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Research Article

Cell "starvation" treatment -- optimization of packaging methods for recombinant adenovirus vectors

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Abstract: ***Objective*** To optimize the condition for transfection of HEK293 cells with exogenous gene in mediation of lipofectamine™2000. ***Methods*** The hTERT-E1A-EGFP was inserted into the shuttle vector pEntry to construct pAd-hTERT-E1A-EGFP vector. pAd--hTERT-E1A-EGFP was then digested by PacI and transfected into the HEK293 cell line by Optimization method. Packaging recombinant adenovirus, cDNA was obtained using reverse transcription after total ***RNA*** was extracted. Results: Our results demonstrated that the adenovirus was successfully packaged by Optimization method. ***Conclusion*** The condition for transfection of HEK293 cells in mediation of lipofectamine™ 2000 was optimized. Compared with the traditional packaging recombinant adenovirus in Lipofectamine™ 2000, optimization method of packing is more effective and convenient, it not only improving the packing efficiency, shortening packaging time, reducing the possibility of cells pollution, but also laing a solid foundation for subsequent adenovirus gene therapy research.

Keywords: Oncolytic virus; Adenovirus; Genome packaging; lipofectamineTM 2000;Opti-MEM® I Reduced-Serum Medium; virus titer

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Adenovirus packaging is to use HEK293 cells with E1 gene as packaging cells, enriching virus particles through multiple ratio amplification[1-3]. As a transfection reagent, Lipofectamine 2000 is widely used in the packaging of recombinant adenovirus vectors because of its stable properties and high transfection efficiency[4]. Its principle is that DNA-Lipofectamine 2000 complexes transfer recombinant adenovirus DNA into cells through endocytosis of HEK293 cells to form endosome, from which DNA is released into cytoplasm and further into nuclear transcription and expression. At present, however LipofectamineTM 2000 was used to transfect HEK293 cells to achieve adenovirus packaging. The time from transfection to subculture and then to collect virus supernatant needed more than 6 days, and many times of subculture were allowed due to the vigorous growth of cells after transfection[5]. On the one hand, subculture during packaging also increases the possibility of contamination. On the other hand, the cell morphology during transfection was not significantly different from the untransfected normal HEK293 cells, Therefore whether the transfection was successful could not judge by morphology changes. Therefore, a large amount of time would be wasted if the packaging was not successful after multiple subculture. Therefore, Opti-MEM medium was used in the transfection process, so that the cells were in the starvation state during the transfection process, and the morphology of HEK293 cells after starvation treatment would change significantly, until the receiving virus supernatant, cells were significantly different from the normal HEK293 cells. Therefore, this study was designed to optimize the conditions for transfection of recombinant adenovirus Ad-hTERT-E1A-EGFP into HEK293 cells mediated by the liposome LipofectamineTM 2000, laying the foundation for efficient transfection of recombinant adenovirus into

HEK293 cells after cell starvation. We herein report a new and simple approach to an efficient and robust packaging of recombinant adenoviral genomes, the method is simple, economical, and efficient, and has excellent applications, which meet the demands of subsequent experiment.

1 Materials and methods

1.1 Materials

Gene sequence, oligonucleotide sequence was synthesized by sangon in Shang Hai(Table 1). Human embryonic kidney cell line 293(HEK293 cell) (Laboratory preservation), pAd vectors (Laboratory preservation), shuttle vector(Laboratory preservation), DMEM basic(1X) and Opti-MEM Medium (Gibco, USA), fetal bovine serum (FBS) (Gibco, USA), Pac I, SacII and XbaI(NEB, USA), Lipofectamine2000(Invitrogen, USA), RNeasy Mini Kit(QIAGEN, Germany), TransScript One-step RT-PCR super MIX (TRANS, China), DNA Plasmid Mini Kit I and Gel Extraction Kit (Omega, USA), Assay kit for adenovirus titer colorimetry (GENMED, Shang Hai).

1.2 Methods

1.2.1 The recombinant adenovirus vector pAd-hTERT-E1A-EGFP was constructed by Gateway technology.

1.2.1.1 Construction and identification of shuttle vector pEntry-hTERT-E1A-EGFP. Preparing the ligation reaction including Homologous recombinant enzyme, Transgene insert DNA, shuttle vector, as follows: 5×CE Ⅱ Buffer 4μL; Exnase Ⅱ 2μL; pEntry 0.9μL; hTERT-E1A-EGFP 0.8μL; add H_2O to 20μL and mix well. Incubate for 30 min in a water bath at 37 ℃. Cool immediately on ice. Thaw chemically competent DH5α E. coli on ice. Add 100 μL of competent cells and 10 μL of diluted ligation reaction to the bottom of the polypropylene round-bottomed tube, places the ligation reactions on ice for 30 min, 42˚C Water bath for 45s. Then incubate on ice for 2min. Add 900μL LB medium. incubate for 1 hours at 37℃ with agitation(200 rpm). Centrifuge the transformed cells at 12000 rpm for 1 min. Then, removes 800 μL of the supernatant, resuspending the bacteria in the remaining 200 μL of medium, and plate the cells on LB agar plates with 50 μg/mL kanamycin and incubate overnight at 37℃. Isolating a single colony from a freshly streaked selective plate, and inoculate a culture of 3 mL LB medium containing the kanamycin antibiotic. Incubate for 14 hours at 37°C with agitation(250 rpm). The plasmids were extracted using Omega's Plasmid Mini Kit, Store DNA at -20°C[6, 7]. Next, the recombinant plasmid was identified by endonucleases Sac Ⅱ and Xba Ⅰ. Analyze 10 μL of the digest by electrophoresis through a 1% agarose gel to confirm the accuracy of the plasmid maps. Simultaneously, the plasmid was sequenced in both directions to accurately clarify the construction. The positive recombinant plasmid was named pEntry-hTERT-E1A-EGFP.

1.2.1.2 Construction of adnovirus expression vectors pAd-hTERT-E1A-EGFP. LR reaction between adenoviral shuttle vector pEntry-hTERT-E1A-EGFP and backbone vector pAd was carried out to establish adenoviral expression vector pAd-hTERT-E1A-EGFP. Prepares the insert and vector backbone DNA fragments, a mixture of the entry cloning containing the target gene, the appropriate target vector and the Gateway LR Clonase enzyme. The ligation reaction included the following: pAd 1.5 μL, pEntry-hTERT-E1A-EGFP 1.5 μL, Gateway™ LR Clonase™ II Enzyme Mix 2μL, ddH_20 5 μL. Incubates for 1 hours in a water bath at 25 ˚C. Add 1μL proteinase K solution incubate on 37℃ for 2min. The digested products were separately transformed into DH5α, incubating the transformation reaction on ice for at least 30 min. 42˚C Water bath for 45s.Then incubate on ice for 2min. Add 900 μL non-resistant LB liquid medium. Incubate for 1 hours at 37˚C with agitation(200 rpm). Centrifuge the transformed cells at 12000 rpm for 1 min. Then, remove 800 μL of the supernatant, resuspend the bacteria in the remaining 200 μL of medium, and plate the cells on LB agar plates with 50 μg/mL ampicillin and incubate overnight at 37˚C. Isolates a single colony from a freshly streaked selective plate, and inoculate a culture of 2mL LB medium containing the ampicillin antibiotic. Incubate for 14 hours at 37°C with agitation(250 rpm). The plasmids were extracted using Omega's Plasmid Mini Kit. the recombinant plasmid was identified by endonucleases Sac Ⅱ and Xba Ⅰ for 1 hour at 37°C. Then, analyzes 10 μL of the digest by electrophoresis through a 1% agarose gel to confirm the accuracy of the plasmid maps. Simultaneously, the plasmid was sequenced in both directions to accurately clarify the construction. The positive recombinant plasmid was named pAd-hTERT-E1A-EGFP.

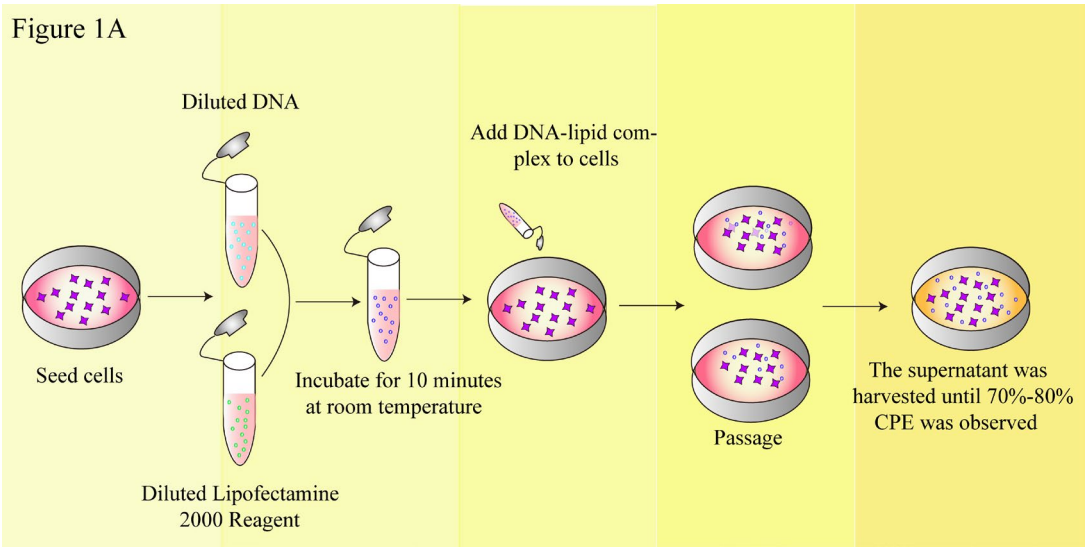
1.2.2 Purification of a large amount of extracted recombinant adenovirus plasmid DNA by linearization. Adenovirus expression vectors pAd-hTERT-E1A-EGFP were linearized by PacI and incubate overnight at 37 ℃ . The linearized reaction included the following: pAd-hTERT-E1A-EGFP 86μL; c Ⅰ 4μL ddH_20 10μL. Next, we gel-extracted the digested products using Omega's Gel Extraction Kit.

1.2.3 Thawing HEK293 Cells, HEK293 cells were cultured in a humidified atmosphere with 5% CO_2 at 37℃ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics. When the fusion degree was about 90% again, the cells were transferred to the 6-well plate and the count was about 5×10^3/ml, cells will be 60%-70% confluent at the time of transfection.

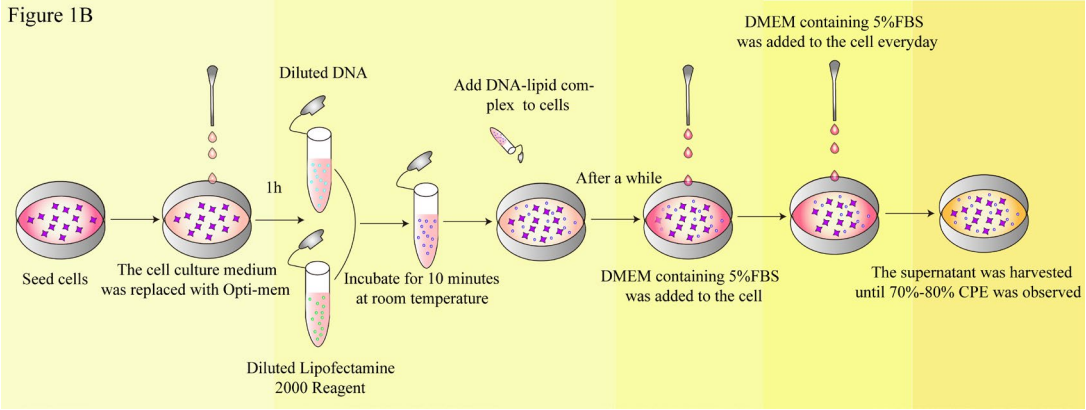
1.2.4 Packaging of the adenovirus

1.2.4.1 Conventional packaging pAd-hTERT-E1A-EGFP. One day before transfection, seed early- or medium-passage HEK293 cells, all the cells were cultured at 37˚C in a humidified incubator with 5% CO_2. On the next day, when cell confluency reaches 50%-60%, proceeds with the following transfection methods. The specific approach is as follows, dilute Lipofectamine 2000 in Opti-MEM Medium 15μL Lipofectamine 2000 + 150μL Opti-MEM Medium; Dilute recombinant adenovirus plasmid DNA pAd-hTERT -E1A-EGFP by linearization in Opti-MEM Medium, 14μg pAd-hTERT-E1A-EGFP + 150 μL Opti-MEM Medium.Add diluted DNA to diluted Lipofectamine 2000 Reagent drop by drop. Incubate for 5 minutes at room temperature. Add DNA-lipid complex to HEK293 cells. When the fusion degree was about 90% again, cell passage cultivation still, the supernatant was harvested until 70% -80% CPE was observed Figure 1A .

Figure 1A



1.2.4.2 Optimized method packaging pAd-hTERT-E1A-EGFP. The specific approach is as follows when cell confluency reaches 50%-60%, siphons off DMEM, at 1 hours before the end of the incubation, gently rinse the cells once with PBS pre-warmed to 37˚C and then add 500 μl of Opti-MEM™ Medium, cultured in a humidified atmosphere with 5% CO_2 at 37 ℃ . dilute Lipofectamine 2000 in Opti-MEM® Medium 15μL Lipofectamine 2000 + 150μL Opti-MEM Medium; Dilute recombinant adenovirus plasmid DNA pAd-hTERT-E1A-EGFP by linearization in Opti-MEM® Medium: 14μg pAd-hTERT -E1A-EGFP + 150 μL Opti-MEM Medium.Add diluted DNA to diluted Lipofectamine® 2000 Reagent drop by drop. Incubate for 5 minutes at room temperature. Add DNA-lipid complex to HEK293 cells. Set 5 groups of starvation time, each group of 3 repeated sample holes, grouped as :0 hour, 0.5 hours, 1 hour, 2 hours, 4 hours. When starvation time is reached, adding 500μL Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) and 0.25% antibiotics. Then add 500μL Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) and 0.25% antibiotics daily. Once 70%-90% of the cell monolayer shows CPE, dislodge any remaining cells by gently tapping the culture dish on the work surface of the cell culture biosafety hood. Transfer the cell suspension to a 15 mL conical centrifuge tube and store it at −80˚C for virus expansion(Figure 1B).



1.2.5 Verify that the virus was successfully packaged, specific measures were took: we collected the cell suspension, The total recombinant adenovirus RNA were extracted using QIAGEN 's RNeasy Mini Kit. Next, the extracted viral total RNA was reversely transcribed by PCR PCR amplifications were performed in 20μL reaction mixture containing pAd-hTERT-E1A-EGFP 3μL Forward Primer 0.4 μL Reverse Primer 0.4 μL, 2×One-step Reaction MIX 10μL, Trans Script One-step 0.4 μL, Rnase-free Water 5.8 μL. The PCR protocol was as follows: 45℃ for 30 min, 94℃ for 5 min followed by 35 cycles at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, and 72℃ extension for 45 s, followed by a final extension at 72℃ for 10 min.

1.2.6 Statistical analysis of virus titers. The result is expressed in terms of x ± s. Adenovirus titer was determined by adenovirus titer colorimetry. Firstly, set experimental background: set microplate reader wave length is 260nm place 1mL Reagent A into separate 1.5mL microcentrifuge tubes, then 10μL adenovirus sample preservation solution to be tested was added, vortex for 30 seconds, 100μL was transferred to the 96 well plate for detection, which was the background blank control. Secondly, place 1mL Reagent A into separate 1.5mL microcentrifuge tubes. then add 10μL adenovirus samples to be measured, vortex for 30 seconds, 100μL was transferred to the 96 well plate for detection, which is the sample reading. Calculate the sample titer: (The sample reading - The background reading)×100(Dilution ratio)×1.1(Standardized Coefficients)×10^{12}= Total number of virus particles /ml. Analysis using Prism 5.0(GraphPad) statistical software.P <0.05 was considered statistically significant.

2 Results

2.1 Construction and identification of shuttle vector pEntry-hTERT-E1A -EGFP. Recombined vectors were identified by restriction enzyme analysis (SacII and XbaI), then throughed a 1% agarose gel to confirm the accuracy of the plasmid maps, correct clone could be cut off to 1870 bp fragments. DNA sequencing results showed that the hTERT-E1A-EGFP gene sequence was completely correct, indicating that the construction of the pEntry-hTERT-E1A-EGFP vector was successful(Figure 2).

Figure 2

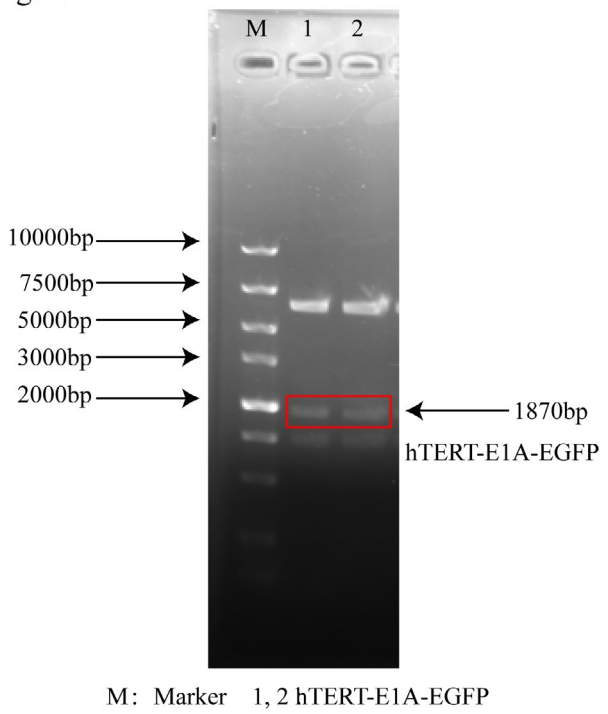
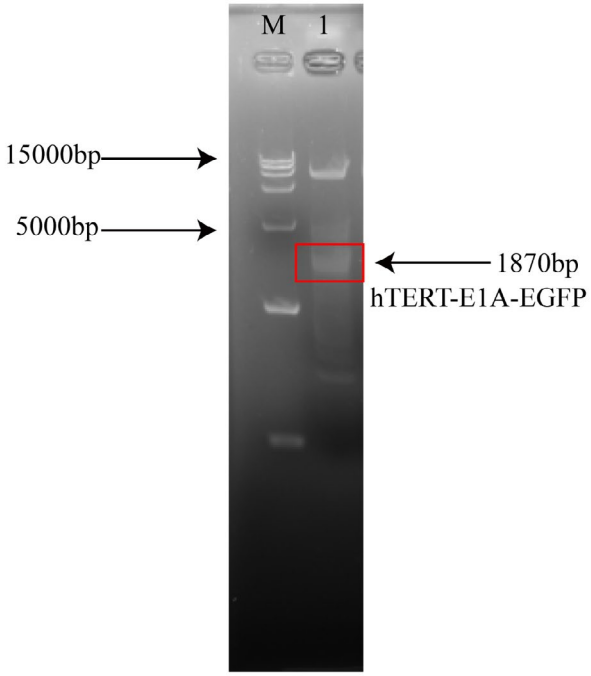


Figure 2. Enzymatic digestion products of the hTERT-E1A-EGFP gene. Lanes 1-2= Agarose gel electrophoresis of hTERT-E1A-EGFP gene Enzymatic digestion products.

2.2 Construction of adnovirus expression vectors pAd-hTERT-E1A-EGFP. LR reaction between adenoviral shuttle vector pEntry-hTERT-E1A-EGFP and backbone vector pAd was carried out to establish adenoviral expression vector pAd-hTERT-E1A-EGFP were identified by restriction enzyme analysis (SacII

and XbaI), correct clone could be cut off to 1870 bp fragments, DNA sequencing results showed that the hTERT-E1A-EGFP gene sequence was completely correct, indicating that the construction of the pAd-hTERT-E1A-EGFP vector was successful(Figure 3).

Figure 3



M: Marker 1:hTERT-E1A-EGFP

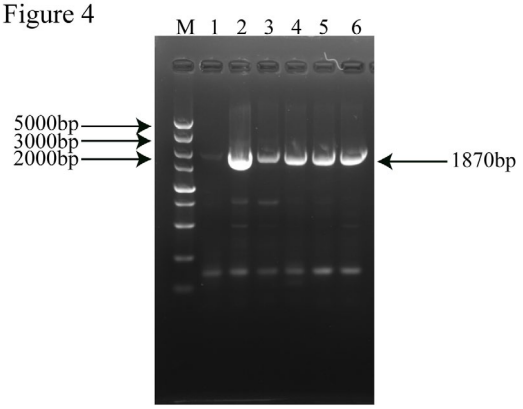
Figure 3. Enzymatic digestion products of the hTERT-E1A-EGFP gene. Lanes 1= Agarose gel electrophoresis of hTERT-E1A-EGFP gene Enzymatic digestion products.

2.3 Verify that the virus was successfully packaged. Digested by PacI, The recycled products were separately transfected into the 293A cell line, When 50% of the cells had detached from the plate, we collected the cell suspension, The total RNA was reversely transcribed to cDNA, and the oligonucleotide sequences used were shown in table 1. The target band can be seen by agarose gel electrophoresis(Figure4). The plasmid was sequenced to accurately clarify the construction. The cell morphology changes significantly with the duration of starvation, which is inversely proportional to the time it takes to receive the poison (Figure5). The virus titer was highest when the cells were starved for 0.5 h (Figure 6). With the prolongation of starvation time, the cell morphology also changed significantly, and the starvation time was inversely proportional to the time of receiving the virus.

Table 1. The primers were designed to reverse the extracted viral total RNA into cDNA

The name of the primer	primer sequence
Forward primer	acaaaaaagcaggctccgcggGACCCCCG GGTCCGCCCG
Reverse primer	gcccacccttaggcctctagaTTACTTGTAC AGCTCGTCCATGCC

Figure 4



M: Marker 1,2,3,4,5,6: pAd-hTERT-E1A-EGFP

Figure 4. RT-PCR products of the hTERT-E1A-EGFP gene. Lanes 1-6= Agarose gel electrophoresis of hTERT-E1A-EGFP gene RT-PCR products.

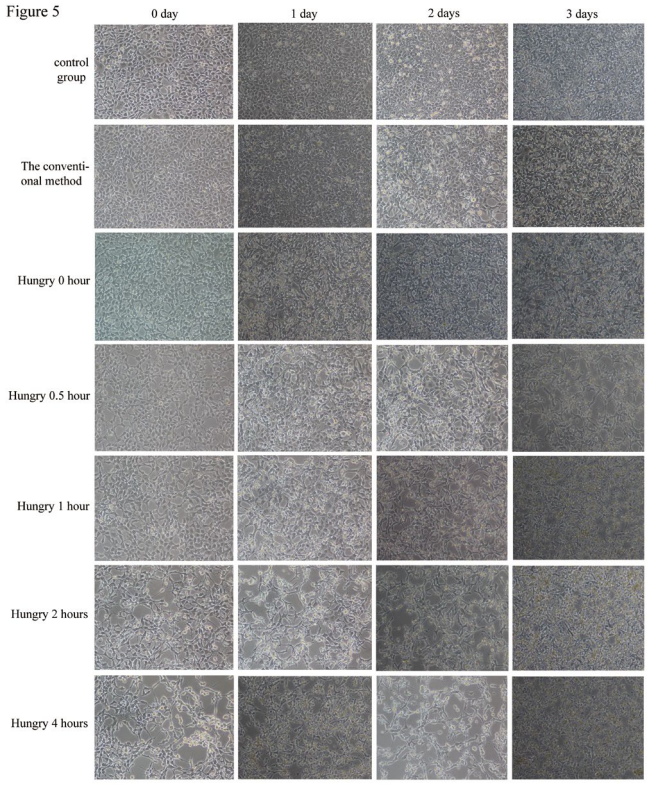


Figure 5. Looking at the cell morphology under a microscope. The intercellular space increased gradually, and the cell morphology changes significantly with the duration of starvation, which is inversely proportional to the time it takes to receive the poison. What'more, the longer the starvation, the more obvious the CPE.

Figure 6

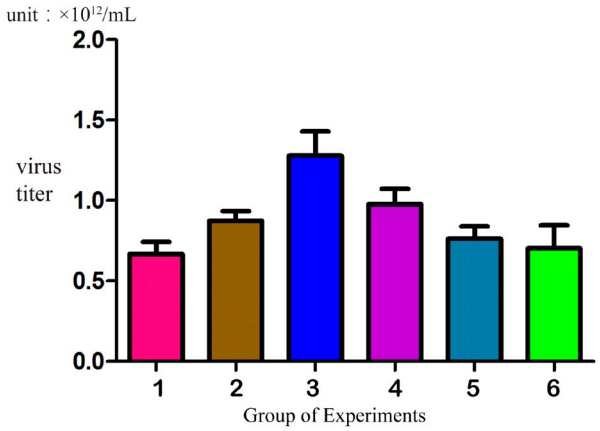


Figure 6. irus packaging was divided into 6 groups, each with 3 repeated sample holes. The first group was used to package pAd-hTERT-E1A-EGFP by conventional method. The second, third, fourth, and fifth groups were 0 h,0.5 h,1 h,2 h, and 4 h respectively.

3 Discussion

Liposome-mediated eukaryotic cell transfection has the characteristics of simple operation, reproducibility and unlimited encapsulation of genes, so it has become a common method for recombinant adenovirus packaging. However, the toxicity and low transfection efficiency of liposomes in this method limited the application of HEK293 cells transfected with liposomes. According to the principle of liposome transfection, the liposome and DNA form a liposome-plasmid complex through electrostatic interaction. The complex enters the cell through membrane endosytosis and releases plasmid DNA in the cell so that foreign genes can be expressed. Therefore, in this experiment, Opti-mem medium was used for cell transfection to improve the packaging efficiency of the virus. Because of the rapid growth of conventional lipid-mediated wall transfection cells during the experiment, the fusion degree reached 100% before the virus reached its maximum replication. Because of the rapid growth of anchorage-dependent cells for conventional liposome-mediated transfection during the experiment, the confluent reached 100% before the virus reached its maximum replication. it not only causes long packaging cycle, but also increases the chances of cross-contamination. In the suspension transfection method, anchorage-depe- ndent cells were more likely to die or be polluted when added the higher toxicity lifectamine 2000 during the suspension state. Therefore, Opti-mem minus serum medium was used to keep the cells in starvation state, the hungry cell is prompted to enter the cell more quickly through membrane endocytosis of the liposome-plasmid complex, releasing plasmid DNA in the cell and allowing foreign genes to be expressed. Since the cells are hungry, the growth state of the cells also decreases. To minimize the possibility of contamination, DMEM medium containing 5% FBS and 0.25% penicillin-streptomycin mixture was used at a later stage. This also shows that the transfection efficiency of lifectamine2000 was not affected by the appropriate amount of antibiotics in the transfection process. When the cells were under hunger state, the growth process was slow, so that the recombinant virus could always be amplified in a culture hole during the whole transfection process, which avoid cell pollution and virus loss caused by cell passage. Therefore, the optimized method could make the recombinant adenovirus quickly, stable and with high yield.

The experimental results showed that the improved liposome transfection mediated recombinant adenovirus Ad-hTERT-E1A-EGFP could achieve higher efficiency in entering HEK293 cells, and the improved method and its optimized parameters could provide experimental basis for further animal experiments and clinical studies.

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