ENHANCING IN-HOUSE PLASMID DNA EXTRACTION USING CUSTOMIZED GLASS FIBER MEMBRANE

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ENHANCING IN-HOUSE PLASMID DNA EXTRACTION USING CUSTOMIZED GLASS FIBER MEMBRANE

By

JAZMI AIMAN BIN JAMEL

Dissertation submitted in partial

fulfilment of the requirements of the

degree of

Bachelor of Health Science (Honours) (Biomedicine)

DEDICATION

I would like to dedicate this dissertation to my parents and family, whose unwavering support and encouragement have been the cornerstone of my academic journey.

DECLARATION

This is to certify that the dissertation entitled ENHANCING IN-HOUSE PLASMID DNA EXTRACTION USING CUSTOMIZED GLASS FIBER MEMBRANE is the bona fide record of research work done by Mr Jazmi Aiman Bin Jamel during the period from August 2024 to December 2024 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

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CERTIFICATE

I hereby declare that this dissertation is the result of my own investigation, except where otherwise stated and duly acknowledged. I also declare that it has not been previously and concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research, and promotional purpose.

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JAZMI AIMAN BIN JAMEL

10/3/2025
DATE

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LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

×	Multiply
±	Plus-minus sign
<	Smaller than
>	Greater than
%	Percentage
°C	Degree Celsius
µg	Microgram
µL	Microlitre
A	Absorbance
Amp	Ampicillin
bp	Base pair
DF	Dilution factor
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia* – 'for example'
etc.	*et cetera*
g	Gravity force
g	Gram
i.e.	*id est* – 'that is'
Inc	Incorporated
kb	Kilobase
kbp	Kilobase pair
LB	Luria-Bertani
M	Molar
mL	Millilitre
mM	Millimole
ng	Nanogram
nm	Nanometre
OD	Optical density

PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Potential of hydrogen
RNA	Ribonucleic acid
rpm	Revolution per minute
s	Second
SDS	Sodium dodecyl sulphate
TAE	Tri-acetate-EDTA
USA	United States
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Voltage

LIST OF APPENDICES

MENINGKATKAN PENGEKSTRAKAN DNA PLASMID DALAMAN DENGAN MENGGUNAKAN MEMBRAN GENTIAN KACA TERSUAI

ABSTRAK

Pengekstrakan dan pemurnian DNA plasmid daripada sel bakteria adalah asas kepada banyak proses bioteknologi. Plasmid yang diekstrak boleh digunakan untuk pelbagai tujuan, termasuk penyelidikan genetik, analisis forensik, dan diagnosis perubatan. Kit pengekstrakan yang dibangunkan secara dalaman, yang lebih kos efektif dan mampu menghasilkan keputusan yang setanding dengan kit komersial, masih giat dikaji oleh ramai penyelidik. Dalam kajian ini, kami bertujuan untuk membangunkan kit pengekstrakan plasmid berasaskan membran gentian kaca secara dalaman dan membandingkan hasil serta ketulenannya dengan kit komersial. Kit rujukan komersial yang digunakan dalam kajian ini ialah kit pengekstrakan plasmid Qiagen. Lajur buatan sendiri dengan saiz liang membran yang berbeza (0.22 µm, 0.45 µm, dan 0.80 µm) serta bilangan lapisan membran (1, 2 dan 6 lapisan) telah dipasang sendiri. Saiz liang membran 0.80 µm menghasilkan kepekatan DNA tertinggi. Lajur yang dipasang dengan 2 lapisan membran GF-0.80 menghasilkan jumlah DNA tertinggi semasa pengekstrakan DNA. Kaedah membran gentian kaca dalaman yang dioptimumkan akhirnya menunjukkan hasil dan ketulenan yang setanding ($P>0.05$) dengan kit pengekstrakan DNA plasmid Qiagen komersial.

ENHANCING IN-HOUSE PLASMID DNA EXTRACTION USING CUSTOMIZED GLASS FIBER MEMBRANE

ABSTRACT

Plasmid DNA extraction and purification from bacterial cells are the foundations of many biotechnological processes. The extracted plasmid can be used for a variety of purposes, including genetic research, forensic analysis, and medical diagnosis. In-house extraction kits that are more cost effective and can produce comparable results to the commercial ones are still being pursue by many researchers. In this study we aimed to develop an in-house glass fiber membrane based plasmid extraction kit and compare its yield and purity with commercial kit. The commercial reference kits used in this study will be the Qiagen plasmid extraction kit. The in-house columns with different pore sizes (0.22 µm, 0.45 µm, and 0.80 µm) and number of membrane layers (1, 2 and 6 layers) were self-assembled. The membrane pore size of 0.80 µm yield the highest DNA concentration. the column assembled with 2 layers of GF-0.80 membrane produced highest DNA yield during DNA extraction. The final optimized in-house glass fiber membrane method showed comparable yield and purity ($P>0.05$) results to the commercial Qiagen plamid DNA extraction kits.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Many different microorganisms, most notably bacteria, have plasmids, which are extra-chromosomal tiny circular DNAs that can replicate without the host's help. During host cell division, they can spread vertically to daughter cells and, in certain situations, horizontally to other bacteria (Dewan & Uecker, 2023). Despite the fact that are not vital components of the host, plasmids can transfer a variety of genes that improve their survival, including those related to reproduction, drug tolerance, and toxins (Barreto et al., 2014). Because of its adaptability and critical uses in fields including genetic engineering, gene therapy, and various other fields of research, plasmid DNA is essential in molecular biology. Therefore, effective plasmid extraction is essential in molecular biology and genetic research.

Effective techniques for isolating the plasmid population directly from the sample under study are necessary for molecular biology research (Delaney et al., 2018). Many methods used in biotechnology rely primarily on the extraction and purification of plasmid DNA from bacterial cells. Various plasmid extraction procedures have been developed over time, including the alkaline lysis method, boiling lysis method, silica column purification, and magnetic bead purification. However, because commercial plasmid extraction kits, which mostly use silica-based columns, are frequently expensive, academic and research institutions with limited funding may not be able to afford them.

Previously, nucleic acid extraction and purification were difficult, time-consuming, labour-intensive, and had a limited overall yield (Tan & Yiap, 2009). In search for more affordable plasmid purification methods is leading to the study of more alternative components and techniques. Glass fibre membranes are one such method that can be used in place of conventional silica columns in plasmid extraction procedures. In terms of DNA yield and purity, glass fibre membranes perform comparably and are more affordable.

Although glass fibre membranes have the potential to be valuable for plasmid purification, there is lack of empirical evidence to demonstrate their efficacy in various settings. The yield and purity of the extracted plasmid DNA are also greatly influenced by the optimisation of the glass fibre membrane's pore size. Reducing pore sizes may promote DNA binding and minimise contaminants (Morán et al., 1999), but the degree of these improvements is not clear.

1.2 Problem Statement

One of the significant challenges in plasmid purification lies in the financial burden associated with the use of commercial kits. While practical and effective, these kits are often expensive, making them less accessible for researchers working with limited budgets, particularly in academic or low-resource settings. In-house kits are a cost-effective alternative but may lack thorough documentation and standardised quality assurance. Hence, researchers are constantly looking for low-cost alternatives to commercial kits that do not compromise the extracted DNA's purity. However, finding the ideal balance between cost-effectiveness and DNA quality remains difficult. Furthermore, there is a significant lack of in-house empirical data to support the effectiveness of alternative plasmid purification techniques. While numerous procedures are available in the scientific literature, their reliability and efficacy frequently vary based on laboratory conditions, equipment, and reagents. This disparity highlights the necessity for performing systematic studies to assess and improve these techniques as well as make sure they fulfil the requirements for present molecular biology applications. Other than that, given the lack of such modular kits on the market, educational institutions are currently dependent on commercially pre-assembled products that could not fully meet their objectives for instruction and training. The upcoming generation of scientists could gain a better grasp of plasmid purification methods through developing kits that are both economical and adaptable for educational purposes.

1.3 Rationale of Study

Glass fibre membranes are used in this study considering they are inexpensive and accessible, making them a financially sensible choice for both research and teaching. Selecting inexpensive materials makes experiments more accessible and sustainable, particularly in environments with limited resources. By comparing the plasmid yields and purity from the commercial and in-house kits, we want to determine any possible differences in extraction efficiency and present insightful information to researchers looking for less expensive options without compromising quality. With continuous collection of empirical data, this in-house method can be upgraded and improved from time to time. If the efficiency of in-house kits is comparable to that of commercial kits, this will give various parties an additional option. Additionally, the study makes use of self-assembled columns, which provide trainees and students with hands-on training. This makes it possible for training and educational activities to involve greater psychomotor practice.

1.4 Significance of Study

This study is crucial as it aims to create an accessible and affordable method for extracting plasmid DNA using glass fibre membranes with different pore size. It allows researchers and academic institutions overcome financial challenges by minimising dependency on expensive commercial kits. The comparison of performance between the in-house column and commercial kits (Qiagen) for DNA extraction in this study will have significant effects on cost efficiency of research studies in laboratories. If in-house columns could perform comparably to other commercial extraction kits (QIAGEN), it could result in significant reductions in costs and the researchers will have broader choices of extraction kits. Other than that, incorporating self-assembled columns into plasmid DNA extraction protocols provides not only a cost-effective option, but also an ideal educational tool. Unlike commercial pre-assembled kits, which require minimal user participation, self-assembled columns allow students and trainees to become actively involved in each step of the purification process promoting critical thinking and technical abilities. Furthermore, the study provides valuable empirical data that supports in-house methods, therefore promoting their broad use and standardisation. By optimising DNA purification processes, the study improves sustainability and inclusivity, allowing more people to participate in molecular biology research and encouraging advancement in DNA extraction methods.

1.5 Research Objectives

1.5.1 General Objectives

To develop glass fiber (GF) membrane-based column for enhanced plasmid DNA extraction.

1.5.2 Specific Objectives

1. To mass produce of *E.coli* Plyss /pET14b-EhCL.
2. To determine the optimum GF membrane pore size for plasmid DNA extraction.
3. To determine the optimum GF membrane layer number for plasmid DNA extraction.
4. To compare the plasmid DNA yield and purity extracted between commercial kit and optimized glass fiber in-house column.

1.6 Hypothesis

1.6.1 Null Hypothesis

There will be no significant changes between the DNA yield and purity from different GF membrane pore size, GF membrane layers and Qiagen kits.

1.6.2 Alternative Hypothesis

There will be significant changes between the DNA yield and purity from different GF membrane pore size, GF membrane layers and Qiagen kits.

CHAPTER 2

LITERATURE REVIEW

2.1 Fundamental of Plasmid DNA in Molecular Biology

The Nobel Prize laureate Joshua Lederberg proposed the term "plasmid" originally to refer to any extrachromosomal genetic particle (Lederberg, 1998). Plasmids are extrachromosomal circular and double-stranded DNA molecules that make about 1% to 5% of the bacterial chromosome which predominantly found in the cytoplasm of bacteria and particular microorganisms. Plasmids are capable of replicating without the help of chromosomal DNA (Rajkumar *et al*., 2020). They can replicate independently within a host cell and range in size from a few kilobases to hundreds of kilobases. Plasmids can greatly improve a bacterium's ability to adapt and survive in a variety of environments. Plasmids primarily contain bacterial genes linked to virulence factors, conjugation, metabolic breakdown, resistance to metal ions and antibiotics, and other adaptive processes (da Silva et al., 2019a).

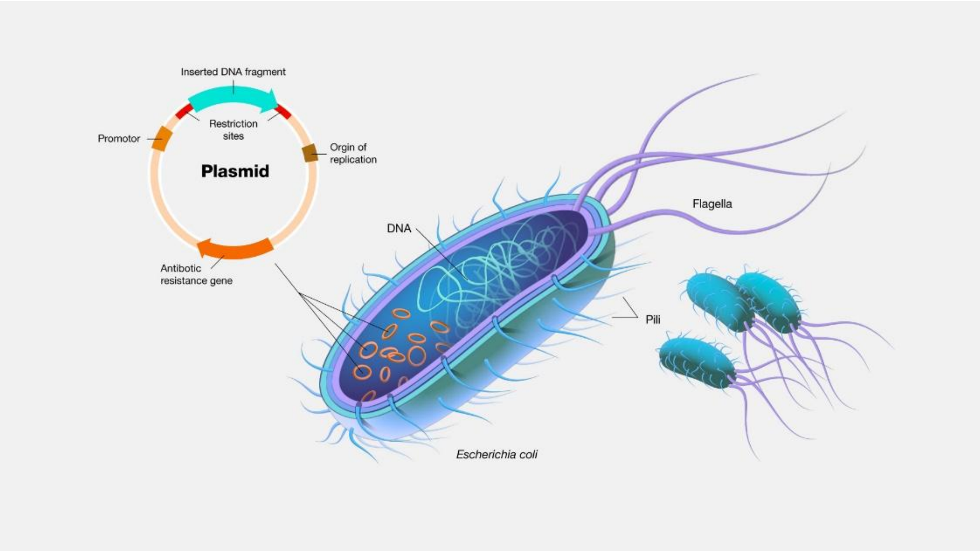

Figure 2.1 Structure of plasmid

The two main reasons plasmids were significant were because of the presence of antibiotic resistance gene and horizontal cell transfer. Through the insertion of an antibiotic resistance gene into a plasmid, researchers can assure that, when cultured in the presence of the related antibiotic, only bacteria that successfully transferred the plasmid remain alive. The isolation and spread of genetically modified organisms depend heavily on this selective pressure. Horizontal gene transfer (HGT), which allows antibiotic resistance genes to spread throughout bacterial populations, is facilitated by plasmids. Resistance traits proliferate rapidly among colonies as a result of this transfer, which takes place through processes like conjugation, in which bacteria directly exchange plasmids (Dewan & Uecker, 2023). Researchers use Recombinant DNA techniques to insert desired genes into a plasmid. The inserted gene is duplicated when the plasmid replicates itself.

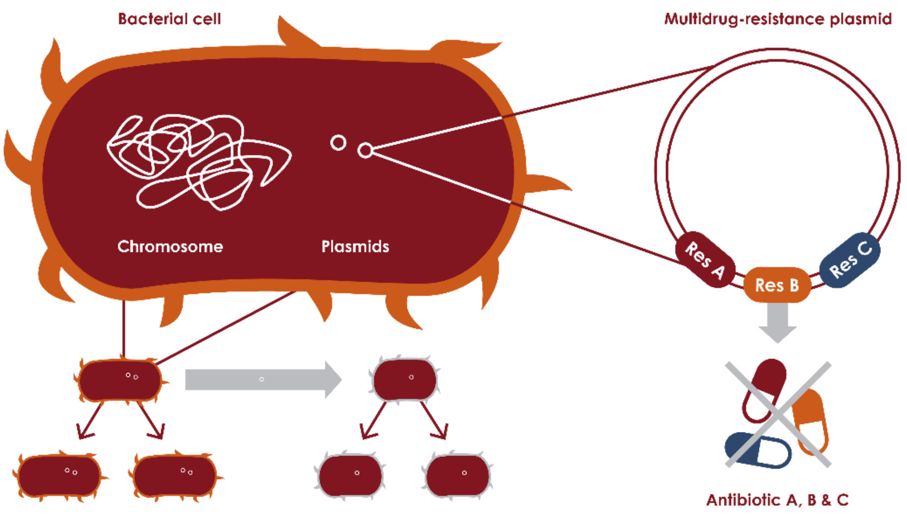

Figure 2.2 Mechanism of multidrug-resistance plasmid transfer in bacteria

Plasmids are becoming an essential instrument in biotechnology and genetic engineering because of their tiny size and simplicity of engineering for biotechnological applications. Clinical applications and medical research both heavily rely on plasmids. They are utilised in the manufacturing of vaccines, gene therapy, and therapeutic proteins. By cloning the matching gene into a plasmid and then transforming a microbial host, it was possible to produce an infinite number of proteins, which established the way for the development of a variety of products and applications that are significant to both industry and medicine (Prazeres & Monteiro, 2014). It has been proven to develop DNA vaccines using plasmids that transfer genes whose products can attract the immune system (Ferraro et al., 2011). DNA vaccines, in contrast to conventional vaccines, induce the body to internally produce antigens. This indicates that the immune system's presentation of these antigens is identical to how it responds to an infection that occurs naturally.

2.2 Techniques for Plasmid DNA Extraction

In 1869, Swiss physician Friedrich Miescher carried out the first rudimentary extraction of DNA. He introduced the name "nuclein" after unintentionally separating DNA from the nucleus while studying leukocyte proteins and discovering that this material exhibited properties that were fundamentally distinct from those of proteins (Dahm, 2005). Plasmid extraction is an essential process in molecular biology that allows for the purification of plasmid DNA from bacterial cells for subsequent applications such as cloning, sequencing, and gene expression research. Over time, various methods have been developed, each with different benefits and limitations in terms of scalability, yield, and purity. As most biotechnology applications require high purity and good integrity of DNA samples as essential components for their usage as starting materials, new plasmid DNA extraction techniques are constantly being developed (da Silva et al., 2019)(da Silva et al., 2019). The current review analyses the development of several DNA extraction techniques, including solvent-based and physical extraction techniques (Jaiswal & Tiwari, 2023).

2.2.1 Phenol-Chloroform Extraction

The phenol-chloroform method is one of the most commonly used organic extraction methods for DNA because it effectively purifies high-quality DNA among the various other methods that are available. Barker et al. introduced the phenol-chloroform DNA extraction method in 1998. This method evolved from prior organic extraction methods that used phenol to denature proteins. Later, chloroform was added to improve phase separation and DNA purity. The technique has been optimized over time to increase yield while decreasing contamination, making it a standard in molecular biology research (J Shetty, 2020). The principle underlying plasmid extraction is the difference in solubility of nucleic acids between aqueous and organic phases.

At first, cells are lysed in lysis buffer containing SDS to break down cellular components. Next, add the phenol-chloroform-isoamyl alcohol reagent (25:24:1) to destroy cellular components and facilitate DNA precipitation (J Shetty, 2020). After centrifuged, the suspension separates into two distinct phases. The spin down process produces an upper aqueous layer containing DNA and an organic layer containing precipitated proteins (Javadi et al., 2014). The plasmid-containing aqueous phase will be extracted with chloroform to remove any remaining phenol or impurities. The combination of phenol and chloroform denatures proteins more effectively than either reagent alone (Soren et al., 2020).

However, the primary problems with the phenol-chloroform extraction method are toxic nature of the phenol and chloroform itself. Exposure to phenol can cause skin irritation where prolonged exposure may damage the kidneys and/or liver. On the other hand, breathing or consuming chloroform can render a person unconscious (Rajkumar et al., 2020). Labour extensiveness while being cautious during the handling of dangerous substances are also part of the limitation. Although it offers a kit-free option, the standard phenol-chloroform extraction technique with ethanol precipitation is more time-consuming due to its multistep nature compared to the silica-based spin columns (Figueroa-Bossi et al., 2022).

2.2.2 Cesium Chloride Gradient Centrifugation

Cesium chloride (CsCl) gradient centrifugation is fundamental method in molecular biology for the purification and analysis of nucleic acids, especially DNA and RNA. This method was first presented in 1957 by Matthew Meselson, Franklin W. Stahl, and Jerome Vinograd, who demonstrated the semi-conservative replication of DNA using CsCl gradient centrifugation (Jaiswal & Tiwari, 2023). This method does not require RNase which means that phenol/chloroform extraction is not required in the procedure (Green & Sambrook, 2018). Thus, plasmid DNA purified by this method is appropriate for practically all biochemical experiments. Researchers can utilise the plasmid DNA extracted using this method to transfect cultured cells, and so on.

DNA sample and CsCl are first mixed, and the mixture is then ultra-centrifuged for over 10 hours at a high speed (10,000 to 12,000 rpm) using a high-speed. Ultracentrifuge that uses centrifugal force to separate components in suspension into bands according to their size and density (Jaiswal & Tiwari, 2023). In order to achieve equilibrium separation, biomolecules migrate to regions along the gradient where their densities match with those of the surrounding medium. By integrating with nucleic acids and changing their densities, ethidium bromide is frequently used to improve the separation of relaxed, linear, and supercoiled DNA molecules. Effective separation is achieved when larger, denser particles or components experience greater centrifugal force and sediment migrate away from the rotor and towards the tube's bottom. Those that are less dense than the solvent, on the other hand, will float. Lastly, each DNA band is examined by visualising it under ultraviolet (UV) light.

Alkaline lysis procedures and column-based kits are two alternative nucleic acid extraction methods that are typically compared with CsCl gradient centrifugation. Although these other techniques could be more rapid and convenient, they frequently fall short of the same purity levels as CsCl gradients (Sasagawa, 2019). CsCl gradient centrifugation has limitations despite its benefits. Specialised equipment that can perform high-speed centrifugation is needed which is expensive, and the procedure is very time-consuming since it requires lengthy period of high-speed ultra-centrifugation Additionally, EtBr which often used with this method is known to induce frame shift mutations and genotoxicity, which will impact subsequent processes including DNA sequencing, PCR, and cloning. Additionally, because EtBr requires UV excitation for detection, it has a high potential to harm DNA samples and burn skin and eyes. Because of these draw backs, this method is not approved for clinical microbiology and is not currently in use in clinical laboratories.

2.2.3 Boiling Lysis Method

Boiling lysis is a plasmid extraction technique that Holmes and Quigle presented in 1981 as an alternative to alkaline lysis. Boiling lysis is widely used in research laboratories in molecular biology due to its simplicity of use and rapidity. This method usually involves minimum equipment and reagents, making it appropriate for laboratories with limited expenses. As alkali denaturation and precipitation process, which were employed in the alkaline lysis procedure, are not used, it needs fewer steps and shorter handling time.

Plasmids greater than 10 kb should be extracted using other methods such as alkaline lysis. This approach yields lower quality plasmids compared to the alkaline lysis method. However, the quality is suitable for restriction digestion analysis and subcloning.

The alkaline lysis technique and the boiling lysis method share many basic principles, but the boiling lysis method adds a stage of heat-induced disruption at 100°C to separate the plasmid from bacterial cells. Lysosome treatment is the first step in the boiling lysis procedure, which weakens the bacterial cell wall, denatures cellular proteins, and partially lyses the cells. As a result, while genomic DNA stays trapped within cell debris, bacterial cells will release their plasmids into the solution. The remaining genomic DNA will next be denatured by a boiling water bath treatment, while chromosomal DNA and bacterial proteins will be precipitated by the following high-speed centrifugation, leaving the plasmids in the solution that can be recovered by precipitation with ethanol or isopropanol (Ralte et al., 2022).

The boiling lysis method has limitations despite its many benefits. Boiling lysis can separate more plasmids than alkaline lysis, however when used for laboratory research-scale preparations, this method is not appropriate due to its inconsistent plasmid yield and purity and operational complexity (Xiaolin et al., 2008). The most significant limitation of this method is the possibility of RNA contamination and the partial separation of chromosomal DNA from plasmid DNA (Sasagawa, 2019). The quality of the isolated plasmid also may be impacted by the destruction of plasmid DNA caused by extended exposure to heat during excessive boiling.

2.2.4 Alkaline Lysis Method

Alkaline lysis is a popular method for extracting plasmid DNA from bacterial cells. The alkaline lysis technique, which was first published by Bimboim and Doly, is based on the theory that alkaline solution selectively denatures high molecular weight chromosomal DNA while leaving covalently attached circular plasmid DNA intact (Wang et al., 2021). Because of its effectiveness, affordability, and simplicity, it continues to be an essential tool of molecular biology. Large-scale plasmid DNA extractions and regular laboratory work are especially well-suited for it because of its reputation for rapidness and simplicity of use in removing plasmid DNA from bacterial cells. Its affordability lies from its ease of use, which enables researchers to extract plasmids using cheap chemicals and little equipment. Furthermore, in comparison to the boiling lysis method, this procedure performs especially effectively for extracting molecules with a high molecular weight (>10 kb) (Gautam, 2022).

The fundamental principle of the alkaline lysis method is the selective denaturation of chromosomal DNA while maintaining the solubility of plasmid DNA. Cell lysis, neutralisation, and plasmid DNA recovery are the three primary processes in the procedure. Buffer P1 is first used to resuspend bacterial cell pellets. This buffer contains RNase A to break down RNA, EDTA to act as a chelator to tie up excess divalent cations needed for DNase activity, and Tris Cl to maintain a steady pH of 8. In order to lyse cell membranes and denaturise proteins, the sample is suspended in buffer P2 which is an alkaline solution that contains NaOH and SDS detergent. Chromosome DNA, which has a larger molecular weight than intact plasmid DNA that is still double-stranded, is selectively denatured by the alkaline environment. The chromosomal DNA then renatures and precipitates out of the solution when buffer N3 which contain guanine chloride and potassium acetate is added to neutralise it. After centrifugation, plasmid DNA in the supernatant can be extracted (Behle, 2016).

Despite the fact alkaline lysis is a well-established technique for plasmid DNA extraction, there are a few known limitations, including plasmid DNA that gets stuck in cell debris, which reduces plasmid DNA recovery, the lengthy process, and volume increase caused on by the buffers used, all of which increase production costs (Haberl et al., 2013). The procedure includes several purification stages, which could result in breakdown of samples and increased technical complexity. As a result, this technique is best suited for bacterial cultures and is unsuitable for complicated samples that contain both bacteria and other elements (Delaney et al., 2018).

2.3 Spin Column-Based Plasmid DNA Extraction

Until recently, the process of extracting and purifying nucleic acids was difficult, time-consuming, tedious, and had a low overall efficiency. There are several specialised methods available presently for extracting pure biomolecules, including solution-based and column-based methods. Despite their effectiveness, nucleic acid (NA) liquid-phase extraction (LPE) methods have several limitations. These methods are labour-intensive, need lengthy processing periods, require an extensive amount of samples, and use organic solvents that limit amplification. They also carry a high risk of contamination and degradation (Li et al., 2022). The idea of using column-based extraction method originated in 1979, when Vogelstein began using silica in DNA extraction. Two years later, McCormick pioneered solid-phase DNA extraction, bringing in a new era of plasmid extraction. Over the years, an increasing number of commercial plasmid DNA extraction kits have become available on the market (Wang et al., 2021).

Spin column is a solid-phase extraction method in which the target molecules (nucleic acids) bond to immobilised solid-phase silica resin under specific conditions. The procedure normally includes cell lysis, DNA binding, washing, and elution. The lysate is suspended on a spin column and centrifuged, allowing the plasmid DNA to cling to the silica membrane while impurities flow through. Proteins, salts, and other contaminants are removed through successive washing stages before the DNA is eluted in a low-salt buffer or water. Spin columns for nucleic acid separation provide many advantages over other methods, including high yield, cost-effectiveness, automated processing, reduced time consumption, environmental friendliness, adaptability, and great scalability.

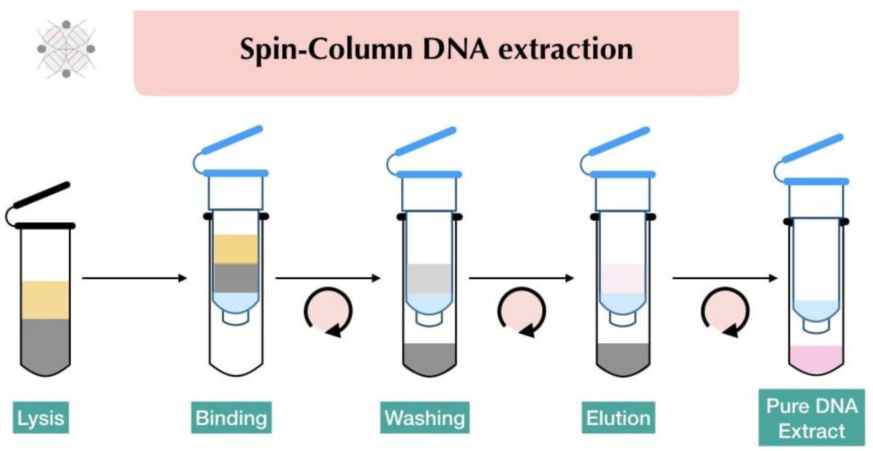

Figure 2.3 General procedures for spin-column DNA extraction

The commercialisation of spin column-based plasmid extraction kits was pioneered by Qiagen, a well-known biotechnology company, which greatly contributed to their widespread adoption in the laboratories. For simple nucleic acid binding, washing, and elution during the purification process, commercial kits typically use spin columns or multiple well spin plates combined with solid-phase nucleic acid binding material. Although commercial spin columns or plates are made to be used just once, their overall cost is higher than that of many conventional purification techniques. By reusing spin columns or plates following cleaning and regeneration using commercial kits, many laboratories aim to lower operational costs (Lemke et al., 2011)

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2.4 Plasmid DNA Yield and Purity

In molecular biology, plasmid DNA yield and purity are crucial factors that impact the outcome of subsequent processes including transfection, sequencing, and cloning. However, it is still difficult to obtain high plasmid DNA yields. Methods for purification are essential for increasing purity. Although they may not totally remove impurities, precipitation techniques including ethanol or isopropanol precipitation aid in the recovery of plasmid DNA (Sharma et al., 2023). Higher purity can be obtained using column-based purification, such as silica-based or anion-exchange columns, which bind plasmid DNA more readily (Yang et al., 2008).

Numerous variables, such as the bacterial strain, growing environments, extraction techniques, and plasmid type, affect the yield and purity of plasmid DNA. In order to obtain high-quality plasmid DNA appropriate for a variety of subsequent uses, it is necessary to optimise these variables. Ineffective extraction may result in DNA loss, plasmid degradation, or contamination with undesirable cell components. The ability to isolate plasmid DNA from genomic DNA and other cellular material varies for different extraction methods. There are numerous factors to consider when choosing an extraction technique for DNA purification. Assessments must be made regarding the quantity of hands-on time and the number of transfer steps needed, the cost-effectiveness of the process, the length of time spent in contact with hazardous chemicals, and the purity of the nucleic acid sample that was acquired (Boesenberg-Smith et al., 2012).

Techniques including agarose gel electrophoresis, fluorescent DNA-binding dyes, and absorbance (optical density) can be used to measure DNA yield. All approaches are helpful, but when selecting a quantitative strategy, it is important to consider their limitations. Spectrophotometry is a standard method for assessing the quality of isolated DNA. A spectrophotometer detects the absorbance of UV light by nucleic acids at 260 nm. DNA concentration is determined using the absorbance at this wavelength. The absorbance ratio between 260 and 280 nm (A260/A280) is used to assess DNA purity. A ratio of around 2.0 suggests pure DNA that is free of protein contamination. Furthermore, the absorbance ratio at 260 nm to 230 nm (A260/A230) aids in determining contamination from organic chemicals or chaotropic salts. A smaller ratio indicates higher levels of contamination, with an optimal value of greater than 1.8 (Díaz et al., 2023).

In this study, DS-11 Spectrophotometer from DeNovix is used to measure the parameters of DNA yield and purity.

CHAPTER 3

METHODOLOGY

3.1 Flow Chart of Study

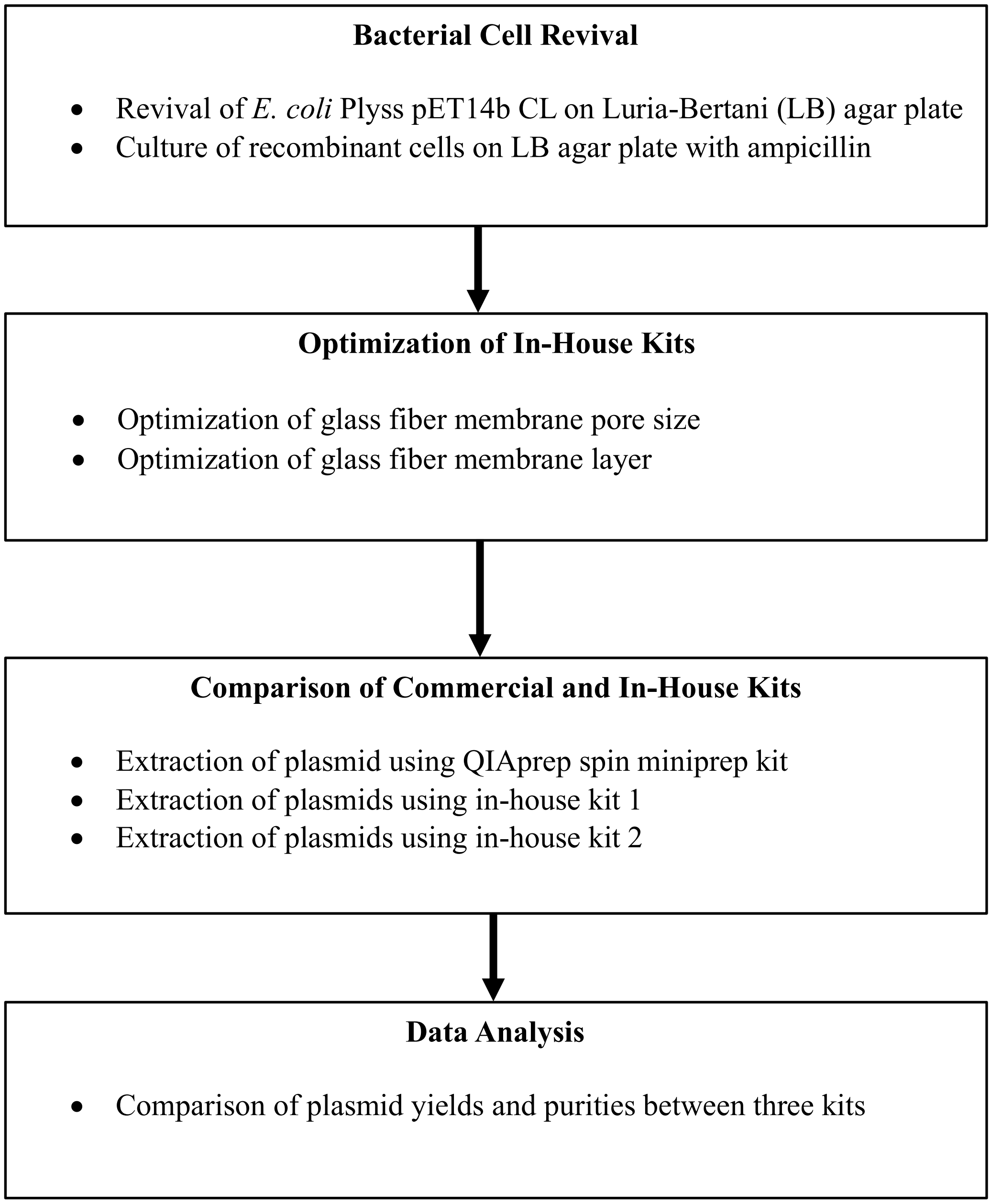

Figure 3.1 Study flow chart

3.2 Materials

3.2.1 Chemical and Reagents

Table 3.1 shows the list of chemicals and reagents used in this study

Table 3.1 List of chemicals and reagents

Chemical / Reagent	Manufacturer
6X DNA loading buffer	Thermo Scientific, USA
Acetic acid (CH_3COOH)	Merck, Germany
Agarose	Vivantis, Malaysia
Ampicilin	Bio Basic, Canada
Deoxyribonucleotide triphosphates (dNTPs)	1st Base, Singapore
Ethanol	Merck, Germany
Ethylenediaminetetraacetic acid (EDTA)	Vivantis, Malaysia
Forward primer/ T7 promoter	1st Base, Singapore
GelStain	TransGen Biotech, China
GeneRuler 1kb DNA ladder	Thermo Scientific, USA
Glucose	Merck, Germany
Glycerol	Merck, Germany
Guanidinium chloride (Gu-HCl)	Merck, Germany
Hydrochloric acid (HCl)	Merck, Germany
Luria-Bertani (LB) broth miller	Merck, Germany
Magnesium chloride ($MgCl_2$)	Thermo Scientific, USA
Magnesium sulfate ($MgSO_4$)	Merck, Germany
Polyethylene glycol (PEG)	Sigma-Aldrich, USA
Potassium acetate (CH3COOK)	Sigma-Aldrich, USA
Reverse primer/ T7 terminator	1st Base, Singapore
RNase A	Merck, Germany
Sodium dodecyl sulfate (SDS)	Bio Basic, Canada
Sodium hydroxide (NaOH)	Merck, Germany
Taq buffer with (NH4)2SO4 (10X)	1st Base, Singapore
Taq DNA polymerase (Recombinant)	1st Base, Singapore
Tris base	Vivantis, Malaysia
Tris-Acetate-EDTA (TAE), 10X	1st Base, Singapore

3.2.2 Kits and Consumables

Table 3.2 shows the list of kits and consumables used in this study

Table 3.2 List of kits and consumables

Consumables	Manufacturer
Centrifuge tube, 1.5 mL	Axygen, USA
Glass fiber filter membrane (0.22 µm, 0.45 µm, 0.80 µm)	
PCR tube	Axygen, USA
Pipette tips (1-10 µL, 10-100 µL, 100-1000 µL)	Axygen, USA
QIAprep spin miniprep kit (250)	Qiagen, Netherland
Spin column (4 layers, 6 layers, 8 layers) with Collection Tube	Haimen Kahotest Citotest Labware, China

3.2.3 Bacteria and Plasmids

Table 3.3 shows the list of bacteria and plasmids used in this study.

Table 3.3 List of bacteria and plasmids

Bacteria and Plasmids	Manufacturer
E. coli Plyss	Thermo Scientific, USA
pET-14b-EhCL	Novagen, USA

3.2.4 Laboratory Apparatus and Equipment

Table 3.4 shows the list of laboratory apparatus and equipment used in this study.

Table 3.4 List of laboratory apparatus and equipment

Chemical / Reagent	Manufacturer
-20°C freezer	SNOW, Malaysia
-80°C deep freezer	ilShin, Korea
Agarose gel electrophoresis set	Major Science, USA
Biophotometer Plus	Eppendorf, Germany
DS-11 Spectrophotometer	DeNovix, USA
Ecotron incubation shaker	INFORS HT, USA
Elite 200 power supply	Wealtec, USA
Forma incubated benchtop orbital shaker	Thermo Fisher, USA
Hot plate & magnetic stirrer	ERLA, Malaysia
Laminar flow hood	ERLA, Malaysia
Microwave oven	Electrolux, Malaysia
MIKRO 120 microlitre centrifuge	Hettich Lab, Germany
Mini spin plus centrifuge	Eppendorf, Germany
Pipette	Eppendorf, Germany
UniBloc analytical balance	Shimadzu, Japan
UNIVERSAL 320 R centrifuge	Hettich Lab, Germany
Veriti 96-well fast thermal cycler	Applied Biosystem, USA
Water bath/incubator	Memmert, Germany

3.2.5 Computer Programme and Software

Table 3.5 shows the list of computer programme and software used in this study

Table 3.5 List of computer application programmes and software

Computer Application Programme and Software	Brands
Molecular Imager® Gel Doc™ XR System with Image Lab™ Software	Bio-Rad, USA
Statistical Product and Service Solutions (SPSS)	IBM Corporation, USA

3.3 Preparation of Buffers, Chemical, and Culture Media

3.3.1 P1 Buffer (Resuspension Buffer)

50 mM Tris-Cl was prepared by dissolving Tris base in 30 mL of distilled water (dH_20), and the pH was adjusted to 8.0 by adding 1 M hydrochloric acid (HCl). The final volume was made up to 50 mL with dH_20. After that, 1 mL of 10 mM EDTA, 2.5 mL Tris-Cl, and 5 mg RNase A were dissolved in 50 mL of dH_20. The buffer P1 was stored at 2-8 °C while it can remain stable for 6 months.

3.3.2 P2 Buffer (Lysis Buffer)

2 g of NaOH pellets were dissolved in 50 mL of dH_20 to produce 200 mM NaOH. After that, 0.50 g of SDS was dissolved in 10 mL of NaOH and the solution was made up to the final volume of 50 mL with dH_20. The buffer P2 was stored at room temperature.

3.3.3 N3 Buffer (Neutralization Buffer)

20.06 g of Gu-HCl was dissolved in 25 mL of autoclaved dH_20. After adding 4.42 g of potassium acetate powder to the mixture, glacial acetic acid was used to bring the solution's pH down to 4.8. Autoclaved dH_20 was used to make up the remaining 50 mL of the solution.

3.3.4 PE Buffer (Wash Buffer)

12.11 g of Tris base was dissolved in 70 mL of dH_20 and thoroughly agitated to formulate 1 M Tris-Cl. 1 M HCl was added to bring the pH of the solution down to 7.5, then dH_20 was added to reach the final volume of 100 mL. 950 µL of dH_20 was then combined with 40 mL of 80% ethanol and 50 µL of 1 M Tris-Cl. Using dH_20, the final volume was increased to 50 mL.

3.3.5 EB Buffer (Elution Buffer)

6.06 g of Tris base was dissolved in 30 mL of dH_20 to produce 50 mM Tris-Cl. 1 M hydrochloric acid (HCl) was then added to bring the pH of the solution down to 8.0. Using dH_20, the final volume was increased to 50 mL. After that, 500 µL of Tris-Cl was pipetted into a blank container, and dH_20 was used to bring the total volume up to 50 mL.

3.3.6 LB Broth

A total of 12.5 g LB powder was dissolved in 500 mL of dH_20 and adjusted to pH 7.0. The solution was autoclaved at 121 °C for 5 minutes and kept at 4 °C. Antibiotic was added accordingly if needed after the medium was cooled to 55 °C.

3.3.7 LB Agar Plate with Ampicillin

After dissolving 5 g of LB powder in 200 mL of dH_2O, the pH was adjusted to 7.0. The solution was then autoclaved for 15 minutes at 121°C after 3 g of bacterial agar powder was added. After cooling the medium to 55°C, 20 µL of ampicillin was added in total. Three petri dishes (20 mL each) were filled with the solution, which was then allowed to solidify for half an hour at room temperature. The plates were incubated for 16 hours at 37°C as part of a sterility test. The plates were stored at 4°C.

3.3.8 TAE Buffer (10X)

400 mL of dH_2O was used to combine and dissolve 24.2 g of Tris base, 5.71 mL of glacial acetic acid, and 20 mL of 0.5 M EDTA, pH 8.0. Using dH_2O, the final amount was increased to 500 mL. Next, one-part 10X TAE was combined with nine parts dH_2O to produce 1X TAE.

3.3.9 Agarose Gel, 2%

20 mL of 1X TAE buffer was combined with 0.20 g of agarose powder. The mixture was heated to the boiling point for one minute at 600 V in a microwave. The bottle was swirled in cool water to allow the fluid to cool to a warm temperature after the agarose suspension had been homogenised. 0.5 µL of gel staining solution was added to the mixture, and the bottle was swirled to homogenise it. For approximately half an hour, the fluid was allowed to solidify at room temperature after being put into the gel casting mold that had been attached to the gel casting comb.

3.4 Revival of Glycerol Stock

Using an inoculating loop, approximately 10 µL of *E. coli* Plyss glycerol stock was streaked in one quadrant of the agar plate after being inoculated on LB agar. Until four sets of streaks were completed, the streaking technique was repeated. To avoid contamination and dehydration, the Petri dish was then covered with parafilm. After that, the sealed plate was incubated for the entire night at 37°C, which was ideal for the growth of *E. coli* Plyss. Following incubation, the plate was checked for well-isolated colonies and bacterial growth.