Development and Application of An Animal-Component-Free Single-Cell Cloning Medium for Chinese Hamster Ovary Cell Lines

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Abstract

Single-cell cloning is a critical process in the generation of recombinant protein-producing mammalian cell lines. This process traditionally requires 10–20% fetal bovine serum (FBS) or other sera. Due to the presence of sera, single-cell cloning is a potential source of contamination from animal viruses and other adventitious agents. In order to address the regulatory needs of the biopharmaceutical industry, we have developed an Animal Component-Free (AF) medium designed for single-cell cloning of Chinese Hamster Ovary (CHO) cells. We utilized Design of Experiment (DOE) methodology to optimize the levels of six groups of nutrients (amino acids, trace metals, plant-derived hydrolysates, lipids, vitamins and selenium). The optimized formulation was tested with three recombinant CHO cell lines and was shown to generate comparable results, in terms of clonal survival and growth (80% of positive control in average), to the 10% FBS control. The clones generated using the AF cloning medium from three recombinant CHO cell lines were successfully scaled up to spinner or shaker flask cultures in Animal Component-Free culture media. The AF cloning process demonstrated improvement of growth and/or productivity. Transfection of a parental CHO K1 cell line and the subsequent selection and cloning processes were also evaluated in this AF cloning medium.

Materials and Methods

Cell Lines and Media

The stock cultures of the test cells lines (parental CHO K1, CHO AP expressing Secreted Alkaline Phosphatase, Recombinant CHO Line 2 expressing recombinant IgG) were maintained in suspension culture in animal-component free media (Sigma-Aldrich C8862 or proprietary formulation). The Basal Medium supplemented with 10% fetal bovine serum (FBS) was the positive control and included for every experiment. The clonal survival and growth results were reported as Wells with Growth (% of Positive Control) from duplicate 96-well plates. All formulations are supplemented with 4mM L-Glutamine unless otherwise specified.

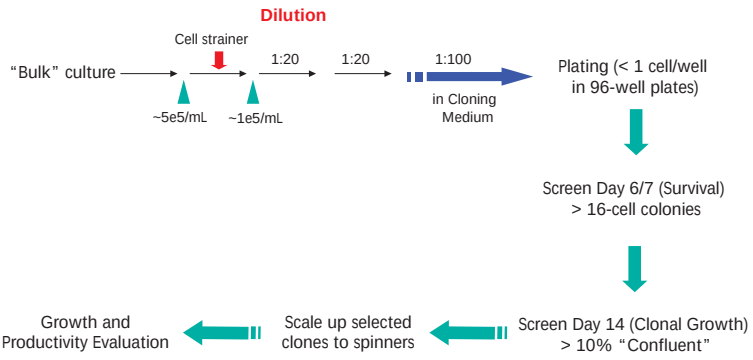


Figure 1. Limiting Dilution Cloning, Screening, and Scale-Up Procedures

Productivity Assays

IgG: HPLC Protein G affinity assay.

Statistical Analysis

In the two Factorial Matrix Experiments, the results were analyzed using Design Expert® software (Stat Ease, Minneapolis, MN). Normal probability plots were used to illustrate the significant factors (**Figure 2**).

Transfection, Selection, Single-Cell Cloning and Clone Evaluation of the CHO K1-GFP Cell Lines

The parental CHO K1 cells were transfected with a proprietary Green Fluorescence Protein (GFP) expression vector using Escort II Transfection Reagent (Sigma-Aldrich L6037) in the AF Cloning Medium (C6366). The transfected cells were selected using 200 mg/mL G418 (Sigma-Aldrich L6037) for two weeks in the AF Cloning Medium (C6366), until all the mock transfected cells were non-viable. The transfectants were transferred to 1:1 mixture of C6366 and CHO DHFR Medium (Sigma-Aldrich, C8862) containing 200 mg/mL G418 for an additional two weeks. The "bulk" stable transfected cells were then single-cell cloned in C6366. Selected GFP-positive clones were expanded sequentially to 24-well, 6-well plates and T75 flasks in the 1:1 mixture of C6366 and C8862. The GFP clones were selected for cyropreservation and further evaluation.

Results and Discussion

Matrix Experiments: Optimizing Amino Acids, Plant-Derived Hydrolysates, Iron, Lipids, Vitamins and Selenium for Cloning

Cell Line	CHO K1	
Basal Medium	An animal-component-free Chinese Hamster Ovary Cell growth medium	
Test Medium Components	Low Test Level (% of Medium A*)	High Test Level (% of Medium A)
Plant-Derived Hydrolysates (A)	0	25%
Amino Acids (B)	Basal Medium Level	10%
Iron (C)	Basal Medium Level	15%

* Medium A: A serum- and animal component-free medium intended for high-density suspension culture of CHO cells

Table 1. Matrix Experiment 1 (2^3) media design

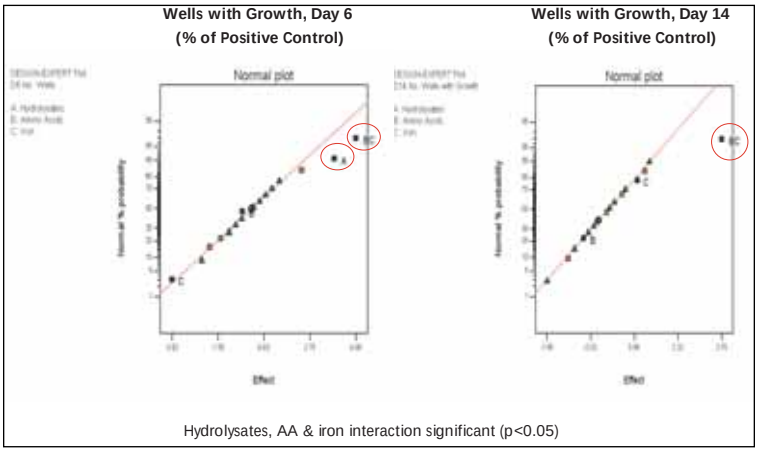


Figure 2. Statistical Analysis of Matrix Experiment 1

Cell Line	Recombinant CHO Line 2	
Basal Medium	Center-Point Formulation from Matrix 1	
Test Medium Components	**Low Test Level**	**High Test Level (% of Medium A)**
Lipids	Basal Medium Level	5%
Vitamins	Basal Medium Level	5%
Selenium	0	5nM

Table 2. Matrix Experiment 2 (2^3) media design

The detailed results of the two matrix experiments were described in a previous poster (available upon request). In brief, in Matrix Experiment 1, hydrolysates, amino acids and iron were statistically significant based on Day 6 growth (ANOVA $p<0.05$). These components are beneficial to clonal survival. The interaction between amino acids and iron was statistically significant based on Day 14 growth (ANOVA $p<0.05$, **Figure 2**). In Matrix Experiment 2, lipids, vitamins, selenium and the interaction between lipids and vitamins were statistically significant based on Day 7 growth (ANOVA $p<0.01$). These components are beneficial to clonal survival. Lipids, selenium and the interaction between lipids and selenium were statistically significant based on Day 14 growth (ANOVA $p<0.01$, Normal plot not shown). In order to confirm the findings from Matrix Experiment 2, selenium was tested at higher concentrations (100%, 200% and 500% of the high test concentration from the matrix, data not shown).

Finalizing the Formulation

Medium Components	Final Formulation (% of Medium A)
Hydrolysates	12.5%
Amino Acids	5%
Iron	7.5%
Lipids	Basal Medium Level
Vitamins	Basal Medium Level
Selenium	50 nM

Table 3. Final Formulation

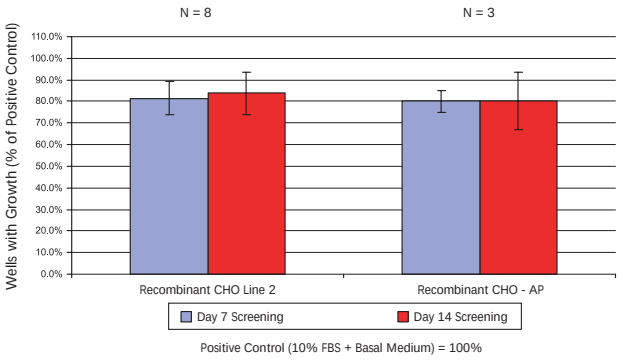


Figure 3. Cloning Performance of the Final Formulation in Two rProtein Producing Cell Lines

The final formulation with optimized chelator concentrations is depicted in Table 3. The final formulation was tested with three recombinant CHO cell lines. The clonal survival and growth performance was 80% in average of the positive control (**Figure 3**).

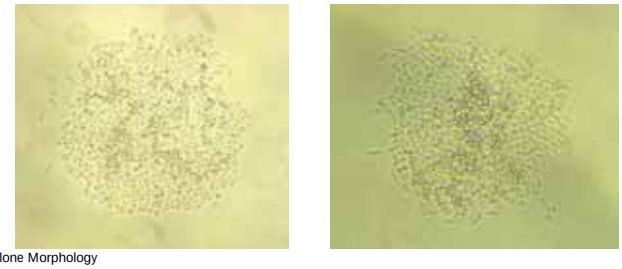


Figure 4. Clone Morphology

Experimental Stage	CHO AP	Recombinant CHO Line 2	CHO K1 GFP
	Number of Clones		
Selected from 96-well plates	37	37	40
Expanded to T-75 flasks	32 (86.5%)	35 (94.6%)	40 (100%)
Expanded to spinner flasks	not performed	28 (75.7%)	not performed

Table 4. Clone Scale-Up Summary

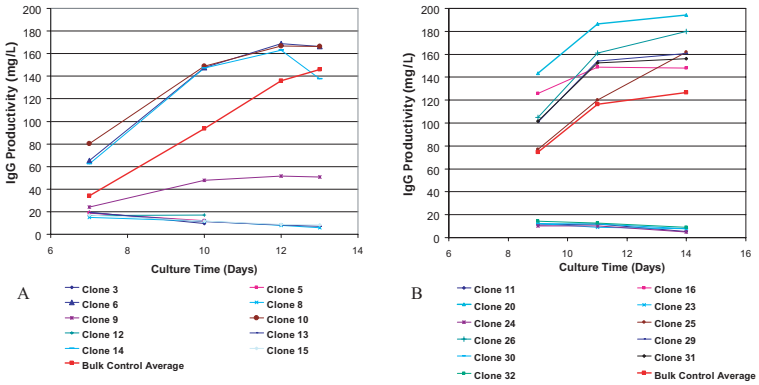


Figure 5. Productivity of the Selected Recombinant CHO Line 2 Clones

Clone Expansion and Growth/Productivity Evaluation

In order to demonstrate that the clones arisen from this Cloning Medium are expandable, and the cloning process using this AF Cloning Medium is able to improve growth and/or productivity comparing to the bulk cultures prior to cloning, selected clones generated from CHO AP, Recombinant CHO Line 2 and CHO K1-GFP cells were expanded in a 1:1 mixture of C6366 and C8862 or the culture media used prior to cloning. Spinner or shaker flask growth and productivity assays were performed in selected clones to evaluate the outcome of the cloning.

As depicted in **Figure 5**, 21 Recombinant CHO Line 2 clones were evaluated for growth and productivity in two separate spinner experiments in presence of selection pressure (200 mg/mL G418). Fourteen out of these 21 clones reached higher peak viable cell density than the bulk control (Data not shown here). Nine out of 21 clones demonstrated higher accumulative IgG productivity (42.8 % of the selected clones).

Transfection, Selection, Single-Cell Cloning and Clone Evaluation of the CHO K1-GFP Cell Line

The CHO K1 cells stably transfected with the GFP expression vector demonstrated heterogeneous fluorescence under the fluorescence microscope. This "bulk" population was single-cell cloned using C6366 four weeks under 200 mg/ml G418 selection. Out of the 40 clones that were expanded to T75 cultures, 36 were visually evaluated under the fluorescence microscope in order to eliminate mixed population cultures and the clones with very low fluorescence. Twenty-seven clones homogeneously expressed GFP. As depicted in Figure 6 (left panel), these clones (in T75 static cultures) were then evaluated for GFP expression levels by measuring mean fluorescence intensity (in Relative Fluorescence Units) on a fluorescence plate reader (Molecular Devices, Spectramax GeminiXS). The RFU values from each clone were normalized by per 10^6 viable cells. 26 out of 27 clones evaluated demonstrated stronger fluorescence per 106 viable cells than the heterogeneous "bulk" culture (**Figure 6**, right panel). The normalized mean fluorescence of the clones was 1.8 to 5.5-fold higher than the bulk culture.

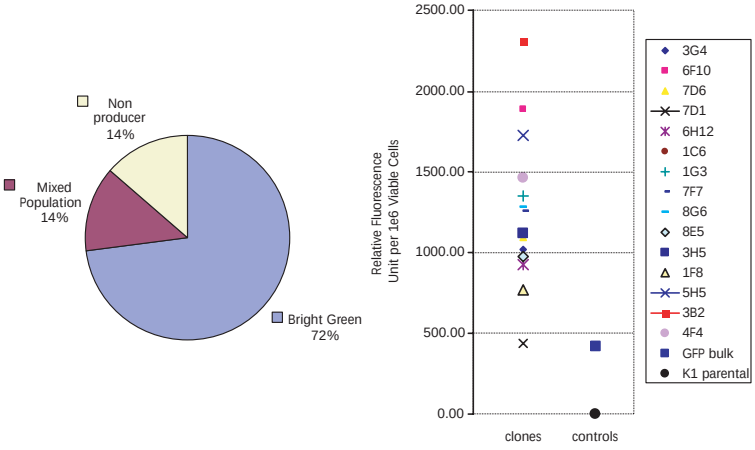
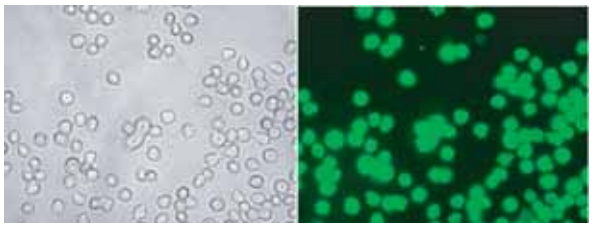
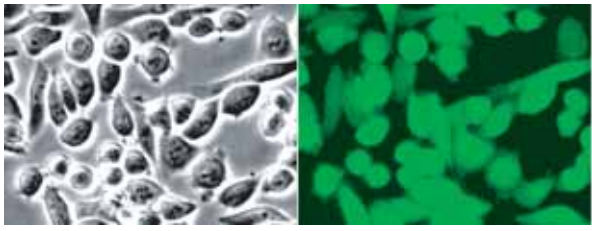


Figure 6. Cloning and Expansion of the CHO K1-GFP Cell Line



A. Heterogeneous culture



B. Clone 1G3

Figure 7A. CHO K1 GFP Bulk Culture

Figure 7B. CHO K1 GFP Clone 1 G3

Conclusions

- The Animal Component-Free cloning medium supports CHO cell clonal survival and growth comparable to 10% FBS supplemented Basal Medium.
- Selected clones from three recombinant CHO cell lines arisen from the AF cloning medium were successfully scaled up and demonstrated to have improved growth or productivity over the heterogeneous "bulk" culture.
- The AF Cloning medium can be used for other cell line generation procedures such as transfection and selection.
- The DOE approach is applicable for custom Animal Component-Free cloning media optimization.

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