 96-well plates allowed simultaneous testing of many different conditions (factors) such as buffers, pH, salt.

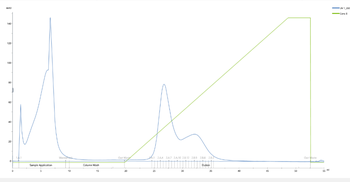
After scouting and screening with PreDictor plates, final verification and purification method optimization was performed with ÄKTA system.

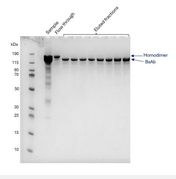
High-throughput screening CEX resin screening using PreDictor plates



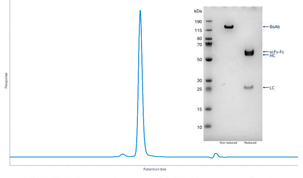
Capto S | SP Sepharose FF | Capto MMC

Step 2 – CEX – Final purification method optimization on AKTA system

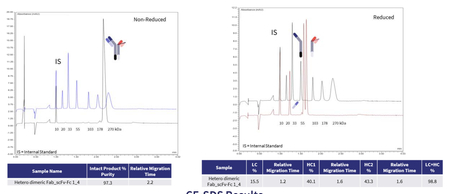




Final sample



SEC HPLC Chromatogram - 96.2% Monomeric Purity



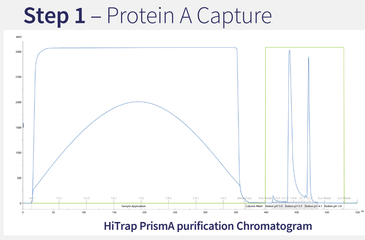
Sample Name	Intact Product % Purity	Relative Migration Time
Hetero-dimeric Fab_scFv Fc 1_4	97.3	2.2

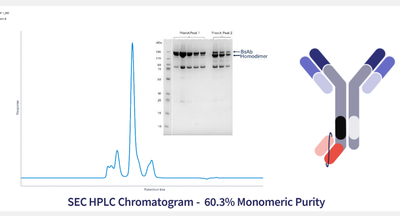
Sample	LC %	Relative Migration Time	HC1 %	Relative Migration Time	HC2 %	Relative Migration Time	LC+HC %
Hetero-dimeric Fab_scFv Fc 1_4	15.5	1.2	40.1	1.6	43.3	1.4	98.8

CE-SDS Results

High-throughput screening of different resins and different purification conditions resulted in a second purification step that increased the **purity of the heterodimer from 41.8% to 96.2%**

Step 1 – Protein A Capture


HiTrap PrismA purification Chromatogram

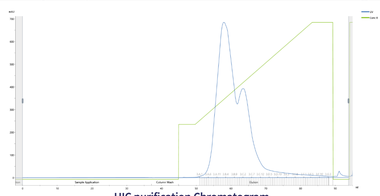


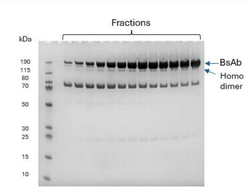
SEC HPLC Chromatogram - 60.3% Monomeric Purity

Standard Protein A affinity chromatography using step pH elutions led to different profiles being observed. As anticipated, a milder elution resulted in increased proportion of bispecific compared to homodimer, whereas harsher elution favoured the homodimer.

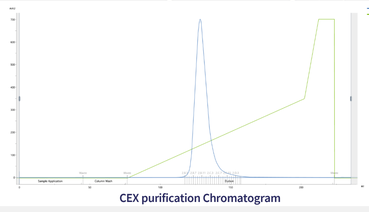
However, additional purification steps were necessary to remove residual homodimer and low molecular weight species (LMWS). Based on several purification method development runs, Hydrophobic Interaction Chromatography (HIC) followed by Cation Exchange (CEX) Chromatography were chosen as successful techniques to separate the target from the contaminants and achieve > 95% monomeric purity of final material.

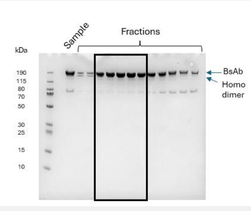
Step 2 – Hydrophobic Interaction Chromatography


HIC purification Chromatogram

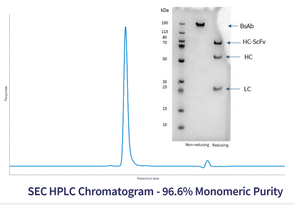


Step 3 – Cation Exchange Chromatography

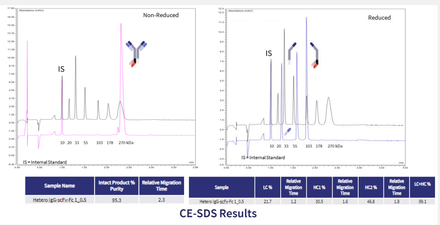

CEX purification Chromatogram



Final sample



SEC HPLC Chromatogram - 96.6% Monomeric Purity



Sample Name	Intact Product % Purity	Relative Migration Time
Hetero IgG-scFv Fc 1_3.5	95.3	2.3

Sample	LC %	Relative Migration Time	HC1 %	Relative Migration Time	HC2 %	Relative Migration Time	LC+HC %
Hetero IgG-scFv Fc 1_3.5	21.7	1.2	30.5	1.4	46.8	1.0	99.1

CE-SDS Results

Introducing two additional purification steps, HIC followed by CEX led to an increase in the **heterodimer purity from 60.3% to 96.6%**

Conclusions

Three bispecific molecules against the same two target antigens were expressed in different formats, incorporating different molecular engineering strategies. These were expressed in the AbZelect™ CHO platform at small scale and purified. For two constructs, purification using pre-developed, platform affinity capture and polishing steps were effective in delivering high purity (>95%) bispecific product. However, for one format, heterodimeric Fab/scFv-Fc, these platform conditions failed to give acceptable purity or yields. To achieve this, high throughput resin screening was performed using the Cytiva PreDictor plates to rapidly develop a new CEX method for effective purification of this construct. It is also noted that activities such as construct design and chain ratios can significantly influence productivity and product quality thus, a one-size-fits-all approach is not always appropriate. In certain situations, generating empirical data may be necessary for optimal cell line development. Following successful purification, these molecules have now progressed to functional assessment.