Optimized transfection protocol for efficient *in vitro* non-viral polymeric gene delivery to human retinal pigment epithelial cells \(ARPE-19)

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Method Article

Keywords: non-viral gene delivery, gene therapy, retinal pigment epithelium, cationic polymer, gene expression

Posted Date: April 23rd, 2009

DOI: https://doi.org/10.1038/nprot.2009.78

Abstract

Introduction

Transfection of therapeutic genes to retinal pigment epithelial cells has many potential applications for the treatment of ocular diseases. This protocol has been systematically optimized to obtain reproducible, high-level gene transfer to retinal pigment epithelial cells \(ARPE-19) _in vitro_, using branched polyethyleneimine \(PEI) as a gene carrier and secreted _Renilla_ Luciferase as a reporter protein. In a first stage of the protocol development, different parameters, including cell density at seeding, PEI/DNA charge ratio, composition of the preparation buffer and nanoparticulate assembly conditions as well as incubation time of the nanoparticulates with ARPE-19 cells, were optimized in a matrix-like fashion. Selection of the most effective conditions for gene transfer led to the finalization of the present protocol, with which transgene expression efficacies of 10-20 ng/ml are typically obtained. Cell viability is 60-80% depending on the incubation time of the nanoparticulates with cells. This protocol has been optimized for ARPE-19 cells and for PEI as gene carrier. However, with minor changes, it should be suitable also for transfection of other cultured cells, as well as for different polymeric carriers.

Reagents

ARPE19 cells \(ATCC accession no. CRL-2302), passage number 25-35 Cell culture media \(see REAGENT SETUP) Coelenterazine 1mM solution \(see REAGENT SETUP) Dulbecco's Phosphate Buffered Saline \(D-PBS) with and without Ca^{2+} and Mg^{2+} \(see REAGENT SETUP) Erythrosin B 0.1% solution \(see REAGENT SETUP) Mes-Hepes buffered saline \(see REAGENT SETUP) Methanol \(Fluka, 65543) \!CAUTION Highly flammable, toxic. Polyethylenimine 2.95 mM stock solution \(see REAGENT SETUP) Renilla Luciferase \(RL) plasmid \(see REAGENT SETUP) Tris-EDTA buffer pH 8 \(TE buffer, see REAGENT SETUP) Trypsin 0.25% \(1X) with EDTA solution \(see REAGENT SETUP) Uptiblue viable cell counting reagent \(Interchim, UP669413) Ethanol 70% \(vol/vol) REAGENT SETUP **DMEM/F12 medium with 10% and 20% serum** DMEM/F12 \(Gibco, 31330), 10% \(or 20%) \(vol/vol) fetal bovine serum \(FBS) \(Gibco, 10270) and 1% \(vol/vol) 100x penicillin-streptomycin, glutamine \(Gibco, 10378). **DMEM/F12 medium without serum** Same as above, but without fetal bovine serum \(FBS). **Dulbecco's Phosphate Buffered Saline \(D-PBS) with $Ca^{2+}$and Mg^{2+}** 1 g/L $CaCl_2$ anhyd., 1 g/L $MgCl_2$-$6H_2O$, 2 g/L KCl, 2 g/L KH_2PO_4, 80 g/L NaCl, 21,6 g/L Na_2HPO_4-$7H_2O$. Dilute 1:10, adjust pH to 7.1. Ready-to-use mixture with Ca^{2+} and Mg^{2+} \(Gibco, 14080). **Dulbecco's Phosphate Buffered Saline \(D-PBS) without Ca^{2+} and Mg^{2+}** 2 g/L KCl, 2 g/L KH_2PO_4, 80 g/L NaCl, 21,6 g/L Na_2HPO_4-$7H_2O$. Dilute 1:10, adjust pH to 7.1. Ready-to-use mixture without Ca^{2+} and Mg^{2+} \(Gibco, 14200). **Mes-Hepes buffered saline** 50 mM Mes hydrate \(Sigma, M2933), 50 mM Hepes \(Sigma, H4034), 75 mM NaCl \(Riedel, 31434) in mqH_2O; adjust pH to 7.2. **Trypsin 0.25% \(1X) with EDTA 4Na solution** 2.5 g/L trypsin \(1:250), 0.38 g/L EDTA-4Na in Hanks' Balanced Salt Solution without $CaCl_2$, $MgCl_2$-$6H_2O$, and $MgSO_4$-$7H_2O$. Contains phenol red. Ready-to-use mixture \(Gibco, 25200). **Coelenterazine 1 mM solution** Dissolve 250 µg coelenterazine \(Promega, S2001) in methanol \(Fluka, 65543) to get a 1 mM solution. **Erythrosin B 0.1% solution** Dissolve

erythrosin B \(Merck, 15936) in mqH_2O while stirring, filter before use. **Polyethylenimine 2.95 mM stock solution** Prepare a 2.95 mM \(0.6136 mg/ml) PEI \(branched, MW 25 kDa, water free, Sigma, 408727) solution in mqH_2O. Adjust pH to 7.2. Sterile filter with PVDF filter \(0.22 µm). In order to prepare the stock solution of a carrier polymer, the mass/charge of the polymer needs to be known. For PEI it is 208 g/mol \(1 positive charge for every 208 Da). Therefore 2.95 mM x 208 g/mol = 0.6136 mg/ml. **_Renilla_ Luciferase \(RL) plasmid \(1 mg/ml) stock solution** Prepare a 1 mg/ml RL plasmid stock solution in TE buffer pH 8. Check the plasmid DNA purity by spectrophotometer \(DNA 260/280 ratio should be > 1.7). **10x Tris-EDTA buffer \(TE buffer) pH 8** Mix 33.3 ml 3 M Tris-HCl pH 8 \(dilute 363.42 g Tris \(ICN 819638) in 1 L mqH_2O, adjust pH to 8) with 20 ml 0.5 M EDTA pH 8 \(dilute 186,12 g EDTA \(J.T.Baker, 1073) in 1 L mqH_2O adjust pH to 8) ad. 1 L mqH_2O, adjust pH to 8. Dilute 1:10.

Equipment

96-well cell culture plate \(black, flat bottom, with lid) \(Greiner, 655090) 96-well plate \(clear, flat bottom, non treated) \(Nunc, 256510) Bürker cell counting chamber Dynamic light scattering \(DLS) apparatus \(Malvern Zetasizer) Inverted microscope \(Olympus CKX31) Labculture Class II Type A2 Biohazard Safety Cabinet \(ESCO) Millex-GV filter unit, 0.22 µm, PVDF \(Millipore, SLGV033RS) pH meter \(Jenway, model 3510) Pipettes and multichannel pipettes \(Finnpipette, 10, 100, 300 and 1000 µl) Spectral scanning multimode reader including fluorescence intensity and luminometric detection technology, with onboard dispenser \(Thermo Scientific Varioskan Flash) Sterile plasticware: 50 ml, 15 ml, 5 ml tubes \(Sarstedt), 1.5 ml micro tubes \(Sarstedt), tips for pipettes Syringes Tissue culture flask 75 cm^2 \(Sarstedt) Tissue culture incubator at 37°C, with humidified, 7% CO_2 atmosphere \(HERAcell 150) UV-visible spectrophotometer \(Spectronic Genesys 10 Bio, Thermo Electron) Vortex mixer \(Finevortex) Water bath at 37°C

Procedure

Seeding ARPE-19 cells \(day 1) 1) Grow ARPE19 cells in a tissue culture flask until they reach 80% confluence. 2) Wash the cells with 10 ml D-PBS \(without Ca^{2+} and Mg^{2+}). 3) Add 3 ml trypsin 0.25% \(1X) with EDTA solution to the flask, mix briefly and immediately discard most of the trypsin \(just leave one drop in the bottle). 4) Incubate the cells for 4 minutes in a tissue culture incubator at 37°C, with humidified, 7% CO_2 atmosphere. 5) Re-suspend the cells in 9 ml DMEM/F12 medium without serum. CRITICAL STEP Make sure by microscopic observation that the cells have detached from the bottom of the flask. 6) Stain dead cells with Erythrosin B 0.1% solution and count the living cells in a Bürker cell counting chamber. 7) Dilute the cells with DMEM/F12 medium without serum at the desired final concentration of 20,000 cells/well. 8) Seed 50 µl cell suspension with a multichannel pipette into a black 96-well cell culture plate, cover the plate with the lid. CRITICAL STEP ARPE19 cell density at seeding affects remarkably the transfection efficiency and cytotoxicity. 9) Incubate the cells for 20 minutes in a tissue culture incubator at 37°C, with humidified, 7% CO_2 atmosphere 10) Add 50 µl DMEM/F12 culture medium containing 20% FBS with a multichannel pipette. 11) Incubate the cells overnight in a tissue

culture incubator at 37°C, with humidified, 7% CO_2 atmosphere. **Preparation of PEI/DNA \(charge ratio 2/1) nanoparticulates \(day 2)** General indications for the preparation of polycation/DNA nanoparticulates at charge ratios 4/1, 2/1 and 1/1 can be found in Table 1. The following procedure is for PEI/DNA charge ratio 2/1. 12) **_Renilla_ Luciferase plasmid \(RL plasmid)**. In a 5 ml tube dilute 12 µl RL plasmid \(1 mg/ml) with 488 µl Mes-Hepes buffered saline. Incubate at room temperature for 5 min. 13) **Polycation**. Meanwhile in a 1.5 ml micro tube dilute 25 µl PEI \(2.95 mM) with 475 µl Mes-Hepes buffered saline. Vortex briefly. 14) Add all the PEI solution to the DNA dilution, mix rapidly twice with the pipette and immediately vortex at 3000 rpm for 2 s. CRITICAL STEP It is important to follow the preparation procedure as indicated. The appearance of the nanoparticle solution must be clear, no precipitation should occur. 15) Incubate the complexes in the safety cabinet for 2 h. CRITICAL STEP If the incubation time is shortened, incomplete nanoparticulate formation may occur. 16) Confirm the formation of nanoparticulates by dynamic light scattering. The size should be ~ 300 nm. **Transfection of ARPE-19 cells with PEI/DNA nanoparticulates** 17) After 2 h incubation time distribute 60 µl complexes into a clear 96-well plate \(4 wells for each charge ratio). CRITICAL STEP This clear plate will have exactly the same filling matrix as intended for transfection. In this way by convenient transfer of the nanoparticulate solutions, cells can be transfected simultaneously. 18) Wash the overnight cultured ARPE-19 cells with 150 µl D-PBS \(with Ca^{2+} and Mg^{2+}) per well. 19) Add 100 µl DMEM/F12 medium without serum per well with a multichannel pipette. 20) Transfer 50 µl nanoparticulates from the clear 96-well plate to the cells with a multichannel pipette. Remember to leave 4 control wells for each of the following: Mes-Hepes buffered saline, free plasmid DNA \(600 ng/well) and free cationic polymer \(767 ng/well, in the case of PEI at charge ratio 2). CRITICAL STEP The cells should be transfected simultaneously in order to obtain consistent results. 21) Stir the plate gently. 22) Incubate for 1 or 2 h in a tissue culture incubator at 37°C, with humidified, 7% CO_2 atmosphere, depending on the desired outcome \(higher transfection versus toxicity). CRITICAL STEP Incubation time of the nanoparticulates with ARPE-19 cells is of crucial importance, in order to get high gene expression levels and minimal cytotoxicity. 23) Wash ARPE-19 cells with 150 µl D-PBS \(with Ca^{2+} and Mg^{2+}) per well. 24) Add 100 µl DMEM/F12 medium with 10% serum per well. 25) Incubate overnight in a tissue culture incubator at 37°C, with humidified, 7% CO_2 atmosphere. **Measurement of _Renilla_ Luciferase activity and cell viability 24 h, 48 h and 72 h after transfection \(days 3, 4 and 5)** 26) Dilute coelenterazine 1 mM solution in D-PBS \(with Ca^{2+} and Mg^{2+}) to achieve a 1.5 µM concentration \(i.e. for a total volume of 40 ml, take 60 µl coelenterazine 1 mM). CRITICAL STEP The coelenterazine dilution needs to be prepared just before the measurement. We suggest to aliquot the coelenterazine 1 mM solution and store it at -20°C until use. 27) **_Renilla_ Luciferase activity.** With a luminometer/fluorometer 96-well plate reader, inject directly 50 µl coelenterazine 1.5 µM solution into the transfected wells. Measure the luminescence for 10 s with a delay of 2 s between injection and measurement. 28) **Cell viability.** i) Add 15 µl Uptiblue \(10% of culture volume) per well with a multichannel pipette. ii) Incubate for 2 h in a tissue culture incubator at 37°C, with humidified, 7% CO_2 atmosphere. iii) Measure fluorescence ex.540/em.590 nm for 0.1 s. 29) Wash ARPE-19 cells with 150 µl D-PBS \(with Ca^{2+} and Mg^{2+}) per well. 30) Add 100 µl DMEM/F12 medium with 10% serum per well. 31) Incubate overnight in a tissue culture incubator at 37°C, with humidified, 7% CO_2

atmosphere. 32) Repeat the measurements on days 4 and 5. 33) Calculate the normalized _Renilla_ Luciferase activity in ng/ml and the cell viability in % relative to buffer treated wells. TROUBLESHOOTING

Timing

The protocol takes 5 days to complete.

Critical Steps

5) Re-suspend the cells in 9 ml DMEM/F12 medium without serum. CRITICAL STEP Make sure by microscopic observation that the cells have detached from the bottom of the flask. 8) Seed 50 µl cell suspension with a multichannel pipette into a black 96-well cell culture plate, cover the plate with the lid. CRITICAL STEP ARPE19 cell density at seeding affects remarkably the transfection efficiency and cytotoxicity. 14) Add all the PEI solution to the DNA dilution, mix rapidly twice with the pipette and immediately vortex at 3000 rpm for 2 s. CRITICAL STEP It is important to follow the preparation procedure as indicated. The appearance of the nanoparticle solution must be clear, no precipitation should occur. 15) Incubate the complexes in the safety cabinet for 2 h. CRITICAL STEP If the incubation time is shortened, incomplete nanoparticulate formation may occur. 17) After 2 h incubation time distribute 60 µl complexes into a clear 96-well plate \(4 wells for each charge ratio). CRITICAL STEP This clear plate will have exactly the same filling matrix as intended for transfection. In this way by convenient transfer of the nanoparticulate solutions, cells can be transfected simultaneously. 20) Transfer 50 µl nanoparticulates from the clear 96-well plate to the cells with a multichannel pipette. Remember to leave 4 control wells for each of the following: Mes-Hepes buffered saline, free plasmid DNA \(600 ng/well) and free cationic polymer \(767 ng/well, in the case of PEI at charge ratio 2). CRITICAL STEP The cells should be transfected simultaneously in order to obtain consistent results. 22) Incubate for 1 or 2 h in a tissue culture incubator at 37°C, with humidified, 7% CO_2 atmosphere, depending on the desired outcome \(higher transfection versus toxicity). CRITICAL STEP Incubation time of the nanoparticulates with ARPE-19 cells is of crucial importance, in order to get high gene expression levels and minimal cytotoxicity. 26) Dilute coelenterazine 1mM solution in D-PBS \(with Ca^{2+} and Mg^{2+}) to achieve a 1.5 µM concentration \(i.e. for a total volume of 40 ml, take 60 µl coelenterazine 1 mM). CRITICAL STEP The coelenterazine dilution needs to be prepared just before the measurement. We suggest to aliquot the coelenterazine 1 mM solution and store it at -20°C until use.

Troubleshooting

See Table 2.

Anticipated Results

Self-assembly of plasmid DNA and carrier is due to electrostatic interaction, induced by the attraction between the anionic DNA and the cationic carrier polymer. The stability of these nanoparticulates

depends on the strength of the electrostatic interaction and thus on the total charge and the charge density of the carrier molecule as well as the ionic strength of the solution used. In general, colloidal stability is being achieved by working with an excess of cationic charge, that is, carrier polymer, to ensure complete coating of the DNA. This creates charged nanoparticulates that are stabilized by the electrostatic repulsion. The hydrodynamic diameter of PEI/DNA charge ratio 2/1 nanoparticulates is ~300 nm, as determined by dynamic light scattering method. DLS also reveals the polydisperse nature of the polyplexes. Complex size is a potentially important property of synthetic gene delivery vectors. DNA complexes tend to form particulate systems \(i.e., size range of 0.05–1 μm) with the exact size depending on a number of factors such as, for example, DNA to carrier ratio, type of carrier, ionic strength of the buffer, and kinetics of mixing. The same PEI/DNA charge ratio 2/1 nanoparticulates, if prepared in 10 mM Hepes buffer without addition of salt, are much smaller in size \(70 nm). Zabner et al. \(J. Biol. Chem. 270, 1995) have shown that whilst a large amount of DNA is effectively delivered to the cell, only a small percentage is released from the endosomes, and even less makes its way from the cytoplasm to the nucleus where it is transcribed. Therefore larger nanoparticulates that sediment faster and can bear a larger DNA dose, may have some advantages over smaller vector systems. Transgene peak expression efficacy typically obtained with this protocol using ARPE19 cells and PEI/DNA charge ratio 2/1 is in the range of 10 ng/ml for 1 h incubation time and 20 ng/ml for 2h incubation \(Figure 1). The profile shows in both cases a peak in expression on the second day. This is probably caused by the CMV promoter in the plasmid. Cell viability is higher if the contact of the nanoparticulates with the cells is minimized; 80% for 1h and 60-70% for 2h incubation times \(Figure 1).

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Acknowledgements

We are grateful to EU-FP6 project funding, PolExGene, Biocompatible non-viral polymeric gene delivery systems for the _ex vivo_ treatment of ocular and cardiovascular diseases with high unmet medical need, project number 019114.

Figures

Polycation/DNA nanoparticulates				
Charge ratio	*RL plasmid*	*Mes-Hepes saline buffer*	*Polycation (i.e PEI)*	*Mes-Hepes saline buffer*
4/1	12 µl	488 µl	50 µl	450 µl
2/1	12 µl	488 µl	25 µl	475 µl
1/1	12 µl	488 µl	12.5 µl	487.5 µl

Figure 1

Table 1 Indications for the preparation of polycation/DNA nanoparticulates Plasmid DNA stock solution is 1 mg/ml. Stock solution concentration of the carrier polymer is calculated from the mass/charge of the polymer itself. For PEI it is 208 g/mol (1 positive charge for every 208 Da). Therefore 2.95 mM x 208 g/mol = 0.6136 mg/ml.

PROBLEM	STEPS	POSSIBLE REASON	SOLUTION
Low yield of luciferase	33	Plasmid purity is too low	Check absorption intensity at 260/280 nm (it should be > 1.7) and eventually purify plasmid DNA by ethanol precipitation
		Cells are unhealthy	Check cell viability
Low cell survival rate	33	Charge ratio may be too high (high amount of free polycation)	For toxic polymeric carriers (like PEI), it is important to chose the lowest effective concentration
		Incubation time of the nanoparticulates with cells too long	Reduce incubation time and keep it as short as possible to preserve cell viability
		Passage number too high	Standardize the passage number of tested cells (in case of ARPE-19 between 25-35)

Figure 2

Table 2 Troubleshooting advice

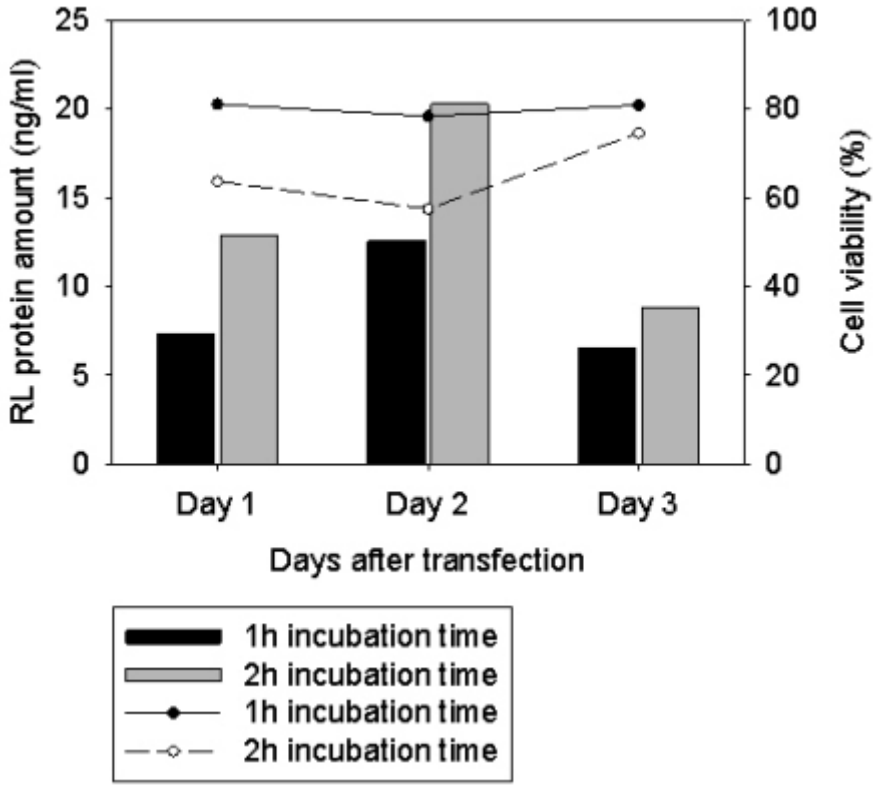

Figure 3

Figure 1 Transgene expression and cell viability profiles 3-day time course of _Renilla_ Luciferase secretion rates after transfection of dividing ARPE-19 cells. RL-DNA (600 ng per well) was complexed with PEI at charge ratio 2/1. Transfection efficacy data for 1h and 2h incubation times are shown (vertical bars). Cell viability (lines) was also monitored during the experiment and it is in reference to buffer-treated cells.