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Screening and Quality Assessment of High-Yield Anti-CD20 Antibody Cell Lines (Postprint)

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Abstract

Purpose: To screen high-expressing monoclonal cell lines and, through optimization of medium and feed, ultimately achieve the goal of improving target protein yield and quality. Methods: Monoclonalization of CHO-S cells transfected with the target protein was performed by limited dilution method, and the antibody expression level of monoclonal cell lines was preliminarily evaluated using a double-antibody sandwich ELISA method. Finally, based on the viability, density, productivity, and metabolic profile of the screened cell lines, 2-3 monoclonal cell lines were selected for culture condition optimization. The obtained fermentation broth was purified and captured, and comprehensive evaluation and analysis were conducted based on antibody protein expression level, glycoform, isoelectric point, purity, acidic and basic peak distribution, etc., to screen out the optimal cell line and optimal culture protocol. Results: After monoclonalization and culture condition optimization, the protein expression level increased from the initial less than 500 mg/L to 2290 mg/L, and the antibody protein purity reached as high as 97.48%. Antibody protein quality analysis results showed that the B1 protocol was the optimal culture protocol for this experiment. Conclusion: Cell line screening and medium optimization can significantly improve antibody protein yield and quality, while also providing certain degree of optimization for antibody protein glycoform, isoelectric point, purity, etc. Therefore, in industrial production, we can completely improve and enhance target protein yield and quality to a certain extent through screening of high-expressing clones and optimization of culture processes. This study provides good guidance significance for subsequent experimental research and industrial protocol development.

Full Text

Screening and Quality Evaluation of High-Yield Anti-CD20 Antibody Cell Lines

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Abstract

Objective: To screen high-expression monoclonal cell lines and optimize culture media and feeds to improve the yield and quality of the target protein. **Methods:** CHO-S cells transfected with the target protein were single-cell cloned using limited dilution. Antibody expression levels were initially evaluated via double-antibody sandwich ELISA. Based on cell viability, density, productivity, and metabolic profiles, 2-3 monoclonal cell lines were selected for culture condition optimization. Fermentation broth was purified and captured, followed by comprehensive evaluation of antibody expression level, glycosylation patterns, isoelectric point, purity, and acidic/basic peak distribution to identify the optimal cell line and culture strategy. **Results:** Following monoclonal selection and culture optimization, protein expression increased from an initial level below 500 mg/L to 2290 mg/L, with antibody purity reaching 97.48%. Quality analysis identified Protocol B1 as the optimal culture strategy. **Conclusion:** Cell line screening and medium optimization significantly enhance antibody yield and quality while improving glycosylation profiles, isoelectric point, purity, and charge variant distribution. These findings demonstrate that high-expression clone selection and process optimization can substantially improve target protein production and quality, providing valuable guidance for future research and industrial development.

Keywords: Monoclonal; Medium Optimization; Quality Evaluation

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Introduction

Non-Hodgkin' s lymphoma (NHL) is a malignant tumor of the blood and lymphatic system, with the vast majority of adult lymphomas originating from B lymphocytes. The CD20 antigen is a B lymphocyte surface differentiation antigen expressed in 95% of B-cell tumors but absent in normal tissues, making anti-CD20 antibodies highly specific for treating malignant lymphoid diseases. Rituximab, as an anti-CD20 antibody drug, has significantly extended survival time for NHL patients. However, clinical studies have revealed that some patients do not respond to or develop resistance against rituximab. Therefore,

improving anti-CD20 antibody drugs to enhance specificity and reduce immunogenicity represents a primary research direction. Currently, three generations of anti-CD20 antibody drugs have been approved for clinical use: the first-generation chimeric antibody rituximab, the murine antibody tositumomab, the second-generation fully human antibody ofatumumab, and the third-generation glycoengineered anti-CD20 antibody obinutuzumab.

This study builds upon previous investigations of the anti-CD20 antibody mutant Thio-2F2, including in vitro affinity assays, complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and in vivo antitumor activity. We conducted systematic optimization of the production process, including screening for high-yield stable cell lines, optimization of culture media and feeds, and comprehensive quality analysis of the antibody protein.

Materials and Methods

1.1 Cells

A stable cell line expressing the anti-CD20 antibody mutant Thio-2F2 was constructed in our laboratory. The constructed plasmid was transfected into CHO-S cells via electroporation at 300 V with a single pulse, yielding an initial post-transfection productivity of 451 mg/L.

1.2 Instruments

Biological safety cabinet (Thermo, MSC-advantage 1.8), shaking incubator (Kuhner, ISF4-XL), CO incubator (SHEL LAB, 2440-2), turbidimeter (IMMAGE 800), automatic cell counter (Inno-Alliance Biotech, Countstar IC100), biochemical analyzer (NOVA Plus100), electrophoresis apparatus (Bio-Rad, DYY-6B), electrophoresis tank (Bio-Rad, mini-sub cell GT), gel imaging system (Shanghai Tianneng, Tanon 1600), microplate reader (Molecular Devices, SpectraMax M5), quantitative PCR instrument (BIO-RAD T100), MabSelect SuRe protein purification system (AKTA-100), high-performance liquid chromatography system (Agilent, Model 200), and capillary electrophoresis system (CE-MDQ).

1.3 Methods

1.3.1 High-Yield Cell Line Screening Stably transfected pCHO1.0 plasmid cells were subjected to monoclonal selection via limited dilution. Double-antibody sandwich ELISA (DAS-ELISA) was used to measure antibody production after 14 days of static culture. Goat anti-human -chain secondary antibody served as the capture antibody, and HRP-labeled goat anti-human IgG Fc fragment as the detection antibody. Clones producing >30 mg/L were sequentially scaled up from 96-well plates to 24-well plates, 6-well plates, and finally 24-well

deep-well plates, cultured at 36.5°C, 8% CO , and 80% relative humidity for 7 days with periodic sampling to monitor cell status. Well-performing clones were further expanded to 50 mL shake tubes. After 3-5 days, cells were counted and, when viability exceeded 90%, seeded at 5×10 cells/mL for fed-batch productivity assessment.

1.3.2 Medium and Feed Optimization Minitab software' s Design of Experiment (DOE) function was employed to optimize culture media and feeds for different cell lines. Eleven experimental groups were established (designated A1-A11 and B1-B11), each with three parallel cultures. Fermentation was terminated when cell viability dropped below 70%, and IgG concentration was determined by immunoturbidimetric rate scattering analysis.

1.3.3 Antibody Quality Analysis **1.3.3.1 Antibody Purification**
Fermentation broth was purified using MabSelect SuRe Protein A chromatography (Column: Hiscreen, 10 cm bed height, 4.7 mL column volume, flow rate: 1.43 mL/min; Equilibration buffer: 20 mM PBS, 0.15 M NaCl, pH 7.0; Elution buffer: 50 mM citrate-citric acid, 0.15 M NaCl, pH 6.0). Impurities were removed selectively, and antibodies were eluted from Protein A. UV spectrophotometry was used for preliminary purity analysis of purified intermediates.

1.3.3.2 Antibody Purity Analysis
1) SDS-PAGE
Polyacrylamide gel electrophoresis was performed under both reducing and non-reducing conditions to analyze antibody purity and molecular weight. Statistical software was used for linear analysis based on separation differences from standards to determine heavy chain (50-55 kDa), light chain (23-25 kDa), and intact antibody (130-150 kDa) molecular weights and purity.

2) HPLC-SEC
High-performance size-exclusion chromatography was conducted using a mobile phase of 0.03 M Na HPO + 0.02 M NaH PO + 0.15 M NaCl + 10% acetonitrile (pH 7.0) at 0.8 mL/min at room temperature. Samples were diluted to 1 mg/mL and separated based on molecular size. Purity was calculated using area normalization.

1.3.3.3 Capillary Isoelectric Focusing (CIEF)
CIEF-WCID was performed using a CE MDQ system. Test samples were prepared as 5-10 mg/mL aqueous solutions. Ten microliters of sample were mixed with 240 L of master mix containing 12 L of pH 3-10 ampholytes, 200 L of 3 M urea-CIEF gel, 2 L of anode stabilizer, 2 L of cathode stabilizer, and 2 L each of pI markers A, B, and C. After centrifugation at 10,000 rpm for 3 min, the supernatant was injected manually. Appropriate focusing parameters were set based on sample characteristics. CEInsight software was used to acquire CMOS whole-column images and convert them to chromatograms at 280 nm. A standard curve of pI versus migration time was constructed using known protein markers to calculate sample isoelectric points and determine percentages of

acidic, main, and basic peaks via area normalization.

1.3.3.4 HPLC-FLR Glycan Analysis

High-efficiency normal-phase chromatography (Column: XBridge Glycan BEH Amide, 130 Å, 3.5 m, 4.6 mm × 250 mm; Column temperature: 60°C; FLR detector: excitation 265 nm, emission 425 nm) was used to separate and analyze released oligosaccharides after glycosidase digestion. Major glycan forms, structures, and molecular weights were identified by comparing retention times and peak patterns with standards.

Results

2.1 Monoclonal Screening Results

Limited dilution yielded 439 monoclonal cell lines, of which 135 produced >30 mg/L. Nineteen clones were selected for fed-batch culture. The highest-expressing clone reached 1530 mg/L, representing a two-fold increase over the parental cell line. Cell density also improved significantly, peaking at 1.8×10 cells/mL, with viability maintained above 95% throughout 15 days of culture. Comparative results for viability, growth rate, and productivity are shown in [Figure 1: see original paper].

2.2 Medium Optimization Results

Based on monoclonal screening, cell lines A and B were selected for process optimization. [Figure 2: see original paper] compares cell growth and productivity across different culture conditions. Expression levels for protocols A1, A4, B1, and B4 reached 1860 mg/L, 1760 mg/L, 2290 mg/L, and 1950 mg/L, respectively—significantly higher than other groups and improved from pre-optimization levels of 1440 mg/L and 1530 mg/L.

2.3.1 Antibody Purification Results

Protein A capture was performed on fermentation broth from protocols A1, A4, B1, and B4. UV spectrophotometry revealed that Protocol B1 yielded the highest protein concentration and capture amount, as detailed in -1.

2.3.2 Antibody Purity Comparison

2.3.2.1 SDS-PAGE Results

Quantity One software was used to integrate and analyze molecular weights and purity via peak area normalization. Electrophoresis results are shown in [Figure 3: see original paper]. All four groups exhibited intact antibody molecular weights between 130-150 kDa, light chains at 23-25 kDa, and heavy chains at 50-55 kDa, consistent with theoretical values. Purity exceeded 90% for all groups.

2.3.2.2 HPLC-SEC Results

Main peak retention times for A1, A4, B1, and B4 were 16.131 min, 16.128 min, 16.129 min, and 16.130 min, respectively, with purities of 95.63%, 98.23%, 97.47%, and 92.86% (all >90%). Aggregate peak areas for A1 and B4 were 3.84% and 6.28%, respectively, while A4 and B1 showed lower aggregation at 1.64% and 2.25%. These results indicate superior purity for A4 and B1, consistent with electrophoresis data. SEC chromatograms are presented in [Figure 4: see original paper].

2.4 Capillary Electrophoresis Results

CIEF results are summarized in -2. Basic peaks resulting from C-terminal lysine were considered part of the main peak as they do not affect efficacy or safety. All four groups showed similar isoelectric points (~8.3), consistent with theoretical values, with no statistically significant differences ($P<0.05$). Acidic peak analysis revealed that A4 and B4 had significantly higher acidic peak percentages (68.57% and 55.14%, respectively) compared to the other two groups. Since these share identical culture media and feeds, different culture conditions significantly impact charge variant distribution. High acidic peaks may reduce pharmacological activity, suggesting that Medium/Feed #1 is more suitable for target antibody expression.

2.5 HPLC-FLR Glycan Analysis Results

Glycan analysis results are shown in [Figure 5: see original paper] and -3. Compared with reference standards, A4 showed significantly lower G0 and G0F content than the other three groups ($P<0.05$), all of which exceeded 50%. G1, G1F, and G2F are galactosylated glycans, with B1 showing the highest galactose content. Notably, only B1 contained sialic acid modification (1.166%). Core fucosylation levels exceeded 85% across all groups, within the acceptable range of 2-13% for non-fucosylated glycans.

Discussion

The CHO expression system offers genetic tractability and enables selection of stable monoclonal cell lines with amplified gene copy numbers through DHFR or GS inhibition. CHO cells also achieve high viability and density in suspension culture, facilitating antibody production. During screening, high ELISA titers in 96-well plates did not always correlate with final productivity. For example, clone 2-71 showed 49 mg/L by ELISA but only 564 mg/L in fed-batch culture, whereas clone 2-95 yielded 32.93 mg/L initially but reached 1530 mg/L finally. This discrepancy may reflect clone-specific optimal conditions for medium composition, temperature, CO , humidity, shaking amplitude, and speed. Expanding the screening scope, particularly for moderately high-producing clones, could address this issue.

Thio-2F2 is a fully human antibody. Humanization reduces immunogenicity and enhances specificity, improving targeting and reducing toxicity. After selecting high-producing clones, various strategies can optimize culture processes to increase protein yield, including medium optimization, temperature reduction, and supplementation with sodium butyrate or DMSO. However, effects vary case-by-case and require comprehensive evaluation. In this study, preliminary optimization increased antibody titer to 2290 mg/L, with Protocol B1 emerging as the optimal strategy. Given antibody structural complexity, diverse mechanisms of action, and manufacturing intricacies, multiple quality control methods are essential for thorough product evaluation.

IgG glycosylation significantly impacts CDC, ADCC activity, half-life, and anti-inflammatory properties. Galactosylation enhances C1q binding and CDC effect but minimally affects ADCC. Increased sialic acid reduces ADCC activity, though low sialic acid content contributes to anti-inflammatory effects—complete removal abolishes this benefit. Non-fucosylated antibodies show markedly increased Fc RIIIa affinity and ADCC activity, with acceptable non-fucosylated glycan levels of 2-13%.

Based on expression level, Protocol B1 is optimal. Purity and charge variant analyses indicate A1 and B1 produce superior antibody quality. Glycan analysis demonstrates that B1 not only maintains high CDC and ADCC activity but also contains low sialic acid for anti-inflammatory effects, making it the most suitable protocol for Thio-2F2 expression. In summary, monoclonal screening and culture optimization yielded high-producing cell lines with improved physicochemical properties, including purity, glycosylation, charge variants, and isoelectric point. This work enhances biopharmaceutical productivity, streamlines processes, and establishes a solid foundation for future research and manufacturing.

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