Video Article

Transfecting RAW264.7 Cells with a Luciferase Reporter Gene

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Abstract

Transfection of desired genetic materials into cells is an inevitable procedure in biomedical research studies. While numerous methods have been described, certain types of cells are resistant to many of these methods and yield low transfection efficiency¹, potentially hindering research in those cell types. In this protocol, we present an optimized transfection procedure to introduce luciferase reporter genes as a plasmid DNA into the RAW264.7 macrophage cell line. Two different types of transfection reagents (lipid-based and polyamine-based) are described, and important notes are given throughout the protocol to ensure that the RAW264.7 cells are minimally altered by the transfection procedure and any experimental data obtained are the direct results of the experimental treatment. While transfection efficiency may not be higher compared to other transfection methods, the described procedure is robust enough for detecting luciferase signal in RAW264.7 without changing the physiological response of the cells to stimuli.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52807/>

Introduction

Transfection of nucleic acids in cells has a diverse application in scientific research. Examples include (1) reporter genes to study the role of different gene elements in gene expression, (2) protein expression plasmids to overexpress the protein of interest, and (3) small interfering RNA to downregulate gene expression. By manipulating the expression level of particular genes and measuring the differential effect of such manipulations, researchers can deduce the gene functions in the chosen biological systems. Not all transfection methods provide the same transfection efficiencies, and even the same transfection method does not transfect all cell types equally¹. Hence, different transfection methods have been developed such as calcium phosphate method, DEAE dextran, cationic lipid transfection, cationic non-lipid polymer transfection, electroporation, and nucleofection^{2,3}.

Transfection into macrophages is especially difficult due to the fact that macrophages are professional phagocytes that are very sensitive to foreign materials including bacteria derived (methylated) DNA⁴. Introduction of foreign DNA activates the Toll-like receptor 9 (TLR9) pathway leading to the production of cytokines and nitric oxide^{5,6}. These activated macrophages may then be less responsive to treatment that the researchers intend to examine.

Our lab routinely transfects the RAW264.7 macrophage cell line with luciferase reporter genes, and we have developed a protocol that is robust enough to have luciferase signal significantly higher than background, but also gentle enough for macrophages to remain at their resting state. The behaviours of the transfected cells were evaluated by a firefly-luciferase reporter gene harbouring the promoter region of I κ B ζ (pGL3- I κ B ζ). I κ B ζ expression is upregulated by the bacterial cell wall component lipopolysaccharide (LPS)^{7,8}, and downregulated by the anti-inflammatory cytokine, Interleukin-10 (IL-10)⁸. To account for transfection variation among wells, we co-transfect a control plasmid containing the Renilla luciferase gene (e.g., pRL-TK) for normalization purposes. The protocol described is optimized after testing various parameters including timing of transfection, type of transfection reagents, amounts of transfection reagents and of plasmid DNA, as well as ratio of transfection reagent to plasmid DNA. The two transfection reagents included in this protocol are (1) a lipid-based transfection reagent and (2) a protein/polyamine-based transfection reagent.

Protocol

1. Plasmid DNA Purification

1. Extract plasmid DNA using a maxiprep kit according to the manufacturer's protocol. Resuspend plasmid DNA in 500 μ l of TE buffer.

2. Perform a phenol:chloroform:isoamyl alcohol extraction and isopropanol precipitation to remove residual bacterial contaminants. The presence of LPS interferes with transfection⁹.
 1. Add 500 μ l of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) to the plasmid DNA and shake vigorously for 15 sec. Phenol causes severe skin burns and is an anesthetic so burns are not always felt until there is severe damage. Perform phenol:chloroform:isoamyl extraction in the fume hood and use caution.
3. Incubate the mixture for 5 min at RT. Centrifuge at 13,000 x g for 10 min at RT. Transfer 350 μ l of upper aqueous phase into a new 1.5 ml collection tube.
4. Add 350 μ l of TE buffer to the tube that contains the lower organic phase. Shake vigorously for 15 sec. Centrifuge at 13,000 x g for 5 min at RT. Transfer 350 μ l of upper aqueous phase to the same 1.5 ml collection tube.
5. Add 70 μ l of 3 M sodium acetate (pH 5.2) and mix well. Add 700 μ l of 100% isopropanol. Mix well and incubate 10 min at RT. Centrifuge at 13,000 x g for 10 min at 4 °C. Remove the supernatant.
6. Add 500 μ l of 75% ethanol to the pellet. Vortex samples to mix and centrifuge at 5,000 x g for 5 min at 4 °C. Remove the supernatant. DNA is in the pellet.
7. Open the cap of the tube and air dry the pellet at RT for 5 - 10 min. The pellet should become translucent. Add 500 μ l of TE buffer to resuspend the DNA pellet. Heat at 50 - 60 °C for 10 min to dissolve the DNA pellet.
8. Measure the absorbance at 260 nm (A260) and 280 nm (A280) with a spectrometer. Calculate the concentration of DNA by multiplying the A260 value by 50 ng/ μ l. Assess the quality of DNA by calculating the A260/A280 ratio. DNA with high quality should give an A260/A280 ratio of 1.8 - 2.0.

2. Cell Culturing and Seeding

1. Culture RAW264.7 cells in DMEM supplemented with 9% fetal calf serum (DMEM/9% FCS) in a 37 °C, 5% CO₂ incubator. During each passage, detach the cells from the plate by continuous pipetting using a Pasteur pipette. Passage the cells every 2 days, and seed 1.5 - 2 million cells on a 10 cm tissue culture treated dish as the stock. Thaw a new stock of cells every 5 - 6 weeks.
2. On the day of transfection, seed 200,000 cells per well in a 24-well plate in a volume of 500 μ l of DMEM/9% FCS. Incubate the cells for 4 hr in the 37 °C incubator.
3. Transfect cells either with the lipid-based reagent (step 3.1) or the protein/polyamine-based reagent (step 3.2).

3. Transfection

1. Lipid-based transfection:
 1. Warm up the transfection reagent to RT in the dark. Warm up serum-free medium and DMEM/9% FCS to 37 °C.
 2. Add 0.5 μ g of plasmid DNA to 50 μ l of serum-free medium in a 1.5 ml tube. Add 2 μ l of transfection reagent with the pipette tip below the surface of the liquid. Vortex for 6 sec.
 3. Leave the reagent/DNA mixture in the dark at RT for 30 min.
 4. During the 30 min incubation, remove the media from the well and add 250 μ l of fresh DMEM/9% FCS to each well.
 5. After 30 min, add 250 μ l of DMEM/9% FCS to the reagent/DNA mixture. Mix well by pipetting up and down.
 6. Add 300 μ l of the diluted mixture to each well. Incubate for 2 - 4 hr in a 37 °C, 5% CO₂ incubator.
 7. Remove transfection solution and add 500 μ l of DMEM/9% FCS. Incubate for 24 - 48 hr in a 37 °C, 5% CO₂ incubator before cell stimulation.
2. Protein/polyamine-based transfection:
 1. Warm up serum-free medium and DMEM/9% FCS to 37 °C.
 2. Add 0.75 μ l of transfection reagent to 18.75 μ l of serum-free medium. Vortex briefly to mix. Incubate at RT for 5 min. Add 0.5 μ l of 1 μ g/ μ l DNA into the diluted transfected reagent. Incubate at RT for 10 min.
 3. During the 10 min incubation, remove media from each well of the 24-well plate and add 250 μ l fresh DMEM/9% FCS to each well.
 4. After 10 min, add 250 μ l of DMEM/9% FCS into the reagent/DNA mixture.
 5. Add 270 μ l of the diluted mixture to the well. Incubate in a 37 °C and 5% CO₂ incubator for 2 - 4 hr.
 6. Remove transfection solution and add 500 μ l of DMEM/9% FCS. Incubate for 24 - 48 hr in a 37 °C, 5% CO₂ incubator.

4. Cell Stimulation and Luciferase Assay

1. Replace media in the well with 250 μ l of fresh DMEM/9% FCS.
2. Add 50 μ l of LPS or LPS+IL-10 at the desired concentrations to the well. Return the plate to the 37 °C, 5% CO₂ incubator. Stimulate for 2 - 6 hr.
3. Remove stimulation solution by aspiration, wash with ice cold PBS, and add 200 μ l of 1x Passive Lysis Buffer. Rock at RT for 30 min. Scrape and transfer the lysate to 1.5 ml tubes and remove cell debris by centrifuging at 20,000 x g for 10 min at 4 °C.
4. Transfer 40 μ l of cleared lysate (supernatant) to a white, clear bottom 96-well plate for the determination of luciferase signals according to the manufacturer's protocol.
5. Read the luciferase signal with a luminometer across the entire visible light spectrum.

5. Data Analysis

1. Calculate the firefly:Renilla ratio by dividing individual values of firefly luciferase by the values of Renilla luciferase from each well.
2. Calculate the fold change by dividing the firefly:Renilla ratio of the treated well by that of the untreated well.

Representative Results

Figure 1 compares the transfection efficiency of the two transfection reagents in RAW264.7. The lipid-based reagent typically gave about 25% transfection rate while the protein/polyamine-based transfection resulted in about 5% efficiency (**Figure 1A**). The difference in transfection efficiency was also observed in luciferase signals in RAW264.7 cells transfected with the pGL3-IkB ζ promoter reporter (**Figure 1B**). Addition of LPS to these transfected cells increased firefly luciferase signal, a direct indication of increased transcription activity of the IkB ζ promoter reporter. In other words, the results suggested that LPS upregulated the expression of the IkB ζ gene, consistent with previous reports^{7,8}. **Figure 2** shows the typical results obtained in our experiments, using lipid-based transfection as an example. After obtaining individual signal values from firefly luciferase and Renilla luciferase (**Figures 2A and 2B**), the firefly luciferase signals were normalized to the Renilla luciferase signal (**Figure 2C**). Normalization is recommended due to well-to-well variation in transfection efficiency. To determine if the treatment conditions (LPS or LPS+IL-10) altered the reporter signals, the firefly:Renilla ratio (*i.e.*, the normalized signals) of the treatment groups were compared with that of the untreated (unstimulated) sample (**Figure 2D**). Our data showed that treating RAW264.7 cells with LPS upregulated the activity of the pGL3-IkB ζ promoter reporter, indicating that LPS increased the transcription level of the IkB ζ gene. In the presence of IL-10, the IkB ζ promoter reporter had lower activity, suggesting that IL-10 was able to inhibit LPS-induced transcription of the IkB ζ gene. The protein/polyamine-based transfection usually gave lower values in both firefly luciferase and Renilla luciferase signals. **Figure 3** compares the length of rest time between transfection and stimulation (24 hr or 48 hr), and shows that luciferase signals decreased over time. The decrease in signal did not interfere with data interpretation when the lipid-based transfection reagent was used (**Figure 3A**); induction by LPS and inhibition by IL-10 were still observed after 48 hr of rest. However, the decrease in signal was more significant when the protein/polyamine-based transfection reagent was used (**Figure 3B**), especially the Renilla luciferase signals. As a result, the difference between treatment groups was not observed after a 48 hr rest.

One undesired effect of transfection is cell death so the impact of each transfection method on the degree of apoptosis was assessed. Cells were transfected, or not, and subjected to Annexin-V and propidium iodide (PI) staining. Lysates prepared from these cells were also analyzed for the presence of intact and cleaved poly ADP ribose polymerase (PARP) protein. Flow cytometric analysis of the Annexin-V/PI stained cells suggested that the lipid-based transfection slightly increased the proportion of Annexin-V positive cells as compared to the untransfected cells, while the protein/polyamine-based transfection did not (**Figure 4A**). Similarly, the lipid-based transfected slightly increased the proportion of cleaved PARP while the protein/polyamine-based transfection did not (**Figure 4B**). However, despite the elevated numbers of apoptotic cells, the morphology of the cells by light microscopy did not change (**Figure 4C**). More importantly, the biological response of the lipid-based transfected cells remained identical to those of the untransfected cells (**Figure 4D**). The cells were stimulated with LPS $\pm$ IL-10, and the amounts of TNF α secreted into the culture supernatant were quantified by ELISA. Both the untransfected and lipid-based transfected cells made similar amounts of TNF α in response to LPS and were inhibited by the addition of IL10. No TNF α was made when the cells were not stimulated (**Figure 4D**) suggesting that the cells remained naïve after transfection.

Figure 1. The lipid-based transfection gave higher transfection efficiency than the protein/polyamine-based transfection. (A) RAW264.7 cells were transfected a GFP-expressing plasmid with either the lipid-based or the protein/polyamine-based transfection reagents. GFP signal was measured by flow cytometry after 24 hr. **(B)** RAW264.7 cells were transfected with the pGL3-luciferase reporter. After 48 hr rest, cells were stimulated with LPS for 6 hr. Firefly luciferase signal was measured according to manufacturer's instructions. [Please click here to view a larger version of this figure.](#)

Figure 2. Typical luciferase assay data. RAW264.7 cells were transfected with TK-Renilla and I κ B ζ promoter reporter. After 24 hr rest, cells were stimulated with LPS $\pm$ IL-10 for 2 hr. (A) Firefly luciferase and (B) Renilla luciferase signals were measured according to luciferase assay manufacturer's instructions. (C) Reporter activity was normalized to the TK-Renilla signal and plotted as Firefly/Renilla ratio. (D) Fold change is calculated by dividing the firefly:Renilla ratio of the stimulated samples by that of the unstimulated sample. [Please click here to view a larger version of this figure.](#)

A Lipid-based Transfection

B Protein/Polyamine-based Transfection

Figure 3. The strength of the luciferase signal is time-dependent. (A) Lipid-based transfected and (B) Protein/polyamine-based transfected cells were rested for either 24 hr or 48 hr prior to stimulation with LPS $\pm$ IL-10 for 2 hr. [Please click here to view a larger version of this figure.](#)

Figure 4. Effect of transfection on RAW264.7 cells. (A) RAW264.7 cells were transfected with the I κ B ζ promoter reporter. After 24 hr rest, cell death was assessed by Annexin-V and propidium iodide (PI) staining on a flow cytometer. (B) Transfected cells were subjected to immunoblotting analysis for PARP (full length and cleaved) and GAPDH (loading control). Band intensities were then quantified using imaging software. L = lipid-based transfection, P = protein/polyamine-based transfection, STP = 0.1 μ M staurosporin. (C) Microscope images of cells transfected with the lipid-based transfection reagent. (D) Cells transfected with the lipid-based transfection reagent were stimulated with LPS $\pm$ IL-10 for 6 hr, and the levels of the pro-inflammatory cytokine TNF α were measured using ELISA. [Please click here to view a larger version of this figure.](#)

Discussion

The protocol described here does not solely focus on transfection efficiency, but aims to strike a balance between efficiency and preservation of the physiological states of the cells. Specifically, our procedure succeeds in minimizing the toxicity of transfection reagent and maximizing luciferase signal.

One critical step in the protocol is the health of the cells. Overgrown cultures are not suitable for transfection as their physiology changes, and continuous culturing of RAW264.7 cells for a long period of time can also change the phenotype and function of the cells¹⁰. Freshly thawed cells that have a low passage number are recommended to use for transfection.

Another important consideration is the choice of transfection reagents. Lipid-based transfection reagents are typically used in research due to its ease of use and commercial availability. However, some of these reagents caused unintended (and usually unwanted) changes in global gene expression in transfected cells^{11,12}. To address this problem, cells are only incubated with the transfection reagents for a few hours instead of the typical O/N, or a non-lipid-based reagent is chosen for transfection. Longer incubation time with the transfection reagent will increase transfection efficiency, but it can also be harmful to the cells either causing cell death or cell activation, both of which can interfere with the experimental design. **Figures 4A** and **4B** show that the protein/polyamine-based transfection did not cause apoptosis or necrosis, compared to untransfected cells. The lipid-based transfection, even with the short incubation time, caused higher level of cell death. It is correlated to the higher transfection efficiency of the lipid-based transfection reagent. However, when the transfected cells were observed under the microscope, there were no notable morphological changes (**Figure 4C**). Activated macrophages usually adopt a sprawling shape, but both the untransfected and transfected cells did not show signs of activation prior to stimulation, indicating that they were in their resting states. In addition, the transfected cells responded similarly to LPS and IL-10 stimulation as the untransfected cells (**Figure 4D**). These observations collectively indicate that this transfection procedure does not alter the cells' natural behaviour.

The time between transfection and experimental treatment (the rest time) is also crucial. Enough time needs to be given for the luciferase genes to express and for the cells to re-establish their resting state; however, a long incubation can reduce luciferase signals, especially when transfection efficiency is not high.

The procedure here applies to the specific luciferase reporter gene used in our lab. Minor adjustments will be needed when another reporter is used. Parameters that need to be tested include different firefly luciferase reporter:Renilla luciferase ratio, the rest time between transfection and treatment, and treatment conditions. A 50:1 ratio of pGL3-lkBZ: phRL-TK is typically used, but the ratio can range from 1:1 to 100:1. It was found that a 24 hr rest between the removal of transfection solution from the cells and the start of experimental treatment gave the best signal. After 48 hr, luciferase signals started declining, and differences between treatment groups were reduced or even abolished.

The described transfection procedure is not limited to luciferase reporter assays. Other experimental designs that do not need a homogenous population will be benefited; for instance, fluorescent microscopy which relies on observations from individual cells. One disadvantage will be to locate the successfully transfected cells among untransfected counterparts, but the benefits of being less time-consuming and labor-intensive may be more desired. In cases where stable cell lines are preferred, our protocol provides a mean for initial experiments in which experimental procedure can be optimized when stable cell lines are being generated.

Disclosures

The authors have nothing to disclose.

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