COMPARATIVE EFFICIENCY OF ACCESSIBLE TRANSFECTION METHODS IN MODEL CELL LINES FOR BIOTECHNOLOGICAL APPLICATIONS

Vorobyev PO[1], Kochetkov DV[1], Vasilenko KV[2], Lipatova AV[1] ✉

[1] Engelhardt Institute of Molecular Biology, Moscow, Russia

[2] Pirogov Russian National Research Medical University, Moscow, Russia

Transient gene expression is one of the most common methods in molecular biology, equally relevant for basic research projects and biotechnological industries. Despite the existence of commercial transfection systems, which afford high transfection efficiency and high expression levels of reporter genes, expanding such systems to industrial scales is often problematic due to high costs of the reagents. The well-described methods of cationic and calcium-phosphate transfection are accessible and ensure reproducible results at much lower costs. This study is aimed at comparative validation of calcium phosphate and cationic (polyethylenimine-based) transfection protocols along with the commercially available TurboFect reagent for mono- and cotransfections on a panel of commonly used cell lines including HEK293T, Huh7, BHK-21, CHO and MRC5. The efficiency of transfection with plasmid constructs encoding different fluorescent proteins was measured by flow cytometry. Of all the tested methods, calcium phosphate transfection afforded the highest efficiency of plasmid DNA delivery in all the cell lines except BHK21, for which the PEI method turned out to be more efficient than calcium phosphate transfection, and CHO, for which both methods showed comparable efficiency.

Keywords: calcium phosphate transfection, polyethylenimine, cotransfection, transfection, lentiviruses

Funding: the project was supported by the Russian Science Foundation (grant number 20-75-10157 of August 14, 2020 "Research on the possibilities of obtaining recombinant strains of oncolytic viruses with tumor-specific replication and immunomodulatory protein expression").

Author contribution: Vorobyev PO, Kochetkov DV, Vasilenko KV and Lipatova AV participated equally in the laboratory experiments, preparation of the figures and interpretation of the results.

Compliance with ethical standards: the study complies with the requirements of the World Medical Association Declaration of Helsinki.

✉ **Correspondence should be addressed:** Anastasia V. Lipatova
Vavilova, 32/1, Moscow, 119991, Russia; lipatovaanv@gmail.com

Received: 22.04.2022 **Accepted:** 30.05.2022 **Published online:** 23.06.2022

DOI: 10.24075/brsmu.2022.031

СРАВНИТЕЛЬНЫЙ АНАЛИЗ ЭФФЕКТИВНОСТИ ОБЩЕДОСТУПНЫХ МЕТОДОВ ТРАНСФЕКЦИИ МОДЕЛЬНЫХ КЛЕТОЧНЫХ ЛИНИЙ ДЛЯ ЗАДАЧ БИОТЕХНОЛОГИИ

П. О. Воробьев[1], Д. В. Кочетков[1], К. В. Василенко[2], А. В. Липатова[1] ✉

[1] Институт молекулярной биологии имени В. А. Энгельгардта, Москва, Россия

[2] Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва, Россия

Краткосрочная экспрессия генов является одним из самых широко используемых методов в молекулярной биологии, как в исследовательских проектах, так и для решения задач биотехнологической промышленности. Несмотря на то что существующие коммерческие трансфекционные агенты позволяют добиться эффективной трансфекции и высокой экспрессии целевых генов в клетках, масштабирование производственного процесса часто затруднительно из-за высокой стоимости таких агентов. Хорошо описанные методы катионной или кальций-фосфатной трансфекции доступны и дают воспроизводимые результаты при значительно меньшей себестоимости. Целью исследования было проверить методы кальций-фосфатной трансфекции, катионной трансфекции (PEI) и коммерчески доступного реагента TurboFect на эффективность монотрансфекции и котрансфекции на панели широко используемых клеточных линий, таких как HEK293T, HUH7, BHK-21, CHO, MRC5. Эффективность трансфекции плазмидными конструкциями, несущими различные флуоресцентные белки, оценивали путем проведения проточной цитофлуориметрии. Среди всех методов кальций-фосфатная трансфекция позволяет добиться максимально эффективной доставки плазмидной ДНК во всех клеточных линиях, использованных в нашем исследовании, кроме BHK21 — для нее катионная трансфекция с использованием PEI оказалась эффективнее, и сопоставима по эффективности с кальций-фосфатным методом в клеточных линиях CHO.

Ключевые слова: кальций-фосфатная трансфекция, полоиэтиленимин, котрансфекция, трансфекция, лентивирусы

Финансирование: проект был поддержан Российским научным фондом (Соглашение № 20-75-10157 от 14 августа 2020 г. «Изучение возможностей получения рекомбинантных штаммов онколитических вирусов с опухоль-специфической репликацией и экспрессией иммуномодулирующих белков»).

Вклад авторов: П. О. Воробьев, Д. В. Кочестков, К. В. Василенко, А. В. Липатова внесли равнозначный вклад в проведение лабораторных экспериментов, подготовку рисунков и интерпретацию результатов.

Соблюдение этических стандартов: исследование проведено в соответствии с требованиями Хельсинкской декларации Всемирной медицинской ассоциации.

✉ **Для корреспонденции:** Анастасия Валерьевна Липатова
ул. Вавилова, д. 32/1, г. Москва, 119991, Россия; lipatovaanv@gmail.com

Статья получена: 22.04.2022 **Статья принята к печати:** 30.05.2022 **Опубликована онлайн:** 23.06.2022

DOI: 10.24075/vrgmu.2022.031

Transfection is the process of exogenous DNA or RNA delivery into eukaryotic cells [1], used for recombinant protein production [2], signal pathway investigation [3–6], etc. This group of methods has emerged in connection with the viral genome infectivity and its self-perpetuation research [7]. By now, transfection protocols are indispensable for the creation of recombinant viral strains, as well as for the production of viral vectors on the basis of lenti- and adeno-associated viruses [8, 9].

The 'chemical' transfection methods are classified by the type of the agent binding nucleic acid molecules to enable their introduction into a cell. Such agents include calcium phosphate, polyethylenimine (PEI) and other cationic polymers

(e.g. chitosan), liposomes and polyamidoamines [10]. Each agent has its own advantages and disadvantages; the choice should depend on the balance of cytotoxicity and transfection efficiency of the agent in a particular experimental setting [11]. Despite the extensive supply of ready-made commercial solutions including Lipofectamine 3000, TurboFect, SuperFect, and FuGENE HD [12], the accessible and well-studied calcium phosphate and cationic transfection methods are still actively used [10].

The calcium phosphate transfection (CPT) was firstly developed in 1973 [13] during studies on the adenoviral genome infectivity, but its mechanism had long remained incomprehensible. With the discovery of direct endosomal transport of CaPi–DNA precipitates to the nucleus in 1990 [14] the method became increasingly widespread. The classical protocol involves co-precipitation of calcium salts and DNA with the formation of hydroxyapatite complexes under precise physical and chemical conditions of oversaturated solution, within a defined range of temperatures and concentrations. The reproducible performance of this technique requires a certain amount of skill [15–18], although some recent modifications of the classical protocol effectively overcome this limitation [19, 20].

The cationic transfections with polyethylenimine (PEI) and its commercially available modifications (e.g. TurboFect™) are popular as well [10, 21]. The critical parameters in these protocols are DNA/PEI ratio and the molecular weight of PEI, a reduction in which hampers cytotoxicity. Commercial versions mostly use 25 kDa PEI [22]; another option is PEI "Max" — a chemically modified 40 kDa PEI with reduced cytotoxicity [10, 23]. The undoubted advantages of PEI include high efficiency, handling simplicity and universal applicability. The sensitivity of dry PEI to oxidation by atmospheric oxygen represents a certain drawback, fairly surmountable by dissolving the reagent in 0.2 M HCl for prolonged storage [24], whereas the cytotoxicity of the reagent can be reduced by using a lactate buffer as a medium for the transfection complexes formation.

The widely used transfection reagent Lipofectamine 3000 is extremely efficient and stable at a 4 °C storage temperature, but prohibitively expensive (50 USD for a single transfection experiment with 107 cells). This price tier of transfection reagents is beyond the scope of this article.

A simultaneous delivery of multiple genetic constructs to a cell is a widespread task often required for multimeric protein synthesis, design of recombinant viral strains [8, 9], lenti- and adeno-associated viral particle production and other biotechnological purposes, sometimes at industrial scales [25]. The use of ready-made commercial kits, whatever expedient and reliable, in large-scale transfections is hampered by high costs and also by logistical problems associated with the lack of domestically manufactured analogs. In this study, we compare the efficiencies of CPT, PEI and TurboFect methods in cotransfections with reporter plasmid constructs and quantitatively assess the effectivity by flow cytometry.

METHODS

Cells

Cell lines HEK293T (ATCC #CRL-3216), BHK21 (ATCC #C-13), MRC5 (ATCC #CCL-171) and CHO K1 (ATCC #CCL-61) were purchased from ATCC (American Type Culture Collection; USA); cell lines CHO DG-44 и Huh7 were generously gifted by A.V. Ivanov (Laboratory of Biochemistry of Viral Infections at the Engelhardt Institute of Molecular Biology; Moscow, Russia). The cells were cultured in DMEM (Gibco; USA) supplemented with 10% fetal bovine serum (FBS, HyClone; USA) and antibiotics to standard concentrations (penicillin 50 U/mL and streptomycin 50 μg/mL); CHO DG-44 cells were cultured in DMEM/F12 (PanEco; Russia) with 10% FBS and antibiotics.

Plasmid constructs and transfection protocols

The coding sequences of Katushka, BFP and eGFP fluorescent proteins were cloned into the pL-CMV-PL4-Puro vector developed previously in the Laboratory of Cell Proliferation (Engelhardt Institute of Molecular Biology). After the verification of successful cloning by sequencing, the plasmids were propagated in the *E. coli* strain TOP-10 (New England Biolabs; USA) using 200 mL LB aliquots and purified with a Plasmid Midiprep 2.0 kit (Evrogen; Russia). The plasmid DNA extraction quality was controlled by spectrophotometry (NanoDrop 2000; ThermoFisher, USA) using the 260/280 nm absorbance ratio; all samples used in the experiments had A260/280 of 1.9 or higher.

The cells were plated in six-well plates 24 h prior to transfection, 1.5×10^5 cells in 4 mL of DMEM supplemented with 10% FBS, and maintained in an incubator at 37 °C, 5% CO_2 and high humidity until use.

The PEI transfection method was conducted as described previously [24]. The dry reagent (Cat# 23966 Polysciences, Inc.; USA) was dissolved in 0.2 M HCl to 5 μg/μL; this stock solution was aliquoted and stored long-term at –80 °C. The lactate buffer (20 mM sodium lactate, 150 mM NaCl, pH 4.0) was stored at 4 °C.

On the day of transfection, 3 μg of plasmid DNA were diluted with 150 μL of the lactate buffer. In a separate tube, 15 μg (3 μL) of PEI were diluted with 150 μL of the lactate buffer. The two mixtures were combined, mixed thoroughly, incubated at room temperature for 10–15 min and added drop-wise to the culture medium. In a modified version of this protocol, DNA and PEI were added to 300 μL of the lactate buffer, which preserved the transfection efficiency (data not shown). The fluorescence measurements were carried out 48 h post-transfection (after addition of DNA-containing complexes to cells).

Calcium phosphate transfections (CPT) were carried out in accordance with the previously published protocol [26]. The culture medium was replaced with DMEM (Gibco) supplemented with 10% FBS (HyClone), 2.25 mL per well, 1 h before the transfection. The DNA/$CaCl_2$/dH_2O solution (125 μL) was prepared from 2M $CaCl_2$ diluted with other components to a final concentration of 148 mM; the total amount of DNA was 3 μg. The DNA/$CaCl_2$/dH_2O solution was combined with the equal volume of 2× HBS buffer (HBS; Table 1) at thorough mixing. The transfection mixture was incubated at room temperature for 10–20 min and added drop-wise to the culture medium. The cultures were placed in an incubator; 6 h or 14 h post-transfection the culture medium was replaced with a fresh portion. The membrane shock procedure was carried out 14 h post-transfection: the culture medium was replaced with 1 mL of 10% DMSO in phosphate-buffered saline (PBS). After a 2.5 min-long incubation the cells were washed twice with 3 mL of PBS and covered with the fresh culture medium.

Transfections with TurboFect™ were carried out according to the manufacturer's recommendations. On the day of transfection, 4 μg of plasmid DNA were diluted with serum-free DMEM and combined with 6 μL of the TurboFect reagent. The mixture was incubated at room temperature for 20 min and added drop-wise to the culture medium.

Cell viability assay

For a standard thiazolyl blue tetrazolium bromide (MTT) viability assay, the cells were transferred to 24-well plates. The tests

were carried out 24 h post-transfection as longer incubation times could blur the differential toxic effects. Dry MTT (Dia-M; Russia) was dissolved in PBS to obtain the solution (5 µg/mL) added directly to the wells. After a 3 h-long incubation, the culture medium was replaced with 300 µL of DMSO (PanEco; Russia). The controls were the corresponding non-transfected cultures. The detection was carried out in a CLARIOstar plate reader (BMG Labtech; USA) by measuring absorbance at 595 nm normalized to 490 nm.

Flow cytometry assay and data processing

All the measurements were carried out in a BD LSRFortessa flow cytometer (Beckman Dickinson; USA) with detection in the PE (561/(586/15) nm — Katushka), FITC (488/(530/30) nm — eGFP) and Pacific Blue (405/(450/50) nm — tagBFP) channels. We used the preinstalled BD FACSDiva software for the compensation adjustment and primary processing of the data and the Flowing Software 2.0 (Perttu Terho, Turku Bioscience Centre; Finland) for the advanced analysis. All experiments were performed in three independent biological replicates.

Lentiviral stock production and titration

Lentiviral component-encoding plasmids pREV, pGAG-pol, p-VSV-G and pL-CMV-eGFP-puro were constructed earlier in the Laboratory of Cell Proliferation (Engelhardt Institute of Molecular Biology). HEK293T cells were plated in Petri dishes to a 35% confluency; on the next day, the cells were transfected with a 2 : 1 : 1 : 4 mass ratio of the plasmids (in the listed order) [27]. After a 16 h-long incubation, the medium was replaced with DMEM containing 2% FBS. The harvesting of lentiviral particles was repeated twice daily, starting at 8 h since the first medium replacement. The titers of the pooled five consecutive viral stocks were evaluated on HEK293T cultures using a modified final dilution method. 10-fold serial dilutions of the viral stocks in serum-free DMEM were added to fresh cultures growing in 48-well cell culture plates at 1.5×10^4 cells per well. The medium was replaced with DMEM containing 2% FBS and the dilutions of lentiviral stocks were added. The experiment was conducted in four technical parallels. After 72 hours, fluorescent plaques were counted in wells with maximum viral dilutions.

Table 1. The 2× hepes buffer saline (HBS) composition

NaCl	280 mM
KCl	10 mM
Na_2HPO_4	1.5 mM
Dextrose/glucose	12 mM
HEPES pH 7.05	50 mM

Statistical analysis

The data were analyzed using the GraphPad Prism 9.0 package (GraphPad Software, Inc.; La Jolla, CA, USA). The differences were validated using one-way ANOVA.

RESULTS

Post-transfection cell viability assay

Fig. 1 shows the results of the MTT assay performed 24 h post-transfection.

The data indicate that over 80% cells survive the transfection procedure, independently of the cell line and protocol. MTT assays performed 48 h post-transfection are non-informative due to the effects of contact inhibition: in control wells, cells become 100% confluent, while transfected cells continue to proliferate.

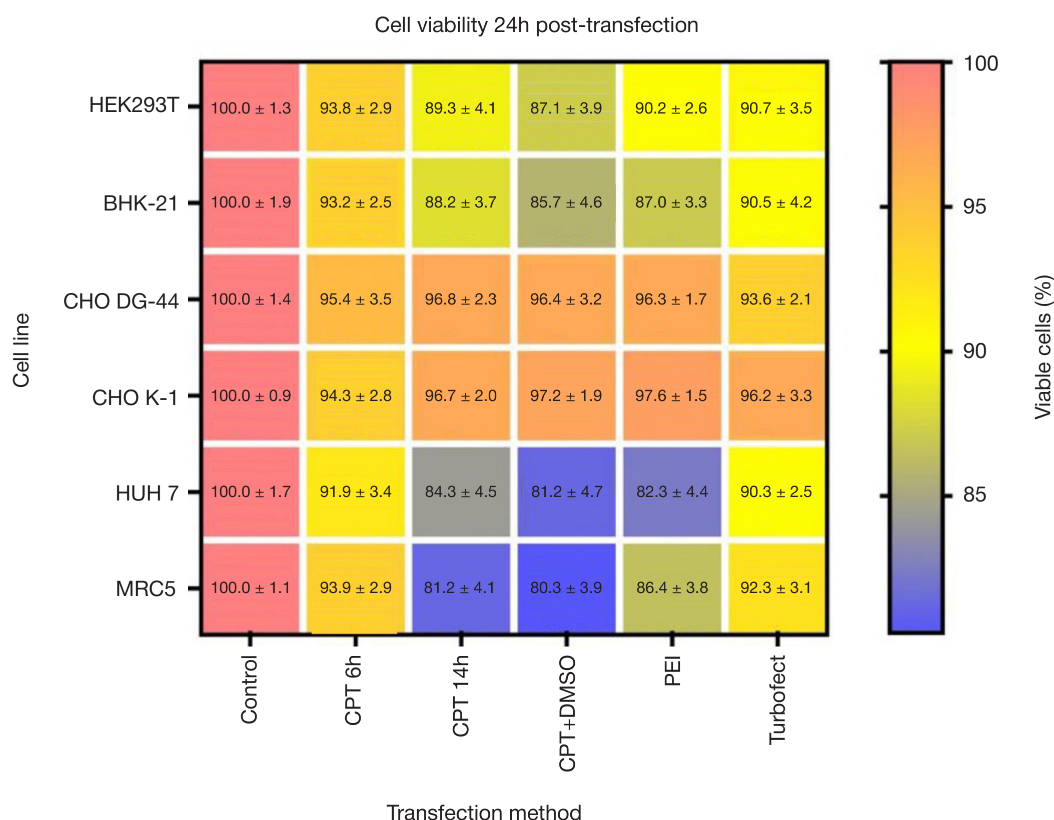

Fig. 1. The post-transfection MTT cell viability assay for PEI, TurboFect and CPT methods; Control — non-transfected cultures

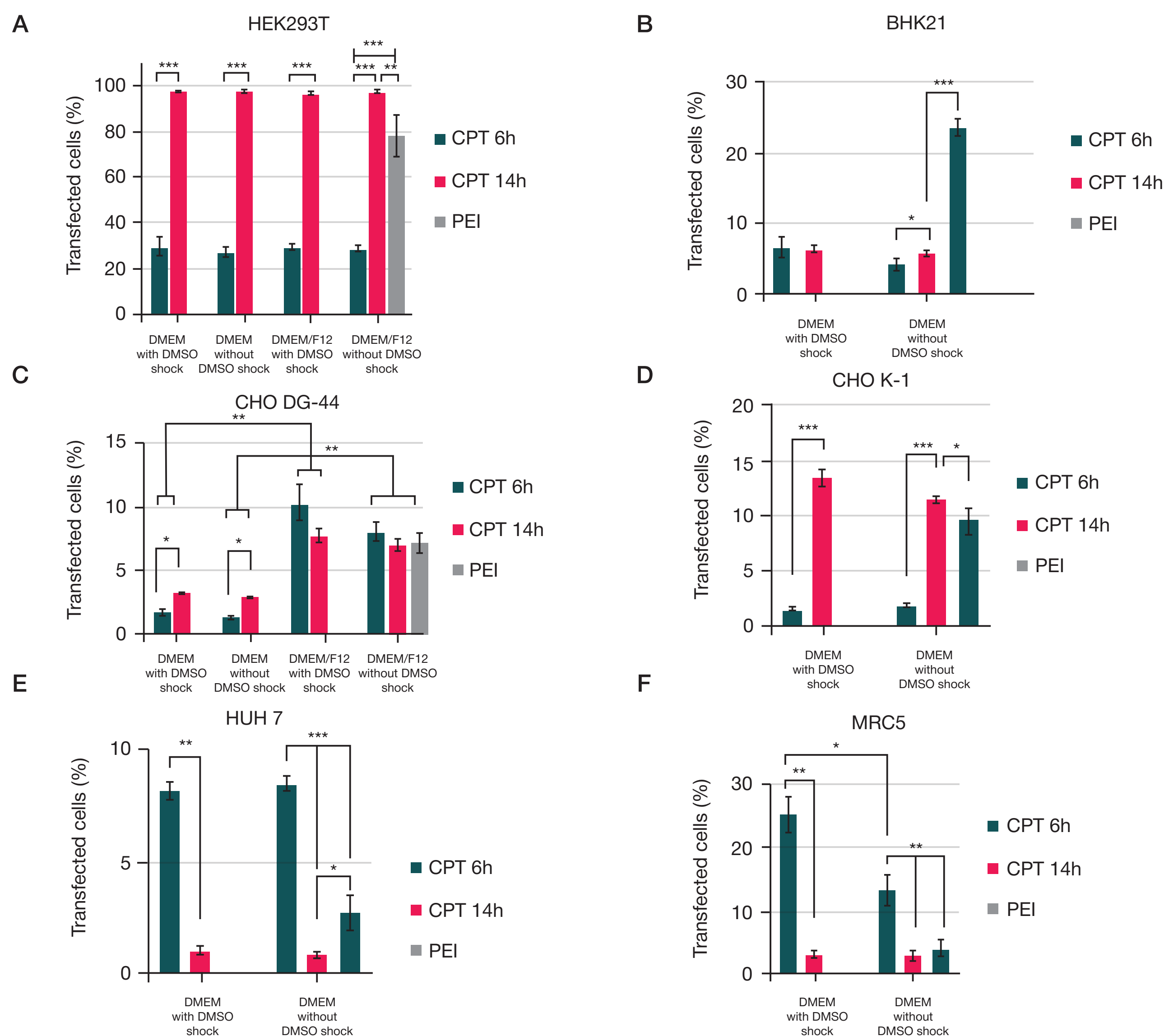

Fig. 2. Comparative efficiency of CPT and PEI monotransfections with Katushka expression plasmid (red fluorescence) in different model cell lines: HEK293T (**A**), BHK-21 (**B**), CHO DG-44 (**C**), CHO K-1 (**D**), Huh7 (**E**) and MRC5 (**F**). CPT 6 h — calcium phosphate transfection with 6 h incubation; CPT 14 h — calcium phosphate transfection with 14 h incubation

Monotransfection efficiency assay

The comparative evaluation of the CPT and PEI methods in terms of the transfection efficiency involved the pL-CMV-Katushka-puro plasmid construct (Fig. 2).

The following conditions were varied for the optimization:

– the duration of incubation with the CaPi–DNA precipitate (6 h and 14 h);

– the impact of the membrane shock procedure (2.5 min exposure of 10% DMSO).

The increase of the incubation time with the CaPi–DNA precipitate from 6 h to 14 h dramatically enhanced the transfection efficiency for the HEK293T and CHO K 1 cell lines: the numbers of reporter-positive cells increased, respectively, from 27% to 97% for HEK293T ($p < 0.01$) and from 1.5% to 13% for CHO K-1 ($p < 0.01$). Importantly, the effect was cell line-dependent: the incubation time with the precipitate produced a drop in the transfection efficiency from 25% to 3% for MRC5 ($p < 0.01$) and from 8% to 1% for Huh7 ($p < 0.01$).

The introduction of the 10% DMSO treatment step (membrane shock) led to a significant increase in the transfection efficiency for the MRC5 cell line only ($p < 0.05$). CPT showed the best results in all the cell lines except BHK-21. The PEI method was also efficient in most cell lines and produced comparable cytotoxic effects.

The influence of the cell culture medium on the CPT efficiency was tested using the HEK293T and CHO DG-44 cell lines (Figs. 2A and 2C). CHO DG-44 cells showed enhanced transfection efficiency in DMEM/F12 ($p < 0.01$). The use of the membrane shock (10% DMSO exposure) provided no significant enhancement in the transfection efficiency in these experiments.

Cotransfection efficiency assay

In the next series of experiments, we cotransfected the cells with plasmid constructs encoding eGFP and Katushka fluorescent proteins (Fig. 3A, Table 2). The highest cotransfection efficiency was observed in HEK293T cells. CPT worked significantly better than the PEI method for all the cell lines except BHK21 ($p < 0.001$). Transfections with TurboFect were the least efficient. Microphotographs of CPT in HEK293T are shown in Fig. 4.

The trends identified for double cotransfections were preserved in triple cotransfections, although the efficiencies (measured as the percentage of cells expressing all reporter

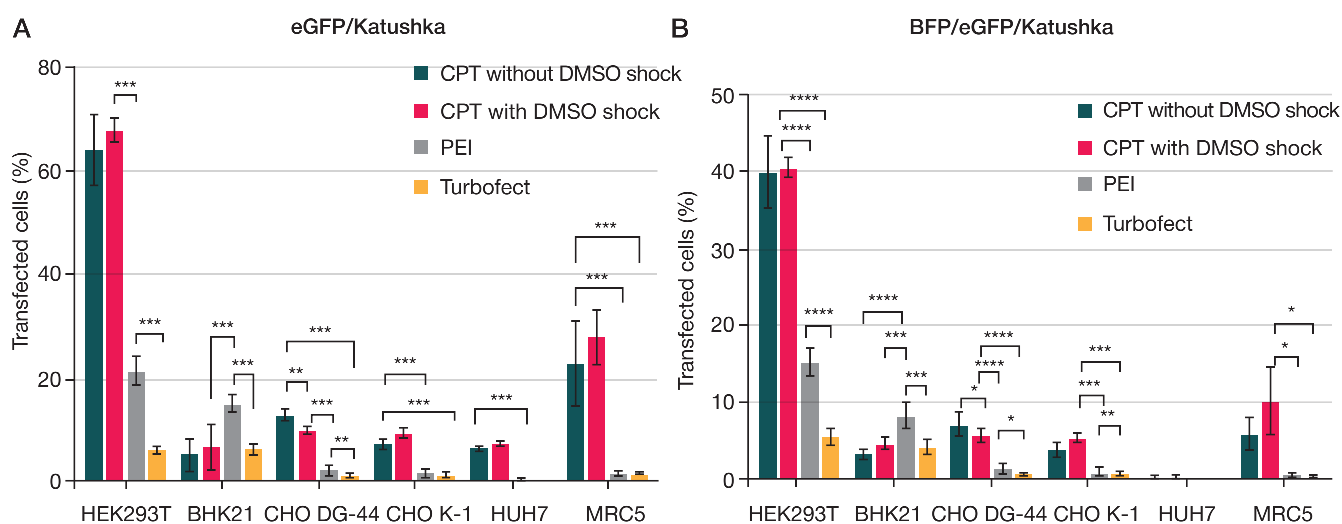

Fig. 3. Comparative efficiency of double cotransfections with eGFP and Katushka encoding plasmids (**A**) and triple cotransfections with BFP, eGFP and Katushka plasmids (**B**) in different model cell lines

proteins) were expectedly lower (Fig. 3B, Table 3). The flow cytometry data analysis for HEK293T and Huh7 is presented in Fig. 5; the positivity thresholds were set in accordance with the contour plots of autofluorescence for the non-transfected control cultures.

Overall, the results demonstrate excellent efficiency of the accessible chemical transfection methods in mono- and cotransfections, comparable with the efficiency of advanced commercially available systems exemplified by FuGENE HD and Lipofectamine 3000 [28].

Assessment of the lentiviral particles' assembly efficiency

Lentiviral transduction is a standard tool for obtaining cell sublines stably expressing exogenous proteins, cell reprogramming and many other purposes in molecular and cell biology. To produce lentiviral stocks, the HEK293T packaging cell line is simultaneously transfected with 3–4 plasmid constructs encoding HIV-1 proteins and VSV-G glycoprotein, as well as a lentiviral genome plasmid carrying a reporter transgene. The yield of viral particles harvested with the supernatant depends

Table 2. Comparative efficiency of double cotransfections with eGFP and Katushka plasmids, measured 48 h post-transfection (best results in each row are highlighted)

Cell line	Double-positive cells, %			
	CPT	CPT + DMSO	PEI	Turbofect
HEK293T	64,27 ± 6,85	68,1 ± 2,29 ($p < 0{,}001$)	21,33 ± 2,78	5,97 ± 0,61
BHK21	5 ± 3,11	6.6 ± 4.40	15 ± 1,85 ($p < 0{,}001$)	6,2 ± 0,86
CHO DG-44	12,87 ± 1,16 ($p < 0{,}001$)	9,85 ± 0,75	2,13 ± 0,92	1,05 ± 0,47
CHO K-1	7,1 ± 0,916	9,43 ± 0,93 ($p < 0{,}001$)	1,43 ± 0,83	1,13 ± 0,51
HUH7	6,93 ± 0,50	7,23 ± 0,55 ($p < 0{,}001$)	0,23 ± 0,16	0,09 ± 0,06
MRC5	22,7 ± 8,15	27,93 ± 5,25 ($p < 0{,}001$)	1,4 ± 0,56	1,55 ± 0,25

Table 3. Comparative efficiency of triple cotransfections with BFP, eGFP and Katushka encoding plasmids, measured 48 h post-transfection (best results in each row are highlighted)

Cell line	Triple-positive cells, %			
	CPT	CPT + DMSO	PEI	Turbofect
HEK293T	39,83 ± 4,55	40,47 ± 1.27 ($p < 0{,}001$)	15,2 ± 1,75	5,57 ± 0,98
BHK21	3,3 ± 0,511	4,63 ± 0,80	8,23 ± 1,70 ($p < 0{,}001$)	4,16 ± 0,92
CHO DG-44	7,2 ± 1.52 ($p < 0{,}001$)	5,73 ± 0,99	1,33 ± 0,72	0,63 ± 0,09
CHO K-1	3,8 ± 0,92	5,4 ± 0,53 ($p < 0{,}001$)	0,93 ± 0,41	0,7 ± 0,19
HUH7	0,13 ± 0,06	0,2 ± 0,13 (ns)	0,09 ± 0,06	0,07 ± 0,06
MRC5	5,8 ± 2,02	10,17 ± 4,33 ($p < 0{,}05$)	0,43 ± 0,15	0,33 ± 0,15

Note: ns — not significant.

on the transfection efficiency and cell viability. Our choice of the CPT + DMSO, PEI and TurboFect protocols for this practical task was based on the preliminary optimization experiments.

Transfections were performed in a 10 cm Petri dish at a 70% confluency (6×10^6 cells).The resulting viral titers evaluated by the final dilution method are presented in Fig. 6.

The highest viral particle production efficiency was achieved with CPT (9×10^5 IFU/mL). The PEI and TurboFect protocols also produced satisfactory yields, albeit the titers were significantly lower (10^5 IFU/mL; $p < 0.05$, and 6×10^4 IFU/mL; $p < 0.01$, respectively).

DISCUSSION

The chemical transfection methods vary in toxicity and transfection efficiency depending on their chemical nature and DNA-to-agent ratio. Transfection efficiency is influenced by multiple parameters which are difficult to unify, as dedicated comparisons between studies are hindered by variability of experimental conditions [16, 24]. Here we report a comparative analysis of the efficiency for three transfection methods in several cell lines of a diverse origin.

HEK293T is one of the best-studied model cell lines, known for its high transfection capacity. These cells are ubiquitously used in the production of recombinant proteins and lentiviral stocks. CPT ensures excellent results in HEK293T cells (> 95% efficiency in monotransfections), with the length of incubation with the CaPi–DNA precipitate being a decisive factor. For the incubation times of 6 h and 14 h, the efficiency constituted 29% и 97%, respectively ($p < 0.01$), whereas the role of the membrane shock was negligible (no significant differences observed). In cotransfections, the efficiencies of the CPT and PEI methods constituted 68% and 21% for two-plasmid and 40% and 15% for three-plasmid systems, respectively ($p < 0.01$ in both cases). The corresponding efficiencies of the PEI and TurboFect methods constituted 21% and 5.9% for two-plasmid and 15% and 5% for three-plasmid transfections ($p < 0.01$).

We further tested the feasibility of multiple cotransfections with lentiviral component-encoding plasmids to produce viral particles encoding a reporter gene (eGFP) by these two methods. The tests involved titration of the harvested lentiviral stocks by the transduction assay with the reporter eGFP

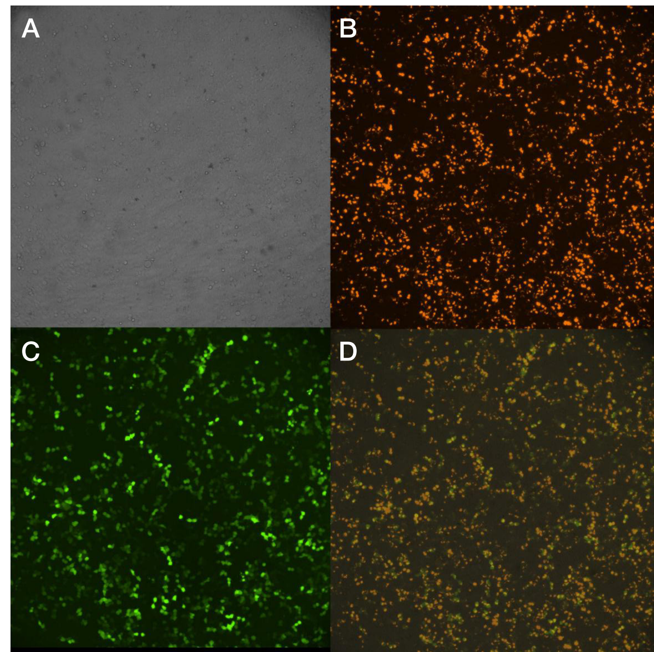

Fig. 4. Microphotographs of cell cultures cotransfected with genetic constructs encoding eGFP and Katushka fluorescent proteins. **A**. Light field. **B**. Katushka. **C**. eGFP. **D**. Merged image (magnification ×40)

fluorescence. In this series of experiments, the lentiviral stocks produced with CPT revealed the highest titers (9×10^5 IFU/mL), whereas the titers produced with the PEI and TurboFect methods were significantly lower (105 IFU/mL and 6×10^4 IFU/mL, respectively; $p < 0.05$).

However, for the BHK-21 cell line, CPT proved to be significantly less efficient than the PEI method, yielding 6.6% vs 15% in double and 4.6% vs 8.3% in triple cotransfections ($p < 0.01$ for both); this result overlaps with the previously published data [24]. The discrepancy in the results can be explained by several critical factors, e.g., the PEI preparation method. The efficiency of the PEI method is compromised by the prolonged storage of the dry reagent, as its oxidation by atmospheric oxygen negatively affects the binding capacity [24]. The PEI method showed better efficiency than TurboFect in double and triple cotransfections (11% vs 6% and 8% vs 4%, respectively; $p < 0.01$

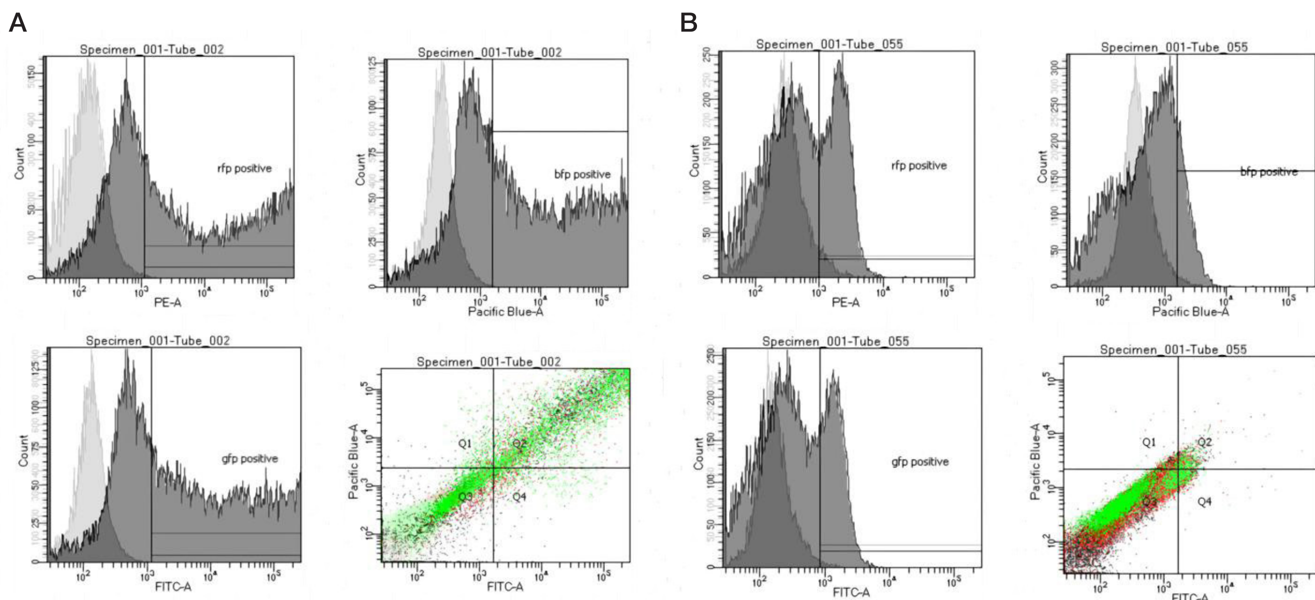

Fig. 5. Flow cytometry data for HEK293T and Huh7 cell lines (respectively, **A** and **B**); *light contour* — non-transfected control, *dark contour* — CPT. Populations single-positive for particular fluorescent proteins (eGFP, Katushka or BFP) or triple-positive (Q2) are indicated

in both cases). Of note, these results should be further verified by using a different batch of the TurboFect transfection reagent, which is highly sensitive to the storage temperature (should be stored at 2–8 °C).

Overall, CPT showed higher efficiency than PEI for most cell lines used in our study ($p < 0.001$ for HEK293T, Huh7, CHO DG-44 and CHO K-1, and $p < 0.05$ for MRC5). It should be noted that the use of the DMSO-induced membrane shock had small effects in both double and triple cotransfections of CHO-K1 ($p < 0.05$) and MRC5 cells. None of the protocols tested by us in this study ensured efficient cotransfections of Huh7; nevertheless, monotransfections of these cells by CPT with membrane shock afforded efficiencies as high as 7%.

One clear advantage of the PEI transfection method over CPT, although beyond the scope of our study, is the ease of use for suspension cell cultures. The Expi293 cell line, widely used for recombinant protein production, can be efficiently transfected using PEI instead of the expensive Expifectamine [29].

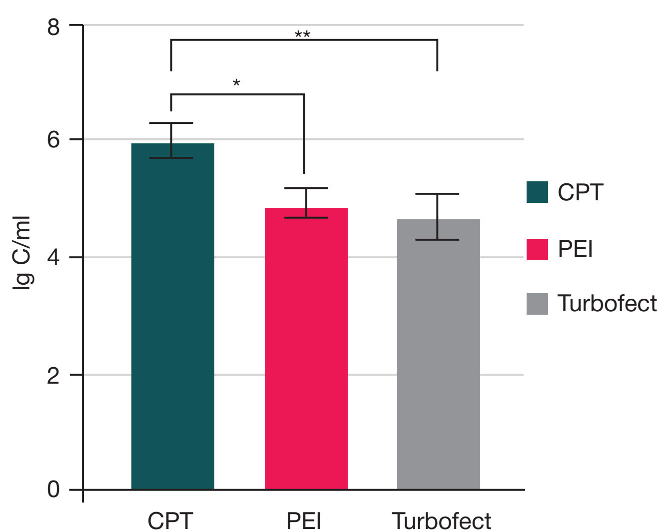

Fig. 6. Concentrations of lentiviral stocks produced with HEK293T cells using different transfection methods

CONCLUSIONS

The calcium phosphate method ensures high transfection efficiency in a panel of cell lines widely used for research and biotechnological tasks. Despite its relative technical complexity, its efficiency cannot be affected with inappropriate storage conditions (a major liability in other protocols) and shows highly reproducible results. The use of the calcium phosphate method for the production of lentiviral stocks in HEK293T cells affords excellent yields (9×10^5 IFU/mL without concentration, which is comparable with custom-made commercial supplies). The cationic transfection with PEI also shows high efficiency in most cell lines, while its protocol is technically unburdened. Handling the reagents in compliance with the storage requirements makes the method easily scalable. The TurboFect protocol ensures acceptable results, although the transfection efficiency is lower; still, the protocol is technically simple and well suited for small research tasks. At the same time, high costs and lengthy delivery times make it an unlikely choice for large-scale projects.

References

1. Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Analytical and Bioanalytical Chemistry. 2010; 397: 3173–8.
2. Teixeira AP, Stucheli P, Auslander S, Auslander D, Schonenberger P, Hurlemann S, et al. CelloSelect — A synthetic cellobiose metabolic pathway for selection of stable transgenic CHO cell lines. Metabolic Engineering. 2022; 70: 23–30.
3. Gu L, Kitamura M. Sensitive detection and monitoring of senescence-associated secretory phenotype by SASP-RAP assay. PLoS One. 2012; 7: e52305.
4. Miura M, Yuan J. Transient transfection assay of cell death genes. Methods in Enzymology. 2000; 322: 480–92.
5. Strebel A, Harr T, Bachmann F, Wernli M, Erb P. Green fluorescent protein as a novel tool to measure apoptosis and necrosis. Cytometry. 2001; 43: 126–33.
6. Boulaiz H, Prados J, Melguizo C, Garcia AM, Marchal JA, Ramos JL, et al. Inhibition of growth and induction of apoptosis in human breast cancer by transfection of gef gene. British Journal of Cancer. 2003; 89: 192–8.
7. Vaheri A, Pagano JS. Infectious poliovirus RNA: a sensitive method of assay. Virology. 1965; 27: 434–6.
8. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA. 2000; 97: 6108–13.
9. Weber K, Bartsch U, Stocking C, Fehse B. A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. Molecular Therapy. 2008; 16: 698–706.
10. Longo PA, Kavran JM, Kim MS, Leahy DJ. Transient mammalian cell transfection with polyethylenimine (PEI). Methods in Enzymology. 2013; 529: 227–40.
11. Jin L, Zeng X, Liu M, Deng Y, He N. Current progress in gene delivery technology based on chemical methods and nano-carriers. Theranostics. 2014; 4: 240–55.
12. Rahimi P, Mobarakeh VI, Kamalzare S, SajadianFard F, Vahabpour R, Zabihollahi R. Comparison of transfection efficiency of polymer-based and lipid-based transfection reagents. Bratisl Lek Listy. 2018; 119: 701–05.
13. Graham FL,van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology. 1973; 52: 456–67.
14. Orrantia E,Chang PL. Intracellular distribution of DNA internalized through calcium phosphate precipitation. Experimental Cell Research. 1990; 190: 170–4.
15. Kingston RE, Chen CA, Okayama H. Calcium phosphate transfection. Current protocols in immunology. 2001; 31: 1–9.
16. Olton D, Li J, Wilson ME, Rogers T, Close J, Huang L, et al. Nanostructured calcium phosphates (NanoCaPs) for non-viral gene delivery: influence of the synthesis parameters on transfection efficiency. Biomaterials. 2007; 28: 1267–79.
17. Pedraza CE, Bassett DC, McKee MD, Nelea V, Gbureck U, Barralet JE. The importance of particle size and DNA condensation salt for calcium phosphate nanoparticle transfection. Biomaterials. 2008; 29: 3384–92.
18. Sokolova VV, Radtke I, Heumann R, Epple M. Effective transfection of cells with multi-shell calcium phosphate-DNA nanoparticles. Biomaterials. 2006; 27: 3147–53.
19. Cao X, Deng W, Wei Y, Su W, Yang Y, Wei Y, et al. Encapsulation of plasmid DNA in calcium phosphate nanoparticles: stem cell uptake and gene transfer efficiency. Int J Nanomedicine. 2011; 6: 3335–49.
20. Nouri A, Castro R, Santos JL, Fernandes C, Rodrigues J, Tomas H. Calcium phosphate-mediated gene delivery using simulated body fluid (SBF). Int J Pharm. 2012; 434: 199–208.
21. Baker A, Saltik M, Lehrmann H, Killisch I, Mautner V, Lamm G, et al. Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery. Gene Therapy. 1997; 4: 773–82.
22. Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk

A. A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. Molecular Therapy. 2005; 11: 990–5.

23. Liu T, Yu X, Kan B, Guo Q, Wang X, Shi S, et al. Enhanced gene delivery using biodegradable poly(ester amine)s (PEAs) based on low-molecular-weight polyethylenimine and poly(epsilon-caprolactone)-pluronic-poly(epsilon-caprolactone). J Biomed Nanotechnol. 2010; 6: 351–9.
24. Fukumoto Y, Obata Y, Ishibashi K, Tamura N, Kikuchi I, Aoyama K, et al. Cost-effective gene transfection by DNA compaction at pH 4.0 using acidified, long shelf-life polyethylenimine. Cytotechnology. 2010; 62: 73–82.
25. Yahalom-Ronen Y, Tamir H, Melamed S, Politi B, Shifman O, Achdout H, et al. A single dose of recombinant VSV-G-spike vaccine provides protection against SARS-CoV-2 challenge. Nat Commun. 2020; 11: 6402.
26. Kwon M,Firestein BL. DNA transfection: calcium phosphate method. Methods Mol Biol. 2013; 1018: 107–10.
27. Chumakov SP, Kravchenko JE, Prassolov VS, Frolova EI, Chumakov PM. Efficient downregulation of multiple mRNA targets with a single shRNA-expressing lentiviral vector. Plasmid. 2010; 63: 143–9.
28. Hasan MM, Ragnarsson L, Cardoso FC, Lewis RJ. Transfection methods for high-throughput cellular assays of voltage-gated calcium and sodium channels involved in pain. PLoS One. 2021; 16: e0243645.
29. Fang XT, Sehlin D, Lannfelt L, Syvanen S, Hultqvist G. Efficient and inexpensive transient expression of multispecific multivalent antibodies in Expi293 cells. Biol Proced Online. 2017; 19: 11.

Литература

1. Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Analytical and Bioanalytical Chemistry. 2010; 397: 3173–8.
2. Teixeira AP, Stucheli P, Auslander S, Auslander D, Schonenberger P, Hurlemann S, et al. CelloSelect — A synthetic cellobiose metabolic pathway for selection of stable transgenic CHO cell lines. Metabolic Engineering. 2022; 70: 23–30.
3. Gu L, Kitamura M. Sensitive detection and monitoring of senescence-associated secretory phenotype by SASP-RAP assay. PLoS One. 2012; 7: e52305.
4. Miura M, Yuan J. Transient transfection assay of cell death genes. Methods in Enzymology. 2000; 322: 480–92.
5. Strebel A, Harr T, Bachmann F, Wernli M, Erb P. Green fluorescent protein as a novel tool to measure apoptosis and necrosis. Cytometry. 2001; 43: 126–33.
6. Boulaiz H, Prados J, Melguizo C, Garcia AM, Marchal JA, Ramos JL, et al. Inhibition of growth and induction of apoptosis in human breast cancer by transfection of gef gene. British Journal of Cancer. 2003; 89: 192–8.
7. Vaheri A, Pagano JS. Infectious poliovirus RNA: a sensitive method of assay. Virology. 1965; 27: 434–6.
8. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA. 2000; 97: 6108–13.
9. Weber K, Bartsch U, Stocking C, Fehse B. A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. Molecular Therapy. 2008; 16: 698–706.
10. Longo PA, Kavran JM, Kim MS, Leahy DJ. Transient mammalian cell transfection with polyethylenimine (PEI). Methods in Enzymology. 2013; 529: 227–40.
11. Jin L, Zeng X, Liu M, Deng Y, He N. Current progress in gene delivery technology based on chemical methods and nano-carriers. Theranostics. 2014; 4: 240–55.
12. Rahimi P, Mobarakeh VI, Kamalzare S, SajadianFard F, Vahabpour R, Zabihollahi R. Comparison of transfection efficiency of polymer-based and lipid-based transfection reagents. Bratisl Lek Listy. 2018; 119: 701–05.
13. Graham FL,van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology. 1973; 52: 456–67.
14. Orrantia E,Chang PL. Intracellular distribution of DNA internalized through calcium phosphate precipitation. Experimental Cell Research. 1990; 190: 170–4.
15. Kingston RE, Chen CA, Okayama H. Calcium phosphate transfection. Current protocols in immunology. 2001; 31: 1–9.
16. Olton D, Li J, Wilson ME, Rogers T, Close J, Huang L, et al. Nanostructured calcium phosphates (NanoCaPs) for non-viral gene delivery: influence of the synthesis parameters on transfection efficiency. Biomaterials. 2007; 28: 1267–79.
17. Pedraza CE, Bassett DC, McKee MD, Nelea V, Gbureck U, Barralet JE. The importance of particle size and DNA condensation salt for calcium phosphate nanoparticle transfection. Biomaterials. 2008; 29: 3384–92.
18. Sokolova VV, Radtke I, Heumann R, Epple M. Effective transfection of cells with multi-shell calcium phosphate-DNA nanoparticles. Biomaterials. 2006; 27: 3147–53.
19. Cao X, Deng W, Wei Y, Su W, Yang Y, Wei Y, et al. Encapsulation of plasmid DNA in calcium phosphate nanoparticles: stem cell uptake and gene transfer efficiency. Int J Nanomedicine. 2011; 6: 3335–49.
20. Nouri A, Castro R, Santos JL, Fernandes C, Rodrigues J, Tomas H. Calcium phosphate-mediated gene delivery using simulated body fluid (SBF). Int J Pharm. 2012; 434: 199–208.
21. Baker A, Saltik M, Lehrmann H, Killisch I, Mautner V, Lamm G, et al. Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery. Gene Therapy. 1997; 4: 773–82.
22. Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk

A. A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. Molecular Therapy. 2005; 11: 990–5.

23. Liu T, Yu X, Kan B, Guo Q, Wang X, Shi S, et al. Enhanced gene delivery using biodegradable poly(ester amine)s (PEAs) based on low-molecular-weight polyethylenimine and poly(epsilon-caprolactone)-pluronic-poly(epsilon-caprolactone). J Biomed Nanotechnol. 2010; 6: 351–9.
24. Fukumoto Y, Obata Y, Ishibashi K, Tamura N, Kikuchi I, Aoyama K, et al. Cost-effective gene transfection by DNA compaction at pH 4.0 using acidified, long shelf-life polyethylenimine. Cytotechnology. 2010; 62: 73–82.
25. Yahalom-Ronen Y, Tamir H, Melamed S, Politi B, Shifman O, Achdout H, et al. A single dose of recombinant VSV-G-spike vaccine provides protection against SARS-CoV-2 challenge. Nat Commun. 2020; 11: 6402.
26. Kwon M,Firestein BL. DNA transfection: calcium phosphate method. Methods Mol Biol. 2013; 1018: 107–10.
27. Chumakov SP, Kravchenko JE, Prassolov VS, Frolova EI, Chumakov PM. Efficient downregulation of multiple mRNA targets with a single shRNA-expressing lentiviral vector. Plasmid. 2010; 63: 143–9.
28. Hasan MM, Ragnarsson L, Cardoso FC, Lewis RJ. Transfection methods for high-throughput cellular assays of voltage-gated calcium and sodium channels involved in pain. PLoS One. 2021; 16: e0243645.
29. Fang XT, Sehlin D, Lannfelt L, Syvanen S, Hultqvist G. Efficient and inexpensive transient expression of multispecific multivalent antibodies in Expi293 cells. Biol Proced Online. 2017; 19: 11.