

Development of an Animal-Component Free Electroporation and Recovery Formulation Using EX-CELL® CHO Cloning Medium

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Introduction

Electroporation is widely used for gene delivery in cell line generation, and is the method of choice for serum-free suspension culture transfection. Electroporation protocols and media can have a major impact on post-electroporation cell viability and transfection efficiency. Many laboratories use Phosphate Buffered Saline (PBS), various buffers or growth media for electroporation, which can lead to variable post-electroporation viabilities and transfection levels. We have optimized a single electroporation and recovery medium in an effort to increase post-electroporation cell viability, recovery viable cell density, and protein expression levels for Chinese Hamster Ovary (CHO) Cells.

We demonstrated that EX-CELL® CHO Cloning Medium (C6366) supports electroporation of plasmid DNA as well as siRNA into CHO K1 cells. We conducted electroporation and recovery media optimization using Design of Experiments (DOE) by mixing EX-CELL CHO Cloning Medium (C6366) with two other SAFC media in order to further improve transfection efficiency and post-electroporation recovery. By optimizing post-electroporation viabilities, viable cell densities and protein expression levels, we were able to create an optimized formulation. This optimized formulation achieved equivalent or higher post-electroporation viabilities, equivalent or higher viable cell densities after 24 hours recovery, and up to five-fold higher transient protein expression levels than other media tested.

Materials and Methods

Cell Lines and Media

The stock cultures of parental CHO K1 cell line (ATCC) were maintained in suspension culture in EX-CELL CHO DHFR Medium (C8862). A stock culture of clonal CHO K1 cells expressing a proprietary Green Fluorescence Protein (GFP) was maintained in EX-CELL ACF CHO Medium (C5467) supplemented with 200 µg/mL G418. All SAFC formulations were supplemented with 4 mM L-Glutamine. Competitor Medium A was supplemented with 8mM L-Gln and 1x HT Supplement according to manufacturer's instructions. Competitor Media B and C were used without additional supplementation.

Plasmid Electroporation

The parental CHO K1 cells were transfected with a proprietary GFP expression vector in the test media by electroporation, using a Gene Pulser II Electroporator (Bio-Rad). 4 mm-gap electroporation cuvettes (Bio-Rad and Sigma-Aldrich) were chilled on ice for 10 minutes prior to transfection. Parental CHO K1 stock culture was centrifuged at 200 RCF for 5 minutes and re-suspended in test media at 5.0×10^6 viable cells/mL. 0.8 mL cell suspension was gently mixed with 50 µg of sterile plasmid DNA in an Eppendorf tube and transferred to an electroporation cuvette. Electroporation was conducted in exponential decay mode (time constant ∞) at 300 Volts and 950 µF capacitance. The electroporated cell suspension was then immediately transferred into 5 mL test medium for recovery. Samples were taken at this point to evaluate post-electroporation viability using a ViCell XR Automated Cell Viability Analyzer (Beckman-Coulter). The remaining suspension was then recovered in T25 tissue culture flasks (Corning) for 24 hours at 37 °C, 5% CO₂.

Cell Recovery and Transient GFP Expression Assay

Twenty-four hours post-electroporation, all cells from each T-25 flask were collected (trypsinization where necessary), centrifuged at 200 RCF for 5 minutes, and re-suspended in 1.3 mL Phosphate Buffered Saline (PBS). 550 µL of the cell suspension was used to perform viable cell density counts using ViCell. Triplicate 200 µL aliquots of this cell suspension were then transferred to a black-walled, clear-bottom 96-well plate (Corning) for fluorescence reading. Mean fluorescence intensity was measured on Spectramax Gemini XS Fluorescence Plate Reader (Molecular Devices) at the following settings: excitation 435 nm, emission 535 nm, cutoff 530 nm, well-scan mode (9 points per well). Viable cell density and RFU values from each test condition were normalized to the value of the C6366 test condition.

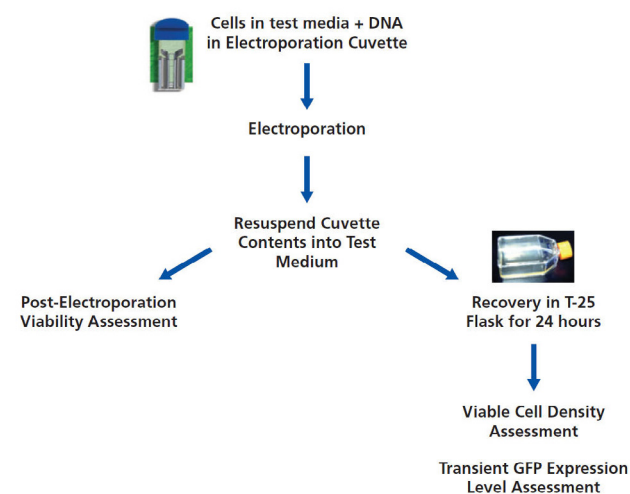


Figure 1: Electroporation and Recovery Procedure

siRNA Electroporation

The CHO K1-GFP cells were transfected with a single siRNA targeting the GFP sequence. siRNA transfections were performed using a Gene Pulser II Electroporator (Bio-Rad) and 4 mm-gap electroporation cuvettes (Bio-Rad). Cells were pelleted by centrifugation and resuspended in either EX-CELL CHO Cloning Medium (C6366) or Competitor C media at a concentration of 2.0×10^6 viable cells per 400 µL. Cells were then transferred to cuvettes and electroporated using a single square wave pulse of 100 µs at 1200 V. Cells were then allowed to recover in the cuvettes at 37 °C for 10 minutes and then transferred to Poly-2-hydroxyethyl methacrylate (P3932) coated 6-well plates. 2 mL EX-CELL ACF CHO Medium (C5467) was added to each well.

Target Gene Knockdown Efficiency Assay

Cells were counted 24 hours post transfection on a ViCell XR Automated Cell Viability Analyzer, and 5.0×10^5 cells were saved in RNA Later (R0901) as per manufacturer's instructions. Total RNA was isolated from cells using GenElute Mammalian Total RNA Miniprep Kit (RTN10). Two-step reverse transcription PCR reactions were setup using MMLV Reverse Transcriptase (M1302) with 200 µg total RNA followed by Realtime PCR using SYBR® Green JumpStart™ Taq ReadyMix™ (S4438). PCR reactions were run on an MXPro 3000P QPCR System (Stratagene). Data was analyzed using MxPro software (Stratagene). Beta-actin was used as a housekeeping gene to normalize GFP expression levels.

Media Optimization by DOE Mixing

Design of Experiments (DOE) mixing experiment was designed using Design Expert® software (Stat Ease, Minneapolis, MN). D-optimal model was used with slight modification. Three formulations were mixed (Table 1) to generate test formulations 1–16. Electroporation and recovery were performed as described above. The results were then analyzed using Design Expert software.

Run	C6366	SAFC Medium A	SAFC Medium B	Run	C6366	SAFC Medium A	SAFC Medium B
1	0%	0%	100%	9	50%	50%	0%
2	33%	33%	33%	10	0%	50%	50%
3	33%	33%	33%	11	100%	0%	0%
4	17%	67%	17%	12	17%	17%	67%
5	100%	0%	0%	13	67%	17%	17%
6	33%	33%	33%	14	0%	100%	0%
7	0%	100%	0%	15	33%	33%	33%
8	50%	0%	50%	16	0%	0%	100%

Table 1: Experimental Design for Mixing Experiments

Results and Discussion

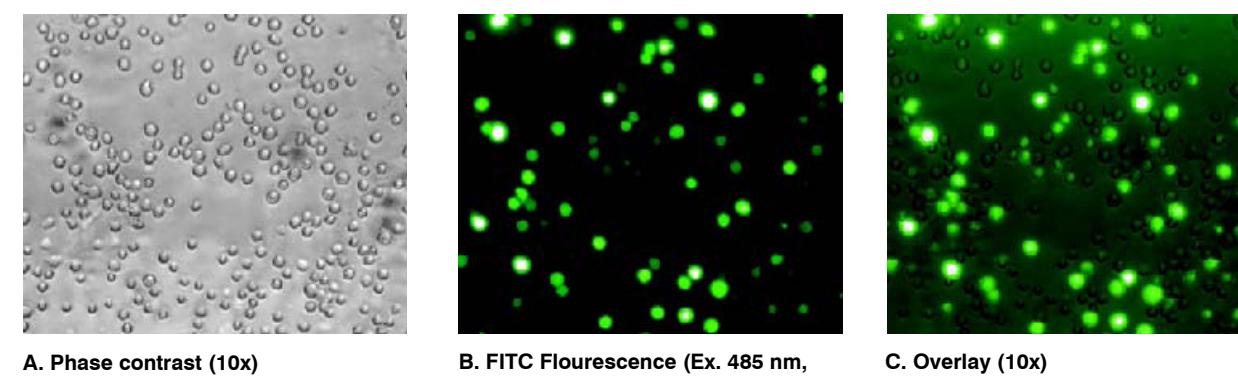


Figure 2: Transient GFP Expression in EX-CELL CHO Clone Cloning Medium (C6366)

CHO K1 parental cells were electroporated and recovered in EX-CELL CHO Cloning Medium (C6366). Recombinant GFP expression after 24 hours was visualized using fluorescence microscopy.

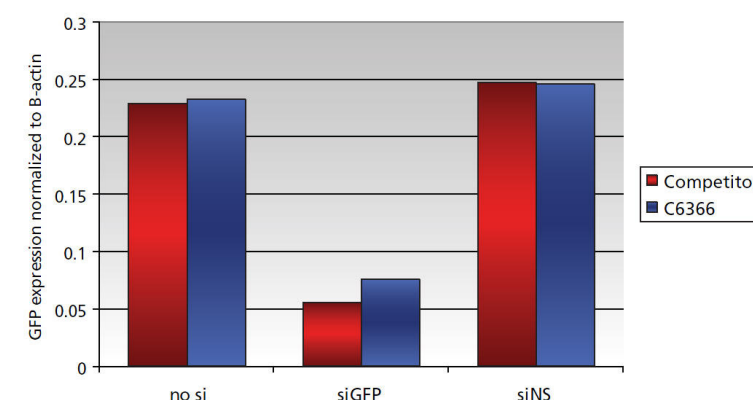


Figure 3: siRNA Knockdown of GFP in C6366 and Competitor's Electroporation Media

Approximately 70% knockdown of the GFP message was achieved by transfecting a siRNA specific to GFP. Transfection of a non-specific siRNA (siNS) had no effect on GFP transcript concentrations. The level of knockdown observed was comparable using CHO Clone AF Cloning Medium (C6366) and Competitor C medium (a medium marketed for enhanced siRNA electroporation).

Name	Goal	Importance
C6366 (X-1)	is in range	3
SAFCB Medium A (X-2)	is in range	3
SAFCB Medium B (X-3)	is in range	3
Post EP Viability	maximize	3
RFU (D1)	maximize	5
VCD (D1)	maximize	5

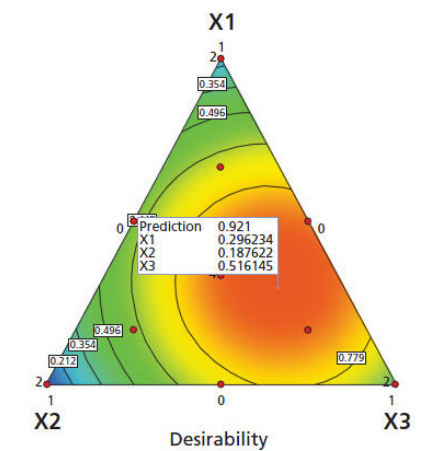
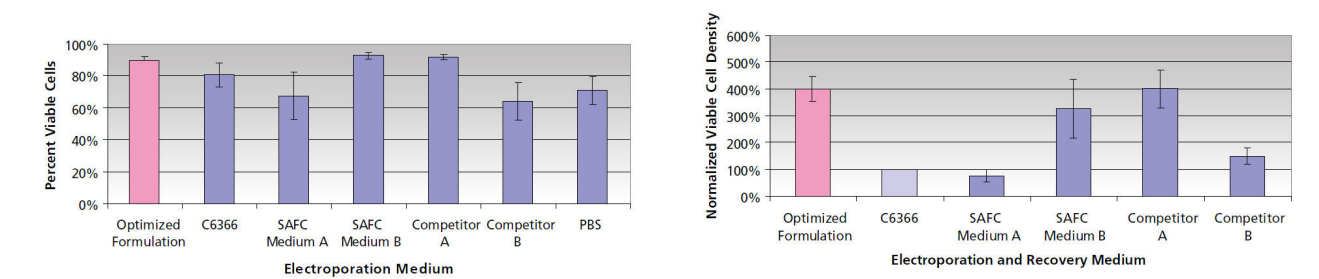


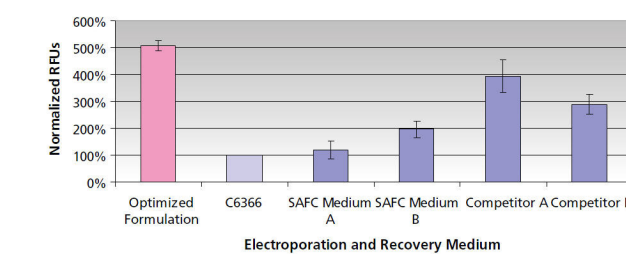
Figure 4: DOE Mixing Statistical Analysis and Numerical Optimization

Post-electroporation viability, average Relative Fluorescence Units (RFU's) from triplicate samples and viable cell density (VCD) after 24 hours recovery were used as parameters to perform statistical analysis (quadrant models selected for all three parameters, model fit $p < 0.01$). Numerical optimization was performed based on the importance assigned to each parameter (depicted in Figure 4, left panel). The optimized formulation was indicated in the contour plot (right panel). Based on the statistical analysis, the optimized mixing ratio was 29.6% EX-CELL CHO Cloning Medium (C6366), 18.8% SAFC Medium A and 51.6% SAFC Medium B.



5A. Post-Electroporation Viability

5B. Normalized Viable Cell Density after 24 Hours Recovery



5C. Normalized Transient GFP Expression Level after 24 Hours Recovery

Figure 5: Confirmatory Studies of the Mixing-Derived Optimized Formulation

As depicted in Figure 5, the optimized formulation (Figure 4) achieved equivalent or higher post-electroporation viabilities and viable cell densities, and up to five-fold higher transient protein expression levels than other media tested, including the three mixture components and two competitor's media.

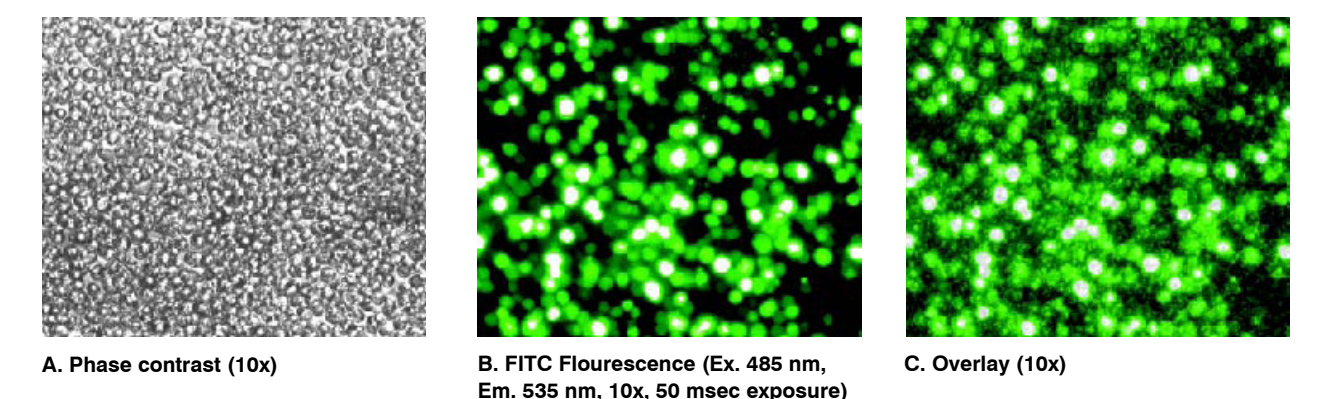


Figure 6: Transient GFP Expression in Mixing-Derived Optimized Medium

CHO K1 parental cells were electroporated and recovered in the optimized formulation. Recombinant GFP expression after 24 hours was visualized using fluorescence microscopy.

Conclusions

- EX-CELL CHO Cloning Medium (C6366) supports electroporation of plasmid DNA and siRNA into CHO K1 cell lines.
- Knockdown efficiency of target gene (GFP) using EX-CELL CHO Cloning Medium (C6366) as the electroporation medium for siRNA is equivalent to the competitor's siRNA transfection medium in a CHO K1 cell line stably expressing GFP.
- An optimized animal-component free electroporation and recovery formulation was derived from DOE mixing experiments using EX-CELL CHO Cloning Medium (C6366) as one of the components. This formulation achieves equivalent or higher post-electroporation viabilities, equivalent or higher viable cell densities after 24 hours recovery and up to five-fold higher transient protein expression levels than other media tested.