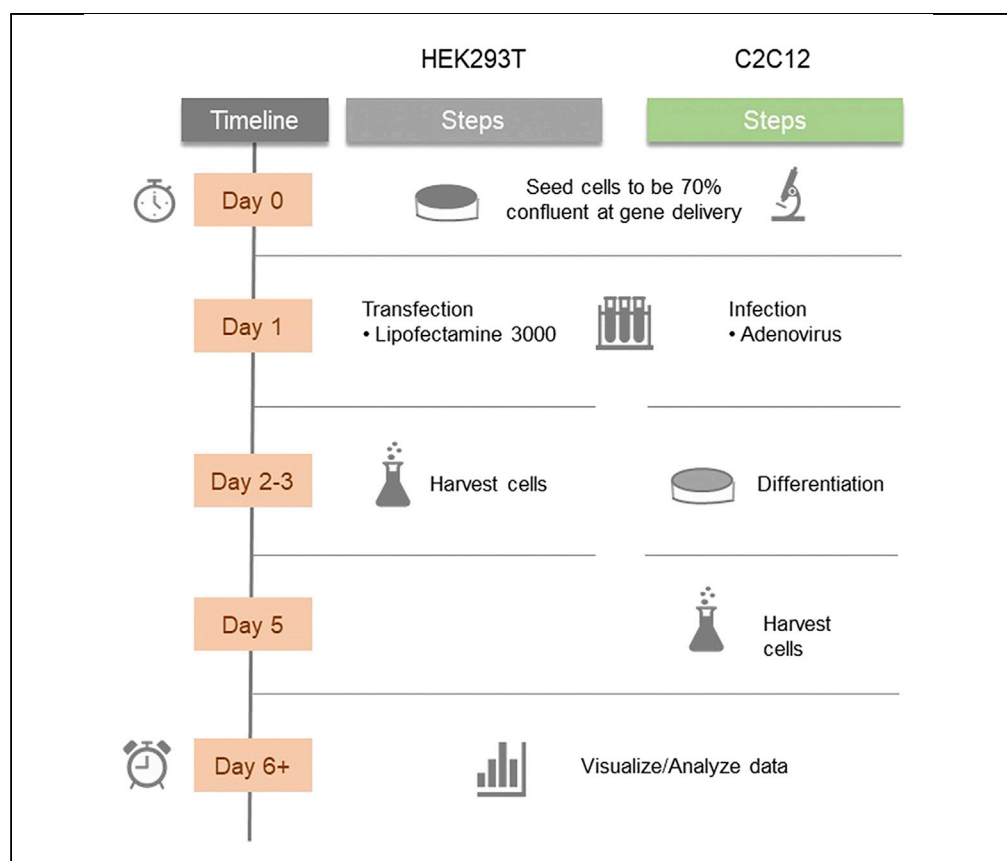


## Protocol

# Protocol for cell preparation and gene delivery in HEK293T and C2C12 cells



Exogenous overexpression of target genes in both general and specific cell types is important for mechanistic studies of gene function. Here, we provide a step-by-step protocol for cell culture, plasmid transfection in HEK293T, and adenoviral infection in C2C12 cells for gene overexpression *in vitro*, using MG53 as an example. This protocol enables sufficient and efficient gene expression for the downstream functional analysis.

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### Highlights

Efficient gene expression in HEK293T by plasmid transfection with Lipofectamine 3000

Robust gene expression in C2C12 myotubes by adenoviral infection

Differentiation of C2C12 myoblasts into myotubes

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## Protocol

# Protocol for cell preparation and gene delivery in HEK293T and C2C12 cells

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## SUMMARY

Exogenous overexpression of target genes in both general and specific cell types is important for mechanistic studies of gene function. Here, we provide a step-by-step protocol for cell culture, plasmid transfection in HEK293T, and adenoviral infection in C2C12 cells for gene overexpression *in vitro*, using MG53 as an example. This protocol enables sufficient and efficient gene expression for the downstream functional analysis.

For complete details on the use and execution of this protocol, please refer to Jiang et al. (2021).

## BEFORE YOU BEGIN

### Plasmid DNA and adenovirus

Preparing high quality, sufficient amount of plasmid DNA or adenovirus for transfection.

The plasmid DNA is better prepared using endotoxin-free plasmid preparation kit (such as PureLink™ HiPure Plasmid Midiprep Kit from Invitrogen). For overexpression of MG53 in HEK 293, we use 2.5 µg of plasmid DNA for transfection of cells in a 3.5 cm dish (or one well in a 6-well plate). The plasmid DNA prepared from 100 mL overnight culture of *E. coli* is over 500 µg, and can be stored at –20°C for a couple of months. The concentration of plasmid DNA can be determined by NanoDrop spectrophotometer from Thermo Fisher.

Purify the virus particle from cell lysate using CsCl continuous density gradient centrifugation. Determine the titer of the purified adenovirus by measuring OD<sub>260</sub> value, and calculate the number of virus particles in every 1 mL sample according to the formula: VP/mL=OD<sub>260</sub>×1.1×10<sup>12</sup>. The adenovirus should be kept in a –80°C freezer in small aliquot of 50–100 µl to avoid frequent freeze-thaw.

### Cell culture

⌚ Timing: 1 week

The protocol below describes the specific steps for maintaining HEK293T cells, including how to start a cell culture from frozen vials of cells in cryogenic storage. Similar procedures can be used for C2C12 myoblasts. However, for C2C12 myoblasts, do not allow the cells to reach 100% confluent



before subculture, as this will deplete the myoblastic population in the culture. Numbering the passage number of C2C12 cells and monitoring their differentiation potential are thus important.

1. Thaw cryopreserved cells
  - a. Put cryogenic vials in a 37°C water bath. To reduce the possibility of contamination, spray the thawed vial with 70% ethanol and wipe carefully. Transfer thawed liquid contents into a 50 mL conical tube prefilled with 5 mL fresh complete culture medium (DMEM supplemented with 10% fetal bovine serum, and the same medium is used for maintaining C2C12 myoblasts).
  - b. Collect cells for plating.
    - i. Centrifuge at 125 × g for 5 min, discard supernatant, and resuspend cells in 10 mL complete medium.
    - ii. Plate  $\sim 5 \times 10^5$  cells in a 10 cm dish and incubate cultures at 37°C.
2. Passage HEK293 cells upon 80%–90% confluency. Subculture ratio is 1:5. C2C12 cells can be subcultured when the cells reach 70% confluency, and the subculture ratio is 1:3~1:5.

**Note:** Culture medium should be prewarmed to 37°C before use to avoid inflicting cold stimulation to cells.

### Cell preparation

⌚ Timing: 2 days

3. Disassociate cells before plating
  - a. Discard culture medium, briefly rinse the cell layer with prewarmed PBS. Remove PBS and add 1 mL 0.25% Trypsin solution (with EDTA) and incubate the cells at 37°C for 2–3 min.
  - b. Observe cells under an inverted microscope until cell layer is dispersed, add 6–8 mL complete growth medium and aspirate cells by gently pipetting.
4. Collect and seed cells
  - a. Transfer the medium containing cells to a 50 mL conical tube.
  - b. Centrifuge at 125 × g for 5 min. Discard supernatant, and resuspend cells in 40–50 mL complete culture medium.
  - c. Seed cells at appropriate density according to the specific requirements of each experiment.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
β-actin	Sigma-Aldrich	Cat# A5441; RRID:AB_476744
MG53	<a href="#">Song et al., 2013</a>	N/A
<b>Bacterial and virus strains</b>		
Adenovirus expressing: β-gal	<a href="#">Song et al., 2013</a>	N/A
Adenovirus expressing: Myc-MG53	<a href="#">Song et al., 2013</a>	N/A
<b>Chemicals, peptides, and recombinant proteins</b>		
Dulbecco's modified Eagle's medium	Invitrogen	12800017
Fetal bovine serum	Invitrogen	10099141
Donor Equine Serum	HyClone	SH30074.03
Trypsin	SIGMA	T4049
1×PBS	N/A	N/A
<b>Critical commercial assays</b>		
Lipofectamine™ 3000 Transfection Reagent	Thermo Fisher	L3000015

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
HEK293T cells	ATCC	CRL-11268
C2C12 cells	ATCC	CRL-1772
Recombinant DNA		
Plasmid: Myc-MG53	(Song et al., 2013)	N/A
Plasmid: Flag-MG53	(Song et al., 2013)	N/A
Software and algorithms		
Image Lab	Bio-Rad Laboratories	<a href="https://www.bio-rad.com">https://www.bio-rad.com</a>
GraphPad Prism 6	GraphPad Software	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>

## STEP-BY-STEP METHOD DETAILS

### Plasmid transfection in HEK293T cells

⌚ Timing: 2–4 days (contingent on time necessary for some of the steps)

The following steps describe the detailed procedures about using Lipofectamine™ 3000 Reagent to transfect HEK293T cells with Myc-MG53 plasmid DNA.

1. Plasmid transfection
  - a. Seed  $4.5 \times 10^5$  -  $6.0 \times 10^5$  cells per 3.5 cm dish (or one well of a 6-well plate) in 2 mL of complete growth medium. Incubate cells overnight at 37°C in 5% CO<sub>2</sub> incubator (Figure 1A).
  - b. The next day, transfect cells with plasmids using Lipofectamine™ 3000 Reagent following [manufacturer's instructions](#). Specifically for HEK293 cells, transfect cells with 1 µg Myc-MG53 plasmid using 2 µL P3000™ Reagent and 3 µL Lipofectamine™ 3000 Reagent per 3.5 cm dish according to manufacturer's instructions. After transfection for 6 h, switch to fresh culture medium. It is not necessary to change medium after transfection unless the culture medium turns yellow. The amount of reagents/plasmid DNA used should be tested to achieve the best transfection efficiency.
2. Harvest cells 24–36 h after transfection. The cells can be lysed in lysis buffer for further experimentation such as co-immunoprecipitation. Western blotting can be performed to determine the amount of target protein overexpressed.

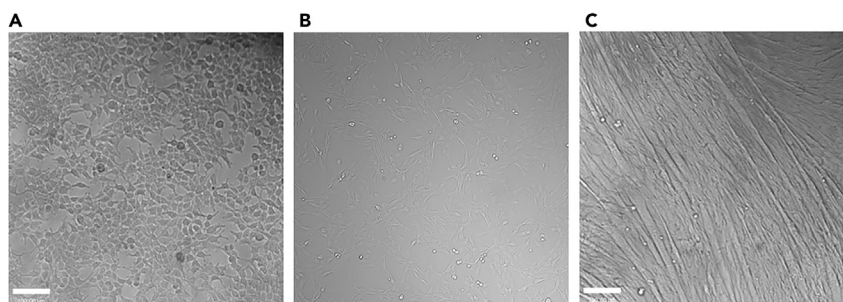
### Adenoviral infection in C2C12 myoblasts

⌚ Timing: 1 week

[Include a brief description about what this major step accomplishes. This will help other researchers repeat and troubleshoot the protocol.]

The following steps describe the detailed procedures for infection of differentiated C2C12 myoblast with adenovirus.

3. Adenoviral infection
  - a. Seed myoblasts in dishes to reach 70%–80% confluence (Figure 1B).
  - b. Replace complete growth medium with differentiation medium (DMEM with 2% donor equine serum). Add adenovirus into the medium and swirl briefly to allow the adenovirus to uniformly distribute in the dish. For C2C12 myotubes, adenovirus is usually used at 15 M.O.I. (multiplicity of infection, referring to the number of infected viral particles per cell) M.O.I. used for each experiment should be determined with a titration as shown in Figure 2B.



**Figure 1. Images of cells in culture**

HEK293 cells at 80%–90% confluency (A). C2C12 myoblasts at 70%–80% confluency (B) and differentiated into myotubes after maintained in differentiation medium for 5 days (C). Scale bar, 100  $\mu$ M

While there is a dose-dependency in the amount of virus used and the amount of target protein overexpressed, the M.O.I. of adenovirus applied should also be determined empirically based on biological effects of the target protein.

- c. Change medium (fresh differentiation medium) 24 h after infection.
4. Myoblast differentiation
  - a. After infection, myoblast cells are cultured in differentiation medium for at least 4 days to induce their differentiation into myotubes (Figure 1C).
  - b. Change medium every 2 days.
5. Harvest cells after differentiation for at least 4 days.

## EXPECTED OUTCOMES

The expression level of target gene can be determined by western blotting (Figure 2). The biological function of the target gene can then be evaluated with appropriate assays.

## LIMITATIONS

Although overexpression is a commonly used approach to study the function of a protein, the procedure is still tricky and needs to be carefully adjusted case by case. Transfection efficiency with Lipofectamine depends on multiple factors, including cell type, cell density, passage number, ratio between plasmids and liposome, etc. Adenoviral infection is more robust in terms of efficiency in various cell types. Nevertheless, viral titer, the vitality of both adenovirus and cells, and the amount of adenovirus used may also have impacts on the results. In additions, some proteins to be expressed have important biological functions that may interfere with the outcome of transfection. For example, MG53 is involved in the process of myogenesis, expressing MG53 at very high level blocks the differentiation of myoblasts and makes it impossible to examine the role of MG53 in the differentiated myocytes. In extreme cases, overexpression of certain genes may cause deleterious effects on cells and even impede their growth. To overcome these situations, optimizing gene delivery in each specific experiment is highly recommended.

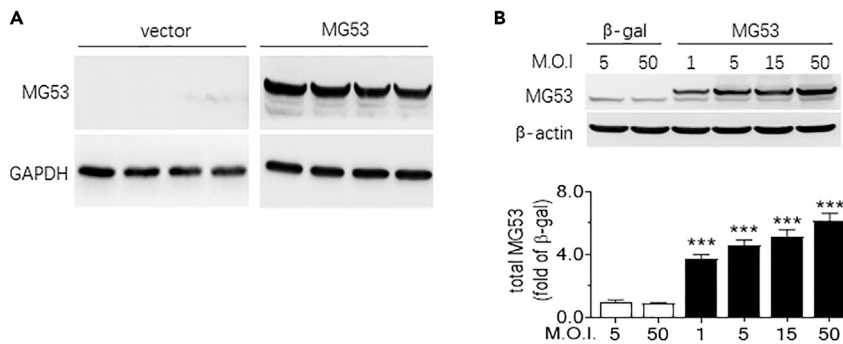
## TROUBLESHOOTING

### Problem 1

Insufficient expression of target proteins.

### Potential solution

This can attribute to different reasons. First, make sure that cells are seeded at appropriate density and in good condition. Second, make sure the plasmid DNA is endotoxin-free and the concentration is accurate. Third, optimize the ratio of Lipofectamine reagents and DNA. In case of adenovirus infection, viral vitality and titer used for infection are important. Polybrene can also be added to



**Figure 2. Representative western blots showing overexpression of MG53**

Overexpression of MG53 in HEK293 cells (A) and C2C12 myotubes (B) after gene delivery. \*\*\*,  $p < 0.005$  as compared with cells infected with adenovirus expressing β-gal.

increase efficiency. Finally, always keep the potential biological function of the target protein in mind before accessing the efficiency and phenotype of overexpression.

## Problem 2

Decreased cell viability after gene delivery.

### Potential solution

Both Lipofectamine and adenovirus can be toxic to cells when too much is applied. Decreased cell viability could be a big issue if cell proliferation or survival is the readout of the subsequent functional assays. To reduce the influence on cell viability, adjust the amount of reagents used or reduced the incubation time of cells with DNA/Lipofectamine or adenovirus.

## Problem 3

Variations in expression level among repeats.

### Potential solution

First, the amount of cells plated in each dish/well should be consistent. For transfection with Lipofectamine, mix plasmid DNA and Lipofectamine for all the repeats and with some extra at once, then add the mixture into each dish/well from this same preparation. For adenoviral infection, dilute the virus in the culture medium, and add about 100 μL of medium containing virus into each dish/well. Change the tips each time after adding DNA/Lipofectamine or adenovirus into culture dish, as the tips may bring up medium and change the concentration of DNA/Lipofectamine or viral particles in the stock solution when dip back into the stock.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rui-Ping Xiao ([xiaor@pku.edu.cn](mailto:xiaor@pku.edu.cn)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate unique code.

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## AUTHOR CONTRIBUTIONS

P.J., L.R., and L.Z. performed the experiments. L.R., P.J., X.H., and R.-P.X. wrote the manuscript. All authors contributed to the manuscript and approved it for publication.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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