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Research Article

Efficient production of bispecific antibodies—optimization of transfection strategy leads to high-level stable cell line generation of a Fabs-in-tandem immunoglobin

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ABSTRACT

Bispecific antibodies (bsAbs) are often composed of more than two component chains, such as Fabs-in-tandem immunoglobin (FIT-Ig) comprising three different component chains, which bring challenges for generating a high proportion of the correctly assembled bsAbs in a stable cell line. During the CHO-K1 stable cell line construction of a FIT-Ig, we investigated the FIT-Ig component chain ratio in transfection, where two sets of expression vectors were designed. Both designs utilized two vectors for co-transfection. Multiple transfections with plasmid ratio adjustment were applied, and the resultant minipools were evaluated for expression titer and quality of produced FIT-Ig. The results suggested that abundant outer Fab short chains (twofold chain genes versus other chains) can promote complete FIT-Ig assembly and therefore reduce the fragmental impurities of FIT-Ig. This adjustment of the component chain ratios at the beginning is beneficial to FIT-Ig stable cell line generation and brings favorable clones to process development.

Statement of Significance: Bispecific antibodies are often composed of more than two component chains, which bring challenges to production, particularly in a stable cell line. These challenges can be addressed by rational vector design and transfection strategy as well as rational screening funnel for high-level stable clones.

KEYWORDS: bispecific antibody; FIT-Ig; stable cell line; vector; transfection; fragmental molecule; process development

INTRODUCTION

Enabling novel mechanisms of action not achievable by monoclonal antibodies alone or in combination, bispecific antibody (bsAb) has been a modality of biotherapeutics and attracting considerable interests [1]. The number of bsAbs in clinical trials is expanding quickly, whereas even more are at various stages of development [2, 3]. With the recent approval of Lunsumio®, a CD20xCD3 T-cell-engaging bispecific antibody, in June 2022 [4], there are a total of five bsAbs in the market for various indications, three of which were approved in the recent 2 years [5, 6], suggesting an imminent burst in this field.

During the long history of bispecific technology development, various technologies have been developed for bsAb generation [7]. Antibody engineering by recombinant approach became the most prevalent approach for generating bsAbs in a single host cell [8]. It has been reported there are more than 100 different bispecific formats at various development stages. Most bispecific antibodies comprise unconventional antibody polypeptide chains. A dual-variable domain immunoglobin has both its heavy chain and light chain longer than those of regular IgG due to the fusion of additional variable regions [9]. Meanwhile, many bsAbs have more than two component

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chains requiring proper assemblance into a single molecule [10]—for instance, CrossMab has four different component chains that require correct pairing between cognate heavy and light chains [11]. Although the art of mAb production is mature and most mAbs can be efficiently produced with a standard process [12], these unconventional properties of bsAb present a challenge for the efficient production of bsAb at the desired level of quality, especially in terms of the proportion of correctly assembled bsAbs, particularly in stably transfected cell lines.

Transient expression in human embryonic kidney (HEK) cell lines is a well-established approach for the rapid generation of recombinant proteins, including bispecific antibody. The expression level of each component chain can be readily adjusted by chain-encoding plasmid ratios in transfection, which were commonly used for improving the expression and quality of the product [13]. However, the generation of high-producing stable CHO cell clones is a time-consuming and difficult process for bispecific antibodies, where a more extensive effort is required to obtain high-producing and high-quality clones.

We previously described FIT-Ig as a versatile bispecific format for engaging multiple therapeutic targets [14]. FIT-Ig is a symmetrical Ig-based bispecific molecule composed of three different component chains (Fig. 1a and b). According to the standard process of mAb generation, FIT-Ig protein can be readily expressed and purified by the co-transfection of three component chain-encoding plasmids in a transient HEK293 cell system. In most cases, a 1:3:3 plasmid ratio (chain #1 to chain #2 to chain #3) transfection followed by one-step affinity chromatography can obtain products at acceptable quantity and quality, which are similar to those of the regular transient production of mAb [15]. Moreover, the performance of transiently transfected HEK293 cells is also suggestive to the titers and quality of products in stable CHO cell clones, as there could be a correlation between transiently expressing HEK293 and stably expressing CHO cell clones [16, 17]. In this study, we generated and investigated stable CHO cell clones for an exemplary FIT-Ig using two transfection strategies with different vector designs and vector ratios.

MATERIALS AND METHODS

Vector constructs

FIT-Ig is composed of three component chains as shown in Fig. 1(b). For transient transfection, the coding sequence for each chain was respectively placed into pcDNA3.1 vector (Invitrogen). For stable transfection, all the vectors were derived from the pQKX vector (Shanghai OPM Biosciences Co., LTD.), and therefore had the same backbone elements including an origin of replication, an ampicillin resistance gene, the gene encoding glutamine synthetase, which is used for selection in the stable transfections, and CMV promoters upstream of each coding gene. For the vector constructions, first, chain #1, chain #2, and chain #3 genes were synthesized with optimized codon and then separately cloned into the pQKX vector, resulting in vectors pC1, pC2, and pC3, respectively. For the construction of design 1 vector pC1C3, the chain #3 expression cassette (involving CMV promoter, coding gene, and SV40 terminal signal) was cut from pC3 by restriction enzyme digestion and was then inserted to pC1, resulting in pC1C3 vector. pC2 and pC1C3 were used for design 1 transfections. Similarly, pC2C3 was constructed from pC2 and pC3 vectors. pC1 and pC2C3 were used for design 2 transfections.

Transient transfection

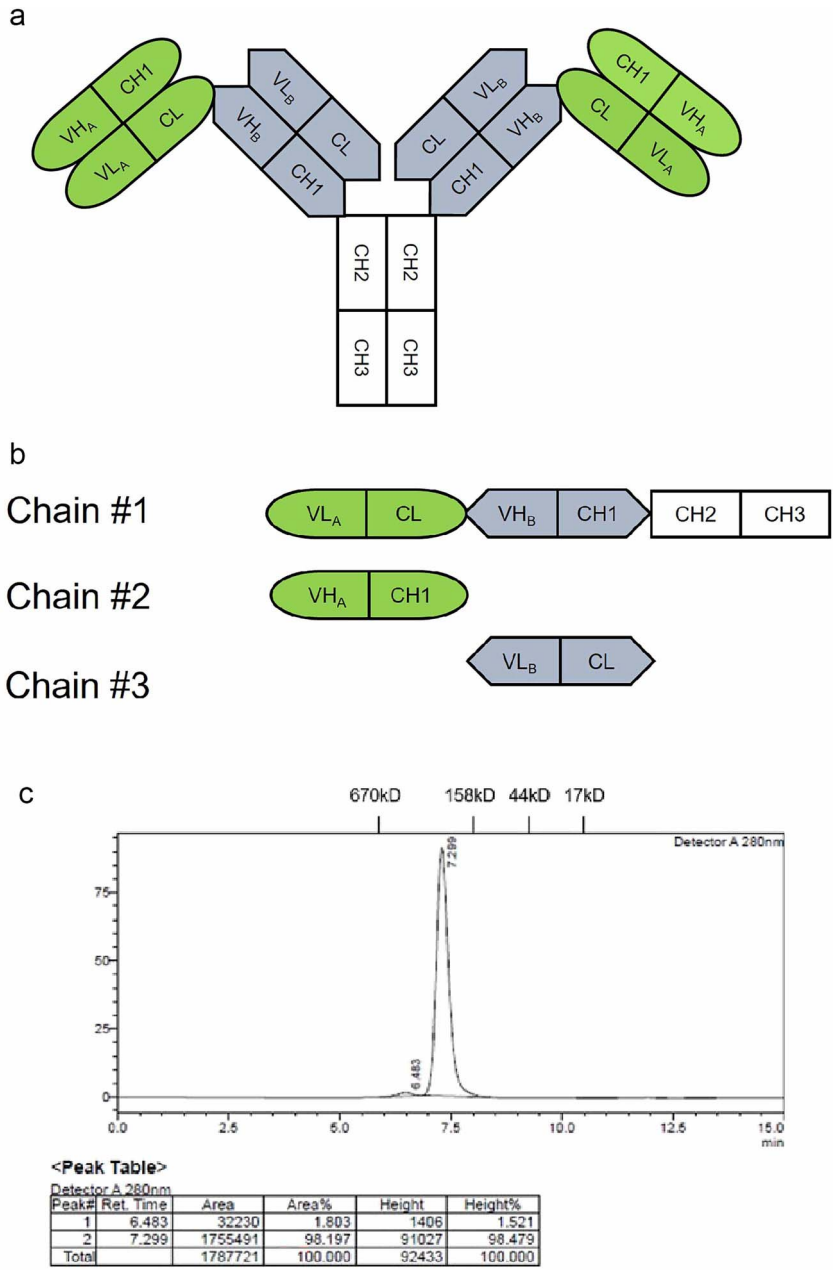
At 1 day before transfection, HEK293 cells were seeded to 0.6×10^6 cells/mL in FreeStyle™ 293 Expression medium and cultured in a shaker at 37°C, 8% CO_2, and 120 rpm. On the day of transfection, the cell density should be around $1.0–1.2 \times 10^6$ cells/mL. For a 100-mL transfection, 100 μg of plasmids (the molar ratio of the plasmids of chain #1, chain #2, and chain #3 was 1:3:3) and 200 μg PEI were, respectively, prepared in a 5-ml medium, kept at room temperature for 5 min, and then mixed. The mixture was incubated for 25 min at room temperature and then added dropwise to the cells. The cells were cultivated for 6–24 h, and then 1/10 volume of 5% peptone was added to the cells. The conditioned medium was harvested on the sixth day after transfection.

Generation of stable cell line

The plasmids at designated ratios were linearized and electroporated into CHO-K1 host cells. The cells were then plated into 96-well plates at 5000 cells/well. After 24 h, 50 μM methionine sulfoximine (MSX) was added as the selection pressure, and the plates were cultured in CD CHO medium (OPM) for minipool screening in an incubator operating at 37°C and 8.0% CO_2. On day 25, the minipools with a cell growth confluency higher than 30% were selected, and titers were examined with ELISA. High-producing minipools were then expanded into 24-well plates and incubated in OPM-CHO CD07 medium supplemented with 50 μM MSX. After 3 days, the expressed antibody level was examined using biolayer interferometry (BLI) method. High-producing minipools were selected and expanded into six-well plates. After a 3-day culture, the antibody expression level was examined by BLI method, and selected minipools were expanded into a 125-ml shake flask and incubated for another 3 days. Top minipools were selected for cell culture in shake flasks under limited fed-batch (LFB) mode. For LFB culture, the cells were inoculated at 5×10^5 cells/ml in 30 ml of culture volume. Viable cell density, cell viability, glucose and lactic acid levels were monitored. The glucose level was maintained at 2–6 g/L by the addition of glucose as needed. After 12 days, the titer was measured by the BLI method.

For each round of subcloning, the selected minipools/clones were plated into 96-well plates at 0.5 cell/well and incubated in CD CHO medium + 10% conditioned medium + 50 uM MSX + supplement medium. Higher-titer clones were evaluated in a shake flask under LFB mode.

The top clones were subjected to a stability study. Briefly, the cells were passaged twice a week for more than 60 days



Peak#	Ret. Time	Area	Area%	Height	Height%
1	6.483	32230	1.803	1406	1.521
2	7.299	1755491	98.197	91027	98.479
Total		1787721	100.000	92433	100.000

Figure 1. Schematic diagram of the FIT-Ig molecule. (a) Molecular structure of FIT-Ig. Fragment of antigen binding (Fab) domains targeting antigen/epitope A are in green, Fab domains targeting antigen/epitope B are in gray, and white for Fc (fragment crystallizing) region comprising of CH2 and CH3. (b) Three component chains of FIT-Ig. Chain #1 is also known as long chain or C1; chain #2 and chain #3 are short chains that are also known as C2 and C3, respectively. (c) The monomer percentage of one-step-purified FIT-Ig was analyzed by SEC-HPLC. The bottom table listed the observed peaks, their retention times, and area percentages; each peak of gel filtration standard with known MW was marked at the appropriate positions.

in CHO CD07 medium with 50 uM MSX. LFB assessment was performed using the cells from different passages. The expression level and protein purity from the LFB harvests were measured.

1000-L manufacture and stability study of FIT-Ig protein

The manufacturing process for FIT-Ig drug substance (DS) consisted of cell culture (upstream process) and purification (downstream process). In the upstream process, banked cells were thawed, expanded, and finally cultured in STR 1000-L bioreactor (Sartorius) at 33°C under stirring at 64 rpm (59–69 rpm). The cell culture was harvested after 14 days of incubation in a 1000-L bioreactor. In the downstream process, the FIT-Ig protein in the clarified bulk harvest was purified by Protein A affinity chromatography and two consecutive ion exchange chromatographic steps (anion and cation or AEX and CEX) and finally formulated at 20.0 ± 2.0 mg/mL. The FIT-Ig drug product (DP) was manufactured by an aseptic filling process of

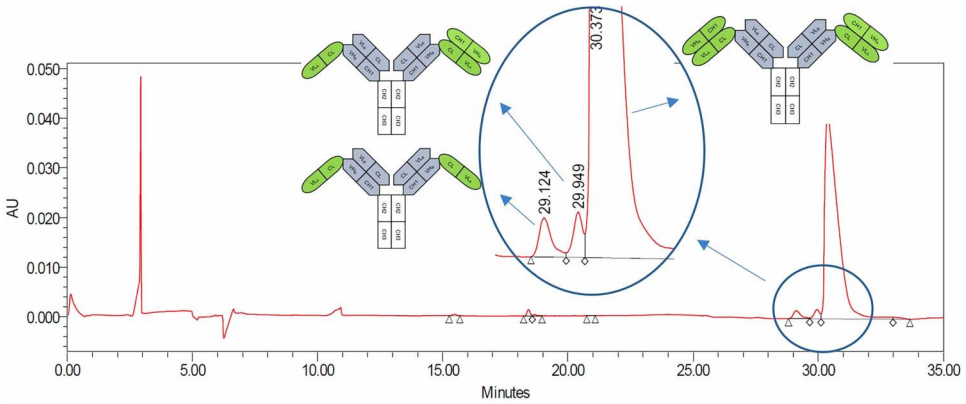


Figure 2. Determination of a fragmental molecule in FIT-Ig. FIT-Ig protein generated from transient transfected HEK293 cells was purified by one-step Protein A purification and then analyzed by non-reducing CE-SDS. The MW of the purified FIT-Ig protein was also detected by mass spectrometry.

DS without formulation or concentration adjustments and stored under 2–8°C.

The long-term stability study of the FIT-Ig DP was conducted under 5 ± 3°C. The FIT-Ig proteins were sampled at different incubation periods (0, 3, 6, 9, 12, 18, and 24 months) and analyzed for quality attributes.

Measurement of expression titers

The expression titers were measured by enzyme-linked immunosorbent assay (ELISA) or BLI-based assay. For the ELISA method, the FIT-Ig proteins in conditioned medium were captured by anti-human IgG Fc antibody (Rockland) coated in highly absorbent 96-well plate and detected by a secondary antibody of horseradish peroxidase-labeled Fab antibody (Sigma). For the BLI assay, the FIT-Ig proteins in conditioned medium were captured by Protein A Biosensor equipped in OctetRed96 (Fortebio), where the responses were detected. The detection of reference FIT-Ig protein in the same way provided a standard curve for the correlation between response and volumetric concentration, by which the concentrations of the test sample were calculated.

Measurement of monomer percentage

Monomer percentage was measured by size exclusion chromatography (SEC-HPLC). Purified protein was applied on a TSKgel G3000SWXL, 300 mm × 7.8 mm I.D. column (TOSOH). An HPLC instrument, model WPS-3000TSL ANALYTICAL (DIONEX), was used for SEC. FIT-Ig proteins were determined using UV detection at 280 nm. The elution was isocratic at a flow rate of 1 mL/min.

Detection of fragmental molecule

Beckman Coulter PA800 Plus Capillary Electrophoresis Unit was used for the detection of fragmental molecules in non-reducing (nr) condition. The test sample was first diluted with sample dilution buffer (PB-CA buffer) to 4.0 mg/mL and then with SDS dilution buffer to 1.0 mg/mL. The diluted sample was then alkylated with the addition of 5 μl N-ethylmaleimide, followed by sequential centrifugation/dry heat at 60°C for 10 min/centrifugation. After that, 90 μl of the prepared sample was transferred into a 200-μl vial and placed in the sample vial holder for CE-SDS analysis. The sample in the CE gel was detected with a PDA detector at 220 nm. The blank sample, prepared by mixing sample dilution buffer with SDS dilution buffer with a volume ratio of 1:3, was also treated and analyzed in the same way as the test sample.

RESULTS

FIT-Ig protein generation in transient transfection system

A FIT-Ig protein was generated by transfecting HEK293 cell with three-component chain-encoding plasmids and purified from the conditioned media using Protein A chromatography. The one-step purified FIT-Ig protein exhibited a high monomer percentage (greater than 95% in SEC-HPLC, Fig. 1c). Furthermore, the fragment byproducts analyzed by nrCE-SDS showed that there were two small peaks preceding the main peak (Fig. 2), indicating the presence of two types of molecules with a smaller molecular weight (MW). To further identify the composition of the fragmental molecules, the MWs of purified FIT-Ig protein were measured by mass spectrometry as described previously and then were compared with the predicted MWs (data not shown) [15]. The MW comparison indicated that the main peak represented the intact FIT-Ig molecule, while fragmental molecules were incomplete FIT-Ig lacking one and two chain #2, respectively (Fig. 2). Although these two fragmental molecules accounted for ~4.1% of the one-step purified protein from this transient transfection, it may be amplified in stable cell line generation. Moreover, fragmental molecules could induce aggregation formation by exposed hydrophobic patches and thus bring challenges to downstream purification.

To improve the percentage of intact molecule assembly, a specific increase of chain #2 ratio could be beneficial and applied to the design of stable transfection.

Table 1. Stable transfections and parameters

Design group	Plasmid name	Transfection no.	Plasmid ratio*	Chain ratio (C1:C2:C3)
1	pC1C3: pC2	1-1	1:1	1:1:1
		1-2	1:2	1:2:1
		1-3	1:3	1:3:1
2	pC1: pC2C3	2-1	1:1	1:1:1

*This was molar ratio calculated based on the molecular weight of each plasmid.

Generation of stably expressing minipools of FIT-Ig protein

CHO-K1 host cell was used to develop a stable cell line for the above-mentioned FIT-Ig molecule. The expression vectors for three component chains were constructed as design 1 and design 2 as described in Materials and Methods. In design 1, placing chain #2 in an individual expression vector pC2 (Fig. 3a) allows the flexible adjustment of its relative abundancy. Design 2 placed the two short chains, chain #2 and chain #3, together in expression vector pC2C3 (Fig. 3b), so the ratio of expressed chain #2 and chain #3 is 1:1, assuming that they have the same expression efficiency. Different transfections with plasmid ratio adjustment were applied (Table 1). As described previously in transient transfection, more short chains are required for promoting the full FIT-Ig molecule assembly, while excessive short chains can be easily removed by using affinity chromatography. To boost such a scenario, transfections 1-2 and 1-3 were probed for short chain usages greater than chain #1 attributed to more abundant chain #2 expressed from more copies of the coding sequence. Even for the cases of 1:1:1 (transfections 1-1 and 2-1), the short chain expressions were also expected to be higher than chain #1 for the short chains of a much smaller size would be more efficiently expressed.

Minipool selection was performed for all four transfections. For design 1 transfections, over a 1000 minipools with >30% cell growth confluence were assessed by using high-throughput expression titer measurement at the 96-well plate stage. At this early stage, the screening focused on expression titers because the cell culture volume was too small to get enough purified protein for quality assessment. The average minipool titers of transfection 1-2 was higher than that of transfections 1-1 and 1-3 (Fig. 4a), suggesting that the chain ratio 1:2:1 was a better design. Minipool selection and evaluation were performed stepwise from 96-well plate, 24-well plate, and 6-well plate to a 125-ml flask based on the expression level observed at each scale. Finally, 11 minipools from design 1, all from transfection 1-2 except for I-887 from transfection 1-3, were cultured and assessed in a shake flask with LFB condition. A total of eight minipools had expression titers greater than 1000 mg/L at the 12th day of cell culture, and I-472 had the highest titer (∼1400 mg/L) (Fig. 4b).

Similarly, six minipools from transfection 2-1 (design 2) were cultured and assessed in a shake flask with limited fed-batch condition, and the final titers were all below 800 mg/L (Fig. 4c). These results suggested that transfection 2-1 in design 2 was less productive than those of design 1.

The FIT-Ig proteins produced by minipools were purified by one-step Protein A chromatography and analyzed for purities using SEC-HPLC and non-reducing CE-SDS. The analytical data showed FIT-Ig proteins generated from I-508, I-548, and I-583 had >80% purity in CE-SDS analysis, where fragmental portions were less than 10% (Table 2). They also had >80% main peak (monomer) portion tested by SEC-HPLC. I-472 exhibited the highest expression, but the FIT-Ig protein purified therefrom contained ∼30% of aggregations and fragmental molecule, therefore deemed less favorable for subsequent subcloning. On the other hand, all of the minipools from transfection 2-1 seemed less desirable since none of the FIT-Ig proteins purified therefrom had a monomeric fraction above 75% (Table 2). Based on the high expression and high purity of the proteins produced, I-508, I-548, and I-583 derived from transfection 1-2 were selected for subcloning.

Generation of stably expressing clones of FIT-Ig protein

To acquire single clones expressing FIT-Ig protein, I-508, I-548, and I-583 minipools were subcloned by two rounds of limited dilution. In each round, the subclones were evaluated for both the expression level of the cell culture and the monomeric fraction of the proteins produced. Based on the expression level and purity of purified protein, four clones were selected in limited fed-batch assessment (Table 3). All of them originated in transfection 1-2, and three of them were from minipool I-548. Lastly, clone I-548-40-138 was selected as the top candidate for its high expression combined with favorable product quality. In limited fed-batch assessment, clone I-548-40-138 expressed over 1200 mg/L FIT-Ig protein with 94.0% monomeric fraction in SEC-HPLC and 81.6% intact molecule in nrCE-SDS. I-548-40-138 cells were continuously passaged twice a week, where the cells were subjected to LFB assessment every five passages. The last LFB assessment at the 21st cell passages, corresponding to ∼70 cell population double levels (PDL), observed a titer decrease of less than 20% and no significant change of quality of the purified protein (Table 4) compared to the first LFB assessment. This indicated that clone I-548-40-138 was stable during multiple cell passaging and competent for large-scale manufacture.

Clone I-548-40-138 was selected for process development, and 1000 L was manufactured, where the expression titer was 3.5 g/L. The DP was supplied as 20 mg/mL

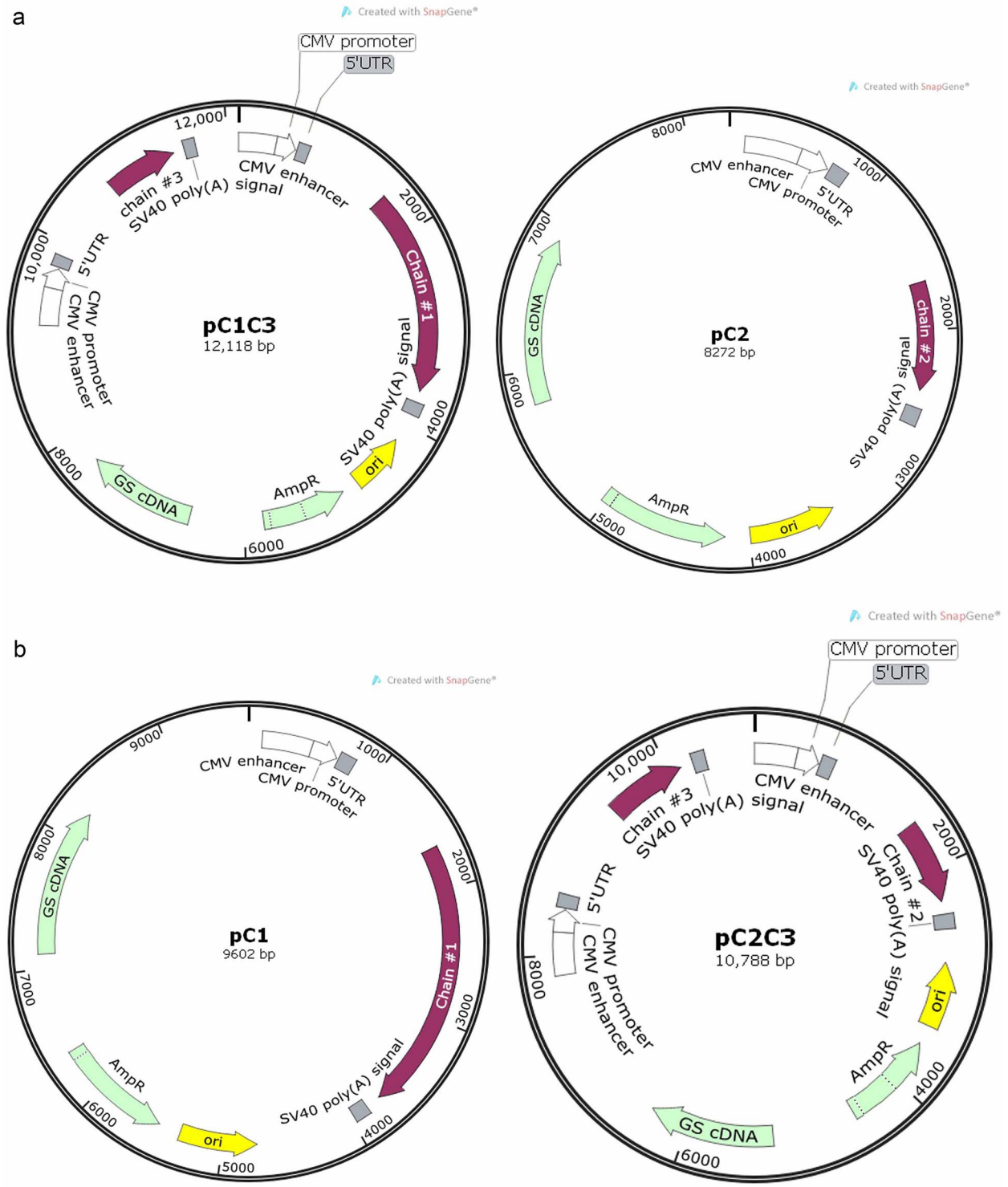


Figure 3. Vector designs for stable transfections. (a) Vector design 1, where coding genes of chain #1 and chain #3 were in pC1C3 vector, and chain #2 was in pC2 vector. (b) Vector design 2, where coding genes of chain #2 and chain #3 were in pC2C3 vector, and chain #1 was in pC1 vector.

injection stored under 2–8°C. A stability study was conducted under long-term (5°C ± 3°C) conditions. The quality attributes, such as monomer percentage in SEC-HPLC and main peak percentage in nrCE-SDS, had no significant change for up to 24 months (Fig. 5a and b). This indicated that this FIT-Ig protein had favorable stability. It is worth noting that the fragmental portion of FIT-Ig protein was not significantly changed, reflecting that few component chains disassociated from the FIT-Ig molecule during 2 years incubation.

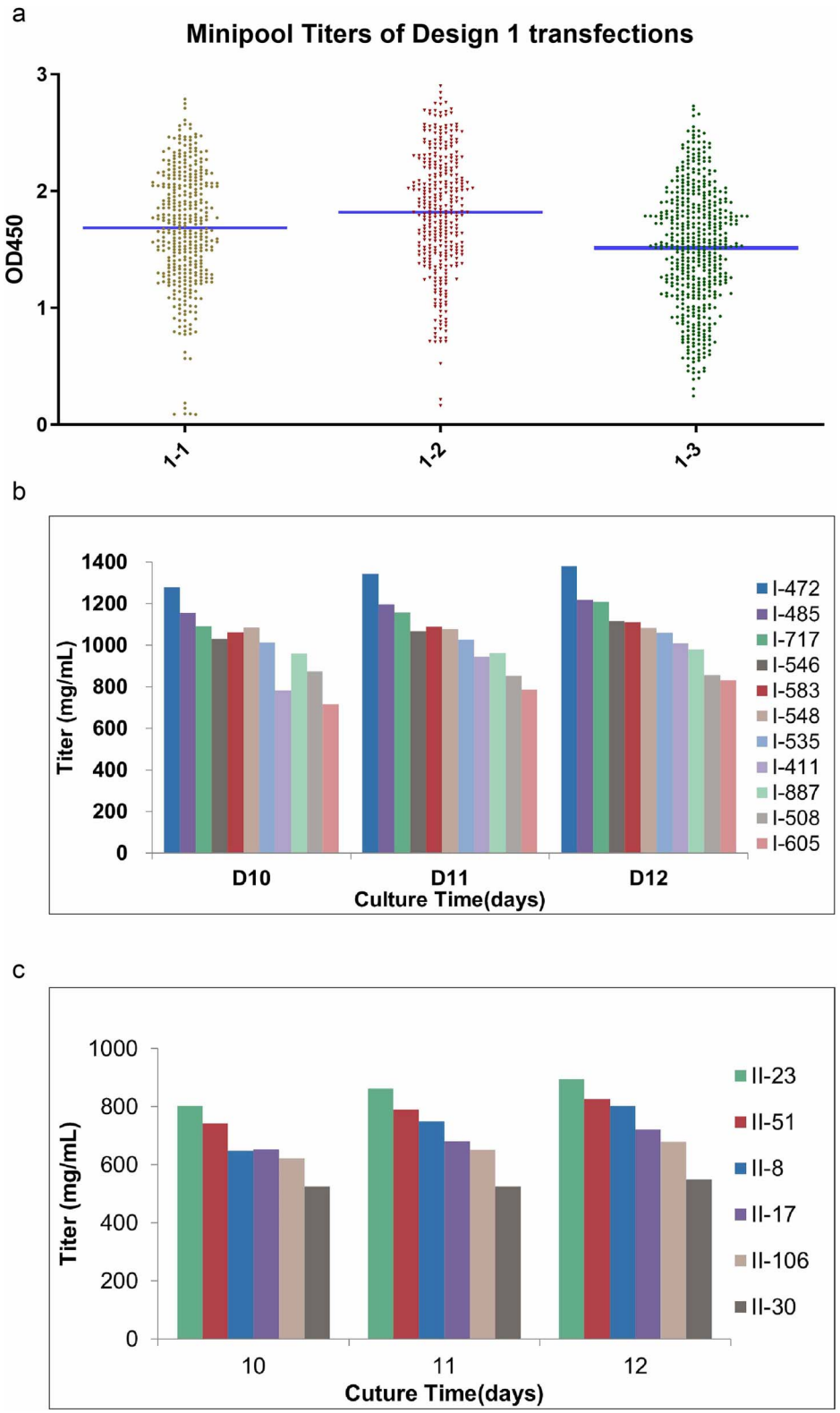


Figure 4. Expression assessment of minipools. (a) Titer levels of minipools from design 1 transfections at 96-well plates measured by the ELISA method. Each spot represented a titer level of one minipool, with 1215 minipools in total. The mean titer level of each transfection was labeled as a line. High titer minipools were further assessed, and top minipools were selected. The expression of 11 minipools from design 1 (b) and six minipools from design 2 (c) transfection were evaluated in LFB. Titers at days 10, 11, and 12 of cell culture were measured by the BLI method.

Table 2. Purity analysis of top minipools from design 1 and design 2 transfections

Minipool #	From transfections	SEC-HPLC main peak[a] (%)	nrCE-SDS main peak[a] (%)	nrCE-SDS fragment (%)[b]
I-411	1-2	64.2	65.1	17.4
I-472		65.0	74.7	12.8
I-485		54.8	59.7	24.3
I-508		89.7	84.6	5.9
I-535		66.7	68.7	12.2
I-546		40.0	48.2	29.7
I-548		87.9	81.9	6.4
I-583		83.7	80.4	8.1
I-605		61.2	72.7	12.1
I-707		42.3	50.9	21.1
I-887	1-3	28.8	29.0	43.3
II-8	2-1	63.2	66.5	19.6
II-17		74.5	71.4	15.8
II-23		39.0	ND	ND
II-30		49.6	ND	ND
II-51		43.0	ND	ND
II-106		58.3	ND	ND

[a]The main peaks of SEC-HPLC and nrCE-SDS were identified based on the retention time of the reference FIT-Ig protein. [b]Fragment percentage represented the amount of fragmental FIT-Ig molecule.

Table 3. Expression level and quality analysis of top clones

Clone #	From transfections	From minipool	Titer* (mg/L)	SEC-HPLC main peak (%)	nrCE-SDS main peak (%)	nrCE-SDS fragment (%)
I-548-35-117	1–2	I-548	1223	90.2	69.6	11.4
I-548-40-138			1258	94.0	81.6	5.7
I-548-45-179			1716	66.6	73.3	11.9
I-583-246-289		I-583	1325	82.6	79.5	7.9

*Titers were measured at Day 11 of the LFB cell culture.

Table 4. Stability results of clone I-548-40-138

Cell culture	Passage	PDL	Titer* (mg/L)	SEC-HPLC main peak (%)	nrCE-SDS main peak (%)
LFB-1	1	0	1285	94.0	81.6
LFB-2	5	11	1249	88.8	89.2
LFB-3	11	38	1212	87.8	87.4
LFB-4	21	76	1044	88.2	80.7

*Titers were measured at Day 11 of the LFB cell culture.

DISCUSSION

Most biologics products including bispecific antibodies are produced in stably transfected mammalian cell lines where the host cells were often CHO cell line [18]. During biologics development, stable cell line generation is the first step and the basis of the whole process development and manufacture. A productive and stable cell line can reduce the manufacturing cost, shorten the production period, and may add value to the product. The generation of high-quality stable clones is a time-consuming and expensive process, where plenty of strategies and clones need to be screened [19]. For regular mAb, a single vector containing both heavy chain and light chain coding genes can be used for generating favorable stable clones by a standard process [20]. However, bispecific molecules composed of three or more component chains may require a subtle vector design for transfections. An article investigated the cell line generation of a CrossMab, which is a bispecific antibody comprising four different component chains. Conventional strategy was tried first, but the clones obtained thereby were not satisfying because of the low quality of the purified protein, where the highest intact molecule purity is only 68.5%. In order to reduce the amount of partial molecules

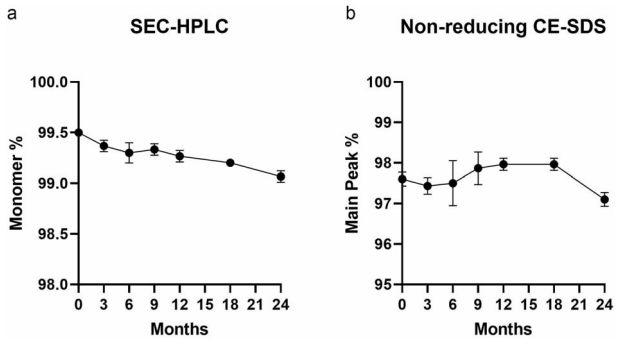


Figure 5. Quality attributes of the FIT-Ig protein in a long-term stability study. FIT-Ig DPs supplied as 20 mg/mL injection were stored under long-term (5°C ± 3°C) conditions. The FIT-Ig proteins were sampled at different incubation periods (0, 3, 6, 9, 12, 18, and 24 months) and analyzed for quality attributes. (a) The monomer percentage was measured by SEC-HPLC and plotted by incubation time. (b) The molecule integrity was detected by CE-SDS in a non-reducing condition. The main peak percentage was plotted by incubation time. Each point represents the mean of three measurements.

missing one or two light chains observed from conventionally generated clones, the investigators further tried over 20 different plasmid configurations and conditions and eventually screened five times the number of clones typically done for a mAb cell line development program [21].

The FIT-Ig molecule is a symmetrical, Ig-based bispecific molecule composed of three component chains. An analysis of FIT-Ig protein from the transient expression system suggested that excessive short chains can promote complete molecule assembly and can be removed readily by Protein A purification. Furthermore, a detailed analysis of impurities indicated that there is a small portion of fragmental molecules lacking one or two chain #2, resulting in the dysfunctional binding ability of outer Fabs. In contrast, fragmental molecules lacking chain #3 were not observed at all. The reason could be that chain #2 is a VH-CH1 polypeptide chain, unlike chain 3 that is just a natural IgG light chain. This is also why chain #2 ratio was adjusted in this study. In addition, the number of fragmental molecules may be amplified in stable cell line generation. The plasmid ratio used in transient transfection can be precisely transformed to expressed chain ratio, which is essential for the correct assembly of a bispecific antibody. However, in stable transfection, coding genes in plasmids need to be incorporated to the genome of host cells, during which the actual expressed chain ratio can be altered and beyond the optimal range due to the different incorporation efficiency and position of each gene. These fragmental molecules may also expose the hydrophobic region and induce aggregation formation, which can compromise the downstream purification efficiency [22]. Although the fragmental portion can be removed by ion exchange chromatography, obtaining high-quality stable clones at the early stage demanding less purification endeavors will be more cost effective, thus more desirable.

In this study, we hypothesized that extra chain #2 expression may increase the opportunity for chain #2 to pair with chain #1 and therefore reduce the percentage of the partial molecules, while too much chain #2 may affect the transfection efficiency of other chain-coding plasmids. So, in design 1, we cloned chain #2 gene into a separate vector to allow its ratio to be adjusted independently. The results suggested that minipools derived from design 1 transfection 1-2 generally exhibited a higher expression and produced a higher quality of FIT-Ig proteins than transfection 2-1 of design 2. Within design 1, transfection 1-2 generated the majority of high-performance minipools, suggesting that a chain ratio of 1:2:1 may be more desirable for the balance of the expression titer and FIT-Ig assembly. Afterward, the selected clone I-548-40-138 had been banked and used for multiple manufacturing runs at a 1000-L scale, where the titers were always above 3 g/L. The purification process was similar to regular IgG. Remarkably, a long-term stability study of FIT-Ig DP had demonstrated that FIT-Ig protein was stable for 2 years in a regular formulation, with no significant increase of both LMW and HMW species. Based on the structure design of FIT-Ig, chain #2 was covalently linked to chain #1 via an interchain disulfide bond, similar to regular Fab. Therefore, chain #2 is unlikely to dissociate from FIT-Ig unless a reducing reagent is present.

Our data demonstrated that a high-performance stable clone could lead to the efficient manufacturing process of FIT-Ig. This is attributed to a thorough understanding of FIT-Ig properties in terms of bispecific structure, component chains, and their assembly. The particular features of a FIT-Ig molecule as well as the essential factors on its efficient assembly were fully considered during the contemplation of the vector design and transfection strategy. During subsequent clone screening, both the expression titer and the quality of the protein produced are considered systematically, so that stably expressing CHO clones with high titer and product homogeneity were identified and retained. For a stable cell line generation of a bispecific antibody with other format, the special features of a bispecific molecule need to be adequately studied and applied to the early strategy and late screening. In conclusion, the strategy described in this study may increase the chance of obtaining high-level CHO cell clones of bispecific antibodies and thereby improve the manufacturing efficiency.

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CONFLICT OF INTEREST STATEMENT

Shiyong Gong and Chengbin Wu are current employees of Shanghai EpimAb Biotherapeutics Co., LTD. Dr. Chengbin Wu holds the position of a Editorial Board Member for Antibody Therapeutics and is blinded from reviewing or making decisions for the manuscript.

AUTHOR CONTRIBUTIONS

Shiyong Gong (Conceptualization-equal, Data curation-lead, Formal analysis-equal, Investigation-lead, Methodology-lead, Project administration-lead, Writing—original draft-lead, Writing—review and editing-equal) and Chengbin Wu (Conceptualization-equal, Investigation-equal, Methodology-equal, Project administration-supporting, Supervision-equal, Writing—review and editing-equal)]

DATA AVAILABILITY STATEMENT

The data underlying this article can be available online.

ETHICS AND CONSENT

Not applicable.

ANIMAL RESEARCH

Not applicable.

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