Optimization of Serum-Free Culture Conditions for HEK293T Cell-Based Production of Humanized Anti-CD147 Antibody

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Abstract

The production of antibodies in mammalian cells is essential for therapeutic development, but optimizing culture conditions remains a challenge for scalable production. To optimize the culture conditions for antibody production in HEK293T cell, humanized anti-CD147 antibody (HuM6-1B9) produced by HuM6-1B9-expressing HEK293T cells was used as a model. The cells (3.0×10^5 cells/mL) were seeded into T25 cell culture flasks. The performance of three serum-free media (SFM)—CDM4HEK293™, CHO-S-SFM II and EX-CELL™ 293—under direct and sequential adaptation were evaluated. Cell viability and cell number were examined using the trypan blue exclusion method and secreted HuM6-1B9 in the culture supernatant was quantified using indirect ELISA.

The results showed that CHO-S-SFM II achieved the highest HuM6-1B9 yield, especially with direct adaptation. It maintained high cell viability (97%) and enhanced per-cell antibody production. A strong positive correlation ($r = 0.893$, $p < 0.05$) between cell number and HuM6-1B9 concentration highlighted the importance of viable cell density. In conclusion, direct adaptation in CHO-S-SFM II maximizes HuM6-1B9 yield per cell, offering a foundation for optimizing recombinant antibody production in HEK293T cells.

Keywords: Antibody production, Mammalian cell expression system, Humanized antibody, CD147, Serum-free media.

Introduction

Recombinant antibodies are monoclonal antibodies produced using recombinant DNA technology in which engineered DNA sequences are introduced into host cells to express the desired proteins[7,22,26]. This technique has become essential for developing antibodies used in immunodiagnostics, therapeutics and biomedical research[2]. Compared to traditional hybridoma-derived monoclonal antibodies, recombinant antibodies offer significant advantages including reduced immunization steps and a minimized risk of human anti-mouse antibody (HAMA) responses due to enhanced humanization[13]. For full-length antibodies that require post-translational modifications such as glycosylation and disulfide bond formation, mammalian cell lines, particularly Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) 293 cells, are widely employed[21].

CHO and HEK293 cells have distinct advantages and limitations. CHO cells are considered the industry standard due to their high protein yield, adaptability to suspension cultures and proven scalability[12,23,28]. However, their glycosylation patterns differ from those of human cells, potentially impacting the efficacy of certain therapeutic antibodies[9]. In contrast, HEK293 cells, which are of human origin, are highly efficient in expressing complex glycosylated proteins with post-translational modifications similar to those found in human tissues[24]. This makes them especially suitable for producing therapeutic antibodies that require precise glycosylation.

As a result, several therapeutic proteins produced in HEK293 cells such as drotrecogin alfa (XIGRIS®; Eli Lilly), recombinant factor IX Fc fusion protein (rFIXFc; Biogen), recombinant factor VIII Fc fusion protein (rFVIIIFc; Biogen), human cell line recombinant factor VIII (NUWIQ®; Octapharma) and dulaglutide (TRULICITY®; Eli Lilly), have been approved by regulatory agencies like the FDA and EMA[6]. In addition, HEK293 cells demonstrate high transfection efficiency and robust protein expression, making them ideal for rapid and flexible biologics production[5,17,20].

Optimizing upstream processes including media selection and culture conditions specific to each cell line and antibody, is critical for efficient recombinant antibody production. Key factors to evaluate include medium composition, serum deprivation, antibiotic use, temperature, pH, CO_2 concentration, medium volume, harvest timing, nutrient supplementation and fed-batch strategies[10].

For instance, the addition of peptone to the medium significantly enhanced light chain production in HEK293E cells[11] while CDM4HEK293™ and SFM4HEK293™ have shown optimal performance for the growth and protein expression of HEK-293 E2-CD154 cells[16]. Additionally, optimized conditions for anti-apoptotic antibody production

by HEK293 Bax Bak double knock-out (DKO) cells were found to be at pH of 7.0 ± 0.3 and an agitation speed of 630 rpm[1].

CDM4HEK293™ and EX-CELL™ 293 are SFM specifically formulated for HEK293 cells, supplying essential nutrients such as amino acids, vitamins, lipids and trace elements to support cell metabolism and protein expression[4,8]. CHO-S-SFM II, although designed for CHO cells, has surprisingly been shown to support higher recombinant protein (CD99HIgG) production in HEK293T cells compared to CDM4HEK293™, suggesting the importance of metabolic adaptation and media optimization over strict cell line compatibility[3,25].

SFM offer several advantages for therapeutic antibody production including reduced contamination risk, improved lot-to-lot consistency and simplified downstream purification. Importantly, SFM eliminates animal-derived components, thus reducing immunogenicity and supporting compliance with FDA regulatory guidelines[27]. Moreover, SFM offers economic and scalability benefits by reducing production costs, improving reproducibility and enhancing large-scale manufacturing efficiency. However, adapting cells to serum-free conditions can be time-consuming optimization and may impact cell proliferation and productivity. Therefore, tailored adaptation protocols are necessary to achieve optimal antibody yields, depending on the host cell type and antibody characteristics.

In this study, we used a humanized monoclonal antibody targeting CD147 (HuM6-1B9) as a model to optimize antibody production in HEK293T cells. Different SFM and adaptation protocols were systematically evaluated for culturing HuM6-1B9-expressing HEK293T cells with high antibody production. Our findings provide valuable insights into optimal serum-free culture conditions for HEK293T cells and support the development of scalable production strategies for recombinant therapeutic antibodies.

Material and Methods

Cell Line and culture media: HEK293T cells were maintained in complete Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 U/mL of penicillin, 100 µg/mL of streptomycin and 2 mM of L-glutamine. Three SFM were used in this study: CDM4HEK293™ (Cytiva, Marlborough, Massachusetts, USA) supplemented with 4 mM L-glutamine, CHO-S-SFM II (Gibco, Thermo Fisher Scientific Inc., WLM, USA) and EX-CELL™ 293 (Merck KGaA, Darmstadt, Germany), supplemented with 6 mM L-glutamine.

Transfection of HuM6-1B9-expressing plasmid into HEK293T cells: HEK293T cells were seeded in 24-well plates at a density of 1×10^5 cells per well and cultured in complete DMEM at 37°C with 5% CO_2. Upon reaching 80% confluence, cells were transfected with 0.5 µg of pVITRO1-HuM61B9-IgG1/κ plasmid using TransIT-X2 dynamic delivery system (Mirusbio, Madison, USA), following the manufacturer's instructions. Plasmid construction was described in a previous study[18]. After 48 hours, cells were subjected to hygromycin B selection at concentrations ranging from 50 to 400 µg/mL to establish stable HuM6-1B9-expressing cell pools. Expression of HuM6-1B9 was confirmed by intracellular immunofluorescence staining and indirect enzyme-linked immunosorbent assay (ELISA).

Intracellular immunofluorescence staining: Stable HuM6-1B9-expressing HEK293T cells were harvested and fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 15 minutes at room temperature. Cells were washed twice with FACS buffer (2% FBS, 0.5 mM EDTA, 0.1% NaN_3 in PBS), then permeabilized using 0.1% saponin in PBS containing 5% FBS and 0.1% NaN_3. Blocking was performed on ice for 30 minutes with 10% FBS in PBS containing 0.1% saponin, 5% FBS and 0.1% NaN_3. Cells were stained with goat F(ab')2 anti-human IgM/G/A (H+L) conjugated with phycoerythrin (PE) at a 1:250 dilution for 30 minutes on ice. After incubation, cells were washed with PBS containing 0.01% saponin, 5% FBS and 0.1% NaN_3 and analyzed using a BD Accuri C6 plus flow cytometer and FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA).

Adaptation of cells to SFM: Stable HuM6-1B9-expressing HEK293T cells (3.0×10^5 cells/mL) were cultured in a T25 cell culture flask containing complete DMEM supplemented with 400 µg/mL of hygromycin B at 37°C, 5% CO_2 for 72 hours prior to SFM adaptation. For direct adaptation, the cells were adjusted 3.0×10^5 cells/mL in SFM supplemented with 400 µg/mL of hygromycin B (Protocol 1) and cultured in a T25 flask at 37°C, 5% CO_2 for 72 hours. For sequential adaptation, the cells (3.0×10^5 cells/mL) were cultured in the current medium until two population doublings were observed, then transferred into sequential SFM supplemented with 400 µg/mL of hygromycin B at 25%, 50%, 75% and 100% SFM (Protocol 2) or at 25%, 50%, 75%, 90% and 100% SFM (Protocol 3). After 72 hours of incubation, supernatants were collected for antibody quantification via indirect ELISA. Then, the cells were harvested to assess viability and cell number using the trypan blue exclusion method with a Countess 3 Automated Cell Counter (Invitrogen, Thermo Fisher Scientific Inc., WLM, USA).

Indirect ELISA: HuM6-1B9 concentration in culture supernatants was determined using indirect ELISA as previously described[18]. Briefly, 50 µL of CD147-BCCP (10 µg/mL) was coated onto a 96-well ELISA plate and incubated overnight at 4°C in a moist chamber. All subsequent steps were performed at room temperature. After washing with 0.05% Tween-20 in PBS, wells were blocked with 2% BSA in PBS containing 0.05% Tween-20 for 1 hour. Supernatants were added and incubated for 1 hour. After washing, HRP-conjugated rabbit anti-human IgG (1:3,000)

was added for 1 hour. The reaction was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and stopped with 1 N hydrochloric acid (HCl). Absorbance was measured at 450 nm using an ELISA microplate reader (Hercuvan Lab Systems, Brixton, London, UK). Antibody concentrations were calculated from a standard curve ranging from 0 to 25 ng/mL.

A linear line of best fit was generated. HuM6-1B9 concentration was determined using the equation: Absorbance at 450 nm = 0.06634 × [HuM6-1B9] (ng/mL) + 0.1349, with an R^2 value of 0.9845.

Statistical analysis: The results were presented as mean ± standard deviation (SD) from three independent experiments. One-way ANOVA with post hoc tests was used to multiple group comparisons. Spearman's rank correlation was applied to analyze the relationship between parameters. Statistical analyses were performed using GraphPad Prism 10, with p-values < 0.05 considered statistically significant.

Results and Discussion

Generation of stable HuM6-1B9-expressing cells: To establish a stable HEK293T cell expressing HuM6-1B9, HEK293T cells were transfected with the pVITRO1-HuM61B9-IgG1/κ plasmid and selected using hygromycin B drug selection (Figure 1A). The expression of HuM6-1B9 in pool stable cells was evaluated by intracellular protein staining and indirect ELISA. As shown in figure 1B, the pool of stable HuM6-1B9-expressing HEK293T cells exhibited 97.9% positive cells, while the untransfected cells were negative. Additionally, the secreted HuM6-1B9 was detected by indirect ELISA using a 10-fold serial dilution of the culture supernatant. The signal remained detectable even at a 1:1,000 dilution, with an optical density (OD) at 450 nm of 0.791 (Figure 1C). These results confirm the successful generation of stable HuM6-1B9-expressing cells that secrete the HuM6-1B9 antibody into the supernatant.

Optimization of serum-free media and adaptation protocols for HuM6-1B9 production: To determine the optimal conditions for HuM6-1B9 production, HuM6-1B9-expressing HEK293T cells were cultured in three different SFM using three distinct adaptation protocols (Figure 2). After 72 hours, the cells were harvested for analysis of viability and cell number and culture supernatants were collected to quantify HuM6-1B9 concentrations by indirect ELISA, with concentrations calculated based on a standard curve. All culture conditions maintained high cell viability (Figure 3A). In term of cell number, cultures in complete DMEM produced significantly higher cell densities, approximately five times greater than those in SFM conditions. However, no significant difference in cell number was observed among the three SFMs across all protocols (Figure 3B).

To evaluate the most suitable culture condition for HuM6-1B9 production, the performance of each 100% SFM condition under the three protocols was compared to complete DMEM (Figure 3C). The antibody concentration observed in protocol 1 and 2 indicated that CHO-S-SFM II resulted in the highest antibody production, followed by CDM4HEK293™ and EX-CELL™ 293. In contrast, protocol 3 did not show significant differences in antibody concentration among the three media. Notably, antibody production in CHO-S-SFM II remained consistent across all protocols, with no significant variation observed. Surprisingly, CHO-S-SFM II, although originally optimized for CHO cells, outperformed HEK293-specific media. This highlights the critical role of media composition over cell line specificity[14,15].

A similar phenomenon was reported in the production of CD99HIgG in HEK293T cells[25]. These results open new avenues for optimizing culture conditions across different host cell systems, with potential implications for the broader field of recombinant antibody manufacturing. Although complete DMEM supported the highest cell growth and total antibody production, the antibody production capacity per cell was lower compared to the CHO-S-SFM II medium under protocol 1 (Figure 3D). This indicates that serum-free conditions may induce metabolic shifts that favor protein synthesis over proliferation. Similar stress-induced responses have been observed in A549 cells where HSPA5 is upregulated in Opti-MEM compared to complete DMEM[19]. Collectively, these findings confirm that direct adaptation to CHO-S-SFM II (Protocol 1) is optimal for HuM6-1B9 production and reduce overall time required for antibody production. Notably, direct adaptation led to partial loss of adherence, suggesting a transition toward suspension culture. Further adaptation to suspension culture could enhance scalability and production efficiency in future studies.

Correlation analysis of cell number and HuM6-1B9 concentration: The relationship between the number of viable cells and the concentration of HuM6-1B9, correlation analysis was performed using data from all experimental conditions. The results showed a significant positive correlation between cell number and HuM6-1B9 concentration, with a correlation coefficient (r-value) of 0.893 (Figure 4). This data suggests that SFM and adaptation protocols supporting high cell viability and cell density contribute to an increase in HuM6-1B9 production.

Conclusion

The optimal conditions for HuM6-1B9 antibody production in stable HuM6-1B9-expressing HEK293T cells were achieved using CHO-S-SFM II medium with direct adaptation. This condition preserved high cell viability and enhanced antibody yield per cell, offering a practical and efficient approach for recombinant antibody manufacturing. These findings underscore the importance of selecting suitable serum-free media and adaptation protocol to enhance protein expression in HEK293T cells.

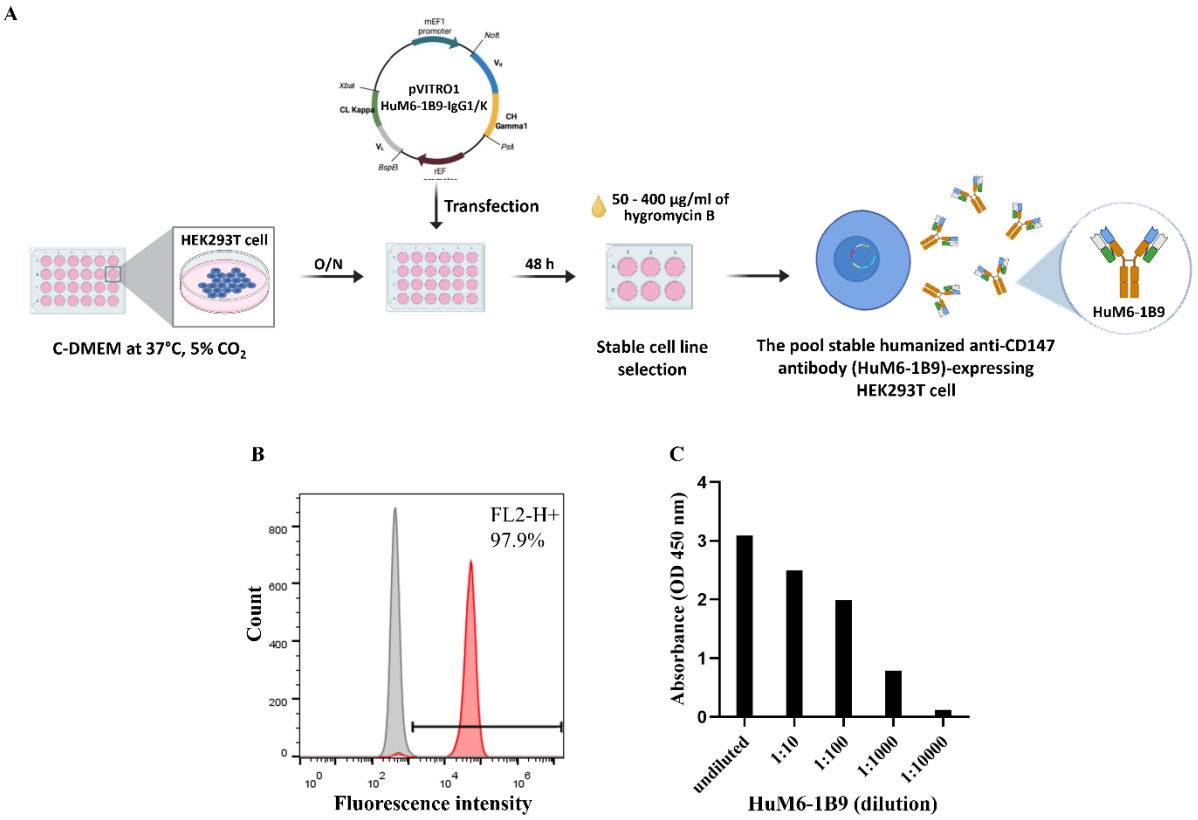

Figure 1: Generation process and expression of the stable HuM6-1B9-expressing HEK293T cell pool.
(A) Schematic workflow showing transfection of HEK293T cells with the pVITRO1-HuM6-1B9-IgG1/κ plasmid followed by hygromycin B selection (50–400 µg/mL). Cells surviving at 400 µg/mL were established as a stable pool of HuM6-1B9-expressing HEK293T cells.
(B) For intracellular HuM6-1B9 expression, untransfected cells (grey line) and the stable HuM6-1B9-expressing HEK293T cell pool (red line) were stained with PE-conjugated anti-human IgG antibodies.
(C) For HuM6-1B9 secretion, the level of HuM6-1B9 in the culture supernatant was detected by indirect ELISA using a 10-fold serial dilution.

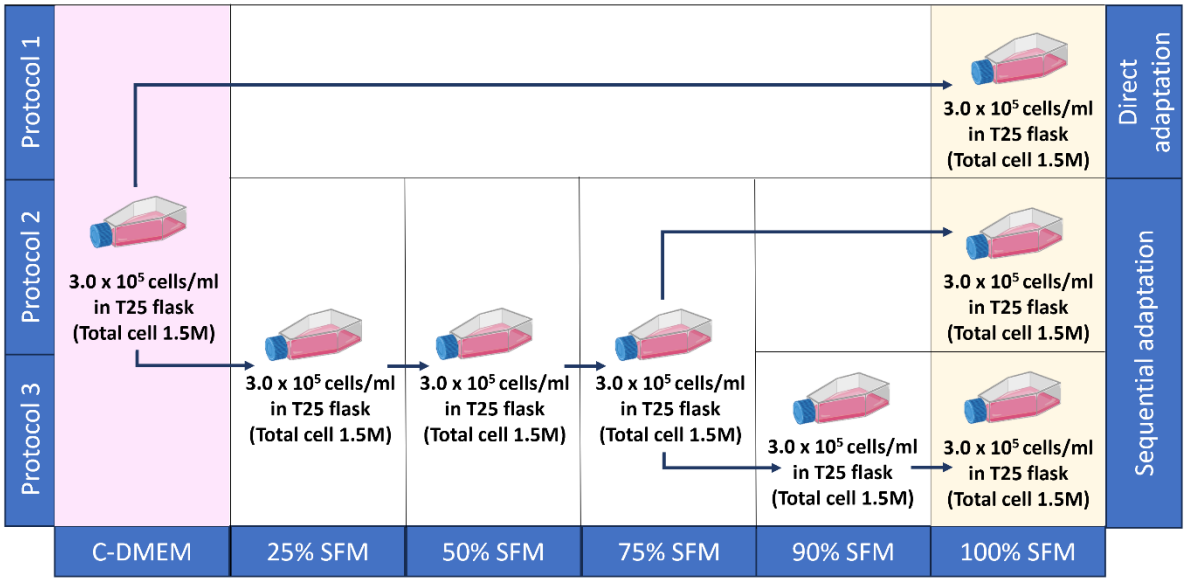

Figure 2: Schematic illustration of cell adaptation protocols. The three adaptation protocols for HuM6-1B9-expressing HEK293T cells include one direct adaptation protocol (Protocol 1) and two sequential adaptation protocols (Protocol 2 and Protocol 3).

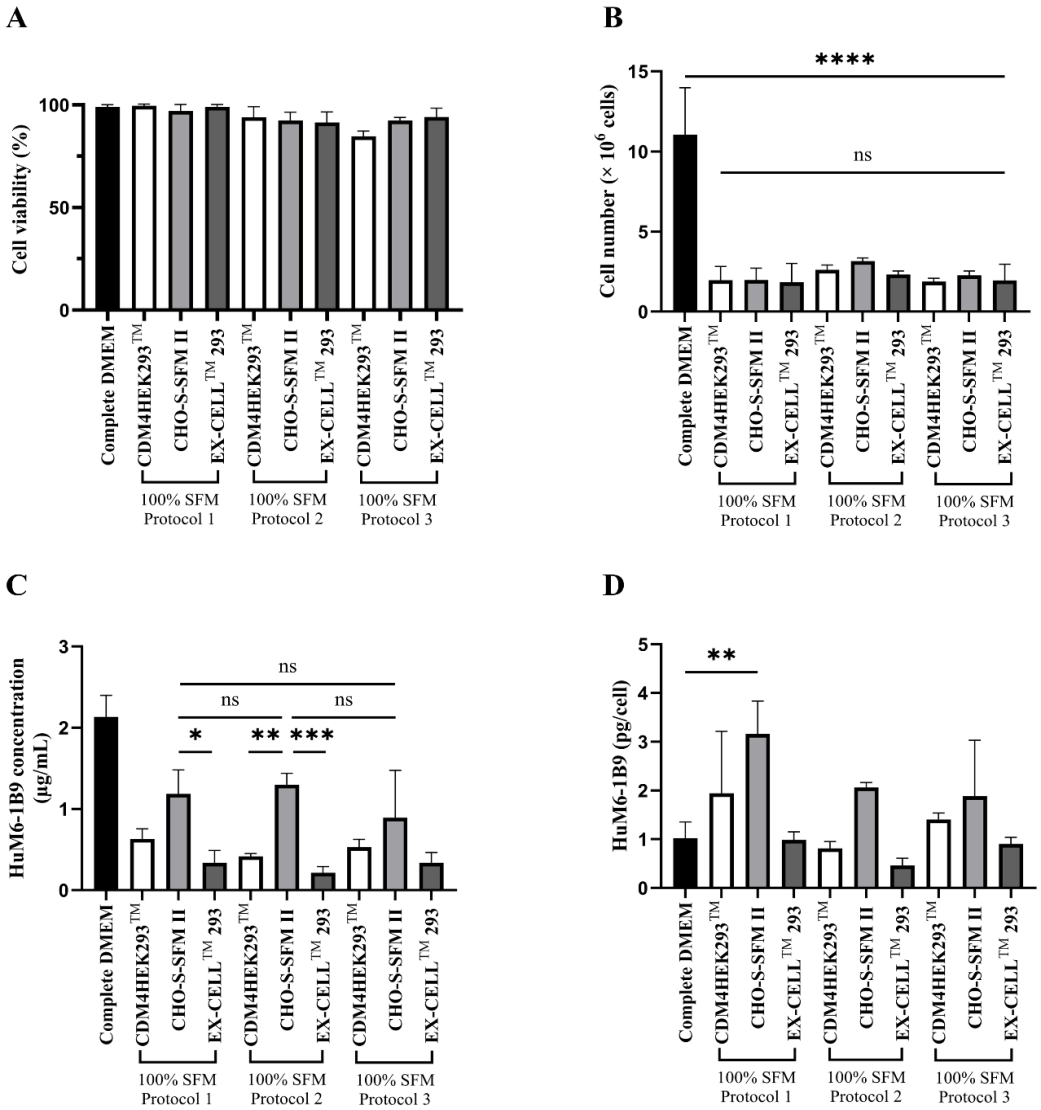

Figure 3: Comparison of the efficiency of SFM and adaptation protocols. (A) Cell viability and (B) the cell number of HuM6-1B9-expressing HEK293T cells after 72 hours of culture was measured using the trypan blue exclusion method. (C) The HuM6-1B9 concentration in the culture supernatant was calculated using a standard curve. (D) The HuM6-1B9 production capacity of the cells was demonstrated as the yield of HuM6-1B9 (pg) per cell. All experiments were performed in triplicate. Statistical analysis was determined using one-way ANOVA. ** indicates $p \leq 0.0001$, *** indicates $p \leq 0.0002$, ** indicates $p \leq 0.0021$, * indicates $p \leq 0.0332$ and non-significant (ns) indicates $p > 0.1234$.**

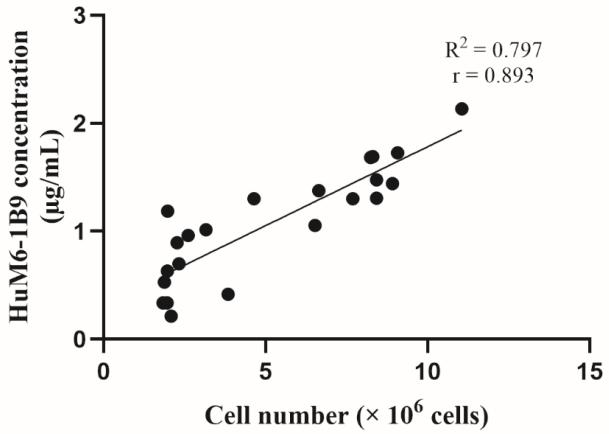

Figure 4: Correlation between cell number and HuM6-1B9 concentration. The relationship between HuM6-1B9 concentration and cell number across all experimental conditions was analyzed by Spearman's rank correlation.

Furthermore, the observed adaptability of HEK293T cells to CHO-S-SFM II suggests that media composition may have a greater impact than cell-line specificity, presenting new opportunities for optimizing culture conditions across different host systems. Future studies should investigate suspension adaptation and fed-batch culture strategies to further enhance scalability and productivity for therapeutic antibody manufacturing.

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