Genes & Diseases (2021) 8, 298–306

Available online at www.sciencedirect.com

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FULL LENGTH ARTICLE

Development of a simplified and inexpensive RNA depletion method for plasmid DNA purification using size selection magnetic beads (SSMBs)

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Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2020.04.013

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Received 10 March 2020; received in revised form 3 April 2020; accepted 22 April 2020
Available online 20 May 2020

KEYWORDS
DNA transfection;
DNA vaccination;
Gene delivery;
Plasmid DNA purification;
RNA depletion;
Size selection magnetic beads

Abstract Plasmid DNA (pDNA) isolation from bacterial cells is one of the most common and critical steps in molecular cloning and biomedical research. Almost all pDNA purification involves disruption of bacteria, removal of membrane lipids, proteins and genomic DNA, purification of pDNA from bulk lysate, and concentration of pDNA for downstream applications. While many liquid-phase and solid-phase pDNA purification methods are used, the final pDNA preparations are usually contaminated with varied degrees of host RNA, which cannot be completely digested by RNase A. To develop a simple, cost-effective, and yet effective method for RNA depletion, we investigated whether commercially available size selection magnetic beads (SSMBs), such as Mag-Bind® TotalPure NGS Kit (or Mag-Bind), can completely deplete bacterial RNA in pDNA preparations. In this proof-of-principle study, we demonstrated that, compared with RNase A digestion and two commercial plasmid affinity purification kits, the SSMB method was highly efficient in depleting contaminating RNA from pDNA minipreps. Gene transfection and bacterial colony formation assays revealed that pDNA purified from SSMB method had superior quality and integrity to pDNA samples cleaned up by RNase A digestion and/or commercial plasmid purification kits. We further demonstrated that the SSMB method completely depleted contaminating RNA in large-scale pDNA samples. Furthermore, the Mag-bind-based SSMB method costs only 5—10% of most commercial plasmid purification kits on a per sample basis. Thus, the reported SSMB method can be a valuable and inexpensive tool for the removal of bacterial RNA for routine pDNA preparations.

Introduction

Plasmid DNA (pDNA) amplification and isolation from bacterial cells is one of the most common and critical steps in molecular cloning and biomedical research, especially in response to rapid advances in the use of pDNA in gene therapy and vaccines.[1—8] Historically, the first DNA isolation was carried out by a Swiss physician, Friedrich Miescher in 1869, who hoped to solve the fundamental principles of life, and to determine the chemical composition of cells.[3,9] For the past 50 years, significant progress has been made in DNA purification. The standard method for large scale pDNA purification prior to the use of solid phase techniques, was to lyse the cells by using alkaline conditions, boiling, or detergent, followed by ultracentrifugation in a CsCl/ethidium bromide gradient.[3,4,8—10]

Regardless of the methods, almost all pDNA purification involves the following basic steps: lysis or disruption of bacterial cells; removal of membrane lipids, proteins and host genomic DNA; purification of pDNA from bulk lysate; and cleanup and concentration of pDNA for downstream applications.[3,4,8,10] Bacterial cell disruption can be accomplished by physical and chemical methods. Chemical disruption methods include osmotic shock, lytic enzymatic digestion, various types of detergents, and alkali treatment, whereas mechanical methods include with homogenization with blade or pestle, ultrasonic treatments, pressure cells/French press, and ball mill with glass/steel beads.[8] Among these methods, alkaline lysis pDNA isolation is one of the most commonly used and least expensive methods.[8,11—15] Alkaline lysis pDNA extraction method, initially described by Bimboim and Doly,[11] is based on the principle that alkaline solution selectively denatures high molecular weight chromosomal DNA, leaving covalently bond circular pDNA intact.[8] Upon neutralization, chromosomal DNA renatures and becomes insoluble precipitates, whereas pDNA remains in the supernatant. Thus, alkaline lysis method can be used for both small and large DNA plasmids. Nonetheless, in a typical alkaline bacterial lysate pDNA only accounts for ~3% of the cleared lysate, while bacterial host proteins account for 55%, RNA for 21%, host cDNA for 3%, LPS for 3% and other host components for 15%.[2]

Numerous solid-phase pDNA purification methods have been developed over the years, representing most of the commercial pDNA extraction kits currently available on the market.[3,8] Solid phase methods will absorb pDNA depending on the pH and salt content of the buffers, in which hydrogen-binding interaction with a hydrophilic matrix under chaotropic conditions, ionic exchange under aqueous

conditions by means of an anion exchanger, and affinity and size exclusion mechanisms.[3,8] Commonly used solid supports for solid phase pDNA extraction include several types of matrices, such as silica matrices, glass particles, diatomaceous earth, anion-exchange carriers, and magnetic beads.[3,8] Similar to liquid-phase pDNA purification methods, solid-phase pDNA isolation also involves four steps, cell lysis, pDNA adsorption, washing, and elution.[3,8]

Magnetic bead-based nucleic acid isolation technology is emerging as popular strategies for isolating genomic, plasmid, and mitochondrial DNA and RNA. Many magnetic carriers are now commercially available.[3,8] In these systems, particles with magnetic charge are removed by using a permanent magnet. In most cases, magnetic carriers with immobilized affinity ligands or prepared from biopolymers, synthetic polymers, porous glass or magnetic particles based on inorganic magnetic materials, showing affinity to the target nucleic acids are used for the isolation process.[3,8] Some of the commonly-used commercial affinity purification kits include the QIAprep Spin Miniprep Kit (QIAGEN), the Monarch® Plasmid Miniprep Kit (NEB), and the Wizard® Plus SV Minipreps DNA Purification Systems (Promega, Madison, WI). In this regard, magnetic particulate materials such as beads are more preferable to be a support for solid phase pDNA isolation due to their larger binding capacity. Magnetic bead-based purification methods avoid centrifugation steps and offer possibility of automation and/or high throughput extraction of pDNA. Several commercial kits based on solid-phase reversible immobilization paramagnetic bead technology are available in market, such as Agencourt AMPure XP (Beckman Coulter), AxyPrep Magnetic (MAG) Bead Purification kits (Corning-AxyGEN), Mag-Bind® UltraPure Plasmid DNA kit (Omega Bio-tek), and sparQ PureMag Beads (Quantabio). While these commercial kits are effective, they are in general expensive and not practical for routine lab uses.

While the above pDNA extraction methods can get rid of most RNA, the final pDNA preparations are still contaminated with varied degrees of bacterial RNA in most cases, which limits the pDNA downstream applications. One of the most commonly-used methods to remove RNA is to digest the pDNA preparations with RNase A, which is an endoribonuclease that specifically degrades single-stranded RNA at C and U residues.[16] While RNase A is effective to remove most of the residual RNA, a significant fraction of bacterial RNA is RNase-resistant and cannot be depleted completely. Thus, more efficient alternative methods are needed.

In order to develop a simple, cost-efficiency, and effective method for RNA depletion, we sought to test whether commercially available size selection magnetic beads (SSMBs), such as Omega Bio-tek's Mag-Bind® Total Pure NGS Kit (referred to it as Mag-Bind thereafter), can be used to achieve complete depletion of bacterial RNA in pDNA preparations. SSMBs have been widely used in next-generation sequencing (NGS) sample preparations due to their size selection ability. Mechanistically, SSMBs are coated with a layer of negatively charged carboxyl molecules and suspended in the buffer containing polyethylene glycol (PEG) and salt. When PEG is added to a DNA solution at saturating condition, DNA forms large random coils. Adding DNA with the right concentration of salt (Na^+) will cause DNA to aggregate and precipitate out. Because the Na^+ can shield the negatively charged phosphate backbones causing DNA to stick together and associate with carboxylated beads. Conversely, adding aqueous solution (e.g., ddH_2O or TE buffer) back will fully hydrate DNA and convert it from an aggregated state back into solution, whereas the negative charged carboxyl beads repel DNA, allowing the recovery of pDNA in the supernatant. Thus, the status of the reversible immobilization of DNA on SSMBs depends on the amount of PEG and NaCl in solution, so the volumetric ratio (v/v) of beads to DNA is critical. The lower the ratio of Beads:DNA, the larger the final molecules (e.g., pDNA) will be obtained at elution, while smaller molecules (e.g., RNA) will be retained in the initial buffer and discarded.

In this proof-of-principle study, using Mag-Bind SSMBs we demonstrated that, compared with RNase A digestion and commercial plasmid affinity purification kits, the SIRD method was user-friendly, reproducible, and highly efficient in removing contaminating RNA from pDNA minipreps. We also demonstrated that the SSMB method completely depleted contaminating RNA from large-scale purified pDNA samples. Comparative studies from gene transfection and bacterial colony formation assays revealed that pDNA purified from the SSMB method had superior quality and integrity to the pDNA cleaned up by RNase A digestion and/or commercial plasmid affinity purification kits. Furthermore, the Mag-bind-based SSMB method costs approximately 5–10% of most commercially available plasmid purification kits. Therefore, the reported SSMB method is valuable tool to remove contaminating bacterial RNA for routine pDNA preparations.

Materials and methods

Cell culture and chemicals

HEK-293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C in 5% CO_2 as described.[17,18] Unless indicated otherwise, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA, USA).

Alkaline lysis pDNA purification

The alkaline lysis pDNA isolation was performed as described previously.[11–15] In Brief, 2 ml plasmid-containing bacterial cells were cultivated in LB medium containing proper antibiotic overnight. Bacterial cells were pelleted into 2.0 ml Eppendorf microfuge tubes, resuspended in 200 μl of BD-I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0), lysed in 200 μl of BD-II (0.2 N NaOH, 1% SDS), and neutralized with 200 μl of BD-III (5 M potassium acetate:10 M acetic acid, v/v). The mixture was centrifuged at top speed on bench top microfuge. The supernatant was transferred to new 1.5 ml tubes, and 700 μl isopropanol was added to precipitate pDNA, followed by 70% ethanol washes. The pDNA was dissolved in 50 μl ddH_2O (~0.2 μg DNA/μl) and kept at −20 °C for downstream

applications. For a large scale pDNA preparation, 1.0—2.0 L of bacterial culture were collected and subjected to the same alkaline lysis protocol except that BD-I, BD-II, and BD-III volumes were scaled up by 50 times. The pDNA was dissolved in 500—1,000 μl ddH2O (~1.0 μg DNA/μl), and kept at −20 °C for downstream applications.

pDNA isolation using commercial DNA affinity purification kits

Two commercial plasmid DNA purification kits, the QIAGEN Plasmid Mini Kit (QIAGEN, Germantown, MD) and the Monarch Plasmid Miniprep Kit (NEB, New England Biolabs, Ipswich, MA), were used for pDNA isolation. 2 ml plasmid-containing bacterial cells were cultivated in LB medium containing proper antibiotic overnight. The pDNA was isolated according to the manufacturers' instructions. Each miniprep pDNA was dissolved in 50 μl ddH_2O and kept at −20 °C for downstream applications.

Qualitative and quantitative analysis of contaminating RNA in pDNA preps

The pDNA samples were resolved on 1% agarose gels and visualized by ethidium bromide staining. High-resolution black/white invert images were captured by using the SYNGENE PXi 6 Access imaging unit (Syngene International Ltd, Biocon Park, SEZ). The images were quantitatively analyzed with ImageJ software.

Bacterial RNA removal by RNase a digestion

The pDNA minipreps were incubated with Monarch RNase A (NEB, 20 mg/ml; final concentration of 0.5 mg/ml) at 56 °C for the indicated durations, followed by phenol-chloroform extraction and ethanol precipitation as previously described.[19] The recovered plasmid DNA was analyzed on 1% agarose gels.

Bacterial RNA removal by size selection magnetic beads (SSMBs)

The commercially available SSMBs Mag-Bind® TotalPure NGS (or Mag-Bind, Omega Bio-tek, Inc., Norcross, GA) were used for the study. Briefly, the miniprep pDNA (approximately 10 μg DNA) and the Mag-Bind beads were mixed at a vol/vol ratio of 5:2 (i.e., Mag-Bind beads were 0.4 times of the pDNA volume). Experimentally, a standard alkaline lysis plasmid miniprep was dissolved in 50 μl ddH_2O, and then added with 20 μl Mag-Bind beads. The DNA-beads mixture was mixed well and sit at room temperature for 10 min. The mixture was subjected to magnet separation and the RNA-containing supernatant was discarded, while DNA-bound beads were washed with 70% ethanol twice. After a brief air-dry for 60 s, the plasmid DNA was eluted from the beads with a desired volume (20—100 μl) of ddH_2O for any downstream use.

The size selection magnetic bead process was also employed to deplete bacterial RNA in large scale pDNA preparation. A total volume of 1.0—2.0 L of pDNA-containing DH10B cells were collected and subjected to alkaline lysis pDNA purification as described above except scaling up.

Transformation (electroporation) of DH10B bacterial cells

Electrocompetent DH10B bacterial cells (NEB® 10-beta Electrocompetent *E. coli*) were mixed with various amounts of pDNA samples (in a total volume of 20 μl), transferred to 1.0 mm cuvettes, and electroporated by using a MicroPulser Electroporator (BIO-RAD Laboratories, Hercules, CA) at 1.8 KV. The transformed cells were resuspended in 500 μl LB medium, and 50 μl of the transformation mix were immediately plated onto replicates of LB-agar/Amp plates. The plates were incubated at 37 °C for 16 h, and then the bacterial colonies were documented.

Transfection of mammalian cells

Subconfluent HEK-293 cells were seeded in 12-well cell culture plates and transfected with pAdTrack or pCMV-eGFP plasmid DNA using the polyethylenimine (PEI) reagents (Polysciences, Inc., Warrington, PA) as previously described.[20,21] At 48 h after transfection, the transfected cells were examined under bright field and fluorescence microscope to document fluorescence signal intensity.

Statistical analysis

The quantitative studies were carried out in triplicate and/or performed in three independent batches. Statistical differences between samples were determined by one-way analysis of variance (ANOVA). A P-value < 0.05 was defined as statistically significant when a comparison was made.

Results and discussion

The complete depletion of contaminating bacterial RNA from pDNA preparation is technically challenging

As RNase A is widely used for enzymatic depletion of bacterial RNA in pDNA preps, we first tested the efficiency of RNase A-mediated RNA removal. We digested one-tenth of one standard alkaline lysis miniprep pDNA pMOK (3.1 kb) (approximately 1 μg DNA) with RNase A for 0 min, 30 min, 60 min, and 90 min, and analyzed on 1% agarose gels. We found that residual bacterial RNA was readily detected after 90 min digestion (Fig. 1A, panel a) although > 95% of contaminating RNA was removed (Fig. 1A, panel b). These

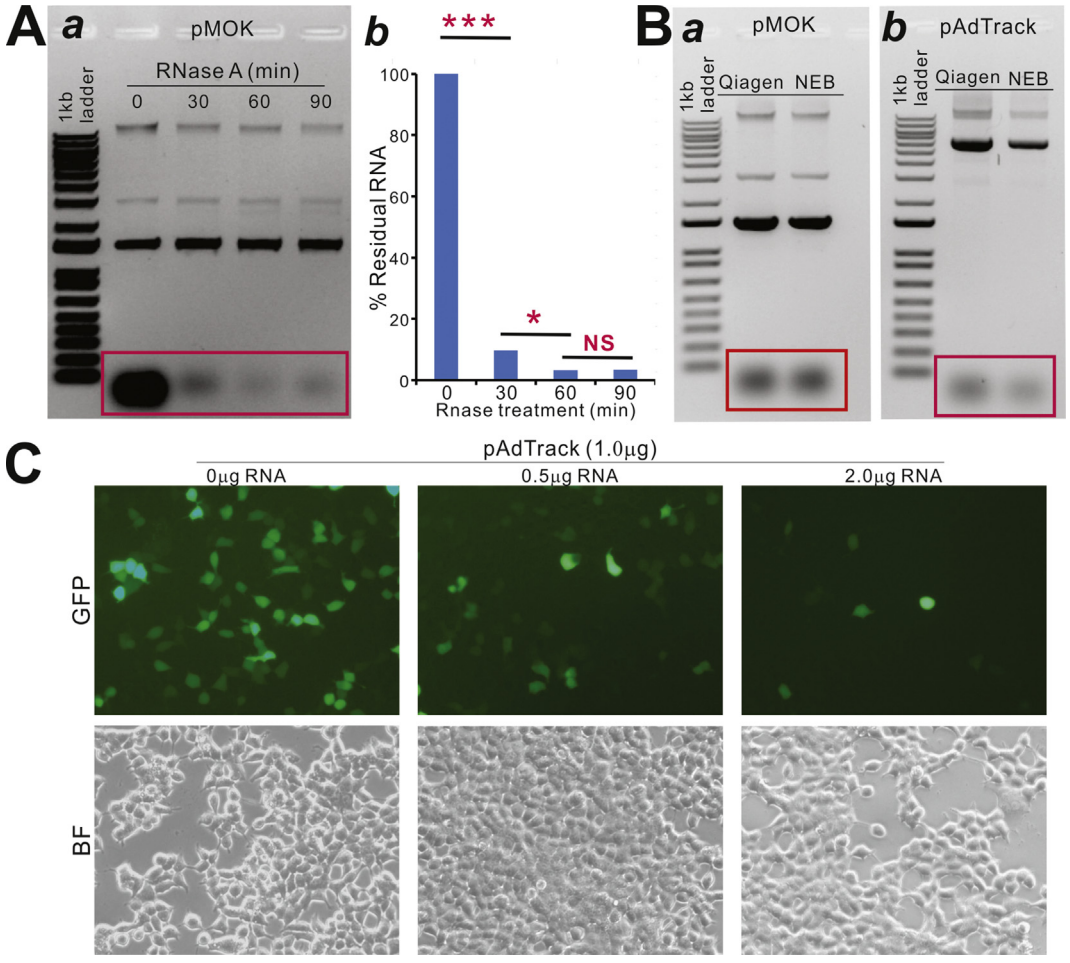

Figure 1 The complete removal of bacterial RNA in most pDNA preparations is technically challenging and significantly hampers downstream applications. **(A)** Incomplete removal of RNA in miniprep DNA by RNase A digestion. One tenth of one standard alkaline lysis miniprep pDNA pMOK (3.1 kb) was digested with equal amount of RNase A in triplicate. The digestion reactions were terminated at the indicated time points, and analyzed on 1% agarose gels (*a*). Representative images are shown. The red box indicates the presence of bacterial RNA. The RNA bands were quantitatively analyzed by using ImageJ software (*b*). "***", $P < 0.001$; "*", $P < 0.01$; NS, $P > 0.05$. **(B)** Incomplete removal of RNA in pDNA by commercial DNA purification kits. Two milliters of overnight DH10B culture for pMOK (*a*) and pAdTrack (9.2 kb) (*b*) was subjected to pDNA purification using the QIAGEN or NEB plasmid extraction kit. One tenth of miniprep pDNA was analyzed on 1% agarose gels. The red boxes indicate the presence of bacterial RNA. Representative images are shown. **(C)** The presence of bacterial RNA significantly diminishes DNA transfection efficiency in mammalian cells. Subconfluent HEK-293 cells were seeded in 12-well cell culture plates and transfected with 1 μg of pAdTack mixed with the indicated amount of total RNA isolated from HEK293 cells. Both bright field (BF) and green fluorescence (GFP) images were recorded at 48 h post transfection. Representative images are shown.

results suggest that the gold standard RNase A-mediated RNA removal may be incomplete.

We further tested two commonly used commercial pDNA affinity purification kits from QIAGEN and New England Biolabs (NEB). Even though the commercial pDNA affinity extraction kits routinely included RNase A in solution, we found that significant amounts of residual bacterial RNA were presented in the pDNA preps for pMOK, a small plasmid of 3.1 kb (Fig. 1B, panel a), and pAdTrack, a larger plasmid of 9.2 kb (Fig. 1B, panel b). Taken together, these results indicate that a complete depletion of contaminating bacterial RNA is a challenging endeavor and can't be easily accomplished with ether RNase A digestion and/or expensive commercial pDNA affinity purification kits.

Contaminating RNA significantly diminishes the transfection efficiency of pDNA in mammalian cells

RNA-free pDNA is important for many applications, including DNA sequencing and transfection of mammalian cells. We tested the effect of RNA on pDNA transfection in a commonly used human cell line, HEK-293. Using the purified pAdTrack pDNA doped with varied amounts of total RNA isolated from HEK-293 cells, we found that more than 80% transfection efficiency in the group without RNA contamination, whereas the transfection efficiency drastically decreased, e.g., approximately 20% and 5% in the groups with contaminating RNA at 0.5 μg and 2.0 μg, respectively (Fig. 1C). These results further highlight the necessity of

complete depletion of bacterial RNA from pDNA preparations.

The use of SSMBs represents an inexpensive and rapid approach to bacterial RNA removal with high efficiency

Various SSMBs have been widely used in NGS library preparations.[19] Such SSMB selections are in general easy to use and have high efficiency, although the beads are usually sold as a part of the NGS kits and rather expensive. In this study, we used the bulk supplied, relatively inexpensive Mag-Bind® Total Pure NGS kit from Omega Bio-tek. Based on the size difference between pDNA and contaminating bacterial RNA, we optimized and developed a size selection protocol for depleting contaminating RNA from pDNA using the Mag-Beads (Fig. 2A). pDNA and Mag-Beads were mixed well at a ratio of 5:2 (v/v, pDNA:Mag-Beads) at room temperature for 10 min (Fig. 2A, panel a). The mixture was then subjected to magnet separation (Fig. 2A, panel b) and the RNA-containing supernatant was discarded, while DNA-bound beads were washed with 70% ethanol twice (Fig. 2A, panel c). After a period of air-dry, the pDNA was eluted from the beads with a desired volume of ddH_2O for any downstream application (Fig. 2A, panel d). It took 15—20 min to complete the whole protocol.

To test whether bacterial RNA could be effectively removed from pDNA by using the SSMBs, we subjected the alkaline lysis mini-prepared pMOK and pAdTrack to the Mag-Bind beads and found that, compared with the input, the eluted pDNA samples from the beads for both plasmids did not contain any detectable RNA, whereas the discarded supernatants contained the contaminating RNA (Fig. 2B, panels ab). Quantitative analyses indicate that the pDNA recovery rates are 93.6% and 96.7% for pMOK and pAdTrack, respectively (Fig. 2C, panel a). On the other hand, $>$ 99% of the contaminating RNA was depleted for both pMOK and pAdTrack pDNA samples (Fig. 2C, panel b). Thus, these results demonstrate that RNA depletion with the Mag-Bind SSMBs is highly effective and reproducible.

SSMBs efficiently remove contaminating RNA from large-scale preparation of pDNA samples and preserve pDNA integrity

Some *in vivo* applications require large-scale pDNA purification, which is usually accomplished by using multiple commercial pDNA affinity extraction columns or CsCl gradient ultracentrifugation.[3,4,9] However, these protocols are usually expensive and/or time consuming. More importantly, the purified pDNA from these procedures usually contains significant amount of contaminating bacterial RNA (data not shown). We further tested whether the Mag-Bind beads could effectively remove bacterial RNA from large-scale alkaline lysis pDNA preparations. Using another homemade GFP-expressing vector pCMV-eGFP, we carried out a large-scale pDNA preparation, and subjected the same amount of pCMV-eGFP to RNase A digestion or Mag-Bind bead purification. We found that compared with the input, the pCMV-eGFP sample subjected to magnetic bead purification did not have any detectable RNA, while approximately 20% of RNA remained in the RNase A treated sample (Fig. 3A).

We also tested the integrity of the pCMV-eGFP plasmid samples treated with RNase A vs. Mag-Bind beads purification by direct transformation of the plasmid preps into DH10B cells. We found that the pDNA from Mag-Bind SSMB purification yielded the highest number of colonies, while the RNase A treatment group produced the lowest number of colonies, even fewer than that of the untreated pDNA sample (Fig. 3B, panels abc). These results suggest that Mag-Bind SSMB based purification may preserve pDNA integrity better with fewer strand breaks and/or loss of DNA. The overall quality of the prepared pDNA samples from these two methods was further assessed by transfecting HEK-293 cells. We found that the pDNA recovered from the Mag-Bind bead selection yielded the highest number of GFP + cells, significantly higher than that of the RNase A treatment group (Fig. 3C). Collectively, these results strongly suggest that the overall quality of the pDNA preps from Mag-Bind bead purification may be superior to that of RNase A treatment in terms of contaminating RNA depletion.

The use of SSMBs may represent a timesaving, inexpensive, and yet effective method to deplete bacterial RNA in pDNA preparations

Plasmid DNA propagation and purification is almost a daily routine in many molecular and cell biology laboratories worldwide. Many labs choose to use commercially available plasmid purification kits, which are unfortunately expensive, approaching two US dollars per sample. Furthermore, the DNA affinity purification procedures usually involves extensive washes with solutions containing inorganic salts and organic solvents, which may create nicked DNA or DNA with broken strands, affecting the overall integrity and quality of the purified pDNA. As our results indicated, such affinity purification procedures cannot always completely remove contaminating bacterial RNA, hampering our ability to obtain optimal results from those studies that require the use of RNA-free pDNA preparations.

While RNase A is a commonly used enzymatic method to remove RNA, our results indicate there may be limitations on how thoroughly contaminating RNA can be removed from pDNA preps. As an endoribonuclease that specifically degrades single-stranded RNA at C and U residues, RNase A cleaves the phosphodiester bond between the 5′-ribose of a nucleotide and the phosphate group attached to the 3′-ribose of an adjacent pyrimidine nucleotide, resulting 2′, 3′-cyclic phosphate that is further hydrolyzed into corresponding 3′-nucleoside phosphate. While this enzyme is effective, it seems that some RNA fragments may be resistant to RNase A digestion in a dose and time-independent fashion. Furthermore, most RNase A of molecular biology grade contains protein impurities, possibly including DNases that would degrade pDNA non-specifically. In order to get rid of RNase A and the protein impurities, RNase A treatment is usually followed by phenol-chloroform extractions and ethanol precipitations, which is more time-consuming and pose more biohazardous risks to investigators.

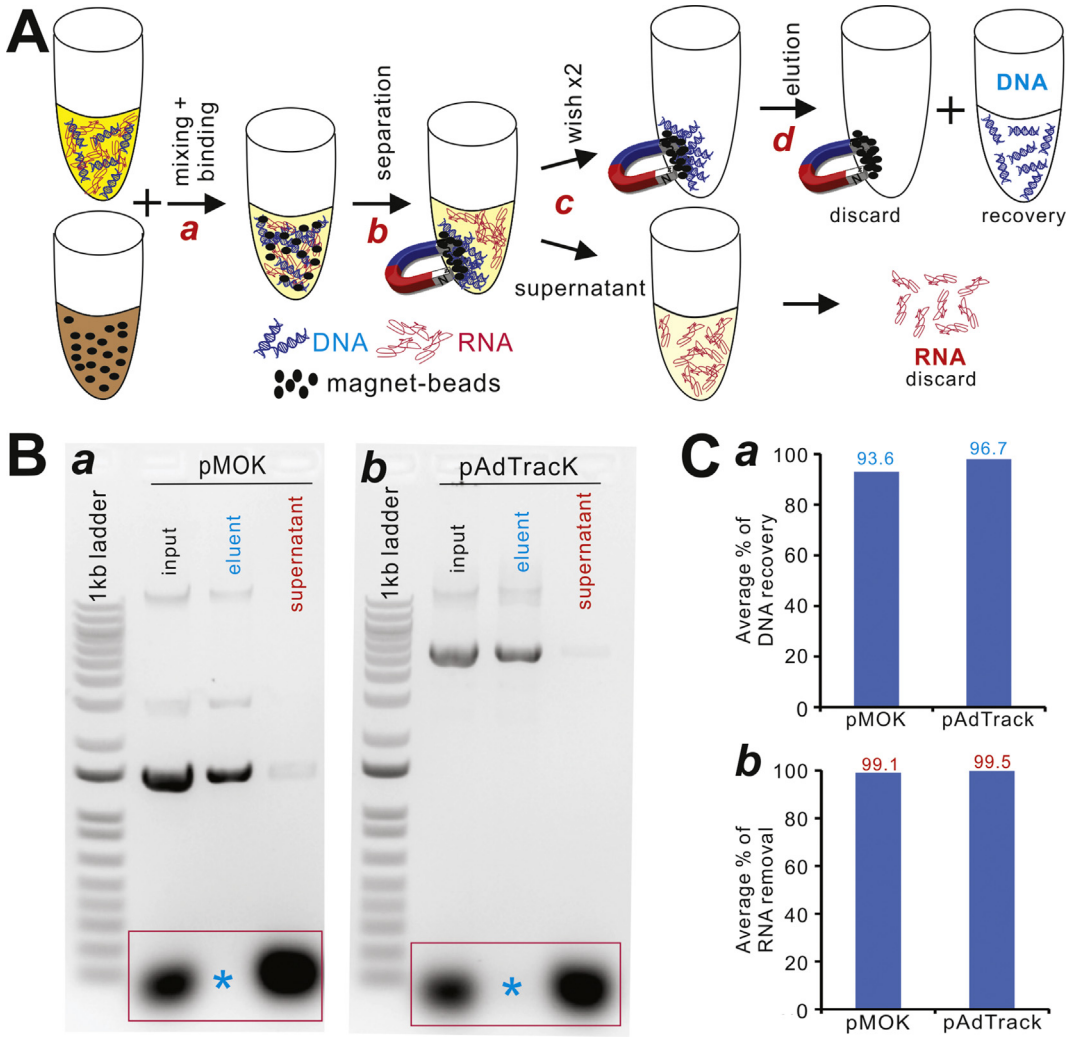

Figure 2 Bacterial RNA in pDNA preparations can be completely depleted by using size selection magnetic beads (SSMBs). **(A)** The schematic representation of the RNA depletion from pDNA process using SSMBs. The pDNA prepared from alkaline lysis protocol is mixed with the Mag-Bind SSMBs at a volume ratio of 5:2 (v/v, DNA: Beads) for 10 min at room temperature (*a*). The mixture is subjected to magnet separation (*b*) and the RNA-containing supernatant is discarded, while DNA-bound beads are washed with 70% ethanol twice (*c*). After air-dry for 60 s, the pDNA is eluted from the beads with a desired volume (20—100 μl) of ddH_2O for any downstream use (*d*). **(B)** A complete removal of contaminating bacterial RNA in pDNA preps. DH10B cells transformed with pMOK (*a*) or pAdTrack (*b*) were grown overnight in 2 ml LB/Kan culture and subjected to alkaline lysis miniprep procedure. The miniprep pDNA was dissolved in 40 μl ddH_2O, mixed with 16 μl Mag-Bind beads, and followed through the process outlined in **(A)**. One tenth of the eluted miniprep pDNA was analyzed on 1% agarose gels, along with the same proportions of respective input samples and the discarded supernatants. The red boxes indicate the presence of bacterial RNA, while the blue asterisks indicate the absence of bacterial RNA. Representative images are shown. **(C)** Quantitative assessment of the DNA recovery (*a*) and RNA removal (*b*) efficiencies of the pDNA purification approach with the SSMBs.

An ideal method for depleting bacterial RNA from pDNA preps should be simple, efficient, reliable, reproducible, and cost-effective, so it can be easily adapted for most common experiments. Here, we developed a simple and efficient RNA depletion protocol using the SSMB Mag-Bind beads. In this proof-of-principle study, we demonstrated that the Mag-Bind-based SSMB method was user-friendly, highly reproducible, and efficient in removing contaminating bacterial RNA from pDNA preps. Based on the results from gene transfection and bacterial colony formation assays, we demonstrated that the quality and integrity of the pDNA purified from the Mag-Bind SSMB method was superior to that of the pDNA samples cleaned up with RNase A digestion and/or commercial plasmid affinity purification kits.

In summary, in order to overcome the technical challenge in the depletion of contaminating bacterial RNA in pDNA preparations, we developed and validated a simplified and inexpensive RNA depletion method by using the Mag-Bind SSMBs. We demonstrate that, compared with RNase A digestion and commercial plasmid affinity purification kits, the SSMB method was user-friendly, reproducible, and highly efficient in removing contaminating RNA from pDNA minipreps. We

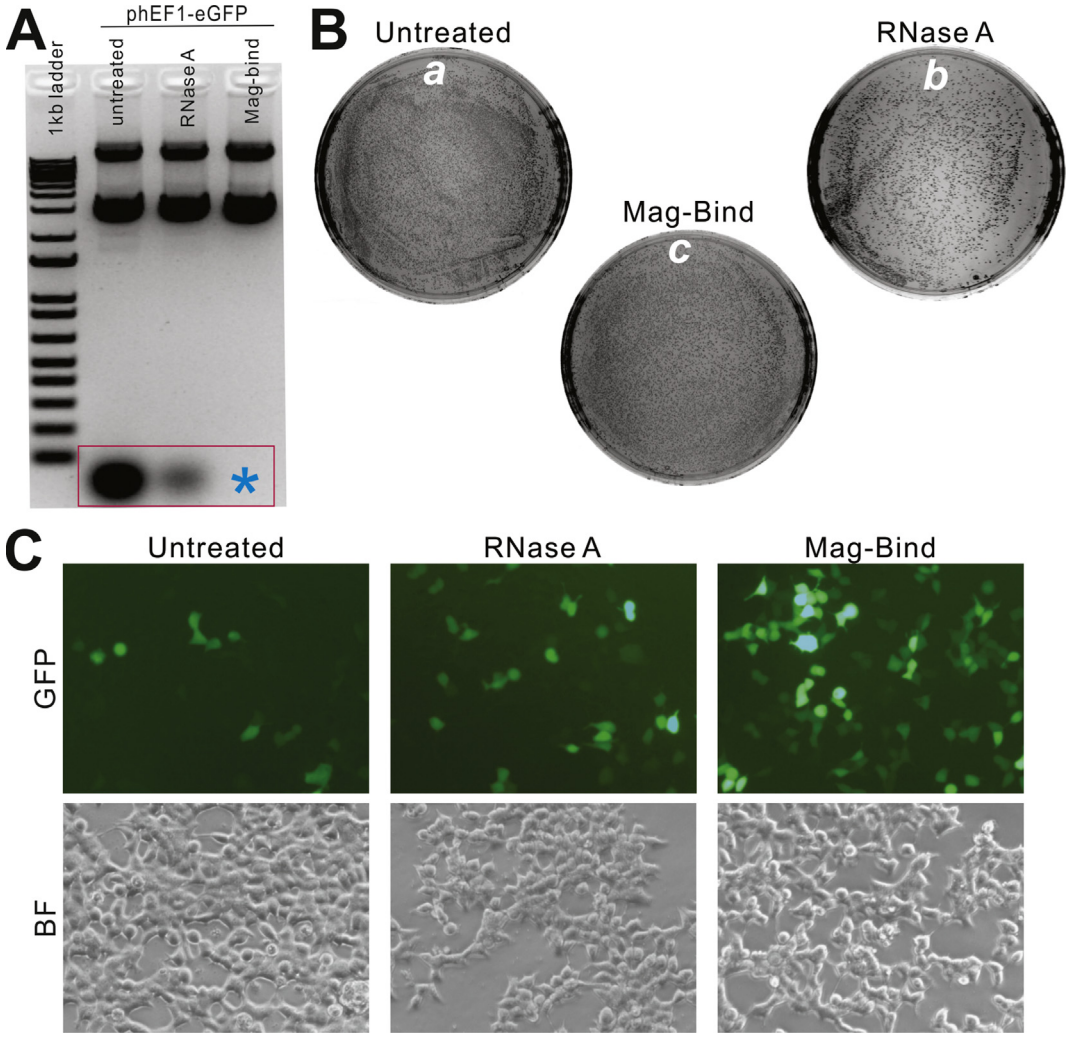

Figure 3 The SSMB purification process preserves pDNA integrity. **(A)** The homemade plasmid phEF1-eGFP (6.9 kb) was isolated from the alkaline lysis large-scale pDNA isolation protocol and subjected to different RNA removal treatments. The red box indicates the presence of bacterial RNA, while the blue asterisk indicates the absence of bacterial RNA. **(B)** Colony forming efficiency. Approximately 5 μg pDNA was untreated (*a*), digested with RNase A for 60 min (*b*), or RNA depleted with Mag-Bind beads (*c*), and transformed into DH10B cells by electroporation, and 10% of the transformation mix was plated onto replicates of LB/Amp plates. Representative images from each treatment are shown. **(C)** The effect of residual RNA on transfection efficiency in mammalian cells. Subconfluent HEK-293 cells were seeded in 12-well cell culture plates and transfected with 10% of one standard phEF1-eGFP miniprep, either untreated, or treated with RNase A digestion or Mag-Bind SSMB depletion. Both bright field (BF) and green fluorescence (GFP) images were recorded at 48 h post transfection. Representative images are shown.

also demonstrated that the SSMB method completely depleted contaminating RNA from large-scale purified pDNA samples. Comparative studies from gene transfection and bacterial colony formation assays revealed that pDNA purified from the SSMB method had superior quality and integrity to the pDNA samples cleaned up with RNase A digestion and/or commercial plasmid affinity purification kits. Furthermore, the Mag-bind-based SSMB method costs only about 5—10% of most commercially available plasmid purification kits. Therefore, the reported SSMB method may be a valuable tool to deplete contaminating bacterial RNA for routine pDNA preparations.

Compliance with ethics requirements

The authors declare that they have complied with all ethics requirements.

Conflict of Interests

The authors declare that they do not have any competing conflicts of interest.

Acknowledgements

The reported work was supported in part by research grants from the China Postdoctoral Science Foundation (2019M663446 to ZZ), and the Postdoctoral Program of the Natural Science Foundation of Chongqing, China (cstc2019jcyj-bsh0006 to ZZ). WW was supported by the Medical Scientist Training Program of the National Institutes of Health (T32 GM007281). This project was also supported in part by The University of Chicago Cancer Center Support Grant (P30CA014599) and the National Center for Advancing Translational Sciences of the National Institutes of Health through Grant Number UL1 TR000430. TCH was supported by the Mabel Green Myers Research Endowment Fund and The University of Chicago Orthopaedics Alumni Fund. Funding sources were not involved in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

References

1. Ismail R, Allaudin ZN, Lila MA. Scaling-up recombinant plasmid DNA for clinical trial: current concern, solution and status. *Vaccine*. 2012;30(41):5914—5920.
2. Stadler J, Lemmens R, Nyhammar T. Plasmid DNA purification. *J Gene Med*. 2004;6(Suppl 1):S54—S66.
3. Tan SC, Yiap BC. DNA, RNA, and protein extraction: the past and the present. *J Biomed Biotechnol*. 2009;2009, e574398.
4. Price CW, Leslie DC, Landers JP. Nucleic acid extraction techniques and application to the microchip. *Lab Chip*. 2009; 9(17):2484—2494.
5. Breyer B, Jiang W, Cheng H, et al. Adenoviral vector-mediated gene transfer for human gene therapy. *Curr Gene Ther*. 2001; 1(2):149—162.
6. Lee CS, Bishop ES, Zhang R, et al. Adenovirus-mediated gene delivery: potential applications for gene and cell-based therapies in the new era of personalized medicine. *Genes Dis*. 2017;4(2):43—63.
7. Hardee CL, Arevalo-Soliz LM, Hornstein BD, et al. Advances in non-viral DNA vectors for gene therapy. *Genes*. 2017;8(2), e65.
8. Ali N, Rampazzo RCP, Costa ADT, et al. Current nucleic acid extraction methods and their implications to point-of-care diagnostics. *BioMed Res Int*. 2017;2017, e9306564.
9. Dahm R. Friedrich miescher and the discovery of DNA. *Dev Biol*. 2005;278(2):274—288.
10. Busso D, Delagoutte-Busso B, Moras D. Construction of a set Gateway-based destination vectors for high-throughput cloning and expression screening in Escherichia coli. *Anal Biochem*. 2005;343(2):313—321.
11. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*. 1979; 7(6):1513—1523.
12. Feliciello I, Chinali G. A modified alkaline lysis method for the preparation of highly purified plasmid DNA from Escherichia coli. *Anal Biochem*. 1993;212(2):394—401.
13. Feng T, Li Z, Jiang W, et al. Increased efficiency of cloning large DNA fragments using a lower copy number plasmid. *Biotechniques*. 2002;32(5):992—994, 996 passim.
14. Jiang W, Zhou L, Breyer B, et al. Tetracycline-regulated gene expression mediated by a novel chimeric repressor that recruits histone deacetylases in mammalian cells. *J Biol Chem*. 2001;276(48):45168—45174.
15. Zhou L, An N, Jiang W, et al. Fluorescence-based functional assay for Wnt/beta-catenin signaling activity. *Biotechniques*. 2002;33(5):1126—1128, 1130, 1132 passim.
16. Cuchillo CM, Nogués MV, Raines RT. Bovine pancreatic ribonuclease: fifty years of the first enzymatic reaction mechanism. *Biochemistry*. 2011;50(37):7835—7841.
17. Fan J, Feng Y, Zhang R, et al. A simplified system for the effective expression and delivery of functional mature microRNAs in mammalian cells. *Canc Gene Ther*. 2020;27(6): 424—437.
18. Yan S, Zhang R, Wu K, et al. Characterization of the essential role of bone morphogenetic protein 9 (BMP9) in osteogenic differentiation of mesenchymal stem cells (MSCs) through RNA interference. *Genes Dis*. 2018;5(2):172—184.
19. Zeng Z, Huang B, Wang X, et al. A reverse transcriptase-mediated ribosomal RNA depletion (RTR2D) strategy for the cost-effective construction of RNA sequencing libraries. *J Adv Res*. 2020;24:239—250.
20. Zeng Z, Huang B, Huang S, et al. The development of a sensitive fluorescent protein-based transcript reporter for high throughput screening of negative modulators of lncRNAs. *Genes Dis*. 2018;5(1):62—74.
21. Wang X, Yuan C, Huang B, et al. Developing a versatile shotgun cloning strategy for single-vector-based multiplex expression of short interfering RNAs (siRNAs) in mammalian cells. *ACS Synth Biol*. 2019;8(9):2092—2105.