

Transfection of Mouse and Human Embryonic Stem Cells by Electroporation Using the Gene Pulser MXcell™ System

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Introduction

Gene targeting in mouse embryonic stem (ES) cells via homologous recombination has been a key scientific breakthrough that enables the generation of knock-out mice and the delineation of gene function in animal models. The clinical applications of this technology may have a significant impact in regenerative medicine, since genetic modification of human ES cells is an important step toward stem cell-based therapies. Since pluripotent human ES cells have the unique property of differentiating into any cell type, successful genetic modification of these cells would enable the repair of defective genes when cells differentiated from ES cells are transplanted into patients.

Electroporation is the method of choice for introducing genes into mouse ES cells. By using well-established electroporation parameters, mouse ES cells may be stably transfected with high efficiency and without deleterious effects on their pluripotency and karyotype stability. Electroporation protocols that have been established for mouse ES cells do not translate well to human ES cells. Compared to mouse ES cells, human ES cells have shown low cell viability and transfection efficiencies after electroporation.

There are only a few publications on electroporation-based gene targeting for human ES cells (Davis et al. 2008, Di Domenico et al. 2008, Irion et al. 2007, Urbach et al. 2004, Zwaka and Thomson 2003). Most reported methods are for specific gene loci, or for genes that are expressed in ES cells. It is unclear if the same method would be effective for targeting any gene at any gene locus. In a recent publication, Ruby and Zheng have reported an electroporation-based method for transfecting human ES cells that have been grown under conditions that involve the use of trypsin to dissociate and passage the cells (Ruby and Zheng 2009). Since most human ES cell lines, such as the widely used H9 cells from WiCell Research Institute, Inc. (Madison, WI), do not re-form colonies after dissociation with trypsin, this method may not be translatable to all stem cell lines. Other strategies of gene targeting in ES cells are a zinc-finger nuclease-based method

with an integrase defective lentiviral vector delivery (Lombardo et al. 2007) and an adenovirus-based vector delivery approach (Suzuki et al. 2008). Although these strategies may be promising for gene delivery, they are technically more demanding and time-consuming than electroporation.

The aim of the present experiments was to optimize electroporation parameters for the transfer of DNA into human ES cells that have been grown under colony-forming and feeder cell-free conditions. Bio-Rad's Gene Pulser® II electroporation system is a reliable and frequently used instrument for the transfection of mouse ES cells, but it is difficult to simultaneously test multiple electroporation conditions on it. We used the Gene Pulser MXcell system, with its 96-well plate format and easily programmable features, to test different electroporation conditions for efficient transfection of human ES cells.

Methods

Pluripotent mouse EZ1 ES cells (Mueller-Oritz et al. 2009) were grown, at passage 11, on mitotically-inactivated murine embryonic fibroblast (MEF) cells and passaged the day before electroporation (Nagy et al. 2003). Prior to electroporation, 2×10^7 cells/ml were dissociated with trypsin/EDTA (0.25%/1 mM), broken up to single cells, and centrifuged at $300 \times g$ for 5 minutes at room temperature. The cellular pellet was resuspended in electroporation buffer (EmbryoMax ES Cell Qualified Electroporation Buffer, Millipore Corporation), washed twice with electroporation buffer, then resuspended in a final volume of 600 μ l of electroporation buffer. Linearized targeting vector DNA (30 μ g/ml), containing the neomycin-resistance gene, was added to the cell suspension and electroporation was performed using either the Gene Pulser II or the Gene Pulser MXcell system (Bio-Rad Laboratories, Inc.). For both electroporators, the voltage, capacitance, and resistance were set at 220 V, 950 μ F, and 1,000 Ω , respectively, with an exponential waveform. In the Gene Pulser II system the cells were electroporated in a cuvette (0.4 cm electrode, Bio-Rad), whereas in the Gene Pulser MXcell system the cells were transfected in a 96-well electroporation plate (Bio-Rad), using 4 wells with 150 μ l of cell suspension in each well.

Following electroporation, the cells were plated onto neomycin-resistant MEFs and incubated at 37°C, 95% humidity, and 5% CO₂. After 24 hr, Geneticin (350 µg/ml active concentration, Invitrogen Corporation) was added to the growth media for positive selection of antibiotic-resistant ES cell colonies. The media was changed every day and Geneticin selection was maintained for 6 days. Antibiotic-resistant ES cell colonies were counted, picked, and split to grow in duplicate for cryopreservation and for screening of gene targeting.

Human H9 ES cells (passage 52) were cultured under feeder cell-free conditions on BD Matrigel (Becton, Dickinson and Company) in mTeSR media (StemCell Technologies). Cells were passaged using Dispase (1 mg/ml, StemCell Technologies) and grown for 5 days on a 6-well plate, until well-developed colonies formed. Prior to electroporation, the cells were dissociated with Dispase and mechanically broken up into small cellular clumps. Since the human ES cells used in this study do not maintain their colony-forming property after dissociation into single cells, no accurate cell count was obtained for the cells. It was estimated that 2–3 x 10⁷ cells were used for the electroporation. The cell suspension was centrifuged at 300 x g for 5 min at room temperature and the cellular pellet was resuspended in Gene Pulser electroporation buffer (Bio-Rad). The cells were washed twice with electroporation buffer, then resuspended in 300 µl of the electroporation buffer. For transient transfection of the cells, pmaxGFP plasmid (25 µg/ml, Amaxa Biosystems GmbH) was added to the cell suspension. Electroporation was performed in a 96-well electroporation plate using the Gene Pulser MXcell system, set at an exponential waveform. Different voltages (250 V and 300 V) and capacitance parameters (100 µF, 200 µF, 300 µF, and 950 µF) were tested at a constant DNA concentration and similar cell densities. After electroporation, cells were plated onto a 6-well plate coated with BD Matrigel and allowed to grow for 48 hr in mTeSR media. Green fluorescent protein (GFP) expression was visualized by viewing the ES cell colonies under a fluorescence microscope (Olympus IX70, Olympus America Inc.).

Results and Discussion

We have successfully transfected mouse and human ES cells by using the Gene Pulser MXcell electroporation system.

In the first part of the study, we transfected mouse ES cells using either the Gene Pulser MXcell system or the routinely used Gene Pulser II system in order to determine if the two instruments were similar in how well they facilitated the transfer of DNA into cells. Table 1 shows that when the same electroporation parameters were used to transfect mouse ES cells, the Gene Pulser II and Gene Pulser MXcell systems were equally effective in transfecting the cells. Following the electroporation, the mouse ES cells were viable, quickly attached to feeder layer cells, and formed fast-growing

colonies that were visible after 24 hr. Using the two different electroporators, the time constants were similar, as were the number of Geneticin-resistant colonies, following antibiotic selection (Table 1).

These results indicate that the Gene Pulser MXcell electroporator, set at a voltage of 220 V, a capacitance of 950 µF, and a resistance of 1,000 Ω, is an efficient system for transfecting mouse ES cells. Experiments examining the gene targeting rates of the Geneticin-resistant ES cell colonies are under way. For the production of knock-out mice, such a transfection experiment has to be scaled up two to three fold. For most genes, 200–300 antibiotic-resistant ES cell colonies have to be screened in order to find the rare homologous recombination events.

Table 1. Comparison of the Gene Pulser II and Gene Pulser MXcell systems for transfecting mouse ES cells.

	Gene Pulser II System	Gene Pulser MXcell System
Voltage	220 V	220 V
Capacitance	950 µF	950 µF
Resistance	1,000 Ω	1,000 Ω
Waveform	Exponential	Exponential
Cell density	2 x 10 ⁷ cells/ml	2 x 10 ⁷ cells/ml
DNA concentration	30 µg/ml	30 µg/ml
Time constant (T ^{1/2})	12.4 ms	15.2 ms
Cell viability	Viable	Viable
Number of Geneticin-resistant ES cell colonies	107	109

In the second part of the study, we examined whether the same electroporation parameters that were used for mouse ES cells may be used to transfect human ES cells. When human H9 ES cells (approximately 2–3 x 10⁷ cells/ml) were transiently transfected with the GFP reporter gene (25 µg/ml) using the Gene Pulser MXcell system (220 V, 950 µF, 1,000 Ω, and exponential waveform), the time constant was very high (T^{1/2} = 73.2 ms) and the cells were not viable.

Since the conditions that have been established for mouse ES cells cannot be used for the transfection of human ES cells, we tested a range of different voltages and capacitance settings. The reported range of optimal voltages is between 200–300 V for the electroporation of other cell types, so we initially set the voltage at 250 V and varied the capacitance (100 µF, 200 µF, 300 µF, and 950 µF). Cell viability was determined by visually assessing the presence of attached and growing ES cell colonies 24 and 48 hr after electroporation. The transient expression of GFP was examined in the transfected cells by viewing the cells under a fluorescence microscope. Table 2 shows that with increased capacitance, cell viability quickly decreased, whereas for GFP expression the capacitance had to be above a minimum level. A capacitance of 200 µF resulted in the most favorable levels of cell viability and GFP expression.

Table 2. Effect of capacitance on cell viability and GFP expression in transfected human H9 ES cells.

Capacitance, μF	Time Constant ($T^{1/2}$), ms	Cell Viability	GFP Expression
100	7.60	Viable	Negative
200	14.20	Viable	Positive
300	23.75	Nonviable	N/A
950	73.20	Nonviable	N/A

Next, we compared the effect of setting the voltage at 250 V versus 300 V, when the capacitance was held constant at 200 μF . The time constants were 14.2 ms and 15.2 ms for the 250 V and 300 V settings, respectively. At both voltages, the cells were viable and showed GFP expression. The number of viable ES cell colonies was slightly higher at the lower voltage (250 V).

The optimal conditions that we have identified for electroporating human ES cells are a voltage of 250 V, a capacitance of 200 μF , a resistance of 1,000 Ω , and an exponential waveform. Under these conditions, 10–30% of cells within ES colonies, showed strong, transient GFP expression (Figure 1). Further studies are needed to determine if these electroporation parameters may be used for stable transfection and gene targeting in human ES cells. Preliminary results have shown that electroporating with targeting vectors, containing the puromycin resistance gene, results in good cell viability and effective antibiotic selection. Experiments measuring targeting rates in human ES cells are currently under way.

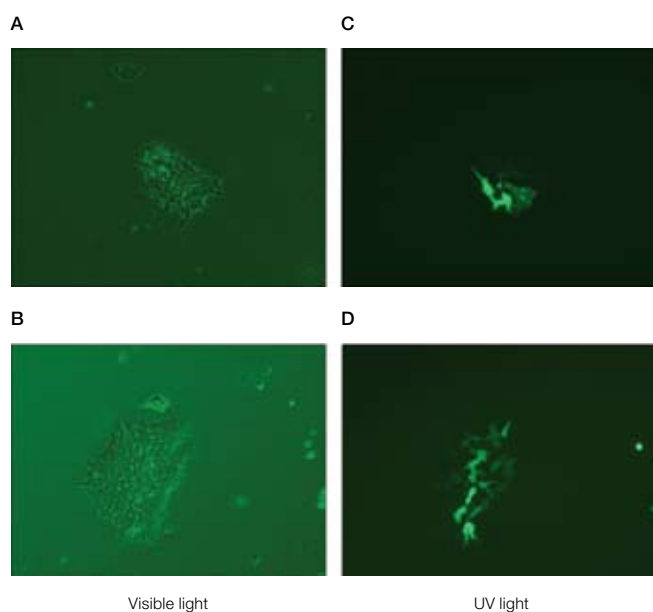


Fig. 1. Transient expression of GFP in human H9 ES cells. Human H9 ES cells (approximately $2-3 \times 10^7$ cells/ml) were transiently transfected with GFP (25 $\mu\text{g}/\text{ml}$) using the Gene Pulser MXcell system. The ES cell colonies were viewed 48 hr after transfection under a fluorescence microscope (Olympus IX70) under visible light (A, B) and under UV (488 nm) light (C, D).

Conclusions

Electroporation-based methods represent a simple and efficient way of delivering DNA into cells. One of the main advantages of the present method, using the Gene Pulser MXcell system, is that the electroporation parameters may be easily manipulated and hence optimized on this instrument. Furthermore, since it is a nonchemical method of gene delivery, it results in reduced cytotoxicity. The fact that this electroporation-based approach works on human ES cells that have been grown under feeder cell-free conditions may be important for future clinical applications, where the presence of feeder cells, of nonhuman origin, would be undesirable. The maintenance of human ES cells without feeder cells also has the clear advantage of reducing the number of cells needed for effective transfection, since all the DNA is available to go into ES cells rather than into the MEFs. Further studies are needed to determine whether the reported electroporation parameters are effective for successful gene targeting in H9 ES cells and other human ES cell lines.

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