Full Length Research Paper

The technology of large-scale pharmaceutical plasmid purification by cetyltrimethylammonium bromide and Tritonx-114

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Many methods for plasmid purification have been developed, and the whole process must be designed to remove the host RNA, protein, genomic DNA and endotoxin. Currently, plasmid is mostly purified by time-consuming chromatographies. As an alternative, a new plasmid purification technology with cetyltrimethylammonium bromide (CTAB) is described. After lysis with alkali, the CTAB was directly titrated into the supernatant for plasmid precipitation, then the coprecipitated pellets were dealt with 3 M KAc and TritonX-114. Quality detection showed that the purified plasmids were free from the contamination of host RNA. In 1 mg purified plasmid, the bacterial genomic DNA, host endotoxin and protein were less than 10 µg/ mg, 50 EU/ mg and 10 µg/mg, respectively. The ratio of OD_{260}/OD_{280} was between 1.75 - 1.85, more than 90% of the prepared plasmid presented in the supercoiled form. Further test demonstrated that the pcDNAlacZ purified with CTAB and authoritative endotoxin-free plasmid Kit had the similar transfection efficiency *in vivo* and *in vitro*. CTAB can be used for plasmid purification; the main advantages of the DNAs purified with CTAB include the avoidance of animal-derived enzymes, toxic substance like chloroform and phenol. More attractive is that the whole process has the predominance of low cost.

Key words: Plasmid, CTAB, TritonX-114, genomic DNA, RNA, endotoxin, protein, transfection.

INTRODUCTION

In recent years, gene therapy and gene vaccination have developed quickly as new ways for disease prevention and treatment. Large-scale processes to manufacture plasmid DNA of high quality are needed for animal experiments, clinical test and sequential application. Plasmids should be produced reproducibly to meet the stringent quality criteria in terms of purity, safety and efficacy. Taking into account the application of animal DNA vaccine, the cost of purification must be decreased. Although commonly used in laboratory-scale methods for the isolation of plasmid DNA, organic reagents, mutagenic and toxic compounds (phenol) and animal derived enzymes (lysozyme, proteinase K, and RNase A) constitute an additional concern for regulatory agencies

and must be avoided (Prazeres et al., 1999). Another challenge in the purification of plasmid is the elimination of cellular components from the host (*Escherichia coli*) that potentially induce immunological and biological responses. Final plasmid (p) preparations should be free from genomic (g) DNA (<0.05 µg gDNA/µg pDNA), host proteins (undetectable by bicinchoninicacid assay or silver-staining gel), RNA (not seen in 0.8% agarose gel) and endotoxins (<0.1 EU/µg pDNA) (Gustavsson et al., 2004). The presence of host impurities and manufacturing related compounds in the final preparations may lead to dose-related toxicity effects during in vivo transfection studies.

Chromatographic techniques, such as size-exclusion chromatography (Li et al., 2007), anion exchange (Prazeres et al.,1998; Eon-Duval et al., 2004) and hydrophobic interaction chromatography (Diogo et al., 2000) have been mostly used at large scale to obtain final pure preparations of plasmid DNA. Affinity chromatography

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phy and aqueous two-phase extraction system have also been described, but for lab-scale applications (Balan et al., 2003; Darby and Hine, 2005; Trindade et al., 2003). Other non-chromatographic techniques, such as tangential flow filtration, superparamagnetic nanoparticles and zwitterionic detergent have been also reported (Eon-Duval et al., 2003; Chiang et al., 2005; Chowdhury and Toshihiro, 2005).

Considering the current techniques for plasmid purification suffer from the complicated processes, and the cost is too high for animal application. So, it is necessary to establish a suitable large-scale purification technology. In the present study, we selected the CTAB as the reagent to precipitate the supercoiled DNA from the supernatant after alkali lysis, while RNA and most host protein and endotoxin still hold in the supernatant. Followed by the precipitation, the plasmids are released form the pellet complex by 3 M KAc, and the endotoxin is removed with TritonX-114 finally.

MATERIALS AND METHODS

General

Unless stated otherwise, all reagents and chemicals were obtained from commercial sources. CTAB (Amresco) was used to precipitate plasmid selectively, EndoFree Plasmid Mega Kit (QIAGEN) for control plasmid, TritonX-114 for endotoxin removal (Sigma) and gel clot tachypleus amebocyte lysate (chinese, Xia men) for endotoxin quantification, BCA protein assay kit (Pierce) for bacterial protein detection, DIG DNA Labeling and Detection Kit (Roche) and β-Galactosidase Enzyme Assay System With Reporter Lysis Buffer (Promega) for host gDNA and β-Galactosidase Enzyme quantification.

Bacterial strains, cells, plasmids and animals

Except pMDT-18 (Takara), plasmid pcDNAlacZ containing β-galactosidase gene, pCpG1, pCPG2, pCpG3 and pCpG4 with different copies CpG oligonucleotide motifs; pETAII and pETIB with gene of *Actinobacillus pleuropneumoniae* protective antigen, eukaryotic expression vector pcDNK with antikanamycin gene all were constructed and preserved in our laboratory.

Alkali lysis of recombinant *Escherichia coli*

Followed by fermentation, the recombinant *E. coli* bacteria were centrifugated at 6000 g, 10 min for collection. The pellets was suspended with solution I [25 mM Tris-HCl (H8.0), 10 mM EDTA, 50 mM Glucose], then the solution II (200 mM NaOH, 1% SDS) and solution III (3 M KAc, 5 M CH_3COOH) were added as described elsewhere (Sambrook et al., 1989). Fifty minutes later, the residue was removed by centrifugation at 6000 g for 10 min and the supernatant was collected.

Selective precipitation of plasmid DNA

In order to precipitate plasmid, 5% (w/v) CTAB was titrated into the supernatant with agitation at 15 r/min at room temperature to final concentration from 0 to 25% (v/v), in which consecutive mixture were sampled. The samples were analyzed by agarose gel electro-

phoresis, BCA and spectrum. Then the plasmids with different concentrations and molecular weight were precipitated with the assumptive concentration of CTAB.

Selective release of plasmid DNA

Pellets of plasmid DNA coprecipitated with CTAB were divided into ten equal parts, and the ten aliquots were dissolved by 10 mM TE (pH 8.0), PBS (pH 7.2), DDW, 3 M NaCl, 5 M NaCl, 1 M NaAc, 3 M NaAc, 0.5 M NH_4Ac, 1 M Na_3PO_4 and 3 M KAc, respectively. After centrifugation at 15°C, 6000 g for 10 min, the supernatants were collected for OD_{260} and OD_{280} analysis, and the plasmid DNA recoveries of released plasmid DNA in the solutions were calculated according to the formula (Recovery = plasmid in supernatant / plasmid in CTAB-DNA complex).

Endotoxin removal

The plasmid DNA purification was concentrated and dissolved in 10 mM TE buffer and then 10% TritonX-114 was added to a final concentration of 1, 2 and 3%. After ice incubation for 10 min, the mixture was immediately transfered to 20, 26, 32 and 42°C water bath for another 10 min, afterwards centrifugation was carried out at 10000 g for 5 min at 20°C. And the plasmid presented in the supernatant was collected. The operation procedure may be repeated when needed.

Plasmid identification with restriction digestion

The purified plasmid pcDNAlacZ was digested with *Hin*dIII and *Bam*HI, *Hin*dIII and *Eco*RI, *Hin*dIII and *Xho*I, respectively at 37°C for 2 h, and the products were submitted to 1% agarose gels electophoresis.

RNA detection

Gel electrophoresis was performed on 0.7% agarose gels in TBE Buffer (0.089 M Tris–borate, pH 8.0, 2 mM EDTA), 15 µL of sample (including 2.5 µL of a 50% solution of glycerol in water was loaded in each well and the samples were run at 60 V for 60 min. When the electrophoresis was completed, the agarose gel was stained with ethidium bromide by soaking the gel in 100 mL TBE buffer containing 1.5 g ethidium bromide for 30 min. The agarose gel was analyzed and photographed using the gel documentation software 418 (Tanon, Shang hai, China).

Host protein detection

Protein concentration was measured by the bicinchoninic acid assay. Final protein concentrations in the purified plasmid fractions were determined by the BCA assay. One mL samples were added to 1 mL BCA reagent and incubated for 60 min at 60°C. Absorbance was measured at 562 nm. 0.5 M NaCl, 1 mM EDTA, 25 mM Tris–HCl (pH 8) was used as blank. Also in this case, bovine serum albumin standards were used to construct a calibration curve. Protein concentrations were then calculated by comparing with a calibration curve of bovine serum albumin standards.

Genome DNA detection

For amplification of the 16 s rRNA fragment, specific primers

(Forward primer:5'-cgttacccgcagaagaagca-3' and Reverse primer:5'-catctgacttaacaaaccgcctg-3') was used, PCR was performed with 30 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 30 s and extension at 72°C for 30 s using a DNA thermal cycler [Applied Biosystems Inc. (ABI), CA, USA]. The fragments were recovered with gel recovery kit (Takara) and labeled with nucleic acid probe according to the direction of DIG DNA Labeling and Detection Kit (Roche).

The gDNA of *E. coli* DH5α (75, 37.5, 18.7, 9.4 and 4.7 ng, respectively) was prepared with Genomic DNA Extraction Kit (OMEGA) and the purified plasmid DNA (0.5 and 1 μg) were digested overnight with *Eco*RI. Following digestion, the products were electrophoresised in a 1.0% Tris acetate-EDTA-agarose gel at 80 V for 80 min and then the products were transferred to the positive charged nylon membrane by siphonage. After UV-Crosslinking (330 nm/2 min), hybridization of nucleic acid was performed according to the direction of DIG DNA Labeling and Detection Kit. The condition of prehybridization (prehybridization solution containing 50% formylamine), hybridization (hybridization solution containing 50% formylamine) and ablution were 42°C for 4 h, 42°C for 6 h and 65°C for 15 min the color reaction was used with NBT/BCIP.

Endotoxin testing and safety assay

Standard endotoxin sample and the purified plasmid DNA were diluted with free pyrogen deionized water into 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.006 EU/μL and 1000, 500, 250, 125 and 62.5 ng/μL respectively. Dilution was repeated twice each sample. Then tachypleus amebocyte lysate was added into 100 μL aliquot of endotoxin standards or plasmid samples for 1 h incubation at 37°C. The results were judged according to the formation and fastness of the gels. The actual endotoxin content was calculated compared to the controls.

Transfection

COS-7, MDCK and Marc-145 cells were cultured in the DMEM supplemented with 10% fetal bovine serum close to confluence cells in 24-well microplate. The purified pcDNAlacZ by established technology and Kit as well as the conventional method were coated with polyetherimide (PEI, 25 kDa, Sigma), and then each 200 μL PEI-DNA mixers were added into wells for incubation at 37°C, 3 h later, the medium was replaced with complete medium supplemented with 10% calf serum. Forty eight hours later, the positive cells were detected by in situ β-galactosidase staining kit (beyotime, China) and the transfection efficiency was calculated.

Mice were randomly divided into 5 groups, 15 mice per group. The purified plasmid pcDNAlacZ were coated with PEI (25 kDa, Sigma). The first group was administrated with PBS as placebo, the second group, each mice was injected with pcDNA3.0 at a dose of 20 μg, while in group III, IV and V, each mice was immunized with 20 μg pcDNAlacZ purified by the established technology, Kit and conventional method. Three mice were killed every 4 days interval. Tissues of heart, liver, spleen, lung and kidney from the mice were collected and preserved in -20°C. The tissues were ground and then dissolved into 1×RLB solution. After centrifugation, the supernatant was collected.

The concentration of protein was tested by bicinchonininc acid Kit which was obtained from Pierce (Rockford, IL, USA) and been adjusted to 5.0 μg/mL with 1×RLB solution. Followed by this, the β-Galactosidase Enzyme activity in each tissue were detected according to the direction of β-Galactosidase Enzyme Assay System With Reporter Lysis Buffer (Promega), and the average value were obtained.
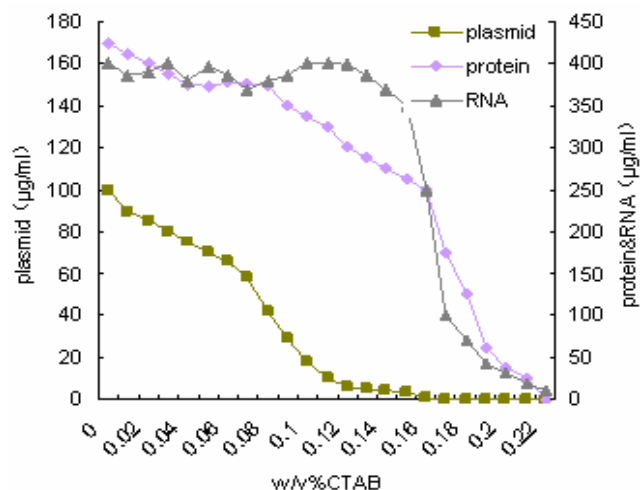

Figure 1. The selective precipitatioin of plasmid by CTAB.

RESULTS

Selective precipitation of plasmid DNA

After fermentation, the *E. coli* containing the pcDNAlacZ were collected for alkali lysis, and CTAB was titrated into the collected supernatant to a final concentration of 0 - 0.25%. After incubation for 10 min at room temperature, the admixture was centrifugated at 6000 g, 15°C for 10 min, the residual supernatant was detected for plasmid, host RNA and protein by spectrophotometry and gel electrophorosis. As shown in Figure 1, when the final CTAB concentration reached to 0.18%, all of the plasmid was precipitated. Meanwhile, total host RNA was still in the supernatant and approximately 28% of the host protein was co-precipitated with the plasmid.

In order to validate whether the final CTAB concentration does work for different size and concentration of plasmid, 0.18% CTAB was added into the supernatant of each recombinant bacteria which contained the pCpG1, pCPG2, pCpG3, pCpG4, pMDT, pETAII and pcDNK preserved in our laboratory. With the same operation, all the plasmids were total precipitated, while host RNA still retained in the supernatant, which indicated that the plasmid could be selectively precipitated effectively by CTAB at the final concentration of 0.18%. Furthermore, more than 90% of the precipitated plasmid DNA was presented as supercoiled form, and no contamination of RNA and host genome DNA were observed (Figure 2).

Selective release of plasmid DNA

The coprecipitated plasmids were dissolved with 10 mM TE (pH 8.0), PBS (pH 7.2), DDW, 3 M NaCl, 5 M NaCl, 1 M NaAc, 3 M NaAc, 0.5 M NH_4Ac, 1 M Na_3PO_4 and 3 M KAc, respectively. Then the solutions were centrifugated
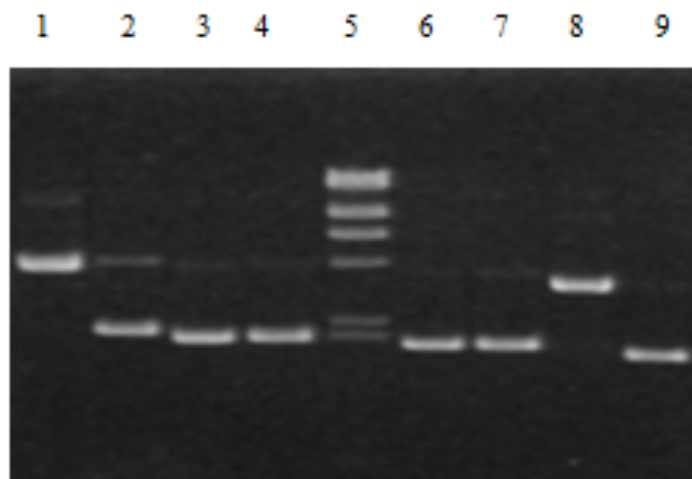
Figure 2. The electrophoresis of the different plasmid purified by CTAB.
Lane 1 = pcDNA*lacZ*, 2 = pCpG1, 3 = pCPG2, 4 = pCpG3, 5 = λ DNA
*Hin*d III Markers, 6 = pCpG4, 7 = pMDT, 8 = pETAII, and 9 = pcDNK.
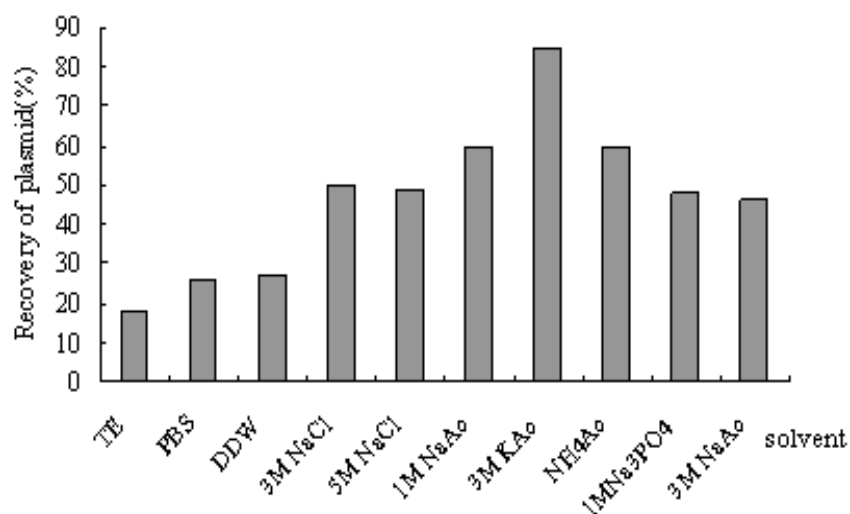
Figure 3. The plasmid recovery after the precipitated plasmids dissolved with
different solvents.

and the supernatants were collected for quantification by spectrophotometry. We could see from Figure 3, the recovery rate of 3 M KAc is as high as 85%, while the other solvent recovery rate is relatively low.

Digestion of the purified plasmids for identification

The purified plasmid pcDNAlacZ was digested with *Hin*dIII and *Bam*HI, *Hin*dIII and *Eco*RI, *Hin*dIII and *Xho*I, respectively. The results of agarose gel electrophoresis suggested that all pcDNAlacZ were correctly digested. Because of *Eco*RI site, pcDNAlacZ was digested into four fragments by *Hin*dIII and *Eco*RI but two by other diges-

tions (Figure 4). Restriction digestion is an effective tool for quality verification of purified plasmid, with the aim of checking whether any unknown changes have taken place of the plasmids. In the present study, we choose pcDNAlacZ for the goal to verify that the plasmid precipitated by CTAB is feasible. The digestion results have proved that the CTAB will not have any impact on the precipitated plasmids.

RNA detection of purified plasmids

The supernatant with and without CTAB precipitation were collected for agarose gel electrophoresis, as can be
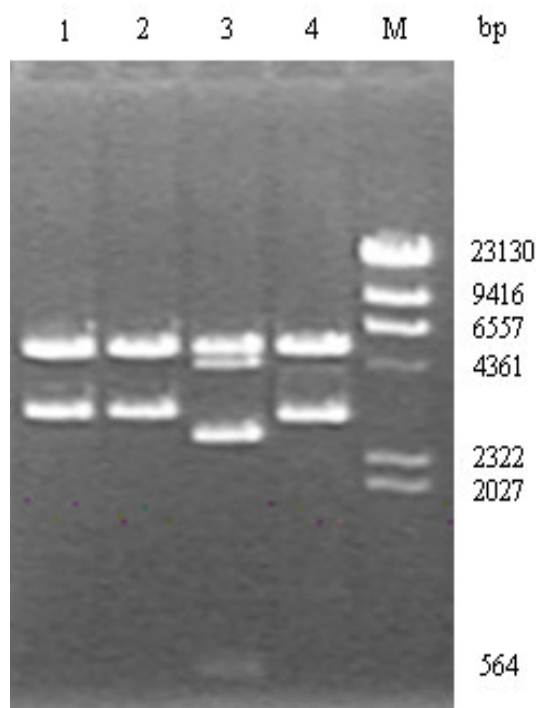
Figure 4. The restriction map of CTAB-purified pcDNAlacZ. Lane 1 = enzyme digestion by *Hind*III and Apa I, 2 = enzyme digestion by *Hind*III and *Xho*I, 3 = enzyme digestion by Hind III and *Eco*RI, 4 = enzyme digestion by Hind III and *Bam*HI, and M = λDNA Hind III Markers.
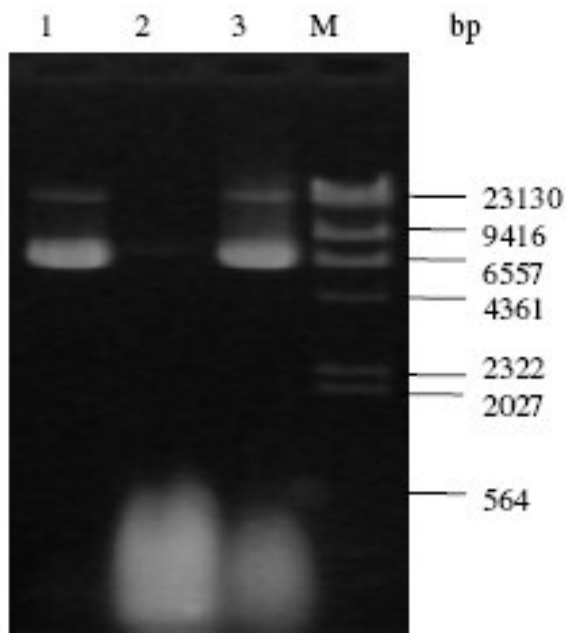
Figure 5. RNA detection by gel electrophoresis. Lane 1 = The pcDNAlacZ precipitated with CTAB, 2 = the supernant after CTAB precipitation, 3 = the plasmid pcDNAlacZ without CTAB precipitation, and M = λ*Hin*dIII DNA Markers.

seen, after CTAB precipitation, all the bacterial RNA are all still retain in the supernatant, while almost all of the plasmid were precipitated, compared with the plasmid before precipitation, the bands shape, size and concentration of the target plasmid did not change (Figure 5).

Host protein detection of purified plasmids

The plasmid pcDNAlacZ purified by CTAB and 3 M KAc was diluted by Sterile DDW at different concertrations for host protein quantification, and the results demonstrated that less than 1 ng host protein was detected in 1 µg plasmid, the residual host protein is very close to the required standards stipulated by WHO (Robertson and Griffiths, 1998).

Bacterial genome detection of purified plasmid

The remnant bacterial genomic DNA in the purified plasmid was detected by Southern blotting with a digoxin labeled 16srRNA fragment as probe, as can be seen from Figure 6, the detection sensitivity of the probe for host gDNA is 10 ng, which just to satisfy the minimum requirement of purified plasmid, and less than 10 ng gDNA were detected in 1 µg purified plasmid, which meet the criterion stipulated by WHO (Robertson and Griffiths, 1998).

Endotoxin detection

The plasmid DNA was detected according to the direction of gel clot tachypleus amebocyte lysate, the results suggested that 1% TritonX-114 would remove the endotoxin efficiently at 42°C (Table 1). Meanwhile, the plasmid recovery rate was more than 98%, and only 50 EU endotoxin was detected in 1 mg plasmid.

Transfection *in vitro*

The pcDNAlacZ were coated with PEI (25 kDa) and transfected into COS-7, MDCK and Marc-145 cell lines respectively. Forty eight hours later, the positive cells were counted for calculating the transfection efficiency, as can be shown from Figure 7, no significant differences of the transfection efficiency were found between the plasmid purified by the established technology (A, 35, 36 and 15%) and endotoxin-free plasmid kit (B, 40, 38 and 16%), but the efficiency were higher than the plasmid prepared by conventional method (C, 15, 16 and 8%). The results illustrate that the plasmid purified by the established technology has the same effect as the authoritative plasmid purification kit.
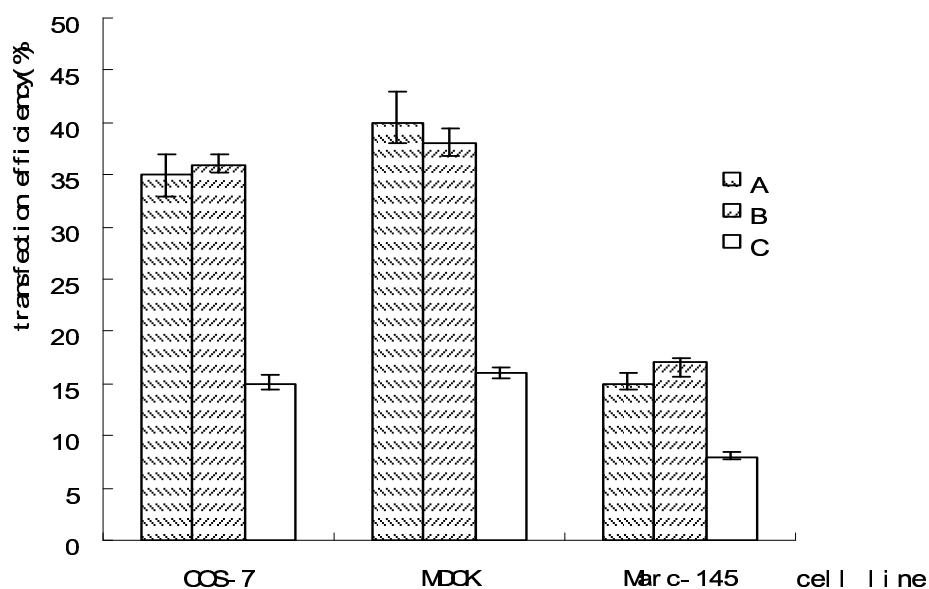
Transfection *in vivo*

Three mice were killed in each group on 2^{nd}d, 4^{th}d, 6^{th}d,

Figure 6. Detection of the host genomic DNA in the purified plasmid by Southern blotting. Lanes 1 – 5 = 75, 37.5, 19, 10 and 5 ng DH5α gDNA, respectively, 6 = 0.5 µg purified plasmid DNA, 7 = 1 µg purified plasmid DNA.

Table 1. Endotoxin detection by limulus amoebocyte lysate.

| TritonX-114 (%)	1				2				3			
Temperature (℃)	20	26	32	42	20	26	32	42	20	26	32	42
Endotoxin (EU/mg)	100	75	125	50	150	125	150	75	175	150	75	75
Plasmid recovery (%)	80	85	78	98	68	75	80	86	70	65	75	80

Figure 7. The transfection efficiency of pcDNAlacZ in cell lines.

8th d and 10th d after they were injected with pcDNAlacZ. The tissues of heart, liver, spleen, lung and kidney were ground and dissolved into the schizolysis buffer of report gene. The concentration of protein was quantified and adjusted to 5.0 µg/mL. The activity of β-galactosidase enzyme in each tissue was detected, and the results showed that the enzyme activity was only detected in heart, spleen and lung tissues. The activities of A group was 54.54, 56.72 and 30.8 mU/mL, respectively. No signi-ficant differences were observed between A and B. But the enzyme expression was higher compared to C (Figure 8). β-galactosidase enzyme activity was detected on the different time after mice were injected with recom-

binant plasmid. The utmost expression was detected on the 4th day and the recombinant enzyme activity reduced significantly. Ten days later, enzyme activity could not be detected (data not shown). This result met the rule of recombinant DNA expression *in vivo*.

DISCUSSION

With the rapid growth of genetic engineering technology, gene therapy and DNA vaccination become increasingly popular as novel ways of dealing with infectious diseases. However, the recombinant plasmids used for gene therapy and DNA vaccination require high quality and the
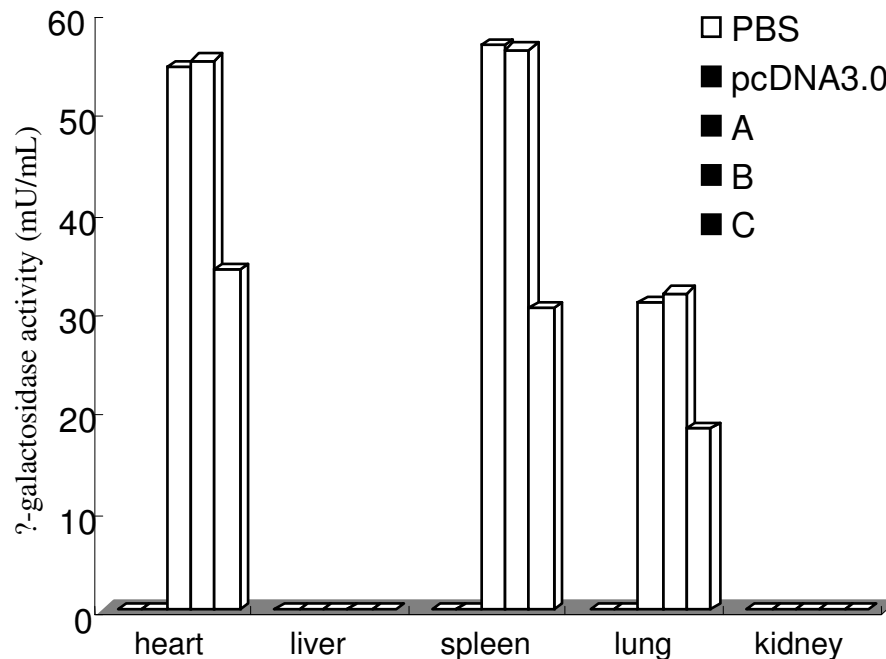
Figure 8. β-Galactosidase activity in different tissues of mice injected with pcDNALacZ purified with different methods.

process which contaminated with animal-derived enzymes, phenol and chloroform must be avoided. Therefore, large-scale plasmid purification becomes the bottleneck of gene therapy and DNA vaccination.

The aim of the plasmid purification is to remove the bacterial RNA, endotoxin, host protein and genomic DNA, etc. In the present study, the RNA removal mainly depend on the tangential flow filtration (TFF) (Eon-Duval et al., 2003) or salt such as $MnCl_2$ and $CaCl_2$ (Wahlund et al., 2004; Shenoy et al., 2003), host genomic DNA removal by nitrocellulose has been described (Levy et al., 2000), and host protein been removed by hydrophobic interaction chromatography can be seen elsewhere (Diogo et al., 2000). Because of the similar charge and molecular weight with plasmid, the endotoxin is relative difficult to separate from the plasmid (Anspach, 2001). Now, methods such as chromatography had good prospects in plasmid purification, which is based on the negative charge and hydrophobicity of plasmids. The shortcomings are the high cost and tedious process. Although the idea of plasmid purification by TFF is very good, the plasmid mainly presented in negative super-coiled form, and its actual molecular weight can not be reflected, so the purification result is not satisfying (Eon-Duval et al., 2003).

In this study, according to the interaction of CTAB with plasmid at low ion concentration, followed by alkali lysis CTAB was directly titrated into the supernatant. An easy and feasible technology for plasmid purification was established with the optimized proportion between the quantity of CTAB and plasmid, combined with 3 M KAc

for DNA release and TritonX-114 for endotoxin removal. The plasmids purified by this technology have characteristics of high-purity, agarose gel electroelectrophoresis detection showed that the purified plasmid was free the contamination of host RNA (Figure 5). More than eighty percent of the prepared plasmids were presented in the supercoiled form (Figure 2), and the host protein, genomic DNA and endotoxin were less than 1, 10 µg and 50 EU in 1 mg plasmids (Figure 6, Table 1). Compared to the conventional large-scale plasmid purification, this technology for plasmid prepara-tion are free of animal-derived enzymes, phenol, chloro-form, the expensive chromatographic column and filtra-tion membrane.

Cell transfection assay is the conventional method to detect the quality of recombinant DNA. To verify the plasmid quality purified with this technology, the COS-7, MDCK and Marc-145 cell lines were transfected with pcDNAlacZ purified in this study and EndoFree Plasmid Mega Kit as well as conventional method, the results demonstrated that the transfection efficiency of the pcDNAlacZ purified by the established technology and Kit are almost similar, higher than the conventional method. Further *in vivo* experiment has showed that the pcDNAlacZ purified by established technology and kit obtained relative high level expression but by conven-tional method (Figure 8).

It is worth mentioning that this technology has the predominance of simple operation, low cost and safety, which can satisfy the demand of DNA vaccine, particularly the animal-used DNA vaccine for the economic cost.

ACKNOWLEDGEMENTS

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