Harran Üniv Vet Fak Derg, 2022; 11 (2): 154-161 *Research Article*
DOI:10.31196/huvfd.1151456

Optimization of High Concentration Plasmid DNA for Use in COVID-19 mRNA Vaccine Development: Comparison of Between Alkaline Lysis Method and Commercial Kit Results

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Geliş Tarihi: 31.07.2022 **Kabul Tarihi: 25.08.2022**

Abstract: While forming the stable IVT mRNA molecule, high concentration and purity plasmid DNA must be obtained to ligase the ORF antigen sequence initially copied from the plasmid DNA with the UTR regions. In this study, in the stage of creating the mRNA molecule, which is the first step of the COVID-19 mRNA vaccine, comparison and optimization of the pDNA containing the ORF target antigen sequence were performed as a result of isolation with alkaline lysis method and commercial kit. Plasmid DNA bacteria containing the target antigen ORF sequence were grown under appropriate conditions. Plasmid DNA was isolated by commercial kit and alkaline lysis method from bacterial cultures stopped at different OD600 nm values (0.02-0.05, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5). After the obtained pDNAs were visualized on an agarose gel, their purity and concentration were measured by spectroscopic measurement. After the stab culture is resuscitated in SOC medium, bands are formed in a single form after isolation with the kit, and in multiple forms (linear, supercoiled, circular) after pDNA isolation by alkaline lysis method. The ideal OD600 nm for both methods was 0.3-0.4. As a result of isolation with the kit, higher purity on the contrary low concentration pDNA was obtained. The ideal OD600 nm value is a critical parameter that affects the concentration and purity of pDNA. The alkaline lysis method is a cheap and powerful technique that can be used as an alternative for mRNA vaccine development compared to kit isolation.
Keywords: *Alkaline lysis, Isolation, Kit, mRNA vaccine, Plasmid DNA.*

COVID-19 mRNA Aşı Geliştirilmesinde Kullanılacak Olan Yüksek Konsantrasyondaki Plazmid DNA'sının Optimizasyonu: Alkalen Lizis Metodu ve Ticari Kit Sonuçlarının Kıyaslanması

Özet: Kararlı IVT mRNA molekülü oluşturulurken başlangıçta plazmid DNA'sından kopyalanan ORF antijen sekansının UTR bölgeleri ile ligaze edilebilmesi için yüksek konsantrasyon ve saflıkta plazmid DNA'nın elde edilmesi gerekmektedir. Bu çalışmada, COVID-19 mRNA aşısının ilk basamağı olan mRNA molekülünün oluşturulması adımında, ORF hedef antijen sekansını içeren pDNA'nın alkalen lizis method ve ticari kit ile izolasyonu sonucundaki kıyaslama ve optimizasyonu yapılmıştır. Hedef antijen ORF sekansını içeren plazmid DNA bakterisi uygun üreme koşullarında çoğaltılmıştır. Farklı OD600 nm değerlerinde (0.02-0.05, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5) durdurulan bakteri kültürlerinden ticari kit ve alkalen lizis yöntemi ile plazmid DNA izolasyonu yapılmıştır. Elde edilen pDNA'lar bir agaroz jelde görüntülendikten sonra, spektroskopik ölçüme tabi tutularak saflığı ve konsantrasyonu ölçülmüştür. Stab kültür SOC medium içerisinde canlandırıldıktan sonra, kit ile izolasyon sonrasında tek formda, alkalen lizis metodu ile pDNA izolasyonu sonrasında birden çok formda (lineer, süper kıvrımlı, sirküler) bant oluşur. Her iki yöntem için de ideal OD600 nm 0.3-0.4 olarak bulunmuştur. Kit ile izolasyon sonucunda daha yüksek saflıkta ancak düşük konsantrasyonda pDNA elde edilmiştir. İdeal OD600 nm değeri, pDNA'nın konsantrasyonunu ve saflığını etkileyen önemli bir parametredir. Alkalen lizis yöntemi, kit izolasyonuna kıyasla mRNA aşı geliştirmeye alternatif olarak kullanılabilecek ucuz ve güçlü bir tekniktir.
Anahtar Kelimeler: *Alkalen lizis, İzolasyon, Kit, mRNA aşısı, Plazmid DNA.*

Introduction

Plasmid DNA (pDNA) (Liu, 2011) and mRNA vaccines that have been popular in recent years (Pardi et al., 2018) provide a platform that can be used for applications ranging from the prophylaxis to therapy and personalized medicine to global health solutions. Both vaccine methods draw attention because they have a faster production cycle and can carry the desired genetic information (Liu, 2019). In developing nucleic acid-based vaccines, creating a gene structure that encodes a recombinant antigen protein, instead of inactivating or weakening the

pathogen, prevents the study's potential live pathogen transmission risks.

Especially in the fight against COVID-19, mRNA vaccines have a critical place. mRNA vaccines are generally preferred because of their safety, high efficacy, ease of production and ability to be produced by IVT (with in vitro transcription) (Duran et al., 2021). mRNA vaccines are divided into two groups (i) self-amplifying (self-replicating) mRNA vaccines and (ii) conventional (non-replicating) mRNA vaccines. Although both vaccine types share a common mRNA structure, self-replicating mRNA vaccines additionally contain several sequences in the coding region for RNA replication (Kim et al., 2021). DNA-level modifications are also possible by using pDNA as a template.

Traditional IVT mRNA vaccines are highly similar to native mRNA, consisting of a 5' cap, 5' untranslated region (UTR), coding region (ORF), 3' UTR, and a poly (A) tail (Duran et al., 2021; Kim et al., 2021; Verbeke et al., 2019). A pDNA template for IVT contains at least one bacteriophage promoter, an ORF region, and a poly (d (A/T)) sequence transcribed into poly(A) (Duran et al., 2021; Schlake et al., 2019). After the linearized pDNA template is obtained, it is transcribed into mRNA with a mixture of recombinant RNA polymerase (T7, T3, or SP6) and free nucleoside triphosphates (dNTPs). Thus, the synthetic mRNA construction is formed, the dinucleotide regular cap analog m7G (5')-ppp-(5') G (Schlake et al., 2012; Konarska et al., 1984) or modified ARCA (5' cap form; Anti-reverse cap) with is included in the structure at the IVT stage (Verbeke et al., 2019). With the successful addition of these elements to the ORF, efficient binding of mRNA to ribosomes is achieved (Duran et al., 2021; Verbeke et al., 2019).

While forming a stable IVT mRNA molecule, high concentration and purity pDNA must be obtained to ligase the ORF antigen sequence copied from the DNA with the UTR regions. In this study, two various methods (alkaline lysis and commercial kit) were used for isolation and optimization at various OD600 nm values (0.02-0.05, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5) to obtain pDNA containing the mRNA ORF target antigen sequence in high purity and concentration.

Materials and Methods

Ethical approval

This study was approved by the KTO Karatay University Non-Pharmaceutical and Medical Device Research Ethics Committee (2021/005 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

Plasmid Selection: In the COVID-19 mRNA vaccine, the codon-optimized 6736 bp plasmid (Addgene pcDNA3-SARS-CoV-2-S-RBD-sfGFP, #141184) containing the target antigen (S-RBD) was preferred. In addition, a plasmid containing GFP (green fluorescent protein) was used, as fluorescent imaging was planned for later transfection steps.

Bacterial Adaptation and Stimulation of Stab Culture: The stab plasmid (Addgene Company, Cambridge, Massachusetts, USA) was taken from the culture with a sterile pipette tip/loop into SOC Outgrowth medium (New England Biolabs, NEB, Ipswich, MA) preheated to 37 °C and inoculated next to the Bunsen burner flames and in a laminar flow cabinet. SOC culture tubes were incubated at 1200 rpm for 2 hours in a shaking oven. Then, 100 µl of the stimulated stab starter culture was taken and inoculated into LB Miller antibiotic-containing medium (Ampicillin, 100 µg/ml, Sigma-Aldrich, USA). After incubation at 37 °C for 16-24 hours, the adapted bacteria were transferred to a solid LB Miller medium. At the time of transfer, 10x dilution was also made and inoculated culture Petri dishes. O/N cultured and pure clones were stored in cryotubes containing 1:1 glycerol at -80 °C.

Reproduce of Plasmids: Single colonies produced from adapted stab plasmids were inoculated in LB Miller broth medium with antibiotics (Ampicillin, 100 µg/ml, Sigma-Aldrich, USA). OD600 nm measurements were made by taking measurements at a wavelength of 600 nm in the spectrophotometer between 6-12 hours from the cultures that were shaken at 37 °C and high speed (200 rpm). Culture bottles taken on ice at various times were isolated at various OD600 nm values (0.02-0.05, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5).

Isolation of pDNA from Bacteria Using Kit: Single colony cultures were centrifuged at 2700 g for 15 min at +4 °C. Bacterial pellets were isolated with the NucleoSpin® Plasmid kit for pDNA (Macherey-Nagel™, MN) according to the manufacturer's instructions. Before the elution step, the pDNA in the spin column was washed two times with ethanol-containing wash buffer and eluted by following a two-step process. Thus, DNA suspended in the spin column was minimized.

Isolation of pDNA from Bacteria Using Alkaline Lysis Method: The following solutions were prepared for pDNA isolation by the alkaline lysis method (Green and Sambrook, 2016).

- Solution 1: 25 mM Tris-Cl (pH 8.0), 50 mM Glucose and 10 mM EDTA (pH 8.0) were mixed and filled to 100 ml with distilled water and autoclaved in 15 psi (1.05 kg/cm^2) liquid cycle for 15 minutes. Immediately after cooling, 100 mg/ml RNase A (Sigma-Aldrich, USA) was added and stored at +4 °C.

•Solution 2: 0.2 N NaOH and 1% SDS (w/v) were dissolved in 100 ml of distilled water and stored fresh at room temperature.

•Solution 3: 5 M 60 ml of potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of distilled water were mixed, and stored at +4 °C. It should be ice cold before use.

•STE Buffer: After mixing 10 mM Tris-Cl (pH 8.0), 0.1 M NaCl and 1 mM EDTA (pH 8.0), it was made up to 100 ml with distilled water and autoclaved in 15 psi (1.05 kg/cm^2) liquid cycle for 15 minutes. Immediately after cooling, it was stored at +4 °C. It should be ice cold before use.

All pellets were brought together and diluted with very cold (≤4 °C) STE buffer, and centrifuged at 2700 g for 10 minutes at +4 °C so that 1.5 ml of the culture remained. The supernatant was discarded and 100 µl of Solution 1 was added to the pellet. After gentle pipetting, 100 µl of freshly prepared 10 mg/ml lysozyme (Hibrigen Biotech R&D, Istanbul, Turkey) was added to the tube. Immediately after, 2 times volume (200 µl) of freshly prepared Solution 2 was added to the medium, and gently pipetting continued. After keeping the tubes at room temperature (25 °C) for 5-10 min, 300 µl of very cold (<4 °C) Solution 3 was added to the medium.

After mixing the tubes gently, they were incubated on ice for 10 min. The tubes were centrifuged at 12.000 g for 5 minutes at +4 °C. The supernatant was carefully taken into a pre-chilled Eppendorf tube, 200 µl of isopropanol (Merck & Co., Inc.) was added and incubated at room temperature for 10 minutes. Then, the tubes were centrifuged at 12.000 g for 15 minutes at 25 °C. Carefully discarding the supernatant, the pellet was washed with 70% ethanol (Merck & Co., Inc.) prepared with sterile nuclease-free water at room temperature, and centrifuged at 7500 g for 10 minutes at 25 °C. After ethanol evaporation, the DNA pellet was dissolved with 50 µl (according to pellet size) 1xTE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) buffer prepared with 2.0 µg/ml DNase-free RNase A (Sigma-Aldrich, USA) and stored at -20 °C.

Visualization in Agarose Gel: All obtained pDNAs were loaded onto agarose gel (1% agarose/0.5xTAE) containing Safe-Red (ABM, Inc.) dye. Marker and all samples were electrophoresed in 1xTAE (Tris-Acetic Acid-EDTA) Buffer at 200 Volts for 15-20 minutes. The bands were visualized under UV light with the Vilber Fusion FX gel documentation system (Vilber Lourmat, France).

Spectroscopic Measurement: Using the µdrop applicator (Thermo Fisher, Scientific, USA) of the MultiskanSky Microplate Spectrophotometer device, the concentration and absorbance values of pDNAs obtained by both methods were recorded at 260, 280, and 230 nm. All measurements were made in triplicate.

Statistical analysis: All statistical analyzes were performed using MATLAB® v.7.8.0 (The MathWorks Inc., Natick, Mass., USA). The p-value and mean standard error was calculated using Student's t-test.

Results

Stab bacteria is resuscitated in SOC medium:

Stab bacteria containing pcDNA3-SARS-CoV-2-S-RBD-sfGFP plasmid were first resuscitated in SOC Outgrowth medium. In the transfer at 10x dilution, more single colony formation was observed than in the undiluted group. One colony was taken from the single colonies formed and transferred to LB Miller medium. After incubation at 37 °C for 16-24 hours, bacterial colony growth was observed (Figure 1).

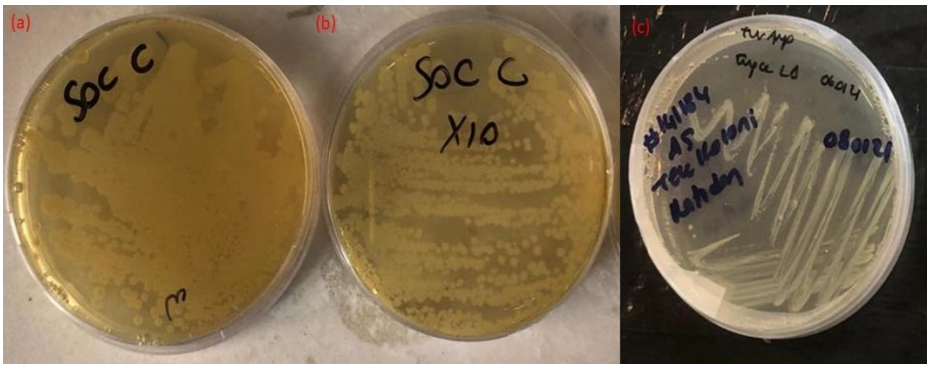

Figure 1. Image of stab bacteria containing pcDNA3-SARS-CoV-2-S-RBD-sfGFP plasmid after resuscitation in SOC Outgrowth medium. (a): Cultivation of undiluted stab bacteria. (b): Cultivation of stab bacteria at 10x dilution. (c): Single colony cultivation of stab bacteria.

Bands are formed in one form after pDNA isolation with the kit, and in multiple forms after pDNA isolation with Alkaline Lysis Method: The pDNAs isolated with the NucleoSpin® Plasmid kit and the pDNAs isolated by the alkaline lysis method were visualized on an agarose gel. After isolation with the kit, there is a single pDNA band of 6736 bp in the gel (Figure 2). However, in pDNA samples isolated by the

alkaline lysis method, more than one form (nicked, linear, supercoiled, circular) band was displayed on agarose gel (Figure 3).

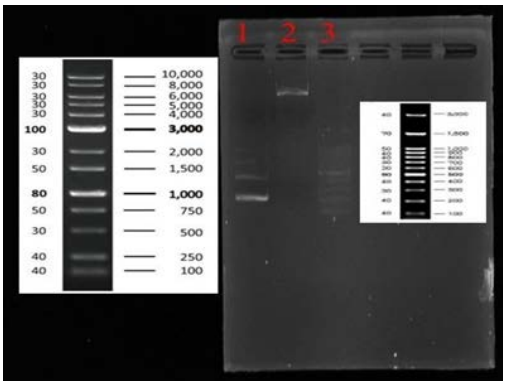

Figure 2. Single band (6736 bp) image of pDNA isolated using the NucleoSpin® Plasmid kit on agarose gel (1st and 3rd well marker, 2nd well pDNA sample).

The ideal OD600 nm value is an important parameter that affects the concentration and purity of pDNA: The concentrations of the samples were also measured after the isolations were made separately at different OD600 nm values. Accordingly, for both isolation methods, the OD600 nm value was in the range of 0.3-0.4, and the maximum concentration of pDNA was obtained. The OD600 nm value was in the range of 0.3-0.4, followed by the concentration value of 0.4-0.5, 0.2-0.3, 0.1-0.2, 0.05-0.1, 0.02-0.05, respectively. The lowest concentration value was found in the range of 0.02-0.05 for both methods (Figure 4). Looking at the A260/280 values, values close to 1.8 were found for both methods, and the OD600 nm value was the closest in the range of 0.3-0.4 (Figure 5). When the A260/230 values were examined, values closer to 2.0-2.2 values were found as a result of the isolation with the kit (Figure 6).

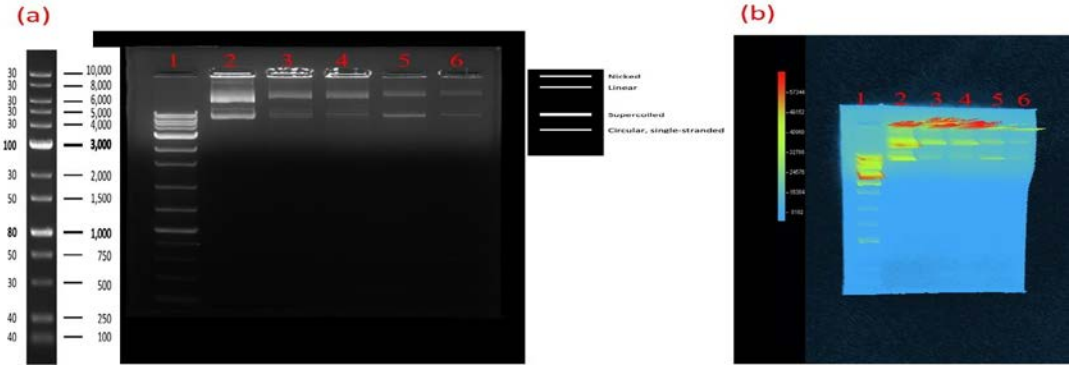

Figure 3. Multiband (nicked, linear, supercoiled, circular) image of pDNA isolated using alkaline lysis method on agarose gel (1st well marker, all following wells are samples of pDNA in different band forms). (a): Two-dimensional gel imaging. (b): Three-dimensional gel imaging.

Table 1. Spectrophotometric measurement results were obtained as a result of plasmid DNA isolation with a plasmid miniprep kit and alkaline lysis method at ideal OD600 nm 0.3-0.4.

Method	Measure	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
Kit	Measure 1	110,76	1,82	2,15
	Measure 2	111,25	1,81	2,16
	Measure 3	110,89	1,82	2,16
	Mean	110,96±0,146	1,81±0,003	2,15±0,003
Alkaline Lysis	Measure 1	2560,56	1,72	1,92
	Measure 2	2565,78	1,71	1,92
	Measure 3	2568,57	1,72	1,91
	Mean	2564,97±2,347	1,71±0,004	1,91±0,004
	p-value	<0.001	<0.001	<0.001

Isolation with the kit results in lower concentration but higher purity pDNA:

At 0.3-0.4, which is the ideal OD600 nm value, both the alkaline lysis and A260/280 purity values of the pDNAs isolated with the kit were found to be close to 1.8, according to the spectrophotometric measurement results. However, the pDNA isolated with the kit was found to be closer to the ideal DNA A260/280 ratio of ~1.8. When A260/230 values are analyzed, the pDNA isolated only with the kit is in the range of ~2.0-2.2 values, which is the ideal A260/230 ratio (Table 1). In contrast, pDNA isolated by the alkaline lysis method has an almost ~20-fold higher concentration (Figure 7). Comparisons made with the parameters specified between the methods were found to be statistically significant (p<0.001).

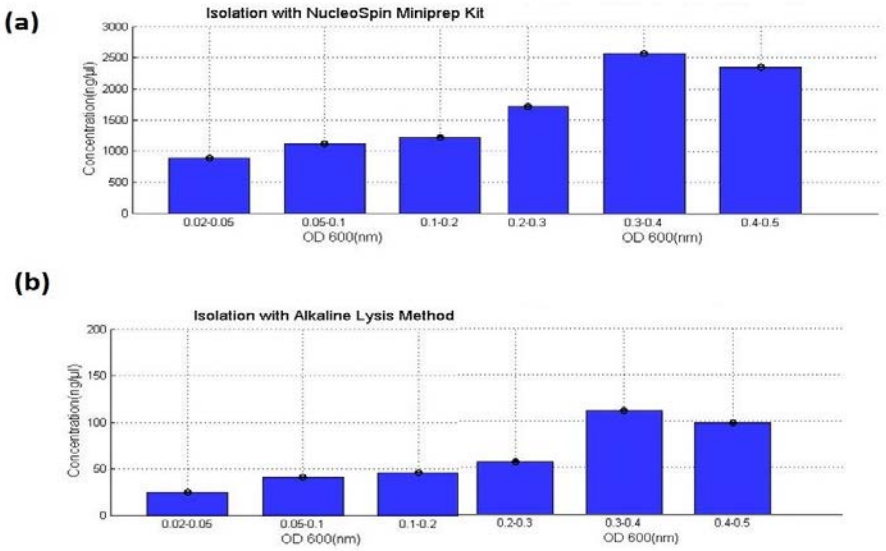

Figure 4. Graph of pDNA concentrations isolated by kit and alkaline lysis method at different OD600 nm values. (a): NucleoSpin® Plasmid kit concentration values. (b): Concentration values of alkaline lysis method.

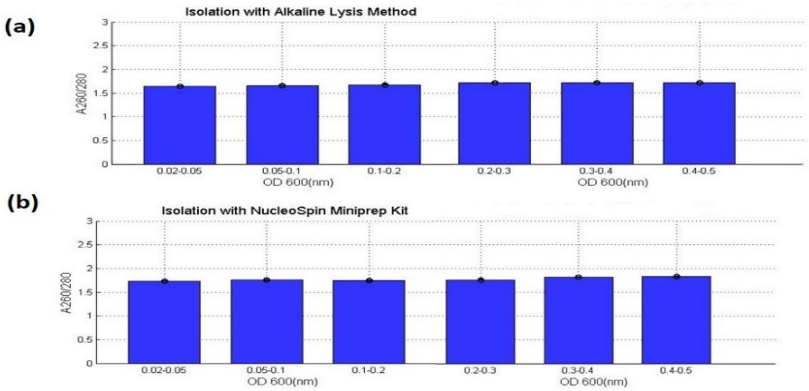

Figure 5. Graph of pDNA A260/280 values isolated by kit and alkaline lysis method at different OD600 nm values. (a): NucleoSpin® Plasmid kit A260/280 values. (b): Alkaline lysis method A260/280 values.

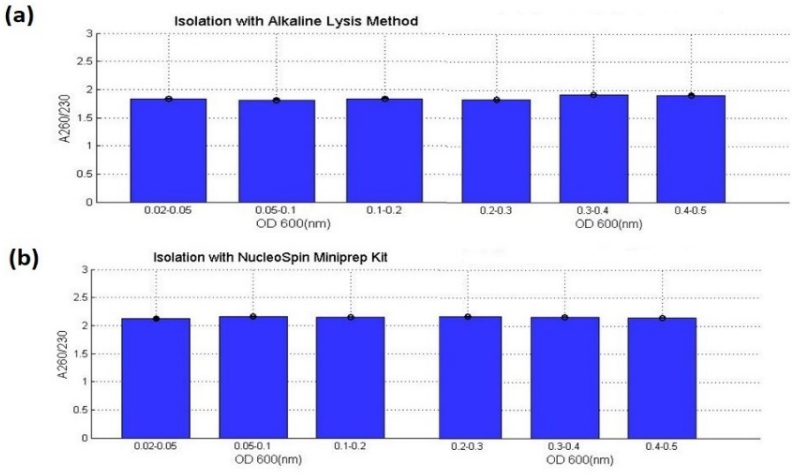

Figure 6. Graph of pDNA A260/230 values isolated by kit and alkaline lysis method at different OD600 nm values. (a): NucleoSpin® Plasmid kit A260/230 values. (b): Alkaline lysis method A260/230 values.

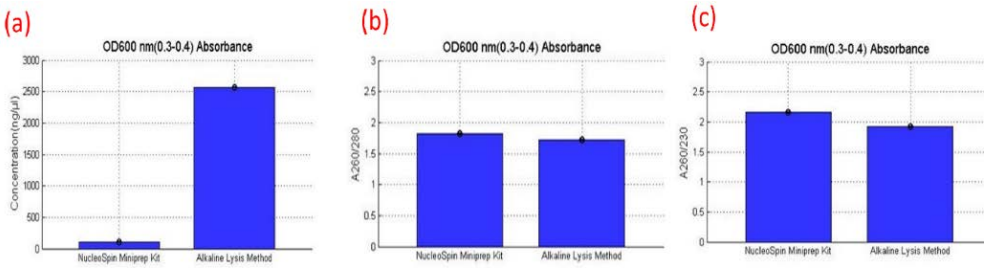

Figure 7. Graph comparing (a): concentration, (b): A260/280, (c): A260/230 values of pDNA isolated by the kit and alkaline lysis method at ideal OD600 nm (0.3-0.4).

Discussion and Conclusion

Since the ’90s, vaccine studies with nucleic acids targeting cancer and infectious diseases have been ongoing (Villarreal et al., 2013; Yang et al., 2014). While mRNA vaccines are developed *in vitro*, the target antigen is frequently obtained on pDNA (Pardi and Weissman, 2017). In particular, using pDNA templates offers researchers a safe option to minimize the risk of infection (Anand and Stahel, 2021). Synthetic mRNA can typically be obtained by in vitro transcription (IVT) of plasmid DNA (pDNA) using a bacteriophage RNA polymerase (Krieg and Melton, 1984; Pascolo, 2006). Thus, the first step in mRNA production is the preparation of pDNA (Schlake, 2012), and it may also seem like the production of the mRNA molecule requires more effort than the production of pDNA. This is undoubtedly related to the quick and easy obtaining of high-quality pDNA.

pDNA contains tiny amounts of bacterial genomic DNA and varying proportions of three forms of pDNA (supercoiled, loose circle, or linear). Therefore, reproducible preparation, ie optimization, of pDNA in pure and invariant form is required to develop a reliable mRNA vaccine (Schlake, 2012). In this study, supercoiled pDNA, which is the most optimized form of pDNA isolated with the alkaline lysis method and kit, was obtained by the alkaline lysis method. However, obtaining pDNA in other forms besides the supercoiled form requires recovery of the supercoiled DNA from the agarose gel. Looking at the literature, the supercoiled form of pDNA resulted in higher transfection activity than nicked circular or linear DNA (Hirose et al., 1985). Moreover, the amount of supercoiled pDNA form in the first step of the mRNA preparation step indicate pDNA stability and activity (Chancham and Hughes, 2001). However, during DNA isolation, the DNA topology can be altered by shear stress to transform the supercoiled plasmid into open circular (single-stranded notched), linear, or even fragmentary (Adami et al., 1998). This conformational change may affect the transfection efficiency of pDNA. During recovery from the gel, care must be taken not to damage the supercoiled pDNA.

Another parameter that determines the quality and purity of pDNA is the OD600 nm value. In the early log stage, the OD600 nm range of 0.3-0.4 is generally accepted as the ideal value (Zou et al., 2012). Considering the results, the ideal OD600 nm value was found to be 0.3-0.4 in this study, which is consistent with the literature. Increased bacterial count in the late exponential growth stage of bacteria and higher concentration were directly related, resulting in a low number of bacteria and low concentration in the early log phase. However, the OD600 nm value at which growth is stopped and isolation is started must be optimized for each plasmid and bacterial species. Because the ideal OD600 nm value is related to the structure of the origin of replication in the plasmid, the plasmid copy number, and the growth rate of the host cell.

At ideal OD600 nm and all other values, the alkaline lysis method resulted in a very high concentration of pDNA (2564.97±2.87 ng/µl) compared to the kit. The alkaline lysis method has been preferred for many years in molecular biological studies with its easy, cheap, and high-concentration product efficiency. However, its purity is slightly lower than that of mass-isolated pDNA. The purity ratio of DNA samples at 260 and 280 nm is used to determine the purity of the DNA (Glasel, 1995) and the A260/280 ratio of ~1.8 is considered “pure” DNA (Hassan et al., 2015). If the ratio of A260/280 is significantly low (≤1.6), it is possible to speak of contamination of proteins and phenol (Lucena-Aguilar et al., 2016). The A260/280 ratio (1.71±0.04) of the pDNA obtained by the alkaline lysis method was found to be lower than the purity of the pDNA isolated with the kit (1.81±0.04), however, it is not at or below the limit of phenolic contamination. Therefore, there is a tolerable relative impurity. On the contrary, the A260/280 value is far from the pure RNA value of ~2.0 (Nouvel et al., 2021), and also indicates the absence of RNA contamination.

In addition, A260/230 is the secondary spectrophotometric parameter commonly used to measure DNA purity (Aleksić et al., 2012; Usman et al., 2014), and DNAs in the ∼2.0-2.2 range are generally considered pure (Lucena-Aguilar et al., 2016). This value was found as 1.91±0.04 and 2.15±0.04 for the pDNA obtained by the alkaline lysis method and the kit, respectively. Since the rate for pDNA isolated by the alkaline lysis method is lower than the expected value, the effectiveness of contaminants such as salts, guanidine HCl, EDTA, lipids, carbohydrates or phenol can be mentioned (Stulnig and Amberger, 1994). However, this value is not a stable DNA quality indicator for our study due to the saline elution buffer used in the final isolation stage with the kit. Because in the isolation method with the kit, there may be a higher salt concentration increase than the DNA concentration in the sample. Consequently, out of two pDNA samples of the same purity, the less concentrated sample will show a lower 260/230 ratio due to the absorbance of the salts at 230 nm. Therefore, the fact that the finding obtained by the alkaline lysis method is slightly far from the ideal value does not mean it is of poor quality.

The target antigen of the recently popular immune weapon mRNA vaccines can be obtained using pDNA. Amplifying target antigen from pDNA provides a safe option for investigators and targeted specific therapies. Especially when working with infectious or zoonotic infectious agents such as COVID-19, plasmids containing the relevant gene present should be primarily preferred. On the other hand, the supercoiled form of pDNA is ideal for vaccine and molecular biology applications. The alkaline lysis method can be preferred if it is desired to obtain cheap/easy pDNA in high concentration and supercoiled form. According to our study, the alkaline lysis method resulted in pDNA of relatively less purity, but a very high concentration than the commercial kit. Before using pDNA samples, DNA purity and quality must be determined by conventional methods. It should also be noted that the excellent OD600 nm value is also essential for pDNA quality.

Similarity Rate

We declare that the similarity rate of the article is 14% as stated in the report uploaded to the system.

Ethical Approval

This study was approved by the KTO Karatay University Non-Pharmaceutical and Medical Device Research Ethics Committee (20.12.2021, 2021/005 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Funding

This study was supported by *Kocaeli University Scientific Research Projects Coordination Office,* (Project Number: 2021/2663).

Acknowledgment

The study was generated from part of the first author's Ph.D. thesis entitled “Development of Lipid Nanoparticle Coated Uniquely Designed mRNA Vaccine for SARS-CoV-2 (COVID-19) Spike Receptor Binding Domain (RBD) Antigen”.

Author Contributions

Motivation / Concept: TD, NC, NK
Design: TD, NC
Control/Supervision: TD, NC, NK
Data Collection and / or Processing: TD, NC, NK, SK
Analysis and / or Interpretation: TD, NC, NK, SK
Literature Review: TD, NC, NK
Writing the Article: TD
Critical Review: TD, NC, NK

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