A ONE STEP METHOD FOR THE RECOVERY OF UNSTAINED DNA FROM AGAROSE GELS USING pBR322 AS A MODEL

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

Advisor — Date: 5/4/93

Barbara Brothers
Dean of the Graduate School — Date: 5/20/93

YOUNGSTOWN STATE UNIVERSITY

June, 1993

Y-10-4
(6)

THESIS APPROVAL FORM

THESIS TITLE: A One Step Method For the Recovery of Unstained DNA Using pBR322 as a Model

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DEGREE: Master of Science in Biological Sciences

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A ONE STEP METHOD FOR THE RECOVERY OF UNSTAINED DNA FROM AGAROSE GELS USING pBR322 AS A MODEL

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Master of Science

Youngstown State University, 1993

ABSTRACT

The isolation of specific fragments of DNA from agarose gels has become a necessity in current molecular biological research. There are, however, several problems associated with the recovery of DNA from the gels, including: 1) contamination of DNA fragments with intercalating dyes and surrounding medium, 2) failure to quantitatively recover high molecular weight DNA, and 3) the need for expensive equipment and time consuming procedures. This study discusses a rapid, simple, and efficient technique for recovery of specific unstained and uncontaminated DNA from agarose gels. Electrophoresis is initiated in a modified casting tray, directly on an ultraviolet transilluminator. The progress of unstained bands are monitored via the use of pre-stained DNA in a parallel lane. The DNA is then recovered by direct pipette aspiration from the preformed wells with high efficiency. Additional purification, concentration, and manipulation of the recovered DNA is not necessary, and the recovered DNA can be utilized immediately.

ACKNOWLEDGEMENTS

I would like to thank the following individuals: Ray Bernat, for his initial work on this project, Eric Huth for his technical assistance in the preparation of some techniques, as well as the preparation of many reagents used in the experimental procedure, Joe Protain for his research assistance, Mrs. Carol McGuinnes and Mr. James A. Mullen Jr. for their assistance in preparing the manuscript, Dr. Carmen J. Leone for his critical review of the manuscript, the Physics Department for the use of its Mechanics Lab, Dr. David Asch, for his assistance, council, and the use of some laboratory materials, Drs. James R. Toepfer and Paul C. Peterson for their evaluation of this thesis, and especially Dr. Anthony E. Sobota for his original ideas and constant leadership throughout this project. His presence and council were instrumental in all stages of this study. I would also like to thank my family and friends whose support and belief in me made this work possible.

DEDICATION

This thesis is dedicated to the loving memory of my cousin

DOMINIC S. LEONE

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CHAPTER 1

INTRODUCTION

In the ever changing field of molecular biology, particularly in nucleic acids research, it is and has always been necessary to break down and analyze new information into its simplest and most readily recognizable components, and by doing so, get a better view of the whole. It became clear, through years of research, that the study of genome structure and function relied heavily on the isolation and analysis of defined DNA fragments (Yang et al., 1979). Thus, to better understand the DNA molecule, it has become necessary to detect minute sequence differences through various digestion, separation, and elution methods which are continuously improving. The general procedure for the isolation of specific fragments of DNA relies upon three interrelated techniques: 1) the use of specific restriction endonucleases to cleave the DNA into well defined fragments, 2) agarose gel electrophoretic methods to resolve the individual fragments, 3) the recovery of the DNA fragment from the gel matrix (McEnery et al., 1985).

A large DNA molecule can be cleaved at specific nucleotide sequences by restriction endonucleases, which serve a major role in the bacteria from which they are derived; that is, they protect the bacteria from invading viruses by destroying the foreign DNA associated with these viruses. The native DNA of the bacteria is not degraded by its own digestive enzymes, because it is protected by methyl groups that are added to specific adenine or cytosine

residues within their recognition sites (Watson et al., 1987). Thus, restriction endonucleases recognize short DNA sequences, and cleave double-stranded DNA at specific sites within or adjacent to the restriction sequences (Ausubel et al., 1987). Prior to the utilization of restriction endonucleases, enzymatic digestions produced random non-specific cuts which were of limited use because of their unpredictability. Thus, restriction endonucleases are of great advantage to molecular biological research, since specific sequences on the DNA to be studied can be targeted and analyzed with relative ease.

Following the enzymatic digestion, the restriction fragments of DNA can be studied in great detail by further separation within various gel matrices, and this is accomplished through agarose gel electrophoresis. Simply, this separation method is applied to the movement of small ions and charged macromolecules in solution under the influence of an electric field (Rickwood et al., 1982). Lodge (1886) is credited with the first electrophoresis attempt, when he tried to measure ionic velocities in a tube of jelly containing phenolphthalein indicator, which eventually led to Tiselius' moving method of determining transfer numbers (Moody, et al., 1975). Today, virtually all protocols in molecular biology require, at some point, the fractionation of nucleic acids of different sizes, and gel electrophoresis has much greater resolution than alternative techniques such as density gradient centrifugation, and thus, is the fractionation method of choice (Ausubel et al., 1987).

The gel itself provides the nucleic acids a favorable medium in which the molecules are able to migrate. The nucleic acids are selectively impeded by the surrounding medium and pH of the gel/buffer mixture when an electrical current is applied.

As the DNA is electrophoretically drawn through the gel matrix, migration rates are inversely proportional to the logarithms of the molecular weights (Aaij et al., 1972), and are also related to the three dimensional configuration of the molecule. It should be noted, however, that gel electrophoresis is not only used as an analytical method, but also in a preparative manner for the purification of specific DNA fragments (Old et al., 1989). Thus, there is an intricate balance between the purity of the DNA to be studied, and the numerous technical methods of studying the separated band fragments of DNA.

Common gel forming materials include polyacrylamide, and agarose, both of which are efficient for rapid separation of DNA fragments of different sizes (Dretzen et al., 1980). Generally, an agarose matrix is employed when DNA fragments are below twenty to twenty-five kilobases, which is normally the case in gel elution studies. In fact, agarose gel electrophoresis is severely limited in its ability to resolve molecules longer than twenty kilobases, since large DNA molecules have a tendency to become more distorted as they move through the pores of an agarose gel (Kornberg et al., 1992).

The percentage of agarose in the gel is a function of the size of the DNA to be studied. Thus, the higher the molecular weight of

the fragments, the lower the agarose concentration (Hackett et al., 1988). In addition, the three dimensional character of the DNA is related to migration. Covalently closed circular fragments will migrate the furthest in the shortest amount of time, while the open nicked or single stranded DNA forms will move slower and not as far through the gel. This aids in the separation and understanding of the various forms of DNA.

Specifically, agarose gel electrophoresis is used for the fractionation of both monomolecular nucleic acids, and multimolecular complexes such as serum lipoproteins and viruses (Serwer et al., 1986). The DNA samples to be separated are diluted to specific concentrations with loading buffer, and are loaded via pipette into preformed wells. These wells are created by placing a comb in the agarose prior to solidification of the medium. The negatively charged nucleic acid samples are loaded next to the cathode in the gel apparatus. Since the nucleic acid DNA has an overall negative charge, the loaded samples will be drawn to the anode when a voltage drop is applied.

The migration of nucleic acids through the agarose matrix is a function of several variables, including 1) size of the nucleic acid, generally measured in base pairs (DNA) or nucleotides (RNA); 2) conformation of the nucleic acid: DNA supercoiled, circular, or linear, while RNA may be folded into varying degrees of secondary structure; 3) base composition of the nucleic acid; 4) density of the agarose; and 5) voltage across the gel (Hackett et.all, 1988).

After separation, it is often necessary to study in greater

detail specific fragments, or bands, as they appear on the gel. The current and most convenient method of visualizing DNA bands in agarose gels is by the use of the fluorescent dye ethidium bromide (Sharp et al., 1973). This particular dye contains a planar group that intercalates between the stacked bases of the DNA. The fixed position of this group and its close proximity to the bases causes dye bound to DNA to display an increased fluorescent yield compared to dye in free solution (Maniatis et al., 1982). Typically, ethidium bromide-stained DNA is viewed on an ultraviolet source.

Since agarose gel electrophoresis has become regarded as the technique of choice in the field of recombinant DNA research, several methods have been developed for the elution of separated DNA fragments from agarose to study them more extensively (Tautz et al., 1983). Initial attempts to recover DNA fragments from agarose gels were rather limited for many reasons. The processes were time consuming, since the fragment extraction procedures were laborious and required specialized equipment, and the resulting DNA was frequently not suitable for subsequent studies (Finkelstein et al., 1978). Also, in many instances, following recovery from the gel, the DNA fragments required further purification. This occurs since most grades of agarose are contaminated by sulfated polysaccharides, which are extracted from the gel together with the DNA. These substances are potent inhibitors of the enzymes (restriction endonucleases, ligases, kinases, polymerases) that are commonly used in subsequent cloning steps (Maniatis et al., 1982). Complete recovery of a fragment of DNA was virtually impossible due

to these extraction and purification processes.

In 1975, Southern proposed an elution method for transferring fragments of DNA from agarose gels to cellulose nitrate filters. The fragments were then hybridized to labeled RNA, and hybrids were detected by radioautography or fluorography. This method was advantageous in that it retained the high resolving power of the gel; however the main disadvantage was that fragments of 500 nucleotide pairs or less gave low yields of hybrid, and such fragments would be under-represented or even missing from the analysis (Southern, 1975).

Other techniques also utilized the concept of molecules binding to filters. Thomas et al., in 1978, devised a non-electrophoretic method of assaying DNA segments bearing tightly bound proteins. This method was based on the observation that proteins bind quantitatively to glass fiber filters, thus indicating that DNA which is tightly bound to the protein will be retained with that protein on the filter, while DNA which is unbound will pass directly through the filter. This method works on the premise that the concentration of salt must be high for the protein/DNA complex to bind efficiently (Chen et al., 1979). Although elution of DNA fragments, at high percentage yields, by this method was possible, the method itself was primarily designed for removing unwanted proteins from DNA solutions, and thereby purifying the DNA sample for further experiments. Later experiments attempted to recover DNA samples by using agarose gel electrophoresis, which is currently the method of choice for gel

elution studies.

A Method was devised which improved upon the previously mentioned method proposed by Thomas et al., (1978) in that it was designed to recover DNA fragments, following electrophoresis, that were not attached to or associated with a binding protein. In this study, DNA fragments could be recovered through solubilization of agarose gels with $NaClO_4$, immediately followed by retention of the DNA on a glass filter, and the subsequent removal of the $NaClO_4$ with ethanol. The DNA could then be extracted in the presence of a low salt buffer. This method was based on two earlier observations: 1) that agarose can be melted by certain "chaotrophic" anions, such as ClO_4-, and 2) that DNA can be quantitatively retained on glass fiber filters provided the salt concentration is high (Chen et al., 1979). Despite the high percentage yields of approximately 80% that were obtained, this method posed a problem, in that a pure sample was not always attainable due to the presence of various contaminants which often remained in the recovered DNA sample. Also, there was no simple procedure for removing the dissolved agarose from the $NaClO_4$ extract (Thuring et al., 1975).

Girvitz et al., (1980) attempted to recover DNA fragments from agarose by the insertion of filter paper strips backed by dialysis membrane, into slits that were cut in the gel preceding the DNA bands. After the strips were set in place, electrophoresis was continued to allow the DNA bands to collect on the filter paper. Finally, through low speed centrifugation, the DNA was eluted from the paper. Despite the high percentage yields that resulted from

this study, there was still the problem with the apparent necessity to physically manipulate the gel before and after electrophoresis. Also, the process itself was time consuming with many steps. Finally, the purity of the eluted fragments was still in question as the recovered DNA from the filter paper was slightly more resistant to restriction endonuclease cleavage than unprocessed DNA (Girvitz et al., 1980). However, despite this problem, the purification of the eluted fragments was for the most part attainable, yielding close to 100% recovery of eluted fragments.

A subsequent study by Clad et al., (1982) described a device for the electrophoretic elution of DNA from gel slices onto a malachite green gel. The procedure involved the insertion of gauze nets in the chamber channels of the apparatus, which together formed bags. The malachite green gel was placed in these bags by Pasteur pipet, and because of grain size of the gauze net, the green gel did not leak through. The major advantage of this technique was the fact that large amounts of DNA could be eluted at a time. This was significant in that prior to this time, many of the gel elution techniques were limited to relatively small amounts of low molecular weight DNA (Finkelstein, 1978). Despite this advantage over previous elution methods, purification steps were once again needed since the elution material, $NaClO_4$, would inhibit subsequent enzymatic steps (Clad et al., 1982). Thus, there would be a chance that each elution by this method would suffer from purification problems.

Subsequently, Tautz et al., 1982, discussed a method by which

bands of interest were cut out of the gel following electrophoresis, and were equilibrated in a neutral salt buffer. The gel slices containing the bands were then centrifuged through a filtration assembly, whereby the DNA-containing buffer was squeezed out. This method was a modification of the "freeze-squeeze" method that was originally proposed by Thuring et al., in 1975, where gel slices were frozen and the liquid containing the DNA was squeezed out. In this study, long DNA, molecular weight ranging from 25 x 10^6 to 6 x 10^6, was recovered with approximately 70% efficiency, and no further purification was subsequently needed following the procedure. Although this method was good in terms of percentage recovery and purity of recovered sample, physical and chemical manipulation of the agarose containing the desired DNA band following electrophoresis was both cumbersome and time consuming.

Another method which yielded a high percentage of eluted DNA involved the use of a disposable affinity column, and low melting agarose. In this method, the bands of interest were excised after electrophoretic separation, and the DNA in the agarose was melted in a low salt buffer, cooled, eluted at high salt concentration, and ethanol precipitated (Schmitt et al., 1983). Although this method yielded 80% or more eluted DNA, it was not rudimentary since it required both the use of chromatography, which can be expensive and time consuming, and further purification of the DNA.

A study done in 1985, required the use of a NENSORB cartridge. In this technique, DNA was electroeluted onto the cartridge matrix

with a subsequent elution of the bound DNA by a methanol (50% v/v) wash. This resulted in the quantitative recovery of the restriction fragment (McEnery et al., 1985). This method posed one notable advantage: the extracted fragment could be cut, following elution, with a second restriction endonuclease. However, residual ethidium bromide was present in the eluted fragment, and thus, retarded the movement of the DNA through the agarose gel (Sharp et al., 1973). Although the presence of ethidium bromide did not seem to affect the digestive action of the restriction enzymes, it remained advantageous to have a completely pure sample, since uncontaminated DNA is necessary for other enzymatic activities. This, therefore, indicated that further purification steps were needed.

A subsequent study by Vogelstein 1986, involved slicing the separated fragments out of the gel with a razor blade. In this study, agarose slices containing DNA were placed in a disposable plastic column, and the DNA was separated from the agarose by centrifugation using a microcentrifuge. This study expanded upon the pre-existing centrifugal techniques, as discussed by Tautz et al., 1983, and Zhu et al., 1985. The advantages of the Vogelstein technique are as follows: 1) construction of the entire apparatus is relatively simple since it is made from a commercially available minicolumn, 2) results are reproducible, 3) problems are limited, 4) time is saved in that no buffer pre- equilibration or freezing of agarose slices was required (Vogelstein et al., 1986). There was, however, one major problem: the gel was manipulated with the razor blade, to obtain the desired fragments. This resulted in a

decrease in the total percentage of DNA recovered.

An additional method discussed by Ahokas, 1987, which built upon the Vogelstein study (1986), attempted to show that electroelution was possible within the microcentrifuge tube itself. A cathode, completely covered with teflon, and an anode, surrounded by a changeable piece of dialysis tubing, were attached to a screw-connector joined to the cap of the tube. DNA samples that were end-labeled were subsequently electroeluted into TBE buffer within the tube. Radioactivity of the eluted fragments varied from 3-17%. Although DNA eluted by this method was able to be digested and ligated, the apparatus itself was complicated, due to the small volume of the microcentrifuge tube, and the many elution steps resulted in poor yields of DNA.

A similar study proposed by Symons et al., 1988, examined the recovery of DNA fragments using a specialized apparatus created especially for the procedure, in which DNA was recovered by direct aspiration with a micropipetter. In this procedure, 250 ng of DNA can be quantitatively recovered from gel slices.

A further method for electroelution of DNA that was much simpler and less expensive than previously mentioned techniques was proposed by Sandhu et al., 1989. The procedure entails cutting the band out of the ethidium bromide stained gel and placing it in a microcentrifuge tube, where the gel slice is held in place by a hypodermic needle. A second needle is then inserted into the lower end of the tube. The tube is filled with buffer, and the upper needle is connected to the negative terminal of a nine-volt

battery, while the bottom needle is attached to the positive terminal. The elution time varies from 30 to 90 minutes depending on the size of the DNA. The gel can be viewed under ultraviolet light, but again, there is the problem of removing the agarose and ethidium bromide, so that the DNA fragments could be further manipulated.

A much simpler procedure requiring basically one single step with no expensive equipment required was proposed by Errington, (1990). The method itself is a modification of Girvitz et. al., (1980), in which several unessential steps were removed from the procedure. In the Errington study, DNA was electrophoresed using an agarose gel. A slit was made in front of the desired pre- stained band to be removed, and a strip of Whatman 3MM paper, about the same width as the desired band, was inserted. The gel was then returned to the electrophoresis tank, for a brief period of electrophoresis, which served to electrically move the desired band onto the previously inserted paper. When the band was seen trapped on the paper, the paper was removed. The paper was then placed in a 500uL microcentrifuge tube, with a small hole in the bottom, and the tube was transferred to a 1.5mL tube and microfuged for 20 seconds to recover the DNA. This method yielded 30 to 40% of the original fragment. This is sufficient for ligation purposes, since only small volumes of the DNA fragment are needed. Despite the relative simplicity of this technique, it suffers from a number of drawbacks, which include the following: 1) less than 50% of the original sample was recovered, which is an insufficient amount for

use in other manipulative techniques besides ligation, 2) there was a possibility that prolonged electrophoresis would result in the band moving right through the paper; and 3) the quantity of DNA recovered by this method was not improved by loading more DNA, unless several strips were inserted together in parallel (Errington, 1990).

A method that also did not require expensive equipment, and was still relatively rapid, involving the use of Sephadex G-50 beads, was proposed by Mukhopadhyay et al 1991. This technique involved the construction of a column using the beads in a 10mL syringe. Subsequently, siliconized wool was placed in 1.5mL microfuge tube which served to seal the tube, and the DNA band of choice was excised out of the stained agarose gel, onto the beads. A series of incubations and chemical treatments were then performed, resulting in a recovery of 95% of the DNA.

Karuppiah et al., 1992, describe a rapid simple microelution technique which can be used for the micro-electroelution of either nucleic acids or proteins from agarose gels. In this study, the desired band, which was previously stained with ethidium bromide, was placed in a 0.5mL microfuge tube, with the bottom end of this tube cut. The tube was then placed within a 1.5mL microfuge tube containing electrophoresis buffer. Platinum wires were then inserted into the tubes; one in the 1.5mL tube, and the other in the 0.5mL tube, and the power source employed was a 9-V battery. After elution, the DNA which was present in the 1.5 mL tube, was ethanol precipitated.

This study proposes a technique that permits almost total recovery of individual unstained fractions of DNA after separation in agarose gels. The sample of DNA is recovered directly, and does not require further purification or concentration unless it is desirable to replace the running buffer.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strain and Plasmid

Escherichia coli, strain RR1, is an rec$^+$ derivative of HB101 constructed by R. Rodriguez. Its genotype is F,- hsdS20(r_B-, m_B-), recA+, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl-1 $supE_{44}$, λ (Maniatis et al., 1982). This *E. coli* strain was purchased from Bio-Rad (Richmond, California), and is noted for its ability to be transformed at high efficiency with plasmid vector annealed to cDNA by homopolymeric tails (Boliver et al., 1977; Peacock et al., 1981).

For the purposes of this project, RR1 was transformed with the plasmid pBR322 (Sutcliffe, 1978, 1979), which codes for a specific protein, ß-lactamase. This protein imparts ampicillin resistance to the bacterial cell. RR1/pBR322 was grown in the presence of ampicillin at a concentration of 50ug/mL to select for the plasmid. LB medium, which contains 10g Tryptone, 5g Yeast extract, 10g NaCl,/Liter of H_2O, was used to culture the *E. coli*.

Chemical Reagents

Unless it is otherwise stated, all chemical and biological reagents were obtained from the SIGMA chemical company, St. Louis Missouri.

DNA Extraction Procedures

A. Phenol-based Method

The plasmid pBR322 was extracted from *E. coli* strain RR1 using a technique provided by Dr. Allen Steggles of the Northeast Ohio Universities College of Medicine. A total of 1.4 mL of an overnight culture of RR1/pBR322 was transferred via pipette, into a non-sterile 1.5mL microcentrifuge tube, and centrifuged in a microcentrifuge at top speed for three minutes to obtain a pellet of cells. The supernatant was removed by vacuum aspiration, and an additional 1.4 mL of the overnight culture was added to the pellet. The sample was then centrifuged again, and the supernatant was removed. To ensure complete removal of excess media, the pellet was resuspended in 1.4mL of STE buffer (10mM Tris . Cl, pH 8.0, 100mM NaCl, 1mM EDTA, pH 8.0), and again centrifuged for three minutes, after which the supernatant was vacuum aspirated. The pellet was then resuspended in 200uL of STE buffer, and 200uL of a 25:25:1 mixture of phenol/ chloroform/isoamyl alcohol was added. The sample was vortexed for 1 minute, and centrifuged for 6 minutes in the cold at 4°C.

Following centrifugation, 150uL of the top (aqueous) phase was removed and transferred to a new non-sterile microcentrifuge tube. The organic phase and the cellular interphase were discarded. The plasmid DNA, in aqueous solution, was then precipitated by the addition of 55uL of 7.5M ammonium acetate, and 250uL of 95%

ethanol. The sample was then placed in ice for 15 minutes, and subsequently centrifuged for 10 minutes to pellet the DNA.

The supernatant was removed by vacuum aspiration, and the pellet was washed with 70% ethanol. Following the removal of the ethanol wash, the pellet was dried in a vacuum desiccator for 30 minutes, and was resuspended in 20uL of TE buffer (10mM Tris pH 7.5, 1mM EDTA).

B. Alkaline Plasmid Screen

In this procedure, a culture of RR1/pBR322 was grown overnight in a shaker at 37°C. The following day, 1.4mL was transferred to a 1.5mL microcentrifuge tube, and centrifuged for 15 seconds at top speed in a microcentrifuge. Following aspiration of the supernatant, the pellet was resuspended in 0.2mL G-Buffer (50mM Dextrose, 25mM Tris, pH 8.0, and 10mM EDTA, pH 8.0), to stabilize the cells. Following resuspension, 0.4mL of Denaturing Solution (0.2N NaOH, 1% SDS), was added, and the sample was placed on ice for 5 minutes. After incubation on ice, 0.3mL of ice cold Neutralizing Solution (3M KOAC45, 2M HOAC) was added to the tube and mixed by vortexing for 10 seconds.

The mixture was then placed on ice for 15 minutes, and centrifuged for 5 minutes in a microcentrifuge. A total of 1mL of the supernatant containing the plasmid DNA was then transferred to a new microcentrifuge tube, and 0.54mL of isopropanol was added. The sample was then centrifuged for 5 minutes, and the pellet was then washed twice with 80% ethanol, and dried in a vacuum

desiccator. The pellet was then resuspended in 50 uL TE buffer, and stored at 4°C.

C. Chloramphenicol Amplification and Lysis by Alkali

1) Chloramphenicol Amplification

Since plasmid DNA is often under stringent control such that only a few copies of the plasmid are present in each bacterial cell, (Novick et al., 1976), chloramphenicol is used to increase the copy number by several thousand (Clewell, 1972). The procedure used involves the amplification of the plasmids in rich medium (Maniatis et al., 1982).

LB medium containing ampicillin (50ug/mL) was inoculated with a 1uL loopfull of RR1/pBR322, and incubated in a shaker at 37°C overnight. Following this initial incubation, 25mL of fresh LB medium, again containing ampicillin (50ug/mL), was inoculated with 0.1mL of the overnight culture, and cultured until late log phase was reached (OD_{600} = 0.6). At that time, the culture was diluted with 500mL of LB medium at 37°C, containing ampicillin (50ug/mL). The sample was then placed on a shaker for 2.5 hours, after which 2.5mL chloramphenicol (34mg/mL in ethanol) was added. The sample was then incubated with shaking overnight at 37°C.

2) Lysis of cells by Alkali

Following the chloramphenicol amplification, the 500mL culture was aliquoted into 50mL samples and centrifuged at 4000g for ten

minutes at a temperature of 4°C. The supernatant was removed from each tube, and the pellets were resuspended in 1mL of reagent 1 (50mM glucose, 25mM Tris·Cl, pH 8.0, 10mM EDTA), containing 5mg/mL lysozyme. The cellular mixture was then transferred to a new 50mL tube, and placed on ice for 5 minutes. Following the ice bath, 2mL of Solution II (0.2N NaOH, 1% SDS) was added to each tube, and mixed. The samples were then incubated in the ice for an additional 10 minutes. After incubation, 1.5mL of a potassium acetate solution (60 mL of 5M potassium acetate, 11.5mL of glacial acetic acid, 28.5mL H_2O, pH 4.8), was added to each tube, and the samples were mixed by vortexing. The tubes were once again placed in the ice bath for 10 minutes, and centrifuged at 32,000g for 20 minutes at 4°C.

Following centrifugation, 3mL of supernatant from each tube was transferred into two 15mL centrifuge tubes, and 1.8mL of isopropanol was added to each tube. After standing at room temperature for 15 minutes, the sample was centrifuged at 12,000g for 30 minutes at room temperature, and the pellet was washed once with 70% ethanol and dried in a vacuum desiccator. The pellets were suspended in 4mL TE buffer and were aliquoted into 1 mL samples and stored in a freezer at -20°C.

Electrophoresis

A) Set up of Apparatus

A mini-gel casting tray (7.1 X 9.3 cm), purchased from Fotodyne Incorporated, New Berlin Wisconsin, was modified, (Figure 1), by cutting four additional comb slots 1 and 1.5cm apart with a band saw, to permit the positioning of extra combs in the tray. The combs (Fotodyne) were modified by removing three of the six teeth. Also, two 8cm length 24 gauge platinum wires (Fisher Scientific, Pittsburgh, Pa) were positioned across both ends of the casting tray. This was accomplished by drilling holes 6mm from the bottom of the tray and 10mm from each end. The wires served as the cathode and anode, and were connected to the power source via alligator clips.

B) DNA Pre-staining

Plasmid pBR322 was mixed at appropriate concentrations with 2X loading buffer (0.1% bromophenol blue, 1mM EDTA, 1% sodiumdodecyl sulfate, 50% glycerol), after which, 1uL of ethidium bromide, .5ug/mL, diluted 1:10 with deionized water, was added, and incubated at room temperature for 30 minutes.

C) DNA Electrophoresis

A 0.8% agarose gel was cast in the modified tray, using both a standard comb (six teeth) and a modified comb (three teeth). The platinum wires were then inserted through the drilled holes. The casting tray was then placed on an ultraviolet transilluminator. The holes were sealed with plumbers putty, and the alligator clips were attached. Subsequently, 60 mL electrophoresis buffer (40mM

Tris acetate, 5mM sodium acetate, 1mM EDTA pH 7.8) was then added to the tray, and the combs were removed. Three 10uL samples of the DNA mixture were loaded into the wells via pipette. The samples were electrophoresed at 45 milliamps for 30 minutes or until the desired band reached a modified well, after which the power source was shut off. A photograph, using Poloroid 667 film, was then obtained by placing a camera (Fotodyne) directly over the modified casting tray and switching on the ultraviolet source.

The visible band present in the modified well was recovered in a single 10uL pipette aspiration and transferred into a new microcentrifuge tube for later use. Also, 10ul was recovered from a neighboring well containing unstained DNA.

Following the recovery of the DNA, electrophoresis was continued for 10 minutes, and a second photograph was taken. The entire gel was stained with 0.5ug/mL of ethidium bromide for 30 minutes and destained in running distilled water. A third photograph was then taken.

Transformation Procedures

A) Transformation of Recovered Plasmid

To test the efficiency of the DNA recovery from the modified wells by pipette aspiration, plasmid pBR322 was electrophoresed into the modified well of the agarose gel. After the covalently closed circular portion of the molecule moved into the modified well, 10uL of buffer containing the unstained DNA band was

recovered by direct pipette aspiration.

A transformation procedure using calcium chloride was then employed (Mandel et al., 1970). Two overnight cultures of E. coli in log phase of growth (OD_{600} of 0.8-0.9) were obtained after 24 hours of an overnight incubation at 37°C. At that time, 18mL of each culture were transferred into 50mL centrifuge tubes, and were subsequently centrifuged for 5 minutes at 4000g. The temperature was set at 4°C for this procedure. After pouring off the supernatant, 9mL of cold sterile 50mM $CaCl_2$ in 10mM Tris was added to the pellet with a sterile ice cold 10mL pipette. The tubes were then placed in ice, and the pellet was resuspended by stirring with the transfer pipette. The cells were then left in ice for a 15 minute period, and pelleted at 4000g for five minutes. The supernatant was discarded, and the cells were resuspended in 1.2mL of cold sterile $CaCl_2$ with a second cold sterile pipette. At that point, the cells were competent to receive the plasmid.

Following these preliminary steps, 0.3mL of the competent cellular mixture was transferred into sterile microcentrifuge tubes, also placed in the ice bath, using a cold 1mL sterile transfer pipette. Ten microliters of both DNA samples to be tested were pipetted into the microcentrifuge tubes containing the competent cells. This mixture was then placed on ice for 90 minutes. To stop the reaction, the cells were heat shocked for 2 minutes by placing them in a water bath that was pre-heated to 42°C.

When heat shock was complete, 1mL of sterile LB media was

added to each microcentrifuge tube, and was subsequently shaken in an incubator set at 37°C for 45 minutes. Finally, 0.1mL of the cells were pipetted into 0.9mL of sterile media, and 0.1mL of the cells were transferred to a second 0.9mL of media.

B) Transformation of Residual DNA

To test the efficiency of the recovery of DNA from the modified well, 10uL of buffer was recovered from a modified well following the movement of the covalently closed circular portion of unstained pBR322 through the well. The transformation procedure was performed, again via the $CaCl_2$ procedure (Mandel et al., 1970).

C) Transformation of Standard pBR322

To test the effectiveness of the $CaCl_2$ method, E coli RR1 was transformed with a standard plasmid pBR322.

Ampicillin Resistance

To test for ampicillin resistance, and thus the presence of the plasmid pBR322, 0.1 mL of each dilution from each transformation was plated on an ampicillin plate. The plates were made by adding 50ug/mL of ampicillin to sterile LA media (LB media + agar at 15g/Liter) and pouring the mixture into sterile petri-plates. After solidifying, the plates were refrigerated until the time of their use in the transformation procedure.

Restriction of Recovered pBR322

To further demonstrate the purity of the unstained recovered sample, digestion was performed with restriction endonucleases. To a sterile microcentrifuge tube, the following reagents were added: Ten microliters of recovered pBR322, 5uL One-Phor-All buffer, 1 unit of restriction endonucleases Hind III, and Pst I, and 33uL sterile water. The mixture was vortexed briefly, and placed in a water bath at 37°C for 2 hours. After incubation, the sample was heat shocked for 10 minutes in a 75°C water bath.

Following restriction digestion, a 0.8% agarose gel was cast, and the entire sample was loaded onto the gel along with appropriate controls. The gel was run using standard electrophoresis for 45 minutes at 50 milliamps, stained for 30 minutes in 0.5ug/mL ethidium bromide, and photographed with Polaroid film 667.

CHAPTER III

RESULTS

pBR322 Extraction Procedure

E. coli RR1 containing the plasmid pBR322, was extracted using an alkali lysis technique, after which pellets containing the nucleic acids were resuspended in a total of 6mL TE buffer, and aliquoted into 1.5mL microcentrifuge tubes.

A 5 and 10 uL sample of the extracted plasmid/buffer mixture was loaded onto a 0.8% agarose gel along with a standard sample of pBR322 (SIGMA), which was used as the control to demonstrate the success of the extraction procedure. The wells were cast and the DNA was loaded nearest the negative electrode, with lane 1 at the top of the gel. The gel was electrophoresed, using a standard electrophoresis chamber, for 45 minutes at 50 milliamps, and a photograph of the gel can be seen in Figure 2. The lanes are numbered in the photographs. Also note the large diffuse RNA band at the lower portion of the gel. The concentrations of DNA in each lane were prepared as follows: Lane 2, 5uL extracted pBR322/5uL 2X loading buffer; Lane 3, 10uL extracted pBR322/2uL 10X loading buffer; Lane 4, 1uL pBR322(SIGMA)/9uL 2X loading buffer. When compared to the standard in lane 4, as well as photographic standards (Hackett), the concentration of pBR322 in lanes 2 and 3 were estimated to be 100ng/uL and 200ng/uL respectively.

A second gel was cast using the same apparatus, materials, and

concentrations as in the first gel (Figure 2) with one exception: all DNA samples were pre-stained with ethidium bromide (1:10 dilution of 0.5ug/mL EtBr) for 30 minutes prior to loading onto the agarose gel. The results can be observed in Figure 3.

Another gel was cast in a modified tray using the modified technique to demonstrate the similarity in results with the standard runs. The results can be seen in Figure 4, with the DNA samples at identical concentrations as the first gel (Figure 2).

Finally, a forth gel was cast, again using the modified technique; however, all DNA samples were pre-stained with ethidium bromide prior to loading onto the gel. These results can be observed in Figure 5, and again, with identical DNA concentrations as the previous 3 gels.

Electrophoresis and Recovery of Plasmid

After an appropriate DNA concentration was determined, a 0.8% agarose gel was cast in a modified electrophoresis tray, and DNA samples were loaded at the following concentrations: Lane 1, 10uL extracted pBR322/2uL 10X loading buffer + 1uL EtBr(1:10 dilution); Lane 3, 10uL extracted pBR322/2uL 10X loading buffer; Lane 5, 10uL extracted pBR322/2uL 10X loading buffer + 1uL EtBr(1:10 dilution). The gel was run for 30 minutes at 50 milliamps (until the bands of choice moved into the modified wells), at which time a photograph was taken. This is seen in Figure 6a. The bands, stained and unstained, were recovered by direct pipette aspiration, and a second photograph was taken to demonstrate the success of the

recovery, as seen in Figure 6b. After the photograph was taken, the gel was stained for 30 minutes in ethidium bromide (0.5ug/mL), and a third photograph, Figure 6c, was taken. Finally, a second gel was cast in the modified electrophoresis tray; however, the modified comb was not inserted. The gel was loaded as follows: Lane 1, 10uL extracted pBR322/2uL 10X loading buffer; Lane 2, 10uL recovered band(pre-stained)/2uL 10X loading buffer; Lane 3, 10uL recovered band(pre-stained)/2uL 10X loading buffer; Lane 4, 10uL recovered band(unstained)/2ul 10X loading buffer. The gel was run under modified conditions for 30 minutes at 50 milliamps and photographed (Figure 6d).

Transformation and Plating

A) Recovered Plasmid

E. coli RR1 was transformed and made competent using a $CaCl_2$ procedure (Mandel et al., 1970). A 10uL sample of covalently closed circular pBR322 was recovered from an agarose gel and added to the competent cells to test the transformation efficiency and purity of the recovered plasmid. A sample containing 0.1 mL of the transformed cells was plated on ampicillin plates at a concentration of 50ug/mL, which would only support the growth of cells transformed with pBR322. The cells were plated at the following concentrations: 1:10, 1:100, 1:1000, and after overnight incubation at 37°C, 36 colonies were found on the 1:1000 dilution ampicillin plate, indicating a transformation efficiency of

0.0021%.

B) Residual DNA

To test efficiency of DNA recovery, 10 uL of buffer was aspirated from a modified well following migration of a covalently closed circular band through the well. Using the same procedure, the sample was subjected to transformation. No transformed colonies were seen on any of the ampicillin plates.

C) Standard pBR322

To demonstrate effectiveness of the procedure, a standard sample of pBR322 was transformed under the same conditions described above two, and after overnight incubation, 48 colonies were seen on the ampicillin plate corresponding to the 1:1000 dilution of transformed cells indicating a transformation efficiency of 0.0028%

Restriction Procedure

To demonstrate purity of the recovered DNA band, a restriction digestion was performed following the recovery of an unstained covalently closed circular band of pBR322. The samples were digested with endonucleases HindIII and PstI, and then subjected to standard agarose electrophoresis against the standard sample The contents of each lane in the gel are as follows: Lane 1, 10uL extracted pBR322/2uL 10X loading buffer; Lane 2, 10uL pBR322 restricted /2uL 10X loading buffer; Lane 3, 10uL recovered

pBR322/2uL 10X loading buffer; Lane 5, 10uL recovered pBR322 restricted/2uL 10X. The gel was run for 45 minutes at 50 milliamps, stained in ethidium bromide, and photographed on a UV source. The photograph can be viewed in Figure 7. It can be observed that digestion of the standard sample (lane 2) and the recovered standard (Lane 5) were both successful.

FIGURE 1

Drawing of Modified Gel Casting Tray

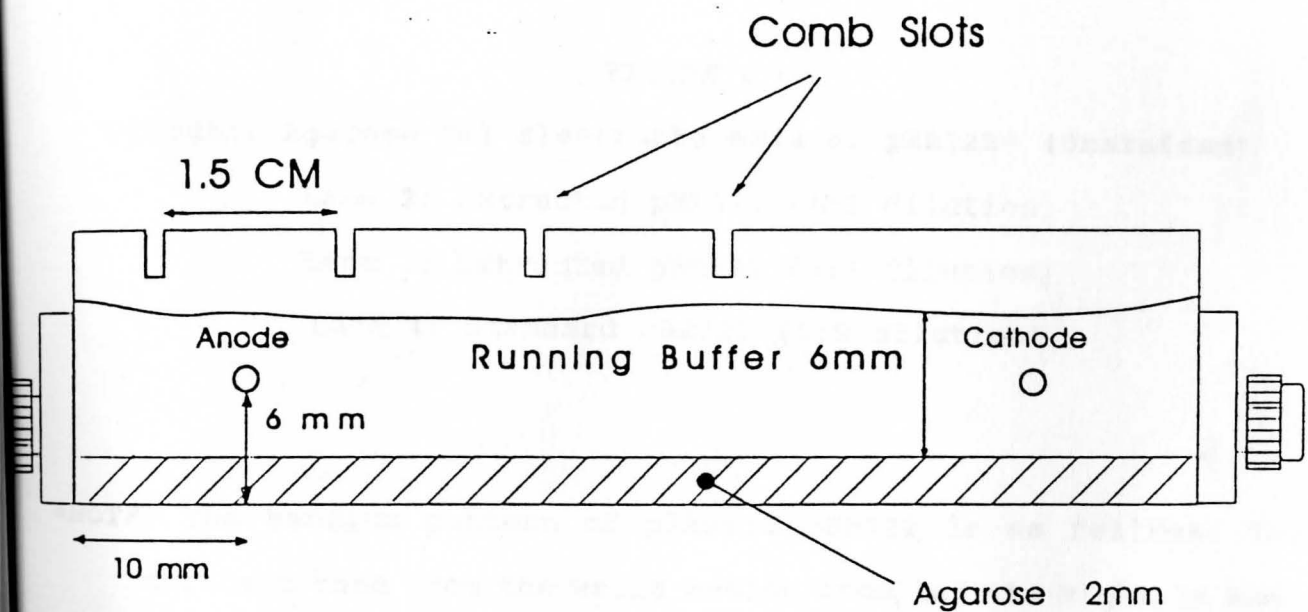
Comb Slots
1.5 CM
Anode
Running Buffer 6mm
Cathode
6 mm
10 mm
Agarose 2mm

FIGURE 2

Standard Agarose Gel Electrophoresis of pBR322* (Unstained)

Lane 2: Extracted pBR322 (1:1 dilution)

Lane 3: Extracted pBR322 (5:1 dilution)

Lane 4: Standard pBR322 (1:9 dilution)

*NOTE: The banding pattern of plasmid pBR322 is as follows: The furthest band from the wells moving from left to right is RNA. The next band is covalently closed circular DNA, followed by the open-nicked circular portion of the plasmid. The band closest to the wells (nearest the left side of the gel), is linear DNA.

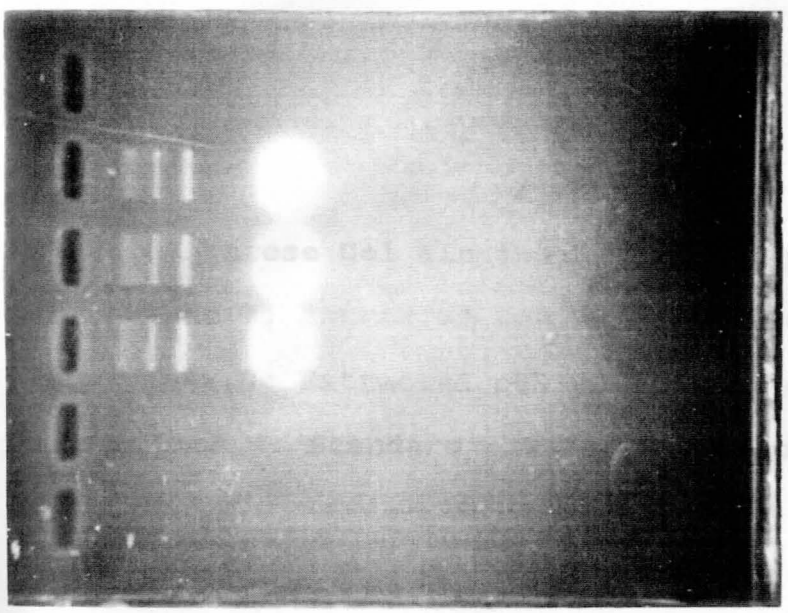
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FIGURE 3

Standard Agarose Gel Electrophoresis of pBR322 (Pre-stained)

Lane 2: Extracted pBR322 (1:1 dilution) + EtBr

Lane 3: Extracted pBR322 (5:1 dilution) + EtBr

Lane 4: Standard pBR322 (1:9 dilution) + EtBr

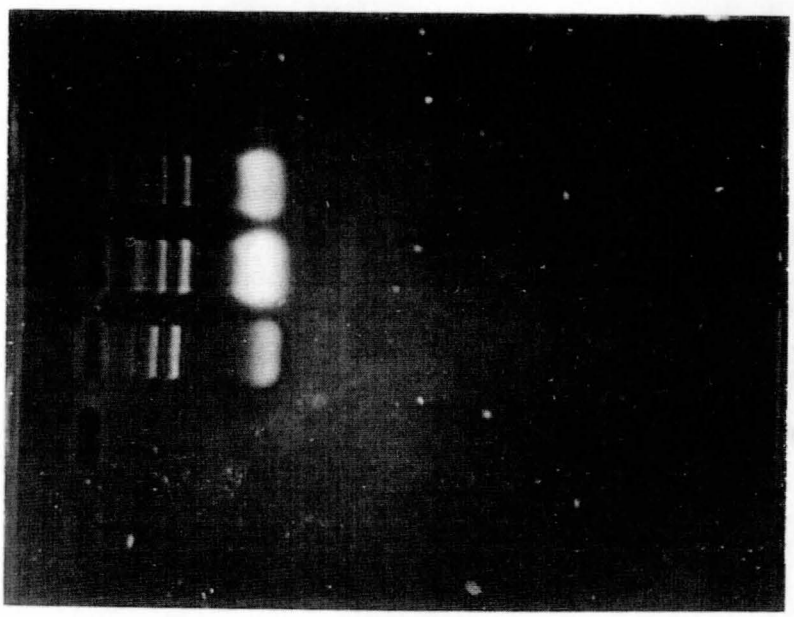

FIGURE 4

Modified Agarose Gel Electrophoresis of pBR322 (Unstained)

Lane 2: Extracted pBR322 (1:1 dilution)

Lane 3: Extracted pBR322 (5:1 dilution)

Lane 4: Standard pBR322 (1:9 dilution)

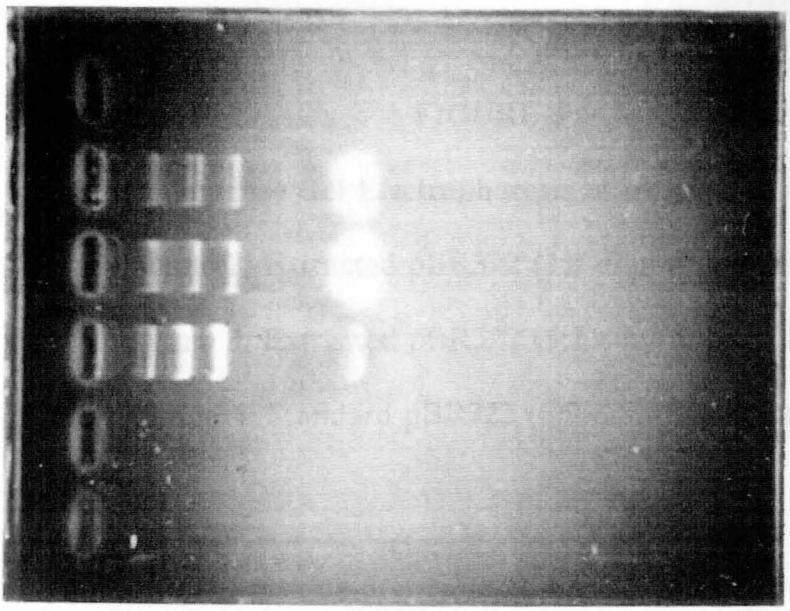
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FIGURE 5

Modified Agarose Gel Electrophoresis of pBR322 (Pre-stained)

Lane 2: Extracted pBR322 (1:1 dilution) + EtBr

Lane 3: Extracted pBR322 (5:1 dilution) + EtBr

Lane 4: Standard pBR322 (1:9 dilution) + EtBr

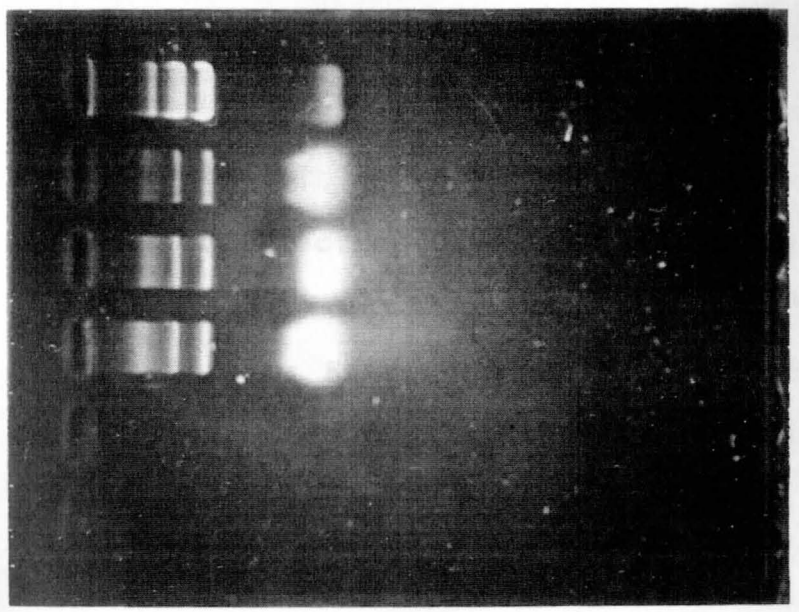
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FIGURE 6a

Agarose Gel Electrophoresis of pBR322 in Modified wells

Lane 1: Extracted pBR322 (5:1 dilution) + EtBr

Lane 3: Extracted pBR322 (5:1 dilution)

Lane 5: Extracted pBR322 Standard (5:1 dilution) + EtBr

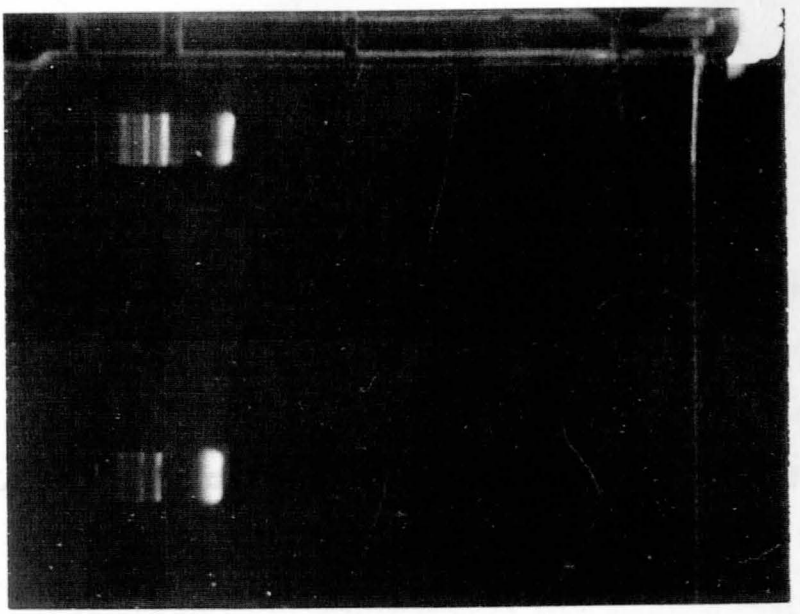

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FIGURE 6b

Agarose Gel Following Recovery of pBR322 Band

Lane 1: Extracted pBR322 (5:1 dilution) + EtBr

Lane 3: Extracted pBR322 (5:1 dilution) + EtBr

Lane 5: Extracted pBR322 Standard (5:1 dilution) + EtBr

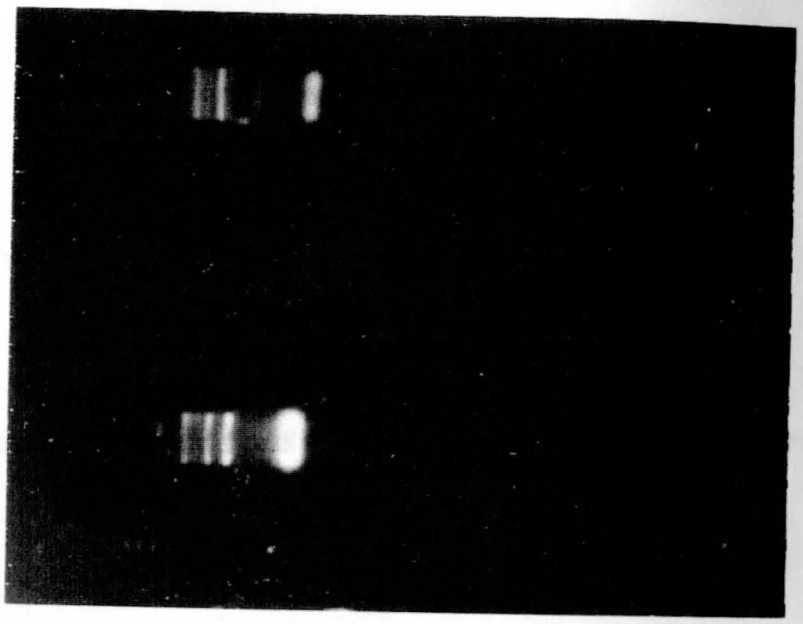

FIGURE 6c

Agarose Gel (6a) Following Ethidium Bromide Staining

Lane 1: Extracted pBR322 (5:1 dilution) + EtBr

Lane 3: Extracted pBR322 (5:1 dilution)

Lane 5: Extracted pBR322 Standard (5:1 dilution) + EtBr

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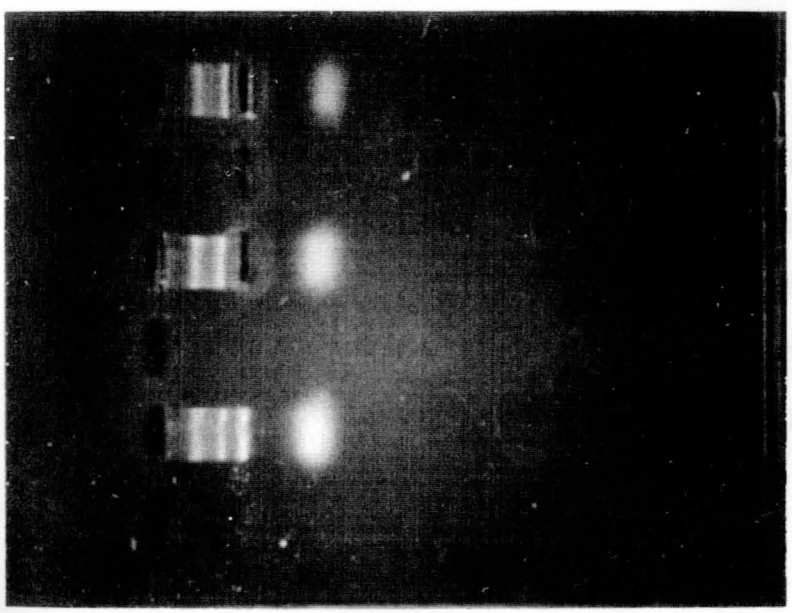

FIGURE 6d

Agarose Gel Electrophoresis of Recovered DNA with Standard

Lane 1: Extracted pBR322 Standard (5:1 dilution)

Lane 2: Recovered Pre-stained pBR322 Band

Lane 3: Recovered Pre-stained pBR322 Band

Lane 4: Recovered Unstained pBR322 Band

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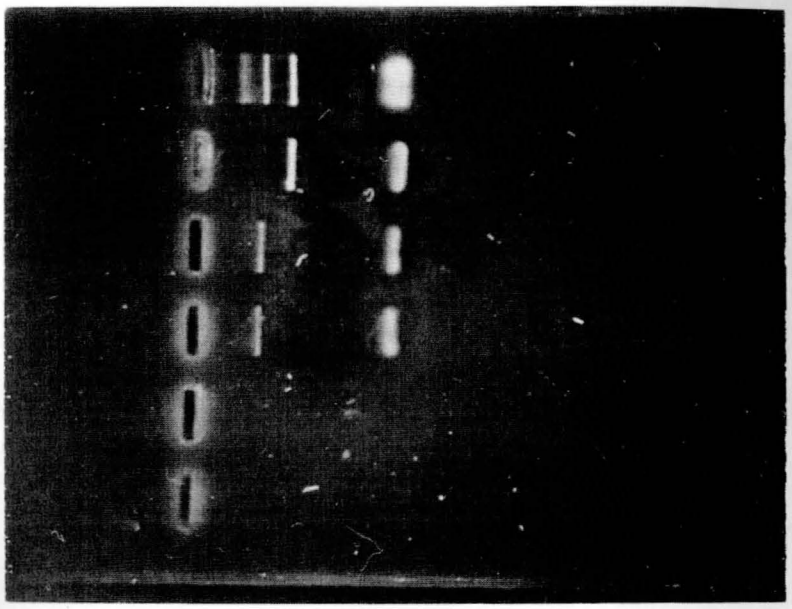


FIGURE 7

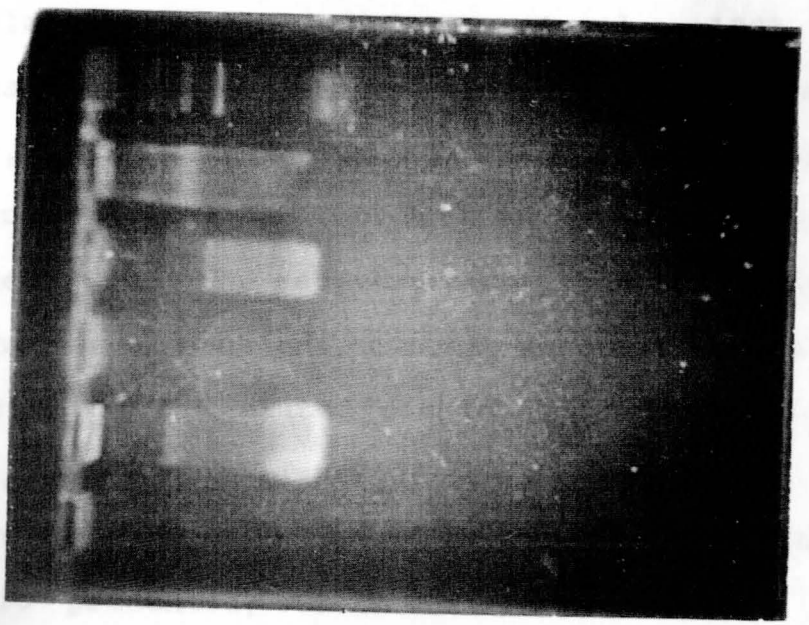
Agarose Gel Electrophoresis of Restricted pBR322 with Standard

Lane 1: Extracted pBR322 (5:1 dilution)

Lane 2: Extracted pBR322 HindIII/PstI Digest

Lane 3: Recovered pBR322 from Modified Well

Lane 5: Recovered pBR322 HindIII/PstI Digest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CHAPTER IV

DISCUSSION

The electrophoresis and recovery of DNA from agarose gels has become an important step in the manipulation of DNA in the molecular laboratory. McEnery et al., 1985, stated that to successfully study DNA fragments it is necessary, among other things, to be able to separate DNA by electrophoresis, and recover particular fragments for further molecular biological studies. Despite the many DNA recovery techniques currently available, there are a number of problems commonly associated with these techniques: 1) purity of the eluted sample, 2) percentage recovery of said sample, and 3) complexity of the procedure.

In this study, a method of recovering DNA bands from agarose gels is presented, which is simple, rapid, and requires no additional purification of the DNA. The DNA is extracted using the alkali lysis technique, since this method yields the best results, as compared to the other two methods discussed in Materials and Methods. Electrophoresis of DNA is performed in a modified casting tray directly on an ultraviolet transilluminator. This allows the samples to be monitored intermittently as they are drawn across the agarose gel. The DNA sample that is pre-stained with ethidium bromide serves as the marker, to permit recovery of pure samples of DNA. The success of recovery of the unstained DNA is dependent on the movement of the pre-stained tracer DNA into a modified well. In

most cases, the pre-stained and unstained bands move at approximately the same speed through the gel. In order to insure that the bands move at the same rate across the gel, the following precautions must be taken: 1) the platinum wires must be perfectly straight across the gel, 2) TAE buffer must be added to the tray, so that the wires are completely covered with buffer, and 3) the ethidium bromide used in the pre-staining step must be at a concentration of 0.05 ug/mL, 4) the amperage must be no greater than 50 milliamps. Thus, it is crucial that all of the above mentioned parameters are met each time the procedure is used in order for recovery of unstained DNA to be successful. For best results when recovering the bands, the pipette tip should be inside the well without touching the bottom, and the band should be recovered slowly by moving the pipet from one side of the well to the other.

In contrast to other techniques, the unstained recovered samples are not contaminated in any way, since the band of interest is recovered by pipette directly from a modified well in the agarose.

To assure that the unstained recovered sample was not contaminated, E. coli RR1 was transformed with a recovered portion of pBR322, and the transformation was successful. Also, a recovered portion of the pBR322 was digested successfully with restriction endonucleases, again demonstrating that the recovered portion of the plasmid was in fact pure.

Despite the purity of the recovered sample, an interesting

artifact was observed upon electrophoresis. Namely, an extra band, which occurred where RNA would normally be seen. The band was resistant to RNAase, and occurred in both standard and modified electrophoretic runs, using both standard pBR322 (SIGMA), and extracted pBR322. Surprisingly, the extra band also appeared following recovery of any one band in the sample. This would seem to indicate that the extra band could possibly be a degradation product of the entire DNA sample. It should, however, be emphasized that the extra band in no way inhibited the digestion of standard and recovered DNA with restriction endonucleases, or the transformation of E. coli RR1 with the recovered band. Therefore, this artifact does not appear to jeopardize the purity of the recovered sample, and thus, it can be hypothesized that this method would perhaps be useful in the purification of Polymerase Chain Reaction fragments away from primers, primer dimers, dNTPs, proteins, and salts from reaction mixtures.

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