

Electroporation of Primary Bone Marrow Cells

Contributed by Elizabeth Eustis-Turf*, Kiu-Mei Wang†, and Lawrence B. Schook, Laboratory of Molecular Immunology, Department of Animal Sciences, University of Illinois, Urbana-Champaign.

The transfer of genes into eukaryotic cells permits the study of many things, such as gene regulation, gene structure, and gene function. The ability to insert a gene into a high percentage of hematopoietic stem cells would also make gene therapy a viable option. Our laboratory has been trying to maximize genetic transfer into non-adherent, murine bone marrow cells using electroporation. We have used the Gene Pulser® apparatus to electro-transfect either a murine MHC class II gene, A_B^b, or a plasmid encoding a mutant dihydrofolate reductase enzyme (*dhfr*) into murine bone marrow cells.¹ In these studies, we determined which parameters worked best for transient expression of genes as well as stable integration of genes. Transient expression is one way for us to study gene regulation, but we are also interested in gene therapy, which can only be successful if there is some kind of selection for stable gene integration. Therefore, we varied buffer, capacitance, and voltage in order to define the optimal conditions for both outcomes.

Bone marrow cells were isolated from the femurs of C3H/HeN and C578B1/6 mice, washed, and resuspended in electroporation medium, at 7-9 x 10⁶ cells/ml. Samples of 800 µl each were placed in 0.4 cm gap Gene Pulser cuvettes and 10 µl of DNA (1.0 µg/ml) added. The cell-DNA suspension was gently agitated and placed on ice for 15 minutes prior to electroporation. After receiving the pulse, the samples were placed on ice for a second 15 minute incubation. Control cultures consisting of cells but no DNA were treated exactly like the test samples. After the second incubation on ice, the control and test cells were cultured in liquid culture in the present of CSF-1. The transient expression of the class II gene was monitored by staining the cells with the appropriate monoclonal antibody (BP 107) and analysis on a Coulter Epics C cytofluorometer. The stable integration of the *dhfr* gene was demonstrated by the acquired ability of cells to survive in selective medium containing the anti-folate drug, methotrexate (MTX). For colony-forming unit (CFU) assays, 0.33% noble agar was added to the medium, and the number of colonies was counted after 10 days in culture. Cells receiving the *dhfr* plasmid were cultured with increasing amounts of MTX, from 0-500 nM. Genomic DNA was isolated from the bone marrow cells for Southern blot analysis.^{2,3}

* Present address: Medical College of Virginia, Department of Preventive Medicine, Box 212, Richmond, VA 23298.

† Present address: Inner Mongolia Medical College, 010059 Huhehouite, Peoples Republic of China

As shown in Table 1, a 3.5-fold increase was seen in the number of bone marrow stem cells able to survive in 500 nM MTX, when 500 µF and 300 V (750 V/cm) Gene Pulser settings were used and a pulse time constant of 10 msec was achieved. Southern blot analysis was done to verify the presence of the *dhfr* gene within the cultured cells. High molecular weight genomic DNA was isolated from cells that had been pulsed seven days previously and cultured in MTX. Figure 1 reveals hybridization of the *dhfr*-containing plasmid to sequences within the test cells, yet no hybridization to control cell DNA. The presence of the *dhfr* gene within the test cells, together with the evidence of expression of that gene due to survival in MTX, indicate the stable integration of the pulsed DNA into the bone marrow genomic DNA.

Table 1. Resistance of CFU-M^a and L929 Cells after Electroporation

	Gene Pulser Settings		Pulse Time Constant ^b (msec)	Mean % survival of CFU-M and L929 in 500 nM MTX	
	Volts	µF		Control	With <i>dhfr</i>
CFU-M	300	250	5.8	6.5	9
CFU-M	250	500	11.1	0.8	4
CFU-M	300	500	10.6	14.0	39
L929	250	25	7.3	5.0	19

- a. 2 x 10⁵ marrow cells were pulsed; CFU-M = marrow cell colony forming units.
- b. Pulse time constants for test cultures. Controls were within 1 msec of test samples.

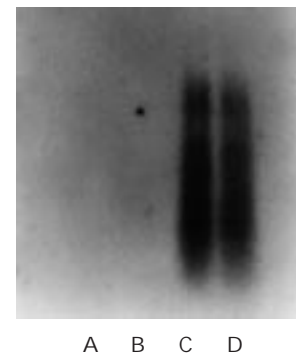


Fig. 1. Southern blot analysis of control and test bone marrow cultures. High molecular weight genomic DNA was digested with *Hind* III, electrophoresed (10 µg/lane), blotted, and probed with ³²P-labeled *dhfr*. Lanes A and B: Control cell DNA; lanes C and D: Test cell DNA. Hybridization of the *dhfr* probe is seen only with the test cell DNA.

We have also used the Gene Pulser apparatus to stably transfect adherent 929 cells with the *dhfr* gene in order to compare the conditions required when an adherent cell rather than a non-adherent one was the target. These cells were cultured in medium containing MTX to determine the percentage of cells expressing the mutant enzyme. The use of the phosphate buffered sucrose (272 mM sucrose, 7 mM PO₄, 1 mM MgCl₂, pH 7.4) allowed a lower capacitance setting of 25 μF; and with a 250 V (650 V/cm) pulse, a time constant of 8-11 msec was optimal. These Gene Pulser settings resulted in the production of the mutant *dhfr* in 19% of the cells capable of forming colonies, a 4-fold increase over controls. Table 2 gives a comparison of the parameters we have found to give optimal results for both adherent and non-adherent cell types.

In our system, the most important variables appear to be field strength (v/cm) and pulse time constant. We found that electroporation conditions of 625-750 V/cm and time constants of 7-11 msec were optimal for transient expression or stable transfection of bone marrow or L929 cells. With phosphate buffered sucrose electroporation medium, the 25 μF capacitor was used to produce a pulse with a time constant in the optimal range. When lower resistance PBS was used, the 250 and 500 μF capacitors of the Capacitance Extender were required. The conditions reported here are now used routinely in our laboratory in our studies of the regulation of class II gene expression and the immune response.

Table 2. Comparison of Optimal Parameters for Electroporation of Adherent and Non-Adherent Cells

	L929	Bone Marrow	Bone Marrow
Electroporation medium	Phosphate buffered sucrose	PBS	PBS
Voltage setting	250 V (750 V/cm)	300 V (650 V/cm)	300 V (650 V/cm)
Capacitor (μF)	25	500	500
Time constant (msec)	7-10	8-11	8-11
Efficiency of transfection	8 x 10 ^{-4a}	1 x 10 ^{-4a}	b

- Stable transfection efficiency was calculated as (number of CFU/total number of cells plated).
- Maximum transient expression (measured as a two-fold increase of fluorescence intensity of samples over controls) was obtained using these conditions. Transient expression was higher when supercoiled plasmid DNA was used rather than linearized plasmid.

References

- The mutant *dhfr* sequence, pFR400, was generously provided by Genentech, Inc., South San Francisco, CA.
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