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ADDRESSING A MANUFACTURING BOTTLENECK IN CAR-T THERAPY: SCALABLE PRODUCTION OF TRANSFECTION-GRADE PLASMID DNA FOR CAR-T VECTOR MANUFACTURING

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ABSTRACT

The rapid advancing of cell and gene therapy has increased the demand for plasmid DNA used in viral vector manufacturing. The production of transfection-grade plasmid DNA remains a critical bottleneck, particularly for academic and emerging manufacturing facilities, due to the high cost and limited scalability of existing purification technologies. The aim of this study was to develop a practical and cost-effective approach for producing transfection-grade plasmid DNA suitable for lentiviral vector manufacturing.

The described acidic phenol extraction method was evaluated using plasmids commonly employed in lentiviral packaging systems and compared with a reference method, CsCl density gradient ultracentrifugation. The resulting plasmid preparations showed efficient removal of genomic DNA and other impurities, including endotoxin – the acidic phenol method consistently reduced endotoxin levels to 1.4–3.8 EU/μg DNA. Moreover, the purified plasmid DNA was predominantly in the supercoiled (covalently closed circular) form. Functional validation in transfection assays confirmed that plasmids purified by the proposed method supported lentiviral vector production with efficiency comparable to the reference method, yielding titers exceeding 5×10^6 TU/mL, with no statistically significant differences in transduction efficiency between the groups.

These findings confirm that the acidic phenol extraction method is a simple, scalable, and economical approach for isolating transfection-grade plasmid DNA for lentiviral vector production. The method represents a practical alternative to ultracentrifugation-based purification and may facilitate broader use of cell and gene therapy by reducing dependence on specialized equipment and costly commercial kits.

Keywords: plasmid DNA, lentiviral vector, CAR-T therapy, gene therapy, transfection-grade DNA, plasmid purification, cell and gene therapy, bioprocessing.

INTRODUCTION

The advent of gene therapy, DNA vaccination, and particularly Chimeric Antigen Receptor T-cell (CAR-T) therapy has created a critical need for the large-scale supply of high-quality plasmid DNA (pDNA) derived from *Escherichia coli* [1, 2]. These plasmids are used directly as gene delivery tools in DNA vaccination and, more critically, as essential intermediate materials for the packaging of viral vectors – the indispensable components of cell and gene therapy (CGT) [3]. Lentiviruses (LV) and adeno-associated viruses (AAV), the primary delivery vehicles for these advanced therapies, depend on high-quality pDNA for their production [3].

LV production is most commonly achieved both in industry and academia using transient plasmid transfection of suitable packaging cell cultures with a mixture of plasmid DNAs containing the transfer vector, which encodes the viral genome, and helper plasmids that express the various components of the packaging system [4, 5]. Plasmid transfection currently is the preferred method because it offers flexibility, speed, and simplicity: different plasmid combinations can be readily adjusted to produce diverse vector constructs without the need to generate stable producer cell lines, which are time-consuming and labor-intensive to establish. This transient approach is particularly advantageous for research settings and academic CAR-T manufacturing, where multiple vector designs may need to be tested and optimized.

Regulatory guidelines mandate stringent purity criteria for

plasmid DNA intended for therapeutic use, requiring pDNA to be predominantly in the covalently closed circular form (>90% cccDNA), with minimal contamination by RNA, protein, bacterial genomic DNA (gDNA), and endotoxin [6, 7]. Endotoxin (lipopolysaccharide from the outer membrane of Gram-negative bacteria) is a pyrogen that can trigger severe inflammatory responses, and its removal is a significant hurdle during purification of GMP-grade plasmids. For clinical applications, acceptable levels are often below 0.1 endotoxin units (EU)/μg of pDNA [8].

Laboratory-scale pDNA purifications are frequently performed using commercial kits due to their simplicity and convenience for the end-user. However, these high-quality purification kits are typically expensive and offer limited scalability, which restricts their application in industrial settings [9, 10].

Industrial pDNA purification processes rely primarily on chromatographic methods [11, 12]. The most commonly used techniques are anion exchange chromatography (AEC) and hydrophobic interaction chromatography (HIC), followed by size exclusion chromatography (SEC), which is commonly employed as a polishing step. AEC effectively removes proteins, low-molecular-weight RNA, and endotoxins, while HIC separates the supercoiled pDNA isoform from open-circular isoforms and from more hydrophobic impurities such as endotoxins and denatured DNA. More recently, thiophilic aromatic adsorption chromatography (e.g., Cytiva's Plasmid-Select Xtra) has emerged as a generic method for purifying

supercoiled covalently closed circular (ccc) pDNA. A well-established industrial process, described by Urthaler et al. [3], employs three chromatographic steps (HIC, AEC, and SEC) followed by tangential flow filtration (TFF) for concentration. Such elaborate multi-step industrial methods produce pDNA with up to 98% cccDNA content, undetectable RNA, protein content below 1 µg/mg pDNA, and endotoxin levels below 0.1 EU/mg pDNA. Novel approaches, such as multimodal chromatography [13], seek to simplify the production line.

The purification step remains the costliest operation during pDNA production, accounting for up to 80% of total manufacturing costs [11]. As plasmid production itself constitutes a significant bottleneck to the global accessibility of CGT products, this has further intensified the demand for more efficient, scalable, and cost-effective pDNA purification methods [14].

While industrial-scale production depends on sophisticated chromatography, academic laboratories have two practical alternatives to commercial kits for isolating transfection-grade pDNA.

The first approach is CsCl density gradient ultracentrifugation, long considered the «gold standard» for isolating ultra-pure plasmid DNA [1, 15]. This method exploits the differential buoyant density of DNA molecules in the presence of an intercalating dye, typically ethidium bromide, which binds to DNA and reduces its density proportionally to its degree of supercoiling [16]. Under ultracentrifugation, covalently closed circular (ccc) plasmid DNA bands at a higher density than linear or nicked forms, enabling efficient separation from chromosomal DNA and other contaminants [17]. First described by Radloff et al. in 1967 [16], CsCl gradient centrifugation has been the classical preparative method for decades and remains widely used in molecular biology. However, this technique requires specialized equipment, such as an ultracentrifuge with a selection of rotors [1, 18].

The second approach, and the focus of this study, is based on extraction of the majority of impurities with buffered (acidic) phenol. This method requires no expensive equipment and offers a simpler, faster, and more economical alternative. The foundation of this protocol, as described by Zasl-off et al. (1978) [19] and subsequently refined by Fisher et al. (1991) [20] and Wang and Rossman (1994) [21], exploits the differential protonation and surface charge of cccDNA versus linear or nicked DNA at controlled pH (4.0–5.5). Specifically, cccDNA molecules with high surface charge remain in the aqueous phase, whereas protonated linear or nicked DNA molecules are extracted into the organic phase. An additional advantage of this procedure is its capacity to efficiently extract endotoxin under the same acidic conditions.

Endotoxin (lipopolysaccharide, LPS) is detrimental to transfection and is a common impurity extracted from bacterial cell walls during pDNA isolation. It is difficult to remove using standard precipitation or adsorptive chromatography because LPS is negatively charged and amphipathic, forming high-molecular-weight micelles that mimic DNA molecules in their physicochemical properties. However, at pH 4.0–5.5, the phosphate groups of LPS become protonated, neutralizing its negative charge and promoting its partitioning into the organic phase during extraction with buffered phenol [22].

The aim of this study was to develop a scalable and

cost-effective method for isolating transfection-grade pDNA that efficiently removes residual genomic DNA and endotoxin while serving as a practical alternative to CsCl gradients. We systematically compared this novel protocol with a reference method, CsCl gradient ultracentrifugation, evaluating yield, purity, endotoxin content (using a chromogenic LAL assay), and functional performance in lentiviral vector packaging transfections.

MATERIALS AND METHODS

Bacterial strain, cells and media

E.coli DH5a strain was used for plasmid propagation and large-scale plasmid isolation. Bacterial cells were grown in Terrific Broth (TB) medium (Sigma Cat. T0918).

Adherent HEK293FT cells (Thermo Fisher Scientific Cat. R70007, Waltham, MA, USA) were used for lentiviral vector packaging and functional titer determination. Cells were cultured in complete DMEM (high glucose; Gibco Cat. 11965092) supplemented with 10% fetal bovine serum (FBS; Gibco Cat. 10091148, Grand Island, NY, USA), 1% penicillin-streptomycin, 1% non-essential amino acids (NEAA), and 2 mM L-glutamine (all from Gibco). Cells were maintained at 37 °C in a 5% CO₂ atmosphere and passaged upon reaching 80–90% confluence.

Plasmids

The following plasmids were used in this study. The lentiviral transfer vector LV/CAR-GFP, encoding a chimeric antigen receptor (CAR) against the human cell surface marker CD19 fused to GFP via an uncleavable linker, was described previously. For lentiviral particle packaging, the second-generation packaging plasmid psPAX2 (Addgene Cat. 12260) and the VSV-G envelope-expressing plasmid pMD2.G (Addgene Cat. 12259) were used.

Biomass production for plasmid DNA isolation

Transformed bacterial cells were grown in TB medium supplemented with appropriate antibiotics (ampicillin or kanamycin, 50 µg/mL). Starter cultures were prepared by inoculating 5 mL of TB medium with a single colony and incubating for 12–16 h at 37 °C with shaking at 150 rpm. For biomass production, 500 mL of TB medium in 2-L Erlenmeyer flasks were inoculated with 5 mL of the overnight starter culture and incubated under identical conditions. Multiple culture batches grown for the same plasmid were pooled, mixed, and divided into equal volumes in centrifuge bottles. Bacterial biomass was collected by centrifugation at 6,000 rpm (JA-10 rotor, Avanti J26S XP centrifuge; Beckman Coulter) for 10 min at 4 °C. The supernatant was discarded, and the resulting cell pellets of equal weight were used for plasmid DNA isolation using different purification protocols.

Plasmid DNA isolation by CsCl density gradient ultracentrifugation

The bacterial pellet was resuspended in buffer BF1 (0.025 M Tris-HCl, 0.1 M NaCl, 0.01 M EDTA, pH 7.5) to approximately 30% (w/v), and the volume was adjusted to 10 mL with the same buffer. Then 20 mL of buffer BF2 (0.2 M NaOH, 1% SDS) was added, and the mixture was mixed until the lysate became clear and homogeneous, indicating cell lysis. The lysate was incubated on ice for 10 min. Next, 15 mL of ice-cold buffer BF3 (3 M potassium acetate, pH 5.5) was

added, mixed, and incubated on ice for 15 min. Insoluble debris was pelleted by centrifugation at 6,000 rpm (JA-10 rotor, Avanti J26S XP centrifuge, Beckman Coulter) for 10 min at 4 °C. The supernatant was transferred to 50-mL tubes, avoiding the pellet. An equal volume of isopropanol was added, mixed, and kept at –20 °C for 15 min to precipitate nucleic acids. The precipitate was collected by centrifugation at 4,000 rpm (A-4-81 rotor, Eppendorf 5810R centrifuge) for 10 min at 4 °C. The supernatant was discarded, and the pellet was dissolved in 2 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). An equal volume of 5 M lithium chloride (LiCl) solution in water was added, vortexed, and left on ice for 30 min to precipitate high-molecular-weight RNA. The RNA was removed by centrifugation at 4,000 rpm for 10 min at 4 °C (same rotor and centrifuge). DNA was again precipitated with an equal volume of isopropanol. After pelleting, the supernatant was removed, and the pellet was dissolved in 1 mL of TE buffer. This crude plasmid preparation was used for further purification by banding in cesium chloride (CsCl) gradients.

To prepare a gradient for ultracentrifugation, 4.3 g of dry CsCl was weighed in a tared 15 mL tube, then 1 mL of TE buffer was added, followed by the plasmid DNA solution (1 mL in TE) and 40 µL of ethidium bromide solution (10 mg/mL). The CsCl was dissolved completely by gentle mixing, and TE buffer was added to bring the total weight to 8.1 g. The solution was transferred to 4.9-mL OptiSeal tubes (Beckman, Cat. 362185). The tubes were sealed, placed in an NVT90 rotor (Beckman), the rotor was packed according to the manufacturer's instructions, and the tubes were centrifuged at 65,000 rpm for 14–18 h at 22 °C using an Optima XPN-90 ultracentrifuge (Beckman Coulter). After centrifugation, the lower band (covalently closed circular plasmid DNA) was collected by puncturing the tube with a needle and aspirating with a syringe. The plasmid solution was transferred to a 15 mL tube, mixed with 1 mL of TE buffer and 4.5 mL of 96% ethanol, and kept at –20 °C for 15 min to precipitate the DNA. The DNA was pelleted by centrifugation at 4,000 rpm (A-4-81 rotor, Eppendorf 5810R centrifuge) for 10 min at 4 °C. The supernatant was discarded, and the pellet was dissolved in 0.4 mL of TE buffer and transferred to a 2-mL microcentrifuge tube. To remove ethidium bromide, 0.4 mL of phenol-chloroform (1:1 mixture; Sigma Cat. 77617) was added, vortexed vigorously, and centrifuged for 10 min at 10,000 rpm. The upper aqueous phase was transferred to a fresh microcentrifuge tube. Then, 0.1 volume of 5 M NaCl and an equal volume of isopropanol were added, mixed, and kept at –20 °C for 15 min. The DNA was pelleted by centrifugation. The pellet was washed with 0.5 mL of 70% ethanol, followed by removal of the liquid and air-drying. Finally, the purified plasmid DNA pellet was dissolved in TE buffer. DNA concentration and purity were assessed by measuring absorbance at 260 nm (A_{260}) and the A_{260}/A_{280} ratio, and the concentration was adjusted to 1 mg/mL. Plasmid integrity and topology were verified by agarose gel electrophoresis of undigested DNA and by restriction analysis with selected endonucleases.

Preparation of buffered phenol (pH 5.5)

Crystallized phenol was distilled using a standard distillation apparatus. All further equilibration procedures were performed at room temperature. The colorless phenol crystals

were saturated with water by mixing with an equal volume of MQ water, followed by vigorous shaking. The mixture was allowed to stand until complete phase separation occurred, upon which the upper aqueous phase was removed. An equal volume of equilibration buffer (50 mM sodium acetate, 75 mM NaCl, pH 5.5) was added to the phenol phase, and the mixture was shaken to ensure thorough mixing. The mixture was again allowed to stand for phase separation. The aqueous phase was then removed, and its pH was measured. This equilibration step was repeated with fresh buffer until the pH of the aqueous phase reached 5.5 ± 0.1 . The resulting buffered phenol was aliquoted into sterile tubes, overlaid with a layer of the same buffer (50 mM sodium acetate, 75 mM NaCl, pH 5.5), and stored at –20 °C until use.

Plasmid DNA isolation by modified alkaline lysis followed by acidic phenol extraction

The bacterial pellet was resuspended in buffer E (40 mM Tris-acetate, 2 mM EDTA, pH 7.9) to approximately 30% (w/v), and the volume was adjusted to 10 mL with the same buffer. Then 20 mL of alkaline SDS buffer (3% SDS, 50 mM Tris, pH 12.6) was added, and the mixture was mixed until homogeneous. The tube was placed in a water bath at 55 °C for 1 h to denature chromosomal DNA. After cooling on ice for 5 min, 15 mL of ice-cold 3 M potassium acetate (pH 5.5) was added, the tube was inverted 10 times to ensure complete mixing of the contents, and incubated on ice for 30 min to precipitate SDS-protein complexes and denatured chromosomal DNA. Subsequently, 5 mL of chloroform was added, the mixture was vigorously shaken, and centrifuged at 6,000 rpm (JA-10 rotor, Avanti J26S XP centrifuge, Beckman Coulter) for 10 min at 4 °C. The aqueous phase was transferred to a fresh tube, and nucleic acids were precipitated with an equal volume of isopropanol upon incubation at –20 °C for 15 min. The crude DNA pellet was collected by centrifugation (4,000×g, 10 min, 4 °C), dissolved in 2 mL of TE buffer, treated with an equal volume of 5 M LiCl on ice for 30 min to precipitate residual high-molecular-weight RNA, and centrifuged (4,000×g, 15 min, 4 °C). The supernatant containing plasmid DNA was transferred to a new tube, precipitated with isopropanol in the presence of 0.1 volume of 5 M NaCl, and the pellet was dissolved in 1 mL of TE buffer. To further degrade residual RNA, RNase A (Sigma Cat. R4875; 10 µg/mL solution in TE) was added, and the mixture was incubated at 37 °C for 30 min.

To remove endotoxin and residual fragments of linear genomic DNA or relaxed (nicked) plasmid DNA, the plasmid solution after RNase treatment was first precipitated with 0.1 volume of 5 M NaCl and an equal volume of isopropanol. The resulting pellet was dissolved in 2 mL of acidic extraction buffer (50 mM sodium acetate, 75 mM NaCl, pH 4.0). This mixture was distributed in 1-mL portions into microcentrifuge tubes. To each tube, 1 mL of water-saturated phenol equilibrated with a buffer (50 mM sodium acetate, 75 mM NaCl, pH 5.5) was added. The tube was vortexed vigorously for at least 1 min to ensure complete mixing and the formation of a fine emulsion, then centrifuged at 10,000×g for 10 min at room temperature. After centrifugation, three distinct phases formed: a lower organic (phenol) phase, a white interphase layer containing endotoxin, denatured proteins, and extracted linear or nicked DNA, and an upper aqueous phase contain-

ing supercoiled plasmid DNA.

The upper aqueous phase was transferred to a new tube, avoiding the interphase. The described procedure of acidic phenol extraction was repeated two more times, each time using 400 µL of fresh phenol equilibrated to pH 5.5. A final extraction was performed using 500 µL of chloroform per tube. Plasmid DNA was then precipitated using 5 M NaCl and isopropanol. The resulting pellets were washed with 500 µL of 70% ethanol, air-dried, and dissolved in 500 µL of TE buffer.

Preparation of nucleic acid impurities extracted into the organic phase for electrophoretic analysis

To monitor the efficiency of nucleic acid contaminant removal, the organic phases remaining after the three rounds of acidic phenol extraction and after the chloroform extraction, were retained. Each organic phase was mixed with an equal volume of TE buffer, vortexed, and adjusted to pH 7.0–7.5 by adding 1 M Tris (pH 12.4). After centrifugation at $10,000 \times g$ for 10 min at room temperature, the upper aqueous phase was transferred to a fresh tube. Nucleic acids were precipitated with 0.1 volume of 5 M NaCl and an equal volume of isopropanol, pelleted by centrifugation ($10,000 \times g$, 10 min, 4 °C), washed with 70% ethanol, air-dried, and dissolved in 30 µL of water. Concentrations were adjusted to 1 mg/mL. One microliter of each sample was loaded onto a 1% agarose gel, stained with ethidium bromide, and visualized under UV light to assess the extraction of genomic DNA and RNA contaminants at each step.

Analysis of plasmid supercoiled content

To quantify the proportion of covalently closed circular (ccc) plasmid DNA, 5 µg of purified plasmid was resolved on a 1% agarose gel, stained with ethidium bromide, and photographed under UV light. Densitometric analysis of the bands corresponding to different plasmid isoforms was performed using Image Lab software (Bio-Rad).

Endotoxin quantification

The endotoxin (lipopolysaccharide from the outer membrane of *E. coli* cells) content was measured using the Toxin-Sensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Cat. L00350) according to the manufacturer's protocol. The lyophilized LAL reagent was reconstituted with 1.7 mL of supplied water. Plasmid DNA samples (100 µL) were mixed with 100 µL of the LAL reagent and incubated at 37 °C for the time specified on the kit's label. Subsequently, 100 µL of chromogenic substrate was added to each vial and incubated for an additional 6 min at 37 °C. The reaction was stopped by addition of color-stabilizers (500 µL of vials #1, #2, and #3), and absorbance was recorded at 545 nm. An endotoxin standard (0.1–1 EU/mL) was used to generate a calibration curve. Endotoxin concentrations were determined by interpolation from the standard curve and are expressed as EU per µg of plasmid DNA.

Lentiviral vector packaging

HEK293FT cells were seeded in 150 mm dishes (P150) at a density of 1×10^5 cells/cm². For each dish, a total of 10 µg of plasmid DNA (pLV/CAR-GFP, psPAX2, and pMD2.G at a molar ratio of 2:1.5:1) was diluted in 2.5 mL of serum-free DMEM. Separately, 30 µL of a 1 mg/mL polyethylenimine (PEI MAX, 25 kDa linear; Polysciences, Cat. 24765-1) stock solution was diluted in 2.5 mL of serum-free DMEM. The di-

luted polyethylenimine solution was added to the diluted DNA solution and mixed briefly by vortexing. The resulting mixture was incubated for 15–20 min at room temperature (RT) to allow polyplex formation.

Before addition of the transfection mixture, the culture medium was removed, and 5 mL of the polyplex mixture was added immediately to the cell monolayer and distributed evenly. The cells were incubated for 4 h at 37 °C in a 5% CO₂ atmosphere. Subsequently, 20 mL of complete DMEM was added to each dish without removing the transfection mixture, and the cells were incubated for an additional 48 h. The culture supernatant was then collected, clarified by centrifugation at $300 \times g$ for 10 min, filtered through a 0.45 µm PES membrane (Pall Corporation Cat. 515-0157, Port Washington, NY, USA), aliquoted, and stored at –80 °C until use. All transfections were performed in triplicate.

Determination of lentiviral functional titer

Lentiviral functional titers (TU/mL) were determined by transducing HEK293FT cells with serial dilutions, followed by flow cytometric analysis. HEK293FT cells were seeded in 6-well plates at 4×10^5 cells per well and allowed to attach overnight. Serial dilutions of LV preparations were prepared in complete DMEM containing 8 µg/mL polybrene (Sigma Cat. H9268) and added to the cells. After 12 h, the polybrene-containing medium was replaced with standard complete DMEM. Preliminary experiments used dilutions from 1:10 to 1:10,000 to estimate the titer range. Subsequently, titrations were performed with dilutions from 1:10 to 1:1,000 to achieve 1–20% transduced cells in the final well. At 72 h post-transduction, cells were harvested for analysis.

For the LV/CAR-GFP vector, transduction efficiency was assessed by quantifying GFP-positive cells. Cells were detached with trypsin-EDTA, washed with PBS, and resuspended in MACSQuant Running Buffer (RB; Miltenyi Biotec Cat. 130-092-747). For the LV/CAR vector (without GFP), non-permeabilized cells were stained sequentially with biotinylated CD19 CAR Detection Reagent (Miltenyi Biotec Cat. 130-129-550) and anti-biotin-PE antibody (Miltenyi Biotec Cat. 130-113-291). Samples were acquired on a MACSQuant 10 flow cytometer (Miltenyi Biotec). At least 50,000 events were recorded per sample. Data were analyzed using MACSQuantify Software (Miltenyi Biotec). Gates for positive cells were set using naïve HEK293FT cells as a negative control, with <0.1% events in the positive gate. MACSQuant Calibration Beads (Miltenyi Biotec Cat. 130-093-607) were used to monitor gate stability across experiments. The functional titer (TU/mL) was calculated as:

$$\text{TU/mL} = (\% \text{ positive cells} / 100) \times (\text{number of cells at transduction}) \times (\text{dilution factor})$$

Transduction of HEK293FT cells for comparison of lentiviral vector functional activity

To compare the functional activity of lentiviral vectors packaged with CsCl-purified and acidic phenol-purified plasmid DNA, HEK293FT cells were seeded in 6-well plates at 4×10^5 cells per well and transduced with LV/CAR-GFP at a multiplicity of infection (MOI) of 0.5 TU/cell in complete DMEM containing 8 µg/mL polybrene. After 12 h, the polybrene-containing medium was replaced with fresh complete DMEM, and cells were incubated for a further 72 h. The per-

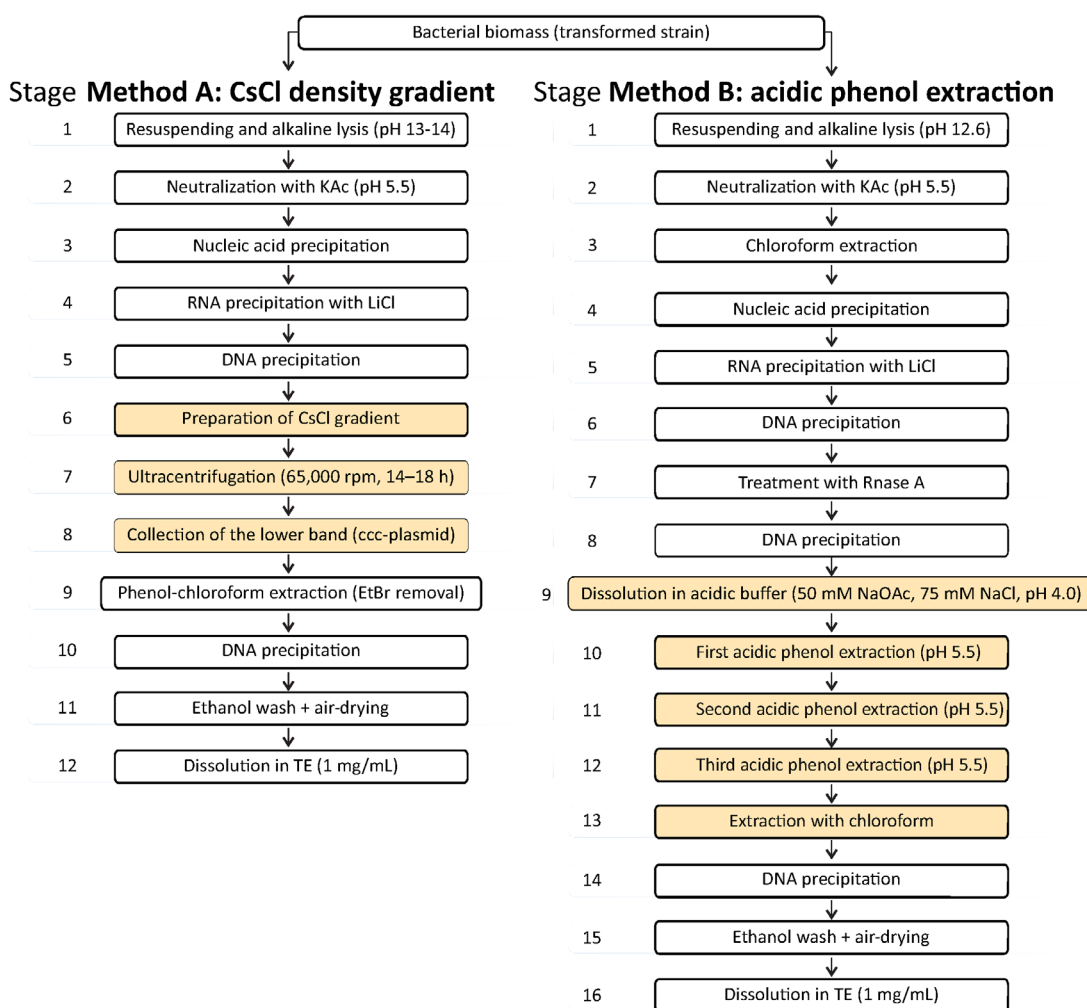


Figure 1. Schematic comparison of the two preparative plasmid DNA isolation methods. Method A (reference) employs CsCl density gradient ultracentrifugation to remove endotoxin, RNA, and genomic DNA, and requires an ultracentrifuge. Method B uses consecutive extractions with acidic phenol (pH 5.5) to remove endotoxin and genomic DNA, requiring no ultracentrifuge or expensive equipment. Quantitative LAL assay showed that Method B reduced endotoxin levels to 1.4–3.8 EU/μg DNA, versus 17.2–25.4 EU/μg DNA for Method A (Table 1). Both methods are scalable to 1–10 mg of plasmid DNA and eliminate the need for commercial purification kits.

centage of GFP-positive cells was then determined by flow cytometry. All transductions were performed in triplicate.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) from three biological replicates. Statistical comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test. All statistical analyses were conducted using GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, CA, USA). Significance levels are denoted as: ns (not significant, $p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$).

RESULTS

Overview of the two purification methods

To compare acidic phenol extraction with classical CsCl gradient ultracentrifugation, we isolated plasmid DNA from parallel bacterial cultures using both protocols. The experimental workflow for both methods is illustrated in Figure 1.

Nucleic acid impurities removal by acidic phenol extraction method

To assess the efficiency of the acidic phenol protocol in removing nucleic acid contaminants, material back-extracted from the organic phases was analysed on 1% agarose gels (Figure 2a). The phenol phase contained high-molecular-weight DNA retained in the wells (lanes 1–6) and a diffuse mid-gel signal (1–3 kb) that intensified with successive extractions (lanes 1–2 to 5–6) and was present in the organic extracts as the major contaminant during chloroform extraction (lanes 7–8). This material likely corresponds to bacterial chromatin fragments complexed with proteins. Its absence from the final plasmid preparations (lanes 9–10) confirmed efficient removal. The final products exhibited a prominent band at ~6 kb, corresponding to pMD2.G, alongside low-molecular-weight species presumably representing residual RNA.

Undigested plasmid samples (lanes 9–10) revealed a clean electrophoretic profile with two distinct bands: a lower, intense band corresponding to the monomeric supercoiled (ccc) form and an upper, weaker band presumably representing the non-covalent dimer (catenane), confirming the high purity of the isolated DNA. Restriction digestion with BamHI (lane 11) and BfuI (lane 12) produced clean fragments of the expected sizes, verifying plasmid integrity.

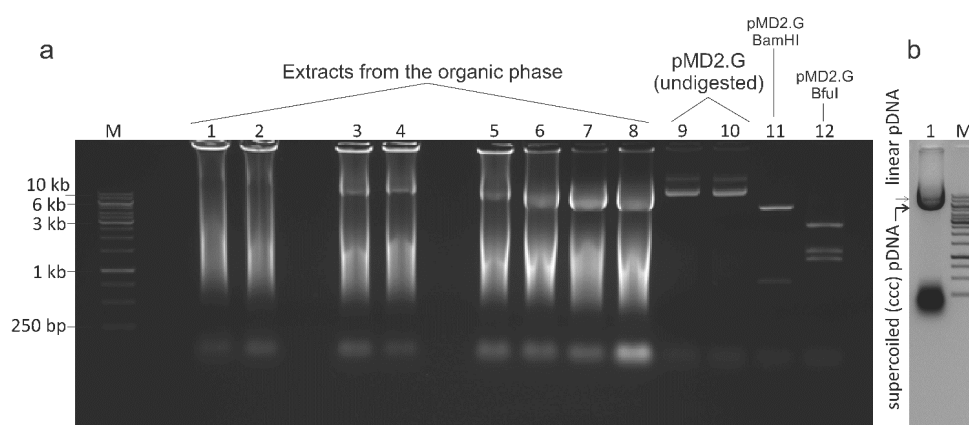


Figure 2. Monitoring of nucleic acid extraction and assessment of supercoiled (ccc) plasmid content. Panel (a): Two independently produced bacterial pellets (Prep #1 and Prep #2) were processed in parallel. Samples collected at each step were resolved on a 1% agarose gel stained with ethidium bromide (lanes 1, 3, 5, 7, 9 – Prep 1; lanes 2, 4, 6, 8, 10 – Prep 2). Lanes 1–2: organic phase after first phenol extraction; lanes 3–4: after second phenol extraction; lanes 5–6: after third phenol extraction; lanes 7–8: after chloroform extraction; lanes 9–10: final pMD2.G preparations. Lane 11: pMD2.G digested with BamHI (expected fragments: 4964, 827, 31 bp); lane 12: digested with BfuI (expected fragments: 2779, 1527, 1318, 198 bp). High-molecular-weight DNA retained in the wells (lanes 1–6) confirms efficient removal of genomic DNA into the organic phase; a diffuse lower band corresponds to residual RNA. After chloroform extraction (lanes 7–8), minimal non-plasmid DNA remains. Final undigested plasmids (lanes 9–10) display a clean supercoiled profile with monomeric supercoiled (lower, intense band) and non-covalent dimer (upper, weaker band) forms. Restriction digests (lanes 11–12) yielded fragments of expected sizes. Lane M: GeneRuler 1 kb DNA ladder (Thermo Scientific, Cat. SM0311). Panel (b): To quantify supercoiled (ccc) plasmid content, 5 µg of acidic phenol-purified plasmid was resolved in a 1% agarose gel stained with ethidium bromide. The regions corresponding to different plasmid forms were subjected to densitometric analysis as described in the Results section.

To quantify the proportion of the covalently closed circular (ccc) isoform, a lane in an 1% agarose gel was overloaded with 5 µg of the purified plasmid. The resolved gel was stained with ethidium bromide and photographed (Figure 2b). A region on the photograph corresponding to plasmid DNA isoforms was subjected to densitometric analysis using Image Lab software (Bio-Rad). Only two isoforms were detected: the dominant supercoiled ccc form and a faint relaxed (open circular) band. The ccc isoform accounted for 91.3% of the total plasmid DNA.

Comparative assessment of plasmid yield and purity

Three plasmids employed in CAR-T vector production - pLV/CAR-GFP, psPAX2 and pMD2.G - were purified by both methods, and their yields and purity (OD_{260}/OD_{280} , endotoxin

content) are summarised in Table 1. Yields varied by plasmid type but remained comparable between the two purification approaches. CsCl gradient centrifugation consistently afforded higher optical purity (1.78–1.92) than acidic phenol extraction (1.70–1.75), indicating more effective removal of protein and other UV-absorbing impurities. However, these biochemical parameters alone are insufficient to assess plasmid quality. In contrast, acidic phenol extraction yielded DNA with endotoxin contents ranging from 1.4 to 3.8 EU/µg DNA, whereas CsCl-purified preparations contained 17.2–25.4 EU/µg DNA (Table 1). These results confirm that acidic phenol extraction, despite leaving slightly higher UV-absorbing impurities than the reference single-round CsCl method, removes endotoxin more efficiently than the reference method.

Table 1. Comparison of plasmid DNA yields and purity obtained by CsCl density gradient ultracentrifugation and acidic phenol extraction.

Plasmid	Purification method	Batch ¹	Cell pellet (wet weight, g)	Total yield (mg)	Specific yield (mg/g)	Purity (OD_{260}/OD_{280})	Endotoxin (EU/µg DNA)
Transfer vector pLV/CAR-GFP	CsCl gradient	#1	2,1	5,4	2,57	1,86	19,8
-/-		#2	2,1	5,3	2,52	1,78	25,4
-/-	Acidic phenol extraction	#1	2,1	5,6	2,67	1,72	3,8
-/-		#2	2,1	5,7	2,71	1,75	2,2
Packaging helper psPAX2	CsCl gradient	#1	3,7	7,4	2,00	1,92	19,0
-/-		#2	3,7	6,7	1,81	1,88	24,8

-/-	Acidic phenol extraction	#1	3,7	6,8	1,84	1,73	2,6
-/-		#2	3,7	7,1	1,92	1,70	1,9
Envelope plasmid pMD2.G	CsCl gradient	#1	4,4	8,2	1,86	1,84	18,1
-/-		#2	4,4	8,5	1,93	1,90	17,2
-/-	Acidic phenol extraction	#1	4,4	9,6	2,18	1,70	2,1
-/-		#2	4,4	8,9	2,02	1,72	1,4

Comments: ¹For each plasmid, data from two independently produced batches are presented.

Functional validation of plasmids in transfections for lentiviral vector packaging

To assess whether acidic phenol-purified plasmids are functionally equivalent to CsCl-purified plasmids for downstream applications, we performed transient transfections with plasmid mixtures in which each component was purified by the same protocol.

Transfected cell cultures (Figure 3a–b) exhibited comparable transfection efficiency between the two purification methods, based on both the proportion of fluorescent cells and GFP fluorescence intensity. Finally, functional lentiviral titers from these transfections were statistically indistinguishable between the two purification methods ($p < 0.05$), reaching above 5×10^6 transducing units (TU/mL) (Figure 3c). These titers are consistent with those routinely obtained in our laboratory for CAR-T lentiviral vectors.

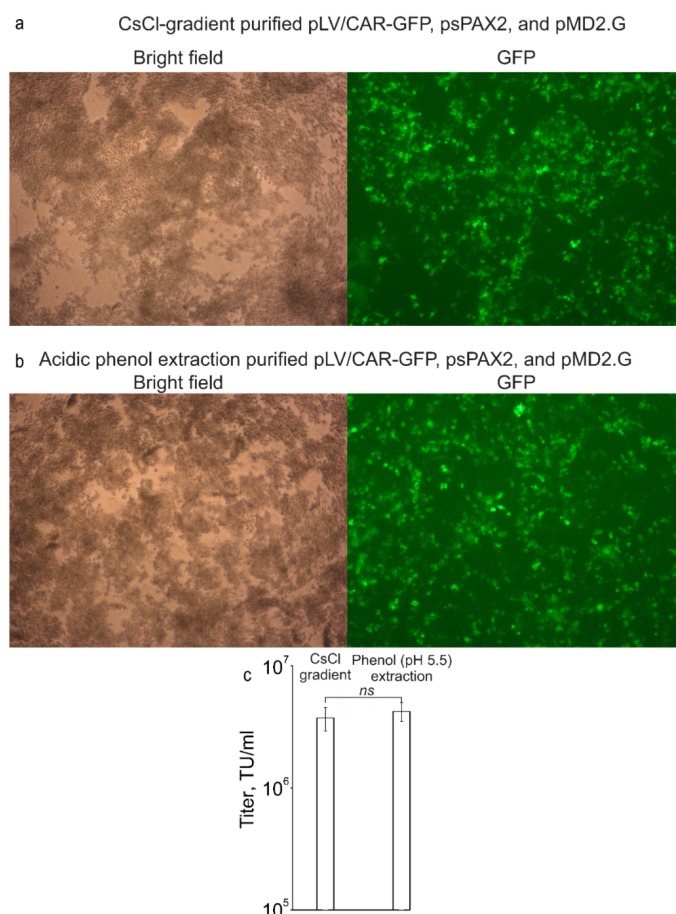


Figure 3. The acidic phenol-based plasmid purification method supports lentiviral vector production as efficiently as CsCl density gradient ultracentrifugation. HEK293FT cells were transfected with plasmid mixtures (pLV/CAR-GFP, psPAX2 and pMD2.G) in which each plasmid was purified by either the traditional CsCl gradient method (a) or the novel acidic phenol extraction protocol (b). Representative phase-contrast (left) and GFP fluorescence (right) images are shown. For both (a) and (b), images were acquired with a $5\times$ objective. (c) Functional titers (transducing units, TU/mL) of LV/CAR-GFP in culture supernatants. Data are mean $\pm$ SD from three biological replicates. Functional titers were assessed by flow cytometry after transduction of HEK293FT cells with the packaged vector. No statistically significant difference was observed between the two purification methods (ns , $p > 0.05$).

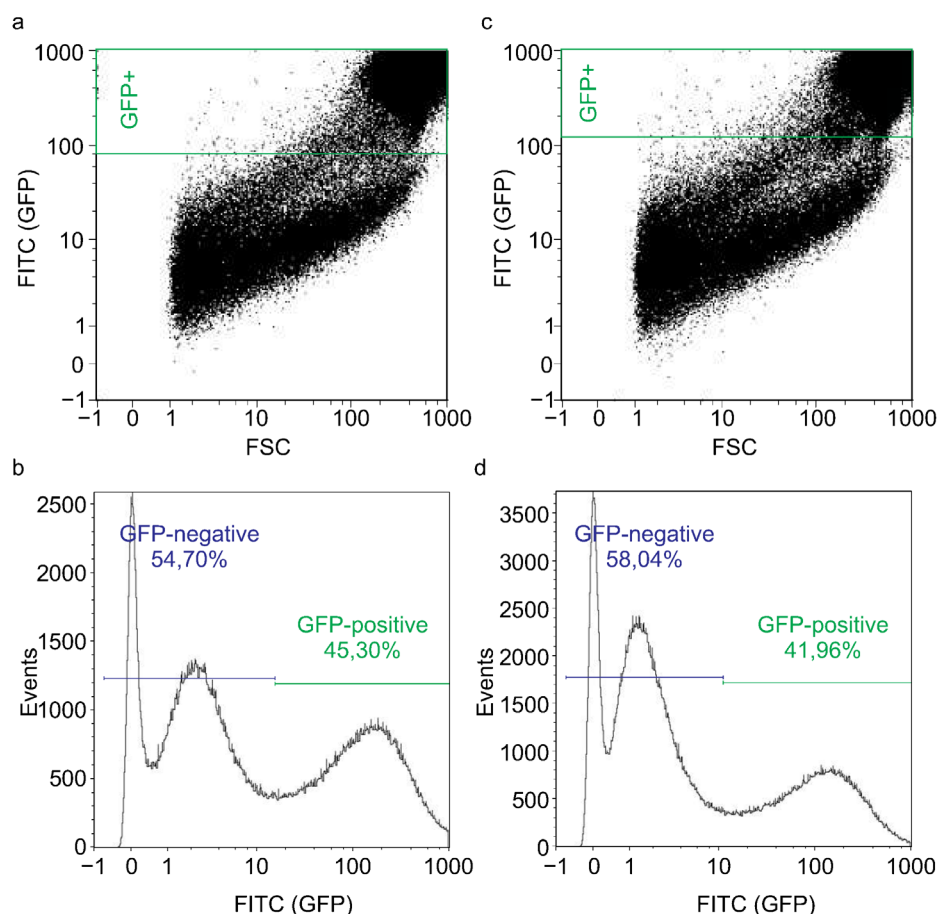


Figure 4. Comparison of transduction efficiency of lentiviral vectors packaged with acidic phenol-purified and CsCl-purified plasmid DNA. HEK293FT cells were transduced with LV/CAR-GFP at a low multiplicity of infection (MOI = 0.5) using vector batches produced with plasmid mixtures purified by either the acidic phenol extraction (a, b) or CsCl gradient method (c, d). GFP expression was assessed by flow cytometry at 72 h post-transduction. Representative flow cytometry data from one experiment are presented as scatter plots (FSC vs. GFP, a, c) and GFP fluorescence histograms (b, d). The percentage of GFP-positive cells was similar between the two groups, confirming equivalent functional quality of the resulting vectors.

Transduction efficiency of lentiviral vectors

To further validate the functional equivalence of the two procedures, we compared the transduction efficiency of lentiviral vectors packaged with plasmid DNA purified by each protocol. HEK293FT cells were transduced with LV/CAR-GFP vector particles at a low multiplicity of infection (MOI = 0.5), and the percentage of GFP-positive cells was determined by flow cytometry.

Representative flow cytometry plots and GFP fluorescence histograms (Figure 4a–d) revealed comparable transduction profiles between the vectors, with similar proportions of transduced (GFP-positive) cells. Quantitative analysis, performed on three biological replicates per vector (Table 2), confirmed

that the vectors exhibited comparable potency, independent of the starter plasmid purification method.

DISCUSSION

Increasing demand for high-quality (GMP-grade) plasmid DNA (pDNA) driven by DNA vaccines and the rapidly expanding field of cell and gene therapy (CGT), notably for lentiviral vector production in CAR-T manufacturing, has placed mounting pressure on GMP facilities and requires expanded production capacities across both industry and academia [1, 2]. While industrial-scale production employs sophisticated multi-step chromatography [3, 11], academic laboratories, which remain largely dependent on commercial

Table 2. Comparison of transduction efficiency of lentiviral vectors packaged with plasmid DNA purified by CsCl density gradient ultracentrifugation and acidic phenol extraction.

Purification method	Repeat 1 (%) ¹	Repeat 2 (%) ¹	Repeat 3 (%) ¹	Mean ± SD (%) ²
CsCl gradient	41,96	44,25	45,13	43,78 ± 1,64
Acidic phenol extraction	45,30	43,70	41,85	43,62 ± 1,73

Comments:¹Transduction experiments were performed in triplicate; data for each biological repeat are shown. ² The efficiency did not differ between vectors packaged with plasmids purified with different methods (p = 0,913; Student's *t*-test).

kits, need practical, low-cost alternatives. Here, we describe the development and validation of a modified alkaline lysis protocol incorporating acidic phenol extraction for transfection-grade pDNA purification. Our results confirm that this method yields plasmid DNA with comparable quantity, purity, and functional performance to the gold-standard CsCl density gradient ultracentrifugation, while offering a simpler, faster, and more cost-effective workflow.

Our protocol is built upon two classical methods. The first is a modified alkaline lysis procedure described by Kado and Liu in 1981 [23], which exploits the differential stability of covalently closed circular (ccc) DNA versus linear DNA under strongly alkaline conditions. Incubation of *E. coli* cells in 3% SDS at pH 12.6 lyses the cells and selectively denatures chromosomal DNA, while supercoiled plasmid DNA remains intact. Subsequent neutralization with 3 M potassium acetate precipitates denatured bacterial chromatin, leaving plasmid DNA in solution [23]. Importantly, however, the original Kado method was neither developed nor validated for the purification of transfection-grade pDNA.

The second foundation is based on the pH-dependent partitioning of conformationally distinct DNA forms, supercoiled cccDNA versus relaxed (linear or nicked) DNA - into water and organic phases, as described by Zasloff et al. (1978) [19], Fisher et al. (1991) [20], and Wang and Rossman (1994) [21]. At acidic pH (4.0–5.5), relaxed DNA molecules possess less surface charge than cccDNA, enabling selective extraction of non-supercoiled DNA into the organic phase while supercoiled plasmid DNA remains in the aqueous phase.

We therefore incorporated three consecutive extractions with phenol buffered to pH 5.5 into our protocol. Under these acidic conditions, lipopolysaccharide (LPS) becomes protonated and partitions into the organic phase, a principle long established in classical phenol-water extraction methods for bacterial endotoxins [24].

We performed a head-to-head comparison of yield and purity with the classical CsCl method, long regarded as the «gold standard» for ultra-pure pDNA isolation [15]. Our comparative assessment (Table 1) demonstrated that the acidic phenol extraction method achieves plasmid yields equivalent to those of the CsCl method across three different plasmids. The specific yields ranged from 1.84 to 2.71 mg plasmid per gram of wet cell pellet for the acidic phenol method and from 1.81 to 2.57 mg/g for CsCl gradients. These values are within the expected range for high-copy-number plasmids (1–3 mg/g wet weight) reported in the literature [1, 18].

We acknowledge that the step-wise recovery and losses during the multiple phenol extractions were not quantitatively assessed in this study. This represents a limitation of the current work; future optimization will focus on determining the recovery at each purification step to further refine the process.

With regard to plasmid optical purity, CsCl gradient centrifugation consistently yielded higher OD₂₆₀/OD₂₈₀ ratios (1.78–1.92) compared with acidic phenol extraction (1.70–1.75), suggesting more efficient removal of protein and other UV-absorbing contaminants by the reference method. At the same time, densitometric analysis of overloaded agarose gels revealed that the acidic phenol method preserved the covalently closed circular (ccc) isoform at 91.3% of total plas-

mid DNA, thereby exceeding the >90% criterion commonly cited for transfection-grade plasmids [6, 7]. A particularly notable finding was the superior endotoxin removal by the acidic phenol method. While CsCl gradient centrifugation is often considered the gold standard for purity, testing our preparations with the ToxinSensor Kit revealed that the single-round CsCl banding was less effective at removing endotoxin than the acidic phenol extraction (Table 1). CsCl-purified plasmids contained 17.2–25.4 EU/μg DNA, whereas the acidic phenol method yielded preparations with only 1.4–3.8 EU/μg DNA. This observation is consistent with the well-established principle that protonated lipopolysaccharide (LPS) partitions into the organic phase at acidic pH [24]. The functional equivalence of plasmids purified by the two methods in lentiviral packaging (Figures 3–4) suggests that either method yields plasmid preparations suitable for successful transfection. However, CsCl gradients require costly, specialized equipment (ultracentrifuge and dedicated rotors) [15], whereas our extraction method requires only standard laboratory equipment. Importantly, both methods use domestically produced reagents and eliminate the need for commercial plasmid isolation kits.

A further key objective was to validate the new method for a critical downstream application: its use in transfections for lentiviral vector packaging. During lentiviral vector packaging experiments, cell cultures transfected with pDNAs purified by either the classical or the novel protocol showed no differences in cell morphology or GFP fluorescence (the used vector expresses both CAR receptor and GFP). More importantly, functional lentiviral titers were statistically similar between the two purification conditions, with both yielding $\sim 5 \times 10^6$ TU/mL of a packaged lentiviral vector, the result consistent with our routine data and with published literature [25, 26].

To further validate functional equivalence, we transduced cell cultures with the infectious vector particles using a low multiplicity of infection (MOI = 0.5), which was chosen to maximize sensitivity for detecting differences in functional activity. Transduction efficiency was quantified by flow cytometry. These transductions demonstrated comparable performance of vectors derived from the differently obtained pDNA preparations, with levels of approximately 44%, in good agreement with the used MOI. We conclude that the acidic phenol extraction method serves as a viable alternative to CsCl ultracentrifugation when the ultimate goal is production of lentiviral vectors for CAR-T.

In the head-to-head comparison, the acidic phenol extraction method proved faster and cheaper, while remaining scalable to 5–10 mg per preparation. Its main limitations are slightly lower OD₂₆₀/OD₂₈₀ ratios and the handling of phenol - a toxic compound classified as a drug precursor in Kazakhstan, necessitating a license for its purchase and use.

Other research groups have developed low-cost alternatives to commercial pDNA purification kits for academic laboratories, including the Filterprep method [27] and magnetic bead-based approaches [28]. However, the scalability of these methods to the preparative scale (5–10 mg pDNA per preparation) remains questionable, as they rely on the adsorption principle that dictates proportional scaling with the amount of DNA processed. By contrast, our acidic phenol extraction method is free of any adsorption matrix; its scalability is lim-

ited only by volumes that can be practically handled.

Future development of the described method is expected to be driven by the demand from DNA vaccination and CGT/CAR-T fields and will focus on its adaptation to GMP-compliant manufacturing.

CONCLUSION

The acidic phenol extraction method is a simple, scalable alternative to both CsCl and industrial chromatography, matching them in yield, purity, and functional lentiviral titers—an enabling tool for academic laboratories expanding into CGT/CAR-T manufacturing.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.A.; Data curation, B.E, N.G., K.V., K.T., I.B., K.A., Z.M., Z.D. and S.A.; Formal analysis, Z.M. and S.A.; Funding acquisition, S.A.; Investigation, B.E, N.G., K.V., K.T., I.B., K.A., Z.M., Z.D. and S.A.; Methodology, B.E, N.G., K.V., K.T., I.B., K.A., Z.M., Z.D. and S.A.; Project administration, Z.M., Z.D. and S.A.; Resources, S.A.; Supervision, Z.M., Z.D. and S.A.; Validation, Z.M., Z.D. and S.A.; Visualization, Z.M., Z.D. and S.A.; Writing—original draft, B.E, N.G., K.V., K.T., I.B., K.A., Z.M., Z.D. and S.A.; Writing—review and editing, B.E, N.G., K.V., K.T., I.B., K.A., Z.M., Z.D. and S.A. All authors have read and agreed to the published version of the manuscript.

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УСТРАНЕНИЕ СДЕРЖИВАЮЩЕГО ФАКТОРА В CAR-T-ТЕРАПИИ: МАСШТАБИРУЕМОЕ ПОЛУЧЕНИЕ ПЛАЗМИДНОЙ ДНК ТРАНСФЕКЦИОННОГО КАЧЕСТВА ДЛЯ ПРОИЗВОДСТВА CAR-T-ВЕКТОРОВ

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АННОТАЦИЯ

Стремительное развитие клеточной и генной терапии привело к росту потребности в плазмидной ДНК, используемой для производства вирусных векторов. Получение плазмидной ДНК трансфекционного качества остается одним из ключевых ограничивающих факторов, особенно для академических и развивающихся производственных площадок, что обусловлено высокой стоимостью и ограниченной масштабируемостью существующих технологий очистки. Целью настоящего исследования являлась разработка практического и экономически эффективного подхода к получению плазмидной ДНК трансфекционного качества, пригодной для производства лентивирусных векторов.

Описанный метод экстракции кислым фенолом был протестирован на плаزمидях, широко используемых в системах упаковки лентивирусов, и сопоставлен с референсным методом - ультрацентрифугированием в градиенте плотности хлорида цезия (CsCl). Полученные препараты плазмидной ДНК показали эффективное удаление геномной ДНК и других примесей, включая эндотоксин - метод с кислым фенолом последовательно снижал уровень эндотоксина до 1,4–3,8 EU/мкг ДНК. Кроме того, очищенная плазмидная ДНК была преимущественно в суперскрученной (ковалентно замкнутой кольцевой) форме. Функциональное тестирование в трансфекции подтвердило, что плазмиды, очищенные предложенным методом, обеспечивают производство лентивирусных векторов с эффективностью, сопоставимой с референсным методом, с титрами, превышающими 5×10^6 TU/мл, и без статистически значимых различий в эффективности трансдукции между группами.

Полученные результаты подтверждают, что метод экстракции кислым фенолом является простым, масштабируемым и экономичным способом выделения плазмидной ДНК трансфекционного качества для производства лентивирусных векторов. Метод является практичной альтернативой очистке с помощью ультрацентрифугирования и может способствовать более широкому внедрению технологий клеточной и генной терапии за счет снижения зависимости от специализированного оборудования и дорогостоящих коммерческих наборов.

Ключевые слова: плазмидная ДНК, лентивирусный вектор, CAR-T-терапия, генная терапия, ДНК трансфекционного качества, очистка плазмидной ДНК, клеточная и генная терапия, биопроцессинг.

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CAR-T-ТЕРАПИЯДАҒЫ ШЕКТЕУШІ ФАКТОРДЫ ЖОЮ: CAR-T ВЕКТОРЛАРЫН ӨНДІРУ ҮШІН ТРАНСФЕКЦИЯЛЫҚ САПАДАҒЫ ПЛАЗМИДАЛЫҚ ДНҚ-НЫҢ МАСШТАБАЛАТЫН ӨНДІРІСІ

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АНДАТПА

Жасушалық және гендік терапияның қарқынды дамуы вирустық векторларды өндіруде қолданылатын плазмидалық ДНҚ-ға деген сұраныстың артуына алып келді. Трансфекциялық сападағы плазмидалық ДНҚ алу қолданыстағы тазарту технологияларының жоғары құны мен шектеулі масштабталуына байланысты, әсіресе академиялық және дамып келе жатқан өндірістік алаңдар үшін маңызды шектеуші факторлардың бірі болып қала береді. Осы зерттеудің мақсаты лентивирустық векторларды өндіруге жарамды трансфекциялық сападағы плазмидалық ДНҚ алудың практикалық және экономикалық тиімді тәсілін әзірлеу болды.

Сипатталған қышқыл фенолмен экстракциялау әдісі лентивирустарды қаптау жүйелерінде кеңінен қолданылатын плазмидаларда сыналып, референс-әдіспен — цезий хлоридінің (CsCl) тығыздық градиентінде ультрацентрифугалау-

мен салыстырылды. Алынған плазмидалық ДНҚ препараттары геномдық ДНҚ мен басқа қоспалардың, соның ішінде эндотоксиннің тиімді жойылғанын көрсетті – қышқыл фенол әдісі эндотоксин деңгейін 1,4–3,8 EU/мкг ДНҚ-ға дейін төмендетті. Сонымен қатар, тазартылған плазмидалық ДНҚ негізінен суперширатылған (ковалентті тұйық сақиналы) пішінде болды. Трансфекциядағы функционалдық тестілеу ұсынылған әдіспен тазартылған плазмидалардың лентивирустық векторларды өндіруді референс-әдіспен салыстырмалы тиімділікпен қамтамасыз ететінін, титрлері 5×10^6 TU/мл-ден асып, топтар арасында трансдукция тиімділігінде статистикалық маңызды айырмашылықтар болмағанын растады.

Алынған нәтижелер қышқыл фенолмен экстракциялау әдісі лентивирустық векторларды өндіруге арналған трансфекциялық сападағы плазмидалық ДНҚ-ны бөліп алудың қарапайым, масштабталатын және экономикалық тиімді тәсілі екенін растайды. Бұл әдіс ультрацентрифугалауға негізделген тазартуға практикалық балама болып табылады және мамандандырылған жабдық пен қымбат коммерциялық жинақтарға тәуелділікті азайту арқылы жасушалық және гендік терапия технологияларын кеңінен енгізуге ықпал ете алады.

Түйін сөздер: плазмидалық ДНҚ, лентивирустық вектор, CAR-T терапиясы, гендік терапия, трансфекциялық сападағы ДНҚ, плазмидалық ДНҚ-ны тазарту, жасушалық және гендік терапия, биопроцессинг.