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Review

Methods for recovering nucleic acid fragments from agarose gels

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ABSTRACT

Agarose gel electrophoresis is a powerful technique for the separation of nucleic acids on the basis of their size and conformation. The development of methods to recover size-fractionated nucleic acids molecules from agarose gels has greatly facilitated recombinant DNA technologies. Although several methods for recovering DNA and RNA molecules have been developed during the past fifteen years, none of them has been universally accepted. In this review we describe, discuss and evaluate the most common procedures with which we have had experience. Our evaluation is based on the criteria of yield, purity, speed, simplicity and low cost. We have considered three different approaches to the problem of recovering nucleic acids: chemical gel dissolution, physical gel disruption and physical extrusion from intact gels.

CONTENTS

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LIST OF ABBREVIATIONS

bp	Base pairs
kb	Kilobases (1 kb = 1000 bp)
LMT	Low melting temperature
PCR	Polymerase chain reaction
TBE	Tris–borate–EDTA buffer
TE	Tris–EDTA buffer
UV	Ultraviolet light

1. INTRODUCTION

Agarose gel electrophoresis is the standard method used to separate and identify nucleic acids on the basis of different size and conformation [1–4]. By varying the agarose concentration, it is possible to reliably separate DNA and RNA molecules in the size range from *ca.* 200 base pairs (bp) to *ca.* 30 kilobases (kb). Its application has become extremely important because restriction endonucleases, which recognize short oligonucleotide sequences in DNA and cleave the DNA at all such sites, have come into wide use [4–8]. A restriction enzyme cuts pure DNA into a reproducible set of fragments that can thus be separated by gel electrophoresis. The successive development of genetic engineering and, therefore, the possibility of transferring exogenous DNAs inside the genome of high reproductive rate microorganisms, have permitted the physical separation of specific DNA segments, their isolation and purification, to become a basic element of such technologies.

Agarose is a linear commercially available polymer, isolated from agar, and even though progress has been made in reducing the amount of contaminants it contains, it is not yet possible to find it really pure. It is contaminated by other polysaccharides, salts and proteins, and the contamination can affect both the migration of the DNA, and the use of the DNA recovered from the gel as a substrate in enzymic reactions. Agarose gels are prepared by melting the agarose in the presence of the appropriate buffer until a clear solution is obtained [4]. As it cools, melted agarose forms a block gel, whose hardening is directly related to the agarose concentration. The mechanism for gelation of agarose was first suggested by Rees [9,10] and demonstrated by Arnott *et al.* [11]. Upon hardening, the agarose gives rise to a continuous filamentous matrix, which forms relatively large pores with diameters that depend on the concentration of the agarose. DNA and RNA molecules, which tend to become oriented in an end-on position [6,12] when subjected to an electric field, because of their negative charge at neutral pH, are forced to pass through the pores of the matrix with a worm-like movement called reptation [13,14]. The migration rate of these molecules is inversely proportional to the logarithm of their molecular mass [6] and also depends on the agarose concentration: a linear DNA molecule migrates at different rates through agarose gels of different concentrations. There is a linear relationship between the logarithm of the electrophoretic mobility of DNA (μ) and the gel concentration (τ), according to the equation [15]:

$$\log \mu = \log \mu_0 - k_r \tau$$

where μ_0 is the free electrophoretic mobility of DNA and k_r is the retardation coefficient, a constant that is related to the properties of the gel and the size and shape of the migrating molecules. The result of such a migration, which is not significantly affected by the nucleic acid base composition [16], is the separation between the various components of different size, in the form of bands detectable under a UV lamp, after staining the gel with a fluorescent dye such as ethidium bromide [7].

For general information on agarose gel electrophoresis we refer to commercially available manuals [4,17]. For a complete survey of electrophoretic methods we refer to two review articles based on historical background [18] and recent developments [19], respectively.

As mentioned above, the development of all the technologies that paved the way to the isolation and cloning of DNA fragments or genes focus on the recovery of DNA fragments separated in agarose gels as a key step in the application of these technologies. In this respect, we refer to Old and Primrose [21] for a general survey on genetic

engineering products which have found an application in the diagnosis of different pathologies and in the production of recombinant proteins approved for clinical use.

2. DNA RECOVERY FROM AGAROSE GELS

From the first approaches [22–24], a myriad of methods have been developed to recover and purify size-fractionated DNA fragments from agarose gels [25–71]; this can be seen as a consequence of no entirely satisfactory protocol being available.

A first step in the recovery of a particular DNA species from a gel is to locate and isolate the band of interest. This can be done by excising a DNA-containing gel slice from the whole gel with a razor blade under a direct visualization of the ethidium bromide-stained DNA band, through the use of long-wave length UV light. This gel slice can be used for the direct recovery of the DNA. The first limiting step of such a technology is recovering the DNA fragments in an intact form, free of contaminants. Even with the most careful handling, some damage to the DNA can always occur. Exposure of the DNA to physical stresses, or to an excess of UV light in the presence of ethidium bromide, could result in damage that would condition further manipulations. Moreover, the presence of contaminants, or of agarose itself, has been demonstrated to have an inhibitory effect on the suitability of such DNAs for subsequent manipulations [20].

Another factor that can condition the choice of a method is the yield of the recovered DNA. Methods that ensure the recovery of uncontaminated intact DNAs often lead to a very low yield and are to be avoided, especially when very low DNA concentrations are to be recovered. The problem of the yield and the purity of DNA is often worsened by increasing agarose concentrations, which are known to allow a reliable separation of molecules of progressively lower molecular mass [4,17]. Methods that give a high yield for DNAs of low molecular mass often become progressively inefficient as the size of the DNA fragments to be recovered increases. Although the yield and the purity of the recovered DNAs remain the main aim of such technologies, other important elements to consider in choosing a recovery procedure are the simplicity and the speed, together with the low cost of the system.

All the conditions mentioned above are necessary in order to select a reliable and efficient method. Particular parameters, such as DNA molecular mass, DNA concentration and agarose concentration, often suggest the choice of one method instead of another, aside from the performance of the method itself. Moreover, the most common methods generally indicated in the molecular biology manuals [4] are not always the easiest and the most efficient. They are surely among the most reliable, and generally allow a satisfactory recovery of uncontaminated DNA. Recently, however, very simple and fast methods have been developed, which ensure satisfactory yield of recovered material, often in a wide range of molecular masses [38,46,59].

In treating this argument, we prefer to consider different approaches to the problem, even though the classification of these techniques, according to categories based on general principles, is difficult and perhaps improper. We shall therefore distinguish the following approaches to DNA recovery from agarose gels: chemical gel dissolution; physical gel disruption; and physical extrusion from an intact gel.

2.1. Chemical gel dissolution

Chaotropic agents, such as potassium iodide, sodium perchlorate and sodium iodide, are able to dissolve gelated agarose when present in high concentrations. It is thus possible to solubilize the agarose matrix under conditions that do not damage the DNA. Such use of chaotropic agents has been suggested by various authors [26–30], and found widespread application in a method developed by Vogelstein and Gillespie [31], which uses NaI. As reported in the original paper, to which we refer for a specification of the procedure, the solubilization of DNA-containing agarose gel slices is obtained by using a saturated solution of NaI at 25°C. The addition of activated glass powder to the incubating solution allows

all the released DNA to be bound to the glass; the DNA binding rate is proportional to the concentration of the glass surface. Glass powder is then collected by centrifugation, washed in a solution of NaI, and reincubated as above to allow residual agarose to diffuse into NaI. The DNA–glass complex is again collected by centrifugation and washed in a mixture of 50% buffer (20 m*M* Tris–HCl, pH 7.2; 0.2 *M* NaCl; 2 m*M* EDTA) and 50% ethanol to remove NaI. DNA is eluted from the glass by incubating the complex at 37°C for 30 min in the presence of a Tris–EDTA (TE) buffer [4]. Glass powder is then removed by centrifugation. Phenol extractions are then performed to purify the DNA from contaminants, and the resultant aqueous phase is precipitated with ethanol to recover DNA.

A modification of this protocol is currently applied in our laboratory, routinely giving an optimized 90% recovery of pure DNA fragments ranging from a few hundred of nucleotides to *ca.* 1.5 kb. This variance involves the solubilization of gel slices at 65°C in the presence of NaI, followed by ice incubation of the solution for 20 min after the addition of glass powder used to bind the gel-released DNA. Two washing steps, as in the original protocol, and incubation of the DNA–glass complex at 65°C in the presence of TE buffer, in order to release the DNA from the glass, are then performed. Phenol extractions and ethanol precipitation are performed according to the original protocol.

Based on the principle of the solubilization of the gel matrix in order to release the DNA, the use of low-melting-temperature (LMT) agarose gels was developed. A number of types of agarose containing hydroxyethyl groups in the polysaccharide chain are available. This modification causes the agarose to melt at 65°C and gelate at 30°C. Because 65°C is well below the melting temperature of most double-stranded DNAs, it is possible to melt DNA-containing gel slices without damaging the nucleic acid itself. This procedure was first reported by Wieslander [32], and involves incubation of DNA-containing gel slices for 5 min at 65°C, in the presence of a TE buffer containing 0.2 *M* NaCl in order to melt the agarose. As the melting of the gel slices is completed, phenol extractions are performed to remove agarose particles together with other contaminants. After ethanol precipitation of the aqueous phase, *ca.* 60% of the DNA is recovered according to this procedure.

This method is very simple and rapid; however, in many cases the recovered DNAs need further purification, *i.e.* by passage through DEAE-Sephacel (see below), to obtain a product screened for the presence of contaminants. Although LMT gels are difficult to handle because of their fragility, and some difficulties in obtaining a pure DNA encountered, this method has a great advantage: LMT agarose remains fluid at 37°C, so that enzymic reactions performed at the same temperature, such as DNA ligation and digestion with restriction enzymes, can be carried out by adding volumes of DNA-containing melted gel solution directly to the reaction mixture (the so-called "in-gel manipulation" [33,34]). Different modifications of this procedure [35–37] were developed to ensure an improved yield of the recovered DNA. Although apparently advantageous, the "in-gel manipulation" technique has never becomes popular because it requires a large amount of enzymes, which are often expensive, for complete reaction.

Recently, an alternative method [38] has been proposed to recover DNA using LMT agarose. This method, besides working well with small DNA molecules, gives an increased yield of high-molecular-mass DNAs, with respect to Wieslander's [32] procedure. It makes use of DNAase-free agarase (now commercially available Gelase, Epicentre Technologies, Madison, WI, USA) to digest the agarose. A gel slice equilibrated in TE buffer containing NaCl is melted at 68°C for 10 min, re-equilibrated to 37°C, then incubated for 12–16 h in the presence of 2 U of agarase per 100 μl of gel. As in the above-mentioned protocol, the DNA-containing melted gel solution may be used at this stage for "in-gel manipulation". Alternatively, the DNA may be purified through two phenol extraction steps followed by ethanol precipitation.

Although many methods based on gel dissolu-

tion presented here give a satisfactory recovery of intact DNA, our opinion is that they have two main limitations. First, most of them need extra purification steps in order to obtain really pure DNA, probably because some contaminants present in the agarose, or agarose itself, are eluted together with DNA. These purifications are known to reduce the yield of recovered DNA, as well as prolonging the procedure. Second, there is a progressively lower recovery when DNA size increases. This effect is worth brief consideration, because it applies to all methods that involve DNA release through dissolution or disruption (see below) of the gel matrix. DNA molecules migrating in agarose gels are trapped between the matrix meshes. The gel matrix dissolution/disruption, even though apparently complete, is never absolutely complete, so that the release of DNA particles is easier for smaller molecules than larger ones. In this view, DNA release from gel occurs in an almost "size-dependent" fashion. High-molecular-mass DNAs are recovered with a very low efficiency using agarose gel dissolution/disruption methods. The use of agarase has partially resolved this disadvantage, probably because the enzymic treatment is added to a previous dissolution of LTM agarose obtained by heating. The main disadvantage of this method is the long incubation needed for a complete digestion of the agarose matrix.

2.2. Physical gel disruption

Based on a first attempt at DNA passive diffusion from crushed gels [22], an example of a rapid and simple method based on physical disruption of the gel matrix with consequent release of DNA is the so-called "freeze–squeeze" procedure [24]. A DNA-containing gel slice is placed and frozen in a small envelope of Parafilm sheet or of plastic wrap. The main benefit of freezing the agarose is to disrupt its structure. The frozen gel slice is then squeezed in its envelope by fingers or against a solid support to complete the disruption of the agarose matrix. The gel compression/disruption allows the DNA to diffuse out together with the gel interstitial buffer. This buffer drop, collected from the envelope with a pipette, contains a relatively high amount of DNA. After centrifugation to remove agarose particles, the resulting supernatant is indicated by the authors to contain 40–70% of recovered DNA, depending on the gel concentration and the DNA size.

A modification of this procedure [39] allows a slightly higher recovery of DNA. According to this protocol, the physical extrusion of DNA is obtained by squeezing the frozen gel slice by centrifugal force. An Eppendorf tube is punctured in the bottom, plunged with siliconized glass wool, and inserted in a second intact Eppendorf tube (Fig. 1A). The gel slice is placed on top of the glass wool and frozen as in the freeze–squeeze procedure. The DNA-containing buffer, eluted by centrifugation, passes through the glass wool, which restrains agarose particles, and is collected in the second tube. The DNA recovered using both of these procedures must be further purified to remove ethidium bromide and other contaminants eluted together with DNA. These purification steps, which obviously lead to an even lower yield of DNA, are, in our opinion, the main limitation in the use of the freeze–squeeze method. Moreover, use of high agarose concentrations and large DNA molecules are reported by the authors to reduce the recovery. Only high DNA concentrations, of a DNA size range of 1–5 kb,

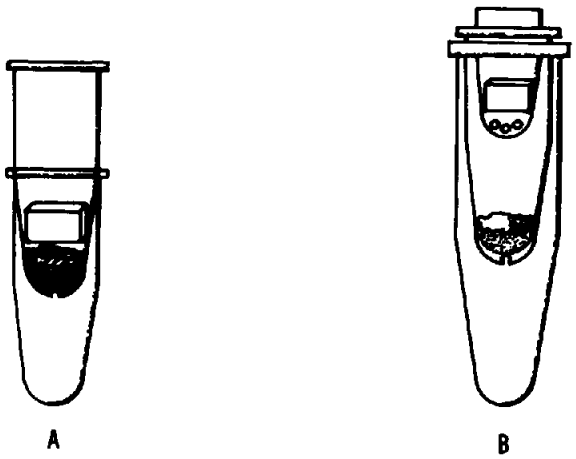

Fig. 1. Schematic drawing of (A) the two 1.5-ml Eppendorf tubes in the method described by Tautz and Renz [39] and (B) the three-tube system described by Vaux [46].

are suggested for a satisfactory application of the freeze–squeeze method.

Based on the principle of DNA extrusion through gel compression by centrifugation, with or without gel freeze, a series of similar procedures have been recently reported [40–46]. The newest one [46] is worth considering because it is simple, fast, and gives an almost satisfactory yield of DNA, related to its simplicity. The bottom half of a 0.5-ml polymerase chain reaction (PCR) tube is punctured four or five times, and placed on top of a second PCR tube with a single bottom hole. A piece of siliconized glass filter paper is pushed to the bottom of this second tube, and the two-tube system is inserted into an intact 1.5-ml Eppendorf tube (Fig. 1B). The gel slice is placed into the top tube and, under centrifugation, is disrupted as it is forced to pass through the tube's holes into the second tube. Here, the glass paper blocks the passage of the agarose but lets the DNA-containing buffer pass into the lower tube. The authors declares a 75% recovery of a 6.6-kb DNA molecule. On our hands this procedure leads routinely to a 50% recovery of DNA fragments in the size range 1–8 kb. Considering the workability of this method, the relatively low yield assumes less importance and can be considered satisfactory.

2.3. *Physical extrusion from an intact gel*

The idea of extruding DNA molecules from the gel without destroying the gel structure was first applied successfully by Tabak and Flavell [25]. According to this procedure, a small trough is cut in the agarose gel just in front of a DNA band and is then filled with hydroxyapatite. When electrophoretic migration is restarted, the DNA moves into the trough and is trapped by the hydroxyapatite, from which it can be eluted by passage through a Sephadex G50 column. For two reasons, a very low amount of DNA is recovered through this simple procedure: not all the migrated DNA is bound to the hydroxyapatite; and not all the hydroxyapatite DNA-bound is eluted by passage through the Sephadex column. Variations of this method involve the insertion of strips of 3MM Whatman filter paper [47] or DEAE-cellulose paper [48], into slits cut just in front of the DNA bands (Fig. 2). As electrophoresis is resumed, DNAs migrate into the paper, from which they are recovered through different elution steps. Although a considerable amount of DNA is found bound to the paper in both procedures, the elution steps, being long and complicated, considerably reduce the yield of recovered DNAs. Indeed, some difficulties may be sometimes encountered in eluting the DNA from the strips, particularly with high-molecular-mass DNAs. Despite these disadvantages, these procedures allow simultaneous recovery of many samples in a single electrophoretic step, a great advantage when different-size DNA components are to be recovered from a single gel. Moreover,

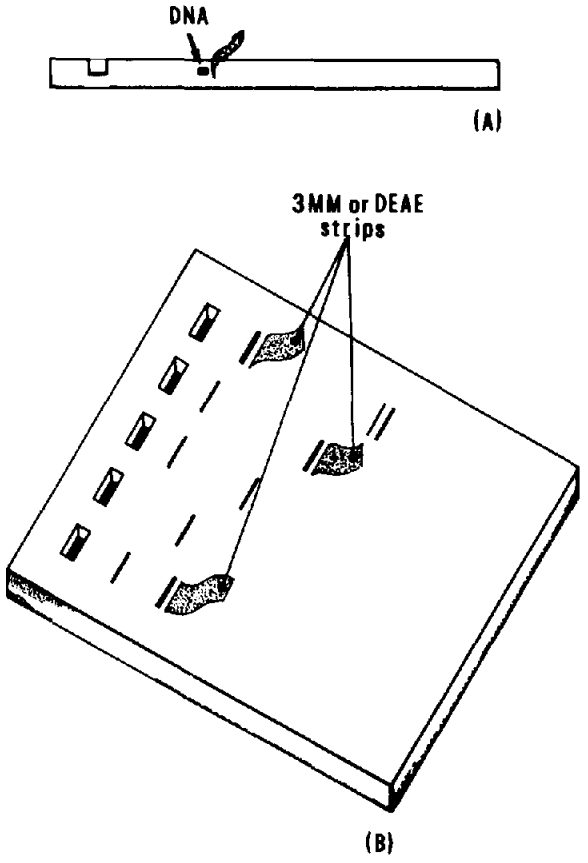

Fig. 2. Different 3MM Whatman filter paper [47] or DEAE-cellulose paper [48] strips inserted into slits cut in front of electrophoretically separated DNA bands: (A) side view; (B) top view.

DNAs recovered from the strips are of high purity, and do not need any further purification. As multiple DNA bands are separated in the same track, another strip can be inserted upstream of the DNA to be recovered, in order to "protect" it from possible contaminations caused by the migration of upper DNA bands. More recently, electroelution of DNA through NACS Prepac columns [49] and NENSORB 20 cartridges [50] has been proposed, but it gives a reduced yield [49] or takes too much time [50] to obtain an uncontaminated DNA.

The principle of eluting DNA from gels in the same buffer in which electrophoresis is performed was suggested first by Patterson *et al.* [23] and later found successful application in a variance developed by McDonell *et al.* [8]. This method, although not entirely satisfactory for the reasons we shall discuss below, has achieved widespread use. A DNA-containing gel slice is placed into a dialysis tube, tied off at one end and filled with a low-conductivity elution buffer, until the gel is completely immersed in it. The dialysis bag is then firmly closed and immersed in an electrophoretic tank filled with the same buffer. The bag is placed between and parallel to the electrodes (Fig. 3). When electrophoresis is started, the DNA is electroeluted out of the gel onto the inner wall of the bag. The current polarity is then reversed for 1–2 min to release the DNA eventually bound to the dialysis tube, and the DNA-containing buffer is collected from the bag using a Pasteur pipette. We suggest that the ethidium bromide-stained gel slice and the dialysis bag should be checked under a UV lamp after electrophoresis, in order to confirm that all the DNA is recovered. In fact, the eluted DNA is often found firmly bound to the inner surface of the dialysis bag, from which it is sometimes really difficult to recover. The impossibility to check DNA migration during the electrophoresis process makes this method unreliable. There is no way to "set" an elution time because it depends on some variable parameter, such as DNA size and agarose concentration. Prolonged electrophoresis, needed for the elution of large DNAs, causes the eluted DNAs to adhere firmly to the bag surface, or to re-enter the gel slice. Indeed, DNAs recovered using this procedure often need further purification. Despite these disadvantages, this method is one of the most effective for the recovery of large DNA molecules.

Based on the principle of electroelution, other methods have been reported [51,52] but do not show better reliability than the method of McDonell *et al.* [8]. Of these, we shall describe a very simple procedure that has been popular for a long time, even though we do not consider it sufficiently reliable. This procedure is similar to the Tabak and Flavell [25] method described above. In this method [53,54] the small trough created in front of the DNA bands is used as a tank filled with electrophoretic buffer (Fig. 4). As electrophoresis is re-started, ethidium bromide-stained

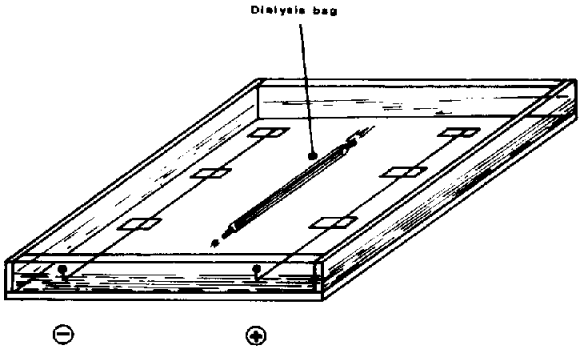

Fig. 3. Schematic drawing of the electrophoretic tank in which dialysis tube containing a gel slice is placed parallel to the electrodes, as described in ref. 8.

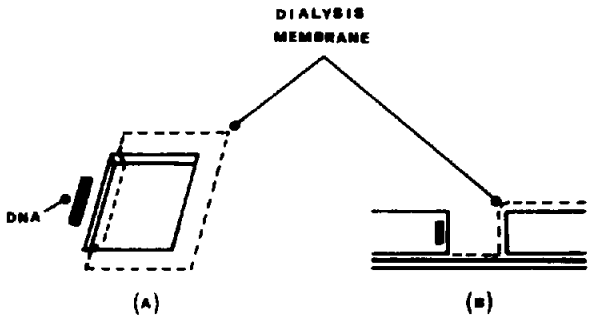

Fig. 4. (A) Schematic drawing and (B) a section through an elution trough [53,54].

DNA moves into the tank. This migration can be followed under a UV lamp. As all the DNA is transferred into the trough and is trapped in it by a dialysis membrane set all around the trough itself, it can be collected using a pipette. Phenol extractions are then needed to purify the eluted DNA which is recovered by ethanol precipitation. The success of this method in recovering the highest possible amount of eluted DNA rests on the skill and speed of the operator. The DNA, if not rapidly captured, can run out of the trough and be dispersed in the surrounding buffer. This is the reason why the procedure is to be considered unreliable.

Many attempts to develop an elution procedure that is not significantly affected by DNA size or by agarose concentration have led to the construction of variously complicated apparatus [55–58] through which DNA recovery has become a really long and expensive procedure. To improve the recovery of large DNA molecules, a procedure that gives a satisfactory recovery of pure DNA of a wide range of sizes in short times, through the use of a very simple apparatus, has been recently developed in our laboratory [59]. It consists of two platinum electrodes, one (positive) circular and the other (negative) linear, perpendicular to the plane of the first (Fig. 5). A DNA-containing gel slice is placed on a Parafilm sheet, and the circular electrode is placed around it. The complex is covered with a drop of a low-conductivity buffer (1 × Tris–borate-EDTA buffer (TBE) [4]), which surrounds the gel slice and adheres to the circular electrode. The linear electrode is placed on top of the gel in order to touch the gel itself. As a continuous potential of 25 V is applied to the system, the electic field created between the two conductors allows the ethidium bromide-stained DNA in the gel to migrate rapidly and radially towards the circular electrode in the surrounding buffer (a migration that can be followed under a UV lamp), from which it is recovered by a pipette.

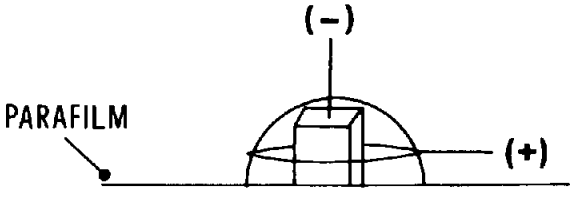

Fig. 5. Schematic drawing of the electroelution system developed by Duro *et al.* [59].

The only disadvantage we have encountered while developing this procedure has been a partial degradation of DNA molecules when a long-time is needed for the elution. We have overcome this by eluting DNAs in several electrophoretic rounds of 1 min each, instead of 3 min as in the original protocol. The eluted DNA is then purified by a single chloroform extraction, and collected by ethanol precipitation. This method allows a satisfactory recovery of pure DNA in the range 0.5–20 kb, and is only marginally affected by the agarose concentration. Higher-molecular-mass DNAs, such as 48-kb λ DNA, are also recovered with a satisfactory yield (*ca.* 50%) using this procedure.

3. PURIFICATION OF DNA

As often mentioned, recovered DNA needs purification before further manipulations. Because the contaminants eluted from agarose gels with DNA during the recovery process are essentially polysaccharides, agarose itself, and ethidium bromide, two different purification processes are suggested to remove these contaminants.

3.1. Extraction with organic solvents

Passage through siliconized glass wool removes small pieces of agarose, and extraction with phenol or phenol–chloroform [4] is used to eliminate other contaminants. Sometimes there is a significant decrease in volume during the first phenol extraction. In such a case it might be necessary to re-extract the organic phase by adding a 0.5 volume of buffer and combining the resultant two aqueous phases. Ethanol precipitation [4] allows the recovery of purified DNA, which may be resuspended in its appropriate volume of buffer. As mentioned above, phenol extraction reduces the yield of the recovered DNA.

3.2. Passage through DEAE-Sephacel or DEAE-Sephadex

In this purification step, the negatively charged DNA is bound to an anionic matrix and the contaminants are eluted through the column. The DNA is then released from the matrix by increasing the ionic strength of the elution buffer. These columns are commercially available, so the reader is referred to manufacturers' specifications for their correct use.

4. RNA RECOVERY

Although there are no theoretical reasons for the application of DNA recovery methods to RNA, many of the methods discussed above cannot be used to isolate RNAs in pure undegraded form, probably owing to the instability of such molecules. The elution method developed in our laboratory [59] gives a very low recovery (10–12%) of partially undegraded RNA molecules. Some methods developed for DNA recovery are indicated by the authors to be applicable to RNA. Even though different methods [32,49,51,60,61] are devoted or applicable to the recovery of RNAs from agarose gels, the LMT agarose procedure [32] is, in our opinion, the most reliable for obtaining undegraded pure RNAs.

5. CONCLUSIONS

Several criteria can be applied to the selection of a particular procedure from the many valid ones available. Generally, the choice depends on the specific experimental parameters (DNA size, DNA concentration, agarose concentration, final volume in which the DNA is recovered). Unavoidably, though, each researcher feels more comfortable working with certain methods rather than with others, so there is a strong subjective component involved. In our laboratory, we naturally prefer to use our own elution procedure [59]. However, our working experience with DNA recovery methods has led us to consider the NaI dissolution gel procedure [31] (for DNA sizes less than 1.5 kb) and, more recently, the three-tube system described by Vaux [46] (for DNA sizes of 1–8 kb). For higher-molecular-mass molecules, we consider our procedure [59] to be the most reliable.

6. ACKNOWLEDGEMENTS

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