

Development 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 in three recombinant CHO cell lines and was shown to generate comparable results, in terms of clonal survival and growth (75%–105% of positive control), to the 10% FBS control. The clones generated using the AF cloning medium from two recombinant CHO cell lines were successfully scaled up to spinner cultures in animal-component free culture media. The AF cloning process demonstrated improvement of growth and/or productivity.

Materials and Methods

Cell Lines and Media:

The stock cultures of the test cell lines (Table 1) were pre-adapted to 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 duplicated 96-well plates. All formulations are supplemented with 4mM L-Glutamine unless otherwise specified.

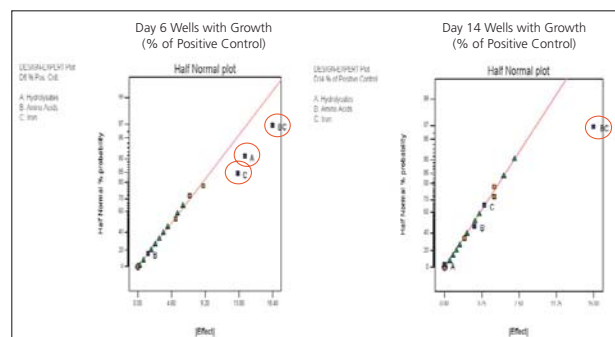


Figure 2: Statistical analysis of Matrix Experiment 1

As depicted in the Half-Normal Probability Plots in Figure 2, Hydrolysates, amino acids and iron (Test Factors A, B and C) were statistically significant based on Day 6 growth (ANOVA $p < 0.05$, left panel, circled in red). These components are beneficial to clonal survival. The interaction between amino acids and iron (Interaction B*C) was statistically significant based on Day 14 growth (ANOVA $p < 0.05$, right panel, circled in red).

Confirmatory Studies of Matrix Experiment 1

Based on the data shown in Figure 2, two formulations from Matrix Experiment 1 were tested in the four model cell lines (Table 2).

Test Medium Components	CL013-8 (% of Medium A)	CL013-9 (% of Medium A)
Plant-Derived Hydrolysates	25%	12.5%
Amino Acids	10%	5%
Iron	15%	7.5%

Table 3: Matrix Experiment 1 derived leading formulations

Cell Line	Cell Line Lineage	Recombinant Protein Produced
CHO K1	CHO K1 parental	None
CHO AP	CHO K1 derived	Secreted Alkaline Phosphatase
Recombinant CHO Line 1	CHO DG44 derived	IgG
Recombinant CHO Line 2	CHO K1 derived	IgG

Table 1: Model cell lines

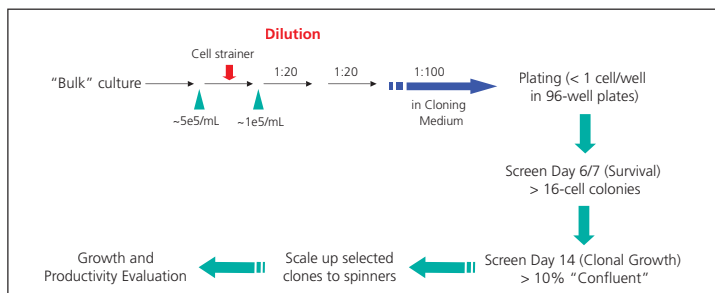


Figure 1: Limiting dilution cloning, screening, and scale-up procedures

Productivity Assays:

IgG: HPLC Protein G affinity assay. Alkaline Phosphatase Activity: Alkaline Phosphatase Activity Fluorescence Kit was used to assay the activity (Sigma-Aldrich AP-F). The procedures were carried out at the manufacturer's protocol with minor modifications.

Statistical Analysis:

In the two Factorial Matrix Experiments, the results of clonal survival (Day 6 Wells with Growth, % of Control) and clonal growth (Day 14 Wells with Growth, % of Control) were analyzed using Design Expert® software (Stat Ease, Minneapolis, MN). Half-Normal Probability Plots were used to choose significant effects. A plot of the ordered values of a sample versus the expected ordered values from the true population are approximately a straight line ("The Line of Chance"). The effects show up as outliers on the half-normal probability plot and have a p -Value less than 0.05 by Analysis of Variance (ANOVA) are statistically significant.

Results and Discussion

Matrix Experiment 1: Optimizing Amino Acids, Plant-Derived Hydrolysates and Iron for Cloning

Cell Line	CHO K1	
Test Medium Components	Low Test Level (% of Medium A)	High Test Level (% of Medium A)
Plant-Derived Hydrolysates	0	25%
Amino Acids	Basal Medium Level	10%
Iron	Basal Medium Level	15%

Table 2: Matrix Experiment 1 (2ⁿ) media design

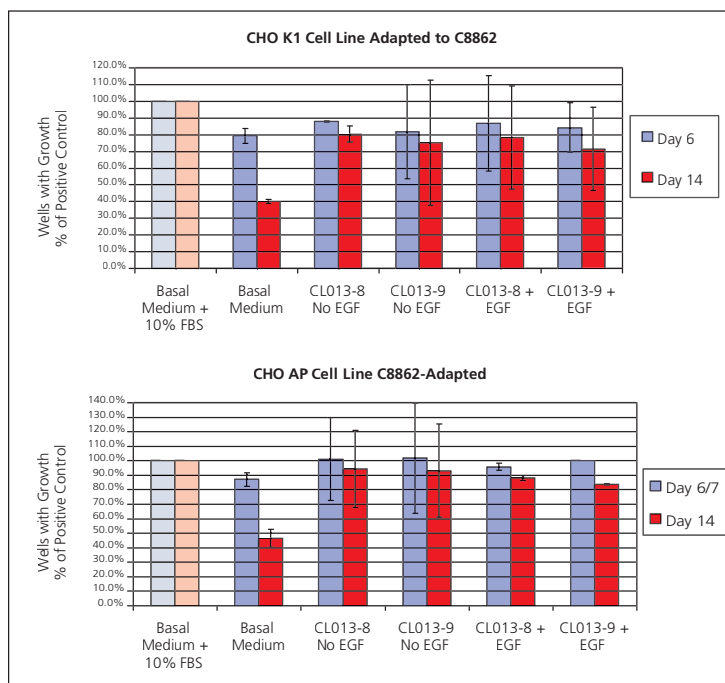


Figure 3: Confirmatory studies of Matrix Experiment 1 using CHO K1 and CHO AP cell lines

In summary, the two leading formulations derived from Matrix Experiment 1 gave comparable performance to the positive control in the CHO K1 and CHO AP cell lines, but not the two recombinant CHO cell lines that produce IgG. The clonal growth on Day 14 was less than 70% of positive control for Recombinant CHO Line 1, and less than 50% of positive control for Recombinant CHO Line 2 (Data not shown).

Matrix Experiment 2: Optimizing Lipids, Vitamins and Selenium for Cloning High-Producing Cell Lines

Cell Line	Recombinant CHO Line 2	
Basal Medium	CL013-9	
Test Medium Components	Low Test Level (% of Medium A)	High Test Level (% of Medium A)
Lipids	Basal Medium Level	5%
Vitamins	Basal Medium Level	5%
Sodium Selenite	0	5nM%

Table 4: Matrix Experiment 2 (2⁷) media design

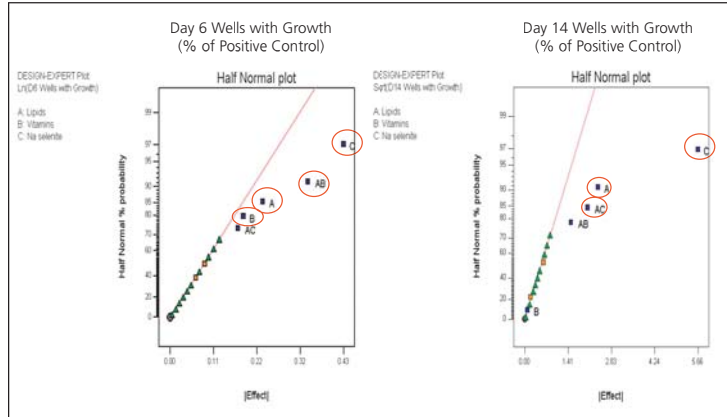


Figure 4: Statistical analysis of Matrix Experiment 2

As depicted in the Half-Normal Probability Plots in Figure 4, lipids, vitamins, selenium and the interaction between lipids and vitamins were statistically significant based on Day 7 growth (ANOVA $p < 0.01$, left panel, circled in red). 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 $p < 0.01$, right panel, circled in red). The clonal survival and growth were improved 20–30% of control comparing to CL013-8 and CL013-9 in previous experiments in this cell line (Data 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). Formulation CL013-9 supplemented with high level of lipids and 25 nM selenium demonstrated the highest average clonal survival and growth with the least variation in Recombinant CHO Line 2. This formulation was selected to be the final formulation. It was confirmed that selenium, in combination with lipids, was beneficial to clonal growth.

Clone Scale-up and Growth/Productivity Evaluation

In order to demonstrate that the clones arisen from this Cloning Medium are expandable, and the cloning process using this Cloning Medium is able to improve growth and/or productivity comparing to the bulk cultures prior to cloning, selected clones generated from CHO AP and Recombinant CHO Line 2 cells were scaled-up in the culture media prior to cloning. 14-day spinner growth and productivity assays were performed in selected scaled-up clones to evaluate the outcome of the cloning. Out of the 18 CHO AP clones that were successfully scaled-up to spinners, 5 clones reached higher peak viable cell density than the bulk control. Out of the nine clones evaluated for alkaline phosphatase activity, one clone demonstrated 141.1% higher peak AP activity than the bulk control. This clone demonstrated moderate growth (peak viable density 2.6e6/mL (Data not shown here).

As depicted in Figure 5, 21 out of 28 successfully scaled-up 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). 14 out of these 21 clones reached higher peak viable cell density than the bulk control. Nine out of 21 clones demonstrated higher accumulative productivity (42.8% of the selected clones).

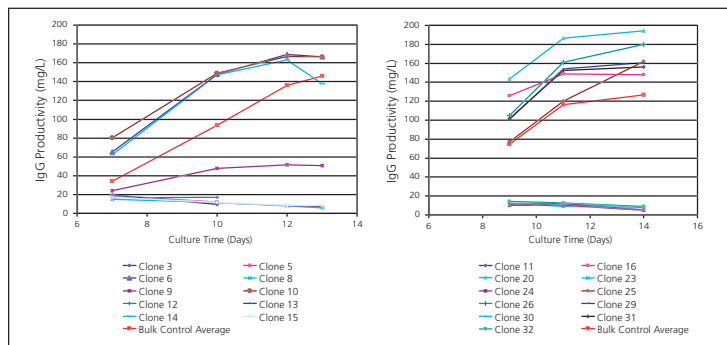


Figure 5: Productivity of the selected recombinant CHO Line 2 clones

Scale-Up Stage	CHO AP Number of Clones	Recombinant CHO Line 2 Number of Clones
Selected from 96-well plates	22	37
24-well plates	22	37
T-75 flasks	18	35
Spinner flasks	18	28
% of Clones Scaled up to Spinners	81.8%	75.7%

Table 5: Clone scale-up summary

Finalizing the Formulation

Based on the results from Matrix Experiment 2, two formulations were tested in three recombinant-protein producing model CHO cell lines to study the differential response amongst the cell lines and finalize the formulation (see Table 6 below).

Medium Components	Final Formulation (% of Medium A)
Hydrolysates	12.5%
Amino Acids	5%
Iron	7.5%
Lipids	5%
Vitamins	Basal Medium Level
Selenium	25 nM

Table 6: Final formulation

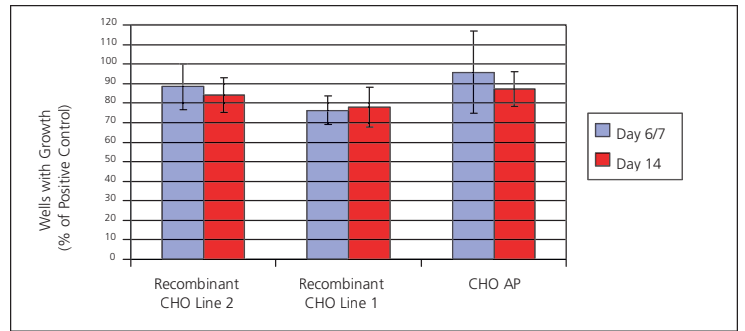


Figure 6: Cloning performance of the final formulation in the producing cell lines

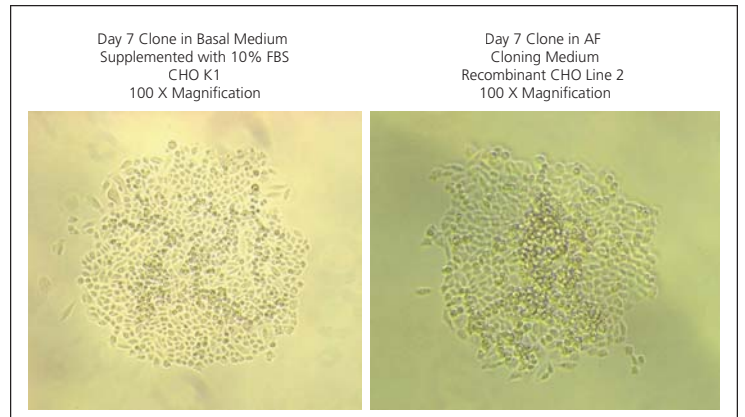


Figure 7: Clone morphology in the AF cloning medium

Conclusions

- The animal-component free cloning medium supports CHO cell clonal survival and growth comparable to 10% FBS supplemented Basal Medium (75%–105% of positive control).
- Selected clones from two recombinant CHO cell lines arisen from the AF cloning medium were successfully scaled up and demonstrated to have improved growth or productivity over the bulk culture prior to cloning.
- The DOE approach is applicable for custom animal-component free cloning media optimization.