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Electroporation of mouse embryonic stem cells with Neon device

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SUMMARY

Mouse embryonic stem cells are widely used as a promising material for producing of new cellular systems with desired properties in cellular and molecular biology, pharmacology, virology, medicine, veterinary medicine and biotechnology. Each type of cells requires different electroporation conditions that are determined experimentally. Therefore, the main goal was to optimize conditions of electroporation with Neon® Transfection System, a new-generation device, by selecting and changing of various parameters (voltage, impulse width and number of impulses) to maximize efficiency of D3 embryonic stem cell line transfection and to maintain cell viability. The following parameters were found to be the most optimal for the said cells: impulse voltage – 1200 V, impulse width – 10 ms, number of impulses – 3. Under given conditions, viability of the cells after electroporation was 91%, and transient transfection efficiency (24 hours after electroporation) assessed based on bacterial *β*-galactosidase production was 88%. It was shown that with higher cell density any electroporation condition tested yielded higher transfection efficiency ranging between 34 and 88%. It was demonstrated that only 5 out of 12 tested protocols with different parameters could be successfully used for insertion of DNA plasmid carrying *lacZ Escherichia coli* gene into D3 cell line. Thus, the experiment results show the more optimal conditions can be selected experimentally taking into account available information on electroporation protocols for similar cell types recommended by the device manufacturer. Electroporation of mouse embryonic stem cells with the new-generation device can be an effective method for *in vitro* insertion of nucleic acids into the cells of interest to the researcher.

Keywords: embryonic stem cells, insertion of exogenous DNA plasmid, Neon electroporation device, *lacZ Escherichia coli* gene, transfection efficiency, viability

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Электропорация эмбриональных стволовых клеток мыши с помощью прибора Neon

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РЕЗЮМЕ

Эмбриональные стволовые клетки мыши широко используются в качестве перспективного материала для создания новых клеточных систем с заданными свойствами в клеточной и молекулярной биологии, фармакологии, вирусологии, медицине, ветеринарии и биотехнологии. Для каждого типа клеток требуются разные условия электропорации, которые должны быть определены экспериментально, поэтому была поставлена цель путем подбора и из-

менения различных параметров (напряжения, ширины импульса и количества импульсов) оптимизировать условия электропорации при использовании электропоратора нового поколения Neon® Transfection System, обеспечивающие высокую эффективность трансфекции эмбриональных стволовых клеток линии D3 и их жизнеспособность. Установлено, что наиболее подходящими параметрами для данных клеток являются: импульсное напряжение – 1200 V, ширина импульса – 10 ms, количество импульсов – 3. При данных условиях жизнеспособность клеток после электропорации составила 91%, а эффективность временной трансфекции (24 ч после электропорации), оцениваемая по продукции бактериальной *β*-галактозидазы, достигала 88%. Показано, что при более высокой клеточной концентрации любые испытанные режимы электропорации обеспечивают более высокую эффективность трансфекции в диапазоне от 34 до 88%. Продемонстрировано, что для введения ДНК плазмиды с геном *lacZ Escherichia coli* в клетки линии D3 из 12 изученных протоколов с разными параметрами можно успешно использовать 5. Таким образом, полученные в эксперименте результаты показывают, что, имея предварительную информацию о режимах электропорации аналогичного типа клеток, которую рекомендует производитель прибора, можно подобрать экспериментальным путем более оптимальные условия. Электропорация эмбриональных стволовых клеток мыши с использованием электропоратора нового поколения может быть эффективным методом введения нуклеиновых кислот в представляющие интерес для исследователя клетки *in vitro*.

Ключевые слова: эмбриональные стволовые клетки, введение экзогенной ДНК плазмиды, электропоратор Neon, ген *lacZ Escherichia coli*, эффективность трансфекции, жизнеспособность

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INTRODUCTION

Since their discovery in 1981 [1, 2] mouse embryonic stem cells (ESCs) have been widely used as a promising material for the creation of new cellular systems with desired properties in cellular and molecular biology, pharmacology, virology, medicine, veterinary medicine and biotechnology. These cells have following features: high rate of doubling, specific marker gene expression confirming their origin, ability to respond to growth factors, causing morphological and biochemical changes leading to the ESCs differentiation into cells with a phenotype similar to more than 225 cell types in culture, and retention of early pre-implantation embryo properties both *in vitro* and *in vivo*.

The introduction of exogenous DNA into mouse ESCs enables genetic modification of their genomes, but also use of such cells for gene-edited animal creation [3]. Methods of recombinant molecule introduction into ESCs require careful selection due to their morphological features: small cells with a large nucleus and a narrow cytoplasm rim that grow in dense colonies, usually on a fibroblast monolayer. Electroporation is considered as the most acceptable way of highly efficient introduction of exogenous DNA into these cells without cellular changes and with retained differentiation capacity [4]. During such transfection, cells are exposed to a high-voltage impulse in the presence of exogenous nucleic acid. High voltage causes short-term permeability of the cell membrane, which allows foreign nucleic acids to enter the cell [5, 6]. Each type of cells requires different electroporation conditions that are determined experimentally. Intensity of the electric field and the impulse duration shall be taken into account, as they are key parameters for achieving maximum transfection efficiency and maintaining cell viability after the procedure. The impulse applied to the cells can be generated in two different waveforms: rectangular and exponential decay. Rectangular waveforms rely on a constant charge being applied to the cells for a set time. The use of rectangular signals allows applying multiple impulses. During the exponential decay waveform, the initial voltage is set, and the attenuation duration (time constant) is the product of capacitance setting and resistance of the circuit including the sample. Since the sample resistance mainly depends on the ionic strength of the electroporation buffer, while the resistance is constant, effect of capacitance setting changing on the impulse can be determined empirically [7]. Buffer solution components are also have an impact on transfection efficiency and cell viability. Earlier, buffer solution with high ionic strength (low resistance), such as phosphate-buffered saline (PBS) or serum-free growth medium, was adapted to for mouse ESC-D3 electroporation with high capacitance [8].

The goal was to optimize the electroporation conditions providing high efficiency of mouse ESCs transfection and viability due to purchasing of Neon® Transfer System (Invitrogen, Thermo Fisher Scientific, USA), new generation electroporator. Unlike previously systems used for electroporation, for example Gene Pulser (Bio-Rad), unique 100 or 10 µL tips are used as an electroporation chamber for electrical impulse exposure instead of cuvettes. It was demonstrated that the above-said device could be used for successful transfection of the foreign

DNA incorporation-incompetent cells, primary and immortalized hematopoietic cells, as well as stem cells and cells of various tissues [9–11].

MATERIALS AND METHODS

Mouse embryonic stem cells (D3 line) were tested. The mouse ESCs were cultivated in DMEM containing 4.5 g/L of glucose, 10% fetal bovine serum (HyClone, USA), essential amino acid solution, 2 мМ *α*-glutamine, 0,1 мМ *β*-mercaptoethanol and antibiotics: penicillin and streptomycin at final concentration of 50 U/mL and 50 μg/mL, respectively (NPP "PanEco", Russia). The ESCs were cultivated in monolayer of mouse diploid embryonic fibroblasts pre-treated with mitomycin C (final concentration: 30 μg/mL for 3 hours) to block mitosis.

pcDNATM3.1/His/lacZ plasmid comprising nucleotide sequence of *lacZ Escherichia coli* marker gene is used for transfection. Foreign DNA was re-precipitated with ethanol (70%) and dissolved in sterile buffer (0.1 мМ EDTA, 1 мМ Tris-HCl, pH 8.0) before transfection.

Transfection was performed by electroporation using Neon® Transfection System device and starter kit of reagents according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, USA). The day before transfection the ESCs were passaged to separate them from feeder layer be taking an advantage of differences in cell adhesion and seeded in Petri dishes (60 mm in diameter) without fibroblasts 24 hours before transfection so that they were in an exponential growth phase on the day of electroporation.

The cells were washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions (PBS-2); 1 mL of trypsin (NPP "PanEco", Russia) was added and the cells were incubated at 37 °C for 2 minutes before electroporation. The cells were resuspended after adding 9 mL of supplemented DMEM and then transferred to sterile 15 mL polypropylene tube (Sarstedt, Germany) and centrifuged at 200 g for 5 minutes. The precipitate was resuspended in 10 mL of PBS and the cells were calculated in Goryaev's chamber. The cell were precipitated again and buffer solution R from the kit for electroporation was added to the precipitate: up to final cell concentration of 1.0×10^7 cells/mL in the first case and two times less concentration (0.5×10^7 cells/mL) in the second case, as well as DNA plasmids, pcDNATM3.1/His/lacZ (final concentration of 1 μg/mL). DNAs and ESCs were mixed in the buffer and 10 μL of the mixture were collected by a pipette with tips for electroporation. The pipette is fixed in the holder for electroporation and exposed to electric current using preliminary set or manually entered protocols in Neon device. The transfected cells were transferred (10 μL) to the well of 24-well tissue culture plate containing 500 μL of suitable growth medium without antibiotics and the procedure was repeated 11 times for each cell concentration only changing the parameters for the electroporator. The plate with transfected mouse ESCs and control (non-transfected ESCs) was incubated at 37 °C in humidified 5% CO_2 atmosphere for 24 hours.

The cells were tested for their viability after electroporation through their staining with trypan blue (0.02% solution). Percentage of viable cells was calculated as the ratio of the number of unstained cells to the total number of cells multiplied by 100.

Transfection efficiency was assessed based on presence of *β*-galactosidase, *lacZ E.* coli gene expression product in the cells. For this purpose, X-gal (Sigma-Aldrich, USA) was used as a substrate. The cells were fixed with cold methanol (–20 °C) on ice for 15 minutes before staining. Proportion of blue-green-stained cells (*β*-galactosidase-producing cells) was calculated providing that at least 1,000 cells were analyzed. Non-transfected ESCs served as negative control.

Table
Optimization of conditions for electroporation of D3 mouse embryonic cell line in 10 μL tip (Neon)

Protocol No.	Impulse voltage, V	Impulse width, ms	Number of impulses	Transfection efficiency/viability, % (m ± SEM)	
				cell concentration: 0.5×10^5	cell concentration: $1,0 \times 10^5$
1	1,000	10	3	29±0.1/70±0.04	39±0.01/83±0.5
2	1,000	20	2	37±0.6/77±0.05	39±0.5/79±0.1
3	1,000	40	1	37±0.7/64±0.3	37±0.01/68±0.7
4	1,200	10	3	74±0.02/86±0.8	88±0.7/91±0.04
5	1,200	20	2	66±0.03/87±0.01	70±0.6/90±0.3
6	1,200	20	3	65±0.04/85±0.1	76±0.5/89±0.7
7	1,200	40	1	44±0.5/58±.001	57±0.4/60±0.3
8	1,400	10	3	71±0.05/70±0.3	78±0.1/88±0.1
9	1,400	20	2	68±0.2/70±0.5	81±0.1/90±0.5
10	1,400	20	3	65±0.4/66±0.6	70±0.7/72±0.6
11	1,400	30	3	33±0.7/59±0.02	57±0.4/66±0.1
11	1,500	10	3	26±0.6/46±0.3	35±0.1/48±0.01
12	1,500	40	1	22±0.07/34±0.4	34±0.2/41±0.2

The cells were visualized with inverted phase-contrast microscope (Carl Zeiss, Germany) using AxioVision Rel. 4.8. software. The experiments were performed in triplicate. Arithmetic mean (m) and standard error of the mean (SEM) were calculated.

RESULTS AND DISCUSSION

Several experiments for introduction of foreign DNA plasmid containing *lacZ E. coli* marker gene in mouse ESCs with electroporation using Neon® Transfection System device designed for mammalian cells and starter reagent kit. Two sets of electroporation parameters recommended by the device manufacturer were used for mouse ESCs transfection (protocols No. 5 and 8 in the Table). Several combinations of voltage (1,000; 1,300; 1,400 or 1,500 V), impulse duration (10, 20, 30, 40 ms) and number of impulses (1–3), as indicated in the Table, were additionally tested. Different cell concentrations, 0.5×10^5 or 1.0×10^5 cells in 10 µl, were used to optimize conditions.

Data given in the Table show that the best results were obtained when protocols No. 4 and 9 were used: transfection efficiency was the highest and proportion of stained cells was higher than that one when the other electroporation parameters were used.

The method produces reproducible results that has been determined by comparing its effectiveness in three repeated experiments. Parameters recommended by the device manufacturer for mouse ESCs (protocols No. 5 and 8 given in the Table) were also efficient but were inferior to the expected parameters. Thus, the manual states that 79% and 88% efficiency of the mouse ESCs transfection with *EGFP* gene-containing plasmid at 99% and 96% cell viability can be achieved after 48 hours using the proposed parameters in accordance with protocols No. 5 and 8, respectively.

Data analysis showed that cell concentration in the suspension was one of the most important variables having an impact on efficiency of the transfection performed with Neon device. Obtained results allows us to conclude that at a higher cellular concentration, in our case two-fold concentration, any tested electroporation protocols provided higher transfection efficiency in the range of 34 to 88%. The said values varied from 22 to 74% when lower cell concentration was used.

Unlike standard cuvette-based electroporation methods, the Neon system uses unique reaction chambers – Neon tips that generate higher electric field for biological samples. The tips maximizes the gap size between two electrodes while minimizing the surface area of each electrode. This results in minimal pH change, less ion formation, and negligible heat generation. This design enhances transfection efficiency and cell viability as well as maintains ergonomic workflow. It would be of interest to assess cell viability after electroporation since the transfection occurred in the mixture microvolume (buffer solution, DNA and cells). Cells subjected to electroporation and not subjected to electroporation (not exposed to foreign DNA and electric impulse) were handled in a similar way. The results showed that ESCs viability (1.0×10^5 cell concentration) after transfection according to protocol No. 4, 5, 6, 8, 9 parameters (Table) was comparable with that one of control ESCs 95 ± 0.2% (ESCs not subjected to electroporation) was as follows: 91, 90, 89, 88 and 90%, respectively. The cell viability was the highest (91%) when the following parameters were used: impulse voltage – 1,200 V, impulse width – 10 ms, number of impulses – 3.

Mouse ESCs, promising for engineering a laboratory model for viral infection studies [12] and being immortalized cells, i.e. immortal in culture, were selected for the experiments. *lacZ E. coli* gene being a part of pCMV-lacZ plasmid was successfully introduced in the said cells with Gene Pulser device (Bio-Red, USA) at transfection efficiency of 35% during previous experiments [8]. Therewith, maximum proportion of the cells survived after electroporation was 82%. Comparative examinations of mouse D3 embryonic stem cell electroporation with Neon device (from 34 to 88%) for electroporation efficiency have shown the advantage of the new-generation electroporator where transfection occurs in tips. It should be noted that the Neon system allows the use of very small volumes for transfection, is miniaturized for the use of tips for electroporation and 10 or 100 µL volumes for transfection.

During the experiment foreign DNA plasmid, pcDNATM3.1/His/lacZ, was successfully introduced into mouse ESCs at transfection efficiency of 88% by changing electroporation parameters. Data on the efficiency of foreign RNA [13–15], DNA and proteins introduction into cells non-competent to take up foreign material using Neon electroporator are currently accumulated [16–18]. The experiment data are consistent with these reports, however, cells shall be tested for their quality and the parameters shall be optimized for each cell culture before transfection. Differences noted in the electroporation protocols published in scientific literature [9, 10, 16] suggest that even minor changes in cell growth conditions could have a significant impact on foreign DNA introduction into them.

CONCLUSION

Thus, the results obtained during the experiment show that more optimal conditions can be selected experimentally, when preliminary information on electroporation modes for similar cell type recommended by the manufacturer is available. Electroporation is shown to be a very effective method for nucleic acid introduction into the cells of interest, including those that are often considered difficult for transfection. The high transfection efficiency for the cells described in this paper is achieved by optimization or determination of electroporation parameters for Neon device. The following parameters are found the most suitable parameters for mouse D3 embryonic stem cells: impulse voltage – 1,200 V, impulse width – 10 ms, number of impulses – 3, at which cell viability after electroporation is 91%, and the temporary transfection efficiency (24 hours after electroporation) assessed based on bacterial protein *β*-galactosidase production is 88%.

The analysis of the obtained results indicates the need to select optimal conditions for electroporation of mouse D3 ESCs due to the peculiarities of their behavior in culture. The concentration of cells in the suspension is shown to be one of the most important variables affecting transfection efficiency under optimized electroporation conditions. Obtained results allows us to conclude that at a higher cellular concentration, two-fold concentration in our case, any tested electroporation protocols provide higher transfection efficiency in the range of 34 to 88%.

The best electroporation conditions for any cell type can be obtained by using a system that allows adjustment of parameters including waveform, voltage, as well as cell density, which can significantly affect both the cell viability after electric current exposure and transfection efficiency. Proportion of viable cells (comparable to that one in control, i.e. non-transfected cells) or transfection efficiency can be increased by changing these parameters. It is demonstrated that only 5 out of 12 tested protocols with different parameters can be successfully used for introduction of DNA plasmid carrying *lacZ Escherichia coli* gene into ESCs-D3. Thus, Neon® Transfection System electroporator (Invitrogen, Thermo Fisher Scientific, USA), allows selection of conditions that are the most suitable for creating cells with the specified properties.

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