# **revvi**

Streamlining the development of bispecific antibodies from expression to quality assessment with Revvity's biotherapeutic workflow solutions.

# Introduction

Recent advancements in antibody engineering have opened a new chapter in immunotherapeutics, leading to the development of bispecific antibodies (bsAbs) with superior stability, specificity and therapeutic potential<sup>1</sup>. Unlike conventional monoclonal antibodies (mAbs) that target a single antigen, these engineered antibodies possess two distinct antigen-binding arms. This unique architecture allows them to simultaneously engage two targets, facilitating diverse therapeutic effects<sup>2</sup>.

The production of bispecific antibodies is a complex and challenging process. Some of the specific challenges in the production of bispecific antibodies include correct chain pairings<sup>3</sup>, homodimer contamination<sup>4</sup>, yield, correct protein folding and post-translational modifications. These challenges have limited the development and approval of bispecific antibodies. However, antibody design-led solutions and the incorporation of innovative process technologies have made it possible to overcome many of these challenges, and there are now several bispecific antibodies in clinical development<sup>5</sup>.

The intricate process of bispecific antibody production demands a robust and reliable expression platform, comprising:

**• Expression vectors:** An ideal expression vector for bispecific or multi-specific antibodies would enable genomic integration and expression of all chains in the optimal ratio. Although multi-cassette vectors can offer convenience, the introduction of each half of the bispecific antibody in separate vectors allows fine-tuning of their individual expression levels for optimal pairing.

- **• Cell line:** The expression of an engineered bispecific antibody requires a robust host cell line (with Chinese Hamster Ovary (CHO) cells being widely used), as it demands not only efficient chain pairing but also correct post-translational modifications, folding and secretion.
- **• Early quality screening:** Early quality analysis during pool and clone screening could help to assess chain pairing efficiency, folding patterns, and potential aggregation risks.
- **• Process optimization:** Even with optimized cell lines and vectors, homodimerization remains a potential hurdle in bispecific production. By optimizing cell culture processes and purification strategies, scientists can further refine bispecific antibody production, ensuring minimal homodimer contamination and a significantly higher yield of the desired molecule.

Revvity's CHOSOURCE™ TnT Transposon Technology can help streamline bispecific antibody production. This comprehensive solution brings together robust CHOSOURCE Glutamine Synthetase (GS) knock-out (KO) and ADCC+ host cell lines, a dual-cassette transposon vector and transposase mRNA for seamless genomic integration that, combined with optimized cell line development processes, favours efficient bispecific expression and pairing. Additionally, the integration of Revvity's LabChip® GXII Touch™ \* protein characterization system allows rapid quantification and early homo/heterodimer differentiation to be incorporated into the biotherapeutics workflow, maximizing efficiency and quality control.

## Expressing bispecific antibodies using CHOSOURCE TnT Transposon Technology

CHOSOURCE TnT Transposon Technology consists of a dual cassette transposon vector with optimized regulatory elements for higher productivity, alongside mRNA encoding a transposase, which is required for integration of the transposon into the host genome (Figure 1). The vector contains a GS cassette for metabolic selection of expressing pools in CHOSOURCE GS KO and ADCC+ cell lines, both of which are GS deficient<sup>6</sup>.



**Figure 1: CHOSOURCE TnT Transposon Technology:** CHOSOURCE Transposon Technology is a two-component system consisting of the TnT transposon vector and TnT transposase mRNA. The genes of interest (GOIs) are cloned into the TnT transposon vector and, together with the GS selection cassette, are flanked by terminal inverted repeats (TIRs). The transposase recognizes the TIRs and integrates the transposon containing the GOIs and the selection cassette into the host cell genome, whilst excluding the vector backbone elements.

In this work, CHOSOURCE TnT Transposon Technology was used to express an asymmetric 4-chain bispecific antibody in the CHOSOURCE GS KO host cell line (Figure 2). The antibody consists of two light chains (LC1 and LC2) and two heavy chains (HC1 and HC2). The antibody has knobsinto-holes (KiH) mutations built into the heavy chains to enable correct chain pairing. The HC1 chain has the "hole" mutation and the HC2 chain has the "knob" mutation.



**Figure 2: An asymmetric 4-chain bispecific antibody with a KiH Fc region was expressed using CHOSOURCE TnT Transposon Technology** [A] An asymmetric 4-chain bispecific antibody that binds to two different antigens. [B] Some of the different incorrect chain combinations and free chains that may be co-expressed as contaminants with the correct heterodimer antibody by cells.

### Materials and methods

#### **Cloning strategy:**

The expression of a 4-chain molecule requires cloning the chains into two CHOSOURCE TnT dual cassette transposon vectors, and co-transfection of the two vectors with CHOSOURCE TnT transposase mRNA (Figure 3). This method relies on a single GS selection system.



**Figure 3: Expression of a bispecific antibody using CHOSOURCE TnT Transposon Technology:** The expression of a 3 or more chain molecule requires the use of two TnT transposon vectors to be co-transfected with TnT transposase mRNA.

The sequence of the 4-chain bispecific antibody was retrieved from a public database and the sequence of each chain was codon optimised for expression in CHO cells using a publicly available codon optimisation tool. Commonly used signal peptides were added to both heavy and light chains to facilitate their secretion from the cells. Optimal expression of the bispecific antibody would require further optimisation, such as trialling different codon optimisation tools and signal peptides for each chain.

The correct assembly of heterodimers requires the expression of all chains in an optimal ratio. The position of each individual chain within the CHOSOURCE TnT transposon vector can affect its expression, hence affecting the final yield of the correctly assembled molecule. Eight vectors were generated for the expression of this 4-chain asymmetric bispecific antibody, and the configurations listed below were tested (Figure 4). The chains cloned into the two multiple cloning sites (MCS) in a CHOSOURCE TnT transposon vector integrate into the CHOSOURCE host cell line genome in a 1:1 ratio. Therefore, to maintain the 1:1 ratio for HC:LC chains, one pair of HC and LC were cloned into each vector - configurations with two chains of the same type (two heavy chains or two light chains) present on the same vector (i.e. LC1LC2, LC2LC1, HC1HC2 and HC2HC1) were not tested.





LC2HC1

LC1HC<sub>2</sub>

#### **Transfection and stable pool generation:**

To generate pools expressing all four chains of the bispecific antibody, the eight vectors (Figure 4) were co-transfected in pairs in a 1:1 ratio (vector 1: vector 2) into the CHOSOURCE GS KO host cell line, along with CHOSOURCE TnT transposase mRNA, using a Neon™ Electroporation System (Thermo Fisher Scientific) (Table 1). Two pools were generated for each co-transfection. Single vectors were also transfected to generate homodimer pools which were used as controls to test the specificity of droplet digital PCR (ddPCR) primers and probes. The protein expressed by these homodimer pools was also used as a control for protein assessment to differentiate heterodimers from homodimers. The transfected pools were subjected to selection in a growth medium without L-glutamine after 48 hours. Un-transfected CHOSOURCE GS KO cells were used as a negative control for the selection process. On day 5, cells were counted, centrifuged, and resuspended in fresh selection medium. Cell growth was monitored every one to two days, and pools were expanded to E125 shake flasks upon recovery.

**Table 1: Transfection conditions:** The 8 vector configurations were tested in 8 different co-transfection reactions.



#### **Copy number assessment:**

To identify whether the integration of all chains of the bispecific molecule was in a desirable ratio, we assessed the number of integrated copies of the individual chains (HC1, LC1, HC2 and LC2) and their ratio to the GS gene using ddPCR. A standard housekeeping gene was used as a reference gene for the assays.

The primers and probes for each chain were designed specifically to the unique regions of the chains. The specificity of the primers and probes was confirmed using homodimer pools (data not shown).

Cell pellets were collected for each pool after recovery from selection. Genomic DNA was extracted from the cell pellets and ddPCR was performed using the designed primers and probes.

#### **Pool fed batch experiment:**

To assess productivity, transfected pools were enrolled in a 14-day non-optimized fed-batch overgrow experiment, once they were fully recovered from selection (with a viability of >95%). The fed-batch experiment was conducted in TPP® TubeSpin bioreactors (Sigma-Aldrich). Cell counts were performed on days 0, 4, 7, 10, 12 and 14 and metabolites were monitored using a BioProfile® FLEX2 (Nova Biomedical). Pools were supplemented with feeds and glucose on days 4, 7, 10 and 12.

Although this non-optimized fed-batch process allowed for initial screening to identify the best vector configuration resulting in the correct assembly of the 4-chain bispecific antibody, further optimization of media and feed composition may be required to improve protein titres.

#### **Pool productivity and quality:**

Supernatants were collected from the pool fed-batch cultures on days 7, 10, 12, and 14 and titers assessed. Subsequently, Protein A columns were used to purify antibodies from the collected supernatants. Finally, microchip electrophoresis (μCE-SDS) analysis using the ProteinEXact™ assay \* on a LabChip GXII Touch HT (Revvity) system was performed to allow detailed characterization of the purified protein alongside a reference molecule. Sample preparation for μCE-SDS analysis followed the manufacturer's instructions for the ProteinEXact assay. A commercially available antibody with 95% purity was used as the reference standard for these assays.

# Results

#### **Pool selection recovery and copy number analysis:**

Analysis of the co-transfected pools following selection (Figure 5) revealed a range of recovery times, with the earliest observable recovery at day 8 and the latest at day 16. Selection was performed in glutamine free media, without any other selection agents such as methionine sulfoximine (MSX). The different recovery times suggest that the antibody chain position within each vector has an impact on the selection recovery of the pools, possibly due to varying expression levels of the antibody chains in different positions.



**Figure 5: Selection recovery profile:** The graphs represent the selection profile of co-transfected pools. Graph (A) represents the viable cell density and graph (B) represents the percentage viability. Each point on the graph represents the average of two independently transfected pools. Error bars represent standard deviation.

The number of integrated copies of the individual antibody chains, as well as the number of integrated copies of the GS gene, were assessed for each selected pool using ddPCR assays. Since the vectors were co-transfected in a 1:1 ratio, an equal number of copies of the four chains were expected to be integrated in each pool. Co-transfection conditions

4, 7, and 8 had an almost equal number of all four chains (Table 2). However, in other conditions, the integration of the two vectors was not in a 1:1 ratio. For example, in co-transfection condition 1, the vector containing the "knob" HC (HC2) integrated fewer copies (approximately half) than the vector with the "hole" HC (HC1) (Table 2). This might be explained by the potential for higher "knob" HC integration to lead to increased production of "knob-knob" homodimers, which could aggregate<sup>7</sup> within the cell and cause cell death. Consequently, cells integrating more copies of "knob" HC-containing vectors may not survive post-transfection.

As expected, the HC and LC genes that were originally cloned in the same expression vector were integrated in a 1:1 ratio within each pool (Figure 6). The observed copy number of the GS gene was higher than that of the individual heavy and light chains, as it is integrated from both vectors. For all pools, the GS gene copy number closely matched the sum of the average copy number of the chains integrated on each vector (Figure 6, Table 2). For example, in co-transfection condition 3, vector 1 contributed 15 copies of the GS gene (the number of copies determined for LC1 and HC1) and vector 2 contributed 11 copies of the GS gene (the number of copies determined for HC2 and LC2), which combined together match the observed 26 copies of the GS gene (Table 2). This further demonstrates that the CHOSOURCE TnT transposase mRNA integrates an intact transposon, consisting of both expression cassettes and the GS gene.



**Figure 6: Copy number variation:** The graph represents the LC1, HC1, LC2, HC2 and GS gene copy number variation as observed in the co-transfected pools following recovery from selection. Each assay was performed in triplicate. Error bars represent standard deviation.

**Co-transfection condition Pool ID LC1 HC1 LC2 HC2 GS Vector 1 Vector 2** 1 HC1LC1 HC2LC2 14 14 8 8 21 2 HC1LC1 LC2HC2 12 13 9 9 21 3 LC1HC1 HC2LC2 15 15 11 11 26 4 LC1HC1 LC2HC2 9 9 9 7 7 1 15 5 HC1LC2 HC2LC1 7 12 11 7 21 6 HC1LC2 LC1HC2 9 13 14 10 23 7 LC2HC1 HC2LC1 7 8 10 6 16 8 LC2HC1 LC1HC2 11 13 14 11 24

**Table 2: Copy number variation:** Table summarising the copy number variation of all four chains (LC1, HC1, LC2, HC2) and the GS gene for each co-transfected pool following recovery from selection.

#### **Pool productivity and quality assessment:**

The productivity profile of the pools followed the same trend as the gene copy number (Figure 7). The antibody yield for six of the co-transfected pools with similar copy number was within the same range, and above 1g/L, while the yield for the two remaining pools was lower. The "LC1HC1\_HC2LC2" (co-transfection condition 3, Table 2) configuration had the highest number of integrated copies and hence led to the highest overall antibody titer. Conversely, the "LC1HC1\_ LC2HC2" (co-transfection condition 4, Table 2) configuration had the lowest number of integrated copies and hence resulted in the lowest titer (Figure 6, Figure 7). Since the different vector configurations led to different numbers of integrations and antibody yields, it can be inferred that the chain arrangement within the vector significantly impacts the integration of the individual chains, their expression and, consequently, antibody assembly. This may be advantageous, since heterodimer formation can potentially be increased not only by adjusting the ratios of the two CHOSOURCE TnT transposon vectors, but also by changing the position of the individual chains within the vectors.

The antibody expressed by all pools was further purified and analysed in denatured (non-reduced) and reduced conditions by microchip electrophoresis analysis using the ProteinEXact assay on the LabChip GXII Touch HT system and compared against the reference bispecific antibody. The analysis performed with denatured samples allows proteins to be separated on the basis of their size. This enables the identification of different conformations of the antibody, such as heterodimers, homodimers, glycosylated



**Figure 7: Pool Fed-batch productivity:** Summary of the stable pool productivity of all eight co-transfected pools

and non-glycosylated variants, and free chains. The analysis performed with reduced samples allows for the identification as well as quantification of the individual antibody chains.

Following the comparison of the electrophoretic profile of the denatured (non-reduced) protein purified from all eight vector configurations, it was found that although the "LC1HC1\_HC2LC2" (co-transfection condition 3) had the highest antibody titer (Figure 7), the expressed antibody profile differed from the reference molecule (Figure 8A). The position of the two heavy and light chains within the

two vectors co-transfected to generate this pool led to reduced expression of the LC2 chain, which likely hampered the correct assembly of the molecule (Figure 8B). However, antibody produced by the "LC2HC1\_LC1HC2" (co-transfection condition 8) pool, which had only a modestly lower titer than condition 3 (1.2g compared to 1.4g, Figure 7), exhibited 94% correct conformation as compared to the reference molecule (Figure 8A, Figure 9A, Figure 9B). Protein purified from the other co-transfection conditions showed an electrophoretic profile that differed from the reference molecule, indicating the presence of mis-paired antibodies or excess free chains (Figure 8A).



**Figure 8: µCE-SDS analysis of expressed protein:** The electrophoretic mobility of both denatured (non-reduced) and reduced expressed proteins from all 8 co-transfected pools was assessed and compared with the reference bispecific antibody using the LabChip GXII Touch HT system. The electrophoretic profile of denatured (non-reduced) protein is shown in [A]. The electrophoretic profile of reduced protein identified individual chains, and concentrations of all chains were calculated based on the electropherogram. The bar graph [B] represents concentration of each chain represented as percentage of total concentration (%).

Furthermore, the electrophoretic profile of the reduced protein purified from the "LC2HC1\_LC1HC2" (co-transfection condition 8) pool showed the four antibody chains were present in a very similar ratio to that of the reference molecule (Figure 8B, Figure 9C, Figure 9D), whereas some of the other co-transfection configurations, such as conditions 1, 2, 5, 6 and 7, had excess HC1 chain as compared to the HC2 chain, in combination with reduced LC2 chain (Figure 8B).

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Figure 9: Protein quality analysis of LC2HC1\_LC1HC2 (co-transfection condition 8) relative to the reference molecule: The peaks observed in the microchip electrophoresis of denatured (non-reduced) and reduced protein expressed by the LC2HC1\_LC1HC2 pool were compared with those of the reference molecule. [A,B] Peaks observed in microchip electrophoresis of denatured (non-reduced) protein expressed by the LC2HC1\_LC1HC2 pool and of the reference molecule. [C,D] Peaks observed in microchip electrophoresis of reduced protein expressed by the LC2HC1\_LC1HC2 pool and of the reference molecule.

Although conditions 4 and 7 showed integration of all chains in a 1:1:1:1 ratio, as observed in condition 8 (Figure 6, Table 2), they did not display the same ratio of chain expression, as assessed from the electrophoretic profile of reduced protein (Figure 8B). Condition 4 showed lower expression of HC2 and LC2 chains as compared to HC1 and LC1 chains, and condition 7 showed equal expression of LC1 and LC2 but lower expression of HC2 as compared to HC1. This suggests that more complex dynamics exist between the number of integrated copies and the expression of the individual chains, which affects the assembly of the final molecule. This is likely partially caused by the position of each individual chain within the CHOSOURCE TnT transposon vector. The electrophoretic profile of the reduced protein from all 8 co-transfections indicated that the HC2 and LC2 chains always had lower expression than the HC1 and LC1 chains, irrespective of the chain positions (Figure 8B). Nonetheless, the profile of condition 8 closely matched the profile of the reference molecule.

In this study, the LC2HC1\_LC1HC2 co-transfection configuration (condition 8) led to genomic integration of an almost equal number of all four chains with a 1:1 co-transfection ratio and achieved 94% correct conformation as compared to the reference molecule. To achieve the optimal integration of all chains and the correct assembly of the bispecific antibody for the other co-transfection configurations tested, it may be necessary to test co-transfection of different ratios of vectors.

#### **Discussion:**

In the present study we expressed a 4-chain asymmetric bispecific antibody using the CHOSOURCE GS KO cell line and CHOSOURCE TnT Transposon Technology. As the CHOSOURCE TnT transposon vector is a dual cassette vector, the expression of a 4-chain molecule required co-transfection of 2 vectors into the CHOSOURCE host cells, along with the CHOSOURCE TnT transposase mRNA. The pairing of the correct chains is often influenced by an interplay between the ratio of genomic integration and level of expression of the chains, as well as the intracellular conditions which influence molecule assembly. In our study, we tested 8 different chain combinations of the 4-chain

bispecific antibody in the two vectors. All 8 combinations led to different selection recovery times, gene copy numbers and antibody yields. Our study demonstrates that the position of the chains within the CHOSOURCE TnT transposon vector affects their integration and expression, hence impacting the correct assembly of the antibody and heterodimer formation (Figure 6, Figure 7, Figure 8). We also observed that the vector containing the "knob" HC gene integrated at lower numbers of copies compared to the vector containing the "hole" HC gene. This could potentially be attributed to "knob" chains being more prone to aggregation due to the exposed tryptophan residue. A higher integration of the "knob" HC may therefore cause more aggregation within cells, causing cell death post-transfection<sup>7</sup>.

In this study, a 1:1 co-transfection ratio between the two vectors yielded to the correct configuration of the antibody for one co-transfection configuration. However, since the dynamics of genomic integration and expression are complex for bispecific antibodies, the level of expression of the different chains may differ for different bispecific molecules and the two vectors may need to be co-transfected in different ratios to generate the correct heterodimer configuration.

For a 4-chain bispecific antibody, if the chains of the bispecific antibody were allowed to randomly pair, it would result in a minimum of 16 different possible combinations<sup>2</sup> (Figure 2). Out of these, only one would be the correct combination. Hence, the identification of the correct heterodimer conformation, as well as the optimal ratio of all chains, remains the holy grail of bispecific antibody production. Therefore, incorporating quality analysis early in the cell line development process becomes a critical step. The pool that attained the highest titer in this study did not ultimately result in the molecule with the correct conformation. Instead pool "LC2HC1\_LC1HC2" (condition 8), which yielded a slightly lower titer, achieved a 94% correct conformation compared to the reference molecule (Figure 8A, Figure 9). This demonstrates that yield and quality assessment are equally important in the identification of optimal pools to be taken forward in the cell line development pipeline.

# Conclusions

In this work, we expressed an asymmetric 4-chain bispecific antibody in its correct heterodimer configuration, as compared to a reference molecule, using CHOSOURCE TnT Transposon Technology. Advances in bispecific antibody design, like knobs-into-holes mutations, have significantly reduced mispairing and homodimer contamination. However, challenges remain regarding "hole-hole" homodimers and co-purified free chains, which can mask true yields during screening. Implementing efficient chain integration strategies and integrating quality checks early in the cell line development process can further refine the selection process and lead to the identification of higher-quality candidates. The CHOSOURCE TnT Transposon technology dual cassette vector provides the flexibility to modulate the expression of different chains of a bispecific antibody. This can be achieved not only by testing co-transfection of different ratios of the two vectors, but also different chain positions. This approach aims to achieve the appropriate configuration of the molecule while minimizing unwanted structures. The partnership of CHOSOURCE TnT Transposon Technology with CHOSOURCE cell lines supports the implementation of a robust and stable cell line development pipeline. This expression platform enables the assessment of protein quality at the pool stage, within about 6 weeks of transfection, contributing to the acceleration of biologics development programs while minimizing risks.

Revvity's LabChip GXII Touch HT protein characterization system can be used to further increase the speed of biologics development programs. In this study, the expressed proteins were rapidly characterized via microchip electrophoresis (μCE-SDS), using Revvity's ProteinEXact assay on the LabChip GXII Touch HT system. The results from the assay allowed a direct comparison of denatured (non-reduced) and reduced antibody profiles against the reference molecule. This enabled early identification of optimal pools during the cell line development process, while excluding pools expressing mis-paired antibodies and excess free chains from the workflow.

In conclusion, the partnership between Revvity's CHOSOURCE expression platform and Revvity's ProteinEXact assay on the LabChip GXII Touch HT system allows streamlining and acceleration of cell line development processes, contributing to the advancement of development and manufacturing of biotherapeutics.

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